Number and function of CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells in patients suffering from multiple sclerosis

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Dedicated to my parents

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Abbreviations

APC	Allophycocyanin
APCs	Antigen presenting cells
BD	Becton Dickinson
BSA	Bovine serum albumin
CD25NEG	Peripheral blood mononuclear cells plus $CD4^+CD25^-$ cells
CD25MIX	CD25NEG plus natural regulatory T cells
CNS	Central nervous system
Cpm	Count per minute
Cpn	Chlamydia pneuemoniae
CSF	Cerebrospinal fluid
Ct	Cycle threshold
CTLA-4	Cytotoxic T lymphocyte-associated antigen-4
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EBV	Epstein barr virus
EDSS	Expanded disability status score
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence assisted cell sorting
FCS	Foetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead box P3
GITR	Glucocorticoid inducible tumor necrosis factor receptor
HCl	Hydrochloric acid
HHV- 6	Human herpes virus-6
HLA	Human leukocyte antigen
ICAM-1	Intracellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immune dysfunction, polyendrocrinopathy, enteropathy, X-linked inheritance

LAG-3	Lymphocyte activation gene-3
MBP	Myelin basic protein
Min	Minutes
MHC	Major histocompatibility complex
MOG	Myelin oligogodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MX	Mitoxantrone
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP	Peridinin chorophyll protein
PerCP-cy5.5	Peridinin chorophyll protein complex-cyanine 5.5
PMTs	Photomultiplier tubes
PP	Primary progressive
PWM	Pokeweed mitogen
RBCs	Red blood cells
RR	Relapsing remitting
RT	Room temperature
SD	Standard deviation
SI	Stimulation index
SP	Secondary progressive
TAC	Tetrameric antibody complex
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TNF	Tumor necrosis factor
T_{reg}	Natural regulatory T cells
T_r1	T regulatory type 1
Th	T helper cells
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4
XLAAD	X-linked autoimmune and allergic dysregulation syndromes

1   Introduction

1.1   Multiple sclerosis (MS): An introduction

Multiple sclerosis has been the oldest described and one of the common organic neuropathy in Europe, for more than 500 years. Even after 160 years since the pathological and anatomical description by Cruveilhier and Carswell, and more than 150 years after the establishment of MS as a pathological and clinical entity by Charcot (Charcot, 1868; Charcot, 1877; Höher PG, 1985), the etiology of this disease is still unknown.

The latest knowledge of the pathogenesis of MS is at the best summarized as a combination of an infection and autoimmunity especially directed against neural antigens e.g. myelin basic protein (MBP), and disturbance of immunoregulation in genetically susceptible humans (Barnett and Sutton, 2006; Ibrahim and Gold, 2005; Thacker et al., 2006). This results in local and temporary disseminated demyelination especially of the white matter and axon loss of the central nervous system (CNS) mainly associated with inflammation; leading to the name multiple sclerosis.

MS is a heterogeneous disease: acute and chronic, exacerbations and remissions, and manifold clinical symptoms.

1.2   MS types

There are two major forms of MS: (1) relapsing remitting (RR) and (2) primary progressive (PP) (Sospedra and Martin, 2005). RR is the most frequent form of MS (85–90 %) which is characterized by discrete clinical "attacks" or "relapses" often followed by subsequent improvement. This type is common in young women (Keegan and Noseworthy, 2002).

After several years of onset, RR may develop into secondary progressive (SP) form. In this condition patients develop a slow, insidiously progressive neurological deterioration over many years with or without clinical "attacks" superimposed.

About 10–15 % of the patients suffer from PP, which is characterized by a progressive course from onset, an absence of clinically evident relapses. The underlying pathology of PP is thought to be different from RR. It could be a degenerative form rather than inflammatory. It is still not clear what factors are responsible for the different courses.

1.3 Pathological hallmarks and neurological symptoms

Four immunopathological subtypes of MS lesion have been reported: (1) inflammation with T cells, B cells and macrophages/microglia, (2) demyelination with oligodendrocyte loss during chronic disease stage and a variable degree of remyelinataion, (3) axonal loss and (4) gliosis with astrocyte proliferation and intensive glial fibre production (Bruck and Stadelmann, 2005).

The mechanisms of tissue damage in MS lesion are not yet fully understood. Gross examination of MS brain tissue reveals multiple, sharply demarcated plaques in the CNS white matter with a predilection to the optic nerves and white matter tracts of the periventricular regions, brain stem, and spinal cord. Example of lesions in the brain of a MS patient is given in **Figure 1**.
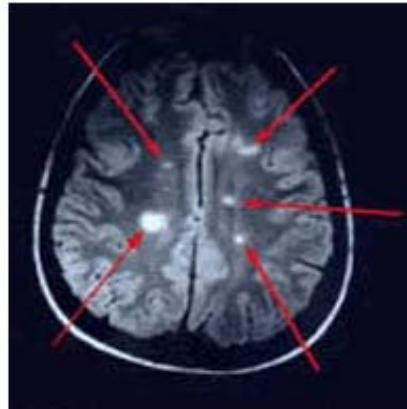
Figure 1 This magnetic resonance imaging scan shows multiple abnormal white areas that correspond to multiple sclerosis plaques (lesions). Each arrow shows the lesion.

However, a new paradigm proposes that oligodendrocyte apoptosis is the earliest change in newly forming lesions and that tissue injury is later on amplified by the subsequent recruitment of a systemic immune response (Barnett and Sutton, 2006).

Neurological symptoms in MS reflect the location of the lesion within the CNS for example visual loss reflects a lesion of the optic nerve; hemi-, para or quadriparesis, with or without bowel/bladder dysfunction reflects a lesion of the spinal cord; vertigo reflects a lesion of the brain stem and ataxia, and cerebellum (Keegan and Noseworthy, 2002). There are other symptoms like debilitating fatigue,

paresthesias on neck flexion (Lhermitte's sign), and heat exacerbated symptomatic worsening (e.g.Uhtoff's symptom) may be present.

1.4   Pathogenesis of MS

1.4.1   Genetic factor

It has been shown that genetic factors play a prominent role in susceptibility to MS. Adoption-study results reported that adoptive relatives, although raised from infancy with the MS patient, were no more likely to develop MS than expected for the general population. This data showed that the familiar aggregation of MS is related to genetic sharing rather than to shared family environment (Dyment et al., 2004). Confirmation of genetic predisposition comes primary from twin and sibling studies (Keegan and Noseworthy, 2002). Several twin studies have been done in MS. It has been shown that the concordance rate for MS in monozygotic twins is 25–30 %, in contrast to 3–5 % concordance between dizygotic twins and non-siblings (Sadovnick et al., 1996). MS risk is higher in offspring from both parents with MS than that of offspring with only one parent with MS (Dyment et al., 2004).

The major histocompatibilty complex (MHC) in humans "human leukocyte antigen" (HLA) is unambiguously associated with MS (Dyment et al., 2004). There are over 200 genes within 4-5 megabase of the MHC. Many of these play an important role in the development, maturation, and composition of the T cell repertoire as well as in immunological processes. The HLA proteins are necessary for adaptive immunity because T cells are recognizing antigens only as peptides presented from antigen-presenting cells (APCs) such as astroglia, microglia, and macrophages in CNS. Whereas, HLA class I molecules present peptides to $CD8^+$ T cells, antigens complexed with HLA class II molecules such as HLA-DR are recognized by $CD4^+$ T helper lymphocytes.

HLA-DR2 haplotype with the MHC on short arm of chromosome 6 is the strongest genetic effect identified in MS, and has been consistently demonstrated in family and case-control studies (Olerup and Hillert, 1991). MS patients from Sweden, Norway, Great Britain, Germany, Mexico, Canada, and Australia show mostly the haplotype DRB1*1501-DQA1*0102-DQB1*0602. In contrast, the MS patients from Asia are associated with the allele HLA-DRB1*0802. However the mechanism by which the MHC affects the MS is still unknown (Haines et al., 2002).

1.4.2 Infectious agent

Association of viral and bacterial agents with MS has been reported but none of the associations are conclusive. The role of human herpes virus-6 (HHV-6) variant A in MS is supported by its higher neurotropism, increased lymphoproliferative response against variant A in MS patients (Soldan et al., 2000), and its deoxyribonucleic acid (DNA) presence in cerebrospinal fluid (CSF) from MS patients. Epstein-Barr virus (EBV) is also linked to MS as it has been reported that anti-EBV antibodies are elevated in MS patients compared with healthy individuals (Wandinger et al., 2000). It was also reported that the risk of MS is close to zero among individuals who are not infected with EBV, intermediate among individuals infected with EBV in early childhood, and highest among individual infected with EBV in adolescence or later in life (Thacker et al., 2006).

Among bacteria, Chlamydia pneuemoniae (Cpn) has been implicated in MS. One study reported the presence of Cpn in the CSF of a large percentage of MS patients compared with healthy individuals (Sriram et al., 1999). Other studies failed to report an association between Cpn and MS (Pucci et al., 2000).

Two main mechanisms have been proposed to explain how infectious agents induce MS (Sospedra and Martin, 2005):

(a) Molecular mimicry: This involves activation of autoreactive T cells by cross reactivity between self and foreign antigens e.g. it has been reported that there is sequence homology between MBP and viruses.

(b) Bystander activation: In this mechanism, autoreactive T cells are activated because of nonspecific inflammatory events that occur during infection.

1.4.3 Immune factor

In principle, all of the immunocompetent cells are believed to participate in the pathogenesis of MS: APCs, T helper (CD4$^+$,Th) cells, and effector cells like antibody-producing B lymphocytes and CD8$^+$ cytotoxic T cells **(Figure 2, page 17)**.

Genetic and environmental factors may facilitate the increased movement of autoreactive Th cells from the systemic circulation into the CNS through disruption of blood-brain barrier. In the CNS, local factors may upregulate the expression of endothelial adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular-cell adhesion molecule-1 (VCAM-1), and E-selectin, which further facilitate the entry of T cells into the CNS (Noseworthy et al., 2000). Th cells penetration from the circulation into the brain parenchyma is enhanced by increased activity of matrix metalloproteinase responsible for the breakdown of extracellular matrix material (Keegan and Noseworthy, 2002).

Th cells are specifically activated in CNS by binding of putative MS antigens such as peptides from MBP, myelin oligogodendrocyte glycoprotein (MOG), proteolipid protein, αβ crystalline, phosphodiesterase and S-100 protein. T cell receptor (TCR) on Th cells recognize these antigens when these are associated with class II MHC molecules on APCs such as astrocytes, microglia, and macrophages (Noseworthy et al., 2000). Then interaction between the co-stimulatory molecules such as CD28 on Th and the proteins B7.1 or B7.2 on APCs further activates these cells. After these interactions, Th cell allow itself to proliferate. It achieves by releasing a potent T cell growth factor called interleukin (IL)-2. Activated Th cell also produce the alpha subunit of the IL-2 receptor (CD25 or IL-2Rα) enabling a fully functional receptor that can bind with IL-2, which in turn activates the T cell proliferation pathways. Th lymphocytes proliferate and can develop under appropriate conditions in cells secreting a relatively proinflammatory cytokine repertoire showing a type 1 helper T (Th1) pattern [tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-12]. This Th1 cytokine repertoire leads to immune-mediated injury to myelin and oligodendrocytes. Alternatively, Th lymphocytes can differentiate in type 2 helper T (Th2) cells secreting transforming growth factor (TGF-β), IL-4 and IL-10. Th2 cells may send anti-inflammatory signals to the activated APCs and stimulate pathologic or repair-enhancing antibody-producing B cells. CD8$^+$ T cells may directly damage the oligodendrocyte and myelin.

It has been shown that MBP-reactive T cells are present in the T cell repertoire of both, MS patients and healthy individuals (Lunemann et al., 2004). This brings into question, why MS patients but not healthy individuals develop inflammatory tissue damage. Two explanations for this discrepancy have been assumed; (1) a higher frequency of MBP reactive T cells in MS patients compared with healthy individuals, and (2) a different pattern of peptide recognition by T cells. The evaluation of the precursor frequency of MBP-reactive T cells (Hong et al., 2004) as well as the analyses of antigen presentation and peptide specificity have been inconclusive (Davies et al., 2005). Therefore, the control of autoreactive T cells may be different in healthy individuals and MS patients.

Disruption of blood-brain barrier may also result in the entry of autoreactive B cells, autoantibodies, and the complement factor into the CNS **(Figure 2, page 17)**. Within CNS, autoreactive B cells may be activated by Th cells in association with antigen and differentiate into antibody-secreting plasma cells. Antibodies against antigens which are located on the surface of the myelin sheath or oligodendrocyte can cause demyelination. These antibodies may gain access to the CNS through disruption of the blood-brain barrier as a consequence of a T-cell initiated inflammatory response (Noseworthy et al., 2000).

Autoantibodies detection against myelin antigens in the serum of MS patients has been elusive. This difficulty may in part be related to a tendency to form immune complexes which hampers detection (Dasgupta et al., 1983). Some studies have reported the elevated level of anti-MBP antibodies in MS compared with healthy individuals (Chamczuk et al., 2002; Reindl et al., 1999; Terryberry et al., 1998) whereas in some studies they are not detected (Colombo et al., 1997; Olsson et al., 1990). MOG is also an important target antigen in MS and anti-MOG antibodies have been also reported (Reindl et al., 1999). Antibody-mediated effector mechanisms may cause damage by opsonization of autoimmune target. This aids macrophages-mediated phagocytosis and by activation of the complement membrane attack complex, which may open pores in myelin membranes (Keegan and Noseworthy, 2002).

Multiple mechanisms of immune-mediated injury of myelin have been postulated; cytokine mediated injury of oligodendrocyte, digestion of surface myelin antigens by macrophages including binding of antibodies against myelin and

oligodendrocyte, complement mediated injury and direct injury of oligodendrocytes by CD4$^+$ and CD8$^+$ T cells **(Figure 2, page 17)**.
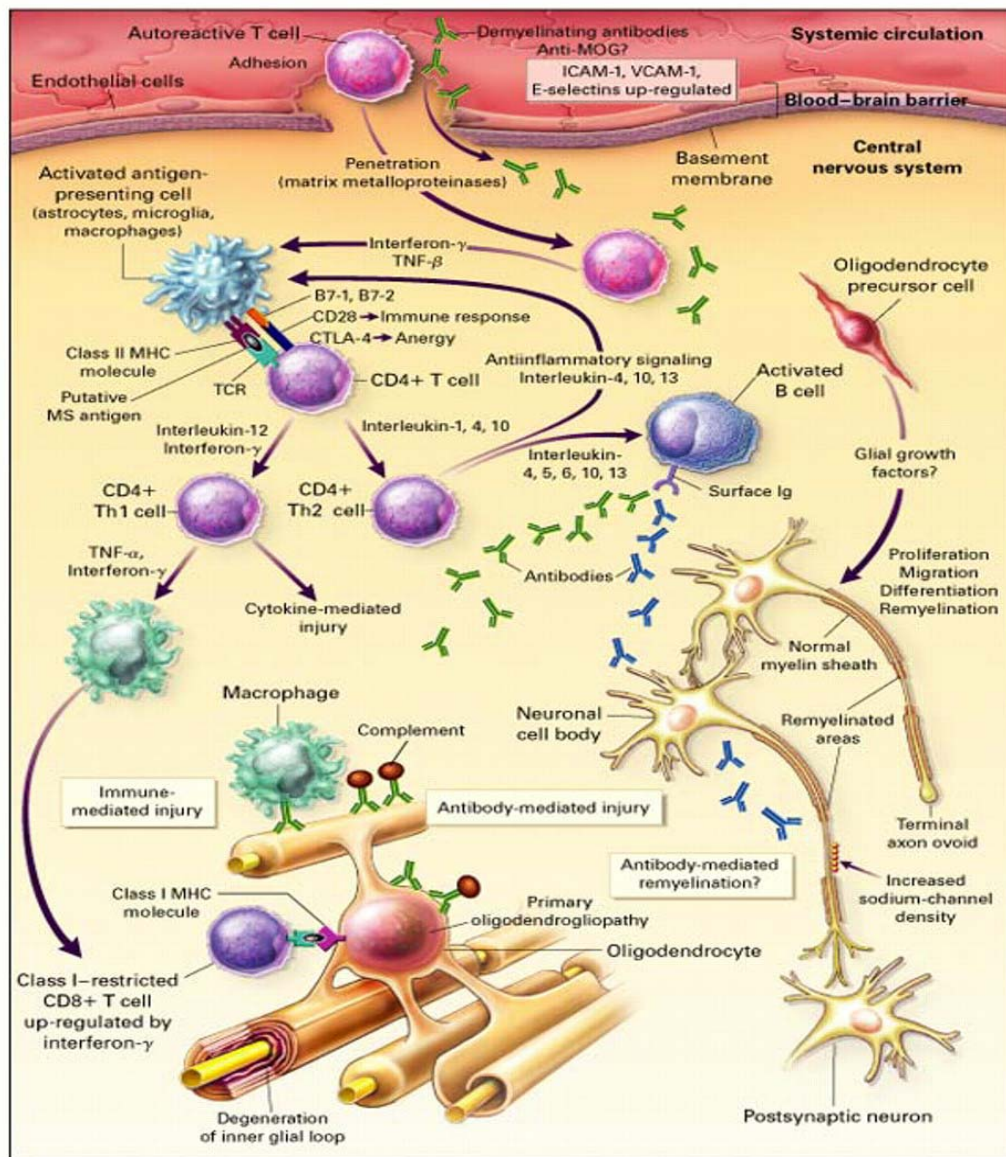
Figure 2   Possible mechanisms of injury and repair in multiple sclerosis.
          This figure is adapted from Noseworthy et al. (2000).

Figure 3 extends the working hypothesis by the inclusion of "molecular mimicry" and regulatory T cells.
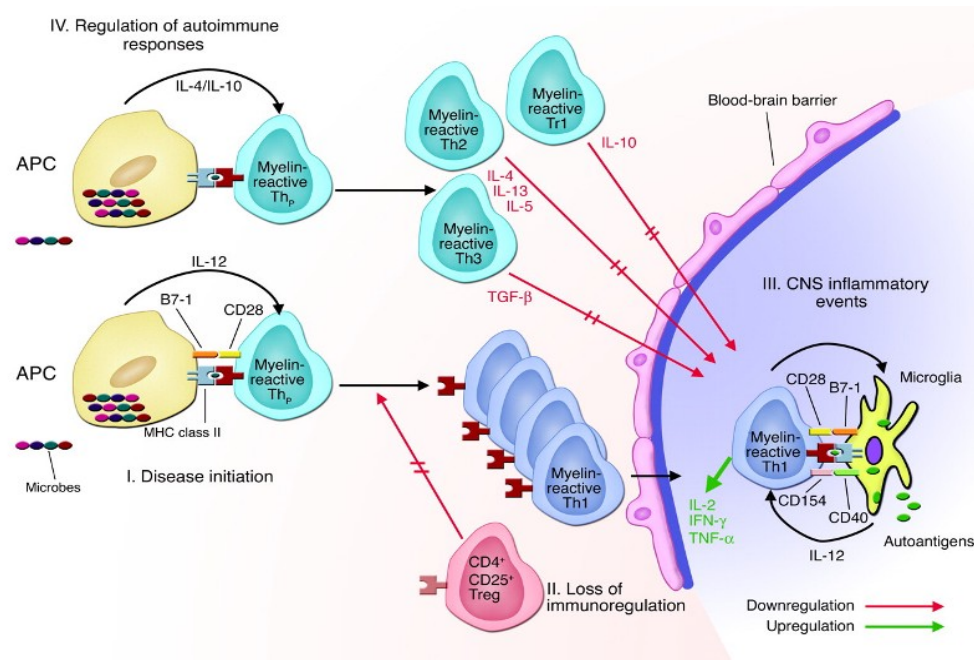
Figure 3 Working hypothesis as to the cause of multiple sclerosis.
(I) In a genetically susceptible host, common microbes activate antigen presenting cells (APCs) through molecules recognized by toll like receptors and protein sequences cross-reactive with self antigens recognized by major histocompatibility complex (MHC) class II molecules. This can be the minimum requirement for inducing multiple sclerosis (MS). (II) Loss of immuneregulation, such as loss of suppressive function of regulatory T cells in MS patients, allows further pathologic activation of autoreactive T cells. (III) Activated myelin reactive T cells migrate into central nervous system (CNS) and recognize antigen presented by microglia, local APCs. This results in secretion of type 1 helper T (Th1) cytokines which initiate an inflammatory cascade. (IV) Naturally occurring mechanisms may exist which regulate autoimmune responses. This includes the induction of autoreactive type 2 helper T (interleukin-4, interleukin-5, interleukin-13), type 3 helper T (transforming growth factor-β), or T regulatory type 1 (Tr1, Interleukin-10) cytokines-secreting T cells that migrate to CNS and downregulate (red arrow) inflammatory Th1 autoreactive T cells (green arrow). This figure is adapted from Hafler (2004).

1.5   Regulatory T cells

Several subsets of regulatory T cells have been described. Inducible regulatory T cells such as T regulatory type 1 (T_R1) or T helper type 3 (T_H3) can develop from conventional Th cells (Beissert et al., 2006; Jonuleit and Schmitt, 2003).

Another subset has been described as natural regulatory T cells (T_{reg}) which is our focus of study. T_{reg} are $CD4^+$ cells which further express the IL-2α chain (CD25). These cells have been the object of intense study because their function appears to be important in maintaining self tolerance.

Recently, thymocytes and peripheral blood T cells with the phenotype $CD8^+CD25^+$ as well as forkhead box P3 (FOXP3) expression has been described (Cosmi et al., 2003; Jarvis et al., 2005). These cells also share regulatory function.

1.5.1   Natural regulatory T cells (T_{reg}): An introduction

T_{reg} have been first described in mice in 1995 (Sakaguchi et al., 1995). Sakaguchi et al. (1995) removed CD25 expressing cells from the splenic $CD4^+$ cell population obtained from healthy BALB/c mice and injected these cells into athymic BALB/c mice; this results in the development of autoimmune gastritis. Addition of purified $CD4^+CD25^+$ cells to $CD4^+CD25^-$ cells at the time of injection protected against the disease. Afterwards, other studies have also shown that co-transfer of T_{reg} with $CD4^+CD25^-$ prevents the development of experimentally induced autoimmune diseases such as colitis, insulin dependent autoimmune diabetes and thyroiditis (Powrie et al., 1997; Read et al., 2000; Salomon et al., 2000). Later, the existence of T_{reg} has also been reported in human peripheral blood (Jonuleit et al., 2001) and thymus (Stephens et al., 2001).

1.5.1.1 Molecular markers of T_{reg}

A significant advancement in more precisely defining the T_{reg} population occurred when the forkhead box P3 (FOXP3) transcription factor has been identified as being necessary for T_{reg} development (Banham et al., 2006; Ziegler, 2007). In contrast to stable expression of FOXP3, other thymocytes, T cells, B cells, natural killer T cells do not express FOXP3 (Sakaguchi, 2005). This indicates that FOXP3 expression is highly specific for $T_{reg.}$

Genetic mutations in the FoxP3 gene have been identified in both human and mice, and results in fatal autoimmune diseases. Human with mutations in FoxP3 gene suffer from a severe and rapidly fatal autoimmune disorder known as immune dysfunction, polyendrocrinopathy, enteropathy, X-linked inheritance (IPEX) syndrome. An analogous disease also occurs in mice, known as 'scurfy' (Le and Chao, 2007).

FOXP3 suppresses IL-2 production and, thus abrogate the proliferation of T_{reg}. In contrast FOXP3 upregulates the expression of other T_{reg} associated molecules such as CD25, cytotoxic T lymphocyte-associated antigen (CTLA)-4 and glucocorticoid-inducible tumor necrosis factor receptor (GITR) (Sakaguchi, 2005). Ectopic expression of FOXP3 in CD4$^+$CD25$^-$ naïve T cell by retroviral gene transfer can convert them to phenotypically and functional T_{reg}. Thus, FoxP3 seems to be a 'master control gene' for the development and function T_{reg} **(Figure 4, page 21)**.

In addition to CD25 and FOXP3, other molecules are also expressed in higher density on cell surface of T_{reg} such as IL-2R β-chain (CD122), CD44, CD54, GITR, neuropillin-1, and lymphocyte activation gene 3 (LAG-3) (Suri-Payer and Fritzsching, 2006). CTLA-4 and CD152 are also constitutively expressed in the cytoplasm of T_{reg}. It has been also described that these cells are HLA-DR positive (Baecher-Allan et al., 2001). Recently, it has been shown that T_{reg} are also described by the lower surface expression of CD127 marker (Hartigan-O'Connor et al., 2007).

CD25 expression of T_{reg} from mouse and human is different. In mouse, T_{reg} are seen as a distinct population of cells that is easily distinguished from CD4$^+$CD25$^-$. In contrast, human CD4$^+$ T cell exhibit a continuous and primary low expression of CD25 (Baecher-Allan et al., 2001; Baecher-Allan et al., 2004).
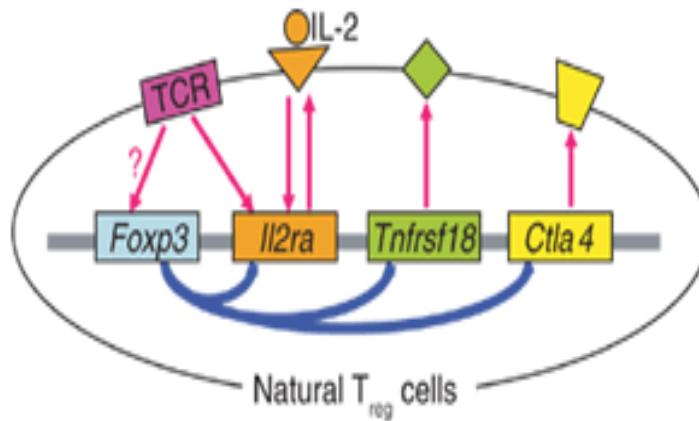
Figure 4 Control of regulatory T cell-associated molecules in natural regulatory T cells.
Forkhead box P3 (FoxP3) seems to control genes which encode natural regulatory T cells-associated molecules such as CD25, glucocorticoid-inducible tumor necrosis factor receptor (GITR) and cytotoxic T lymphocyte-associated antigen-4 (CTLA-4). T cell receptor signals and also binding of interleukin-2 to interleukin-2 receptor enhance CD25 expression. This figure is adapted from Sakaguchi (2005).

1.5.1.2 Function of T_{reg}

Upon activation of T_{reg} via their TCR, the main function of these cells is to suppress the proliferation of effector cells such as Th, CD8$^+$ cells, B cells, NK cells as well as APCs. Accumulating evidences suggest that T_{reg} are actively engaged in variety of physiological and pathological immune responses. These cells not only prevent the development of autoimmune diseases but are also responsible for the induction of immunological tolerance to non-self antigens (such as transplantation tolerance), negative control of aberrant immune responses (such as allergy and immunopathology), and enhancement of host defense (such as tumor immunity and microbial immunity) (Sakaguchi, 2005) **(Figure 5, page 22)**.
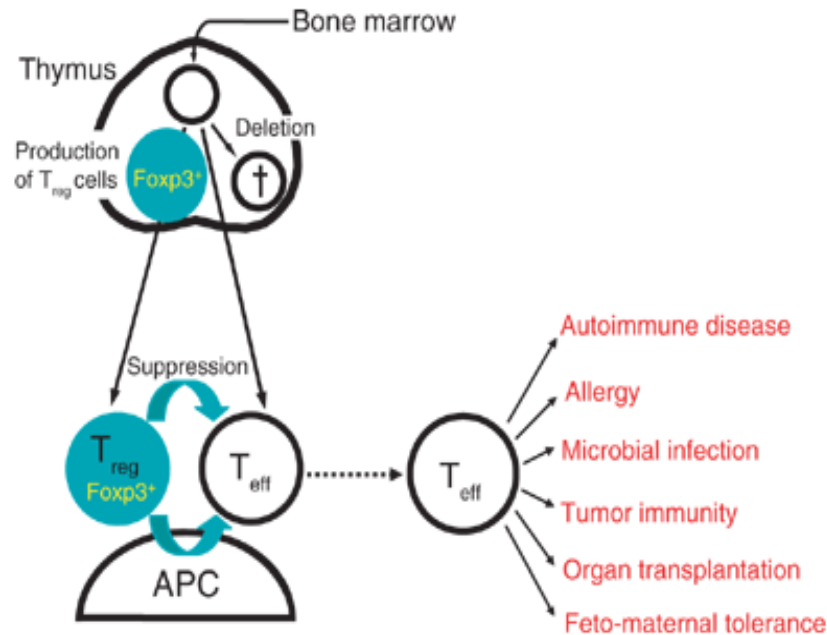
Figure 5   Linkage of central and peripheral tolerance by thymic production of natural regulatory T cells that contributes self tolerance.
A decrease in natural regulatory T cells (T_{reg}) number or impairment in suppressive function may result in autoimmunity, tumor immunity, microbial immunity and allergy. On the other hand, increase in T_{reg} number or augmentation of their suppressive activity may result in transplantation tolerance and maintain feto-maternal tolerance. T_{reg} may control effector T cells (T_{eff}) either directly or indirectly through antigen presenting cells. This figure is adapted from Sakaguchi(2005).

1.5.1.3 Mechanisms of suppression of effector T cells proliferation by T_{reg}

The mechanism of suppressive activity of T_{reg} is less understood. *In vitro* studies have shown that suppression of effector T cells proliferation requires direct cell contact between T_{reg} and the target effector T cells and is cytokine independent (Le and Chao, 2007; Piccirillo et al., 2002; Suri-Payer and Cantor, 2001; Takahashi et al., 1998). APCs are also not essential as suppression of effector T cell proliferation occurs in APC-free cultures (von Boehmer, 2005). It appears that T_{reg} must be activated in order to perform suppressive function, and has been shown by two separate studies in which fixed, bead isolated T_{reg} could suppress effector T cell proliferation, only if T_{reg} is preactivated before the fixation (Baecher-Allan et al., 2004).

Cytokines involvement in T_{reg} suppression is controversial. It has been shown that T_{reg} produce IL-10 *in vivo*, and it has been established that certain form of autoimmunity such as colitis can be suppressed by T_{reg} and require IL-10. Whereas, other studies have reported that autoimmune disease can be suppressed independently of IL-10 (von Boehmer, 2005). Involvement of transforming growth factor (TGF)-β is difficult to say as some studies have reported that T_{reg} isolated from the blood shows no effect on the suppression of effector T cells proliferation by blocking TGF-β while others have reported that neutralizing TGF-β reduces the suppression (Annunziato et al., 2002; Nakamura et al., 2004).

The estimation of the proliferation of mitogen-activated T lymphocytes is a common measure for T cell reactivity. We (Mueller et al., 2003) and Bo et al. (2001) have found that impaired proliferative responses to mitogens may be due to a high local production of tumor necrosis factor (TNF)-α . Recently, it has been shown that anti-TNF-α therapy in rheumatoid arthritis generates naïve population of T_{reg}, which compensates for the defective T_{reg} (Nadkarni et al., 2007).

Recent, *in vitro* studies on immunosuppression have suggested the function of CD80 and CD86 ligands that are present on activated Th cells. These molecules are essential in T_{reg} mediated suppression as it is found that their absence results in marked reduced susceptibility to suppression by T_{reg} compared with wild-type of effector T cells (von Boehmer, 2005). This hypothesis of mechanism of suppression of effector T cells proliferation is described in **Figure 6, page 24**.
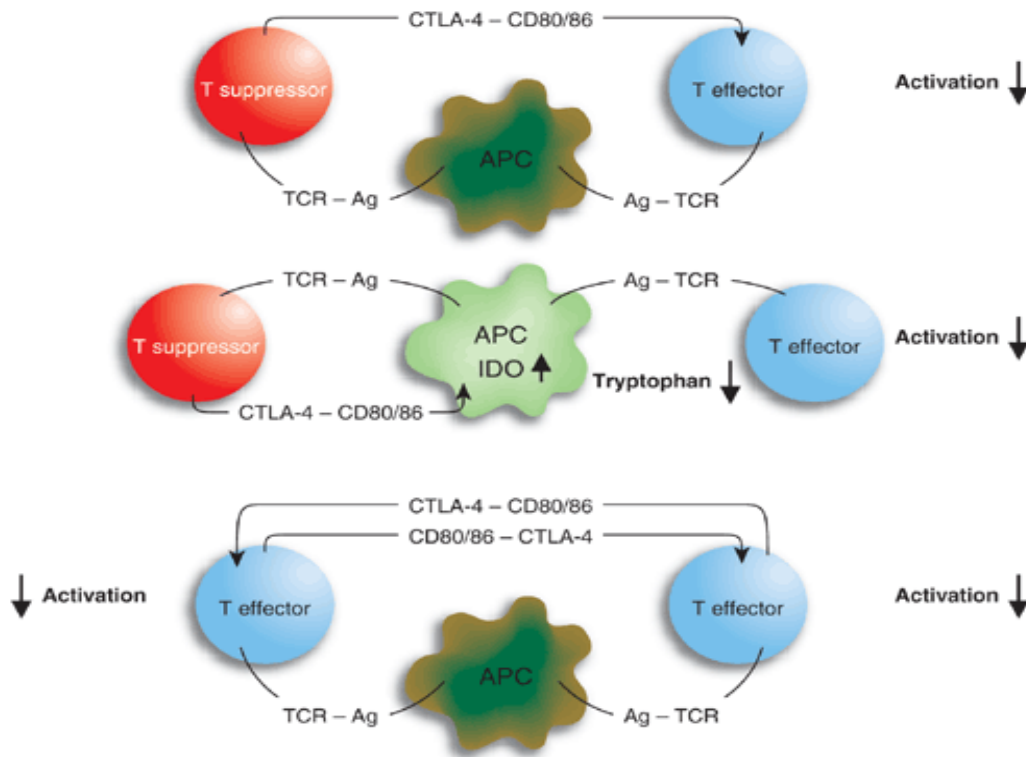
Figure 6 Cytotoxic T lymphocyte-associated antigen 4 and CD80 and/or CD86 in suppression of effector T cells proliferation by natural regulatory T cells.

(A) Natural regulatory T (T suppressors) and T effector cells meet at an antigen presenting cells (APCs) and facilitate binding of cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) on T suppressors to CD80 and/or CD86 (CD80/CD86) on activated effectors. 'Outside signaling' by CD80 and/or CD86 then prevent the activation of effectors.

(B) T suppressors could activate indolamine 2, 3 dioxygenase (IDO) when CTLA-4 on T suppressors binds to CD80 and/or CD86 on APCs. The IDO dependent metabolism of tryptophan would prevent activation of T effectors cells.

(C) In the absence of T suppressors, mutual binding of CTLA-4 and CD80 and/or CD86 by effector T cells cause interference with their further activation through cell-autonomous inhibitory signals by CTLA-4.

This figure is adapted from von Boehmer (2005) with required modification.

1.5.1.4 Number and function of T_{reg} in MS

MS is believed to be an autoimmune disease so it was interesting to study T_{reg} function and cell numbers in this disease.

Only a few studies have been done on T_{reg} number with different results using different definitions for T_{reg}. It has been reported that compared with healthy individuals, the number of circulating T_{reg} was decreased (Khoury et al., 2000) or unchanged (Haas et al., 2005; Putheti et al., 2004; Venken et al., 2006; Viglietta et al., 2004) in MS patients.

Recently, three reports have been published regarding T_{reg} function. In two of the studies CD25^{high} expressing cells are used. According to Viglietta et al. (2004) mitogen-stimulated lymphocytes of MS patients have showed an impaired suppressive function of T_{reg}. Moreover, reduced antigen-specific suppression are described in myelin oligodendrocyte glycoprotein (MOG) stimulated lymphocytes (Haas et al., 2005). The third study used the same reagents as us and has found also a reduced T_{reg} function toward mitogen-induced proliferation in RR MS patients compared with healthy individuals (Venken et al., 2006). But no studies have been performed using the most abundant protein in CNS, the MBP.

1.6   Therapy

In view of the likely autoimmune pathogenesis of MS, the therapy of this disease is based on the use of agents influencing the immune system, e.g. interferon (IFN) as cytokines, an antigenic substance resembling MBP in the amino acid composition (glatiramer acetate), antagonists of adhesion [anti-very late antigen-4 (VLA-4) antibody], and agents which suppress proliferation of immune cells.

There are six "Food and Drug Administration" (FDA) approved treatments for relapsing remitting MS: two IFN-β1a agents (Avonex$^®$ and Rebif$^®$), one IFN-β1b (Betaseron$^®$), glatiramer acetate (Copaxone$^®$), anti-VLA-4 monoclonal antibody (Tysabri$^®$) and mitoxantrone (Novantrone$^®$) for progressing advanced cases (Hafler et al., 2005). Mitoxantrone (MX) and other anti-proliferative agent cyclophosphamide are prescribed for secondary progressive MS.

Interferon-1β is naturally occurring cytokine. Although, its exact mode of action is not known but it has been found to suppress T cell proliferation, reduce T cell migration from the systemic circulation into the CNS, and alter Th1 to Th2 response.

Copaxone is a synthetic peptide of four amino acids (L-glutamic acid, L-lysine, L-alanine, and L-tyrosine). Its structure resembles MBP. It is an immunomodulator that appears to block myelin-specific autoimmune responses and also alters the immune response from Th1 to Th2 (Keegan and Noseworthy, 2002).

Mitoxantrone (MX) is an anthracenedione derivative of doxyrubicin which is highly effective in the treatment of progressive MS (Hartung et al., 2002). It reduces both attack and progression rates and has been approved for the patients with secondary progressive and worsening forms of MS (Jain, 2000; Neuhaus et al., 2006). MX is a DNA-intercalating agent which interacts with topoisomerase-2, and inhibits DNA and RNA synthesis (Smith, 1983). Furthermore, MX acts as an immunosuppressant targeting proliferating immune cells. It suppresses the proliferation of T lymphocytes, B cells, and macrophages (Fidler et al., 1986a; Fidler et al., 1986b; Wang et al., 1986). It also inhibits B cell function and antibody production (Fidler et al., 1986a). Despite its widespread use in the treatment of MS, the immunological effects of MX are not fully understood. *Ex vivo* analyses in MS patients have revealed that MX induces suppression of mononuclear cell proliferative responses and alterations of lymphocyte subsets (Chan et al., 2005; Gbadamosi et al., 2003). These studies mainly examined acute or short-term effects of MX.

Treatment with MX and cyclophosphamide are effective but associated with significant toxicity. Moreover, all therapies have a limited effect. Thus there is a need to develop new therapies with low toxicity and higher efficiency. An ideal therapy could be to prevent the initiation of such attacks and reduce progression by correcting the dysregulated immune system.

1.7   Aim of the study

Multiple sclerosis (MS) is an inflammatory autoimmune disorder of the central nervous system which is thought to result from aberrant T cell immune response to self antigens e.g. myelin basic protein (MBP). Active suppression by natural $CD4^+CD25^+$ regulatory T cells (T_{reg}) plays a key role in the control of self-antigen-reactive cells and in the induction of peripheral tolerance *in vivo*.

Only a few publications have been reported describing the number and function of T_{reg} in MS. In these reports, T_{reg} are defined mainly by $CD25^{high}$ expression. These cells are only available after time and cost-consuming use of a fluorescence-activated cell sorter. With the introduction of reagents using magnetic separation of $CD4^+$ lymphocytes in $CD25^+$ and $CD25^-$ cells it is now possible to get in a relatively short time, large number of T_{reg} for functional studies. Therefore, we used this separation technique for studying T_{reg} (mixture of $CD25^{high}$ and $CD25^{intermediate}$) regarding function in an *in vitro* proliferation assay using most abundant protein in CNS, MBP, as an antigen. Moreover, we estimated number of T_{reg} as well as leukocytes and their subpopulations in MS patients and healthy individuals. FOXP3 and HLA-DR are other markers for T_{reg}. To characterize our T_{reg}, it was also important to see the expression of FOXP3 and HLA-DR on these cells.

In order to elucidate the possible mechanisms for suppressive activity of T_{reg}, we also looked for the role of cytokines e.g. TNF-α.

Antibody production is the last step in the immune response and MS seems to be associated with increased concentration of anti MBP antibodies, we also measured these autoantibodies in MS.

Mitoxantrone (MX) is a potent immunosuppressive agent in the treatment of active relapsing remitting and secondary progressive MS. Therefore, our next aim of study was to analyze the effect of this drug on number and function of T_{reg}.

2 Materials and methods

2.1 Patients and healthy individuals

The study was approved by the local ethics committee. All MS patients and blood donors (healthy individuals, Institut fuer Transfusionsmedizin, Universitaetsklinikum Essen) gave written informed consent prior to the study.

During the time of the study, we included all patients at our MS center of the Department of Neurology, University hospital, University of Duisburg-Essen, who fulfilled our predefined inclusion criteria: (i) patients had to suffer from clinical definite relapsing remitting or secondary progressive MS according to the McDonald criteria (McDonald et al., 2001), (ii) they had to be untreated for at least 6 months, (iii) they had to be relapse free for at least 8 weeks and (iv) they had not received treatment for at least 8 weeks.

Forty millilitre heparinized blood, 5 ml ethylene diamine tetraacetic acid (EDTA) blood and 10 ml blood without anticoagulants for getting serum was collected by venipuncture in the morning from each individual.

For enumeration and function of T_{reg} in MS patients and healthy individuals, 35 patients (13 males and 22 females; RR = 25 and SP = 10) and 34 healthy (17 males, 17 females) were enrolled in our study. The mean and standard deviation (mean$\pm$SD) was calculated for patients age (40±10 years, range 20-69 years), disease duration (7.4±5.9 years, range 0.5-25 years) and expanded disability status score (EDSS; 3.7±2, range 1.0-7.5). The age (mean$\pm$SD) of the healthy individuals was 40±12 years, range 21-62 years. There was no significant difference for age and sex between MS patients and healthy individuals.

For MX follow up study, twenty MS patients (5 males and 15 females; RR = 6, SP = 12 and PP = 2) were included. Their age was 41±8 years (range 24-57 years), the disease duration was 10±5 years (range 3-19 years) and their EDSS values 5.6±1.3 (range 3-8). Eleven patients from this group were already included for testing of enumeration and function of T_{reg}. All patients received MX *de novo*. MX was given at a standardized dose of 10 mg/m^2 in all patients. Intravenous application was performed after antiemetic treatment during one hour. Blood was always collected before treatment and at intervals of 3 months up to one year immediately before infusion of MX to investigate long-term effects of the applications.

For antibody analysis, 39 MS patients (11 males and 28 females; RR = 25, SP = 14) and 39 healthy individuals (15 males and 24 females) were enrolled. Thirty two MS patients from this group were included for testing of enumeration and function of T_{reg} and rest 7 were included for MX follow up study. The age of the healthy individuals was 42±8 years, range 24-60 years. Only four healthy individuals from this group were included for testing of enumeration and function of T_{reg}. There was no significant difference for age and sex between MS patients and healthy individuals.

2.2 Chemicals and reagents

All the chemicals and reagents used throughout this work were purchased from the different companies as given in **Table 1.** If not otherwise stated the chemicals and reagents were bought in Germany.

Table 1 List of Chemicals and reagents

Chemicals/Reagents	Company
Bovine seum albumin (BSA)	Merck , Darmstadt
Carbonate/bicarbonate buffer capsule	Sigma-Aldrich,steinheim
Ficoll-Paque plus	Amersham Biosciences, Uppsala, Sweden
Foetal Calf Serum (FCS)	Biochrom, Berlin
7.25 % Hydro chloric acid (HCl)	Braun Melsungen, Melsungen
Heparin (Liquemin N25000)	Roche, Grenzach-wyhlen
L-glutamine	Sigma-Aldrich
Myelin Basic Protein (MBP)	Acris Antibodies, Hiddenhausen
Penicilin	Gibco, Karlsruhe
Phosphate buffered saline (PBS)	Gibco
Pokeweed Mitogen (PWM)	Sigma-Aldrich
Rosewell Park Memorial Institute (RPMI) 1640 medium	Gibco
ß-Mercaptoethanol	Sigma-Aldrich
Streptomycin	Gibco
Trypan blue	Sigma-Aldrich
Tween 20	Merck Schuchardt, Hohenbrunn

2.3 Antibodies for flow cytometry

All antibodies as given in **Table 2.a** and **b** were purchased from Becton Dickinson (BD) Biosciences, Heidelberg, Germany, except tumor necrosis factor (TNF)-α antibody conjugated with fluorescein isothiocyanate (FITC) which was purchased from BD Pharmingen, Heidelberg, Germany.

Table 2.a List of four and three color antibodies

FITC/PE/PerCP/ PerCPCy5.5*/ Antibody	Clone	Order.Nr.	Amount/tube
MultiTEST CD3/8/45/4	SK7/SK1/2D1/SK3	342417	20 µl
MultiTEST CD3/16+56/45/19	SK7/B73.1/NCAM16.2/ 2D1/SJ25C1	342416	20 µl
Oncomark CD4/25/3	SK3/2A3/SK7	341134	20 µl
Tritest CD4/8/3	SK3/SK1/SK7	342414	20 µl

* Fluorescein isothiocyanate (FITC)/ phycoerythin (PE)/ peridinin chlorophyll protein (PerCP)/ peridinin chlorophyll protein cyanine 5.5 (PerCPCy5.5)

Table 2.b List of single color antibodies

Antibody/IgG class	Clone	Order Nr.	Amount/tube
CD14 APC[#]/IgG2b	MoP9	345787	2 µl
CD8 APC/IgG1	SK1	345775	2 µl
HLA-DR APC /IgG2a	L243	347403	1 µl
CD4PE/IgG1	SK3	345766	10 µl
CD3PerCP/IgG1	SK7	345769	10 µl
TNF-αFITC/IgG1	MAb11	554512	5 µl
MouseIgG1FITC	X40	345815	5 µl

[#] Allophycocyanin (APC)

2.4   Culture medium

RPMI 1640 medium was used for all cultures containing 25 mM HEPES, supplemented with 2 mM L-glutamine, 200 U/ml penicillin and 200 µg/ml streptomycin. The medium was further supplemented with 10 % heat–inactivated pooled human serum (Institut fuer Transfusionsmedizin, Universitaetsklinikum Essen).

2.5   Myelin basic protein (MBP) reconstitution

One milligram of myelin basic protein (MBP) was reconstituted in 10 mM HCl. MBP powder was first dissolved in 100 µl of HCl (7.25 % HCl was added in 2 ml sterile water) and then diluted with 900 µl PBS. Final concentration of MBP was 1 mg/ml, which was used for stimulation.

2.6   Isolation of T_{reg} and $CD4^+CD25^-$ cells

2.6.1   Principle

Isolation kit from CellSystems Biotechnologie (St. Katharinen, Germany) was used which involves RosetteSep and EasySep to isolate T_{reg} and $CD4^+CD25^-$ cells. RosetteSep is a rapid and easy cell separation procedure for the isolation of highly purified cells directly from whole blood. Utilizing antibody-based tetrameric antibody complex (TAC) technology, RosetteSep turns a simple buoyant density centrifugation into a specific, antibody-mediated cell enrichment system. In this procedure, sample is incubated with the antibody-based enrichment cocktail at room temperature (RT), followed by standard buoyant density separation. The antibody-based enrichment cocktail crosslinks unwanted cells ($CD8^+$, $CD16^+$, $CD19^+$, $CD36^+$, $CD56^+$, and $CD66b^+$) to multiple red blood cells (RBCs) to pellet them along with free red blood cells by density centrifugation (negative selection for $CD4^+$). This increases the density of the unwanted cells and the pellet down along with the free RBCs when centrifuged over a buoyant density medium such as Ficoll-Paque. Desired cells are not labelled with antibody and are easily collected as a highly enriched population at the interface between plasma and density medium.

EasySep involves labelling of target cells with dextran-coated magnetic nanoparticles using bispecific TAC. These complexes recognize both dextran and the target cell surface antigen **(Figure 7, page 32)**. The small size of the magnetic dextran

iron particles allows for efficient binding to the TAC-labelled cells. These magnetic labelled cells are then separated from unlabeled cells.
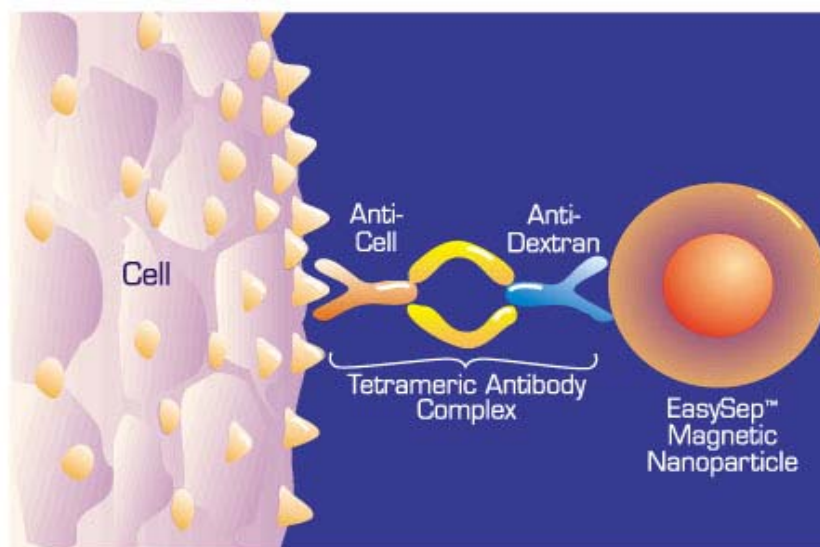
Figure 7 Magnetic isolation of natural regulatory T (CD4$^+$CD25$^+$) cells.
This figure is adapted from StemCell technologies (www.stemcell.com).

2.6.2 Procedure

Thirty millilitre of heparinized venous blood samples were used for isolation of T$_{reg}$ and CD4$^+$CD25$^-$ cells. Forty microlitre "RosetteSep^{TM} CD4$^+$ cell enrichment cocktail" (CellSystems Biotechnologie) was added per millilitre of blood and incubated for 15 minutes (min). After incubation, blood samples were diluted with an equal volume of PBS enriched with 2 % FCS. Diluted blood samples were layered onto 15 ml Ficoll-Paque^{TM} plus density medium and centrifuged [1,200xg, 20 min at room temperature (RT)]. Cells removed from the density medium/plasma interface contained enriched CD4$^+$ cells. They were washed 2 times with PBS plus 2 % FCS (400xg, 10 min at RT). After washing, cells were resuspended at 1 x 10^7 cells per 100 µl PBS plus 2 % FCS and incubated with 100 µl of "EasySep^{TM} CD25 positive Selection Cocktail" per ml of sample for 15 min. CD25$^+$ cells were isolated by adding 50 µl of EasySep^{TM} SA Magnetic Nanoparticles (CellSystems Biotechnologie) per millilitre of sample. After incubation for 10 min, 2.1 ml PBS plus 2 % FCS was added. The tube was placed in the EasySep magnet (CellSystems Biotechnologie) for 5 min. Thereafter, the supernatant containing the CD4$^+$CD25$^-$ cells was poured into another tube by inverting magnet and tube in one continuous motion. This magnetic

step was repeated two times more to get pure T_{reg} and all supernatants were combined to receive the $CD4^+CD25^-$ cells. These $CD4^+CD25^-$ cells were centrifuged (400xg, 5 min at RT) and resuspended in 1 ml RPMI 1640 medium. The tube containing T_{reg} was removed from the magnet and the cells were also resuspended in 1 ml RPMI 1640 medium.

For FOXP3 expression, Isolated T_{reg} and CD4+CD25- cells were first snap frozen in liquid nitrogen and then finally stored in -80°C until use.

2.7 Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from seven millilitre of heparinized whole blood by Ficoll-Paque^{TM} plus density gradients. Blood samples were diluted with an equal volume of phosphate buffered saline and then diluted blood samples were layered onto 15 ml Ficoll-Paque^{TM} plus density medium and centrifuged (400xg, 30 min at RT). PBMCs were removed from the density medium/plasma interface and were washed twice with PBS (400xg, 10 min at RT).

2.8 Cell proliferation assays

2.8.1 Optimization of the antigen concentration and cell numbers for our proliferation assay

To optimise MBP concentrations, 1×10^5 PBMCs/well were stimulated with 0.5, 1, 2, 4 and 8 µg MBP/well.

For PBMCs cell number optimisation, 0.5×10^5, 1×10^5, 1.5×10^5, 2×10^5 and 2.5×10^5 cells/well were stimulated with 4 µg/well of MBP. Isolated T_{reg} and $CD4^+CD25^-$ cell numbers were also optimised using different cell numbers. 2.5×10^3, 5×10^3, 7.5×10^3 and 10×10^3 cells/well were added into 1×10^5 PBMCs and stimulated with 4 µg/well of MBP.

2.8.2 Function of T_{reg}

To assess the functional activity of T_{reg}, 1×10^5 PBMCs plus 1×10^4 $CD4^+CD25^-$ cells were cultured in absence (CD25NEG) or presence of 1×10^4 T_{reg} (CD25MIX) in 230 µl RPMI 1640 in U-bottom wells (96-well plate; BD Labware, Heidelberg, Germany). We used $CD4^+CD25^-$ cells in order to increase the number of MBP specific responder T cells. Furthermore, during isolation procedure, some cells

may get activated. Hence as a control, we added isolated $CD4^+CD25^-$ cells in PBMCs. Then we estimated the effect of T_{reg} on PBMCs plus $CD4^+CD25^-$ cells.

Cells (CD25NEG or CD25MIX) were cultured in 230 µl RPMI 1640 medium without any stimulation (autologous cultures) or stimulated with either 5 µg/well of MBP (antigen) or 1 µg/well of pokeweed mitogen (PWM, mitogen) in U-bottom wells. Cultures were set up as triplicates and incubated at 37°C in a humidified atmosphere with 5 % CO_2.

After 5 days of cultures, 1 µCi of ^{3}H-thymidine (Amersham Buchler, Braunschweig, Germany) per well was added and incubated for further 16 hours. Using a multiple automatic sample harvester (Mach III Harvester96; Tomtec, Hamden, UK), cells were harvested on glass fiber paper and counted in scintillation fluid (Betaplate-Scint; Wallac, Turku, Finland). ^{3}H-thymidine incorporation was measured as counts per minute (cpm). The stimulation index (SI) was calculated by dividing the mean (cpm) of triplicate samples (MBP or PWM-induced proliferation) by the mean (cpm) of triplicate autologous cultures. The percentage of suppression of T_{reg} (CD25MIX) was calculated by setting the SI values of CD25NEG cultures to 100 % proliferation.

2.9   Counting of Cell numbers

Isolated cells were counted using haemocytometer counting chamber. In brief, cells were diluted in ready trypan blue solution. Then the diluted suspension was introduced in this chamber which was covered with cover slip and counted under the microscope in all four corners.

Cell numbers = (total cell counted/4) x dilution factor x 10^4 cell per ml.

2.10   Flow cytometry

2.10.1 Principle

Flow cytometry uses the principle of light scattering, light excitation and emission of fluorochrome molecules for the measurement of characteristics of single cells. Cells suspended in sheath fluid that allows the cells to flow in a defined stream of single file pass under hydrodynamic pressure. The moving cell hits the focused beam of laser lights and the light is scattered in the forward direction (FSC, indicates size of the cell) and in the side direction (SSC, indicates contents and the granularity of the cell) with respect to the laser beam. The laser beam excites the dyes bound to the monoclonal antibodies marking the cells to fluorescence. A series of

photomultiplier tubes (PMTs) detectors collect these informations and converted into a form suitable for computer storage and subsequent analysis **(Figure 8)**.
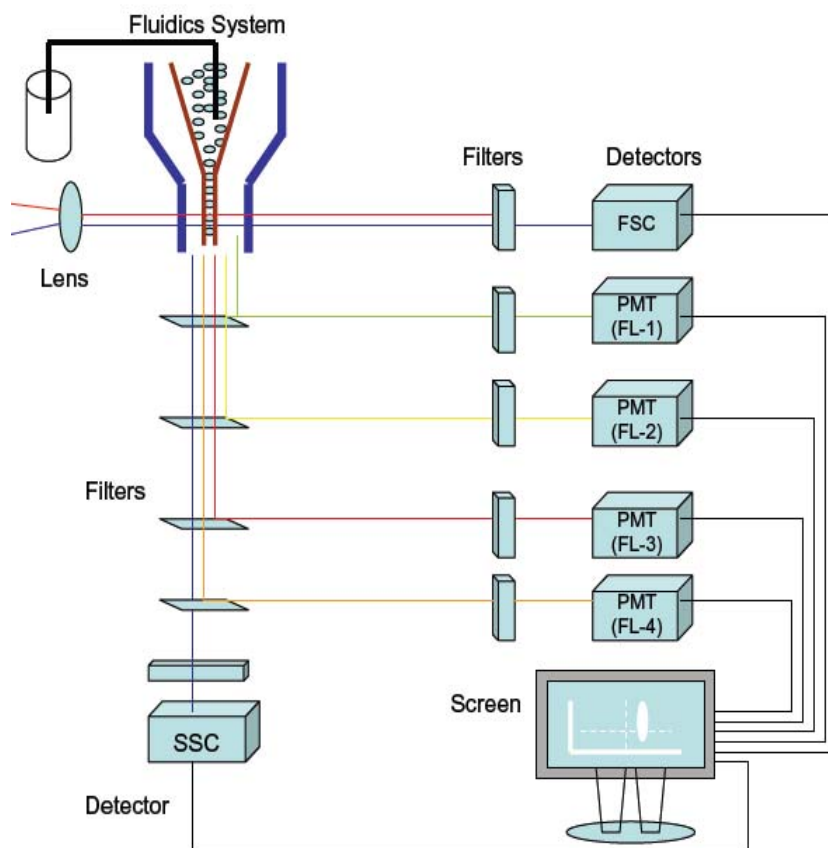
Figure 8 Schematic diagram of flow cytometry.

2.10.2 Procedure

Four color flow cytometry was used for enumeration of whole blood and different subpopulation of leukocytes.

2.10.2.1 Surface staining

To stain cells of interest, first different antibodies were added. After addition of antibodies, 50 µl of whole blood or isolated cells were added and incubated for 15 min at RT. Thereafter, 500 µl of BD FACS lysing solution (BD Biosciences) was

added, mixed and incubated for 15 min. Samples were measured in a FACSCalibur and analyzed utilizing CellQuest software (BD Biosciences).

True count beads (BD, Heidelberg, Germany) containing MultiTEST CD3$^+$FITC/CD8$^+$PE/CD45$^+$PerCP/CD4$^+$APC and CD14$^+$APC were used to count the absolute number of leukcocytes. Relative number of lymphocytes, T cells (CD3$^+$) and their subpopulations (CD4$^+$ and CD8$^+$) were enumerated using MultiTEST CD3$^+$FITC/CD8$^+$PE/CD45$^+$PerCP/CD4$^+$APC and CD14$^+$APC. To enumerate relative number of B lymphocytes cells, MultiTEST CD3$^+$FITC/CD16$^+$+56$^+$PE/ CD45$^+$PerCP /CD19$^+$APC were used.

Relative number of CD25 expressing CD3$^+$ cells in whole blood cells and purity of isolated CD4$^+$, T$_{reg}$, and CD4$^+$CD25$^-$ cells were measured using BD Oncomark^{TM} CD4$^+$FITC/CD25$^+$PE/CD3$^+$PerCP-Cy5.5 and CD8$^+$APC. HLA-DR expression of CD3$^+$ cells was measured in whole blood using Tritest CD4$^+$ FITC/ CD8$^+$ PE/CD3$^+$ PerCP and HLA-DR$^+$ APC.

To analyze, B lymphocytes, CD3$^+$ cells and their subpopulations, lymphocytes were gated. For analysis of CD25 and HLA-DR expressing CD3$^+$ cells, CD3$^+$ cells were gated. Three thousand events were acquired on gated cells. HLA-DR positivity was defined as described in Ditschkowski et al. (1999) The relative number of B and CD3$^+$ lymphocytes are given as a percentage of lymphocytes. For relative number of T cells expressing CD4$^+$, CD8$^+$, CD25$^+$ or HLA-DR$^+$, percentage of CD3$^+$ in whole blood was calculated. To obtain the absolute number of B cells, first lymphocytes number were calculated from relative lymphocyte number and leukocytes number. Then from lymphocytes number, absolute number of B cells was calculated using the relative B cell number.

2.10.2.2 Intracellular staining

First cells were collected after 5 days of cultures and centrifuged (400xg, 5 min at RT). Supernatant was discarded and 1 ml of 1 % BSA + PBS was added for washing (centrifuge at 400xg, 5 min at RT). After washing the cells were diluted in 1 % BSA + PBS according to number of tube (100 µl per tube). For staining, 200 µl PBS was first added in each tube and then different antibodies (CD4$^+$PE, CD3$^+$PerCP and CD45$^+$FITC/CD14$^+$PE) were added. Then 100 µl cells were added in each tube and incubated for 15 min. After incubation, 1 ml of 1 % BSA + PBS were added for washing (centrifuge at 400xg, 5 min at RT).

After washing, 200 µl BD cytofix + cytoperm (BD Biosciences) were added and incubated for 20 min at 4°C. Wash was repeated with 1 ml of 1 % BDPerm/Wash (BD Biosciences). 1 % BDPerm/Wash was prepared by diluting BDPerm/Wash in sterile water.

Meanwhile antibodies were diluted as 5 µl antibody + 50 µl of 1 % BDPerm/Wash per tube. TNF-α FITC and Mouse IgG1 FITC (isotype control) were used for measurement of TNF-α expression.

Fifty microlitre of diluted antibodies were added in each tube and incubated for 30 min at 4°C. After incubation, 1 ml of 1 % BDPerm/Wash was added for washing. After washing, stained cells were resuspended in 200 µl PBS and then were measured in a FACSCalibur and analyzed utilizing CellQuest software.

2.11   Evaluation of FOXP3 expression

2.11.1 Isolation of RNA

Isolation of ribonucleic acid (RNA) from T_{reg} or CD4$^+$CD25$^-$ cells was performed using the RNeasy Kit (Qiagen, Hilden, Germany), including the optional DNase digest (RNase-free DNase set). Isolated cells stored at -80°C were flicked to loosen the pellets. This step and the following procedure were performed at RT.

In RLT (company designed buffer) buffer, 1 % β-mercaptoethanol was added first and 600 µl of this RLT buffer was added to the cells and mixed it well by vortexing. Then lysate was pipetted directly into a QIAshredder spin column which was placed in a 2 ml collection tube and centrifuged (10,000xg, 2 min at RT). 600 µl of 70 % ethanol was added to the homogenized lysate and then 600 µl of the sample was pipetted into QIAamp spin column placed in a 2 ml collection tube and centrifuged (10,000xg, 15 sec at RT). The flow through was then discarded and rest 600 µl of the sample was pipetted again into QIAamp spin column and centrifuged for 15 sec at maximum speed. After discarding the flow through, 350 µl RWI buffer (company designed buffer) was added and centrifuged (10,000xg, 15 sec at RT).

Ten microlitre Dnase 1 stock solutions was added to 70 µl buffer RDD (company designed buffer) and this Dnase incubation mix (80 µl) was pipetted directly onto the RNeasy silica-gel membrane and left on it for 30 min. Thereafter, 350 µl RWI buffer was again added and centrifuged (10,000xg, 15 sec at RT) and the

QIAamp spin column transferred into a 1 ml eppendorf tube and 30 µl of RNase free water was pipetted directly onto the QIAamp membrane and centrifuged (10,000xg, 1 min at RT). Again 30 µl of RNase free water pipetted directly onto the QIAamp membrane and centrifuge (10,000xg, 1 min at RT). The eppendorf contained RNA in volume of 60 µl.

2.11.2 cDNA synthesis from isolated RNA

Utilizing the Gold RNA PCR core kit (Applied Biosystems, Foster City, CA, USA), 150-600 ng of RNA were reverse transcribed in a total volume of 20 µl in a Gene-Amp PCR system 9700 (Applied Biosystems).

The reaction mix for cDNA synthesis was as follows.

5xRT (company designed buffer)	4 µl
25 mM MgCl$_2$	2 µl
10 mM dNTP (Deoxyribonucleotide triphosphate)	2 µl
RNase inhibitor	0.5 µl
10 mM DTT (dithiothreitol)	2 µl
Random Hexamer oligonucleotide	0.5 µl
Reverse Transcriptase	0.3 µl
	11.3 µl

8.7 µl of RNA (150-600 ng) and water were added to make final volume of 20 µl. Conditions for reaction were as follows: 25°C for 10 min, 42°C for 15 min, 95°C for 5 min, and 5°C for 5 min.

2.11.3 Real time PCR to evaluate FOXP3 expression

2.11.3.1 Principle

Real time polymerase chain reaction (PCR) is a method for quantitative detection of the initial amounts of DNA template in a sample. Most commonly used Real time PCR methods are TaqMan and SYBR Green methods, which utilize fluorescent-based DNA methodology.

TaqMan methodology is based on the 5' nuclease assay first described by Holland et al. (1991), which uses the 5'-3' exonuclease activity of *Taq* DNA polymerase to cleave a dual-labeled probe annealed to a target sequence during PCR-

amplification **(Figure 9)**. The TaqMan probe contains both a fluorescent dye at the 5'-end and a quencher dye at the 3'-end. The quencher can only quench the reporter fluorescence when the two dyes are close to each other which are only in case of an intact probe. Once amplification occurs, the probe is degraded by the 5'-3' exonuclease activity of *Taq* DNA polymerase, and the fluorescence will be detected by a laser in integrated in the sequence detector (ABI 7000 Real-Time PCR system, Foster City, CA, USA). The PCR cycle number at which fluorescence reaches a threshold value of 10 times the standard deviation of baseline emission is used for quantitative measurement. This cycle number is called the cycle threshold (Ct) and it is inversely proportional to the starting amount of the target cDNA.

TaqMan® Probe Method
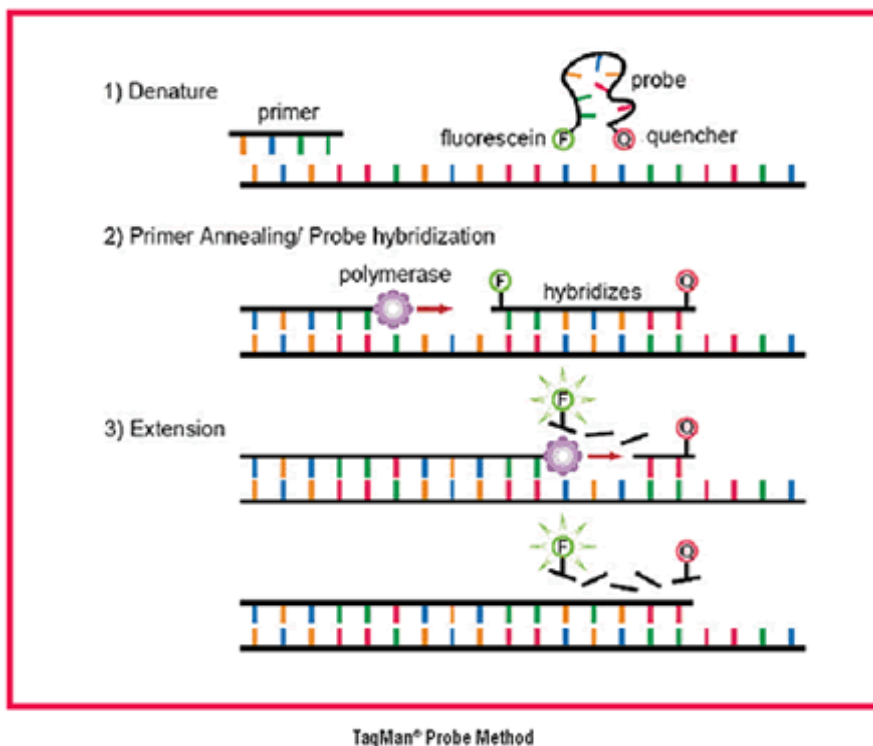
Figure 9 Mechanism of TaqMan method.
This figure is adapted from Takara Mirus Bio, Madison, Wisconsin (www.takarabiousa.com).

2.11.3.2 Procedure

The amount of FOXP3 and β-actin (ACTB; house keeping gene) message was quantified with an ABI 7000 Real time PCR system using TaqMan Gene Expression Assays (Assay IDs: Hs00203958_m1 for FOXP3, Hs99999903_m1 for ACTB; Applied Biosystems). Twenty five microlitre reaction mixtures containing 2.5 µl cDNA were run in duplicates.

The reaction mix was as follows:

Assays	1.25 µl
2xMix (PCR Mix)	12.5 µl
H_2O	8.75 µl
cDNA	2.50 µl

ΔCt values for FOXP3 were calculated taking the house keeping ACTB as standard gene. The difference in FOXP3 expression between T_{reg} and $CD4^+CD25^-$ cells was calculated as fold difference $= 2^{\Delta\Delta Ct}$ where $\Delta\Delta C_t = (\Delta C_t\ CD4^+CD25^-) - (\Delta C_t\ T_{reg})$.

2.12   Enzyme linked immunosorbent assay (ELISA) to detect antibodies against MBP

2.12.1 Principle

Enzyme linked immunosorbent assay (ELISA) is a commonly used immunoassay which combines the specificity of antibodies with the sensitivity of simple enzyme assay, by using antibodies or antigens coupled to an easily assayed enzyme. ELISA can be used to detect the presence of antigens that are recognized by an antibody or to detect antibodies that are recognized by an antibody.

To quantitate the human antibodies directed against MBP, we used a modified method described by Chamczuk et al. (2002). The standard curve was obtained using a monoclonal antibody (first antibody) from the mouse directed against human MBP. This monoclonal antibody was marked by a second antibody (coupled with an enzyme) directed against the immunoglobulin (Ig) G class of the first antibody.

The human antibodies were detected in the same indirect way that means by a second enzyme coupled antibody directed against the human IgG.

Our ELISA is a five-step procedure **(Figure10)**

a) Coating of microtiter plate wells with antigen,

b) blocking of all unbound sites to prevent false positive results,

c) addition of serum sample containing antibodies to well,

d) addition of secondary antibody conjugated to enzyme and

e) reaction of a substrate with the enzyme to produce a colored product.
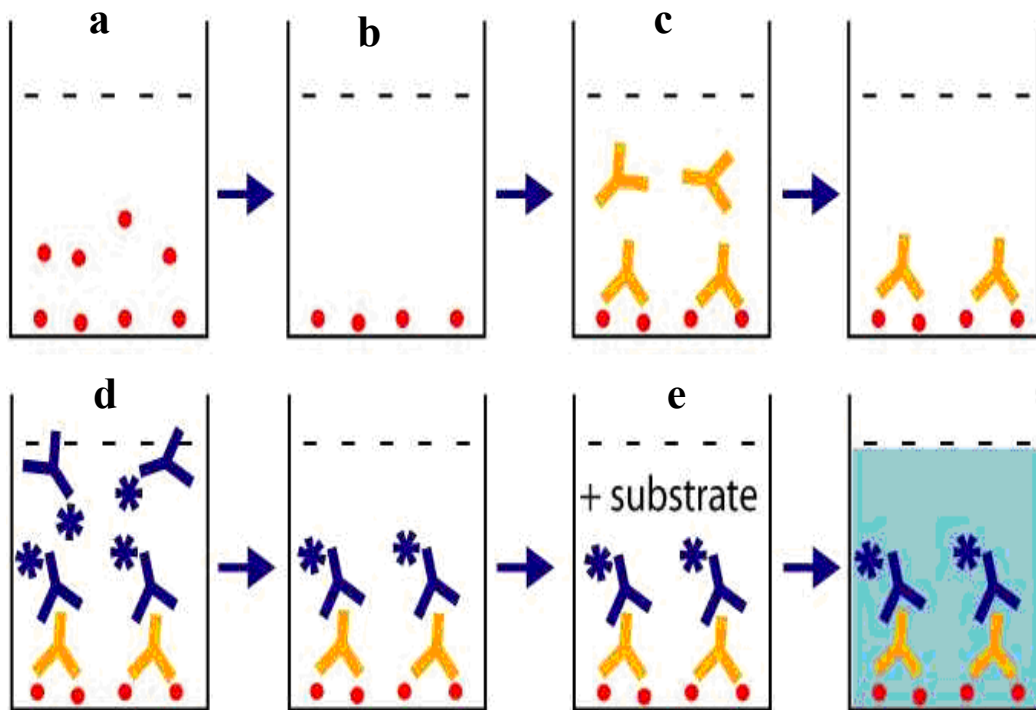
Figure 10 Schematic diagram of indirect enzyme linked immunosorbent assay.

2.12.2 Procedure

Different buffers were prepared for ELISA.

1) Carbonate / bicarbonate buffer

According to instructions of manufacturer, 2 capsules of carbonate / bicarbonate were diluted in sterile water to get a concentration of 100 mM and pH 9.6.

2) Blocking buffer

2 % BSA was added in carbonate / bicarbonate buffer and heparin (10 µl per ml of buffer) was added to this buffer.

3) Washing buffer and dilution buffer 1

0.05 % Tweeen 20 was added to PBS. This washing buffer was also used as dilution buffer 1.

4) Dilution buffer 2

2 % BSA was added in the PBS.

Ninety-six well microtiter plates (Multisorp catalogue no: 446490, Nunc, Wiesbaden, Germany) were coated with 125 µl of MBP solution (1 µg MBP diluted in 125 µl of carbonate/bicarbonate buffer). After coating, plates were sealed with ELISA plate sealer (IBL, Hamburg, Germany) and incubated at $4^°C$ overnight. After incubation, plate was washed 3 times with 300 µl of washing buffer per well by ELISA Plate washer (Tecan, Crailsheim, Germany). Then blocking was done with 250 µl of blocking buffer and incubated for 2 hours at RT. After washing, 100 µl of 3.125, 6.25, 12.5, 25, and 50 ng/ml of monoclonal mouse anti MBP antibody, diluted in dilution buffer 1 (Acris Antibodies, Hiddenhausen, Germany) were added in duplicate to generate standard curve. In other wells, 100 µl of serum or plasma samples diluted 1:667 in dilution buffer 2 were added in duplicate and incubated for 1 hour at RT. Following washing, 100 µl of Biotin-SP-Conjugated AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgG, F(ab')$_2$ Fragment Specific (Dianova/Jackson, Hamburg, Germany) diluted 1:40,000 in dilution buffer 1, was added to each well containing monoclonal mouse anti MBP antibody. Moreover, 100 µl of Biotin-SP-Conjugated AffiniPure Goat Anti-Human IgG, F(ab')$_2$ Fragment Specific (Dianova/Jackson) diluted 1:40,000 in dilution buffer 1, was added to wells containing serum samples. All wells were then incubated for 1 hour at RT. Thereafter, all wells were washed and 100 µl of the enzyme horseradish peroxidase (HRPO) conjugated streptavidin (GE healthcare, Buckinghamsphire, United Kingdom) diluted

1:8,000 in dilution buffer 1, was added in each well and incubated for 30 min. After washing, 100 µl of substrate tetramethyl benzidine (TMB; Human, Wiesbaden, Germany) was added to each well and stored in dark for 2-5 minutes. Then reaction was stopped with 100 µl/well with stop solution (Human, Wiesbaden, Germany). The optical density of colored product generated by the bound, enzyme-linked detection reagents was measured spectrophotometrically using an ELISA plate reader (Tecan) at a wavelength of 450 nm with the reference wavelength at 620 nm. The concentration of anti MBP antibodies was calculated by generating standard curve using concentration of monoclonal mouse anti-myelin basic protein against optical density (Systat software, San jose, CA, USA).

2.13   Statistics

If not otherwise mentioned, data are given in mean ± standard deviation (SD). The comparison of age, sex, number of T lymphocytes and their subpopulations e.g. T_{reg} as well as SI values of MS patients with the appropriate values of healthy individuals was performed using Wilcoxon two sample tests (Mann Whitney U test). Kruskal Wallis test was performed to analyze all parameters for comparison during follow up of mitoxantrone therapy in MS patients. Analysis of differences of SI values between CD25NEG and CD25MIX within the group of MS patients or healthy individuals was calculated by {[(cpm CD25NEG/cpm autologous CD25NEG)/ (cpm CD25MIX/cpm autologous CD25MIX)] − 1} and evaluated using the signed rank Wilcoxon test. The level of significance was designated as $p < 0.05$ (two-tailed tests). A trend of increase assigned as p value is 0.05-0.1. Spearman's coefficient of correlation was used for correlation analysis. The Statistical Analysis System software (SA, Cary, NC, USA) was used to calculate statistical significance.

3   Results

3.1   Molecular markers of T_{reg}

3.1.1   CD25 expression

In human, T_{reg} population exhibits low expression of CD25 to high expression as shown in **Figure 11, page 45**. Therefore, it was difficult to define a distinct population using a threshold channel for CD25 expression of T_{reg}.

Based on previous measurements done in our laboratory, the threshold value of CD25 expression to define T_{reg} was set to > 369 channels (fluorochrome PE). But the resulting cell population was a mixture of CD25^{high} and CD25^{intermediate} expressing cells as **Figure 11, page 45** illustrates. CD25^{high} expression was defined according to the CD25 fluorescence intensity of CD8$^+$ T cells in whole blood. We analyzed the CD25 expression of CD8$^+$ T cells in all MS patients and healthy individuals. In each individual, the highest channel for CD25 expression of CD8$^+$ T cells was defined as CD25^{high}. All cells showing higher CD25 expression were CD25^{high} expressing cells. Thus, this threshold was used to estimate the percentage of CD25^{high} expressing cells of the appropriate T_{reg} population. CD25^{intermediate} was defined between the threshold of > 369 channels and CD25^{high} expressing cells.

To validate the threshold of T_{reg} > 369 channels, we analyzed isolated T_{reg} and CD4$^+$CD25$^-$ cells from 10 healthy individuals (**Figure 12, page 45**). This analysis confirmed that most of the T_{reg} are located above the > 369 channels.

In our study, we used a magnetic bead separation method to isolate $T_{reg.}$ First, CD4$^+$ cells were isolated and then isolated CD4$^+$ cells were used to obtain T_{reg} and CD4$^+$CD25$^-$ cells. The purity of these isolated cells were analyzed using four color flow cytometry and the above defined threshold as shown in **Figure 13a, b and c, page 46**. Purity of isolated T_{reg} (> 369 channels) was calculated from 20 healthy individuals and purity was found to be greater than 82 %.
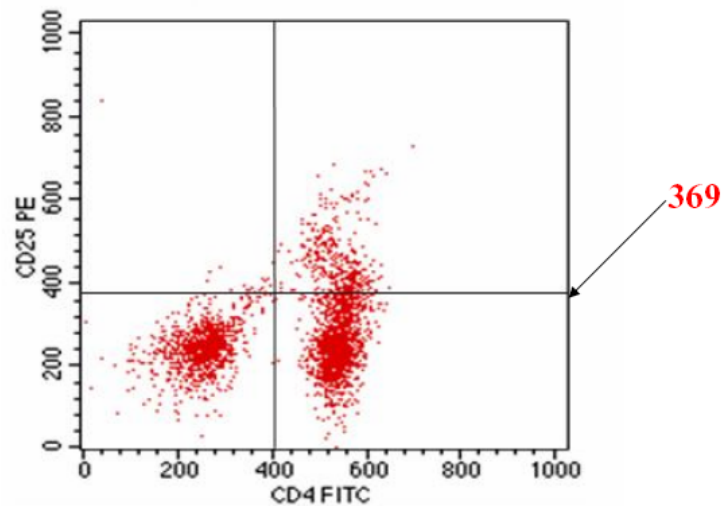
Figure 11 A representative example of flow cytometric analysis in whole blood showing the threshold of CD25 expression of natural regulatory T cells. Threshold defined for natural regulatory T cells to > 369 channels. For this analysis CD3$^+$ cells were gated.
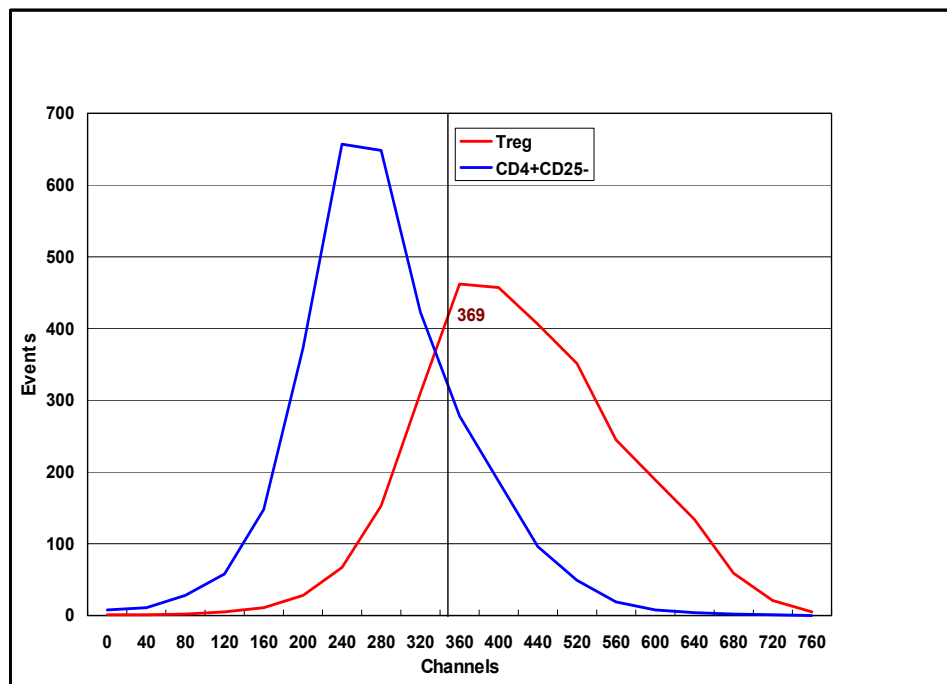
Figure 12 CD25 expression of natural regulatory T cells and CD4$^+$CD25$^-$ cells below and above the threshold of > 369 channels.
Isolated natural regulatory T and CD4$^+$CD25$^-$cells were stained with CD4 fluorescein isothiocyante and CD25 phycoerythrin. Events of CD25 expression were counted in every 40 channels for both isolated cells. Mean of events from 10 healthy individuals were taken and then mean was plotted against the channels.
T$_{reg}$: Natural regulatory T cells
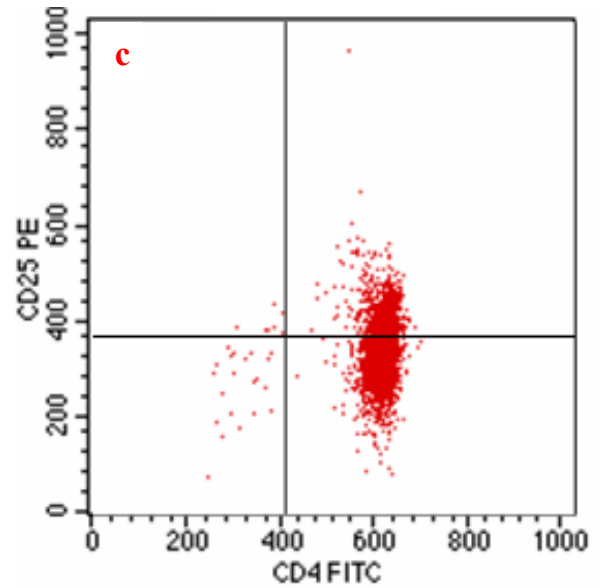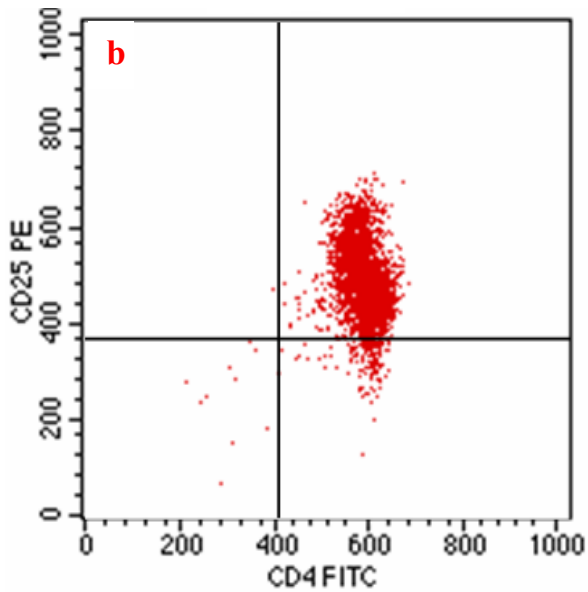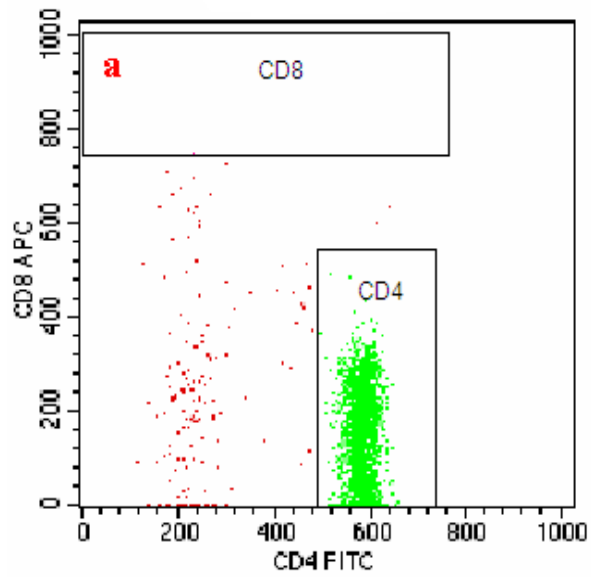
Figure 13 Flow cytometric analysis showing the purity of isolated cells.
After isolation, natural regulatory T cells (T_{reg}) and $CD4^{+}CD25^{-}$ cells were
analyzed for their purity by flow cytometry
(a) Isolated $CD4^{+}$
(b) Isolated T_{reg}
(c) Isolated $CD4^{+}CD25^{-}$ cells

3.1.2 HLA-DR expression

MS patients may have higher number of activated cells, thus "diluting" the number of T_{reg} account for the apparent deficient function in *in vitro* assays. HLA-DR expression of T ($CD3^+$) lymphocytes is an important parameter for activation. In order to characterize activation of our isolated T_{reg}, we measured HLA-DR expression in our T_{reg} and $CD4^+CD25^{high}$ cells from a MS patient. We found that 25 % of isolated T_{reg} were HLA-DR positive while 37 % of CD4+CD25^{high} cells were HLA-DR positive. This showed that not only $CD4^+CD25^{high}$ cells were HLA-DR positive but also our CD25^{intermediate} T_{reg} population was HLA-DR positive **(Figure 14 a to d, page 48)**. In order to compare the whole number of activated T cells, we measured number of HLA-DR expressing T cells and their $CD4^+$ and $CD8^+$ subpopulations in whole blood of MS patients and healthy individuals. We did not find differences in relative number of HLA-DR expressing T cell and their subpopulations between MS patients and healthy individuals **(Table 3, page 49)**. Thus, it seems unlikely that our *in vitro* results may be influenced by a "dilution effect".
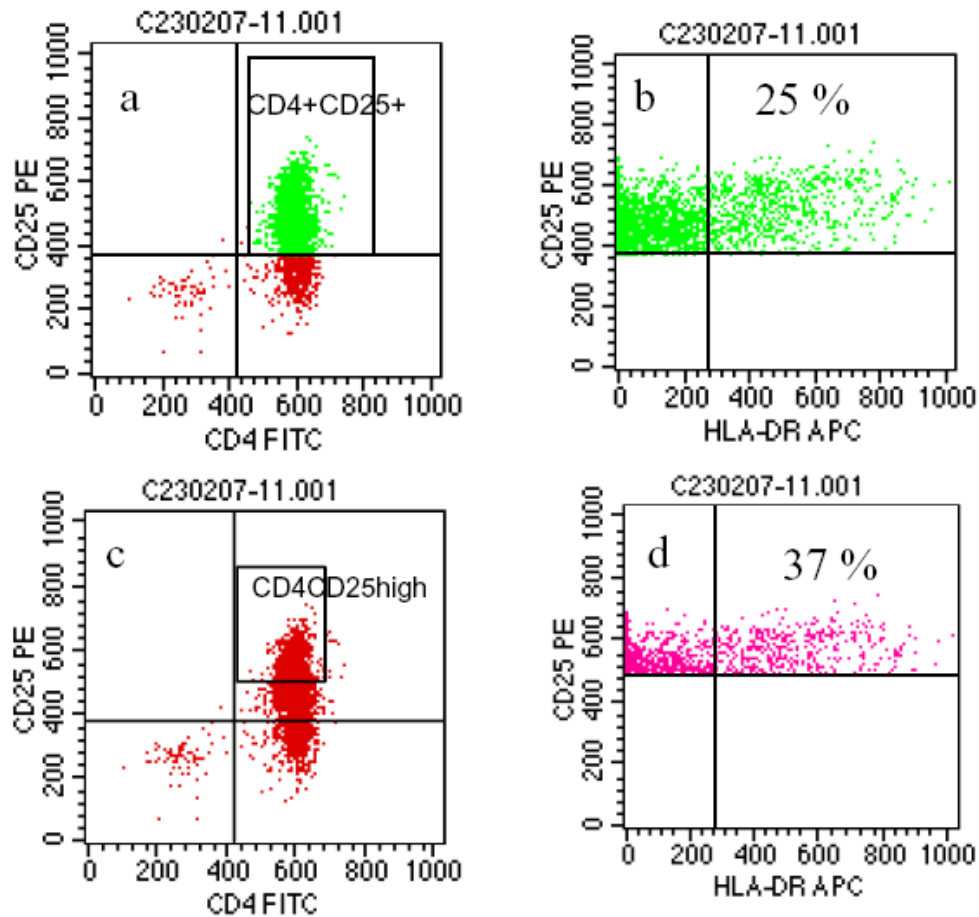
Figure 14 Flow cytometric analysis showing the HLA-DR expression in natural regulatory T (CD4$^+$CD25$^+$) cells and CD4$^+$CD25^{high} (CD4CD25high).

(a) CD25$^+$ expression of natural regulatory T cells (T$_{reg}$) and gate for HLA-DR measurement

(b) HLA-DR expression of the appropriate gate of (a)

(c) CD25^{high} expression of regulatory T cells (T$_{reg}$) and gate for HLA-DR measurement

(d) HLA-DR expression of the appropriate gate of (c)

Table 3 Relative number of HLA-DR expressing T lymphocytes and their subpopulations in whole blood of 33 healthy individuals and 34 patients suffering from multiple sclerosis

T lymphocytes	Healthy individuals	MS patients[a]	p value
$CD3^+HLA\text{-}DR^+$ [%]	2.4 ± 0.3[b]	2 ± 0.2	ns[c]
$CD3^+CD4^+HLA\text{-}DR^+$ [%]	1.2 ± 0.1	1.2 ± 0.1	ns
$CD3^+CD8^+HLA\text{-}DR^+$ [%]	0.9 ± 0.1	0.7 ± 0.1	ns

[a] MS: Multiple sclerosis
[b] All values are given as mean±standard deviation
[c] ns: Not significant

3.1.3 FOXP3 mRNA expression

Recently, it was described that FOXP3 is a unique marker for T_{reg}. Therefore, it was important to look for the expression of FOXP3 in our isolated T_{reg}. Thus, we measured FOXP3 mRNA expression in T_{reg} and $CD4^+CD25^-$ cells using real time PCR. An example of real time PCR results of mRNA expression in isolated T_{reg} and $CD4^+CD25^-$ is given in **Figure 15, page 50**. In both MS patients and healthy individuals, T_{reg} expressed about 13 fold more FOXP3 gene transcript than $CD4^+CD25^-$ cells **(Figure 16, page 51)** and confirming again that the isolated T_{reg} carry this unique marker.

FOXP3 is important for the development and function of T_{reg}. Therefore, different FOXP3 amounts could influence the suppressive activity of T_{reg}. Thus, it was important to measure FOXP3 gene transcript in MS patients and healthy individuals. The amount of FOXP3 gene transcript was not significantly different between MS patients and healthy individuals. This showed that changes in T_{reg} function between MS patients and healthy individuals were not influenced by FOXP3.
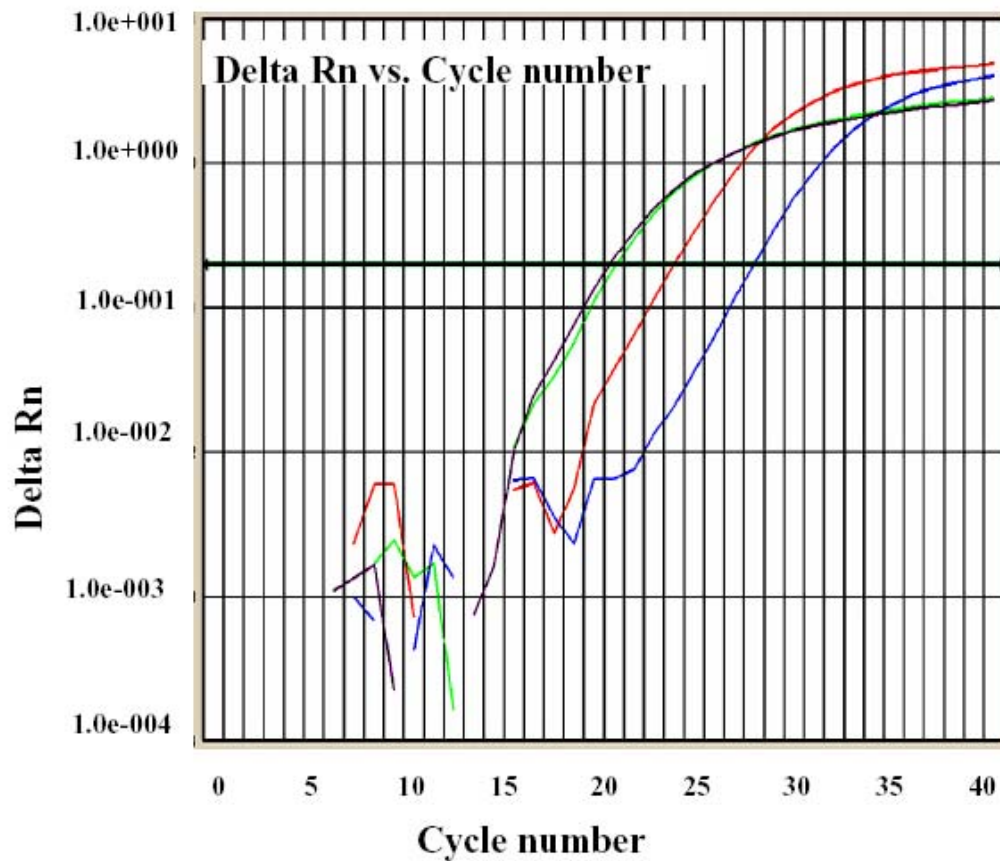
Figure 15   An example of real time PCR results showing the threshold cycle value
of natural regulatory T and CD4$^+$CD25$^-$ cells.
Natural regulatory T cells (T$_{reg}$) have less threshold cycle (Ct) value than
CD4$^+$CD25$^-$ for FOXP3. Violet color for CD4$^+$CD25$^-$ beta-actin, green
color for T$_{reg}$ beta-actin, red color for CD4$^+$CD25$^-$ FOXP3 and blue
color for T$_{reg}$ FOXP3.
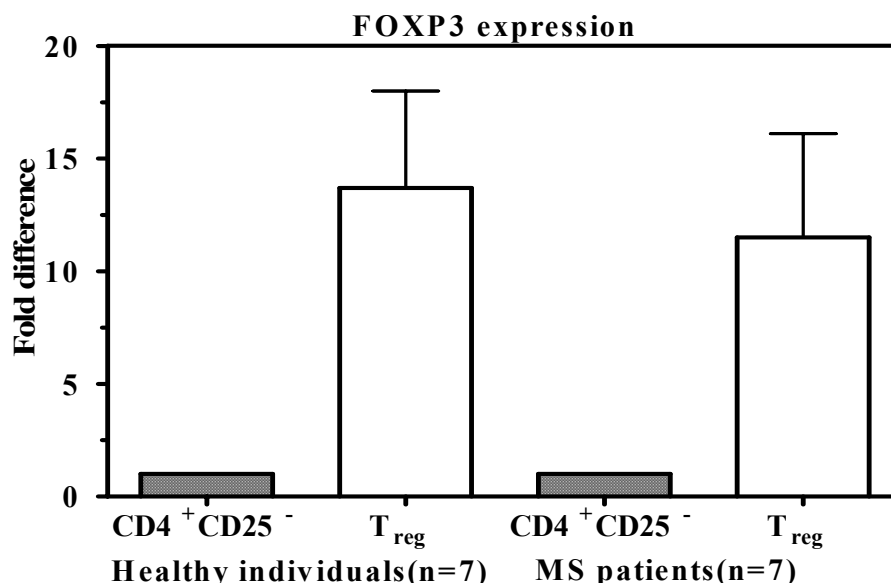
Figure 16 Expression of FOXP3 in natural regulatory T cells (T_{reg}) and CD4$^+$CD25$^-$ cells of healthy individuals and multiple sclerosis (MS) patients.
The difference in FOXP3 expression was calculated between T_{reg} and CD4$^+$CD25$^-$ cells as fold difference.

3.2 Number of leukocytes and their subpopulations

3.2.1 Comparison of cell numbers between healthy individuals and MS patients

Different absolute number of T_{reg} between MS patients and healthy individuals may have an impact on the function of these cells. Therefore, it was necessary to estimate the absolute T_{reg} number using the number of leukocytes and the relative amount of lymphocytes. Leukocyte numbers were found significantly increased while lymphocyte numbers significantly decreased in whole blood of MS patients compared with healthy individuals **(Table 4, page 52)**. But these changes neither influenced the relative number of T lymphocytes and their CD4$^+$ or CD8$^+$ subpopulations nor the B lymphocytes. No significant differences could be found in these cells between MS patients and healthy individuals in whole blood.

T_{reg} control autoimmune reactions so it was important to measure the number of T_{reg} in MS. It was described that CD8$^+$CD25$^+$ share the phenotypic and functional features with T_{reg}. Thus, it was also interesting to measure CD8$^+$CD25$^+.$ Relative number of both CD25 positive T lymphocytes (CD3$^+$CD25$^+$) and T_{reg} (CD3$^+$CD4$^+$CD25$^+$) were significantly increased in whole blood of MS patients

compared with healthy individuals. The relative number of $CD3^+CD8^+CD25^+$ lymphocytes tend to be increased in MS patients compared with those of healthy individuals. Because some authors had described that $CD4^+CD25^{high}$ population as T_{reg}, we also measured number of $CD4^+CD25^{high}$ cells. But, we did not find significant difference between healthy individuals and MS patients.

Although the leukocyte numbers of the patients was significantly increased and the relative amount of lymphocytes was decreased in MS patients compared with healthy individuals, the T_{reg} number was increased. That means that a diminshed T_{reg} function in the MS patients could not be explained by a decreased number of these cells.

Table 4 Number of leukocytes and their subpopulations in whole blood of 33 healthy individuals and 34 patients suffering from multiple sclerosis

Parameter	Healthy individuals	MS patients[a]	p value
Leukocytes [cells/µl]	6091 ± 1917[b]	7887 ± 2750	0.0012
Lymphocytes [%]	31 ± 7	24 ± 8	0.0004
B [cells/µl]	235 ± 152	260 ± 144	ns[c]
B [%]	12 ± 5	15 ± 6	ns
$CD3^+$ [%]	71 ± 1	72 ± 1	ns
$CD3^+CD4^+$ [%]	46 ± 1	47 ± 2	ns
$CD3^+CD8^+$ [%]	23 ± 1	23 ± 1	ns
$CD4^+/CD8^+$	1.4 ± 1	1.1 ± 0.8	ns
$CD3^+CD25^+$ [%]	17 ± 1	22 ± 2	0.016
T_{reg}[d] [%]	15 ± 1	20 ± 1	0.029
$CD3^+CD4^+CD25^{high}$ [%]	1 ± 0.5	0.8 ± 0.6	ns
$CD3^+CD8^+CD25^+$ [%]	1.4 ± 0.2	2.3 ± 0.4	0.05

[a] MS:Multiple sclerosis
[b] All values are given as mean±standard deviation
[c] ns: Not significant
[d] T_{reg}: Natural regulatory T cells ($CD3^+CD4^+CD25^+$)

3.2.2   Effect of mitoxantrone (MX) therapy on cell numbers of MS patients

Mitoxantrone (MX) is an anthracenedione derivative of doxyrubicin which is highly effective in the treatment of progressive MS (Hartung et al., 2002). MX acts as an immunosuppressant which target proliferating immune cells. To clarify which cell type is influenced by MX, we analyzed the effect of MX on number of T_{reg} and other immune cells and did follow up studies up to one year with interval of 3 months.

Leukocytes number and relative number of lymphocytes remain unchanged during therapy **(Table 5, page 54)**. There were no significant changes in relative number of T cells and their subpopulations ($CD4^+$ and $CD8^+$) and the ratio of $CD4^+/CD8^+$ following treatment of MX**.** We also analyzed CD25 expression on T cells and their subpopulations. Relative number of $CD3^+CD25^+$, T_{reg} and $CD8^+CD25^+$ cells were also not affected by MX treatment.

Interestingly, there was a significant decrease in relative and absolute B cell numbers at 3 months compared with baseline (before treatment) values and this decrease was more pronounced over period of time **(Figure 17a and b, page 55)**. Based on these finding, it seems that a persistent and selective reduction of B cell numbers occurred following MX treatment but other lymphocytes subpopulations remain unaffected by MX therapy.

Table 5 Number of leukocytes and their subpopulations in whole blood of patients suffering from multiple sclerosis before and after treatment of mitoxantrone

Parameter	Baseline[a] (n^b=20)	3 months (n=18)	6 months (n=12)	9 months (n=6)	1 year (n=5)
Leucocytes [cells/µl]	8985±4618[c]	9974±5333	11386±7428	11816±6338	6632±1861
Lymphocytes [%]	23±8	20±8	21±12	21±13	22±9
$CD3^+$ cells [%]	74±8	74±10	75±15	75±12	79±9
$CD3^+ CD4^+$ [%]	55±8	54±8	54±12	57±6	56±10
$CD3^+ CD8^+$ [%]	18±4	21±7	21±7	18±6	21±3
$CD4^+/CD8^+$	3.3±1.2	3±0.9	2.8±0.8	3.5±1.4	2.7±0.7
$CD3^+CD25^+$ [%]	28±19	26±9	26±7	28±10	23±8
T_{reg}^{d} [%]	26±9	24±9	23±6	26±9	22±8
$CD3^+ CD8^+CD25^+$ [%]	2.5±2.2	2.3±1.9	2.6±2.6	2.4±1.9	1.3±0.7

[a] Baseline (before treatment)
[b] n: Number of patients with multiple sclerosis
[c] All values are given as mean±standard deviation;
[d] T_{reg}: Natural regulatory T cells ($CD3^+CD4^+CD25^+$)
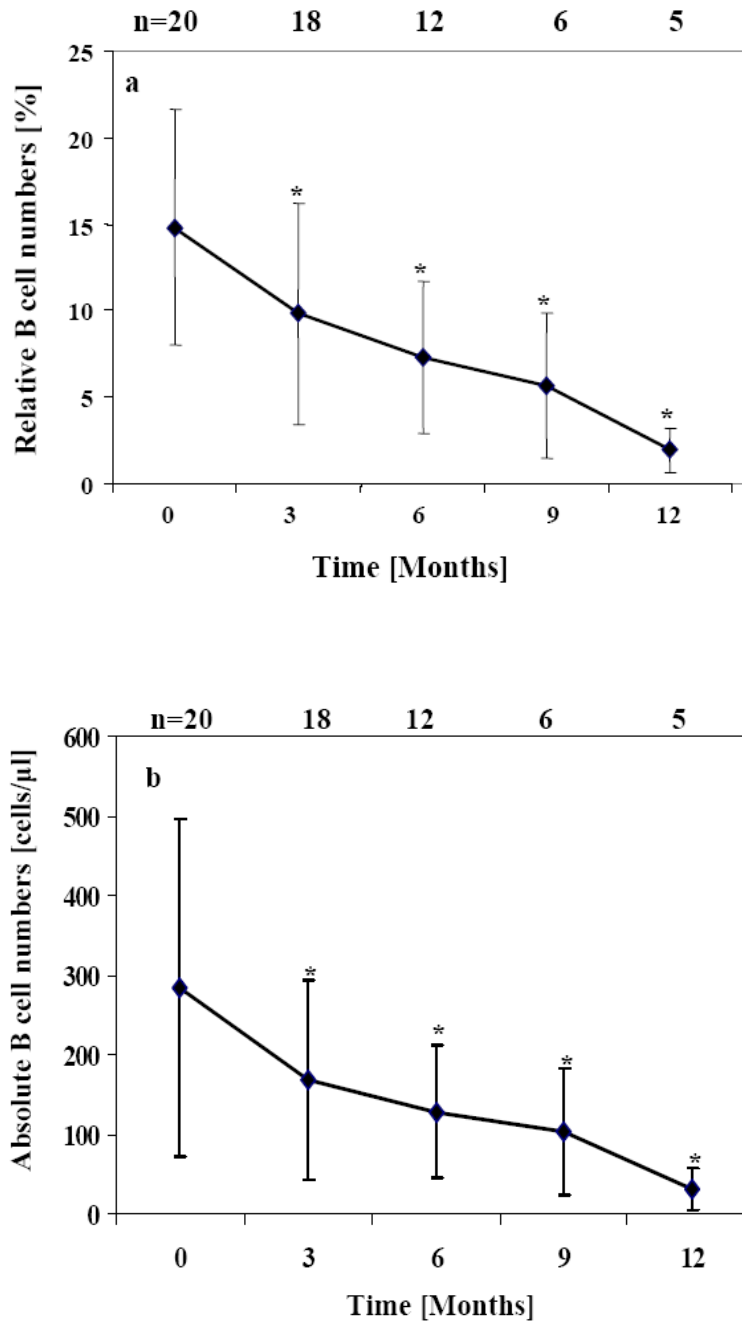
Figure 17 Relative **(a)** and absolute **(b)** number of B cells of multiple sclerosis patients before and during therapy with mitoxantrone.
Numbers were shown as mean±standard deviation. Zero months represents before treatment and 3, 6, 9, 12 months represents after treatment of mitoxantrone. n represents number of patients.
* $p < 0.05$ vs. zero months

3.3 Function of T_{reg}

T_{reg} control autoimmune reactions so it was important to examine the function of these cells in MS patients. As a possible target of this autoimmune reaction, myelin basic protein (MBP) has been the most widely studied myelin antigen. Thus we used MBP as an antigen and as a control pokeweed mitogen (PWM) for stimulation.

3.3.1 Optimization of the antigen concentration and cell numbers for the proliferation assay

3.3.1.1 Determination of MBP concentration

We tested different concentrations of MBP in four healthy individuals. For this purpose, peripheral blood monocytes (PBMCs) were stimulated with different concentrations of MBP and proliferation was measured by ^{3}H-thymidine incorporation as counts per minute. We included as a control PBMCs without any antigenic or mitogenic stimulation (autologous) in order to estimate the intrinsic capacity of proliferation because some of the T cells included in the PBMCs were preactivated. To take this autologous proliferation in consideration, the stimulation index (SI) equal to the values of stimulated cultures/autologous cultures was calculated. Four μg/well of MBP concentration was the best concentration for stimulation **(Figure 18)**. In our cultures, we used 5 μg MBP/well.
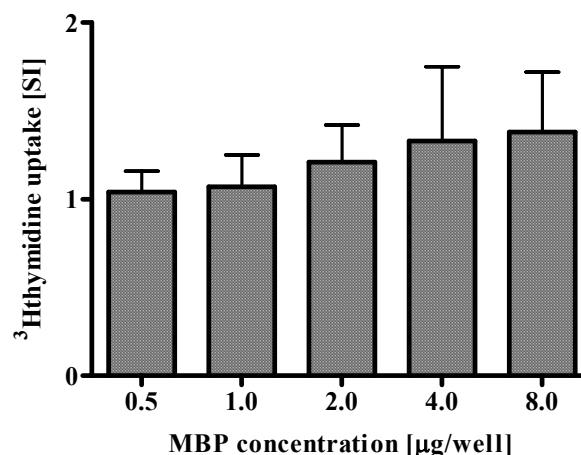
Figure 18   Myelin basic protein induced proliferation of peripheral blood mononuclear cells.
1 x 10^5 peripheral blood mononuclear cells were stimulated with different concentrations of myelin basic protein (MBP). Data are shown as mean+SD.
SI: Stimulation index

3.3.1.2 Determination of PBMCs number

We tested different number of PBMCs using MBP for stimulation in one healthy individual. 2×10^5 cell numbers showed the best proliferation. But, these cell numbers were always difficult to obtain from the MS patients **(Figure 19)**. Therefore, we should use less PBMCs in the cultures. 0.5×10^5 and 1×10^5 PBMCs/well showed measurable proliferation. Because, we needed some more cells to measure additional parameters e.g. TNF-α expression, we chose 1×10^5 PBMCs/well in the proliferation assay.
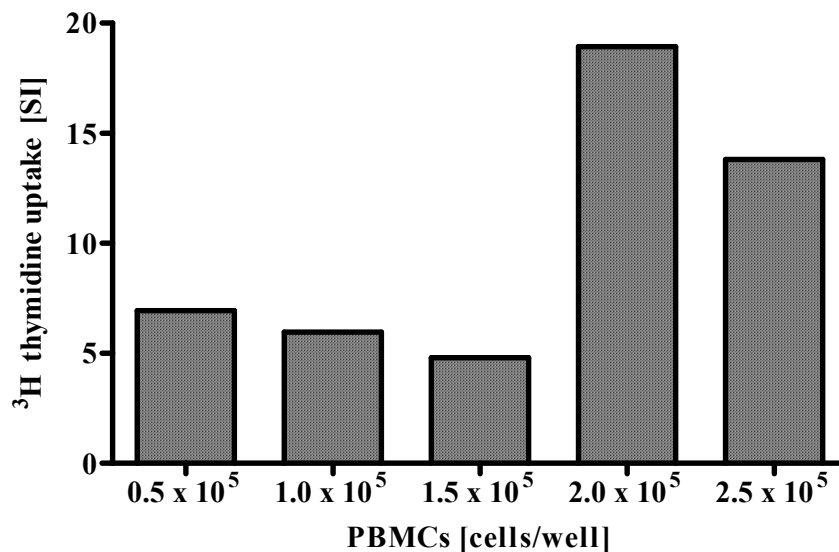
Figure 19 Proliferation of peripheral blood mononuclear cells from one healthy individual.
Different number of peripheral blood mononuclear cells was stimulated with 4 μg/well of myelin basic protein.
PBMCs: Peripheral blood mononuclear cells
SI: Stimulation index

3.3.1.3 Determination of isolated T_{reg} and CD4$^+$CD25$^-$ numbers

We tested different number of isolated T_{reg} or CD4$^+$CD25$^-$ for proliferation in seven healthy individuals. First, we added 1×10^5 PBMCs per well and then we added different numbers of isolated T_{reg} or CD4$^+$CD25$^-$ cells **(Figure 20 and 21, page 58)**. 2.5×10^3 and 10×10^3 of T_{reg} or CD4$^+$CD25$^-$ cells showed the best proliferation. We used 10×10^3 of both cells in order to increase the effect of theses cells.
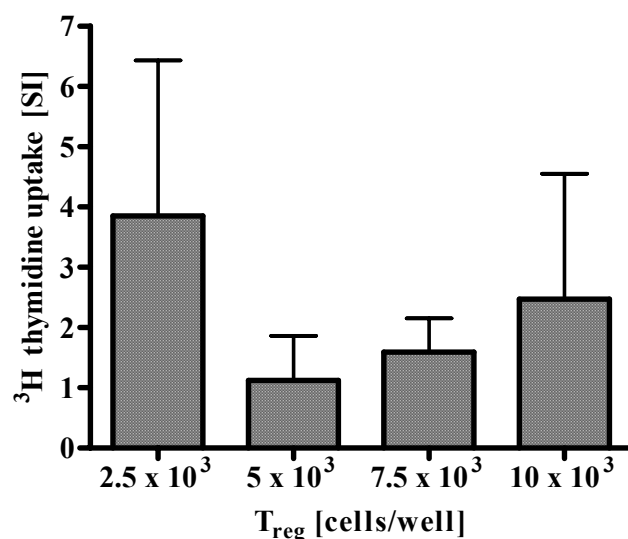
Figure 20 Proliferation of natural regulatory T cells stimulated with 4 μg/well of myelin basic protein.
In all wells, first, $1x10^5$ peripheral mononuclear cells were added and then different cell number of natural regulatory T cells was added. Data are shown as mean + SD.
SI: Stimulation index
T_{reg}: Natural regulatory T cells
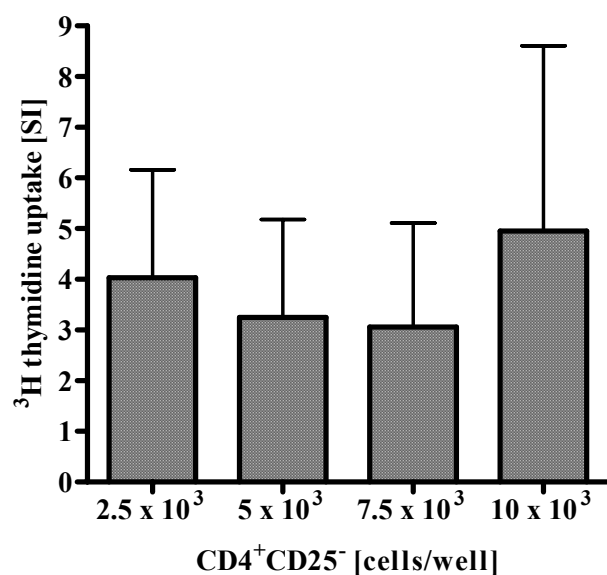
Figure 21 Proliferation of $CD4^+CD25^-$ cells stimulated with 4 μg/well of myelin basic protein.
In all wells, first, $1x10^5$ peripheral mononuclear cells added and then different cell numbers of $CD4^+CD25^-$ cells were added. Data are shown as mean + SD.
SI: Stimulation index

3.3.2 Suppressive activity of T_{reg}

To address the question that T_{reg} are proliferative in nature, T_{reg} from two healthy individuals and two MS patients were cultured alone without any stimulation (autologous) and with antigen (MBP) or mitogen (PWM). We found no difference in proliferation between cultures with and without MBP addition. In contrast, PWM-induced proliferation was increased compared with autologous values. So, T_{reg} proliferation was dependent from the conditions.

In order to establish that the antigen MBP and the mitogen PWM-induced proliferation values of PBMCs plus $CD4^+CD25^-$ (CD25NEG) should be different from the autologous values, we compared these values. Indeed, statistical analysis revealed that mitogen (PWM) and antigen (MBP)-induced proliferation of CD25NEG from MS patients as well as healthy individuals was significantly different from the appropriate autologous values **(Table 6)**. This was also true if we added T_{reg} in CD25NEG (**Table 7, page 60**).

Table 6 Proliferation of peripheral blood mononuclear cells plus $CD4^+CD25^-$ (CD25NEG) cell without (autologous) and with addition of myelin basic protein or pokeweed mitogen from 19 healthy individuals and 29 multiple sclerosis patients

	^{3}H-thymidine uptake [cpm[a]]		
	Autologous	MBP[b]	PWM[c]
Healthy individuals	363 ± 317[d]	1074 ± 1252[e]	53398 ± 22755[e]
MS[f] patients	327 ± 268	940 ± 1072[e]	63089 ± 36940[e]

[a]cpm: Counts per minute
[b]MBP: Myelin basic protein
[c]PWM: Pokeweed mitogen
[d]All values are given as mean±standard deviation
[e]$p<0.05$ compared with appropriate autologous values
[f]MS: Multiple sclerosis

Table 7 Proliferation of peripheral blood mononuclear cells plus $CD4^+CD25^-$ and T_{reg} (CD25MIX) without (autologuous) and with addition of myelin basic protein or pokeweed mitogen from 19 healthy individuals and 29 multiple sclerosis patients

	^{3}H-thymidine uptake [cpm^a]		
	Autologous	MBP^b	PWM^c
Healthy individuals	482 ± 411^d	797 ± 687^e	49299 ± 24633^e
MS^f patients	373 ± 264	894 ± 804^e	63274 ± 34355^e

^{a}cpm: Counts per minute
^{b}MBP: Myelin basic protein
^{c}PWM: Pokeweed mitogen
^{d}All values are given as mean±standard deviation
^{e}p<0.05 compared with appropriate autologous values
^{f}MS: Multiple sclerosis

Now, we looked if there was a difference in proliferation in MS patients and healthy individuals between cultures without (CD25NEG) and with (CD25MIX) T_{reg}. Proliferation was significantly reduced in cultures containing T_{reg} compared to those without addition of T_{reg} in healthy individuals. This was true for the antigen MBP as well as mitogen (PWM)-induced proliferation **(Figure 22 a and b, page 61)**. These results confirmed that the isolated T_{reg} were able to suppress antigen and mitogen-induced proliferation and fulfilled the functional requirements for isolated T_{reg}.

In contrast to healthy individuals, we found that MBP stimulated cultures containing T_{reg} of MS patients did not show significantly reduced proliferation compared with those without T_{reg}. Although, PWM-induced proliferation was significantly diminished in T_{reg} containing cultures compared with those without T_{reg}; this effect was less pronouced compared with healthy individuals. Thus, T_{reg} in MS patients showed reduction or loss of suppressive activity.

There was another difference between MS patients and healthy individuals in PWM-induced proliferation. The proliferation of T_{reg} containing cultures of the MS patients was significantly increased compared with the appropriate values of the healthy individuals. That may be due to an increased proliferative activity of T_{reg}.
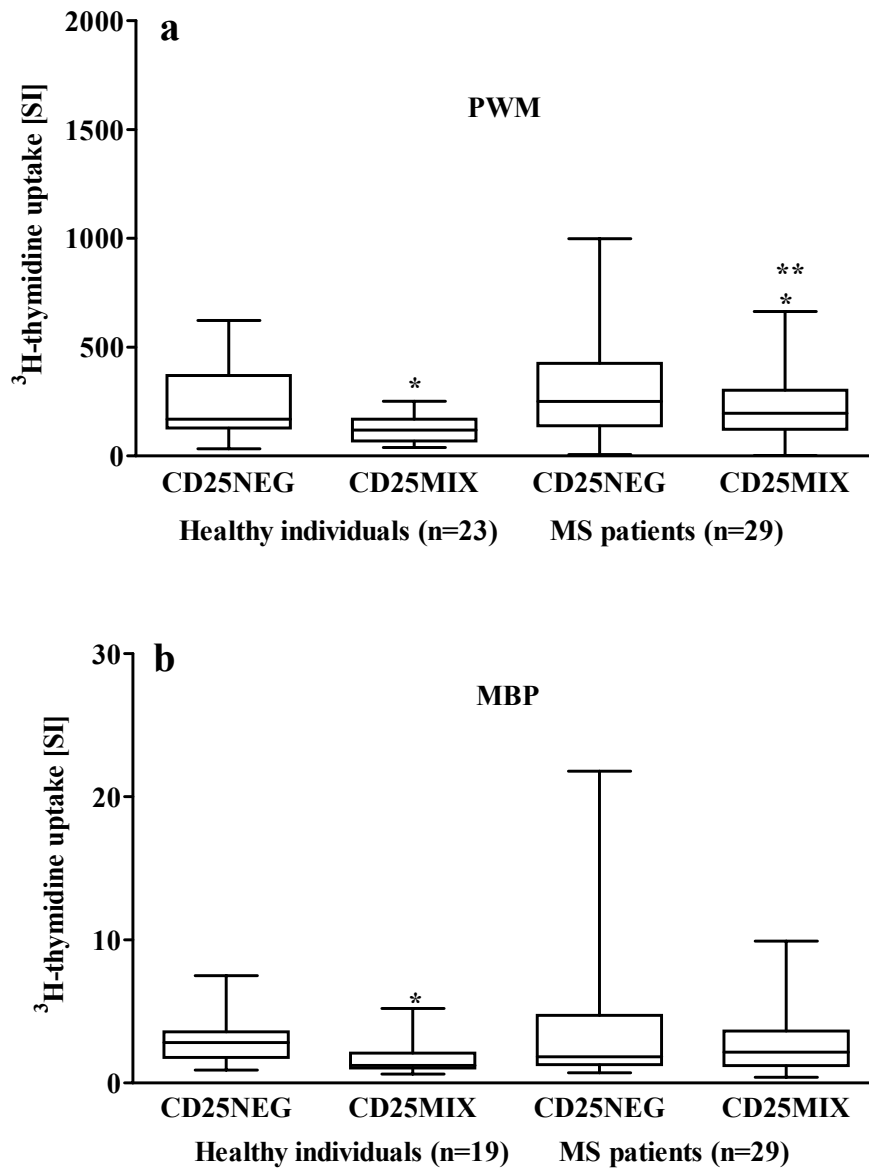
Figure 22 Pokeweed mitogen (a) and myelin basic protein (b) induced proliferation of CD25NEG and CD25MIX.

$1x10^5$ peripheral blood mononuclear cells plus $1x10^4$ CD4$^+$CD25$^-$ cells were cultured in absence (CD25NEG) or presence of $1x10^4$ natural regulatory T cell (CD25MIX). The bars extend from the 25th percentile to the 75th percentile with a horizontal line at the median. Whiskers extend to the smallest value up to the largest.

n represents number of multiple sclerosis (MS) patients and healthy individuals.

PWM: Pokeweed mitogen

MBP: Myelin basic protein

*p < 0.05 vs. CD25NEG

** p < 0.05 vs. CD25MIX of healthy individuals

SI: Stimulation index

Data obtained from MS patients displayed a broad range in proliferation and suggested that the functional activity of T_{reg} may differ between individual MS patients. Therefore, the suppressive activity of T_{reg} was calculated individually and compared. The percentage of suppression of T_{reg} (CD25MIX) was calculated by setting the SI values of CD25NEG cultures to 100 % proliferation.

In PWM-induced co-cultures, T_{reg} from healthy individuals (21 of 23 cases, 91 %) showed a trend to higher suppressive activities (chi square test; p = 0.086) compared with MS patients (20 of 29 cases, 69 %) **(Figure 23, page 63)**. In addition, T_{reg} of 13 out of 29 MS patients (45 %) suppressed MBP-induced proliferation **(Figure 24, page 64)**, this was significantly (chi square test; p = 0.022) less than T_{reg} from healthy individual (17 out of 19, 89 %). In contrast to healthy individuals, T_{reg} of 55 % (MBP-induced proliferation) and 31 % (PWM-induced proliferation) MS patients showed loss of suppression.

T_{reg} suppressive function using PWM and MBP-induced proliferation was significantly correlated in healthy individuals (r = 0.49, p = 0.0349) as well as in MS patients (r = 0.61, p = 0.0005).

PWM

Figure 23 Suppressive function of natural regulatory T cells in pokeweed mitogen induced proliferation.

To assess the functional activity of regulatory T cells (T_{reg}), 1×10^5 peripheral blood mononuclear cells plus 1×10^4 $CD4^+CD25^-$ cells were cultured in absence (CD25NEG) or presence of 1×10^4 T_{reg} (CD25MIX) in healthy individuals or multiple sclerosis (MS) patients. Pokeweed mitogen (PWM) was used for stimulation.

n represents number of multiple sclerosis patients and healthy individuals.

MBP
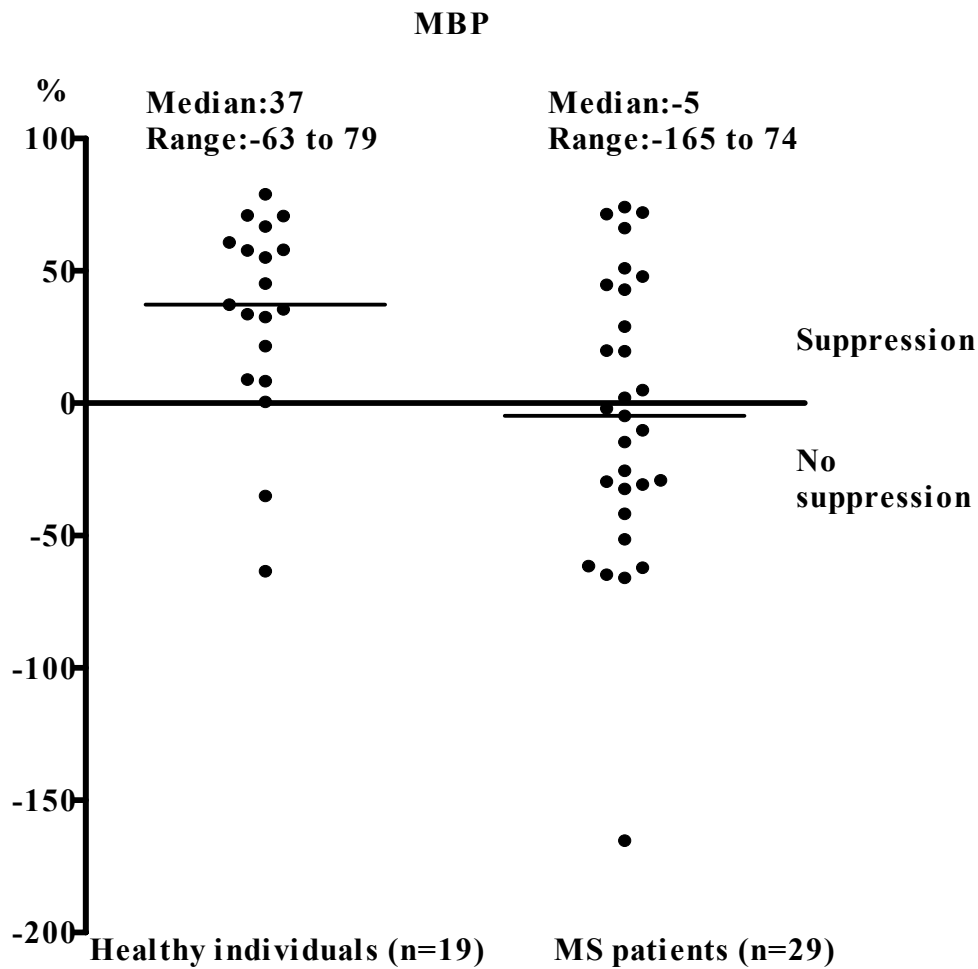
Figure 24 Suppressive function of natural regulatory T cells in myelin basic protein induced proliferation.

To assess the functional activity of regulatory T cells (T_{reg}), 1 x 10^5 peripheral blood mononuclear cells plus 1 x 10^4 $CD4^+CD25^-$ cells were cultured in absence (CD25NEG) or presence of 1 x 10^4 T_{reg} (CD25MIX) in healthy individuals or multiple sclerosis (MS) patients. Myelin basic protein (MBP) was used for stimulation.

n represents number of multiple sclerosis patients and healthy individuals.

3.3.3 Effect of MX on suppressive activity of T_{reg}

As described earlier in section 3.3.2, we found the loss of suppressive activity of T_{reg} in MS patients. Hence, we assumed that MX therapy may restore the loss of suppressive activity of T_{reg}.

We did the proliferation analysis to measure the suppressive activity up to 9 months after MX therapy and compared it with the baseline (before treatment) values. We did not find any significant difference in suppressive activity of T_{reg} in MBP and PWM-induced proliferation **(Figure 25 and figure 26, page 66)** during therapy compared with baseline values. Thus, MX therapy did not restore the loss of suppressive activity in MS patients.
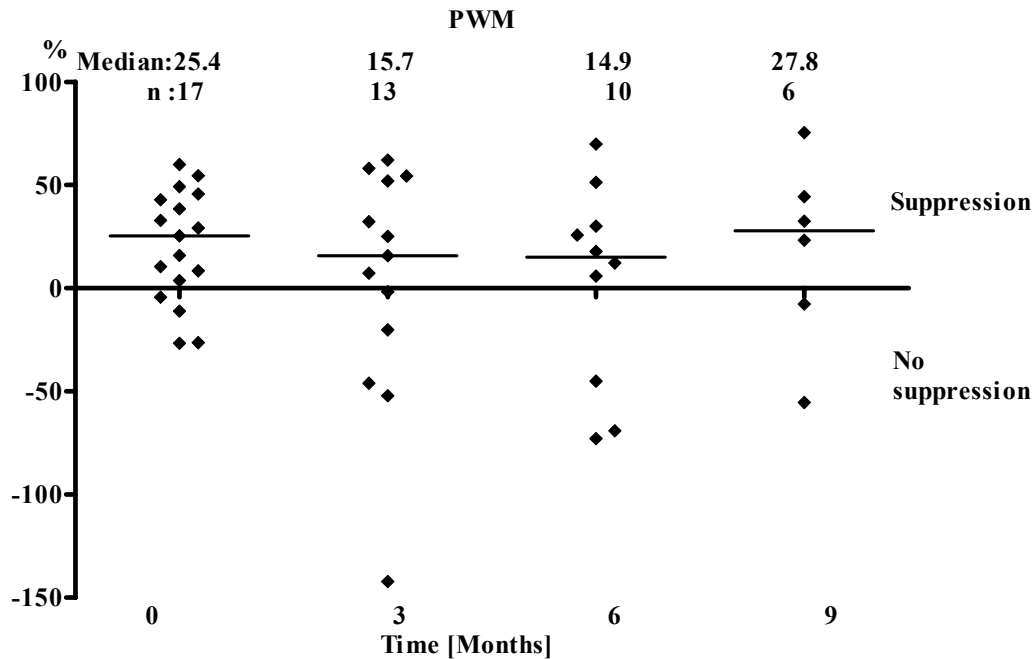
Figure 25 Suppressive function of natural regulatory T cells in pokeweed mitogen induced proliferation.
To assess the functional activity of natural regulatory T cells (T_{reg}), 1 x 10^5 peripheral blood mononuclear cells plus 1 x 10^4 CD4$^+$CD25$^-$ cells were cultured in absence (CD25NEG) or presence of 1 x 10^4 T_{reg} (CD25MIX) in multiple sclerosis (MS) patients. Pokeweed mitogen (PWM) was used for stimulation.
Zero months represents before treatment.
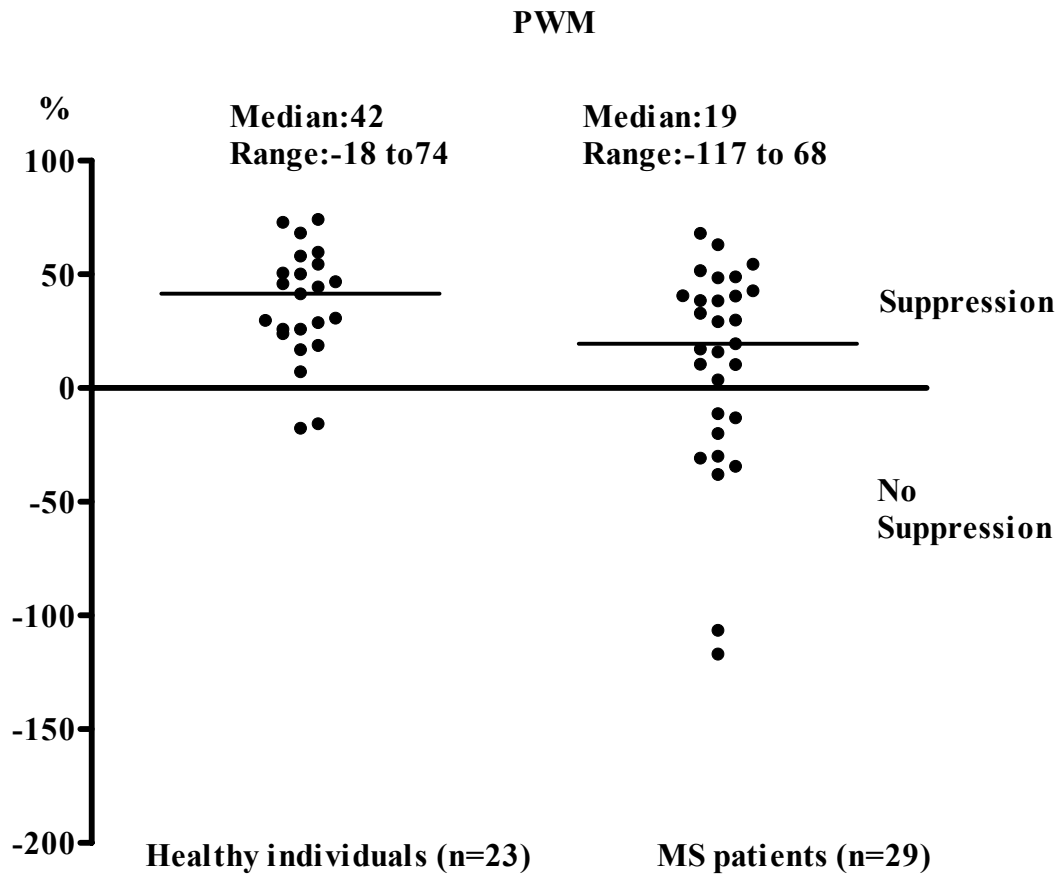 n represents number of MS patients

Figure 26   Suppressive function of natural regulatory T cells in myelin basic protein induced proliferation.
To assess the functional activity of natural regulatory T cells (T_{reg}), 1 x 10^5 peripheral blood mononuclear cells plus 1 x 10^4 $CD4^+CD25^-$ cells were cultured in absence (CD25NEG) or presence of 1 x 10^4 T_{reg} (CD25MIX) in multiple sclerosis (MS) patients. Myelin basic protein (MBP) was used for stimulation.
Zero months represents before treatment.
n represents number of MS patients.

3.4   Role of TNF-α production in T_{reg} function

Mechanism of T_{reg} mediated suppression is still less understood. So in order to elucidate the mechanism of T_{reg} mediated suppression, we measured the TNF-α production in $CD3^+$ and $CD4^+$ cells in four MS patients and five healthy individuals. First, PBMCs plus $CD4^+CD25^-$ cells were cultured in absence (CD25NEG) or presence (CD25MIX) of T_{reg} and then stimulated with PWM. After 5 days of cultures, we collected the cultured cells and then performed intracellular staining to measure the TNF-α expression in proliferating $CD3^+$ and $CD4^+$ cells in cultures with and without T_{reg}. We also tried to measure it in autologous cultures and MBP stimulated cultures, but we could not measure TNF-α production because of less proliferation. An example of flow cytometric measurement of TNF-α is shown in **Figure 27, page 68**. We also did proliferation assay to measure T_{reg} mediated suppression as described in section 3.3.

In MS patients, we found correlation between T_{reg} mediated suppression and TNF-α production. In three MS patients, loss of suppression is related to increase in TNF-α production while in one patient, there is T_{reg} mediated suppression with less TNF-α production **(Table 8, page 69)**.

In contrast to MS patients, we did not find any correlation between T_{reg} mediated suppression and TNF-α production. Out of four healthy individuals with having suppression, two had increased and two had decreased TNF-α production **(Table 8, page 69)**. We had seen earlier in section 3.3.2 that 91 % of healthy individuals showed T_{reg} mediated suppression in PWM-stimulated cultures. Thus, we chose one healthy individual with no suppression to look for TNF-α production and we found a decrease in TNF-α production. In this healthy individual, percentage of suppression is not correlated with TNF-α production as we had found in MS patients. These results demonstrated that involvement of TNF-α production in T_{reg} mediated suppression may differ between MS patients and healthy individuals.
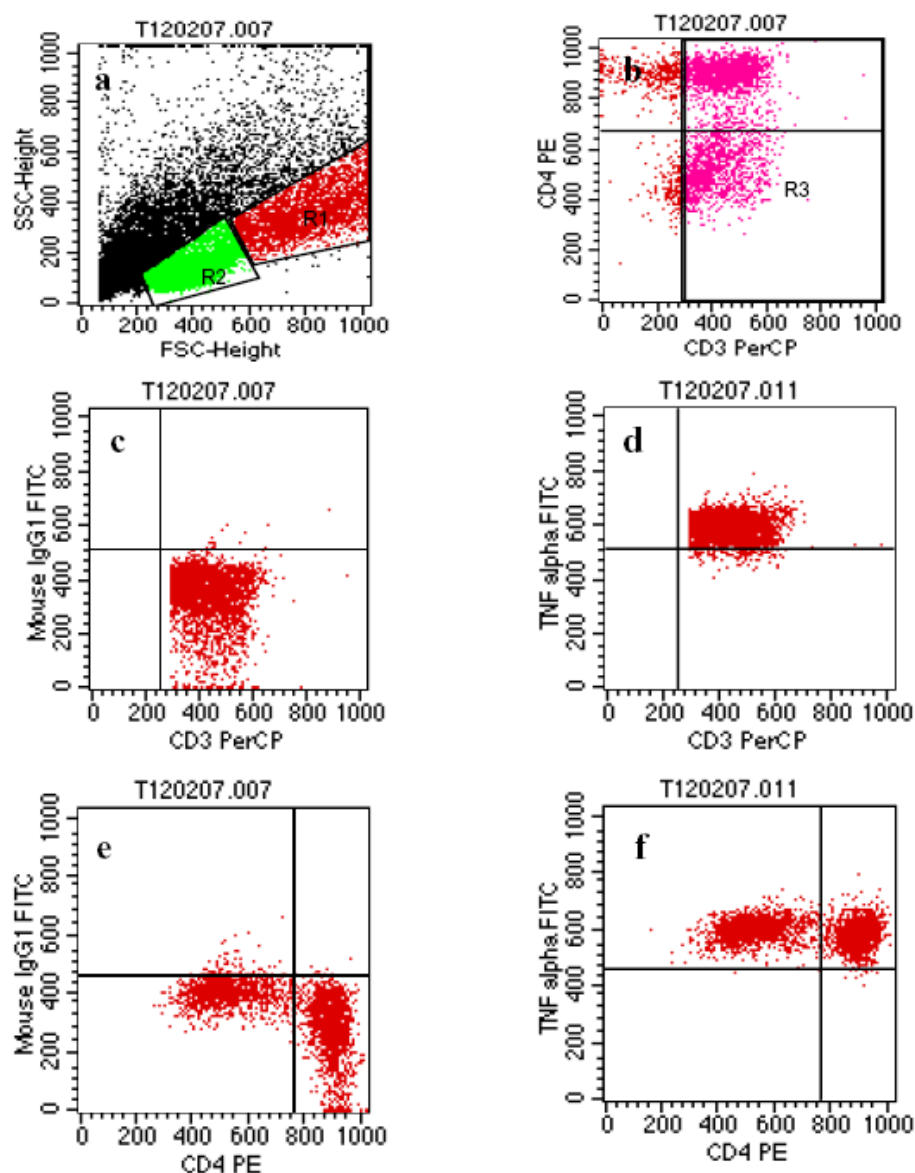
Figure 27 An example of flow cytometric measurement of tumor necrosis factor-α expression.

(a) R1 and R2 gate represents proliferating and non-proliferating cells.

(b) R3 gate represents CD3 positive cells which are gated from R1

(c) CD3^{+} against isotype control (Gated on R1 and R3)

(d) CD3^{+} against tumor necrosis factor TNF-α which shows TNF-α positive cells CD3^{+} cells (Gated on R1 and R3)

(e) CD4^{+} against isotype control (Gated on R1 and R3)

(f) CD4^{+} against TNF-α which shows TNF-α positive cells CD4^{+} cells (Gated on R1 and R3)

Table 8 Difference in relative numbers of tumor necrosis factor-α positive $CD3^+$ and $CD4^+$ cells on the peripheral blood mononuclear cells plus $CD4^+CD25^-$ without (CD25NEG) and with (CD25MIX) T_{reg} population after proliferation with pokeweed mitogen-stimulated cultures and percentage of suppression

Patient/Healthy	[a]% of $\Delta CD3^+$	[b]% of $\Delta CD4^+$	[c]Suppression
Patient1	14	18	No
Patient2	4	5	No
Patient3	19	17	No
Patient4	-54	-59	Yes
Healthy1	36	45	Yes
Healthy2	-15	-16	Yes
Healthy3	48	49	Yes
Healthy4	-4	-0.5	Yes
Healthy5	-5	-14	No

[a]$\Delta CD3^+$ = (TNF-α positive $CD3^+$ of CD25MIX) - (TNF-α positive $CD3^+$ of CD25NEG)

[b]$\Delta CD4^+$ = (TNF- α positive $CD4^+$ of CD25MIX) - (TNF-α positive $CD4^+$ of CD25NEG)

[c] Suppression is percentage of suppression of PWM induced proliferation

3.5   Detection of antibodies against MBP

Autoimmunity is also detectable by measurement of auto antibodies. Thus, we compared concentrations of autoantibodies the CNS-restricted antigens (MBP) in serum of MS patients and healthy individuals. At the time when we established this assay, no human anti MBP antibody was available. Therefore, we quantitated these autoantibodies from a standard curve generated using a monoclonal antibody (first antibody) from the mouse directed against human MBP. An example of this standard curve is shown in **Figure 28**. Co-efficient of intra-assay variation was calculated by running plasma sample 10 times in one assay. The co-efficient of intra-assay variation was 12 %.

We did not find significant difference in anti MBP antibodies concentration between serum of healthy individuals and MS patients **(Figure 29, page 71).** Although, MBP antibodies is involved in the pathogenesis of MS but their concentrations were not elevated in MS patients compared with healthy individuals. We compared the anti MBP antibodies in MS patients and percentage of T_{reg} mediated suppression but we did not find any correlation.
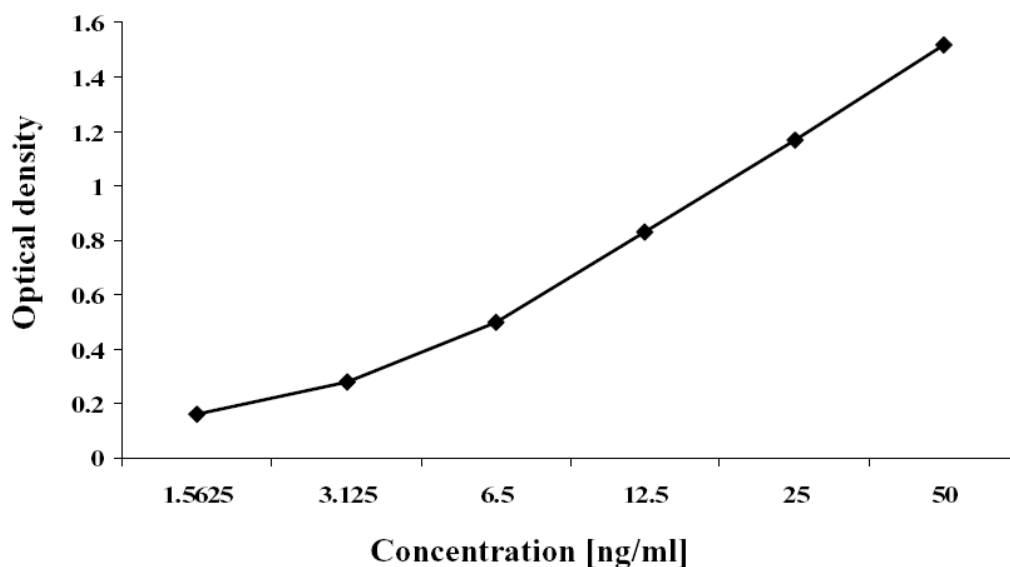
Figure 28 An example of standard curve generated using different concentrations of monoclonal mouse anti-myelin basic protein against optical density.
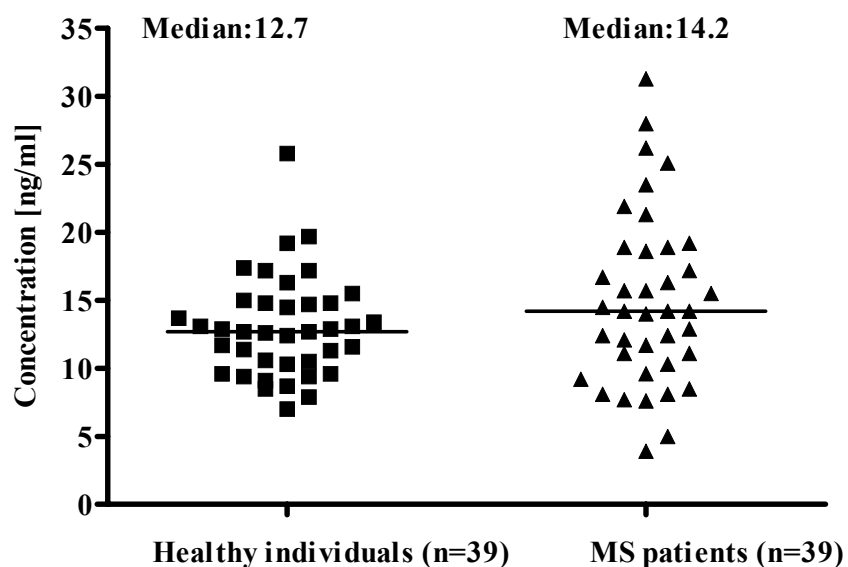
Figure 29 Concentration of anti myelin basic protein antibodies in multiple sclerosis patients and healthy individuals.
Concentration of anti myelin basic protein antibodies in serum were estimated from standard curve generated from monoclonal mouse anti-myelin basic protein.
n represents number of multiple sclerosis (MS) patients or healthy individuals.

4 Discussion

In this study, we proved that natural regulatory T cells (T_{reg}) obtained from a magnetic bead isolation method has phenotypical and functional characteristics. We found a reduction or loss of suppressive activity of T_{reg} in the patients suffering from multiple sclerosis (MS) compared with healthy individuals using an optimized antigen [myelin basic protein (MBP)] and mitogen [pokeweed mitogen (PWM)]-induced proliferation assay. This may be explained by a reduction in number of these cells and/or a disruption of T_{reg} function of a single cell.

4.1 Comparison of cell numbers between healthy individuals and MS patients

Diminished suppressive activity of T_{reg} cannot be due to a lack of these cells because when compared with healthy individuals, MS patients had a significantly higher relative number of T_{reg} in their peripheral blood. Moreover, it cannot be explained by alterations in T cell populations as we did not find significant differences in number of T cells and their subpopulations ($CD4^+$ and $CD8^+$ and their ratio $CD4^+/CD8^+$).

Our finding of an increase in relative number of T_{reg} in MS patients compared with healthy individuals, were in contrast to previous findings. Three studies (Haas et al., 2005; Putheti et al., 2004; Viglietta et al., 2004) did not report significant changes in frequency of $CD25^{high}$ cells between MS patients and healthy individuals. When the analyses were extended to $CD25^{intermediate}$ cells (Haas et al., 2005) or $CD4^+CD25^+$ (including all $CD25^+$) cells (Putheti et al., 2004), comparable number of T_{reg} in MS patients and healthy individuals were found. On the other hand, it was shown that MS patients had significantly reduced relative number of $CD4^+CD25^+$ T cells (Khoury et al., 2000).

One possible explanation may be that different subgroups of MS patients were included in the studies. The MS patients analyzed in our study had a mean age of 40 years, an average disease duration of 7.4 years and a mean EDSS of 3.7. The clinical features are representative for MS patients with a relapsing remitting or secondary progressive MS (Limmroth et al., 2006).

Another possible explanation for this discrepancy could be the definition of T_{reg} based on the level of CD25 expression. It has always been a matter of discussion

to define T_{reg} in human because CD25 expression of T_{reg} is not distinct. Some studies have shown that only CD25^{high} expressing cells are T_{reg}. We defined T_{reg} by a threshold for CD25 expression of > 369 PE fluorescence channels. Using this threshold, we did not include only CD25^{high} cells expressing but also CD25^{intermediate} cells. When we restricted our analysis to CD4^{+}CD25^{high} cells which was only 1 % of CD3^{+} (CD25^{high} were gated according to > than CD25 fluorescence intensity of CD8^{+} T cells), we also did not find significant differences in CD4^{+}CD25^{high} cells in MS patients compared with healthy individuals.

T_{reg} may be defined by additional markers such as HLA-DR or FOXP3. Baecher-Allan et al. (2001) reported that expression of HLA-DR was restricted to CD25^{high} lymphocytes so we measured HLA-DR expression on our isolated T_{reg} and our defined CD25^{high} expressing T_{reg} population. We found that 25 % of our isolated T_{reg} were HLA-DR positive while 37 % of CD4^{+}CD25^{high} cells were HLA-DR positive. This concluded that in our isolated $T_{reg,}$, the CD25^{high} and CD25^{intermediate} expressing cells were HLA-DR positive.

In our study, determination of FOXP3 mRNA message in T_{reg} revealed a 13-fold higher expression compared to the appropriate CD4^{+}CD25^{-} cell population of MS patients as well as healthy individuals. Our results were in concordance with the findings by Haas et al. (2005) who reported nearly the same ratio of FOXP3 mRNA expression between CD25^{high} as T_{reg} and CD4^{+}CD25^{-} cells.

An interesting aspect of definition of T_{reg} is the recently published observation that T_{reg} showed decrease in CD127 expression (Hartigan-O'Connor et al., 2007).

Aside from the increased number of T_{reg} in MS patients compared with healthy individuals, we also found a tendency of increased in numbers for the CD8^{+}CD25^{+} cell population. This may be of interest because CD8^{+}CD25^{+} thymocytes with regulatory function and a phenotype similar to T_{reg} including FOXP3 expression was previously described (Cosmi et al., 2003). This activity also found in peripheral blood T cells, too (Jarvis et al., 2005).

4.2   Function of T_{reg}

An important aspect of our study was the measurement of suppression activity of T_{reg}. Previous studies have shown that an autoimmune T-cell response to myelin antigens is thought to be involved in MS pathogenesis (Martin and McFarland, 1995; Ota et al., 1990). As a possible target of this autoimmune reaction, MBP has been the most widely studied myelin antigen, so we used MBP as an antigen and as a control PWM as mitogen in an optimised assay using ^{3}H-thymidine uptake as a measure for suppression of proliferation.

For our *in-vitro* assay, we used peripheral blood mononuclear cells (PBMCs) plus $CD4^+CD25^-$ cells, which were cultured in absence (CD25NEG) or presence (CD25MIX) of T_{reg}. We added $CD4^+CD25^-$ cells in PBMCs for two reasons; first, in order to increase the number of MBP specific responder T cells and second, as a control because some cells may get activated during isolation procedure.

We had used immunodensity and immunomagnetic separation procedure which involves anti-CD25 antibody-coupled magnetic beads to isolate our T_{reg} population. In T_{reg} consisting of a mixture of $CD25^{high}$ and $CD25^{intemediate}$ cells, we observed suppressive activities up to 80 % in co-cultures from healthy individuals. These data suggested but finally could not prove that the functional activity of $CD25^{high}$ and a mixture of $CD25^{high}$ and $CD25^{intemediate}$ cells may be comparable. But, recently one group also used the same isolation procedure and reported that bead-sorted which is a mixture of $CD25^{high}$ and $CD25^{intemediate}$ and FACS sorted $CD25^{high}$ T cells showed comparable levels of suppression for both MS patients and healthy individuals (Venken et al., 2006).

Suppressive activity of the mixture of $CD25^{high}$ and $CD25^{intemediate}$, is also supported by the findings of Wing et al. (2003), who analyzed $CD4^+CD25^+$ expressing cells. They reported that 40 % of the cells showing bright immunofluorescence (i.e. including intermediate CD25 expressing cells) were probably suppressive for myelin oligogodendrocyte glycoprotein (MOG)-induced proliferation in healthy individuals. They used an immunomagnetic separation procedure to obtain T_{reg} by positive selection, while we isolated $CD4^+$ lymphocytes by a negative selection procedure. Due to the low number of T_{reg} in the peripheral blood

and the selection procedure it was not possible to compare the functional activity of T_{reg} with high or intermediate CD25 expression directly.

4.2.1 Mitogen and antigen- induced proliferation assays

A significant decrease in the suppressive activity of T_{reg} lymphocytes expressing CD25^{high} has been shown in mitogen e.g. anti-CD3 and anti-CD28 polyclonal stimulated cultures of lymphocytes from MS patients (Viglietta et al., 2004). We also observed an overall decrease in the functional activity of T_{reg} in mitogen (PWM)-stimulated cultures in 31 % MS patients. This finding was also confirmed in antigen (MOG)-stimulated cultures using isolation procedure similar to us (Haas et al., 2005). Moreover, in our MBP (antigen)-stimulated co-cultures, T_{reg} obtained from about 89 % of healthy individuals displayed suppressive activity whereas 55 % of MS patients showed no suppressive T_{reg} function.

Mitogen stimulation may be less efficient to detect impaired activity of T_{reg} in MS patients. As shown in our study, suppressive activity was lost to a higher degree in MBP-antigen induced cultures than in PWM stimulated cultures. We cannot judge from the publication by Haas et al. (2005), whether in MOG-driven responses functional activity of T_{reg} is lacking in all patients. A failure in the regulation of MBP-specific responses may have more impact on the pathogenic process than regulation of MOG-specific responses. This was underlined by our observation that in contrast to antigen-induced proliferation, the proliferation of T_{reg} containing cultures stimulated with mitogen of the MS patients was significantly increased compared with the appropriate values of the healthy individuals. This may be explained by different mechanisms of suppressive activity of T_{reg} in antigen or mitogen-induced proliferation.

A loss of the functional activity of T_{reg} in MBP-stimulated cultures from MS patients may explain the manifestation and maintenance of the disease. So far, we have no explanation for the failure of T_{reg} in MS patients.

Several factors could account for the deficient function of T_{reg} in MS patients. First, a higher frequency of MBP reactive T cells in MS patients compared with healthy individuals may explain the loss of suppression in MBP-induced proliferation. But so far, according to Hong et al. (2004), estimation of MBP-reactive T cells has been inconclusive. Another explanation may be that MS patients have higher number

of activated cells, thus "diluting" the number of T_{reg} may account for the apparent deficient function in *in vitro* assays. Therefore, we measured HLA-DR expression of T lymphocytes as an important parameter for the activation. We did not find significant differences in the relative number of HLA-DR expressing T cells between MS patients and healthy individuals so it seems unlikely that our *in vitro* results may be explained by a "dilution effect".

Huan et al. (2005) reported a higher proliferation rate of T_{reg} from MS patients compared with healthy individuals. Moreover, the authors provide evidence that proliferating T_{reg} are less suppressive than non-proliferating T_{reg}. We also found that cultures containing T_{reg} (CD25MIX) cells have higher PWM-induced proliferation compared with healthy individuals. These findings may explain a surplus of proliferation in those MS patients who present with a complete loss of the functional activity of T_{reg}. These data support our observation that MS patients compared with healthy individuals harbour proliferating albeit non-functional T_{reg}.

One possible explanation may also be that suppression is influenced by demographic parameters. Therefore, we compared suppressing activity of T_{reg} with diseases duration, age of onset and EDSS of MS patients but we did not find any correlation.

FOXP3 is important for the development and function of T_{reg}. Therefore, different FOXP3 amounts could influence the suppressive activity of T_{reg}. Thus, it was important to measure FOXP3 gene transcript in MS patients and healthy individuals. The amount of FOXP3 gene transcript was not significantly different between MS patients and healthy individuals. This showed that changes in T_{reg} function between MS patients and healthy individuals were not influenced by FOXP3. Haas et al. (2005) also found no difference in FOXP3 expression between MS patients and healthy individuals. However, there was one report on decreased FOXP3 levels of $CD4^+CD25^+$ lymphocytes in MS patients (Huan et al., 2005). The possible explanation for this discrepancy might be that the MS patients they analyzed probably belonged to a different subgroup. These patients had average disease durations of 15.3 years compared with 7.4 ± 6.9 years in our study.

Another aspect is that aside the natural $CD3^+CD4^+CD25^+FoxP3^+$ T_{reg} several other $FoxP3^+$ and non-$FoxP3^+$ subsets of induced suppressor T cells (Tr1, Th3) are also players of the immune tolerance network (Wing et al., 2006). Since, we used

PBMCs in our *in vitro* system we could not absolutely exclude that suppression of T_{reg} might be influenced by other such cells.

4.2.2 Role of TNF-α production in T_{reg} function

The exact mechanism of suppression by T_{reg} is not well understood. Accumulating evidences suggest that T_{reg} suppress effector T cell proliferation through a cell contact dependent manner and cytokine independent mechanism (Piccirillo et al., 2002; Suri-Payer and Cantor, 2001; Takahashi et al., 1998). But still it is not clear whether cytokines are involved in T_{reg} suppression or not. In order to elucidate the mechanism of suppression, we measured TNF-α production in cultures with and without T_{reg} using intracellular staining by flow cytometry.

Due to less proliferation in autologous and MBP-induced cultures, it was difficult to detect TNF-α production. So, we measured TNF-α production in PWM-induced cultures. Interestingly, we found different results in MS patients and healthy individuals. Out of four MS patients, three patients showed increased in TNF-α production after addition of T_{reg}. In these patients, we found loss of suppression. While in one patient we found decrease in TNF-α production after addition of T_{reg}, and patients showed suppressive activity of T_{reg}. This shows that TNF-α production had an inverse relation with T_{reg} function. This result was in concordance with one recent study in which it was shown that anti-TNF-α therapy in rheumatoid arthritis generates a newly differentiated population of T_{reg}, which compensates for defective T_{reg}. We (Mueller et al., 2003) and Bo et al.(2001) found that impaired proliferative responses to mitogens may be due to a high local production of TNF-α. With these result it can be deduced that defect in T_{reg} function may be due to increase in TNF-α production.

In contrast to MS patients, we found different results in healthy individuals. We did not find any correlation between percentage of suppression and TNF-α production. This indicated that involvement of TNF-α production in T_{reg} mediated suppression may differ between MS patients and healthy individuals.

4.2.3   Antibodies against MBP

Antibody production by mature B lymphocytes, plasma cells, is the final stage of an immune response. Thus, antibody production should also be influenced by T_{reg} function. MBP is one of the target proteins in MS pathogenesis. Therefore, we developed an assay for measurement of anti MBP antibodies.

Accumulating evidences suggest that an antibody-mediated process may have an important role in the pathogenesis of MS (Raine et al., 1999; Storch et al., 1998). Autoantibodies recognizing several myelin proteins including MBP (Newcombe et al., 1985; Olsson et al., 1990; Paterson et al., 1981; Sellebjerg et al., 1995), proteolipid protein (Sellebjerg et al., 1995; Warren and Catz, 1994) and Myelin oligogodendrocyte glycoprotein (MOG) are present in MS patients (Baig et al., 1991; Moller et al., 1989), but their role in disease progression is enigmatic and controversial.

MBP is one of the widely studied target proteins in MS pathogenesis hence we analyzed autoantibody against MBP. The detection of anti MBP antibodies in serum of MS patients has been elusive. This difficulty may in part be related to a tendency to form immune complexes which hampers their detection (Dasgupta et al., 1983). We referred to the established ELISA method utilized by Chamczuk et al. (2002) and performed some modification in order to increase the sensitivity of the assay.

In our study we did not detect any significant difference in anti MBP antibodies between serum of healthy individuals and MS patients. This result was in contrast to the group who established this ELISA method where they reported that 77 % MS patients had elevated levels of anti MBP antibodies (Chamczuk et al., 2002). Using western blot techniques, anti MBP antibodies were detected in 28 % MS patients (Reindl et al., 1999). Other studies also reported the presence of anti MBP antibodies (Terryberry et al., 1998; Vojdani et al., 2003), while in some studies they were not detected (Brokstad et al., 1994; Colombo et al., 1997; Olsson et al., 1990). One possible explanation for this discrepancy may be that either MS patients we analyzed in our study probably belong to a different subgroup or we have taken a small cohort of MS patients.

We did not detect any correlation between anti MBP antibodies and T_{reg} mediated suppression.

4.3 Effect of mitoxantrone on number of T_{reg} and other immune cells as well as on T_{reg} function

In the second part of our study, we investigated the long-term effect of mitoxantrone (MX), a potent disease modifying drug for MS treatment, on the number of T_{reg} and other immune cells in MS patients. We assumed that MX therapy may restore the impaired function of T_{reg}, hence, we measured effect of MX on suppressive activity of T_{reg}.

4.3.1 Cell numbers

Several investigations were performed in order to elucidate the mode of action of MX. It was reported that MX induces decrease in lymphocytes number by induction of apoptosis (Chan et al., 2005). However, the data on whether MX selectively affects specific subpopulations is contradictory. Chan et al. (2005) and Gbadamosi et al. (2003) reported preferential cell death of $CD8^+$ T cells. In this study, we showed that MX did not lead to a longstanding reduction of number of $CD4^+$ or $CD8^+$ T cells, neither in the beginning (3 months post first infusion) nor in the later course of the treatment. The main difference of our study compared to the previous investigations was that we performed our analyses 3 months after MX was applied while others focused on short-term effects (1 hour up to 14 days) (Chan et al., 2005; Gbadamosi et al., 2003). It assumed that a decrease of $CD8^+$ cells occurs along with general leukopenia in the first weeks after MX treatment, but that these cells recover afterwards. Acute leukopenia generally resolved one month post infusion. This assumption was supported by recent finding (Pelfrey et al., 2006) who came to a similar conclusion when they analyzed MX effects one month after infusion and did not find a reduction of $CD4^+$ or $CD8^+$ T cells in patients with primary progressive MS. A previous study that focused on short term effects of mitoxantrone also reported some long-term *in vivo* effects of MX in MS (Gbadamosi et al., 2003). The investigators did not find a significant change of $CD8^+$ T cells up to 6 months. More data on $CD8^+$ cells were from a longitudinal study by Gonsette (1996). This group found a selective reduction of $CD4^+CD45^+$ cells with relative sparing of $CD8^+$ cells, which resulted in a decreased $CD4^+/CD8^+$ ratio during MX therapy up to 3 years. But in our study, we did not find significant difference in $CD4^+/CD8^+$ ratio.

MX is known to cause a transient leukopenia, but its effect on leukocyte subsets are less understood. Therefore, we examined number of leukocytes and relative number of lymphocytes but we did not find effect of MX on number of leukocytes and lymphocytes. This showed that long-term effect of MX was not associated with leukopenia. Pelfrey et al. (2006) also showed the same as they did not find change in number of lymphocytes.

Our longitudinal data showed that MX led to a significant decrease of B cell numbers that could be measured as early as 3 months after the first cycle (mean reduction; relative number: 34 % and absolute number: 41 %). This effect was not only sustained during additional treatment cycles but it became more significant (mean reduction; relative number: 87 % and absolute number: 89 %) after 12 months. The finding of a preferential reduction of B cells was in line with several previous investigations. A preferential reduction of B cells was also found by Gbadamosi et al. (2003) who reported on 8 patients with 3-4 MX cycles at 2-3 months intervals. In contrast to our study, the effect reached statistical significance only after 6 months, which might be due to the fact that the patient number in the earlier study was small. Mauch et al. (1992) and also Gonsette (1992) reported a decrease of B cell numbers after one year during or/and also after cessation of MX therapy. Although the treatment protocols in some of the earlier studies were different from the most widely used ones nowadays, it seems that the current treatment regimen had a comparable effect on lymphocyte subpopulations.

In agreement with previous findings, our study underlines that the long-term effects of MX in the treatment of MS could preferentially be mediated by targeting B cells. Fidler et al. (1986) demonstrated that MX reduces splenic B lymphocytes in mice with inhibition of antibody response to thymic dependent and thymic independent antigens (Fidler et al., 1986a). *In vitro* treatment of malignant leukemia cells with MX revealed that MX induces apoptosis (Bellosillo et al., 1998; Bhalla et al., 1993). Inhibition of non-antigen-specific proliferation of activated peripheral blood lymphocytes (both T and B cells) as well as antigen-specific T cell lines by MX in dose dependent manner were reported (Neuhaus et al., 2005). This group also showed that MX induces apoptotic cell death at low concentrations and necrotic cell death at high concentrations. In our study we did analysis with numbers of T cells but did not find influence of MX on these cells. Based on these finding, it seems that a

persistent and selective reduction of B cell numbers occur but other lymphocytes subpopulation remain unaffected by MX therapy.

We also measured the behaviour of T_{reg} number during MX therapy. We had shown earlier that relative number of T_{reg} was increased in MS patients compared to healthy individuals so; we assumed that MX might effect relative number of T_{reg}. On the contrary, we did not find any significant difference in T_{reg} frequency during MX treatment *in vivo.* Thus far, the effects of disease modifying drugs on T_{reg} in peripheral blood have not much known. One publication (Putheti et al., 2004) in agreement with us reported that T_{reg} remain unaffected during ongoing treatment with IFN-ß-1a or GA or IFN-ß-1a+GA. They also did follow-up of MS patients before and during IFN-ß-1a treatment but there were no changes in the proportions of T_{reg}. Recently, another publication showed that there was trend of increasing proportions of T_{reg} after 6 months of IFN-ß-1a therapy when compared with the baseline (de Andres et al., 2007).

4.3.2 Suppressive function of T_{reg}

We had also measured the effect of MX on suppressive capacity of T_{reg}. As we described earlier that in PWM and MBP-stimulated cultures, there was decrease or loss in functional activity of T_{reg} in MS patients. So, we assumed that MX may restore the T_{reg} suppressive activity. Interestingly, we did not observe any persistent effects of MX on functional activity of T_{reg} up to 9 months compared with baseline values. The knowledge about the effect of immunomodulatory drug on T_{reg} function is very much limited. Recently, it has been reported that suppressive activity of T_{reg} is significantly enhanced after 3 and 6 months of IFN-ß-1a therapy (de Andres et al., 2007). But, still effect of MX on function and frequency of T_{reg} in MS patients has not reported.

The comparison of T_{reg} mediated suppressive function between healthy individuals and MS patients revealed that MS patients had impaired function, although B cell numbers and anti MBP antibodies between both groups were not different. So, it could be that impaired T_{reg} function in MS patients effects interaction between effector cells or interaction between antigen presenting cells and not between B cells. In contrast, we found that MX influences the B cell numbers and not the T_{reg} function.

As a summary, it can be concluded that MX does not exhibit a persistent effect on the numbers or suppressive capacity of T_{reg} but that its potent effect on MS disease activity is predominantly, although not exclusively, mediated by a suppression of humoral immunity.

5   Summary

Multiple sclerosis (MS) is considered to be an autoimmune disorder directed against self antigens of the central nervous system e.g. myelin basic protein (MBP). Natural $CD4^+CD25^+FoxP3^+$ regulatory T cells (T_{reg})-mediated active suppression is an essential mechanism in the control of self-antigen reactive cells and in the induction of peripheral tolerance *in vivo*.

In this study, we proved that our isolated T_{reg} has phenotypical and functional characteristics. We found a reduction or loss of suppressive activity of T_{reg} in the patients suffering from multiple sclerosis (MS) compared with healthy individuals.

In our study, we set the threshold for CD25 expression of T_{reg} to > 369 channels which was a mixture of $CD25^{high}$ and $CD25^{intemediate}$ expressing cells, T_{reg} may be defined with the other markers such as FOXP3 and HLA-DR. Analyzing HLA-DR expression on our isolated T_{reg} and $CD25^{high}$ showed that 25 % of our isolated T_{reg} were HLA-DR positive while 37 % of $CD4^+CD25^{high}$ cells were HLA-DR positive. This concludes that our $CD25^{intermediate}$ T_{reg} population was also HLA-DR positive. In our study, determination of FOXP3 mRNA message in T_{reg} revealed a 13-fold higher expression compared to the appropriate CD25 negative cell population of MS patients as well as healthy individuals.

We studied whether changes in the suppressive function of a mixture of $CD25^{high}$ and $CD25^{intemediate}$ expressing T_{reg} in myelin basic protein (MBP)-and PWM-induced proliferation occurred in untreated MS patients compared with healthy individuals. Suppression of MBP-induced proliferation was observed in 13 out of 29 (45 %) MS patients; this was significantly less compared with 17 out of 19 (89 %) healthy individuals. In pokeweed mitogen-induced co-cultures, T_{reg} from healthy individuals (21 of 23 cases, 91 %) showed a trend to higher suppressive activities (p = 0.086) compared with MS patients (20 of 29 cases, 69 %).

We also looked for changes in relative T_{reg} number and found that they were significantly increased in MS patients (mean±SD; 20±8 %) compared with healthy individuals (15±5 %).

The exact mechanism of suppression by T_{reg} is not well understood. In order to elucidate the mechanism, we measured tumor necrosis factor (TNF)-α production intracellulary after 5 days in PWM stimulated cultures. We found that in MS patients, TNF-α production had an inverse relation with T_{reg} function. It seems that defect in

T$_{reg}$ function may be due to increase in TNF-α production. In contrast to MS patients, we found different results in healthy individuals. We did not find any correlation between percentage of suppression and TNF-α production. This showed that involvement of TNF-α in T$_{reg}$ mediated suppression mechanism might differ in MS patients and healthy individuals.

Antibody production by mature B lymphocytes and plasma cells is the final stage of an immune response. Thus, antibody production should also be influenced by T$_{reg}$ function. MBP is one of the target proteins in MS pathogenesis. Therefore, we developed as assay for measurement of anti MBP antibodies. We did not find any significant difference in anti MBP antibodies between healthy individuals and MS patients.

In the second part of our study, we investigated the long-term effect of mitoxantrone (MX), a potent disease modifying drug for MS treatment, on the number of T$_{reg}$ and other immune cells in MS patients. We found a persistent and selective reduction of B cell numbers occur during therapy but other lymphocytes subpopulation remains unaffected.

We assumed that MX therapy may restore the impaired function of T$_{reg}$ so we measured effect of MX on suppressive activity of T$_{reg}$. But we did not find a persistent effect of MX on functional activity of T$_{reg}$ up to 9 months compared with baseline.

Comparative analysis of T$_{reg}$ mediated suppressive function between healthy individuals and MS patients revealed that MS patients had impaired function, although B cell numbers and anti MBP antibodies between both groups were not different. Thus, it could be that impaired T$_{reg}$ function in MS patients effects interaction between effector cells or interaction between antigen presenting cells but not between B cells. In contrast, we found that MX influences the B cell numbers and not the T$_{reg}$ function.

As a summary, it can be demonstrated that MX does not exhibit a persistent effect on the number or suppressive capacity of T$_{reg}$ but its potent effect on MS disease activity is predominantly, although not exclusively, mediated by a suppression of humoral immunity.

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Publications

Kumar, M., Putzki, N., Limmroth, V., Remus, R., Lindemann, M., Knop, D., Mueller, N., Hardt, C., Kreuzfelder, E., Grosse-Wilde, H., 2006. $CD4^+CD25^+FoxP3^+$ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. J Neuroimmunol 180, 178-184.

Putzki, N., **Kumar, M.,** Kreuzfelder, E., Grosse-Wilde, H., Diener, HC., Limmroth, V. Mitoxantrone does not restore impaired function of $CD4^+CD25^+$ regulatory T cells in myelin basic protein induced proliferation. (Manuscript to be submitted)

Published abstracts

Kumar, M., Putzki, N., Remus, R., Lindemann, M., Limmroth, V., Kreuzfelder, E., Hardt, C., Grosse-Wilde, H., 2005. Failure of $CD4^+CD25^+$ Foxp3$^+$ T cells to suppress myelin basic protein induced proliferation in multiple sclerosis patients. Immunobiol. 210: P 400.

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Strassburger, K., **Kumar, M.,** Vago, S., Kreuzfelder, E., Limmroth, V., Putzki, N. 2006. Immunological profile of patients with multiple sclerosis associated fatigue. Multiple Sclerosis 12 (Suppl 1): P 256.

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Erklärung:

Hiermit erkläre ich, gem. § 6, Abs. 2, Nr. 6 der Promotionsordnung Math.-Nat.-Fachbereiche zur Erlangung des Dr.rer.nat., dass ich die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den.........................   ....
                                                                  Manoj Kumar
                                                                  (Doktorand)

Erklärung:

Hiermit erkläre ich, gem. § 6, Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung der Dr.rer.nat., dass ich das Arbeitgebiet, dem das Thema „Number and function of $CD4^+CD25^+FoxP3^+$ regulatory T cells in patients suffering from multiple sclerosis" zuzuordnen ist, in Forschung und Lahre vertrete und den Antrag von Herrn Kumar Manoj befürworte.

Essen, den.......................   ....
                                                                  Prof. Dr.rer.nat. E. Kreuzfelder
                                                                  (Betreuer der Arbeit)

Erklärung:

Hiermit erkläre ich, gem. § 6, Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr.rer.nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner Fakultät abgelehnt worden ist.

Essen, den ....
                                                                  Manoj Kumar
                                                                  (Doktorand)