REGULATORY CD4+ AND CD8+ T CELLS AS CANDIDATES FOR REGENERATIVE THERAPIES IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

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Abbreviations

APC	antigen presenting cell
AT	atorvastatin
α-hu	anti-human
α-m	anti-mouse
BBB	blood brain barrier
BSA	bovine serum albumin
CFA	complete Freund`s adjuvant
CFSE	carboxyfluorescein succinimidyl ester
CNS	central nervous system
ConA	concanavalin A
cpm	counts per minute
CSF	cerebrospinal fluid
DC	dendritic cells
EAE	experimental autoimmune encephalomyelitis
EGFP	enhanced green fluorescent protein
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
GA	glatiramer acetate
GM-CSF	granulocyte monocyte colony stimulating factor
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HMGCRI	HMG-CoA reductase inhibitors
iDC	immature dendritic cells
IL	interleukin
IL-10 Treg	IL-10-producing regulatory T cells
IFN	interferon
IR	infrared
MACS	magnetic cell sorting
MBP	myelin basic protein
MHC	major histocompatibility complex
MM	mouse cell culture medium
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis

NK cell	natural killer cell
OPO	optical parametric oscillator
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
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
RRMS	relapsing-remitting multiple sclerosis
SA	streptavidin
SHG	second harmonic generation
SPMS	secondary progressive multiple sclerosis
Th cell	T helper cell
TMEV	Theiler`s murine encephalomyelitis virus
TPLSM	two-photon laser scanning microscopy
Treg	naturally occurring regulatory T cells
WM	mouse washing medium

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS) in Europe and North America, leading to devastating disability in young adults with only limited treatment options available so far. In 1867, the disease was systematically and scientifically analyzed by the French neurologist Jean-Martin Charcot (1). Charcot described episodically occurring neurological dysfunctions in patients, which remitted after days to weeks. He and his students 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". Later on he also described the loss of axons and neurons as additional features of the disease. Up to the present date, these findings represent the hallmarks of multiple sclerosis.

1.1.1 Epidemiology and etiology

MS is most prominent in young adults, manifesting itself typically between 20 and 40 years of age. Women are affected twice as often as men, and people in the northern hemisphere more frequently than in equatorial regions, with a prevalence of 155/100.000 inhabitants in northern Europe (2). In Germany, the number of MS-patients is estimated to 67.000-138.000 (3). Although intensive research was performed in the field of MS, leading to many new insights, the cause of the disease, as well as the exact pathogenic mechanisms are not well understood so far. Complicating the picture is the fact, that MS is a multifactorial disease and that aside from gender, age, and geographical aspects, also genetic aspects (e.g. Major Histocompatibility Complex (MHC) class II alleles) as well as a history of certain viral infections (e.g. by Epstein-Barr virus or Herpes viruses) seem to influence the susceptibility to MS (4). It is assumed, that an autoimmune response led by activated pro-inflammatory T cells specific for CNS-antigens induces the inflammation and the observed damage in the CNS, leading to defects in the motor, sensory, visual and autonomous nervous system, and to the clinical manifestations of MS. The most frequent symptoms are paralysis, sensory disturbances, optic neuritis, ataxia, bladder dysfunction, fatigue, and cognitive deficits (5).

Three different clinical disease course patterns for MS exist. The "Relapsing-Remitting MS" (RRMS) is the most frequent course of the disease, with about 80% of patients affected by it. RRMS is characterized by relapsing-remitting episodes of neurologic deficits that last for several weeks and then remit partially or completely (Fig.1A). After 10 years, about 50% of the patients suffer from (secondary) progressive and creeping deterioration in between relapses and recover much worse from relapses than before. In this stage, motor function of the lower limbs is primarily affected, which is defined as "Secondary Progressive MS" (SPMS, Fig.1B). About 10-15% of patients show a "Primary Progressive MS" (PPMS) disease course, they deteriorate from the beginning without any improvement and most of these patients never experience clear-cut relapsing episodes (Fig.1C). Several therapies are available to treat MS nowadays, however, their effectiveness is limited. These drugs reduce the relapse rate and delay progression of disability to a certain degree in RRMS and SPMS, but do not cure the disease (2,6). Furthermore, the more effective the therapy regimen is, the higher is also the risk to develop side effects, as long-term immunosuppression increases the rate of serious infections and malignancies. This is an important issue considering that most patients are in their early twenties when started on a medication and need long-term treatment of a disease, which per se is not reducing their life span dramatically. Therefore, more specific treatment approaches are needed with better risk-benefit ratio.
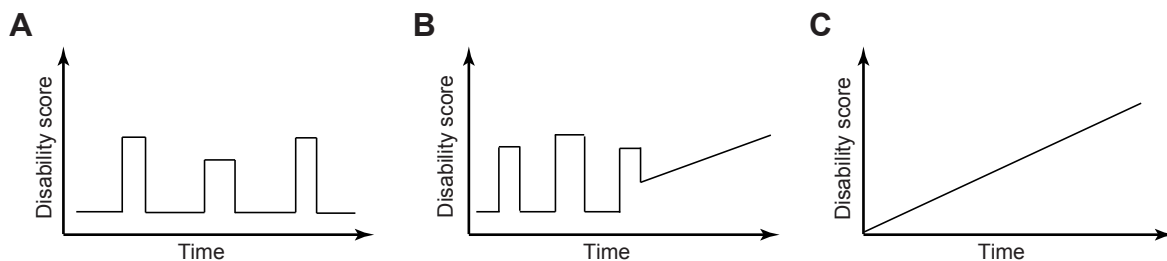
Figure 1. Different clinical progression patterns for MS: (A) relapsing-remitting MS, (B) secondary progressive MS, (C) primary progressive MS. Adapted from Lublin *et al.* (6).

1.1.2 Pathogenesis

As the study of disease pathogenesis in MS patients is limited to tissue samples from biopsies and autopsies of MS patients as well as magnetic resonance imaging, most knowledge about the pathogenesis of the disease is derived from studies of the animal model experimental autoimmune encephalomyelitis (EAE). 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, which are characterized by inflammatory infiltrates - consisting mostly of T cells, B cells, and macrophages - demyelination, gliosis, and axonal damage (7–9). The consensus view is, that MS is a misguided immune response, initiated by CNS-reactive T cells (10,11). Autoreactive T cells are not by themselves a pathological concern, but are present also in the healthy immune system. However, in the case of autoimmunity, the mechanisms normally containing an immune response against self are not properly working, leading to an immune attack against self-structures.

According to the present knowledge, circulating myelin-specific T cells are activated and expanded outside the CNS in the periphery. These activated T cells upregulate adhesion molecules and chemokine receptors on their surface, and are attracted to the blood brain barrier (BBB) by a chemokine gradient (12). Here, they adhere, role along, and finally transmigrate through the endothelium (13). In the perivascular compartment, the myelin-specific T cells are reactivated by local antigen presenting cells (APC) presenting their target antigens, mostly myelin structures, produce inflammatory cytokines, and gain access to the CNS parenchyma (14). 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 (15). B cells enter the CNS, which aggravates the immune attack (16). Furthermore, peripheral macrophages and resident microglia contribute to the inflammation (17). These events finally lead to inflammatory demyelination (Fig.2). However, demyelination is not the only process contributing to pathogenesis, also direct neuronal and axonal damage take place. Hereby, neurotoxic substances such as reactive oxygen species released by T cells and macrophages, as well as cell contact-dependent mechanisms might contribute to the damage (18).
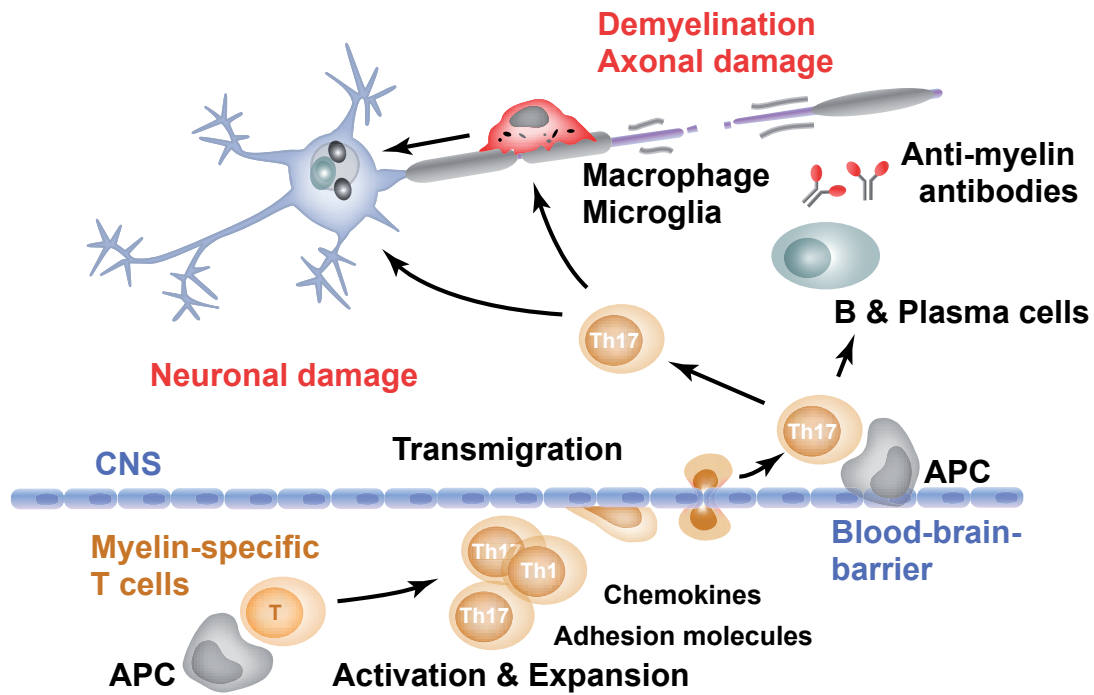
Figure 2. Disease model of multiple sclerosis. Peripherally activated myelin specific T cells transmigrate through the blood brain barrier (BBB) via upregulation of adhesion molecules. In the central nervous system (CNS), T cells are reactivated by local antigen presenting cells (APC), secrete proinflammatory cytokines, mostly of a Th17 or Th1 subtype, and gain access to the parenchyma. This initiates an immune response directed against myelin, 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.* (19).

1.2 The animal model EAE

Many insights into autoimmune diseases are based on animal models that mimic the human disease. To study MS as an inflammatory autoimmune disease, the animal model experimental autoimmune encephalomyelitis (EAE) is employed. EAE has clinically and pathologically common features with the human disease 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 (20).

EAE can be induced in several species, such as mice, rats, rabbits, or primates, the most frequently used model however is the EAE induced in mice. Two different ways to induce the disease in genetically susceptible animals are known (21). "Active" EAE is induced by subcutaneous immunization with myelin protein or myelin peptides, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), or proteolipid protein (PLP).

The peptides have to be mixed with complete Freund`s adjuvant (CFA) containing mineral oils and inactivated mycobacterium tuberculosis as a strong inflammatory stimulus, otherwise tolerance is induced. Generally after immunization, the priming phase of T cells 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 (22). "Passive" EAE, also called adoptive-transfer EAE, is induced by injection of myelin-specific CD4+ T cells, derived from peptide-immunized or myelin-T-cell-receptor transgenic animals, into naïve recipients (21). The autoimmune attack on the target organ and the modes of chronification of the disease process differ between strains and species pertaining to disease progression, immune cells involved and tissue damage. This allows investigation of different aspects of the pathology in extrapolation of the human disease (20).

Two mouse strains are predominantly used to study EAE and were also used in this thesis: C57BL/6 mice and SJL mice. In C57BL/6 mice, EAE can be induced with MOG_{35-55} or MOG-specific CD4+ T cells (23), which leads to a severe attack followed by incomplete recovery and, frequently, a secondary progressive stage of permanent clinical deterioration, as found in later stages of MS. The inflammation in this case is mostly concentrated on the spinal cord and brain stem. In SJL mice, immunization with $PLP_{139-151}$-peptide or transfer of PLP-specific CD4+ T cells (24), leads to a relapsing-remitting disease course, which is the closest model of the situation in human RRMS. In this thesis, passive EAE with MOG_{35-55}-specific CD4+ T cells was also performed in $Rag1^{-/-}$ mice, which have a C57BL/6 background, but do not possess any T or B cells by themselves (25). 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 (26).

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

T cells are critical for the pathogenesis of MS and EAE. However, the importance of different T cell subsets is highly controversial. The prevailing opinion is, that CD4+ T cells are the essential mediators of the disease. This assumption is supported by the following findings: In

EAE, transfer of myelin specific CD4+ T cells into naïve recipient animals was shown to be sufficient to induce the disease (27,28). In MS, there is a strong correlation of MS susceptibility with certain alleles of the major histocompatibility complex (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 (29). Further, myelin specific CD4+ T cells have been isolated from the cerebrospinal fluid (CSF) of MS patients (30)(30)(30)(30). Nevertheless, there are also data supporting an important role for cytotoxic CD8+ T cells in the damaging cascade of chronic neuroinflammation (31). CD8+ T cells are present, and in some biopsies even outnumber CD4+ T cells in chronically inflamed MS plaques (32,33). In addition, there is abundant expression of MHC class I on target cells in the CNS, which present antigen to CD8+ T cells (34). The first direct evidence for the implication of MHC-I genes and CD8+ T cells in the pathogenesis of MS was presented only recently, by showing that transgenic expression of the human HLA-A allele HLA-A*0301 (encoding the MHC-I molecule HLA-A3) and an HLA-A3-restricted myelin-specific T cell receptor in mice is capable of inducing an MS-like disease in these animals (35). On the other hand, the HLA-A allele HLA-A*0201 (encoding the MHC-I molecule HLA-A2) is associated with a reduced risk of MS (35,36).

In parallel to the effector role of T cells in chronic neuroinflammation, also a regulatory role for T cells is described. T cells do not only induce the disease, but are also important players in the control mechanisms that contain autoimmune responses. In very early experiments, it was shown, that depletion of certain T cells resulted in the onset of autoimmune diseases in mice, whereas co-transfer of the same T cells prevented the development of experimentally induced autoimmune diseases, indicating a protective role for these T cells in autoimmunity (37). Today it is well established, that regulatory T cells of the CD4- and CD8-subtype exist, which are essential for the control of autoimmune responses in healthy individuals and which are disturbed in their function in MS and EAE (38). Best characterized of these regulatory T cells are naturally occurring FoxP3-expressing CD4+ Treg cells and IL-10-producing CD4+ Treg cells, but also various suppressor CD8+ T cells have been 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.

1.3.1 Innate vs. adaptive immune system

The immune system, as a specialized system to fight pathogenic viruses, bacteria, and other parasites, is divided into two parts, the innate and the adaptive immune system. From an evolutionary point of view, the innate immune system developed first. It provides a first line of defense against many common microorganisms. It is a very fast, but not very specific way to eliminate common bacterial pathogens. The main mechanism is the recognition of pathogens by pathogen recognition receptors, such as Toll-like receptors, and the elimination of the pathogens by macrophages and granulocytes. Toll-like receptors recognize molecular structures that are unique to pathogens, which contributes to the decision if an immune response should be mounted or not, thereby preventing immune responses against self-structures or commensal bacteria. This mechanism is called peripheral tolerance. In vertebrates, the adaptive immune system developed, which is a very effective and specific way to eliminate pathogens. Here, the main players are T- and B-lymphocytes, which express specialized receptors to recognize antigen, the T cell receptor or B cell receptor. B cells can recognize antigen independent of other cells, whereas T cells only recognize antigenic peptides presented by MHC on APC. Each lymphocyte expresses receptors of a strong specificity on its surface. Upon recognition of this specific antigen, clonal expansion of the lymphocyte takes place, leading to activation of further immune cells and finally to an immune response directed specifically against the recognized pathogen.

Generally, the type of antigen influences the type of immunological response. Cytokines produced by the cells of the innate immune system shape the adaptive immune response, ensuring that no immune response against self or commensal bacteria is mounted in the healthy host. Virus-infected cells express viral particles on MHC class I on their surface which can be recognized by cytotoxic CD8+ T cells with the ability to directly kill virus-infected cells. Intracellular bacterial pathogens and extracellular pathogens coated with B-cell antibodies are internalized by APC and presented on MHC class II to CD4+ T cells. When a CD4+ T cell recognizes the presented antigenic peptides together with co-stimulatory molecules expressed on the APC, either a cell mediated immune response by Th1 cells is induced, which produce TNF-α, IFN-γ, IL-2 and thereby activate macrophages to fight intracellular pathogens, or a humoral immune response by Th2 cells is induced, which produce IL-4, IL-5, IL-10 and thereby activate B cells to produce antibodies against extracellular pathogens. In the absence of co-stimulatory molecules, i.e. in the absence of

infection/inflammation, the corresponding T cell is not activated but becomes anergic. This mechanism contributes to peripheral tolerance of self-antigens and is important to avoid autoimmune responses. A prominent feature of the adaptive immune system is further the immunological memory, which allows a faster and more effective response upon subsequent encounters with the same antigen (39). Effective immune responses against pathogens are sometimes accompanied by strong inflammatory reactions. To minimize damage to self, the activation of the immune system also triggers anti-inflammatory circuits, such as the induction of regulatory T cells, which contribute to peripheral tolerance. Both inflammatory and anti-inflammatory reactions are normal components of the same immune response, which coordinately fight infections while preventing immune pathology (40).

1.3.2 CD4+ T cells

The role of CD4+ T cells, also known as Th cells, is to identify dangers for the integrity of the organism and to activate and coordinate other cells of the immune system, most importantly B cells and macrophages, in order to trigger an effective and tightly regulated immune response. Furthermore, they provide immune memory for future more rapid immune reactions against a similar threat.

Naïve CD4+ T cells, which have not yet encountered their antigen, are activated upon their first encounter with peptide-MHC-complex on the surface of an activated APC. Upon activation, naïve CD4+ T cells differentiate into effector Th cells. As described above, 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, including the cytokines present and the nature and amount of the antigenic peptide (39). In *vitro*, 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. The different Th cell subtypes have specialized effector functions and produce a specific set of cytokines, assuring that the pathogen is cleared in the most effective way. 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. In recent years several novel CD4+ T cell subsets, including Th17 and Treg cells, have been identified (Fig.3).
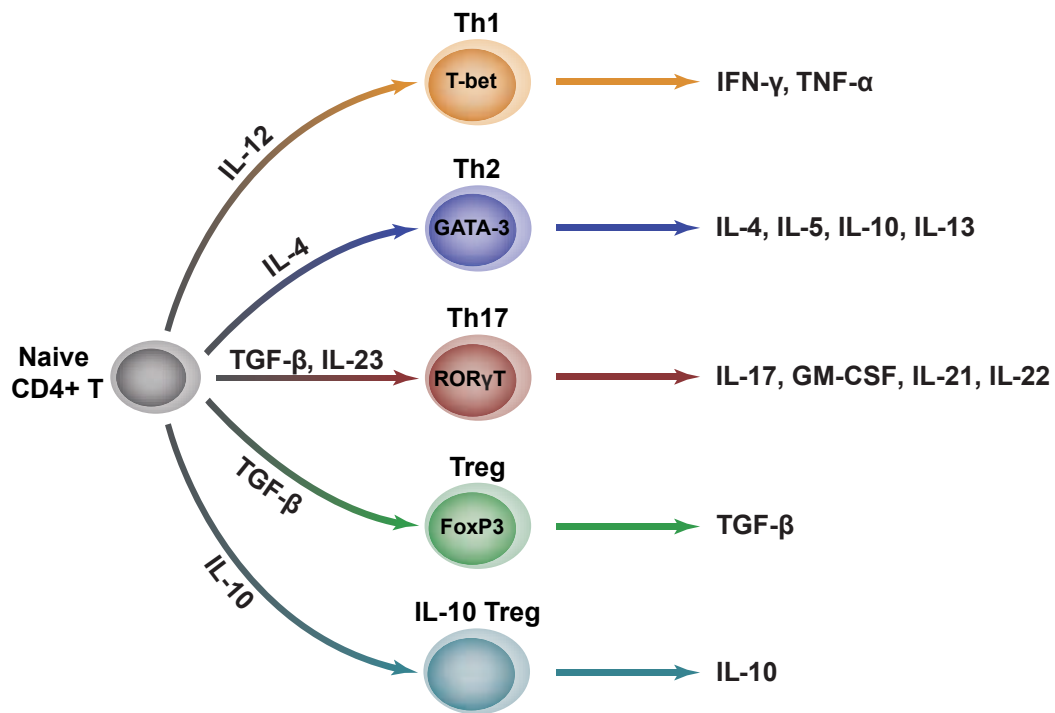
Figure 3. Diagram of the different CD4+ T cell subsets. Shown are the main factors determining their phenotype, their lineage specific transcription factors, and the main cytokines secreted by them. Adapted from Steinman *et al.* (41).

1.3.2.1 Th1 vs. Th17

For many years, Th1 cells were considered to be the proinflammatory T cell subset, which is responsible for the induction of EAE and potentially MS. Encephalitogenic T cells in EAE were shown to have a Th1 profile and Th1 clones specific for myelin antigens induced EAE upon adoptive transfer (42,43). Neutralization of interleukin (IL)-12, which is required for the differentiation of Th1 cells, protected against EAE (44). Furthermore, administration of IFN-γ, the main cytokine produced by Th1 cells (Fig.3), to MS patients worsened the disease (45). Puzzling however was the fact, that IFN-γ showed more immunoregulatory than proinflammatory functions in EAE, as IFN-γ-knockout and IFN-γ-receptor knockout mice showed more severe EAE (46,47). In recent years, it was shown, that not IL-12, but IL-23, a heterodimeric cytokine that shares the p40 subunit with IL-12, is the critical factor in the development of EAE (48). The IL-23-dependent step in chronic neuroinflammation was found in the differentiation of the Th17 lineage (49–51). Th17 cells contribute to different autoimmune diseases by the highly proinflammatory cytokines IL-17, GM-CSF, IL-21, IL-22,

and TNF-α (Fig.3) (52–54). In EAE, knockdown of the key transcription factor RORγT of the Th17 lineage abolished EAE susceptibility (51), whereas depletion or neutralization of the effector molecule IL-17 lead to contradictory results (55,56). In MS, enhanced IL-17 and IL-23 expression by human peripheral blood mononuclear cells (PBMC) and mononuclear cells in the CSF of patients was reported, suggesting an involvement of Th17 cells also in the human disease (57,58). It is assumed, that myelin specific Th1 and Th17 cells both can induce EAE, but that the two subsets induce disease with a different pathological phenotype, which might explain the pathological heterogeneity observed in MS lesions (59).

1.3.2.2 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 (Fig.3), exhibiting anti-inflammatory properties especially in parasitic infections. Th2 and Th1 cells are counter-regulated, as IL-4 strongly inhibits Th1 differentiation, and IL-12 and IFN-γ on the other hand inhibit Th2 differentiation (52). Th2 cells are involved in allergic hyperreactions, i.e. allergic asthma. In the field of EAE it was shown, that myelin specific Th2 clones are not able to transfer EAE to recipient mice (59,60). 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 (52).

1.3.2.3 Treg

Tolerance is one of the most intriguing events happening in the immune system and it is an active process involving fine-tuned surveillance processes. Therefore, a specialized CD4+ T cell population is generated early on in the life of animals and humans: the so-called thymus-derived naturally occurring regulatory T (Treg) cells. These cells are characterized by the constitutive expression of the IL-2 receptor (CD25, IL-2 receptor alpha chain) and the transcription factor FoxP3 (Fig.3) (61,62). In early experiments it was shown, that depletion of CD4+CD25+ suppressor T cells resulted in the onset of systemic autoimmune diseases in mice. Co-transfer of these cells with CD4+CD25- T cells prevented the development of experimentally induced autoimmune diseases (63). The FoxP3-knock-out mouse finally proved, that the transcription factor FoxP3 is necessary for Treg development and function, as

mutation or deletion of the FoxP3 gene caused fatal autoimmune disease in these mice (64). The human equivalent of this multi-organ autoimmunity is IPEX syndrome (65). In contrast, gene transfer of FoxP3 was able to convert naïve CD4+CD25- T cells into regulatory cells (61). In naïve specifically-pathogen-free mice, FoxP3 is a reliable marker for Treg. The human T cell population however is more heterogeneous than that of the mouse, as both CD25 and FoxP3 expression can be induced in naïve CD4+ T cells by cell activation in humans, which constantly happens as they are continuously in exchange with microbiota from the environment. In humans, only T cells with highest CD25 expression (CD25^{high}) are considered regulatory, as FoxP3 is strongly expressed in these cells. The search for alternative surface markers to identify and purify human Treg has yielded several markers, such as CD127, CD27, CD62L, CTLA4, GITR, none of which is a specific and exclusive marker for human Treg, but in combination adds to narrowing this subset (38).

CD4+CD25+FoxP3+ Treg are generated as an individual lineage within the thymus. In addition, conversion of FoxP3- CD4+ T cells into FoxP3+ Treg can take place under certain circumstances in the periphery *in vivo* or upon activation in the presence of TGF-β *in vitro*. Suppression by Treg cells *in vitro* is cell contact dependent, but many reports indicate that cytokines such as IL-10 and TGF-β may be involved in mediating suppression in *vivo* (66). Treg cells proliferate poorly in *vitro* and require high amounts of IL-2 for survival.

The potential of Treg cells to control CNS-autoimmunity has been well documented in experimental models. It was shown, that adoptive transfer of Treg cells can significantly reduce EAE severity (67,68). In contrast, depletion of Treg enhanced disease severity and mortality (69). In MS, no difference in Treg numbers between MS patients and healthy subjects was detected (70). However, Treg from patients with RRMS showed decreased FoxP3 levels, which correlated with impaired Treg function (70–72). The important regulatory role that Treg cells play in EAE makes them a major potential target for human immunotherapy. However, better Treg specific cell surface markers are needed to identify and isolate these cells for clinical applications.

1.3.2.4 IL-10-producing Treg

Another regulatory T cell subset are IL-10 producing regulatory T cells, also known as IL-10 Treg. In contrast to CD4+CD25+ naturally occurring Treg, these cells do not express the transcription factor FoxP3, but are a more heterogeneous cell population defined by the

production of the anti-inflammatory cytokine IL-10 (73) (Fig.3). IL-10 Treg can be induced *in vitro* and *in vivo* under particular conditions of antigenic stimulation. In mice, these cells share functional properties with naturally occurring Treg in that they can inhibit the proliferation of naïve CD4+ T cells independently of IL-10 production via cell-cell contact *in vitro* (74). However, IL-10 Treg inhibit T cell expansion *in vivo* and in EAE via an IL-10 dependent mechanism (75,76). As also other cell types such as naturally occurring Treg cells, Th1 cells, CD8+ T cells and B cells can produce IL-10 under certain conditions, it is difficult to narrow down the subset of IL-10 Treg (40).

It was shown, that IL-10 Treg cells can be induced in *vitro* upon priming of naïve human and murine T cells with antigen in the presence of IL-10. These cells were anergic in *vitro* and suppressed CD4-mediated immune responses and colitis (77). Later on it was shown, that the exogenous cytokines IL-10 and IFN-α are important for induction (78), and IL-15 and IL-2 for growth and expansion of human IL-10 Treg in *vitro* (79). Another way to induce human IL-10 Treg cells in *vitro* was described by Jonuleit et *al.* (80). They repetitively stimulated naïve human T cells with allogeneic immature human dendritic cells (DC), which resulted in anergic IL-10 producing T cells with the ability to suppress the antigen-driven proliferation of Th1 cells in *vitro*. The stimulation of murine and human CD4+ T cells in the presence of vitamin D3 and dexamethasone also induced IL-10 Treg cells in *vitro*. These cells retained their proliferative capacity while producing high amounts of IL-10 and suppressed EAE in an IL-10 dependent way (76).

1.3.3 CD8+ T cells

CD8+ T cells, also known as cytotoxic T cells, have the possibility to kill target cells via different mechanisms, which leads to the assumption, that they might contribute to the observed oligodendrocyte and neuronal death in EAE and MS. CD8+ T cells recognize antigen presented by MHC class I, which can be expressed on target cells within the CNS under inflammatory conditions. One way for CD8+ T cells to kill target cells is the release of lytic granules, which contain the cytotoxic proteins perforin and granzymes. Perforin inserts into the target cell membrane to form pores, through which granzymes enter the target cells, which can lead to the induction of apoptosis via caspase-activation. The second cytotoxic pathway involves the binding of Fas (CD95) on the target cell membrane by the Fas-ligand (CD95L) on activated CD8+ T cells, also leading to caspase activation and induction of apoptosis in the target cells. Furthermore, CD8+ T cells can also exert an effector function via

the secretion of cytokines such as IFN-γ or TNF-α (39). In contrast to CD4+ T cells, there are no specific markers for CD8+ T cells available by which clearly defined subsets of CD8+ T cells with specific functions could be distinguished. Early studies of CD8+ T cells in EAE preferentially describe a regulatory rather than proinflammatory role for CD8+ T cells, whereas in MS a contribution of CD8+ T cells to the disease is assumed.

1.3.3.1 Proinflammatory CD8+ T cells

A role for CD8+ T cells as inducers of EAE has emerged from two animal studies in which EAE was induced in recipient mice by adoptively transferring cytotoxic, myelin-specific CD8+ T cells (81,82). From a histological point of view, CD8-induced EAE differs significantly from conventional EAE as there is enhanced and prolonged meningeal involvement, extensive neutrophil recruitment, and signs of necrotic cell damage, all of which suggests a somewhat unspecific cytotoxic effect, as seen in other animal models of bystander damage (83). However, a recent publication showed the potential of CD8+ T cells to induce antigen-specific oligodendrocyte lysis in *vivo* (84) and thereby shows that CD8+ T cells can actually contribute to the pronounced loss of oligodendrocytes observed in MS plaques. In this study a mouse model system was designed, ensuring specific expression of the *influenza* hemagglutinin peptide in oligodendrocytes. Transfer of preactivated hemagglutinin-specific CD8+ T cells into these mice led to inflammatory lesions in the optic nerve, spinal cord and brain, and resulted in a mild EAE-like disease. The lesions were characterized by CD8+ T cell infiltration, demyelination and microglia-activation, resembling active MS-lesions.

In MS, several indications for a proinflammatory role of CD8+ T cells in the disease exist. There is a strong predominance of CD8+ T cells over CD4+ T cells in MS lesions, and the CD8+ T cells are directly enriched at the site of actively demyelinating lesions (33). Cytotoxic mediators like granzyme B were found to be upregulated in MS lesions. In *vitro* studies have shown that CD8+ T cells recognizing their correct peptide have the potential to kill all CNS cell subtypes, including microglia, astrocytes, oligodendrocytes and neurons (85). The CD8+ cells found in MS lesions are often of oligoclonal origin, a conclusive confirmation of a CNS antigen has however not been reported to date (86).

1.3.3.2 Regulatory CD8+ T cells

In the first descriptions of EAE experiments it was noted, that rats that recovered from EAE were protected from a second induction of EAE (87). It was later shown that CD8+ T cells are

responsible for this protection, as CD8+ T cells isolated from EAE-mice after a first disease phase could prevent the disease in recipient mice (88). Furthermore, CD8-depleted mice were no longer protected from a second induction of the disease (89). EAE in CD8 knockout mice lead to more relapses than in wild-type mice, suggesting a regulatory role for CD8+ T cells in EAE (90). These protective CD8+ T cells could be induced by T cell vaccination with antigen-activated attenuated encephalitogenic T cells (91,92), an approach that was later promisingly tested in the treatment of MS (93,94).

Confirmatory evidence also came from experiments, where CD8+ T cells isolated from EAE-recovered mice were shown to specifically suppress autoreactive CD4+ T cells in a Qa-1 (non-classical MHC-I molecule) restricted way (95). The regulatory role of these Qa-1-restricted CD8+ cells in protecting from subsequent relapses was confirmed in several studies. The human homologue of Qa-1, HLA-E, was shown to mediate the suppressive effect in humans (96). Interestingly, the model of Qa-1 restricted CD8+ T cells is based on suppression of intermediate avidity effector CD4+ T cells and is not dependent on antigen specificity. It further seems to be based on suppression via perforin-dependent pathways (97). Recently, CD122+CD44+Ly49+ Qa1-restricted CD8+ T cells were described, which were found to be defective in an animal model of systemic lupus erythematosus and which inhibited follicular T helper cells, thereby preventing the development of systemic lupus erythematosus (98,99). The identification of these surface markers might help to describe and study the population of Qa-1 restricted suppressor CD8+ T cells in more detail.

Additionally, several other populations of naturally occurring or in *vitro* induced regulatory CD8+ T cells were described in inflammatory settings. Problematic however is the fact that no distinct markers for CD8+ regulatory T cells have been found. It is therefore not clarified whether the found or generated regulatory CD8+ T cells are distinct populations, or if they overlap. CD8+CD122+ cells were described, which suppress autoreactive CD4+ T effector cells via IL-10, and which were shown to be essential for the recovery from EAE (100). CD28-expressing regulatory CD8+ T cells were described, that also regulate via IL-10 expression (101,102). It was reported, that regulatory IFN-γ-producing CD8+ T cells could be generated in *vitro* by co-incubation with autologous monocytes in the presence of IL-2 and GM-CSF, and that this CD8+ subset was functionally impaired in MS patients (103). Other studies have shown, that regulatory CD8+ T cells could be induced by CD40L-activated plasmacytoid DC (104) or TGF-β-treated APC (105). Furthermore, the existence of naturally

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occurring CD8+CD25+FoxP3+ T cells sharing phenotype, function, and mechanisms of action with CD4+ Treg was reported in humans and mice (106,107).

1.4 Antigen presenting cells

Dendritic cells (DC), macrophages and B cells carry MHC-II molecules and are thus professional antigen presenting cells (APC). They present peptide antigens on MHC-II and MHC-I molecules to CD4+ and CD8+ T cells respectively. APC are activated by danger signals and present peptide antigens together with the co-stimulatory molecules CD80 and CD86. T cells recognize antigen presented in this context and are activated. DC are the most powerful of the APC, as they constitutively express high levels of MHC-II and co-stimulatory molecules, the two signals required to prime naïve CD4+ T cells (39).

DC not only play an important role in triggering adaptive immune responses, but also in preserving tolerance against self-antigens (108). For a long time it was assumed that induction of immunity or tolerance by DC depends on their maturation state. Immature DC (iDC), i.e. steady-state DC in the absence of any proinflammatory stimulus such as toll-like-receptor ligands, were associated with tolerance. On the other hand, mature DC (after encounter with toll-like receptor ligands) induced strong immune responses (109). iDC efficiently capture antigens for processing and presentation to naïve T cells, but they are poor promoters of T cell activation as they lack the necessary signal 2, i.e. co-stimulation by surface molecules (B7 family, CD40) and cytokines (IL-6, IL-12, IL-23) (39). Thereby they shift immune responses and induce active tolerance, for example by inducing Treg or IL-10 Treg. Inflammatory environments promote DC maturation, characterized by (i) MHC-II stabilization, (ii) upregulation of co-stimulatory molecules and inflammatory cytokine production, (iii) up-regulation of CCR-7 and migration to secondary lymphoid organs, and (iv) induction of specific immune responses upon encounter with antigen-specific T cells (39). In recent years however it was shown, that under low inflammatory conditions, DC can become "regulatory" (110). These "regulatory" or "tolerogenic" DC are different from iDC, as they are mature and highly activated, but they promote peripheral tolerance, for example by inducing regulatory T cells (111,112). Therefore, immature as well as mature DC can suppress T cell responses.

The role of DC in autoimmune neuroinflammation is controversially debated. On the one hand, severity of EAE and the number of MS plaques seems to correlate with the presence and functional status of DC (14,113). It was clearly shown, that DC are able to induce and

amplify EAE (114,115). On the other hand, DC seem to be regulating CNS inflammation, as it was shown that depletion of certain DC subsets resulted in exacerbation of EAE (116) and that transfer of certain DC subsets prevented EAE (111,117). This regulatory effect of DC can, at least in part, be attributed to an induction of Treg and IL-10 Treg cells (111,118).

1.5 Two-photon laser scanning microscopy

Two-photon laser scanning microscopy (TPLSM) has revolutionized 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 (119,120).

First applied to studies of immune cell behavior in lymph nodes (121,122), it can also be applied to visualize immune cell dynamics and cellular interactions within the complex networks of the inflamed CNS. The use of fluorescent labeling techniques and transgenic animals expressing different fluorescent proteins (123) permits the monitoring of specific subpopulations of cells during the course of EAE directly in the target organ of living anesthetized mice. This led for example to the current knowledge on the behavior of self-reactive CD4+ T cells during EAE. It was shown, that shortly before onset of EAE self-reactive CD4+ T cells roll along and then crawl along the intraluminal side of the leptomeningeal vessels before they finally extravasate (124). In the extraluminal space, the CD4+ T cells show a vessel-associated behavior and make contacts with perivascular APC as they get reactivated (125). After reactivation the T cells detach from the vessel and invade the CNS parenchyma (124). In full-blown EAE, sustained interactions between myelin-specific

Th17 cells and neurons were observed, which induced extensive axonal damage in these neurons (126).

By leading to a better understanding of immune cell behavior and function in *vivo*, intravital imaging hopefully contributes to the development of safer and more targeted therapeutic approaches for autoimmune diseases in the future.

1.6 Therapeutic immune modulation

The modulation of inflammatory immune responses, such as the interference with T-DC interaction or the induction of regulatory T cells, might be an effective way to treat inflammatory or autoimmune diseases. This can either be achieved by special culture conditions of cells in *vitro*, as described above for the induction of Treg, IL-10 Treg, regulatory CD8+ T cells, and tolerogenic DC, or by the help of immunomodulatory drugs. Immunomodulatory drugs were successfully applied in *vivo* and in *vitro* to induce tolerogenic DC or to induce and expand regulatory T cells. Treatment of murine and human CD4+ T cells with vitamin D3 and dexamethason induced IL-10 Treg (76), whereas rapamycin was shown to expand murine and human Treg cells (127,128). Also interferon-β (IFN-β), glatiramer acetate (GA), and atorvastatin (AT) have immunomodulatory effects on T cells and APC, as described in the following.

1.6.1 Immunomodulatory drugs in MS therapy

In MS therapy, the immunomodulatory drugs IFN-β and GA are the therapy of choice today for RRMS. They reduce the relapse rate and can delay the progression of disabilty, but they do not arrest the disease.

GA was shown to mediate its effect by inducing a shift from Th1 to Th2, by expanding Treg cells, and by influencing APC (129,130). GA induces the proliferation of GA-specific T cell cells, which show a Th2 phenotype and can suppress EAE (131). Subsequently, it was found that glatiramer acetate specific T cell lines isolated from glatiramer acetate treated MS patients show a Th2 phenotype, whereas T cells from untreated patients show a Th1 phenotype (132). Treatment with glatiramer acetate also restored Treg function in patients with MS. It promoted the conversion of CD4+CD25- into CD4+CD25+FoxP3+ T cells and led to a significant increase in the FoxP3 expression of CD4+ T cells (133). GA also induces proliferation of cytotoxic CD8+ T cells and restores their reduced suppressor activity found in

MS patients (134,135). When bone marrow-derived monocytes are incubated in *vitro* with GA, they release less inflammatory cytokines and more anti-inflammatory cytokines, such as IL-10 and TGF-β. This again polarizes T cells towards a Th2 or regulatory phenotype. Adoptive transfer of these GA-treated monocytes into mice increased Foxp3 expression and the number of Treg cells in these mice, and reversed neurological signs in EAE (136).

The therapeutic effect of IFN-β is not as well understood, but is attributed to a reduction of inflammatory cytokine production, leukocyte proliferation, and MHC-II antigen presentation (137,138). Early experiments showed, that IFN-β reduces the secretion of the inflammatory cytokine IL-12 by DC, inhibits Th1 development, and induces Th2 cells (139,140). Newer experiments in mice with a conditional knockout of the type 1 IFN-receptor indicate, that the effect of IFN-β is mediated via myeloid cells, including macrophages, DC and microglia (141). In these cells IFN-β suppresses the production of inflammatory cytokines (TNF-α, IL-23), downregulates MHC-II, decreases phagocytic activity, and increases the production of the anti-inflammatory cytokine IL-27. As a secondary effect, this leads to reduced Th1 and Th17 cell differentiation and expansion (138).

1.6.2 Statins: lipid-lowering and immunomodulatory drugs

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (HMGCRI), also known as statins, are presently among the most widely used lipid-lowering drugs to treat cardiovascular disease. By blocking the HMG-CoA reductase, these drugs inhibit the conversion of HMG-CoA to L-mevalonate, and thereby block downstream cholesterol synthesis (142). The discovery and further development of statins began in the 1970s (143). Today, statins are generally divided in two groups: 1) statins from natural compounds, such as mevastatin, lovastatin, pravastatin, and simvastatin, and 2) fully synthetic statins, such as fluvastatin, rosuvastatin, pitavastatin, and atorvastatin (142). The first evidence for an anti-inflammatory property of HMGCRIs came from a study reporting an association between decreased natural killer cell cytotoxicity and cardiac transplantation success independent of altered cholesterol levels (144). This finding was followed by several studies that identified immunoregulatory and anti-inflammatory properties of statins. It was shown, that atorvastatin reduced T cell activation and inhibited MHC-II expression in several cell types (145). It was further shown, that statins block the function of the integrins VLA-4 and LFA-1 on T cells, which are important for adhesion properties of the cells, and thereby influence their

circulation and extravasation (146,147). These findings lead to speculations on a potential therapeutic role for statins in the treatment of neuroinflammatory diseases such as MS.

In EAE, the administration of atorvastatin drastically reduced disease severity (148,149). The beneficial effect of atorvastatin was attributed to a reduced expression of the pro-inflammatory cytokines IFN-γ, TNF-α, IL-12, and IL-2, to an increased expression of the anti-inflammatory cytokines IL-4, IL-10, and TGF-β, and to T cell anergy induced by a block in cell cycle progression (148). In addition, a reduced expression of MHC-II and co-stimulatory molecules on APC was detected. Passive EAE demonstrated, that encephalitogenic T cells from atorvastatin-treated animals could prevent the disease in recipient animals (149). Atorvastatin was further shown to enhance the Th2-promoting effects of glatiramer acetate in EAE (150), indicating that a combination of statins with established immunomodulators may be a concept for future clinical trials.

Several clinical trials with RRMS patients showed a beneficial effect of statins, either alone or in combination with IFN-β (151). It is however debated if the combination therapy has advantages over the monotherapy with IFN-β, as also potential interfering pharmacological effects must be considered especially concerning hepatotoxicity of both drugs (152).

1.7 Aim of this work

The disadvantage of in *vivo* application of immunomodulatory drugs is that they have pleiotropic and often counter-acting effects, not all of them known, resulting also in unwanted side effects. The better approach to treat autoimmune neuroinflammation therefore would be to specifically modulate cells in *vitro* and transfer them for therapeutic applications. This means, we should learn from pleiotropic drugs the main mechanisms of action in a specific disease and find new ways to only address this pathway. The key role that regulatory CD4+ T cells play in the EAE model identifies these cells as a major potential target for immunotherapy. The role of CD8+ T cells in EAE is still controversially debated, and it is presently not satisfyingly clarified, if CD8+ T cells are effectors contributing or even initiating the disease, or if they are naturally occurring or inducible regulators of the disease.

The overall aim of this work was to better understand the phenotype and the role of regulatory T cells of the CD4 and CD8 subset and to study the phenotype of these cells in *vitro* and in *vivo* in order to apply them as a therapeutic approach in EAE.

To address this aim, this thesis is divided into three parts:

1.7.1 Pharmacological generation of regulatory CD4+ T cells *in vitro*

It is well established, that regulatory CD4+ T cells, such as FoxP3+CD4+ Treg and IL-10-producing Treg exist, and a regulatory role for these cells in EAE and MS has been described (67,69,70). Previous work from our group and others has shown, that the HMGCRI atorvastatin has a beneficial effect in EAE, which is attributed to a direct influence of atorvastatin on T cell proliferation and a shift from Th1 to Th2 (148). Furthermore, atorvastatin was shown to reduce MHC-II and co-stimulatory molecules on APC, which is likely to impact their stimulatory capacity and therefore the direction in which they prime T cells (149). However, the effects of atorvastatin on the modulation of the T cell response, in particular for the induction and expansion of regulatory CD4+ T cells, had not been examined at the time point when this thesis was started.

The following questions were addressed in this part:

- *Does atorvastatin modulate the T cell response of naïve CD4+ T cells?*

- *Does atorvastatin modulate the T cell response in such a way that T cells adopt a regulatory function or that their regulatory potential is increased?*

- *Can the anti-proliferative effect of atorvastatin be utilized to specifically expand regulatory T cells?*

- *Does atorvastatin influence differentiation of T cells indirectly by affecting DC?*

1.7.2 Contribution of CD8+ T cells to MOG-induced EAE

T cells are required for EAE and most likely also for the induction and propagation of MS. However, as the role of different T cell subsets in EAE is still controversially debated, the aim in this part of the thesis was to determine the individual contribution of the different T cell subsets, namely CD4+ and CD8+ T cells in MOG-induced EAE. While for CD4+ T cells it is well established that specific subsets with specific functions exist, less is known about the role and subsets of CD8+ T cells in EAE. As no specific markers are available to distinguish between CD8+ T cells with specific functions, here, the distribution and phenotype of CD8+ T cells in the different disease phases and their potential to induce or attenuate EAE was

examined. As also their primary interaction partners in the CNS are not known, the behavior of CD8+ T cells and their interaction with possible target cells directly at the site of inflammation in the CNS was studied using TPLSM. Previous work from our group on the behavior of CD4+ and CD8+ T cells in the CNS was derived from analysis of in *vitro* activated T cells in a hippocampal slice model, where it was shown, that CD8+ T cells show a distinctly different behavior from CD4+ T cells. Here, performing intravital imaging of the brainstem of EAE-affected living anesthetized mice allowed to study the actual situation in *vivo*.

The following questions were addressed in this part:

- *What phenotype do CD8+ T cells in MOG-induced EAE have in the different disease phases?*

- *What is the influence of CD8+ T cells on the disease course in MOG-induced EAE?*

- *What behavior do CD8+ T cells show in the CNS of EAE-affected mice?*

- *With which target cells do CD8+ T cells interact in the CNS?*

- *How do CD8+ T cells influence the behavior of CD4+ T cells in the CNS?*

1.7.3 Isolation and expansion of suppressor CD8+ T cells with regulatory potential

Already early EAE-studies gave evidence that CD8+ T cells with a regulatory role in EAE exist. Mice depleted of CD8+ T cells are no longer protected from a second induction of EAE (89). Furthermore, EAE in CD8 knockout mice lead to more relapses than in wild-type mice (90). These findings indicate, that regulatory CD8+ T cells are probably generated during a first disease phase and exert their regulatory effect only in a secondary disease phase. Therefore, it was assumed here, that if CD8+ T cells with regulatory potential exist, they are presumably found in mice that are in remission of a first disease peak. Therefore, in this part of the thesis, the hypothesis that it might be possible to isolate and expand CD8+ T cells with regulatory potential from EAE-recovered mice was tested, and the suppressive behavior of these cells was examined in *vitro* and in a therapeutic approach in EAE in *vivo*.

The following questions were addressed in this part:

- *Do CD8+ T cells isolated from EAE-recovered mice exhibit regulatory potential?*

- *Is it possible to generate or expand CD8+ T cells with regulatory potential in vitro from CD8+ T cells isolated from EAE-recovered mice? If yes, what is their mechanism of suppression?*

- *Can these in vitro generated suppressor CD8+ T cells be used in a therapeutic approach in EAE? And what is the behavior of these in vitro generated suppressor CD8+ T cells in the CNS of EAE-affected mice in vivo?*

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% Hepes 1M, 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% Hepes 1M
5% FCS
100 µg/ml streptomycin
100 U/ml penicillin

Human cell culture medium (FCS-medium)	RPMI 1640 + 5% Hepes, Gibco Invitrogen, Germany
	10% FCS
	1% L-glutamin
	1% penicillin-streptomycin, PAA, Germany
Human washing medium	RPMI 1640 + 5%
	5% FCS
	1% penicillin-streptomycin
Freezing medium	FCS
	20% DMSO, Sigma, Germany
Iscove's Mod Dulbecco's Medium (IMDM)	Gibco Invitrogen, Germany
Lysis-buffer	PBS
	1% $KHCO_3$, Merck, Germany
	8,29% NH_4Cl, Roth, Germany
	37,2% Na_2EDTA, Sigma, Germany
Paraformaldehyde (PFA)-solution	0.1 M PBS, Gibco, Germany
	2% or 4% PFA, Roth, Germany

2.1.2 Reagents and chemicals

Agarose	Serva, Germany
Atorvastatin	Pfizer, Germany
Brefeldin A	Sigma-Aldrich, Germany
Carboxyfluorescein succinimidyl ester (CFSE)	Molecular Probes, Germany
Collagenase	Sigma, Germany
Collagenase-Dispase	Roche, Germany
Complete Freund`s adjuvant (CFA)	BD Difco, Germany
Concanavalin A (ConA)	Sigma, Germany

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DNase	Roche, Germany
Ethanol	Merck, Germany
Ficoll	Biochrom, Germany
FITC-dextran	Sigma, Germany
H37RA	Difco, Germany
^{3}H-thymidine	Amersham, Germany
Isoflurane	Abbot, Germany
Isotonic Ringer solution	Braun, Germany
Ketamin	Curamed, Germany
MBP$_{85-99}$	Pepceuticals, UK
(EKPKYEAYKAAAAPA)	
MOG$_{35-55}$	Pepceuticals, UK
(MEVGWYRSPFSRVVHLYRNGK)	
NaCl-solution (0.9%)	Braun, Germany
Ovalbumin (OVA)	Sigma, Germany
OVA$_{323-339}$	Pepceuticals, UK
(ISQAVHAAHAEINEAGR)	
Percoll	Sigma, Germany
Pertussis toxin (PTX)	List Biologicals, USA
PLP$_{139-151}$	Pepceuticals, UK
(HSLGKWLGHPDKF)	
Propidium iodide (PI)	Sigma, Germany
Trypan blue (0.4%)	Biochrom, Germany
Xylazinhydrochloride (2%)	Bayer Health Care, Germany

2.1.3 Consumables

Cannulas, syringes	Braun, Germany
Cell strainers (100 μm pore size)	BD Biosciences, Germany
Cell scrapers	Corning, 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

Heparin tubes	Sarstedt, Germany
MACS LS-columns	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

β-scintillation counter (Wallac MicroBeta)	Perkin Elmer, 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
Vortexer	Scientific Industries, UK

2.1.5 FACS antibodies

Tab.1: Anti-human antibodies

Name	Clone	Company
Surface antibodies		
α-hu CD4 FITC	RPA-T4	BD Biosciences, Germany
α-hu CD4 PerCP	SK3	BD Biosciences, Germany
α-hu CD4 V450	RPA-T4	BD Biosciences, Germany
α-hu CD8 FITC	HIT8a	BD Biosciences, Germany
α-hu CD11c APC	B-ly6	BD Biosciences, Germany
α-hu CD14 APC	MΦP9	BD Biosciences, Germany
α-hu CD25 APC	M-A251	BD Biosciences, Germany
α-hu CD45RA APC	HI100	BD Biosciences, Germany
Intracellular antibodies		
α-hu FoxP3 PE	259D/C7	eBioscience, USA
α-hu IL-10 APC	JES3-19F1	BD Biosciences, Germany

Tab.2: Anti-mouse antibodies

Name	Clone	Company
Surface antibodies		
α-m CD3 APC	145-2C11	BD Biosciences, Germany
α-m CD4 FITC	RM4-5	BD Biosciences, Germany
α-m CD4 AlexaFluor 647	RM4-5	Invitrogen, Germany
α-m CD8 PE	53-6.7	BD Biosciences, Germany
α-m CD8 biotin	53-6.7	BD Biosciences, Germany
α-m CD11b Pacific Blue	M1/70	Biolegend, USA
α-m CD11c APC	N418	Biolegend, USA

α-m CD25 APC	PC61	BD Biosciences, Germany
α-m CD44 APC	IM7	BD Biosciences, Germany
α-m CD62L APC	MEL-14	BD Biosciences, Germany
α-m CD122 PE	TM-Beta 1	BD Biosciences, Germany
α-m CD127 PE	SB/199	BD Biosciences, Germany
α-m Ly49 FITC	14B11	eBioscience, USA
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	FJK-16s	eBioscience, USA
α-m GranzymeB PE	16G6	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
α-m TNF-α APC	MP6-XT22	BD Biosciences, Germany

Tab.3: Secondary antibodies and isotype controls

Name	**Clone**	**Company**
mouse α-hu IgG$_1$ PE	MOPC-21	eBioscience, USA
rat α-m IgG2a PE		eBioscience , USA
α-m Fcγ III/II (CD16/CD32)	2.4G2	BD Biosciences, Germany
human IgG, Fc fragment		Calbiochem, Germany
SA-FITC		BD Biosciences, Germany
SA-PE		BD Biosciences, Germany
SA-PerCP		BD Biosciences, Germany
SA-APC		BD Biosciences, Germany
SA-PacificBlue		Invitrogen, Germany

2.1.6 Cytokines, stimuli, blocking antibodies

rh GM-CSF	R&D Systems, Germany
rh IL-1β	R&D Systems, Germany
rh IL-2	Chiron Therapeutics, USA
rh IL-4	R&D Systems, Germany
rh IL-10	R&D Systems, Germany
rh IL-15	PeproTech, Germany
rh IL-23	R&D Systems, Germany
rh IFN-α-2a	R&D Systems, Germany
rh TGF-β	R&D Systems, Germany
rm GM-CSF	selbst gemacht mit HEK-Zellen, wie angeben?
rm IL-2	R&D Systems, Germany
rm IL-6	R&D Systems, Germany
rm IL-12	R&D Systems, Germany
rm IL-23	R&D Systems, Germany
Purified α-hu CD3 (OKT3)	Janssen-Cilag, Germany
Purified α-hu CD28	R&D Systems, Germany
Purified α-m CD3	BD Biosciences, Germany
Purified α-m CD28	BD Biosciences, Germany
Purified α-m IL-4	Miltenyi Biotec, Germany
Purified α-m Qa-1b	Santa Cruz Biotechnology, USA

2.1.7 Software

Adobe Illustrator CS4	Adobe Systems Inc., USA
FACSDiva	BD Biosciences, Germany
FlowJo	Tree Star Inc., USA
GraphPad Prism 5	GraphPad Software, USA
ImageJ	Wayne Rasband, NIH, USA
Imaris	Bitplane, Switzerland
Imspector	LaVision Biotec, Germany
SPSS 12.0	SPSS, Germany
Volocity	Improvision, Germany

2.2 General cell biological methods

2.2.1 Cell culture

Cell culture preparations and handling were performed under a laminar flow hood under aseptic conditions. Murine cells were cultured in mouse cell culture medium (MM), whereas human cells were cultured in FCS-medium in incubators at 37°C in a 5% CO_2 atmosphere and 95% humidity. All material for cell culturing was sterilized or disinfected with 70% alcohol before use. Waste was autoclaved at 120°C for 20 min at 1 bar.

2.2.2 Cell counting

To determine cell numbers, cells were resuspended in a defined volume and an aliquot of this cell suspension was mixed with trypan blue in a ratio of 1:1, 1:5 or 1:10. This mixture was applied to a Neubauer-hemocytometer and the cells in the 16 fields of one quadrant (n) were counted under a light microscope. Dead cells were excluded, as they can be distinguished from viable cells by their blue appearance due to the uptake of trypan blue. The total number of living cells was calculated as following:

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

2.2.3 Cell isolation

2.2.3.1 Human PBMC

PBMC were isolated by Ficoll Hypaque density gradient centrifugation from buffy coats (German Red Cross, Berlin) or heparinized peripheral blood of healthy donors taken in accordance with the local ethics committee. Ficoll is an iso-osmotic sucrose-polymer, which during centrifugation separates different cell types according to their density. Erythrocytes, dead cells, and granulocytes (high density) pass through the Ficoll, whereas monocytes, T cells, B cells, and NK cells (low density), the PBMC, accumulate in the interphase. It is important to perform all steps of the isolation at room temperature. Buffy coats were washed with PBS and the eluate was filled up to 300ml with PBS. Heparinized peripheral blood from 2 blood tubes was combined and filled up to 50 ml with PBS. 25 ml of blood-PBS suspension, from either buffy coat or heparinized peripheral blood, was then carefully layered on top of 15 ml Ficoll in 50 ml falcon tubes. Following centrifugation at 760g for 40 min without break, the PBMC-containing interphase was carefully collected, transferred to another tube

containing PBS, and centrifuged for 20 min at 560g. The pellet was resuspended in PBS and cells were centrifuged for 15 min at 250g, followed by another centrifugation step for 10 min at 250g. The cell pellet was then resuspended in human washing medium for cell counting.

2.2.3.2 Murine spleen and lymph node cells

Mice were sacrificed by cervical dislocation and disinfected with 70% EtOH. Spleen and lymph nodes (usually inguinal and axial) were removed and transferred separately to 50 ml falcon tubes on ice containing 5 ml WM. Under the laminar flow hood, the removed organs were meshed through a cell strainer in a petri dish to generate a single cell suspension. The sive was rinsed with WM, the cell suspension then transferred to a 50 ml falcon tube and centrifuged at 550g for 5 min at 4°C. The lymph node cells were then directly resuspended in WM for counting, whereas the spleen cells were subjected to an additional step to lyse the erythrocytes. Therefore, the spleen cells were resuspended in 10 ml lysis buffer, then 5 ml WM was added, and the suspension was centrifuged at 550g for 5 min at 4°C. The spleen cells were resuspended in WM, centrifuged again at 550g for 5 min at 4°C, and resuspended in WM for counting.

2.2.3.3 Lymphocytes from mouse CNS

Mice were lethally anaesthethized with an intraperitoneal injection of ketamin/xylazine-mixture (415 mg/kg / 9.7 mg/kg). 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 20 ml ice-cold PBS. Brain and spinal cord were isolated and transferred to a petri dish. The CNS tissue was cut into small pieces using a scalpel and diluted in 5 ml IMDM-medium substituted 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. 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 700g (room temperature) without break, mononuclear cells were collected from the interphase of the gradient, washed with WM, and counted.

2.2.3.4 Murine bone marrow cells

Mice were sacrificed by cervical dislocation and disinfected with 70% EtOH. Femurs were removed and placed in a petri dish with PBS. Under the laminar flow hood, BM cells were isolated by flushing femurs with PBS containing 0.5% BSA. The cell suspension was put through a cell strainer on top of a 50 ml falcon tube and afterwards centrifuged at 550g for 5 min at 4°C. Cells were resuspended in WM and counted.

2.2.3.5 Murine PBMC

Blood from the tail vein of mice 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 550g, 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 human T cell response, T cells were stimulated 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) and T cell response calculated as an index of stimulation (SI) following alloantigen or unspecific stimulus ($cpm_{stimulated}/cpm_{unstimulated}$), which was then transformed into percentages.

2.2.5 CFSE proliferation assay

To measure murine T cell proliferation, T cells were labeled with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE). Therefore, T cells were preincubated for 15-30 min at 37°C in MM and subsequently washed twice in pre-warmed RPMI+1% Hepes. Cells were then resuspended in 10 ml pre-warmed RPMI+1% Hepes containing 2.5 µM CFSE and incubated for 10 min at 37°C in the dark. The labeled cells were washed twice with cold MM, counted, and cultured in 48-well plates for 3-4 days. Target cells were thereby stimulated either polyclonally with anti-CD3 and anti-CD28 antibodies, with APC and the corresponding antigen (ratio targets to APC 1:3), or with iDC and the corresponding antigen (ratio targets to

iDC 10:1). After this culture period, cells were harvested, washed with FACS buffer, stained with FACS antibodies for T cell markers and measured at a FACSCanto II.

2.2.6 ^{3}H suppression assay of human T cells

To examine the suppressive capacity of potentially regulatory human CD4+ T cells, these cells were co-cultured with ConA pre-activated autologous CD4+ T cells, which were isolated from PBMC using CD4+ microbeads, in varying ratios. Inhibition of proliferation of pre-activated CD4+ T cells was detected by a standard ^{3}H-thymidine proliferation assay.

2.2.7 CFSE suppression assay of murine T cells

To study the suppressive capacity of potentially regulatory murine CD4+ and CD8+ T cells, these cells were co-cultured with murine CFSE-labeled CD4+ target cells. The CFSE-labeling was performed as described above (chapter 2.2.5). Target cells were then stimulated with APC and the corresponding antigen. The potentially regulatory cells were added to the CFSE-labeled pre-activated target cells in different ratios, and cells were cultured and measured as described above for the CFSE-proliferation assay.

2.2.8 Cytotoxicity assay

Cytotoxicity of potentially regulatory human and murine CD8+ T cells was assessed as following: target cells (mouse CD4+ 2d2/OT2 Th17 cells or human MBP$_{85-99}$-specific CD4+ Th17 cells) were stained with anti-mouse CD4-AF647 or anti-human CD4-Horizon and subsequently with 1 μM CFSE. CFSE-labeling was performed as described in chapter 2.2.5. Target cells were stimulated polyclonally with anti-CD3 and anti-CD28. CD8+ T cells were added in a 4:1-ratio to the targets and cells were incubated for 18-24 hr. Cells were then harvested and directly transferred to FACS-tubes for measuring at the flow cytometer. As a positive control for dead or dying cells, some harvested cells were incubated with saponin-buffer for 5 min to permeabilize the cell membrane and washed with FACS-buffer before measuring. Immediately before measuring, 1 μl PI (0.1 μg/μl) was added to each sample to visualize dead and dying cells. Samples were then acquired at a FACSCanto II.

2.3 Immunological methods

2.3.1 Flow cytometry

Flow cytometry (FACS = fluorescence activated cell sorting) is a very efficient method for the characterization of individual immune cell populations. Hereby, morphological characteristics, such as cell size and granularity, but also the expression of specific molecules on the cell surface or within the cells can be analyzed. Fluorescently labeled monoclonal antibodies that bind to specific molecules are used to mark individual cell populations. Furthermore, biotinylated monoclonal antibodies are available, which can be combined with a secondary fluorescently labeled 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 fluorescences bound to each cell are excited by the corresponding laser beam and emit a signal that is detected by the flow cytometer. 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 fluorescences in addition to cell size (FSC = forward scatter) and cell granularity (SSC = sideward scatter). Analysis of flow cytometric data was performed using FlowJo analysis software.

2.3.1.1 Cell surface stainings

For cell surface stainings, usually 0.5-1.5 x 10^6 cells were used per sample. Cells were washed with FACS-buffer in a 1.5 ml eppendorf tube. Centrifugation steps were always carried out at 550g for 5 min at 4°C. The supernatant was discarded and cells stained with 50 μl of antibody-mixture (antibodies diluted in FACS-buffer) for 10-20 min at 4°C in the dark. After the incubation, cells were washed with 1 ml FACS-buffer, and if needed stained with 50 μl of secondary antibody for 10-20 min at 4° C in the dark. Cells were washed twice with 1 ml FACS-buffer and resuspended in 300-400 μl FACS-buffer. Cells were then transferred to FACS-tubes and measured at a FACSCanto II.

In some cases, 1 μl PI (0.1 μg/μl) was added to the sample shortly before measuring to exclude dead and dying cells. This dye can enter only cells with damaged cell membrane, intercalating with their DNA, and thereby allowing differentiation of dead and living cells.

To determine absolute cell numbers in one sample, a defined number of BD TruCOUNT Beads was added to the sample shortly before measuring. From the number of acquired beads (beads$_{acq}$), the total number of beads added to the sample (beads$_{total}$), and the number of acquired cells (cells$_{acq}$), the total number of cells (cells$_{total}$) in each sample can be calculated according to the following equation:

cells$_{total}$ = (beads$_{total}$ / beads$_{acq}$) x cells$_{acq}$

2.3.1.2 Intracellular stainings

To stain intracellular cytokines, T cells were first 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 (1-3x10^6 cells/ml) to induce cytokine production. 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, cells were harvested and transferred to 2 ml eppendorf tubes. Surface staining of the cells was performed as described above, but with 100 μl antibody mixture instead of 50 μl, due to the different tube size. After the surface staining, cells were washed once with PBS, then fixed with 2% PFA for 20 min at 4°C. Cells were washed once with PBS and once with saponin-buffer. Cells were then incubated for 10 min at 4°C with 70 μl α-m Fcγ III/II in saponin-buffer to block unspecific binding. After the incubation, 20 μl of antibody-mixture (intracellular antibodies in saponin-buffer) was added and the cells incubated for 20 min at 4°C. Cells were subsequently washed once with saponin-buffer and once with FACS-buffer, resuspended in 300-400 μl FACS-buffer, and transferred to FACS-tubes for measuring at the FACSCanto II.

To stain the intranuclear molecule FoxP3, the FoxP3-staining kits for mouse or human cells from eBioscience were used according to the manufacturers instructions.

2.3.2 Isolation of immune cells by magnetic sorting

The isolation of immune cell subsets from mouse spleen and lymph node cells or human PBMC was accomplished by magnetic cell sorting (MACS), using kits from Miltenyi Biotec and according to the manufacturers instructions. Hereby, two basic principles can be distinguished. One is the direct labeling of the target cell population with antibodies coupled to magnetic beads, which is called positive sort. The cell suspension is run on a MACS column in the magnetic field of a MACS separator, which after rinsing retains only the

magnetically labeled target cells within its magnetic field, which subsequently can be eluted. This results in a target cell population with magnetic beads on its surface. The beads however fall off the cells after several days in culture. The other principle is an indirect or negative sort, where not the target cell population is labeled with magnetic beads, but all the unwanted cells. Therefore, a biotinylated antibody-mixture labeling all cells except the target cell population is applied. In a second labeling step, anti-biotin antibodies coupled to magnetic beads are added, which bind to the primary antibodies. After running over the column and rinsing it, all unwanted cells are retained in the magnetic field of the MACS separator, whereas the unlabeled 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 Human T cells and monocytes

The isolation of human CD4+ or CD8+ T cells and monocytes was achieved by positive sorting. Therefore, isolated PBMC were washed once with MACS-buffer. The cell pellet was resuspended in 80 μl MACS-buffer per 10^7 cells, followed by the addition of 20 μl CD4, CD8, or CD14 MicroBeads per 10^7 cells. The suspension was incubated for 15 min at 4°C, washed with 50 ml MACS-buffer, and resuspended in 500 μl MACS-buffer per 10^8 cells. The cell suspension was applied onto a MACS LS-column, which had previously been rinsed with 3 ml of MACS-buffer. After the cell suspension had passed through the column, the column was washed 3 times with 3 ml MACS-buffer to remove any unlabeled cells. The column was then removed from the MACS-separator and placed on top of a 15 ml falcon tube. 5 ml MACS-buffer were applied onto the column and the magnetically labeled cells were immediately flushed out by pushing the plunger into the column.

The isolation of naïve human CD4+ T cells was achieved by negative sorting. Isolated PBMC were washed with MACS-buffer and the cell pellet resuspended in 40 μl of MACS-buffer per 10^7 cells. 10 μl of Naïve CD4+ T Cell Biotin-Antibody Cocktail II was added per 10^7 cells and the cell suspension was incubated for 10 min at 4°C. Cells were washed with 50 ml of MACS-buffer and the cell pellet resuspended in 80 μl of MACS-buffer and 20 μl of Anti-Biotin MicroBeads per 10^7 cells. The cell suspension was incubated for an additional 15 min at 4°C. Cells were then washed with 50 ml MACS-buffer and resuspended in 500 μl MACS-buffer per 10^8 cells. The cell suspension was applied onto a MACS LS-column, which had previously been rinsed with 3 ml of MACS-buffer, in the field of a MACS-separator,

followed by 3 washing steps with 3 ml MACS-buffer each. The flow-through containing the unlabeled naïve CD4+ T cells was collected.

The purity of the isolated cell populations was checked by FACS surface stainings with CD4-, CD8-, CD14-, and CD45RA-antibodies and measured at a FACSCanto II. The purity was usually around 90-95 %.

2.3.2.2 Murine T cells

CD8+ T cells used for cell culture were isolated by positive sorting. Therefore, murine spleen and lymph node cells were washed once with MACS-buffer. The cell pellet was resuspended in 90 µl MACS-buffer per 10^7 cells, followed by the addition of 10 µl CD8 MicroBeads per 10^7 cells. The suspension was incubated for 15 min at 4°C, washed with 50 ml MACS-buffer, and resuspended in 500 µl MACS-buffer per 10^8 cells. The cell suspension was applied onto a MACS LS-column, which had previously been rinsed with 3 ml of MACS-buffer. After the cell suspension had passed through the column, the column was washed with 3 x 3 ml MACS-buffer to remove any unlabeled cells. The column was then removed from the MACS-separator and placed on top of a 15 ml falcon tube. 5 ml MACS-buffer were applied onto the column and the magnetically labeled CD8+ T cells were immediately flushed out by pushing the plunger into the column.

CD4+ and CD8+ T cells that were used for direct application in *vivo* were isolated by negative sorting. Murine spleen and lymph node cells were washed with MACS-buffer and the cell pellet resuspended in 40 µl of MACS-buffer per 10^7 cells. 10 µl of CD4+ T Cell Biotin-Antibody Cocktail or CD8+ T cell Biotin-Antibody Cocktail was added per 10^7 cells and the cell suspension was incubated for 10 min at 4°C. Then, without washing, 30 µl of MACS-buffer and 20 µl of Anti-Biotin MicroBeads per 10^7 cells were added and the cell suspension was incubated for an additional 15 min at 4°C. Cells were then washed with 50 ml MACS-buffer and resuspended in 500 µl MACS-buffer per 10^8 cells. The cell suspension was applied onto a MACS LS-column, which had previously been rinsed with 3 ml MACS-buffer, in the field of a MACS-separator, followed by 3 washing steps with 3 ml MACS-buffer each. The flow-through containing the unlabeled CD4+ or CD8+ T cells was collected.

For the isolation of naïve murine CD4+ T cells, a negative sort for CD4+ T cells was performed as described above to receive untouched CD4+ T cells, followed by a positive sort

for CD62L, a marker for naïve T cells. Therefore, the untouched CD4+ T cells collected from the flow through of one column were 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. The cells were washed with 50 ml MACS-buffer and then resuspended in 1 ml MACS-buffer. The cell suspension was applied onto a MACS LS-column, which had previously been rinsed with 3 ml of MACS-buffer. After the cell suspension had passed through the column, the column was washed with 3 x 3 ml MACS-buffer to remove any unlabeled cells. The column was then removed from the MACS-separator and placed on top of a 15 ml falcon tube. 5 ml MACS-buffer were applied onto the column and the magnetically labeled CD62L+ T cells were immediately flushed out by pushing the plunger into the column.

The purity of the isolated cell populations was checked by FACS surface stainings with CD4-, CD8-, and CD62L-antibodies and measured at a FACSCanto II. The purity of the positively isolated CD8+ T cells was usually around 95%, of the negatively isolated CD8+ and CD4+ T cells around 80-90%, and of the CD62L+ cells above 95%.

2.3.2.3 Murine APC

APC were isolated from mouse spleen cells by depletion of all T cells (CD90+). This was achieved by a positive sort for CD90+ cells using CD90.2 MicroBeads and the collection of the flow-through from the column, containing all spleen cells except the T cells, which were retained in the magnetic field of the column. The labeling procedure corresponded to the one for the positive sort of murine CD8+ T cells in chapter 2.3.2.2, but with CD90.2 MicroBeads instead of CD8 MicroBeads.

2.3.3 Generation of human and murine iDC

2.3.3.1 Human iDC

Monocytes isolated from PBMC of healthy donors were cultured at 4 x 10^6 cells/ml in FCS-medium in 6-well plates (2 ml/well) in the presence of rhGM-CSF (50 ng/ml) and rhIL-4 (20 ng/ml). On day 3, 1 ml fresh medium with GM-CSF and IL-4 was supplemented. On day 7, the generated iDC were harvested. Therefore, the medium with floating cells was transferred to a falcon tube. Then, ice-cold PBS was added to the plate, which was incubated on ice for several minutes, followed by washing with ice-cold PBS to remove the adherent cells. The

cells were added to the falcon tube containing the previously removed medium, centrifuged and resuspended in FCS-medium.

2.3.3.2 Murine iDC

Murine bone marrow cells were grown in 100 mm Petri dishes in MM supplemented with GM-CSF containing supernatant from a transfected 293FT HEK cell line. GM-CSF concentration of the supernatant was measured by ELISA, normalized and used in a final concentration of 10 ng/ml. Per dish, 4×10^6 cells in 10 ml medium were cultured. On day 3, 10 ml fresh medium and GM-CSF (10 ng/ml) were added. On day 6, 10 ml of medium were carefully removed from the dish and replaced with 10 ml fresh medium and GM-CSF (10 ng/ml). On day 8, 10 ml of medium were carefully removed from the dish and replaced with 10 ml fresh medium and the double concentration of GM-CSF (20 ng/ml). iDC were harvested on day 10 by harvesting the medium with the floating cells and by removing the adherent cells from the dish with a cell scraper. Cells were centrifuged and resuspended in MM.

2.3.3.3 Treatment with atorvastatin during iDC generation

During the generation of iDC, atorvastatin was added in different concentrations (1 μM, 2 μM, 5 μM) to the cultures, always on the days when fresh medium and GM-CSF was added. During the generation of human iDC, atorvastatin was added on days 0 and 3. During the generation of murine iDC, atorvastatin was added to the culture on days 0, 3, 6, and 8.

2.3.4 Stimulation and culture of human and murine T cells

2.3.4.1 Polyclonal stimulations

Mouse T cells were stimulated with plate-bound α-m CD3 (3 μg/ml) and plate-bound α-m CD28 (2.5 μg/ml) in 48-well-plates or 96-well-plates (round bottom). Therefore, wells were coated with 120 μl or 50 μl antibody-mixture per well overnight at 4°C. The antibody-mixture was removed shortly before the cells were added to the wells for stimulation. For intracellular FACS stainings, murine T cells were polyclonally stimulated for 4 hours at $1\text{-}3 \times 10^6$ cells/ml/well in 48-well-plates.

To study the direct effect of atorvastatin on naïve murine CD4+ T cells, naïve CD4+ T cells were polyclonally stimulated at 0.4×10^6 cells/200μl/well in 96-well-plates in the presence of

different concentrations of atorvastatin for 3 days, followed by flow cytometric analysis of IL-10, FoxP3 and CFSE.

As a control for murine CD8+ T suppressor cells, CD8+ T cells isolated from B6 or B6.RFP mice were polyclonally stimulated at 1×10^6 cells/ml/well in 48-well-plates, transferred to 24-well-plates on day 2, and cultured in medium supplemented with 100 U/ml rhIL-2 for a culture period of 7-10 days.

Human T cells were stimulated with plate-bound α-hu CD3 (10 µg/ml) and soluble α-hu CD28 (5 µg/ml) in 24-well-plates. Therefore, wells were coated with 500 µl anti-CD3 per well overnight at 4°C or for 3 hours at 37°C. Shortly before cells were added to the wells for stimulation, the antibody was removed and wells were washed twice with PBS. Cells were added to the wells together with the anti-CD28 antibody. Another method used to polyclonally activate human CD4+ T cells was the addition of ConA (2 µg/ml) directly to the T cells.

To study the direct effect of atorvastatin on naïve human CD4+ T cells, naïve CD4+ T cells isolated from PBMC were polyclonally stimulated with ConA at 1×10^6 cells/ml in the presence or absence of atorvastatin for 3 days, followed by a ^{3}H suppression assay with autologous CD4+ target cells.

As a control for human CD8+ T suppressor cells, CD8+ T cells isolated from PBMC by magnetic sorting were polyclonally stimulated with anti-CD3/anti-CD28 and cultured in medium supplemented with 10 U/ml rhIL-2 for 7-10 days.

2.3.4.2 Antigen-specific stimulation of murine CD4+ T cells

For the culture of murine CD4+ T effector cells in the presence of atorvastatin to enrich Treg cells, PLP-specific CD4+ T cells were generated. Therefore, cells from draining lymph nodes of PLP$_{139-151}$-immunized SJL mice were isolated on day 10 after immunization and cultured with 12.5 µg/ml PLP$_{139-151}$ and different concentrations of atorvastatin for 3 weeks in MM supplemented with 100 U/ml rhIL-2. Cells were restimulated on days 7 and 14 with freshly prepared irradiated (30 Gy) APC from SJL mice (ratio 1:3 - 1:10) and 12.5 µg/ml PLP$_{139-151}$, and further incubated with or without atorvastatin. Every 3-4 days cells from the culture were stained for FoxP3 and analyzed by flow cytometry.

To study the direct effect of atorvastatin on naïve CD4+ T cells, naïve CD4+ T cells isolated from B6.OT2 mice were cultured with freshly isolated irradiated (30 Gy) APC from C57BL/6 mice (ratio 1:3 - 1:10) and 0.6 μM OVA$_{323-339}$ for 3-4 days in the presence of different concentrations of atorvastatin, followed by flow cytometric analysis of IL-10, FoxP3 and CFSE.

To study the indirect effect of atorvastatin on naïve CD4+ T cells, naïve CD4+ T cells isolated from B6.OT2 mice were cultured with OVA-lodaded iDC generated from mouse bone marrow cells in the presence or absence of atorvastatin. Therefore, the iDC were loaded with 25 μM OVA$_{323-339}$ for 1h at 37°C, washed, and then incubate with the naïve CD4+ T cells (ratio iDC to T cells 1:10) for 3-4 days, followed by flow cytometric analysis of the CD4+ T cells for IL-10, FoxP3 and CFSE.

To generate MOG- or OVA- specific CD4+ Th17 cells, naïve CD4+ T cells were isolated from B6.2d2-, B6.OT2-, or B6.2d2.EGFP-transgenic mice by magnetic sorting. Naïve T cells were stimulated with irradiated (30 Gy) APC from C57BL/6 mice (ratio 1:3 - 1:10), the corresponding peptide (12.5 μg/ml MOG$_{35-55}$, 0.6 μM OVA$_{323-339}$), and the cytokines rmIL-6 (20 ng/ml), rmIL-23 (20 ng/ml) and rhTGF-β (3 ng/ml) at 3 Mio cells/ml in 24-well-plates. Cells were splitted on days 3 and 5 with fresh MM supplemented with rhIL-2 (50 U/ml) and rmIL-23 (10 ng/ml). On day 7, cells were restimulated with freshly isolated irradiated APC, peptide and cytokines (10 ng/ml IL-6, 20 ng/ml IL-23, 0.75 ng/ml TGF-β), and again cultured in MM supplemented with IL-2 and IL-23, as in the week before. On day 14, cells were restimulated again with freshly isolated irradiated APC, peptide and cytokines (5 ng/ml IL-6, 20 ng/ml IL-23). On day 3 after the second restimulation, cells were harvested and used for in *vitro* experiments and induction of passive EAE in Rag1$^{-/-}$ recipient mice. Expression of cytokines on this day was checked by intracellular staining with anti-CD4, anti-IL-17, and anti-IFN-γ antibodies and analyzed by flow cytometry, routinely yielding 15-30% IL-17 and no IFN-γ.

To generate MOG-specific CD4+ 2d2 Th1 cells, a single-cell suspension was generated from spleens of B6.2d2.RFP-transgenic mice. Cells were incubated with 12.5 μg/ml MOG$_{35-55}$ and the cytokines IL-12 (10 ng/ml) and anti-IL-4 (20 μg/ml) at 2.5 Mio cells/ml in 6-well-plates. Cells were splitted every second day with fresh MM supplemented with rhIL-2 (100 U/ml). On days 7 and 14, cells were restimulated with freshly isolated irradiated APC, peptide and

IL-12 (10 ng/ml). On day 3 after the second restimulation, cells were harvested and used for induction of passive EAE in B6.Rag1$^{-/-}$Thy1.21.EGFP recipient mice. Expression of cytokines on this day was checked by intracellular staining with anti-CD4 and anti-IFN-γ antibodies and analyzed by flow cytometry, routinely yielding >50% IFN-γ.

For CFSE-suppression assays, CD4+ 2d2 or OT2 Th17 cells from day 7 or day 14 of the culture were used as target cells. The Th17 cells were labeled with CFSE and stimulated with APC (ratio 1:2) and the corresponding peptide (12.5 μg/ml MOG$_{35-55}$, 0.6 μM OVA$_{323-339}$).

2.3.4.3 Antigen-specific stimulation of human CD4+ T cells

To generate MBP-specific CD4+ Th17 cells, PBMC were depleted of CD8+ T cells by positive magnetic sorting for CD8+ T cells. CD8-depleted PBMC were cultured with MBP$_{85-99}$ (50 μg/ml) and the cytokines rhIL-1β (12.5 ng/ml), rhIL-23 (10 ng/ml), and rhTGF-β (0.2 ng/ml) at 3 Mio cells/ml in 24-well-plates. Cells were cultured in medium supplemented with 10 U/ml rhIL-2 and 10 ng/ml rhIL-23. On day 6, cells were harvested and sorted for CD4+ T cells. The CD4+ T cells were restimulated with irradiated CD8-depleted PBMC, MBP$_{85-99}$ (25 μg/ml) and the cytokines rhIL-1β (6.25 ng/ml), rhIL-23 (10 ng/ml), and rhTGF-β (0.05 ng/ml) in a ratio of 1:3 to 1:5. Cells were cultured in medium supplemented with 5 U/ml rhIL-2 and 5 ng/ml rhIL-23. On day 13, one part of the CD4+ T cells was irradiated and used for the co-culture with CD8+ T cells, whereas the other part of the CD4+ T cells was restimulated again as described above. On day 20, these cells were harvested and used as targets in the cytotoxicity assay.

2.3.4.4 Generation of murine suppressor CD8+ T cells

CD8+ T cells were isolated by magnetic sorting from spleens and lymph nodes of MOG$_{35-55}$-immunized C57BL/6 or B6.RFP mice in remission. At the corresponding time points, CD8+ T cells were isolated from C57BL/6 or B6.RFP mice immunized with OVA-protein, and from non-immunized C57BL/6 or B6.RFP mice as controls. *Ex vivo* isolated CD8+ T cells were either directly used for experiments, or expanded *in vitro* in a co-culture with irradiated (30 Gy) CD4+ 2d2 Th17 or CD4+ OT2 Th17 cells (ratio 1:3) for 7-10 days in MM supplemented with 100 U/ml IL-2. Control CD8+ T cells were stimulated polyclonally with α-m CD3/CD28, and cultured in medium supplemented with 100 U/ml IL-2 for 7-10 days. *Ex vivo* CD8+ T cells and *in vitro* expanded CD8+ T cells were characterized by flow cytometric

analysis using the following anti-mouse antibodies: CD8-bio, CD25-APC, CD44-APC, CD122-PE, CD127-PE, IL-10-APC, IFN-γ-PE, IL-17-PE, TNF-α-APC, Streptavidin-PerCP and Ly49-FITC, GrzB-PE, FoxP3-APC. CD8+ T cells were used for *in vitro* experiments (CFSE suppression assay and cytotoxicity assay) or transferred as treatment into mice with EAE on day 10 after induction of EAE.

2.3.4.5 Generation of human suppressor CD8+ T cells

CD8+ T cells were isolated from PBMC by magnetic sorting. Sorted CD8+ T cells were co-cultured with autologous irradiated (30 Gy) MBP$_{85-99}$-specific CD4+ Th17 cells for 7 days at a ratio of 1:3 to 1:5 in FCS medium supplemented with 10 U/ml rhIL-2. Control CD8+ T cells were stimulated polyclonally with α-hu CD3/CD28, and cultured for 7 days in medium supplemented with 10 U/ml rhIL-2. CD8+ T cells were then used for *in vitro* cytotoxicity assays.

2.3.4.6 Generation of human IL-10-producing Treg cells

Naïve CD4+ T cells were sorted from PBMC by magnetic sorting and co-cultured with previously generated allogeneic iDC at a ratio of 20:1 at a resulting cell concentration of 1 x 10^6 cells/ml in FCS-medium. On day 0, a cytokine cocktail consisting of rhIL-10 (100 U/ml), rhIFN-α-2a (675 U/ml), rhIL-15 (20 U/ml) and rhIL-2 (16 U/ml) was added to the culture. Every three days fresh medium, IL-2 and IL-15 were supplemented. On day 7 cells were restimulated with allogeneic DC and cytokines as on day 0 and cultured for additional 7 days. Cells were then used for a ^{3}H-suppression assay.

2.3.4.7 Incubation of T cells with atorvastatin

Murine and human T cells were incubated with atorvastatin in concentrations from 0.1-25 μM. Atorvastatin was usually added at the beginning of the T cell culture and the final concentration was maintained until the end of the culture. Therefore, when cells were splitted or fresh medium was added, atorvastatin was supplemented.

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.OT-2 (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. 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 (153) 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. B6.RFP mice are C57BL/6 mice with omnipresent tdRFP-expression under a *ROSA26* promotor (154). B6.EGFP mice are C57BL/6 mice with omnipresent EGFP-expression under the *β-actin* promotor (originally derived from the Jackson Laboratory, USA). 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. B6.2d2 mice and B6.EGFP mice were crossed to generate double transgenic B6.2d2.EGFP mice. All animal experiments were approved by local authorities (LaGeSo Berlin: G0148/05, G0106/05, G0029/08, G0255/08, T0271/08) and conducted according to the German Animal Protection Law.

2.4.2 Anesthesia of mice

Before perfusion, mice were anesthetized with an intraperitoneal injection of ketamin/xylazine-mixture (415 mg/kg / 9.7 mg/kg). For in *vivo* imaging experiments, mice were anesthetized with 1.5% isoflurane in oxygen/nitrous oxide (2:1) via a 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 immunization 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 immunization and 48 hr later.

Immunization of C57BL/6 mice with ovalbumin was performed as follows: 250 μg OVA-protein and 800 μg H37RA emulsified in CFA were injected subcutaneously, followed by two intraperitoneal doses of 400 ng PTX in PBS at the time of immunization and 48 hr later.

Induction of active EAE in Rag1$^{-/-}$ or Rag1$^{-/-}$Thy1.21 mice reconstituted with CD4+ and/or CD8+ T cells was induced by 100 μg MOG$_{35-55}$-peptide or 50 μg OVA$_{257-264}$-peptide and 400 μg H37RA emulsified in CFA, followed by two intraperitoneal doses of 200 ng PTX in PBS at the time of immunization and 48 hr later.

Induction of active EAE in SJL mice was induced by subcutaneous immunization with 250 μg PLP$_{139-151}$ peptide and 800 μg H37RA emulsified in CFA. PTX (200 ng) was injected intraperitoneally at the time of immunization and 48 hr later when the disease course was studied, but was omitted when lymph node cells were isolated to generate PLP-specific CD4+ T cells *in vitro*.

2.4.3.2 Induction of passive EAE

Passive EAE was induced in Rag1$^{-/-}$ or Rag1$^{-/-}$Thy1.21 mice by intravenous transfer of 3-5 Mio MOG$_{35-55}$-specific CD4+ 2d2 Th17 cells on day 3 after second in *vitro* restimulation of the cells.

2.4.3.3 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.

2.4.3.4 Therapeutic measurements

CD8+ T cells with potentially regulatory properties were transferred into mice with active or passive EAE before onset of clinical signs on day 10 after induction of EAE. 10 x 10^6 CD8+ T cells were intravenously transferred per mouse.

2.5 Two-photon laser scanning microscopy

2.5.1 Setup and imaging

Operation procedures and two-photon laser scanning microscopy were performed as previously described by Siffrin *et al.* (125,126). Mice were anesthetized with 1.5% isoflurane in oxygen/nitrous oxide (2:1) via a facemask. The mice were then tracheotomised and continuously respirated with a Harvard Apparatus Advanced Safety Respirator. The anesthetized 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 (155). 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 anesthesia 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 (LaVision BioTec, Germany) previously described by Herz *et al.* (123), 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 colocalized 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 Data analysis

Intravital images were post-processed using acquisition software Imspector (LaVision Biotec). 3D presentation, quantitative cell tracking analysis, and cell-cell contact determination were performed with the software ImageJ (NIH), Volocity (Improvision), and Imaris (Bitplane).

2.5.2.1 Angle calculation

Quantification of axon-associated motility of CD8+ T cells was achieved by analyzing the trajectory vectors, which are the connecting line between starting point and end point of each cell track and which represent the direction of displacement of individual cells, as described

by Siffrin et *al.* (125). Next, the vectors were mirrored onto the axonal axis and the smallest angle between vector and axons was calculated, which resulted in a nominal value between 0° and 90° for each track, with 45° indicating no alignment. This measure is independent of polarized directionality and absolute displacement, making it useable as a tool for describing tangential movement along an axis (125).

2.5.2.2 Cell-cell contact determination

Contacts between EGFP+ and RFP+ cells within the brain stem of imaged mice were analyzed as described by Siffrin et *al*. (126). In short, double-positive EGFP/RFP voxels were highlighted by standard image analysis software. Using automated identification of double-positive voxels, a co-localization 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 labeled 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 as well as their contact-volume and the total imaging-volume, the contact index k can be calculate, which is independent of the absolute number of cells. k is a measure for the co-localization and therefore for the interaction of two cell populations.

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

2.6 Statistical analysis

Statistical analysis was carried out with GraphPad Prism 5 and SPSS 12.0. 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 Mann-Whitney-U test or Kurskal-Wallis test were used. P-values < 0.05 were considered significant.

3 Results

3.1 Pharmacological generation of regulatory CD4+ T cells in *vitro*

Several groups, including ours, studied the effect of atorvastatin on CD4+ T cells. Atorvastatin was shown to induce T cell anergy in effector CD4+ T cells as a result of blocking cell cycle progression and to shift the differentiation status of CD4+ T cells from a Th1 towards a Th2 phenotype (148). This has a beneficial outcome in an autoimmune setting such as EAE, as self-reactive effector CD4+ T cells, probably the main players in autoimmune neuroinflammation, are controlled. Previous findings from our group also showed, that atorvastatin-induced T cell anergy is mediated via early phosphorylation of the Map-Kinase ERK1 and increased expression of the anti-inflammatory cytokine IL-10 (156). Furthermore, it was shown that atorvastatin downregulates MHC-II and co-stimulatory molecules on APC, which led to a slight decrease in their T cell stimulatory capacity (157). These findings indicate, that atorvastatin might not only impact T cell proliferation, but also modulate the T cell response either directly or indirectly via APC in such a way, that T cells adopt a regulatory function or that their regulatory potential is increased. Here, it was investigated if atorvastatin can generate T cells with a regulatory phenotype, namely FoxP3+ Treg or IL-10-producing Treg cells. Therefore, the effects of atorvastatin on priming and differentiation of naïve CD4+ T cells were examined, as well as the effects of atorvastatin on the expansion and regulatory potential of existing regulatory T cells. Furthermore, it was investigated if atorvastatin exerts its immune modulatory effects directly on T cells, or indirectly via APC.

3.1.1 Effect of atorvastatin on the proliferation of naïve CD4+ T cells

3.1.1.1 Atorvastatin impacts T cell proliferation of naïve CD4+ T cells

Though the effect of atorvastatin on proliferation of effector CD4+ T cells has been studied extensively, its effect on naïve CD4+ T cells remains controversial. Using freshly isolated T cells from human PBMC (mixture of naïve and effector T cells) it was shown, that atorvastatin reduces polyclonal proliferation of these cells (156). Here, as a starting point for future experiments murine T cells were used, which were sorted for naïve CD4+CD62Lhi T cells to study the direct effect of atorvastatin on T cell priming. The murine model has the advantage, that T cell receptor transgenic mice can be used. In these mice, high numbers of identical T cells allow physiologic stimulation with antigen for these studies, which is not

possible for human T cells due to the low precursor numbers. The proliferation of naïve antigen-specific murine T cells after polyclonal or antigen-specific activation in the presence of different concentrations of atorvastatin was examined. Naïve CD4+ T cells were isolated from spleen and lymph nodes of OT-2 transgenic mice, expressing a T cell receptor specific for the ovalbumin (OVA)-peptide 323-339. The naïve CD4+ T cells were labeled with the fluorescent dye CFSE and activated either in an antigen-unspecific way using anti-CD3 and anti-CD28 antibodies, or in an antigen-specific way with irradiated APC and $OVA_{323-339}$ peptide. Atorvastatin was added to the cells in different concentrations (0, 2, 5, 25 μM) and cells were incubated for 3 days. Subsequently, cells were harvested and the dilution of CFSE-intensity in proliferated cells was analyzed by flow cytometry as a measure of T cell proliferation (exemplarily shown in Fig.4A). The percentage of cells in each gate was further analyzed for the different atorvastatin concentrations (Fig.4B and Fig.4D). Atorvastatin inhibited proliferation of naïve CD4+ T cells in a dose-dependent manner. For the polyclonally activated T cells effects were already visible at the lowest concentration of 2 μM atorvastatin, visualized here by an increased percentage of cells that underwent no or only one division and decreased percentages of cells that underwent more than one division (Fig.4B). With antigen-specific stimulation, the effects were most robust for higher concentrations of atorvastatin, probably due to the generally increased proliferation of the CD4+ T cells following this mode of stimulation (Fig.4D). The addition of 25 μM atorvastatin to the cultures showed a strong inhibition of T cell proliferation and an important cytotoxic effect, as demonstrated by inclusion of propidium iodide (PI, Fig. 4C). These experiments show a direct inhibitory effect of atorvastatin on T cell proliferation of naïve CD4+ T cells, which is independent of APC.
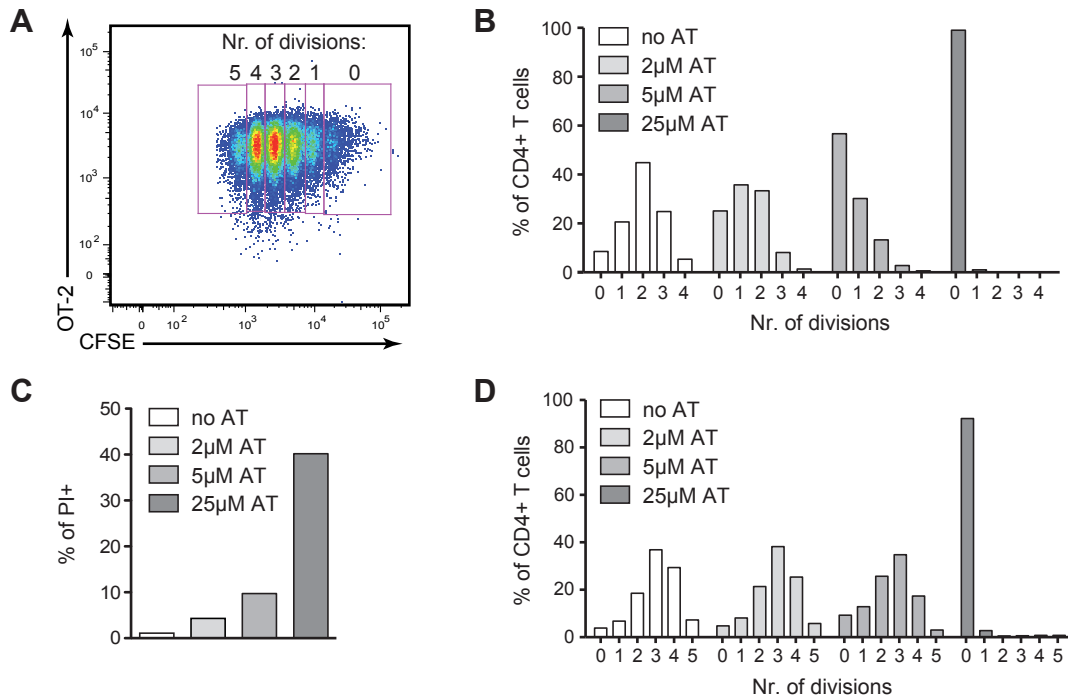
Figure 4. Atorvastatin (AT) inhibits proliferation of naïve OVA-specific CD4+ T cells. (A) Proliferation of naïve OT-2 CD4+ T cells is characterized by a decrease in CFSE-intensity in the proliferated cells. The starting cell population is found in gate "division 0", when cells divide once, they are found in gate "division 1" and so on. It was gated on living CD4+ T cells. (B) Analysis of T cell proliferation 3 days after activation with anti-CD3/anti-CD28 and addition of different concentrations of atorvastatin. (C) Percentage of dead/dying (PI+) cells of the culture shown in (B). (D) Analysis of T cell proliferation 3 days after activation with APC and OVA$_{323-339}$ and addition of different concentrations of atorvastatin. One representative experiment of three is shown.

3.1.1.2 Atorvastatin has no indirect effect on the proliferation of naïve CD4+ T cells

Next, the effect of atorvastatin on T cell priming as a consequence of atorvastatin treatment of APC was examined. iDC, as the professional APC, were generated from bone marrow cells of C57BL/6 mice in the absence or presence of different atorvastatin concentrations. Subsequently, the iDC were loaded with OVA$_{323-339}$ peptide and used to stimulate naïve CFSE-labeled OT-2 CD4+ T cells *in vitro*. Of note, in this experiment atorvastatin was not present during the 4 day DC-T cell culture, which allowed to study the effect of atorvastatin solely on the APC-compartment. iDC generated in the presence of atorvastatin had the same capacity to stimulate naïve CD4+ T cells as iDC generated in the absence of atorvastatin (Fig.5).
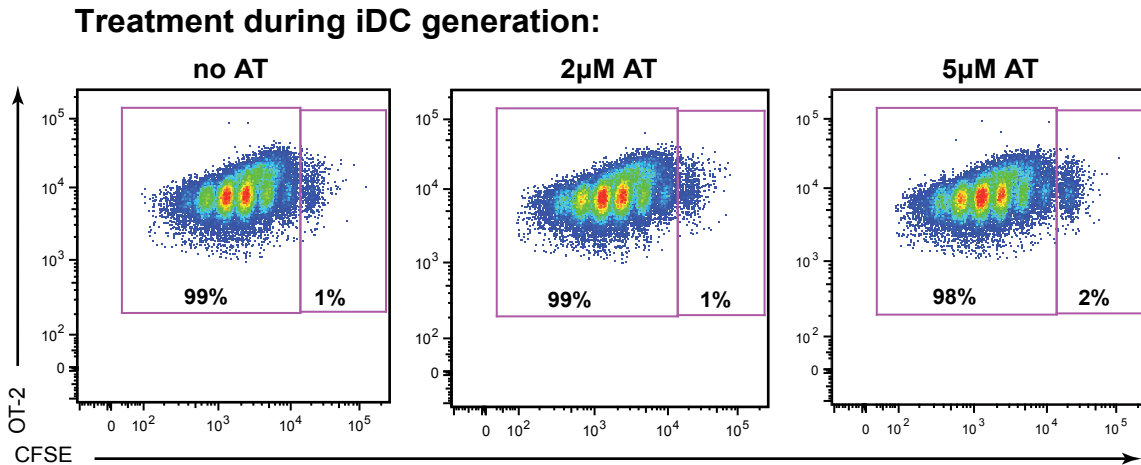
Treatment during iDC generation:

Figure 5. Atorvastatin (AT) has no indirect effect on the proliferation of naïve OVA-specific CD4+ T cells. Addition of atorvastatin during the generation of iDC from bone marrow cells has no effect on the stimulative capacity of the iDC and therefore on the proliferative response of the CFSE-labelled naïve OT-2 CD4+ T cells. One representative experiment of three is shown.

3.1.2 *De novo* generation of regulatory T cells using atorvastatin

To examine if naïve CD4+ T cells are influenced by atorvastatin to commit to a certain phenotype, the following question was approached: Can atorvastatin induce de novo generation of regulatory T cells from naïve CD4+ T cells? On the one hand the generation of FoxP3+ Treg cells was examined, on the other hand the induction of IL-10-producing Treg cells.

3.1.2.1 Atorvastatin does not induce regulatory T cells from naïve murine CD4+ T cells

First, naïve CD4+ T cells were isolated from spleen and lymph nodes of OT-2 transgenic mice, activated using anti-CD3 and anti-CD28 antibodies, and cultured for 3 days *in vitro* with different concentrations of atorvastatin in the culture. After 3 days, stainings for FoxP3 and IL-10 were performed, but no significant difference in the percentage of FoxP3+ and IL-10+ cells was observed for CD4+ T cells cultured in the presence or absence of atorvastatin (Fig.6A). The T cells were expanded by addition of 100 U/ml IL-2, and cultured for another 3 days in the presence of different atorvastatin concentrations. On day 6, cells were harvested and their suppressive capacity was examined by a classical T cell suppression assay using CFSE-labeled CD4+CD25- target cells isolated from spleens of OT-2 mice (Fig.6B). The ratio of effector to target T cells was varied from 1:1, 1:5 to 1:10, but no effect of the

atorvastatin treatment during the generation of the effector T cells was observed on the proliferation of the target cells.
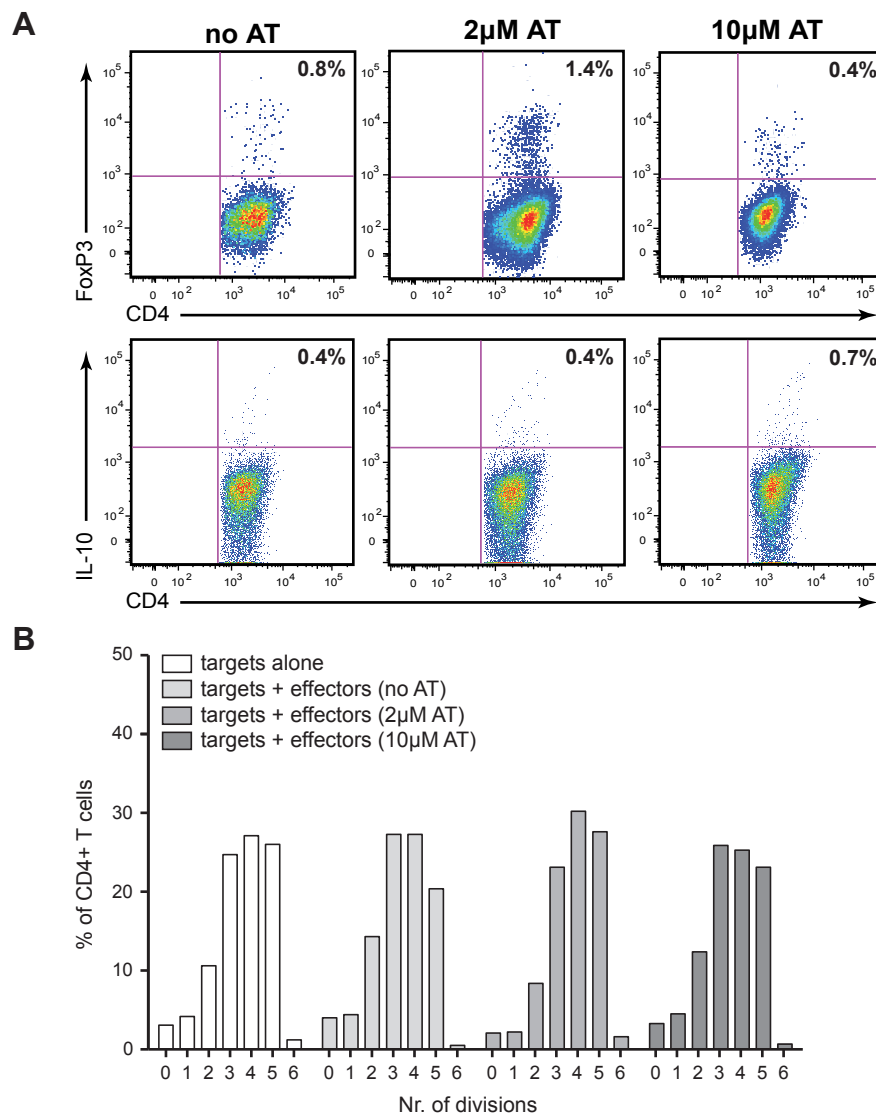

Figure 6. Murine CD4+ T cells stimulated in the presence of atorvastatin (AT) do not show a regulatory phenotype. (A) Flow cytometric analysis of the regulatory markers FoxP3 and IL-10 on OT-2 T cells cultured for 3 days in the presence or absence of atorvastatin. (B) Analysis of suppressive capacity of OT-2 T cells cultured in the presence or absence of atorvastatin. Shown is the proliferation of CFSE-labeled CD4+ OT-2 target T cells co-cultured for 4 days with CD4+ effector T cells (ratio 1:1), which were generated from naïve CD4+T cells in the presence or absence of atorvastatin. One representative experiment of three is shown.

3.1.2.2 Atorvastatin does not induce regulatory T cells from naïve human CD4+ T cells

To examine the effect of atorvastatin on the differentiation of human CD4+ T cells, naïve human CD4+ T cells were isolated from PBMC by magnetic sorting. The T cells were polyclonally stimulated with ConA and cultured for 3 days in the presence or absence of different atorvastatin concentrations. These effector T cells were then harvested and co-cultured with autologous CD4+ target cells. ^{3}H-thymidine was added to the culture and the suppressive capacity of the effector T cells generated in the presence or absence of atorvastatin was examined by measuring the amount of incorporated ^{3}H-thymidine in a standard ^{3}H-thymidine proliferation assay after 3 days (Fig.7). The ratio of target to effector T cells was varied from 10:1 to 2:1, but no effect of the atorvastatin treatment during the generation of the effector T cells was observed on the proliferation of the target cells. This indicates that atorvastatin does not modulate T cell differentiation in a way that T cells acquire a regulatory phenotype, neither in the murine nor in the human system.
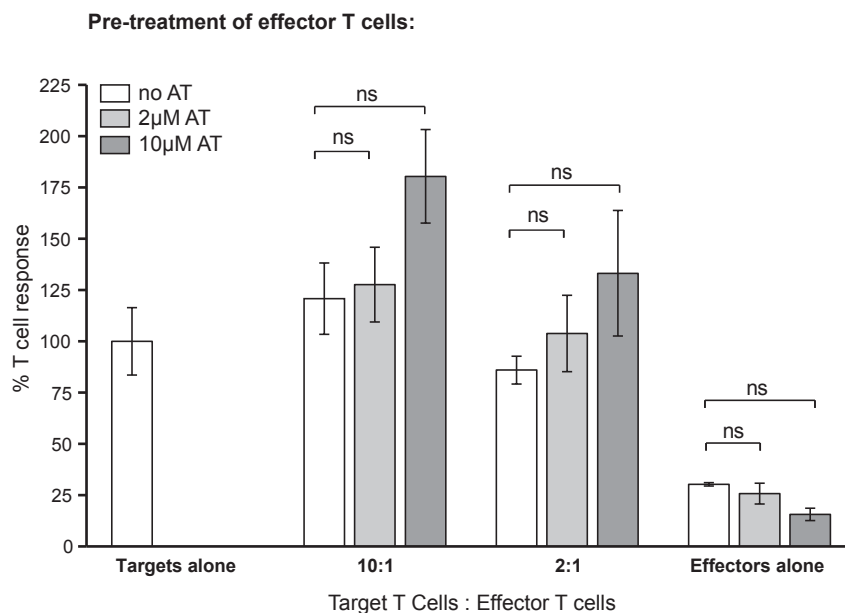

Figure 7: Human CD4+ T cells stimulated in the presence of atorvastatin (AT) do not show a regulatory phenotype. The suppressive capacity of naïve CD4+ T cells activated in the presence or absence of atorvastatin was analyzed by co-culturing them with autologous CD4+ target cells. ^{3}H was added to the culture and the amount of incorporated ^{3}H was measured on day 3. Data are shown as MEAN +/- SEM and were analyzed by unpaired t-test: ns p>0.05.

3.1.3 Effect of atorvastatin on the expansion of FoxP3+ Treg cells *in vitro*

Previous findings and the results presented above show, that atorvastatin inhibits proliferation of naïve and effector T cells (148). Furthermore, it is known that FoxP3+CD25^{hi}CD4+ Treg cells do not proliferate very strongly *in vitro*, as they require high amounts of IL-2 for proliferation (66). Based on these two facts, the idea was pursued that the anti-proliferative effect of atorvastatin could be used to specifically expand Treg cells from a mixture of T cells, as the proliferation of the strongly proliferating T cells might be inhibited more effectively by atorvastatin than the proliferation of the Treg cells. Furthermore, Battaglia *et al.* had shown, that it is possible to selectively expand Treg cells using rapamycin, an immunosuppressive compound (127,128). As FoxP3 is not an exclusive marker for Treg cells in the human system, but is up-regulated following activation, the experiment was performed in the murine system, in which FoxP3 is specifically expressed by Treg cells.

SJL-mice were immunized with a peptide from proteolipid protein (PLP$_{139\text{-}151}$). After 10 days, the draining lymph nodes were extracted, a single cell suspension was generated, and the cells were incubated with PLP$_{139\text{-}151}$-peptide and different concentrations of atorvastatin for 3 weeks. Cells were restimulated every week with freshly prepared antigen presenting cells and PLP$_{139\text{-}151}$, and further incubated with or without atorvastatin. Every 2-3 days IL-2 was added to the cultures and every 3-4 days some cells from the culture were analyzed for the expression of CD4, CD25, and FoxP3 by flow cytometry (Fig.8). On day 7 of the culture, a significant increase in the percentage of Treg cells in the atorvastatin treated groups could be observed compared to the group cultured without atorvastatin (Fig.8A). The highest increase in the percentage of Treg was induced by a concentration of 3μM atorvastatin, which was also the case on day 11 of the culture (Fig.8A). The absolute cell counts show, that the numbers of T cells dramatically decrease over time – also Treg cells in the culture decrease but less timely – independent of the atorvastatin treatment (Fig.8B).

In conclusion, it was shown that all T cells in the culture are inhibited in their proliferation by atorvastatin, independent of their phenotype. However, the effector T cells are inhibited more effectively than the Treg cells, presumably because they are stronger proliferators than Treg cells. This led to a relative increase of Treg cells compared to effector T cells after 1 week of culture, but not to an increase in absolute Treg numbers, as generally the number of Treg in the culture decreased drastically over time.
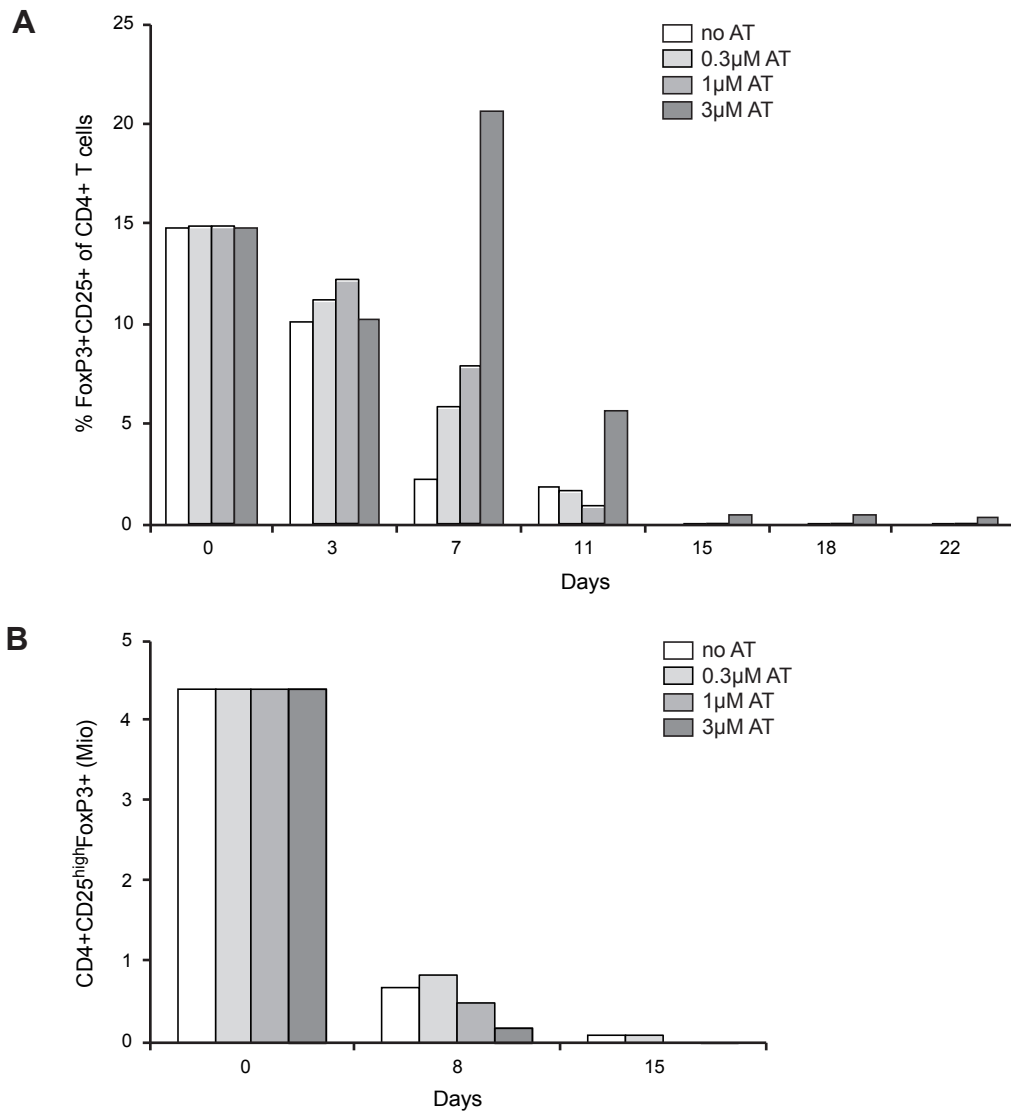
Figure 8. Flow cytometry data of murine PLP-specific T cells cultured *in vitro* with different concentrations of atorvastatin (AT) for a period of 22 days. (A) Percentages of FoxP3+CD25+ cells of CD4+ T cells are shown. (B) Absolute cell numbers of Treg in the culture at selected time points. One representative experiment of two is shown.

3.1.4 Atorvastatin enhances the suppressive capacity of IL-10-producing Treg cells

As shown above, atorvastatin does not induce T cells with a regulatory phenotype and expands Treg cells only as percentage, but not as total number. The next question addressed was, if atorvastatin has the ability to enhance the suppressive capacity of T cells with a regulatory phenotype. Therefore, a protocol for the induction of IL-10-producing Treg cells from human PBMC was established based on previous publications, and the direct and

indirect effects of atorvastatin on the suppressive capacity of these cells was then examined. A study by Jonuleit *et al.* had shown, that repetitive stimulation of naïve human T cells with allogeneic iDC induces IL-10-producing Treg cells (80). It was further shown, that the cytokines IL-10 and IFN-α are important for the induction of IL-10-producing Treg cells (78), and that the cytokines IL-2 and IL-15 are required for the expansion of these cells in culture (79). Here, a protocol was established to induce IL-10-producing CD4+ T cells with a suppressive phenotype from naïve human CD4+ T cells, and to study the effect of atorvastatin on these suppressor T cells (Fig. 9).
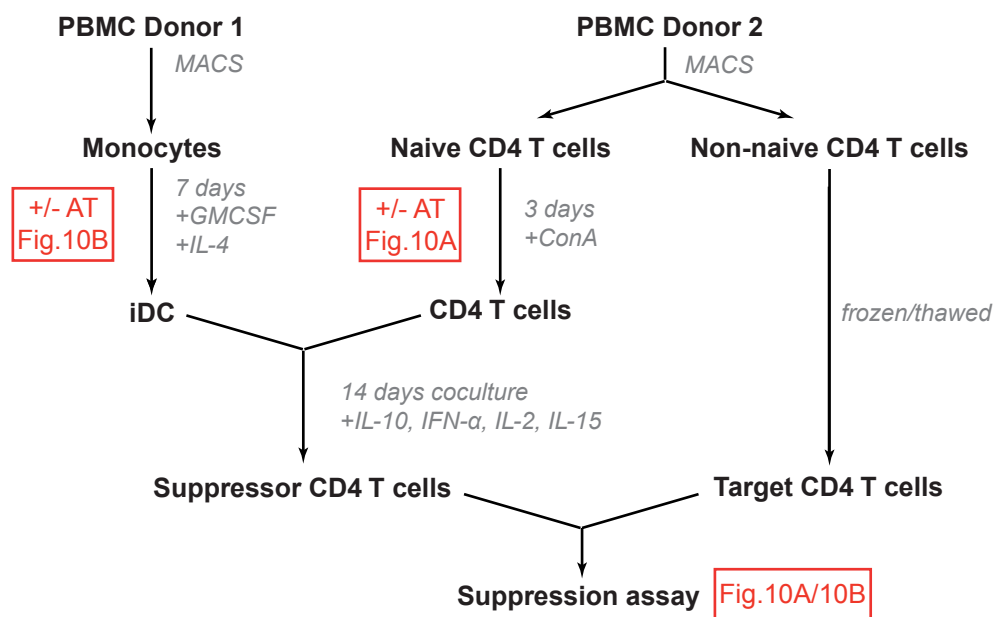

Figure 9. Setup for the generation of IL-10 Treg cells and suppression experiments of Fig.10 A and B.

In short, naïve CD4+ T cells isolated from human PBMC were preactivated with ConA for 3 days and subsequently co-cultured with allogeneic iDC, generated *in vitro* from isolated monocytes, and the cytokines IL-10, IFN-α, IL-2, IL-15. After 7 days, the CD4+ T cells were restimulated with fresh iDC and cytokines, and after 14 days the CD4+ T cells were harvested. The suppressive effect of the resulting suppressor CD4+ T cells was examined in a ^{3}H-thymidine suppression assay with autologous CD4+ T cells as targets. To examine the effect of atorvastatin on the suppressive capacity of the suppressor CD4+ T cells, atorvastatin was added either during the activation of naïve CD4+ T cells or during the generation of iDC from monocytes.
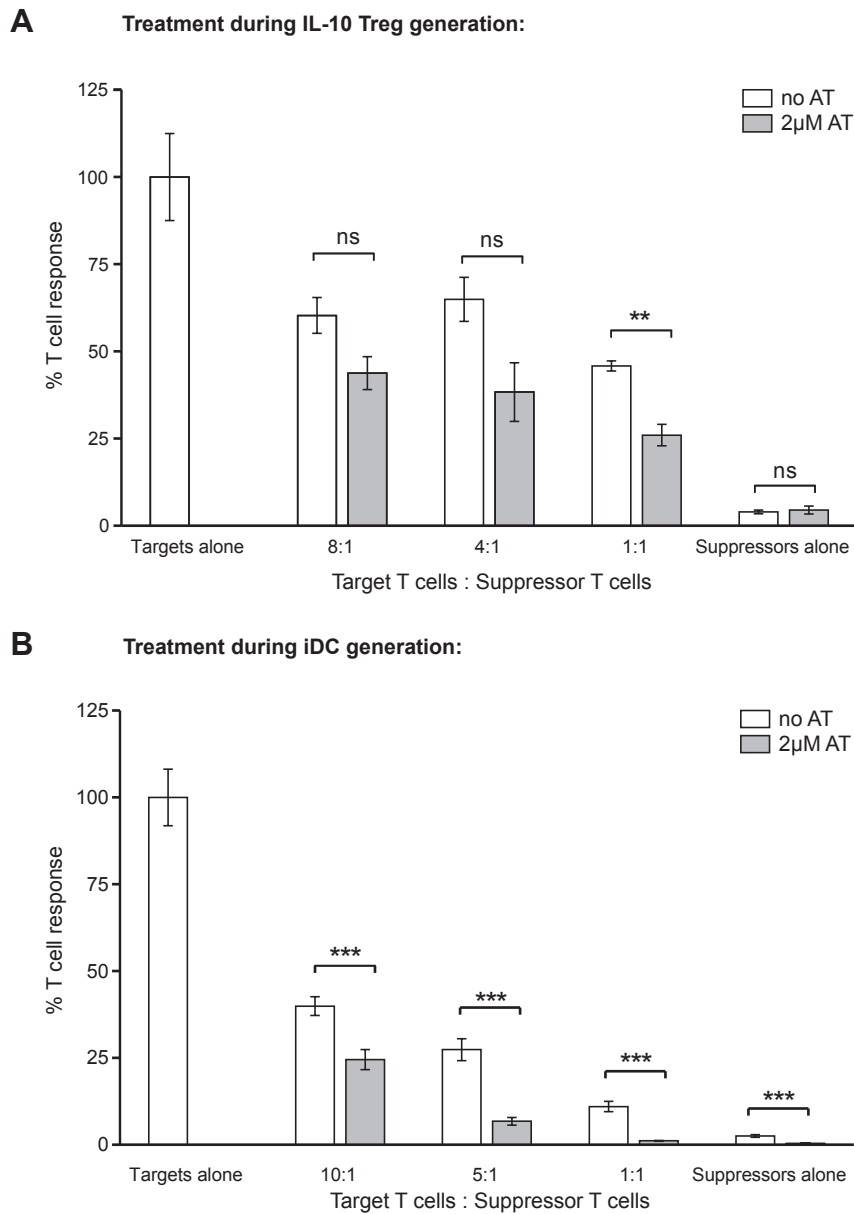
A **Treatment during IL-10 Treg generation:**

B **Treatment during iDC generation:**

Figure 10. Atorvastatin (AT) enhances the regulatory capacity of IL-10 Treg cells. (A) Atorvastatin was applied to naïve CD4+ T cells during the generation of IL-10 Treg cells. IL-10 Treg cells were then added in different ratios to autologous CD4+ target cells. ^{3}H was added to the culture and the amount of incorporated ^{3}H was measured on day 3. (B) Atorvastatin was applied only during iDC generation. IL-10 Treg cells were then added in different ratios to autologous CD4+ target cells. ^{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 analyzed by unpaired t-test: ** $p<0.01$, *** $p<0.001$.

The addition of atorvastatin during the activation of naive CD4+ T cells had no or only a very mild effect on the suppressive capacity of the suppressor CD4+ T cells (Fig.10A). Only a ratio of 1:1 of target to suppressor T cells showed an increased suppression of the measured T

cell proliferation, but this effect was not visible in all of the performed experiments. Higher target to suppressor ratios showed no effect of atorvastatin on the suppressive capacity of the suppressor T cells. On the other hand, the presence of atorvastatin during the generation of iDC from monocytes lead to a significant and reproducible increase in suppression of target T cells compared to the suppression by suppressor T cells generated with untreated iDC (Fig. 10B). Even with a high target to suppressor ratio of 10:1, the effect of atorvastatin was still visible. Interestingly, the proliferation of the suppressor T cells alone showed, that the iDC generated in the presence of atorvastatin induced a more anergic type of suppressor T cell than untreated iDC. Of note, in this part of the experiment atorvastatin was not present in the T cell culture, but only during the generation of iDC, indicating an indirect effect of atorvastatin on T cells via DC.

The observed effect of atorvastatin on DC was further pursued in another thesis in our lab. As the effect of atorvastatin *in vivo* in EAE had already been studied by our group, and was further examined in a clinical trial, the focus of this thesis was shifted from regulatory CD4+ T cells to CD8+ T cells and their possible regulatory role in EAE.

3.2 Contribution of CD8+ T cells to MOG-induced EAE

T cells are required for EAE and most likely also for the induction and propagation of MS. However, as the role of different T cell subsets in EAE is still controversially debated, the first overall aim was to determine the individual contribution of the different T cell subsets, namely CD4+ and CD8+ T cells in MOG-induced EAE. While for CD4+ T cells it is well established that specific subsets with specific functions exist, less is known about the role and subsets of CD8+ T cells in EAE. In MS, a contribution of CD8+ T cells to the disease is assumed, whereas in EAE the CD4+ T cells are acknowledged as the main inducers of the disease, and the CD8+ T cells are thought to play a rather regulatory role. As no specific markers are available to distinguish between CD8+ T cells with specific functions, here, the distribution and phenotype of CD8+ T cells in the different disease phases and their potential to induce or attenuate EAE was examined. As also their primary interaction partners in the CNS are not known so far, the behavior of CD8+ T cells and their interaction with possible target cells directly at the site of inflammation in the CNS was studied. The opportunity to use a TPLSM and use it for intravital imaging of the brainstem of EAE-affected living anesthetized mice opened a wide range of new possibilities in this field. Before this work started, our group had already described the behavior of CD4+ and CD8+ T cells in a

hippocampal slice model, where it could be shown, that activated effector CD4+ T cells show a CXCR4-dependent vessel associated behavior independent of their antigen specificity. This might be to enhance contact with perivascular APC expressing MHC-II, which is required for CD4+ T cells to recognize their antigen. CD8+ T cells in contrast did not show this vessel-associated behavior. They moved freely through the parenchyma, which might be due to the fact, that MHC-I is more widely expressed on cells within the inflamed CNS, and therefore, the CD8+ T cells are not dependent on contacts with perivascular APC for their restimulation. These studies were however only performed with OVA-specific CD8+ T cells, as no system to generate CNS-specific CD8+ T cells for visualization in the hippocampal slice model was available. Furthermore, in the slice model, the underlying movement patterns of individual T cell populations can be investigated, which give hints for the situation *in vivo*, however, they do not describe the actual situation *in vivo*, as the blood brain barrier does not have to be overcome. Here, a system was established to visualize CD4+ and CD8+ T cells with different fluorescences in the CNS of EAE-affected mice *in vivo*. This was used to analyze the movement of the CD4+ and CD8+ T cells as well as their interactions with each other and of CD8+ T cells with neurons as possible target cells in the CNS.

3.2.1 Distribution and phenotype of CD8+ T cells in mice with MOG-induced EAE

In order to examine the role of CD8+ T cells in EAE, in a first approach the distribution and phenotype of CD8+ T cells during the course of EAE in MOG_{35-55}-immunized C57BL/6-mice was analyzed. Therefore, immune cells from lymph nodes, spleen, and CNS were isolated in the peak of disease and during remission. Flow cytometry of isolated immune cells revealed that CD8+ T cells are present in the inflammatory infiltrates in the CNS, but represent a smaller population than the $CD4^+$ T cells (Fig.11A). Interestingly, no regulatory markers known from CD4+ T cells, such as FoxP3 or IL-10 were expressed in significant amounts by CD8+ T cells in this model, neither in the periphery nor in the CNS, and neither in the peak nor in the remission phase (Fig.11B and C). CD8+ T cells produced high amounts of IFN-γ, peaking in maximum disease, but did not show any production of the pro-inflammatory cytokine IL-17 (Fig.11D and E).
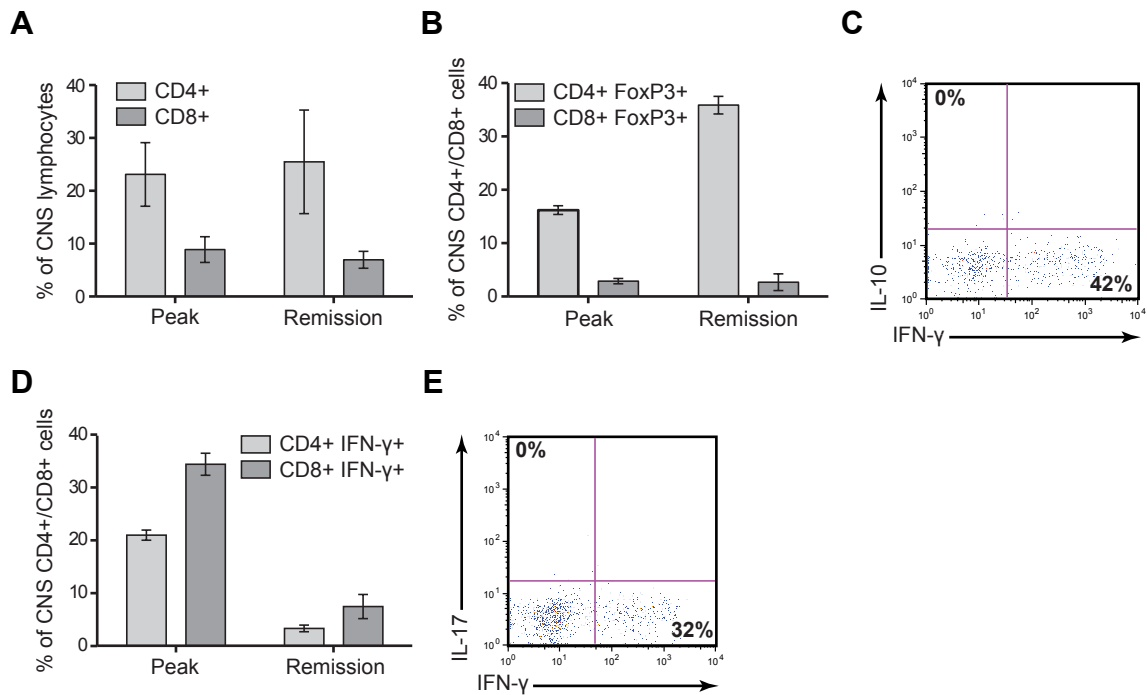
Figure 11. Immune cells isolated from the CNS of C57BL/6 mice immunized with MOG_{35-55} at the peak of disease and in remission. (A) CD8+ T cells are present in lower numbers than CD4+ T cells in the CNS of EAE-affected mice. (B) Only very low numbers of CD8+FoxP3+ cells are present in the peak and in the remission phase compared to CD4+FoxP3+ cells. (C) CD8+ T cells in the CNS produce no IL-10 at the peak of the disease. (D) CD8+ T cells in the CNS produce high amounts of IFN-γ in the peak of the disease, but no IL-17 (E). Data from three mice were pooled and shown as MEAN+/- SEM.

3.2.2 CD8+ T cells are poor inducers or attenuators of MOG-induced EAE

To explicitly distinguish between the influence of CD4+ and CD8+ T effector cells on the course of EAE, a system was developed where lymphocyte deficient Rag1^{-/-} mice were reconstituted with CD4+ T cells alone, CD8+ T cells alone, or a mixture of the two cell types, isolated by magnetic sorting from spleens of wild-type C57BL/6 mice. A period of 4 weeks was allowed for homeostatic engraftment (158). To check if the reconstitution of the mice with T cells was successful, a FACS-staining for CD4 and CD8 was performed with cells isolated from peripheral blood of the reconstituted mice 4 weeks after transfer of the T cells (Fig.12). The reconstitution was successful, with 15-25% CD4/CD8 T cells of the total lymphocytes in the blood. Due to technical limitations of MACS sorting, about 4% contaminating CD8+ T cells were present in the mice that had received only CD4+ T cells and also 4% contaminating CD4+ T cells were present in the mice that had received only

CD8+ T cells. The purity of the transferred cell populations was usually between 90% and 95%.
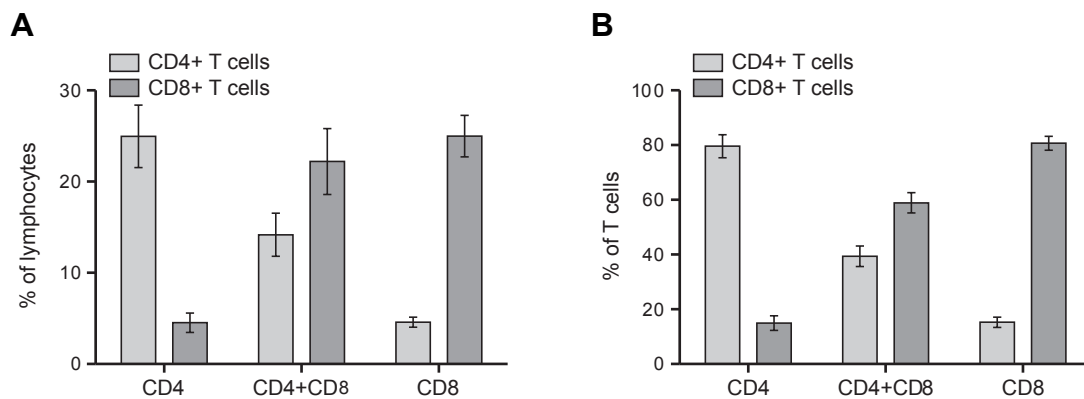

Figure 12. Staining for CD4+ and CD8+ T cells from the blood of Rag1$^{-/-}$ mice 4 weeks after reconstitution with T cells. On the x-axis, the three different groups of mice are shown, either reconstituted with CD4+ T cells alone, CD4+ and CD8+ T cells, or CD8+ T cells alone. (A) Percentage of CD4+ and CD8+ T cells within the lymphocyte gate. (B) Percentage of CD4+ and CD8+ T cells of total T cells. Data from 7 mice per group were pooled and shown as MEAN+/- SEM.

The T cell replenished animals were actively immunized with MOG$_{35-55}$ peptide 4 weeks after T cell transfer. The disease course in the three different groups was monitored and the phenotype of the T cells within the CNS of the mice in the three groups was analyzed between days 26 and 36 after immunization. No significant difference in disease incidence, onset and severity was observed between the groups that had received CD4+ T cells alone or a mixture of CD4+ and CD8+ T cells (Fig.13). The influence of the CD8+ T cells in the remission phase of the disease could not be studied in these two groups in this approach, as the disease course was very severe and non-remitting. Interestingly, mice that had received CD8+ T cells alone showed a drastically reduced disease incidence, and only a very mild disease course with delayed onset (Fig.13).
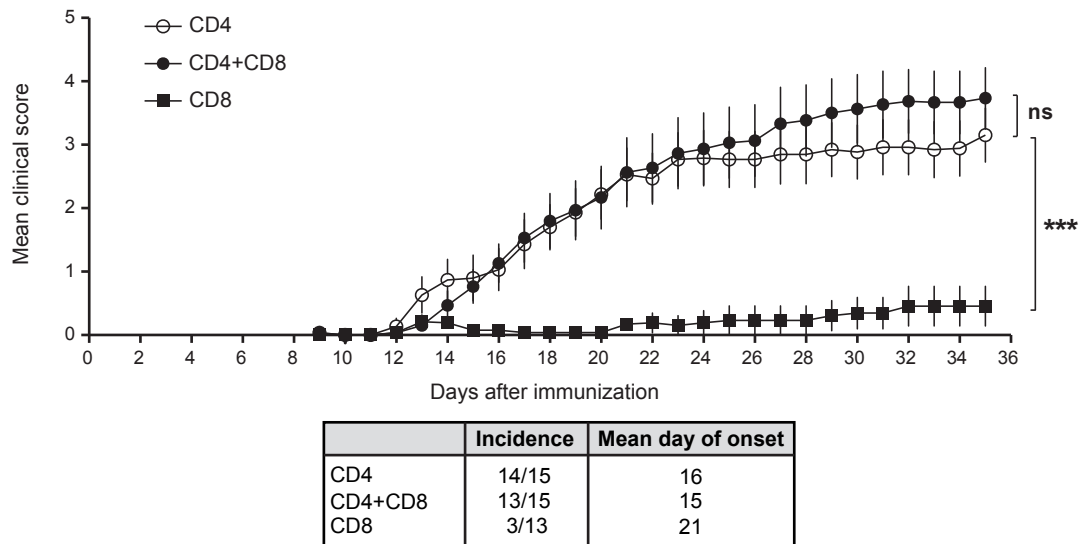
	Incidence	Mean day of onset
CD4	14/15	16
CD4+CD8	13/15	15
CD8	3/13	21

Figure 13. Disease course of Rag1^{-/-} mice replenished with CD4+ and/or CD8+ T cells and subsequently immunized with MOG_{35-55} (Day 0). Reconstitution with CD8+ T cells alone and subsequent immunization led to reduced EAE incidence and severity, with delayed disease onset. Data are shown as MEAN+/-SEM, pooled from three independent experiments, and analyzed by Kruskal-Wallis test and Dunn`s Multiple Comparison post test: p<0.05 was considered significant, *** p<0.001.

To investigate more closely the phenotype of the T cells within the three different groups of the reconstituted Rag1^{-/-} mice, lymphocytes were isolated from the CNS of the mice between days 26 and 36. Flow cytometric analysis of the isolated cells revealed, that activated effector (CD62L-) CD4+ and CD8+ T cells were present in the CNS, with high amounts of CD4+ T cells in the CD4-group, high amounts of CD8+ T cells in the CD8-group, and more CD4+ than CD8+ T cells in the group that had received both cell types (Fig.14A), supporting the previous findings from Fig.11. The CD4+ and CD8+ T cells isolated from the CNS produced high amounts of IFN-γ, the CD8+ T cells even more than the CD4+ T cells (Fig.14B). However, a striking difference in phenotype was the production of the pro-inflammatory cytokine IL-17 by CD4+ T cells compared to CD8+ T cells (Fig.14C). The expression of this highly inflammatory cytokine, which was shown to be a prerequisite for the induction of EAE (55), correlates with disease severity in this EAE model. As visible from Fig.13, only the groups that received the IL-17-expressing CD4+ T cells showed clinical signs of disease, whereas the expression of IFN-γ by CD4+ and CD8+ T cells did not correlate with disease severity.
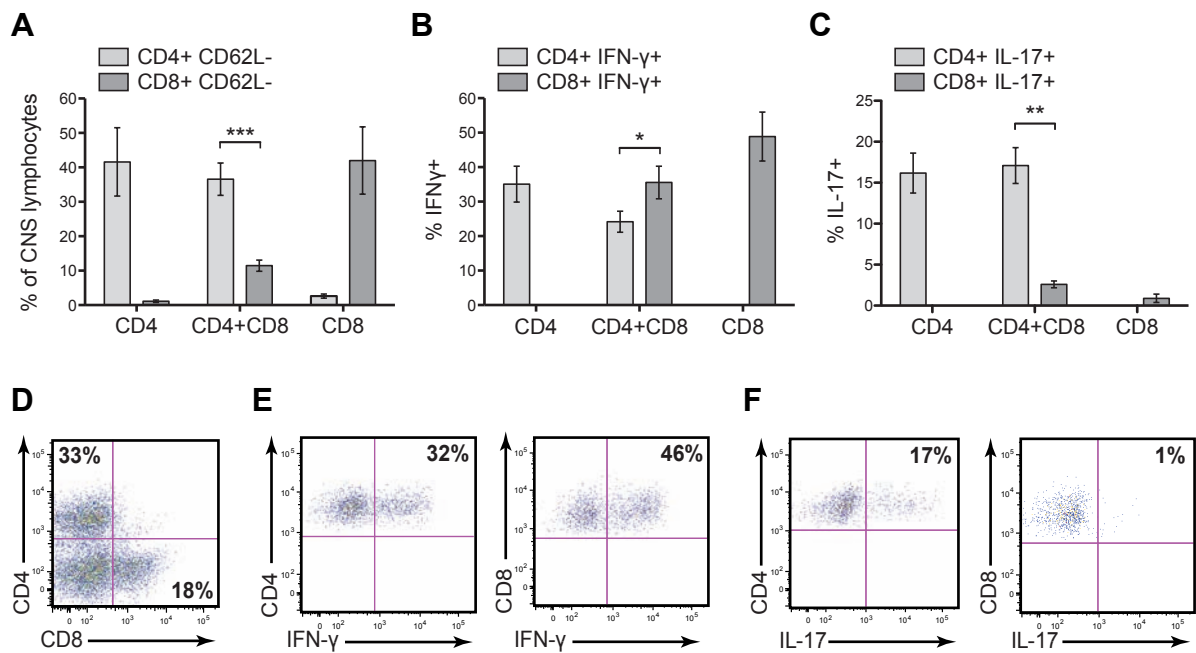
Figure 14. Flow cytometry data of CNS-derived immune cells isolated from MOG$_{35-55}$-immunized Rag1$^{-/-}$ mice previously replenished with CD4, CD8, or CD4+CD8 T cells. (A) Percentage of effector (CD62L-) CD4+ and CD8+ T cells in the CNS of mice from the three different groups. (B), (C) Percentage of IFN-γ+ and IL-17+ cells of the CD4+ and CD8+ T cells isolated from the CNS of mice of the three different groups. Data from 5 mice per group were pooled, shown as MEAN+/-SEM, and analyzed by Mann-Whitney-U test: * p<0.05, ** p<0.01, *** p<0.001. (D), (E), (F) FACS raw data from CNS-derived immune cells of one mouse reconstituted with CD4+CD8 T cells.

3.2.3 Monitoring the behavior of CD8+ and CD4+ T cells in the CNS of EAE-affected mice using TPLSM

T cell dynamics can be very rapid and very interactive, which makes it difficult to track down the different steps in the cascade of damage. As the primary interaction partners of T cells within the CNS during EAE were not known at the time point of these experiments, the behavior of CD8+ T cells and their interactions with CD4+ T cells and axons was investigated using intravital TPLSM of the brain in anesthetized mice with EAE. The approach described above was adapted for the use with TPLSM. Therefore, Rag1$^{-/-}$ mice were replenished with green fluorescent (EGFP) CD4+ T cells and/or red fluorescent (RFP) CD8+ T cells, isolated from spleens of B6.EGFP and B6.RFP mice respectively. After an engraftment period of 4 weeks, mice were immunized with MOG$_{35-55}$. Shortly after onset or at the peak of disease (between day 20 and 30), TPLSM of the upper brainstem, which is a major target site in EAE,

was performed. This approach allowed imaging the behavior of CD4+ and CD8+ T cells in living mice with EAE, independently of each other and also in combination.

In Rag1$^{-/-}$ mice that were replenished with CD4+ and CD8+ T cells, the CD4+ T cells showed a vessel associated behavior (Fig.15A left), whereas the CD8+ T cells did not show this confined movement, instead they moved rapidly through the parenchyma (Fig.15A right). These findings are in line with previous findings from our group using a hippocampal slice model, where the same differential behavior of CD4+ and CD8+ T cells was shown. The movement of the CD8+ T cells through the parenchyma here seemed not random, but also directional (Fig.15A right). The reason for this directed CD8-movement however is not clear, but matches the direction of axons frequently seen in experiments using mice with green fluorescent neurons (Fig.15B).
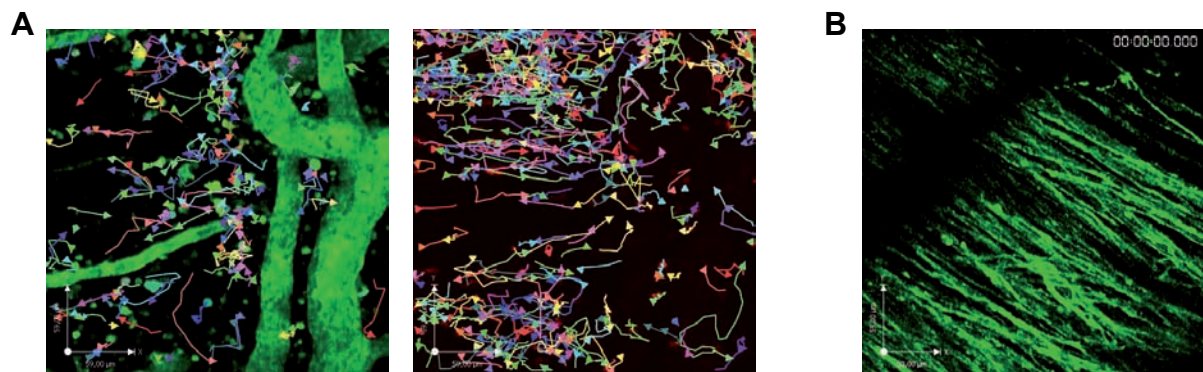

Figure 15. Analysis of CD8 and CD4 trajectories in Rag1$^{-/-}$ mice reconstituted with CD4-EGFP and CD8-RFP cells, and subsequently immunized with MOG$_{35-55}$. (A) Tracks of CD4+ T cells recorded over time show a vessel-associated behaviour (left). Vessels were stained by perfusion of the mouse with FITC-dextran. Tracks of CD8+ T cells show a directional movement of the CD8+ T cells in the parenchyma (right). One example of 9 imaged mice is shown. (B) Direction of axons visualized in a mouse with green fluorescent neurons (B6.Thy1.21.EGFP).

Analysis of the movement patterns of CD4+ T cells and CD8+ T cells in mice reconstituted with CD4+ and CD8+ T cells revealed, that the mean velocity of both cell types was dependent on the disease stage, with cells at the onset of disease showing a significantly higher mean velocity than cells at the peak of the disease (Fig.16A). At the onset of disease, CD4+ T cells (0.085+/-0.002 μm/s) moved faster than CD8+ T cells (0.076+/-0.01 μm/s), whereas at the peak of disease no difference between CD4+ T cell velocity (0.059+/-0.002 μm/s) and CD8+ T cell velocity (0.061+/-0.002 μm/s) was observed (Fig.16A). Similar

behavior of CD4+ and CD8+ T cells was observed regarding the displacement rate of the cells (Fig.16B). The displacement rate is a measure for how fast an object moves away from its starting point, so in comparison to velocity it also includes a measure of directionality of the movement. For cells moving in a directional way, the displacement rate is higher than for cells that show a spatially confined movement. Here, displacement rates for CD4+ and CD8+ T cells were higher at the onset of disease than at the peak of disease (Fig.16B). At the onset of disease, the displacement rate of CD4+ T cells (0.047+/-0.002 μm/s) was higher than of the CD8+ T cells (0.036+/-0.001 μm/s), whereas no difference between the two cell types was observed at the peak of disease (CD4: 0.029+/-0.002 μm/s; CD8: 0.027+/-0.001 μm/s).
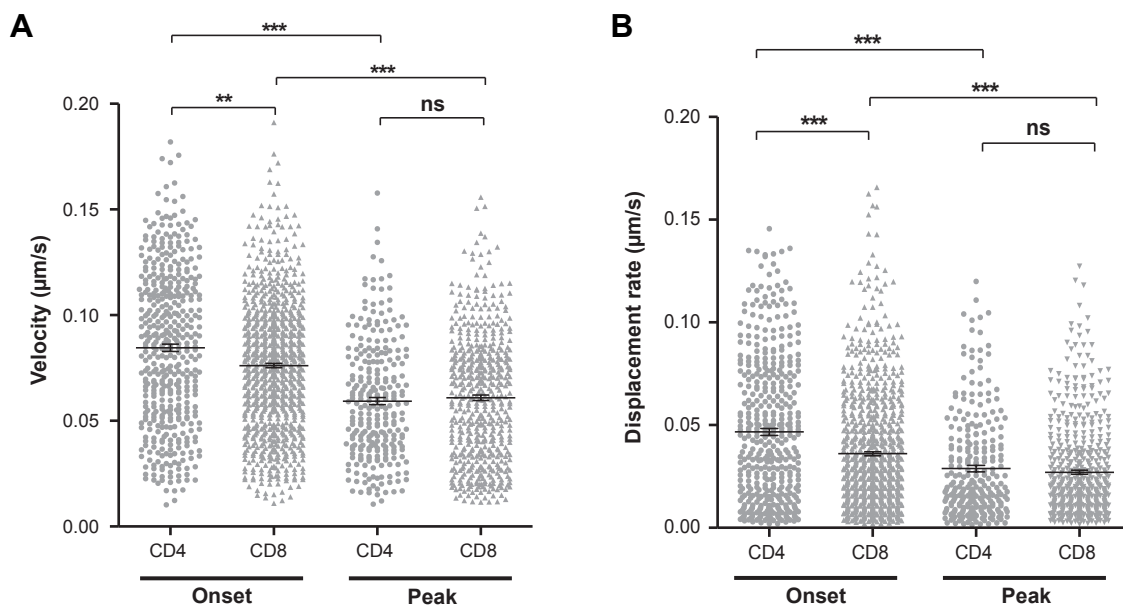

Figure 16. Analysis of CD8 and CD4 movement in Rag1^{-/-} mice reconstituted with CD4-EGFP and CD8-RFP cells and subsequently immunized with MOG$_{35-55}$. Velocity (A) and displacement rate (B) of CD4+ and CD8+ T cells were analyzed at the onset and at the peak of disease. Cell-tracks of 4 movies per time point were pooled, shown as MEAN+/-SEM, and analyzed by Mann-Whitney-U test: p<0.05 was considered significant, ** p<0.01, *** p<0.001.

To examine, if the presence of CD8+ T cells influences the behavior of CD4+ T cells, cell movement of CD4+ T cells in Rag1^{-/-} mice reconstituted with CD4+ and CD8+ T cells was compared with movement of CD4+ T cells in Rag1^{-/-} mice reconstituted with CD4+ T cells alone. Mice from the two groups were immunized with MOG$_{35-55}$ 4 weeks after T cell transfer and imaged at the peak of disease. The mean velocity and displacement rate of the CD4+ T cells were comparable in both groups (Fig.17). In the mice that had been reconstituted with

CD4+ and CD8+ T cells (CD4+CD8), the mean velocity of the CD4+ T cells was 0.059+/-0.002 μm/s compared to a mean velocity of 0.063+/-0.002 μm/s in the mice reconstituted with CD4+ T cells only (CD4 only) (Fig.17A). The mean displacement rate of the CD4+ T cells in the CD4+CD8 group was 0.029+/- 0.002 μm/s compared to 0.026+/-0.002 μm/s in the CD4 only group. The presence of CD8+ T cells therefore did not influence the behavior of the CD4+ T cells, indicating that they did not specifically interact with the CD4+ T cells.
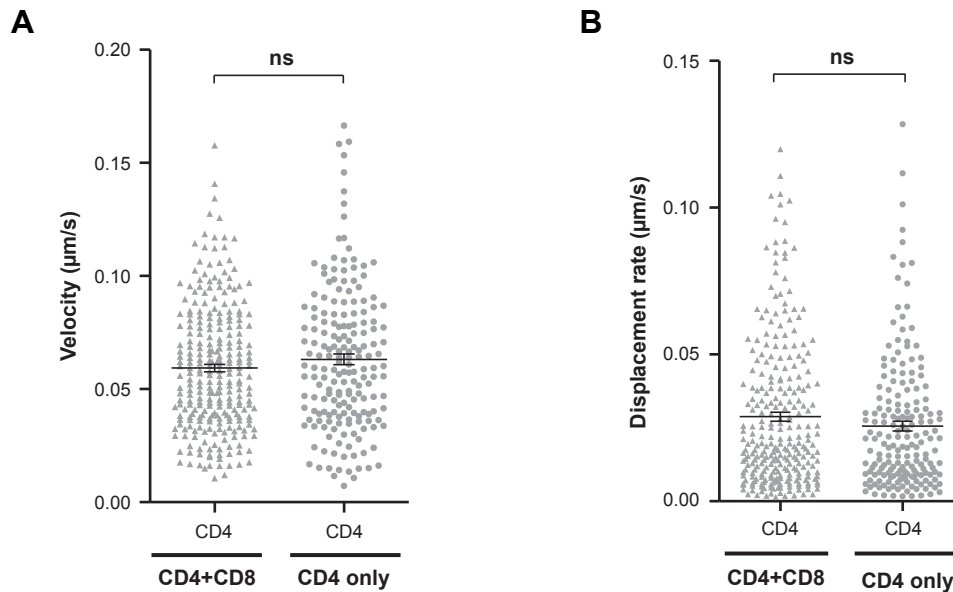

Figure 17. Analysis of CD4 movement in Rag1^{-/-} mice reconstituted with CD4-EGFP and CD8-RFP cells (CD4+CD8) or CD4-EGFP cells only (CD4 only) and subsequently immunized with MOG_{35-55}. Velocity (A) and displacement rate (B) of CD4+ T cells in the two groups were analyzed at the peak of disease. Cell-tracks of 4 movies per time point were pooled, shown as MEAN+/-SEM, and analyzed by Mann-Whitney-U test: $p<0.05$ was considered significant.

To explicitly study the interactions between CD8+ T cells and CD4+ T cells as possible target cells in the CNS, the recorded movies were further analyzed for interactions between CD8-RFP T cells and CD4-EGFP T cells, applying a method developed in our group (126). Thereby, the fact of non-overlapping spectra of EGFP and RFP are exploited to measure the extent of contact formation of green and red fluorescent cells, which can be visualized in a third color channel, here shown in white (Fig.18A). The area of contact formation (voxels positive for green and red fluorescence only in areas of close proximity) can subsequently be followed over time and afterwards quantified. To describe the interactivity of two differently labeled cell populations, our group has developed a means of contact quantification, which is based on the law of mass action (126). By considering the volumes of the two different

fluorescences (Voxel A and Voxel B) as well as their overlap-volume (Voxel coloc AB) and the total imaging-volume (Voxel all), the contact index k can be calculate, which is independent of the absolute number of cells. A high value for k indicates more interactions than a low value for k (Fig.18B). Here, from movies of four different mice, very low k-values between 0.273+/-0.042 and 0.439+/-0.045 were calculated, indicating only very few long-lasting specific contacts between CD4+ and CD8+ T cells at the peak of disease (Fig.18A).
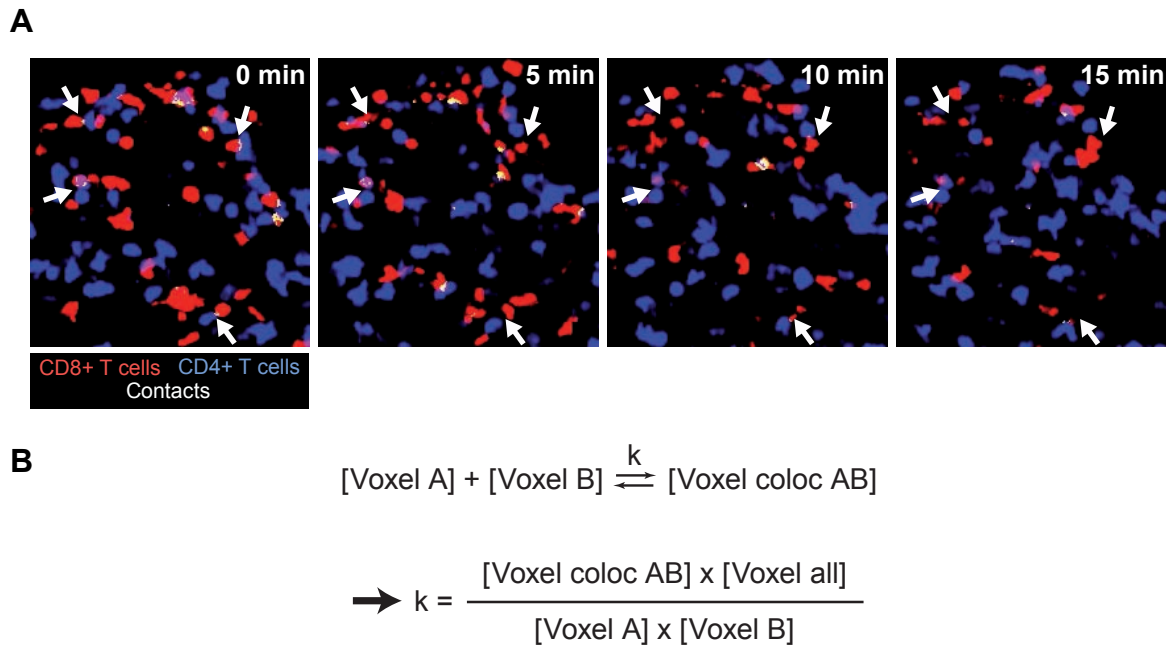
A

B

$$[\text{Voxel A}] + [\text{Voxel B}] \underset{}{\overset{k}{\rightleftharpoons}} [\text{Voxel coloc AB}]$$

$$\rightarrow k = \frac{[\text{Voxel coloc AB}] \times [\text{Voxel all}]}{[\text{Voxel A}] \times [\text{Voxel B}]}$$

Figure 18. (A) Visualization of contacts (white; some indicated by white arrows) between CD8-RFP cells (red) and CD4-EGFP cells (blue) in Rag1^{-/-} mice reconstituted with CD4+CD8 T cells and subsequently immunized with MOG$_{35-55}$, imaged at the peak of disease. (B) Calculation of the contact index k as a means of contact quantification of two differently labelled cell populations A and B.

To study in more detail the behavior of CD8+ T cells in the CNS, mice that had been reconstituted with CD8+ T cells alone and subsequently immunized with MOG$_{35-55}$ were imaged and analyzed. Based on the previously observed behavior of the CD8+ T cells (Fig.15), CD8-RFP cells were injected into mice that express EGFP in neurons and neuronal processes (B6.Rag1^{-/-}Thy1.21.EGFP) in order to also study interactions between CD8+ T cells and neurons as possible target cells in the CNS. Mice were imaged using TPLSM between days 28 and 30 after immunization. CD8-RFP cells were present in the CNS of these mice (Fig.19), although the mice showed no clinical signs of disease at all, corresponding to the data from Fig.13 and Fig.14. However, in contrast to the rapid and directed movement of the CD8+ T cells within the parenchyma in mice reconstituted with CD4+ and CD8+ T cells

(Fig.15), the CD8+ T cells in the mice reconstituted with CD8+ T cells only showed slow movements, thus static behavior (Fig.19). The fact that the neurons were intact in all of the acquired movies indicates, that this static behavior of the CD8+ T cells is probably due to an improper activation of the CD8+ T cells rather than to specific cytotoxic contacts of the CD8+ T cells with the neurons.
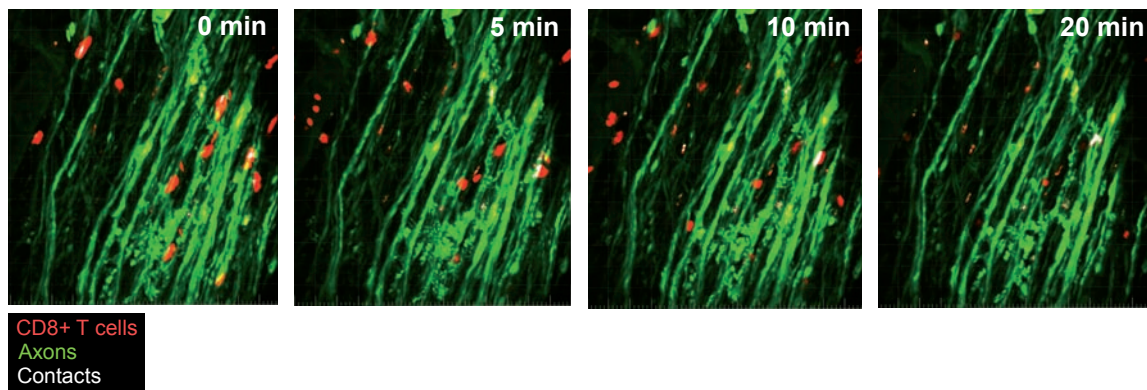

Figure 19. Visualization of CD8-RFP cells (red), EGFP-expressing axons (green), and their co-localization (white) in B6.Rag1$^{-/-}$Thy1.21.EGFP mice reconstituted with CD8-RFP cells and subsequently immunized with MOG$_{35-55}$. Mice were imaged on day 28-30 after immunization.

As a comparison, to visualize the interaction of highly encephalitogenic T cells and neurons, another model developed in our group was applied, where passive EAE was induced in B6.Rag1$^{-/-}$Thy1.21.EGFP mice by transferring *in vitro* differentiated RFP+ MOG-specific (2d2) CD4+ Th17 cells. These cells are highly encephalitogenic and induce severe clinical symptoms in the recipient mice. TPLSM of the CD4+ 2d2 Th17 cells at the peak of disease showed a highly dynamic behavior of these cells in the CNS, not a low migratory behavior as observed for the CD8+ T cells. Albeit the fast and dynamic behavior of the CD4+ 2d2 Th17 cells, many interactions of CD4+ T cells and neurons were observed (Fig.20A). Generally, the neurons showed signs of axonal dysmorphology, such as axonal varicosities and ellipsoid bodies. The interactions were mainly of a dynamic kind with CD4+ 2d2 Th17 cells scanning axons especially in areas of axonal dysmorphology. Tracking of the contacts between the CD4+ 2d2 Th17 cells and neurons over time and quantification of the contacts as described by Siffrin *et al.* (126) showed very high k-values of 4.98+/-0.22 (Fig.20B). Interestingly, *in vitro* differentiated CD4+ 2d2 Th1 cells showed significantly reduced k-values of 1.82+/-0.10

for their interaction with neurons (Fig.20B), indicating that the Th17 cells showed more long-lasting interactions with neurons than the Th1 cells.
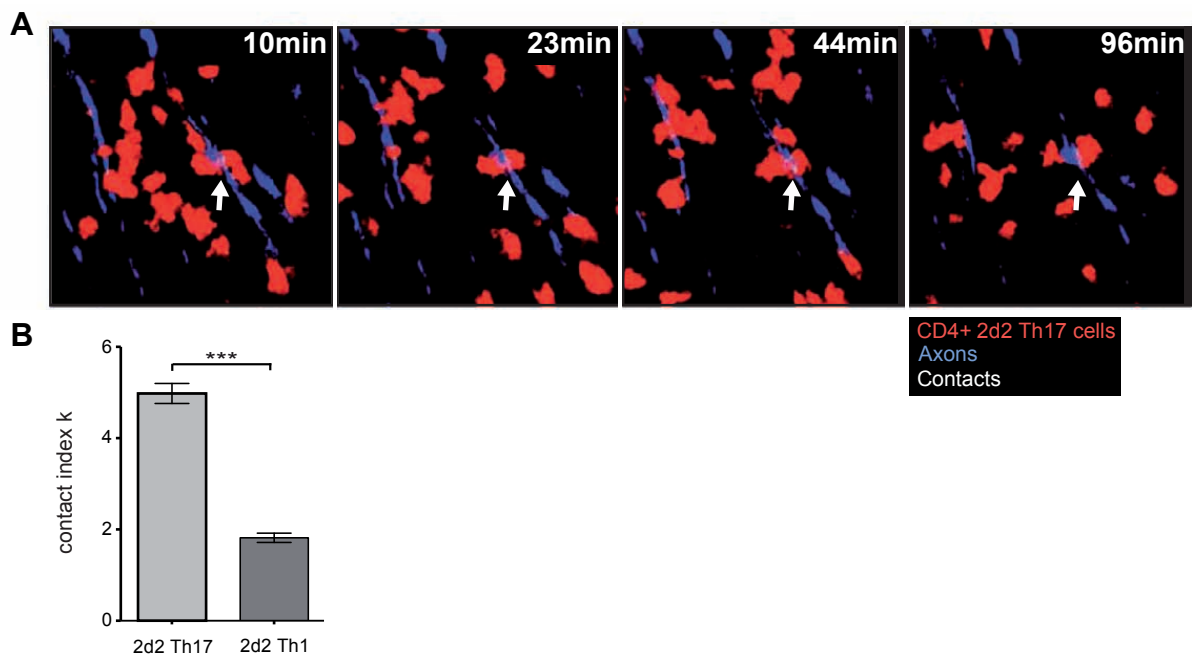

Figure 20. Visualization and quantification of contacts between MOG-specific CD4+ T cells and neurons. (A) Passive EAE was induced in B6.Rag1$^{-/-}$Thy1.21.EGFP mice with EGFP-expressing axons (blue) by transferring RFP+ CD4+ 2d2 Th17 cells (red). Contacts are shown in white and one long-lasting contact is indicated by arrow. (B) Comparison of contact index k for contacts between CD4+ 2d2 Th17 cells and neurons, as well as for CD4+ 2d2 Th1 cells and neurons. Data are shown as MEAN+/-SEM, analyzed by Mann-Whitney-U test: ***p<0.001. Adapted from Siffrin *et al.* (126).

In summary, in this part of the thesis it was shown, that in MOG$_{35-55}$-induced EAE, CD8+ T cells did not induce the disease or contribute to the disease. The disease course correlated with the presence of IL-17-producing CD4+ T cells, which is supported by the observation of long-lasting contacts between CD4+ 2d2 Th17 cells and neurons. However, the CD8+ T cells in the here presented setup did also not attenuate the disease, which is in line with the fact, that no long-lasting interactions of the CD8+ T cells with the self-reactive CD4+ T cells could be observed in the peak of the disease. Since CD8+ T cells with regulatory phenotype were described in the literature, in the next part of the thesis it was investigated, if CD8+ T cells with regulatory potential can be generated or expanded by specific culture conditions in *vitro*.

3.3 Isolation and expansion of suppressor CD8+ T cells with regulatory potential

Early EAE-studies already showed, that CD8+ T cells with a regulatory role in EAE exist. It was shown, that mice depleted of CD8+ T cells are no longer protected from a second induction of EAE (89). Furthermore, active EAE in CD8 knockout mice led to more relapses than in wild-type mice (90). These findings indicate, that regulatory CD8+ T cells are probably generated during a first disease phase and exert their regulatory effect only in a secondary disease phase. This assumption is also supported by newer findings, where it was shown that Qa-1 restricted CD8+ T suppressor cells are generated during a first disease occurrence and exert their regulatory effect in a secondary immune response (159). Therefore, it was assumed here, that CD8+ T cells with regulatory potential are presumably found in mice that underwent a first disease peak. Although many different subtypes of CD8+ T cells with regulatory potential were described, it is so far not possible to specifically isolate and expand one of these subtypes in *vitro*. Here, a protocol was established for the in *vitro* expansion of CD8+ T cells based on the theory of vaccination. Already early EAE-studies had shown, that protective CD8+ T cells could be induced by T cell vaccination with antigen-activated attenuated encephalitogenic T cells (91). This knowledge was used here to expand CD8+ T cells isolated from EAE-recovered mice in *vitro* by co-culturing these cells with irradiated CNS-specific CD4+ T cells. The phenotype and behavior of the generated CD8+ T cells was then examined in *vitro* as well as in *vivo* in a therapeutic approach in EAE.

3.3.1 Characterization of CD8+ T cells isolated from EAE-recovered mice

3.3.1.1 *Ex vivo* CD8+ T cells

C57BL/6 mice were immunized with MOG_{35-55} to induce active EAE, and CD8+ T cells were isolated from the spleens and lymph nodes of those mice showing remission, which is the case in about 50% of the EAE-affected animals in this EAE model. The phenotype of the isolated CD8+ T cells was characterized by FACS-stainings and in a CFSE-based suppression assay using MOG-specific (2d2) CD4+ Th17 cells as targets. It was found that *ex vivo* CD8+ T cells expressed the surface markers CD44, CD122, and Ly-49 (Fig.21A), markers that were recently shown to describe a population of Qa-1 restricted regulatory CD8+ T cells in an animal model of autoimmune lupus erythematosus (99). The isolated CD8+ T cells also

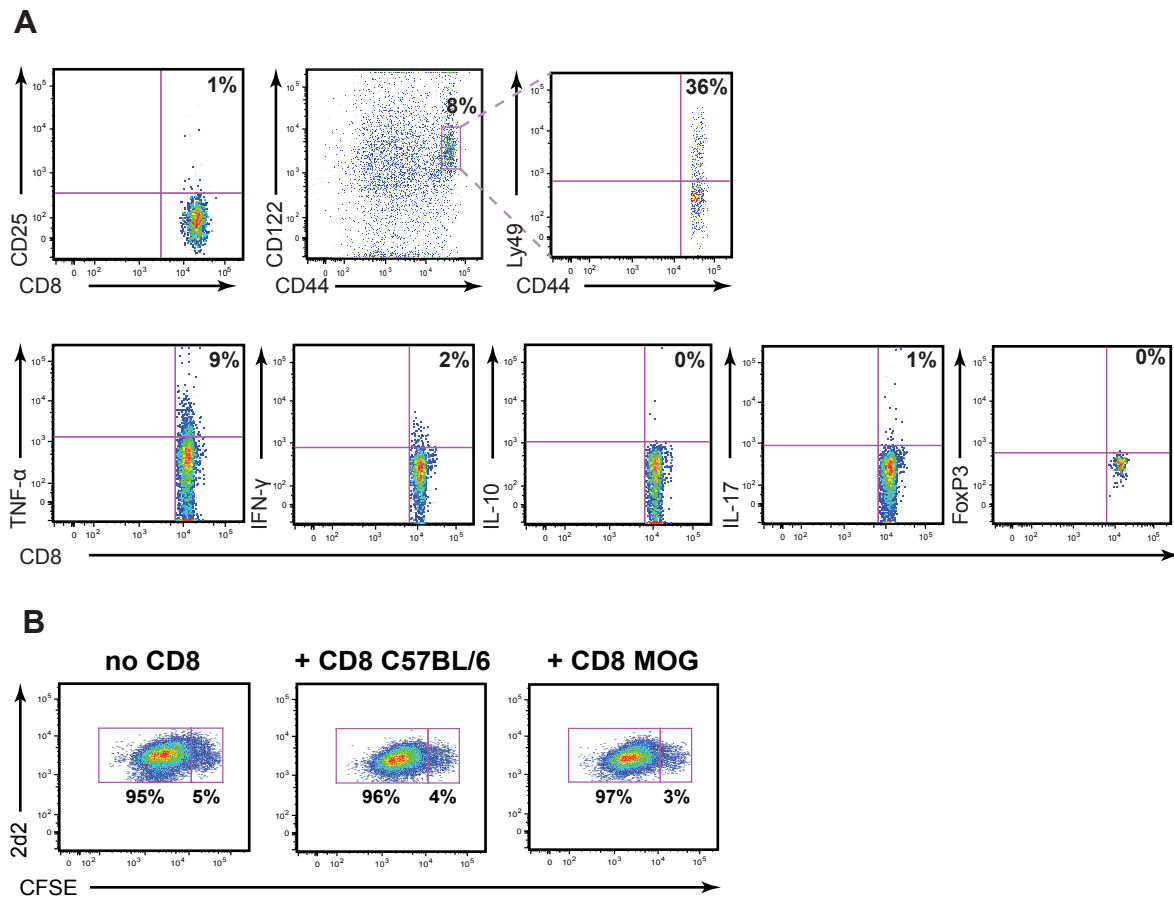produced low amounts of TNF-α, but no IFN-γ, IL-10, IL-17, or the Treg marker FoxP3 (Fig.21A).

Figure 21. Flow cytometry data of *ex vivo* CD8+ T cells isolated from spleens of MOG-immunized C57BL/6 mice after remission. (A) Surface staining shows that the CD8+ T cells are not activated (CD25-), but contain a population of CD44+CD122+Ly-49+ cells of potentially regulatory cells. Intracellular staining reveals almost no cytokine production and no FoxP3 expression by the CD8+ T cells. (B) CFSE-labeled CD4+ 2d2 Th17 cells were cultured with MOG-loaded APC in the presence or absence of *ex vivo* CD8+ T cells for 3 days, in a ration of 1:1. CD8+ T cells isolated from MOG-immunized mice in remission (CD8 MOG) do not suppress the proliferation of the CFSE-labeled target cells, comparable to control CD8+ T cells from non-immunized C57BL/6 mice (CD8 C57BL/6) or target cells alone without any CD8+ T cells (no CD8). One representative experiment of at least three is shown.

To examine the suppressive capacity of the *ex vivo* CD8+ T cells from C57BL/6 mice after remission, CD4+ 2d2 Th17 cells were labeled with CFSE and cultured *in vitro* with MOG-loaded APC in the presence or absence of the CD8+ T cells. As an additional control to exclude unspecific effects of the CD8+ T cells, such as a competition for space or stimuli, CD8+ T cells from non-immunized C57BL/6 mice were used in the experiments. The ratio of

CD8+ to CD4+ T cells was varied from 3:1 to 1:1 to 1:3, and the cells were cultured for 3 days. No decrease of the CFSE-signal was observed when the CD8+ T cells from MOG$_{35-55}$ immunized C57BL/6 mice in remission (CD8-MOG) or the control CD8+ T cells from non-immunized C57BL/6 mice (CD8 C57BL/6) were added to the CFSE-labeled CD4+ 2d2 Th17 target cells (Fig.21B). The proliferation of the target cells with addition of CD8+ T cells in any ratio was comparable to the proliferation of the targets without CD8+ T cells (no CD8), showing that the *ex vivo* CD8+ T cells did not suppress proliferation of the target cells *in vitro*.

3.3.1.2 *In vitro* expanded CD8+ T cells

It was assumed that if CD8+ T cells with suppressive capacity were present, they would most likely require expansion for acquisition of suppressor activity. Therefore, based on the idea of T cell vaccination, the *ex vivo* CD8+ T cells were expanded *in vitro* by co-culturing them with irradiated CD4+ 2d2 Th17 cells for 10 days and their phenotype was assessed thereafter. Flow cytometric analysis showed, that the expanded CD8+ T cells (CD8 MOG) expressed high amounts of CD25, CD44, IFN-γ, TNF-α, some Ly-49 and IL-10, but no CD122, IL-17, or FoxP3 (Fig.22A and B). These results did not differ from the expression of the markers by polyclonally expanded control CD8+ T cells isolated from C57BL/6 mice (CD8 C57BL/6), except for Ly-49, which was not expressed at all by the control cells (Fig.22B). These findings indicate, that no expansion of the previously described Qa-1 restricted CD44+CD122+Ly-49+ regulatory CD8+ T cells, or an induction of FoxP3+ regulatory CD8+ T cells took place in the culture. Interesting is the increased expression of Ly-49 and the anti-inflammatory cytokine IL-10 compared to the *ex vivo* data in Fig.22A, which however is inconsistent with the increased expression of the pro-inflammatory cytokines IFN-γ and TNF-α.

To examine the suppressive capacity of the *in vitro* expanded CD8+ T cells, again an *in vitro* suppression assay was performed with CFSE-labeled CD4+ 2d2 Th17 cells as targets, as described above. The *in vitro* expanded CD8+ T cells (CD8 MOG), previously isolated from MOG$_{35-55}$-immunized mice in remission, almost completely suppressed the proliferation of CFSE-labeled CD4+ 2d2 Th17 target cells (Fig.22C).
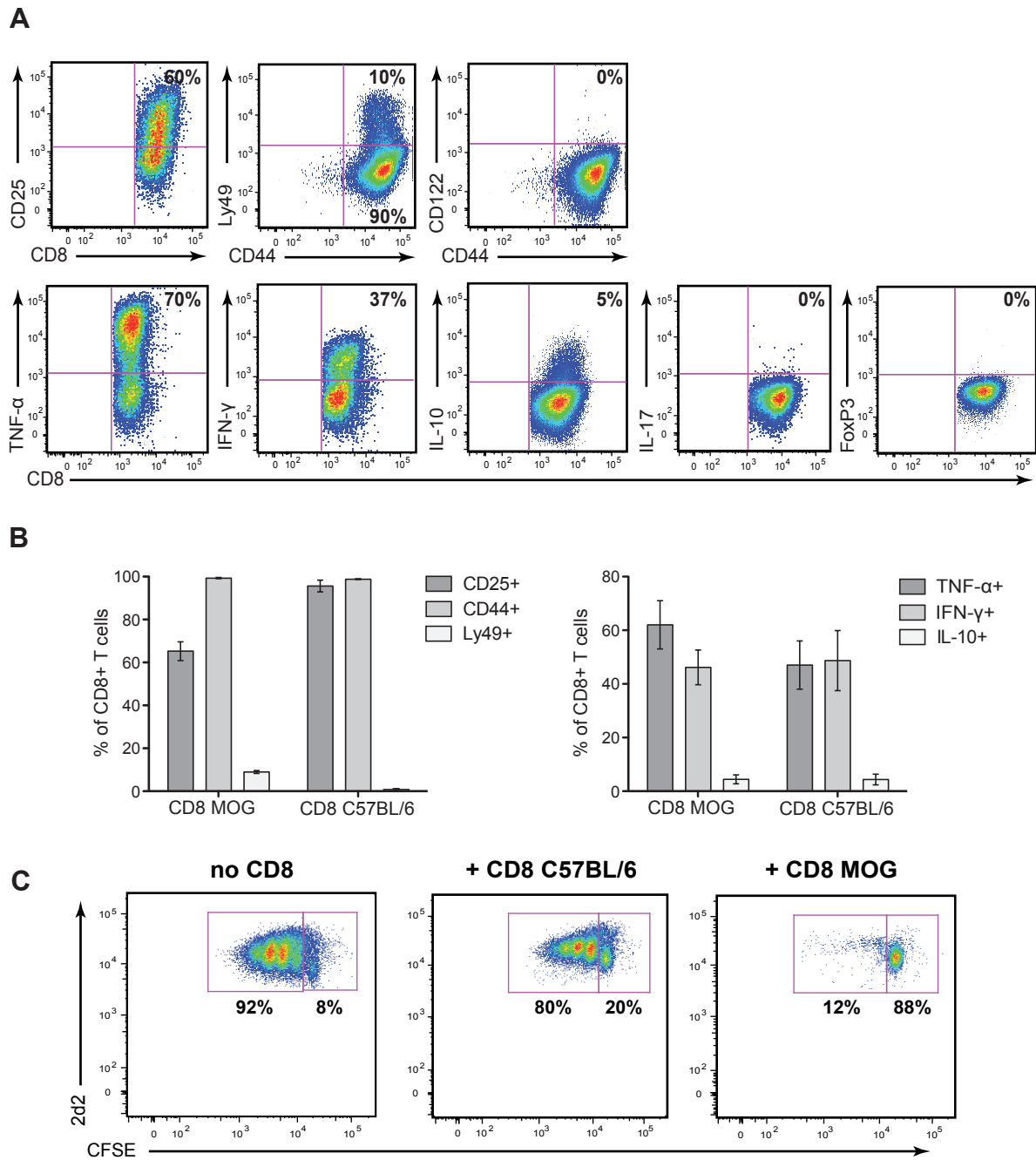
Figure 22. Flow cytometry data of *in vitro*-expanded CD8+ T cells on day 10 after isolation from spleens of MOG-immunized C57BL/6 mice in remission (CD8 MOG) or control mice (CD8 C57BL/6). (A) CD8 MOG cells express CD25, CD44, and Ly49 on their surface, and produce the cytokines TNF-α, IFN-γ, and IL-10. (B) Comparison of CD8 MOG and CD8 C57BL/6 control cells. (C) *In vitro* suppression assay with CFSE-labelled CD4+ 2d2 Th17 target cells cultured with MOG-loaded APC for 3 days, in the presence or absence of *in vitro* expanded CD8 T cells. CD8+ T cells isolated from MOG-immunized C57BL/6 mice in remission and expanded in co-culture with irradiated CD4+ 2d2 Th17 cells (CD8 MOG) suppress proliferation of CFSE-labeled target cells (1:1 ratio shown). *In vitro* polyclonally expanded control CD8+ T cells from non-immunized C57BL/6 mice (CD8 C57BL6) suppress proliferation of target cells only slightly compared to targets without CD8+ T cells (no CD8). One representative experiment of at least three is shown.

The *in vitro* expanded control CD8+ T cells (CD8 C57BL/6), previously isolated from non-immunized C57BL/6 mice, suppressed the proliferation of the target cells only to a very low extent, indicating that the *in vitro* expanded CD8 MOG cells really induced a specific and significant suppression of the target cells (Fig.22C). The suppression could be observed at a ratio of CD8+ to CD4+ T cells of 5:1 and 1:1, but not when less CD8+ than CD4+ T cells were present in the wells.

After having shown, that the suppressor CD8+ T cells reduce proliferation of MOG-specific CD4+ target cells *in vitro*, the next addressed question was if these suppressor CD8+ T cells have a beneficial effect *in vivo* in EAE. To investigate this, passive EAE in lymphocyte deficient Rag1$^{-/-}$ mice was induced by intravenously transferring 5 Mio CD4+ 2d2 Th17 cells per mouse. On day 10, before onset of clinical signs, 10 Mio suppressor CD8+ T cells or control CD8+ T cells were then transferred into these mice. There was no significant difference in the disease courses of animals that had received suppressor CD8+ T cells (CD8 MOG) compared to those that had received the control CD8+ T cells (CD8 C57BL/6, Fig.23). Also, mean day of onset, incidence, and mortality did not differ significantly between the two groups (Fig.23).

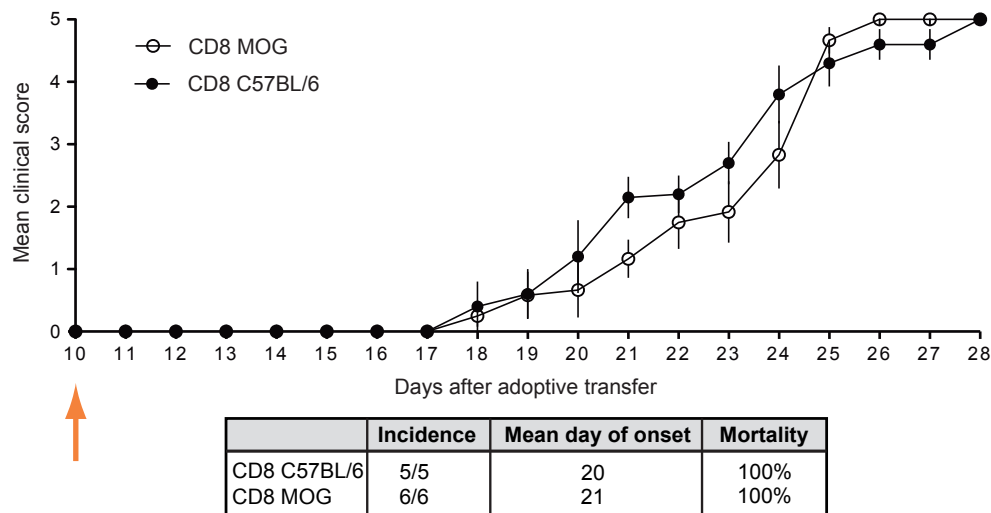|	Incidence	Mean day of onset	Mortality
CD8 C57BL/6	5/5	20	100%
CD8 MOG	6/6	21	100%

Figure 23. Passive EAE was induced in Rag1$^{-/-}$ mice (day 0) and CD8 MOG or control CD8 C57BL/6 cells were transferred before onset of disease on day 10, indicated by red arrow. One representative experiment of three is shown. Displayed are MEAN+/-SEM.

Next, a potential therapeutic effect of the suppressor CD8+ T cells was examined in active EAE. Therefore, active EAE was induced in C57BL/6 mice by immunization with MOG_{35-55}-peptide, followed by transfer of 10 Mio suppressor CD8+ T cells (CD8 MOG) or control CD8+ T cells (CD8 C57BL/6) into these mice before onset of disease on day 10. Incidence and disease severity were comparable in both groups, only in the remission phase of the disease a positive effect of the suppressor CD8+ T cells could be observed (Fig. 24). A very mild regulatory effect of the suppressor CD8+ T cells is therefore visible in *vivo*.

	Incidence	Mean day of onset	Mortality
CD8 C57BL/6	6/8	14	0%
CD8 MOG	6/8	14	0%
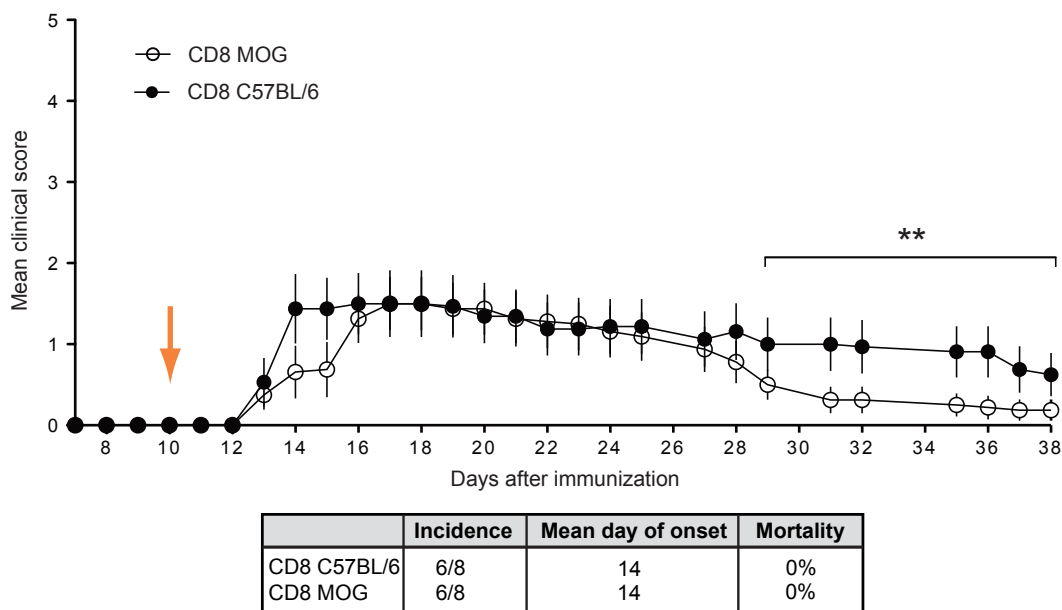
Figure 24. C57BL/6 mice were actively immunized with MOG_{35-55}, followed by transfer of CD8 MOG or CD8 C57BL/6 cells before onset of disease on day 10, indicated by red arrow. Data from one experiment, shown as MEAN+/- SEM, analyzed by Mann-Whitney-U test: ** p<0.01.

3.3.2 Mode of suppression by *in vitro* expanded suppressor CD8+ T cells from EAE-recovered mice

3.3.2.1 Cell-cell contact dependency

To test if the observed suppression *in vitro* was cell-cell contact dependent, the *in vitro* suppression assay was performed with the supernatants from the CD8-culture instead of the in *vitro* expanded CD8+ T cells themselves. Supernatants from the *in vitro* expanded CD8 MOG cells alone however did not suppress proliferation of the target cells (Fig.25). These data indicate, that CD8+ T cells with regulatory potential are present and can be expanded or

generated from EAE-recovered mice, and presumably exert their suppressive function via a contact dependent mechanism.

Figure 25. *In vitro* suppression assay with CFSE-labeled CD4+ 2d2 Th17 target cells cultured with MOG-loaded APC for 3 days and supernatants from *in vitro* CD8 T cell cultures. Supernatants from control CD8+ T cells (CD8 C57BL6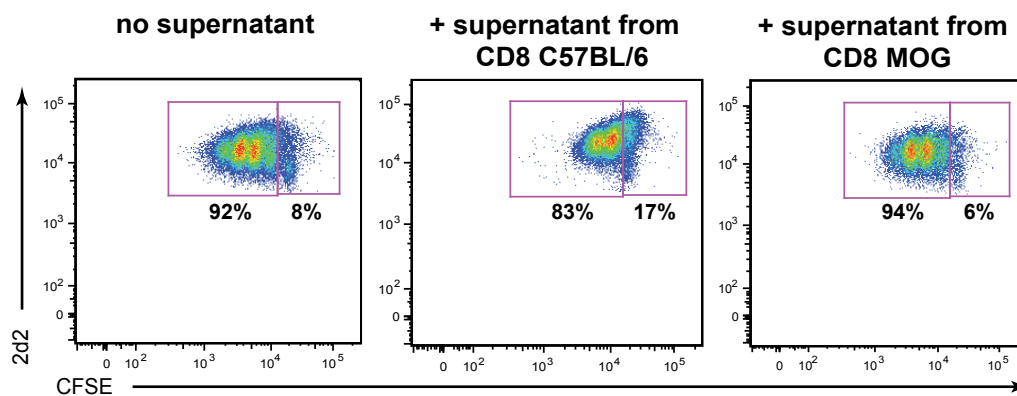) and supernatants from CD8+ T cells isolated from MOG-immunized C57BL/6 mice in remission and expanded in co-culture with irradiated CD4+ 2d2 Th17 cells (CD8 MOG) had no effect on the proliferation of the target cells. One representative experiment of two is shown.

3.3.2.2 Antigen specificity

To test whether the observed suppression by the CD8+ T cells is antigen-dependent, CD8+ T cells from C57BL/6 mice immunized with OVA-protein were isolated and expanded as described above and used in the suppression assay, which was performed as described above with a 1:1 ratio of CD8+ T cells to target cells. CD8+ T cells isolated and expanded from OVA-immunized mice (CD8 OVA) suppressed proliferation of CFSE-labeled CD4+ 2d2 Th17 target cells comparable to expanded CD8+ T cells from MOG$_{35-55}$-immunized mice (CD8 MOG, Fig.26A). As controls, again, targets alone (no CD8) and with expanded CD8+ T cells from non-immunized C57BL/6 mice (CD8 C57BL/6) were used, which suppressed proliferation of the target cells only slightly (Fig.26A). To further investigate the antigen specificity of the suppression, OVA-specific (OT2) CD4+ Th17 cells were used as target cells in the suppression assay. These OT2 cells differ from 2d2 cells not only in their antigen-specificity, but also in their avidity, as they are derived from a high avidity clone, compared to 2d2 cells that are derived from an intermediate avidity clone. Both, CD8+ T cells from MOG$_{35-55}$- and OVA-immunized mice (CD8 MOG, CD8 OVA) however had no suppressive effect on CFSE-labeled OT2 CD4+ Th17 target cells (Fig.26B). In summary it can be concluded, that CD8+ T cells suppress the proliferation of the encephalitogenic CD4+ 2d2

Th17 cells independently of their specificity, but they do not suppress proliferation of CD4+ OT2 Th17 target cells.
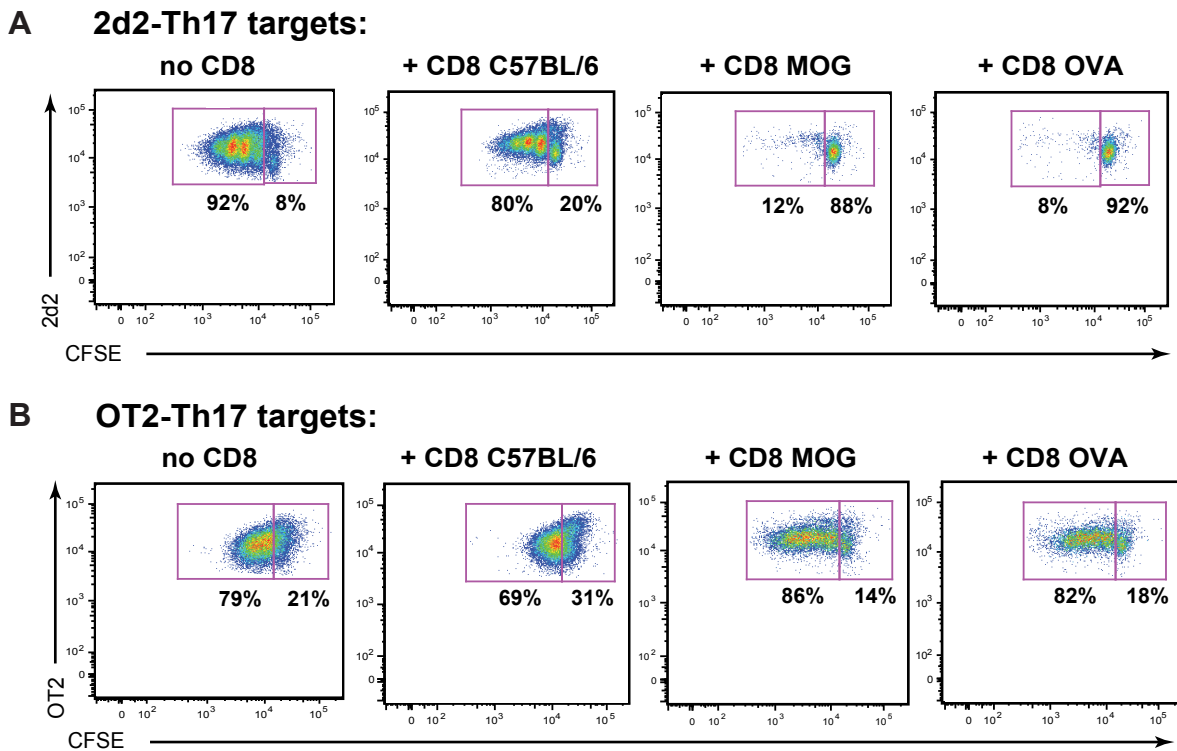
Figure 26. Suppressor CD8+ T cells selectively decrease proliferation of myelin specific CD4+ target T cells. (A) *In vitro* expanded CD8+ T cells isolated from MOG$_{35-55}$- (CD8 MOG) or OVA- (CD8 OVA) immunized C57BL/6 mice suppress proliferation of CFSE-labeled MOG-specific CD4+ 2d2 Th17 target cells equally well compared to control CD8+ T cells (CD8 C57BL6). (B) OVA-specific CD4+ OT2 Th17 target cells are not suppressed in their proliferation by *in vitro* expanded CD8+ T cells from MOG- (CD8 MOG) or OVA- (CD8 OVA) immunized C57BL/6 mice. One representative experiment of at least three is shown.

3.3.2.3 Qa-1 dependency

To test if the observed suppression by the CD8+ T cells in *vitro* is dependent on the expression of Qa-1 on the CD4+ target cells, it was examined if a blocking antibody against Qa-1 abrogates the observed suppression of proliferation. Therefore, the CFSE-labeled CD4+ 2d2 Th17 target cells were preincubated with 20 μg/ml of the Qa-1 blocking antibody before the CD8+ T cells were added. The blocking antibody was also present during the three days of culture. Proliferation of the target cells was suppressed by the in *vitro* expanded CD8+ T cells from MOG-immunized mice (CD8 MOG), but not by the control CD8 C57BL/6 cells, in the presence or absence of the Qa-1 antibody (Fig.27). Therefore, the observed suppression by the CD8+ T cells is not Qa-1-dependent.
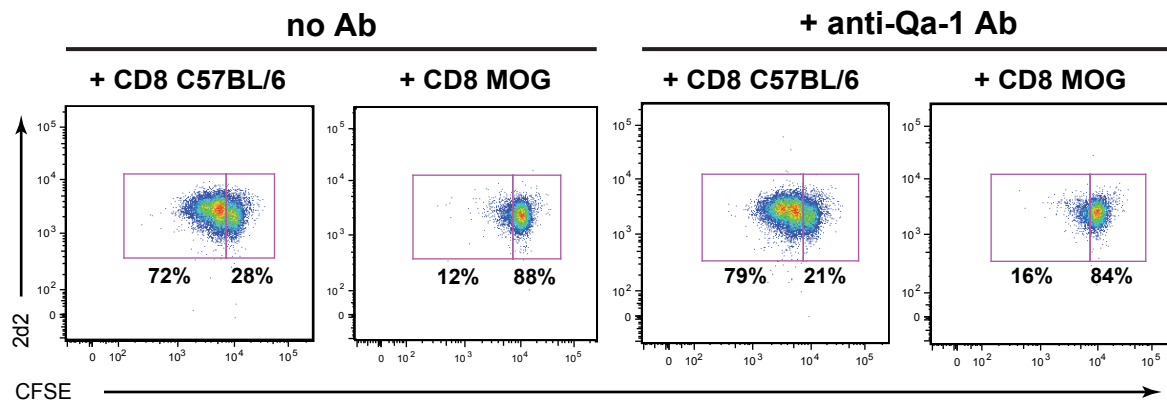
Figure 27. Suppression of CD4+ 2d2 Th17 target cells by i*n vitro* expanded CD8+ T cells isolated from MOG$_{35-55}$-immunized C57BL/6 mice (CD8 MOG) is not abrogated by a blocking antibody against Qa-1. One representative experiment of three is shown.

3.3.2.4 Effect on cytokine secretion

To test if the CD8 suppressor cells affect also cytokine secretion of the CD4+ 2d2 Th17 target cells, in addition to the measurement of the CFSE-signal, an intracellular cytokine staining of the target cells was performed after the 3 day-culture with the CD8-suppressor cells. Analysis at the flow cytometer revealed, that not only the proliferation of CD4+ 2d2 Th17 target cells was impaired by the CD8 MOG and CD8 OVA cells, but also their expression of the pro-inflammatory cytokine IL-17 as compared to the controls without CD8 cells or with CD8 C57BL/6 cells (Fig.28).
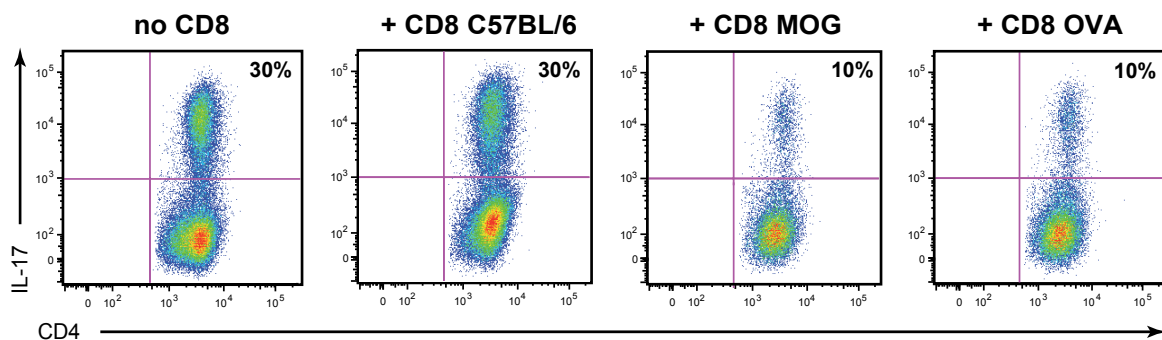
Figure 28. IL-17 secretion of MOG-specific CD4+ 2d2 Th17 target cells is suppressed by CD8 MOG and CD8 OVA cells compared to control CD8 C57BL/6 cells. One representative experiment of two is shown.

3.3.2.5 Cytotoxicity

Because CD8+ T cells have the ability to kill target cells, it was further investigated if the expanded suppressor CD8+ T cells not only suppress the proliferation and cytokine secretion of the CD4+ 2d2 Th17 target cells, but also kill these target cells. To test this, a FACS-based cytotoxicity assay was applied, where the target cells were not only labeled with CFSE, but also with propidium iodide (PI) to label dead and dying cells. CD8+ suppressor T cells and CD4+ target T cells were co-incubated for 24 hours in a 4:1 ratio, followed by flow cytometric analysis, where the percentage of PI+ cells of the CFSE+ target cells was determined. As expected from the results of the suppression assays, the suppressor CD8+ T cells from MOG- and OVA-immunized mice increased the percentage of PI+ cells from the CFSE-labeled CD4+ 2d2 Th17 target cells compared to control CD8+ T cells (Fig.29A). Surprisingly, also the percentage of PI+ CFSE-labeled CD4+ OT2 Th17 target cells was increased by the suppressor CD8+ T cells (Fig.29A), indicating that the suppressor CD8+ T cells also killed the non-myelin specific high avidity CD4+ T cells, which stands in contrast to the results of the proliferation assay in Fig.26B. However, a quantitative analysis of the proliferation data from Fig.26B using BD TruCOUNT Beads, which allow to calculate the total amount of cells in the acquired sample, revealed that the total amount of CD4+ OT2 Th17 target cells also there is highly reduced by the suppressor CD8+ T cells, although the proliferation of the surviving CD4+OT2 Th17 target cells is not affected (Fig.29B).

To transfer the results into the human system, CD8+ T cells and CD4+ T cells were isolated from the peripheral blood of a HLA-DR2+ donor, as the MHC class II allele HLA-DR2 is associated with an increased susceptibility of MS. MBP_{85-99}-specific CD4+ Th17 cells were established *in vitro*, which were then used for co-culture during the expansion of the CD8+ T suppressor cells and as targets in the FACS-based cytotoxicity assay. As in the murine system, the human suppressor CD8+ T cells increased the percentage of PI+ CFSE-labeled MBP_{85-99} -specific CD4+ Th17 target cells (Fig.29C).

Thus, it is possible to generate suppressor CD8+ T cells that kill myelin specific and non-myelin specific CD4+ effector T cells.
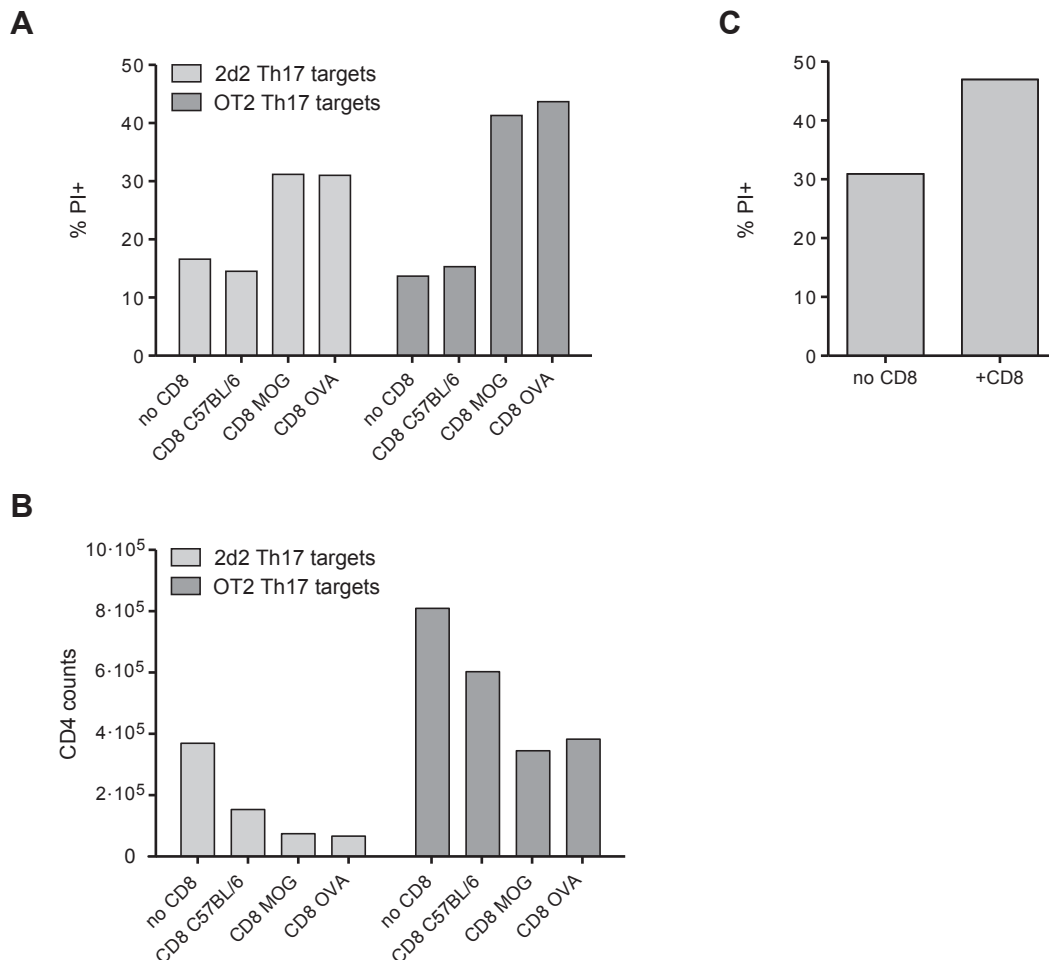
Figure 29. (A) Murine cytotoxicity assay: percentage of PI+ CFSE-labeled CD4+ 2d2/OT2 Th17 target cells co-cultured for 24 hours without CD8+ T cells (no CD8), or with *in vitro* expanded CD8+ T cells from non-immunized C57BL/6 mice (CD8 C57BL/6), or with suppressor CD8+ T cells from MOG_{35-55}- or OVA-immunized mice (CD8 MOG, CD8 OVA). (B) Murine proliferation assay using BD TruCOUNT Beads: absolute cell counts of CFSE-labeled CD4+ 2d2/OT2 Th17 target cells after 3 days of co-culture with the different CD8+ T cells. (C) Human cytotoxicity assay: percentage of PI+ CFSE-labeled MBP_{85-99}-specific CD4+ Th17 cells co-cultured for 24 hours with or without *in vitro* expanded CD8+ T cells from the same donor. One representative experiment of at least two is shown.

3.3.3 Monitoring the behavior of suppressor CD8+ T cells within the CNS of EAE-affected mice using TPLSM

In order to investigate the behavior of the suppressor CD8+ T cells directly at the site of inflammation, TPLSM was applied in the brainstem of EAE-affected mice. Adoptive transfer of EGFP+ CD4+ 2d2 Th17 cells was used to induce EAE in Rag1$^{-/-}$ mice. CD8-RFP T cells were isolated from MOG_{35-55}-immunized or non-immunized B6.RFP mice, expanded *in vitro*,

and transferred into the EAE mice before onset of clinical signs, as described above for Fig.23. After onset of disease, TPLSM of brainstem lesions was performed in these mice. CD8-RFP T cells were present and highly motile, as were the encephalitogenic CD4+ 2d2 Th17 EGFP cells, within inflamed lesions of these animals (Fig.30A).
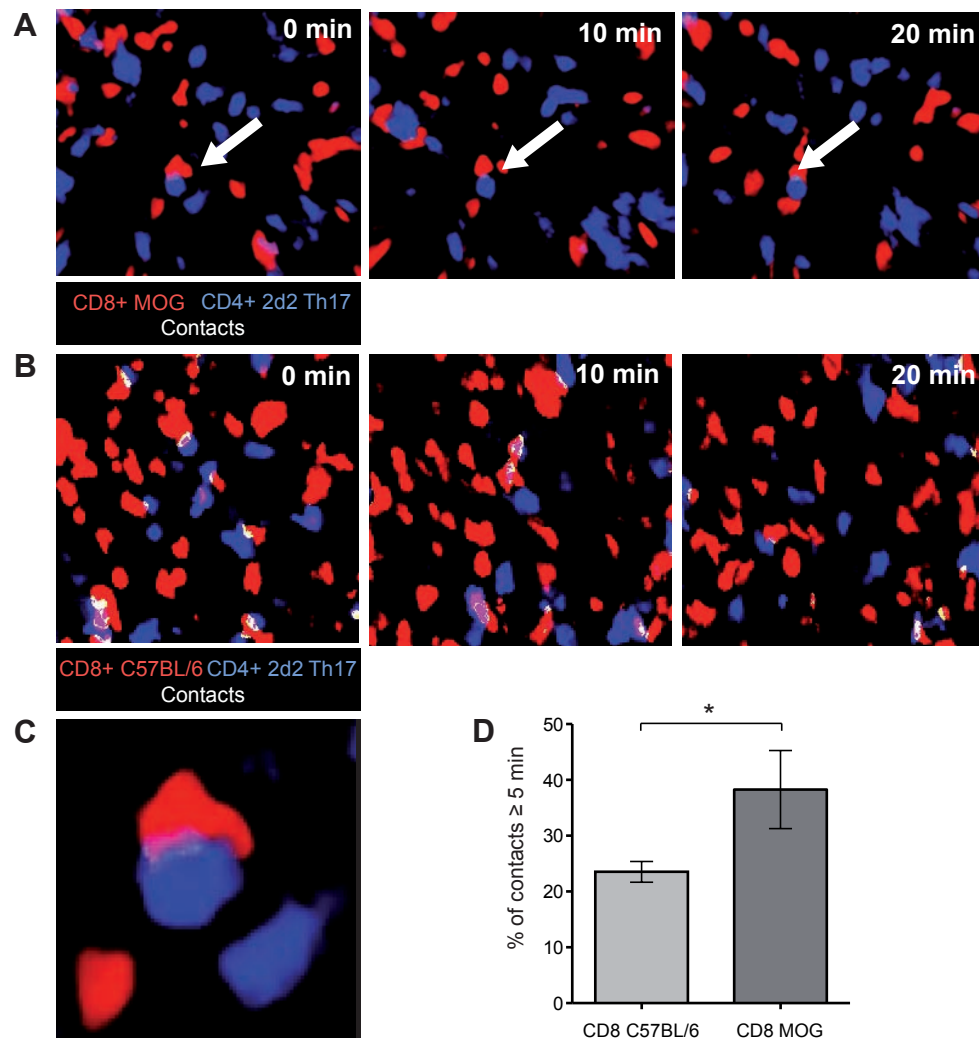
Figure 30. Contacts between EFGP+ CD4+ 2d2 Th17 and CD8-RFP MOG suppressor cells (A) or control CD8-RFP C57BL/6 cells (B) within inflamed lesions of EAE-affected Rag1$^{-/-}$ mice are visualized in white and tracked over time. One long-lasting contact is indicated by white arrow. (C) Zoom of interaction between CD8-RFP MOG suppressor and EFGP+ CD4+ 2d2 Th17 cell, as indicated by white arrow in (A). (D) Percentage of long-lasting contacts between CD4+ 2d2 Th17 cells and CD8+ T cells is higher for CD8 MOG cells than control CD8 C57BL/6 cells. Cell-cell contact durations between CD4+ and CD8+ T cells were determined for 4 movies per group. The percentage of contacts ≥5 min are shown as MEAN+/-SEM and were analyzed by Mann-Whitney-U test: * p<0.05.

As *in vitro* suppression experiments had indicated a potentially contact dependent way of suppression, contacts between the EGFP+ CD4+ 2d2 Th17 cells and the suppressor or control RFP+ CD8+ T cells were visualized and analyzed. Hereby, the method described by Siffrin *et al.* (126), described in detail in chapter 3.2.3, was applied to visualize contacts between EGFP+ and RFP+ cells and to automatically track established contacts over time (Fig.30A and 30B). Suppressor CD8+ T cells (CD8 MOG) showed significantly more long-lasting contacts with the encephalitogenic CD4+ Th17 cells than control CD8+ T cells (CD8 C57BL/6) (Fig.30C and Fig.30D). Although no effect on the disease course was observed in this model *in vivo* (Fig.23), this supports the *in vitro* data from Fig.25, suggesting a cell-cell-contact dependent way of suppression also *in vivo*.

4 Discussion

Multiple sclerosis is a chronic inflammatory disease of the CNS. It is assumed, that self-reactive myelin-specific T cells, which have escaped the control mechanisms of the immune system, are responsible for the disease (11). These self-reactive cells are activated in the periphery and transmigrate through the BBB into the CNS, where they need to be reactivated by local APC in order to recruit further lymphocytes from the periphery, which leads to demyelination, axonal damage, and neuronal cell death (19). The quest for the disease-inducing T cell subset is a point of ongoing debate, since CD4+ T cells as well as CD8+ T cells were able to induce the disease in the animal model EAE. Most importantly, also a regulatory role for T cells is acknowledged. Regulatory T cells of the CD4- and CD8-subtype are mediators of peripheral self-tolerance and ensure the inhibition of autoimmune responses under normal circumstances. In several autoimmune diseases, including MS, these regulatory mechanisms were shown to be disturbed in their function (38).

The current therapies available for MS are not satisfying. The immunomodulatory drugs IFN-β and GA, which are first-line treatments for the therapy of RRMS, reduce relapse rate and delay disease progression, but they do not stop the disease progression. Furthermore, most of the therapies applied today for the treatment of MS have pleiotropic ways of action and therefore also unwanted and sometimes severe side effects – in particular if they are very potent. Stronger therapy regimens, like the monoclonal antibody Natalizumab or the S1P1 agonist Fingolimod, carry stronger risks for the patients concerning opportunistic infections and most likely also malignancies. It is therefore important to better understand the underlying mechanisms of the disease in order to develop more specific therapeutic approaches. Regulatory T cells are a major potential target for immunotherapy and strategies to induce, expand, or modulate these cells *in vitro* and transfer them for therapeutic applications is a promising approach for future therapies. The overall aim of this work was to better understand the phenotype and the role of regulatory T cells of the CD4 and CD8 subset by studying and modifying the phenotype of these cells in *vitro* and in *vivo* in order to apply them as a therapeutic approach in EAE.

The results of this thesis show, that the immunomodulatory drug atorvastatin directly inhibited proliferation of naïve CD4+ T cells, but did not modulate the T cell response in a way that the T cells acquired a regulatory phenotype. This is keeping with recent reports about a rather marginal effect of statin-treatment in MS patients (160). We found here that a

relative expansion of Treg cells, but no absolute enrichment was achieved by incubation of a T cell mixture with atorvastatin. It was further shown that in MOG_{35-55}-induced EAE, CD4+ T cells – here especially in association with IL-17 production - are relevant for the manifestation of clinical signs of the disease, whereas CD8+ T cells alone did not induce disability in the animals. CD8+ T cells did not enhance or attenuate the CD4-mediated disease, and did not affect the behavior of the CD4+ T cells in the CNS of EAE-affected mice, as shown by TPLSM. A distinctly different motility pattern was observed for the CD8+ T cells compared to the CD4+ T cells, and no long-lasting interactions between the two cell types were observed in the CNS using TPLSM. However, it was further possible to expand CD8+ T cells with a regulatory phenotype *in vitro* using a newly established protocol. These suppressor CD8+ T cells have yet no specific markers. Functionally, they suppressed proliferation and IL-17 production of myelin-specific CD4+ Th17 cells *in vitro* in a contact-dependent way, and further showed a cytotoxic effect. When transferred into C57BL/6 mice before onset of active EAE, the *in vitro* expanded CD8+ suppressor T cells had a mild beneficial effect on the disease course, which was most likely due to contact-dependent suppression of CD4+ T cells in the CNS, as shown by TPLSM.

4.1 Anti-inflammatory effect of statins in autoimmunity

4.1.1 Anti-inflammatory action of statins in autoimmune disease via naïve and regulatory T cells

It is assumed that autoimmune diseases, such as MS, are closely linked to defects in immune regulation. Several populations of T cells with regulatory properties have been described, the most intensively studied being CD4+ naturally occurring Treg cells and IL-10-producing Treg cells. These cell types contribute to immune tolerance by suppressing the CNS-specific self-reactive T cells that are responsible for the pathology in EAE and MS (11). In the mouse model, the importance of regulatory T cells is well documented. In EAE, the depletion of Treg cells enhanced disease severity, whereas adoptive transfer of Treg cells had a beneficial effect on the disease course (67,69). Transfer of IL-10-producing Treg cells before immunization prevented EAE (76). Furthermore, MS patients with RRMS were shown to have impaired Treg function (70). Therefore, the induction and expansion of regulatory T cells *in vitro* followed by transfer of these cells into patients represents an interesting future therapeutic approach.

Various substances were shown to exert their immunomodulatory effects by directly influencing T cells, either by inducing a shift in cytokine production or by inducing and expanding regulatory T cells. The exact underlying mechanisms however are still not fully understood. As many of the immunomodulatory drugs have pleiotropic and often adverse side effects, it is important to dissect their ways of action in order to apply them more specifically and thereby avoid the unwanted side effects. GA, a standard treatment for RRMS, was shown to induce a Th2 shift in T cells (129). Treatment with GA also restored Treg function in patients with MS. It promoted the conversion of CD4+CD25- into CD4+CD25+FoxP3+ T cells and led to a significant increase in the FoxP3-expression of CD4+ T cells (133). Activation of naïve CD4+ T cells in the presence of vitamin D3 and the synthetic corticosteroid dexamethasone led to reduced numbers of Th1 and Th2 cells, but increased numbers of IL-10-producing Treg cells (76). Battaglia *et al.* showed that the immunosuppressive compound rapamycin selectively expands murine CD4+CD25+FoxP3+ Treg cells *in vitro*, and that these Treg suppress proliferation of target T cells (127). They further showed, that rapamycin inhibits proliferation of murine and human CD4+ T cells, but does not induce anergy or cell death in those cells. Based on these findings, they established a protocol for the induction of CD4+CD25+FoxP3+ Treg cells from human CD4+ T cells (128). T cells treated with the HMG-CoA reductase inhibitor atorvastatin prevented EAE in recipient mice (149). This was attributed to a shift in cytokine production of T cells from a Th1 to a Th2 phenotype, to the induction of T cell anergy in effector T cells (148,156), and to a reduced expression of MHC-II and co-stimulatory molecules on APC (149). It was also shown in clinical studies, that atorvastatin has a beneficial effect in the treatment of RRMS (151).

In this thesis, it was investigated how atorvastatin modulates the T cell response. To examine the effect of atorvastatin on proliferation of naïve CD4+ T cells, naïve murine CD4+ T cells were stimulated polyclonally or in an antigen-dependent way in the presence or absence of atorvastatin. It could be clearly shown, that atorvastatin directly inhibits proliferation of naïve CD4+ T cells, already in low concentrations. The inhibitory effect was independent of APC, as both polyclonally stimulated naïve CD4+ T cells as well as naïve CD4+ T cells stimulated with APC and the corresponding peptide were suppressed in their proliferation. This is in line with previous findings from our group, where it was shown that atorvastatin reduces proliferation of human and murine effector T cells independently of APC (148,156). This reduced proliferation of effector T cells was attributed to an interference of atorvastatin with

cell cycle progression, as atorvastatin-treated T cells failed to downregulate the anergy-factor p27kip, and not to apoptosis-induction. Here, it was shown that a high dose of atorvastatin of 25 μM had a cytotoxic effect on the naïve CD4+ T cells, highlighting the importance to work with physiological concentrations of the compound, which induce anergy, but not apoptosis. In summary, it was shown here for the first time, that atorvastatin directly inhibits proliferation of naïve CD4+ T cells, in a similar extent as is known for CD4+ T effector cells.

To examine the effect of atorvastatin on the *de novo* induction of regulatory T cells, naïve murine and human CD4+ T cells were stimulated polyclonally in the presence or absence of atorvastatin, followed by analysis of their FoxP3 and IL-10 expression, and their ability to suppress CD4+CD25- target cells. After this short-time culture of 3-6 days, no effect of atorvastatin on the expression of the regulatory markers FoxP3 and IL-10 could be observed. Neither the murine nor the human naïve CD4+ T cells activated in the presence of atorvastatin showed an enhanced capacity to suppress target T cells compared to control cells. This indicates, that atorvastatin does not induce *de novo* regulatory T cells. Recent findings in the field of statins however have shown, that atorvastatin and simvastatin are able to induce FoxP3+ Treg cells. It was shown, that culture of human CD4+CD25- T cells with atorvastatin increases the percentage of CD4+CD25+FoxP3+ T cells after 96 hours compared to control cells cultured without atorvastatin. However, in the same study no increase in Treg cells was observed in C57BL/6 mice after treatment with statins, indicating that the observed effect is restricted to human T cells (161). In this context it has to be considered, that FoxP3 is not an exclusive marker for Treg in the human system as compared to the mouse system, since it is upregulated in human CD4+ T cells upon activation (162). Another study showed, that atorvastatin increased Treg numbers and function in rheumatoid arthritis patients. Also here, the increased Treg numbers were attributed to a *de novo* induction of Treg cells from CD4+CD25- T cells (163). Simvastatin on the other hand was shown to induce murine FoxP3+ Treg cells from CD4+CD25- T cells. One study observed this effect in the absence of TGF-β, but noted a synergistic effect with TGF-β, leading to even higher numbers of Treg cells (164). In another report, TGF-β was required for the induction of Treg cells from murine CD4+CD25- T cells (165). It therefore might be interesting to study the effect of atorvastatin on the induction of Treg cells in the presence of TGF-β.

As an anti-proliferative effect of atorvastatin was clearly shown here for naïve CD4+ T cells and is well established for effector CD4+ T cells (148,156), it was next asked, if this anti-

proliferative effect of atorvastatin can be employed to enrich Treg cells from a mixture of CD4+ T cells. The experiments were performed with mouse CD4+ T cells, which were activated polyclonally or in an antigen-specific way. The advantage of the murine system being, that FoxP3 is an exclusive marker for Treg cells. CD4+ T cells were activated in the presence or absence of atorvastatin, kept in medium supplemented with IL-2, and restimulated every week. The percentage and total number of CD4+FoxP3+ Treg cells was followed over time. Here it could be shown, that all T cells in the culture were inhibited in their proliferation by atorvastatin, independent of their phenotype. However, the effector T cells were inhibited more effectively than the Treg cells, presumably because they are stronger proliferators than Treg cells. This led to a relative increase of Treg cells compared to effector T cells mostly after 1 week of culture, but not to an increase in absolute Treg numbers, as generally the number of Treg in the culture decreased drastically over time. Similar experiments have been performed using rapamycin, which selectively blocked expansion and proliferation of human CD4+ effector T cells, but spared and promoted growth of CD4+ Treg (128). However, also in this study the total number of CD4+ T cells was reduced by rapamycin at the end of the culture, indicating rather a percental enrichment in Treg than in total numbers. Murine CD4+ T cells activated in the presence of rapamycin showed a delayed kinetic of proliferation compared with control cells (127). However, after three weeks of culture the number of CD4+ T cells was not reduced, indicating that rapamycin does not block proliferation of murine CD4+ T cells. The total number of CD4+ Treg cells was also increased after 3 weeks of culture with rapamycin in the murine system, representing a genuine expansion of Treg cells.

To examine if atorvastatin enhances the suppressive capacity of existing regulatory T cells, a protocol for the induction of IL-10 Treg cells from human PBMCs was modified and applied here (80). In short, human CD4+ T cells were co-cultured for 14 days with allogeneic immature dendritic cells generated from human monocytes. The suppressive capacity of the generated IL-10 Treg cells was then analyzed in a ^{3}H-thymidine suppression assay, in a co-culture with allogeneic CD4+ target cells. Atorvastatin was added to the CD4+ T cells before they were co-cultured with the allogeneic iDC, to study its effect on the thereby generated IL-10 Treg cells. It could be shown, that atorvastatin however did not significantly increase the suppressive capacity of IL-10 Treg cells in a direct way. A recent study has shown a direct effect of atorvastatin on the suppressive capacity of Treg cells (163). It has been shown, that CD4+CD25+FoxP3+ Treg induced from CD4+CD25- T cells by atorvastatin *in vitro* exhibit an enhanced inhibitory function. Furthermore, atorvastatin

restored suppressive function of Treg cells in patients with rheumatoid arthritis and reduced clinical disease activity in these patients. Also in MS the suppressive function of Treg cells is impaired and it has been shown that for example treatment with GA increased Treg numbers and reversed the defect in Treg function in RRMS patients (166). So far, nothing is known about the effect of statins on Treg or IL-10 Treg function in the context of multiple sclerosis. Several clinical studies with atorvastatin in MS were conducted. Two preliminary observational studies reported a positive effect of statins on relapse rate as well as lesion number and volume in RRMS patients (167,168), supporting the findings from EAE experiments, where atorvastatin significantly reduced disease severity (148,149). However, most of the more recent controlled studies did not find a significant reduction of relapses or disease progression in RRMS patients treated with statins alone or as add-on to IFN-β (160,169,170). One trial showed that combining atorvastatin with IFN-β reduced the number and volume of lesions (151), while another trial showed that the combination of these drugs increased both relapses and new lesions (171). Several trials assessing statins as monotherapy or as combination-therapy with IFN-β for MS are still ongoing. So far, in contrast to the findings in EAE, statins seem to have no or only a marginal effect in the therapy of multiple sclerosis. The reasons for the different conclusions relative to those of animal studies, as well as among the human studies, are unclear but may include differences in study design, in statin types and dosages, variation in IFN-β treatment, and the incomplete correlation between murine EAE and human MS.

In summary, the findings of this part of the thesis indicate, that atorvastatin has a strong direct effect on T cell proliferation, which was shown here for the first time for naïve CD4+ T cells, and which is independent of APC. In the performed experiments, atorvastatin did not otherwise directly influence the differentiation of T cells. That these findings contradict more recent findings, where an induction of Treg cells by atorvastatin was reported might be attributed to technical differences or the above-mentioned difference between enrichment (pseudo-expansion) and genuine expansion in numbers. Here, existing regulatory T cells were not expanded by atorvastatin and their suppressive capacity was not directly influenced by atorvastatin.

4.1.2 Modulation of T cells via atorvastatin treatment of DC

As a beneficial effect of atorvastatin in EAE is well established and an effect of atorvastatin on APC has been shown before (148,149), it was further examined if atorvastatin has indirect

effects on T cells via APC. DC are uniquely well equipped APC that initiate and regulate immune responses. DC initiate immune responses by activation of naïve B and T cells and the stimulation of natural killer cells. They control immunity through their ability to induce antigen-specific unresponsiveness of lymphocytes in primary and secondary lymphoid tissues by mechanisms that include induction of regulatory cells. Their unique role in immunity and tolerance makes them ideal targets for pharmacological modulation of immune responses. Several studies have shown, that immunosuppressive drugs such as corticosteroids, vitamin D3 and rapamycin, as well as other immunomodulatory drugs such as atorvastatin, GA or IFN-β target DC biology at various stages, affecting DC differentiation, antigen uptake and processing, migration, and maturation (172). This again affects the outcome of immune responses, as experimental evidence indicates that immature DC and tolerogenic DC can induce tolerance, presumably by the induction of Treg and IL-10 Treg cells (80,109).

For a long time, the immunosuppressive effects of corticosteroids have been ascribed mainly to the suppression of T cell activation. Later, it was shown that corticosteroids and vitamin D3 reduce DC numbers *in vivo* by blocking DC differentiation, increasing DC apoptosis and altering DC migration, leading to impaired T cell stimulation (173,174). Rapamycin was shown to reduce growth-factor-induced expansion of DC populations quantitatively, but does not impair DC differentiation qualitatively. Furthermore, rapamycin induces apoptosis in DC (175).

Antigen uptake and presentation by DC are tightly regulated mechanisms: iDC have the hightest capacity to take up antigen but have low T cell stimulatory activity, whereas mDC downregulate endocytic activity and are strong T cell stimulators (39). Corticosteroids, vitamin D3, and acetylsalicylic acid suppress DC maturation and enhance their endocytic capacity (173,176,177). Rapamycin on the other hand inhibits DC endocytosis in a DC-maturation-independent manner (178). These manipulations of antigen uptake influence endolysosomal processing of exogenous antigens and thereby MHC-II-restricted antigen presentation, leading to reduced immunostimulatory function and hence to reduced T cell activation. Preliminary results from our group indicate that atorvastatin also inhibits endocytosis in DC. DC maturation is triggered by numerous endogenous and exogenous stimuli, such as pro-inflammatory cytokines or exogenous microbial products. It is characterized by the downregulation of endocytic capacity, the upregulation of co-stimulatory molecules (CD40, CD80, CD86) and MHC-II molecules, the production of IL-12 and TNF-α,

and changes in migratory behavior. After maturation, DC become professional APC with strong capacity to stimulate T cells (39). Many different immunosuppressive and anti-inflammatory agents interfere with DC maturation, resulting in reduced expression of co-stimulatory and MHC-II molecules, reduced cytokine expression, and subsequently reduced T cell proliferation (172). The simple concept that immature DC are tolerogenic and mature DC immunogenic has however been revised by several reports showing that phenotypically mature but IL-12p70 low DC can induce regulatory T cell responses *in vitro* and *in vivo* (179). Pharmacological strategies to induce such semi-mature DC are therefore also likely to promote tolerance induction.

Rapamycin was reported to impair expression of co-stimulatory and MHC-II molecules on DC, and to inhibit proliferation of OVA-specific T cells when stimulated with rapamycin-pretreated DC and OVA-peptide (180). By having simultaneous inhibitory effects on DC and interacting T cells, as described above, rapamycin might achieve a greater immunosuppressive effect than if acting on either cell alone. The treatment of developing DC with IL-10 promotes the generation of immature tolerogenic DC that induce anergic CD4+ and CD8+ T cells *in vitro* with antigen-specific suppressor activity (181). GA and IFN-β decrease the expression of MHC-II and co-stimulatory molecules on DC. GA-treatment of DC was further shown to promote their secretion of anti-inflammatory type II cytokines, whereas IFN-β reduces the secretion of inflammatory cytokines by DC. This contributes to the differentiation of T cells into Th2 cells or regulatory T cells, which is thought to mediate the beneficial effect of GA and IFN-β in MS (182). Atorvastatin was shown to suppress expression of MHC-II molecules in microglia and of MHC-II and co-stimulatory molecules on APC. APC treated with atorvastatin *in vitro* or *in vivo* showed reduced capacity to stimulate T cell proliferation, although the effect on proliferation was not as pronounced as the direct effect of atorvastatin on T cell proliferation (149). In this thesis, the effect of atorvastatin-treatment specifically on the stimulatory and regulatory capacity of DC was studied. To study the stimulatory effect, atorvastatin was added to the culture during the generation of iDC from murine bone marrow cells, and the iDC were then harvested and co-cultured with T cells in the absence of atorvastatin. No suppression of proliferation of naïve OVA-specific (OT-2) CD4+ T cells was observed when they were stimulated with iDC generated in the presence of atorvastatin compared to iDC generated in the absence of atorvastatin. iDC loaded with OVA-peptide were generally very strong stimulators for naïve OT-2 T cells. These results seem somehow surprising, as previous unpublished work by our

group indicated a reduced expression of MHC-II and co-stimulatory molecules on atorvastatin-treated iDC. Moreover, Youssef *et al.* (149) described a decrease in stimulatory capacity of AT-treated APC. A possible explanation could be that the OVA-peptide was used here, which does not need to be processed intracellularly, or that the stimulative capacity of a pure DC population, as compared to a mixture of APC, is so strong that with a saturation of OVA-peptide, subtle differences in the stimulatory capacity cannot be detected.

To examine the regulatory capacity of iDC treated with atorvastatin, a protocol for the induction of IL-10-producing Treg cells from human PBMCs was applied (80), as described in the previous chapter. In short, human CD4+ T cells were co-cultured for 14 days with allogeneic immature dendritic cells generated from human monocytes. The suppressive capacity of the generated IL-10 Treg cells was then analyzed in a ^{3}H-thymidine suppression assay. To study the effect of atorvastatin specifically on the APC-compartment, atorvastatin was added during the generation of iDC from monocytes, before these iDC were harvested and co-cultured with the allogeneic CD4+ T cells. Here, a significant and reproducible increase in suppression of the target cells was observed when IL-10 Treg cells were generated with iDC that were generated in the presence of atorvastatin compared to IL-10 Treg cells generated with iDC in the absence of atorvastatin. Moreover, the IL-10 Treg cells generated with atorvastatin-treated iDC were even more anergic than the "normal" IL-10 Treg cells. These results indicate that atorvastatin enhances the capacity of iDC to induce more potent IL-10 Treg cells. Preliminary results from our group further indicate, that this effect might be attributed to an increased IL-10 production by atorvastatin treated iDC. The requirement for IL-10 in the induction of IL-10 Treg cells has already been described by Levings *et al.* (78).

In summary, the results presented here indicate, that a regulatory effect of atorvastatin on T cells is mediated indirectly via the modulation of DC. Although the capacity of iDC to stimulate T cell proliferation is not affected by atorvastatin, atorvastatin modulates DC in a way that they become "tolerogenic", inducing regulatory T cells with enhanced suppressive capacity. The effects of atorvastatin on DC and the modulation of DC-T cell interactions are very interesting as a use for future therapies and are further pursued in our group.

4.2 Do CD8+ T cells induce, aggravate, or attenuate EAE?

MS has long been considered a prototypic CD4+ T cell-mediated autoimmune disease, whereas the role of CD8+ T cells in MS and EAE is still controversially debated. The reason

for this CD4-bias is the relatively strong association of MS-susceptibility with MHC class II alleles and the fact, that CD4+ T cells are the main effector T cells in animal models of MS, such as EAE or Theiler`s murine encephalomyelitis virus (TMEV)-induced demyelinating disease. In general, CD8+ T cells, in contrast to CD4+ T cells, are equipped with the mechanisms to directly kill target cells, either via the Fas-FasL interaction or via secretion of lytic granules containing perforin and granzymes (39). A pathogenic role for CD8+ T cells in MS is suspected, as CD8+ T cells outnumber CD4+ T cells in inflammatory brain lesions and demonstrate oligoclonal expansions in MS brain and CSF (32,33). A detrimental function of CD8+ T cells is further supported by the fact that MHC class I can be expressed under inflammatory conditions on potential target cells within the CNS, such as neurons and oligodendrocytes (34), and the ability of CD8+ T cells to kill oligodendrocytes and neuronal cells *in vitro* (183,184). A direct evidence for CD8+ T cells as inducers of MS has however not been presented to date. An open question in this context is, which cells the target cells of CD8+ T cells within the CNS are – if there are target cells at all. Do CD8+ T cells kill neurons and oligodendrocytes and thereby contribute to the disease, or do they kill the self-reactive CD4+ T cells, thereby regulating the disease? Or are they just an epiphenomenon as intrathecal antibody production?

TMEV-induced demyelinating disease is the most relevant of the available virus-induced animal models of immune-mediated demyelination and serves as a good system to assess the potential contribution of viral infections to anti-myelin autoimmune responses in MS. In this model, infection with the natural mouse pathogen TMEV has been shown to induce CNS autoimmunity by causing bystander activation of myelin-specific CD4+ T cells, resulting in a chronic CD4+ T cell-mediated demyelinating disease with a clinical course and histopathology similar to that of chronic progressive MS (185). Only recently a model was described in which an infectious agent abrogated tolerance in myelin-specific CD8+ T cells, which generally seems to be more challenging than breaking CD4-tolerance (186). In this study, an infection of mice with vaccinia virus activated CD8+ T cells expressing dual T cell receptors that were able to recognize both MBP and viral antigens. The CD8-mediated immune response was characterized by infiltration of CD8+ T cells and macrophages, activated microglia in brain and spinal cord, and clinical signs as weight loss and ataxia. In the most common EAE models, CD4+ T cells serve as the main inducers of the disease, since these models are induced by immunization with myelin peptides, such as $PLP_{139-151}$ or MOG_{35-55} that are prototypic CD4-peptides in adjuvant, which mainly activates CD4+ T cells

leading to a CD4-mediated autoimmune response (23,24). Although MOG_{35-55} was shown to partially activate CD8+ T cells, shorter peptides such as MOG_{37-46} were determined that optimally activate CD8+ T cells and can induce a CD8-mediated disease (187). Two recent EAE-studies clearly showed, that CD8+ T cells can induce an MS-like disease with MS-like lesions, which however differed from conventional CD4-mediated EAE in disease course and pathology. Pre-activated CD8+ T cells specific for an *influenza* hemagglutinin peptide, which were transferred into transgenic mice with hemagglutinin-expressing oligodendrocytes, induced mild clinical symptoms, such as weight loss, but no pronounced paralysis (84). The pathology was characterized by CD8+ T cell infiltration and demyelination in the optic nerve, spinal cord and brain. In another study, a humanized mouse model was generated, where the MS-associated MHC class I molecule HLA-A3 and a myelin-specific autoreactive T cell receptor derived from a CD8+ T cell clone of an MS patient were expressed in mice (35). Here, CD8+ T cells also induced an MS-like disease with infiltration of CD8+ T cells in the optic nerve, spinal cord and brain, but no or only mild clinical symptoms. CD4+ T cells were shown to be essential for progression of the disease and the development of more severe clinical symptoms in this model. As the interest in CD8+ T cells as inducers of MS and its animal models is just emerging, new models specifically studying disease inducing CD8+ T cells are being developed. In earlier EAE-models, CD8+ T cells were rather seen as regulators than as inducers of the disease. This was supported by the findings, that EAE in CD8 knockout mice led to more relapses than in wild-type mice, and that CD8-depleted mice were no longer protected from a second induction of the disease (89,90). Several CD8+ T cells with regulatory properties have been described since then, some of which were shown to play a role in the control of EAE, such as Qa-1 dependent CD8+ T cells or IL-10-producing CD8+ T cells (95,100). Regulatory CD8+ T cells, in contrast to regulatory CD4+ T cells are however not well-defined cell populations, and their role in EAE is not well understood so far.

In some models of EAE, CD8+ T cells thus convincingly serve as beneficial regulators of the pathology and reduce disease severity whereas in other models CD8+ T cells are destructive effector cells relevant for CNS pathology. Problematic is the fact, that distinctive or indicative cellular markers to distinguish regulatory and pathogenic CD8+ T cells in EAE are lacking so far. One of the aims of the here presented work was to clarify the role of CD8+ T cells in MOG_{35-55}-induced EAE in mice with C57BL/6 background by describing their phenotype and behavior, and by clearly distinguishing between the contribution of CD4+ and CD8+ T cells to the disease, using a new approach. TPLSM was used to study the behavior of CD8+ T cells

directly at the site of inflammation in the CNS and to study their interaction with possible target cells, namely CD4+ T cells and neurons, within the CNS.

4.2.1 CD8+ T cells as inducers of EAE?

As it is difficult to distinguish the individual contribution of different T cell subsets in conventional EAE models, a new experimental approach was established to study CD4+ and CD8+ T cells individually. Therefore, T cell deficient $Rag1^{-/-}$ mice were reconstituted with CD4+ T cells (EGFP) and CD8+ T cells (RFP) or each cell type alone, and after 4 weeks for homeostatic engraftment, the mice were immunized with MOG_{35-55} peptide to induce EAE. With this approach it could be shown, that CD8+ T cells did not induce EAE by themselves, and that CD8+ T cells did not influence the clearly CD4-mediated disease.

The fact that CD8+ T cells alone did not induce EAE is surprising, as the CD8+ T cells were present in the CNS of mice reconstituted with CD8+ T cells only, as shown by FACS-staining and TPLSM, although these mice showed no clinical signs of disease. The flow cytometric analysis further showed an activated effector phenotype of these CD8+ T cells, as they were CD62L- and produced high amounts of IFN-γ, indicating that the CD8+ T cells were activated by the immunization with MOG_{35-55}. This corresponds to previous findings showing, that MOG_{35-55} does activate CD8+ T cells, although it is not the ideal peptide, as it is the immunodominant peptide for CD4+ T cells (187). The fact, that also CD8+ T cells specific for MOG_{37-46}, the immunodominant epitope for CD8+ T cells, need CD4+ T cells for induction of severe disease and sustained CNS inflammation during chronic EAE (188), might be an explanation for the absence of clinical signs here. TPLSM of mice that had received CD8+ T cells only showed low migratory capacity of the CD8+ T cells in the CNS of these mice. This contradicts previous findings from our group using the hippocampal slice model, where it was shown that CD8+ T cells move rapidly through the parenchyma. As the studies in the slice model were only performed with OVA-specific CD8+ T cells, the behavior observed here could be indicative for antigen recognition by the CD8+ T cells and show long-lasting contacts between CD8+ T cells and neurons. However, TPLSM revealed that the neurons in the mice that received CD8+ T cells only were intact, which correlates with the absence of clinical signs of disease. The static behavior of the CD8+ T cells might therefore rather be an indication for the CD8+ T cells not being properly activated, than an indication for specific cytotoxic contacts between the CD8+ T cells and the neurons. It is possible, that suboptimal activated CD8+ T cells do not find enough migratory signals in the absence of

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CD4+ T cells, as already shown for naïve CD4+ T cells by our group (189). It was shown, that for example extracellular matrix structures detected by second harmonic generation (SHG) signals, which guide lymphocyte motility in lymphoid tissues, could only be detected under inflammatory conditions in the CNS (189,190). It was also shown, that these structures can provide chemotactic signals for CD8+ T cells in the CNS during parasitic infection (190). The question however is, how the CD8+ T cells enter the CNS if they are not properly activated. Is the presence of the low numbers of contaminating CD4+ T cells in the mice that received CD8+ T cells alone enough to open the BBB and allow the CD8+ T cells to enter the CNS, but not enough to induce the disease? As the flow cytometric analysis presented here clearly indicates an activation of the CD8+ T cells also in the mice that received CD8+ T cells only, this point clearly needs further clarification. To answer the question, if CD8+ T cells are activated by MOG_{35-55} in the absence of CD4+ T cells, an *ex vivo* proliferation assay with CD8+ T cells isolated from the reconstituted immunized $Rag1^{-/-}$ mice should be performed. Also, the MOG_{37-46}-peptide should be used as a positive control. Furthermore, a comparative analysis of chemokine/cytokine expression profile of CNS lesions in the different conditions would complete the data set.

Interestingly, in the presence of CD4+ T cells, the CD8+ T cells showed a differential behavior than in the absence of CD4+ T cells. When CD4+ T cells were present, the CD8+ T cells showed the behavior of activated CD8+ effector T cells and moved rapidly through the parenchyma, as was previously observed by our group in the hippocampal slice model. The movement of the CD8+ T cells was not random, but directed. The reason for this directed behavior is not clear, but the directionality matches the direction of axons frequently observed in our experiments. However, also inflammation-induced SHG structures could provide migratory signals for CD8+ T cells here. The CD4+ T cells in contrast show a vessel-associated behavior. This indicates a dominant role of the perivascular area for CD4+ T cells and was further shown to be dependent on the chemokine receptor CXCR4 (125). It is assumed, that CD4+ T cells screen and interact with perivascular APC in this area, whereby they get reactivated (124). As CD8+ T cells recognize antigen on MHC-I, which can be expressed on various cell types within the CNS, this vessel-associated motility might not be important for their reactivation. The finding, that at the onset CD4+ T cells move faster and in a more directed manner than CD8+ T cells, whereas at the peak no difference in the behavior of CD4+ and CD8+ T cells could be observed supports these findings. The behavior of both, the CD8+ T cells as well as of the CD4+ T cells, is dependent on the disease stage. Both cell

types show a higher mean track velocity and displacement rate at the onset than at the peak of the disease. For CNS-specific CD4+ T cells, these motility changes have been interpreted as a stop signal in the process of antigen recognition and were described to correlate with the progression and severity of the clinical disease in EAE (126,191). The finding that CD8+ T cells show the same behavior could therefore be interpreted as antigen recognition within the CNS. However, from the EAE disease courses of the mice that received CD4+ and CD8+ T cells compared to the mice that received CD4+ T cells only, it is evident that the CD4+ T cells are the inducers of the disease and that the CD8+ T cells do not aggravate the CD4-mediated disease. The CD8+ T cells are present in the CNS in high numbers in these mice, are activated and show rapid, directed motility, but they seem not to be involved in the damaging cascade. Of note, the flow cytometric analysis of the CNS-lymphocytes showed that only the CD4+ T cells produced IL-17, whereas the CD8+ T cells produced IFN-γ only. This could be an explanation for CD8+ T cells not inducing EAE and not contributing to the CD4-mediated disease in this model, although they are present in the CNS, clearly activated, and most likely recognize antigen within the CNS. Although CD8+ T cells are able to kill oligodendrocytes in the absence of IL-17 (84), at least for CD4+ T cells, it could be clearly shown here, that only IL-17-producing CD4+ Th17 cells, but not IFN-γ-producing CD4+ Th1 cells establish long-lasting immune-neuronal contacts. These long-lasting contacts were further shown to lead to axonal and neuronal damage in EAE (126). The existence of IL-17-producing CD8+ T cells has been described, and these cells were also found among infiltrating cells in active MS lesions (192,193). As their role in EAE is so far unclear, they received only marginal attention in contrast to the well-established encephalitogenic Th17 cells. Two recent publications suggest, that the cytokine GM-CSF, an effector cytokine of Th17 cells, might be the critical factor determining pathogenicity of auto-aggressive T cells (53,54). GM-CSF produced by CNS-infiltrating CD4+ T cells is required for sustained CNS-inflammation during the effector phase of EAE (53). The production of GM-CSF is driven by the transcription factor RORγT and the cytokine IL-23, but suppressed by the cytokines IFN-γ and IL-12 (54). It is therefore mainly produced by Th17 cells, and only to some extent by Th1 cells. Although CD8+ T cells are in principle able to produce GM-CSF, as for example shown for virus-specific CD8+ T cells (194), nothing is known about the secretion and regulation of it in the context of EAE.

4.2.2 CD8+ T cells as suppressors of EAE?

Studies on CD4+ Treg cells in EAE have shown, that they play a major role in the recovery from actively induced EAE, as Treg depletion prevented the normal progression to the recovery phase. Treg-accumulation in the CNS further correlated with recovery (69). In several studies regulatory CD8+ T cells exerted their suppressive effect only after a first disease incidence (31). Here, a regulatory role for CD8+ T cells was not observed in the CD4+CD8 replenished lymphopenic hosts in the first bout of the disease. The group that received CD4+ and CD8+ T cells did not show an alleviated disease course compared to the group that received CD4+ T cells alone. The CD4-mediated disease course was very severe and non-remitting in the applied model, so that no remission phase could be observed. In general, CD8+ T cells have different mechanisms to exert a regulatory function. Expression of regulatory markers and cytokines, such as FoxP3 and IL-10, were shown to mediate a regulatory effect of CD8+ T cells in inflammatory settings (100,107). Another way for CD8+ T cells to exert a regulatory effect might however not be via the expression of regulatory markers and the secretion of regulatory cytokines, but via their "normal" cytotoxic mechanisms directed against the self-reactive CD4+ T cells. These cytotoxic mechanisms include the secretion of perforin and granzymes, or Fas/FasL-interactions. Perforin for example was shown to mediate the suppressive effect of Qa-1 restricted regulatory CD8+ T cells in EAE (195). Flow cytometric analysis of CD8+ T cells from C57BL/6 mice in remission showed no expression of FoxP3 and IL-10 on the CD8+ T cells. The only cytokine that was consistently detected is IFN-γ, which is a typical cytokine produced by CD8+ T cells. The role of IFN-γ is however very controversially discussed in EAE and MS, and not fully understood so far. It is assumed, that IFN-γ-producing Th1 cells can induce EAE, although they are not as encephalitogenic as Th17 cells (42,43). In MS, administration of IFN-γ worsened the disease (45). However, also IFN-γ-producing regulatory CD8+ T cells were described in humans, and these cells were further shown to be defective in patients with chronic progressive MS (103). In EAE, IFN-γ contributed to the suppressive effect of Qa-1 restricted regulatory CD8+ T cells (195). The expression of cytotoxic markers by CD8+ T cells is no indication for the role of these cells in EAE, as it is dependent on the target cell (i.e. self-reactive CD4+ T cell or neuron), if the cytotoxic mechanism has a regulatory or pro-inflammatory effect. Therefore, the presence of the CD8+ T cells on the behavior of the CD4+ T cells as well as the interaction of CD8+ T cells and CD4+ T cells was monitored using TPLSM in the approach described above. The CD8+ T cells had no influence on the

migratory behavior of the CD4+ T cells, as the CD4+ T cells showed the same mean velocity and displacement rate in the presence or absence of CD8+ T cells. A change in CD4+ T cell motility would be an indication for a possible direct or indirect regulatory effect of the CD8+ T cells. Using TPLSM, it has been shown that Treg cells influence the behavior of autoantigen-specific Th cells in the lymph node (196,197). In these publications, the first indication for a regulatory effect of the Treg cells was the changed behavior, i.e. increased speed, of the autoreactive effector T cells in the presence of Treg cells compared to their absence. Although the mechanism of regulation in this studies was not attributed to a direct interaction of the Treg and effector T cells, the Treg compromised the antigen-dependent arrest of the effector T cells by reducing their interactions with DC. In the experiments presented here, no long-lasting contacts between CD4+ and CD8+ T cells were observed in the CNS of EAE-affected mice, as indicated by low values for the contact index k. High k values, as calculated for the interaction of CD4+ 2d2 Th17 cells and neurons, indicate long-lasting specific interactions of the two observed cell populations. However, the problem that the remission phase could not be imaged was also a problem for the TPLSM, as regulatory CD8+ T cells might only accumulate during this disease phase.

4.3 Induction and expansion of suppressor CD8+ T cells

Peripheral tolerance mechanisms protect the body from detrimental effects after activation of self-reactive T cells. Active down-regulation of self-reactive T cell responses by regulatory T cells is one of the key mechanisms for the maintenance of self-tolerance and protection from autoimmune disease (39). Although it is not fully understood which role CD8+ T cells play in EAE and MS, there are convincing data for the existence of regulatory CD8+ T cells that contribute to self-tolerance (31,198). An interesting therapeutic approach for EAE and possibly also MS therefore is to expand those cells *in vitro* and transfer them as a therapy. Problematic is the fact, that CD8+ regulatory T cells are not a well defined cell population as for example CD4+ Treg, therefore no distinctive markers are available by which only the regulatory cells could be identified for subsequent isolation. Many human and rodent CD8+ regulatory T cells have been shown to express cell surface markers characteristic of activated T cells, including CD122, CD25, CD45RC^{low}. Upon activation, the majority of T cells upregulate CD25 (IL2 receptor α chain) and/or CD122 (IL2 receptor β chain), therefore, differentiating regulatory T cells from activated conventional T cells on the basis of these molecules is problematic (198). Expression of the transcription factor FoxP3 has also been

shown to be present in several CD8+ regulatory T cells, such as CD8+CD28- or CD8+CD25+ T cells (107,199). However, FoxP3 is also upregulated in conventional human T cells upon activation and might not be restricted purely to T cells displaying regulatory function (162). Furthermore, CD8+CD122+ regulatory T cells and Qa-1 restricted CD8+ regulatory T cells are FoxP3 negative (200,201). In addition, regulatory CD8+ T cells might have special requirements for expansion *in vitro*, as shown for CD4+ Treg (202), which are not understood so far. Here, a protocol was established for the in *vitro* expansion of CD8+ T cells based on the theory of vaccination. Already early EAE-studies had shown, that protective CD8+ T cells could be induced by T cell vaccination with antigen-activated attenuated encephalitogenic CD4+ T cells (91). As discussed in the previous chapter, CD8+ T cells with regulatory potential are suspected to accumulate after a first disease peak. This knowledge was combined here to expand CD8+ T cells isolated from EAE-recovered mice in *vitro* by co-culturing these cells with irradiated CNS-specific CD4+ T cells. The phenotype and behavior of the generated/expanded CD8+ T cells was then examined in *vitro* as well as in *vivo* in a therapeutic approach in EAE.

It was shown here, that *ex vivo* isolated CD8+ T cells from MOG_{35-55}-immunized C57BL/6 mice in remission did not express any of the regulatory markers known from CD4+ Treg. A population of CD44+CD122+Ly49+ T cells was detected, which was recently shown to define a population of Qa-1 restricted suppressor CD8+ T cells in an animal model of systemic lupus erythematosus (98,99). However, during in *vitro*-expansion with irradiated myelin-specific CD4+ 2d2 Th17 cells, no expansion of the CD44+CD122+Ly49+ T cell population took place, as CD122 was not upregulated. Also, no upregulation of other regulatory markers was observed. Furthermore, the upregulation of IFN-γ and the anti-inflammatory cytokine IL-10 was not higher than in control cells. Interestingly, Ly49 was expressed to a higher extent on the expanded CD8+ T cells as compared to control cells. Ly49 describes a family of receptors recognizing MHC-I molecules and is primarily expressed on natural killer (NK) cells. Recognition of MHC-I molecules by Ly49+ NK cells transmits inhibitory signals that prevent NK cells from mediating cytotoxicity, which has been proposed as a regulatory mechanism to prevent the lysis of normal host cells (203). It was subsequently shown, that Ly49 is also be expressed on a fraction of self-specific CD8+ T cells if CD4+ T cells are present (204). Chronic antigenic exposure from endogenous inducers seems to be required for the induction of these cytolytic CD8+ T cells. It was further shown, that the CD8+CD44+Ly49+ T cells comprise a mixed population of IL-15-dependent

(CD122^{high}) and IL-15-independent (CD122^{low}) cells (205). This is interesting, as in the experiments performed here no IL-15 was present in the culture and the cells did not express CD122. The exact role of these CD8+ populations however remains to be elucidated.

As no expansion of a clear-cut CD8+ T cell population was observed, the suppressive function of the expanded cells was examined *in vitro* and *in vivo*. It could be shown, that *in vitro* expanded CD8+ T cells isolated from MOG-immunized mice in remission clearly suppressed proliferation and IL-17 production of MOG-specific CD4+ 2d2 Th17 target cells *in vitro,* whereas *ex vivo* CD8+ T cells did not. This indicates that the expansion step *in vitro* is required for the CD8+ T cells to either acquire their regulatory phenotype, or to increase the amount of regulatory cells to a number where an effect becomes visible. The suppressive effect on CD4+ 2d2 Th17 cells could however not be observed *in vivo*, as EAE induced in Rag1$^{-/-}$ mice by transfer of CD4+ 2d2 Th17 cells was not suppressed or attenuated by the suppressor CD8+ T cells. The advantage of this passive EAE model in Rag1$^{-/-}$ mice is, that only the T cells transferred into the mice are present in these mice, as they do not possess any T or B cells by themselves. Therefore, the effect of the transferred T cells can be very clearly studied, without any intervening cells. The disadvantage of the model however is a very severe disease, with 100% incidence and mortality, whereby a mild effect of the CD8+ T cells might not be visible. One idea to overcome this problem is to use less CD4+ 2d2 Th17 cells for disease induction. Preliminary results from experiments performed in our group however indicate, that this rather affects disease incidence and onset than disease progression itself, as the mice that get the disease still develop a very severe disease course. An explanation might be, that Treg cells are missing here that normally control the disease and contribute to remission (69). Another advantage of the approach however is, that it allows TPLSM with defined T cell populations, such as EGFP+ self-reactive CD4+ T cells and RFP+ suppressor CD8+ T cells. Both, CD4+ and CD8+ T cells were highly motile in the CNS of EAE-affected mice. Furthermore, contacts between the CD4+ and CD8+ T cells were visible, which were not stationary but dynamic and could be tracked over time. As described in the previous chapter, long-lasting contacts between the two cell types might be an indication for a regulatory interaction. More long-lasting interactions between CD8-MOG cells and self-reactive CD4+ 2d2 Th17 cells were observed than between control CD8-C57BL/6 cells and CD4+ 2d2 Th17 cells. This indicates, that the CD8-MOG T cells differ in their behavior from the control CD8-C57BL/6 T cells, although the two cell types did not significantly differ in their phenotype, as shown by FACS-staining, or in their potential to control EAE in Rag1$^{-/-}$

mice. That direct cell-cell contact seems to be required for suppression was also confirmed by *in vitro* experiments*,* as supernatants from the CD8-MOG culture did not suppress the proliferation of CD4+ 2d2 Th17 target cells. Regulatory mechanisms that require direct cell-cell contact are for example Fas-FasL interactions, as well as granzyme or perforin mediated cytotoxicity. However, at least for CD4+ Treg it is assumed that cell-cell contact dependent mechanisms (i.e. expression of CTLA-4) and independent mechanisms (i.e. secretion of TGF-β and IL-10) operate in cooperation (206). To circumvent the problem of the severe EAE-course in Rag1$^{-/-}$ mice, the effect of the suppressor CD8+ T cells was also examined in active EAE. Here, a mild regulatory effect of the CD8-MOG cells was observed in the remission phase, compared to CD8-C57BL/6 control cells. However, these *in vivo* findings need to be confirmed.

To study the mechanism of suppression in more detail further *in vitro* experiments were performed, as a clear suppressive effect had been observed. The *in vitro* suppression assay therefore constituted a good approach to investigate the underlying mechanism and narrow down the regulatory subpopulation. Surprisingly, not only CD8+ T cells from MOG$_{35-55}$-immunized mice, but also from mice immunized with OVA-protein suppressed the myelin-specific CD4+ 2d2 Th17 cells. However, none of these CD8+ T cells suppressed proliferation of CD4+ OT2 Th17 cells. These findings are consistent with the theory, that suppression by Qa1-restricted regulatory CD8+ T cells is determined by avidity rather than specificity. The concept of Qa-1 restricted CD8+ T cells is based on the "avidity model of peripheral T cell regulation" (207). The major mechanism of self-tolerance is thymic negative selection, where self-reactive T cells expressing T cell receptors of high avidity to the majority of self-antigens are deleted (39). However, while releasing innocuous self-reactive T cells with low avidity, this process also allows a fraction of self-reactive T cells with intermediate avidity to be released into the periphery. These cells present a potential danger of pathogenic autoimmunity. It is therefore suggested, that self-nonself discrimination must continue in the periphery after thymic negative selection by selective down-regulation of autoimmune responses without damaging the normal responses to foreign pathogens. As anti-infection immunity is largely mediated by high-avidity T cells, this can be achieved by selective down-regulation of intermediate-avidity T cells, independent of their specificity, by Qa-1/HLA-E-restricted CD8+ T cells (207). Qa-1 in mice and its human homologue HLA-E are non-classical MHC-I molecules expressed on the surface of activated CD4+ T cells. A surrogate target structure, the Qa-1/Hsp60sp complex was identified, which is preferentially expressed

on CD4+ T cells of intermediate avidity regardless of which antigens the target CD4+ T cells were triggered by, and which can be recognized by Qa-1 restricted CD8+ T cells (208). Hence, it could be shown in mice, that peptide vaccination with 1-9NacMBP (normally protecting from subsequently induced EAE), as well as the unrelated peptide P277 (normally protecting from a spontaneous development of type 1 diabetes) induced cross-protection mediated by Qa-1 dependent CD8+ T cells, as both vaccination strategies protected from subsequently induced EAE as well as type 1 diabetes independently of each other (97). In this context, an explanation for the results of the experiments performed here could be, that immunization with MOG_{35-55} as well as OVA-protein generates intermediate avidity CD4+ T cells that can be recognized by Qa1-restricted CD8+ T cells, which then suppress the intermediate avidity CD4+ 2d2 Th17 cells, whereas the high avidity OT2 CD4+ Th17 cells are not suppressed. The application of an anti-Qa-1 blocking antibody however did not prevent suppression of the CD4+ 2d2 Th17 target cells by the CD8+ suppressor cells, indicating that the observed suppression is not Qa-1 dependent. Experiments using Qa-1 knockout mice would give definitive clarity on this subject.

Finally, it could be shown here, that the in vitro expanded suppressor CD8+ T cells not only suppressed proliferation and IL-17 production of CD4+ 2d2 Th17 target cells, but also had a cytotoxic effect on these target cells. This could also be transferred to the human system, where CD8+ suppressor T cells from an HLA-DR2+ donor were shown to kill autologous myelin-specific CD4+ target cells. Surprisingly, in the cytotoxicity assay not only CD4+ 2d2 Th17 cells were killed by the suppressor CD8+ T cells, but also non-myelin specific high avidity CD4+ OT2 Th17 target cells. It is therefore likely, that two different mechanisms are taking place here. One that suppresses proliferation and is directed against myelin-specific/intermediate-avidity cells, and one that kills target cells independently of their antigen specificity or avidity. The exact underlying mechanisms clearly need further clarification. Also here, the use of knockout mice, such as IL-10-ko, IFNγ-ko, or perforin-ko mice, will give more insights and these experiments are already in progress in our group.

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Summary

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS) in Europe and North America, leading to devastating disability in young adults with only limited treatment options available. Although intensive research has been performed in the field of MS, leading to many new insights, the cause of the disease, as well as the exact underlying pathogenic mechanisms are not well understood so far. The consensus view is, that MS is a misguided immune response, initiated by CNS-reactive T cells. These T cells are activated in the periphery and transmigrate through the blood brain barrier (BBB) into the CNS, where they get reactivated by local antigen presenting cells (APC). Thereafter, together with other lymphocytes recruited from the periphery, they lead to demyelination, axonal damage, and neuronal cell death. Most knowledge about the pathogenesis of the disease is derived from studies in the animal model experimental autoimmune encephalomyelitis (EAE), which allows to examine the role of different cell types in the disease in more detail. The prevailing opinion is that CD4+ T helper (Th) cells of the Th1 or Th17 subtype are the essential mediators of the disease, whereas the contribution of CD8+ T cells is controversially debated. In parallel to the pathogenic role of T cells in MS and EAE, also a regulatory role for T cells is acknowledged. Regulatory T cells of the CD4- and CD8-subtype contribute to peripheral self-tolerance by controlling autoimmune responses. In MS, regulatory T cells are disturbed in their function and transfer of regulatory T cells into EAE-affected animals was able to significantly reduce disease severity.

Several therapies are available to treat MS nowadays, however, their effectiveness is limited. These drugs reduce the relapse rate and delay progression of disability to a certain degree, but do not cure the disease. As most of the treatments are based on a general immunosuppression, they are accompanied by severe side effects. It is therefore required to better understand the underlying mechanisms of the disease in order to develop more specific treatment approaches with better risk-benefit ratio. Regulatory T cells (Treg/IL-10 Treg) are a major potential target for immunotherapy and strategies to induce, expand, or modulate these cells *in vitro* and transfer them for therapeutic applications is a promising approach for future therapies. The overall aim of this work was to better understand the phenotype and the role of regulatory T cells of the CD4- and CD8-subset, in order to apply them as a therapeutic approach in EAE. Therefore, the phenotype of these cells was studied and modified in *vitro,* and their behavior was monitored in *vivo* using two-photon laser scanning microscopy (TPLSM).

In the first part of this thesis it was examined if and how the immunomodulatory drug atorvastatin can shift the T cell response into a regulatory direction. It was shown, that atorvastatin directly inhibited proliferation of naïve CD4+ T cells, but did not directly modulate the T cell response in a way that the T cells acquired a regulatory phenotype. The presence of atorvastatin however allowed the percental expansion of CD4+ Treg cells from a mixture of CD4+ T cells, presumably by inhibiting proliferation of effector T cells. In contrast, treatment of the APC-compartment with atorvastatin had a regulatory effect, as the co-culture of immature dendritic cells (DC), generated in the presence of atorvastatin, with T cells induced CD4+ IL-10 Treg cells with increased suppressive potential. The treatment of DC with atorvastatin and the modulation of DC-T cell interactions might therefore be a promising starting-point for future therapies. Taken together, we found only a subtle immunomodulatory effect on adaptive immune responses, which is in keeping with a probably only modest beneficial effect in autoimmunity *in vivo*.

In the second part of this thesis, the role of CD8+ T cells in MOG-induced EAE was investigated. Therefore, an experimental approach was established, where Rag1$^{-/-}$ mice were reconstituted with CD4+ and/or CD8+ T cells before immunization with MOG$_{35-55}$. It could be shown, that CD4+ T cells clearly are the inducers of the disease. CD8+ T cells did not induce the disease by themselves and had no effect on the CD4-mediated disease. Interestingly CD8+ T cells were present in high numbers in the CNS and showed an activated effector phenotype, expressing high amounts of IFN-γ, no IL-17 and no regulatory markers. This is in line with the finding, that only IL-17-expressing MOG-specific CD4+ Th17 cells, but not IFN-γ-expressing MOG-specific CD4+ Th1 cells established long-lasting contacts with neurons, leading to neuronal cell death. TPLSM revealed a distinctly different motility pattern for CD8+ T cells compared to CD4+ T cells, as the CD8+ T cells invaded the parenchyma, whereas the CD4+ T cells showed a vessel-associated behavior, presumably induced by scanning and contacting perivascular APC. CD8+ T cells did not affect CD4-motility in the CNS, and no long-lasting interactions between the two cell types were observed, supporting the assumption that CD8+ T cells are neither regulatory nor pathogenic in this approach.

In the third part of this thesis it was examined, if it is possible to generate or expand CD8+ T cells with regulatory potential *in vitro* from EAE-recovered mice. It could be shown here, that co-culture with irradiated myelin-specific CD4+ T cells generated CD8+ T cells with the

potential to specifically suppress proliferation and IL-17 production of myelin-specific CD4+ Th17 cells *in vitro* in a contact-dependent way. These suppressor CD8+ T cells further showed an unspecific cytotoxic effect. When transferred into C57BL/6 mice before onset of active EAE, the *in vitro* expanded CD8+ suppressor T cells had a beneficial effect on the disease course. This indicates that the generation/expansion of suppressor CD8+ T cell is possible and an approach worthwhile following for the therapy of EAE, however, the exact mechanisms of suppression still need to be investigated further.

Zusammenfassung

Multiple Sklerose (MS) ist die häufigste chronisch-entzündliche Erkrankung des zentralen Nervensystems (ZNS) in Europa und Nordamerika. Die Erkrankung führt zu schwerwiegenden Behinderungen bei jungen Erwachsenen und bis jetzt gibt es nur beschränkte Therapiemöglichkeiten. Trotz intensiver Forschung auf dem Gebiet der MS sind die genauen Ursachen der Krankheit sowie die zu Grunde liegenden Mechanismen nur unvollständig verstanden. Es wird angenommen, dass MS eine fehlgeleitete Immunantwort ist die durch ZNS-spezifische T Zellen ausgelöst wird. Diese T Zellen werden in der Peripherie aktiviert und wandern danach über die Blut-Hirn-Schranke ins Hirngewebe ein, wo sie von lokalen Antigen-präsentierenden Zellen (APZ) reaktiviert werden. Zusammen mit anderen Immunzellen, die ebenfalls ins ZNS rekrutiert werden, führen sie zu Demyelinisierung, axonaler Schädigung und neuronalem Zelltod. Das heutige Wissen über die Pathogenese der Erkrankung stammt zum grössten Teil aus Studien des Tiermodells der MS, der Experimentellen Autoimmunen Enzephalomyelitis (EAE). Dieses Modell ermöglicht es die Rolle einzelner Zelltypen im Detail zu untersuchen. Es wird angenommen, dass CD4+ T Zellen des Subtyps Th1 oder Th17 für die Krankheit verantwortlich sind, wohingegen die Rolle von CD8+ T Zellen umstritten ist. Neben der pathogenen Rolle von T Zellen in der MS und der EAE, ist aber auch eine regulatorische Rolle der T Zellen bekannt. Regulatorische CD4+ und CD8+ T Zellen halten Autoimmunantworten in Schach und tragen dadurch zu peripherer Toleranz bei. Es konnte gezeigt werden, dass die Funktion regulatorischer T Zellen in der MS gestört ist und dass der Transfer von regulatorischen T Zellen in der EAE einen positiven Einfluss auf den Krankheitsverlauf hat.

Es stehen heutzutage mehrere Therapiemöglichkeiten für MS zur Verfügung, deren Wirkungen aber leider beschränkt sind. Sie führen in der Regel zu einer Reduktion der Schubrate und können ein Fortschreiten der Krankheit verzögern, aber nicht verhindern. Da die meisten zur Verfügung stehenden Medikamente zu einer generellen Unterdrückung des Immunsystems führen, haben sie auch starke Nebenwirkungen. Es ist deshalb wichtig, die zu Grunde liegenden Mechanismen der Krankheit besser zu verstehen und spezifischere und sicherere Therapien zu entwickeln. Regulatorische T Zellen (Treg/IL-10 Treg) sind ein vielversprechender Ansatzpunkt für neue Immuntherapien, wobei Strategien diese Zellen *in vitro* zu generieren, zu verändern oder zu vermehren, um sie danach *in vivo* als Therapie anwenden zu können, im Mittelpunkt stehen. Das übergeordnete Ziel der vorliegenden Arbeit

war es, den Phänotyp und die Rolle von regulatorischen T Zellen des CD4 und CD8 Subtyps besser zu verstehen um sie als Therapie in der EAE einsetzen zu können. Dazu wurde der Phänotyp dieser Zellen *in vitro* untersucht und modifiziert, sowie das Verhalten der Zellen *in vivo* mittels 2-Photonenmikroskopie untersucht.

Im ersten Teil der vorliegenden Arbeit wurde untersucht, ob und wie das immunmodulatorische Medikament Atorvastatin T Zellen in eine regulatorische Richtung lenken kann. Es konnte gezeigt werden, dass Atorvastatin die Proliferation von naiven CD4+ T Zellen direkt inhibiert, diese aber nicht in eine regulatorische Richtung lenkt. Mit Hilfe von Atorvastatin konnten CD4+ Treg Zellen aus seiner Mischung von CD4+ T Zellen prozentual angereichert werden, vermutlich durch Hemmung der Proliferation von Effektor T Zellen. Im Gegensatz dazu führte die Behandlung des APZ-Kompartements mit Atorvastatin zu einem regulatorischen Effekt. Die Kokultur von T Zellen mit unreifen dendritischen Zellen (DC), die in der Anwesenheit von Atorvastatin generiert wurden führte zu CD4+ IL-10 Treg Zellen mit höherem suppressorischem Potential. Die Behandlung von DC mit Atorvastatin und die Modulation von DC-T Zell Interaktionen könnten deshalb vielversprechende Ansatzpunkte für neue Therapiemöglichkeiten sein. Zusammenfassend kann man sagen, dass hier nur ein schwacher immunmodulatorischer Effekt von Atorvastatin beobachtet werden konnte, was mit einem schwachen positiven Effekt, der in klinischen Studien beobachtet wurde, einhergeht.

Im zweiten Teil der vorliegenden Arbeit wurde die Rolle von CD8+ T Zellen in der MOG-induzierten EAE genauer untersucht. Dazu wurde ein neuer experimenteller Ansatz entwickelt, bei dem Rag1$^{-/-}$ Mäuse mit CD4+ und/oder CD8+ T Zellen rekonstituiert und anschliessend mit MOG$_{35-55}$ immunisiert wurden. Es konnte klar gezeigt werden, dass CD4+ T Zellen die Auslöser der Krankheit sind. CD8+ T Zellen lösten keine Krankheit aus und hatten auch keinen Einfluss auf die CD4-vermittelte Krankheit. Interessanterweise waren viele CD8+ T Zellen mit einem aktivierten Effektor-Phänotyp im Hirn zu sehen. Diese Zellen produzierten viel IFN -γ aber kein IL-17 und keine regulatorischen Marker. Dies stimmt mit dem Befund überein, dass nur IL-17-produzierende MOG-specifische CD4+ Th17 Zellen aber nicht IFN-γ-produzierende MOG-specifische CD4+ Th1 Zellen langanhaltende Kontakte mit Neuronen eingehen, die schliesslich zum neuronalen Zelltod führen. Mittels 2-Photonenmikroskopie konnte ausserdem gezeigt werden, dass CD8+ T Zellen und CD4+ T Zellen ein unterschiedliches Bewegungsmuster im Hirn zeigen. CD8+ T Zellen dringen ins

Hirngewebe ein, wohingegen sich CD4+ T Zellen entlang der Gefässe bewegen, vermutlich zur Kontaktaufnahme mit perivaskulären APZ. CD8+ T Zellen beeinflussten das Bewegungsmuster von CD4+ T Zellen im Hirn nicht und es konnten auch keine langen Interaktionen zwischen den zwei Zelltypen beobachtet werden. Dies spricht dafür, dass CD8+ T Zellen in diesem Ansatz weder regulatorisch noch pathogen wirken.

Im dritten Teil der vorliegenden Arbeit wurde untersucht, ob von CD8+ T Zellen, die aus Mäusen in der Remissionsphase einer EAE isoliert wurden, *in vitro* regulatorische Zellen generiert oder expandiert werden können. Die Kokultur von CD8+ T Zellen mit bestrahlten Myelin-spezifischen CD4+ T Zellen führte zur Gewinnung regulatorischer CD8+ T Zellen. Diese konnten die Proliferation und IL-17-Produktion von Myelin-spezifischen CD4+ Th17 Zellen gezielt unterdrücken. Ausserdem zeigten diese regulatorischen CD8+ T Zellen einen unspezifischen zytotoxischen Effekt. Als die *in vitro* generierten CD8+ T Zellen vor dem Beginn der Erkrankung in aktiv immunisierte C57BL/6 Mäuse gespritzt wurden, hatten sie einen schwachen positiven Effekt auf den Krankheitsverlauf. Dies verdeutlicht, dass es möglich ist regulatorische CD8+ T Zellen zu generieren/expandieren und dass dies einen vielversprechenden Therapieansatz in der EAE darstellen könnte. Um die zu Grunde liegenden Mechanismen der CD8-vermittelten Suppression genau zu verstehen, bedarf es aber noch weiterer Untersuchungen.

List of publications

(* equally contributing authors)

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Curriculum Vitae

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

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