Role of Regulatory T cells in Experimental Autoimmune Encephalomyelitis

A Functional and Imaging Study

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SUMMARY

CD4$^+$Foxp3$^+$ regulatory T cells (Treg) are essential for maintaining immune tolerance and play a critical role in the control of autoimmune responses. During actively induced Experimental Autoimmune Encephalomyelitis (EAE), an animal model for Multiple Sclerosis, Treg accumulate in the central nervous system (CNS), paralleling the recovery from the disease. However, whether Treg are essential for recovery from EAE, as well their function *in vivo*, especially in the target organ CNS, remains to be elucidated. In the present study, the functional role of Treg cells in CNS autoimmunity was investigated, using combinations of transgenic mouse strains and EAE models, as well as intravital 2-photon imaging.

To evaluate the role of Treg in recovery from EAE, selective and efficient depletion of Treg at the peak of EAE in mice immunized with myelin oligodentrocyte glycoprotein (MOG) was performed. Interestingly, ablation of Treg not only prevented recovery, but led to fatal disease. The severe exacerbation of the disease was characterized by enhanced numbers of IFNγ- and IL-17-producing effector T cells (Teff) in immunization-site draining lymph nodes (LN) and the CNS. In parallel, elevated numbers of macrophages were found in the CNS infiltrates of Treg-depleted mice, while lower frequency of regulatory (M2) macrophages was observed in LN. These findings suggest that Treg mediate recovery from EAE by controlling Teff and macrophage in LN or the CNS, or both.

To visualize the function of Treg within the CNS during EAE, intravital 2-photon imaging was performed in the spinal cord meninges of MOG-immunized mice at different phases of the disease. Treg were significantly more motile and displayed shorter interactions with Teff and local APC at the onset, while they showed decreased motility and formed prolonged contacts with APC at the peak of EAE, indicating that Treg exhibit their suppressive function during this phase. In contrast, the motility of Teff and their contact duration with APC did not change drastically throughout the disease. Importantly, depletion of Treg at the peak of EAE resulted in decreased motility of Teff, although the duration and number of Teff-APC contacts was not affected. Therefore, either prevention of stable Teff-APC contacts is not the suppression mechanism that Treg use in the CNS or Treg control the autoimmune response in the peripheral immune organs, rather than in the CNS.

The role of Treg was also examined in a spontaneous EAE (sEAE) model, the spontaneous opticospinal EAE (OSE) mice, 50% of which develop sEAE. Intriguingly, no difference was observed in the frequency, antigen-specificity or suppressive capacity of Treg between affected and resistant OSE mice. Nonetheless, MOG-specific Treg appeared in strikingly high frequency in the CNS infiltrates of affected OSE mice already at the disease onset and persisted throughout the disease course. Furthermore,

depletion of Treg in OSE mice, starting before the onset of sEAE, increased the incidence, but not severity, of the disease. These observations indicate a protective role for Treg also in sEAE and point to the periphery as the site of Treg-mediated suppression.

In summary, both the functional and imaging data presented in this thesis establish a definitive role for Treg in recovery from EAE.

ZUSAMMENFASSUNG

CD4$^+$Foxp3$^+$ regulatorische T-Zellen (Treg-Zellen) sind ein wichtiger Bestandteil des Immunsystems, dienen sie doch der Aufrechterhaltung der Selbsttoleranz und somit der Vermeidung von Autoimmunerkrankungen. Im Krankheitsverlauf der aktiv induzierten experimentellen autoimmunen Enzephalomyelitis (EAE), einem Tiermodell der Multiplen Sklerose, kommt es zu einer Ansammlung von Treg-Zellen im zentralen Nervensystem (ZNS) und in Folge dessen zu einer Verbesserung der Krankheitssymptome. Jedoch ist die Funktion von Treg-Zellen *in vivo* (v.a. im ZNS), sowie der Zusammenhang von Treg-Zellen mit der Verbesserung der EAE-Symptome noch unklar. In der vorliegenden Arbeit wurde die Rolle der Treg-Zellen in einer ZNS-spezifischen Autoimmunerkrankung anhand transgener Mausstämme, verschiedenen Krankheitsmodellen, sowie intravitaler 2-Photonen Mikroskopie untersucht.

Um den Einfluss der Treg-Zellen auf die Genesungsphase der EAE zu untersuchen, wurden Treg-Zellen am Höhepunkt der Erkrankung in Mäusen immunisiert mit Myelin Oligodendrozyten Glykoprotein (MOG) zielgerichtet und effektiv depletiert. Interessanterweise verhinderte die Depletion der Treg-Zellen nicht nur die Genesung, sondern hatte auch einen drastischen Fortschritt der EAE zur Folge. Die dramatische Verschlechterung der Krankheitssymptome war gekennzeichnet durch eine gesteigerte Anzahl an IFN-γ- und IL-17-produzierenden Effektor-T-Zellen (Teff) sowohl in den Lymphknoten als auch im ZNS. Außerdem wurden vermehrt Makrophagen in den ZNS-Infiltraten von Treg-depletierten Mäusen, sowie weniger regulatorische (M2) Makrophagen in den Lymphknoten nachgewiesen. Dies lässt vermuten, dass Treg-Zellen während der EAE eine Funktion in der Kontrolle von Teff-Zellen und Makrophagen in den Lymphknoten sowie dem ZNS übernehmen und dadurch den Verlauf der Krankheit positiv beeinflussen.

Um die Funktion der Treg-Zellen im ZNS zu veranschaulichen, wurde intravitale 2-Photonen-Mikroskopie an den Gehirnhäuten des Rückenmarks von MOG-immunisierten Mäusen in verschiedenen Stadien der Krankheit durchgeführt. Zu Beginn der EAE zeigen Treg-Zellen eine höhere Mobilität sowie kürzere Interaktionen mit Teff-Zellen und lokalen Antigen-präsentierenden Zellen. Am Höhepunkt der Krankheit hingegen zeigen sie eine reduzierte Beweglichkeit. Ein zusätzlicher Hinweis auf eine supprimierende Rolle der Treg-Zellen ergibt sich aus längeren Kontakten mit Antigen-präsentierenden Zellen. Im Gegensatz dazu zeigten Teff-Zellen keine drastischen Veränderungen bezüglich ihrer Beweglichkeit bzw. Kontaktdauer mit Antigen-präsentierenden Zellen während verschiedenen Stadien der EAE. Mögliche Erklärungen wären zum einen, dass die Treg-vermittelte Suppression nicht über die verhinderte

Kontaktausbildung erfolgt, oder zum anderen, dass die Treg-vermittelte Kontrolle nicht wie vermutet im ZNS sondern in den peripheren Immunorganen stattfindet.

Abschließend wurde die Rolle der Treg-Zellen zusätzlich in einem spontanen EAE (sEAE) Modell untersucht. Fünfzig Prozent der optikospinalen EAE (OSE) Mäuse entwickeln spontan EAE. Interessanterweise konnte kein Unterschied zwischen kranken und resistenten OSE Mäusen in Bezug auf die Frequenz, die Antigen-Spezifität oder die hemmende Wirkung der Treg-Zellen festgestellt werden. Bereits zu Beginn der Krankheit konnten große Mengen an MOG-spezifische Treg-Zellen in den ZNS-Infiltraten von OSE Mäusen nachgewiesen werden. Eine Depletion der Treg-Zellen in OSE Mäusen vor Beginn der EAE hatte eine Erhöhung der Inzidenz-Rate, jedoch keine Verschlechterung der Krankheit zur Folge. Auch diese Ergebnisse weisen darauf hin, dass Treg-Zellen in OSE Mäuse ihre schützende Funktion in der Peripherie und weniger im ZNS entfalten.

Zusammenfassend lässt sich anhand der funktionellen sowie Mikroskopie-Daten, die in der vorliegenden Arbeit vorgestellt wurden, eine Rolle der Treg-Zellen in der Erholungsphase der EAE vermuten.

INTRODUCTION

1.1   Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system (CNS), characterized by focal infiltration of lymphocytes that leads to inflammation, demyelination and axonal degeneration. First described by Jean-Martin Charcot in 1868, MS is today the most common inflammatory disorder of the CNS in the Western World, with approximately 500,000 affected individuals in Europe and more than 2 million worldwide (Flachenecker and Stuke, 2008).

MS symptoms include a variety of motor, sensory, visual and autonomic dysfunctions, depending on the location of demyelination sites and the size of neuronal damage. Hypoesthesia, muscle weakness and spasms, visual problems, fatigue, pain and cognitive impairment, as well as difficulty in moving, coordination and balance, are the main clinical features, few of which, however, are disease-specific. The diagnosis is supplemented by detection of abnormalities in white matter, using MRI (95% of the patients), and the presence of oligoclonal immunoglobulin bands in the cerebrospinal fluid after protein electrophoresis (90% of the patients) (Compston and Coles, 2008). After an initial acute episode, the majority of MS patients display a relapsing course (relapsing-remitting MS; RRMS), with not more than 1-5 episodes per year. However, with time recovery from each attack becomes incomplete, leading eventually to accumulation of symptoms and a progressive disease phase (secondary progressive MS; SPMS). In fewer cases, the disease can be progressive from the beginning, lacking the relapsing phase (primary progressive MS; PPMS) (Compston and Coles, 2008) (Lassmann et al., 2007). In all MS types, the clinical course spans over several decades and the median time to death is approximately 30 years after the onset (Compston and Coles, 2008).

The pathogenesis of MS is believed to be immune-mediated, although a neurodegenerative hypothesis cannot be entirely excluded. Under physiological conditions, the CNS is inaccessible to immune cells, due to the presence of the blood-brain barrier (BBB) and the lack of lymphatic vessels (Engelhardt and Ransohoff, 2005). However, during MS, massive number of immune cells manages to infiltrate the CNS causing inflammation, BBB disruption and demyelination. Inflammation is dominated by CD8$^+$ T cells and activated macrophages or microglia and is accompanied by local secretion of proinflammatory cytokines and chemokines. Abundant demyelination is followed by axonal injury and degeneration, which cause the clinical symptoms. Although extensive remyelination occurs in 20% of MS patients, continuous demyelination and remyelination seems to exhaust the tissue repair capacity (Patrikios et al., 2006).

There is strong evidence supporting the autoimmune pathogenesis of MS, including the lesion domination by immune cell infiltrates, the presence of oligoclonal bands in the CSF, the association of HLA polymorphisms with disease susceptibility and the effect of T and B cell targeted immunotherapy (Wekerle, 2008). However, the antigen specificity of the immune response remains unresolved.

 The etiology of MS, although not clear, is considered to be a combination of genetic and environmental factors. Despite the fact that MS is not regarded as a hereditary disease, monozygotic twins show concordance in 30% of the cases compared to 3-5% in first degree relatives. The main genetic risk factor is the major histocompatibility complex class II (HLA), as there is a strong and consistent association between MS and HLA DRB1*1501, DQA1*0102, and DQB1*0602 extended haplotype (Dyment et al., 2004). Regarding the environmental factors, the global distribution of the disease indicates an increased risk of MS in higher geographic latitude. In agreement with the previous observation, correlation between MS and low sunlight exposure, as well as vitamin D deficiency, has been repeatedly reported (Handel et al., 2010; Marrie, 2004). Moreover, dietary habits, especially animal fat consumption, have been suggested been suggested to play a role in MS incidence (Schwarz and Leweling, 2005), which could possibly explain the rise in the disease prevalence in Japan since 1950, where diet has been shifted from traditional one to western style (Kira et al., 1999). Finally, several infectious agents, including Epstein-Barr virus (EBV) and *Chlamydia pneumoniae*, have been associated with MS, but none has been definitively proven so far (Marrie, 2004).

1.2   Experimental Autoimmune Encephalomyelitis

Due to the limited access to *ex vivo* MS patient material (especially CNS biopsy), the disease heterogeneity and the need for investigating new therapeutic approaches, animal models for MS are required. Experimental Autoimmune Encephalomyelitis (EAE) is the most commonly used animal model for MS, providing an attractive setup of studying clinical, neuropathological and immunological aspects of MS, as well as of testing possible therapeutic treatments.

EAE was first described in 1925, with rabbits developing paralysis and spinal cord inflammation after immunization with human spinal cord tissue (Koritschoner and Schweinburg, 1925). Few years later, CNS inflammation and demyelination was induced in rhesus monkeys by repeated injection of rabbit CNS tissue, in an attempt to study the neurological complications after rabies vaccination in humans (Rivers et al., 1933). The development of a mineral oil-based adjuvant by J. Freund (Freund and McDermott, 1942), mixed with brain tissue, allowed EAE induction with only a single injection (Kabat et al., 1947). In the decades that followed, this technique was expanded in several animals (mice, rats, guinea pigs,

macaques, marmosets etc.). In the 1980's, the autoimmune nature of this experimental model was proven, when EAE was induced by transfer of myelin-specific T cells to naïve syngeneic rats (Ben-Nun et al., 1981). Murine EAE models were first described in the 1950's, but their use was limited because of lower disease incidence and higher clinical heterogeneity compared to rats and guinea pigs (Gold et al., 2006). Only after the introduction of pertussis toxin to enhance disease induction and the use of more susceptible mouse strains (Bernard and Carnegie, 1975; Levine and Sowinski, 1973), murine EAE models became of wider use. The development of genetic manipulation techniques to generate transgenic and knock-in/out mice, allowing studying the contribution of a plethora of genes in the disease development and progression, made mice the most predominant experimental animal for EAE studies (Handel et al., 2011).

In "classic" EAE phenotype, the clinical symptoms start with a loss of the tail tone. The paralysis progresses in caudal to rostral direction, first to hind legs and then upper limbs, accompanied by intense weight loss. The lesions are mainly located in the spinal cord and are dominated by CD4$^+$ T cells and macrophages (Stromnes and Goverman, 2006a). The disease may spontaneously resolve (acute monophasic) or continue into a chronic paralysis (chronic). Alternatively, EAE can obtain a fluctuating course, with disease episodes followed by partial recovery (relapsing-remitting) (Wekerle, 2008). In contrast, untypical EAE phenotype is characterized by ataxia, spasticity, inability to walk straight and rolling in an axial-rotary manner (Stromnes and Goverman, 2006a). The disease symptoms, severity and course depend on the mouse model.

EAE is the best available animal model to study MS, as individual variants of EAE reflect different aspects of the human disease (Krishnamoorthy and Wekerle, 2009). However, despite the obvious relevance of EAE to MS, there are several key differences between the human disease and its animal model. Firstly, while MS is a spontaneously occurring disease and the antigen(s) is not yet known, in EAE the experimental mice are immunized with specific myelin proteins or peptides. Even in recently developed spontaneous EAE models, the T cells bear myelin antigen-specific T cell receptor (TCR). Secondly, in most of EAE protocols, strong immune adjuvants are used to induce the disease, while under physiological conditions such intense immune system activation is unlikely to occur (Gold et al., 2006). Moreover, for practical reasons and for experimental reproducibility, the animals used in EAE studies are highly inbred, genetically homogeneous and housed in pathogen-free, controlled environment, thus not reflecting the genetic heterogeneity of human populations and the environmental factors that contribute to the MS susceptibility (Handel et al., 2011). In addition, while EAE is a CD4$^+$ T cell-mediated disease, MS lesions are dominated by CD8$^+$ T cells and macrophages, with CD4$^+$ T cells being infrequent (Sriram and Steiner,

2005). Lastly, some therapeutic approaches that have been successful in EAE, such as tumor necrosis factor (TNF) blocking, have failed dramatically in MS clinical trials (Probert and Akassoglou, 2001; Wekerle, 2008). In total, EAE is a useful tool for studying the basic mechanisms of MS such as BBB disruption, lymphocyte trafficking, CNS inflammation and demyelination, as well as for validating possible therapeutic compounds. Indeed, the knowledge obtained by studying different EAE models has fundamentally shaped our understanding of not only MS mechanism, but also aspects of self-tolerance and autoimmune responses in general. However, the differences with MS should be always considered when attempting to extrapolate the results from EAE studies to the human disease.

1.2.1 Induced EAE Murine Models

The most common way of EAE induction is by active immunization (actively induced EAE – aEAE), with subcutaneous injection of myelin proteins or peptides, emulsified in complete Freund's adjuvant (CFA). CFA consists of heat-inactivated *Mycobacterium tuberculosis*, which enhances the innate immune response, in mineral oil, allowing gradual but constant release of the antigen. Additionally, pertussis toxin is injected intraperitoneally on day 0 and day 2 post immunization, which is considered to facilitate the BBB opening and subsequent immune cell infiltration into the CNS (Stromnes and Goverman, 2006a). The type of the disease course (acute, chronic, relapsing-remitting) and pathology depend on the mouse strain and the antigen used for immunization (see Table 1.1). Myelin basic protein (MBP), myelin oligondendrocyte glycoprotein (MOG) and proteolipid protein (PLP), which are all myelin components, and peptides derived from them are the most frequently used antigens. Active immunization of C57BL/6 mice with MOG_{35-55} is the main model in the present study.

Mouse strain	MHC	Peptide	Peptide sequence	Clinical course
B10.PL	$H-2^u$	MBP Ac1-9	Ac-ASQKRPSQR	Relapsing-remitting
C57BL/6	$H-2^b$	MOG_{35-55}	MEVGWYRSPFSRVVHLYRNGK	Monophasic
SJL/J	$H-2^s$	$PLP_{139-151}$	HCLGKWLGHPDKF	Relapsing-remitting
		$PLP_{178-191}$	NTWTTCQSIAFPSK	Relapsing-remitting
		MBP_{84-104}	VHFFKNIVTPRTPPPSQGKGR	Relapsing-remitting
		MOG_{92-106}	DEGGYTCFFRDHSYQ	Relapsing-remitting

Table 1.1 Commonly used actively induced murine EAE models [adapted from (Krishnamoorthy and Wekerle, 2009; Stromnes and Goverman, 2006a)]

Alternatively, EAE can be induced in recipient naïve animals by adoptive transfer of encephalitogenic $CD4^+$ T cells, isolated from actively immunized donor mice and stimulated *ex vivo* (passively transferred EAE – pEAE). This EAE induction technique has the advantage that, firstly, the autoaggressive T cells can be labeled prior to the adoptive transfer, in order to distinguish them from the host cells and to monitor

their location and activity. Moreover, pEAE offers the possibility of *ex vivo* manipulation (e.g. polarization) of the cells to be transferred, allowing the investigation of the pathogenicity of different T cell phenotypes (Stromnes and Goverman, 2006b). Finally, pEAE develops faster and permits the study of the effector phase independently from the initiation phase of the disease.

1.2.2 Spontaneous EAE Murine Models

The CFA inoculation and the bulk transfer of *ex vivo* stimulated T cells are important drawbacks of the induced EAE models, as disease triggering factors cannot be examined. The generation of myelin-specific TCR transgenic mice led to the development of spontaneous EAE mouse models, which circumvent the artificial disease initiation of the induced models. The first mice with T cells expressing transgenic TCR that recognize myelin antigens were MBP-specific TCR transgenic mice, which develop spontaneous EAE only when housed in non-sterile facility (Goverman et al., 1993) or crossed to immunodeficient background (Lafaille et al., 1994). In contrast, TCR transgenic mice for the $PLP_{139-151}$/I-A^s epitope in SJL background develop spontaneous CNS autoimmunity in high incidence in specific pathogen free (SPF) conditions, without the need of RAG deficiency (Waldner et al., 2000).

Our group has described two distinct spontaneous EAE models: First, the "opticospinal EAE" (OSE) mouse, a double transgenic mouse in C57BL/6 background which occurs by crossing the 2D2 (TCR^{MOG}) mouse, where T cells bear TCR specific for MOG_{35-55} (Bettelli et al., 2003), with the Th (IgH^{MOG}) mouse, where B cells express B cell receptor (BCR) that carries a rearranged heavy chain of a MOG-specific antibody (Litzenburger et al., 1998). In the OSE model, MOG-specific T cells and B cells cooperate to induce spontaneous chronic opticospinal EAE at the age of four to eight weeks, with 50% incidence (Bettelli et al., 2006a; Krishnamoorthy et al., 2006). The second spontaneous EAE model is the "relapsing-remitting" (RR) mouse, a single transgenic mouse in SJL/J background, where T cells carry TCR that recognizes MOG_{92-106}. Interestingly, in this model the transgenic T cells recruit MOG-specific B cells from the endogenous repertoire, leading to relapsing-remitting EAE at the age of two months, with 80% incidence (Pollinger et al., 2009). These spontaneous EAE models, where T and B cell cooperation is inducing the disease without experimental manipulation, resemble closely two distinct types of MS (opticospinal and RR MS), providing a powerful tool to elucidate the factors determining the outbreak and course of the human disease.

1.3 Immune Cell Infiltration into the CNS

1.3.1 Disease Mechanism

In EAE, autoaggressive CD4^{+} T cells are primed in the periphery by professional antigen presenting cells (APC) of the dendritic cell lineage (Gold et al., 2006). Once activated, the CD4^{+} T cells are able to cross the BBB, which consists of highly specialized endothelial cells of low pinocytotic activity and firmly connected with tight junctions, supported by an underlying endothelial basement membrane, that contains pericytes, and the *glia limitans* (Engelhardt and Coisne, 2011). First, T cells are captured and firmly adhered to the endothelial cells by α4-integrin/VCAM-1 interactions. Their subsequent crawling on the endothelium preferentially against the blood flow, until they find a site for diapedesis, is mediated by αLβ2 (LFA-1)/ICAM-1 interaction (Engelhardt and Coisne, 2011). T cells extravasate only at the level of post-capillary venules (Engelhardt and Ransohoff, 2005) to reach the perivascular space, where they get re-activated by encountering APC, that can be either infiltrating dendritic cells (DC) and perivascular macrophages or local microglia and astrocytes (Dittel, 2008). Despite the low numbers of DC in the CNS, vessel-associated CD11c^{+} DC have been shown to be sufficient to induce EAE (Greter et al., 2005), while peripheral derived CNS myeloid CD11b^{+} DC can polarize effector T cells to Th17 cells (Bailey et al., 2007). During EAE, perivascular macrophages upregulate MHC II and costimulatory molecule expression, turning into potent APC (Fabriek et al., 2005). Similarly, while microglial cells have poor antigen presenting capacity in resting state, they upregulate most immunophenotypical markers during CNS inflammation (Galea et al., 2007). The activated microglia resembles macrophages both phenotypically and morphologically, thus it is difficult to be differentiated from the infiltrating macrophages during EAE (Dittel, 2008). In contrast, MHC II[+] astrocytes are detected rarely and only under strong inflammatory conditions, but they influence the inflammatory response by producing cytokines and chemokines (Aloisi, 2001; Goverman, 2009). In total, the T cell reactivation in the perivascular space not only determines the amount of subsequent parenchymal inflammation, but also results in the secretion of a variety of cytokines and chemokines, that play a crucial role in the local milieu modification and the outcome of the immune response (Gold et al., 2006; Goverman, 2009).

The re-activated CD4^{+} T cells secrete proinflammatory cytokines, including interferon γ (IFNγ), interleukin 17 (IL-17), granulocyte-macrophage colony-stimulating factor (GM-CSF), or tumor necrosis factor α (TNFα), that recruit further immune cells and activate macrophages and microglia. These cells mediate oligodendrocyte damage or direct neuronal toxicity, by producing TNFα and IL-1β, as well as effector molecules such as nitric oxide (NO) and reactive oxygen species (ROS) (Herz et al., 2010).

Autoantibodies against myelin proteins can also contribute to myelin sheath destruction. Eventually, the CNS inflammation leads to demyelination and axonal degeneration, resulting in the clinical symptoms.
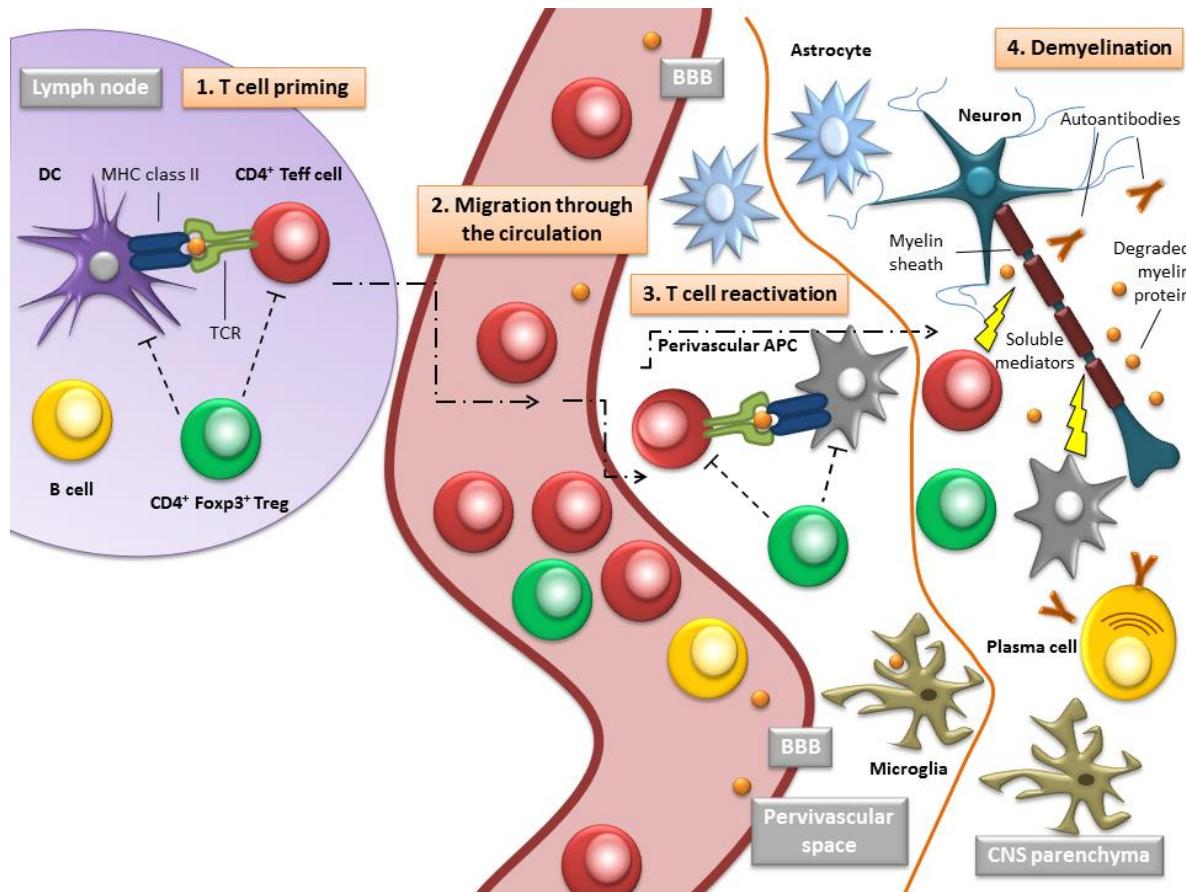
Figure 1.1 Immunopathogenesis of MS and EAE [adapted from (Domingues, 2009)]

1.3.2 T and B lymphocytes in EAE

T cell subsets in EAE

Both in MS and EAE, T cells play the most important role in the disease development. Although susceptibility to MS is strongly associated with HLA class II genes, CD8^{+} T cells are more abundant than CD4^{+} T cell and show clonal expansion in human MS lesions, while CD8^{+} memory T cell are enriched in the CSF and blood of MS patients, compared to CD4^{+} memory T cells (Friese and Fugger, 2005; Goverman, 2009). However, only few reports have shown that CD8^{+} T cell can induce EAE (Huseby et al., 2001; Sun et al., 2001). In contrast, in the vast majority of EAE models, the disease is mediated by CD4^{+} T cells, while CD8^{+} T cells are found in very low frequency in the CNS infiltrates and their role is poorly understood.

Distinct subsets of CD4$^+$ T cells have been described, defined by their cytokine profile and expression of particular transcription factors. First, the T helper 1 and 2 (Th1 and Th2) paradigm was introduced (Mosmann et al., 1986). More recently, two newly characterized subsets, with key roles in autoimmunity, were added: Th17 cells (Park et al., 2005) and regulatory T cells (Treg) (Fontenot et al., 2003; Hori et al., 2003). In the last few years, additional T cell subtypes were suggested based on their signature cytokines, such as Th9 (Veldhoen et al., 2008) and Th22 (Eyerich et al., 2009), although it is still not clear whether they represent stable cell lineages. Th1 and Th17 are now considered as the effector T cells involved in EAE pathology, while Treg are crucial for the maintenance of immunosuppression. The importance of Treg in EAE is the main topic of this thesis and will be addressed in detail in further sections.

Th1 cells need IL-12 for their differentiation, express the transcription factor T-bet and are characterized by the secretion of mainly IFNγ, but also IL-2 and TNFα. They enhance pro-inflammatory cell-mediated immunity and induce delayed-type hypersensitivity (DTH) responses, as well as production of opsonizing isotypes of IgG by B cells (Qureshi et al., 2011). Th1 cells were until some years ago the sole mediators in EAE, as CNS infiltrating T cells produce high levels of IFNγ and adoptive transfer of myelin-specific Th1 cells to naïve animals induces disease (Goverman, 2009). In addition, T-bet deficient mice are resistant to EAE induction (Bettelli et al., 2004) and neutralizing IL-12 p40 subunit prevents the disease (Leonard et al., 1995).

However, paradoxically to this concept, *Ifng*$^{-/-}$ (Ferber et al., 1996) and *Ifngr*$^{-/-}$ (Willenborg et al., 1996), as well as *Il12 p35*$^{-/-}$ (Becher et al., 2002) mice were found to develop more severe EAE. In contrast, *Il23 p19*$^{-/-}$ mice, which are deficient for IL-23 but not IL-12, are completely resistant to EAE (Cua et al., 2003). Since IL-12 and IL-23 share p40 subunit, the inhibition of p40 results in suppression of both interleukins. However, *Il12 p35*$^{-/-}$ or *Il23 p19*$^{-/-}$ mice lack IL-12 or IL-23, respectively. Therefore, these observations suggest the existence of unknown effector T cell subset. IL-23 promotes the secretion of IL-17 in CD4$^+$ T cells, together with transforming growth factor-β (TGF-β) and IL-6 (Veldhoen et al., 2006). Since they were not fitting to the Th1/Th2 paradigm, these IL-17-producing CD4$^+$ T cells were termed Th17 cells, with RORγt as their key transcription factor (Ivanov et al., 2006). Th17 cells are present in the CNS infiltrates and adoptive transfer of Th17 cells induces strong disease, suggesting a pathogenic role for Th17 cells in EAE (Goverman, 2009; Petermann and Korn, 2011). IL-17 is also highly upregulated in MS lesions (Petermann and Korn, 2011; Zhou et al., 2009a). However, IL-17 deficient mice are still susceptible to EAE presumably due to the contribution of other factors secreted by Th17 cells. To

complicate things further, T-bet, a transcription factor associated with Th1 cells, is also necessary for the generation of pathogenic Th17 cells (Yang et al., 2009)

B cells in EAE

B cells play a prominent role in MS, as clearly suggested by the positive effects of B cell depletion using anti-CD20 antibody (Rituximab) (Liang et al., 2008) and the presence of oligoclonal bands in the CSF in MS patients. B cells can produce proinflammatory cytokines and chemokines, function as APC and secrete autoantibodies that mediate demyelination (Huang et al., 2004). However, despite the profound contribution of B cells in MS pathogenesis, their role in the majority of EAE models is debated. On the one hand, aEAE induction by immunization with myelin peptides is antibody-independent and on the other hand, transfer of encephalitogenic T cells in recipient animals is sufficient to induce pEAE without any antibody involvement. Intriguingly though, despite B cell-deficient mice develop EAE upon MOG_{35-55} immunization, they are resistant to the disease when immunized with recombinant MOG (Yu et al., 2009), while injection of MOG-primed serum restores their susceptibility (Solomon et al., 2011). Additionally, while the aforementioned MOG-specific BCR knock-in mice (Th) mice do not develop spontaneous EAE, despite their high titers of MOG-specific IgG antibodies, they show accelerated and exacerbated disease upon immunization (Litzenburger et al., 1998). In contrast, other studies have revealed that B cells are essential for mediating recovery from EAE by producing IL-10 (Lyons et al., 2008; Wolf et al., 1996; Zheng et al., 2009) and regulating Treg via B-7 (Chaudhry et al., 2009). Therefore, different subtypes of B cells appear to have distinct effects in induced EAE (Matsushita et al., 2008).

Interestingly, B cells are pivotal in two spontaneous EAE models described in previous section, the OSE and RR mouse. Firstly, while MOG-specific TCR transgenic mice (2D2) develop spontaneous EAE in very low incidence (5%) (Bettelli et al., 2003), once crossed to Th mice, the double transgenic OSE mice display spontaneous EAE incidence around 50%, suggesting a cooperation between T cells and B cells that are specific for the same antigen (Bettelli et al., 2006a; Krishnamoorthy et al., 2006). Secondly, in the RR model, the MOG-specific T cells recruit MOG-specific B cells from the endogenous repertoire and guide them to produce pathogenic autoantibodies that contribute to the development of spontaneous relapsing-remitting EAE in high incidence, characterized by demyelination. Moreover, anti-CD20 antibody-mediated ablation of B cells in neonatal RR mice suppressed the disease development (Pollinger et al., 2009). Therefore, B cells play fundamental role in these spontaneous EAE models.

1.4 Regulatory T cells

CD4^{+} Forkhead box P3^{+} (Foxp3^{+}) regulatory T cells (Treg) are critical for maintaining immune homeostasis and self-tolerance, as well as modulating immune responses in the contexts of infection, autoimmunity, cancer and transplantation (Campbell and Koch, 2011). Already in the 80's, several studies reported that T cells have the capacity to prevent or suppress deleterious immune reactions (Hall, 1985; Kong et al., 1989; Sakaguchi et al., 1982). Nevertheless, the existence of a suppressor T cell subtype was underestimated, because of poor characterization of these cells and lack of specific markers. This changed in 1995, when a population of IL-2 receptor α-chain (CD25)-expressing CD4^{+} T cells was described to preserve self-tolerance and regulate immune responses against self and non-self-antigens (Sakaguchi et al., 1995). In addition, CD4^{+}CD25^{+} T cells were further shown to suppress T cell proliferation *in vitro* (Thornton and Shevach, 1998). However, as activated CD4^{+} T cells also upregulate CD25 expression, CD4^{+}CD25^{+} regulatory T cells could not be identified as a distinct T cell subset. Therefore, the next major breakthrough in understanding Treg cell biology was the finding that patients with immune dysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome, a rare fatal autoimmune disorder affecting a variety of organs, as well as mice with scurfy phenotype, carry mutations in the X chromosome-encoded *Foxp3* gene (Bennett et al., 2001; Brunkow et al., 2001; Chatila et al., 2000). Soon after, Foxp3 was identified as the master transcription factor of Treg, essential for their differentiation and suppressor function, thus establishing Treg as a separate T cell lineage (Fontenot et al., 2003; Gavin et al., 2007; Hori et al., 2003; Khattri et al., 2003).

The primary function of Treg has been originally defined as prevention of autoimmunity by maintaining immunologic self-tolerance (Sakaguchi et al., 1995). Indeed, Treg depletion in neonatal or adult mice was found to result in fatal multi-organ autoimmunity, underscoring the importance of this T cell subset for preserving peripheral tolerance throughout the lifespan of an individual (Kim et al., 2007; Lahl et al., 2007). Furthermore, the ability of Treg to prevent or suppress autoimmune responses has been demonstrated in several experimental disease models, such as murine systemic lupus erythematosus (SLE) models (Scalapino et al., 2006; Wolf et al., 2005), collagen-induced arthritis (Morgan et al., 2003), autoimmune diabetes (Sgouroudis and Piccirillo, 2009) and colitis (Makita et al., 2007). In parallel, decreased numbers or impaired suppressive capacity of Treg have been reported in patients with systemic or organ-specific autoimmune diseases, including SLE (Kuhn et al., 2009), rheumatoid arthritis (Oh et al., 2010), type-1 diabetes (Zhang et al., 2012) and inflammatory bowel disease (Boden and Snapper, 2008). The studies on Treg role in MS and EAE will be discussed in detail in a separate chapter (see Treg in CNS Autoimmunity). Additionally, over the last years, further beneficial functions have been

attributed to Treg, such as modulating allergic responses (Braga et al., 2011), mediating tolerance against alloantigens after transplantation (Wood, 2011) and preventing fetal rejection during pregnancy (Leber et al., 2010). However, Treg function can be a double-edged sword during infections, as on the one hand they favor pathogen survival, but on the other hand they restrain immunopathologic reactions that can be deleterious to the host (Sanchez and Yang, 2011). Finally, Treg are considered key players in tumor immunity, as they infiltrate tumors and limit antitumor immune responses, promoting progression of cancer (Nishikawa and Sakaguchi, 2010).

The instrumental role of Treg in the control of immune responses has placed this T cell subset in the center of scientific interest, as clinical implementation of their regulatory activity is challenging. Firstly, Treg number or activity could be modified in patients, depending on the clinical condition, by *in vivo* administration of specific agents. Alternatively, Treg could be isolated from patients, expanded *ex vivo* and reinfused, offering a personalized treatment (Riley et al., 2009). However, apart from technical and ethical issues, such approaches require in depth knowledge of Treg differentiation and stability, as well as *in vivo* suppression mechanisms. These issues will be discussed in the following sections.

In conclusion, it is of vital importance to improve our understanding of Treg biology so that it can be potentially translated into therapeutic applications.

1.4.1 Treg Differentiation

Treg can be generated *in vivo* either in the thymus (natural Treg – nTreg) or differentiate from naïve CD4$^+$ T cells in periphery (induced Treg – iTreg). A commonly used marker to distinguish nTreg and iTreg is the transcription factor Helios that is expressed only in nTreg (Thornton et al., 2010), although the reliability of this marker has been questioned recently (Verhagen and Wraith, 2010). The majority of the circulating Treg are of thymic origin (Geiger and Tauro, 2012; Josefowicz et al., 2012a). Nevertheless, nTreg and iTreg display distinct specificity, stability and genetic profile and serve different functions in immune homeostasis and responses (Geiger and Tauro, 2012).

Treg Differentiation in the Thymus

Foxp3 induction and Treg differentiation is instructed by TCR signaling in the thymus (Josefowicz et al., 2012a). Sequence analysis studies that compared Treg and non-Treg cell TCR repertoires revealed that Treg express TCRα of broad variety that overlap only partially with non Treg TCRα sequences (Hsieh et al., 2004; Pacholczyk et al., 2006; Pacholczyk and Kern, 2008; Wong et al., 2007). Interestingly, transfer of Teff transfected with Treg TCR into lymphopenic mice resulted in rapid *in vivo* expansion of Teff and

induction of wasting disease, indicating increased self-reactivity of Treg TCRs. However, these Treg TCR-expressing Teff mounted only weak responses *in vitro* against syngeneic APC, contrary to robust responses against the transgenic TCR-recognized foreign ligand, suggesting that the affinity range of non-Treg TCR that recognize a foreign antigen is higher than the range of affinities of Treg TCR for self antigens (Hsieh et al., 2004). Moreover, reduced MHC II expression in medullary thymic epithelial cells (mTEC) led not only to partially impaired negative selection, but also increased frequency of Treg (Hinterberger et al., 2010).

In addition, the increased negative selection in TGF-β receptor (TGF-βR) II-deficient mice resulted in reduced Treg number (Ouyang et al., 2010). Furthermore, in Foxp3-deficient mice, activated T cells preferentially used TCR that are normally expressed in Treg in Foxp3-sufficient mice, indicating that on the one hand these self-reactive TCR contribute to the pathology seen in these mice and on the other hand that these T cells can escape from negative selection despite not expressing Foxp3 (Hsieh et al., 2006). Similarly, in mice expressing Foxp3 reporter null allele (Gavin et al., 2007) or a truncated Foxp3 protein (Lin et al., 2007), CD4$^+$ T cells expressing these nonfunctional alleles as well as self-reactive TCRs, are not deleted but become activated and induce autoimmune disease similar to Foxp3-deficient mice. In conclusion, Treg selection is instructed by TCR signals of intermediate strength, in the range between the weaker TCR affinities that mediate positive selection and the stronger signals that lead to negative selection of CD4$^+$ single positive (SP) T cells in the thymus, while Foxp3 expression is not required for escaping from negative selection (Josefowicz et al., 2012a). Importantly, the broad Treg TCR repertoire has been shown to be a result of intense intraclonal precursor competition that restricts the differentiation of Treg cells expressing TCR of identical specificity (Bautista et al., 2009; Leung et al., 2009).

The fact that the same TCR can be expressed both by Treg and non-Treg cells suggests that TCR signaling alone is not sufficient to induce Foxp3 expression and Treg differentiation. Indeed, essential additional signals for Treg fate determination include mainly IL-2, as IL-2 or IL-2Rα-deficient mice have a significant decrease in Foxp3$^+$ T cells in the thymus. In contrast, loss of IL-7 or IL-15, that act also through the common gamma-chain (γc) cytokine receptors, does not affect Treg generation. Nonetheless, combined ablation of IL-2, IL-7 and IL-5 or deficiency in γc cytokine receptor prevents completely Treg differentiation both in thymus and periphery (Burchill et al., 2007a; Fontenot et al., 2005; Josefowicz et al., 2012a). Therefore, as CD25$^+$Foxp3$^-$ thymocytes were identified as precursors of Treg in the neonatal thymus, a two-step model of thymic Treg generation was suggested (Burchill et al., 2008; Lio and Hsieh, 2008), according to which strong TCR signals lead to CD25 upregulation in Treg precursors, increasing

responsiveness to IL-2 signaling that subsequently mediates Foxp3 induction. Interestingly, STAT5, a transcription factor that is activated downstream of IL-2 and other γc cytokine receptors, has been shown to modulate Foxp3 expression, by binding to the Foxp3 promoter and the *Foxp3*-CNS2 element. Indeed, *Stat5* ablation in DP thymocytes leads to strong reduction in Foxp3^{+} thymocytes, whereas expression of constitutively active STAT5 increases Treg numbers even in the absence of IL-2 (Burchill et al., 2003; Burchill et al., 2007b; Yao et al., 2007). In contrast, while TGF-βR signaling plays a pivotal role in peripheral Treg differentiation (see next section), within the thymus it does not induce Foxp3 expression but only promotes Treg precursor survival (Josefowicz et al., 2012a).
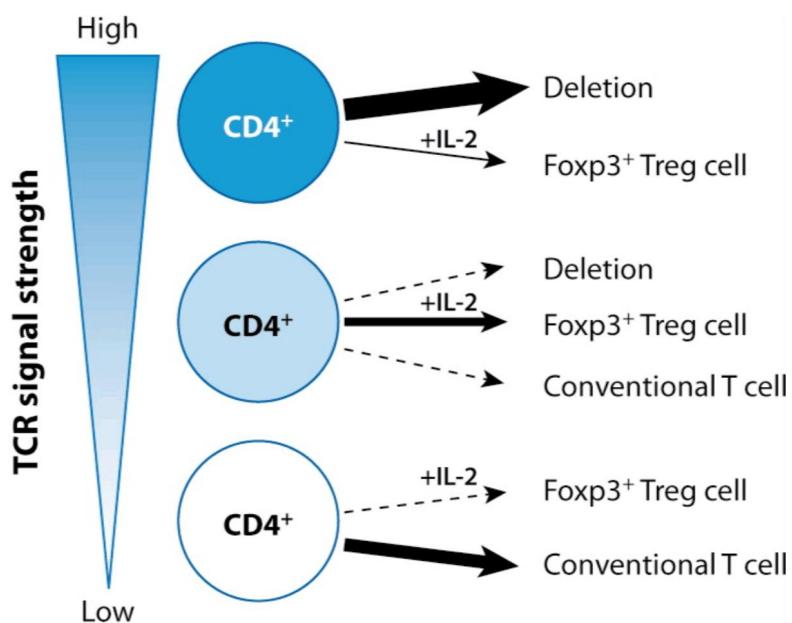
Figure 1.2 TCR signal strength in thymus instructs CD4^{+} T cell fate and Treg differentiation [from (Josefowicz et al., 2012a)]

Besides TCR and cytokine stimulation, CD28 costimulatory signaling is critical for thymic Treg generation, as CD28-, as well as CD80-CD86-deficient mice display significant decrease in Treg frequencies, while the lck-binding domain of the CD28 cytoplasmic tail is essential for Foxp3 induction (Salomon et al., 2000; Tai et al., 2005). Furthermore, TCR/CD28-downstream transcription factors, including NFAT, AP-1 and NF-κB, have been implicated in Treg differentiation. In fact, NFAT and AP-1 can bind to the Foxp3 promoter (Mantel et al., 2006), while targeted ablation or mutations of genes involved in the NF-κB signaling pathway, such as PKCθ, CARMA1, Bcl10, IκB kinase (IKK) 2 and p-105 encoding gene, result in impaired Treg generation. Additionally, several studies have underscored the crucial role of c-Rel, a NF-κB family member that can bind to the intronic conserved noncoding element Foxp3-CNS3, increasing the probability of Foxp3 induction in Treg precursor cells (Isomura et al., 2009; Ruan et al., 2009; Visekruna

et al., 2010; Zheng et al., 2010). Intriguingly, the role of cytotoxic T lymphocyte associated antigen-4 (CTLA-4), a surface molecule exclusively and constitutively expressed by Treg, in Treg thymic development is heavily debated, with contradictory evidence from several studies (Verhagen et al., 2009; Verhagen et al., 2013; Zheng et al., 2006). Finally, further factors have been reported to promote Foxp3 expression in thymocytes, including loss of maintenance DNA methyltransferase I (Dnnmt1) activity, deficiency in mTOR or sphingosine-1 phosphate receptor type 1 (S1P1), and reduction of PI3K signaling (Josefowicz et al., 2012a).

Peripheral Treg Differentiation

In contrast to nTreg cells that are generated in the highly controlled microenvironment of the thymus, promoted by high affinity interactions with self-peptide-MHC II complexes, iTreg differentiation takes place under various conditions and most likely in response to non-self-antigens (Josefowicz et al., 2012a). iTreg generation can occur in gut associated lymphoid tissue (GALT), spleen, inflamed and transplanted tissues, though many aspects of the *in vivo* processes remain to be elucidated. Nevertheless, *in vitro* and *in vivo* studies have established the basic requirements for Treg differentiation from naïve T cells, that include TCR stimulation along with the cytokines TGF-β and IL-2 (Curotto de Lafaille and Lafaille, 2009).

Extrathymic differentiation of Treg cells has been demonstrated to depend on TCR ligand density and affinity, as low dose of strong TCR ligand leads to most efficient Foxp3 induction *in vivo* (Gottschalk et al., 2010). Although iTreg differentiate from naïve CD4^{+} T cells, they use distinct TCRs compared to non-Treg CD4^{+} T cells. In fact, when Foxp3^{-} CD4^{+} T cells are transferred in lymphopenic mice, the TCR repertoire of Foxp3^{+} T cells that occur has been shown to be different from the TCR repertoire of the remained Foxp3^{-} cells, suggesting that TCR of certain specificities promote iTreg differentiation (Lathrop et al., 2008). Moreover, colonic iTreg display TCRs that are reactive to local antigens, derived mainly from commensal microbiota, and are different from TCRs from Treg in other locations (Lathrop et al., 2011). Therefore, iTreg differentiation is supported by TCRs specific for antigens to which an organism is chronically exposed under physiological conditions, such as commensal microflora. Importantly, the unique iTreg TCR repertoire implies that iTreg have different functions from nTreg, that can be determined by their antigen specificity.

In addition to TCR stimulation, cytokine signaling plays a critical role in peripheral Treg generation. TGF-β has been consistently shown to be indispensable for *in vitro* and *in vivo* iTreg differentiation. Addition of TGF-β to cultures of naïve CD4^{+} T cells along with TCR stimulus, induced Foxp3 expression and led to acquisition of suppressive function both *in vitro* and *in vivo* (Chen et al., 2003). Moreover, blocking TGF-β

in vivo has been reported to impair the differentiation of antigen-specific Treg (Mucida et al., 2005) and inhibit the protection against colitis (Oida et al., 2003). TGF-β signaling can induce iTreg differentiation and development through different mechanisms, such as binding of Smad3 to *Foxp3*-CNS1 together with NFAT and opposition of Dnnmt1 recruitment (Josefowicz et al., 2012a). Furthermore, IL-2 is also essential for TGF-β-mediated induction of Foxp3 in CD4$^+$ T cells *in vitro* (Davidson et al., 2007; Horwitz et al., 2008). IL-2 not only acts through STAT5 to activate the *Foxp3* locus while promoting cell survival, but also inhibits polarization to Th17 phenotype (Laurence et al., 2007).

Further co-stimulation requirements have been described for iTreg generation. Interestingly, CTLA-4, independently of its role for Treg development in the thymus, has been shown to be essential for *in vitro* iTreg differentiation (Zheng et al., 2006). Moreover, retinoid acid (RA), that can be also produced by CD103$^+$ DCs, has been demonstrated to promote iTreg differentiation and growth, while inducing a gut-homing phenotype (Benson et al., 2007). In contrast, CD28 cross-linking has been reported to inhibit iTreg development (Kim and Rudensky, 2006).

As extrathymic Treg differentiation has specific prerequisites, iTreg generation is limited to particular tissues and microenvironments (Josefowicz et al., 2012a). GALT serves as the main site of iTreg induction, as it provides constitutively antigens derived from commensal microbiota and food. In fact, germ-free mice have been shown to have reduced numbers of Treg in their colon, while colonization with specific bacteria led to a significant increase in the Treg number (Atarashi et al., 2011). Additionally, the gut and mesenteric LN harbor large numbers of CD103$^+$ DCs, which mediate Treg conversion by producing TGF-β and RA (Coombes et al., 2007; Sun et al., 2007). The profound presence of iTreg in the GALT raises questions regarding their possible function in this niche. Interestingly, iTreg cells have been recently demonstrated to restrain allergic-type inflammation at mucosal interfaces rather than Th1/Th17 autoimmune responses, as iTreg-deficient mice develop spontaneously Th2-type pathologies in lungs and gastrointestinal tract (Josefowicz et al., 2012b). Besides the GALT, Treg differentiation occurs also in the spleen and has been reported to be mediated by CD8$^+$DEC205$^+$ DCs that produce TGF-β (Yamazaki et al., 2008).

Foxp3 expression and stability

Foxp3 has an instrumental role for Treg identity and function. The contribution of Foxp3 in the transcriptional and functional characteristics of Treg was investigated in aforementioned studies using mice harboring a Foxp3 null allele (Gavin et al., 2007) or a truncated version of the Foxp3 protein lacking the DNA-binding domain (Lin et al., 2007), which showed that Foxp3 is indispensable for the suppressive

capacity, proliferative activity and metabolic fitness of Treg cells. Moreover, Foxp3 represses production of proinflammatory cytokines, such as IL-2, TNFα, IFNγ, IL-4 and IL-17, the latter one by interacting directly with RORγt, the key transcription factor of Th17 cells, and modulating its transcriptional activity (Zhou et al., 2008). Furthermore, as mentioned earlier, T cells bearing TCR normally expressed by Treg are inducing aggressive autoimmune syndrome in Foxp3-deficient mice (Hsieh et al., 2006). Additionally, ablation of a conditional *Foxp3* allele in mature Treg led to production of Th1 cytokines and loss of suppressive capacity (Williams and Rudensky, 2007). In parallel, decreased Foxp3 expression in Treg resulted in acquisition of Th2 phenotype by these cells and caused severe immune disease (Wan and Flavell, 2007). In total, these findings clearly suggest that continuous Foxp3 expression is absolutely necessary not only for the maintenance of Treg suppressor function, but also for preventing conversion of Treg to effector T cell types. Thus, given that Treg-expressed TCR display increased affinity for self-peptides, the latter function of Foxp3 is of critical importance for maintaining immune homeostasis and preventing potential autoimmune responses.

The stability of Foxp3 expression and, therefore, of Treg phenotype has been actively debated, creating a complicated picture. Several *in vitro* studies have reported loss of Foxp3 expression in Treg following exposure to IL-6 (Xu et al., 2007; Yang et al., 2008), OX40 (So and Croft, 2007; Vu et al., 2007) or dectin-1 activated DC (Osorio et al., 2008). Moreover, another report suggested that the instability of Foxp3 expression in Treg *in vivo* results in the generation of an ex-Treg population that has memory phenotype and the potential to induce autoimmune response (Zhou et al., 2009b). However, following transfer of CD4$^+$Foxp3$^+$ T cells to lymphopenic mice, the majority of Treg was demonstrated to display stability in Foxp3 expression, while only a minor fraction of the CD25$^-$ subpopulation lost it (Komatsu et al., 2009). Importantly, by using noninvasive inducible labeling of Foxp3-expressing cells and tracking their fate *in vivo*, Treg were shown to exhibit notably stable expression of Foxp3 under physiologic, lymphopenic and inflammatory conditions (Rubtsov et al., 2010).

The mechanisms underlying the stable Foxp3 expression in mature Treg are of great interest. CpG dinucleotide methylation at the *Foxp3* locus, at the promoter and at Foxp3-CNS2, has been repeatedly demonstrated to regulate Foxp3 expression. Indeed, demethylation of CpG motifs at the Foxp3 locus has been correlated with stable Foxp3 expression in human and mouse nTreg, while these elements remain methylated in *in vitro* generated iTreg with unstable expression of Foxp3 (Baron et al., 2007; Floess et al., 2007; Polansky et al., 2008). In parallel, runt-related transcription factor 1 (Runx1) and core-binding factor β (Cbfβ) have been reported to be essential for maintenance of high levels of Foxp3 expression (Bruno et al., 2009; Kitoh et al., 2009; Klunker et al., 2009). Importantly, CNS2 has been shown to bind

Foxp3/Runx1/Cbfβ, CREB/ATF, NF-κB and Ets-1 protein complexes in a CNS2 CpG DNA demethylation-depended manner (Polansky et al., 2010; Zheng et al., 2010). Therefore, Foxp3 protein complexes bind to the demethylated CNS2 to maintain stable Foxp3 expression, forming an autoregulatory loop that defines Treg lineage stability (Josefowicz et al., 2012a).

1.4.2 Treg Suppression Mechanisms

Despite the rapidly accumulating knowledge on Treg development and regulation of the immune homeostasis and responses, our understanding on the actual suppression mechanisms used by Treg remains rather limited and is based mostly on *in vitro* studies. Treg are considered capable of suppressing CD4$^+$ Teff, CD8$^+$ T cells, B cells, NK cells, monocytes and DC in both cell-to-cell contact-dependent and independent ways (Gasteiger et al., 2013; Lourenco and La Cava, 2011; Shevach, 2009; Sitrin et al., 2013). However, the major target cells of Treg are Teff and DC.

Teff as Targets of Treg-mediated Suppression

Treg-secreted cytokines, including IL-10, IL-35 and TGF-β, have been implicated in Treg-mediated suppression. IL-10 production by Treg has been shown to be required for limiting immune responses at environmental surfaces, such as colon and lungs, although not essential for the control of systemic autoimmunity (Rubtsov et al., 2008). Similarly, IL-35-deficient Treg have been demonstrated to display decreased suppressive capacity *in vitro* and are unable to restrain homeostatic proliferation and cure inflammatory bowel disease *in vivo* (Collison et al., 2007). Moreover, Treg-produced TGF-β1 has been reported to be indispensable for inhibiting Th1 differentiation and inflammatory bowel disease in a transfer model (Li et al., 2007).

Cytolytic mechanisms reported to be used by Treg are granzyme A-, granzyme B- and perforin-mediated. Activated human Treg have been reported to express granzyme A upon CD3 and CD46 stimulation and can lyse CD4$^+$ and CD8$^+$ T cells (Grossman et al., 2004). However, murine Treg were found to highly upregulate granzyme B, not granzyme A, expression (Josefowicz et al., 2012a). In fact, Treg have been demonstrated to induce apoptosis of Teff or antigen-presenting B cells *in vitro* via granzyme B (Gondek et al., 2005; Zhao et al., 2006). Importantly, granzyme B$^+$ Treg were shown to be critical *in vivo*, both for skin graft tolerance and for suppressing tumor clearance (Cao et al., 2007; Gondek et al., 2008). There is contradictory evidence on the role of perforin as a Treg suppression mechanism. Although in some of the aforementioned studies Treg have been observed to act in a perforin-independent manner (Gondek et al., 2008; Gondek et al., 2005), others have underscored the importance of perforin in Treg suppression

(Cao et al., 2007; Grossman et al., 2004; Zhao et al., 2006). Moreover, Treg have been reported to induce cell cycle arrest and apoptosis to Teff by galectin-1, a β-galactosidase binding protein that is preferentially expressed in Treg and is upregulated upon TCR activation (Garin et al., 2007). However, stable Treg-Teff interactions have not been detected in explanted lymph nodes (Tang et al., 2006).

Treg-mediated suppression by metabolic disruption of target cells has been also proposed. As already mentioned, Treg, as well as activated Teff, express high levels of IL-2R α chain (CD25). The constitutive high expression of IL-2R by Treg has been suggested to deprive Teff of IL-2 and, therefore, inhibit their proliferation and induce apoptosis (Pandiyan et al., 2007). This mechanism of suppression has been recently shown to be used by Treg to suppress NK cells (Gasteiger et al., 2013; Sitrin et al., 2013). Furthermore, Treg have been reported to suppress Teff by directly transferring the inhibitory second messenger cyclic adenosine monophosphate (cAMP) into Teff via gap junctions (Bopp et al., 2007). Additionally, the ectoenzymes CD39 and CD73, which are highly expressed on Treg, have been repeatedly shown to contribute to suppression of activated Teff, that express the adenosine A2A receptor, by generating pericellular adenosine from extracellular nucleotides that inhibits Teff proliferation (Deaglio et al., 2007; Kobie et al., 2006).
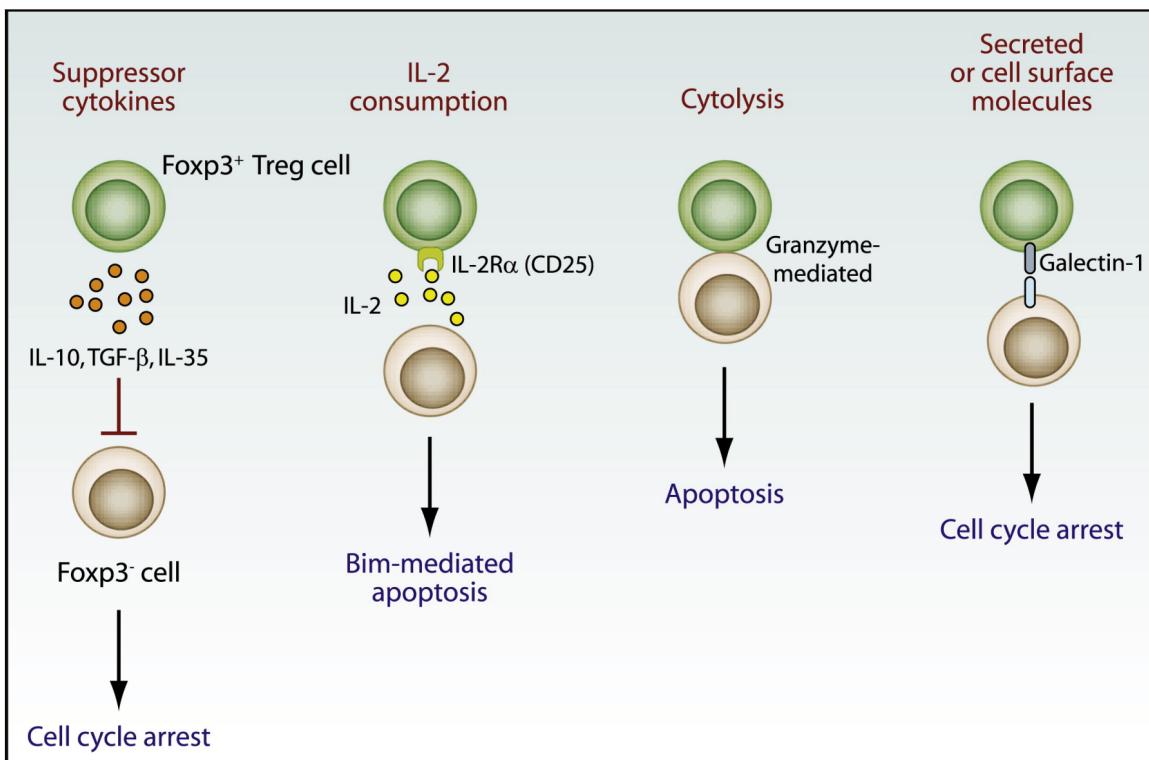

Figure 1.3 Main mechanisms by which Treg can directly suppress Teff [from (Shevach, 2009)]

Interestingly, expression of different Th lineage-specific transcription factors by Treg is required for suppressing the respective Teff subtypes. For example, Treg-specific ablation of STAT3, a key transcription factor Th17 differentiation, impaired Th17 suppression by Treg and resulted in fatal intestinal immunopathology (Chaudhry et al., 2009). Similarly, in Treg, the amount of interferon regulatory factor-4 (IRF4), a transcription factor critical for Th2 induction, is dependent on Foxp3 expression, while ablation of a conditional *Irf4* allele in Treg led to selective dysregulation of Th2 responses (Zheng et al., 2009). Consistent with these findings, Treg upregulation of T-bet, the Th1-specifying transcription factor, in response to IFNγ, promoted expression of the chemokine receptor CXCR3, which enabled Treg to migrate and accumulate at the sites of Th1 responses (Koch et al., 2009). Interestingly, T-bet deficient Treg, despite not showing strongly reduced suppressor capacity, fail to regulate Th1, but not Th2 or Th17 responses. Therefore, these observations suggest expression of Teff lineage-specific transcription factors in Treg cells can tailor their function to suppress distinct Teff responses, by inducing either specialized suppression capacities or acquisition of selective migration properties (Josefowicz et al., 2012a).

DC as Targets of Treg-mediated Suppresion

Treg have been proposed to directly affect APC function through several mechanisms. Firstly, CTLA-4, a Treg-specific surface molecule, has been repeatedly shown to be crucial for Treg-mediated suppression (Friedline et al., 2009; Read et al., 2006; Read et al., 2000; Takahashi et al., 2000). In fact, CTLA-4 has been demonstrated to induce down-regulation of CD80 and CD86 on DC via trans-endocytosis (Qureshi et al., 2011; Wing et al., 2008), in agreement with the notable expansion of DC observed early upon Treg depletion (Kim et al., 2007). Additionally, Treg have been found to form aggregates on DC *in vitro* in a LFA-1-dependent manner and down-modulate CD80/CD86 expression on DC in a LFA-1- and CTLA-4-dependent manner (Onishi et al., 2008). Interestingly, interactions between Treg and immature DC can be promoted *in vitro* by neuropilin-1 (Nrp1), which is expressed on Treg but not on naïve Teff (Sarris et al., 2008). Moreover, Treg have been suggested to inhibit DC maturation and costimulatory capacity by engagement of MHC molecule on immature DC to lymphocyte activation gene-3 (LAG-3) (Liang et al., 2008), a CD4 homolog that binds MHC II molecules with high affinity, which is considered to be essential for Treg suppressive activity (Huang et al., 2004). Furthermore, Treg can also modulate DC function through the surface protein TIGIT, a novel Ig family member expressed at high levels on Treg and activated Teff, which induces IL-10 and TGF-β production by DC (Yu et al., 2009). Importantly, by imaging lymph nodes using two-photon microscopy, stable contacts between Treg and DC in lymph nodes have

been visualized *ex vivo* (Tang et al., 2006), while Treg have been observed to inhibit stable contacts between Teff and DC *in vivo*.
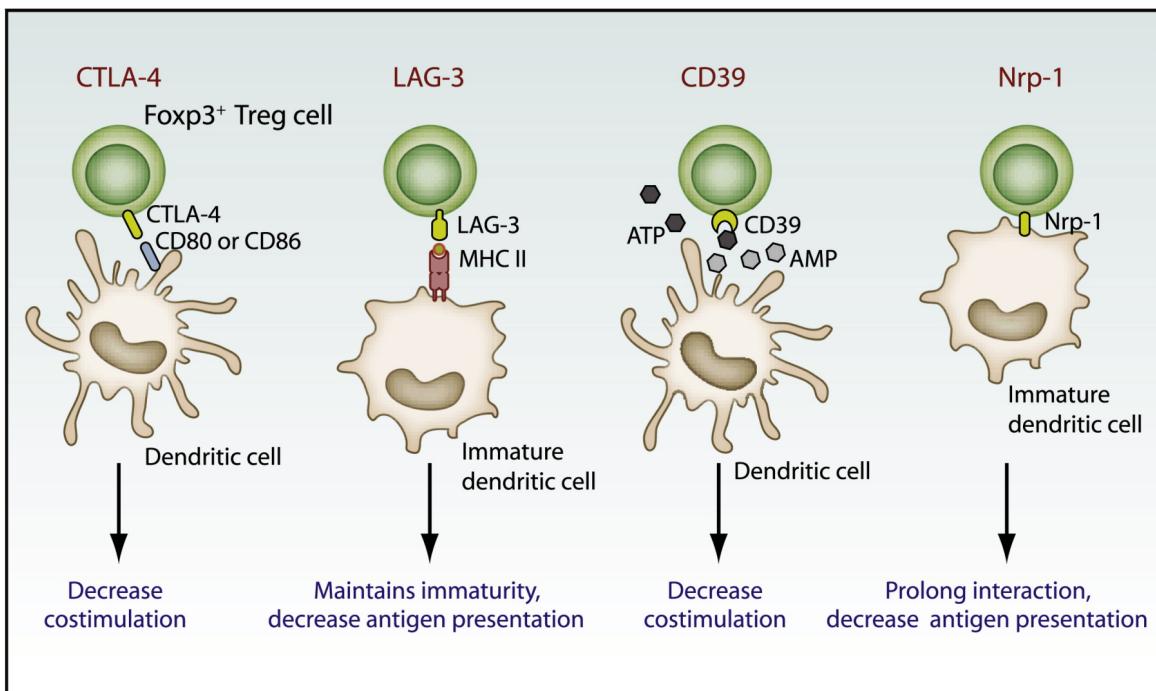
Figure 1.4 Main mechanisms by which Treg can directly suppress APC [from (Shevach, 2009)]

1.4.3 Treg in CNS Autoimmunity

Treg in MS

Soon after the establishment of Treg as a distinct T cell subset with protective or suppressive role in autoimmunity, the Treg homeostasis and function in MS patients was investigated in several studies, with often contradictory results. Several groups have independently observed that the frequency of CD4$^+$CD25^{high} Treg does not differ in peripheral blood of MS patients and healthy controls (Feger et al., 2007; Haas et al., 2005; Putheti et al., 2004; Viglietta et al., 2004), although decreased CD4$^+$CD25$^+$FOXP3$^+$ levels in blood from RR-MS patients has been also described (Venken et al., 2008b). Other studies have reported changes in specific Treg subpopulations in MS patients, such as decrease of CD39$^+$ Treg in RR-MS patients (Borsellino et al., 2007) or increase of CD25$^-$ Treg during relapse (Fransson et al., 2010).

The functionality of Treg in MS patients has been also assessed. CD4$^+$CD25^{high} Treg isolated from blood of patients with RR-MS were shown to have compromised capacity to suppress CD4$^+$CD25$^-$ T cells upon anti-CD3 activation (Viglietta et al., 2004). These findings were confirmed by stimulating also with MOG and MBP (Haas et al., 2005; Kumar et al., 2006). Importantly, by using Teff from both MS patients and

healthy controls in these experiments, the defect in MS patients' Treg function was verified to be intrinsic to Treg and not related to an increased activation status or resistance of Teff (Venken et al., 2010). Yet again, another study disagrees with the concept of impaired Treg suppression in RR-MS patients, reporting normal Treg function when IL-7 receptor α-chain (CD127)-expressing cells are removed from the analysis, as this marker is present on activated T cells but not Treg (Michel et al., 2008). Nevertheless, diminished FoxP3 expression in both mRNA and protein level has been described in RR-MS patients (Huan et al., 2005; Venken et al., 2008b), while patients with SP-MS show normal Treg suppressive function and FOXP3 expression (Venken et al., 2006). In summary, while there is accumulating evidence for an impaired Treg function in MS patients, it is not yet clear whether Treg dysfunction has a causal role in MS or whether it reflects a general defect of the immune system as a result of the autoimmune disorder (Zozulya and Wiendl, 2008).

The mechanisms that underlie the observed Treg defect in MS patients remain unclear. A possible explanation could be an altered generation of Treg in the thymus of MS patients. In fact, the number of recent thymic emigrant Treg (RTE-Treg, defined by the expression of CD31 and CD45RA) was observed to be reduced in the blood of both early and chronic MS patients, due to lower thymic development and migration of Treg to peripheral circulation (Haas et al., 2007; Venken et al., 2008a). Moreover, the decreased RTE-Treg frequency is accompanied with compensatory expansion of memory Treg, resulting in an unaffected total Treg number. Importantly, the MS-derived RTE-Treg display diminished suppressive properties and contracted T cell receptor Vβ repertoire, consistent with impaired clonal expansion of Treg that was described before (Viglietta et al., 2004). Interestingly, long-term treatment of RR-MS patients with glatiramer acetate (GA) was reported to expand the RTE-Treg and restore their suppressive function (Haas et al., 2009). A more recent study suggests that decreased IL-7 receptor α-chain expression contributes to impaired Treg neogenesis in MS (Haas et al., 2011).

The question whether Treg migrate to the CNS during MS is also important to address. Treg are enriched in the CSF of RR-MS but not SP-MS patients, as compared with peripheral blood (Feger et al., 2007; Fritzsching et al., 2011; Venken et al., 2008b). However, Treg are detected in only 70% of brain biopsies from MS patients and their number is rather low (Fritzsching et al., 2011), while they are not detectable by immunostaining of postmortem MS brain lesions (Tzartos et al., 2008). Therefore, the presence of Treg within the CNS during MS, as well as their possible role there, remains to be elucidated.

Treg in EAE

Treg were implicated in EAE before the discovery of Foxp3 as their master transcription factor, defined by their expression of CD25 in CD4$^+$ T cells. Early reports in the development of spontaneous EAE in MBP-specific TCR transgenic mice when they are crossed to RAG-1-deficient background (T/R$^-$ mice) was attributed to the lack of CD4$^+$TCR$^{\alpha\beta}$ T cells with suppressing capacities in these mice (Olivares-Villagomez et al., 1998). Indeed, transfer of CD4$^+$CD25$^+$ T cells from wild-type or RAG-1-competent (T/R$^-$) mice prevented the spontaneous EAE development (Hori et al., 2002). Thus, Treg are protective in this model of spontaneous CNS autoimmunity.

Furthermore, several studies have reported a suppressive role for Treg in induced EAE. Adoptive transfer experiments have shown that large quantities of CD4$^+$CD25$^+$ T cells injected before aEAE induction can reduce the disease severity in MOG$_{35-55}$-immunized C57BL/6 mice (Kohm et al., 2002), as well as in PLP$_{139-151}$-immunized SJL mice (Zhang et al., 2004). In addition, transfer of CD4$^+$CD25$^+$ Treg ameliorates also pEAE in C57BL/6 mice (Kohm et al., 2002). Interestingly, low numbers of CNS-derived CD4$^+$CD25$^+$ T cells are sufficient to ameliorate aEAE, while they show higher suppressive capacity *in vitro* compared to CD4$^+$CD25$^+$ T cells isolated from LN of naïve or EAE mice (McGeachy et al., 2005). Similarly, PLP-specific (5B6) CD4$^+$CD25$^+$ T cells were able to suppress PLP-induced EAE in SJL mice, as well as pEAE in RAG-deficient SJL mice (Yu et al., 2005), while naïve (CD62L^{high}) MPB-specific (Tg4) CD4$^+$CD25$^+$ T cells protect MBP-immunized B10.PL mice against aEAE (Stephens et al., 2009). In both studies, low numbers of myelin-reactive Treg were transferred prior to EAE induction. Therefore, antigen-specific Treg may have higher potency of downregulating the CNS autoimmune response. Importantly, therapeutic approaches have shown that transfer of myelin-specific Treg at the peak of the disease or during the first EAE remission enhances recovery or reduces the severity of the disease during relapse, respectively (Fransson et al., 2012; Mekala and Geiger, 2005; Stephens et al., 2009), providing evidence that Treg cell therapy can be effective in CNS autoimmunity.

In parallel, CD25$^+$ T cell ablation experiments were performed to further assess the Treg role in EAE. Treatment with anti-CD25 antibody either prior to immunization (McGeachy et al., 2005; Montero et al., 2004) or following immunization (but before EAE onset) (Gartner et al., 2006) led to disease exacerbation. Similar results were observed when anti-CD25 antibody treatment was performed during recovery phase in PLP$_{139-151}$-immunized SJL mice (Zhang et al., 2004). Moreover, post-recovery, anti-CD25 treatment was shown to restore susceptibility to EAE reinduction (McGeachy et al., 2005). In contrast, another study reported no influence in EAE disease progression in anti-CD25 antibody treated PLP$_{139-151}$-immunized SJL mice, using a different antibody clone, though (Kohm et al., 2004). Nevertheless, these

results should be interpreted with caution, as anti-CD25 antibody treatment does not lead to specific Treg depletion, but also affects the activated Teff population that also expresses CD25.

Whether Treg exert their suppressive function at the peripheral lymphoid organs or within the inflamed CNS remains controversial. Interestingly, Treg are able to migrate to and accumulate in the CNS of mice with EAE and Treg frequency in the CNS has consistently been shown to correlate with recovery from the disease (Korn et al., 2007; McGeachy et al., 2005; O'Connor et al., 2007). Moreover, the latter two studies revealed that the increased Treg numbers in the CNS during the recovery phase is a result of local proliferation. In fact, MOG-specific Treg are expanded after MOG-immunization, as shown by using MOG$_{35-55}$/IA^{b} tetramers (Korn et al., 2007). In total, these results indicate that Treg could mediate immune suppression within the CNS during EAE. However, despite the fact that CNS-isolated Treg can suppress *in vitro* MOG-specific (2D2) naïve T cells or T cells isolated from spleen of EAE mice, they fail to inhibit *in vitro* the proliferation of CNS-derived encephalitogenic Teff, suggesting that Treg are unable to control the autoimmune response within the target organ (Korn et al., 2007).

Despite Treg have been observed to suppress different models of EAE in several studies, our understanding on how this suppression is accomplished remains poor. Although *in vitro* Treg-mediated suppression has been reported to be IL-10 independent (McGeachy et al., 2005), CD4^{+}CD25^{+} T cells from IL-10-deficient mice were unable to suppress active EAE, suggesting that IL-10 is crucial for the Treg function *in vivo* (Zhang et al., 2004). Similarly, TGF-β has also been shown to be involved in the recovery from EAE, as Treg expressing TGF-β latency-associated peptide (LAP) display increased frequency during the recovery phase and *in vivo* blocking of TGF-β prevented recovery (Zhang et al., 2006). In addition, glucocorticoid-induced TNFR (GITR), expressed on resting Treg, is related with their function, as EAE exacerbation has been observed upon treatment with anti-GITR antibody (Kohm et al., 2004). Recently, Nrp1 expression in Treg has been demonstrated to attenuate EAE progression. Nrp1 deficiency in CD4^{+} T cells has been shown to increase EAE severity, along with higher Th17 frequency and impaired Treg function (Solomon et al., 2011). Intriguingly, against aforementioned studies, CTLA-4 has been suggested to be dispensable for Treg suppressive capacity *in vivo* in an EAE setting, as CTLA-4-deficient mice with MBP-specific TCR transgenic T cells do not develop spontaneous lymphoproliferative disease (as the CTLA-4 KO mice do) nor EAE, but show resistance to disease induction, along with higher frequency of Treg in spleen and thymus (Verhagen et al., 2009). In contrast, reduced Treg frequency both in periphery and the CNS of LFA-1-deficient mice, due to impairment in Treg generation, results in enhanced EAE severity upon MOG$^{35-55}$-immunization (Gultner et al., 2010). Finally, Treg have been reported to prevent

Teff trafficking in the target organ, possibly by modulating the expression of CXCR4, syndecan and S1P1 in immunized mice (Davidson and Shevach, 2011).

OBJECTIVES

Treg have been identified as key players in prevention and suppression of autoimmunity in various diseases and experimental models. In EAE, the animal model for MS, the role of Treg remains unclear. On the one hand, Treg accumulation in the CNS of mice with EAE has been shown to parallel recovery from the disease. Moreover, elimination of Treg population by anti-CD25 Ab treatment has been observed to exacerbate EAE, while transfer of Treg to mice with EAE has been proven beneficial. On the other hand, CNS-infiltrating Treg have been reported to be unable to control the ongoing inflammation in the target tissue. Nonetheless, little is known about the site of Treg-mediated suppression, as well as the mechanisms Treg use to restrict the autoimmune response during EAE.

Therefore, the primary aim of the present study is to examine the function of Treg in MOG-induced aEAE, firstly by investigating the effect of selective and effective depletion of the Treg population, not only on the course of EAE, but also on immune cells that are possible targets of Treg-mediated suppression. Secondly, intravital two-photon microscopy is used to visualize and analyze the locomotion of Treg within the inflamed CNS, as well as their possible interactions with other key immune cells, such as Teff and APC, during different phases of EAE. In addition, this cutting-edge technique allows the assessment of the motility pattern of Teff and their interactions with APC, with or without the presence of Treg. In total, this study aims to elucidate the mechanisms that Treg avail of to mediate recovery from EAE, as well as to identify the site of Treg-mediated suppression of the autoimmune response. Lastly, an important aim of this study is to decipher the role of Treg in a spontaneous EAE model, the OSE mouse. As only half of the OSE mice develop spontaneous EAE (sEAE), the possible contribution of Treg in resistance to the disease is investigated, on the one hand, by comparing the Treg frequency, antigen-specificity and suppressive capacity between sEAE-affected and resistant mice, and on the other hand, by studying the effect of chronic Treg depletion in incidence and severity of sEAE.

MATERIAL & METHODS

2.1   Material

2.1.1   Mice

All animals used in this study were bred in the animal facilities of the Max Planck Institutes of Biochemistry and Neurobiology. The animal procedures were in accordance with guidelines of the committee on animals of the Max Planck Institute for Neurobiology, and with the license of the Regierung von Oberbayern.

Transgenic Mouse Lines (all C57BL/6 background)

- **Foxp3-GFP.KI (Bettelli et al., 2006b)**

  Treg-GFP reporter mouse: GFP expression under the Foxp3 promoter.

- **DEREG (Lahl et al., 2007)**

  "Depletion of Regulatory T cell" mouse: Bacterial artificial chromosome-transgenic mouse expressing a diphtheria toxin receptor-GFP fusion protein under the control of the *foxp3* gene locus.

- **T-Red (Mempel et al., 2006)**

  T cell-RFP reporter mouse: expression of dsRedII under the control of the murine CD4 promoter and proximal enhancer.

- **2D2 (Bettelli et al., 2003)**

  MOG-specific TCR transgenic mouse: $CD4^+$ T cells express a transgenic TCR recognizing MOG_{35-55} peptide in the context of $I-A^b$.

- **Th (Litzenburger et al., 1998)**

  MOG-specific BCR knock-in mouse: B cells express a rearranged Ig heavy chain of a MOG-specific antibody.

2.1.2   Buffers and Reagents

Phosphate buffered saline (PBS)

10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 140 mM NaCl, 2.7 mM KCl

Erythrocyte lysis buffer

0.83 % NH$_4$Cl

ACK buffer

150 mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA

Percoll (GE Healthcare)

- Stock Isotonic Percoll (SIP): 9 parts (v/v) Percoll + 1 part (v/v) of NaCl 1.5 M (d=1.123)

- Percoll d=1.080: 10 ml SIP + 5.7 ml PBS

Cell culture medium

RPMI 1640 medium (Sigma-Aldrich) was complemented with 100 µM MEM non-essential amino acids, 1 mM sodium pyruvate, 50,000 units penicillin, 50 mg streptomycin, 2 mM L-glutamine (all Gibco), and 10 % heat inactivated fetal calf serum (FCS) (Biochrome), 200 µM β-Mercaptoethanol. Prior to use, FCS was inactivated for 1 hour at 56 °C. Medium was sterilized by filtration (pore size 0.2 µm). Quantities refer to 500 ml of medium.

Flow Cytometry buffers

- FACS staining buffer: 1 % BSA, 0.1 % sodium azide in PBS

- Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience)
- Permeabilization Buffer (10X) (eBioscience)

Fluorescence Immunohistochemistry buffers

- Blocking buffer: 4 % BSA, 4 % goat serum in PBS

- Staining buffer: 4 % BSA, 1 % goat serum, 0.1 % Triton 100x in PBS

- Wash buffer: 1 % goat serum, 0.3 % Triton 100x in PBS

Antibodies for Flow Cytometry

Flow cytometry antibodies were labeled with eFluor® 450, FITC, PE, PerCP-Cy5.5, PE-Cy7, APC, APC-eFluor 780 or Alexa Fluor 647, or were biotinylated and used in conjunction with streptavidin-coupled fluorophores.

Specificity	Clone	Antibody class	Provider
CD4	RM4-5	Rat IgG2a, κ	eBioscience
CD11b	M1/70	Rat IgG2b, κ	BD
CD19	1D3	Rat IgG2a, κ	BD
CD25	PC61.5	Rat IgG1, κ	eBioscience
CD44	IM7	Rat IgG2b, κ	BD
CD45	30-F11	Rat IgG2b, κ	eBioscience
CD45R (B220)	RA3-6B2	Rat IgG2a, κ	eBioscience
CD86	GL1	Rat IgG2a, κ	eBioscience
CD152	UC10-4B9	Armenian Hamster IgG	eBioscience
CD206	C068C2	Rat IgG2a, κ	BioLegend
Foxp3	FJK-16s	Rat IgG2a, κ	eBioscience
I-A	NIMR-4	Rat IgG2b	eBioscience
IFNγ	XMG1.2	Rat IgG1, κ	eBioscience
IgM[a]	DS-1	Mouse IgG1, κ	BD
IL-17A	eBio17B7	Rat IgG2a, κ	eBioscience
Ki-67	B56	Mouse IgG1, κ	BD
TNFα	MP6-XT22	Rat IgG1	BD
Vα3.2 TCR	RR3-16	Rat IgG2b, κ	BD
Vβ11 TCR	RR3-15	Rat IgG2b, κ	BD
Streptavidin	-	-	eBioscience

Primers for Real-Time quantitative PCR

Gene	Primer name	Oligo sequence (5'→ 3')	References
GAPDH	Sense	TCACCACCATGGAGAAGGC	Designed by Primer 3 software
	Anti-sense	GCTAAGCAGTTGGTGGTGCA	
	Probe	ATGCCCCCATGTTTGTGATGGGTGT	
IFNγ	Sense	TCAAGTGGCATAGATGTGGAAGAA	(Giulietti et al., 2001)
	Anti-sense	TGGCTCTGCAGGATTTTCATG	
	Probe	TCACCATCCTTTTGCCAGTTCCTCCAG	
IL-17A	Sense	AACTCCCTTGGCGCAAAAGT	Designed by Primer 3 software
	Anti-sense	GGCACTGAGCTTCCCAGATC	
	Probe	CCACGTCACCCTGGACTCTCCACC	

Antibodies for Fluorescent Immunohistochemistry

Fluorescent Immunohistochemistry antibodies were labeled with FITC, Alexa Fluor 568, Alexa Fluor 647, or were biotinylated and used in conjunction with streptavidin-coupled fluorophores.

Specificity	Clone	Antibody class	Provider
CD4	RM4-5	Rat IgG2a, κ	BD
CD4	RM4-5	Rat IgG2a, κ	BioLegend
CD11b	M1/70	Rat IgG2a, κ	BD
Foxp3	FJK-16s	Rat IgG2a, κ	eBioscience
Rat IgG (H+L)	-	Goat Ig	Life Technologies
Streptavidin	-	-	Life Technologies

Fluorescent Dextran Conjugates for Intravital 2-p Imaging

Fluorophore	MW	Provider
Alexa Fluor 488	10 kDa	Life Technologies
Alexa Fluor 647	10 kDa	Life Technologies
Alexa Fluor 680	10 kDa	Life Technologies

2.2   Methods

2.2.1 Mouse Routine

Genotyping

Peripheral blood mononuclear cell (PBMC) were isolated from transgenic mice and analyzed by FACS for fluorescent protein expression (GFP for Foxp3-GFP.KI and DEREG, dsRedII for T-Red), or transgenic TCR (2D2) or BCR (Th) by antibody staining specific for transgene.

Leukocyte Isolation from Peripheral Blood

4 to 6 droplets of blood were collected from anesthetized mice by retro-orbital bleeding into 100 µl of 200 U/ml heparin (Sigma-Aldrich) in PBS. Erythrocytes were lysed by incubation in 1 ml ACK buffer (5 min, RT) and leukocytes were spun down (500 rcf, 5 min, 4 °C). After repeating once more the ACK incubation and spin down, leukocytes were finally resuspended in 150 µl FACS buffer.

EAE induction

EAE was induced by injecting the mice subcutaneously into the flanks with 200 µl of emulsion containing 200 µg MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) and 500 µg *M. tuberculosis* H37 Ra (Difco) in incomplete Freund Adjuvant oil (Difco). In addition, the mice received 400 ng pertussis toxin (List Biological Laboratories) intraperitoneally (i.p.) on days 0 and 2 after immunization.

Clinical signs of EAE were assessed daily according to the following score: score 0 – no disease; score 0.5 – reduced tail tonus; score 1 – limp tail; score 1.5 – limp tail and ataxia; score 2 – limp tail, ataxia and hind limb weakness; score 2.5 – at least one hind limb paralyzed/weakness; score 3 – both hind limbs paralyzed/weakness; score 3.5 – complete paralysis of hind limbs; score 4 – paralysis until hip; score 5 – moribund or dead.

Diphtheria Toxin (DTx) Treatment

For depletion of Treg in DEREG mice, DTx (Sigma-Aldrich) (200 ng in 100 µl PBS) was injected both i.p. and intravascularly (i.v.). For chronic depletion of Treg in OSE x DEREG mice, 100ng DTx (in 100 µl PBS) was injected i.p. twice per week for 5 weeks.

Mononuclear Leukocyte Isolation from Organs

Mice were anesthetized and perfused transcardially through left ventricle with 20 ml cold PBS. Lymphoid organs (spleen, inguinal and axillary lymph nodes), brain and spinal cord were dissected.

- Lymphoid organs

  Single cell suspension was prepared in RPMI by using 40 µm cell strainers (BD). Cells were centrifuged (500 rcf, 10 min, 4 °C) and the cell pellet was resuspended in complemented RPMI for further analysis.

  For spleen preparation, erythrocyte lysis was performed by resuspending and incubating the cells in 0.83 % NH_4Cl for 3 min at RT. Cells were washed with RPMI, centrifuged (500 rcf, 10 min, 4 °C) and resuspended in complemented RPMI.

- CNS

  Single cell suspension was prepared in RPMI by using 100 µm cell strainers (BD). After centrifugation (500 rcf, 10 min, 4 °C), the cell pellet was resuspended in 5 ml RPMI plus 2.16 ml Stock Isotonic Percoll (SIP) and was overlaid on 5 ml Percoll d=1.080. The gradient was centrifuged (1200 rcf, 20 min, RT) and the interface, containing the mononuclear cells, was collected, washed with complemented RPMI and resuspended in complemented RPMI for further analysis.

2.2.2 Flow Cytometry (FACS)

Surface Staining

Cells were transferred in 96-well V bottom plate (Nunc), spun down (250 rcf, 10 min, 4 °C) and washed in 200 µl FACS buffer. They were resuspended in 50 µl of a mix of labeled antibodies in FACS buffer at optimized dilutions and incubated in dark (20 min, 4 °C). After incubation, cells were washed twice in 200 µl FACS buffer. When biotin antibodies were used, a secondary staining using fluorescently conjugated Streptavidin followed, using the same protocol.

Intracellular Staining

For intracellular cytokine staining (IFNγ, IL-17, TNFα), cells were activated with 50 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma) in the presence of 5 µg/ml brefeldin A (Sigma) for 4 h at 37 °C. For Foxp3 and Ki-67 staining no PMA/ionomycin stimulation was performed. Upon completion of the surface staining, cells were fixed and permeabilized by incubation with Foxp3 Fixation/Permeabilization Buffer (eBioscience) in dark (30-60 min, RT). After washing twice in Permeabilization Buffer (eBioscience), cells were resuspended in 50 µl of a mix of labeled antibodies in Permeabilization Buffer at optimized dilutions and incubated in dark (30-60 min, 4 °C). Finally, cells were washed twice in Permeabilization Buffer and resuspended in 150 µl FACS buffer.

Acquisition and Data Analysis

For cell number quantification, 10^4 FACSuite FC Beads (BD) were added per sample prior to acquisition. Samples were acquired in FACS Calibur or FACS Verse (both BD). FACS data were analyzed using FlowJo 7.6.5 software (TreeStar).

Cell Sorting

Isolated splenocytes pooled from 2-4 mice were suspended in 2-4 ml RPMI, filtered through 100 µm filter and sorted to GFP$^+$ and GFP$^-$ cell using a MoFlo XDP cell sorter (Beckman Coulter).

2.2.3 Cell Culture

T cell Purification

For purification of T cells from total splenocytes, MagCellect Cell Selection Kit for mouse naïve CD4$^+$ T cells (R&D Systems) was used according to manufacturer's instructions.

Suppression Assay

10^5 Teff, 5 x 10^4 Treg and 2 x 10^5 irradiated (50 Gy) splenocytes per well were plated in 96-well round-bottom plates in a total volume of 200 µl complemented RPMI in triplicates. Anti-CD3e (BD) (0.5 µg/ml) was added. After a culture period of 48 hours, 1 µCi ^{3}H-labeled thymidine (PerkinElmer) was added per well. Samples were harvested 16 hours later and tritium incorporation was measured on a Matrix 9600 Direct Beta Counter (Packard).

2.2.4 RNA Techniques

RNA extraction & Reverse Transcription

Total RNA was isolated with TRI Reagent (Sigma-Aldrich). Whole tissue or cells were placed in 1 ml TRI Reagent and stored at -20 ^{o}C until RNA extraction. For RNA extraction, 200 µl of chloroform were added; samples were vortexed vigorously and centrifuged at 19000 rcf for 15 min at RT. The aqueous phase was removed and RNA was precipitated with 500 µl of isopropanol. RNA was washed in 1 ml of 75 % ethanol and finally dissolved in RNase-free water. cDNA was generated from 1 µg total RNA using the Verso cDNA Kit (Thermo Fisher Scientific), according to the manufacturer´s instructions.

Quantitative Real-Time PCR

Sense and antisense primers in combination with FAM/TAMRA TaqMan fluorescent probes (all from Metabion) were used for quantitative PCR analysis. Where possible, the primer/probe sequence combinations spanned contact sequences of subsequent exons. For amplification, the Absolute QPCR mix was used (ABgene). Each reaction was run in triplicate on a 7900HT Fast Real-Time PCR System (Applied Biosystems) and was normalized to housekeeping gene GAPDH transcripts. Primary data was analyzed using Gene-Amp SDS 2.3 software (Applied Biosystems).

2.2.5 Fluorescent Immunohistochemistry

Mice were anesthetized and perfused transcardially through left ventricle with 20 ml cold PBS and 20 ml 4 % paraformaldehyde (PFA) in PBS. Organs were dissected and stored in the same fixative overnight at 4 °C, washed with PBS, and finally kept at 4 °C in 25 % sucrose in PBS solution.

Tissues were embedded in Tissue-Tek O.C.T Compound (Sakura) and frozen in -20 $^{\circ}$C. Frozen tissue sections (10-20 μm) were performed using Cryostat CM3050 S (Leica). Tissue sections were thawed and incubated with blocking buffer for 1-2 hours at RT. Next, incubation with primary antibody (1:200) in staining buffer was performed for 1-2 hours at RT or overnight at 4 °C and the sections were washed 3 times with wash buffer. Incubation with secondary antibody (1:200 - 1:400) in staining buffer followed for 1-2 hours at RT in the dark, before sections were eventually washed 3 times with wash buffer and embedded in Gel Mount Aqueous Mounting Medium (Sigma-Aldrich). Images were acquired on a SP5 confocal microscope (Leica), using 20x air-immersion (N.A. 0.70) or 63x oil-immersion (N.A. 1.4) objective. Images were processed using Image J (NIH) and Photoshop CS5 software (Adobe Systems).

2.2.6 Intravital two-photon Imaging

Image Acquisition

Time-lapse two-photon laser-scanning microscopy was performed using a SP2 confocal microscope (Leica) equipped with a 10 W Millenia/Tsunami laser (Newport). The excitation wavelength was tuned to 880 nm and routed through a 20x water-immersion objective (N.A. 0.95, Olympus) or 25x water-immersion objectives (N.A. 0.95, Leica). 1x or 1.5x zoom was used and 40-80 μm z-stacks were acquired with 3-6 μm z-step. The acquisition rate was set to 25.219 s interval time and images were line-averaged twice. The acquisition time was approximately 1 hour per experiment. Fluorescent signals were detected using non-descanned photomultiplier tube detectors (Hamamatsu) equipped with 440/40 nm (for

detection of second harmonic), 525/50 nm (for detection of GFP and Alexa Fluor 488), 579/34 nm (for detection of tetramethylrhodamine dextran), 630/69 nm (for detection of dsRedII), and 685/40 nm (for detection of Alexa Fluor 647 and 680) band-pass filters (Semrock).

Animal Preparation

Animals were anesthetized by intraperitoneal injection of fentanyl/midazolam/medetomidine (50 µg/kg, 5 mg/kg, and 500 µg/kg bodyweight, respectively) mix in PBS, tracheally intubated and ventilated with 1 % isoflurane. Animals were placed on custom-designed microscope stage (Bartholomaus, 2011) and body temperature was regulated at 36.5 - 37.5 °C by using a heating pad and temperature sensor. Electrocardiogram and physiological parameters, such as concentrations of inspiratory and expiratory gases and ventilation pressure, were constantly monitored and recorded during imaging.

Spinal cord Imaging

For spinal cord imaging, a spinal cord window was prepared at level thoracic-12 / lumbar-1. After midline skin incision, the paravertebral musculature was detached from the spine and 3 successive spine discs were fixed to reduce breathing and heart beating artifacts. Laminectomy was performed on the middle spine disc using a dental drill (FOREDOM). Agarose ring was mounted around the spinal cord window to retain the buffer in which water objective was embedded.

Local Injections in Imaging Window

In some experiments, fluorescent conjugated dextran was injected into the spinal cord meninges to visualize local APC. A microcapillary (13 µm tip diameter, Biomedical Instruments) was used for injection in the subarachnoid space. The capillary was inserted, using a microcapillary manipulator (Luigs & Neumann), through a hole (app. 20 µm diameter) created by applying shortly high-energy laser on the arachnoidea. Fluorescent dextran conjugate (10 ng/µl) was injected by CellTram vario (3-4 µl) (Eppendorf).

Image analysis

Time-lapse images were acquired using Leica LCS software (Leica), and subsequently processed and analyzed by ImageJ (NIH). To obtain two-dimensional movies, a Gaussian blur filter was applied and maximum intensity z-projections were made. In some videos, the contrast was adjusted by liner rescaling, and the noise was removed additionally using a median filter. The position of T cells in the two-dimensional space was analyzed using Imaris software (Bitplane). Cell trajectories and motility parameters were calculated from the obtained position coordinates using Excel (Microsoft). Trajectory

lines were drawn based on the obtained coordinate information. The instantaneous T cell velocity was calculated by combining the cell coordinates with the time interval of imaging (25.219 sec). The average velocity per T cell was the average of the instantaneous velocity values. The linearity index is defined as the sum of total displacement divided by the path length of a T cell (Bartholomaus, 2011). A cell was considered to be in stationary phase when its average velocity for three frames (75.66 sec) was less than 3 µm per min. Treg/Teff – APC and Treg – Teff contacts were analyzed manually and all of the physical interactions between Treg, Teff and labeled meningeal APCs in the three-dimensional volume were defined as contacts.

2.2.7 Statistical Analysis

The statistical evaluation was performed using Prism software (GraphPad). A nonparametric t-test (Mann-Whitney) for one pair and one-way ANOVA (Kruskal Wallis test, – Dunn's multiple comparison test) or two-way ANOVA (Bonferonni post test) for more than one pair, were used. In addition, survival curves were analyzed by Log-rank (Mantel-Cox) test. P<0.05 was considered significant. "n.s." indicates no significance.

RESULTS

3.1 Treg Depletion during aEAE

3.1.1 Increased Treg Frequency in the CNS of Mice with aEAE during Recovery

Treg have been reported to accumulate in the CNS of mice with aEAE, paralleling the recovery from the disease (Korn et al., 2007; McGeachy et al., 2005; O'Connor et al., 2007). To confirm these findings, a time-kinetic experiment analyzing Treg frequencies in peripheral immune tissues and the CNS was performed. Foxp3-GFP.KI B6 mice were immunized with MOG_{35-55} in CFA and the frequency of Treg cells was assessed during different phases of the disease (Fig. 3.1.1 a) using flow cytometry. In agreement with the aforementioned studies, Treg were present in the spinal cord throughout the course of EAE, but their frequency was significantly increased during the recovery phase. In contrast, Treg proportions in draining inguinal and axillary lymph nodes (LN) remained unaltered (Fig. 3.1.1 b,c).
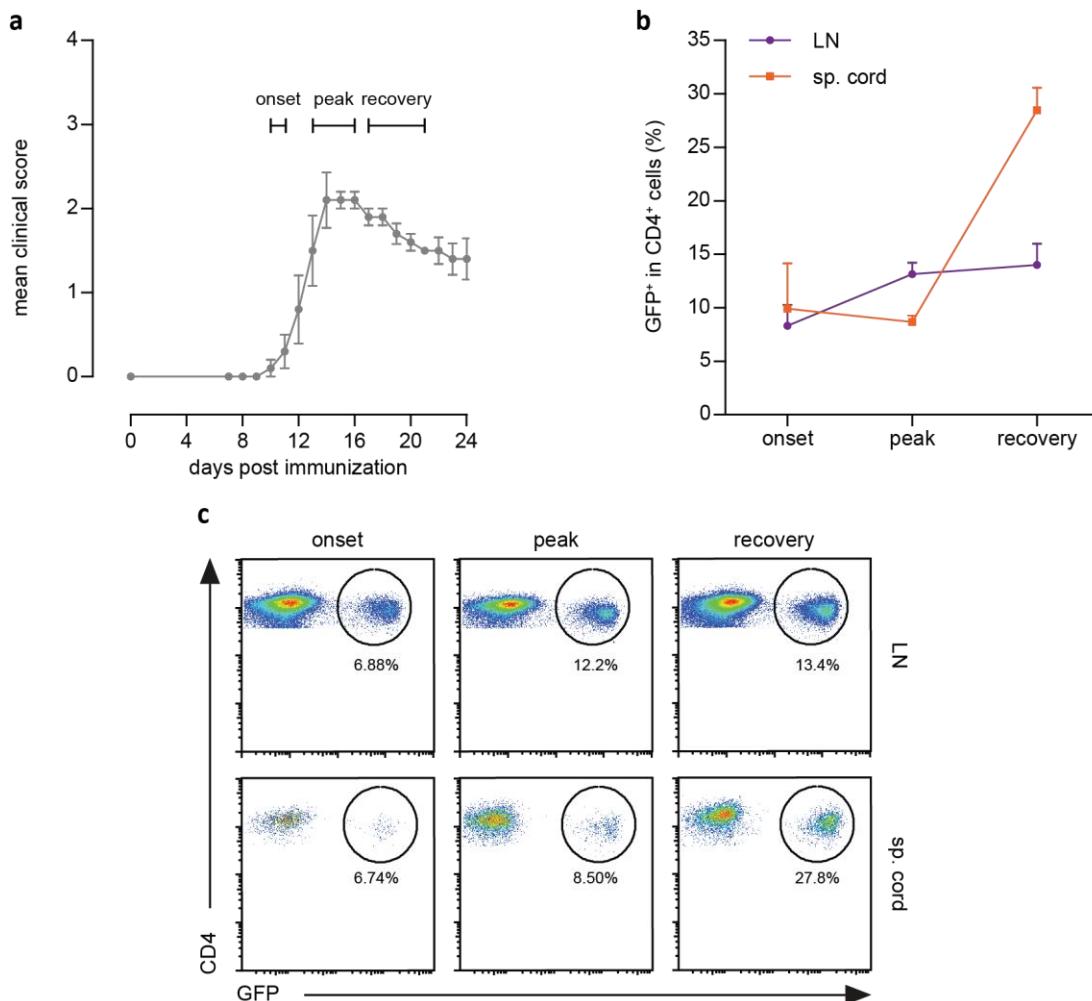
Figure 3.1.1 Treg accumulation in the CNS during recovery from EAE. Foxp3-GFP.KI B6 mice were immunized with MOG$_{35-55}$ in CFA. **(a)** Mean clinical score (± s.e.m.) following immunization and timepoints (onset, peak and recovery) were mice were sacrificed (*n=5* mice) **(b)** Mean frequency (+ s.e.m.) of GFP$^+$ in CD4$^+$ T cells in pooled inguinal and axillary lymph nodes (LN, purple line) and spinal cord (orange line) (*n=3* mice per group, representative data from two independent experiments. *******P*<0.01, 2way ANOVA between onset, peak and recovery). **(c)** Representative flow cytometry plots of CD4$^+$ gated lymphocytes. Treg population and its percentage within the CD4$^+$ compartment are indicated.

Additionally, infiltration of Treg into the spinal cord during EAE and their enhanced frequency during recovery was confirmed by confocal microscopy of spinal cord sections (Fig. 3.1.2).

In summary, the accumulation of Treg in the CNS during recovery from EAE indicates a possible role for this T cell subset in disease remission.
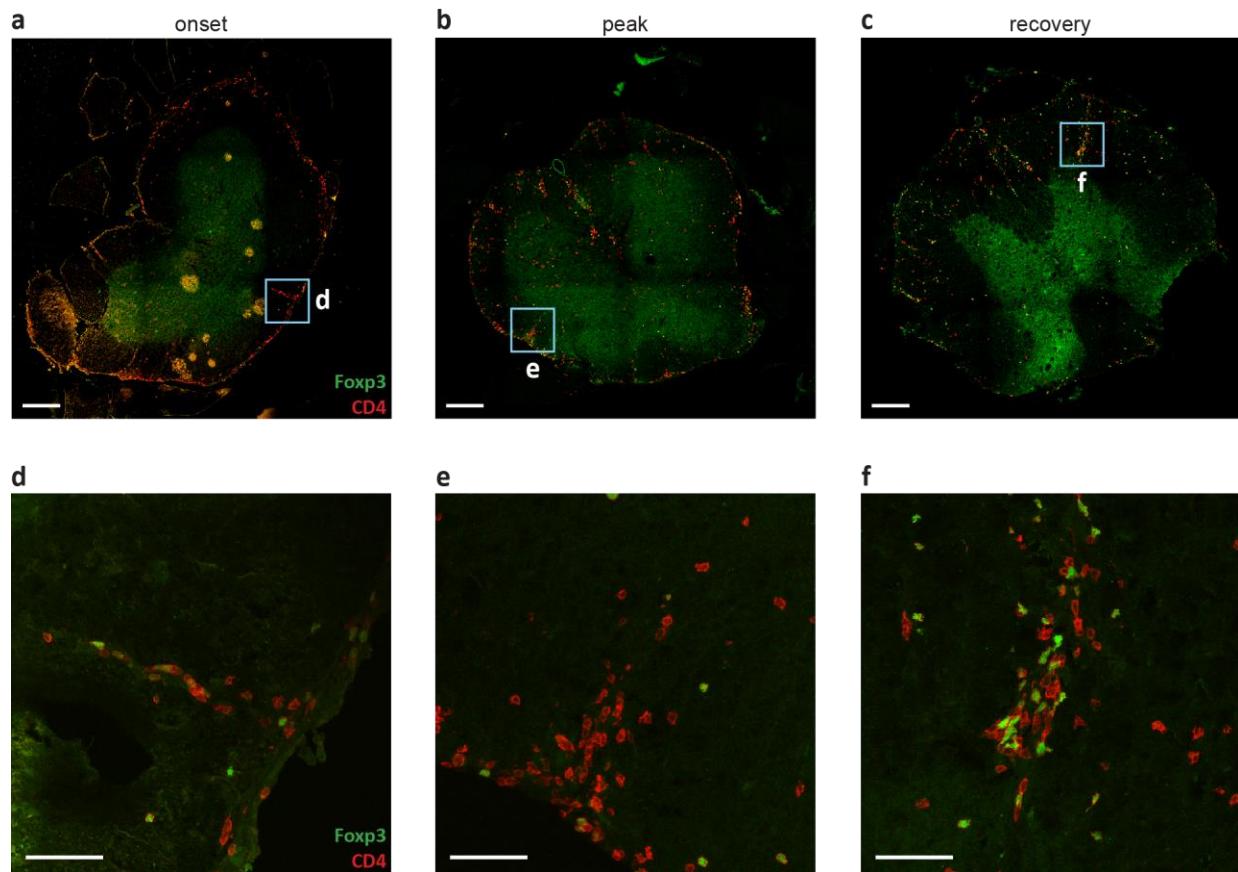
Figure 3.1.2 Treg accumulation in spinal cord during different phases of EAE. Foxp3-GFP.KI B6 mice were immunized with MOG$_{35-55}$ in CFA and sacrificed at day 1 (onset), day 3-5 (peak) or day 7-10 (recovery) post EAE onset. Spinal cord cryosections were stained with anti-CD4 Ab (red). Foxp3$^+$ cells express GFP and are depicted in green. Images were acquired by confocal microscopy. **(a)**, **(b)**, **(c)** Representative panoramic pictures of spinal cord cross sections at the indicated disease phase, composed by approximately 10 pictures acquired with 20x objective lens (scale bar: 200 μm). **(d)**, **(e)**, **(f)** Magnified pictures of the indicated regions of the spinal cord cross sections (a), (b) and (c), acquired with 63x objective lens (scale bar: 50 μm).

3.1.2 Acute Treg Depletion Prevents Recovery and Leads to Fatal EAE

Targeted ablation of the Treg population could elucidate the role of Treg during the recovery phase of EAE. As discussed before (see Introduction – Treg in EAE), although treatment with anti-CD25 Ab has been performed in several studies, it does not result in specific elimination of Treg, as CD25$^+$ Teff are also affected. In order to target Treg efficiently, the transgenic DEREG mice were selected for this study, as they express a diphtheria toxin (DTx) receptor-enhanced GFP fusion protein, under the control of the foxp3 gene locus, permitting specific depletion of Treg by treatment with DTx (Lahl et al., 2007). Therefore, DEREG and non-transgenic littermate control (NTL) B6 mice were immunized with MOG$_{35-55}$ in CFA and treated with DTx or PBS four days post onset.

The efficiency of the Treg ablation in peripheral immune organs and the CNS was tested by flow cytometry two days after the treatment. Indeed, staining for Foxp3 revealed that Treg population was almost completely lost in DTx-treated DEREG mice as compared to the control animals (Fig. 3.1.3 a,b).
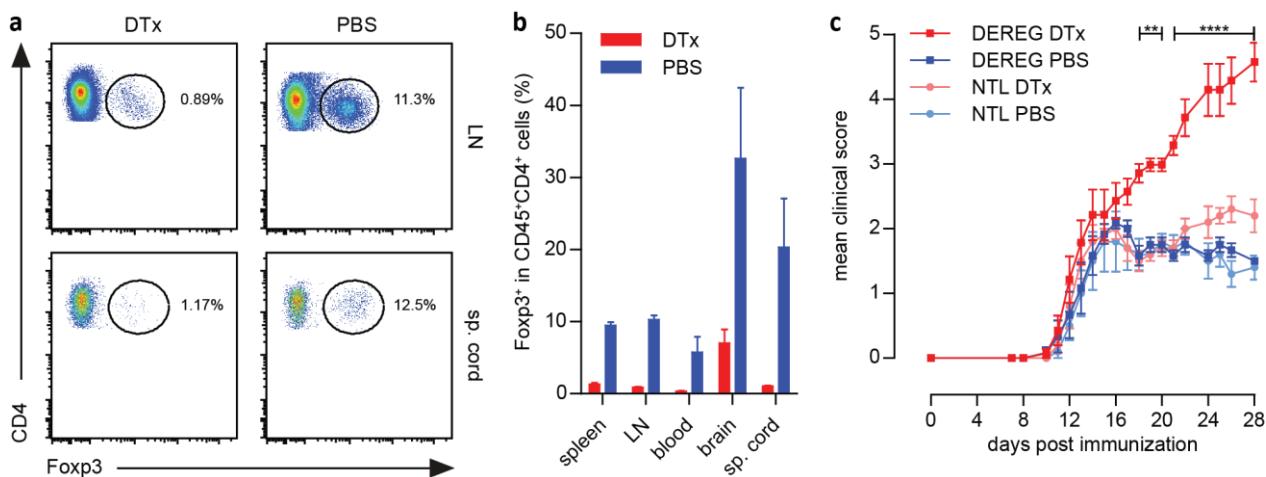

Figure 3.1.3 Prevention from recovery and exacerbation of EAE after efficient Treg depletion at the peak of the disease. DEREG and NTL mice were immunized with MOG$_{35-55}$ in CFA and treated with DTx or PBS four days after the onset of EAE. **(a)** Representative flow cytometry plots of CD45$^+$CD4$^+$ gated lymphocytes isolated from LN and spinal cord of DEREG mice sacrificed two days after DTx or PBS treatment. Treg population and its percentage within the CD4$^+$CD45$^+$ compartment are shown. **(b)** Mean frequency (+ s.e.m.) of Foxp3$^+$ in CD45$^+$CD4$^+$ T cells in peripheral immune tissues and the CNS of DEREG mice, two days after the DTx/PBS treatment (*n*=3-4 mice per group, representative data from three independent experiments) **(c)** Mean clinical score (+ s.e.m.) of mice following immunization and DTx or PBS treatment (day 4 post EAE onset). (*n*=5-7 mice per group, representative data from two independent experiments. *P<0.05, **P<0.01, ****P<0.0001, 2way ANOVA between DEREG DTx and DEREG PBS groups)

Next, immunized and treated mice were monitored daily for clinical score to assess the effect of Treg depletion on EAE pathogenesis. DTx-treated DEREG mice not only showed no recovery from the disease, but also developed severe and, eventually, fatal EAE. In sharp contrast, the PBS-treated DEREG, as well

as DTx and PBS-treated NTL mice, partially recovered from EAE (Fig. 3.1.3 c). These finding suggests that Treg are essential for recovery from aEAE.

3.1.3 Treg Depletion Effect on Teff

The dramatic outcome of Treg ablation during the course of EAE raised the question of how the disease exacerbation is mediated. As discussed earlier, Treg are believed to directly suppress Teff. Therefore, the effect of Treg depletion on Teff numbers and phenotype was examined by flow cytometry. Interestingly, lymph nodes and spinal cord of Treg-depleted mice exhibited significantly increased numbers of Teff compared to control mice (Fig. 3.1.4 a). In addition, the activation status of Teff was tested by staining with CD44 and CD25. Surprisingly, no difference was observed in the percentage of activated Teff (defined as CD44^{high}CD25$^+$) in periphery as well as the CNS of DTx-treated compared to PBS-treated DEREG mice (Fig. 3.1.4 b).
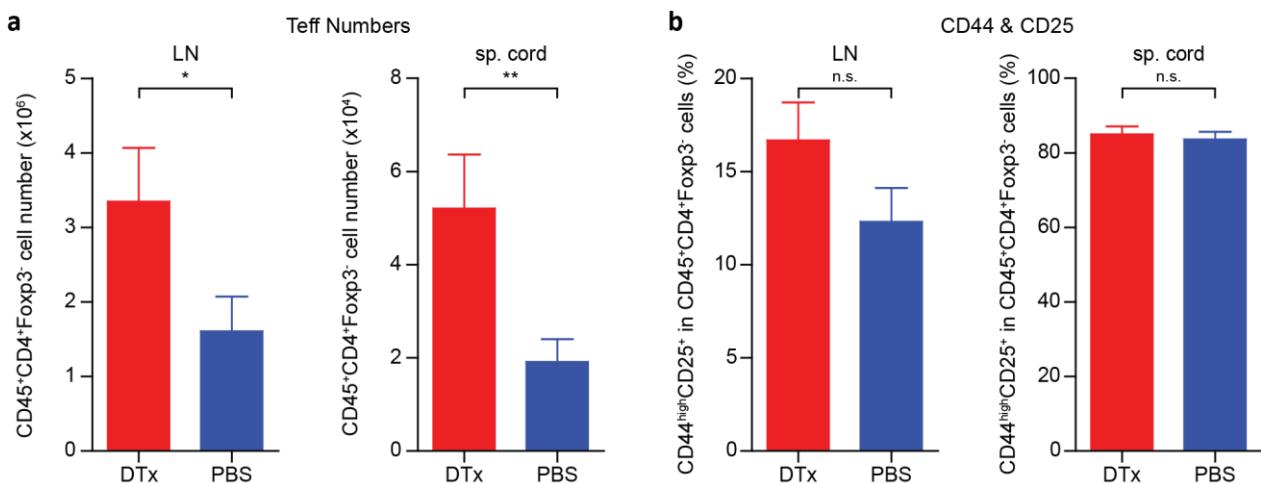
Figure 3.1.4 Increased numbers, but not higher activation status, of Teff after Treg depletion at the peak of EAE. DEREG B6 mice were immunized with MOG$_{35-55}$ in CFA and treated with DTx or PBS four days after the EAE onset. Two days later they were sacrificed and lymphocytes were isolated from LN and spinal cord and analyzed by flow cytometry. **(a)** Mean absolute numbers (+ s.e.m.) of Teff (CD45$^+$CD4$^+$Foxp3$^-$ cells) (n=8 mice per group, pooled data from three independent experiments). **(b)** Mean frequency (+ s.e.m.) of activated (CD44^{high}CD25$^+$) Teff in DTx- and PBS-treated mice (n=6-7 mice per group, pooled data from two independent experiments). (*P<0.05, **P<0.01, t-test)

Next, the cytokine-producing profile of Teff was examined. Notably, Treg depletion led to increased frequency of IFNγ$^+$ Teff in the LN, but not in the spinal cord (Fig. 3.1.5 b). In contrast, the fraction of IL-17$^+$ Teff was comparable in Treg-depleted and Treg-intact mice, both in LN and spinal cord (Fig. 3.1.6 b).

Nevertheless, DTx-treated mice displayed augmented absolute numbers of these proinflammatory cytokine-producing Teff. In fact, significantly more IFNγ-producing Teff were found in LN and spinal cord of DTx-treated DEREG mice (Fig. 3.1.5 a). This finding was corroborated by real-time PCR analysis, which

showed significantly elevated IFNγ mRNA levels in LN of Treg-depleted mice (Fig. 3.1.5 c). Similarly, more IL-17+ Teff were observed in the spinal cord of Treg-depleted mice (Fig. 3.1.6 a), paralleled by increased IL-17 mRNA levels (Fig. 3.1.6 c).

Figure 3.1.5 Increased numbers of IFNγ-producing Teff and eleveated IFNγ mRNA levels after Treg depletion at the peak of EAE. DEREG mice were immunized with MOG_{35-55} in CFA and treated with DTx or PBS four days after the EAE onset. Two days later they were sacrificed and lymphocytes were isolated from axillary and inguinal lymph nodes (LN) and spinal cord and analyzed by flow cytometry (a,b) or total mRNA was extracted from LN and spinal cord (c). **(a)** Mean absolute numbers (+ s.e.m.) of $CD45^+CD4^+Foxp3^-IFNγ^+$ cells **(b)** Mean frequency (+ s.e.m.) of $IFNγ^+$ in $CD45^+CD4^+Foxp3^-$ cells (n=6-7 mice per group, pooled data from two independent experiments). **(c)** Expression of IFNγ (mRNA relative to GAPDH quantified by real-time PCR (n=5-7 mice per group). (*P<0.05, **P<0.01, t-test)

In summary, the exacerbated EAE pathology observed in Treg-depleted mice could be attributed to the elevated numbers of Teff both in LN and spinal cord, leading to enhanced inflammation as indicated by the increased levels of IFNγ and IL-17.

a

b

c
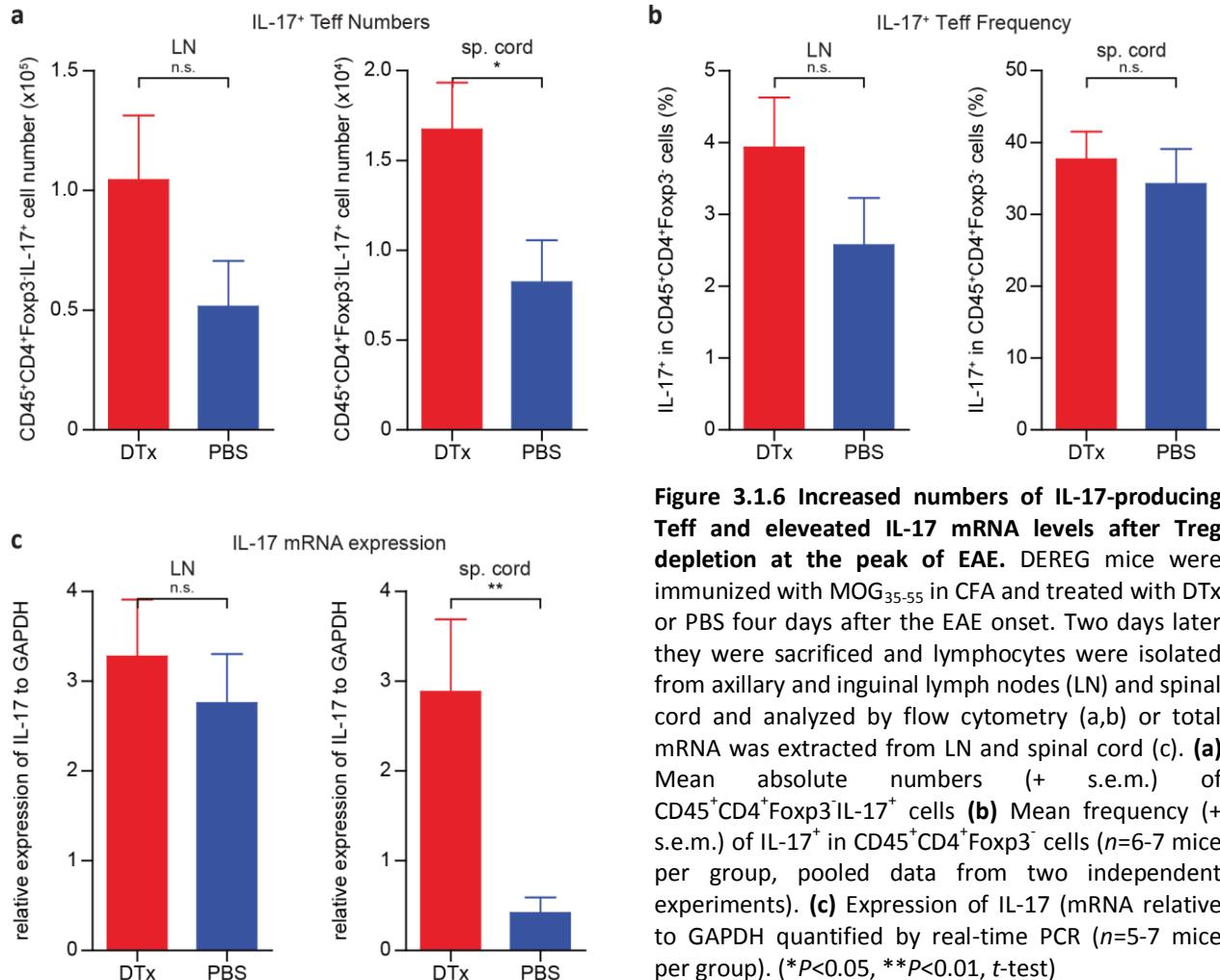
Figure 3.1.6 Increased numbers of IL-17-producing Teff and eleveated IL-17 mRNA levels after Treg depletion at the peak of EAE. DEREG mice were immunized with MOG_{35-55} in CFA and treated with DTx or PBS four days after the EAE onset. Two days later they were sacrificed and lymphocytes were isolated from axillary and inguinal lymph nodes (LN) and spinal cord and analyzed by flow cytometry (a,b) or total mRNA was extracted from LN and spinal cord (c). **(a)** Mean absolute numbers (+ s.e.m.) of $CD45^+CD4^+Foxp3^-IL-17^+$ cells **(b)** Mean frequency (+ s.e.m.) of $IL-17^+$ in $CD45^+CD4^+Foxp3^-$ cells (n=6-7 mice per group, pooled data from two independent experiments). **(c)** Expression of IL-17 (mRNA relative to GAPDH quantified by real-time PCR (n=5-7 mice per group). (*$P<0.05$, **$P<0.01$, t-test)

3.1.4 Treg Depletion Effect on Macrophages

Treg can possibly exert their suppressive capacity also on cells of the innate immune system. Macrophages are not only potent APC, but also main mediators of the disease pathology. Therefore, the macrophage numbers in EAE-affected Treg-depleted mice were assessed by using flow cytometry. Significantly elevated numbers of macrophages were found in the spinal cord infiltrates of DTx-treated compared to control animals, while their numbers in LN remained similar(Fig. 3.1.7 a).
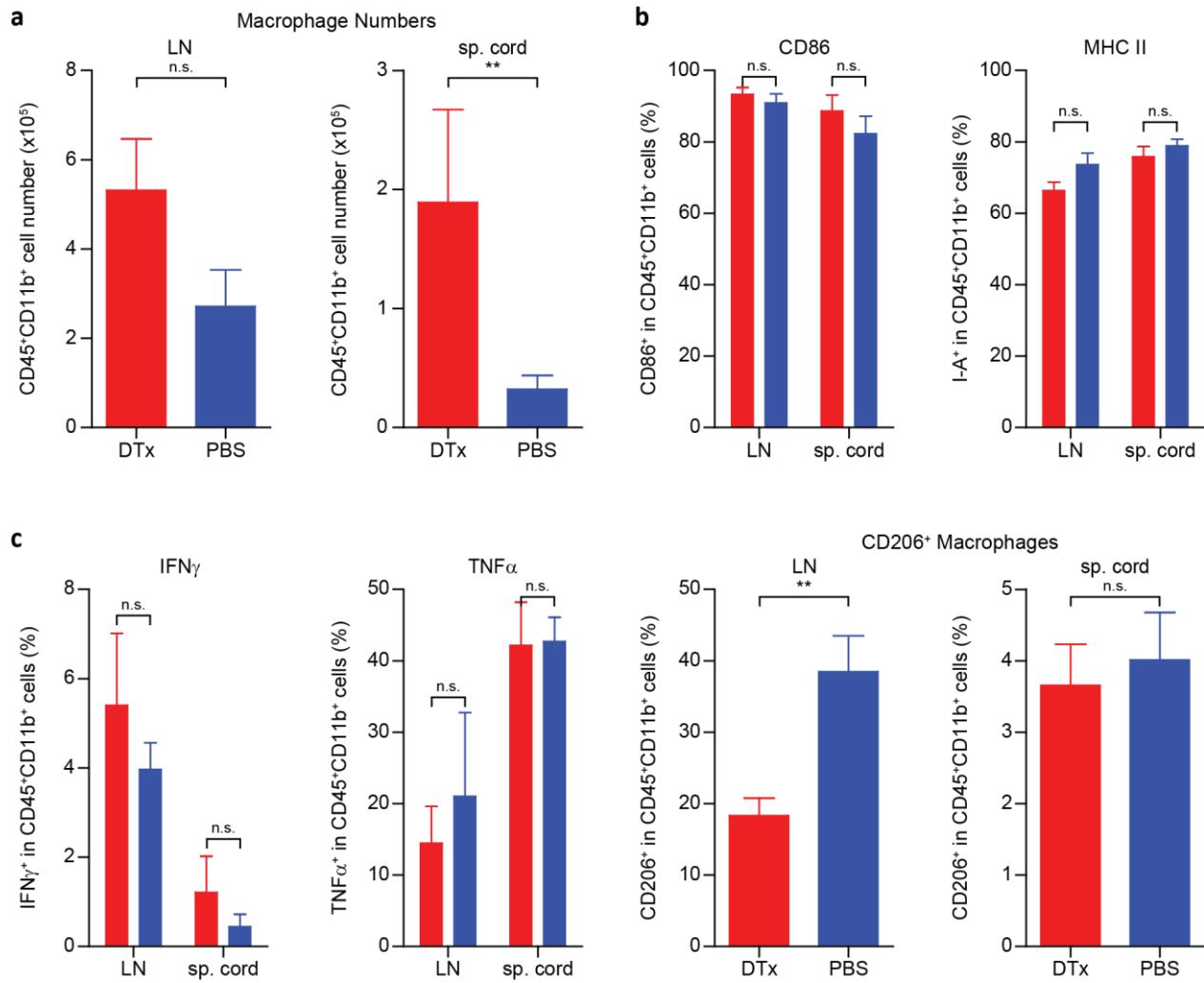
Figure 3.1.7 Increased numbers of CD11b$^+$ macrophages after Treg depletion at the peak of EAE. DEREG mice were immunized with MOG$_{35-55}$ in CFA and treated with DTx or PBS four days after the EAE onset. Two days later they were sacrificed and lymphocytes were isolated from axillary and inguinal lymph nodes (LN) and spinal cord and analyzed by flow cytometry. **(a)** Mean absolute numbers (+ s.e.m.) of macrophages (CD45$^+$CD11b$^+$ cells) (*n*=8 mice per group, pooled data from three independent experiments) **(b)** Mean frequency (+ s.e.m.) of CD86$^+$ and MHC II$^+$ in CD45$^+$CD11b$^+$ cells in DTx-(red bars) and PBS-treated (blue bars) mice (*n*=8 mice per group, pooled data from three independent experiments). **(c)** Mean frequency (+ s.e.m.) of IFNγ$^+$ and TNFα$^+$ in CD45$^+$CD11b$^+$ cells in DTx-(red bars) and PBS-treated (blue bars) mice (*n*=3-4 mice per group). **(d)** Mean frequency (+ s.e.m.) of CD206$^+$ in CD45$^+$CD11b$^+$ cells (*n*=8 mice per group, pooled data from three independent experiments). (*$P<0.05$, **$P<0.01$, *t*-test)

Next, the effect of Treg depletion on the macrophage phenotype was examined. Surprisingly, ablation of Treg did not lead to changes in the activation status of macrophages, as shown by staining for CD86 and MHC II (Fig. 3.1.7 b), or their cytokine-producing capacity, including IFNγ and TNFα (Fig. 3.1.7 c). However, intriguingly, LN from DTx-treated DEREG mice showed significantly reduced frequency of mannose-receptor-expressing (CD206$^+$) macrophages (Fig. 3.1.7 d). CD206$^+$ is considered to be a surface

marker of the alternatively-activated macrophages (M2 macrophages), which have immunoregulatory functions (Sica and Mantovani, 2012b).

Thus, these findings indicate that Treg depletion leads to more severe EAE pathology on the one hand by increasing the numbers of macrophages found in the CNS infiltrates and on the other hand by decreasing the frequency of regulatory macrophages in peripheral LNs.

Finally, the enhanced accumulation of Teff and macrophages in spinal cord of Treg-depleted mice was confirmed by immunohistochemistry. Indeed, spinal cord sections from DTx-treated DEREG mice displayed highly increased numbers of infiltrating Teff and macrophages compared to PBS-treated animals (Fig. 3.1.8)
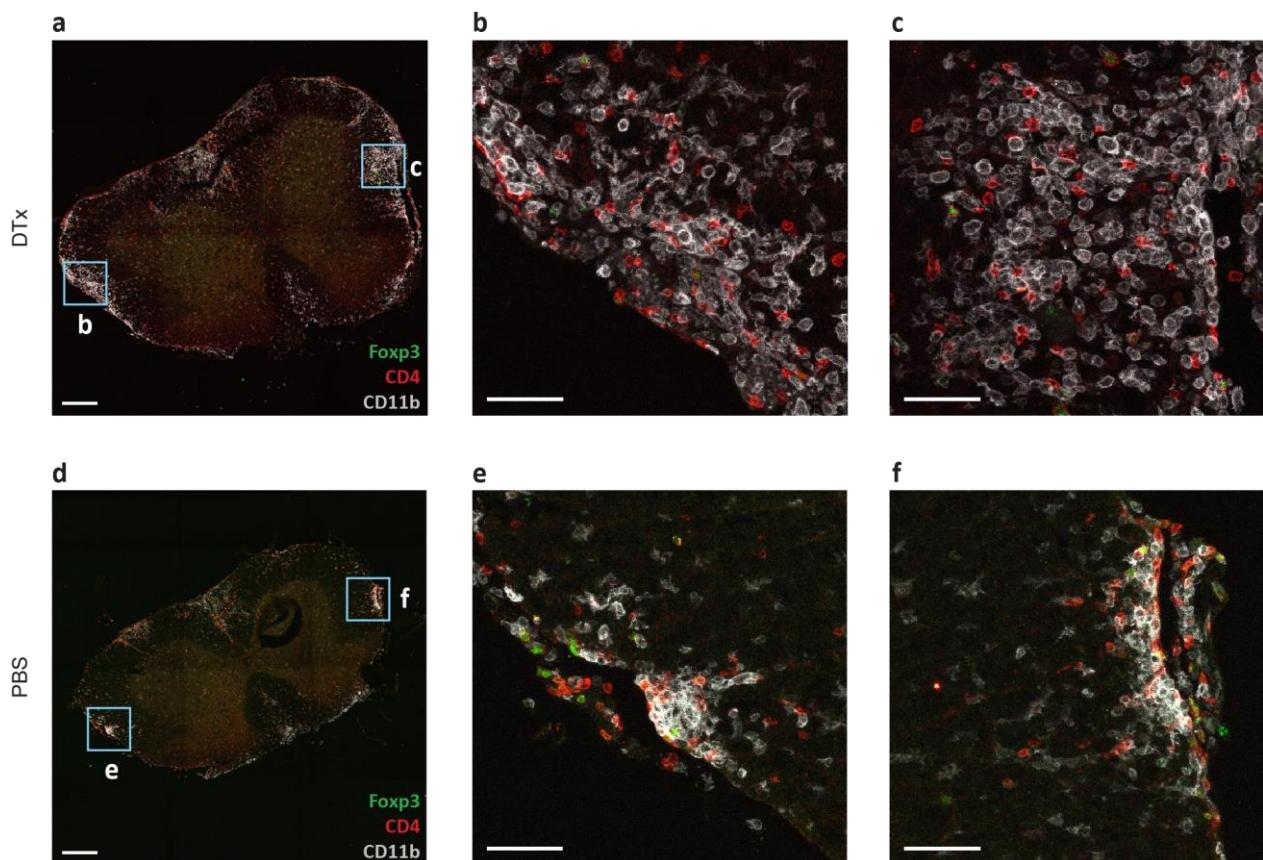

Figure 3.1.8 Increased numbers of Teff and macrophages in spinal cord after Treg depletion at the peak of EAE. DEREG mice were immunized with MOG_{35-55} in CFA and treated with DTx or PBS four days after the EAE onset. Two days later they were sacrificed and spinal cord cryosections were prepared and stained with anti-Foxp3 (green), anti-CD4 (red) and anti-CD11b Ab (grey). Images were acquired by confocal microscopy. **(a)**, **(d)** Representative panoramic pictures of spinal cord cross sections from mice treated with DTx (a) or PBS (d), composed by approximately 10 pictures acquired with 20x objective lens (scale bar: 200 μm). **(b)**, **(c)**, **(e)**, **(f)** Magnified pictures of the indicated regions of the spinal cord cross sections (a) and (d), acquired with 63x objective lens (scale bar: 50 μm).

In summary, Treg infiltrated the CNS during aEAE and their levels were enhanced during recovery from the disease. Selective depletion of Treg at the peak of EAE prevented recovery and led to severe exacerbation of the disease, characterized by enhanced numbers of IFNγ- and IL-17-producing Teff both in draining LN and the CNS, as well as elevated numbers of macrophages in the CNS. In total, these findings suggest that Treg can mediate recovery from EAE by controlling Teff and macrophage in the draining LN or the CNS, or both.

3.2 Visualizing the Function of Treg in the CNS during aEAE

Intravital two-photon imaging is a powerful tool to visualize cell motility and interactions *in vivo*. This technique has been used in the last few years mainly in pEAE models, to elucidate T cell migration into the CNS and their subsequent reactivation (Bartholomaus et al., 2009; Kawakami et al., 2012; Pesic et al., 2013). Although Treg function in peripheral immune organs has been assessed using *in vivo* or *ex vivo* 2-photo imaging (Mempel et al., 2006; Tang et al., 2006), little is known about their locomotion and function within the CNS during EAE.

3.2.1 Teff and Treg Motility and Contacts with APC in the CNS during aEAE

As shown in the previous section, Treg are constantly present in the CNS of mice during EAE. However, their frequency is highly increased during recovery from the disease, suggesting that CNS-infiltrating Treg could contribute to amelioration of EAE. To shed light on the function of Treg within the inflamed CNS tissue, intravital two-photon imaging in the spinal cord meninges was performed. To visualize both Treg and Teff, Foxp3-GFP.KI mice, in which Treg express GFP, were crossed to T-Red mice, whose T cells express the red fluorescent protein dsRedII. T-Red x Foxp3.GFP.KI mice were immunized with MOG_{35-55} in CFA and intravital two-photon imaging in the CNS was carried out at the onset, peak and recovery. Taken that perivascular and meningeal APC are not only necessary for T cell reactivation within the CNS by cell-to-cell contact (Lodygin et al., 2013; Pesic et al., 2013), but also possible targets of Treg-mediated suppression mechanism, they were also visualized by injection of fluorescent-labeled Dextran in the subarachnoid space. Subsequently, semi-automated tracking of both Teff and Treg was performed in the acquired videos, allowing the calculation of motility parameters, including average track velocity, linearity index and stationary phase (see Material and Methods). In addition, as CNS-infiltrating T cells are known to establish contacts with local APC in order to get reactivated, contact number and duration between T cells and labeled APC were analyzed.

a

onset   peak   recovery

b

onset   peak   recovery

c

d

e

f

g

51
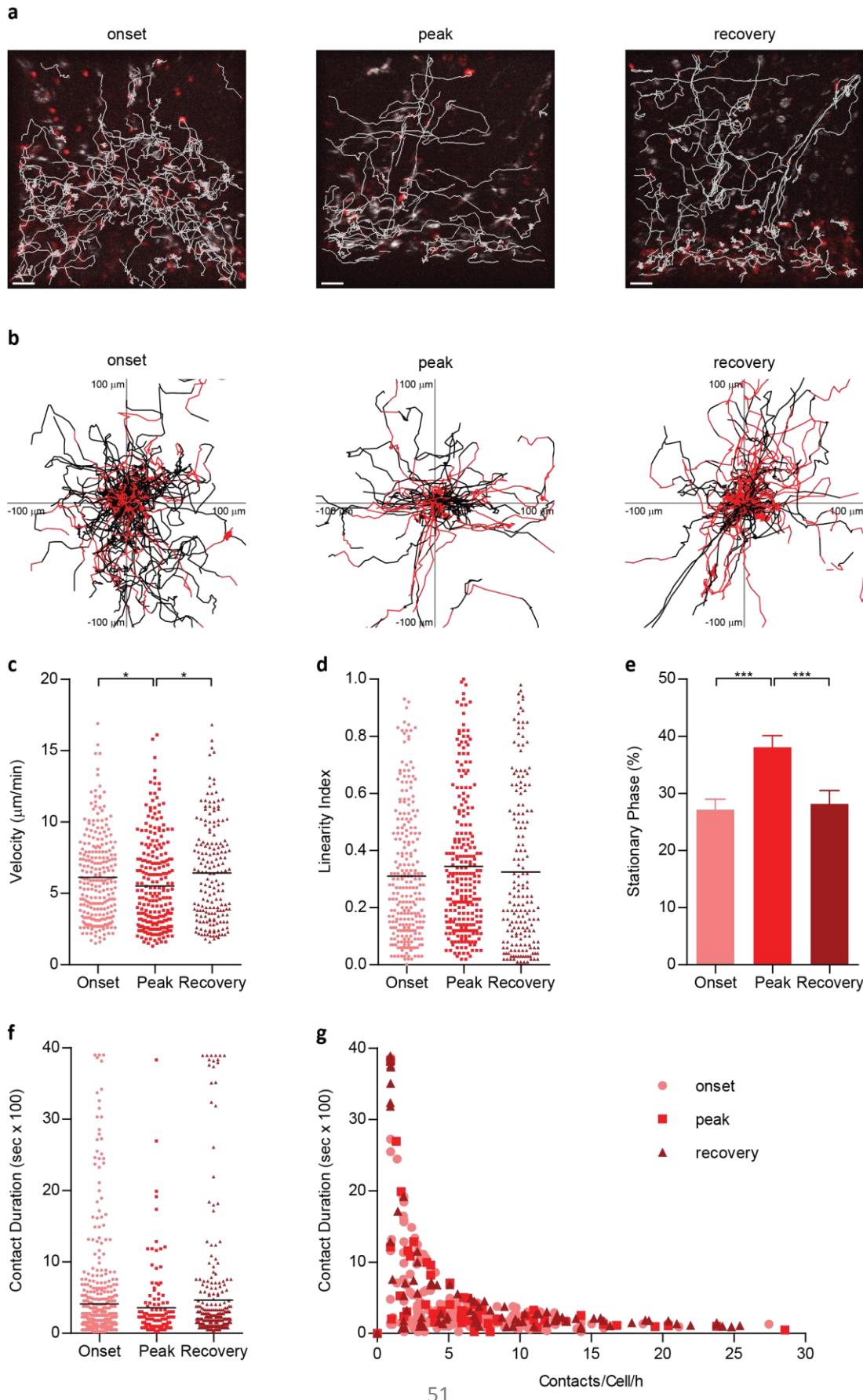
Figure 3.2.1 Teff locomotion and contacts with APC in spinal cord meninges during aEAE. T-Red x Foxp3-GFP.KI mice were immunized with MOG_{35-55} in CFA and intravital two-photon imaging was performed in the spinal cord meninges at onset, peak and recovery. Local APC were labeled by injection of Dextran-Alexa Fluor 680 in the subarachnoid space. **(a)** Trajectories (white lines) of dsRedII[+] Teff overlaid with snapshots from representative videos. One representative out of three independent experiments per disease stage is shown (red: Teff, grey: APC, scale bar: 30 µm) **(b)** Superimposed trajectories of Teff movements during onset, peak and recovery. Timepoints with contacts with APC are indicated in red. One representative out of three independent experiments per disease stage is shown. **(c)** Average velocity, **(d)** linearity index, **(e)** stationary phase (+ s.e.m.) of Teff and **(f)** contact duration with APC during onset, peak and recovery (pooled data from three independent experiments. *$P<0.05$, ***$P<0.001$, 1way ANOVA, Kruskal Wallis test – Dunn's multiple comparison test). **(g)** Scattered plot of Teff-based contacts with APC where each dot represents a single Teff plotted for the number of contacts per hour and average duration of contacts (pooled data from three independent experiments).

Initially, the locomotion of Teff, as well as their contacts with APC between the different phases of EAE were compared, as they could be affected by the differential presence of Treg in the CNS. Surprisingly, the trajectories of Teff did not show major changes during different disease stages (Fig. 3.2.1 a,b). In detail, their velocity was only marginally decreased at the peak, in agreement with the increased percentage of stationary phase at this timepoint (Fig. 3.2.1. c,e). However, these differences were not depicted in the linearity index, which remained unaltered as the disease progressed (Fig. 3.2.1. d). Importantly, the duration and number of Teff – APC contacts were indistinguishable between the three phases of EAE (Fig. 3.2.1 f,g).

Taken together, these observations indicate that Teff motility and contact with APC is not strongly affected neither by the frequency of Treg in the CNS, which is higher during recovery, nor by the changes in the inflammatory milieu during the three distinct phases of EAE.

Next, to elucidate the function of Treg within the CNS, their motility pattern and contacts with local APC at the three phases of EAE were assessed, as APC can be targets of Treg-mediated suppression. Treg showed increased motility at the onset of the disease: their trajectories were more linear (Fig. 3.2.2 a,b), in agreement with higher linearity index (Fig. 3.2.2 d). In parallel, at this timepoint their velocity was higher (Fig. 3.2.2 c), while they also displayed significantly shorter stationary phase (Fig. 3.2.2 e). Additionally, Treg contacted APC for shorter time during this phase (Fig. 3.2.2 f). In sharp contrast, Treg are less motile at the peak of EAE, displaying decreased velocity and increased stationary phase (Fig. 3.2.2 c,d,e). Moreover, Treg formed longer contacts with labeled APC during this phase (Fig. 3.2.2 f). Lastly, Treg showed intermediate motility and contact duration during recovery phase.
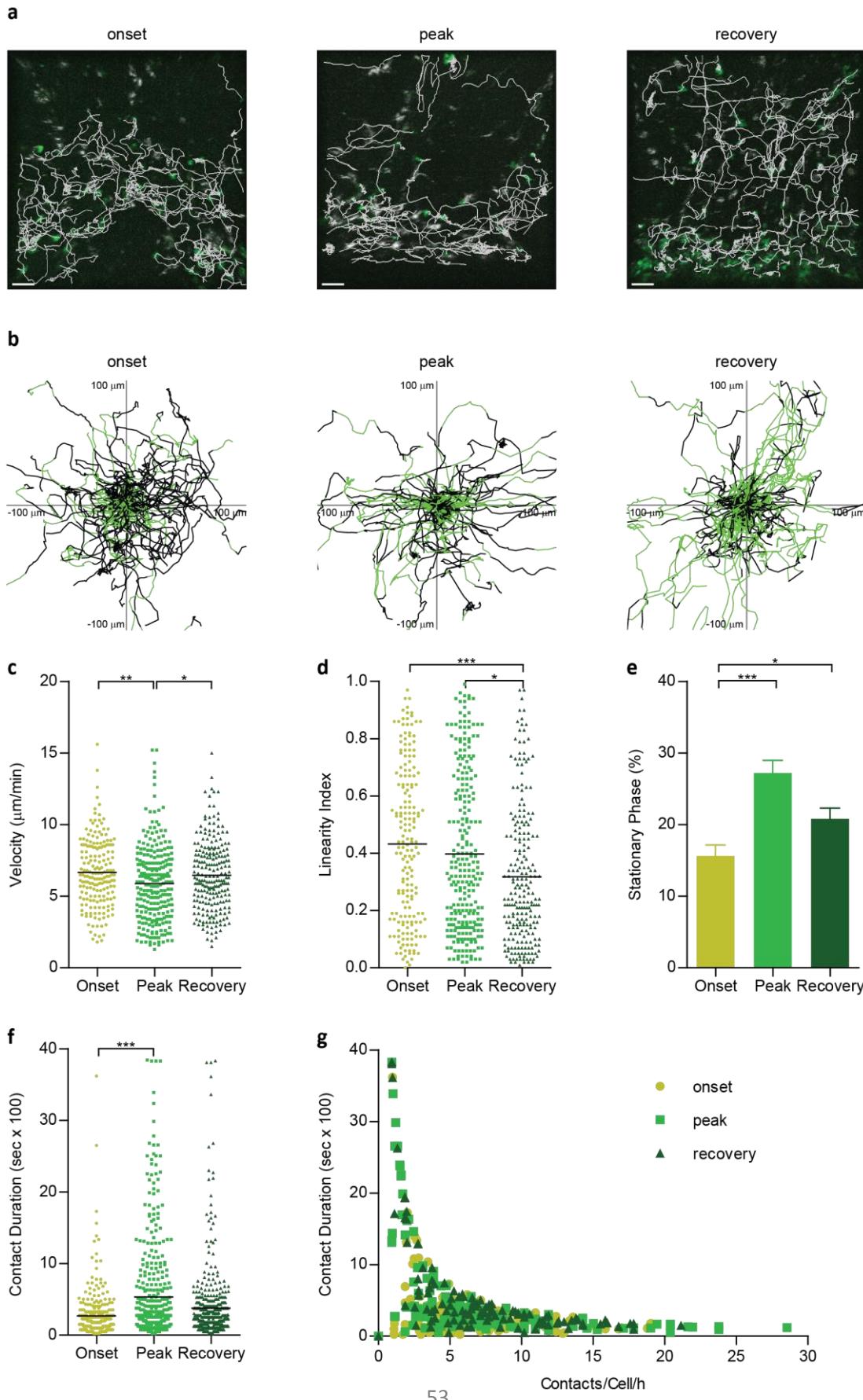
Figure 3.2.2 Treg locomotion and contacts with APC in spinal cord meninges during aEAE. T-Red x Foxp3-GFP.KI mice were immunized with MOG$_{35-55}$ in CFA and intravital two-photon imaging was performed in the spinal cord meninges at onset, peak and recovery. Local APC were labeled by injection of Dextran-Alexa Fluor 680 in the subarachnoid space. **(a)** Trajectories (white lines) of GFP$^+$ Treg overlaid with snapshots from representative videos. One representative out of three independent experiments per disease stage is shown (green: Treg, grey: APC, scale bar: 30 µm) **(b)** Superimposed trajectories of Treg movements during onset, peak and recovery. Timepoints with contacts with APC are indicated in green. One representative out of three independent experiments per disease stage is shown. **(c)** Average velocity, **(d)** linearity index, **(e)** stationary phase (+ s.e.m.) of Treg and **(f)** contact duration with APC during onset, peak and recovery (pooled data from three independent experiments. *P<0.05, **P<0.01, ***P<0.001, 1way ANOVA, Kruskal Wallis test – Dunn's multiple comparison test). **(g)** Scattered plot of Treg-based contacts with APC where each dot represents a single Treg plotted for the number of contacts per hour and average duration of contacts (pooled data from three independent experiments).

As discussed previously, a possible mechanism of Treg-mediated suppression is direct cell-to-cell contact with Teff. Therefore, Treg-Teff contacts within the CNS were analyzed and compared between the different phases of EAE. At the onset of the disease, Treg contacted Teff for shorter time compared to peak and recovery (Fig. 3.2.3 c). In parallel, Treg – Teff interactions were more numerous during this phase (Fig. 3.2.3 b,d). In contrast, Treg-Teff contacts lasted longer during peak and recovery (Fig. 3.2.3 c), while they appear to be less frequent (Fig. 3.2.3 b,d). Thus, on the one hand, these observations further support a restricted role for Treg at the onset of EAE. On the other hand, they indicate that Teff can be also a target for Treg-mediated suppression in the CNS.

In total, provided that Treg suppress immune reactions by direct contact with APC, these findings suggest a limited role for Treg within the CNS during the onset of EAE, when higher motility and shorter contacts with APC were observed. On the opposite, Treg displayed decreased motility and prolonged interactions with APC at the peak, indicating that they could exert their suppressive function during this phase of the disease.
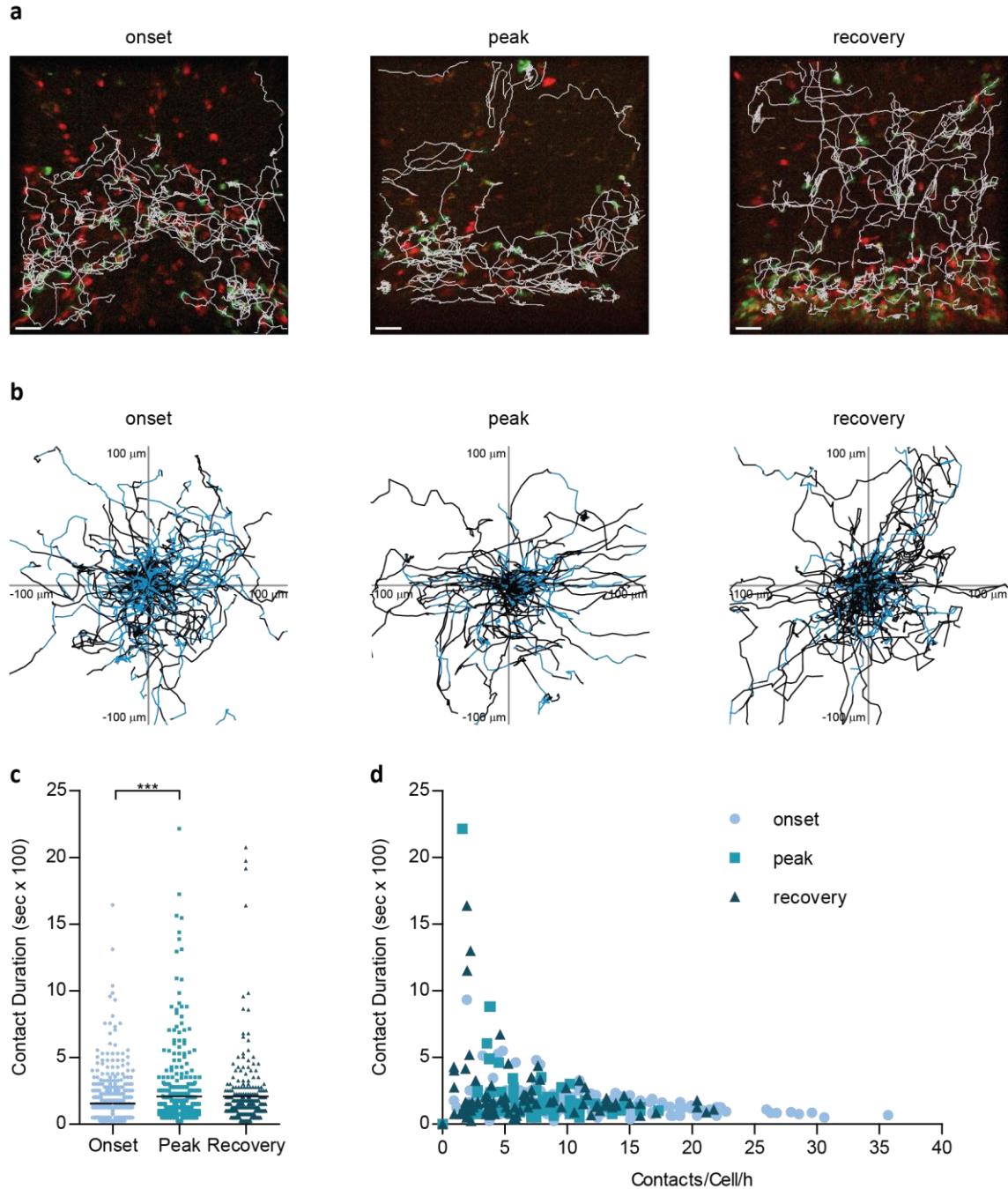
Figure 3.2.3 Contacts between Treg and Teff in spinal cord meninges during aEAE. T-Red x Foxp3-GFP.KI mice were immunized with MOG$_{35-55}$ in CFA and intravital two-photon imaging was performed in the spinal cord meninges at onset, peak and recovery. **(a)** Trajectories (white lines) of GFP$^+$ Treg overlaid with snapshots from representative videos. One representative out of three independent experiments per disease stage is shown (green: Treg, red: Teff, scale bar: 30 µm) **(b)** Superimposed trajectories of Treg movements during onset, peak and recovery. Timepoints with contacts with Teff are indicated in cyan. One representative out of three independent experiments per disease stage is shown. **(c)** Contact duration between Treg and Teff during onset, peak and recovery (pooled data from three independent experiments. ***$P<0.001$, 1way ANOVA, Kruskal Wallis test – Dunn's multiple comparison test). **(d)** Scattered plot of Treg-based contacts between Treg and Teff where each dot represents a single Treg plotted for the number of contacts per hour and average duration of contacts (pooled data from three independent experiments).

3.2.2 Treg Depletion Effect on Teff Motility and Contacts with APC in the CNS

Intravital two-photon imaging studies have shown that, in the absence of Treg, CD4$^+$ T cells formed prolonged contacts with DC in lymph nodes (Tadokoro 2006, Tang 2006). In order to examine how the ablation of Treg can affect the motility pattern of Teff and their contacts with local APC within the CNS in aEAE model, T-Red mice were crossed to DEREG mice, which allow effective depletion of Treg by treating with DTx. Subsequently, T-Red x DEREG mice were immunized with MOG$_{35-55}$ in CFA and treated with DTx or PBS on day 4 after the EAE onset. Two days after the treatment, intravital two-photon imaging was carried out in the spinal cord meninges. Local APC were again visualized by injection of fluorescent-labeled Dextran in the subarachnoid space.

Semi-automated cell tracking of the dsRedII$^+$ Teff revealed that in the DTx-treated mice, Teff displayed more confined trajectories compared to the PBS-treated mice (Fig. 3.2.4 a,b). Indeed, analysis of the Teff tracks showed that, in the spinal cord of Treg-depleted mice, Teff moved significantly slower than in PBS control group (Fig. 3.2.4 c). In addition, the linearity index of Teff was decreased, while the stationary phase of the cells increased (Fig. 3.2.4 c,d). However, no difference was observed in the contact duration, or number, of Teff with APC between the two groups (Fig. 3.2.4 f,g). Thus, Treg depletion did not seem to affect the Teff-APC contacts in the CNS. Nevertheless, in DTx-treated mice, many Teff trajectories appeared to be stationary but not accompanied by contact with APC (Fig. 3.2.4 b left), indicating possible interactions either with non-labeled APC or other cell types or structures within the spinal cord meninges.

In conclusion, Treg depletion led to decreased Teff motility without changing contact duration with APC within the CNS. These observations suggest that, on the one hand, the primary target of Treg-mediated suppression is not the APC, but the Teff; on the other hand, the site where Treg control the autoimmune response may not be within the CNS, but rather in the peripheral immune organs. Nonetheless, the possibility that Teff establish contacts with non-labeled APC or other cell types and structures within the CNS, in the absence of Treg, cannot be excluded. Alternatively, the decreased motility of Teff following Treg depletion could be attributed to the highly inflammatory environment in the spinal cord of DTx-treated mice.
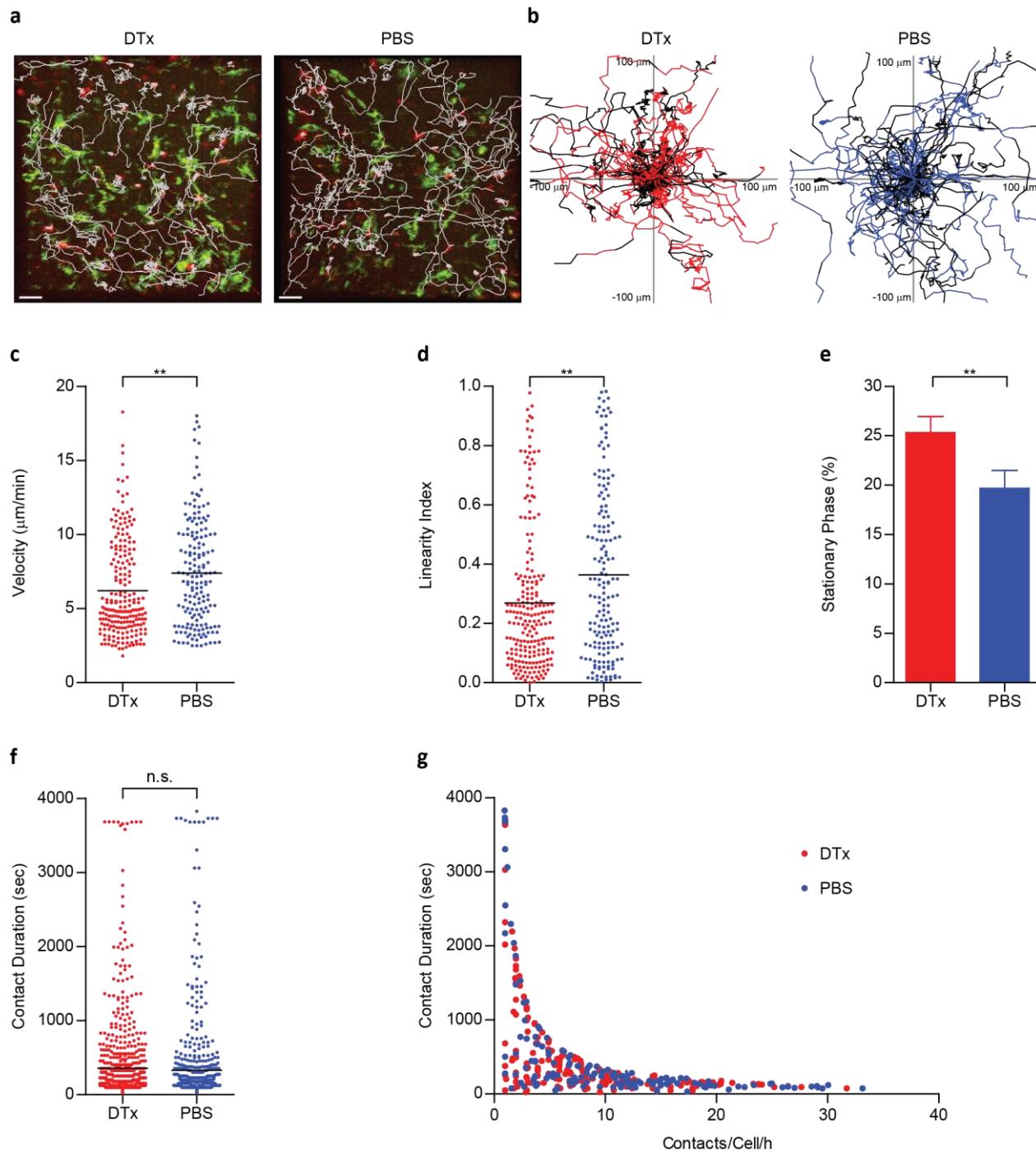
Figure 3.2.4 Effect of Treg depletion on Teff motility and contacts with APC in spinal cord meninges during aEAE. T-Red x DEREG mice were immunized with MOG_{35-55} in CFA and treated with DTx or PBS four days after the EAE onset. Two days later, intravital two-photon imaging was performed in the spinal cord meninges. Local APC were labeled by injection of Dextran-FITC in the subarachnoid space. **(a)** Trajectories (white lines) of dsRedII[+] Teff overlaid with snapshots from representative videos. One representative out of three independent experiments per treatment condition is shown (red: Teff, grey: APC, scale bar: 30 μm) **(b)** Superimposed trajectories of Teff movements after DTx or PBS treatment. Timepoints with contacts with APC are indicated in red or blue, respectively. One representative out of three independent experiments per treatment condition is shown. **(c)** Average velocity, **(d)** linearity index, **(e)** stationary phase (+ s.e.m.) of Teff and **(f)** contact duration with APC after DTx or PBS treatment (pooled data from three independent experiments. **P<0.01, t-test). **(g)** Scattered plot of

Teff-based contacts with APC where each dot represents a single Treg plotted for the number of contacts per hour and average duration of contacts (pooled data from three independent experiments).

3.3   Treg in Spontaneous Opticospinal EAE

3.3.1 Treg Frequency, Ag-specificity and Suppressive Capacity in OSE mice

Spontaneous EAE (sEAE) models provide an ideal setup to examine disease triggers compared to induced EAE models, as they do not require inoculation with adjuvants or transfer of *ex vivo* stimulated T cells. The Opticospinal Spontaneous EAE (OSE) mouse is one of the sEAE models that were developed in our lab (Bettelli et al., 2006a; Krishnamoorthy et al., 2006). OSE mice develop sEAE with 50 % incidence, raising the question whether Treg contribute to the disease resistance in 50% of transgenic mice.

In order to address this question, frequency and Ag-specificity of Treg in the spleen of sEAE-affected and resistant OSE mice were compared using flow cytometry. Interestingly, both sEAE-affected and resistant OSE mice harbored similar percentage of Treg within the CD4^{+} T cell compartment (Fig. 3.3.1 a left). Additionally, the frequency of MOG-specific (Vα.3.2^{+}Vβ11^{+}) Treg was not different in OSE mice with sEAE compared to healthy animals (Fig. 3.3.1. a right).
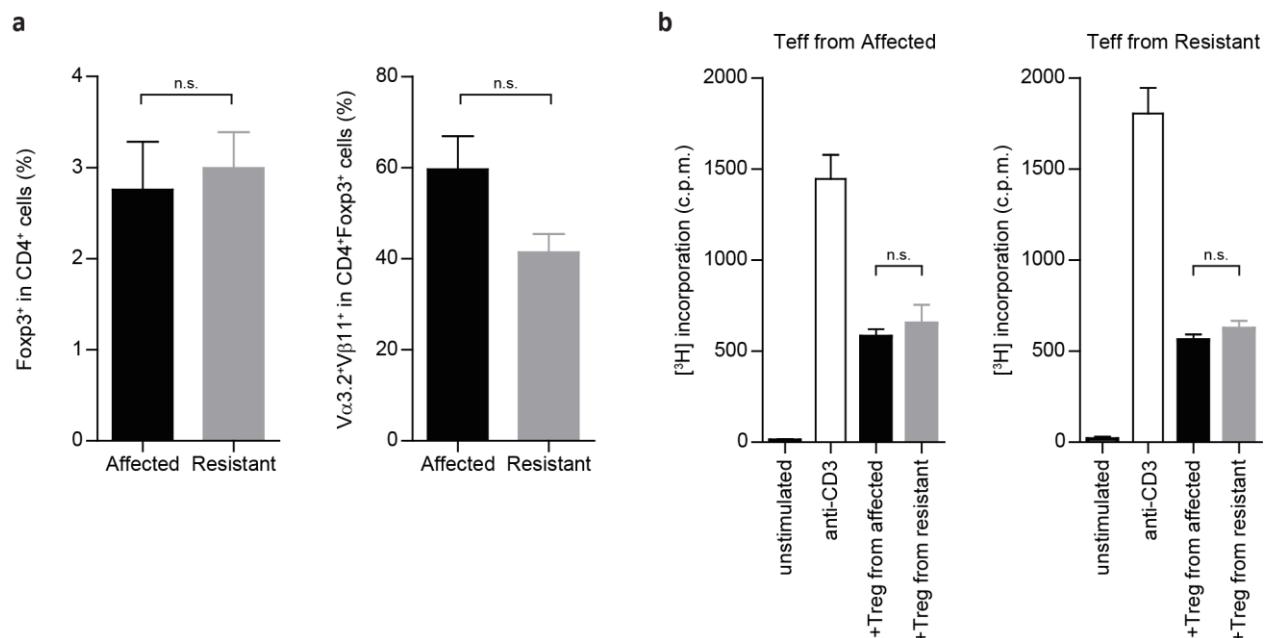

Figure 3.3.1 Treg frequency, Ag-specificity and suppressive capacity in spleen of affected or resistant OSE mice. **(a)** Mean frequency (+ s.e.m.) of Foxp3^{+} in CD4^{+} T cells (left) or Vα3.2^{+}Vβ11^{+} in Foxp3^{+}CD4^{+} T cells (right) (*n*=8-11 mice per group, pooled data from five independent experiments) (n.s. = not significant, *t*-test) **(b)** GFP^{+} and GFP^{-} cells were isolated from splenocytes of affected or resistant OSE x Foxp3-GFP.KI mice (*n*=4 mice per group) and CD4^{+} T cells were purified. 10^5 Teff were cultured with or without 5×10^4 Treg in the presence of 2×10^5 irradiated syngeneic splenocytes as APC and anti-CD3e Ab. After 48 hours, ^{3}H-labeled thymidine was added and its incorporation was measured 16 hours later. Mean ^{3}H incorporation is indicated as c.p.m. (+ s.e.m) in triplicated wells (n.s. = not significant, *t*-test between "+Treg from affected" & "+Treg from resistant groups").

Next, the suppressive capacity of Treg from affected and resistant OSE mice was examined by co-culturing Treg with Teff from both groups. Treg from both groups suppressed Teff proliferation to the same extent (Fig. 3.3.1 b).

In summary, no difference was detected in the proportion, Ag-specificity and suppressive capacity of spleen-isolated Treg between affected and resistant OSE. Taken together, these results suggest that susceptibility of OSE mice to sEAE is correlated neither with reduced frequency and Ag-specificity, nor with defective suppressive function of peripheral Treg.

3.3.2 MOG-specific Treg Accumulation in the CNS during sEAE

As shown earlier, Treg accumulate in the CNS of MOG-immunized mice during the recovery phase (Fig. 3.1.1). However, little is known about Treg frequencies in both the periphery and the CNS during sEAE. Therefore, a kinetic experiment analyzing the percentages of Treg in CD4$^+$ T cell population in spleen and spinal cord of OSE mice by flow cytometry was performed. Interestingly, while Treg levels in the spleen of OSE mice were low, independently of the disease phase, Treg were abundant in the spinal cord already from the onset of sEAE. Importantly, the frequency of Treg in the spinal cord paralleled the clinical score of affected OSE mice (Fig. 3.3.2 a). In addition, while in spleen only around half of Treg express the transgenic MOG-specific TCR, most of the Treg infiltrating the spinal cord are expressing MOG-specific TCR (Fig. 3.3.2 b). Thus, the accumulation of MOG-specific Treg in the spinal cord of affected OSE mice indicates a possible role for this T cell subset in this sEAE model like MOG-induced EAE.
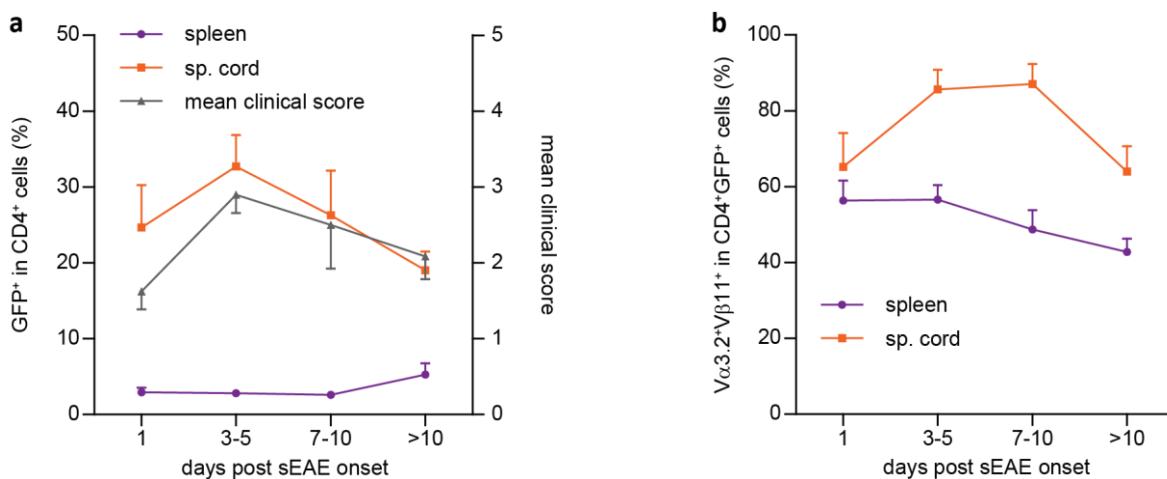

Figure 3.3.2 MOG-specific Treg accumulation in the CNS during sEAE. OSE x Foxp3-GFP mice were sacrificed at different time points after the onset of sEAE and lymphocytes were isolated from spleen and spinal cord. **(a)** Mean clinical score (+ s.e.m., grey line) and mean frequency (+ s.e.m.) of Foxp3$^+$ (GFP$^+$) in CD4$^+$ T cells (Treg) in spleen

(purple line) and spinal cord (orange line) (n=4-9 mice per time point). **(b)** Mean frequency (+ s.e.m.) of Vα3.2$^+$Vβ11$^+$ in CD4$^+$GFP$^+$ T cells in spleen (purple line) and spinal cord (orange line) (n=4-9 mice per group).

3.3.3 Chronic DTx Treatment in OSE x DEREG mice Increases sEAE Incidence

Depletion of the Treg population in OSE mice could be used to evaluate the role of Treg in sEAE incidence and severity. For this reason, OSE mice were crossed with DEREG mice and OSE x DEREG mice were treated with DTx (or PBS as control) twice per week, starting from the age of 21 days - prior to the earliest onset of sEAE – until the age of 52 days (Fig. 3.3.3 a). During the five-week treatment period, the efficiency of Treg depletion was analyzed twice by quantification of CD4$^+$GFP$^+$ T cells in blood using flow cytometry. Indeed, GFP$^+$ cell population was drastically reduced in the blood of DTx-treated mice (Fig. 3.3.3 b).
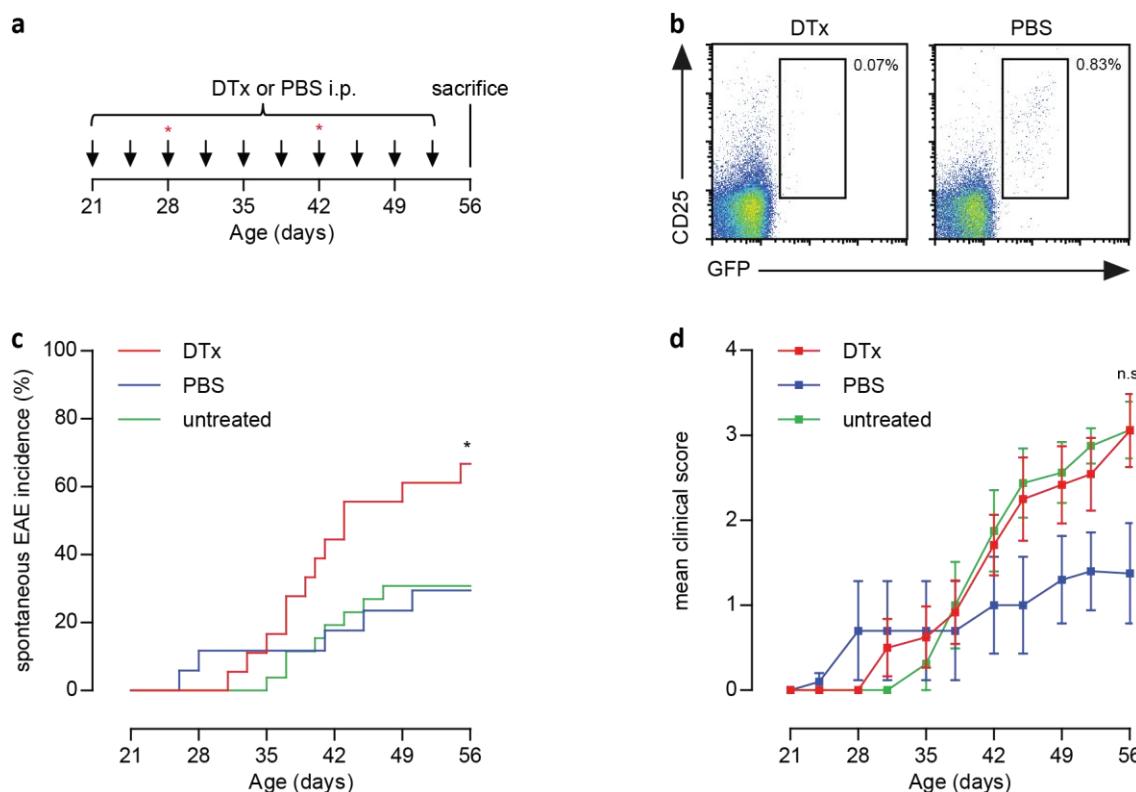
Figure 3.3.3 Increased sEAE incidence in chronically DTx-treated OSE x DEREG mice. (a) Schematic representation of experimental setup. Arrows indicate treatment with DTx (100 ng in 100 µl) or PBS (100 µl). Red asterisks indicate testing the efficiency of Treg-depletion in blood. **(b)** Representative flow cytometry plots of CD4$^+$ gated lymphocytes isolated from blood of 28-days old OSE x DEREG mice. Treg population and its percentage within the CD4$^+$ compartment are indicated. **(c)** Incidence of spontaneous EAE in DTx- (n=18) and PBS-treated (n=17), as well as untreated (n=27) OSE x DEREG mice (*P<0.05, Log-rang (Mantel-Cox) Test). **(d)** Mean clinical score (+ s.e.m.) in DTx- (n=12) and PBS-treated (n=5), as well as untreated (n=8) OSE x DEREG mice. (n.s. = not significant, 2way ANOVA between all groups)

Intriguingly, OSE x DEREG mice treated chronically with DTx showed significantly increased sEAE incidence compared to the PBS treated or untreated animals (Fig. 3.3.3 c), suggesting that Treg are necessary for preventing spontaneous disease. However, the mean clinical score of DTx-treated OSE x DEREG mice was similar to those of the control groups (Fig. 3.3.3 d), indicating that Treg depletion does not affect the sEAE severity.

3.3.4 Effect of DTx Treatment on Splenocyte Composition and Cytokine Production

In order to evaluate the effect of chronic DTx treatment on the immune system of OSE x DEREG mice, the splenocyte composition was examined by staining with cell lineage markers and analyzed using flow cytometry. No differences were observed in the percentages of CD4$^+$ cells or dendritic (CD11c$^+$) cells, while a marginal increase in frequencies of CD8$^+$ cells and macrophages (CD11b$^+$) was found in DTx-treated mice. In contrast, B (B220$^+$) and NK (NKp46$^+$) cell frequencies were significantly reduced in spleens of DTx-treated mice (Fig. 3.3.4 a).
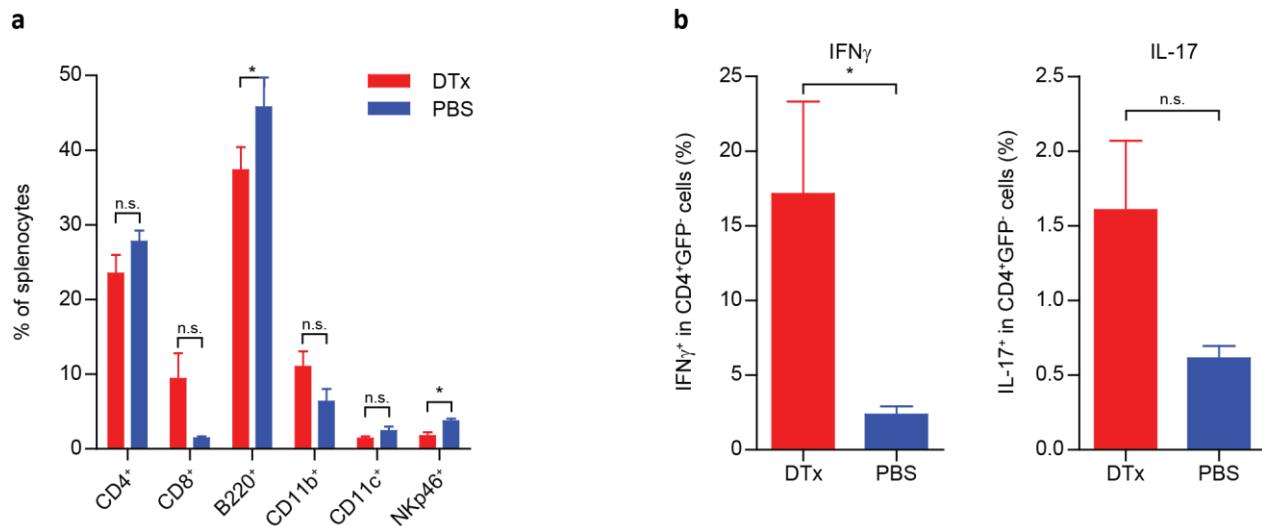
Figure 3.3.4 Effect of chronic DTx treatment on splenocyte populations and cytokine-production profile in OSE x DEREG mice. OSE x DEREG mice were chronically treated with DTx or PBS (as shown in Fig. 3.3.3 a) and sacrificed after 5 weeks of treatment. Splenocytes were isolated and analyzed by flow cytometry. **(a)** Mean frequency (+ s.e.m.) of indicated immune cell populations in total splenocytes (*n*=5 mice per group). **(b)** Mean frequency (+ s.e.m.) of IFNγ$^+$ or IL-17$^+$ in CD4$^+$Foxp3$^-$ T cells (*n*=3-4 mice per group). (*$P<0.05$, *t*-test)

Next, the cytokine production of splenic Teff following chronic DTx treatment was assessed by flow cytometry. Interestingly, DTx treated OSE x DEREG mice showed significantly increased frequency of IFNγ$^+$ Teff, as well as marginally elevated levels of IL-17$^+$ Teff (Fig. 3.3.4 b). This finding suggests that Treg

depletion affects the cytokine production by splenic Teff, pointing again to a modulatory role of Treg in the spleen of OSE mice.

3.3.5 Incomplete Treg Depletion after Chronic DTx Treatment

The efficiency of Treg depletion by DTx treatment was initially tested by quantification of the GFP$^+$ T cells in OSE x DEREG mice. However, Foxp3 staining revealed the unexpected presence of a significant proportion of Foxp3$^+$GFP$^-$ Treg in mice treated chronically with DTx (Fig. 3.3.5 a). In contrast, this population is absent in mice which received only a single injection of DTx (Fig. 3.3.5 b). The presence of Foxp3$^+$GFP$^-$ Treg in OSE x DEREG mice can be attributed to downregulation of the transgene in a minor fraction of Foxp3$^+$ cells. When OSE x DEREG mice are treated chronically with DTx, the Foxp3$^+$GFP$^-$ Treg population presumably expands homeostatically, due to the complete and continuous absence of GFP$^+$Foxp3$^+$ Treg. Therefore, the aforementioned findings in the present section, from experiments where chronic DTx treatment was performed, should be interpreted carefully, as Treg were absent only in the initial phase of the treatment and not until the end of the experimental procedure.
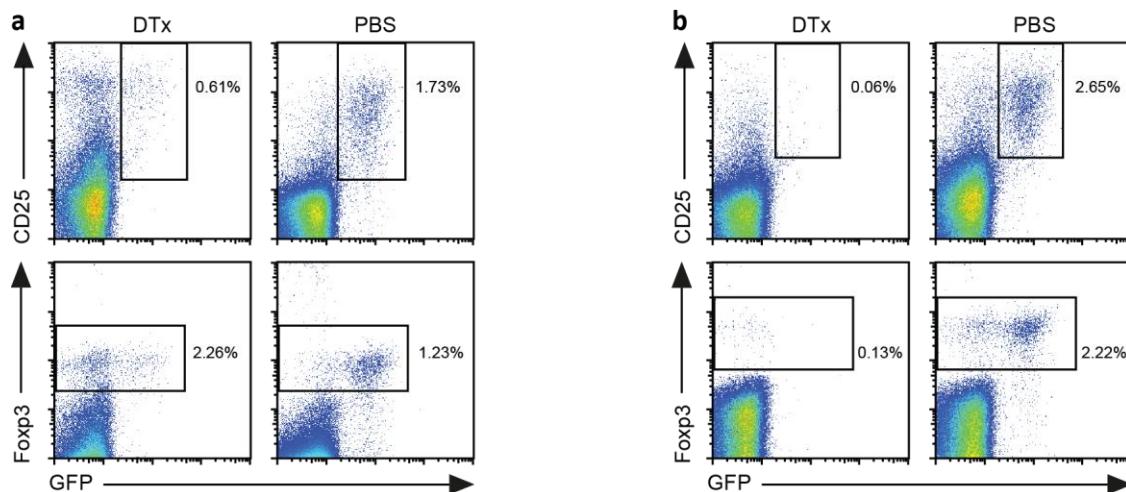

Figure 3.3.5 Incomplete Treg depletion after chronic treatment with DTx in OSE x DEREG mice. Representative flow cytometry plots of CD4$^+$ gated lymphocytes isolated from inguinal lymph nodes of OSE x DEREG mice treated **(a)** for 5 weeks (as shown inf Fig. 3.3.3 a) or **(b)** one day with DTx or PBS.

DISCUSSION

4.1 Treg Depletion during aEAE

The role of Treg in various EAE models has been investigated in several studies, by following two main approaches: On the one hand, transfer of Treg has been shown to ameliorate EAE or enhance recovery from the disease in both active and passive EAE models (Fransson et al., 2012; Kohm et al., 2002; McGeachy et al., 2005; Mekala and Geiger, 2005; Stephens et al., 2009; Yu et al., 2005; Zhang et al., 2004). On the other hand, ablation of Treg population has been attempted by different groups by treatment with anti-CD25 antibody and resulted in exacerbation of EAE (Gartner et al., 2006; McGeachy et al., 2005; Montero et al., 2004; Zhang et al., 2004). However, a major drawback of the latter approach is that CD25 is not a Treg-specific marker, but is also expressed by activated T cells. Thus, anti-CD25 treatment could eliminate not only the Treg population, but also a crucial fraction of Teff, making the interpretation of these findings rather complicated.

The generation of mice with Foxp3-dependent expression of diphtheria toxin receptor, such as DEREG (Lahl et al., 2007) and Foxp3-DTR (Kim et al., 2007), allows precise targeting and specific depletion of Treg. In these studies, the importance of Treg for the maintenance of immune homeostasis and prevention of autoimmunity throughout the lifespan was convincingly shown. Therefore, these mice appear to be ideal for evaluating also the contribution of Treg to suppression of EAE.

Indeed, DEREG mice were used in the present study. Treatment with DTx was performed at the peak of the EAE (four days after the onset). This timepoint was chosen because first, as Treg frequency in the CNS of mice with EAE has been reported to be increased at the recovery from the disease (a finding confirmed in this study), ablation of Treg during this phase was of great interest. Second, depletion of Treg shortly before the onset of EAE did not affect the course of the disease (data not shown). Third, DTx treatment combined with CFA and PTx injection proved to have lethal effects (Meyer Zu Horste et al., 2010). Interestingly, treatment of MOG_{35-55}-immunized DEREG mice with DTx at the peak of EAE not only prevented recovery from the disease, but also led to exacerbated and, eventually, fatal EAE. This finding strongly suggests that an intact Treg population is indispensable for recovery from EAE.

In order to identify the mechanism of EAE exacerbation following Treg depletion, EAE-affected DEREG mice were sacrificed two days after treatment with DTx or PBS, thus before the difference in their clinical score became prominent. As Teff are considered to be main targets of the Treg-mediated suppression (see 1.4.2), the effect of Treg depletion on numbers, activation status and cytokine production of Teff

population was investigated. Importantly, highly increased numbers of Teff were observed in axillary and inguinal lymph nodes (LN), the draining lymph nodes of the immunization site, as well as in the spinal cord of Treg-depleted mice. This could be attributed to either higher proliferation rate or enhanced migration of Teff in the absence of Treg. However, no difference in the frequency of proliferating (Ki-67$^+$) Teff was found between DTx- and PBS-treated mice (data not shown). In addition, blood-circulating Teff from both DTx- and PBS-treated mice expressed similar levels of integrin α4 chain (data not shown), that together with β1 compose VLA-4, suggesting that the increased Teff numbers in the CNS were not a result of higher expression of this migratory molecule. In addition, Teff from Treg-depleted mice were expressing similar levels of activation markers, such as CD25 and CD44, compared to Treg-intact animals, indicating that Treg do not modulate the activation status of Teff. In contrast, the cytokine profile of Teff was altered in the absence of Treg. Indeed, more IFNγ$^+$ Teff were detected in LN and spinal cord of Treg-depleted mice, accompanied with augmented levels of IFNγ mRNA. In parallel, increase in IL-17$^+$ Teff numbers and IL-17 mRNA levels was observed in the spinal cord of DTx-treated mice. In conclusion, specific depletion of Treg during EAE resulted in significantly elevated numbers of Teff and enhanced proinflammatory cytokine production by Teff, both in periphery and the CNS, which is highly likely to contribute to the exacerbated pathology that follows.

Apart from Teff, macrophages are also possible targets of the Treg-mediated suppression, as they can act as APC (see Introduction – Treg Suppression Mechanisms). Therefore, the effect of Treg ablation on macrophage number and phenotype was examined. Notably, spinal cord infiltrates of Treg-depleted mice displayed higher numbers of macrophages, as early as two days after treatment with DTx. As macrophages are major mediators of EAE pathology, by damaging oligodentrocytes and myelin sheath or causing directly neuronal toxicity (Herz et al., 2010), their enhanced presence in the spinal cord can further confer to the increased disease severity following ablation of Treg.

Macrophages can undergo two different forms of activation, resulting in distinct functional phenotypes: On the one hand, classical activation by TLR ligands and IFNγ leads to M1 phenotype, classified by the production of proinflammatory cytokines and reactive nitrogen and oxygen species that promotes Th1 reactions. On the other hand, alternative activation by IL-4/IL-13 results in M2 phenotype, which is characterized by production of ornithine and polyamines through the arginase pathway, expression of mannose and galactose receptors and enhanced phagocytic capacity. While M1 macrophages are associated with promoting and sustaining of inflammation, M2 macrophages are considered to exert immunoregulatory functions (Sica and Mantovani, 2012a). Intriguingly, Treg-depleted mice showed decreased frequency of mannose-receptor (CD206) expressing macrophages in their LN. Therefore, this

observation indicates that Treg ablation could lead to diminished levels of M2-like macrophages in LN that could also contribute to the exacerbated pathology. In contrast, the expression of M1-related markers, including CD80/CD86, MHC II, IFNγ and TNFα, was not altered in macrophages from Treg-depleted animals. Nevertheless, the possibility that the elevated number of macrophages observed in Treg-depleted mice could occur as a result of increased levels of cell debris cannot be excluded. However, the decreased frequency of mannose-receptor expressing macrophages in LN of these animals does not point to this direction, as M2 macrophages are implicated in clearance of dead cells.

In summary, specific depletion of Treg at the peak of EAE prevented recovery and led to exacerbated and, eventually, fatal disease, underlying the protective role of Treg during EAE. Treg-depleted mice were found to have increased number of Teff in both LN and spinal cord, accompanied with elevated levels of proinflammatory cytokines, such as IFNγ and IL-17. In parallel, ablation of Treg led to higher numbers of macrophages in spinal cord infiltrates, combined with reduced frequency of M2-like macrophages in LN. Thus, during EAE, Treg can act on Teff, limiting their expansion and production of proinflammatory cytokines, as well as on macrophages, restricting their presence in the CNS and possibly promoting M2 phenotype in periphery.

4.2   Visualizing the Function of Treg in the CNS during aEAE

Although Treg have been in the focus of scientific research in the last decade, our knowledge on the mechanisms they use to suppress autoimmune responses is rather limited and derives mainly from *in vitro* studies. Moreover, whether Treg exert their suppressive function within the target tissue of the autoimmune response or in peripheral immune organs, has not been answered to date. Intravital two-photon imaging allows visualization of direct cell-to-cell contacts of Treg with Teff or APC that could shed light on the *in vivo* function of Treg.

An indispensable step for T cell activation is direct contact with an APC, which allows the productive recognition of the target antigen through the immune synapse (Henrickson and von Andrian, 2007). Specifically within the CNS, reactivation of T cells has been shown to occur via interaction with perivascular and meningeal APC and this procedure is a prerequisite for initiation of the autoimmune response (Kawakami et al., 2012; Lodygin et al., 2013; Pesic et al., 2013). Earlier reports using two-photon microscopy have demonstrated that CD4$^+$CD25$^-$ T cells established longer contacts with DC in lymph node in the absence of Treg (defined as CD4$^+$CD25$^+$ T cells), while in Treg-sufficient environment these contacts were inhibited (Tadokoro et al., 2006; Tang et al., 2006). In fact, the latter study was carried out in an autoimmunity setup, where imaging of islet-specific Teff cells and DC bearing antigens

derived from pancreas was performed in the pancreatic lymph nodes of non-obese diabetic mice. Therefore, Treg can suppress an autoimmune response in the draining lymph nodes of the target organ, by restricting prolonged interactions between Teff and DC. In contrast, direct cell-to-cell interactions between Treg and Teff (Tang et al., 2006) or CTL (Mempel et al., 2006), have been proven unnecessary for suppressing the immune reaction.

In the present study, the motility pattern of Treg, as well as and their interactions with Teff and APC during the EAE course within the CNS, were examined. The higher velocity and linearity index of Treg at the onset, combined with shorter stationary phase as well as contacts with APC and Teff, strongly suggest a limited role for Treg at this phase of the disease. In contrast, Treg appeared to be slower, more confined and stationary at recovery and, mainly, at the peak of EAE, when they also displayed prolonged interactions with APC and Teff. These observations indicate that, firstly, Treg are likely to exert their suppressive function in the CNS predominantly at the peak of EAE. Secondly, since Treg formed stable contacts with APC, importantly of similar duration to Teff – APC contacts, local APC can be targets of their suppressive function. Unfortunately, in the experimental setup used, the distinction between relevant and irrelevant interactions is not possible, as the CNS-infiltrating T cells are polyclonal. This would require T cells of defined antigen-specificity, as well as APC loaded with known antigen. Thirdly, as Treg interacted also with Teff, the possibility that they can directly suppress Teff by cell-to-cell contact cannot be excluded.

Since Treg frequency in the CNS increases during recovery from EAE, Teff motility pattern and contacts with APC were also assessed throughout the disease. Interestingly, velocity and linearity index of Teff did not show striking variations between the different phases of EAE. A previous study has demonstrated higher velocity during remission, in case of adoptive transfer of polyclonal Teff, and lower velocity during peak, when 2D2 Teff were transferred, although the linearity index values did not vary accordingly (Siffrin et al., 2010). However, these discrepancies could be attributed to the differences in the experimental models (aEAE versus pEAE) and the imaged region (spinal cord meninges versus brain stem). Nevertheless, while Teff appeared to be more stationary at the peak, the Teff – APC contact duration and frequency was undistinguishable between the three phases of EAE. Therefore, the enhanced presence of Treg in the CNS during recovery did not affect the motility pattern of Teff and their interactions with APC. Additionally, the behavior of Teff is similar to Treg in all the phases, suggesting that inflammatory status is most likely the reason for the slowing of Teff at the peak.

As Treg depletion at the peak of EAE prevented recovery and increased dramatically the severity of the disease, the motility pattern of Teff and their interactions with APC following Treg ablation were

investigated. Intravital two-photon imaging in the spinal cord meninges, carried out two days after Treg depletion in mice with EAE, revealed decreased velocity and linearity index of Teff, combined with increased stationary phase. In other words, Teff were slower and more confined in the absence of Treg. Surprisingly, however, no difference was observed in the Teff – APC contact duration in the spinal cord meninges of Treg-deficient and Treg-intact mice. This finding indicates that preventing contacts between Teff and perivascular and meningeal APC is not the major mechanism that Treg use to suppress the ongoing inflammation in the CNS. Thus, the decreased motility of Teff following ablation of Treg population could be attributed to longer interactions between Teff and other cell types or structures in the CNS. For example, Th17 cells have been reported to contact neurons directly and cause axonal damage during EAE (Siffrin et al., 2010). Alternatively, the change in the Teff motility in the CNS of Treg-depleted mice could occur due to the enhanced inflammation observed in these mice, characterized by higher levels of proinflammatory cytokines and increased numbers of macrophages in spinal cord.

However, technical limitations of the experimental model and setup used in this study should be also taken into consideration during the interpretation of the results. For example, incomplete labeling of perivascular and meningeal APC cannot be excluded, as they are only visualized by incorporation of fluorescently-conjugated Dextran which is injected in the subarachnoid space prior to imaging. Thus, APC with lower phagocytic activity would not be visible. Moreover, in this model, Teff are polyclonal and not specific for a defined antigen. Therefore, the myelin-reactive Teff would be only a fraction of the CNS-infiltrating Teff population, which means that an important percentage of the observed Teff – APC interactions can be unspecific.

Nevertheless, CNS-infiltrating Treg have been demonstrated to be unsuccessful in suppressing MOG-specific Teff isolated also from the CNS of mice with EAE (Korn et al., 2007). Therefore, a scenario where Treg do not suppress the ongoing inflammation within the CNS, but rather in peripheral immune organs, could also explain the undistinguishable Teff – APC contact duration and numbers in the spinal cord meninges during presence or absence of Treg. To answer this, intravital imaging in the inguinal and axillary lymph nodes, that drain the immunization site, or the cervical lymph nodes, that drain the CNS, should be performed.

In summary, intravital two-photon imaging in the spinal cord meninges during different phases of EAE revealed increased motility of Treg, accompanied with shorter interactions with APC and Teff, at the onset. In contrast, Treg displayed lower motility and prolonged contacts with APC and Teff mainly at the peak of EAE, suggesting that they exert their suppressive function during this phase. Specific depletion of Treg at the peak resulted in decreased motility of Teff in the CNS, although the Teff – APC contact

duration and frequency was not affected. Therefore, either preventing interactions of Teff with CNS-located APC is not a main mechanism that Treg use to suppress EAE or the control of the autoimmune response by Treg occurs in peripheral immune organs.

4.3   Treg in Spontaneous Opticospinal EAE

As discussed earlier, the primary function of Treg that has been described is the prevention of autoimmunity (Sakaguchi et al., 1995). In fact, patients suffering from IPEX syndrome, as well as scurfy mice, bear mutations in the *Foxp3* gene that lead to the dysfunction of Treg (Bennett et al., 2001; Brunkow et al., 2001; Chatila et al., 2000). In addition, specific depletion of Treg in neonatal or adult mice leads to terminal multi-organ autoimmunity (Kim et al., 2007; Lahl et al., 2007). However, although IPEX syndrome patients, scurfy mice and Treg-depleted animals display severe autoimmune responses in several tissues, the CNS remains, intriguingly, spared.

The factors that contribute to triggering of autoimmune responses can be better studied in spontaneous disease models, which are independent of injection of immune adjuvants. In a sEAE model of MBP-specific TCR transgenic mice in RAG-1-deficient background, the susceptibility to spontaneous disease has been attributed to the lack of Treg, (Olivares-Villagomez et al., 1998). Importantly, adoptive transfer of Treg to those mice prevents the development of sEAE (Hori et al., 2002). Thus, Treg can protect the CNS from autoimmune attack, even in the presence of myelin-specific T cells.

In the OSE mouse model, MOG-specific T cells and B cells cooperate to induce sEAE to 50% of the animals, with the remaining 50% being resistant to spontaneous disease. Therefore, the possible contribution of Treg in the resistance to sEAE was investigated. Initially, the frequency and Ag-specificity of Treg in the spleens diseased and resistant OSE mice were compared. Interestingly, both OSE mice with sEAE and healthy animals displayed similar frequency of Treg and MOG-specific TCR-expressing Treg. In addition, Treg from both sEAE-affected and resistant OSE mice showed undistinguishable suppressive capacity. These findings suggest that susceptibility of OSE mice to sEAE cannot be attributed to decreased Treg levels, differential Ag-specificity or impaired suppressive function of Treg in the peripheral immune organs.

However, despite the low percentage of Treg in the peripheral immune organs of OSE mice (less than 3% of $CD4^+$ T cells) independently of the disease status, increased Treg frequency (more than 20% of $CD4^+$ T cells) was observed in the CNS of OSE mice with sEAE, already from the early phase of the disease. The percentage of Treg in the CNS infiltrates remained stable throughout the sEAE course, unlike the aEAE model where Treg are abundant in the CNS only during the recovery phase. Moreover, most of the CNS-

infiltrating Treg express the MOG-specific transgenic TCR, in contrast to Treg in the periphery where only half of them are expressing TCR^{MOG}. Therefore, the accumulation of MOG-specific Treg in the CNS of OSE mice during sEAE points to a role for Treg within the target organ in this model.

Selective ablation of the Treg population in OSE mice could elucidate the role of Treg in incidence and severity of sEAE. To perform this, OSE mice were crossed to DEREG mice and OSE x DEREG mice were treated chronically with DTx or PBS for 5 weeks, starting from 3 weeks of age. Interestingly, the DTx-treated mice developed EAE in higher incidence compared to the PBS-treated control group. However, no difference was observed in the disease severity between the two groups. Moreover, DTx-treated mice showed significantly higher levels of IFNγ-producing Teff in their spleens compared to the PBS-treated animals, while the frequency of IL-17-producing Teff was only marginally increased. These observations further support a protective role for Treg in OSE mice. Importantly, the increase in disease incidence, but not burden, in Treg-depleted OSE mice on the one hand suggests that Treg contribute to protection from sEAE and on the other hand indicates that Treg act rather in peripheral immune tissue, and not in the CNS, to control the autoimmune response.

However, the chronic DTx-mediated depletion of Treg proved to be, unexpectedly, incomplete. While short-term treatment with DTx leads to efficient ablation of Foxp3$^+$ cells, chronic treatment with DTx results in the expansion of a CD4$^+$Foxp3$^+$GFP$^-$ population that is resistant to the toxin, as these cells do not express the DTx receptor fused to GFP. These DTR$^-$GFP$^-$ Treg, the numbers of which are negligible in untreated or single DTx-treated OSE x DEREG mice, could occur due to silencing of the transgene by few cells. The observed increase of this population could be a result of homeostatic expansion, in order to replace the continuously depleted DTR-GFP-expressing Treg population. Thus, the findings of the experiments where OSE x DEREG mice were treated chronically with DTx should be interpreted with caution, as these mice harbor a significant number of non-DTR-expressing Treg. The previously described increase in the sEAE incidence in DTx-treated OSE x DEREG mice could be attributed to the absence of Treg in the initial days of the experiment. Nevertheless, an expansion of DTR$^-$GFP$^-$Foxp3$^+$ Treg was also documented in a recent study, where irradiated low-density lipoprotein receptor-deficient (*Ldlr-/-*) mice receiving DEREG bone marrow were chronically treated with DTx (Klingenberg et al., 2013). Despite the incomplete elimination of Treg using DTx treatment, highly increased proliferation of lymph node cells from mice treated with DTx versus PBS was observed in this study. Therefore, these newly expanded Treg may have less suppressive capability or harbor a different TCR repertoire, possibly reflecting that they are not fully differentiated Treg.

In order to overcome the problem of the inefficient Treg depletion after chronic treatment with DTx, other genetically modified mice with depletable Treg, such as the Foxp3-DTR knock-in mouse (Kim et al., 2007), could be used. Alternatively, single treatment with DTx could be performed in OSE x DEREG mice with sEAE, to investigate the effect of short-term Treg depletion in disease severity. Nonetheless, the aforementioned findings suggest a protective role for Treg in the OSE mouse model. While the increase in sEAE incidence, but not severity, indicates that Treg contribute protection from the disease in the periphery, the enhanced presence of Treg in the CNS of affected OSE mice points also to a possible role within the target tissue.

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ABBREVIATIONS

aEAE	actively induced Experimental Autoimmune Encephalomyelitis
APC	Antigen-Presenting Cell(s)
BBB	Blood-Brain Barrier
BCR	B Cell Receptor
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CFA	Complete Freund's adjuvant
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T Lymphocyte(s)
CTLA-4	Cytotoxic T Lymphocyte Associated Antigen 4
DC	Dendritic Cell(s)
DTR	Diphtheria Toxin Receptor
DTx	Diphtheria Toxin
EAE	Experimental Autoimmune Encephalomyelitis
FACS	Fluorescence-Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
Foxp3	Forkhead box P3
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GFP	Green Fluorescent Protein
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HLA	Human Leukocyte Antigen
HRP	Horseradish Peroxidase
ICAM-1	Inter-Cellular Adhesion Molecule 1

IFNγ	Interferon Gamma
Ig	Immunoglogulin
IL	Interleukin
i.p.	Intraperitoneal
iTreg	Induced Regulatory T cell
i.v.	Intravenous
LFA-1	Lymphocyte Function-Associated Antigen 1
LN	Lymph Nodes
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MOG	Myelin Oligodendrocyte Glycoprotein
MS	Multiple Sclerosis
NK	Natural Killer
n.s.	Not Significant
nTreg	Natural Regulatory T cell(s)
OSE	Opticospinal Experimental Autoimmune Encephalomyelitis
OVA	Ovalbumin
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
pEAE	Passively transferred Experimental Autoimmune Encephalomyelitis
PerCP	Peridinin Chlorophyll A Protein
PFA	Paraformaldehyde
PLP	Proteolipid Protein
PMA	Phorbol 12-Myristate 13-Acetate
rcf	Relative Centrifugal Force

RFP   Red Fluorescent Protein

RPMI   Roswell Park Memorial Institute

RR   Relapsing-Remitting

RT   Room Temperature

sEAE   Spontaneous Experimental Autoimmune Encephalomyelitis

s.e.m.   Standard Error of Mean

TCR   T Cell Receptor

Teff   Effector T cell(s)

TGF-β   Transforming Growth Factor Beta

TNFα   Tumor Necrosis Factor Alpha

Treg   Regulatory T cell(s)

VCAM-1   Vascular Cell Adhesion Molecule 1

RESOURCES & CONTRIBUTIONS

Foxp3-GFP.KI mice were provided by Vijay Kuchroo.

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T-Red mice were provided by Ulrich von Andrian.

Acquisition of confocal microscopy pictures was performed by PD Dr. Naoto Kawakami.

Programs for analysis of two-photon imaging data were provided by Dr. Ingo Bartholomäus.

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PUBLICATIONS

Participation in other group project:

Berer K, Mues M, **Koutrolos M**, Al Rasbi Z, Boziki M, Johner C, Wekerle H, Krishnamoorthy G; Commensal microbiota and myelin autoantigen cooperate to trigger autoimmune demyelination; **Nature** 2011 479(7374),538–541

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