CD4^{+} CD25^{HIGH} regulatory T cell homeostasis and function in healthy individuals and patients with multiple sclerosis

Pieter Meuwissen

promotor :
Prof. dr. Pieter STINISSEN

co-promotor :

dr. Niels HELLINGS

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Preface

Although my first day at the university seemed yesterday, my time as a student is already ending. 4 years past in flash. Although it had good and bad moments, I can say that these years were incredible! By finishing this thesis I am closing this nice chapter of my life. Now, I realize that I could not have made it this far without the help and support of many people. For this reason I would like to use this page to thank them.

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List of abbreviations

7-AAD :	7-amino-actinomycine D
AICD:	antigen induced cell death
APC :	antigen presenting cell
BBB:	blood brain barrier
BFA :	brefeldin A
BSA :	bovine serum albumine
CaI :	calciumionomycine
CD :	cluster of differentiation
cDNA :	complementary deoxyribonucleic acid
CFSE :	carboxy fluorescein succinimidyl diacetate ester
CTLA-4 :	cytotoxic T lymphocyte antigen-4
CNS :	central nervous system
CMV:	cytomegalovirus
DNA:	deoxyribonucleic acid
DMSO :	dimethyl sulfoxide
EAE :	experimentele autoimmune encefalomyelitis
EDTA :	ethyleen diamine tetra acetate acid
FACS :	fluorescence activated cell sorter
FITC :	fluoresceine isothiocyanaat
FCS :	fetal bovine serum
FoxP3 :	Forkhead-Box P3
FRET:	fluorescence resonance energy transfer
GA:	glatiramer acetate
GITR :	glucocorticoid-induced tumor necrosis factor receptor family related-gene
HC :	healthy control
HLA :	human leukocyte antigen
HPLC :	high pressure liquid chromatography
IFN-β:	interferon-β
IFN-γ:	Interferon-γ
IL:	interleukin
ILT:	immunoglobulin-like transcript
IPEX :	immune dysregulation polyendocrinopathy enteropathy X-linked syndrome
iTreg:	inducible regulatory T cell
mAb:	monoclonal antibody
MACS:	magnetic cell sorting
MBP :	myelin basic protein

MHC :	major histocompatibility complex
MOG :	myelin oligodendrocyt protein
MS :	multiple sclerosis
nnTreg:	natural naive regulatory T cell
NK T-cel :	natural killer T-cel
PBMC :	peripheral blood mononuclear cells
PBS :	phosphate buffered saline
PCR :	polymerase chain reaction
PE :	phycoerythrine
PECAM-1 :	platelet endothelial cell adhesion molecule
Percp :	peridinine- chlorophyll protein
PMA :	phorbol 12-myristate 13-acetate
PP-MS :	primary progresive MS
RA:	Rheumatoid Arthritis
RNA :	ribonucleic acid
RR-MS :	relapsing-remitting multiple sclerosis
RT-PCR:	real-time polymerase chain reaction
SI:	stimulation index
SLE:	Systemic Lupus Erythematosus
sjTRECs:	signal joint T cell receptor excision circles
SP-MS :	secundair progressive multiple sclerosis
TAQ :	Thermus aquaticus
TCR :	T cell receptor
TGF-β:	transforming growth factor β
TNF :	tumor necrosis factor
T_R1 cell :	type 1 regulatory T cell
T_H3 cell :	T helper 3 cell
Treg :	$CD4^+CD25^+$ regulatory T cell
TT :	Tetanus Toxin

Summary

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). T cells specific for components of the myelin sheath are considered to be initial effectors in the autoimmune reactions observed in MS. $CD4^{+}CD25^{high}$ regulatory T cells play an important role as suppressors of T cell mediated immune reactions. The suppressive capacity of Tregs is reduced in patients with relapsing-remitting MS (RR-MS). In contrast, patients with a secondary progressive disease course (SP-MS) show a normal Treg function. It remains unknown why the Treg function is aberrant in RR- and SP-MS patients. The functional characteristics of Tregs in MS patients were further evaluated in this study. In this respect the suppressive capacity of conventional Tregs and the recently described naive Tregs were evaluated in MS patients. In addition, parameters of T cell homeostasis (sjTRECs, CD31 expression and T cell receptor BV usage) were measured on Tregs to gain more insight in the dynamics of this T cell population in HC and MS patients.

The frequency of functional naive Tregs and memory Tregs were both reduced in RR-MS patients as compared to HC. The number of sjTRECs was lower in naive and memory Tregs of RR-MS patients. Moreover, memory Tregs show a decreased expression of CD31. In addition, the TCR BV gene usage of these cells is altered. These results provide indications of a disturbed Treg homeostasis in MS patients. The suppressive capacity of nnTregs and memory Tregs was reduced in RR-MS patients. Interestingly, memory Tregs of RR-MS patients with a long disease duration and SP-MS patients showed a normal suppressive capacity, comparable with healthy individuals. In contrast, the suppressive capacity of nnTregs remained reduced in these patients. Indications are provided that the functional memory Tregs are induced from conventional T cells in the periphery of RR-MS patients.

The Treg suppressive capacity towards myelin specific responses was also evaluated. Tregs of healthy individuals were able to suppress responses towards myelin antigens. Adequate suppression of myelin reactive T cells may be importance as this population contains potentially pathogenic Th1 and Th17 cells.

The results of this study could contribute to an enhanced insight into the role of Tregs in the pathogenesis of MS. Further research of the function and the processes which affect Treg homeostasis is needed before any attempts are made to manipulate these cells therapeutically.

Samenvatting

MS is een chronisch inflammatoire demyeliniserende ziekte van het centrale zenuwstelsel. T cellen gericht tegen componenten van de myeline schede worden beschouwd als de initiële effectors van de autoimmune reacties die kenmerkend zijn aan MS. $CD4^+CD25^+$ regulatoire T cellen (Tregs) spelen een belangrijke rol in het behoudt van perifere tolerantie aangezien ze de activiteit van andere imuuncellen onderdrukken. De onderdrukkende capaciteit van deze cellen is echter sterk verlaagd in RR-MS patiënten. In tegenstelling tot RR-MS patiënten vertonen SP-MS patiënten wel een normale Treg functie. Het is tot op heden onbekend waarom de Treg functie verschilt tussen RR- en SP-MS patiënten. De functionele karakteristieken van Tregs in MS patiënten werden verder bestudeerd in deze studie. Hierbij werd de suppressieve capaciteit van conventionele Tregs alsook de recentelijk beschreven naïeve Tregs geëvalueerd. Daarnaast werd de homeostase van Tregs in MS patiënten bestudeerd. Hiertoe werden parameters, gerelateerd aan T cel homeostase (sjTRECs, CD31 expression and T cell receptor BV usage) bestudeerd op Tregs.

De frequenties van functionele naïeve en geheugen Tregs waren lager in RR-MS patiënten en vergelijking met gezonde controles. Het aantal sjTRECs was lager in zowel naïeve als geheugen Tregs van MS patiënten. Bovendien vertoonden geheugen Tregs ook een verlaagde CD31 expressie. Daarnaast werd ook een vernauwd TCR BV repertoire waargenomen op Tregs van RR-MS patiënten. Deze resultaten indiceren dat de homeostase van Tregs verstoord is in RR-MS patiënten. De onderdrukkende capaciteit van zowel naïeve Tregs als geheugen Tregs verlaagd in RR-MS patiënten in een vroeg ziekte stadium. De gedaalde onderdrukkende capaciteit van geheugen Tregs werd niet waargenomen in SP-MS patiënten. De onderdrukkende capaciteit van naïeve Tregs was echter wel sterk verlaagd in deze patiënten. Tregs van RR-MS patiënten in een laat ziektestadium vertoonden dezelfde functionele eigenschappen als Tregs van SP-MS patiënten. Zeer waarschijnlijk dragen geheugen Tregs, die geïnduceerd worden in de periferie bij tot de herstel van de Treg suppressieve capaciteit in een laat ziektestadium.

De onderdrukkende capaciteit van Tregs ten opzichte van myeline specifieke T cel reacties werd eveneens bepaald. Tregs van gezonde controles waren instaat om myeline specifieke $CD4^+$ T cel reacties te onderdrukken. Een adequate suppressie van myeline reactieve T cellen is belangrijk aangezien deze potentieel pathogene Th1 en Th17 cellen bevatten.

De bevindingen van deze studie dragen mogelijk bij tot een groter inzicht van de rol die Tregs spelen in de pathogenese van MS. Verder onderzoek naar de functie en de processen die Treg homeostase beïnvloeden zijn echter nodig voordat pogingen ondernomen worden om deze cellen therapeutisch te manipuleren.

1 Introduction

1.1 Multiple sclerosis

MS is a chronic inflammatory demyelinating disease of the CNS. The disease usually develops during early adulthood (between 20 and 40 years of age). The disease can result in many different outcomes among affected individuals. Common symptoms include visual and sensory impairment, paralysis and other neurological deficits, sometimes accompanied with considerable cognitive dysfunction. The disease appears in two major forms: RR-MS and primary progressive (PP) MS. RR-MS is the most frequent form. The RR form of MS occurs in 85-90% of patients and affects women about twice as often as men and is characterized by a series of clinical attacks, which result in varying degrees of disability from which patients recover partly or completely. Over time, about one-third of the RR-MS patients develop into a progressive form known as SP-MS. About 10-15% of patients suffer from PP-MS, which is characterized by a gradual progression of the disease from its onset with no superimposed relapses and remissions. It is not clear which factors are responsible for the different disease courses [1].

Pathologically, MS is characterized by the appearance of large, multifocal lesions with reactive glial scar formation within the white matter of the CNS. These lesions result from the local loss of myelin and are found throughout the entire CNS. The majority of lesions however, appear within the periventricular regions, optical nerves, brainstem and spinal cord [2].

1.2 Immunopathogenesis of MS

It is thought that MS is an autoimmune disease, mediated by T cells specific for components of the myelin sheath. Supporting this notion is the observation that injection of myelin antigens in susceptible animals leads to the development of experimental autoimmune encephalomyelitis (EAE), a $CD4^+$ T cell mediated autoimmune disease resembling MS [3]. Moreover, EAE can also be induced by adoptive transfer of myelin reactive T cells to naive recipients [3,4]. The role of $CD4^+$ T cells in MS is supported indirectly by the fact that polymorphisms of certain human leukocyte antigen (HLA) class II genes (HLA-DR and HLA-DQ) are associated with an increased susceptibility for MS, presumably via their antigen presenting role towards $CD4^+$ T cells [1,2].

The current hypothesis regarding the pathogenesis of MS suggests that the disease initiates with the activation of myelin reactive T cells in the periphery. Supporting this hypothesis are studies that report an increased frequency of activated myelin reactive T cells in the peripheral blood of MS-patients as compared to healthy controls (HC)[5,7]. Once activated, these autoreactive T cells are thought to expand and traffic to the CNS,

as MS patients show an increased frequency of myelin reactive T cells in the cerebrospinal fluid (CSF) [2,8]. Moreover, these CSF derived myelin reactive T cells are derived from the population of myelin reactive T cells in the blood, as evidenced by their comparable T cell receptor (TCR) BV repertoire and epitope specificity [7]. Within the CNS, myelin reactive T cells become reactivated once they encounter their specific myelin epitope presented by antigen presenting cells (APC): microglia cells and perivascular macrophages. The reactivated T cells will produce proinflammatory cytokines which promote local activation of resident macrophages and further influx of immune cells. It is within this local inflammatory environment that myelin- and axonal damage occur. The damage to myelin is probably caused by the combined effects of cytotoxic cells (macrophages, $CD8^+$ T cells and γδ T cells), oxygen radicals, demyelinating auto-antibodies and cytokine induced toxicity (e.g. interferon-gamma [IFN-γ] and tumor necrosis factor) [2].

The exact molecular mechanisms triggering the *in vivo* activation of autoreactive T cells are still unknown. Several possible mechanisms have been proposed over the past years. First, autoreactive T cells may become activated by 'molecular mimicry'. This process involves activation of T cells by viral- or bacterial-encoded epitopes which show a high sequence homology with myelin antigens. Upon infection, presentation of these viral antigens in the periphery by APCs may cross-activate autoreactive T cells [9]. Exposure to viral- or bacterial-encoded 'superantigens' has also been proposed as an activation mechanism. Superantigens crosslink MHC class II molecules on APCs with specific TCR BV chains on T cells, activating these T cells independently of their antigen specificity [10]. Apart from these direct mechanisms through which infectious agents may induce MS, autoreactive T cells are also thought to become activated because of nonspecific inflammatory events that occur during infections. This mechanism, also called 'bystander activation' involves TCR independent activation of autoreactive T cells by high concentrations of pro-inflammatory cytokines [11].

The presence of activated myelin reactive T cells in peripheral blood is however not sufficient to cause MS. Molecular mimicry, superantigen activation and bystander activation occur generally and myelin reactive T cells are part of the normal T cell repertoire. However, the frequency of activated myelin reactive T cells is higher in MS patients as compared to healthy individuals [6,7]. It is hypothesized that disturbances in tolerance mechanisms, which prevent the peripheral activation of autoreactive T cells and maintain peripheral tolerance, causes the increased myelin specific T cell activity in MS patients.

1.3 Immunologic tolerance

The concept of self-tolerance can be defined as the ability of the immune system to recognize and respond to foreign antigens but not to self antigens. Establishment and maintenance of tolerance to self-antigens is complex and involves both central and peripheral mechanisms. Central T cell tolerance is established in the thymus. Immature T cells with receptors having a high avidity for self peptides are forced to undergo apoptosis in the thymus in a process called clonal deletion. This process, also called 'negative selection', generates a peripheral T-cell repertoire which is largely self-tolerant [12]. Negative selection is imperfect however, and potentially hazardous self-reactive T cells are present in the periphery of most individuals [6,7].

Peripheral tolerance includes both passive and active mechanisms. The principal passive mechanisms are apoptotic cell death (deletion) and functional inactivation without cell death (anergy) upon encounter with self-antigens. Furthermore, self-reactive T cells may fail to be activated because of low avidities of their TCR, lack of costimulation or immunological ignorance [12]. In addition to these passive mechanisms of suppression, there is unequivocal evidence that regulatory T cells actively downregulate the activation and expansion of self-reactive T cells [13]. Different subsets of regulatory T cells, which use different mechanisms of suppression have been identified. An overview is provided in table 1.1. It has been shown that these specific T cell populations act individually or in concert to actively downregulate the activity of pathogenic autoreactive T cells [14]. In this study, we will focus on the natural occuring $CD4^+CD25^+$ regulatory T cells.

1.4 Natural $CD4^+CD25^+$ regulatory T cells

Natural $CD4^+CD25^+$ regulatory T cells (Tregs) were originally identified by their high surface expression of the IL-2 receptor α-chain (CD25) [15]. These cells play an important role in the maintenance of self-tolerance. Experimental evidence for this comes from animal models of autoimmune diseases. For example, Sakaguchi et al. showed that adoptive transfer of $CD4^+CD25^-$ T cells in athymic nude mice induced multiple organ autoimmune manifestations. However, cotransfer of a small number of $CD4^+CD25^+$ T cells completely prevented the development of autoimmunity in these animals [16]. In an other study, depletion of Tregs from healthy animals leads to spontaneous development of various autoimmune manifestations, whereas reconstitution of Tregs prevents these disorders [17]. In addition, it has also been shown that depletion of Tregs enhances immune responses to invading or commensal microbes, allergic responses and tumor immunity to autologous tumor cells. Thus Tregs not only inhibit autoimmune responses but also modulate a variety of physiological and pathological immune responses to non-self antigens [18].

Table 1.1: Different subsets of regulatory T cells

Regulatory T cells	phenotype	Suggested immunosuppressive mechanism	Ref.
Natural Treg	$CD4^+$ $CD25^{high}$ $FoxP3^+$ $CD127^{low}$	Direct cell-cell contact dependent: - CTLA-4, - GITR, - cell surface TGF-β, - cytotocixity	[15]
iTreg	$CD4^+$ $CD25^{high}$ $FoxP3^+CD127^{low}$	IL-10, TGF-β secretion	[19]
T_R1 T cells	$CD4^+$ $CD25^{int}$ $FoxP3^-$ $IL\text{-}10^{high}$	IL-10 secretion	[20]
T_H3 T cells	$CD4^+$ $CD25^{int}$ $FoxP3^-$ $TGF\text{-}\beta^{high}$	TGF-β secretion	[13]
NK T cells	VαJ24JαQ, Vβ11	IL-4, IL-10, TGF-β, IFN-γ secretion	[21]
CD8+	Qa1/HLA-E restricted $CD8^+$	Direct cell-cell contact-dependent, Cytotoxicity	[22]
	$CD8^+CD28^-$	IL-10 secretion, ILT-3 and ILT-4 upregulation on APCs	[13]

Treg: $CD4^+CD25^+$ regulatory T cel; iTreg: inducible regulatory T cell; T_R1 T cell: type 1 regulatory T cell; T_H3 T cell: type 3 regulatory T cell. NK T cell: natural killer T cell; CD: cluster of differentiation; IL: interleukin; TGF-β: transforming growth factor β; CTLA-4: cytotoxic T lymphocyte-associated antigen 4; GITR: glucocorticoid-induced tumor necrosis factor receptor; IFN-γ interferon gamma; ILT: immunoglobulin-like transcript.

1.4.1 Thymic development and homeostasis of Tregs

The majority of Tregs are produced by the thymus as a functionally distinct T cell subpopulation. The thymus generates Tregs as a naive population, expressing CD45RA. Evidence for this comes the observation that Tregs in cord blood are mainly $CD45RA^+/CD45RO^-$. It is thought that these natural naive Tregs (nnTregs) are precursors of Tregs that have an activated/memory phenotype. Despite the naive phenotype, nnTregs have the same suppressive capacity as their memory counterparts. Interestingly, part of these nnTregs persist during adulthood. In healthy adult individuals, the population of nnTregs comprises approximately 30% of the total Treg population in peripheral blood. However, frequencies of Tregs are highly variable. This variability is partly age dependent, with the number of nnTregs decreasing with age [23,24].

- *Thymic development and antigen specificity of Tregs*

Tregs develop in the thymus by the high affinity interaction of the TCR with self-peptides [25]. Other factors such as CD80 and CD86, ligands for the co-stimulatory receptor CD28, and IL-2 were also shown to be required for the thymic development of Tregs [26]. The fact that the thymus expresses only self antigens implies that Tregs are self-reactive. Indeed, it has been reported that these cells are highly self reactive [15]. The high affinity nature of Tregs does not appear to skew the TCR repertoire as these cells share an equally complex and comparable BV gene usage [27]. The high self reactivity and broad TCR BV repertoire of Tregs ensures their role to dominantly control pathogenic immune responses against self antigens.

- *Homeostasis of Tregs: peripheral mechanisms*

As thymic output declines with age, peripheral T cell and Treg numbers have to be maintained by thymus independent mechanisms. There are three potential mechanisms which explain how Treg cells are maintained in vivo. First, there may be naturally occurring Tregs of thymic origin that are resistent to cell death. However, most Treg cells in adults express CD45RO and these cells are highly susceptible to apoptosis. Second, Tregs may be maintained by continuous proliferation of resident thymic-derived Treg cells in the periphery (homeostatic proliferation). Third, Tregs can be generated from activated effector/memory $CD4^{+}CD25^{-}$ T cells in the periphery (adaptive or induced Tregs) [28].

It is evident that both thymic generation and peripheral mechanisms of Treg are very tightly regulated to maintain a constant peripheral Treg pool under normal circumstances. Notably, disturbances within mechanisms which maintain T cell homeostasis, like increased peripheral expansion of T cells, can have detrimental consequences. For example, studies have reported an association between lymphopenia followed by compensatory homeostatic proliferation leading to the development of autoimmunity [29]. Interestingly, a disturbed T cell homeostasis has also been observed in several autoimmune diseases [29], including MS [30,31]. It is assumed that premature immunosenescence occurs in MS patients. This means that the aging of the immune system occurs much faster in patients with MS as compared to healthy individuals. Whether the homeostasis of Tregs is also disturbed in MS patients is currently unknown. Over the past years, specific Treg markers and characteristics have been identified which allow specific purification and functional analysis of these cells which can lead to a better insight into the extent by which homeostatic processes affect Tregs in MS.

1.4.2 FoxP3: master regulator of the development and function of Tregs

One significant progress in the study of Tregs has been the discovery of the transcription factor FoxP3. The gene FoxP3 was identified in the scurfy mouse strain. Scurfy is an X-linked recessive mouse mutant with lethality in hemizygous males within a month after birth. The disease is caused by a loss of function mutation in the FoxP3 gene and is clinically characterized by hyperproliferation of $CD4^+$ T cells, extensive multi-organ infiltration and overproduction of numerous proinflammatory cytokines [32]. Similarly, mutations in the human FoxP3 gene were found to be the cause of immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), an X-linked immunodeficiency disease characterized by autoimmunity against multiple endocrine organs (such as type 1 diabetes and thyroiditis), inflammatory bowel disease, severe allergy and infections [33].

FoxP3 is crucial for the development and function of natural Tregs. These cells express FoxP3 constitutively and FoxP3 expression correlates with regulatory capacity [34]. Interestingly, FoxP3 expression appears to be sufficient to convert $CD4^+CD25^-$ T cells into phenotypically and functionally $CD4^+CD25^+$ Treg cells in mice. Moreover, FoxP3 deficient mice fail to develop $CD25^+CD4^+$ Tregs and suffer from a scurfy-like inflammatory disease [35]. Using mouse models, it was shown that Tregs acquire FoxP3 expression in the thymus. Consistent with this finding, FoxP3 expression is not upregulated following *in vivo* or *in vitro* TCR stimulation in mice [34]. These data point to FoxP3 as not only a faithful marker for Tregs in mice, but also necessary and sufficient for their development and function.

Many human studies have supported the conclusions from animal studies. However contrasting findings have been reported on the dynamics and functional implications of FoxP3 expression in activated Tregs in humans. For example, upregulation of FOXP3 expression after TCR activation of $CD4^+CD25^-$ T cells has been observed [35]. Likewise, some studies indicate that FOXP3 expression in human T cells does not necessarily associate with immune regulation [34,36]. These observations show that the regulation of FoxP3 is different in humans and rodents.

Despite contrasting observations, FOXP3 remains the best and most specific marker of Tregs as to this moment. FOXP3 expression can be used to both quantify Tregs. However, because FOXP3 is an intracellular molecule, which requires cell fixation and permeabilisation for detection, it is not a marker that can be used to purify Tregs for functional studies.

1.4.3 Surface phenotype of Tregs

In mice $CD4^+CD25^+$ T cells form a population that is readily distinguished from the $CD4^+CD25^-$ T cell population and comprise approximately 10% of $CD4^+$ T cells [15].

Unfortunately, the ability to accurately distinguish human Tregs on the basis of CD25 expression is rather difficult, as recently activated $CD4^+$ T cells also express CD25. It has been shown that in humans, only those T cells with a very high CD25 expression are suppressive [37]. However, there is no uniformly defined consensus as to where the boundary between $CD25^{high}$ and $CD25^{intermediate}$ expression is set.

Other surface markers, which are constitutively expressed on the surface of human Tregs include cytotoxic T lymphocyte-associated antigen 4 (CTLA-4; CD152), glucocorticoid-induced tumor necrosis factor receptor (GITR) and OX40 (CD134). In healthy adult individuals, most Tregs have an activated/memory phenotype, predominantly expressing CD45RO, indicating that these cells have previously encountered antigen *in vivo* [38]. In addition, Tregs express specific chemokine and homing receptors which allow specific migration to lymph nodes, organs, and inflamed tissues. For example, expression of the CD62L lymph node homing selectin [39] and the αEβ7-integrin (CD103), which is present on inflammation seeking Tregs [40], have been described. Despite their level of expression and constitutive nature, the above mentioned surface proteins are not very useful markers for Tregs, as they are also expressed by activated $CD4^+$ T cells and/or other immune cells.

The quest to identify molecules, especially cell surface markers, that uniquely define Tregs has led to the recent identification of an additional potent marker: the IL-7 receptor alfa chain (CD127). The expression of CD127 is inversely correlated with FoxP3 expression and with the suppressive function of the $CD4^+FoxP3^+$T cells. It is important to note that CD127 is downregulated in all human T cells after activation. However, while CD127 is re-expressed on the majority of effector and memory T cells, it remains low or even undetectable in $CD4^+FoxP3^+$ Tregs. These features make CD127 a good marker to isolate of functional Tregs [41].

1.4.4 Functional characteristics of natural Tregs

Tregs possess several immunological features relevant to their key role in the maintenance of self-tolerance. *In vitro* studies have indicated that Tregs can suppress both the activation and/or expansion of multiple types of immune cells. Tregs suppress the activation and function of $CD4^+$ and $CD8^+$ T cells. Furthermore, Tregs have also been shown to suppress B-cell proliferation, immunoglobulin production and class switching. In addition, they inhibit NKT cells, the cytotoxic functions of natural killer cells and the function and maturation of dendritic cells [42]. In conclusion, Tregs possess several immunological features relevant to their key role in the maintenance of self-tolerance.

- *Tregs require TCR stimulation to exert suppression*

Stimulation via the TCR is required for Tregs to exert suppression. Antigen-specific as well as polyclonal TCR stimulation in contrast to irrelevant antigens can activate Tregs to

mediate suppression. Interestingly, Tregs are highly sensitive to antigenic stimulation. That is, they need much less antigen to become suppressive than $CD4^+CD25^-$ T cells need for activation [15]. However, Tregs stimulation via their TCR is not sufficient to mediate suppression. It is thought that a second signal, provided by the pro-inflammatory cytokine IL-2 is needed [43]. Once activated, Tregs not only suppress the proliferation of T cells with the same antigen specificity but also other T cells specific for other antigens (bystander suppression) [44].

- *Tregs are anergic in vitro, but proliferative in vivo*

Although $CD25^+CD4^+$ regulatory T cells require antigenic stimulation for their functional activation, they remain non-proliferative upon *in vitro* antigenic stimulation. This anergic state is closely linked with their suppressive capacity [15]. For example, abrogation of the anergic state by TCR stimulation in the presence of a high dose of IL-2 or CD28 ligation results in the loss of suppressive capacity *in vitro*. Removal of IL-2 or addition of CD28 antibody reverts Tregs to their original anergic and suppressive state, illustrating that this feature is reversable [45]. In contrast to the non-dividing state *in vitro*, Tregs are highly proliferative upon antigenic stimulation *in vivo*. Moreover, a fraction of Tregs in normal naive mice is continuously proliferating without exogenous antigenic stimulation, presumably by recognizing self antigens in the periphery [15]. The reason why Tregs are anergic *in vitro* is still unknown. It may simply be that the currently used culture conditions do not fulfill the requirements to grow these cells.

1.4.5 The molecular basis of Treg mediated suppression

Even though immunoregulation is the main functional characteristic of Tregs, the exact mechanisms of suppression remain to be clarified. Nevertheless, various molecular and cellular events have been described.

- *Direct cell-to-cell contact*

It is well established that Tregs mediate their suppressive effects by a cell-to-cell contact dependent mechanism. This notion is supported by the finding that Tregs are not capable to suppress $CD25^-CD4^+$ responder T cells, when physically separated by a semi-permeable membrane. Moreover, supernatants recovered from activated Tregs failed to suppress the response of $CD25^-CD4^+$ T cells [42]. Several possible mechanisms have been described over the past years to explain the underlying mechanism of this cell-contact dependent suppression. For example, some studies show that Treg cell are able to downmodulate the expression of co-stimulatory molecules CD80 and CD86 on APCs, attenuating their antigen presenting capacity. Other studies demonstrated that activated Tregs mediate their suppressive functions directly on responder T cells. Several T-cell

accessory molecules such as CTLA-4 expressed by Tregs, and CD80 and CD86 expressed by APCs are thought contribute to the contact-dependent suppressive mechanism [15,18,42].

- *Secretion of inhibitory cytokines*

In contrast to the requirement of cell contact for *in vitro* suppression, there are many reports that indicate that cytokines such as IL-10 and TGF-β are needed for suppression. IL-10 and TGF-β which are known to suppress the activity of effector T cells and APCs [42]. The contribution of these cytokines to Treg mediated suppression is however controversial. Several *in vivo* and *in vitro* studies support their involvement in Treg mediated suppression. For example, Tregs have been reported to be involved in the recovery of EAE by local secretion of IL-10 in the CNS [47]. In contrast, the use of anti-IL-10 and anti-TGF-β antibodies failed to inhibit suppression in many studies. In addition, Tregs isolated from neonatal TGF-β knockout mice exhibit a normal suppressive activity *in vitro* and *in vivo*. Furthermore, TGF-β and IL-10 are rarely found in the supernatans of *in vitro* suppression assays [42]. Alternatively, it was shown that Tregs cells may act as an IL-2 sink, depriving autoreactive cells of autocrine IL-2 [46].

- *Cytotoxicity*

Recent studies have indicated that cytotoxicity can be a mechanism by which T cells suppress other cells. For example, murine Tregs express granzyme B upon activation and elicit granzyme B dependent, perforin independent cytotoxicity towards stimulated B cells. This cytotoxic property of Tregs has also been reported for human Tregs. However, the mechanism used by human Tregs differs from murine Tregs. For instance, activated human Tregs express granzyme A but very little granzyme B and display perforin-dependent cytotoxicity against autologous target cells, including activated $CD4^+$ and $CD8^+$ T cells, monocytes, and both immature and mature dendritic cells [48].

None of the above mentioned mechanisms can explain all aspects of Treg suppression. It is probable that various combinations of several mechanisms are operating depending on the milieu and the type of immune responses. It is also probable that subpopulations of Tregs exist with distinct mechanisms of suppression [49]. In addition, there might also be a single key mechanism that has not been found yet.

1.4.6 Tregs in human autoimmune diseases

Tregs have several immunological features for inducing or attenuating immunological tolerance to self- or non-self antigens, which make them important protectors against autoimmune tissue damage. This implies that either deficient generation or a reduced

effector function of Tregs may play a role in the activation of self-reactive T cells, promoting autoimmunity. Indeed, defects of Tregs, either quantitatively (frequency) or qualitatively (suppressor function), have been reported for different human autoimmune diseases. For example, studies reported altered frequencies and/or functional capacity of Tregs patients suffering from systemic lupus erythematosus (SLE), autoimmune hepatitis, rheumatoid arthritis (RA), type 1 diabetes and MS [50].

1.6 Loss of functional suppression of Tregs in MS

Over the past years, evidence emerged which suggests that the immunoregulation by Tregs in MS patients is dysfunctional. While the frequency of $CD4^+CD25^{high}$ Tregs in patients with RR-MS is similar as compared to those of healthy control subjects, the suppressive capacity is reduced. Results from our research institute and others showed a reduced suppressive capacity of Tregs isolated from patients with RR-MS towards proliferation of polyclonal stimulated effector T cells [51,52,53]. It was further demonstrated that Tregs have a reduced suppressive capacity towards T cell proliferation in response to myelin antigens myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG) [53,54]. Reduced suppressive properties of Tregs in RR-MS patients result from a functional impairment rather than a resistance of effector T cells towards suppression, as shown by cross-over *in vitro* proliferation assays [51,52,53]. The Treg function is more affected in the early phase of the disease process, indicating that the suppressive capacity of MS-derived Tregs is inversely correlated with disease duration [51]. In contrast to RR-MS patients, patients with SP-MS show a normal Treg suppressive capacity [51].

As mentioned above, FoxP3 is a key regulatory molecule in the development and function of natural Tregs. In this regard, it was hypothesized that the reduced Treg suppression in RR-MS patients is associated with an abnormal FoxP3 expression. Indeed, studies have shown a reduced FOXP3 expression in $CD4^+CD25^{high}$ Tregs of patients with RR-MS [51]. Consistent with the functional analysis, SP-MS patients showed normal levels of FoxP3 mRNA [51]. In addition, recent research at BIOMED showed a significant reduced number of $CD4^+CD25^{high}FOXP3^+$ T cells as compared to HC and patients with SP-MS. Moreover, a significant lower FOXP3 expression per cell was detected in RR-MS patients [55].

Cerebrospinal fluid (CSF) has also been used as specimen to study the role of Tregs in the pathogenesis of MS. Recently, an increased percentage of Tregs in the CSF of MS patients in comparison to their peripheral blood has been reported [56]. It remains unknown why RR-MS patients show an abnormal Treg suppressive capacity and FoxP3 expression. A further profound characterization of Tregs in patients with RR-MS and SP-MS is needed.

1.6 Study aim

This study aims to investigate whether an altered Treg homeostasis underlies the disturbed Treg function in patients with RR-MS.

First nnTregs and memory Tregs, isolated from peripheral blood of RR-MS and SP-MS patients and HC, are quantified and sorted on the basis of markers CD25, CD127 and CD45RA using flow cytometry and fluorescence activated cell sorting (FACS) respectively. The sorted $CD25^{high}CD127^{low}CD45RA^{+}$ (nnTregs) and $CD25^{high}CD127^{low}CD45RA^{-}$ (memory Tregs) will then be brought into co-culture with polyclonaly stimulated responder cells to evaluate their suppressive capacity. In addition, the suppressive capacity of the total Treg population against myelin specific responses will be determined using an indirect 5,6-carboxyfluoresceine diacetate succinimidyl esther (CFSE)-based suppression assay [54]. Briefly, the suppressive capacity of Tregs is measured indirectly by comparing the antigen reactivity of total $CD4^{+}$ T cells including Tregs and $CD4^{+}$ T cells depleted of $CD4^{+}CD25^{high}$ Tregs. CFSE labeled total $CD4^{+}$ (including Tregs) and $CD4^{+}CD25^{-}$ T cells (depleted of Tregs) are cultured in the presence of irradiated autologous PBMCs pulsed with myelin antigens MBP and MOG, tetanus toxin (TT; control antigen) or no antigen (background proliferation).

Our second objective is to determine whether MS patients have an altered Treg homeostasis. Studies suggested that an altered T cell homeostasis may play an important role in the pathogenesis of MS [30,31]. An abnormal generation of nnTregs by the thymus could be relevant for development of MS. Consequently, we studied the thymic production of nnTregs by quantifying signal joint T cell receptor excision circles (sjTRECs) in FACS-sorted nnTregs and memory Tregs from MS patients and age-matched HC. In parallel, expression of CD31 (platelet endothelial cell adhesion molecule; PECAM-1) will be analyzed using flow cytometry. CD31 is a differentiation antigen, which is expressed on $CD4^{+}$ T cells that are newly generated by the thymus. CD31 is lost upon TCR triggering of cells *in vitro*. Moreover, CD31 expression is related to the proliferative history of T cells [57]. In addition, the TCR BV usage of nnTregs and memory Tregs will be evaluated using quantitative PCR and compared between MS patients and HC to detect disturbances of BV-gene usage.

Results of this study may possibly contribute to an enhanced insight in the mechanisms underlying the disturbed Treg function in patients with RR-MS and the development of new therapies aimed to restore this regulatory function.

2 Materials and Methods

2.1 Patients and healthy controls

Peripheral blood was collected from a total of 55 MS patients. 40 patients had RR-MS. The mean age of these patients was 42 (range: 16-56). The mean EDSS was 2 (range: 1-4.5) and the mean disease duration was 9 years (range: 1-28). The remaining 15 patients had SP-MS, with a mean age of 51 years (range: 31-63), a mean EDSS of 5,25 (range: 3.5-6.5) and a mean disease duration of 15 years (range: 8-28). In addition, blood was drawn from 40 randomly selected HC, with a mean age of 37 years (range: 20-55). Informed consent was obtained from all subjects volunteering for this study.

Table 2.1 Characteristics of MS patients and healthy controls

Subject	Sex	Age	Disease[a]	Medication[b]	Duration	EDSS	HLA-DR2[c]
Healthy Controls							
HC 1	M	20	HC				ND
HC 2	M	21	HC				ND
HC 3	V	21	HC				ND
HC 4	V	21	HC				-
HC 5	M	21	HC				ND
HC 6	V	23	HC				ND
HC 7	V	24	HC				-
HC 8	M	24	HC				-
HC 9	M	27	HC				ND
HC 10	M	30	HC				-
HC 11	V	31	HC				ND
HC 12	V	31	HC				-
HC 13	V	34	HC				ND
HC 14	M	43	HC				-
HC 15	V	44	HC				ND
HC 16	M	46	HC				ND
HC 17	V	47	HC				ND
HC 18	V	47	HC				-
HC 19	V	49	HC				-
HC 20	V	49	HC				+
HC 21	M	50	HC				ND
HC 22	M	50	HC				ND
HC 23	V	55	HC				+
HC 24	V	57	HC				ND
HC 25	M	49	HC				-
HC 26	V	29	HC				ND
HC 27	M	52	HC				-
HC 28	V	27	HC				-
HC 29	V	26	HC				-
HC 30	V	45	HC				+
RR-MS patients							
RR-MS 1	V	36	RR		10	1.5	ND
RR-MS 2	M	46	RR		23	4.0	-
RR-MS 3	V	48	RR		7	4.5	+
RR-MS 4	V	54	RR		22	4.0	+
RR-MS 5	F	16	RR		1	1.5	ND

RR-MS 6	F	24	RR		4	2	-
RR-MS 7	F	27	RR		1	1	-
RR-MS 8	F	27	RR		1	1	+
RR-MS 9	M	31	RR		6	1.5	+
RR-MS 10	F	31	RR		8	1	+
RR-MS 11	M	33	RR		0.18	2	+
RR-MS 12	F	35	RR		5	2.5	+
RR-MS 13	F	44	RR		6	3.5	+
RR-MS 14	M	44	RR		1	1	+
RR-MS 15	F	45	RR		1	4	+
RR-MS 16	F	46	RR		2	2.5	-
RR-MS 17	M	47	RR		7	4.5	+
RR-MS 18	F	49	RR		9	3	-
RR-MS 19	F	50	RR		6	1	-
RR-MS 20	F	52	RR		2	1	-
RR-MS 21	F	52	RR		0.5	1	-
RR-MS 22	F	48	RR		10	3	-
RR-MS 23	V	40	RR	GA	18	3.5	-
RR-MS 24	M	36	RR	GA	16	4.0	-
RR-MS 25	M	41	RR	GA	17	4.0	+
RR-MS 26	V	40	RR	GA	7	ND	-
RR-MS 27	V	39	RR	IFN-β	12	3.5	+
RR-MS 28	V	54	RR	IFN-β	12	4.0	-
RR-MS 29	V	56	RR	IFN-β	6	ND	-
RR-MS 30	V	43	RR	IFN-β	2	4.0	+
RR-MS 31	V	37	RR	IFN-β	4	4.0	ND
RR-MS 32	M	55	RR	IFN-β	5	ND	+
RR-MS 33	M	39	RR	IFN-β	24	3.5	ND
RR-MS 34	V	29	RR	IFN-β	3	4.5	+
RR-MS 35	M	55	RR	IFN-β	11	4.0	-
RR-MS 36	V	48	RR	IFN-β	22	4.0	+
SP-MS patients							
SP-MS 1	V	45	SP		28	5.5	-
SP-MS 2	V	62	SP		16	ND	+
SP-MS 3	V	58	SP		16	6	ND
SP-MS 4	V	48	SP	GA	ND	ND	ND
SP-MS 5	V	49	SP	GA	20	5.5	+
SP-MS 6	M	55	SP	GA	9	6	-
SP-MS 7	V	44	SP	IFN-β	19	6.5	+
SP-MS 8	V	53	SP	IFN-β	14	3.5	+
SP-MS 9	M	31	SP	IFN-β	3	6.5	-
SP-MS 10	V	56	SP	IFN-β	16	5.5	-
SP-MS 11	V	49	SP	IFN-β	16	4	+
SP-MS 12	V	47	SP	IFN-β	17	4	+
SP-MS 13	M	53	SP	IFN-β	25	6.5	-
SP-MS 14	V	63	SP	IFN-β	8	6	-
SP-MS 15	M	43	SP	IFN-β	8	4	+
SP-MS 16	M	53	SP	IFN-β	8	4	-

[a]MS type at time of sampling: RR-, relapsing-remitting; SP-, secondary progressive.

[b]Medication during the last three months before sampling. IFN-β, interferon-beta; GA: Glatiramer Acetate.

[c]Patients were scored positive (+) or negative (-) for the HLA-DR2 haplotype as determined by PCR (see section 2.3.1). ND= not determined.

2.2 Cell Culture techniques

2.2.1 Cell culture reagents

$CD4^+$ T cells were cultured in RPMI 1640 medium supplemented with 1% sodium pyruvaat, 1% nonessential amino acids (all from Life Technologies, Paisley, Scotland, UK), 10% fetal bovine serum (FBS, Hyclone Europe, Erembodegem, Belgium), 50U/ml penicillin and 50µg/ml streptomycin (Invitrogen, Merelbeke, Belgium). Human MBP was purified from white matter of human brain as described earlier (Deibler et al., 1972). Tetanus toxoid (TT) was obtained from the 'Rijksinstituut voor Volksgezondheid en Milieu' (RIVM, Bilthoven The Netherlands). Synthetic MOG peptides (aminoacids 1-22, 34-56, 64-86, 74-96) were synthesized and purified by *High pressure liquid chromatography* (HPLC) (Severn Biotech Ltd, Worcester, UK). The amino acid sequences of the MOG peptides are shown in table 2.2.

Table 2.2 Amino Acid Sequence of Synthetic myelin peptides

MOG (1-22)	GQFRVIGPRHPIRALVGDEVEL
MOG (34-56)	GMEVGWYRPPFSRVVHLYRNGKD
MOG (64-86)	EYRGRTELLKDAIGEGKVTLRIR
MOG (74-96)	DAIGEGKVTLRIRNVRFSDEGGF

2.2.2 Isolation of peripheral blood mononuclear cells (PBMCs) and T cell subsets

PBMCs were isolated from peripheral blood by Ficoll density gradient centrifugation (Histopaque, Sigma diagnostics, St. Louis, USA). Isolation of $CD4^+$ T cells from whole blood samples was performed indirectly by means of rosetting of unwanted haematopoietic cells ($CD8^+$, $CD16^+$, $CD19^+$, $CD36^+$ and $CD56^+$ cells; Rosettesep^{TM} $CD4^+$ T cell enrichment cocktail, Stemcell technologies, Grenoble, France). When frozen PBMCs were used as starting material, $CD4^+$ T cells were isolated using a magnetic associated cell sorting (MACS) $CD4^+$ T cell purification kit (Miltyeni biotec, Senver TECH, Boeghout, Belgium). $CD4^+CD25^{high}CD127^{low}CD45RA^+$ (nnTregs), $CD4^+CD25^{high}CD127^{low}CD45RA^-$ (memory Tregs), $CD4^+CD25^-CD127^{high}CD45RA^+$ (naive $CD4^+CD25^-$ T cells) and $CD4^+CD25^-CD127^{high}CD45RA^+$ (memory $CD4^+CD25^-$ T cells) T cell subsets were isolated by means of FACS-sorting (see fig. 2.1). $CD4^+$ T cells were stained with Fluorescein isothiocyanate (FITC)-labelled anti-CD127 (e-Bioscience), Allophycocyanin (APC) labelled anti-CD45RA (Immunotools), Phycoerythrin (PE) labelled anti-CD25, and Peridin chlorophyll protein (PERCP) labelled anti-CD4 antibodies (BD biosciences, Erembodegem, Belgium) for 30 minutes at 4ºC. T cell subsets were sorted using a high speed cell sorter (FACSAria^{TM}, BD Biosciences, Erembodegem, Belgium) in collaboration with the Virga Jesse hospital Hasselt. The purity of the FACS sorted T cell subsets were routinely >

95%. After sorting, the cells were collected and directly used for suppression assays and/or frozen at -80ºC for subsequent molecular analysis.

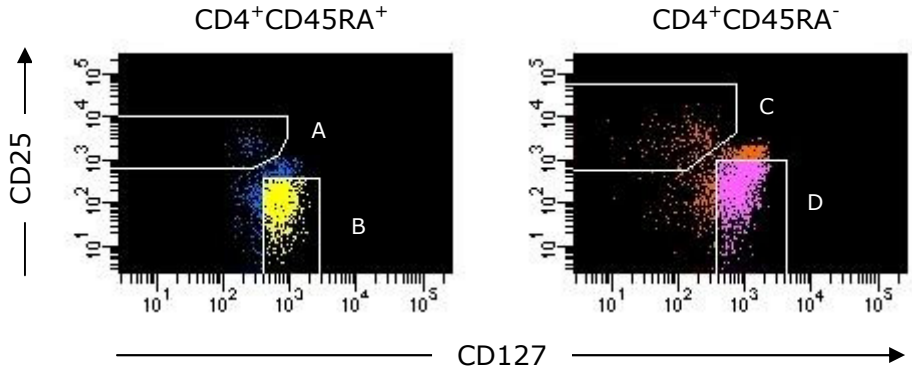


Figure 2.1: Isolation of nnTregs, memory Tregs, $CD4^+CD25^-$ naive T cells and $CD4^+CD25^-$ memory cells by means FACS sorting. Cells were labelled with anti-CD127 FITC, anti-CD4-PERCP, anti-CD25-PE and anti-CD45RA-APC mAb. The CD25 and CD127 signal is measured on gated $CD4^+CD45RA^+$ and $CD4^+CD45RA^-$ cells. **(A)** nnTregs: $CD4^+CD25^{high}127^{low}CD45RA^+$; **(B)** memory Tregs: $CD4^+CD25^{high}127^{low}CD45RA^-$; **(C)** naive T cells: $CD4^+CD25^{low}127^{high}CD45RA^+$; **(D)** memory T cells: $CD4^+CD25^{low}127^{high}CD45RA^-$.

2.2.3 Immunophenotypic analysis by flowcytometry

For phenotypic analysis, purified PBMCs were stained with fluorescent-conjugated monoclonal antibodies (mAb) specific for cell surface markers and analysed by flow cytometry using a FACSCalibur™ (Becton Dickinson, San Diego, CA, USA). Statistical analysis was performed using Cellquest® software. Naive and memory Tregs were quantified by staining PBMCs with a combination of anti-CD4-PERCP, anti-CD25-PE, anti-CD127-FITC and anti-CD45RA-APC.The percentage of naive and memory Tregs within the $CD4^+$ T cell population was then determined. For analysis of CD31 expression on the different T cell populations, PBMCs were stained with anti-CD31-FITC (immunotools), anti-CD25-PE, anti-CD4-PERCP and anti-CD45RA-APC. After gating on CD4 and CD45RA, the percentage of CD31 positive cells was determined within the $CD25^{high}$ and $CD25^{low}$ population.

2.2.4 CFSE labeling

A CFSE stock (10 mM in DMSO; Invitrogen, Merelbeke Belgium) stored at -20ºC, was thawed and diluted in phosphate buffered saline (PBS) to a final concentration of 4µM. Freshly purified T cells were suspended in PBS [0,1% bovine serum albumin (BSA)] at $2x10^6$ cells/ml and incubated with the CFSE solution (final concentration: 2 µM) for 7 minutes at 37ºC. Cells were washed and resuspended in culture medium for 15 minutes to stabilize the CFSE staining. After a final washing step, cells were resuspended in culture medium.

2.2.5 Treg suppression assays

-Direct suppression assay

The ability of Tregs to suppress the proliferation of polyclonally activated of $CD4^+CD25^-$ responder T cells was assessed by a direct suppression assay. CFSE labelled $CD4^+CD25^-CD127^{high}$ T cell responders were cultured in duplo in 96-well round bottom plates (Nunc, Roskilde Denmark) at 2 x 10^4 cells/well, alone or in the presence of varying amounts of $CD4^+CD25^{high}CD127^{low}CD45RA^+$ nnTregs or $CD4^+CD25^{high}CD127^{low}CD45RA^-$ memory Tregs ($T_{responder}/T_{reg}$ ratios were 1/0; 1/0.25; 1/0.5 and 1/1). Autologues irradiated PBMCs (10^5 cells/well) were added as feeders. In parallel, CFSE labeled and unlabeled nnTregs and memory Tregs were cultured alone. All cultures were stimulated with anti-CD3 mAb (2µg/ml) (Clone2G3, BIOMED, Diepenbeek, Belgium). The cells were cultured for 5 days at 37°C (5% CO_2), harvested after 5 days of culture, stained with anti-CD4-PE mAb and 7-amino actinomycin D (7-AAD) and analyzed by flow cytometry. The suppressive capacity of Tregs towards responder cells in coculture was calculated as the relative inhibition of the percentage of $CFSE^{low}$ cells [100 x 1- (% $CFSE^{low}$ $CD4^+CD25$ T cells in coculture / % $CFSE^{low}$ $CD4^+CD25^-$ T cells alone)].

-Indirect suppression assay

Myelin antigen specific proliferation assays were performed as decribed before [54]. Briefly, CFSE labeled total $CD4^+$, $CD4^+CD25^-CD127^{high}CD45RA^+$ (naive $CD4^+$ responder T cells) and $CD4^+CD25^-CD127^{high}CD45RA^-$ (memory $CD4^+$ responder T cells) cells (2 x 10^5 cells/well) were cultured in duplo in 48 well flat bottom plates with 2.5 x 10^5 irradiated autologous PBMCs that were pulsed for 3 hours with MBP (100 µg/ml), MOG peptides (30 µg/ml) or tetanus toxoid (TT) (20 LF/ml). Cells were also cultured in the presence of unpulsed irradiated PBMCs to asses the background proliferation for each experiment. After 10 days of culture, cells were harvested and incubated for 30 minutes at 4ºC with 7-AAD (BD, Erembodegem, Belgium) and anti-CD4-PE mAB (BD, erembodegem, Belgium). The CFSE signal of viable $CD4^+$ cells was analyzed by flow cytometry using a FACSCalibur™. The proliferating fraction (ΔPF) was calculated by subtracting the mean background proliferation from the mean proliferating fraction in response to antigen: ΔPF = [%$CFSE^{low}CD4^+$ T cells $_{(antigen)}$] – [%$CFSE^{low}CD4^+$ T cells $_{(no\ antigen)}$]. The stimulation index (SI) was calculated as the percentage of $CFSE^{low}CD4^+$ T cells $_{(antigen)}$ divided by the percentage $CFSE^{low}CD4^+$ T cells $_{(no\ antigen)}$. An antigenic response was considered significant when ΔPF was higher than 1% and SI higher than 3.

2.2.5 Cloning of myelin reactive T cells

$CD4^+CD25^-$ T cells were isolated from PBMCs by means of negative selection using a $CD25^+$ selection kit (Easysep™, Stemcell technologies, Grenoble, France). $CD4^+CD25^-$ T

cells were labelled with CFSE and cultured in the presence of 2.5 x 10^5 irradiated autologous PBMCs that were pulsed for 3 hours with MBP, MOG peptides or TT (as described above, 2.2.4). After 10 days, the cells were collected and stained with anti-CD4 mAb and 7-AAD. Using a FACSaria^{TM}, viable $CFSE^{low}CD4^+$ cells were sorted directly into a 96-well round bottom plate containing culture medium at 1, 3 or 5 cells/well. These cells were then stimulated with phytohemaglutinin (PHA; 200 ng/ml; Sigma) and 1 x 10^5 irradiated allogeneous PBMCs, followed by supplementation of recombinant interleukin 2 (10 U/ml twice a week; Roche Diagnostics, Brussels, Belgium). After 2-3 weeks of culture, myelin reactivity of the generated clones was determined by performing split well assays. Briefly, the proliferating cells were divided into 4 wells. Two wells received unpulsed irradiated autologous PBMCs. The other wells received PBMCs pulsed with MBP, MOG or TT. After 2 days, 1µCi [^{3}H]-thymidine (Amersham, Buckingham, England) was added to the cells. After 16 hours of culture, the cells were harvested with an automatic cell harvester (Pharmacia, Uppsala, Sweden) and uptake of radioactivity (cpm) was measured in a β-plate scintillation counter (PerkinElmer Lifesciences, Wellesly, USA). The SI index was calculated as the mean cpm of cells which were cultured with pulsed PBMCs divided by the mean cpm of cells cultured in the presence of unpulsed PBMCs. A clone was considered reactive when the SI ≥ 3.

2.2.6 Evaluation of cytokine production

Cytokine production of myelin or TT reactive $CD4^+CD25^-CD127^{high}CD45RA^+$ and $CD4^+CD25^-CD127^{high}CD45RA^-$ T cells was determined at day 10 of culture by means of intracellular flow cytometric analysis. Cells were stimulated for 4 hours at 37°C with phorbol-12-myristate-13-acetate (25ng/ml) in DMSO, calcium-ionomycine (CaI, 1 µg/ml) (both from Sigma-Aldrich; Bornem, Belgium) and brefeldine A (BFA; 10 ng/ml) (Pharmingen, Erembodegem, Belgium). The cells were pre-stained with 7-AAD to discriminate between viable and dead cells. After an additional washing step, the cells were fixed and permeabilized in cytofix/cytoperm solution (pharmingen, Erembodegem, Belgium) and subsequently stained for 30 minutes at 4°C with IFN-γ-PE, IL-4-PE, IL-17-PE mAb or with isotype control antibody IgG1-FITC/IgG2a-PE (BD biosciences, Erembodegem, Belgium). T cell activation was checked by intracellular staining with anti-CD69-PE mAb (BD biosciences, Erembodegem, Belgium). After staining, the cells were washed twice in perm/wash solution (Pharmingen, erembodegem, Belgium) and analyzed by flow cytometry.

2.3 Molecular biological techniques

2.3.1 HLA-DR2 genotyping

Genomic DNA (gDNA) was isolated from PBMCs by incubating the cells in 500 µl lysisbuffer (1.2 g/l TRIS; 23.3 gl NaCl;, 0.7 g/l EDTA), 17 µl 20% sodium dodecyl sulphate (SDS) and 90 µl proteinase K solution (10 g/l SDS; 0.7 g/l EDTA; 2 g/l proteinase K) overnight at 37ºC. gDNA was extracted using a chloroform based extraction method, followed by ethanol precipitation and was suspended in 25 µL H_2O. Polymerase chain reaction (PCR) was used to identify MS patients and HC positive or negative for HLA-DR2 (DRB*0301). PCR of a total volume of 50 µL was performed with 1µl gDNA, 225 µmol $MgCl_2$, 10 µmol dNTPs, 1 pmol forward primer (5-TTCCTGTGGCAGCCTAAGAGG-3), 1 pmol reverse primer (5-CCGCTGCACTGTGAAGCTCTC-3) and 1.5 U Taq polymerase. The PCR program consisted of an initial 5 minutes at 95ºC, followed by 30 cycles of repeated denaturation (20 sec at 95ºC), annealing (20 sec at 60ºC) and elongation (40 sec at 72ºC). PCR products were separated on a 1% agarose gel. For DR2 positive individuals, a PCR product of 261 base pairs was detected.

2.3.2 Quantification of sjTRECs by quantitative RT-PCR

sjTREC (explained in figure 2.1) numbers were quantified from FACS-sorted $CD4^+CD25^{high}CD127^{low}CD45RA^+$, $CD4^+CD25^{high}CD127^{low}CD45RA^-$, $CD4^+CD25^-CD127^{high}$ $CD45RA^+$ and $CD4^+CD25^-CD127^{high}CD45RA^+$ T cells. gDNA was extracted using the QiaAmp Blood Mini Kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. TREC numbers were quantified by quantitative real-time PCR on a LightCycler system (Roche diagnostics, Mannheim, Germany) as described before [58]. In brief, primers (5'-TCC CTT TCA ACC ATG CTG-3' and 5'-GGC TTG GGA TAG CTG T-3') were constructed to amplify a fragment including the δRecψ-Jα signal joint. The TREC 5'probe (5'-CCC CCT GTC TGC TCT TCA-3') was labelled at the 3' end with fluorescein, whereas the TREC 3' probe (5'-CAC CGT TCT CAC GAG TTG-3') was labelled at the 5' end with Lightcycler Red 640. For amplicon detection, the Lightcycler FastStart DNA Master Hybridization Probes kit (Roche Diagnostics GmbH) was used. PCR of 10 µL total volume was performed with 1µl gDNA, 7.5 µmol $MgCl_2$, 0.5 pmol primers, and 0.2 pmol probes. The PCR program consisted of an initial 10 minutes at 95ºC for FastStart Taq DNA polymerase activation, followed by 50 cycles of repeated denaturation (3 sec at 95ºC), annealing (10 sec at 59ºC) and chain extension (15 sec at 72ºC). A serial dilution of TREC containing plasmids was used as a standard for absolute quantification.

Detection of the amplified DNA is based on fluorescence resonance energy transfer (FRET). The probes described above, bind to an internal sequence of the amplified fragment during the annealing phase of the amplification cycle. Hybridisation of the

probes to the DNA fragment brings the two fluorescent dyes into close proximity. Fluorescein is excited by the light source of the Lightcycler, which causes it to emit green fluorescent light. The emitted energy then excites Lightcycler Red 640 by FRET. The red fluorescence emitted by the second probe is measured at the end of each annealing step and is a measure of the amount of DNA which has been amplified. After annealing, an increase in temperature leads to elongation and displacement of the probes. The displaced probes in solution are too far apart to allow FRET to occur.

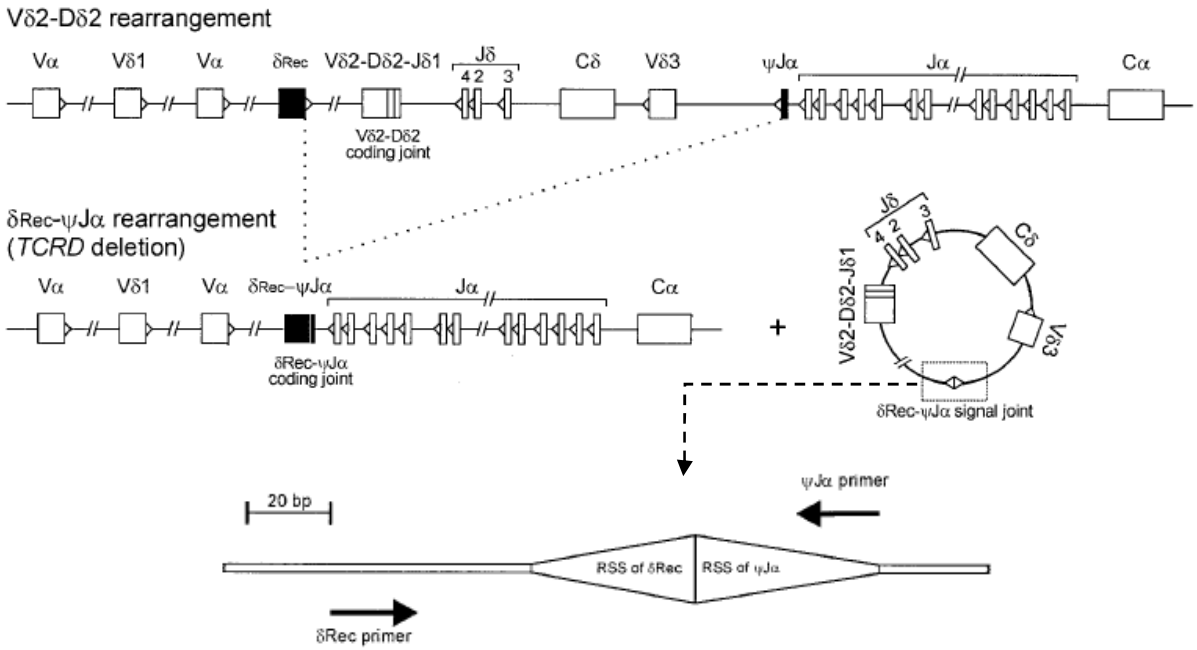


Figure 2.1: Schematic overview of the process generating the sjTREC. During the development of T cells in the thymus, TCR genes are rearranged to form a functional TCR. The SjTREC is formed during the the δRec-ψJα rearrangement (TCRD deletion). In brief, TREC formation is mediated by recombination activation gene 1 and 2 proteins (RAG1 and RAG2), which recognize specific recombination signal sequences (RSS) sequences in the DNA. RSS sequences flank the DNA region which needs to be cut out of the gene. RAG enzymes cleave the DNA between the RSS sequence and the coding end of the involved gene. This results in a hairpinned coding end and a blunt 5' phosphorylated end. Ligation of the hairpinned coding ends results in a coding joint. The ligation of the signal ends results in the formation of an extrachromosomal circular excision product, containing two coupled RSS [59]. Insert position of the RT-PCR primers for the detection and quantification of sjTRECS are given in the bottom panel of the figure. Figure adapted from Hazenberg MD et al. 2001 [59].

2.3.3 TCR BV gene usage

- RNA isolation and cDNA synthesis

TCR BV usage was determined in sorted nnTregs and memory Tregs. RNA was isolated using the QiaAmp Blood Mini Kit (Qiagen, Venlo, The Netherlands). cDNA was synthesized using the reverse transcriptase system (Promega, Madison, USA). The reaction mixture contained 10 mM $MgCl_2$, 2 x reverse transcriptase buffer, 2 mM dNTP, 5% RNAse inhibitor, 5% AMV reverse transcriptase and 10 % oligodT primer. The cDNA

was purified using phenol/chloroform/isoamylalcohol extraction and precipitated overnight in 10 µL Na-acetate (3M) and 200 µL 100% ethanol. After washing 2 times with 70% ethanol, the purified cDNA was dissolved in 25 µL H_20.

- *realtime-RT-PCR*

The TCR BV usage was evaluated by means of quantitative RT-PCR. PCR amplification was performed as described before, with one out of 23 TCR BV specific forward primers and a TCR BC reverse primer [60]. Real-time PCR was performed on the lightcycler system. The FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, germany) was used for amplicon detection. PCR of 10 µL total volume was performed with 1 µl gDNA, 4 mM $MgCl_2$ and 2 pmol primers. The PCR program consisted of an initial 10 minutes at 95ºC, followed by 50 cycles of repeated denaturation (95ºC), annealing (3 sec at 60ºC) and elongation (10 sec at 72ºC). A melting curve analysis was performed after the PCR program to discriminate between amplified DNA fragments and non-specific products such as primer-dimers.

The amplified DNA fragments are detected by measurement of the SYBR Green fluorescence signal. SYBR Green intercalates into the DNA double helix. In solution, the unbound dye exhibits very little fluorescence. Upon DNA-binding, the fluorescence signal is greatly enhanced. Therefore, during the PCR reaction, the increase in SYBR Green fluorescence is directly proportional to the amount of double stranded DNA generated.

2.4 Statistical analysis

Differences between different groups were calculated by Student's t-tests. Correlations were evaluated via Pearson's correlation tests. P-values < 0.05 were considered significant.

3 Results

3.1 Evaluation of Treg homeostasis

3.1.2 Reduced frequency of nnTregs and memory Tregs in RR-MS patients as compared to HC and SP-MS patients

The percentage of $CD4^+CD25^{high}CD45RA^-CD127^{low}$ (nnTregs) and $CD4^+CD25^{high}CD45RA^-CD127^{low}$ (memory Tregs) cells of the total number of $CD4^+$ T cells was measured by means of flow cytometry. PBMCs were isolated from peripheral blood of HC (n=30), untreated RR-MS patients (n=22) and SP-MS patients (n=15). The mean percentages (+SEM) of nnTregs and memory Tregs are given in figure 3.1. Untreated RR-MS patients showed a significantly reduced percentages of total Tregs (4.92 ± 0.34%) as compared to HC. Both the frequencies of nnTregs (1.43 ± 0.15%; $p < 0.05$) and memory Tregs (2.47 ± 0.14%; $p < 0.0001$) were reduced in RR-MS patients as compared to HC (respectively 2.16 ± 0.27% and 4.20 ± 0.30%). In contrast, SP-MS patients showed a normal frequency of nnTregs (2.30 ± 0.30%) and memory Tregs (4.15 ± 0.35%).

Age is a confounding factor, influencing the relative amount of naive and memory T cells within the $CD4^+$ T cell population. Correlation analysis indicates that the frequency of nnTregs is inversely correlated with the age of HC ($p<0.001$ r= -0.61; fig. 3.2 (A)) and RR-MS patients ($p<0.05$; r=-0.59; fig. 3.2 (A)).

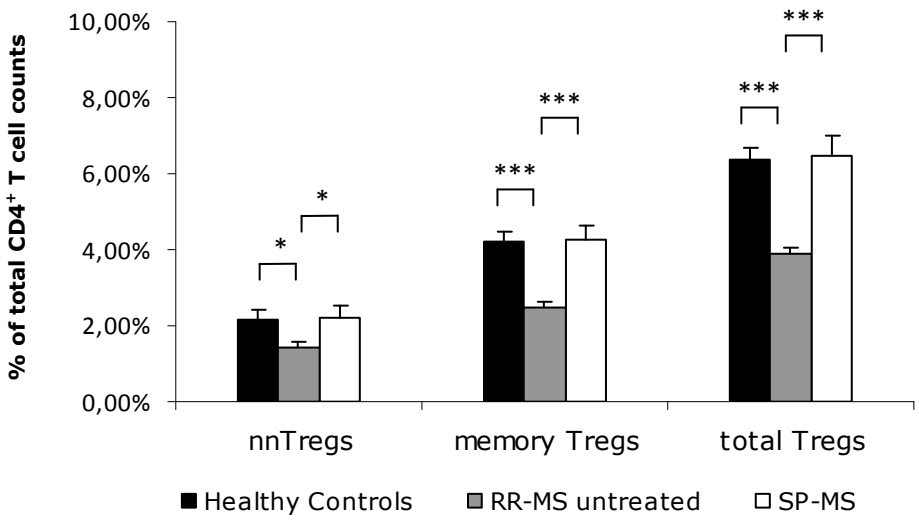
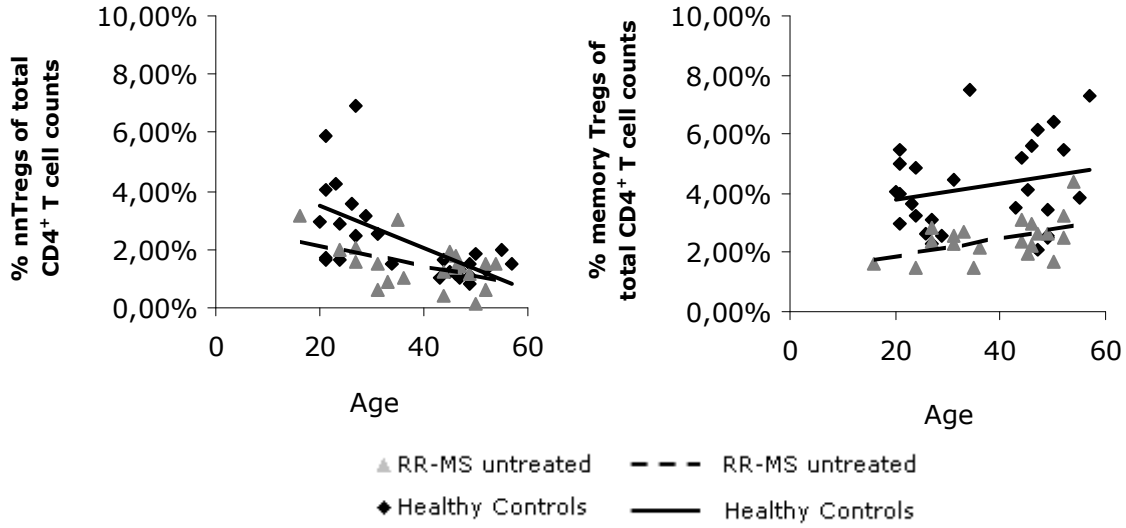
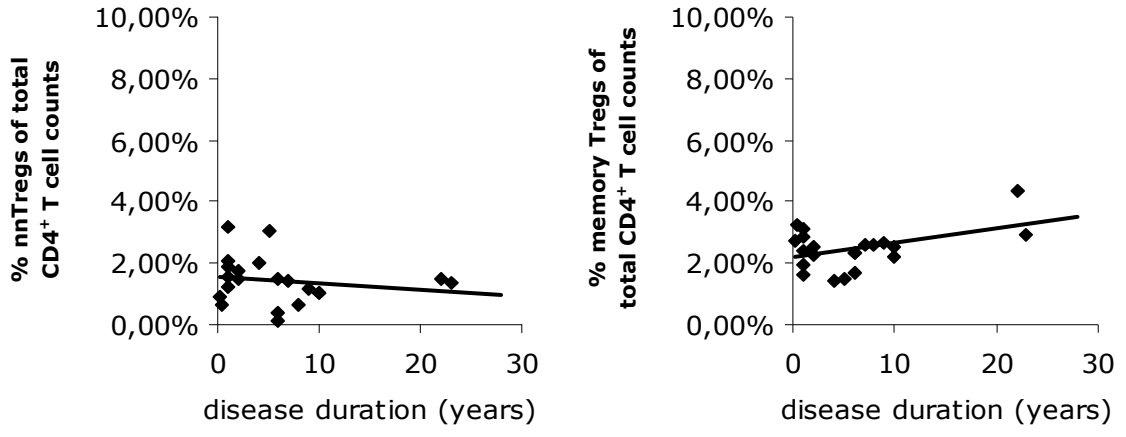


Figure 3.1: Percentages of nnTregs, memory Tregs and total Tregs in peripheral blood of untreated RR-MS patients, HC and SP-MS patients. Percentages of nnTregs, memory Tregs and total Tregs were determined in peripheral blood of 31 HC, 22 RR-MS patients and 15 SP-MS patients by means of flow cytometry. The bars represent the mean percentages (+SEM) of nnTregs ($CD4^+CD25^{high}CD127^{low}CD45RA^+$) and memory Tregs ($CD4^+CD25^{high}CD127^{low}CD45RA^-$) of total $CD4^+$ T cells. The total Treg percentage for each individual was calculated by adding the the frequencies of the nnTregs and memory Tregs. (* $p < 0.05$; *** $p<0.0001$).

In contrast, no significant correlation was found between age and the proportion of memory Tregs in HC and RR-MS patients. The same observation was made for SP-MS patients (data not shown). We also analyzed whether frequencies of naive and memory Tregs were related with disease duration of MS patients. The percentage of $CD4^+CD25^{high}CD127^{low}CD45RA^-$ memory Tregs from RR-MS patients were significantly correlated with disease duration ($p<0.05$, $r= 0.45$; fig. 3.2 (B)). The percentage of $CD4^+CD25^{high}CD127^{low}CD45RA^+$ nnTregs was not correlated with disease duration. No significant correlation could be found between disease duration and nnTreg or memory Tregs for SP-MS patients (data not shown).



(A)



(B)

Figure 3.2 nnTreg and memory Treg frequencies in function of age and disease duration of RR-MS patients. (A) Age associated changes of nnTreg and memory Treg frequencies. nnTreg and memory Treg percentages were plotted against the age (years) of RR-MS patients (n=22) and HC (n=31). **(B)** nnTreg and memory Treg frequencies in function of disease duration of RR-MS patients. The percentages nnTregs and memory Tregs were plotted against the disease duration (years) of untreated RR-MS patients. Dots correspond to individual samples tested. Correlations were measured using Pearson's correlation tests.

3.1.2 sjTREC analysis of nnTregs and memory Tregs in MS patients and HC

In view of a reduced percentage of nnTregs and memory Tregs in RR-MS patients relative to HC, we questioned whether this could result from an altered thymic output of Tregs. In this respect, thymic output of Tregs was estimated by measuring the TREC content of nnTregs. TRECs are generated during TCR rearrangements in the thymus. TRECs are stable, but are not replicated during mitosis and consequently diluted during every cell division. The sjTREC, which results from the recombination of V(D)J gene segments, has been described as a good marker for thymic output [59]. sjTRECs were measured in FACS sorted nnTreg and memory Treg subsets. Figure 3.3 shows the mean number of TRECs which were measured in 10 ng DNA.

The mean concentrations of TRECs in nnTregs were lower in HC, aged between 40-60 years (48145 TRECs/10 ng DNA) than in HC, aged between 20 and 40 years (87454 TRECs/10 ng DNA). For both age groups, the TREC amounts was lower in memory Tregs as compared to nnTregs (age 20-40= 22758 TRECs/10 ng DNA; age 40-60:= 17128 TRECs/10 ng DNA; $p<0.05$). RR-MS patients of both age groups had lower numbers of TRECs in nnTregs (age 20-40= 41135 TRECs; age 40-60= 18524 TRECs) and in memory Tregs (age 20-40= 8988 TRECs; age 40-60= 9674 TRECs) as compared to HC. These differences were not significant however.

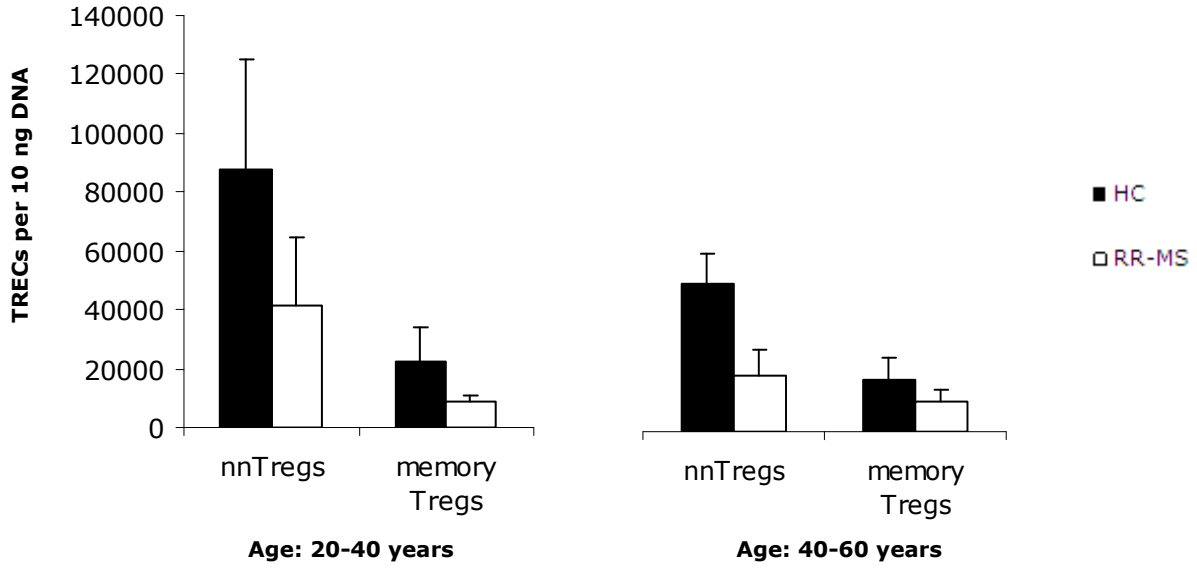


Figure 3.3: Number of TRECs in nnTregs and memory Tregs. TREC numbers were determined in FACS sorted nnTregs and memory Tregs of HC (n=8) and RR-MS patients (n=6) by means of quantitative RT-PCR. Bars represent the mean number of TRECs per 10 ng DNA (+SEM).

3.1.3 CD31 expression is reduced on memory Tregs and CD25$^-$CD45RA$^-$ T cells in RR-MS patients as compared to HC and SP-MS patients

The percentage of CD31 expression allows to determine the amount of recent thymic emigrants within a given T cell population, but also provides a measure of the

proliferation history of T cells [57]. The expression of CD31 was evaluated on nnTregs, memory tregs, $CD4^+CD25^-$ naive T cells and $CD4^+CD25^-$ memory T cells from HC (n=30), untreated RR-MS (n=22) and SP-MS patients (n=15) by means of flow cytometry. The mean percentage of CD31 expressing cells within the indicated T cell populations are given in figure 3.4.

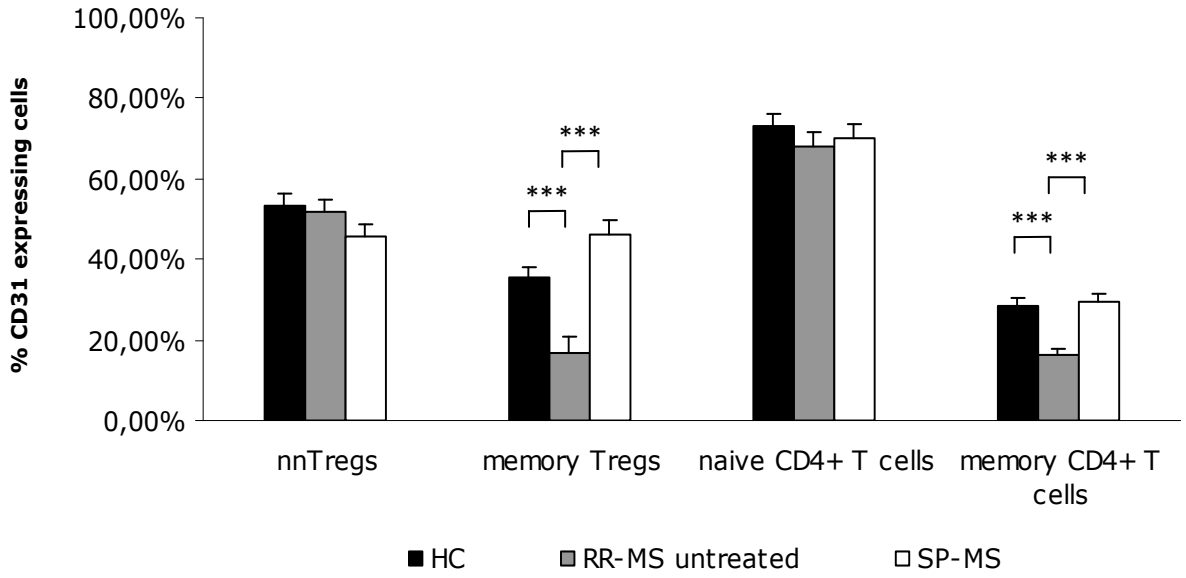


Figure 3.4: CD31 expression of different $CD4^+$ T cell populations in HC and MS patients. CD31 expression was determined on different T cell populations in peripheral blood by means of flow cytometry. Bars represent the mean percentages (+ SEM) of CD31 positive cells within the nnTreg, memory Treg, $CD4^+CD25^-$ T cell and memory $CD4^+CD25^-$ T cell populations of HC (n=33; mean age=37; range: 20-55; black bars), untreated RR-MS patients (n=21; mean age=39; range: 16-52; gray bars) and SP-MS patients (n=15; mean age= 53; range:31-63; white bars). (*** $p<0.0001$).

The percentage of CD31 expressing memory Tregs was significantly lower in RR-MS patients (26.32% ± 3.21%) as compared to HC (35.75% ± 2,33%) ($p<0.0001$). Correspondingly the number of CD31 positive $CD4^+CD25^-$ memory T cells was also reduced (20.35% ± 1.74%) as compared to HC ($p<0.0001$). No difference was observed between RR-MS patients and HC for the number of CD31 expressing nnTregs and naive $CD4^+CD25^-$ T cells. In contrast to RR-MS patients, SP-MS patients showed no altered number of CD31 expressing memory Tregs and memory t cells. However, SP-MS patients showed a reduced frequency of CD31 expressing nnTregs was, although this difference was not significant ($p=0.09$).

We also evaluated whether the percentage of CD31 expressing cells was associated with age (see fig. 3.5). Correlation analysis indicates that CD31 expression of nnTregs is inversely correlated with age in HC ($r=-0.69$; $p<0.001$). No such correlation was found for RR-MS patients and SP-MS patients (data not shown). The percentage of CD31 expressing memory Tregs was not correlated with age in HC, RR-MS and SP-MS patients.

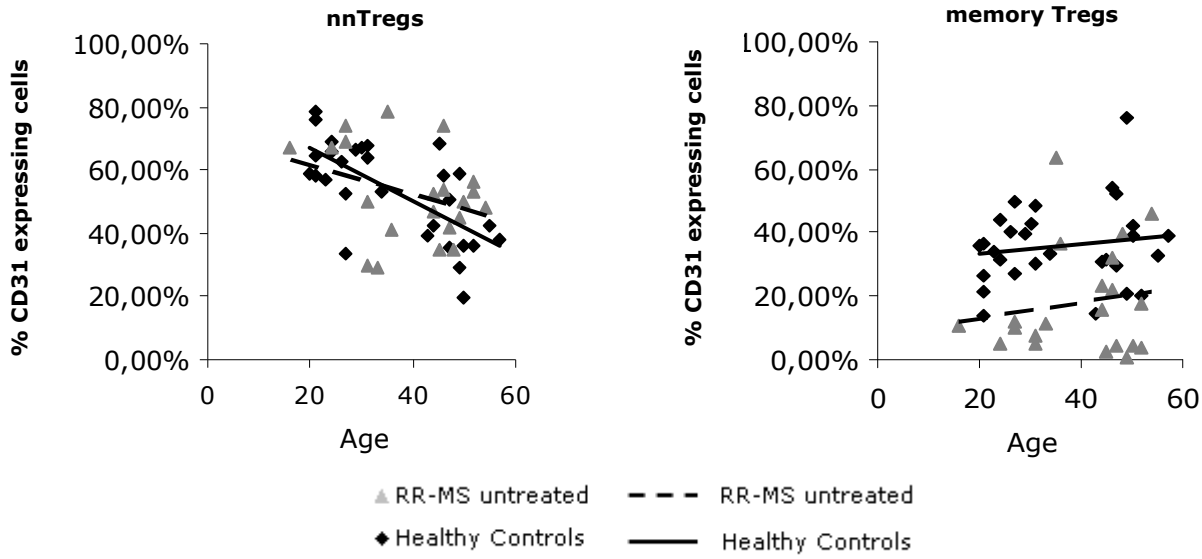


Figure 3.5: Age associated changes of nnTreg and memory percentages. Percentages of CD31 expressing nnTregs and memory Tregs were plotted against the age of RR-MS patients (n=22) and HC (n=31). Dots correspond to individual samples tested. Correlations were measured using Pearson's correlation tests.

3.1.4 HLA-DR2 and CD31 expression

An increased frequency of the common HLA-DR2 haplotype was reported in MS patients [1,2]. Thymic selection of $CD4^+$ T cells is influenced by HLA class II gene expression. In addition, peripheral naive $CD4^+$ T cell survival requires MHC class II restriction [29]. For this reasons the possibility that HLA-DR2 expression influences naive- and memory Treg CD31 expression was investigated. Two of 13 HC (15.38%), 10 of 20 RR-MS patients (50%) and 6 of 11 SP-MS patients (54.5%) were HLA-DR2 positive. Correlation analysis between DR-2 expression and CD31 expression was performed between age-matched groups, as age also influences the CD31 expression.

HLA-DR2 positivity did not correlate with naive and memory Treg CD31 counts in HC and SP-MS patients (data not shown). In contrast, DR-2 expression correlated with CD31 expression levels in RR-MS patients. Figure 3.6 gives the percentage of CD31 expressing nnTregs and memory Tregs in RR-MS patients based on the presence of the HLA-DR2 haplotype. The mean percentage of CD31 expressing nnTregs was significantly lower in DR2 positive RRMS patients (44.21%) than in DR2 negative RR-MS patients (60.87%) ($p<0.001$). CD31 expression was also significantly lower on naive $CD4^+CD25^-$ T cells of DR2 positive- (63.27%) as compared to DR2 negative patients (75.20%) ($p<0.05$). In contrast, DR-2 positivity was not associated with CD31 expression on memory Tregs and memory $CD4^+CD25^-$ T cells.

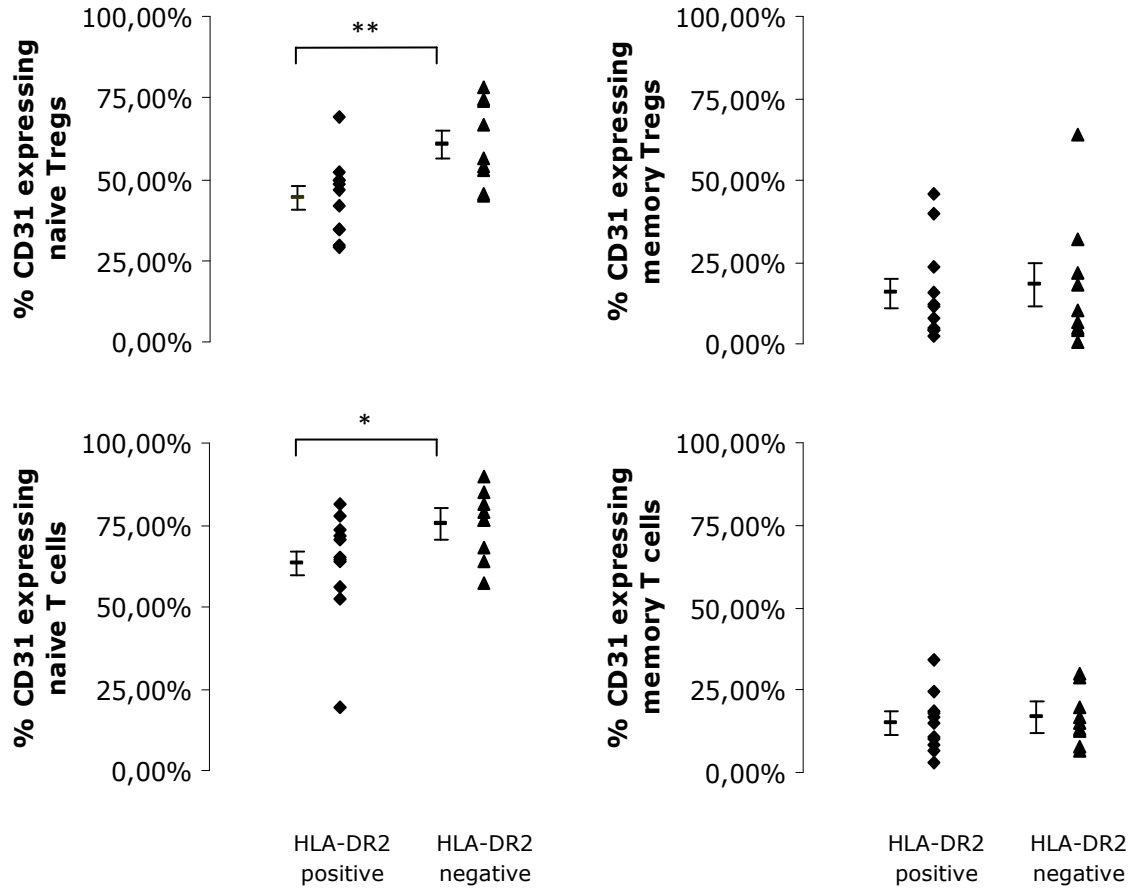


Figure 3.6: CD31 expression on different T cell subpopulations based on HLA-DR2 positivity in RR-MS patients. Dots represent the percentage CD31 expressing cells of the analyzed individuals. The mean percentage (±SEM) of each patient group is also given. The mean age the HLA-DR2 positive RR-MS patients was 40 years (range 27-50). The mean age of the HLA-DR2 negative patient group was 41 years (range 23-52). (A) percentages of CD31 expressing nnTregs; (B) percentages of CD31 expressing memory Tregs; (C) percentages of CD31 expressing naive $CD4^+CD25^-$ T cells; (D) percentages of CD31 expressing memory $CD4^+CD25^-$ T cells.

3.1.5 TCR BV usage

To evaluate the composition of the nnTreg and memory Treg subsets, TCR diversity was investigated in FACS sorted cells from 3 HC and 3 RR-MS patients. The expression of TCR BV genes within these Treg subsets was qualitatively evaluated by means of real time-PCR analysis using a panel of 23 different BV specific primers. The relative number of BV genes that were expressed in each Treg population for all individuals analyzed is given in figure 3.7. Although interindividual differences were observed, nnTregs and memory Tregs from HC show a very broad TCR BV repertoire. Moreover, for all HCs analyzed, the profiles of the TCR BV usage from nnTregs were strikingly similar to those of their memory counterparts (data not shown). In contrast, nnTregs and memory Tregs from 2 of 3 analyzed RR-MS patients (RR-MS 1 and RR-MS 2) showed a more restricted BV gene

usage. Interestingly these patients had a very long disease duration (> 10 years), whereas the other patient (RR-MS 3) had a much shorter disease duration (2 years).

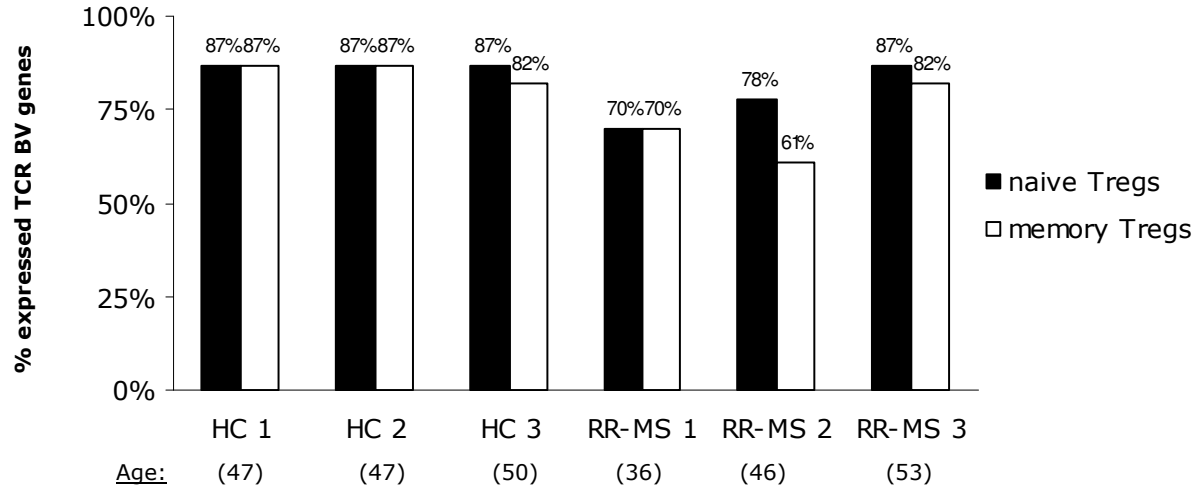


Figure 3.7: TCR BV gene expression in sorted nnTregs and memory Tregs. Expression of 23 different TCR BV genes was qualitatively determined in sorted nnTregs and memory Tregs of 3 and 3 RR-MS patients by means of RT-PCR. The relative number of BV genes that were expressed within each sample was calculated.

3.1.6 Conventional MS therapies affect Treg and T cell homeostasis in RR-MS patients

Commonly used therapies for MS, such as interferon-β (IFN-β) and Glatiramer Acetate (GA), have been shown to increase Treg numbers and function *in vitro* and *in vivo* [61,62]. From this point of view, we evaluated whether these therapies affect parameters of T cell homeostasis in MS patients. First, the frequencies of the nnTregs and memory Tregs were compared between untreated RR-MS patients (n=22) and RR-MS patients treated with GA (n=4) and IFN-β (n=10) (figure 3.8 (A)). In addition, the expression of CD31 was evaluated on the different Treg and $CD4^+$ T cell populations of the same individuals (figure 3.8 (B)).

Total Treg and memory Treg percentages were both significantly higher in GA treated and IFN-β treated RR-MS patients as compared to untreated patients ($p<0.0001$). The percentage of nnTregs was significantly higher in GA treated ($p<0.01$) but not in IFN-β treated patients. The percentage of CD31 expressing cells within the different $CD4^+$ T cell populations also differed between untreated and treated patients. The number of CD31 expressing memory Tregs was significantly higher in GA and IFN-β treated patients ($p<0.0001$). This observation was also made for memory $CD4^+CD25^-$ T cells ($p< 0.001$ and $p<0.05$ for respectively GA and IFN-β treated RR-MS patients). The number of of CD31 expressing nnTregs and naive $CD4^+CD25^-$ T cells was not altered in GA treated patients. In contrast, the number of CD31 expressing nnTregs was found to be significantly lower in IFN-β treated patients as compared to untreated patients ($p<0.05$).

The influence of therapies on parameters of Treg and T cell homeostasis was also evaluated in SP-MS patients. However, IFN-β and GA did not significantly alter nnTreg and memory Treg frequencies in these patients, nor did they affect the number of CD31 expressing cells (data not shown).

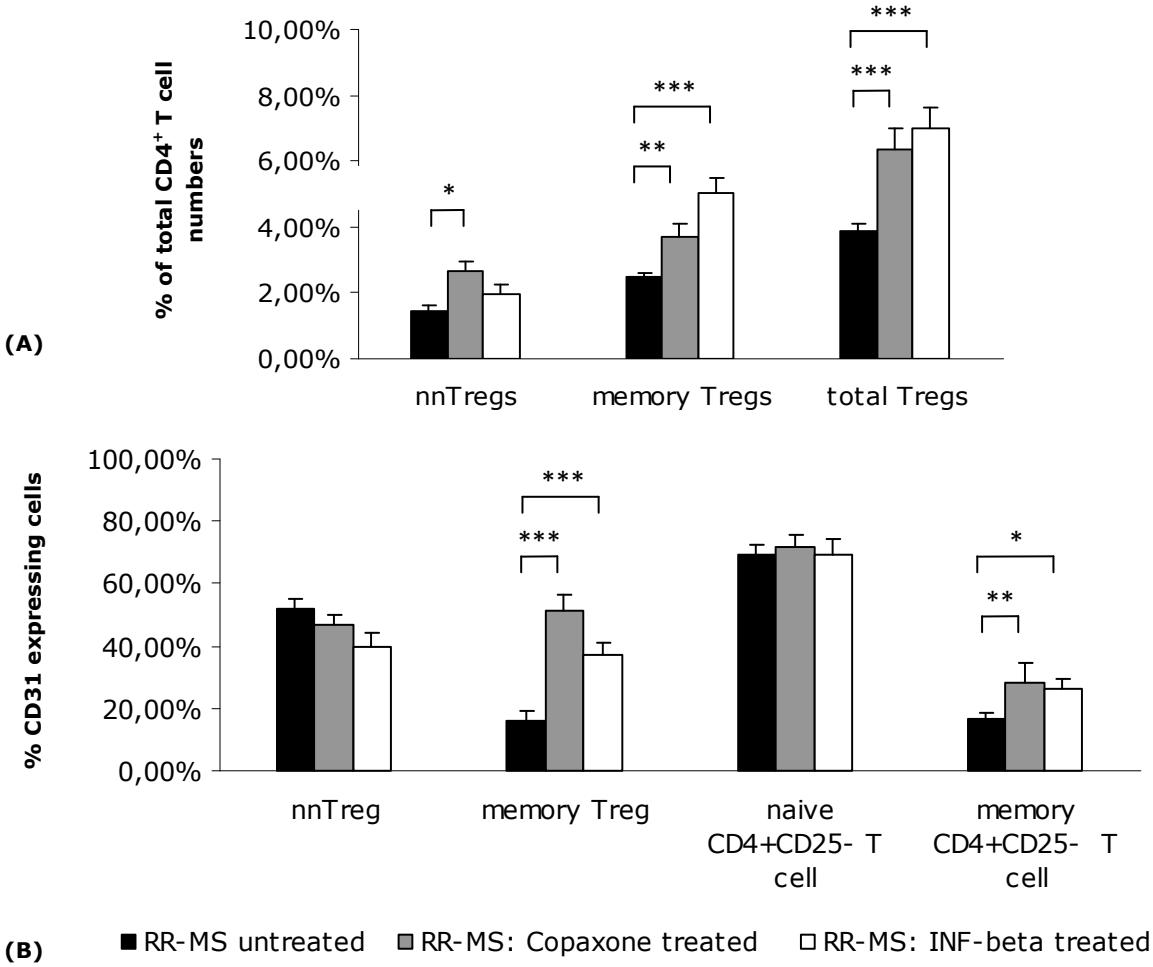


Figure 3.8: Conventional MS therapies affect Treg and T cell homeostasis in RR-MS patients. The frequency of nnTregs and memory Tregs and their expression of CD31 was evaluated by means of flow cytometry and compared between untreated RR-MS patients (n=22), and patients treated with GA (n=4) or IFN-β (n=10). (A) Average percentages (+SEM) of nnTregs, memory Tregs and total Tregs in untreated, IFN-β- and GA treated RR-MS patients. (B) Average percentages (+SEM) of CD31 expressing cells within the indicated T cell populations in untreated, IFN-β-and GA treated RR-MS patients. (* $p < 0.05$; ** $p < 0.001$; *** $p<0.0001$).

3.2 Evaluation of Treg functionality

3.2.1 The suppressive capacity of nnTregs and memory Tregs is reduced in RR-MS patients

As alterations were observed in the frequencies of naive and memory Tregs in RR-MS patients, the suppressive capacity of these Tregs subsets was evaluated. The suppressive capacity of nnTregs and memory Tregs was evaluated *in vitro* by performing co-culture experiments. nnTregs and memory Tregs were isolated by means of FACS-sorting. The

gating strategy which was used excluded T cells at an intermediate stage of maturation, which express both CD45RO and CD45RA. CD45RA positive cells were $CD28^+$, $CD27^+$ and $CD62L^{high}$ (data not shown). A fixed number of CFSE labeled $CD4^+CD25^-CD45RA^+$ T cells (responders) were co-cultured with varying amounts of FACS-sorted nnTregs and memory Tregs and anti-CD3 mAb was added as a polyclonal T cell stimulus. The proliferation of CFSE-labeled responders was evaluated by means of flow cytometry at day 5 after stimulation. Representative CFSE histograms of 1 HC are shown in figure 3.9.

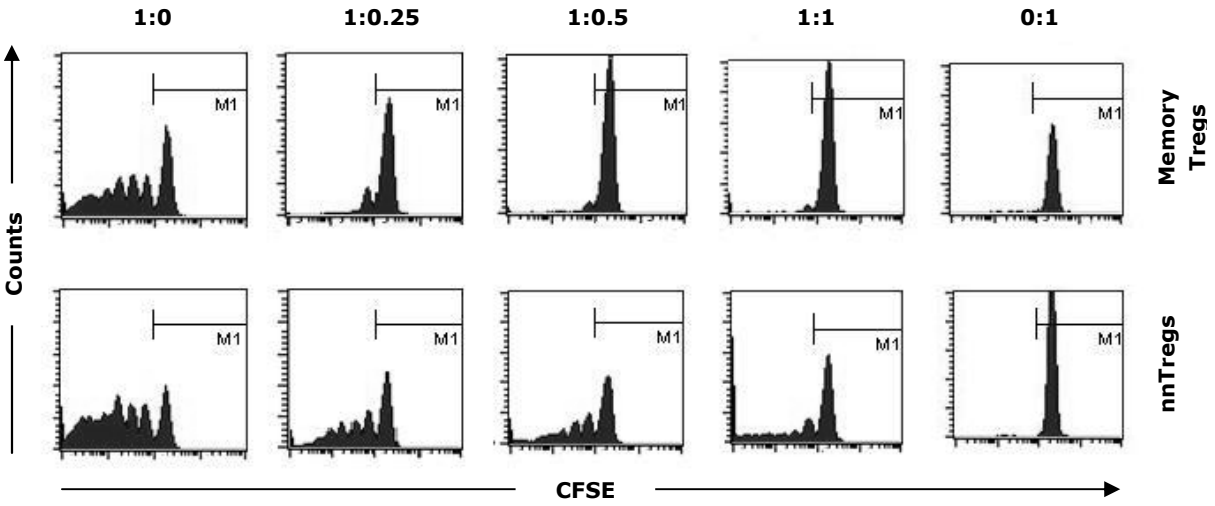


Figure 3.9: $CD4^+CD25^-$ T cell proliferation monitored using CFSE labeling. FACS-sorted nnTregs and memory Tregs were co-cultured with CFSE labeled $CD4^+CD25^-CD45RA^-$ responder T cells at the indicated responder/suppressor cell ratios. CFSE labeled nnTregs and memory Tregs were also cultured alone. Autologues irradiated PBMCs were added as feeders. All cultures were stimulated with anti-CD3 mAb. The proliferation of CFSE-labeled cells was evaluated by flow cytometry at day 5 after stimulation. The figure shows CFSE histograms of one representative HC. Cells under M1 represent non divided cells.

When responder cells were cultured, without a TCR stimulus, only a minor amount of cells proliferated (data not shown). However, when anti-CD3 mAb was added, a strong proliferation (as detected by CFSE dilution) of responders was observed. nnTregs and memory Tregs of HC were hyporesponsive towards TCR stimulation when cultured alone. A dose dependent Treg suppression of responder cells was observed for HC (n=5). Tregs of RR-MS patients (n=4) and SP-MS patients (n=2) showed the same anergic and suppressive phenotype. An overview of the mean suppressive capacity of both nnTregs and memory Tregs in HC, RR-MS patients and SP-MS patients is provided in figure 3.10.
For HC, the suppressive capacity of nnTregs (mean suppression: 59,98% at responder/suppressor ratio 1:1) was lower as compared to memory Tregs (mean suppression: 80,26%), but this difference was not significant. The mean suppressive capacity of RR-MS patients was significantly lower as compared to HC ($p<0.05$). Both SP-MS patients that were analyzed showed a significant reduced nnTreg suppressive capacity (mean suppression: 27.03%) as compared to HC ($p<0.05$). In contrast, the

suppressive capacity of memory Tregs (mean suppression: 86.89%) did not differ between SP-MS patients and HC. Interestingly, Tregs of 1 RR-MS patients showed the same functional features as tregs of SP-MS patients (e.g. the suppressive capacity of nnTregs was reduced, while memory Tregs showed a normal suppressive capacity; data not shown). The disease duration of this RR-MS patient (10 year) was much longer then the disease duration of the other RR-MS patients (1-2 years).

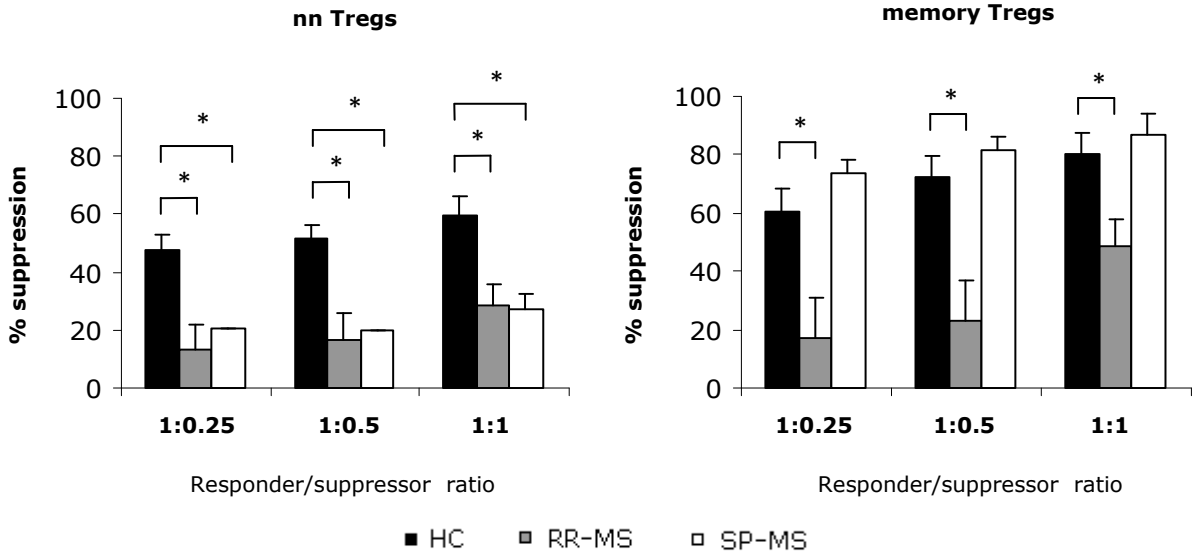


Figure 3.10: *In vitro* suppressive capacity of nnTregs and memory Tregs. Direct suppression assays were performed to evaluate the suppressive capacity of FACS-sorted nnTregs and memory Tregs. Proliferation of $CD4^+CD25^-$ responder T cells was determined using CFSE labeling (as described above). The suppressive capacity of Tregs was expressed as the relative inhibition of the percentage of $CFSE^{low}$ cells [100 x 1- (% $CFSE^{low}$ $CD4^+CD25$ T cells in coculture / % $CFSE^{low}$ $CD4^+CD25^-$ T cells alone)]. The bars represent the mean suppressive capacity (+SEM) of nnTregs and memory Tregs from HC (n=5), RR-MS patients (n=4) and SP-MS patients (n=2). (* $p < 0.05$).

3.2.2 In vitro Treg depletion results in higher myelin specific responses in HC

It is thought that myelin reactive T cells are the initial effector cells in the pathogenesis of MS. From this point of view, the suppressive capacity of the total Treg population towards these myelin specific T cell responses was evaluated. The frequency of myelin reactive T cells in peripheral blood is very low [6]. For this reason, a standard co-culture setup could not be performed, as this assay uses a limited number of responder cells. An alternative way to evaluate auto-antigen specific responses is an indirect CFSE based proliferation assay [54], which allows the analysis of a higher amount of cells. Treg suppression is determined indirectly by comparing the antigen reactivity of total $CD4^+$ T cells (including Tregs) and naive- and memory $CD4^+CD25^-$ T cells (depleted of $CD4^+CD25^{high}CD127^{low}$ Tregs) (figure 3.11 (A)). The mean percentage of total $CD4^+$ T

cells, naive- and memory T cells which proliferated in response to MBP, MOG and TT for HC is given in figure 3.11 (B).

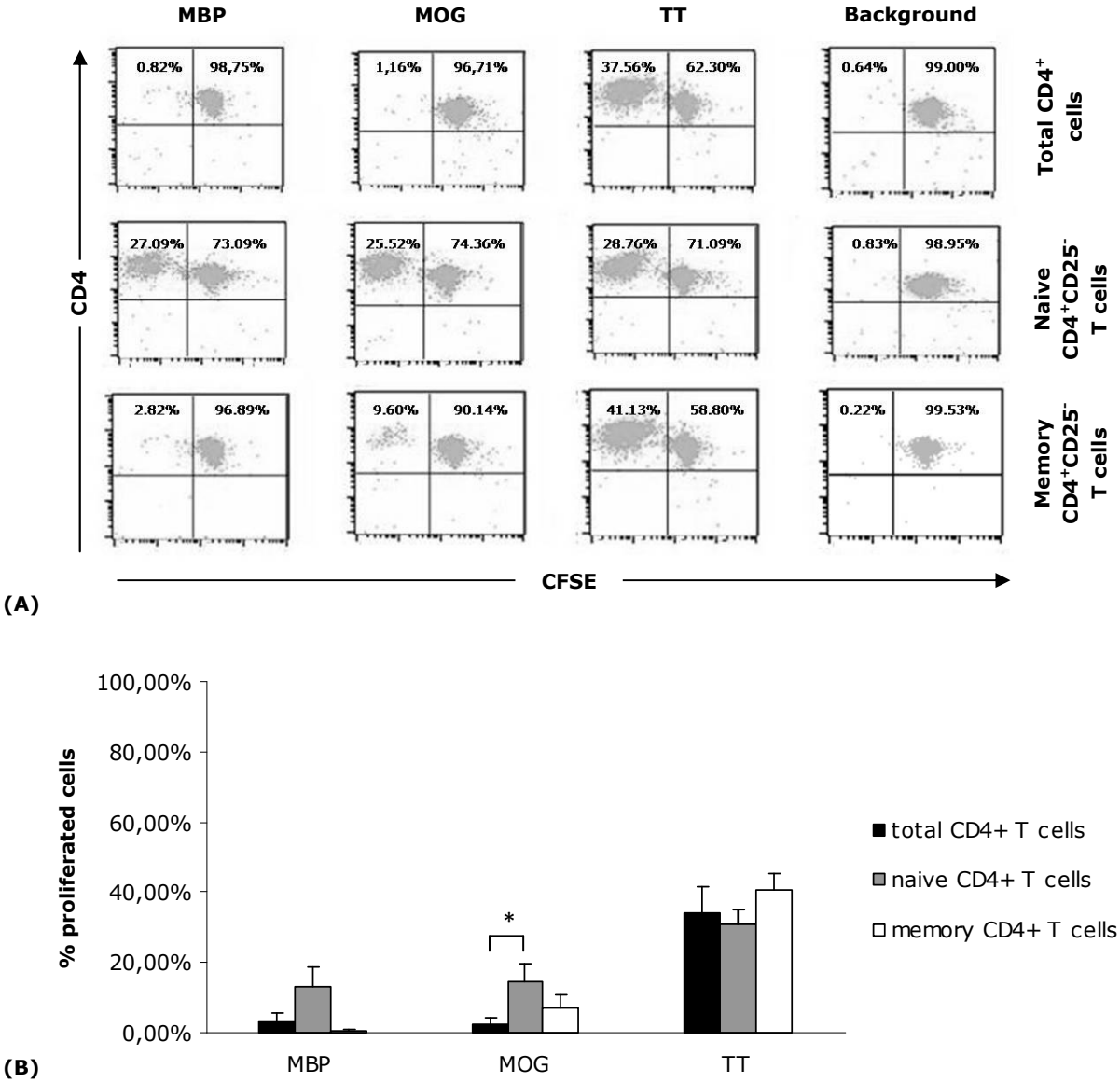


Figure 3.11: Antigen specific reactivity of total CD4$^+$ T cells (including Tregs) and naive- and memory CD4$^+$CD25 T cells (depleted of Tregs) in HC. Total CD4$^+$ T cells (including Tregs), naive- and memory T cell responders were labeled with CFSE and cultured in the presence of irradiated autologous PBMCs pulsed with MBP, MOG and TT or no antigen (background). At day 10 of culture, the CFSE fluorescence of viable cells (7-AAD negative) was evaluated. The proliferating fraction (ΔPF) was calculated by subtracting the mean background proliferation from the mean proliferating fraction in response to the antigen. **(A)** Representative CFSE fluorescence plots at day 10 of culture. **(B)** Overview of the proliferating fraction of total CD4$^+$ T cell (including Tregs) and naive and memory CD4$^+$CD25$^-$ T cells (depleted of Tregs). The bars represent the mean proliferating fraction (± SEM) of total CD4$^+$ T cells (black bars) and naive- (grey bars) and memory CD4$^+$CD25$^-$ T cells (white bars) depleted of Tregs from HC (n=8). (* $p < 0.05$)

Total CD4$^+$ T cells of HC respond towards MBP (mean ΔPF: 3.31 ± 5.08%) and MOG (2.45 ± 4.10%). CD4$^+$CD25^{high}CD127^{high}CD45RA$^-$ T cells (memory responder cells) also proliferated towards MBP (0.52% ± 1.62%) and MOG (6.90 ± 9.63%). In contrast,

higher antigen responses were demonstrated for $CD4^+CD25^{high}CD127^{high}CD45RA^+$ T cells (naive responder cells). The mean percentage of naive $CD4^+CD25^-$ T cells that proliferated in response to MOG was significantly higher (14.34 ± 5.45%; $p<0.05$) as compared to total $CD4^+$ T cells. MBP responses were higher (13.01 ± 5.83%), but this was not significant ($p=0.12$). Responses towards TT were higher (naive T cells: 30.64 ± 4.62%; memory T cells: 40.46 ± 4.92%) than myelin specific responses and did not differ significantly between total $CD4^+$, naive- and memory responders. Of note, memory $CD4^+CD25^-$ T cells respond stronger to TT than naive $CD4^+CD25^-$ T cells, although this difference was not significant ($p=0.09$). Attempts were also made to evaluate the myelin specific suppressive capacity of Tregs in MS patients. However, due to high background none of the responses showed a SI ≥ 3 (data not shown).

To further investigate why TT specific T cell responses were not inhibited in the total (non-Treg depleted) $CD4^+$ T cell fraction, we compared the suppressive capacity of unmanipulated memory Tregs with the suppressive capacity of memory Tregs which were pre-stimulated anti-CD3 mAb as a polyclonal T cell stimulus. Unmanipulated and pre-stimulated memory Tregs were added in increasing numbers to a fixed amount of memory $CD4^+CD25^-$ T cells and stimulated with TT pulsed irradiated PBMCs. Interestingly, both pre-stimulated and unmanipulated Tregs were able to suppress the proliferation of TT specific memory T cell responses at responder/suppressor ratios 1:0.25; 1:0.5 and 1/1. However, the suppressive capacity of Tregs progressively declined with lower suppressor/responder ratios (data not shown).

Table 3.1 Cloning efficiency and antigen reactivity of proliferated $CD4^+CD25^-$ T cells.

antigen	Cell density	# proliferated clones/ total # wells	# antigen positive clones/ # screened clones
MBP	1 cell/well	9/60 (15 %)	5/6 (83.33 %)
	3 cells/well	8/60 (13.33 %)	1/5 (20 %)
	5 cells/well	ND	ND
MOG	1 cell/well	ND	ND
	3 cells/well	9/60 (15 %)	1/4 (25 %)
	5 cells/well	ND	ND
TT	1 cell/well	3/60 (5 %)	ND
	3 cells/well	20/60 (33.33 %)	2/13 (15.38%)
	5 cells/well	16/60 (26.67 %)	0/13 (0 %)

MBP, MOG or TT reactive cells were collected and sorted into a 96-well round bottom plate at 1, 3 or 5 cells/well. These cells were stimulated with PHA and cultured in medium supplemented with IL-2 in the presence of irradiated allogenous PBMCs. After 3 weeks, antigen reactivity was assessed by means of split well assays. A clone was considered positive when the SI ≥ 3. Figures represent the percentage of wells showing proliferation and percentage of proliferating wells with actual antigen specific reactivity. MBP: myelin basic protein; MOG: myelin oligodendrocyt glycoprotein; TT: tetanus toxoid; ND: not determined.

We further tested whether $CFSE^{low}$ T cells were antigen specific by clonally expanding FACS sorted $CFSE^{low}$ cells (for each antigen). Subsequently, the antigen reactivity was tested in a split well assay. Table 3.1 provides an overview of the cloning efficiency and antigen reactivity of proliferated $CD4^+CD25^-$ T cells isolated from peripheral blood of 1 HC. The cloning efficiency (proportion of wells proliferating in respons to PHA and IL-2 stimulation) was correlated with the sorted cell density (table 3.1). The antigen specificity of the proliferating wells was evaluated by means of split well assays. The proliferating cells were divided into 4 wells. Two wells received unpulsed irradiated autologues PBMCs (no antigen). The other wells received PBMCs pulsed with MBP, MOG or TT. The proportion of clones showing a SI ≥ 3 towards MBP was 83.33% (1 cell/well) and 20 % (3 cells/well). For MOG, 25% of wells showed antigen reactivity. The proportion of wells showing TT reactivity ranged from 0% (5 cells/well) to 15.38% (3 cells/well).

3.3.3 Evaluation of the cytokine production of antigen reactive cells

To determine the T helper profile of the antigen reactive cells, a comparison was made between the number of IFN-γ expressing (Th1) and IL-4 expressing (Th2) cells. The results of this experiment are summarized in figure 3.12.

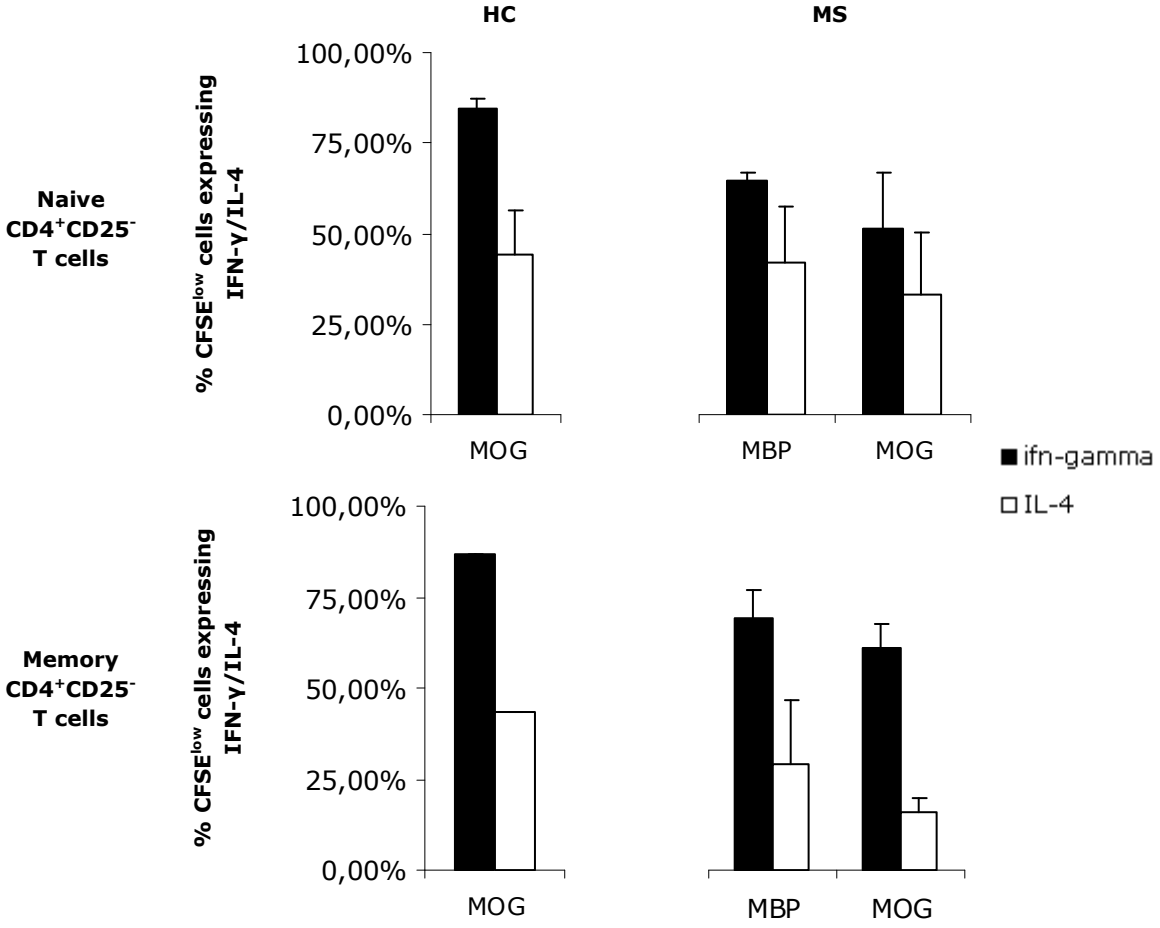


Figure 3.12: Evaluation of the T helper profile of antigen reactive T cells. Production of IFN-γ (Th1 phenotype) and IL-4 (Th2 phenotype) was measured of antigen reactive naive $CD4^+CD25^-$ T cells populations of HC (n=2) and MS patients (n=3). The cytokine production of memory $CD4^+CD25^-$ T cells was evaluated for 1 HC and MS patients (n=3). Cytokine production of viable cells was measured by means of intracellular flow cytometry using fluorescent labeled IFN-γ and IL-4 mAb. Bars represent the mean percentage (+SEM) of cytokine secreting cells within the proliferated cell fraction ($CFSE^{low}$).

The cytokine production was evaluated of MBP and MOG responding $CD4^+CD25^-CD45RA^+$ and $CD4^+CD25^-CD45RA^+$ T cells of HC (n=2) and MS patients (n=3) by means of intracellular flow cytometry. MOG reactive naive $CD4^+CD25^-$ T cells of HC (n=2) contained both IFN-γ and IL-4 producing cells, which is indicative for a Th0 phenotype. Memory $CD4^+CD25^-$ T cells of HC showed the same pattern of cytokine expression. Naive MBP and MOG reactive T cells of MS patients also showed a Th0 phenotype. In contrast, MBP and MOG reactive memory T cells of RR-MS patients seemed to contain less IL-4 producing cells relative to the amount of IFN-γ producing cells, indicating a Th1 phenotype.

The proliferated T cell fraction was also evaluated for the presence of Th17 cells (producing IL-17) [63]. The proportion of Th17 cells within the proliferated naive- and memory T cell fractions are given in Figure 3.13. For HC as well as for MS patients, almost no IL-17 expressing cells were detected within the proliferated naive T cell fraction. Higher numbers of Th17 cells were detected within the proliferated memory T cell fraction of HC and MS patients. Moreover MOG reactive memory $CD4^+CD25^-$ T cells of MS patients contained a higher percentage of IL-17 expressing cells as compared to HC, although this was not significant.

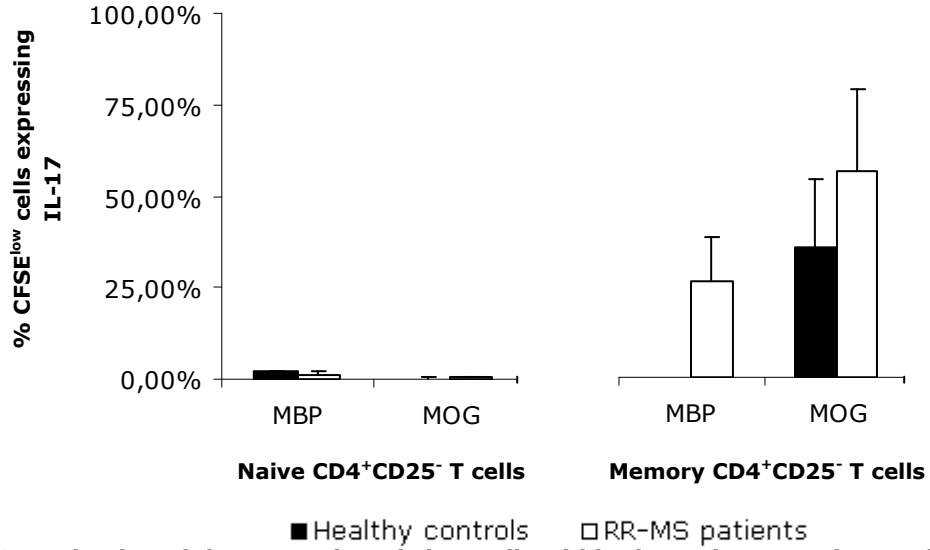


Figure 3.13: Evaluation of the proportion of Th17 cells within the antigen reactive T cell population. The proportion of Il-17 secreting cells was measured within antigen the reactive naive and memory $CD4^+CD25^-$ T cell populations ($CFSE^{low}$ population) of HC (n=2) and MS patients (n=3). Cytokine production of viable cells was measured by means of intracellular flow cytometry using fluorescent labeled anti-IL-17 mAb. The bars the mean percentage (+SEM) of IL-17 expressing cells within the proliferated T cell population.

4 Discussion

Myelin reactive T cells are thought to initiate the pathogenesis of MS. These autoreactive cells are present in the peripheral blood of MS patients as well as HC, but are in a more activated state in MS patients [7]. It is hypothesized that disturbances in immunological tolerance mechanisms are causative for the increased myelin specific T cell activity in MS patients. Accumulating evidence indicates that Tregs play a dominant role in the suppression of autoreactive T cells. In MS, however, the suppressive capacity of Tregs is reduced [51,52,53]. The functional characteristics and the dynamics of Tregs in HC and MS patients were investigated in this study.

Recently, studies reported the existence of a population of nnTregs in peripheral blood of healthy individuals, next to memory Tregs [23,24]. As to this moment, studies of nnTregs in autoimmune diseases have not yet been performed. It is known that a low expression of CD127 is associated with a high $FoxP3^+$ expression and suppressive capacity of Tregs [41]. Therefore, we have used this marker to quantify and isolate functional nnTregs and memory Tregs in HC and MS patients. The gating strategy which was used excluded T cells at an intermediate stage of maturation, which express both CD45RO and CD45RA (data not shown). Moreover, CD45RA positive cells were also positive for CD28 (=co-stimulatory molecule), CD27 (=TNF-receptor family member) but positive for CD62L (L-selectin), indicating that 'CD45RA revertants' were also excluded (data not shown). CD45RA revertant cells are antigen experienced cells which revert from expression of CD45RO to CD45RA as they undergo terminal differentiation to effector cells and are characterized by a low expression of CD28, CD27 and CD62L [64]. In addition, FACS-sorted nnTregs and memory Tregs from both HC and MS patients were anergic after TCR stimulation with anti-CD3 mAb, indicating that effector $CD4^+$ cells (which also have a high CD25 expression) were excluded. This allows proper comparison of nnTregs and memory Tregs between HC and MS patients.

The frequencies of nnTregs and memory Tregs were compared between HC, untreated RR-MS patients and SP-MS patients. Of note, the SP-MS patient group included both treated and untreated patients, as our results (and previous observations) indicated that therapies had no effect on the evaluated parameters in these patients. Total $CD4^+CD25^{high}CD127^{low}$ Treg numbers were significantly reduced in RR-MS patients as compared to HC. This is in agreement with earlier studies which reported that the frequency of FOXP3 expressing cells and the FOXP3 expression per cell is reduced in RR-MS patients [51,55]. Both the frequencies of nnTregs and memory Tregs were found to be decreased in RR-MS, whereas the frequency of these Tregs in SP-MS patients was

comparable with that of HC. The finding that nnTreg numbers decrease progressively with age in both HC and RR-MS patients indicates that thymic involution is mainly influencing the size of the nnTreg population in peripheral blood. The frequency of memory Tregs does not decline with age, which shows that peripheral processes, like homeostatic proliferation, mainly influence the persistence of memory Tregs in the periphery.

An important issue is the underlying cause of the reduced Treg numbers observed in RR-MS patients. In healthy individuals, T cell numbers are tightly regulated, such that the size of the peripheral T cell pool is constant throughout life. The mechanisms that maintain homeostasis within the T cell compartment are multifaceted and involve thymic production, homeostatic proliferation and cell survival and apoptosis signals [29]. Over the past years, evidence emerged for a disturbed T cell homeostasis in MS-patients, suggesting that premature immunosenesence occurs in these patients [30,31,58,65]. This indicates that the aging of the immune system occurs much faster in patients with MS as compared to healthy individuals. For example, frequencies of TRECs and CD31 expressing $CD4^+$ and $CD8^+$ T cells are markedly reduced in RR-MS patients as compared to age matched healthy individuals [30,31]. Second, patients with RR-MS show a skewed TCR BV repertoire as compared to healthy individuals [66]. Whether an altered homeostasis also affects the Treg population was investigated in this study.

A central aspect of the aging of the immune system is thymic involution, during which the epithelial space of the thymus (where T-cell maturation and selection take place) is replaced by fatty tissue. This restructuring process causes a decline in the output of new T cells from the thymus [30,58]. TREC numbers declined progressively with age in nnTregs and memory Tregs of HC, indicating an age-dependent decrease in thymic output. TREC numbers in memory Tregs were much lower than in nnTregs, which can be explained by their high *in vivo* turnover rate [15,28]. nnTregs and memory Tregs of RR-MS patients had a reduced TREC content as compared to HC of the same age group, suggesting that the homeostasis of these cells is disturbed in RR-MS patients. Interindividual differences and the fact that only a low number of patients and HC were analyzed may explain why these differences were not significant. The lower TREC numbers observed in RR-MS patients, can be explained by a reduced thymic output of nnTregs. However, increased homeostatic proliferation, a 'space-driven' autoproliferative mechanism which restores peripheral T cell numbers under conditions of T cell loss [29], may also account for this.

To further analyze this, the expression of CD31 on these cells was determined. CD31 is a differentiation antigen, which is expressed on $CD4^+$ T cells that are newly generated by the thymus. CD31 is no longer expressed on naive $CD4^+$ T cells that have undergone

homeostatic proliferation [57]. CD31 has the advantage that it's expression can be determined at the single cell level whereas TRECs are measured on a population level. The finding that CD31 expression decreases on nnTregs (and naive $CD4^+CD25^-$ T cells) with increasing age in HC and MS patients indicates that thymic production mainly influences these naive T cell populations. The mean percentage of CD31 expressing nnTregs and (naive $CD4^+CD25^-$ T cells) did not differ significantly between HC and RR-MS-patients. These data, together with the lower TREC numbers observed in nnTregs, indicate that the thymic output of nnTregs is reduced in RR-MS patients.

The mean percentage of CD31 expressing memory Tregs (and memory T cells) was significantly lower in RR-MS patients as compared to HC. Age has no influence on the frequency of CD31 expressing memory Tregs, which indicates that peripheral processes, like increased homeostatic proliferation account for the low TREC numbers and percentage of CD31 expressing memory $CD4^+$ T cells observed in RR-MS. The narrowed BV gene usage in memory Tregs of RR-MS patients supports this hypothesis.

Human MHC class II molecules play a role in the positive selection of $CD4^+$ T cells in the thymus and are required for the peripheral survival of naive $CD4^+$ T cells. Self-MHC recognition also appears to be essential for homeostatic proliferation of naive T cells [29]. From this point of view, we evaluated whether the nnTreg pool was influenced by the expression of HLA-DR2, as the expression of this haplotype is associated with an increased susceptibility for MS [1]. HLA-DR2 positive RR-MS patients have a significant lower percentage of CD31 expressing nnTregs (and naive $CD4^+CD25^-$ T cells). These data indicate that peripheral processes next to a reduced thymic output may also alter nnTreg and homeostasis in a subset of RR-MS patients. Increased homeostatic proliferation of nnTregs may explain the lower percentage of CD31 expressing cells in HLA-DR2 positive RR-MS patients. These findings corroborate with an earlier study, performed by Duszczyszyn et al., which focused on naive $CD4^+CD25^-$ T cells [31]. No association was found between HLA-DR2 expression and CD31 expression on memory Tregs and memory $CD4^+CD25^-$ T cells. The difference between memory and naive T cell subsets can be explained by the fact that both populations are maintained independently in the periphery. Memory and naive $CD4^+$ T cells require different signals for their persistence. Unlike naive T cells, memory T cells are less dependent on interactions with selfpeptide/ MHC complexes for survival or homeostatic proliferation. Moreover, both T cell populations require different cytokines for their persistence. For example, survival and proliferation of naive T cell mainly requires IL-7, whereas memory T cells mainly depend on IL-15 [29]. It remains unclear, however, as to why HLA-DR2 expression would influence peripheral nnTreg and naive $CD4^+CD25^-$ T cell homeostasis in RR-MS patients, but not in healthy individuals and SP-MS patients.

It seems contradicting that the number of memory Tregs is reduced if an increased homeostatic proliferation of memory Tregs occurs. Two possible mechanisms could explain this issue. First, a large part of the proliferated memory Tregs may have undergone apoptosis. Indeed, the tendency towards replecative senescence in memory Tregs together with their high susceptibility to apoptosis would explain why these cells do not accumulate *in vivo* [28]. However, Fritzing et al. recently showed that the sensitivity of (total) Tregs towards CD95 ligand-mediated cell death and spontaneous apoptosis was similar in RR-MS patients as compared to HC *in vitro* [68]. Although an increased occurrence of antigen induced cell death (AICD), associated with the high turnover rates of these cells, can not be excluded. Alternatively, the decreased number of memory Tregs in peripheral blood of RR-MS patients may result from an increased migration of these cells into the CNS, the site were the pathological inflammation takes place. This assumption is supported by a recent study performed by Feger et al. (and our unpublished data), which show that frequency of $CD4^+CD25^{high}CD45RO^+$ memory Tregs is significantly elevated in the CSF of MS patients in comparison to peripheral blood. Interestingly, nearly all T cells in the CSF show a memory phenotype [56]. In addition, evaluation of the expression of adhesion molecules showed a significantly higher percentage of $CD103^+$ and VLA-4^+ cells in the blood circulating Treg population in RR-MS patients, which can reflect an increased migration capacity of Tregs towards inflammatory lesions in the CNS [55].

Surprisingly, SP-MS patients showed a normal frequency of both nnTregs and memory Tregs. This seems contradictory in view of our hypothesis that the thymic production of these cells is reduced at the onset of the disease, as thymic function does not restore with aging. It is thought that the progressive phase of MS is associated with a decrease of peripheral T cell dependent inflammation, which are substituted by neurodegenerative features [1]. Possibly, the balance between Tregs/Teffectors may be restored in patients with SP-MS. Alternatively, increased homeostatic proliferation of nnTregs in SP-MS patients may also explain this finding. The reduced amount of CD31 expressing nnTregs in SP-MS patients as compared to HC and RR-MS patients (although not significant) supports this.

The low CD31 expression on memory $CD4^+CD25^-$ T cells may also have some functional consequences. CD31 signaling in T cells was proposed to be a candidate negative regulatory pathway that increases the threshold for T-cell activation, and prevents stimulation by low-strength TCR signals [69]. The low percentage of CD31 expressing memory $CD4^+CD25^-$ T cells may possibly explain the increased frequency of activated myelin reactive T cells in RR-MS [2,7]. Whether CD31 also affect Treg function is not known. It would be interesting to further study the relation between homeostatic proliferation, CD31 expression and function of Tregs and $CD4^+$ T cells in MS.

Next to homeostatic parameters, the suppressive capacity of the Tregs subsets was also evaluated. Functional analysis revealed that the suppressive capacity of both nnTregs and memory Tregs was reduced in RR-MS patients as compared to HC. SP-MS patients showed a defective suppressive capacity of nnTregs, but a normal memory Treg function. Interestingly, 1 RR-MS patient, which had a very long disease duration as compared to the other RR-MS patients, showed the same pattern of Treg suppression as the SP-MS patient with long disease duration. These findings are supported by previous research, which showed that Tregs of RR-MS patients had a lower FOXP3 expression per cell [55]. As SP-MS patients have a relapsing-remitting disease course at onset of disease and memory Treg activity appears to increase with disease duration, it seems that the memory Treg function is restored during the progression of the disease.

A correlation between disease duration and the Treg suppressive function has been reported earlier [51]. Interestingly, the frequency of memory Tregs increases with the disease duration of RR-MS patients. No such correlation was observed for nnTregs and disease duration. It seems that only memory Tregs are responsible for the restoration of Treg function in SP-MS patients. It is possible that these memory Tregs derive directly out of the thymus as $CD4^+CD25^+FOXP3^+$ T cells are present in the thymus as a $CD45R0^+$ population. However, as for conventional T cells, Tregs probably leave the thymus as a naive $CD45RA^+/RO^-$ population, as the majority of Tregs in human cord blood are $CD45RA^+$ [24]. This does not exclude the possibility that some of the $CD45RO^+$ Tregs escape this final maturation step. It is however unlikely that the increase in memory Tregs is caused by an increased production of these thymic premature $CD45RO^+$ Tregs, as the thymus progressively involutes with age. An alternative and more favorable possibility is that Tregs are induced from $CD4^+CD25^-$ T cells in the periphery (induced Treg cells). Studies show that Tregs can indeed be generated from $CD4^+CD25^-$ T cells under certain conditions. Cytokines such as TGF-β and IFN-γ have been shown to induce the conversion of human naive $CD4^+CD25^-$ T cells into memory $CD4^+CD25^-$ Tregs by induction of FOXP3 *in vitro* [70]. In addition, under certain circumstances, Tregs can also be induced from effector/memory $CD4^+CD25^-$ T cells in an antigen dependent manner. For example, induction of anergy in activated $CD4^+CD25^-$ T cells by providing a TCR signal in the absence of co-stimulation induces the development of regulatory T cells *in vitro* [71]. Of note, certain environmental factors may be linked with the induction of Tregs in the periphery. For example, infection with viruses, such as cytomegalovirus (CMV), that induce large scale differentiation of human memory T cells would be expected to also induce the conversion of $CD4^+CD25^-$ T cells into memory Tregs [28]. Of note, in SP-MS patients, the restored Treg function may also be a consequence of the restored balance between pro- and anti-inflammatory cytokines, which was observed in

these patients [1]. Pro-inflammatory cytokines such as IL-2 or TNF-α have been shown to abrogate Treg suppression *in vitro* [42].

It would be interesting to study whether the increasing frequency and functionality of memory Tregs with disease duration in RR-MS patients results from induced Tregs. However, it is difficult to discriminate between induced and natural Tregs, as their phenotype and function is very similar. One possibility to evaluate the presence of induced Tregs in MS patients is to compare the TCR BV repertoire of memory Tregs and memory $CD4^+CD25^-$ T cells in MS patients and HC. The increased presence of induced Tregs theoretically results in an increased similarity of the TCR BV repertoires of memory Tregs and memory $CD4^+CD25^-$ T cells [28]. Our data already show that memory Tregs of RR-MS patients show an altered BV gene usage as compared to HC.

In summary, these results provide indications that the premature aging of the immune system also affects the Treg population in RR-MS patients. Genetic factors, such as HLA-DR2, but possibly also environmental factors (like CMV infection), are associated with a disturbed homeostasis of Tregs. Memory Treg numbers increase with disease duration of RR-MS patients. Most probably, these Tregs are induced from $CD4^+CD25^-$ T cells in the periphery. The increase in memory Tregs seems to be associated with a restoration of the suppressive capacity of the memory Treg population towards polyclonal T cell responses. It remains to be determined whether these induced Tregs efficiently suppress myelin specific T cell responses as a disproportionate representation of Tregs specific for antigens, other than myelin antigens (like virus antigens) could still lead to a decreased protection towards autoinflammatory T cell responses. Based on data from literature and own observations, we have constructed a model for the disturbed Treg homeostasis in MS patients (figure 4.1).

To evaluate the suppressive potential of the total Treg population towards myelin antigen specific responses, an indirect CFSE based proliferation assay was performed. Treg suppression was measured indirectly by comparing the proliferation of total $CD4^+$ T cells and $CD4^+$ T cells depleted of Tregs [54]. Our results provide evidence that a defective Treg function can play a role in the peripheral activation of myelin reactive T cells. In healthy individuals, $CD4^+$ T cell responses against MBP and MOG were increased in Treg depleted $CD4^+$ T cells as compared to total $CD4^+$ T cells. This suggests that myelin specific Tregs occur within the $CD4^+$ T cell population and that they are activated by their cognate antigens during culture and suppress the myelin reactive $CD4^+CD25^-$ T cells. Our results show that the increased myelin reactivity mainly results from the naive T cell population, as naive $CD4^+$ T cell responses towards MBP and MOG were higher as compared to total $CD4^+$ T cells after depletion Tregs.

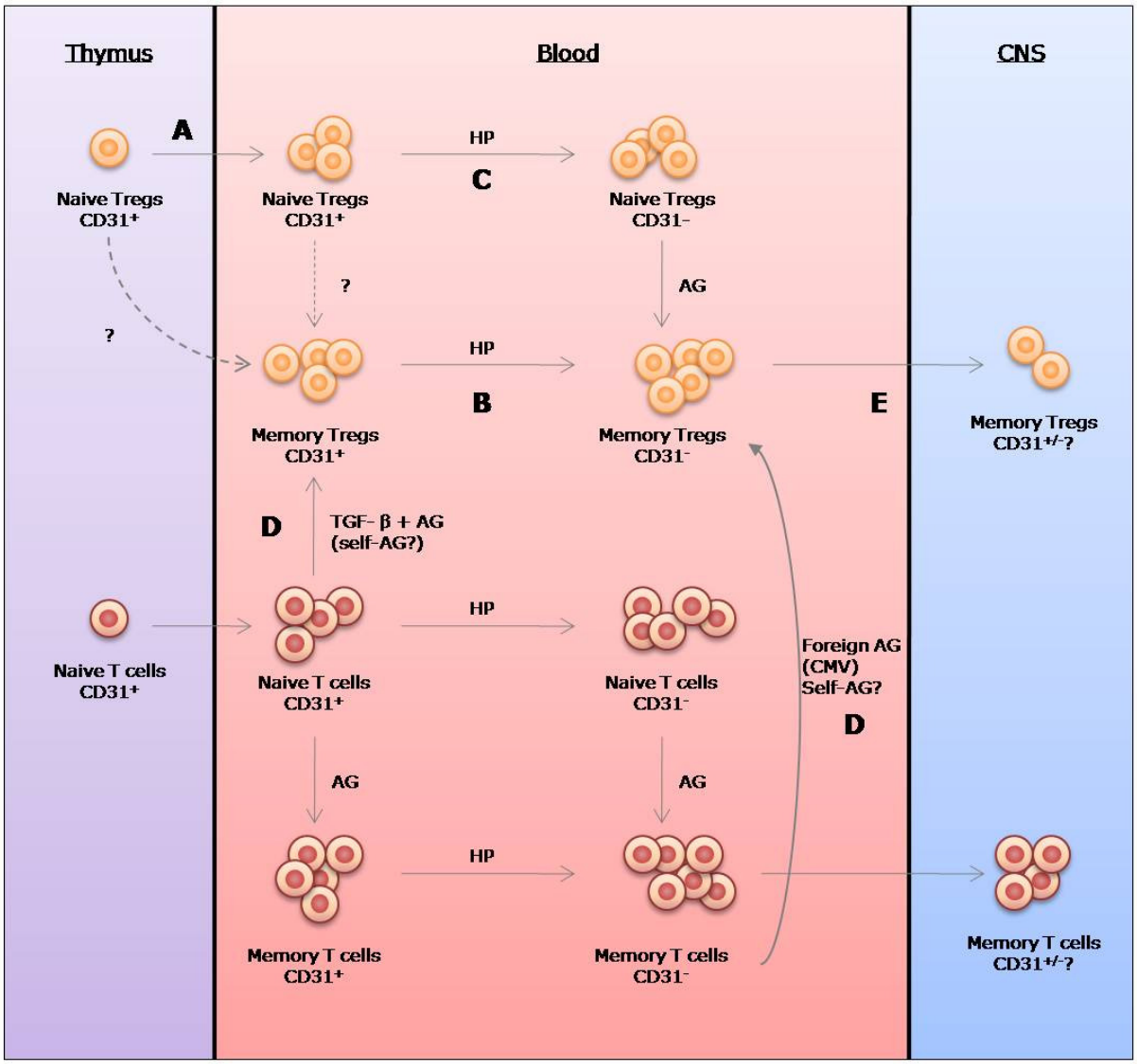


Figure 4.1: Model for the disturbed Treg homeostasis in MS patients. (A) Thymic output of nnTregs (and other T cells) is reduced in patients with RR-MS. **(B)** The reduced frequency of Tregs (and other T cells) leads to increased homeostatic proliferation of memory Tregs. **(C)** Increased homeostatic proliferation of nnTregs occurs in genetically susceptible RR-MS patients (HLA-DR2) and possibly also in SP-MS patients. **(D)** In addition, Tregs may also be converted from naive and antigen experienced $CD4^+CD25^-$ T cells. Environmental factors such as chronic viral infections (e.g. cytomegalovirus [CMV]) may affect this process. **(E)** Increased migration of memory Tregs into the central nervous system (CNS) may explain why memory Tregs do not accumulate in peripheral blood of RR-MS patients. AG= antigen; HP= homeostatic proliferation.

Myelin specific memory $CD4^+$ T cell responses were however not enhanced after Treg depletion. A possible explanation for this could be that myelin-reactive memory T cells circulate in lower numbers in the blood and have a lower functional avidity towards myelin antigens than naive T cells [72]. Our data and of others, do not exclude however that in HC, memory $CD4^+$ T cells reacting against myelin antigens exist. Despite these remarks, we can conclude that myelin reactive $CD4^+$ T cells are kept under control by antigen specific regulatory T cells in healthy individuals. Moreover, our observations

suggest that both the initial activation of naive T cells as well as the effector T cells are suppressed.

TT specific T cell responses were not inhibited in the total (non-Treg depleted) $CD4^+$ T cell population. Tregs have a self-specific TCR repertoire and require TCR stimulation to become suppressive [15]. Therefore, a possible explanation for this observation is that Tregs are not TT reactive. The suppressive capacity towards TT responses was compared between unstimulated Tregs and Tregs which were pre-stimulated with anti-CD3. Interestingly, both non-stimulated and pre-stimulated Tregs could effectively suppress memory T cell responses induced by TT. Possibly, the Treg/responder ratios, which were much higher than in physiological circumstances, may account for this. Indeed, the Treg suppressive capacity progressively declined at lower Treg/responder ratios. There are many contradictory reports regarding the suppressive potential of Tregs towards foreign antigens. However, our results indicate that TT reactivity is indeed suppressed by Tregs.

In our indirect CFSE based assay, antigen reactivity of T cells was measured by means of proliferation of T cells towards antigens. Proliferation of T cell does however not prove antigen specificity of these cells. Proliferation can also result from non-specific processes such as bystander activation (due to production of high levels of cytokines, e.g. IL-2). To verify the antigen specificity of our assay, a cloning experiment cells was performed to evaluate the antigen specificity of proliferated cells. T cells which proliferated in respons to MBP and MOG were specific for the stimulating antigen. Surprisingly, the majority of TT reactive clones were not antigen specific. One explanation can be that the memory/recall respons, which is induced by TT, results in bystander activation. On the other hand, a high background was measured in the split well assays, which possibly influenced the result. Further screening is needed to clarify this issue. Nevertheless, we can conclude that the CFSE-based assay is an accurate method to evaluate low prevalent antigen specific responses, such as myelin antigen specific responses. This is supported by other reports [73]. In future experiments, this assay will be used to determine the Treg suppressive capacity towards myelin reactive T cell responses in MS patients to further explore the Treg dysfunction towards myelin antigens in these patients. Due to a technical problem, this could not be evaluated in this study.

Our CFSE based approach allowed us to investigate the cytokine profile of antigen reactive ($CFSE^{low}$) T cells. We analyzed the expression of IFN-γ and IL-4, as these cytokines are representative for respectively Th1 and Th2 phenotypes. Myelin antigen (MBP and MOG) reactive $CD4^+CD25^-$ T cells of HC and naive ($CD4^+CD25^-$) T cells isolated from MS patients showed a Th0 phenotype. In contrast, the cytokine profile of MBP and MOG reactive memory ($CD4^+CD25^-$) T cells from MS patients indicated a more pronounced Th1 response. The percentage of Th17 cells within antigen reactive population was also determined. IL-17 is a pro-inflammatory cytokine and can play an

important role in the development of autoimmunity. This notion is supported by studies in EAE mice, which indicate that the Th17, rather than Th1 cells are responsible for EAE induction [63]. We observed almost no Th17 cells within the naive $CD4^+CD25^-$ T cell population of HC as well as RR-MS patients. This was consistent with previous observations that IL-17 is produced exclusively by activated memory T cells [63]. Interestingly, the proportion of IL-17 expressing MOG reactive memory T cells in our study was higher in MS-patients as compared to HC, indicating that these cells may play a role in the pathogenesis of MS. This finding is supported by a study performed by Matusevicius et al, which showed that the expression of IL-17 mRNA in PBMCs of MS patients was higher as compared to healthy individuals. Interestingly, MS patients during clinical exacerbation showed even higher numbers of IL-17 expressing PBMCs as compared to patients in remission [74]. Further experiments are needed to confirm these observations.

In summary, Tregs of HC are able to suppress $CD4^+$ T cell responses induced by myelin antigens MBP and MOG. Depletion of Tregs *in vitro* results in higher MBP and MOG activity of myelin specific $CD4^+CD25^-$ responder T cells. Therefore, the reduced Treg suppressive capacity which is observed in patients with RR-MS may indeed play a role in the peripheral activation of myelin antigen reactive T cells, resulting in a higher amount of potentially pathogenic Th1 and Th17 cells.

From a therapeutic point of view, it would be challenging to restore Treg dysfunction in RR-MS patients. Therefore, the influence of current therapies for the treatment of MS, namely GA and IFN-β, on T cell homeostasis was evaluated. Although both therapies have been approved for treatment, their underlying mechanisms of action are not fully clarified. Of note, differences between untreated and treated MS patients was not performed in age-matched groups. However, the same results were obtained when age-matched groups were compared (data not shown). Both GA and IFN-β were able to modulate Tregs in RR-MS patients. The percentages of nnTregs and memory Tregs which are lower in untreated RR-MS patients as compared to HC and SP-MS patients seem to be restored in GA and IFN-β treated patients. Also the frequency of CD31 expressing memory Tregs was restored in IFN-β and GA treated RR-MS patients. These observations indicate that GA and IFN-β influence Treg homeostasis. This is supported by other studies which investigated the effect of GA and IFN-β on Treg function. GA was shown to induce the conversion of peripheral $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ regulatory T cell trough the activation of FoxP3 *in vitro* and *in vivo*. Moreover, the $CD4^+CD25^+$ T cells generated by COP-1 show *in vitro* regulatory potential [61]. Another study reported that $CD4^+CD25^{high}$ Treg numbers significantly increased during IFN-β therapy in RR-MS patients [62]. Of note, the increase in memory Tregs observed in patients treated with

GA or IFN-β can also result from the effects of these therapies on the migration of memory Tregs through the blood brain barrier (BBB). INF-β and GA modulate BBB transmigration of T cells by their effect on expression of adhesion molecules on T cells [75]. Therefore, the increased percentage of memory Tregs in peripheral blood can explained by the fact that they can not longer enter the CNS. Further research is needed to clarify the underlying mechanism by which GA and IFN-β affect the homeostasis of Tregs.

In conclusion, the reduced Treg suppressive capacity which is observed in patients with RR-MS may play a role in the peripheral activation of myelin reactive T cells. Our results show for the first time that the homeostasis of Tregs is disturbed. These cells are also affected by the premature aging of immune system in MS patients. Genetic factors, such as HLA-DR2, are associated with an accelerated aging of the immune system. nnTreg and memory Treg frequencies in RR-MS patients were reduced and showed a reduced *in vitro* function in the early disease phase. However, during the course of the disease, the suppressive capacity of memory Tregs restores, most probably by the induction of Tregs in the periphery. Further research of the function of induced Tregs and the mechanisms by which they are generated would be interesting from a therapeutic point of view, as this process may be exploited to restore the Treg dysfunction in the early disease phase.

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