INVESTIGATING THE ROLE OF TH17 CELLS IN THE INITIATION AND CHRONIFICATION OF AUTOIMMUNE CNS DEMYELINATION

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Abbreviations

°C	degree Celsius
AF	Alexa Fluor
APC	antigen presenting cells
α-m	anti-mouse
BBB	blood-brain barrier
BSA	bovine serum albumin
CD	Cluster of differentiation
CFA	complete Freund's adjuvant
CNS	central nervous system
ConA	concanavalin A
cpm	counts per minute
CSF	cerebrospinal fluid
DC	dendritic cells
DMSO	Dimethyl sulfoxide
EAE	experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGFP	enhanced green fluorescent protein
EtOH	Ethanol
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	forkhead box P3
FSC	Forward Scatter
Gd-DTPA	Gadopentetate dimeglumine
GM-CSF	granulocyte monocyte colony stimulating factor
H37RA	Mycobacterium tuberculosis
HE	hematoxylin and eosin
HLA	Human leukocyte antigen
IFN	interferon
IL	interleukin
IMDM	Iscove's Mod Dulbecco's Medium
IR	infrared

i.v.	intravenous
Ko	knockout
MACS	magnetic activated cell sorting
MBP	myelin basic protein
MHC	major histocompatibility complex
MM	mouse cell culture medium
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
OPO	optical parametric oscillator
OVA	ovalbumin
PBS	phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PFA	paraformaldehyde
PI	propidium iodide
PLP	proteolipid protein
PPMS	primary progressive multiple sclerosis
PTx	pertussis toxin
RFP	red fluorescent protein
rh	recombinant human
rm	recombinant murine
RPMI	Roswell Park Memorial Institute medium
RRMS	relapsing-remitting multiple sclerosis
SA	streptavidin
SPMS	secondary progressive multiple sclerosis
TGF-β	transforming growth factor beta
TCR	T cell receptor
Th cell	T helper cell
TPLSM	two-photon laser scanning microscopy
Treg	naturally occurring regulatory T cell
U	unit
WM	mouse washing medium

1 Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) also known as encephalomyelitis disseminata is the most common chronic inflammatory disease of the central nervous system (CNS) in Europe and North America. It leads to devastating disability in both the young and older population with only limited treatment options available so far. Most commonly, young adults between 20 and 40 years of age are affected (1). In Germany, the number of MS patients is estimated to 67.000 - 138.000 (2). In general, there is a female predominance in prevalence (2:1 ratio) (3,4).

In 1867, the disease was comprehensively described by the French neurologist Jean-Martin Charcot (5). Charcot reported episodically occurring neurological dysfunctions in patients, which remitted after days to weeks. He correlated this clinical manifestation with disseminated focal cell infiltrates in the white matter of the CNS, loss of myelin in the areas of inflammation and glial scar formation, the latter defining the name of the disease “sclérose en plaques”. The more recent observation by Elvin Kabat in 1948 of increased oligoclonal immunoglobulin in the cerebro spinal fluid (CSF) of patients with MS provided evidence for an inflammatory nature to the disease (6). Elvin Kabat’s observations as well as the insights in recent years led to an autoimmunity hypothesis when it comes to MS. It is assumed that, in genetically susceptible individuals, autoreactive pro-inflammatory T cells, which are specific for CNS antigens, are activated in the periphery due to failure of immunological control mechanisms. These T cells are therefore able to pass the blood-brain barrier (BBB) and lead to damage not only of the myelin sheaths but also neurons and axons (4).

Despite intense research in the last decades, the aetiology of multiple sclerosis is still not known. Geographical variations were observed for the occurrence of MS. The disease is affecting people in the northern hemisphere more often than in equatorial regions. Furthermore, genetic factors seem also to play an important role as the risk for relatives of MS patients to develop the disease is increased in comparison to adopted children. Adopted children as well as individuals related by marriage do not have an increased risk in families with disease occurrence. Twin studies did show that the risk to develop disease is six times higher in identical than non-identical twins (7). Thus, MS is a common complex disease with

partial genetic aetiology. By now, many genes are known that can be associated with MS but as single variations play only a minor role in predisposition for the disease, this rather adds up to disease susceptibility. Various results in MS family studies confirm that the major histocompatibility complex (MHC) determining region confers susceptibility to MS (8). Epidemiologic studies showed that environmental factors also play a role in the disease process making MS a multifactorial disease in which genetic as well as environmental influences contribute to the manifestation and the development of the disease (9).

Occurring symptoms can vary from patient to patient. There is a broad range of symptoms connected with the disease that extend from sensory loss over limb ataxia and ataxia of the gait and trunk to complete paralysis in later stages of disease. Moreover, signs and symptoms include optic neuritis, optic atrophy, fatigue, vertigo, cognitive changes, euphoria as well as depression, spasticity, bladder disturbance, and sexual disturbance (10). In general, however, life expectancy of MS patients is just slightly reduced compared to healthy individuals.

There is not only a variety in disease symptoms but also in the clinical disease course (11). The majority of patients experience their disease in relapses. There are four different disease courses that can be differentiated (Figure 1). The most frequent course of disease is relapsing-remitting MS (RRMS) (Figure 1A). 80 – 90 % of the MS patients present with RRMS, which is characterised by a relapsing pattern of acute exacerbations followed by periods of stability. Most patients with RRMS develop a secondary progressive MS (SPMS) with time presenting with progressive deterioration in between relapses and worse recovery from the relapse (Figure 1B). 10 – 15 % of patients present with a primary progressive MS (PPMS) (Figure 1C). They deteriorate from the beginning without any improvement and most of these patients never experience clear-cut relapsing episodes (1). The fourth disease course is described as benign MS and is characterised by no progression of symptoms during the course of disease (Figure 1D) (12).

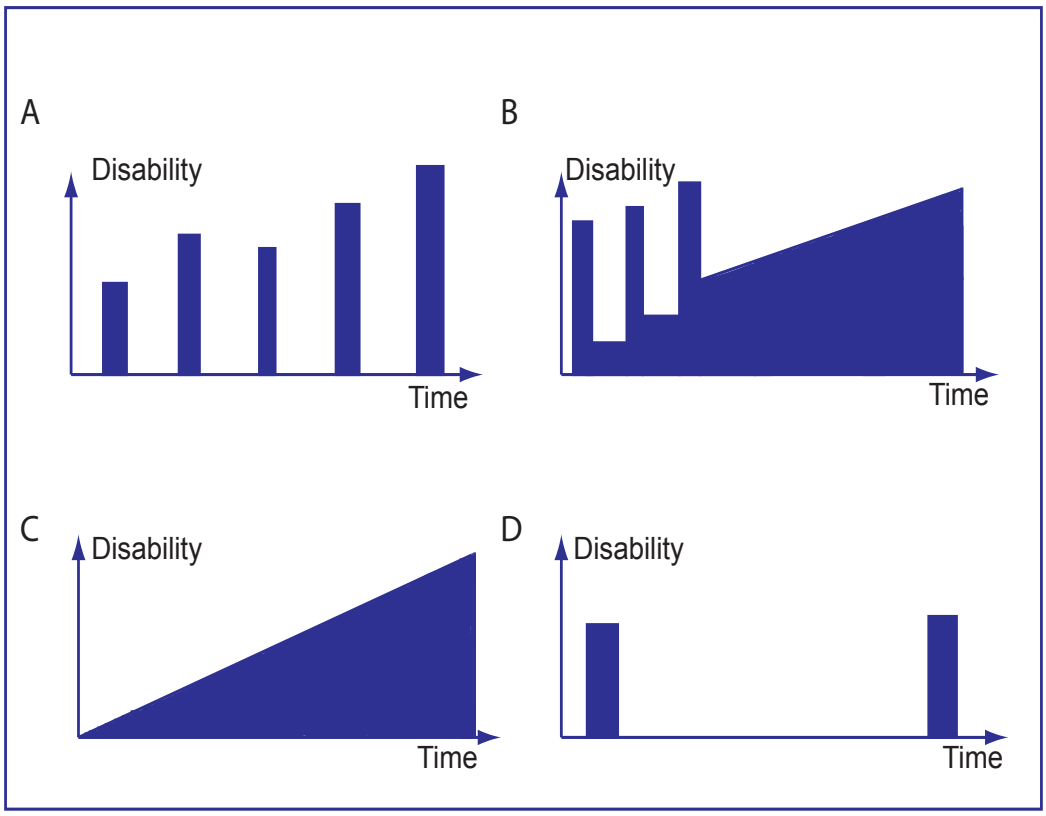


Figure 1. Different clinical disease courses for MS. (A) relapsing-remitting MS, (B) secondary progressive MS, (C) primary progressive MS, (D) benign MS.

Although several therapies are available to treat MS nowadays, like e.g. interferon-β and glatiramer acetate (GA), which are immunomodulatory drugs used as the first line of treatment in RRMS, their effectiveness is limited. The drugs that have been developed so far only succeed in reducing the relapse rate and delaying the disability to a certain degree in RRMS and SPMS patients. None of the drugs available right now is able to cure the disease (4,13). Furthermore, the more effective therapies come with a higher risk for the patient to develop side effects (14). Therefore more effective and specific treatment approaches are needed with better risk-benefit ratio.

1.1.1 Exacerbations in Multiple Sclerosis

Numerous exogenous events have been proposed as triggering factors for exacerbations of MS, e.g., stress, trauma, infection, immunisation, climatic changes, and physical exertion (15). Several lines of evidence have suggested that MS, like other autoimmune diseases, may be triggered by bacterial or viral infections (16). Infections may also be a potent trigger of MS attacks (17). However, attempts to establish a direct epidemiologic association between

infections and MS aetiology have been unsuccessful so far. This might be due to the fact that a complete clearance of the implicated infectious agent occurred long before immunopathology or autoimmunity evolves. Nevertheless, clinical and epidemiologic evidence still supports a link between the development of infections and the onset or the exacerbation of autoimmune diseases such as MS (11,16). Infectious agents and their products are suspected of inducing autoimmune disease development through different immune-mediated mechanisms, including molecular mimicry, production of molecules with super-antigen characteristics, release of hidden self-antigen, up-regulation of presenting and co-stimulatory molecules, epitope spreading and bystander activation of resting encephalitogenic T cells (18–20). As 80 – 90 % of MS patients present with a relapsing-remitting disease course, it is important to further investigate the link between infection and exacerbations (21). Exacerbations can affect the normal daily life of MS patients substantially through the impact of sudden, and above all, unexpected impairment. The unpredictable character of onset and duration of impairment provokes uncertainty. Frequently, recovery is incomplete, resulting in sustained neurological deterioration (22). In addition to the short-term impact of exacerbation activity, an even greater problem for MS patients is the long-term deterioration. Sustained clinical deterioration occurs after irreversible demyelination or loss of axons in a clinically eloquent neuroanatomical area (23). Buljevac et al. could show that exacerbations in the context of systemic infection lead to more sustained damage (22).

The relationship between exacerbations and long-term disease progression is incompletely understood. A high exacerbation rate during the early course is associated with worse prognosis (24), although this correlation seems to be lost in the secondary progressive phase (25).

1.2 Pathogenesis of Multiple Sclerosis

Multiple Sclerosis is characterised by inflammatory and neurodegenerative changes in the CNS. However, the exact mechanisms underlying the disease are still not fully understood. In tissue samples from MS patients disseminated lesions are found in the white and grey matter. Inflammatory infiltrates consisting mostly of T cells, B cells, and macrophages, demyelination, gliosis, antibody and complement deposition as well as axonal/neuronal damage characterise these lesions (23,26,27). The general hypothesis regarding MS is that it is a misguided immune response, initiated by CNS-reactive T cells recognising autoantigens

like e.g. myelin basic protein (MBP), proteolipid protein (PLP), and myelin oligodendrocyte glycoprotein (MOG) (28). Axons as well as neurons were only recently identified as major targets in the CNS. Early axonal pathology can be found in MS patients, correlating with the number of infiltrating immune cells. Evidence from animal experiments indicates that autoreactive myelin-specific CD4+ T helper (Th) 17 and Th1 cells play a major role in the pathogenesis of MS (29).

Generally, autoreactive T cells are present in the healthy immune system without causing any damage. However, in the case of autoimmunity, the mechanisms normally containing an immune response against self are misguided, leading to an immune attack against self-structures.

As mentioned above, it is assumed that MS is triggered by a viral infection (16,30,31). Hereby, the activation of T cells takes place presumably via two mechanisms: molecular mimicry and bystander activation. The molecular mimicry hypothesis suggests that immune responses are raised against pathogenic epitopes with structural similarity to endogenous CNS molecules. The pathogens are cleared from the body but the CNS will be attacked (32–35). In the latter, it is assumed that the activation of autoreactive T cells takes place via non-specific inflammatory processes that occur during the infection (1).

The invasion of the CNS by encephalitogenic T cells through the blood-brain barrier (BBB) is considered an initiatory event of the autoimmune pathology in MS and its animal model, experimental autoimmune encephalomyelitis (EAE), where most knowledge about the pathogenesis is derived from. The CNS was long believed to be both immunologically inert and immunologically separated from the peripheral immune system (36,37). In recent years, this view changed and the CNS is now believed to be both immune competent and actively interacting with the peripheral immune system (38). Activated autoreactive T cells are able to transmigrate the BBB (39). They are activated in the periphery, up-regulate adhesion molecules and chemokine receptors on their surface and are attracted through a chemokine gradient to the BBB (40). The presence of endothelial tight junctions in the intact BBB has long been presumed to prevent leukocyte movement from the blood into the CNS (41,42). However, through interaction of the autoreactive T cells with endothelial adhesion molecules, they are capable to transmigrate the BBB (43). In the perivascular compartment the T cells encounter their target antigen, mostly myelin structures, which is presented to them by CNS-resident antigen presenting cells (APC) like microglia and macrophages. Through this

encounter the T cells are reactivated, begin to secrete pro-inflammatory cytokines and cytotoxic compounds like interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), Fas ligand (CD95L) and TNF-related apoptosis-inducing ligand (TRAIL), and gain access to the CNS parenchyma (44). This leads to the initiation of an immune response directed against myelin, which is accompanied by a breakdown of the BBB and recruitment of additional immune cells (45). More activated T cells but also other immune cells like B cells and monocytes are recruited from the periphery through constant release of chemokines. This leads to an increased self-sustaining inflammatory reaction (46). In the end, impairment of the myelin creating oligodendrocytes, demyelination, and axonal/neuronal damage are induced (47) (Figure 2). B cells contribute to the demyelination by the production of myelin-specific antibodies and free radicals like nitric oxide (NO) (1). NO is also secreted by macrophages, astrocytes and microglia after stimulation with interferon-γ and destroys electrically active axons (48).

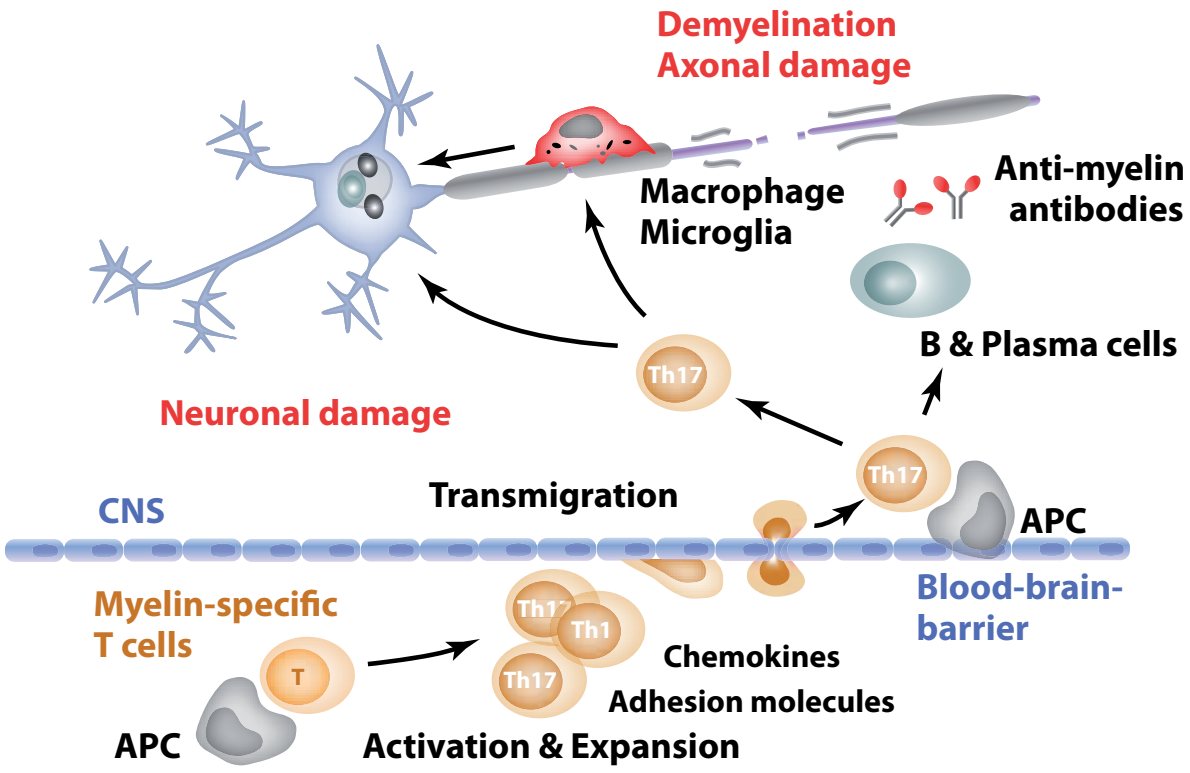


Figure 2. Disease model of multiple sclerosis. Peripherally activated myelin specific T cells transmigrate the blood-brain barrier (BBB) after up-regulation of adhesion molecules. In the central nervous system (CNS), T cells are reactivated by resident antigen presenting cells (APC), secrete pro-inflammatory cytokines, mostly of a Th17 or Th1 subtype, and gain access to the parenchyma. This initiates an immune response directed predominately against the myelin sheath, accompanied by a breakdown of the BBB and recruitment of additional

immune cells. Finally, this leads to inflammatory demyelination, and neuronal and axonal damage. Adapted from Aktas et al. (49).

1.3 The animal model experimental autoimmune encephalomyelitis

Many of the insights gained on MS could not have been made without utilising the animal model of MS, EAE. Studies have shown EAE to be a useful animal model for understanding the mechanisms of immune-mediated CNS pathology of this human autoimmune disease (50).

EAE shares many histopathological features with MS. Also in EAE perivascular infiltration of mononuclear cells in the CNS is observed, as well as demyelination and axonal and neuronal damage, accompanied by the clinical syndrome of paralysis (51). Disease can be induced in genetically susceptible animals by immunisation with myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP), or myelin oligodendrocyte glycoprotein (MOG). The most commonly used animal is the mouse. There are two strategies to induce disease in these animals: active EAE and passive EAE (52). Active EAE requires subcutaneous immunisation with the myelin proteins or peptides in pro-inflammatory adjuvants e.g. complete Freund's adjuvant (CFA) containing mineral oils and inactivated mycobacterium tuberculosis, otherwise tolerance is induced. Generally, active EAE is used to investigate the induction phase of disease. Activation and priming of T cells during disease can be investigated. The priming phase is followed by an appearance of CNS-specific T helper (Th) cells, mainly of the Th1 and Th17 subtype, in the secondary lymphoid organs and with onset of clinical signs also in the brain (53). For passive or adoptive transfer EAE encephalitogenic myelin-specific T cells are derived from immunised animals or transgenic animals with T cell receptors specific for CNS antigens, expanded *in vitro*, and then transferred to naïve recipients (50,54). Passive EAE is useful to study the effector phase of the disease without the influence of immunisation outside the CNS. It is possible to manipulate the T cells *in vitro* prior to transfer, which allows studying the influence of different subtypes of T cells involved in the disease. Passive EAE can also be used to look at the migration of T cells into the CNS more closely.

The most commonly used mouse strains, of which one was also utilised in this thesis, are C57BL/6 and SJL mice. Chronic neuroinflammation can be investigated in the C57BL/6 mouse strain as shown in this thesis, in which immunisation with MOG or its

immunodominant epitope $MOG_{35–55}$ induces a severe attack followed by incomplete recovery and a secondary-progressive stage of silent deterioration, as found in the later stages of MS (55). The inflammation in this case is mostly concentrated in the spinal cord and brain stem. The transfer of encephalitogenic MOG-specific CD4+ T cells into C57BL/6 mice to induce EAE has so far shown little success as only a small number of mice present with the disease after adoptive transfer.

In SJL mice, the animals present with a relapsing-remitting disease course upon immunisation with PLP or its immunodominant epitope $PLP_{139-151}$ or after adoptive transfer of encephalitogenic CD4+ T cells recognising $PLP_{139-151}$. This model is thought to be the closest model of the situation in human RRMS (56).

Additionally, in this thesis passive EAE with MOG_{35-55}-specific CD4+ T cells was also performed in Rag knock-out ($Rag1^{-/-}$) mice. $Rag1^{-/-}$ mice have a C57BL/6 background, but do not possess any T or B cells by themselves (57). This approach allows to effectively study the effect of the transferred T cells, as no other intervening cells are present, and leads to a severe monophasic mostly lethal disease. The most prominent clinical signs in all the described EAE mouse models is an ascending paralysis, which first affects the tail, then the hind limbs and in later stages also the forelimbs. Typically, mice are assigned a disease score according to the severity of the clinical signs. There is a strong correlation of T cell numbers with EAE clinical scores. The T cells disappear from the brain with complete resolution of clinical signs (58).

For many years, EAE was believed to be a Th1-induced autoimmune disease because of the increased expression of Th1 cytokines in the affected CNS and because injection of myelin-specific CD4+ Th1 but not Th2 cells into immunocompetent mice was sufficient to induce EAE (59,60). More recently, IL-17-producing T helper (Th17) cells have been implicated in the pathogenesis of EAE (61–64). Th17 cells have been shown to contribute to several autoimmune diseases by the highly pro-inflammatory cytokines IL-17A (IL-17), IL-17F, tumour necrosis factor (TNF), IL-6, GM-CSF and IL-22 (65–68). Furthermore, CNS-specific Th17 cells seem to possess a much higher potential to induce EAE (63).

1.4 Different T cell subsets and their role in MS and EAE

There is no doubt that T cells are critical for the pathogenesis of MS and EAE. However, the importance of different T cell subsets is still highly controversial.

The commonly received opinion is that CD4+ T helper (Th) cells are the essential mediators of the disease although there is conflicting evidence about the numbers of CD4+ and CD8+ T cells and their ratio in the histopathology of active MS lesions (26,69,70). Nevertheless, findings in EAE support this assumption. In EAE, transfer of myelin-specific CD4+ T cells into naïve recipient animals is sufficient to induce the disease (71,72). In MS, there is a strong correlation of MS susceptibility with certain alleles of the MHC class II (strongest for the allele HLA-DRB1*1501 of the HLA-DRB1 gene, encoding the MHC-II molecule HLA-DR2), which presents antigen to CD4+ T cells (73). Nevertheless, there are also data supporting an important role for cytotoxic CD8+ T cells in the damaging cascade of chronic neuroinflammation (74).

Not only do T cells present with an effector role in chronic neuroinflammation, but also a regulatory role for T cells is described. The different subtypes of T cells, which are important for the understanding of the mechanisms in MS and EAE, will be described in more detail in the following chapters.

1.4.1 CD4+ T cells

The role of CD4+ T cells is to assist in immunological processes. These processes include the maturation of B cells and the activation of cytotoxic T cells and macrophages. They also have to identify dangers for the integrity of the organism and trigger an effective and tightly regulated immune response by activating the afore-mentioned other immune cells. They themselves become activated when presented with antigens by MHC class II molecules by APC. Furthermore, they provide immune memory for future more rapid immune reactions against a similar threat.

The differentiation of naïve CD4+ T cells, which have not yet encountered their antigen, into effector T helper cells is initiated by engagement of their T cell receptor (TCR) (signal 1) and co-stimulatory molecules (signal 2) in the presence of specific cytokines produced by the innate immune system. The subtype of effector Th cell into which a naïve CD4+ T cell develops is determined by the immunological surrounding at the time point of activation. Upon encounter of intracellular pathogens, IFN-γ and IL-12 initiate the differentiation of Th1 cells that are characterised by high production of IFN-γ and are indispensable for clearing these pathogens. In contrast, IL-4 triggers the differentiation of Th2 cells. Th2 cells are known for organising host defence against extracellular pathogens. The initial source of the

differentiation factors for both Th1 and Th2 cells are cells of the innate immune system responding to microbial antigens, parasitic antigens, or allergens (68). A specific effector Th cell subtype can be generated by activation of naïve CD4+ T cells with antigen in the presence of differentiation-committing cytokines *in vitro*. An important molecular marker to distinguish Th cell subtypes is a subtype-specific transcription factor. For a long time only Th1 and Th2 cells were distinguished. More recently, several novel CD4+ T cell subsets were described including Th17, Th9 and Treg cells (Figure 3).

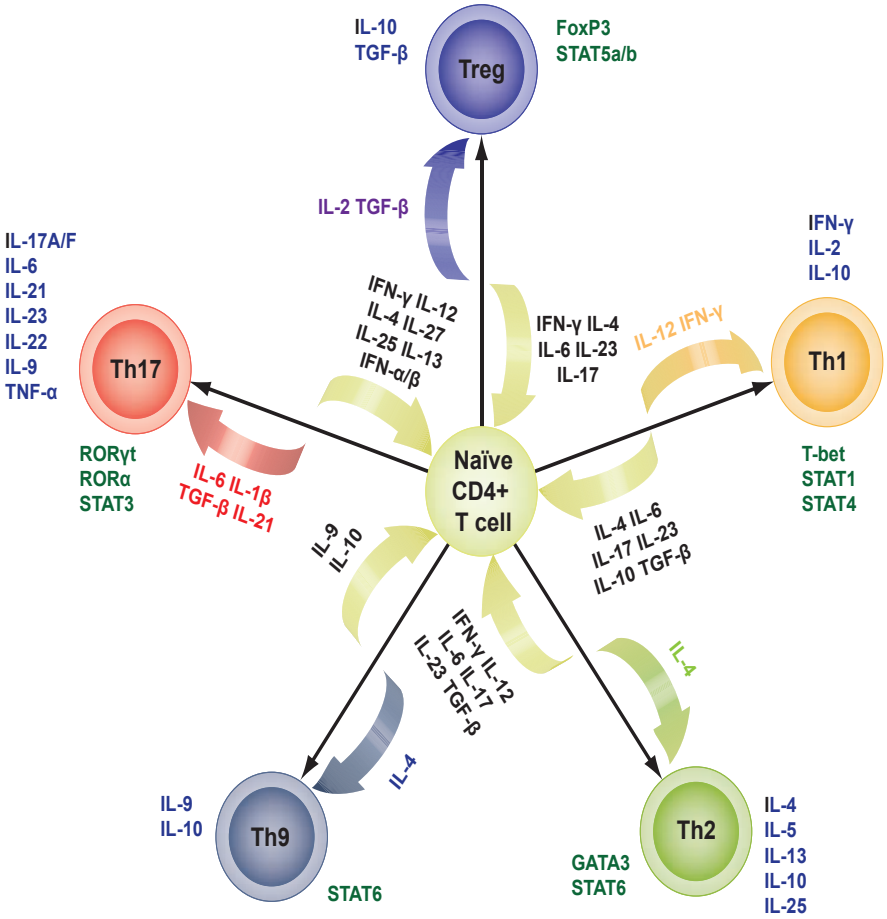


Figure 3. Differentiation of naïve CD4+ T cell precursors to various CD4+ T cell lineages is controlled through several regulatory factors such as, specific transcription factors (green) and stimulatory (red, yellow, green, purple) and inhibitory (black) cytokines. Adapted from Jadidi-Niaragh and Mirshafiey (75).

1.4.1.1 Th1

Until recently, the primary effector T cell in the pathology of both EAE and MS was thought to be a CD4+ Th1 cell. The CD4+ Th1 cell requires IL-12 for its differentiation and is characterised by the secretion of IFN-γ, IL-2 and TNF-α (76). This view was supported not only by the finding that increased clinical activity in MS correlated with the expression of IFN-γ and IL-12 in the CNS and CSF of MS patients, but also by the observation that MS was exacerbated by the administration of IFN-γ (15,77). In EAE, a key role for Th1 cells was supported by the secretion of IFN-γ by CNS-infiltrating T cells and the detection of IL-12p40, a subunit of IL-12, in inflammatory lesions (78). The ability to induce disease by adoptive transfer of Th1 cells also contributed to the common view that MS as well as EAE are Th1-mediated diseases (79). IFN-γ induces MHC class II expression in the CNS, triggers the production of chemokines that attract macrophages and monocytes and activates macrophage function, which is consistent with the idea that Th1 cell-mediated responses establish pro-inflammatory responses in the CNS. However, IFN-γ knock-out mice or signal transducer and activator of transcription-1 (STAT1) knock-out mice, next to T-bet the most important transcription factor of Th1 cells, that are lacking Th1 cells were found to develop more severe EAE (80,81). The discovery of IL-23 led to partial resolution of these paradoxes. IL-23 is structurally related to IL-12. IL-23 shares the p40 subunit with IL-12, which is associated with either a separate p19 or p35 subunit for IL-23 and IL-12, respectively. IL-23p19 knock-out and IL-12p40 knock-out mice were found to be resistant to EAE, whereas IL-12p35 knock-out mice were susceptible (61). IL-23 was shown to be necessary to drive the induction or expansion of a novel distinct subset of CD4+ T cells that secretes IL-17, termed Th17 cells (62).

1.4.1.2 Th17

Th17 cells are characterised by the production of IL-17A (also called IL-17), IL-17F, GM-CSF and IL-22 and are thought to clear extracellular pathogens not effectively handled by either Th1 or Th2 cells. They are also characterised by their expression of the key transcription factor, orphan nuclear receptor RORγt, which was identified to initiate the differentiation of the Th17 lineage. In RORγt-deficient mice, the clinical symptoms of EAE were delayed and mild, but the mice presented with extensive inflammatory infiltrates in the spinal cord expressing high amounts of IFN-γ (66). Because Th17 cells produce large

quantities of IL-17A, most of the Th17-mediated effects are attributed to this cytokine (82). IL-17A is the prototypic cytokine of the IL-17 family, which includes six members: IL-17A, B, C, D, E and F (83). In addition to IL-17A, Th17 cells co-produce IL-17F (62,84). IL-17A and IL-17F have similar functions among others the induction of pro-inflammatory cytokines.

A pathogenic role for Th17 cells is implicated in both animals and humans. They contribute to various autoimmune diseases by the production of the highly pro-inflammatory cytokines IL-17, IL-22, GM-CSF and TNF (65,84). The transfer of myelin-specific IL-17-producing T cells expanded *in vitro* induces severe EAE (62) but conversely IL-17 knock-out mice develop attenuated EAE (63) showing that the role of IL-17A in EAE is still highly controversial, with findings ranging from its complete dispensability to its necessity (60,85). Increased levels of IL-17 have been observed in patients with rheumatoid arthritis and multiple sclerosis (86,87). IL-17 and IL-22 were further shown to disrupt tight junctions between endothelial cells of the blood-brain barrier (88).

A combination of the immunoregulatory cytokine TGF-β and the pro-inflammatory and pleiotropic cytokine IL-6 is required for the differentiation of naïve T cells to Th17 cells (89). In addition, IL-21, an IL-2 family member, was also discovered as an inducer of IL-17 in naïve T cells. As Th17 cells are a major source of IL-21, an autocrine amplification loop was proposed by Korn et al. (90). Although IL-23 promotes the production of IL-17 by activated T cells, it is not involved in the initial differentiation of Th17 cells (91). It rather seems to be important for sustaining the differentiation status (92).

1.4.1.3 Th2

The differentiation of naïve T cells into Th2 cells is driven by the cytokine IL-4, which leads to activation of the transcription factor GATA3 and thereby to the production and secretion of the Th2 cytokines IL-4, IL-5, IL-10, and IL-13. The Th2 cytokines exhibit anti-inflammatory properties especially in parasitic infections. Th2 cytokines inhibit Th1 as well as Th17 differentiation and expansion (93). Th2 cells are involved in eosinophilic inflammation and IgE production in allergic reactions and asthma. In the field of EAE it was shown, that myelin-specific Th2 clones are not able to transfer EAE to recipient mice (78). In contrast, induction of a Th2 response or administration of Th2 cytokines during an ongoing immune

response can be of therapeutic value in EAE, which might be due to the potential of Th2 cells to cross-regulate the generation of other pro-inflammatory Th subtypes (93).

1.4.1.4 Th9

IL-9 was first described as a Th2-associated cytokine but it has recently become apparent that its regulation and biology are unlike that of the other Th2 cytokines (94). *In vitro*, IL-9-secreting T cells seem to be a distinct subset from the Th2 cells. They are dependent on TGF-β signalling and have been recently recognised as an independent T helper cell subset, termed Th9 (94). TGF-β can reprogram Th2 cells to lose their characteristic profile and switch to IL-9 secretion or, in combination with IL-4, can drive the differentiation of Th9 cells directly (94,95). Driven by the combined effect of TGF-β and IL-4, Th9 cells produce large amounts of IL-9 and IL-10. IL-9 together with TGF-β can also contribute to Th17 cell differentiation and Th17 themselves can produce IL-9 (96). IL-25, a member of the IL-17 cytokine family, has been identified to play an important role in the regulation of Th9 cells (97). Th9 cells do not express any well-defined transcription factors like T-bet, GATA3, RORγt, or Foxp3, emphasising that Th9 cells are different from Th1, Th17, and Treg populations. Thus, the existence of a specific transcription factor that is uniquely expressed by Th9 cells remains to be discovered (98).

Th9 cells are capable of inducing tissue inflammation in a colitis model and have been shown to induce EAE after adoptive transfer into C57BL/6 recipients with similar severity but distinct pathological phenotype compared to Th1 and Th17 cells (95,99).

1.4.1.5 Treg

Besides the differentiation into effector subsets, CD4+ T cells can also differentiate into distinct regulatory subsets characterised by their ability to suppress adaptive T cell responses and prevent autoimmunity (100). Regulatory T cells (Treg) stably express the high-affinity component of the IL-2 receptor, CD25. Several studies showed that the forkhead-winged helix transcription factor forkhead box P3 (FoxP3) is uniquely expressed by Tregs and is required for their development (101–104). There are two classes of Treg. One class of regulatory T cells, nTregs, develops intrathymically. Evidence indicates that other Tregs develop from naïve CD4+ T cell precursors in the periphery. They are so called induced Tregs (iTregs) (105). At least two types of iTregs have been described. One, called Tr1, develops under control of IL-10-conditioned DCs and is marked by high amounts of

IL-10 production but does not express the transcription factor FoxP3 (106,107). Another is induced from naïve precursors under the influence of TGF-β. These cells are FoxP3+ and display suppressive activities that are indistinguishable from nTregs, although they develop extrathymically (108). The importance of antigen-specific Treg in conferring genetic resistance to organ-specific autoimmunity (109) and in limiting autoimmune tissue damage has also been documented in the disease model of multiple sclerosis (110). In humans, a decreased frequency of occurrence of Treg or defects in their suppressor function has been demonstrated in MS patients (108).

In myelin-specific CD4+ TCR transgenic mouse models FoxP3+ regulatory T cells prevent spontaneous EAE by suppressing the activation of myelin-specific CD4+ T cells in the periphery (76,109). Nevertheless, the function of Treg in preventing inflammation within the CNS is still highly controversial. Although a correlation between the presence of IL-10-producing FoxP3+ Treg cells in the CNS and disease recovery was reported (110), Tregs seem ineffective in suppressing effector T cells in the CNS until the local levels of IL-6 and TNF decrease (108).

1.4.1.6 Non-CNS-specific T cells

T cells are critical for the pathogenesis of MS and its animal model EAE. The invasion of encephalitogenic T cells through the BBB is considered as the initiatory event of the autoimmune pathology. However, antigen-specificity seems not to be essential for the transmigration step as non-CNS-specific T cells are also capable of entering the CNS without promoting any CNS pathology. Nevertheless, the role of non-CNS-specific T cells in the cellular neuropathology is less understood. Data suggests that activated ovalbumin (OVA)-specific T cells are capable of entering the CNS after altering the BBB (111,112). They are also able to damage brain vascular endothelial cells *in vitro* (113). In contrast, Archambault et al. showed that non-CNS-specific T cells are recruited to the CNS but fail to cross the vascular endothelium (114). These controversial findings indicate that the role of non-CNS-specific T cells needs to be investigated in more detail in chronic neuroinflammation.

1.4.2 CD8+ T cells

MS has been viewed historically as a CD4+ T cell-mediated autoimmune disease, due in part to the genetic association of MS with MHC class II alleles. However, an association with

MHC class I alleles has also been reported (115). Furthermore, the frequency of CD8+ T cells is greater than that of CD4+ T cells in inflamed plaques, and CD8+ T cells show oligoclonal expansion in plaques, CSF and blood of MS patients, which suggests a pathogenic role for CD8+ T cells in MS (116). A few studies involving cell transfer have suggested that CD8+ T cells are pathogenic in EAE (117). However, there is also evidence of a regulatory role for CD8+ T cells in EAE. EAE is more severe in mice deficient in or depleted of CD8+ T cells and disease severity has been correlated inversely with the frequency of CD8+ T cells (117). A recent study demonstrated that CD8+ regulatory T cells suppressed EAE via a TGF-β-dependent mechanism (118).

1.5 Plasticity of T cells

Differentiation of CD4+ T cells into functionally distinct helper T cell subsets is crucial for efficient host defense and normal immunoregulation (42,119). These subsets are specified by extrinsic and intrinsic cues, and the resultant cell populations acquire seemingly stable phenotypes, which are reinforced by epigenetic modifications (120,121). Consequently, these subsets have been viewed as lineages, defined by expression of selective signature cytokines and transcription factors (122). Originally, CD4+ T cells were grouped into Th1 cells, which express T-bet and selectively produce IFN-γ and Th2 cells, which express Gata3 and produce IL-4 (42,119). Regulatory T (Treg) cells are another CD4+ lineage with essential immunosuppressive functions that express the master transcription factor FoxP3 (123). Comprising both thymic derived natural, nTreg cells and peripherally induced iTreg cells, the identification of Treg cells was a key discovery in refining our understanding of mechanisms of autoimmunity. The recognition of cells that selectively produce IL-17 and the transcription factor RORγt (Th17 cells) led to refined views of the genesis of autoimmune disease (129,130). Newer fates for helper T cells continue to be identified, with nomenclature based on production of their signature cytokines: Th9 and Th22 cells (126). Although CD4+ T cell subsets have elements of stability and have been referred to as distinct lineages, there are increasing evidences pointing to substantial phenotypic flexibility of the ‘newer’ helper T cells and indeed, previously identified subsets also appear to be more plastic than originally recognised (121,126,127).

T helper cells can change their phenotype (Figure 4). Although IL-17-secreting helper T cells were initially suggested to represent a new lineage because they do not produce the other lineage-defining cytokines, IFN-γ and IL-4, it is now appreciated that Th17 cells often

become IFN-γ producers (128,129). Treg cells have also been reported to lose their FoxP3 expression and acquire the capacity to produce pro-inflammatory cytokines (130,131). Perhaps the most dramatic example is that Gata3+Th2 cells can be reprogrammed to express T-bet and IFN-γ in the setting of viral infection. Interestingly, also type I interferons are important drivers of reprogramming (132).

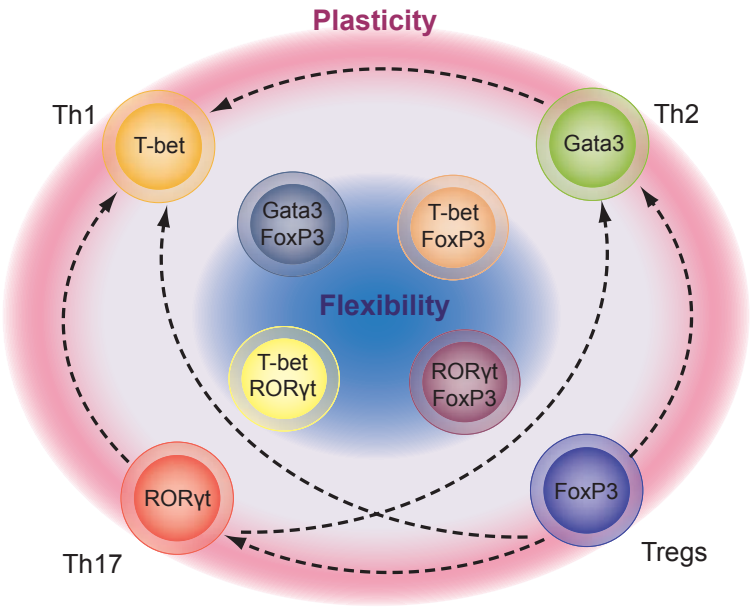


Figure 4: Flexibility and plasticity of T helper cells. Flexibility in expression of signature transcription factors, signature cytokines and gene expression for all T helper subsets has been described. Adapted from Nakayamada et al (133).

1.6 Aim of this work

In the pathogenesis of MS and its animal model EAE T cells play an important role. The invasion of encephalitogenic T cells through the BBB is considered as the initiatory event of the autoimmune pathology. However, antigen-specificity seems not to be essential for the transmigration step as non-CNS-specific T cells are also capable of entering the CNS without promoting any CNS pathology. It has recently been shown that Th17 cells are critically involved in the initiation of EAE. However, it is not completely clear yet, if the Th17 cell, although shown to be very pathogenic, is the only prerequisite for the induction of the disease.

The overall aim of this thesis was to investigate the role of Th17 cells - CNS-antigen-specific or CNS-antigen-non-specific - in chronic neuroinflammation in order to better understand the processes that lead to neuropathology.

To address this aim the thesis is divided into two parts:

1.6.1 T cell differentiation requirements for stable induction of Experimental Autoimmune Encephalomyelitis

It is controversially debated if EAE and MS are rather Th1 or Th17 mediated diseases (134–136). There is strong evidence that IL-23, which is strongly associated with the Th17 phenotype, is the crucial factor for the development of EAE (66). If IL-23 (p19) is knocked out in mice, they are resistant to the induction of EAE, whereas IL-12 knock out animals are not (61). The differentiation towards the Th17 phenotype *in vitro* is dependent on the presence of the pro-inflammatory cytokines IL-6 and IL-23 in the context of TGF-β, whereas their induction *in vivo* seems to rely on the presence of Toll-like receptor (TLR) ligands (65,66). Furthermore, our group could show an up-regulation of Bdkrb1, bradykinin, des-Arg^9-bradykinin, kallikrein-1 and kallikrein-6 as well as low-molecular-weight kininogens (KNGL) in CNS tissue and the cerebrospinal fluid (137). It was shown that the Bdkrb1 agonist R838 (Sar-[D-Phe]des-Arg^9-bradykinin) markedly decreases the clinical symptoms of experimental autoimmune encephalomyelitis (EAE) in SJL mice (138–140), whereas the Bdkrb1 antagonist R715 (Ac-Lys-[D-βNal^7,Ile^8]des-Arg^9-bradykinin) resulted in earlier onset and greater severity of the disease. In line with these rather beneficial findings in the SJL model, the role of Bdkrb1 was investigated more closely in chronic neuroinflammation.

The following questions were addressed:

- What are the requirements for stable encephalitogenic MOG-specific CD4+ Th17 cells?
- How can the *in vitro* generated encephalitogenic MOG-specific CD4+ Th17 cells be applied in vivo?
- What are the differences between MOG-specific CD4+ Th17 and MOG-specific CD4+ Th1 cells in chronic neuroinflammation?
- What are the mechanisms underlying these differences?

1.6.2 The role of non-central nervous system-specific Th17 cells in experimental autoimmune encephalomyelitis

In MS, the clinical disease course is highly variable (11). Typically MS patients present with a relapsing pattern of acute exacerbations followed by periods of stability. Exacerbations can be triggered by exogenous events, e.g. viral or bacterial infections (11,15,19). Bystander activation of resting encephalitogenic T cells or molecular mimicry in response to viral infection might be a possible pathways connecting infection to autoimmunity (11,20). In MS and its animal model, EAE, the transmigration of encephalitogenic T cells through the BBB is considered an initiatory event of the autoimmune pathology. The role of non-CNS-specific T cells in the cellular neuropathology is less understood. Data suggests that activated OVA-specific T cells are capable of entering the CNS after altering the BBB (111,112). Presumably, in a compromised brain, such as in MS and EAE, bystander perturbations of the BBB caused by non-CNS-specific effector cells may have pathological consequences and contribute to disease aggravation as can be seen in patients suffering from an ordinary infection. This hypothesis was investigated in more detail in this part of the dissertation.

Hence, the following questions were addressed in this part of the thesis:

- Are OVA-specific CD4+ Th17 cells able to induce disease exacerbations in a chronic model of EAE?
- Do OVA-specific CD4+ Th17 cells promote pathology in healthy animals?
- Do OVA-specific CD4+ Th17 cross react with myelin antigens?
- What behaviour do OVA-specific CD4+ Th17 show in the CNS of EAE affected mice?

2 Materials and Methods

2.1 Lab supplies

2.1.1 Buffers, solutions, cell culture media

Phosphate buffered saline (PBS)	PAA Laboratories, Austria
FACS buffer	PBS 0.5 % bovine serum albumin (BSA), Serva, Germany
MACS buffer	PBS 0.5 % BSA 2 mM EDTA, Sigma-Aldrich,Germany
Saponin buffer	PBS 0.5 % BSA 0.5 % Saponin, Roth, Germany
Mouse cell culture medium (MM)	RPMI 1640, Gibco Invitrogen, Germany 1 % 1 M Hepes, Gibco Invitrogen, Germany 10 % fetal calf serum (FCS), Biochrom, Germany 100 µg/ml streptomycin, Gibco Invitrogen, Germany 100 U/ml penicillin, Gibco Invitrogen, Germany 2 mM L-glutamin, Gibco Invitrogen, Germany

Mouse washing medium (WM)	RPMI 1640 1 % 1 M Hepes 5 % FCS 100 μg/ml streptomycin 100 U/ml penicillin
Freezing medium	FCS 10 % DMSO, Sigma, Germany
Iscove's Mod Dulbecco's Medium (IMDM)	Gibco Invitrogen, Germany
Lysis buffer	Distilled water 10 mM $KHCO_3$, Merck, Germany 0.15 M NH_4Cl, Roth, Germany 0.1 M $Na_2EDTA*2H_20$, Sigma, Germany
Paraformaldehyde (PFA) solution	0.1 M PBS, Gibco, Germany 4 % PFA, Roth, Germany

2.1.2 Reagents and chemicals

Agarose	Serva, Germany
Brefeldin A	Sigma-Aldrich, Germany
CellTracker™ Orange CMTMR	Invitrogen, Germany
Collagenase	Sigma, Germany
Collagenase-Dispase	Roche, Germany
Complete Freund`s adjuvant (CFA)	BD Difco, Germany
Concanavalin A (ConA)	Sigma, Germany
DNase	Roche, Germany
Ethanol	Merck, Germany
FITC-dextran	Sigma, Germany

Gd-DTPA (Magnevist®)	Bayer Schering, Germany
Glucose	Braun, Melsungen
H37RA	Difco, Germany
^{3}H-thymidine	Amersham, Germany
Isoflurane	Abbot, Germany
Isotonic Ringer solution	Braun, Germany
Ketamin	Curamed, Germany
MBP_{85-99} (EKPKYEAYKAAAAPA)	Pepceuticals, UK
MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK)	Pepceuticals, UK
Mounting Medium "Neo-Mount"	VWR Int. GmbH, Germany
NaCl-solution (0.9 %)	Braun, Germany
$OVA_{323-339}$ (ISQAVHAAHAEINEAGR)	Pepceuticals, UK
Percoll	Sigma, Germany
Pertussis toxin (PTx)	List Biologicals, USA
$PLP_{139-151}$ (HSLGKWLGHPDKF)	Pepceuticals, UK
Propidium iodide (PI)	Sigma, Germany
Object slides Superfrost Plus	Menzel GmbH & Co KG, Germany
Tissue-Tek ® Compound	Sakura, USA
Trypan blue (0.4 %)	Biochrom, Germany
Saccharose	Merck, Darmstadt
Xylazinhydrochloride (2 %)	Bayer Health Care, Germany
Xylol "Neo-Clear"	VWR Int. GmbH, Germany

2.1.3 Consumables

Cannulas, syringes	Braun, Germany
Cell strainers (100 μm pore size)	BD Biosciences, Germany
Cryo tubes	Nunc, Germany
EDTA tubes	Greiner bio-one, Germany
Eppendorf tubes	Eppendorf, Germany
FACS tubes	BD Biosciences, Germany

Falcon tubes	BD Biosciences, Germany
MACS LS columns	Miltenyi Biotec, Germany
MACS Pre-separation filter	Miltenyi Biotec, Germany
Petri dishes	BD Biosciences, Germany
Pipettes	Eppendorf, Germany
Pipette tips	VWR, Germany
Scalpels	Braun, Germany
6-, 12-, 24-, 48-, 96-well-plates	BD Biosciences, Germany

2.1.4 Instruments

7 Tesla rodent scanner (Pharmascan 70/16AS)	Bruker, Germany
β-scintillation counter (Wallac MicroBeta)	Perkin Elmer, Germany
Cryostat "CM1900"	Leica Microsystems GmbH, Germany
Eppendorf Centrifuge 5416 and 5417R	Eppendorf, Germany
Flow Cytometer (FACSCanto II)	BD Biosciences, Germany
Harvard Apparatus Advanced Safety Respirator	Hugo Sachs, Germany
Incubators	Binder, Germany
Laminar flow hood	Heraeus Kendro, Germany
Light microscope	Leica, Germany
MACS separator	Miltenyi Biotec, Germany
MACS rotator	Miltenyi Biotec, Germany
Magnetic stirrer	Eppendorf, Germany
Megafuge 1.OR	Heraeus Kendro, Germany
Microscapnograph CI-240	Columbus Instruments, USA
Micropipettes	Eppendorf, Germany
Neubauer-hemocytometer	Brand, Germany
Optical parametric oscillator (OPO)	APE, Germany
Photomultiplier tubes H7422-40	Hamamatsu, Japan
Pipette aid	Hirschmann, Germany
Precision scales	Mettler, Germany
Scan head (TriMScope)	LaVision Biotec, Germany

Surgical Instruments	Aesculap, Germany
Ti:Sa Laser Mai Tai HP	Spectra Physics, USA
Two-photon laser scanning microscope (whole setup)	LaVision Biotec, Germany
Upright microscope BX-51WI (for TPLSM)	Olympus, Germany
Water bath	Medingen, Germany
Vibratome (NVSLM1)	Motorized Advance Vibroslice
Vortexer	Scientific Industries, UK

2.1.5 FACS antibodies

Table 1: Anti-mouse surface antibodies

Name	**Clone**	**Company**
Surface antibodies		
α-m CD3 APC	145-2C11	BD Biosciences, Germany
α-m CD4 biotin	RM4-5	Caltag, Invitrogen, Germany
α-m CD4 FITC	RM4-5	BD Biosciences, Germany
α-m CD4 AlexaFluor 647	RM4-5	Invitrogen, Germany
α-m CD45 PerCP	30-F11	BD Biosciences, Germany
α-m CD8 PE	53-6.7	BD Biosciences, Germany
α-m CD62L APC	MEL-14	BD Biosciences, Germany
α-m Vβ11 TCR biotin	RR3-15	BD Biosciences, Germany
α-m Vα2 TCR biotin	B20.1	Invitrogen, Germany
Intracellular antibodies		
α-m FoxP3 PE/APC	FJK-16s	eBioscience, USA
α-m IFN-γ PE	XMG1.2	BD Biosciences, Germany
α-m IFN-γ V450	XMG1.2	BD Biosciences, Germany
α-m IL-10 APC	JES5-16E3	BD Biosciences, Germany
α-m IL-17 PE	TC11-18H10	BD Biosciences, Germany
α-m IL-17 APC	17B7	eBioscience, USA

Table 2: Secondary antibodies and isotype controls

Name	Clone	Company
rat α-m IgG2a PE		eBioscience , USA
α-m Fcγ III/II (CD16/CD32)	2.4G2	BD Biosciences, Germany
SA-PerCP		BD Biosciences, Germany
SA-PacificBlue		Invitrogen, Germany

2.1.6 MACS MicroBeads and Kits

Table 3: MACS MicroBeads and Kits

Name	Company
α-m CD4+ T cell Isolation Kit	Miltenyi Biotech, Germany
α-m CD62L MicroBeads	Miltenyi Biotech, Germany
α-m CD90.2 MicroBeads	Miltenyi Biotech, Germany
Mouse IL-17 Secretion Assay	Miltenyi Biotech, Germany

2.1.7 Cytokines and blocking antibodies

Table 4: Cytokines and blocking antibodies

Name	Company
rh IL-2	Chiron Therapeutics, USA
rh TGF-β	R&D Systems, Germany
rm IL-2	R&D Systems, Germany
rm IL-6	R&D Systems, Germany
rm IL-12	R&D Systems, Germany
rm IL-18	R&D Systems, Germany
rm IL-23	R&D Systems, Germany
Purified α-m CD3	BD Biosciences, Germany
Purified α-m CD28	BD Biosciences, Germany
Purified α-m IL-4 (Clone 11B11)	DRFZ, Germany

2.1.8 Software

Adobe Illustrator CS4	Adobe Systems Inc., USA
FACSDiva	BD Biosciences, Germany
FlowJo	Tree Star Inc., USA
GraphPad Prism 5	GraphPad Software, USA
Imaris	Bitplane, Switzerland
Imspector	LaVision Biotec, Germany
Mipav	Center for Information Technology, USA

2.2 General cell biological methods

2.2.1 Cell culture

Cells were cultured and handled under a laminar flow hood under sterile conditions. Murine cells were cultured in mouse cell culture medium (MM) in incubators at 37 °C in a 5 % CO_2 atmosphere and 95 % humidity. All material for cell culturing was sterilised or disinfected with 70 % alcohol before use. Waste was autoclaved at 121 °C for 20 min at 1 bar.

2.2.2 Cell counting

In order to determine cell numbers, cells had to be taken up in a defined volume. An aliquot of the cell suspension was mixed with trypan blue in a ratio of 1:1, 1:5, or 1:10. The mixture was applied to a Neubauer hemocytometer and the cells in the 16 fields of one quadrant (n) were counted under a light microscope. Due to the uptake of trypan blue, dead cells could be excluded. They can be distinguished from viable cells by their blue appearance under the light microscope. The total number of living cells was calculated as follows:

Total cell number = n x dilution factor (trypan blue) x ml cell suspension x 10^4

2.2.3 Cell isolation

2.2.3.1 Murine spleen and lymph node cells

For isolation of spleen and/or lymph node cells, mice were sacrificed by cervical dislocation. The fur was disinfected with 70 % EtOH prior to opening the animal and removing the organs. Spleen and lymph nodes (usually inguinal, brachial, and axillary) were removed and transferred either separately (for lymphocyte isolation) or together (for magnetic sorting) to 15 ml falcon tubes on ice containing 5 ml mouse washing medium (WM). Next, the organs were meshed through a cell strainer in a petri dish to generate a single cell suspension. This and all the following steps were performed under the laminar flow hood. The cell strainer and the petri dish were rinsed with WM and the single cell suspension was transferred to a 50 ml falcon tube. The single cell suspension was centrifuged at 550 g for 5 min at 4 °C. The lymph node cells were directly taken up in WM for counting. The spleen cells alone or when mixed with lymph node cells were lysed in order to remove the erythrocytes. For this, the spleen cells or the spleen and lymph node cell mixture were taken up in 10 ml lysis buffer. Afterwards 5 ml WM were added immediately to stop the lysis and the cell suspension was centrifuged at 550 g for 5 min at 4 °C. The spleen cells were taken up in WM, centrifuged again at 550 g for 5 min at 4 °C and resuspended in WM or MACS buffer for counting.

2.2.3.2 Lymphocytes from mouse CNS

For isolation of lymphocytes from the central nervous system, mice were lethally anaesthetised with an intraperitoneal injection of a ketamin/xylazine-mixture (415 mg/kg / 9.7 mg/kg). For transcardial perfusion the animal was disinfected with 70 % EtOH and the fur was cut open. Next, the sternum was removed to expose the heart. The right atrium was opened, a 20-gauge needle was inserted into the left ventricle, and mice were transcardially perfused with 25 ml ice-cold PBS. Brain and spinal cord were removed and placed in a 15 ml Falcon tube on ice containing 5 ml IMDM. All the following steps were performed under a laminar flow hood. First, the brain and spinal cord were transferred along with the 5 ml IMDM into a petri dish. The CNS tissue was cut into small pieces using a scalpel and retransferred along with the 5 ml IMDM into the 15 ml Falcon tube. The IMDM medium was then supplemented with 360 U/ml collagenase, 200 U/ml DNAse, and 5 µg/ml collagenase/dispase. After incubation for 30 min at 37 °C under continuous rotation on a MACS-rotator, the CNS tissue was put through a cell strainer and washed with cold IMDM

medium. Next, the gradient for the isolation of the lymphocytes was prepared. Therefore, the pellet was resuspended in 5 ml 40 % percoll-solution (percoll diluted in IMDM) and carefully layered on top of 5 ml 70 % percoll-solution (percoll diluted in PBS) in a 15 ml falcon tube. After centrifugation for 30 min at 750 g at room temperature without break, mononuclear cells were collected from the interphase of the gradient, washed with WM, and counted.

2.2.3.3 Lymphocytes from mouse blood

For detection of lymphocytes in the blood of a mouse, the blood from the tail vein was collected in blood collection tubes containing EDTA. The blood was transferred to 2 ml eppendorf tubes and erythrocytes were lysed with 1 ml lysis buffer. After centrifugation for 5 min at 550 g, cells were washed once with FACS buffer for the subsequent FACS staining of the lymphocytes.

2.2.4 ^{3}H-thymidine proliferation assay

To measure the level of T cell response to different antigens (whole brain homogenate, $OVA_{323-329}$, MOG_{35-55}, $PLP_{139-151-}$ and MBP_{85-99}), OVA-specific T cells were stimulated with those antigens or with ConA (2µg/ml) and cultured for three days in 96-well round-bottom plates, followed by incubation for 18 h with ^{3}H-thymidine at a final concentration of 100 µCi/ml. ^{3}H-thymidine incorporation was measured in a β-scintillation counter. Results (means of triplicate cultures) were expressed as counts per minute (cpm).

2.3 Immunological methods

2.3.1 Flow cytometry

Flow cytometry (FACS = fluorescence activated cell sorting) is a technique for characterising and examining individual immune cell populations.

When using flow cytometry, two aspects can be utilised. Firstly, the natural scattering of light by the cell structures and secondly, the fluorescence of molecules bound to specific antibodies that can bind to the cells. Therefore, it is possible to analyse morphological characteristics, such as cell size and granularity, but also the expression of specific molecules on the cell surface or within the cells. For this purpose either monoclonal antibodies that are coupled with fluorochromes can be used or biotinylated monoclonal antibodies. With the directly coupled antibodies it is possible to mark individual cell populations by direct binding of these

antibodies to specific molecules. When utilising the biotinylated monoclonal antibodies, they have to be combined with a secondary fluorescently labelled streptavidin antibody that binds to the primary biotinylated antibody. When measured at a flow cytometer (e.g. FACSCanto II), the cells pass several laser beams with different wavelengths in a single-cell stream. The fluorescent molecules bound to each cell are excited by the corresponding laser beam and emit a signal that is detected by the flow cytometer. The detected signal intensity is proportional to the concentration of the fluorescent molecules present. Depending on the number of lasers within the flow cytometer, the simultaneous detection of a certain number of parameters on each cell is possible. A FACSCanto II (3 lasers) allows the simultaneous detection of 8 different fluorochromes in addition to cell size (FSC = forward scatter) and cell granularity (SSC = sideward scatter). The analysis of flow cytometric data was performed using the FlowJo analysis software.

2.3.1.1 Cell surface stainings

In order to stain molecules on the surface of cells, usually 0.5 - 2.0 x 10^6 cells were used per sample. The cells were washed with FACS buffer in a 2 ml eppendorf tube. Centrifugation steps were always carried out at 550 g for 5 min at 4 °C. The supernatant was discarded and the cells were stained with 100 µl of an antibody-mixture for 10 min at 4 °C in the dark. The antibody-mixture was prepared prior to staining. In this mixture, the antibody or antibodies, if more than one marker was stained simultaneously, were diluted in FACS buffer. After the incubation time, the cells were washed with 2 ml of FACS buffer, and if needed stained with 100 µl of secondary antibody for 10 min at 4 °C in the dark. After the secondary antibody staining, the cells were washed once again with 2 ml of FACS buffer and taken up in 200 - 300 µl FACS-buffer. In a final step, the cells were transferred to FACS tubes and measured at a FACSCanto II.

If differentiation of dead and living cells was needed, 1 µl PI (0.1 µg/µl) was added to the sample shortly before measuring to exclude dead and dying cells. PI can only enter cells with a damaged cell membrane, where it intercalates with their DNA and thereby allows differentiation of dead and living cells.

2.3.1.2 Intracellular stainings

For staining intracellular cytokines, T cells had to be stimulated for 4 hours with plate-bound purified α-m CD3 (3 µg/ml) and α-m CD28 (2.5 µg/ml) in a 48-well-plate (1 - 3 x 10^6 cells/ml) to induce cytokine production first. After 2 hours of stimulation, brefeldin A (5 µg/ml) was added to the cells to inhibit the release of cytokines from the cells. After 4 hours of incubation, the cells were harvested and transferred to 2 ml eppendorf tubes. A surface staining of the cells was performed as described above. After the surface staining, the cells were washed once with PBS, then fixed with 2 % PFA for 20 min at 4 °C in the dark. Next, the cells were washed once with PBS and once with saponin buffer. All the following staining and washing steps were performed with saponin buffer. Saponins can form pores in cell membranes and make them herewith reversibly permeable. After washing with saponin buffer, the cells were incubated for 10 min at 4 °C in the dark with 70 µl α-m Fcγ III/II to block unspecific binding. After the incubation, 20 µl of a mixture of the intracellular antibodies was added and the cells were incubated for 20 min at 4 °C in the dark. Afterwards, the cells were washed once again with saponin-buffer and once more with FACS buffer, resuspended in 250 - 300 µl of FACS buffer, and transferred to FACS tubes for measuring at the FACSCanto II.

To stain the intranuclear molecule FoxP3, the FoxP3-staining kit for mouse cells from eBioscience was used according to the manufacturers instructions.

2.3.2 Isolation of immune cells by magnetic sorting

For the isolation of immune cell subsets from mouse spleen and lymph node cells magnetic cell sorting (MACS) was used. Kits from Miltenyi Biotec were utilised mainly according to the manufacturers instructions. Two basic principles can be distinguished when applying the magnetic cell sorting technique. There is either the direct labelling of the target cells, which is also called a positive sort, or the indirect or negative sort, in which everything but the target cells are labelled. When applying the direct labelling technique, antibodies that are directly coupled to magnetic beads are used. Those are specific for the wanted cell population. After coupling the cells to the magnetically labelled antibodies, they are put on a MACS column in the magnetic field of a MACS separator. After rinsing the column, it contains only the magnetically labelled target cells within its magnetic field. Subsequently, the target population can be eluted. The target population is still labelled with the magnetic beads on its

surface, however the beads fall off of the cells after several days in culture. For the indirect or negative sort, all unwanted cells are labelled with magnetic beads and the target population stays “untouched”. In order to achieve this, a mixture of biotinylated antibodies labelling all cells except the target cell population is applied. In a second labelling step, an anti-biotin antibody coupled to magnetic beads is added. This magnetically labelled secondary antibody binds to the primary antibodies. After running the cell suspension over the column and rinsing it, all unwanted cells are retained in the magnetic field of the MACS separator, whereas the unlabelled target cells pass through the column and can be collected. This results in an untouched target cell population, with no bead-coupled antibodies on its surface.

2.3.2.1 Naïve Murine T cells

In order to isolate naïve murine CD4+ T cells two sorts needed to be performed. At first, a negative sort for the isolation of CD4+ T cells was conducted according to the manufacturers instructions. In short, murine spleen and lymph node cells were washed with MACS buffer and the cell pellet was taken up in 40 µl of MACS buffer per 10^7 cells. 10 µl of CD4+ T Cell Biotin-Antibody Cocktail was added per 10^7 cells and the cell suspension was incubated for 10 min at 4 °C. Afterwards, 30 µl of MACS-buffer and 20 µl of Anti-Biotin MicroBeads per 10^7 cells were added without washing and the cell suspension was incubated for an additional 15 min at 4 °C. The cells were washed with 50 ml of MACS buffer and taken up in 500 µl MACS buffer per 10^8 cells. The labelled cell suspension was applied onto a MACS LS column, which was equilibrated with 3 ml of MACS buffer prior to the application of cells, and placed in the field of a MACS separator. The column was washed three times with 3 ml of MACS buffer after the cell suspension had passed through the column. The flow-through containing the unlabelled and herewith untouched CD4+ T cells was collected. This procedure was followed by a positive sort for CD62L, which is a marker highly expressed on naïve T cells. For the positive CD62L sort, the flow-through of one column from the CD4+ untouched sort was washed with MACS buffer and then incubated with 960 µl of MACS buffer and 40 µl of CD62L MicroBeads for 15 min at 4°C. After labelling, the cells were washed with 50 ml of MACS buffer and taken up in 1 ml of MACS buffer. The cell suspension was applied onto a MACS LS column. The column was equilibrated with 3 ml of MACS buffer prior to the application of the cell suspension. After the cell suspension had passed through the column, the column was washed three times with 3 ml of MACS buffer in order to remove all unlabelled cells. The column was then removed from the MACS separator

and placed on top of a 15 ml falcon tube in order to remove the magnetically labelled CD4+CD62L+ target population. Therefore, 5 ml of MACS buffer were applied onto the column in order to flush the magnetically labelled CD62L+ T cells out. This was achieved by pushing a plunger into the column. This final removal step was repeated once.

The purity of the isolated cell population was checked by flow cytometry. A surface staining with CD4-, CD3-, and CD62L-antibodies was performed and measured at a FACSCanto II. The purity of the negatively isolated CD4+ T cells was usually around 85 - 90 %, and of the CD62L+ cells above 95 %.

2.3.2.2 Murine APC

For obtaining APC from mouse spleen cells, a depletion of all T cells (CD90+) was conducted. In short, a positive sort for CD90+ cells using CD90.2 MicroBeads was performed. For this purpose, spleen cells were washed with MACS buffer and the cell pellet was taken up in 95 µl of MACS buffer per 10^7 cells. 5 µl of CD90.2 MicroBeads were added per 10^7 cells and the cell suspension was incubated for 15 min at 4 °C. The flow-through from the column, containing all spleen cells except the T cells, was collected.

The purity of the isolated cell population was controlled with a FACS surface staining with a CD3 antibody and measured at a FACSCanto II. The purity of the flow through (CD90- cells) was usually above 97 %.

2.3.3 Stimulation and culture of T cells

2.3.3.1 Polyclonal stimulations

For the stimulation of T cells plate-bound α-m CD3 (3 µg/ml) and plate-bound α-m CD28 (2.5 µg/ml) were used. Therefor, 48-well-plates or 96-well-plates (round bottom) were coated with 120 µl or 25 µl per well, respectively, of a mixture of both antibodies in PBS usually overnight at 4 °C or in rare cases for 3 hours at 37 °C. The antibody-mixture was removed prior to adding the cells to the wells for stimulation. For intracellular FACS stainings, the T cells were polyclonally stimulated for 4 hours at 1 – 3 x 10^6 cells/ml/well in 48-well-plates.

In order to have a positive control for the proliferation assay with different antigens, differently differentiated OVA-specific CD4+ T cells were polyclonally stimulated with ConA at 1 x 10^6 cells/ml for 3 days, followed by a ^{3}H proliferation assay.

2.3.3.2 CD4+ Th17 cells

In order to generate MOG- or OVA-specific CD4+ Th17 cells, naïve CD4+ T cells were isolated from B6.2d2, B6.OT2, B6.2d2.EGFP, B6.OT2.EGFP, B6.2d2.tdRFP or B6.OT2.tdRFP transgenic mice by magnetic sorting as described above. The naïve T cells were stimulated with irradiated (30 Gy) APC from C57BL/6 mice (ratio 1:5 - 1:10) and addition of the corresponding peptide (12.5 μg/ml MOG_{35-55} or 0.6 μM $OVA_{323-339}$). Furthermore, the following cytokines needed to be added to shift the naïve T cells to the Th17 phenotype: rm IL-6 (20 ng/ml), rm IL-23 (20 ng/ml) and rh TGF-β (3 ng/ml). The cells were plated at 3 Mio cells/ml in 24-well-plates. On days 3 and 5 usually, the cells were split and fresh MM supplemented with rh IL-2 (50 U/ml) and rm IL-23 (10 ng/ml) was added. On day 7, the CD4+ Th17 cells were restimulated with freshly isolated irradiated APC (ratio 1:3 -1:5), peptide and cytokines (10 ng/ml IL-6, 20 ng/ml IL-23, 0.75 ng/ml TGF-β). The cells were again usually split on days 3 and 5, and MM supplemented with IL-2 and IL-23 was added as described before. On day 14, the cells were restimulated for a second time with freshly isolated irradiated APC, peptide and cytokines (5 ng/ml IL-6, 10 ng/ml IL-23). On day 3 after the second restimulation, the CD4+ Th17 cells were harvested and used for adoptive transfer EAE in $Rag1^{-/-}$ or C57BL/6 recipient mice. For investigating the influence of the activation status of the generated MOG-specific CD4+ Th17 cells on the disease course/on the induction of EAE, the cells were harvested on day 3 after stimulation, first restimulation, and second restimulation and adoptively transferred into $Rag1^{-/-}$ recipient mice. The expression of cytokines was checked throughout the course of the culture and also on the day of harvesting by intracellular staining with anti-CD4, anti-IL-17, and anti-IFN-γ antibodies and analysed by flow cytometry, routinely yielding 15-50 % IL-17- and no IFN-γ-producers.

2.3.3.3 CD4+ Th1 cells

In order to generate MOG- or OVA-specific CD4+ Th1 cells, naïve CD4+ T cells were isolated from B6.2d2- or B6.2d2.tdRFP-transgenic mice by magnetic sorting as described above. The naïve T cells were stimulated with irradiated (30 Gy) APC from C57BL/6 mice (ratio 1:5 - 1:10) and addition of the corresponding peptide (25 μg/ml MOG_{35-55}). Furthermore, the following cytokines and blocking antibodies needed to be added to shift the naïve T cells to the Th1 phenotype: rm IL-12 (50 ng/ml), rm IL-18 (25 ng/ml) and anti-IL4 (10 μg/ml). The cells were plated at 3 Mio cells/ml in 24-well-plates. On days 3 and 5 usually,

the cells were split and fresh MM supplemented with rh IL-2 (100 U/ml) was added. On day 5, the CD4+ Th1 cells were restimulated with freshly isolated irradiated APC (ratio 1:3 - 1:5), peptide and cytokines (10 ng/ml IL-12, 10 ng/ml IL-18, 100 U/ml IL-2). The cells were again usually split on days 3 and 5, where MM supplemented with IL-2 was added as described before. On day 10, the cells were restimulated for a second time with freshly isolated irradiated APC, peptide and cytokines (10 ng/ml IL-12, 10 ng/ml IL-18, 100 U/ml IL-2). On day 3 after the second restimulation, the CD4+ Th1 cells were harvested and used for adoptive transfer EAE in $Rag1^{-/-}$ or C57BL/6 recipient mice. The expression of cytokines was checked throughout the course of the culture and also on the day of harvesting by intracellular staining with anti-CD4, anti-IL-17, anti-IL-10 and anti-IFN-γ antibodies and analysed by flow cytometry, routinely yielding over 50 % of IFN-γ- and no IL-17- and IL-10-secreting cells.

2.3.4 IL-17 Secretion Assay

MOG-specific IL-17-secreting CD4+ T cells were enriched *in vitro* using an IL-17 secretion assay in order to analyse their potential to induce EAE in $Rag1^{-/-}$ recipient mice *in vivo*. The IL-17 secretion assay was performed mostly according to the manufacturers instructions. The mouse IL-17 secretion assay is designed for the isolation, detection, and analysis of viable IL-17A-secreting mouse leukocytes. In short, single-cell preparations are restimulated for a certain period of time with the desired stimulus. Subsequently, an IL-17-specific Catch reagent is attached to the cell surface of all culture cells. The cells are then incubated to allow cytokine secretion. The secreted IL-17 binds to the IL-17 Catch reagent on the positive, secreting cells. These cells are subsequently labelled with a second IL-17-specific antibody, the Mouse IL-17 Detection antibody conjugated to biotin and Anti-Biotin-PE for sensitive detection by flow cytometry. The IL-17-secreting cells can now be magnetically labelled with Anti-PE MicroBeads and enriched over a MACS column, which is placed in the magnetic field of a MACS separator. The magnetically labelled cells are retained in the MACS column while the unlabelled cells run through. After the column has been removed from the magnetic field, the magnetically retained cells can be eluted as positively selected cell fraction, enriched for cytokine-secreting cells. The cells can now be used for cell culture or analysis.

Specifically, B6.2d2.EGFP Th17 cells were prepared from naïve CD4+ T cells as described above. On the day of second restimulation, the MOG-specific CD4+ Th17 cells were

harvested. 100 Mio B6.2d2.EGFP Th17 cells were used for the IL-17 secretion assay. For antigen-specific stimulation of the Th17 cells (ratio 1:3), APC were sorted and subsequently irradiated as described above. The freshly isolated APC were plated in 6-well-plates. Prior to plating, the APC were supplemented with 50 µg/ml MOG_{35-55} and incubated for 30 min at 37 °C. Next, the T cells supplemented with IL-6 (5 ng/ml) and IL-23 (20 ng/ml) were added to the APC. The cell concentration was 10 Mio cells/ml. The cells were incubated for 4 hours at 37 °C. After the incubation time, the cells were carefully harvested and distributed into 50 ml Falcon tubes with 50 Mio cells each. Afterwards, the actual secretion assay was performed. The cells were washed with ice-cold MACS buffer and centrifuged at 500 g for 5 min at 4 °C. The Th17 cells were taken up in cold WM and the mouse IL-17 Catch reagent was added. The amount of WM and mouse IL-17 Catch reagent was calculated for 10^7 total cells and differed with the amount of expected IL-17-secreting cells. Here, over 20 % of IL-17-secreting cells were expected, so the cells were resuspended in 400 µL cold WM and 100 µL mouse IL-17 Catch reagent per 10^7 total cells. The cells were incubated for 5 min on ice. Afterwards, they were washed with warm WM and centrifuged at 500 g for 5 min at 4 °C. The cell pellet was taken up in 50 ml warm WM and 10 ml of the cell suspension was distributed into freshly prepared new 50 ml falcon tubes. Each tube contained 10 x 10^6 cells for the secretion period. The cells were diluted with warm WM to a concentration of 10^5 cells/ml. For this purpose, the tubes were filled with 50 ml warm MM. The cells were incubated for 45 min at 37 °C in the water bath. The falcon tubes were turned every five minutes during the incubation period to avoid settling of the cells. Afterwards, the cells were centrifuged at 500 g for 5 min at 4 °C and put on ice. The cells were resuspended and put back together so that the cell number was 50 Mio cells/falcon tube again. The tubes were rinsed and centrifuged again as described above. The cells were resuspended in 400 µL cold MACS buffer and 100 µL mouse IL-17 Detection antibody (Biotin) per 10^7 total cells. The cells were mixed and incubated for 10 min on ice. Then, they were washed by adding 50 ml cold MACS buffer and centrifuged at 500 g for 5 min at 4 °C. The supernatant was removed and the cells were resuspended in 400 µL cold MACS buffer and 100 µL Anti-Biotin-PE per 10^7 total cells. The cells were mixed and incubated for 10 min on ice. Then, they were washed by adding 50 ml cold MACS buffer and centrifuged at 500 g for 5 min at 4 °C. The magnetic labelling with anti-PE MicroBeads followed this procedure. The cells were again resuspended in 400 µL cold MACS buffer and 100 µL Anti-PE MicroBeads per 10^7 total cells. The cells were incubated for 15 min at 4 °C in the refrigerator and afterwards washed with 50 ml of

cold MACS buffer and centrifuged at 500 g for 5 min at 4 °C. The pellet was taken up in 2 ml of cold MACS buffer. Two LS columns were prepared by putting them into two 15 ml Falcon tubes and equilibrating them with 3 ml of cold MACS buffer each. These columns were used as sham columns without the magnetic separator to get rid off the debris in the cell suspension. The flow-through contained the cells and the debris was retained in the sham column. An aliquot was taken before enrichment for the flow cytometric analysis. The flow-through was centrifuged as described before and taken up in 1 ml of cold MACS buffer for the magnetic separation. For enriching IL-17-secreting cells, it is necessary to always perform two consecutive column runs to achieve the best results. The LS column was placed in the magnetic field of a MACS separator and equilibrated with 3 ml of cold MACS buffer. For removal of all cell clumps a pre-separation filter was placed on top of the column prior to equilibration, on which the cell suspension was placed. The LS column was washed three times with 3 ml of MACS buffer and the flow-through, which contained the unlabelled cells, was collected. Afterwards, the LS column was taken out of the MACS separator and put into a 15 ml Falcon tube, 5 ml of MACS buffer were added and the magnetically labelled IL-17-secreting cells were flushed out with a plunger. The flushing out was repeated once more. The positive and negative fraction were centrifuged at 500 g for 5 min at 4 °C. The cells from the positive fraction were taken up in 1 ml of cold MACS buffer and the cells from the negative fraction in 2 ml. Another LS column was prepared for the positive fraction by placing it in the magnetic field of the MACS separator and equilibrating it with 3 ml of cold MACS buffer. The cell suspension was applied onto the column, the flow-through was collected and the column was washed three times with 3 ml of cold MACS buffer. The LS column was removed from the separator and placed in a 15 ml Falcon tube, 5 ml MACS buffer were pipetted in and the cells retained in the column were flushed out using a plunger. The IL-17-secreting cells were in this cell fraction. The procedure for the negative fraction was similar as described for the positive fraction. Two consecutive LS columns were run as described above. In contrast to the positive fraction, the flow-through was collected and the run was performed with a 20-gauge needle at the bottom of the column. Flow cytometric analysis showed that percentage of IL-17-secreting cells in the positive fraction was over 85 % and in the negative fraction below 0.5 %.

The cells from positive and negative fraction were plated at a cell concentration of 2 Mio cells/ml in a 48-well-plate with the addition of 1 µg/ml anti-mouse CD28 and

incubated for three days at 37 °C. On the third day, the cells were harvested, another cytokine check was performed and the cells were injected into $Rag1^{-/-}$ recipient mice.

2.4 Animal experiments

2.4.1 Mouse strains

Mice were bred under specifically pathogen free (SPF) conditions at the central animal facility of the Charité – Unversitaetsmedizin Berlin (FEM) and the University Medical Center of the Johannes Gutenberg University Mainz, and kept in-house for experiments in individually ventilated cages (IVC) under SPF conditions. C57BL/6 mice were purchased from Charles River (Germany) and Janvier (France). B6.$Rag1^{-/-}$ (B6.$129^{S7\text{-}Rag1tm1Mom/J}$) mice, which do not have any B or T cells, and B6.OT2 (C57BL/6-$Tg^{(TcraTcrb)425Cbn/J}$) mice, in which all CD4+ T cells are $OVA_{323\text{-}339}$-specific were originally derived from The Jackson Laboratory. B6.2d2 mice (C57BL/6-$Tg^{(Tcra2D2,Tcrb2D2)1Kuch/J}$), in which all CD4+ T cells are $MOG_{35\text{-}55}$ specific, were originally generated by the Kuchroo lab and obtained from Ari Waismann, Mainz. B6.OT2 and B6.2d2 mice were crossed with B6.tdRFP and B6.EGFP mice to generate double transgenic B6.OT2.tdRFP and B6.OT2.EGFP mice or B6.2d2.tdRFP and B6.2d2.EGFP, respectively. B6.tdRFP mice are C57BL/6 mice with ubiquitous tdRFP-expression under a *ROSA26* promotor (141). B6.EGFP mice are C57BL/6 mice with ubiquitous EGFP-expression under the *β-actin* promotor (originally derived from the Jackson Laboratory, USA). For B6.$Rag1^{-/-}$Thy1.21.EGFP mice, B6.$Rag1^{-/-}$ mice were crossed with B6.Thy1.21.EGFP mice. B6.Thy1.21.EGFP mice were originally derived from P. Caroni (142) and are C57BL/6 mice in which a *thy1* promotor drives the expression of EGFP that is targeted to the axonal cell membrane via a palmytoylated site. BDKRB1 deficient mice on SV129 background were backcrossed to C57BL/6 to set up F10 offsprings. All animal experiments were approved by local authorities (LaGeSo Berlin: G0147/05, G0029/08, G0255/08, T0271/08) and conducted according to the German Animal Protection Law.

2.4.2 Anaesthesia of mice

Before perfusion, mice were anaesthetised with an intraperitoneal injection of ketamin/xylazine-mixture (415 mg/kg / 9.7 mg/kg). For *in vivo* imaging experiments with the two-photon microscope, mice were anaesthetised with 1.5 % isoflurane in oxygen/nitrous oxide (2:1) via a facemask. For MR imaging, mice were anaesthetised with 2 – 3 % isoflurane

in 100 % oxygen and kept in anaesthesia with 1 – 1.5 % isoflurane with addition of 100 % oxygen via facemask.

2.4.3 Experimental autoimmune encephalomyelitis (EAE)

2.4.3.1 Induction of active EAE

Active EAE in C57BL/6 mice was induced by subcutaneous immunisation with 250 µg MOG_{35-55}-peptide and 800 µg H37RA emulsified in CFA, followed by two intraperitoneal doses of 400 ng PTX in PBS at the time of immunisation and 48 hours later.

2.4.3.2 Induction of passive EAE

Passive EAE was induced in $Rag1^{-/-}$ or $Rag1^{-/-}$Thy1.21 mice by intravenous transfer of 0.5 - 15 Mio MOG_{35-55}-specific CD4+ 2d2 Th17 cells on day 3 after second in *vitro* restimulation of the cells. Passive EAE was induced in C57BL/6 mice by intravenous transfer of 30 Mio MOG_{35-55}-specific CD4+ 2d2 Th17 cells from day 3 after the second *in vitro* restimulation of the cells.

2.4.3.3 Induction of relapse in active EAE

Relapses were induced in actively immunised C57BL/6 mice in remission by transfer of 20 - 30 Mio $OVA_{323-329}$-specific CD4+ OT2 Th17 cells from day three after the second *in vitro* stimulation.

2.4.3.4 Scoring

After induction of EAE, mice were scored daily starting from day 7 (active EAE) or day 10 (passive EAE). Clinical signs usually started on day 10 - 14 after induction of EAE, and in classical EAE manifested themselves as ascending paralysis, starting at the tail, then affecting the hind limbs, and in later stages also the forelimbs. Clinical signs of classical EAE were translated into clinical scores as follows:

0 = no detectable signs of EAE

1 = complete tail paralysis

2 = partial hind limb paralysis

3 = complete bilateral hind limb paralysis

4 = total paralysis of forelimbs and hind limbs

5 = death

In some cases an atypical clinical course was observed. These clinical signs were translated into scores as follows:

1= tail paralysis, hunched appearance, unsteady walk

2 = ataxia, head tilt, hypersensitivity

3 = severe ataxia, spasticity or knuckling, severe proprioception defects

4 = moribund

5 = death

Mice with a score above 3 were killed, according to animal protection law.

2.5 Two-photon laser scanning microscopy

Two-photon laser scanning microscopy (TPLSM) has revolutionised our view of cellular and molecular dynamics, especially in terms of immune and neural processes in health and disease. TPLSM is based on the simultaneous absorption of two photons twice the wavelength employed in conventional one-photon excitation microscopy. The probability of such an event is very small under normal circumstances and increases only with high photon-density (high light-intensity). Therefore, a high-power, pulsed laser sending out photon pulses in the infrared (IR) is required to generate the required photons in a sufficient density. The simultaneous absorption of two photons is confined to a focal spot, as only there two photons simultaneously hit one fluorochrome, providing three-dimensional sectioning without absorption and, thus, without photobleaching and phototoxicity above and below the focal plane. Additionally, long wavelength excitation light is less scattered, leading to increased tissue penetration. TPLSM therefore allows high resolution imaging in great tissue depth (several hundred μm) without the severe disadvantages of conventional (one-photon excitation) microscopy techniques, such as photobleaching and phototoxicity, making it the method of choice for intravital imaging studies (143,144).

First applied to studies of immune cell behaviour in lymph nodes (145,146), it can also be applied to visualise immune cell dynamics and cellular interactions within the complex networks of the inflamed CNS. The use of fluorescent labelling techniques and transgenic animals expressing different fluorescent proteins (147) permits the monitoring of specific subpopulations of cells during the course of EAE directly in the target organ of living anaesthetised mice.

2.5.1 Setup and imaging for intravital micrsocopy

Operation procedures and two-photon laser scanning microscopy were performed as previously described by Siffrin et al. (148,149). Mice were anesthetized with 1.5% isoflurane in oxygen/nitrous oxide (2:1) via a facemask. The mice were then tracheotomised and continuously ventilated with a Harvard Apparatus Advanced Safety Respirator. The anaesthetised animal was transferred to a custom-built operation and microscopy table, and fixed in a hanging position. The preparation of the imaging field was performed according to adapted protocols for cortical imaging (150). In brief, the brain stem was exposed by carefully removing musculature above the dorsal neck area and removing the dura mater between the first cervical vertebra and occipital skull bone. The head was inclined for access to deeper brain stem regions, and the brain stem was superfused with isotonic Ringer solution. A sterile agarose patch (0.5 % in 0.9 % NaCl solution) was installed on the now-exposed brain surface to reduce heartbeat and breathing artifacts. During surgery and microscopy, body temperature was maintained at 37 °C. The depth of anaesthesia was controlled by continuous CO_2 measurements of exhaled gas and recorded with a CI-240 Microscapnograph.

Imaging was performed using a specialized two-photon laser scanning microscope previously described by Herz et al. (151), which allows for dual NIR (700-1020 nm) and IR (1050-1600 nm) excitation, i.e. pulsed NIR radiation is generated by an automatically tunable Ti:Sa laser, 10 % of which is coupled into a scan head. 90 % of Ti:Sa laser power is coupled into a synchronously pumped optical parametric oscillator (OPO). The generated OPO beam first passes a system of spectral filters, is entering the scan head and overlapping the Ti:Sa beam. The co-localised beams are coupled into an upright microscope towards the objective lens (20x, NA 0.95). Fluorescence is collected by the same objective lens and directed to a spectrally resolving detection unit containing the respective dichroic mirrors, interference filters and up to three non-descanned photomultiplier tubes for spectral separation of EGFP and RFP.

XYZ stacks were typically acquired in 1 min intervals over a period of 1-2 hours. Imaging depth was between 20-140 µm, with a usual stack covering 70 µm. The imaging field was 300x300 µm in the xy-range.

2.5.2 Preparation and imaging of brain slices

Brain slice cultures were obtained as described in Nitsch et al. (152). Herefore, 10 day-old C57BL/6 mice were anaesthetised with isoflurane and FITC-dextrane was injected into the right ventricle. Mice were then decapitated, brains were removed quickly, and 400 µm-thick slices were cut with a Vibratome. Slices were transferred to a thermo-regulated recording chamber, which was continuously perfused with aerated (95 % O_2, 5 % CO_2) artificial CSF (ACSF) containing (in mM): NaCl, 124; NaH_2PO_4, 1.25; $NaHCO_3$, 26; KCl, 3; $CaCl_2$, 1.6; $MgSO_4$, 1.8 and glucose, 10, pH 7.35. Acute slices were allowed to recover for at least 1 hour at room temperature. Before imaging Th17 cells were pre-treated either with R838 (500 nM), R715 (500 nM) or PBS for additional 4 h. Cells were visualised in brain slice cultures by a two-photon system SP2 equipped with an upright microscope fitted with a 20x water-immersion objective. Fluorescent dyes were excited simultaneously by a mode-locked Ti-sapphire laser at wavelength 840 nm. Fluorescence from FITC-dextrane and CellTracker™ Orange CMTMR was collected using two external non-descanned detectors. XYZ stacks were typically collected in a depth of 60 – 120 µm below the surface over a period of 1-2 hours (z-stack with a thickness of 60 µm, z-plane distance typically 1.8 µm).

2.5.3 Data analysis

Intravital images were post-processed using acquisition software Imspector (LaVision Biotec). For intravital and brain slice data, cell recognition, movement tracking, 3D representation and average cell velocity were calculated using the software Imaris (Bitplane) and Volocity® (Improvision).

2.5.3.1 Cell-cell contact determination

Contacts of 2d2.tdRFP Th17 and OT2.tdRFP Th17 with neurons (EGFP+) within the brain stem of imaged mice were analysed as described by Siffrin et al. (148). In short, double-positive EGFP/RFP voxels were highlighted by standard image analysis software. Using automated identification of double positive voxels, a co-localisation channel was generated and added to the EGFP-blue and RFP-red color-coded channels to visually

highlight the contact areas as white regions. To describe and compare the contact duration between EGFP+ and RFP+ cells, all individual contact areas were tracked over time. To quantitatively describe the interactivity of two differently labelled cell populations, our group has developed a means of contact quantification, which is based on the law of mass action. By considering the volumes of the two different fluorescences (V_{EGFP}/V_{RFP}) as well as their contact-volume (V_{coloc}) and the total imaging-volume (V_{total}), the contact index k can be calculate, which is independent of the absolute number of cells. k is a measure for the co-localisation and therefore for the interaction of two cell populations.

$k = (V_{coloc} \times V_{total})/(V_{EGFP} \times V_{RFP})$

2.6 Magnetic resonance imaging

Magnetic resonance imaging (MRI) has presented itself as an elaborate *in vivo* tool to non-invasively observe migration of labelled cells (153–155). MRI has become an established tool to diagnose MS and to monitor its evolution. In patients at presentation with clinically isolated syndromes suggestive of MS, MRI criteria for MS diagnosis have been proposed and are updated on a regular basis (156,157). MRI is sensitive in monitoring MS pathology and facilitates evaluation of potential new treatments (158). In the past, *in vivo* MR studies of the animal model were hampered by technical limitations such as insufficient spatial resolution. The development of high resolution MR sequences using high magnetic field strength in dedicated rodent scanner systems facilitates investigation of BBB breakdown in mice after transfer of pathogenic T cells, a suitable model for diagnostic as well as therapeutic strategies in MS.

2.6.1 Setup

MRI was performed before and after induction of passive or active EAE on various days during the disease course as described by Smorodchenko et al. (112). There were measurements performed pre-onset of disease, in the peak of disease and in disease remission. MRI was performed on a 7 Tesla rodent scanner applying a 20 mm RF-Quadratur-Volume head coil. Mice were placed on a heated circulating water blanket to ensure a constant body temperature of 37 °C. During image acquisition, mice were anaesthetised with 1 – 1.5 % isoflurane with addition of 100 % oxygen under constant ventilation monitoring. Axial and coronal T1-weighted images (MSME; TE 10.5 ms, TR 322 ms, 0.5 mm slice thickness,

Matrix 256 x 256, FOV 2.8 cm) were acquired before and after intravenous injection of 0.5 mmol/kg Gd-DTPA.

2.7 Histology

2.7.1 Preparation of tissue

For the generation of tissue sections, mice were lethally anaesthetised with an intraperitoneal injection of a ketamin/xylazine-mixture (415 mg/kg / 9.7 mg/kg). For transcardial perfusion the animal was disinfected with 70 % EtOH and the fur was cut open. Next, the sternum was removed to expose the heart. The right atrium was opened, a 20-gauge needle was inserted into the left ventricle, and mice were transcardially perfused with 120 ml PBS followed by perfusion with 120 ml 4 % paraformaldehyde. Brain and spinal cord as well as spleen were removed and post-fixed overnight in 4 % PFA at 4 °C. Next, the organs were rinsed with water for 1 hour and kept in PBS overnight. In the following two days, the organs were dehydrated in glucose solution (first 15 % glucose in PBS followed by 30 % glucose after 24 hours). Afterwards the organs were frozen. For this, the spinal cord was divided into three parts: cervical, thoracic, and lumbar. The spinal cord parts and the brain were placed separately in molds filled with Tissue-Tek®, an embedding medium, and shock frozen by slowly putting them in liquid nitrogen.

2.7.2 Generation of tissue sections

For histological analysis, tissue sections of the tissue of interest were prepared at a cryostat. The tissue sections were usually prepared with a thickness of 10 µm and collected on object slides. They were dried for at least two hours at room temperature and subsequently directly stained or stored at – 20 °C.

2.7.2 Hematoxylin and eosin stain

The hematoxylin and eosin stain is a standard procedure in histology. It is used to differentiate between different tissue structures. For the staining, hemalum needs to be applicated. Hemalum is a complex formed from aluminium ions and oxidised hematoxylin. It colours the nuclei of cells blue. A counterstaining with eosin, which colours other structures in red, usually follows the hematoxylin stain.

For the hematoxylin and eosin stain, the tissue section were taken out of the freezer and dried for 30 min. This was followed by ten minutes incubation in ice-cold methanol. Next, the object slides with the tissue of interest were bathed in distilled water followed by 10 minutes staining with hematoxylin. Afterwards, the object slides were bathed for 2 min in distilled water followed by bathing the slides in a solution consisting of 1 % of hydrochloric acid and 70 % EtOH. Next, the object slides were bathed again in water. A staining with eosin for 0.5 – 1 minute followed the washing step. The eosin was washed off with water and the washing step was followed by another washing step with increasing alcohol solutions (70 %, 96 % and 99 %). Finally, the object slides were put in xylol and afterwards the cover glass was mounted.

2.8 Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5. Data are usually presented as Mean +/- SEM. To compare two means, unpaired t-test or Mann-Whitney-U test were used. To compare EAE-curves, the non-parametric Kurskal-Wallis test or Mann-Whitney-U test were used. P-values < 0.05 were considered significant.

3 Results

3.1 T cell differentiation requirements for stable induction of Experimental Autoimmune Encephalomyelitis

There is a controversial debate around how far EAE and MS are rather Th1 or Th17 mediated diseases (134–136). There is strong evidence that IL-23, which is strongly associated with the Th17 phenotype, is the crucial factor for the development of EAE (66). If IL-23 (p19) is knocked out in mice, they are resistant to the induction of EAE, whereas IL-12 knock out animals are not (61). In addition, it has become clear that Th1-like (IFN-γ-expressing) cells might develop from originally IL-17-expressing T cells ("ex-Th17"), which express the Th1 cytokine IFN-γ later in the CNS and display distinct features compared to classic Th1 cells (128). However, 90 % of these T cells that infiltrated the CNS were identified to have initially expressed IL-17. These Th1-like "ex-Th17" cells have been shown to be different from classic Th1 cells. In MS, acute clinical exacerbations have been associated with an increase of IL-17-producing /Th17 cells (88,159,160).

To investigate the requirements for stable induction of experimental autoimmune encephalomyelitis (EAE), myelin-specific T cell receptor (TCR) transgenic CD4+ T cells underwent different conditions before being adoptively transferred in wild type and/or lymphopenic ($Rag1^{-/-}$) recipient mice. The focus of experimental investigations was activation status, rounds of restimulation and differentiation (Th1 vs. Th17 cells). Myelin oligodendrocyte glycoprotein (MOG)-specific (2d2-TCR transgenic) T cells were completely primed *in vitro* to generate "pure" Th1 or Th17 cells arising from naïve T cells. This is different from the established models with polyclonal MOG-specific T cells from actively immunised mice (traditional passive EAE), which are restimulated with cognate peptide and combinations of cytokines, e.g. IL-12, IL-23 and IL-18. This rather "dirty" approach (to isolate Th1/Th17 cells and shift them *in vitro* to Th17 cells) uses peptide/CFA pre-immunised mice as donors for polyclonal MOG-specific T cells, that are restimulated before adoptive transfer and can be shifted by cytokine addition in several directions. So far these classic approaches to induce adoptive transfer EAE with MOG-specific CD4+ Th17 cells have yielded low encephalitogenicity by unknown reasons and necessitated often irradiation of the recipient mice.

The differentiation towards the Th17 phenotype *in vitro* is dependent on the presence of the pro-inflammatory cytokines IL-6 and IL-23 in the context of TGF-β, whereas their induction *in vivo* seems to rely on the presence of Toll-like receptor (TLR) ligands (65,66). Requirements for the induction of stable encephalitogenic MOG-specific CD4+ Th17 cells will be identified first. These Th17 cells will be compared with conventional *in vitro* differentiated "pure" Th1 cells. Next, those stable MOG-specific CD4+ Th17 and Th1 cells will be investigated for their potential to induce adoptive transfer EAE in susceptible recipients. Then, transferred T cells will be re-isolated from the CNS of affected animals and analysed for the differentiation markers. Once established, the generated MOG-specific Th17 cells can be used for a variety of applications that will help us come closer to understanding the mechanisms involved in the initiation of autoimmune diseases like MS.

3.1.1 Requirements for stable encephalitogenic MOG-specific CD4+ Th17 cells

3.1.1.1 Impact of activation status of CD4+ Th17 cells on their ability to produce IL-17

2d2 transgenic T cells that express a T cell receptor (TCR) that specifically recognises the MOG-peptide 35-55 (2d2 mice) (161) were used to obtain sufficient numbers of CD4+ Th17 cells. The protocol for *in vitro* differentiation of CD4+ Th17 cells was optimised regarding the activation status. For this purpose, naïve CD4+ T cells were isolated from spleen and lymph nodes of 2d2 transgenic mice, sorted for CD4+CD62Lhi naïve cells and cultivated with the Th17 polarising cytokines IL-6 and IL-23 in the presence of TGF-β as described in the methods section 2.3.4.1. It was investigated if the activation status has an influence on the differentiation of the *in vitro* generated CD4+ Th17 cells. For this, restimulation protocols for the naïve CD4+ T cells were developed. CD4+CD62Lhi T cells were either only stimulated with antigen presenting cells (APC) for three days or cultivated longer with one and two restimulations with fresh APC after 7 days in culture, respectively. Afterwards, the cells were analysed for their ability to produce IL-17 *in vitro* by flow cytometry. It could be observed that with longer cultivation and repeated stimulation more CD4+ T cells differentiated into IL-17 producing cells (Figure 5D). After three days in culture a substantial amount of IL-17 producers (7.8 %) was detected already (Figure 5A). After first restimulation IL-17 production increased to almost 10 % (Figure 5B), after second restimulation Th17 production culminated in about 20 % (Figure 5C). In repeatedly stimulated Th17 cultures 15-50 % of the cells produced IL-17 on average (Figure 5D). The

in vitro generated CD4+ Th17 cells were largely free of contaminating IFN-γ- and FoxP3-producing cells. A reduction of FoxP3-producing cells could be observed with repeated restimulation (Figure 5E). More than 90 % of the MOG-specific CD4+ Th17 cells expressed the Vβ11 transgenic TCR chain (Figure 5F). Repetitive stimulation of initially 2d2 CD4+CD62Lhi T cells was associated with a stronger IL-17-producing and less FoxP3-expressing Th17 population but when restimulated more than twice *in vitro* the MOG-specific CD4+ Th17 cells lost their previous differentiation and started producing high amounts of IFN-γ besides IL-17 (Figure 5G).

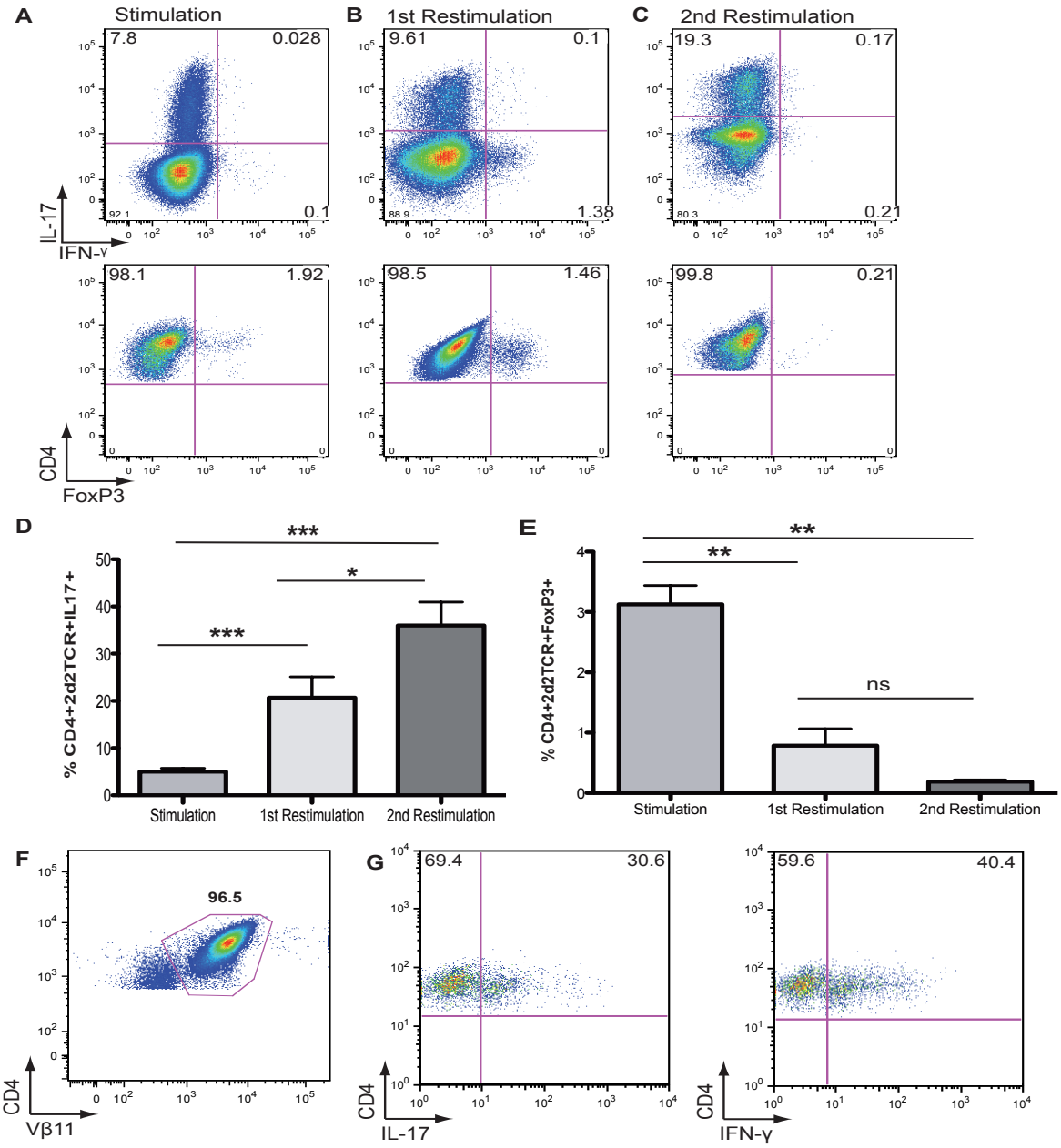
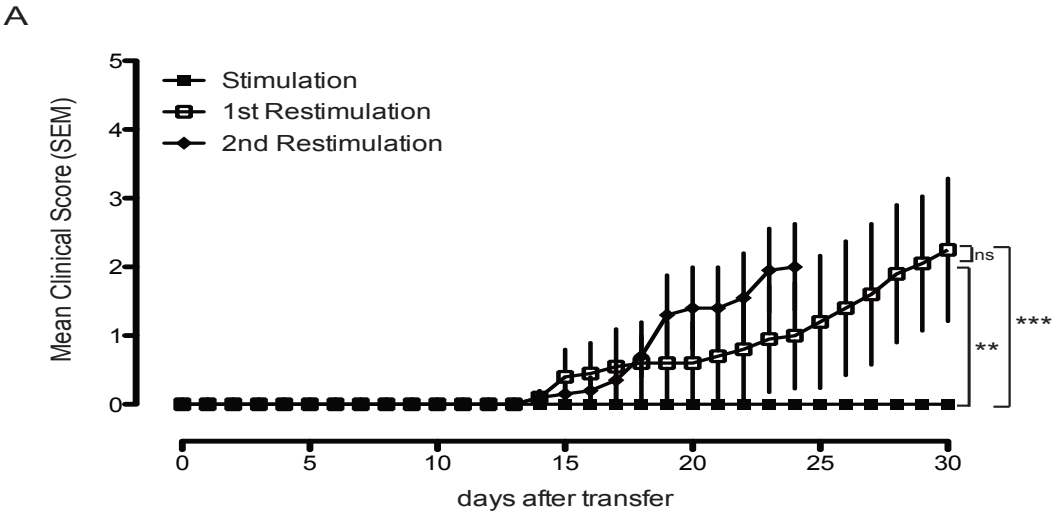


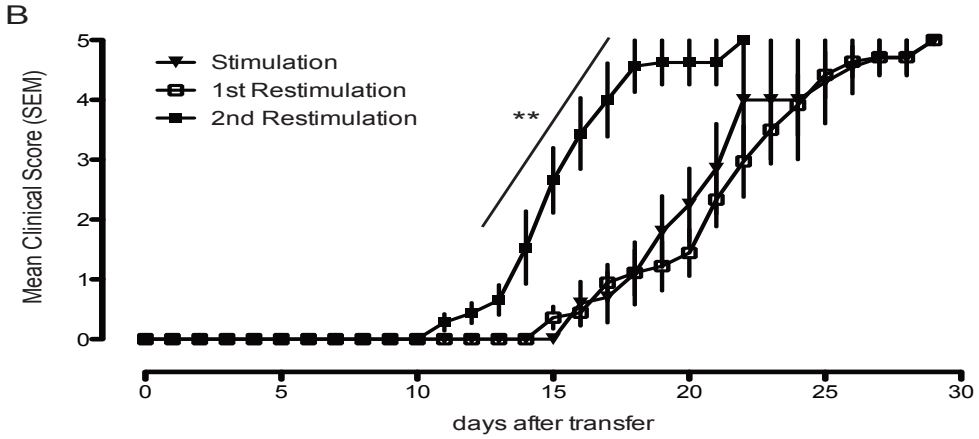
Figure 5. Differentiation of naïve CD4+ T cells from 2d2 mice under Th17 polarising conditions. CD4+CD62Lhigh T cells were kept in culture (A) for 3 days (Stimulation), (B) 10 days with one restimulation with fresh APCs after 7 days in culture (1st Restimulation) and (C) 17 days with two restimulations with fresh APC at day 7 and day 14 (2nd Restimulation), respectively. (A-C) Intracellular IL-17 and IFN-γ cytokine expression and intranuclear FoxP3 expression was assessed by flow cytometry. Data shown are from one representative experiment and are gated on the MOG-specific CD4+ population. (D) Amount of IL-17 producers increased with each restimulation whereas (E) FoxP3-production decreased. Data from several experiments (twelve for IL-17 production and 6 for FoxP3 production) are shown as Mean+/-SEM, and analysed by Mann-Whitney-U test: $p<0.05$ was considered significant, *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns not significant. (F) Representative dot plot of Vβ11 transgenic TCR chain expression of CD4+ Th17 cells throughout all experiments. (G) Representative dot plots of IL-17 production (left) and IFN-γ production (right) of MOG-specific CD4+ Th17 cells restimulated for a third time.

3.1.1.2 Impact of activation status of CD4+ Th17 cells on their pathogenicity *in vivo*

In order to get a better understanding of the role of MOG-specific CD4+ Th17 cells in EAE, the potential of the previously described *in vitro* generated 2d2 CD4+ Th17 cells to induce disease in susceptible animals was investigated. For this purpose, C57BL/6 wild type mice and lymphocyte deficient $Rag1^{-/-}$ mice (57) were adoptively transferred with 2d2 CD4+ Th17 cells three days after stimulation, first restimulation and second restimulation, respectively. C57BL/6 wild type mice received 30 Mio and $Rag1^{-/-}$ mice 10 Mio 2d2 CD4+ Th17 cells each. Notably, we did not use pertussis toxin to avoid any confounding effects on T cells. Pertussis toxin is often used as a pleiotropic adjuvant in active and adoptive transfer EAE. The 2d2 CD4+ Th17 cells that were injected after first and second restimulation respectively showed the potential to induce EAE in both C57BL/6 wild type and $Rag1^{-/-}$ mice very efficiently (Figure 6). Adoptive transfer of 2d2 Th17 cells into wild type recipients led to severe, non-remitting clinical EAE in about 50 % of transferred animals. Transferred 2d2 Th17 cells after initial stimulation failed to induce EAE in the wild type recipients (Figure 6A). In the lymphocyte deficient $Rag1^{-/-}$ mice adoptive transfer of the repeatedly stimulated as well as the stimulated 2d2 CD4+ Th17 cells led to fulminant and severely progressive EAE which resulted in death in all animals. In the $Rag1^{-/-}$ mice activation status correlated with the onset of disease (Figure 6B). It could be observed that EAE developed faster when the 2d2 CD4+ Th17 cells were more activated and produced higher amounts of IL-17 after 2nd restimulation (Figure 7). Also in the wild type recipients an earlier onset of disease and a higher disease incidence but no difference in disease severity was observed when the injected cells were more activated (Figure 6A).



	Incidence	Mean day of onset	Mortality
Stimulation	0/5	-	0/5
1st Restimulation	3/5	23	0/5
2nd Restimulation	4/5	17.5	0/5



	Incidence	Mean day of onset	Mortality
Stimulation	5/5	18	5/5
1st Restimulation	9/9	17	9/9
2nd Restimulation	8/8	12	8/8

Figure 6. Disease course of C57BL/6 mice (A) and Rag1$^{-/-}$ mice (B) after being adoptively transferred with MOG-specific CD4+ Th17 cells after stimulation, 1st restimulation and 2nd restimulation, respectively. (A) C57BL/6 wild type mice show a severe, non-remitting clinical EAE after being transferred with 30 Mio 2d2 CD4+ Th17 cells after first and second restimulation in about 50 % of the animals. 2d2 CD4+ Th17 cells after stimulation failed to induce clinical symptoms in the wild type mice. (B) In Rag1$^{-/-}$ mice adoptive transfer of the stimulated as well as repeatedly stimulated MOG-specific CD4+ Th17 cells led to fulminant and severely progressive EAE which resulted in death in all animals. Data are shown as Mean+/-SEM, either from one representative experiment of two or pooled from two independent experiments, and analysed by Kruskal-Wallis test and Dunn`s Multiple Comparison post test: $p<0.05$ was considered significant, *** $p<0.001$, ** $p<0.01$.

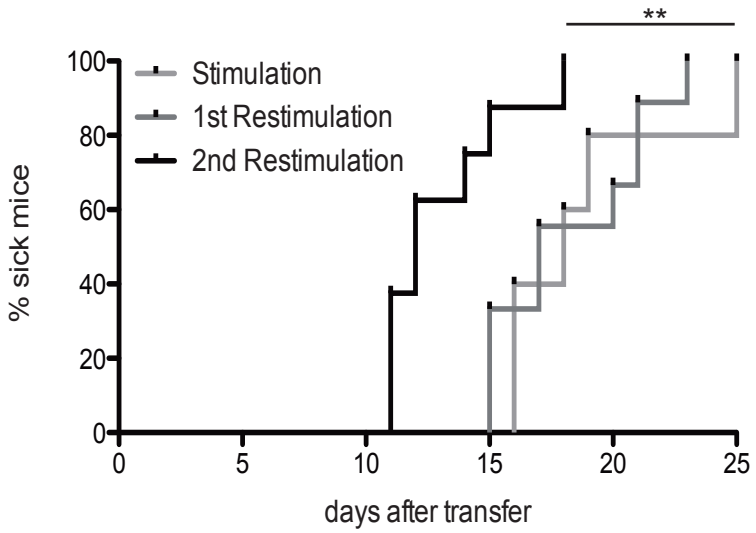


Figure 7. Onset of disease in Rag1$^{-/-}$ mice that were transferred with 2d2 CD4+ Th17 cells three days after stimulation, 1st restimulation and 2nd restimulation, respectively is presented in a Kaplan-Meier survival curve as percentage of sick mice. Disease onset correlates with activation and differentiation status of 2d2 CD4+ Th17 cells as the animals that were transferred with the IL-17 producing CD4+ effector cells after 2nd restimulation, showed an earlier disease onset compared to the animals that were transferred with the MOG-specific CD4+ Th17 cells after 1st restimulation and stimulation. Data shown are pooled from two independent experiments, and analysed by Log-rank (Mantel-Cox) test, $p<0.05$ was considered significant, ** $p<0.01$.

To correlate pre-transfer flow cytometric profiles of the adoptively transferred 2d2 CD4+ Th17 cells, lymphocytes were isolated from the CNS of the diseased mice between days 16 and 28. Flow cytometric analysis of the isolated cells revealed, that the 2d2 CD4+ Th17 cells could still be found in high numbers in the wild type recipients after transfer after 1st and 2nd restimulation, respectively. There were hardly any IFN-γ-producing cells detectable in the C57BL/6 mice. As expected regulatory FoxP3-producing cells could also be found. CNS lymphocytes from C57BL/6 mice that were transferred with 2d2 CD4+ Th17 cells after stimulation were not isolated, as these mice did not develop disease. In the CNS of the Rag1$^{-/-}$ mice MOG-specific CD4+ cells were also found. The adoptively transferred IL-17-producing cells showed plasticity as they seemingly converted to IFN-γ-producing Th1-like cells in high numbers. Regulatory CD4+ T cells were not found in the CNS of the lymphocyte deficient recipients (Figure 8 and Figure 9).

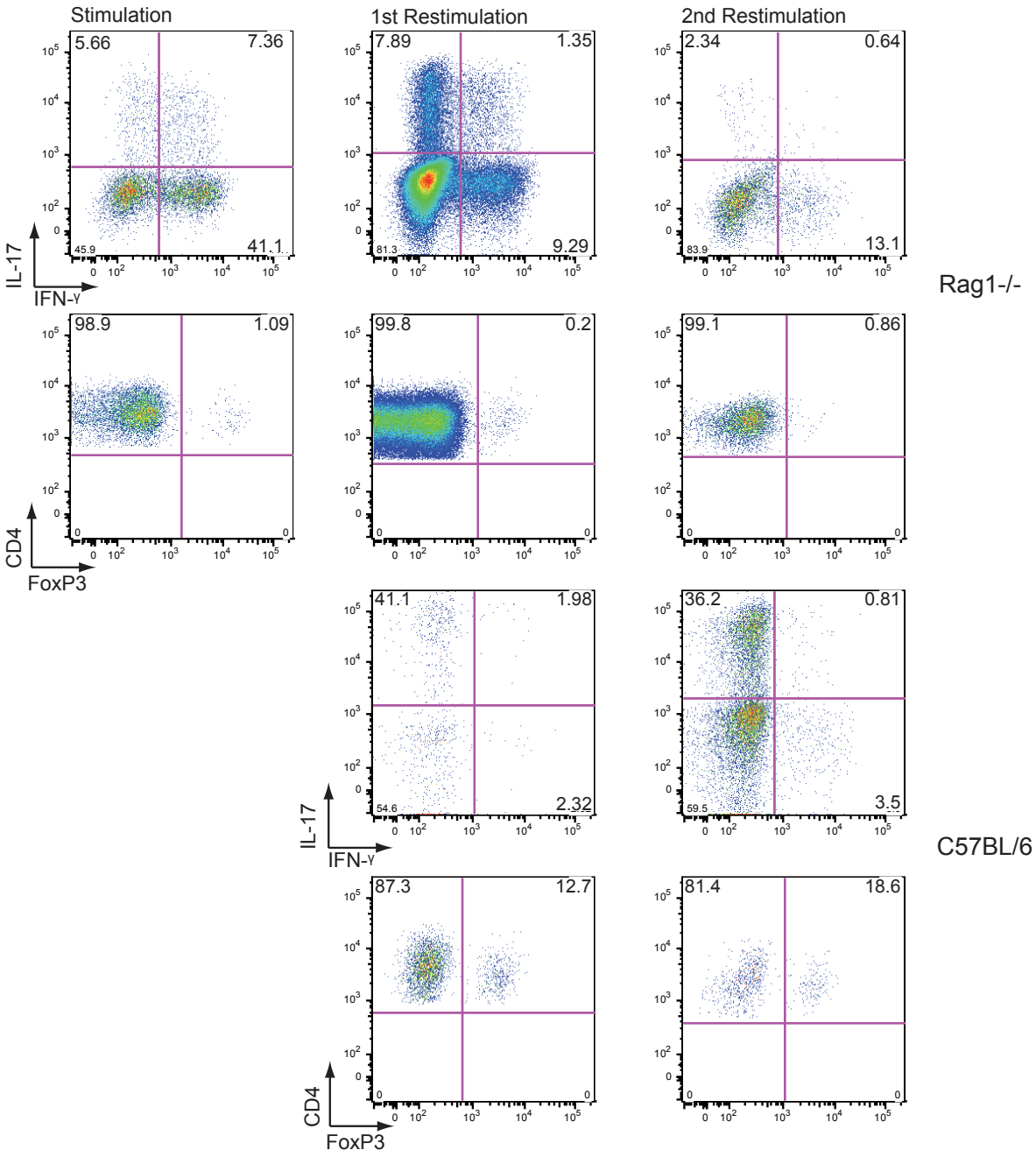


Figure 8. Immune cells isolated from the CNS of Rag1$^{-/-}$ and C57BL/6 mice adoptively transferred with 2d2 CD4+ Th17 cells after stimulation, 1st restimulation and 2nd restimulation, respectively. In the Rag1$^{-/-}$ mice as well as in the C57BL/6 mice the transferred MOG-specific CD4+ Th17 cells could be detected. In the C57BL/6 mice the isolated CD4+ T cells were still predominantly IL-17-producers. Hardly any IFN-γ-producing CD4+ T cells were found. FoxP3-producing regulatory CD4+ T cells were also found. In the Rag1$^{-/-}$ mice the isolated CNS lymphocytes showed a diminished number of IL-17-producing CD4+ T cells and an increased number of IFN-γ-producers when compared with the pre-transfer data. Hardly any regulatory CD4+ FoxP3-producing T cells were detected in the lymphocyte deficient recipients. Data from one

representative mouse each is shown from one of two independent experiments. Cells shown are all gated on 2d2TCR (MOG_{35-55}-specific T cell receptor)+ CD4+ T cells.

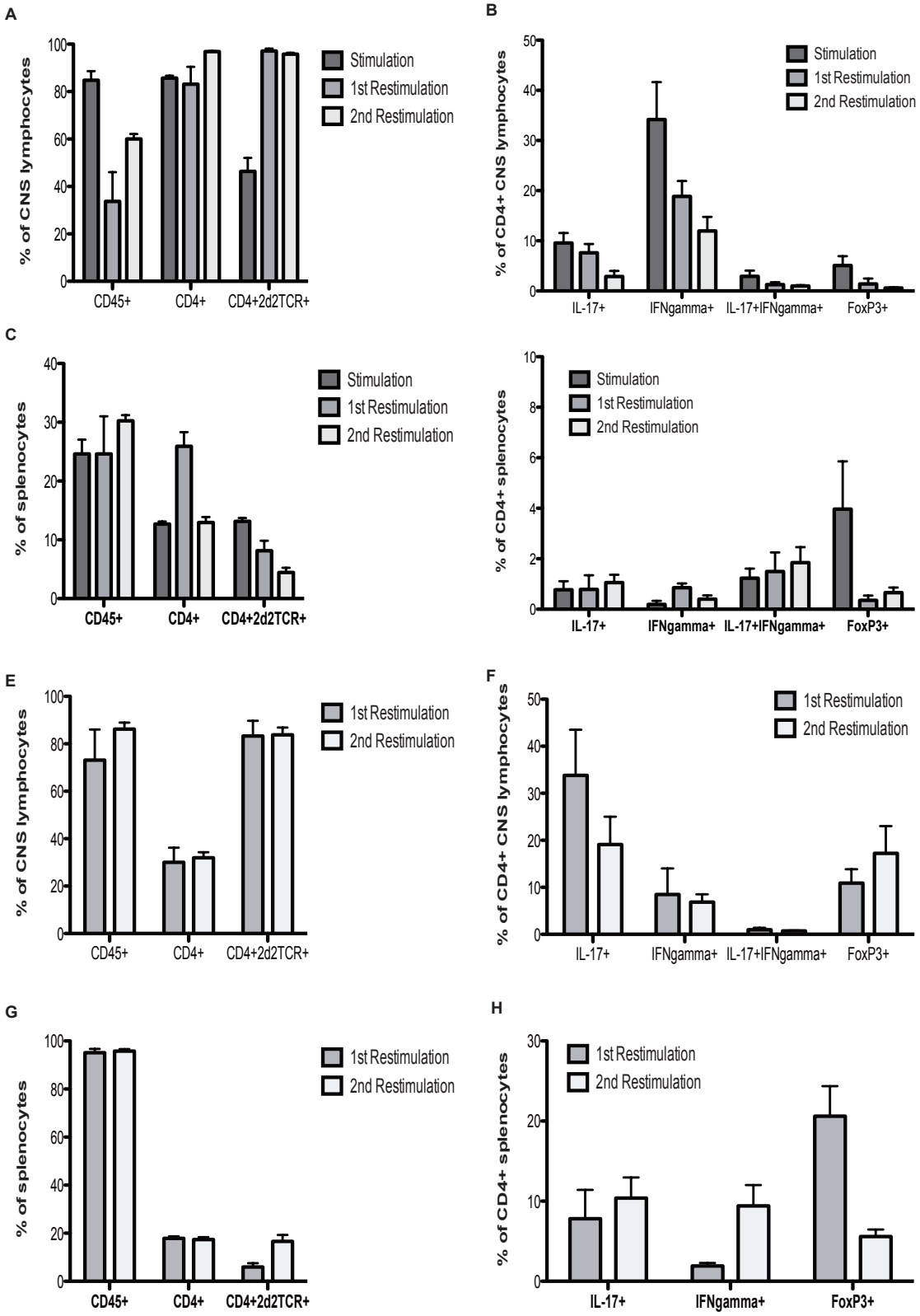


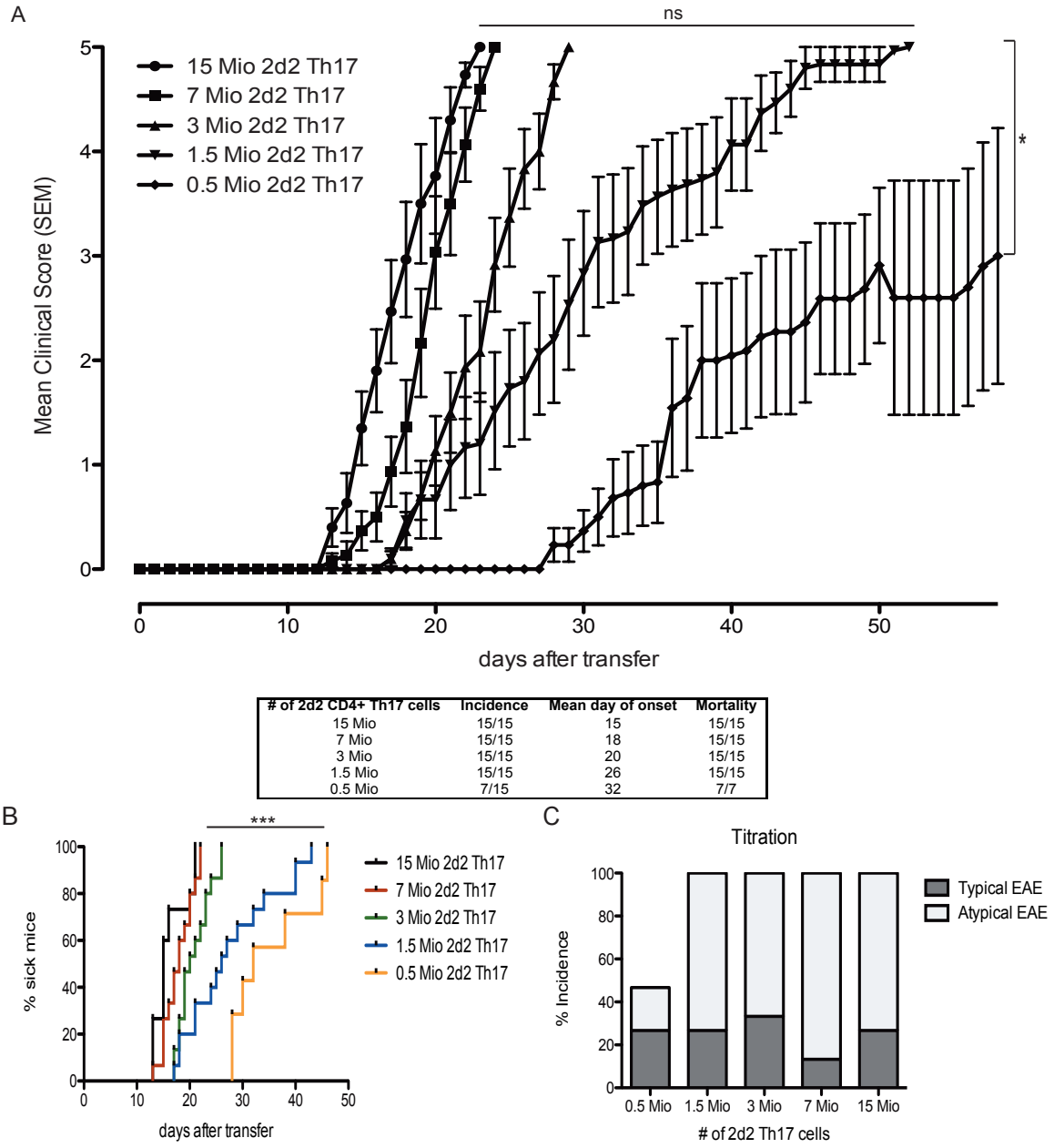
Figure 9. Immune cells isolated from the CNS and spleen of $Rag1^{-/-}$ (A-D) and C57BL/6 (E-H) mice adoptively transferred with 2d2 CD4+ Th17 cells after stimulation, 1st restimulation and 2nd restimulation, respectively.

(A) Percentages of CD45+, CD4+ and MOG-specific (2d2TCR+) cells of CNS lymphocytes from Rag1$^{-/-}$ mice are shown. (B) Percentages of IL-17+, IFN-γ+, IL-17+ IFN-γ+ and FoxP3+ cells of CD4+ T cells from the CNS of Rag1$^{-/-}$ mice are shown. (C) Percentages of CD45+, CD4+ and MOG-specific (2d2TCR+) cells of lymphocytes from the spleen of Rag1$^{-/-}$ mice are shown. (D) Percentages of IL-17+, IFN-γ+, IL-17+ IFN-γ+ and FoxP3+ cells of CD4+ T cells from the spleen of Rag1$^{-/-}$ mice are shown. (E) Percentages of CD45+, CD4+ and MOG-specific (2d2TCR+) cells of CNS lymphocytes from C57BL/6 mice are shown. (F) Percentages of IL-17+, IFN-γ+, IL-17+ IFN-γ+ and FoxP3+ cells of CD4+ T cells from the CNS of C57BL/6 mice are shown. (G) Percentages of CD45+, CD4+ and MOG-specific (2d2TCR+) cells of lymphocytes from the spleen of C57BL/6 mice are shown. (H) Percentages of IL-17+, IFN-γ+, IL-17+ IFN-γ+ and FoxP3+ cells of CD4+ T cells from the spleen of C57BL/6 mice are shown. Data from one representative experiment of three are shown, and analysed by Mann-Whitney-U test: $p<0.05$ was considered significant; $p>0.05$ not significant.

3.1.1.3 Impact of cell number of CD4+ Th17 cells on their potential to induce EAE

To investigate the importance of the amount of 2d2 CD4+ Th17 cells for induction of disease in lymphocyte deficient Rag1$^{-/-}$ mice, a titration with the afore-mentioned cells was performed. For this purpose, MOG-specific CD4+ Th17 cells after second restimulation were used and adoptively transferred into Rag1$^{-/-}$ recipient mice. Indicated as being the most potent to induce EAE in the susceptible rodents in the previously described experiments, the cells after second restimulation were chosen. For the titration of the 2d2 CD4+ Th17 cells the following cell numbers were injected intravenously into the Rag1$^{-/-}$ mice: 0.5 Mio, 1.5 Mio, 3 Mio, 7 Mio and 15 Mio.

It could be observed that with transfer of higher numbers of 2d2 CD4+ Th17 cells disease onset was quickened (Figure 10A and Figure 10B). Only the transfer of 0.5 Mio MOG-specific CD4+ Th17 cells led to fulminant and severely progressive EAE that resulted in death in only about 50 % of the animals (Figure 10A). In all the other animals that were injected with higher Th17 cell numbers, the transfer led to severely progressive disease that resulted in death in all animals. Furthermore, most of the animals transferred with the highly IL-17-producing CD4+ T cells developed atypical disease. However, only about half of the animals transferred with 0.5 Mio 2d2 CD4+ Th17 cells developed those atypical symptoms. Most of the animals transferred with the lowest chosen number of Th17 cells developed typical EAE (Figure 10C).



# of 2d2 CD4+ Th17 cells	Incidence	Mean day of onset	Mortality
15 Mio	15/15	15	15/15
7 Mio	15/15	18	15/15
3 Mio	15/15	20	15/15
1.5 Mio	15/15	26	15/15
0.5 Mio	7/15	32	7/7

Figure 10. Disease course, onset and incidence of Rag1$^{-/-}$ mice after being adoptively transferred with different numbers of MOG-specific CD4+ Th17 cells after second restimulation. (A) Adoptive transfer of 15 Mio, 7 Mio, 3 Mio and 1.5 Mio MOG-specific CD4+ Th17 cells led to fulminant and severely progressive EAE which resulted in death in all animals. When transferred with 0.5 Mio MOG-specific CD4+ Th17 cells only about 50 % of the animals developed EAE. (B) Disease onset in Rag1$^{-/-}$ mice after being adoptively transferred with different numbers of MOG-specific CD4+ Th17 cells after second restimulation shown as percentage of sick mice. (C) Disease incidence in Rag1$^{-/-}$ mice after adoptive transfer with different numbers of MOG-specific CD4+ T cells. Only about half of the animals injected with 0.5 Mio 2d2 CD4+ Th17 cells developed signs of EAE in contrast to the animals that received 1.5 Mio, 3 Mio, 7 Mio and 15 Mio cells, respectively. Those animals all developed EAE and predominantly of the atypical disease type. Data are shown as Mean+/-SEM, either from one representative experiment or pooled from three independent experiments, and analysed by Kruskal-Wallis test

and Dunn's Multiple Comparison post test and by Log-rank (Mantel-Cox) test for disease onset: $p<0.05$ was considered significant, *** $p<0.001$, * $p<0.05$, ns=$p>0.05$.

Pre-transfer flow cytometric profiles of the adoptively transferred 2d2 CD4+ Th17 cells were correlated with cytokine profiles from 2d2 CD4+ T cells isolated from the CNS of sick mice. Lymphocytes were isolated from the CNS of the mice between days 18 and 28. In $Rag1^{-/-}$ mice that showed signs of typical as well as atypical EAE flow cytometric profiles from before and after transfer did not match. Upon re-isolation from the CNS of the sick mice, the CD4+ Th17 cells producing IL-17 were lower in numbers and increased numbers of IFN-γ-producing CD4+ T cells were detected in typically diseased animals as well as in atypically diseased animals. The lower the number of transferred 2d2 CD4+ Th17 cells, the higher the number of IFN-γ-producers after isolation from the CNS. Here, it did not matter whether the animals showed signs of typical or atypical disease (Figure 11 and Figure 12). Very low numbers of FoxP3-producing regulatory T cells were detected in the CNS of the diseased animals (Figure 11C).

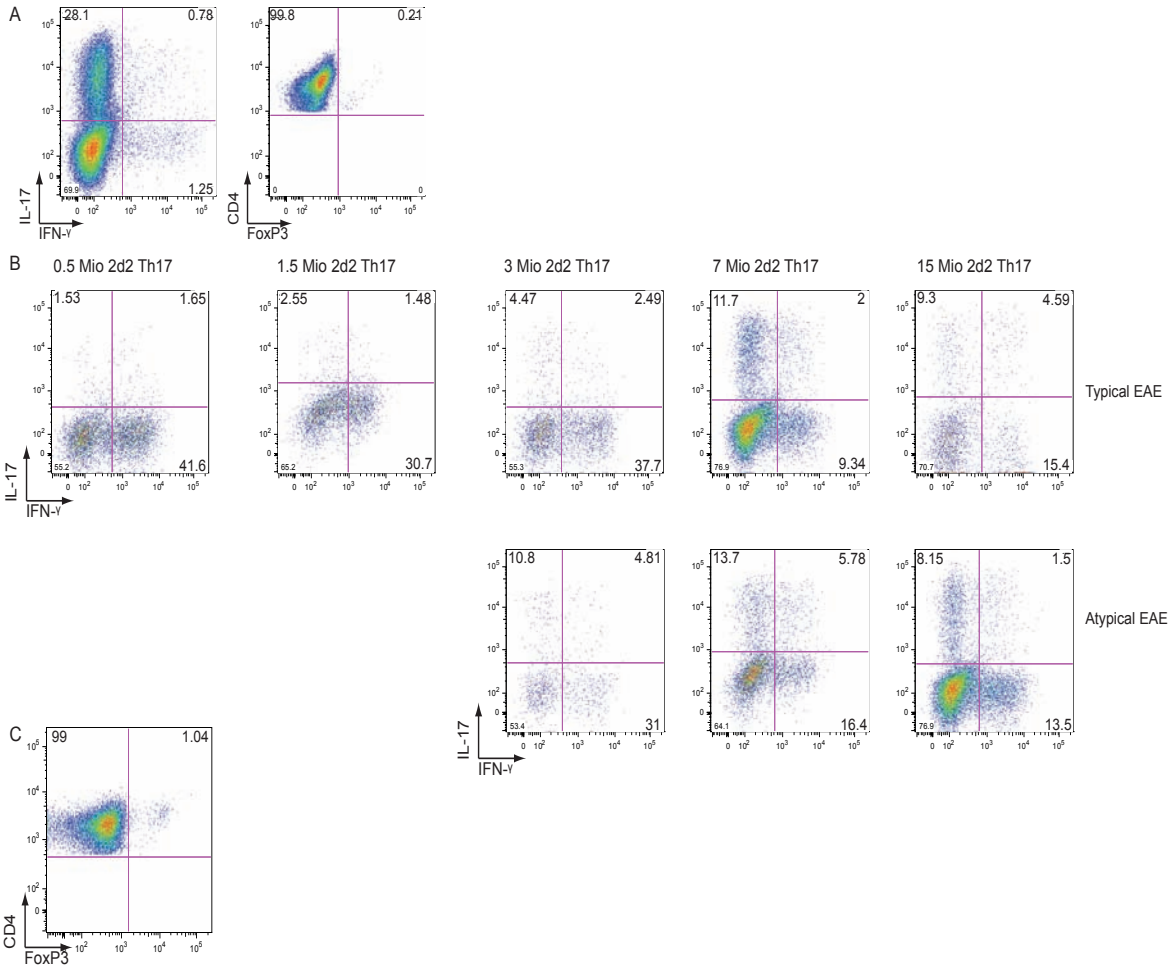


Figure 11. Immune cells isolated from the CNS of Rag1$^{-/-}$ mice adoptively transferred with different numbers of 2d2 CD4+ Th17 cells after 2nd restimulation. (A) Pre-transfer cytokine expression of 2d2 CD4+ Th17 cells on day 3 after the 2nd restimulation. (B) Flow cytometric data from Rag1$^{-/-}$ mice that were transferred with 0.5 Mio, 1.5 Mio, 3 Mio, 7 Mio and 15 Mio 2d2 Th17 cells, respectively. IL-17- vs. IFN-γ-producing CD4+2d2TCR+ T cells are shown in mice with typical and atypical disease. (C) Representative dot plot of FoxP3 expression of CD4+2d2TCR+ T cells isolated from the CNS. Dot plot shows FoxP3 expression in the CNS of a Rag1$^{-/-}$ mouse adoptively transferred with 15 Mio 2d2 CD4+ Th17 cells. Data from one representative mouse each are shown.

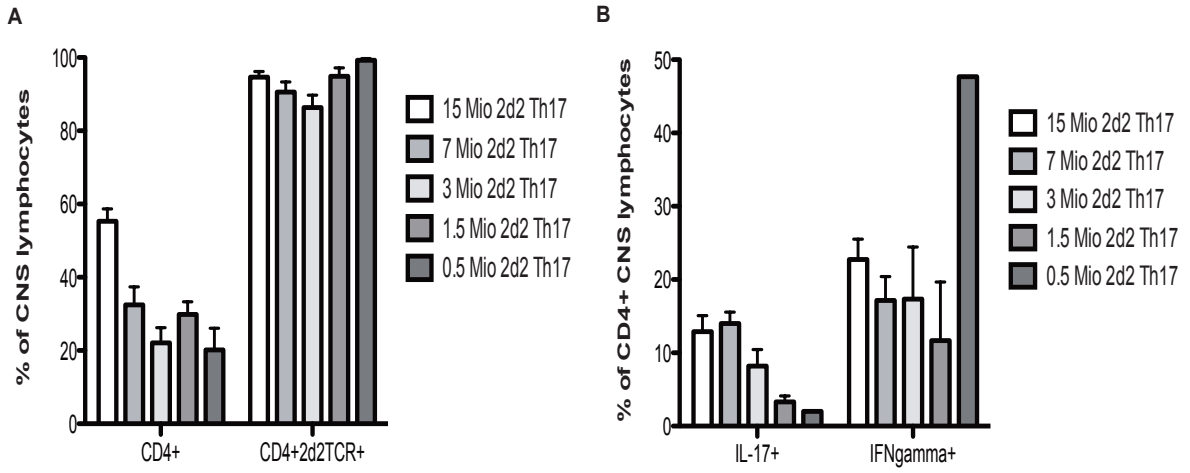


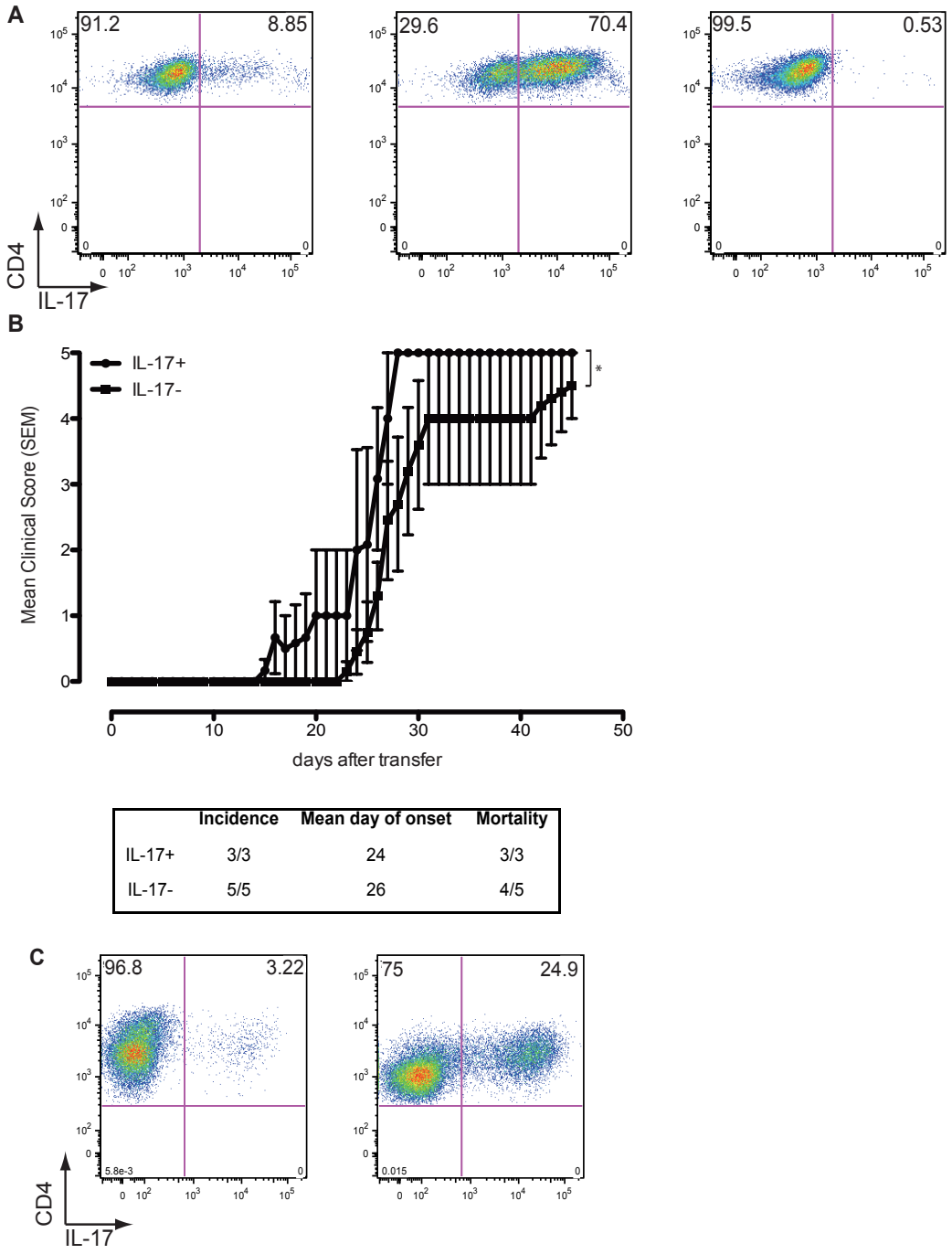
Figure 12. Immune cells isolated from the CNS of Rag1$^{-/-}$ mice adoptively transferred with different numbers of 2d2 CD4+ Th17 cells after 2nd restimulation. (A) Percentages of CD4+ and CD4+2d2TCR+ cells isolated from the CNS of sick mice. (B) Cytokine profiles of CD4+2d2TCR+ T cells isolated from the CNS. Hardly any IL-17+IFN-γ double producers and FoxP3+ T cells could be detected. Data from one representative experiment of three are shown, and analysed by repeated measures ANOVA: $p<0.05$ was considered significant; $p>0.05$ and not significant.

3.1.1.4 Impact of IL-17 production of CD4+ Th17 cells on their potential to induce EAE

Generally, 15-50 % of the cultivated 2d2 CD4+ Th17 cells produced IL-17 after second restimulation. Although there was a variability in the amount of IL-17-secreting cells generated, the number of IL-17-producers was always sufficient to induce disease in lymphocyte deficient recipients, which indicates that rather not the absolute number of IL-17 producers was relevant but the absence of FoxP3-expressing and IFN-γ-expressing cells. In C57BL/6 mice the amount of cells and their activation status seemed to be more important than the percentage of IL-17-secreting cells transferred.

To actually investigate the influence of the amount of IL-17-producers on the disease course, an IL-17 secretion assay was performed. A commercially available secretion assay (IL-17A secretion assay, Miltenyi Biotec) made it possible to highly enrich CD4+ IL-17-secreting T cells and separate them from the non- or less-secreting CD4+ T cells. The secretion assay was performed with 2d2 CD4+ Th17 cells before 2nd restimulation. The CD4+ T cells were stimulated with APC to initiate IL-17 secretion. IL-17-secreting cells were labelled with a catch reagent that recognises the IL-17 producers in a mixture of cells and was provided with the assay. This was followed by a second two-step labelling procedure where a biotinylated

detection antibody was added that was in turn detected with magnetically labelled streptavidin-PE beads. The labelled IL-17-secreting cells were separated from the non-IL-17-secreting cells in a magnetic field. After performing the IL-17 secretion assay, the cells from the positive fraction, which basically contains the enriched IL-17-producers, as well as the negative fraction were cultivated for three days before transfer with anti-CD28. Both, cells from positive and negative fraction, were transferred into $Rag1^{-/-}$ mice in equal cell numbers. The enriched IL-17-secreting T cells (>70 % IL-17A) and IL-17-depleted (<1 % IL-17A) 2d2 Th17 cells were transferred à 1.6 Mio into $Rag1^{-/-}$ mice (Figure 13A). Mice in both groups developed clinical EAE, both fulminant, severely progressive EAE that ended in death for all animals. Animals in both groups started developing disease at almost the same time (Figure 13B). Upon re-isolation from the CNS of the sick mice, T cells showed diminished numbers of IL-17- secreting CD4+ T cells (<70 % IL-17A) whereas the non-IL-17-secreting CD4+ T cells showed a slight gain in numbers (>1 % IL-17A) (Figure 13C). Due to technical difficulties at that time point a staining for IFN-γ was not performed.



	Incidence	Mean day of onset	Mortality
IL-17+	3/3	24	3/3
IL-17-	5/5	26	4/5

Figure 13. Influence of IL-17 secretion on the potential of MOG-specific CD4+ to induce EAE. (A) MOG-specific CD4+ Th17 cells prior to 2nd restimulation were used for the IL-17 secretion assay (left). After the IL-17 secretion assay two cell fractions were won: the enriched IL-17-secreting cells (middle) and the non-IL-17-secreting cells (right). (B) Disease course of Rag1$^{-/-}$ mice after transfer of enriched IL-17-secreting cells (IL-17+) and non-IL-17-secreting cells (IL-17-). (C) Lymphocytes isolated from the CNS of a mouse transferred with the enriched IL-17-secreting cells (right) and the non-IL-17-secreting cells (left). Data are shown as Mean+/-SEM, from one representative experiment and analysed by Mann-Whitney-U test: $p<0.05$ was considered significant, * $p<0.05$.

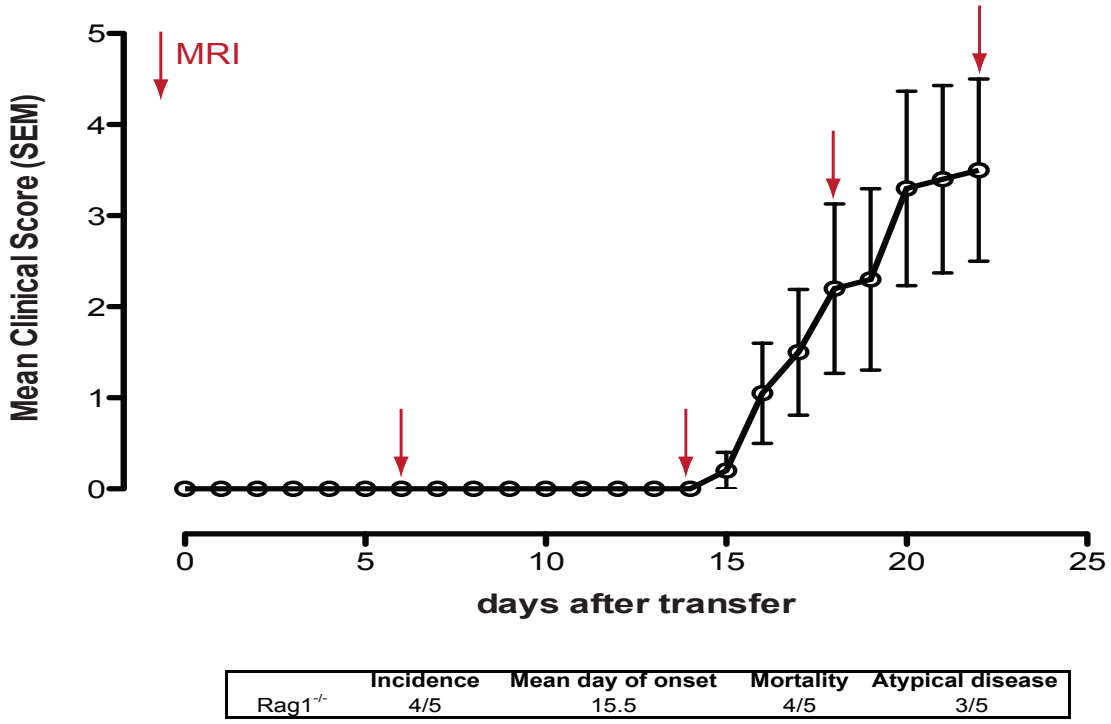
3.1.2 Application for the *in vitro generated* stable encephalitogenic MOG-specific CD4+ Th17 cells *in vivo*

3.1.2.1 Magnetic Resonance Imaging in adoptive transfer EAE

The disruption of the blood-brain barrier (BBB) is regarded as a critical step in the pathogenesis of inflammatory diseases of the CNS like MS and EAE. Encephalitogenic CD4+ T cells that are activated *in vitro* can transmigrate the healthy BBB into the CNS and start a cascade of destruction leading to demyelination and axonal damage (111,162). A better comprehension of the mechanisms involved in the migration of T cells across the BBB will help to understand many aspects of chronic inflammatory diseases. Magnetic resonance imaging (MRI) enhanced with gadopentetate dimeglumine (Gd-DTPA) is the gold standard for defining disease activity in MS patients today. However, BBB disruption does not necessarily reflect clinical disability. Animal models are not only necessary to investigate the BBB breakdown but also to examine what is responsible for inflammation as with animal models the *in vivo* studies can be correlated with histopathological findings. Moreover, MRI is more sensitive in detecting disease severity compared with clinical examination, which in rodents predominantly assesses spinal cord pathology (163).

The potential of the MOG-specific CD4+ Th17 cells generated with the previously described protocol to induce EAE was more closely investigated utilising MRI. $Rag1^{-/-}$ animals were transferred with 8 Mio 2d2 CD4+ Th17 cells after 2^{nd} restimulation and monitored for clinical signs of disease daily. MRI scans were performed with or without contrast agent firstly to get a baseline before transfer and on the following days after transfer: 6, 14, 18 and 22.

After adoptive transfer of the 2d2 CD4+ Th17 cells 4 of 5 $Rag1^{-/-}$ mice developed clinical signs of EAE. The first symptoms arose on day 15. In the $Rag1^{-/-}$ mice disease started with loss of tail tonus and impaired righting reflex. As previously described the $Rag1^{-/-}$ mice developed a fulminant and severely progressive EAE and some of them had to be sacrificed for histological analysis before the last MRI (Figure 14).



	Incidence	Mean day of onset	Mortality	Atypical disease
Rag1$^{-/-}$	4/5	15.5	4/5	3/5

Figure 14. Disease course of Rag1$^{-/-}$ mice after being adoptively transferred with 8 Mio MOG-specific CD4+ Th17 cells after second restimulation for MRI. Adoptive transfer of MOG-specific CD4+ Th17 cells led to fulminant and severely progressive EAE in Rag1$^{-/-}$ mice. Red arrows indicate the days that the animals were scanned in the MRI. Data are shown as Mean+/-SEM, from one experiment.

Lesion development was assessed by the presence of Gd-DTPA directly after contrast agent application. In almost all Rag1$^{-/-}$ animals Gd-DTPA leakage was observed prior to the development of clinical symptoms. In contrast, in a non-sick C57BL/6 wild type animal that was used as a control no Gd-DTPA leakage was visible (Figure 15). In order to correlate the Gd-DPTA leakage with the infiltration of cells to the brain, a histological analysis was performed.

To get an overview over the infiltration of cells, a hematoxylin and eosin stain was performed. For this, 10 µm thick coronal or axial sections of the brains of the animals in the experiment were prepared and stained. Correlating to the Gd-DPTA leakage, infiltrations of

cells were visible in the corresponding sections of the brain (Figure 16).

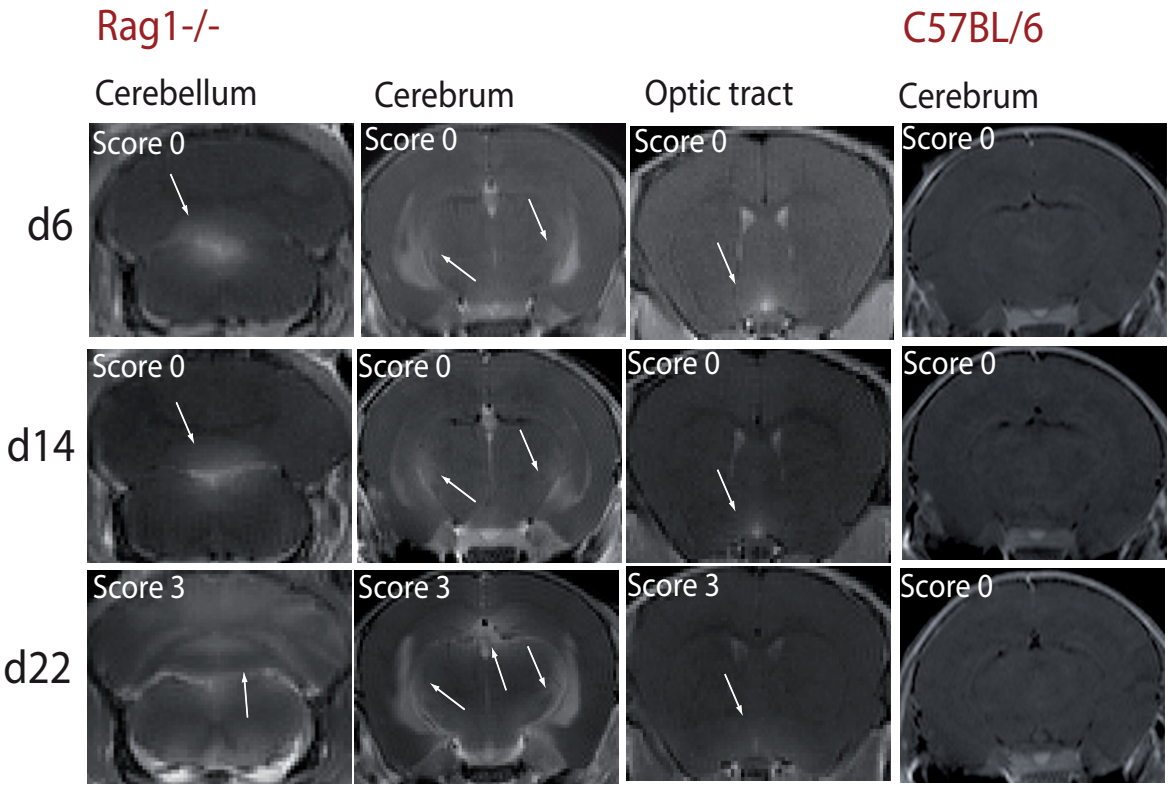


Figure 15. T1-weighted images immediately after Gd-DTPA administration taken at different time points (day 6, 14 and 22) after adoptive transfer of 2d2 CD4+ Th17 cells into Rag1$^{-/-}$ mice. A non-sick C57BL/6 wild type mice was used as a control throughout the experiment. In the Rag1$^{-/-}$ mice, Gd-DTPA leakage is detectable throughout the disease course in different regions of the brain. The BBB breakdown can be visualised prior to onset of clinical signs of disease. In the C57BL/6 mice no Gd-DTPA leakage is detectable throughout the observation period. Data shown are from one representative experiment of one Rag1$^{-/-}$ and one C57BL/6 mouse. Gd-DTPA leakage is indicated with the arrows.

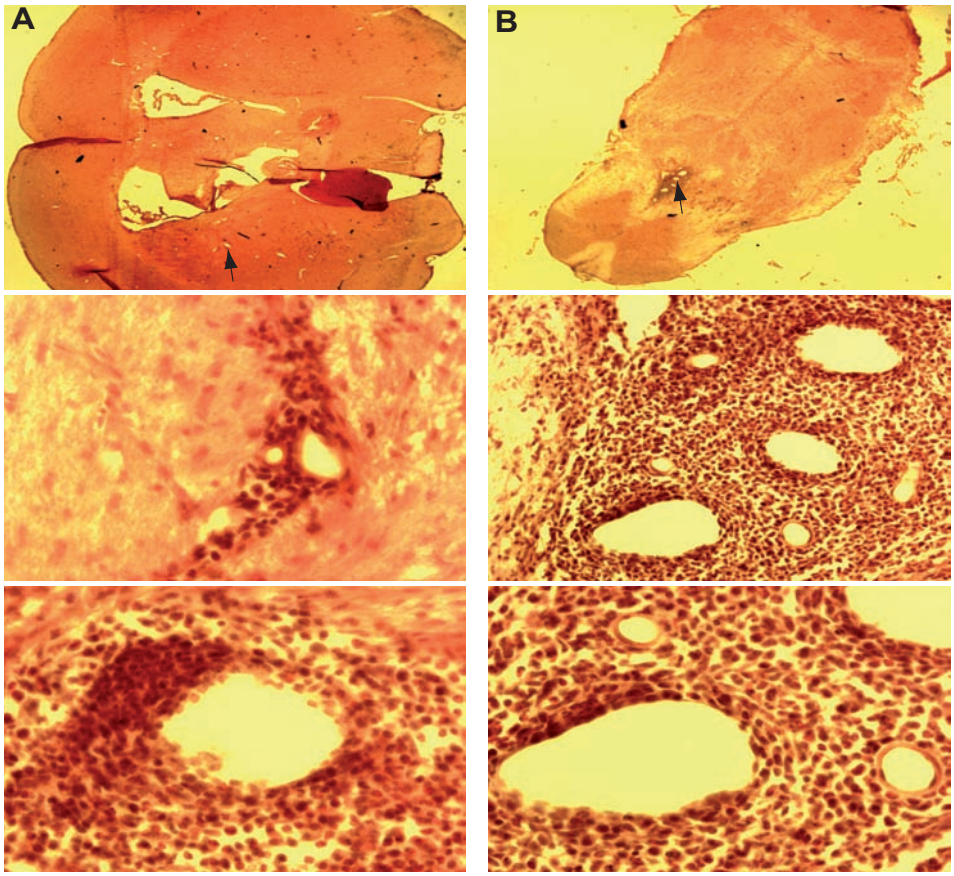


Figure 16. Coronal (A) and axial (B) histological section of brains of Rag1$^{-/-}$ mice that were adoptively transferred with 2d2 CD4+ Th17 cells and measured in the MRI. The sections were stained with hematoxylin and eosin to visualise infiltrating cells. In the upper panel, an overview over the coronal and axial section is shown, respectively. The middle and lower panel show magnifications of cell infiltrates.

3.1.2.2 Intravital microscopy in chronic neuroinflammation

Since the first application of two-photon microscopy ten years ago, the number of studies using this advanced technology has increased dramatically. The two-photon microscope allows long-term visualisation of cell motility in the living tissue with minimal phototoxicity (147) and has not only led to a better understanding of generation and priming of many immune cells, but also of the basics of immune regulation.

With help of the two-photon microscopy questions on how encephalitogenic T cells get access to the CNS and how these T cells interact with resident cells to initiate inflammation can be answered. Utilising two-photon microscopy, our group revealed a highly dynamic nature of immune cells in inflammatory infiltrates that form the characteristic perivascular cuffs. This observation stood in contrast to the general belief that vessel cuffs are the result of limited lymphocyte motility in the CNS (149). Siffrin et al. could show that lymphocyte

motility is an actively promoted mechanism, which can be blocked by CXCR4 antagonism, and that in vivo interference with CXCR4 in EAE disrupted dynamic vessel cuffs and resulted in tissue-invasive migration. The focus of the two-photon microscopy work did not only lie on effector T cells but also naïve T cells were looked on more closely (146). It could be shown by Herz et al. that naïve T cells cannot find sufficient migratory signals in healthy, non-inflamed CNS parenchyma which stands in contrast to the high motility of naïve CD4+ T cells in lymphoid organs. A highly motile migration pattern for naïve T cells as compared to effector CD4+ T cells in inflamed brain tissue of living EAE-affected mice was observed highlighting the versatile potential the two-photon microscope has to offer.

Neuronal damage in autoimmune neuroinflammation is the correlate for long-term disability in MS patients (164). A number of potential triggers of neurodegeneration in inflammatory CNS disease have been suggested, e.g., bystander damage caused by excitotoxicity, reactive oxygen species, myelin breakdown products, or the death ligand TRAIL (47,148,165). The mechanisms involved in vivo, however, remain largely unknown. Our group could recently show that MOG-specific CD4+ Th17 cells can directly interact with neuronal cells in demyelinating lesions which was associated with extensive axonal damage (148) highlighting the central role of the Th17 cell effector phenotype for neuronal dysfunction.

The role of immune-neuronal interaction was investigated *in vivo* in EAE by two-photon laser scanning microscopy (TPLSM). To specifically address the role of encephalitogenic CD4+ T cells in the context of CNS damage processes, red fluorescent MOG-specific CD4+ Th17 cells generated with previously described protocols were utilised. Lymphopenic (B6.Rag1$^{-/-}$Thy1.21.EGFP) recipient mice received 5 Mio 2d2.tdRFP Th17 cells which resulted in a severe, non-remitting EAE (Figure 17A and 17B). Two-photon imaging was performed in living anaesthetised mice, which were continuously monitored for vital signs. Automated cell tracking revealed the highly dynamic and tissue-invasive nature of the encephalitogenic 2d2.tdRFP Th17 cells in adoptive transfer EAE (Figure 17C and 17D). Quantitative analyses of the immune dynamics of 2d2.tdRFP Th17 cells demonstrated a differential motility pattern in different disease phases. 2d2.tdRFP Th17 cells slowed down with clinical worsening and disease progression (Figure 17E) to reduced mean velocities in the peak and chronic disease phase. Acute axonal damage in 2d2.tdRFP Th17 cell-induced B6.Rag1$^{-/-}$Thy1.21.EGFP EAE and active EAE in B6.tdRFP/B6.Thy1.EGFP was assessed with TPLSM. For this, the extent of remaining EGFP-positive voxels in the EAE lesions, which correlates with neuronal cell density (166) was analysed. There was a substantial

decrease of remaining neuronal tissue as defined by EGFP loss, which indicates stronger neurodegeneration in the presence of 2d2 Th17 cells (Figure 17F).

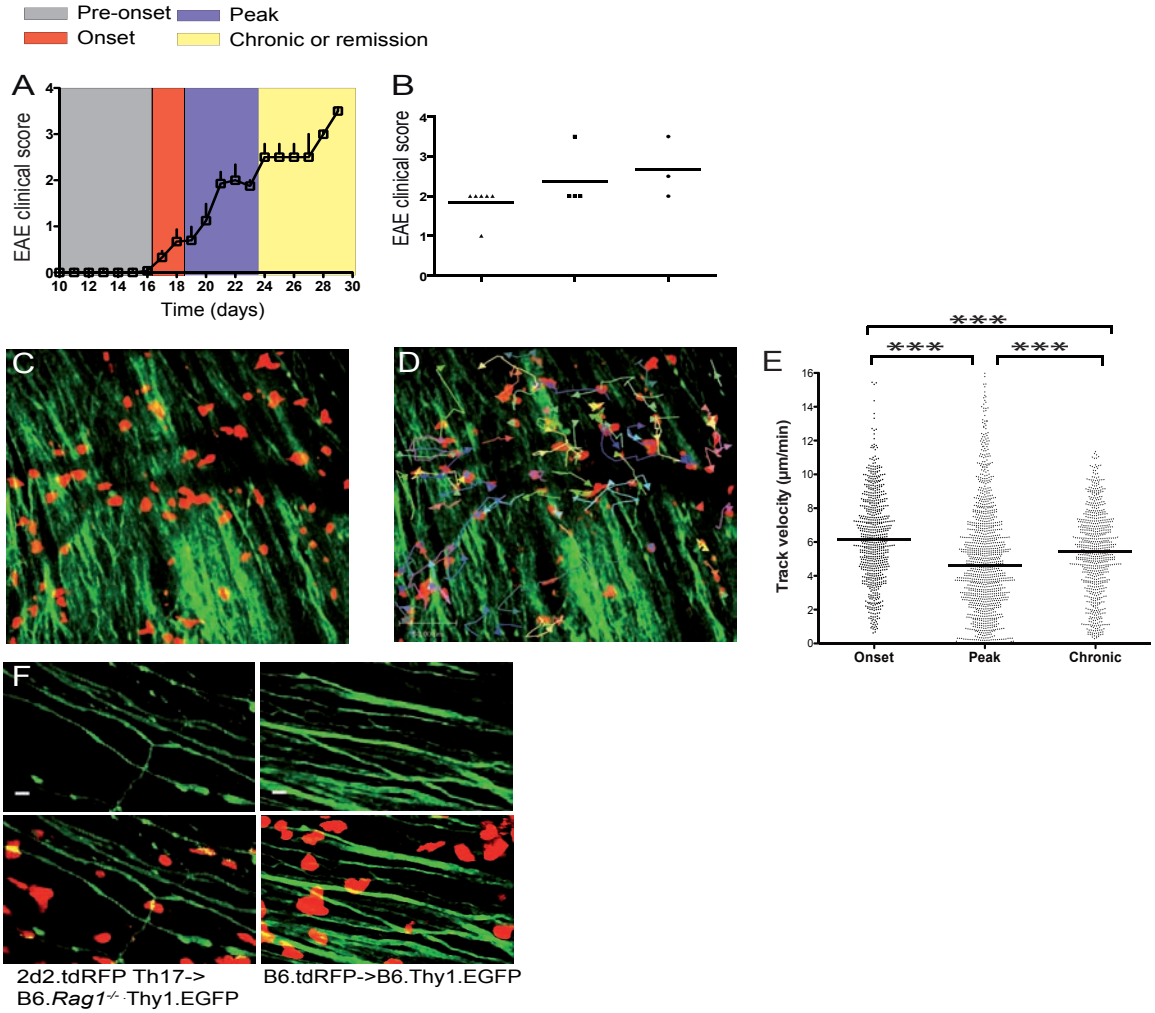


Figure 17. Visualisation of CD4+ 2d2.tdRFP Th17 cells (red) in the brainstem of diseased B6.Rag1$^{-/-}$ Thy1.21.EGFP mice. (A) Mean clinical scores (±SEM) of 2d2.tdRFP Th17 cell-induced adoptive-transfer EAE in B6.Rag1$^{-/-}$Thy1.21.EGFP mice; pre-onset (gray), onset (red), peak (blue), and chronic or remission (yellow). (B) Clinical EAE scores of individual mice investigated by intravital TPLSM at the day of TPLSM for 2d2.tdRFP Th17 cells-induced EAE in B6.Rag1$^{-/-}$Thy1.21.EGFP mice (C) TPLSM of a representative brainstem lesion in the onset of 2d2.tdRFP Th17 cells (red)-induced EAE in B6.Rag1$^{-/-}$Thy1.21.EGFP (green axons) mice. (D) Automated single-cell tracking of 2d2.tdRFP Th17 cells. (E) Mean track velocity (±SEM) of 2d2.tdRFP Th17 cells in adoptive transfer EAE: onset: 6.30 ± 0.1 mm/min, n = 680; peak: 5.02 ± 0.1 mm/min, n = 1167; chronic disease phase 5.36 ± 0.1 mm/min, n = 708. (F) Comparison of loss of axons quantified by reduction of EGFP positive voxels (as measured by intravital TPLSM) in EAE lesions of B6.tdRFP/B6.Thy1.EGFP and 2d2.tdRFP Th17 cell-induced EAE lesions in B6.Rag1$^{-/-}$Thy1.EGFP. Adapted from Siffrin et al. (148).

3.1.3 MOG-specific CD4+ Th17 cells vs. MOG-specific CD4+ Th1 cells in chronic neuroinflammation

While it is well established that T cells with specificities for CNS antigens are crucial for the induction of EAE, it is still widely discussed which T cell subpopulations are the "villains" in the initiation of autoimmunity. It is generally accepted that T cells with a Th1 phenotype transfer EAE whereas Th2 cells were found incapable of doing so. Until recently Th1 cells were regarded as essential initiators of EAE as they secrete IFN-γ in large amounts. IFN-γ has been detected in inflammatory lesions (59) and was regarded as a key molecule in autoimmunity until it was shown that IFN-γ-deficient mice were not only resistant but also even more susceptible to EAE (60). Herewith, the doubt about the role of Th1 cells in EAE began and let to the discovery of another subpopulation of T cells: the Th17 cell. There are many studies about Th1 vs. Th17 cells and while some show that Th1 but not Th17 cells induce EAE others claim that Th17 cells are more pathogenic than Th1 cells.

It has been demonstrated in the sections above that the MOG-specific naïve-derived Th17 cells reliably induce a strong disease phenotype. In addition, our group previously demonstrated that IL-17A expression is associated with a strongly pathogenic, neurodegenerative phenotype that is also found in classic (Th1/Th17) active EAE (148). Moreover, it was shown, also in the sections above, that the transferred Th17 cells had shifted partly to IFN-γ producers ("ex-Th17") when re-isolated from the CNS of EAE affected animals in adoptive transfer EAE. Nevertheless, the question of the encephalitogenicity of completely *in vitro*-differentiated 2d2 Th1 cells is still open. Therefore 2d2 Th1 cells were generated similar to the 2d2 Th17 cells with naïve-sorted transgenic T cells (2 restimulations in vitro with IL-12 and/or IL-18). Pre-transfer cytokine expression showed that the *in vitro* generated 2d2 Th1 cells produced high amounts of IFN- γ and TNF-α (Figure 18B). The cells were able to induce disease lymphopenic hosts ($Rag1^{-/-}$) and, the disease course was very mild when compared to adoptive transfer EAE induced with 2d2 Th17 cells which showed rapid onset and a very severe disease course (Figure 18A). When the Th1 cells were re-isolated from the CNS of the diseased $Rag1^{-/-}$ animals a reduced production of IFN-γ was observed but no IL-17 production was detected (Figure 18C and 18D).

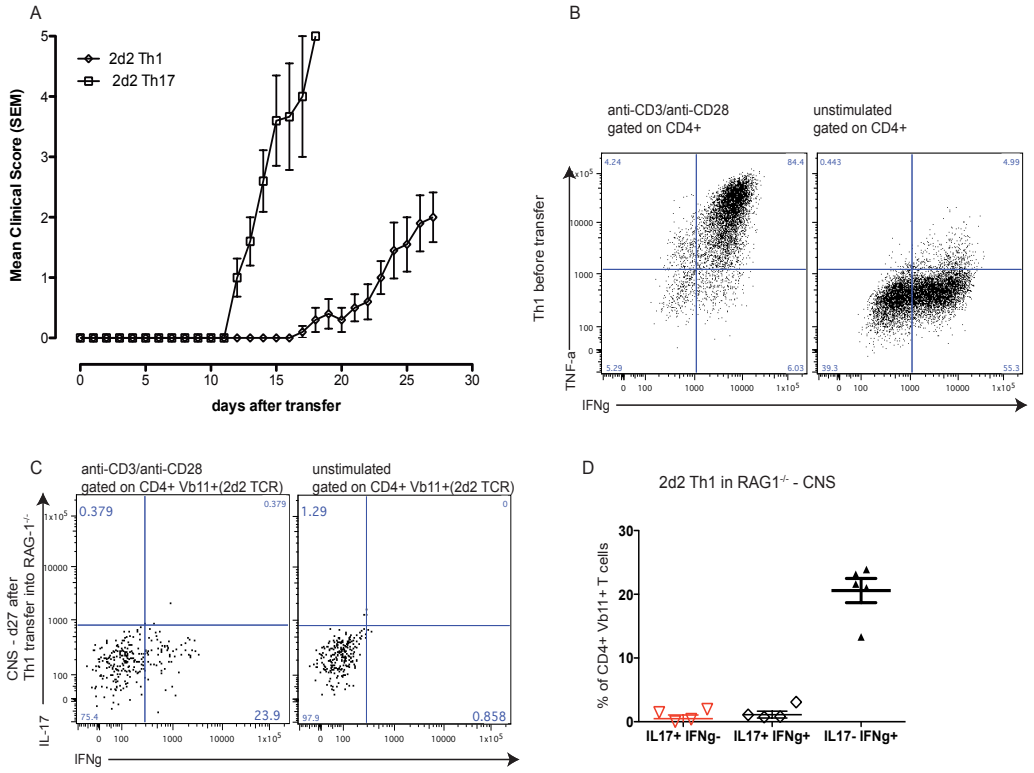
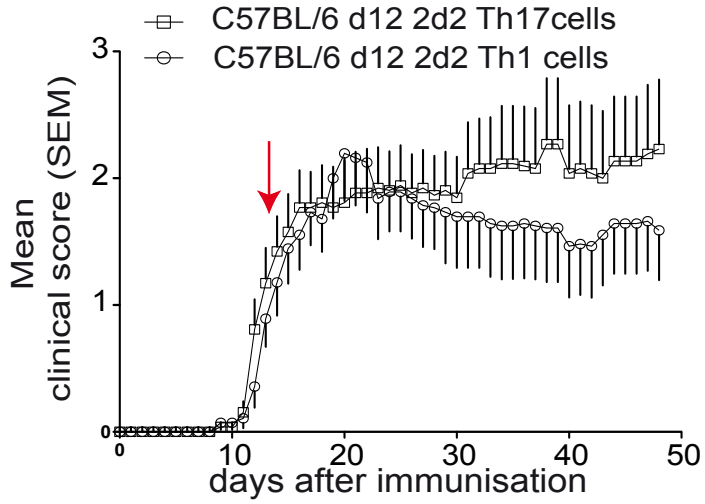


Figure 18. 2d2 Th1 vs. 2d2 Th17 transfer EAE in Rag1$^{-/-}$ mice. (A) 2d2 Th1 cells were transferred into Rag1$^{-/-}$ mice. 2d2 Th17 transfer into Rag1$^{-/-}$ mice was used as a positive control. (B) 2d2 Th1 cultures were prepared analogously to the 2d2 Th17 cultures with the following differences: instead of IL-23, IL-6 and TGF-β, IL-12 and IL-18 in the presence of anti-IL-4 were used. Similarly, as with 2d2 Th17 cells, CD4+CD62Lhi-sorted naïve 2d2 T cells were the starting point. 2d2 Th1 cells were restimulated twice and yielded high IFN-γ production already in unstimulated FACS analysis, increasing strongly upon anti-CD3/anti-CD28 stimulation and co-expressing the pro-inflammatory cytokine TNF-α. IL-17 was not detected over the whole culture period. (C) *Ex vivo* isolated and stimulated/unstimulated CNS-derived CD4+ T cells showed isolated IFN-γ production (IL-17 could not be detected). (D) Quantification of samples as in C from 5 animals.

3.1.3.1 Damage formation in the CNS by Th1 vs. Th17 cells in EAE

To investigate the role of Th1 and Th17 cells in chronic neuroinflammation more closely, *in vitro* generated 2d2 CD4+ Th17 and 2d2 CD4+ Th1 cells were transferred into actively immunized C57BL/6 mice after onset of clinical signs on day 12. Animals were observed for the development or remission of clinical signs over a period of 60 days. Over the observation period more of the actively immunized C57BL/6 mice that were transferred with the 2d2 Th1 cells showed remission of clinical signs. In contrast, the animals that were

transferred with the 2d2 Th17 cells exhibited a higher mortality rate. Overall, the transfer of 2d2 CD4+ Th17 cells promoted a non-remitting and more progressive disease in the immunised mice compared to the transfer of 2d2 CD4+ Th1 cells (Figure 19).



	T cells transferred (day 12)	
	2d2 Th1 cells (n=14)	2d2 Th17cells (n=13)
Remission	9/12 (75%)	6/11 (54.5%)
Mortality	1/14 (7.1%)	3/13 (23.1%)
Cumulative score (day 51)	66.37	77.9

Figure 19. Disease course of actively immunised C57BL/6 mice being adoptively transferred with 7.5 Mio 2d2 Th1 versus 7.5 Mio 2d2 Th17 cells on day 12 (after onset of clinical signs), respectively. Animals were observed for 50 days for the development or remission of clinical signs. Red arrow indicates the day that the animals were adoptively transferred with the transgenic T cells. Data are shown as Mean+/-SEM, from two independent experiments and analysed by Mann-Whitney-U test: $p<0.05$ was considered significant, ** $p<0.01$. Adapted from Siffrin et al. (148).

3.1.4 Mechanisms underlying Th17-mediated pathology

The next objective was to identify mechanisms responsible for the pathologic Th17 accumulation inside the CNS. Proteomic and transcriptional analyses of multiple sclerosis lesions (137,167–169) revealed a modulation of the renin-angiotensin and the opposing kallikrein-kinin pathways, two pathways that are known mainly for their role in blood pressure regulation and are proposed to counterbalance each other (170). Our group recently demonstrated the presence of B1 mRNA on peripheral blood-derived lymphocytes from healthy donors as well as from patients with MS. So far, several *ex vivo* correlative studies

have established a possible link between the kinin system and the autoimmune reaction in MS and EAE (171,172).

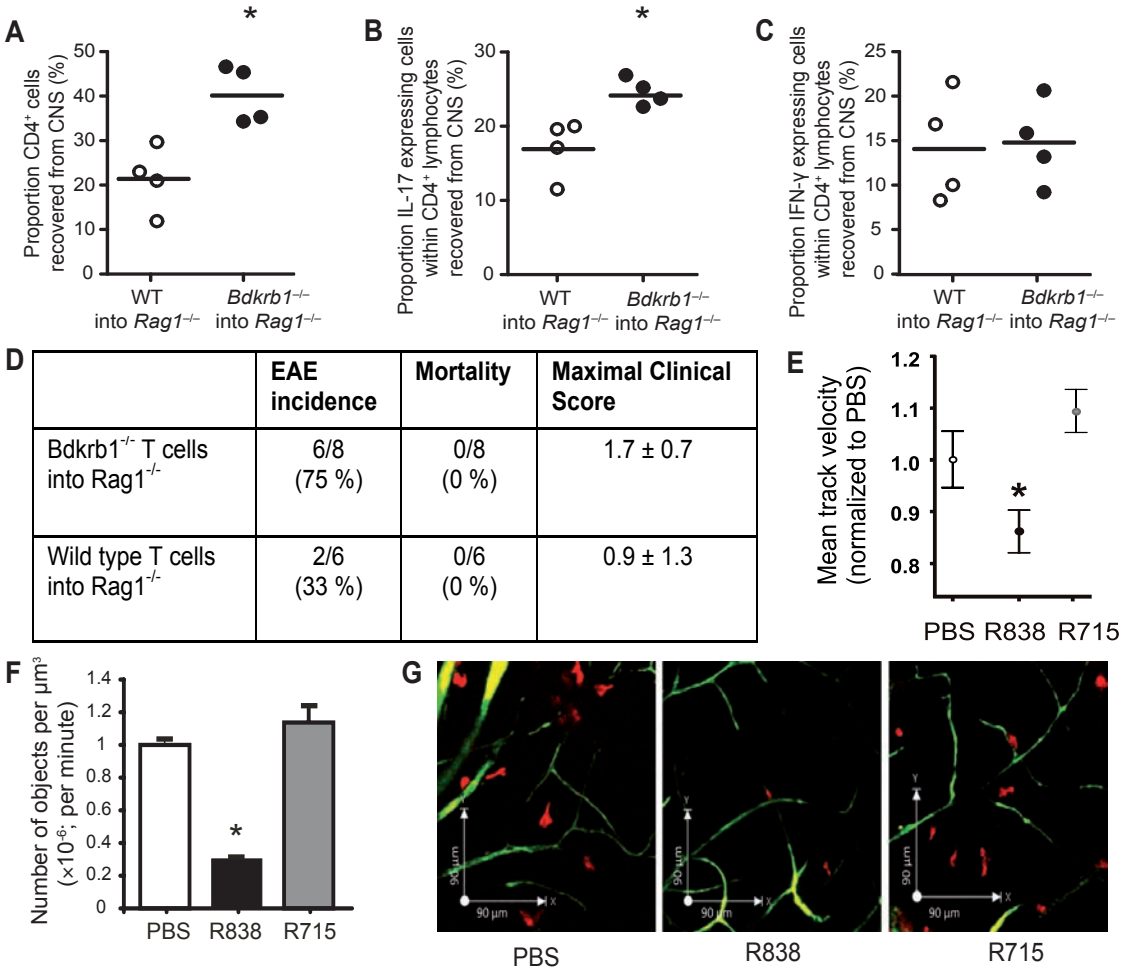
Immunohistochemical investigation of the blood brain barrier showed the pronounced upregulation of the B1 receptor in brains from MS patients, but not in healthy controls (173). In a further study, our group simultaneously compared kinin B1 receptor mRNA levels on peripheral CD3+ T lymphocytes with dynamic clinical and MRI measures in MS patients, and found that B1 expression is correlated positively with neurological disability, clinical relapses, expression of the IL-2 receptor (CD25) and MHC class II molecules on CD4+ T lymphocytes (174). Thus, the role of kinin B1 receptor was investigated more closely in chronic neuroinflammation.

3.1.4.1 The role of kinin receptor B1 in the recruitment of Th17 cells in EAE

Kinins belong to a family of bioactive octa- to decapeptides generated from kininogens in a stepwise cleavage process (175). Kinins exert their biological activities by activating the two pharmacologically different heterotrimeric G-protein coupled receptors, B1 and B2. B2 is constitutively found on a number of cells, such as vascular endothelial cells or macrophages and predominantly mediates homeostatic responses. B1 expression is under the control of the pro-inflammatory transcription factor NF-κB and is up-regulated in the context of inflammation only (176,177). So far, limited attempts to investigate the expression of kinin receptors on lymphocytes have been mostly based on the analysis of lymphocyte responses to *in vitro* stimulation with exogenous kinins. These early studies (178) already suggested the presence of the B1 receptor on T lymphocytes.

Encephalitogenic Bdkrb1$^{-/-}$ T cells and wild type T cells were generated by immunising either Bdkrb1$^{-/-}$ or C57BL/6 mice with 200 µg MOG_{35-55} with subsequent administration of 200 ng PTx. After twelve days, the draining lymph nodes and spleen were removed, depleted of CD8+ T cells by MACS and re-stimulated for 72 hours with 15 µg/ml MOG_{35-55}. Those encephalitogenic T cells were then transferred à 10 Mio cells/mouse intravenously into Rag1$^{-/-}$ recipient mice. More CD4+ T cells were found in the CNS when encephalitogenic Bdkrb1$^{-/-}$ T cells were injected as opposed to wild type T cells (Figure 20A). Animals that received the Bdkrb1$^{-/-}$ had a higher disease incidence. 75 % of the Rag1$^{-/-}$ mice injected with Bdkrb1$^{-/-}$ T cells developed symptoms of disease but only 33 % of the animals that were

transferred with wild type CD4+ T cells showed signs of EAE (Figure 20D). The proportion of CD4+ IL-17-secreting T cells was greater in the sick mice that received Bdkrb1$^{-/-}$ T cells, whereas the proportion of IFN-γ-producing T cells was comparable in mice that got Bdkrb1$^{-/-}$ T cells and wild type T cells, respectively (Figure 20B and 20C). To demonstrate an influence of Bdkrb1 signalling on the migration pattern of Th17 cells, fluorescence-labelled CD4+ Th17 lymphocytes were treated with the Bdkrb1 modulators mentioned above or vehicle before allowing them to infiltrate into syngeneic hippocampal slice cultures (152). Two-photon microscopy analysis revealed a lower mean velocity (Figure 20E) and reduced infiltrative behaviour upon Bdkrb1 activation (Figure 20F and 20G). Taken together, these results suggest that the kallikrein-kinin system is involved in the regulation of CNS inflammation, limiting encephalitogenic T lymphocyte infiltration into the CNS.



	EAE incidence	Mortality	Maximal Clinical Score
Bdkrb1$^{-/-}$ T cells into Rag1$^{-/-}$	6/8 (75 %)	0/8 (0 %)	1.7 ± 0.7
Wild type T cells into Rag1$^{-/-}$	2/6 (33 %)	0/6 (0 %)	0.9 ± 1.3

Figure 20. (A-C) Bdkrb1 activation primarily targets the invasion of Th17 cells. (A) Proportions of CD4+ T cells in the CNS and (B,C) of IL-17+ and IFN-γ+ cells within the CD4+ T cell population in Rag1$^{-/-}$

recipient mice with adoptive transfer EAE induced by injection of Bdkrb1$^{-/-}$ or wild type T cells. (D) EAE disease course data. EAE incidence, mortality and maximal clinical score shown as Mean± s.d. are displayed. (E) Th17 cells incubated with Bdkrb1 agonist R838, antagonist R715 or vehicle (PBS) were applied to hippocampal slice cultures for analysis with TPLSM. Mean track velocity per single cell (n=121 for PBS, n=71 for R838, n=105 for R715) was calculated. Shown are mean ± SEM; *p<0.05, Mann-Whitney-U-test. (F,G) Activation of Bdkrb1 decreased the average number of Celltracker Orange–labeled Th17 cells after application to hippocampal slice cultures for TPLSM. (F) The average number of T cells per minute and per defined volume (between 60–120 mm depth) over time is shown, including quantification and (G) representative overviews. Data shown are Mean±SEM; *p<0.05, Mann-Whitney-U-test. Adapted from Schulze-Topphoff et al. (137).

3.2 The role of Non-Central Nervous System-specific Th17 cells in Experimental Autoimmune Encephalomyelitis

In Multiple sclerosis, the clinical disease course is highly variable (11). Typically MS patients present with a relapsing pattern of acute exacerbations followed by periods of stability. Exacerbations can be triggered by exogenous events, e.g. viral or bacterial infections (11,15,19). They are two to three times more likely to occur during or shortly after common respiratory, gastrointestinal, or urological infections (15,17,179,180). Exacerbations in the context of systemic infection lead to more sustained damage (22). Bystander activation of resting encephalitogenic T cells or molecular mimicry in response to viral infection might be a possible pathways connecting infection to autoimmunity (11,20). In MS and its animal model, EAE, the invasion of encephalitogenic T cells through the blood-brain barrier (BBB) is considered an initiatory event of the autoimmune pathology. The role of non-CNS-specific T cells in the cellular neuropathology is less understood. Data suggests that activated Ovalbumin (OVA)-specific T cells are capable of entering the CNS after altering the BBB (111,112). They are also able to damage brain vascular endothelial cells in vitro (113). Presumably, in a compromised brain, such as in MS and EAE, bystander pertubations of the BBB caused by non-CNS-specific effector cells may have pathological consequences and contribute to disease aggravation like can be seen in patients suffering from an ordinary infection. This hypothesis will be investigated in more detail in the following part of the dissertation.

3.2.1 Investigating the role of non-CNS-specific T cells in the disease course in a chronic model of EAE

To investigate the role of differentiated (effector) non-CNS-specific T cells in chronic neuroinflammation, EAE was induced in C57BL/6 mice by standard immunisation protocol with MOG_{35-55}. 25 days after disease onset green fluorescent OVA-specific CD4+ Th17 were injected intravenously into EAE mice. Additionally, some of the EAE mice were injected with MOG-specific CD4+ Th17 cells and PBS, respectively. The OVA- and MOG-specific CD4+ Th17 cells were generated with the previously established restimulation protocol and injected after 2nd restimulation. Independent of antigen specificity almost 50 % IL-17 producing cells were generated in culture. Prior to transfer no IFN-γ- or IL-10-producers were detected (Figure 21).

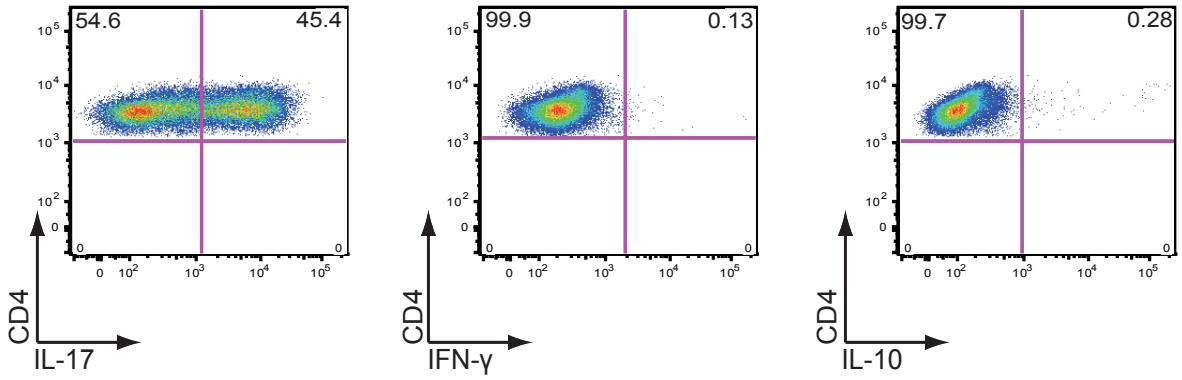
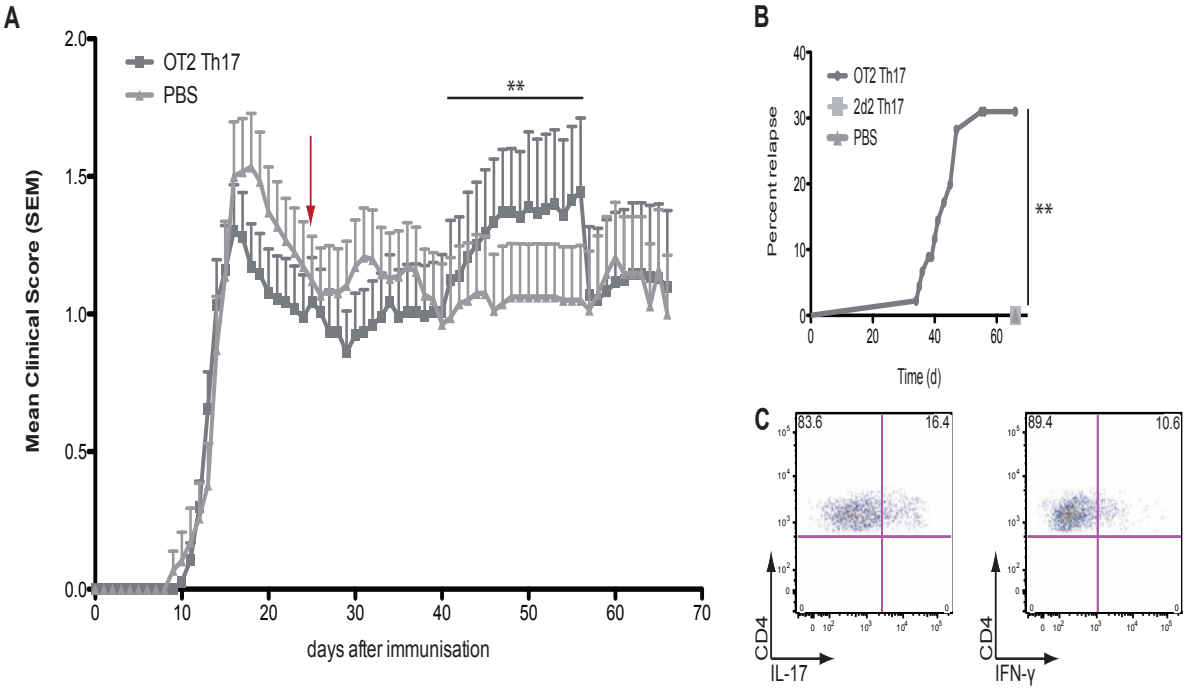


Figure 21. Naïve CD4+ T cells from OT2 mice were activated under Th17 polarising conditions and kept in culture for 17 days with two restimulations with fresh APCs at day 7 and day 14, respectively. Intracellular IL-17, IFN-γ and IL-10 cytokine expression was assessed by flow cytometry. Data shown are from one representative experiment of four independent experiments and are gated on the respective TCR-specific CD4+ population.

After the transfer of OVA- and MOG-specific CD4+ Th17 cells as well as PBS, the disease course was monitored over 40 days. The distribution, location and phenotype of the transferred cells were determined by flow cytometric analysis.

Results show that OVA-specific Th17 cells are in fact able to induce disease aggravation in EAE mice (Figure 22A). 30 % of the EAE mice injected with OVA-specific CD4+ Th17 cells in remission showed signs of disease exacerbation (Figure 22B). None of the animals injected with PBS developed a relapse. Interestingly, also none of the animals injected with

MOG-specific CD4+ Th17 cells showed signs of disease aggravation. First analysis revealed that OVA-specific CD4+ GFP+ Th17 cells were present in the CNS of relapsed mice. The ex vivo examination of these infiltrated cells demonstrated that they did not maintain their original effector phenotype but shifted to a Th1-like effector phenotype characterised by production of not only IL-17 but also IFN-γ (Figure 22C).



	Transfer	EAE incidence	Disease onset (range)	Max. Score	Max. Mean score	Relapse
EAE mice	20 Mio OT2 GFP Th17 cells	33/45 (74 %)	Day 9-18	5	2.4	Yes; Incidence: 12/45 (30 %)
EAE mice	200 µl PBS i.v.	22/29 (76 %)	Day 9-34	3	2.1	No

Figure 22. Influence of non-CNS-specific T cells on the disease course in a model of chronic EAE. (A) Disease course of EAE mice injected with OVA-specific CD4+ Th17 cells and PBS, respectively. The red arrow indicates the day of transfer of the aforementioned cells and PBS. Data are shown as Mean+/-SEM, from four independent experiments and analysed by Mann-Whitney-U test: $p<0.05$ was considered significant, ** $p<0.01$. (B) Occurrence of relapse in EAE mice after transfer of OVA-, MOG-specific CD4+ Th17 cells and PBS is presented in a Kaplan-Meier survival curve as percentage of relapse. Data shown are pooled from three independent experiments, and analysed by Log-rank (Mantel-Cox) test, $p<0.05$ was considered significant, ** $p<0.01$. (C) Flow cytometric analysis of lymphocytes derived from a mouse with relapse after transfer of OVA-specific CD4+ Th17 cells. IL-17- and IFN-γ-producing CD4+OT2TCR+ T cells are shown. Data from one representative mouse from four independent experiments is shown.

3.2.2 Impact of non-CNS-specific T cells in healthy animals

As demonstrated in the previous chapter, the *in vitro* generated OT2 CD4+ Th17 cells were able to induce relapses in chronically sick mice where the MOG-specific CD4+ Th17 cells failed to do so. In order to show that it was rather a targeted process, healthy C57BL/6 mice as well as lymphocyte deficient Rag1$^{-/-}$ mice were adoptively transferred with 20 Mio OT2 CD4+ Th17 cells after second restimulation. For the purpose of proving that the cells are not able to induce signs of disease by themselves, the healthy animals were observed for 50 days after transfer. During this observation time neither the C57BL/6 mice nor the Rag1$^{-/-}$ mice developed any signs of disease (Table 5). After the observation period some of the Rag1$^{-/-}$ mice were sacrificed and blood, spleen, lymph nodes and CNS were examined for the location of the OT2 CD4+ Th17 cells with help of flow cytometry. As the Rag1$^{-/-}$ mice are not able to generate T and B cells by themselves, chances to locate the transferred T cells seemed to be higher than in the C57BL/6 wild type mice. In the blood, spleen and lymph nodes of the healthy animals small numbers of OVA-specific CD4+ T cells were found (Figure 23).

Table 5. Overview of disease occurrence in healthy C57BL/6 and Rag1$^{-/-}$ mice after transfer of OT2 Th17 cells.

	Transfer	EAE incidence
C57BL/6	20 Mio OT2 Th17	0/10
Rag1$^{-/-}$	20 Mio OT2 Th17	0/10

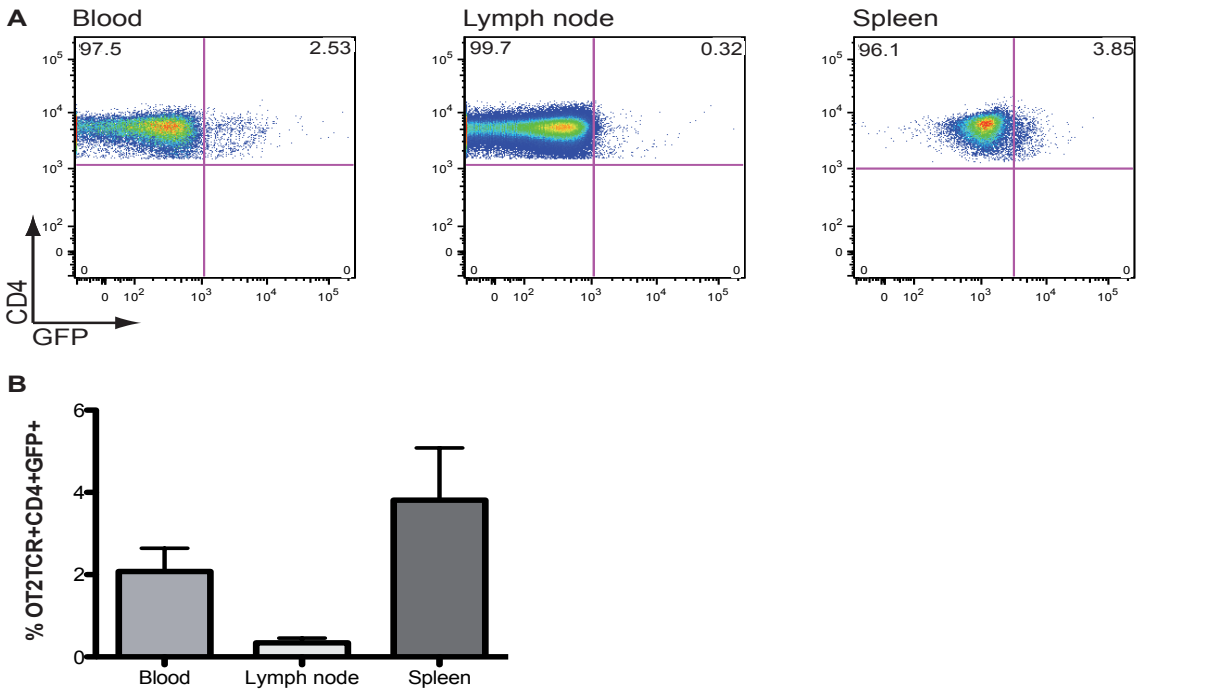


Figure 23. Non-CNS-specific Th17 cells in healthy Rag1$^{-/-}$ mice. (A) Flow cytometric analysis of lymphocytes derived from a healthy Rag1$^{-/-}$ mouse after transfer of OVA-specific CD4+ Th17 cells. CD4+OT2TCR+GFP+ T cells are shown. Data from one representative mouse is shown. (B) Percentage of OT2TCR+CD4+GFP+ cells derived from blood, lymph node and spleen from healthy Rag1$^{-/-}$ mice. Data from one representative experiment of two are shown.

3.2.3 Cross-reactivity of non-CNS-specific T cells

To examine the effect of CNS-specific antigens on the differentiation of the generated Ovalbumin-specific Th17 cells, naïve T cells were isolated from OT2 mice by magnetic sorting. The T cells were differentiated into Th17 effector cells and a proliferation assay was conducted with Ovalbumin$_{323-329}$, whole brain homogenate and the following CNS antigens: MOG$_{35-55}$, MBP$_{85-99}$ and PLP$_{139-151}$ with naïve OT2 CD4+ T cells and OT2 CD4+ Th17 cells upon 1st and 2nd restimulation. For this purpose, the OT2 Th17 cells were stimulated with either Concanavalin A **(**ConA) as a positive control for stimulation or APCs with the addition of the different antigens or whole brain homogenate and cultured for three days. ^{3}H was added to the culture on day 2. The effect of the added antigens on the proliferation of the Ovalbumin-specific Th17 cells at different differentiation time points was examined by measuring the amount of incorporated ^{3}H in a standard ^{3}H-thymidine proliferation assay 18 hrs after addition (Figure 24). The antigens as well as the whole brain homogenate were used in three different concentrations: 12.5 µg/ml, 25 µg/ml and 50 µg/ml. A proliferative effect on the OT2 Th17 cells was only observed when Ovalbumin$_{323-329}$ (OVA) was added or in the positive control with ConA independent of the differentiation status of the OT2 CD4+ Th17 cells. Little to no proliferation was observed when CNS-antigens or whole brain homogenate were added to the differently differentiated non-CNS-specific Th17 cells.

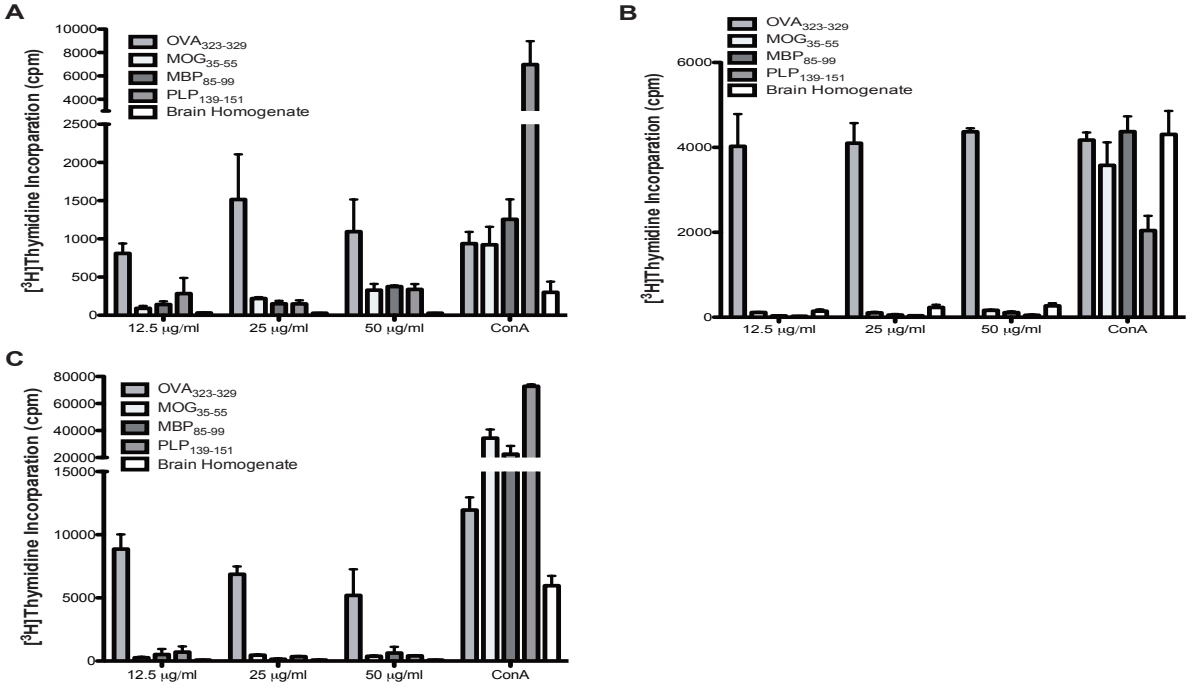


Figure 24. Murine transgenic Ovalbumin-specific CD4+ T cells stimulated in the presence of Ovalbumin$_{323-329}$ (OVA), MOG$_{35-55}$ (MOG), MBP$_{85-99}$ (MBP), PLP$_{139-151}$ (PLP) and whole brain homogenate (Brain Homogenate). (A) Naïve OT2 CD4+ T cells were cultured with either APCs with different antigens in different concentrations or with Concanavalin A **(**ConA) as a positive stimulation control. (B) OT2 CD4+ Th17 cells upon 1st restimulation were cultured with either APCs with different antigens in different concentrations or with Concanavalin A **(**ConA) as a positive stimulation control. (C) OT2 CD4+ Th17 cells upon 2nd restimulation were cultured with either APCs with different antigens in different concentrations or with Concanavalin A **(**ConA) as a positive stimulation control. The proliferative capacity of naïve and differentiated OT2 CD4+ T cells stimulated in the presence of OVA, whole brain homogenate and different CNS-antigens was analysed. ^{3}H was added to the culture and the amount of incorporated ^{3}H was measured on day 3. Data are shown as Mean+/-SEM and were analysed by unpaired t-test: ns not significant $p>0.05$.

3.2.4 Two-Photon Imaging in chronic neuroinflammation

In the context of investigating MOG-specific CD4+ Th17 cells and their interaction with neuronal cells (148), OVA-specific CD4+ Th17 cells were also analysed. They were used to specifically have a look at the interactions of immune cells and neurons. For this purpose, CNS-specific (2d2.tdRFP) and non-CNS-specific (OT2.tdRFP) CD4+ T cells were transferred into B6.Rag1$^{-/-}$Thy1.21.EGFP recipient mice seven days after induction of adoptive transfer EAE with non-fluorescent 2d2 Th17 cells. In order to correlate the differentiation status of the co-transferred cells to their interactive behaviour, 2d2.tdRFP Th17 (Figure 25A),

2d2.tdRFP Th1 (Figure 25B) and OT2.tdRFP Th17 (Figure 25C) cells were compared. Similar motility characteristics of 2d2.tdRFP Th17 cells and OT2.tdRFP Th17 cells were observed, all of which differed from 2d2.tdRFP Th1 cells by higher mean velocity (Figure 25D). Interestingly, within the CNS tissue, for the OT2.tdRFP Th17 cells, there were similar interaction patterns with neuronal pathology (Figure 25E) as for the 2d2.tdRFP Th17 cells. The OT2.tdRFP Th17 cell-neuronal interaction was similarly localised along dysmorphic axons.

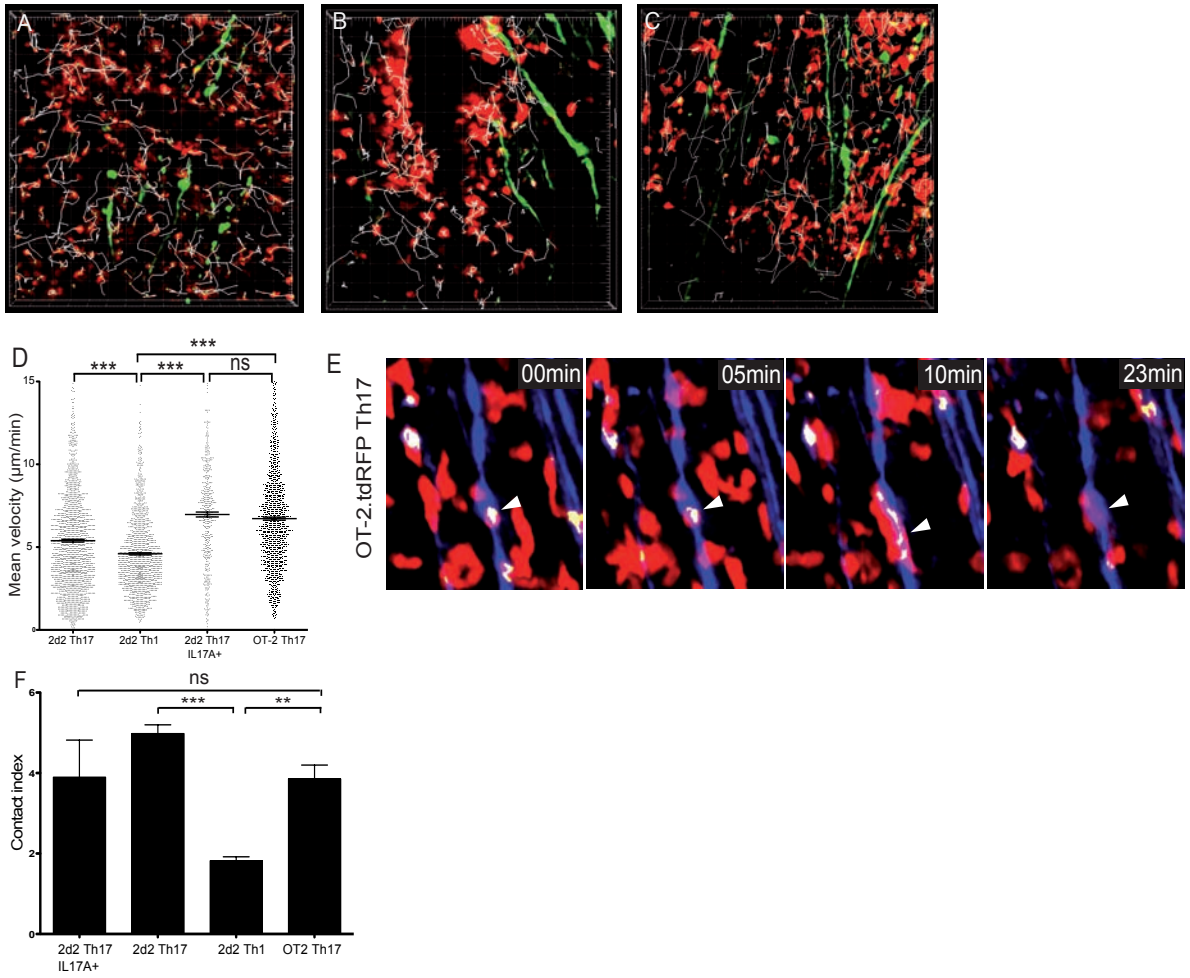


Figure 25. The Interaction of 2d2.tdRFP Th17, 2d2.tdRFP Th1 and OT2.tdRFP Th17 Cells with Neuronal Pathology. Time-lapse TPLSM of *in vitro* differentiated Th17 and Th1 cells, which had been co-transferred on day 7 into 2d2 Th17 cell-induced passive EAE in B6.Rag1$^{-/-}$Thy1.21.EGFP mice. (A) Automated tracking of 2d2.tdRFP Th17 cells shown as a connecting line of the preceding 20 time-lapse positions in EAE lesions in peak disease. (B) Automated tracking of 2d2.tdRFP Th1 cells (red) show less pronounced tissue invasive migration paths. (C) OT2.tdRFP Th17 cells enter the CNS lesions similarly as 2d2.tdRFP Th17 cells. (D) Quantitative cell tracking analysis shows that all Th17 T cell populations (including MACS enriched 2d2.tdRFP Th17 cells) exhibit a higher mean track velocity than 2d2.tdRFP Th1 cells (E) Contact formation of co-transferred OT2.tdRFP Th17 cells in B6.Rag1$^{-/-}$Thy1.21.EGFP mice. Interaction with neuronal pathology in a

similar manner as 2d2.tdRFP Th17 cells with static (indicated by an arrowhead, rounded OT2.tdRFP Th17 cell contacts axonal swelling) and dynamic contacts (indicated by an arrow, scanning OT2.tdRFP Th17 cell). (F) Comparison of contact index k (mean ± SEM) showed significant lower probability for 2d2.tdRFP Th1 cells to contact neurons compared to OT2.tdRFP Th17 cells, IL-17A-MACS-enriched 2d2.tdRFP Th17 cells and 2d2.tdRFP Th17 cells (***p<0.0001; Kruskal-Wallis Test, post-test Dunn's Multiple Comparison Test). Adapted from Siffrin et al. (148).

In order to visualise the contact formation of those OVA-specific CD4+ Th17 cells, the fact of non-overlapping spectra of EGFP and tdRFP fluorescence was exploited to measure the extent of contact formation of the different T cell subsets. The area of contact formation (voxels positive for green and red fluorescence only in areas of close proximity; Figures 26A and 26B) was quantified and in order to circumvent the bias due to variation of cell numbers (both immune and neuronal), a contact index k was computed, derived by applying the law of mass action, which objectifies the probability of tdRFP-positive and EGFP-positive cell populations engaging in close contact formation irrespective of the actual cell numbers (Figures 26C and 26D).

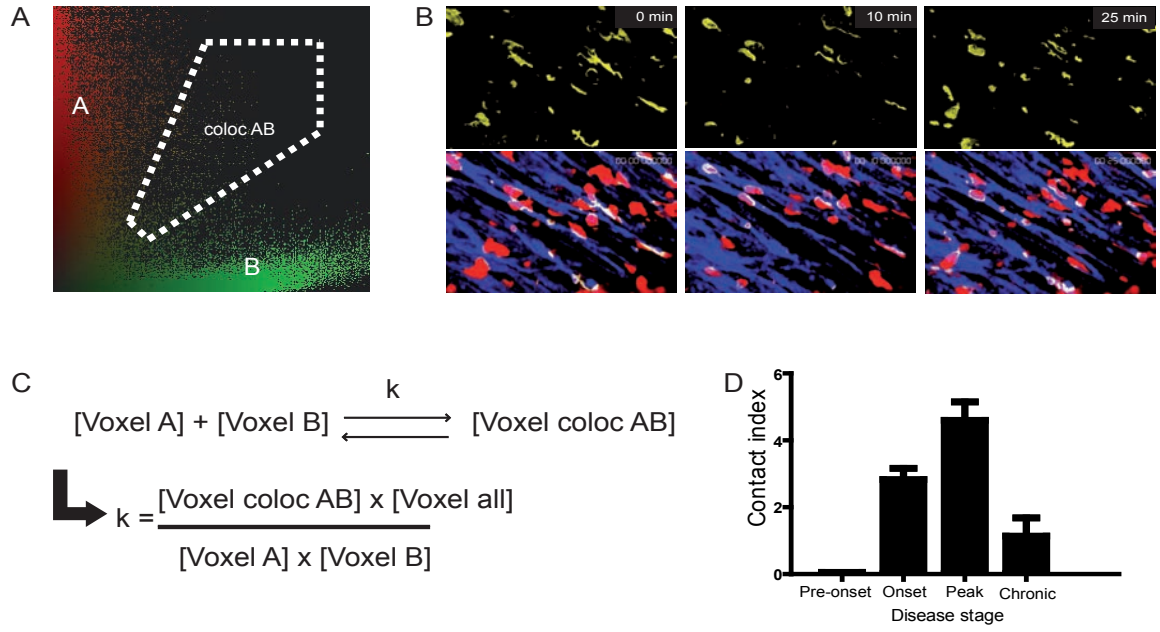


Figure 26. Determination of contact formation using TPLSM. (A) To define areas of close proximity between red-fluorescent (tdRFP) immune cells and green-fluorescent (EGFP) neuronal processes, the co-localisation mode of Volocity imaging software was used to identify double positive voxels in 2D intensity plots, as shown (gate on upper right quadrant). (B) Identified co-localisation voxels were used to generate a co-localisation channel, which identifies areas of close proximity in each time point and which was added as third channel to the original two data channels (EGFP - blue, tdRFP - red). (C) To compare different recordings and cell subsets, a co-localisation index k was computed, which defines the probability of the different fluorescent populations to

form close contacts by making use of the law of mass action. This allows to compare different recordings with divergent EGFP and tdRFP cell concentrations. (D) The contact index k of immune cells with neuronal cells showed a positive correlation for disease severity). Adapted from Siffrin et al. (148).

During the peak of EAE, the contact index k of 2d2.tdRFP Th17 cells (k = 4.98; SEM 0.22), IL-17$_+$ 2d2.tdRFP Th17 cells (k = 3.9; SEM 0.92), and OT-2.tdRFP Th17 cells (k = 3.85; SEM 0.35) was highly increased compared to 2d2.tdRFP Th1 cells (k = 1.82; SEM 0.10), which indicates that immune-neuronal interaction is a feature predominantly found in the Th17 cell subset (Figure 25F) highlighting the central role of the Th17 cell effector phenotype for neuronal dysfunction.

4 Discussion

Multiple sclerosis is the most common chronic inflammatory disease of the CNS in the western world, leading to devastating disability in young adults with only limited treatment options available so far (1). The invasion of encephalitogenic T cells through the BBB is considered the initiatory event of the autoimmune pathology whose characteristics are focal areas of inflammation, demyelination, remyelination and axonal/neuronal damage (neurodegeneration) in MS and its animal model EAE. These demyelinating plaques are typically found in subcortical but also cortical regions and their typical features are CD4+ and CD8+ T cells, activated macrophages and microglial cells, and antibody and complement deposition. Axons as well as neurons were only recently identified as major targets in the CNS. Early axonal pathology can be found in MS patients, correlating with the number of infiltrating immune cells. Evidence from animal experiments indicates that autoreactive myelin-specific CD4+ Th17 and Th1 cells play a major role in the pathogenesis of MS (29). Right now, there is a controversial debate around how far EAE and MS are rather Th1 or Th17 mediated diseases (134–136). IL-23, which is strongly associated with the Th17 phenotype, is the crucial factor for the development of EAE (66). Cua *et al*. showed in 2003 that a knock-out of IL-23 (p19) in mice leads to resistance to the induction of EAE. A knock out of IL-12, however, did not (61). IL-12 is the major cytokine associated with Th1 cells, which were up to then the common "villians" when it came to MS and EAE (77,181) but now seem to have been superseded by the Th17 cell as the encephalitogenic population in autoimmune inflammation (125).

In the first part of this thesis, the role of myelin-specifc Th17 cells in the initiation, chronification and damage processes of autoimmune CNS demyelination was investigated. For this, a differentiation protocol was established with which stable encephalitogenic Th17 and Th1 cells were generated. These *in vitro*-generated cells were investigated with focus on their activation status, the amount of IL-17 production and numbers of cells transferred when it comes to the MOG-specific Th17 cells. Results show that the encephalitogenic potential is dependent on all the aforementioned factors. Furthermore, MOG-specific Th17 and Th1 cells were compared with the focus on the potential to induce disease in lymphopenic and wild type mice and to damage formation in the compromised CNS after adoptive transfer. Results show that MOG-specific CD4+ Th17 cells but not MOG-specific CD4+ Th1 cells are capable to induce severe, non-remitting EAE not only in lymphopenic but also in wild type recipient

mice when all the factors come together rightly which stands in line with the common belief that the Th17 cell is “the new bad guy in town”. Furthermore, the potential use of the generated Th17 cells was exploited for TPLSM and MRI, with which the processes of initiation, chronification and damage formation can be visualised.

In the second part of this thesis, the role of non-CNS-specific T cells in autoimmune inflammation was looked at more closely. Non-CNS-specific T cells have been known to be able to enter the CNS for decades (111). Their role in neuropathology, however, is far less understood. Here, the observation made in MS patients, where exacerbations of disease can be correlated with systemic infections (15), was to be translated to the animal model EAE with the help of non-CNS-specific T cells mimicking the presence of large numbers of activated T cells as in an infection. As most of the patients diagnosed with MS present with a relapsing-remitting disease course, in which a relapsing pattern of acute exacerbations followed by periods of clinical stability can be observed, it is important to understand the underlying mechanism. Data on non-CNS-specific T cells suggests that activated Ovalbumin (OVA)-specific T cells are capable of entering the CNS after altering the BBB (111,112). They are also able to damage brain vascular endothelial cells *in vitro* (113). Presumably, in a compromised brain, such as in MS and EAE, bystander perturbations of the BBB caused by non-CNS-specific effector cells may have pathological consequences and contribute to disease aggravation as can be seen in patients suffering from an ordinary infection. Here, the protocol for the induction of stable encephalitogenic T cells was utilised to induce a Th17 phenotype in OVA-specific CD4+ T cells. These cells were injected into actively immunised C57BL/6 wild type mice in remission to mimic the situation present in the patient. It was shown that the OVA-specific Th17 cells were able to induce relapses in mice in remission where the vehicle control failed to. Furthermore, it was shown that by themselves the cells failed to induce symptoms of disease in healthy wild type and lymphopenic recipients. In a compromised brain, they were able to form contacts with neurons similar to MOG-specific CD4+ Th17 cells demonstrating a potential role in the neuropathology.

EAE shares many histopathological features with MS and is therefore a commonly used animal model of this human autoimmune disease (51). Disease can be induced in genetically susceptible animals by immunisation with myelin proteins such as myelin oligodendrocyte glycoprotein (MOG). There are two strategies to induce disease in those animals. Active EAE requires subcutaneous immunisation with the myelin proteins or peptides in pro-inflammatory adjuvants. For passive or adoptive transfer EAE encephalitogenic myelin-specific T cells are

derived from immunised animals and expanded in vitro before being transferred into naïve recipients (50,54). This latter approach has so far only been successful in the SJL/J mouse model. This model is thought to be the closest model for the situation in human MS as it presents with a relapsing-remitting disease course upon induction with PLP or adoptive transfer of encephalitogenic CD4+ T cells recognising the PLP peptide 139-151 (56). Chronic neuroinflammation can be investigated in the C57BL/6 mouse strain, in which immunisation with MOG or its immunodominant epitope 35–55 induces a severe attack followed by incomplete recovery and a secondary-progressive stage of silent deterioration, as found in the later stages of MS (55).

For many years, EAE was believed to be a Th1-induced autoimmune disease because of the increased expression of Th1 cytokines in the affected CNS and because injection of myelin-specific CD4+ Th1 but not Th2 cells into immunocompetent mice was sufficient to induce EAE (59,60). Only recently, IL-17-producing T helper (Th17) cells have been implicated in the pathogenesis of EAE (61–64). Th17 cells have been shown to contribute to several autoimmune diseases by the highly pro-inflammatory cytokines IL-17A (IL-17), IL-17F, tumour necrosis factor (TNF), IL-6, GM-CSF, IL-21 and IL-22 (65–68). They also possess a much higher potential to induce EAE (63). The pleiotropic cytokines IL-6 and TGF-β are crucial for the commitment to the Th17 lineage whereas IL-23 which was thought to play a pivotal role is rather important in the stabilisation of IL-17 production (89,99,108,110). Thus, the clinical disease severity is not only dependent on the quantity of infiltrating cells and the amount of pro-inflammatory cytokines but also on the differentiation status of the disease-initiating CNS-specific CD4+ cells (134,182).

To investigate the requirements for stable induction of EAE, myelin-specific TCR transgenic CD4+ T cells underwent different conditions before being adoptively transferred in wild type and/or lymphopenic ($Rag1^{-/-}$) recipient mice. The focus of the experiments conducted was on the activation status, rounds of restimulation and differentiation (Th1 vs. Th17 cells). MOG-specific (2d2-TCR transgenic) T cells were completely primed *in vitro* to generate "pure" Th1 or Th17 cells arising from naïve T cells. This is different from the established models with polyclonal MOG-specific T cells from actively immunised mice (traditional passive EAE), which are restimulated with cognate peptide and combinations of cytokines, e.g. IL-12, IL-23 and IL-18 (183,184). So far these classic approaches to induce adoptive transfer EAE with MOG-specific CD4+ Th17 cells have yielded low encephalitogenicity by unknown reasons and necessitated often irradiation of the recipient mice.

In this thesis, the influence of the activation status of myelin-specific CD4+ Th17 cells on their ability to produce IL-17, the signature cytokine for these cells, was investigated first. To examine whether the activation status has an influence on the production of IL-17, naïve T cells were sorted from 2d2 mice and skewed with TGF-β, IL-6 and IL-23 towards a Th17 phenotype for either three, ten or 17 days with restimulations with fresh APC after seven days in culture. It could be clearly shown, that the longer the cells were in culture the more IL-17 producers were detected. At neither stimulation time point contaminating IFN-γ producers were detected in the culture. It was observed that FoxP3-expression decreased with each restimulation. This is in line with reports of a reciprocal relationship between Th17 and regulatory T cells. Regulatory T cells as well as Th17 cells are dependent on TGF-β for their differentiation from naïve T cells. In the presence of IL-6 the development of FoxP3+ regulatory T cells is disturbed and naïve T cells differentiate into Th17 cells (89). Due to the fact that the generated MOG-specific CD4+ Th17 cells after two restimulations *in vitro* yielded the highest amount of IL-17 production and the lowest of FoxP3 production, it was decided to utilise those cells for further experiments. Furthermore, it was observed that the more activated the cells were the earlier they were able to induce EAE at least in the lymphopenic mice. Jäger et al. also observed this effect with transfer of Th17 cells into C57BL/6 mice with differently activated cells (99). More than three restimulations caused the ready differentiated Th17 cells to produce high amounts of IFN-γ in addition to the still present IL-17 production. The specification of T helper cell subsets is controlled by networks of lineage-specifying transcription factors, which bind to regulatory elements in genes that encode cytokines and other transcription factors (185). Recent findings report a more dynamic view when it comes to T cell lineage commitment (186). Epigenetic modifications of developmentally regulated genes have been thought to reinforce transcription factor networks as a basis for T lineage specification (185), however, emerging data indicate that epigenetic modifications are more dynamic than previously appreciated, enabling shifts in expression or silencing of 'signature' genes that characterise distinct T cell subsets (186). A recent study of the Th17 to Th1 transition has been particularly informative, as it has provided a model for defining both the activation and repression of distinct effector cytokine gene loci: the IFN-γ locus and the IL-17A–IL-17F locus, respectively (187). It was demonstrated that Th17 cells derived *ex vivo* for example can retain low-level expression of the IL-12Rβ2 chain, resulting in responsiveness to IL-12 that induces transition into effectors that share many features with classical Th1 cells (129,188,189). The IL-12–induced transition of Th17 precursors involves the rapid extinction of the Th17 genes with reciprocal induction of genes associated with the

Th1 differentiation program (129), and appears to be irreversible. These reports of T cell lineage plasticity are in line with the observation of a T cell lineage shift after more than two restimulations.

Generally, myelin-specific Th17 cells are obtained from immunised mice and expanded *in vitro* with IL-23. These *ex vivo*-generated cells have been shown to be sufficient to induce EAE in naïve recipients (62,183). Both of these reports used the SJL model for induction of passive EAE, which as mentioned before is the "flagship model" of passive EAE. Other reports, like for example O'Connor et al., showed that Th17 cells generated *in vitro* from naïve MBP TCR transgenic T cells induce very little or no disease in recipients treated with Pertussis toxin (PTx) (184). The reasons behind these discrepancies are not clear but O'Connor *et al*. used *ex vivo*-generated Th17 cells and transferred them into C57BL/6 wild type mice which is known to be a far less successful compared to the transfer of *ex vivo*-generated T cells in the SJL model as shown by the low number of reports on passive EAE in C57BL/6 mice without irradiation of the recipients. In line with the stimulation/restimulation protocols developed in this thesis, another group recently published the development of restimulation protocols for MOG-specific Th17 cells as well as Th1 cells in order to circumvent the rather "dirty" approaches for MOG-specific Th17 cells described before (99). Here, naïve CD4+CD62L^{hi} T cells were isolated, primed with TGF-β, IL-6 and IL-23 and harvested for adoptive transfer after 3, 10 or 17 days. An antigen-dependent restimulation was always conducted on day 7 of culturing. The generation of Th17 cells *in vitro* that is proposed by Jäger et al. differs from what as been proposed in this thesis. There, they also used MOG-specific CD4+CD62L^{hi} T cells to differentiate Th17 cells. After initial activation in the presence of the polarising cytokines TGF-β and IL-6 and neutralising antibody, anti-IFN-γ, for 2 days, Th17 cells were further supplemented either with IL-23 or with low doses of IL-2. After 6 days of culture primary Th17 plus IL-2, and Th17 plus IL-23 cells were either collected for transfer or restimulated with antibodies to CD3 and CD28 for 2 days to generate highly activated secondary Th17 plus IL-2, and Th17 plus IL-23 cells (99). Their *in vitro*-generated Th17 cells undergo one antigen-independent restimulation after six days in culture with two more subsequent days of culturing, which is in contrast to the protocol developed in this thesis. The results presented in this thesis show that the MOG-specific CD4+ Th17 cells that were injected into either C57BL/6 wild type or B6.Rag1$^{-/-}$ mice are indeed encephalitogenic. The MOG-specific Th17 cells were capable to induce EAE in the Rag1$^{-/-}$ mice independent of the activation status of the cells. Rag1$^{-/-}$ mice

developed fulminant, non-remitting EAE in all animals regardless of whether the cells were injected after stimulation, 1st restimulation or 2nd restimulation. In the wild type mice the observation was different. Here, only animals that were transferred with Th17 cells after 1st or 2nd restimulation showed signs of clinical disease. When transferred with cells after stimulation, i.e. after three days in culture, the animals developed no signs of the disease throughout the observation period. In line with these results, the MOG-specific Th17 cells generated by Jäger et al. succeeded in inducing EAE in C57BL/6 wild type mice (99). Pertussis toxin was abandoned for all experiments with adoptive transfer EAE in this thesis because of the pleiotropic effects on the immune system. Consistent with this approach are the reports by Jäger et al. that demonstrated that the use of PTx has a not beneficiary effect as with the application of PTx none of the transferred animals developed disease at all (99). When correlating the pre-transfer data with flow cytometric profiles from lymphocytes isolated from the CNS of diseased wild type or $Rag1^{-/-}$ mice, results show that *in vivo* the *in vitro*-generated Th17 cells underwent a T cell lineage switch especially when looking at the data from the lymphopenic recipient. These IFN-γ producers could either originate from previously uncommitted cells present within the transferred cell population that then differentiated into Th1 cells *in vivo* or shifted due to an inherent plasticity of Th17 cells as described above. Or these IFN-γ producers could originate from cells that were once IL-17 producers *in vitro* and acquired the ability to produce IFN-γ or IFN-γ together with IL-17 when transferred *in vivo* (99). It has been shown that IL-17-producing T cells can begin to produce IFN-γ when cultured with IL-12 *in vitro* (188), and a recent study in the diabetes model has demonstrated that Th17 cells can become IFN-γ producers *in vivo* (189). In the wild type animals, the amount of IL-17 producers seems to have increased slightly but no T cell lineage switch was observed which is in line with the data that Jäger et al. present in their report (99). Very low numbers of FoxP3 regulatory T cells were detected in the lymphopenic recipient, which indicates that a plasticity towards the Treg phenotype is not seen under these conditions. In the wild type mice FoxP3-expressing regulatory T cells were detected which is in line with them belonging to the normal T cell repertoire in wild type animals and being enriched in EAE in the CNS (108). The plasticity of T cells that was observed in the $Rag1^{-/-}$ mice has been picked up in many reports. When it comes to plasticity, it is important to remember that although some cytokines are selectively produced by different subsets of effector T cells, others are broadly expressed (133). The changing of the phenotype of T helper cells has also been reported especially in the context of Th17 cells becoming IFN-γ producers (128,129).

While it is well established that T cells with specificities for CNS antigens are crucial for the induction of EAE, it is still widely discussed which T cell subpopulations are the "villains" in the initiation of autoimmunity. It is generally accepted that T cells with a Th1 phenotype transfer EAE whereas Th2 cells were found incapable of doing so. Until recently Th1 cells were regarded as essential initiators of EAE as they secrete IFN-γ in large amounts. IFN-γ has been detected in inflammatory lesions (59) and was regarded as a key molecule in autoimmunity until it was shown that IFN-γ-deficient mice were not only resistant but also even more susceptible to EAE (60). This led to the discovery of the Th17 cell subset. While IL-17-producing T cells expanded with IL-23 were originally reported to be more pathogenic than IFN-γ-producing T cells expanded with IL-12 (10), another report found that only Th1 cells, but not Th17 cells, induce EAE (11). These conflicting observations might be due to differences in the capacity of Th1 versus Th17 cells to induce EAE. However, since each of these studies used a different T cell differentiation protocol and T cells of different TCR specificity, the conflicting data might be due to variations in the cytokine profiles of the T cell subsets or to different antigen specificities of the effector T cells. It has been demonstrated in this thesis that the MOG-specific naïve-derived Th17 cells reliably induce a strong disease phenotype. The question of the encephalitogenicity of completely *in vitro*-differentiated 2d2 Th1 cells is still open. Therefore 2d2 Th1 cells were generated similar to the 2d2 Th17 cells with naïve-sorted transgenic T cells (2 restimulations in vitro with IL-12 and/or IL-18). Pre-transfer cytokine expression showed that the *in vitro* generated 2d2 Th1 cells produced high amounts of IFN- γ and TNF-α. The cells were only able to induce disease lymphopenic hosts ($Rag1^{-/-}$) and, the disease course was very mild when compared to adoptive transfer EAE induced with 2d2 Th17 cells which showed rapid onset and a very severe disease course. This stands in line with a recent report by Jäger et al. where they generated multiple different effector T cell subsets (Th1, Th17 and Th9) and showed that they can induce EAE independently of each other. By inducing EAE with highly pure *in vitro* generated T cell subsets they have demonstrated that there is not only one effector T cell subset that can induce EAE, but that there are in fact several effector T cell subsets and different mechanisms by which each T cell subset can induce EAE (99). Furthermore, Domingues et al. demonstrate in line with the results of this thesis and with the report by Jäger et al. that both Th1 and Th17 lineages possess the ability to induce CNS autoimmunity but can function with complementary as well as differential pathogenic mechanisms (190). In contrast to what was observed in this thesis, they propose that Th17 cells are required for the generation of atypical EAE whereas IFN- γ producing Th1 cells induce classical EAE. Although the phenomenon of

Th17 cells inducing atypical disease has also been described in this thesis and seen in most of the animals transferred with Th17 cells, it is not an isolated event. Mice receiving Th17 cells might as well develop classical EAE. Discrepancies in this context might probably be due to differences in the generation of cells. In contrast to the observations made in this thesis Domingues et al. did not see any difference in onset and severity of EAE induced with either cell type. None of the animals injected with their Th17 cells developed very severe EAE symptoms leading to death. Also a factor that might speak for the discrepancies as the Th17 described in that report most obviously have a lower pathogenic potential than the Th17 cells described in this thesis (187).

The influence of the cell number transferred in order to induce adoptive transfer EAE in lymphopenic recipients was investigated. Therefore, the $Rag1^{-/-}$ mice were transferred with different numbers of MOG-specific CD4+ Th17 cells after second restimulation ranging from 0.5 Mio to 15 Mio cells. Results show that even small cell numbers (0.5, 1.5 and 3 Mio) are able to induce fulminant, non-remitting EAE leading to death in almost all animals. Only in about 50 % of the animals that were transferred with 0.5 Mio 2d2 Th17 cells after two restimulations *in vitro* disease developed. Here, all the animals that showed signs of disease also died/had to be sacrificed because of severe clinical disease. Most of the animals that developed disease that was induced with higher numbers of 2d2 Th17 cells (7 and 15 Mio) but also lower numbers showed atypical signs of EAE in contrast to the classical signs. Even in the animals that received 0.5 Mio cells in about half of them atypical disease was detected typically presenting with ataxia, hunched appearance and spasticity (134). The characteristic clinical feature of EAE is ascending paralysis, commencing at the distal end of the tail and moving to affect the whole tail, hindlimbs and sometimes the forelimbs (191,192). Neuropathological analyses typically show that the CNS inflammatory and demyelinating lesions are located predominantly in the spinal cord (192,193). In atypical EAE, the initial neurological abnormality is the turning of the head to one side. This head turning becomes more pronounced until at the most severe stage of disease the animal can not stand upright and rolls with an axial rotatory movement indicating involvement of the brain (192). Other symptoms characterising atypical EAE are proprioception defects, ataxia, spasticity and hyperreflexivity (134). A predominant brain stem and cerebellar involvement was described for atypical EAE (134,192).

As the disruption of the BBB is regarded as a critical step in the pathogenesis of inflammatory diseases of the CNS like MS and EAE, MRI is one of the more important tools investigating

these processes as BBB leakage can be visualised with contrast agents that are only able to surpass the BBB in case of disruption. A better comprehension of the mechanisms involved in the migration of T cells across the BBB will help to understand many aspects of chronic inflammatory diseases. The focal inability of the BBB to protect the CNS by preventing intravascular macromolecular substances from entering the brain parenchyma can be visualised with gadopentate dimeglumine (Gd-DTPA) enhanced MRI, which is today's gold standard for defining disease activity in MS patients. However, BBB disruption does not necessarily reflect clinical disability, a phenomenon described today as the clinico-radiological paradox with respect to MRI detectable lesions (194). Nevertheless, MRI enhanced with Gd-DTPA is the gold standard for defining disease activity in MS patients today. Animal models are not only necessary to investigate the BBB breakdown but also to examine what is responsible for inflammation as with animal models the *in vivo* studies can be correlated with histopathological findings.

In the biological sciences, many studies using mouse models are limited by the inability to gather anatomical and physiological information non-invasively in a longitudinal manner. MRI is a non-invasive imaging modality because it does not rely on ionising radiation and offers a spatial resolution of tens of microns (195). Many studies have been made in the field of EAE utilising the MRI. With the help of the MRI it was shown that BBB leakage is an early event and precedes cellular infiltration in the development of acute EAE (196). As the manipulation of T cells *ex vivo* requires the use of passive EAE most studies in this field have utilised the SJL model or use EAE in rats as the brain is a target site there as opposed to classical chronic EAE which presents with spinal cord inflammation (157,197–199). In this thesis, it was shown that the transfer of the generated encephalitogenic T cells led to BBB in the recipient mice. Clinical symptoms were visible prior to disease onset in different regions of the brain. All of the mice that were monitored with the MRI developed atypical EAE. As chronic EAE when induced with active immunisation is rather a spinal cord disease, MRI could not be utilised for investigations in this model because (a) there is no major pathology in the brain in an active EAE in the C57BL/6 mouse that can be visualised by MRI, (b) spinal cord imaging is not that far technically advanced in the field of mouse imaging as it struggles with very small anatomical regions and (c) passive EAE in the C57BL/6 model is not successful. As the use of the C57BL/6 mice bears a lot of advantages as many transgenic animals are bred on that background, it would be feasible to have a model that works with the MRI. With the use of transgenic *in vitro* manipulated cells a lot of opportunities for studying

different aspects of disease formation would open up. In this thesis, it is shown that the proposed adoptive transfer EAE model can be used to visualise BBB disruption. Contrast agent enhancement could be detected in the optic tract, the cerebrum and the cerebellum. Lesion size increased with disease progression. This data suggests that the proposed adaptive transfer model where the majority of the mice present with atypical EAE can most certainly be utilised for more advanced studies using the MRI.

A faster onset of disease was also observed in dependence of the cell number transferred and again T cell plasticity was observed when the lymphocytes were re-isolated from the CNS of diseased animals. Here, it did not matter whether the animals showed a typical or atypical disease course. The switch to IFN-γ producers was observed independent of that. These results presented can neither be contradicted nor supported with reports from the literature. Present studies on adoptive transfer in C57BL/6 mice are very heterogeneous when it comes to the animals used, the differentiation protocols of the encephalitogenic T cells and cell numbers. Different studies show that adoptive transfer EAE can be induced with 1, 5, 10 or 20 Mio cells (99,134,182,190,200). These studies have either used Th17 cells differentiated from naïve T cells or splenocytes, also were they either transferred into wild type or lymphopenic recipients highlighting the aforementioned heterogeneity when it comes to investigations concerning adoptive transfer EAE with MOG-specific T cells. None of the aforesaid reports shows such a high encephalitogenic potential for the MOG-specific CD4+ Th17 cells as presented in the results of this thesis. The "pure" *in vitro* generated CD4+ Th17 cells were able to induce very severe EAE reliably in all experiments making the experiment setting a reliable model of EAE. The model presented differs from other EAE models as it does not reflect the common situation that we are presented with in MS patients. With the rapid progress of disease here, it is possible to investigate the neurodegenerative aspect of the disease, but also to evaluate the efficacy of new treatment concepts for inflammation and neuroprotection. With a reliable disease model like this, many opportunities for investigation are open. The atypical disease course that most of the mice in this model present with is due to brain inflammation in contrast to the spinal cord inflammation observed in classical EAE (201–204). IFN-γ deficiency has been shown to cause certain myelin-specific T cells to preferentially induce brain inflammation (205,206). These studies raise the possibility that specific sites of inflammation in the CNS may reflect T cell specificity, as well as the ability to produce IFN-γ (134). And indeed, was it shown by Stromnes *et al*. that the Th17:Th1 ratio of infiltrating cells determines where the inflammation occurs in the CNS. Independent of this

ratio myelin-specific T cells infiltrate the meninges throughout the CNS. In the brain parenchyma, infiltrated were only found when Th17 cells outnumbered Th1 cells. Spinal cord inflammation was observed with T cells showing a wide range of Th17:Th1 ratios (134). In contrast, it was reported that clinical symptoms of spontaneous disease changed from classical to atypical when transgenic MBP-specific TCR transgenic $Rag1^{-/-}$ mice were bred to the IFN-$\gamma^{-/-}$ background (206). This report shows that the site of pathology was shifted from the spinal cord to the cerebellum and brain stem, resulting in disturbances in balance and coordination, suggesting that characteristics of the initiating T cells other than antigen specificity play an important role in directing the site of pathogenesis. In line with this report, it was shown by Lees et al. that transfer of IFN– $\gamma^{-/-}$ Th1-polarised CNS antigen-specific activated CD4+ T cells resulted in an atypical clinical course of EAE (207). However, they showed that *in vitro* polarisation to the Th1 or Th17 phenotype played no role in determining the clinical outcome of transferred IFN- $\gamma^{-/-}$ cell lines, with equivalent atypical clinical manifestations observed in both IFN- $\gamma^{-/-}$ cell lines regardless of previous conditioning (207). Rothhammer et al. investigated the role of α4 integrin in EAE as heterodimeric α4β1 (VLA-4) has been identified to be essential for encephalitogenic T cells to invade the CNS (78). They could show that the amount of α4 integrin expression was significantly lower in MOG-specific Th17 cells than in Th1 cells. Accordingly, EAE was completely inhibited by blockade of α4 in Th1 transfer EAE but not in EAE adoptively transferred by Th17 cells. T cell conditional $\alpha 4^{-/-}$ mice were not protected from actively induced EAE and developed an atypical disease with predominant brain stem and forebrain infiltrates, whereas the spinal cord was spared. In contrast, wild-type littermates always showed classical EAE with predominant spinal cord infiltrates. Together, the clinical and immunopathological phenotypes of actively immunised $\alpha 4^{-/-}$ mice were reminiscent of adoptive transfer EAE induced with *in vitro* differentiated Th17 cells. In contrast, Th1-induced EAE consistently resulted in spinal cord syndromes (208). These observations explain the fact why so many of the animals used in this study presented with atypical EAE. What remains to be looked at more closely is the lineage switch that these brain inflammation inducing Th17 cells perform in vivo and how this might also effect the course of disease.

When talking about Th17 in this thesis or in other reports, it is striking that about 15 – 50 % IL-17 producers in a culture (99,148,190), which is different for e.g. Th1 cells. Despite this variability and incomplete IL-17 production in Th17 it was always possible to induce EAE with the generated cells. This indicates that IL-17 is rather a marker of the pathogenic Th17

subset but not the most relevant effector cytokine (209). To investigate the role of highly IL-17-producing CD4+ Th17 cells a secretion assay was performed to separate the IL-17 producing cells from the non-IL-17 producing cells in culture. Both of these cell types were then used to induce EAE in $Rag1^{-/-}$ mice. Results show that the enriched IL17-producing T cells (>70 % IL-17 production) as well as the depleted (non-IL-17-producers) T cells (<1 % IL-17 production) were able to induce disease in the susceptible animals leading to the possible conclusion that IL-17 production might be dispensable or not the only important factor for the induction of disease. As shown in all previous results, the expression of FoxP3 and IFN-γ was absent when T cells were generated. This might be one factor contributing to the disease potential of the *in vitro* generated CD4+ Th17 cells. As shown in many studies and as also discussed here before IFN-γ was shown to be not required for EAE induction (61,77,210–212). Also the reciprocal development of FoxP3 Tregs and Th17 cells was described in detail. These facts contribute to the assumption that Th17 cells might be more potent disease inducers when those cytokines are lacking. However, also IL-17A and IL-17F are not required for the development of EAE (58,60,213–215) However, IL-23 - the Th17 stabilising factor (68,216) - and the Th17 transcription factor RORγt elicit an encephalitogenic program, which leads to the production of an pathogenic mediator (217). Codarri et al. among others reported GM-CSF (granulocyte-macrophage colony-stimulating factor) to be a possible candidate for the role of an encephalitogenic cytokine (217–219). In this thesis, the role of GM-CSF on the Th17 cell encephalitogenicity was not further elucidated but with the recent reports it will be definitely worth looking into it in future experiments.

Since the first application of two-photon microscopy ten years ago, the number of studies using this advanced technology has increased dramatically. The two-photon microscope allows long-term visualisation of cell motility in the living tissue with minimal phototoxicity (147) and has not only led to a better understanding of generation and priming of many immune cells, but also of the basics of immune regulation.

Neuronal damage in autoimmune neuroinflammation is the correlate for long-term disability in MS patients (164). By visualising the immune and neuronal compartment, it was shown here that immune infiltrates in demyelinating lesions are highly dynamic and show different motility pattern in distinct disease stages. The complexity of immune dynamics is much more diversified in the target organ than thought up to now (144,220). The unexpected attack of Th17 cells on neurons during disease, which has not been seriously considered to date,

revealed a close interaction of Th17 cells and neurons similar to that seen in immune synapses or kinapses. Synapses, both the immune and neuronal subtype, have been shown to ensure a highly selective signal transduction (148,221). Here, we used TPLSM *in vivo* to specifically address the role of encephalitogenic CD4+ T cells in the context of CNS damage processes. Th17 cells were shown to attack neurons during disease, which so far has not been considered as a modus operandi. Not only the CNS-specific 2d2 Th17 cells but also the non-CNS-specific OT2 Th17 cells showed contact to axons. This contact formation was not as pronounced with 2d2 Th1 cells highlighting the importance of the phenotype (148).

Next, we focused on the kinin system in the EAE in order to dissect mechanisms contributing to chonic neuroinflammation. So far, limited attempts to investigate the expression of kinin receptors on lymphocytes have been mostly based on the analysis of lymphocyte responses to *in vitro* stimulation with exogenous kinins. Kinins belong to a family of bioactive octa- to decapeptides generated from kininogens in a stepwise cleavage process (175). Their biological activities are mediated via two pharmacologically distinct G protein–coupled receptors: kinin receptor B1 (Bdkrb1), which under physiological conditions is not found in immune cells, and the ubiquitously expressed kinin receptor B2 (Bdkrb2). B1 expression is under the control of the pro-inflammatory transcription factor NF-κB and is upregulated in the context of inflammation only (176,177). In this thesis, the role of the kinin receptor B1 (Bdkrb1) was investigated more closely. In order to do so a classical approach for passive EAE induction was used. It was shown that Bdkrb1 seems to affect CNS inflammation and control the migration of pro-inflammatory T cells across the BBB. Transfer of Bdkrb1-deficient T cells into lymphopenic recipients revealed that the proportion of CD4+ T cells within the CNS-infiltrating immune cells was higher than in animals that only received wild type T cells. Not only was the proportion of CD4+ T cells but also the proportion of IL-17-producing T cells within that CD4+ T cell population increased. An influence of Bdkrb1 signalling on the migration pattern of Th17 cells in syngeneic hippocampal slice cultures was observed. Two-photon microscopy analysis revealed a lower mean velocity and reduced infiltrative behaviour upon Bdkrb1 activation. These results were in agreement with previous data showing that the Bdkrb1 agonist R838 (Sar-[D-Phe]des-Arg^{9}-bradykinin) markedly decreases the clinical symptoms of EAE in SJL mice (138–140), whereas the Bdkrb1 antagonist R715 (Ac-Lys-[D-βNal^{7},Ile^{8}]des-Arg^{9}-bradykinin) resulted in earlier onset and greater severity of the disease. However, our results stand in contrast with a recent study, where the expression of Bdkrb1 and Bdkrb2 and the effect of their inhibition on the disease course, BBB integrity

and T cell migration following MOG_{35-55}-induced EAE were investigated (222). It was shown that the severity of EAE was significantly alleviated in Bdkrb1-deficient mice when compared with wild type controls. Treatment of wild type mice with the Bdkrb1 antagonist R715 before and after disease onset was equally effective while Bdkrb1 activation by R838 promoted EAE contradicting the results generated by our group (137). The contradicting results implicate that the role of B1 in CNS autoimmunity needs to be investigated in detail. Nevertheless, the results shown in this thesis suggest that the kallikrein-kinin system is involved in the regulation of CNS inflammation, limiting encephalitogenic T lymphocyte infiltration into the CNS.

Numerous exogenous events have been proposed as triggering factors for exacerbations of MS, e.g., stress, trauma, infection, immunisation, pregnancy, climatic changes, and physical exertion (15). Clinical and epidemiologic evidence still supports a link between the development of an infection and the onset or the exacerbation of autoimmune diseases such as MS (11,16). Infectious agents and their products are suspected of inducing autoimmune disease development through different immune-mediated mechanisms, including molecular mimicry, production of molecules with super-antigen characteristics, release of hidden self-Ag, upregulation of presenting and co-stimulatory molecules, epitope spreading and bystander activation of resting encephalitogenic T cells (18–20). As 80 – 90 % of MS patients present with a relapsing-remitting disease course, it is important to investigate the link between infection and exacerbation further (21). There are hardly any animal models for the investigation of these infection-associated exacerbations. Although many animal models exist to investigate different aspects of MS (223), few reports describe a model in which exacerbations of disease are studied (179,224,225) although it is a common problem that many MS patients are confronted with. As most patients present with a relapsing-remitting disease course, models for this aspect of disease exist (56). A naturally occurring relapse, however, does not stand in line with relapses induced by systemic infections. In this thesis, a model, in which exacerbations of disease in the context of infections can be investigated, was established. Surrogate for an actual infection, non-CNS-specific T cells were utilised to mimic the situation of a large number of activated T cells as seen in acute infections. Non-CNS-specific T cells were chosen because their role in the cellular neuropathology is so far only poorly understood. Data suggests that activated Ovalbumin (OVA)-specific T cells are capable of entering the CNS after altering the BBB (111,112). They are also able of damaging brain vascular endothelial cells *in vitro* (113) highlighting the fact that these cells

are able to exert damage by themselves or through bystander activation. Apart from this, it is possible to utilise a transgenic mouse, the OT2 mouse, in which all CD4+ T cells express a TCR that recognises $OVA_{323-329}$ and which makes it possible to manipulate the CD4+ T cells *in vitro* like skewing them in different OVA-specific T cell subsets. To mimic a systemic infection like in the MS patients, C57BL/6 mice were immunised with MOG_{35-55}. In the meantime, the OT2 Th17 cells were generated with the previously established protocol. It was easily possible to skew the naïve OT2 cells into a highly IL-17 producing cell population. After developing disease, when in remission, the wild type animals were transferred with OT2 Th17 cells. As a control vehicle (PBS) was used in order to exclude that the C57BL/6 mice developed spontaneous relapses, which have however not been described for this model. Animals were monitored for a time period of 60 days. Results indicate that OT2 Th17 cells are able to induce relapses in a compromised EAE brain. This could not be observed for the animals that received just PBS. Plasticity of the OT2 Th17 cells was also observed when the cells were taken out of the brains of relapsing animals. Initially only producing high amounts of IL-17, the cells produced high amounts of IFN-γ when re-isolated from the CNS. In order to validate the model with regard to the potential of the OT2 Th17 cells to actually induce exacerbations in chronically sick animals some more inquests were made. Firstly, the potential of the OT2 Th17 cells to induce disease by themselves was investigated. C57BL/6 and $Rag1^{-/-}$ recipients were transferred with OT2 Th17 cells. As previously mentioned, OVA-specific T cells are able to enter the CNS after altering the BBB integrity (111,157) and though it was also reported that they do not promote any neuropathology (112) , that is the only report so far dealing with the problem. In this thesis, it could be shown that OT2 Th17 cells were not able to induce disease if transferred in non-EAE animals. In line with that no cross-reactivity of the OT2 Th17 cells against myelin peptides and whole brain homogenate was found in proliferation assays.

In line with experiments conducted with 2d2.tdRFP Th17 cells for the TPLSM, also OT2.tdRFP Th17 cells were investigated for their potential to form contacts with neurons. Interestingly, the OT2.tdRFP Th17 cells were able to interact with axons in the same extent as 2d2.tdRFP Th17 cells indicating that the phenotype rather than the antigen-specificity seem to be important (148).

These data emphasise the relevance of CD4+ Th17 cells in chronic neuroinflammation independent of their antigen-specificity and demonstrate detailed requirements regarding the

Th17 phenotype. Future activities will be to analyse how exactly CNS-specific and non-CNS-specific T cells interact within the CNS.

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Summary

T cells are critical for the pathogenesis of multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE). The invasion of encephalitogenic T cells through the blood-brain barrier (BBB) is considered as the initiatory event of the autoimmune pathology. However, antigen-specificity seems not to be essential for the transmigration step as non-CNS-specific T cells are also capable of entering the CNS without promoting any CNS pathology. It has recently been shown that Th17 cells are critically involved in the initiation of EAE. Their differentiation *in vitro* is dependent on the presence of the pro-inflammatory cytokines IL-6 and IL-23 in the context of TGF-β. But there is a still a controversial debate around how far EAE and MS are rather Th1 or Th17 mediated diseases.

In the first part of this thesis, the T cell differentiation requirements for a stable induction of EAE were investigated. The focus of experimental investigations was activation status, rounds of restimulation and differentiation (Th1 vs. Th17 cells). Myelin oligodendrocyte glycoprotein (MOG)-specific T cells were completely primed *in vitro* to generate "pure" Th1 or Th17 cells arising from naïve T cells. Results show that the *in vitro* generated MOG-specific Th17 cells are very capable of inducing EAE in wild type and lymphopenic recipients. Adoptive transfer of differentially stimulated IL-17 producing CD4+ T cells into C57BL/6 mice wild type recipients led to severe, non-remitting clinical EAE resulting in death in a small number of animals, whereas the lymphopenic $Rag1^{-/-}$ recipient mice presented with a fulminant and severely progressive EAE resulting in death in all animals transferred with the MOG-specific Th17 cells. High numbers of the MOG-specific Th17 cells were found in the CNS of the $Rag1^{-/-}$ and C57BL/6 mice. It was observed, that the lymphocytes re-isolated from the CNS of diseased animals showed a T cell lineage shift as they showed also increased IFN-γ production. Titration of MOG-specific Th17 cells showed that even low cell numbers could induce severe disease in the $Rag1^{-/-}$ recipient mice. Only repetitive *in vitro* stimulation ensured disease induction. Utilising magnet resonance imaging (MRI) blood-brain barrier (BBB) breakdown detected by contrast-agent (Gd-DTPA) enhancement was observed only in the $Rag1^{-/-}$ mice and was correlated to lymphocyte recruitment to the CNS using immunohistochemistry. Two-photon laser scanning microscopy (TPLSM) visualised the potential of the MOG-specific CD4+ Th17 cells to promote neurodegeneration by directly contacting neurons. A comparison of the disease inducing potential of MOG-specific Th17 and Th1 in chronic neuroinflammation was made showing

that also Th1 cells were able to induce EAE in Rag1$^{-/-}$ mice. The disease course was very mild when compared to adoptive transfer EAE induced with 2d2 Th17 cells.

Additionally, alternative ways to induce adoptive transfer EAE were explored in the context of the investigation of the role of kinin receptor B1 (Bdkrb1). Here, encephalitogenic Bdkrb1-deficient and wild type T cells were transferred into Rag1$^{-/-}$ recipient mice. The transfer of these encephalitogenic T cells led to increased CD4+ T cell numbers in the CNS of mice transferred with encephalitogenic Bdkrb1$^{-/-}$ T cells. The proportion of CD4+ IL-17-secreting T cells was greater in the sick mice that received Bdkrb1$^{-/-}$ T cells, whereas the proportion of IFN-γ-producing T cells was comparable in mice that got Bdkrb1$^{-/-}$ T cells and wild type T cells, respectively. CD4+ Th17 cells treated with Bdkrb1 modulators or vehicle before allowing them to infiltrate into syngeneic hippocampal slice cultures showed a reduced infiltrative behaviour upon Bdkrb1 activation in TPLSM.

In the second part, it was investigated if non-CNS-specific T cells that are able to enter the CNS may contribute to pathological processes such as induction of new clinical episodes in a bystander way. Background of this hypothesis is the epidemiological evidence that MS patients which suffer from trivial systemic infection, e.g. of the upper respiratory tracts, are highly prone to consecutively develop a relapse of the CNS disease. It could be demonstrated that OVA-specific Th17 cells are able to induce relapses in chronically sick mice when compared to vehicle control. They are not able to do so in healthy wild type and lymphopenic mice. The OVA-specific Th17 cells show no cross-reactivity with myelin-antigens and therefore seem to contribute to neuropathological processes in the bystander way. OVA-specific Th17 cells have also been shown to directly contact neurons and induce damage much similar to MOG-specific Th17 cells.

Taken together, these results indicate that repetitive restimulation of MOG-specific Th17 cells is necessary to yield a highly encephalitogenic Th17 subset, which is a potent inducer of EAE in an adoptive transfer model and that also non-CNS-specific Th17 cells can induce relapses in animals with pre-existing CNS pathology and that the kallikrein-kinin system is involved in the regulation of CNS inflammation, limiting encephalitogenic T lymphocyte infiltration into the CNS.

Zusammenfassung

T-Zellen spielen eine entscheidende Rolle in der Pathogenese der Multiplen Sklerose (MS) und ihres Tiermodells, der Experimentellen Autoimmunen Enzephalomyelitis (EAE). Die Invasion von enzephalitogenen T-Zellen über die Blut-Hirn-Schranke wird als Anfangspunkt für die autoimmune Pathologie betrachtet. Antigenspezifität scheint für diesen Prozess keine Rolle zu spielen, da sowohl Zellen die spezifisch wie auch unspezifisch für Antigene im Zentralen Nervensystem (ZNS) sind, die Blut-Hirn-Schranke passieren können. Kürzlich, konnte gezeigt werden, dass Th17-Zellen an der Initiierung von EAE beteiligt sind. Ihre Differenzierung *in vitro* ist abhängig von den proinflammatorischen Zytokinen IL-6 und IL-23 im Zusammenhang mit TGF-β. Im Moment gibt es eine kontroverse Debatte darüber, ob MS und EAE Th1- oder Th17-Zellvermittelte Krankheiten sind.

Im ersten Teil der vorliegenden Dissertation wurde untersucht, welche Voraussetzungen in der Differenzierung von T-Zellen für die Induktion von EAE nötig sind. Dabei wurde ein Schwerpunkt auf den Aktivierungsstatus der Zellen, die Anzahl der Restimulationen und den Differenzierungsstatus (Th1- vs. Th17-Zellen) gelegt. Aus naiven Myelin Oligodendrozyten Glykoprotein (MOG)-spezifischen T-Zellen wurden *in vitro* "reine" Th1- oder Th17- Zellen generiert. Die Ergebnisse zeigen, dass adoptiver Transfer von unterschiedlich oft stimulierten IL-17-produzierenden CD4+ T-Zellen zu einem schweren Krankheitsverlauf sowohl in C57BL/6 Wildtyp Mäusen, als auch in $Rag1^{-/-}$ Mäusen führten. MOG-spezifische Th17-Zellen wurden im ZNS der $Rag1^{-/-}$ und C57BL/6 Mäuse wiedergefunden und zeichneten sich hier durch einen Phänotypwechsel aus, da sie eine erhöhte Produktion von IFN-γ aufzeigten. Eine Titration der Zellen zeigte, dass bereits geringe Zellzahlen in der Lage sind EAE in $Rag1^{-/-}$ Mäusen auszulösen. Mithilfe von Magnetresonanztomographie konnte die zellverursachte Zerstörung der Blut-Hirn-Schranke in $Rag1^{-/-}$ Mäusen beobachtet werden und mit der Rekrutierung von Lymphozyten immunohistologisch korreliert werden. Zwei-Photonen Mikroskopie wurde verwendet um das Potential der MOG-spezifischen CD4+ Th17-Zellen zur Neurodegeneration durch direkten Kontakt mit Neuronen zu visualisieren. Ein Vergleich MOG-spezifischer Th1- und Th17-Zellen in chronischer Neuroinflammation veranschaulichte, dass Th1-Zellen zwar fähig sind EAE in $Rag1^{-/-}$ Mäusen zu induzieren, dass aber in sehr viel abgeschwächterer Form als Th17-Zellen. Zusätzlich wurde die Rolle des Kininrezeptor B1 (Bdkrb1) untersucht. Hierfür wurde eine klassische passive EAE induziert. Bdkrb1-defiziente T-Zellen wurden in $Rag1^{-/-}$ Mäuse transferiert und mit Wildtyp T-Zellen

verglichen. Es konnte eine erhöhte Anzahl CD4+ T-Zellen im ZNS der Tiere, die die Bdkrb1-defizienten T-Zellen erhalten haben, nachgewiesen werden. Der Anteil IL-17-produzierender Zellen in dieser CD4+ Fraktion war ebenfalls erhöht. CD4+ T-Zellen, die mit Modulatoren für Bdkrb1 behandelt wurden, zeigten ein weniger invasives Verhalten in hippocampalen Gehirnschnitten im Zwei-Photonen Mikroskop.

Im zweiten Teil der Arbeit wurde das Verhalten ZNS-unspezifischer T-Zellen untersucht. Dabei sollte gezeigt werden, ob diese ZNS-unspezifischen T-Zellen in der Lage sind ins ZNS zu gelangen und dort zerstörerische Prozesse anzuregen. Basierend auf dem Hintergrund, dass in MS Patienten, die an einer gewöhnlichen Infektion leiden, Schübe beobachtet werden können, sollte das Verhalten der ZNS-unspezifischen T-Zellen untersucht werden. Es konnte gezeigt werden, dass ZNS-unspezifische T-Zellen in der Lage sind Schübe in chronisch kranken Mäusen zu induzieren. Die T-Zellen waren nicht in der Lage EAE in gesunden Tieren auszulösen und zeigten keine Kreuzreaktivität zu ZNS Antigenen auf, was darauf hinweist, dass sie eine sekundäre Rolle in der Zerstörungskaskade der EAE haben, obwohl aufgezeigt werden konnte, dass sie durchaus direkt auch Neuronen angreifen können.

Zusammengenommen zeigen die Ergebnisse auf, dass wiederholte Stimulation MOG-spezifischer Th17-Zellen essentiell ist um eine hochenzephalitogene Zellpopulation zu schaffen, die EAE sehr effizient induzieren kann. ZNS-unspezifische T-Zellen sind in der Lage Schübe in chronisch kranken Tieren zu induzieren und der Kininrezeptor B1 scheint eine Rolle in der Regulation von entzündlichen Prozessen im ZNS zu spielen, indem er die Transmigration von T-Zellen ins ZNS verhindert.

Curriculum Vitae

For data protection reasons the curriculum vitae is not included in the online version.

List of publications

(* equally contributing authors)

Paterka M, Leuenberger T, Waiczies H, Wuerfel J, Infante-Duarte C, Zipp F, Siffrin V. Th17 cells induce experimental autoimmune encephalomyelitis with predominant brain stem and cerebellar involvement. *In preparation.*

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