$CD4^+$ $CD25^+$ T regulatory cells in multiple sclerosis

Doctor of Philosophy (Ph.D) thesis

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The front and back cover illustrates Mohandas Karamchand ‘Mahatma’ Gandhi (1869-1948) memorial in Delhi, India, showing the various groups, which supported the salt protest. Mahatma Gandhi, was the most influential freedom fighter, for his strong principles of peace and non-violence; the inspiration for Nobel Laureate Martin Luther King Jr's fight for racial equality, and Burmese dissident and Nobel Laureate Aung San Suu Kyi's fight for democracy in Burma. Gandhi*ji*, was one such great personality, for whom, the world missed an opportunity to award the Nobel Peace prize.

Relevance of the cover picture: Requirement of a good balance between animal protection and the necessity of medical research

To my lovely family,

and Internet

ABSTRACT

Multiple sclerosis (MS) is a chronic, inflammatory, and demyelinating disease of the central nervous system (CNS), which is likely to be mediated by autoaggressive immunity. Treatments of MS that may beneficially influence the disease course consist of Avonex (interferon-β; IFN-β), and Copaxone (glatiramer acetate; GA). Studies in experimental autoimmune encephalomyelitis (EAE), a commonly used experimental model of human MS, have demonstrated a crucial role of $CD4^+CD25^+$ T regulatory (Tr) cells in suppressing autoreactive T cells and promoting peripheral tolerance. Recent studies have shown that Tr cells inhibiting the proliferation and cytokine secretion by $CD4^+$ T cells, are enriched in the $CD25^{high}$ cells among $CD4^+$ T cells. It is not yet known if circulating $CD4^+CD25^{high}$ Tr cells are involved in MS.

The aims of this study were to:

1. identify and quantify $CD4^+CD25^+$ and $CD4^+CD25^{high}$ Tr cells among blood $CD4^+$ T cells, and proportions of $CD4^+CD25^{high}$ Tr cells expressing cell surface and intracellular molecules in patients with untreated MS *vs* MS treated with either IFN-□-1a or GA or the combination of IFN-□-1a+GA, *vs* healthy controls (HC);
2. to correlate the properties of circulating $CD4^+CD25^+$ Tr cells with lesion load (LL) and gadolinium (Gd) enhancement detected by magnetic resonance imaging (MRI) of the brain in untreated MS patients.
3. analyse the *in-vitro* influence of GA upon $CD4^+CD25^{high}$ Tr cells from MS patients *vs* from HC.
4. examine whether a functional deficit inherent to $CD4^+CD25^+$ Tr cells occurred in untreated MS patients vs HC, in terms of the ability of Tr cells to suppress the production of pro-inflammatory cytokines by blood mononuclear cells (MNC) upon auto- or foreign-antigenic stimulation.

These studies demonstrated that *i)* there are no alterations of frequency of circulating $CD4^+CD25^+$ and $CD4^+CD25^{high}$ Tr cells nor of the proportions of $CD4^+CD25^{high}$ Tr cells expressing CD45RO, HLA-DR, CD95, CTLA-4 or IL-10 in MS patients compared to HC; *ii)* the frequency of $CD4^+CD25^+$ Tr cells, and the proportions of IL-10 expressing $CD4^+CD25^+$ Tr cells are elevated in MS patients with Gd-enhancing lesions; *iii)* the proportion of $CXCR3^+$ $CD4^+$ T cells is lower in the group of patients with Gd-enhancing lesions on T1-weighted images, as compared to the group with no Gd-enhancing lesions; *iv)* GA, *in vitro* elevates the levels of IL-10 producing blood $CD4^+CD25^{high}$ Tr cells in both MS patients and HC, and; *v)* $CD4^+CD25^+$ Tr cells augments the secretion of Th1 cytokines IFN-γ and IL-2, but not of Th2 cytokine IL-13 in response to MBP, equally in MS patients and HC, suggesting that the functional activity of $CD4^+CD25^+$ Tr cells in terms of controlling Th1 and Th2 cytokine secretions in response to MBP, is not significantly altered in MS.

CONTENTS

ABBREVIATIONS

APC	Antigen-presenting cells
BBB	Blood-brain barrier
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CNS	Central nervous system
CSF	Cerebralspinal fluid
CXCR	CXC-chemokine receptor
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded disability status scale
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
GA	Glatiramer acetate
Gd	Gadolinium
HC	Healthy controls
HLA	Human leukocyte antigen
IDDM	Insulin-dependent diabetes mellitus
IFN	Interferon
IL	Interleukin
IP-10	Interferon-γ-inducible protein of 10 kDA
LL	Lesion load
LPS	Lipopolysaccharide
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MNC	Mononuclear cells
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NK	Natural killer cells
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
RA	Rheumatoid arthritis
RR	Relapsing remitting
PP	Primary progressive
PR	Progressive relapsing
TCR	T cell receptor
TGF	Tumour growth factor
Th	T helper
TLL	Total lesion load
Tr	T regulatory
TNF	Tumour necrotic factor

PREFACE

This thesis is based on the following articles and manuscript referred to by Roman numbers:

I. **Putheti P,** Pettersson A, Soderstrom M, Link H and Huang YM. Circulating $CD4^+CD25^+$ T regulatory cells are not altered in multiple sclerosis and unaffected by immunotherapy. *J Clin immunol* 2004;24:155-161

II. **Putheti P,** Morris M, Stawiarz L, Teleshova N, Kivisakk P, Pashenkov M, Kouwenhoven M, Wiberg MK, Bronge L, Huang YM, Soderstrom M, Hillert J, Link H. Multiple Sclerosis: A study of chemokine receptors and regulatory T cells in relation to MRI variables. *Eur J Neurol* 2003;10:529-35.

III. **Putheti P,** Soderstrom M, Link H, Huang YM. Effect of glatiramer acetate (Copaxone) on $CD4^+CD25^{high}$ T regulatory cells and their IL-10 production in multiple sclerosis. *J Neuroimmunol* 2003;144:125-31.

IV. **Putheti P,** Pashenkov M, Huang YM, Soderstrom M, Link H. Multiple sclerosis: No evidence of functional deficit of $CD4^+CD25^+$ T regulatory cells. ***Manuscript.***

1 INTRODUCTION

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is characterised by multiple, relatively firm (sclerotic) plaques in the brain and spinal cord tissue, hence the name of the disease. Charcot, Carswell, Cruveilhier, and others illustrated the clinical and pathological characteristics of MS (Compston et al., 1998), 100 years ago; however, in-spite of considerable amount of valuable research, the cause of MS remained uncertain. Evidences suggest MS as possibly an autoimmune disease. When the cause for another autoimmune disease like Myasthenia gravis, had been discovered (Fambrough et al., 1973; Lindstrom et al., 1976), it should have been possible to unveil the cause for MS also, if the cause is equally straightforward; hence hinting the involvement of multiple factors in MS. On the other hand, our limited knowledge about the brain might be self limiting, in our attempts to discover the cause for MS. Researchers have reported various factors, that could be involved in causing MS, for instance genetic, viral and environmental factors. But, none of the factors alone could be correlated as 'the cause' for the vast number of MS cases tested, at any given time.

Medical research has suggested MS as a chronic, demyelinating disease of the central nervous system (CNS) (Raine 1994), occurring more frequently at higher latitudes than in places closer to the equator, in both northern and southern hemispheres. The prevalence of MS is 1-2 per 1000 in Northern Europe (Ebers and Sadovnick, 1998), striking between the ages of 20 and 40. Symptoms of MS include impairment of muscle strength and coordination, impairment of -vision, -sensation, and of -cognitive processes. Each patient is affected differently by the disorder, and the physical and emotional progression of MS is unpredictable.

MS has been sub-grouped into four major categories depending upon disease progression, namely relapsing-remitting (RR) MS, primary-progressive (PP) MS, secondary-progressive (SP) MS and finally, progressive-relapsing (PR) MS.

Despite some controversies, the most accepted view of MS is that this is probably an autoimmune disease, in which the immune system mounts an abnormal response against self-

antigens such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP), or myelin proteolipid protein in the CNS. In MS patients, activated autoreactive T cells enter the CNS and initiate autoimmune inflammation that eventually leads to destruction of myelin and axons (Couraud, 1998).

1.1.1 Treatments for multiple sclerosis

After being considered as an untreatable disease for decades, two classes of disease modulatory drugs, the interferons (IFN)-β (Avonex™, Betaseron®), Rebif® and glatiramer acetate (GA; Copaxone®), were approved for the treatment of relapsing MS. Through double-blind and placebo-controlled clinical trials, these drugs have been shown to reduce the relapse rate and to slow the progression of disability in MS (The IFNB Multiple Sclerosis Study Group, 1993; Jacobs and Multiple Sclerosis Collaborative Research Group (MSCRG), 1995; Johnson et al., 1995; PRISMS Study Group, 1998; Panitch & the EVIDENCE Study Group, 2002).

Interferon-beta

IFN-β is a type I interferon, that is highly species specific. In humans, the IFN-β polypeptide is produced and secreted by fibroblasts, but virtually all mammalian cells can produce IFN-β upon stimulation. IFN-β is a cytokine that suppresses T-cell proliferation, reduces IFN-γ-induced upregulation of MHC class II expression, reduces synthesis of T helper 1 (Th1) cytokines, induces the production of Th2 cytokines, and inhibits monocyte activation (Neuhaus et al., 2003). Two recombinant IFN-β preparations, IFN-β1a and IFN-β1b are currently available as treatments for MS. The trade names for IFN-β1b are Betaseron and Betaferon, and for IFN- β1a the names are Avonex and Rebif.

Glatiramer acetate

GA is a synthetic mixture of 4 amino acid copolymers: **L**-alanine, **L**-lysine, **L**-glutamic acid, and **L**-tyrosine in a molar ratio of 4.2, 3.4, 1.1, and 1.0. Although the peptide has varying amino acid sequence, the composition and the molar ratios of the amino acids are fixed. The copolymers range from 40 to 90 amino acids in length and the molecular weight ranges between 4.7 and 10 kDa.

GA suppresses the onset of acute experimental autoimmune encephalomyelitis (EAE), the experimental animal model for MS, and has significant therapeutic effect in chronic relapsing EAE. Vaccination of animals with GA, results in its uptake by dendritic cells (DC) in the skin and lymphoid tissue. GA prevents the activation of autoreactive T-cells, or may drive those cells into a state of anergy. More likely, the drug induces regulatory Th2 and $CD8^+$ T-cells that suppress autoimmune responses systemically and in the CNS, as suggested by EAE studies (bystander suppression). Additional neuroprotective and repair mechanisms may ensue as a result of accumulation of GA-reactive T-cells in the CNS (Dhib-Jalbut, 2003).

1.1.2 Th1, Th2 and Th3 cytokines

It is widely accepted that Th1-mediated responses are involved in the pathogenesis of MS (Beck et al., 1988; Lu et al., 1993; Rieckmann et al., 1995). This is based on the association of MS with genes involved with the immune system, the immunopathology of the disease, the clinical response of MS patients to immunomodulatory and immunosuppressive treatments, and similarities with experimental immune-mediated demyelinating diseases in animals, such as EAE. Nevertheless, the Th1/Th2 paradigm has been useful for understanding the mechanism of immunomodulatory therapies for MS (Rudick et al., 1998; Duda et al., 2000).

While the counter-regulatory roles of Th-1 and Th-2 cytokines are well-established in murine systems, this dichotomy is more ambiguous in humans (Sinigaglia et al., 1999). As per the literature to date, helper T-cells (Th cells) have been designated as Th1, Th2, and Th3. Th precursor (Thp) cells are supposed to be the precursor cells of Th0 cells, which then develop into either Th1 or Th2 cells (Figure 1). Th1 cells stimulate strong cellular immunity but only weak and transient antibody responses. These cells produce a number of cytokines known as Th1 cytokines or Type-1 cytokines and include interleukin (IL)-2, IFN-γ, IL-12, tumour necrosis factor (TNF)-α and β.

Th2 cells evoke especially strong antibody responses, but relatively weak cellular activity. Th2 responses are usually elicited by free-living bacteria and other parasites. Th2 cells produce cytokines that are known as Th2 cytokines or Type-2 cytokines and include IL-4, IL-5, IL-6, IL-10, and IL-13.

Th3 cells are $CD4^+$ regulatory cells associated with immune mechanisms involving oral tolerance towards antigens. These cells are characterised, among other things, by the secretion of tumour growth factor (TGF)-β and they have suppressive properties for Th1 and Th2 cells

(Weiner et al., 2001). Th1 and Th2 cells can influence and regulate each other by the cytokines they secrete. For example, IFN-γ, secreted by Th1 cells, can inhibit the proliferation of Th2 cells IL10; secreted by Th2 cells, can suppress Th1 functions by inhibiting cytokine production.

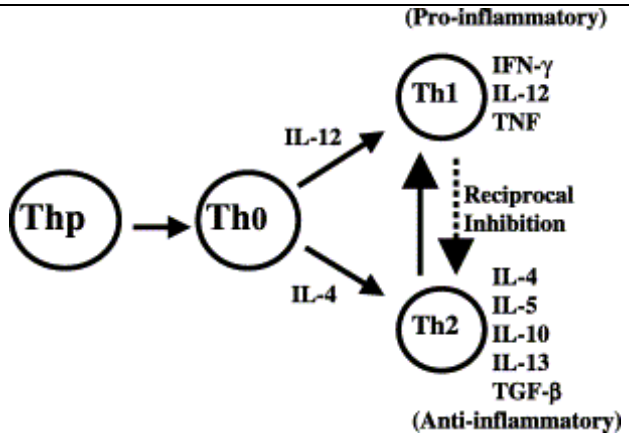

Fig. 1. Schematic representation of T helper (Th) 1 and Th2 cell generation from Th precursor (Thp) cells

$CD4^+CD25^+$ T cells, which will be dealt in detail within this section, have characteristics of Th2 cells in terms of cytokine profiles, along with other unique characteristics. In recent years, several studies have investigated the influence of $CD4^+CD25^+$ T cells on Th1 and Th2 cell responses. In Paper-IV, we have examined the influence of $CD4^+CD25^+$ T cells on Th1 and Th2 cell responses in MS patients *vs* in healthy controls (HC).

On the whole, research in MS has illustrated various chemokines, pro-inflammatory cytokines, and (auto)antigens such as MBP, MOG, MAG, and PLP etc, as potential mediators in the pathogenesis of MS during the progression of lesions.

1.2 $CD4^+CD25^+$ T regulatory cells: General aspects

The induction of specific tolerance is critical for the maintenance of the immune homeostasis and for the prevention of abnormal autoimmunity. There are several mechanisms by which the immune system distinguishes self and non-self peptide antigens or innocuous and harmful foreign antigens. In the thymus, central tolerance is a well-known mechanism, which involves deletion of self-reactive T cells (Kisielow et al., 1988). However, as there is evidence for the presence of autoreactive T cells in healthy subjects without causing any

autoimmune disease, it is speculated that there exists a peripheral control of T cells also for the prevention of autoreactivity, and for the regulation and termination of adverse immune responses. Hence, it is clear that there are certain autoreactive T cells specific for the self-antigens, which escape the deletion mechanism in the thymus. However, in the periphery, interactions between the T-cell receptor (TCR) and MHC-peptide complexes may elicit five distinct functional outcomes: no response (ignorance), productive T cell activation, activation-induced cell death (peripheral deletion), the induction of unresponsiveness, so called anergy (Jenkins et al., 1987; Gett et al., 2003) to subsequent antigen, and active suppression by $CD4^+CD25^+$ T regulatory (Tr) cells. In recent years, increasing attention has been devoted to $CD4^+CD25^+$ Tr cells, due to their unique ability to prevent autoimmune pathologies (Shevach, 2000), intestinal inflammation (Groux et al., 1999), and allograft rejection (Waldmann et al., 2001). Exploring the biology of $CD4^+CD25^+$ Tr cells and the mechanisms that control their differentiation may be of key importance for the development of new therapeutic strategies in MS.

1.2.1 $CD4^+CD25^+$ T regulatory cells in animals

1.2.1.1 In autoimmunity

$CD4^+$ T cells expressing the CD25 molecule, *i.e.* the IL-2 receptor α-chain, constitute 5-10 % of peripheral $CD4^+$ T cells and less than 1 % of $CD8^+$ T cells in normal naïve mice (Sakaguchi et al., 1995). Transfer of BALB/c splenic cell suspensions depleted of $CD4^+CD25^+$ Tr cells, produce histologically and serologically evident autoimmune diseases such as thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis, polyarthritis, and insulitis/insulin-dependent diabetes mellitus (IDDM). Similarly, in rat models, deletion of $CD4^+CD25^+$ Tr cells, produce IDDM. Reconstitution of $CD4^+CD25^+$ Tr cells with $CD4^+CD25^-$ cells prevents these autoimmune developments (Fowell et al., 1993; Sakaguchi et al., 1995). This spontaneous development of autoimmune disease following deletion of $CD4^+CD25^+$ Tr cells can hardly be attributed to T lymphocytopenia and consequent susceptibility of the host to microbial infection, since the deletion reduces the number of T cells only by 5-10 %.

$CD4^+CD25^+$ Tr cells confer significant protection from the development of MOG(35-55)-induced EAE, possibly by promotion of protective Th2 responses and decreased homing of autoreactive cells to the CNS (Kohm et al., 2003). Studies in rodents have suggested that $CD4^+CD25^+$ Tr cells are smaller in size (Peng et al., 2004) and are generated in the thymus,

possibly as a consequence of escape from negative selection (Itoh et al., 1999; Jordan et al., 2001). Their peripheral development and functional importance in controlling immune responses to foreign antigens is suggested by their ability to suppress inflammatory bowel disease and allergy (Thorstenson et al., 2001; Szanya et al., 2002; McHugh et al., 2001; Maloy and Powrie 2001). The molecular basis for the development and function of CD4$^+$CD25$^+$ Tr cells remains unclear. Recently, the forkhead transcription factor (FoxP3) has been implicated in the development and function of CD4$^+$CD25$^+$ Tr cells. In both mice and man, intracellular FoxP3 has been shown to be expressed specifically in CD4$^+$CD25$^+$ Tr cells, and not in any other T cell subsets (Hori et al., 2003; Khattri et al., 2003). Furthermore, the ectopic expression of Foxp3 in CD4$^+$CD25$^-$ T cells, is sufficient for such cells to perform a suppressive function towards T cell responses, suggesting that Foxp3 is at least one of the transcription factor critical for the development of CD4$^+$CD25$^+$ Tr cells (Hori et al., 2003; Khattri et al., 2003).

Functional characteristics of CD4$^+$CD25$^+$ Tr cells

CD4$^+$CD25$^+$ Tr cells in normal naïve mice exhibit *in vitro* suppressive activity against autoreactive T cell functions (Itoh et al., 1999; Kuniyasu et al., 2000). CD4$^+$CD25$^+$ Tr cells perform suppressive activity *in vitro* by antigenic stimulation in absence of co-stimulation through CD28 ligation or a high concentration of IL-2. To perform suppressive function, CD4$^+$CD25$^+$ Tr cells require stimulation via TCR, and antigen presentation by immature DCs *in vivo* can lead to the activation of CD4$^+$CD25$^+$ Tr cells (Bell, 2003)**.** Hence, DC and CD4$^+$CD25$^+$ Tr cell interaction may be crucial for the CD4$^+$CD25$^+$ Tr cell in performing suppressive activity. It has been reported that, CD4$^+$CD25$^+$ Tr cells engaged in controlling self-reactive T cells in the normal internal environment, may recognize self-antigens and can be stimulated by them (Takahashi et al., 1998).

CD4$^+$CD25$^+$ Tr cells are induced *in vivo* and they are *antigen nonspecific* in function (Bell, 2003). Thus, once the CD4$^+$CD25$^+$ Tr cells are stimulated via an antigen-specific (TCR-mediated) mechanism, the suppression they mediate is completely antigen non-specific; which means that they suppress the proliferation of antigen-specific T cells as well as, the proliferation of the T cells, that are specific for other antigens (Thornton et al., 2000). Histocompatibilty between CD4$^+$CD25$^+$ Tr cells and effector T cells, is not required for

effective suppression. $CD4^+CD25^+$ Tr cells can also suppress the activation and proliferation of $CD8^+$ T cells (Itoh et al., 1999).

$CD4^+CD25^+$ Tr cells are *highly differentiated* in their function and exert their immediate specific function at any moment upon the exposure to stimulating antigens. This was illustrated by the finding that $CD4^+CD25^+$ Tr cells freshly isolated from naïve mice or TCR-trangenic mice mediate suppression from the first day of *in vitro* TCR stimulation (Takahashi et al., 1998).

In mice, IL-10 and membrane-bound TGF-β (Powrie et al., 1996; Asseman et al., 1999) are reported to play a critical role in suppressing auto-reactive T cell functions *in vivo,* against intestinal inflammation and colitis. The pathway of these cytokine-mediated suppressive actions is assumed to be via various ways including de-activating antigen-presenting cells (APC) (Fiorentino et al., 1991), direct inhibition of pathological T cell responses (Gorelik et al., 2000) and by cell-cell contact (Nakamura et al., 2001). In rats, $CD4^+CD25^+$ Tr cells expressing $CD45RC^{low}$ have been shown to mediate protective regulatory activity against IDDM (Stephens et al., 2000).

Although $CD4^+CD25^+$ Tr cells require antigenic stimulation for their functional activation, they themselves are *non-proliferative* (*i.e.* anergic) to *in vitro* antigenic stimulation, and this anergic state is closely linked with suppression (Thornton et al., 1998; 2000). $CD4^+CD25^+$ Tr cells fail to proliferate as they undergo cell cycle arrest at the G0/G1 stage (Thornton et al., 2000).

1.2.2 Human $CD4^+CD25^+$ T regulatory cells

1.2.2.1 In autoimmunity

Regulatory T cells or suppressor cells were first described in the early 1970s (Gershon et al., 1970). However, the failure to characterize these cells and to identify the mechanism by which these cells exert their suppressive activity, led to the demise of the field in the mid 1980s (Moller, 1988). Recently, $CD4^+$ Tr cells were demonstrated to constitutively express CD25 (Sakaguchi et al., 2000). In humans, such $CD25^+$ cells represent 10%–15% of the total number of $CD4^+$ T cells (Levings et al., 2001; Jonuleit et al., 2001; Dieckmann et al., 2001). Human $CD4^+CD25^+$ Tr cells are anergic, and suppress proliferation of conventional-naïve and -memory $CD4^+$ T cells (Levings et al, 2001). The failure to understand the mechanism by

which these cells exert their suppressive activity was in part due to the fact that human peripheral blood $CD4^+CD25^+$ T cells represent a heterogeneous population containing not only regulatory T cells, but also activated effector T cells (Baecher-Allan et al., 2001). Recently, it was reported that, human Tr cells predominantly express intracellular FoxP3. Unlike their rodent counterparts, human $CD4^+CD25^+$ T cells generated as a consequence of *in vitro* stimulation of $CD4^+CD25^-$ T cells, express FoxP3 and acquire Tr function. Recent studies have also shown, that $CD4^+CD25^{high}$ Tr cells, expressing *high* surface IL-2 receptor α chain, totally inhibit proliferation and cytokine secretion by $CD4^+$ T cells. These cells differ from $CD4^+CD25^{low}$ T cells in their higher expression levels of surface CD45RO and HLA-DR (Baecher-Allan et al, 2001).

From the current information, it can be derived that:

All $CD4^+$ Tr cells are $CD25^+$,
But, not all $CD25^+$ T cells are $CD4^+$ Tr cells.

Expression of surface and intracellular molecules by $CD4^+CD25^+$ Tr cells

$CD4^+CD25^+$ Tr cells are smaller in size and constitutively express intracellular CTLA-4 (Dieckmann et al, 2001; Annunziato et al, 2002; Peng et al., 2004), which negatively regulates T cell activation. $CD4^+CD25^+$ Tr cells express CD45RO and HLA-DR (Baecher-Allan et al., 2001; Dieckmann et al., 2001; Levings et al., 2001; Ng et al., 2001), but these molecules are also present on the surface of effector T cells. CD62L has also been found to be expressed on $CD4^+CD25^+$ Tr cells (Ng et al., 2001). Recently it has been reported that $CD4^+CD25^+$ Tr cells express the chemokine receptors CCR4 and CCR8 and are chemo-attracted by the respective ligands, CCL17/TARC, CCL22/MDC, and CCL1/I-309 (Iellem *et al*. 2001).

Suppressive function of $CD4^+CD25^+$ Tr cells

$CD4^+CD25^+$ T cells have been reported to be anergic, and that they suppress the proliferation of conventional naïve and memory $CD4^+$ T cells (Levings et al, 2001). $CD25^+$ T cell depletion study (Wing et al., 2003) suggested that $CD25^+$ cells consisting of ~80% of $CD4^+CD25^+$ Tr cells, do not control the responses of cord blood cells to self-protein like MOG, but among adult healthy individuals they suppress the peripheral blood autoreactive T cell proliferation and IFN-γ production. Hence it was suggested that $CD4^+CD25^+$ Tr cells might be immature in the cord blood. However, the mechanism for suppressive function performed by $CD4^+CD25^+$ Tr cells is unclear. Several studies agree that the suppressive

activity of $CD4^+CD25^+$ Tr cells require cell-to-cell contact and is cytokine independent (Dieckmann et al., 2001; Jonuleit et al., 2001; Ng et al., 2001). A possible role for CTLA-4 and PDL1 in T cell–T cell regulation has recently been suggested (Baecher-Allan et al., 2001). Apart from direct suppressor effect on effector $CD4^+$ T cells, $CD4^+CD25^+$ Tr cells may also perform similar function in a DC mediated pathway (Misra et al., 2004).

Characterization of human $CD4^+CD25^+$ thymocytes

$CD4^+CD25^+$ thymocytes produce IL-10 and express surface -CTLA-4, -tumor necrosis factor type 2 receptor (TNFR2), -CCR8, and -TGF-β1. $CD4^+CD25^+$ thymocytes represent a population of regulatory cells that migrate in response to the chemokine CCL1/I-309 and exert their suppressive function via the inhibition of IL-2R alpha-chain (CD25) in target T cells, induced by the combined activity of CTLA-4 and membrane TGF-beta1. These cells have mainly been observed in the context of the fibrous septa of the thymus with prevalent perivascular localization (Annunziato et al., 2002).

Role of the thymus in production of $CD4^+CD25^+$ Tr cells and maintenance of immunological self-tolerance

The available experimental work suggests that immature $CD4^+$ T cells, activated by their respective autoantigen in the thymus, may become Tr cells, provided that they have recognized the autoantigen with avidity, that is sufficient for activation, but not for deletion (Shevach, 2001). Wing et al (2003) suggested a pathway of $CD4^+CD25^+$ Tr cell activation from their observations that autoantigen-specific $CD4^+CD25^+$ Tr cells are selected in the thymus by the self-antigens that are presented on thymic epithelial cells. These autoantigen-specific $CD4^+CD25^+$ Tr cells leave the thymus in a naive state and are then re-stimulated (and possibly expanded) in the periphery by self-antigen-bearing DC in the draining lymph nodes. Upon this restimulation they convert to the memory phenotype and perform their suppressive function.

Evidences that $CD4^+CD25^+$ Tr cells develop in the thymus do not rule out the possibility for their generation in periphery. It is unlikely, that the thymus can replenish the peripheral pool of $CD4^+CD25^+$ Tr cells throughout the life span in humans. With thymic involution, it is not possible for $CD4^+CD25^+$ Tr cells to maintain their stable frequency in periphery; hence there need to be some ongoing mechanism of peripheral maintenance. At least in rats, there is

evidence supporting the peripheral generation of CD4$^+$CD25$^+$ Tr cells. It was shown, that TGF-β plays an important role in the generation of FoxP3-expressing CD4$^+$CD25$^+$ Tr cells *in vivo* (Peng et al., 2004).

Migration of CD4$^+$CD25$^+$ Tr cells to sites of inflammation

The recognition of organ-specific antigens by CD4$^+$CD25$^+$ Tr cells may be the most important factor that would require specific signals to direct CD4$^+$CD25$^+$ Tr cells to sites of inflammation. Chemokines are the logical candidates to direct the recruitment of suppressor T cells. Only one study (Iellem *et al*. 2001), so far, has shown that CD4$^+$CD25$^+$ T cells express the chemokine receptors CCR4 and CCR8. One difficulty with the interpretation of this study is, that CCR4 and CCR8 are expressed also by activated CD4$^+$CD25$^-$ T cells.

1.2.2.2 *In vitro* generation and expansion of CD4$^+$CD25$^+$ Tr cells

Despite the high expression of IL-2 receptor (IL-2R), human CD4$^+$CD25$^+$ Tr cells are non-proliferative *in vitro*. It has recently been reported that IL-2R stimulation results in G1 cell cycle arrest, as well as an increase in cellular size and survival (Bensinger et al., 2004). However, human CD4$^+$CD25$^+$ Tr cells can be generated *in vitro* (Yamagiwa et al., 2001), and rodent CD4$^+$CD25$^+$ Tr cells *in vivo* (Peng et al., 2004), by culturing relative naïve peripheral blood lymphocytes in the presence of TGF-β. However, it is unknown if these cells can regulate responses to alloantigens, similar to autoantigens. Apart from TGF-β, 1α,25-dihydroxyvitamin D3 and dexamethasone in combination, can also lead to the *in vitro* generation of IL-10-producing CD4$^+$CD25$^+$ Tr cells (Barrat et al., 2002). IL-10 has been shown to result in the clonal expansion of CD4$^+$CD25$^+$ Tr cells without any loss of function. Though initial studies have shown that anti-CD3 and IL-2 based culture can also cause expansion of the functional murine CD4$^+$CD25$^+$ Tr cells in numbers (Takahashi et al., 1998; Thornton et al., 2000), later studies demonstrated that multiple re-stimulation of murine CD4$^+$CD25$^+$ Tr cells can lead to loss of function (Chai et al., 2002). However, recently, *in vitro* stimulation of CD4$^+$CD25$^+$ Tr cells with anti-CD3 and anti-CD28 in presence of IL-2, have been shown to result in marked expansion in cell number, with enhanced suppressive function (Godfrey et al., 2004).

Whether these strategies lead to the generation of the same population of $CD4^+CD25^+$ Tr cells, and whether such *in vitro*-generated $CD4^+CD25^+$ Tr cells are identical to those that develop *in vivo*, is not entirely clear at the moment.

1.2.2.3 Therapeutic benefits from manipulating $CD4^+CD25^+$ Tr cell function

Induction and expansion of $CD4^+CD25^+$ Tr cells *in vivo* and *in vitro* could have important implications not only in the field of autoimmunity, but also in cancer immunity and transplantation tolerance (Waldmann et al., 2001). As elimination of $CD4^+$ $CD25^+$ Tr cells in mice helps in destruction of tumors, it will be interesting to investigate whether or not these cells also prevent the generation of effective anti-tumour immune responses in humans affected with cancer. Human $CD4^+CD25^+$ Tr cells are being examined to check, if they can be used as a surrogate marker for tolerance in transplantation, and as cellular therapeutic agent in the clinic. $CD4^+CD25^+$ Tr cells specific for donor-alloantigen may control aggressive $CD4^+$ and $CD8^+$ T cells, preventing rejection and inducing hyporesponsiveness (Wood et al., 2003).

Since $CD4^+CD25^+$ Tr cells can exert bystander suppression in an antigen-non-specific manner; their recognition of one local tissue-expressed autoantigen would be sufficient to inhibit local autoimmune pathology. Identification of the sites of action, mechanism of action of $CD4^+CD25^+$ Tr cells, and mechanisms of their antigen reactivities will be of help in designing new treatment strategies for various inflammatory and autoimmune diseases. Strategies altering $CD4^+CD25^+$ Tr cell proportions may have important clinical benefits in the induction of protective immunity. Depletion of $CD4^+CD25^+$ Tr cells can induce immune action against tumours and result in tumour protection (Shimizu et al., 1999). However, such approaches need to be verified keenly, since long-term depletion of $CD4^+CD25^+$ Tr cells may induce the development of autoimmunity (Taguchi et al., 1996). There may be a delicate balance between enhancing immune responses to tumours or infectious agents while avoiding autoimmunity.

1.2.2.4 Existence of suppressor $CD4^+CD25^-$ T cells

Although it is well established that naturally occurring $CD4^+CD25^+$ Tr cells are 'professional' suppressor cells, various studies in both mice and rats have provided some evidence for $CD4^+CD25^-$ suppressor T cells. In PVG rats, $CD4^+CD25^-CD45RC^-$ donor cells from thoracic duct lymphocytes have been shown to protect thymectomized recipients from

IDDM (Stephens et al., 2000). It has been reported that TCR-transgenic mice that express a specific receptor for the autoantigen MBP do not develop EAE. Conversely, when these mice are bred onto a recombination-activating gene (*Rag)*-deficient background, EAE develops spontaneously and rapidly (Olivares-Villagomez et al., 1998). The regulatory T-cell population that is present in the TCR-transgenic mice on a conventional background is $CD4^+CD25^-$ and expresses TCRs that are encoded by the endogenous TCR α- and β-chain loci (Olivares-Villagomez et al., 1998). Mouse $CD4^+CD25^-$ T cells have also been discovered to mediate protection from inflammatory bowel disease (Annacker et al., 2000). $CD25^+$ and $CD25^-$ cells have been shown to inhibit the development of autoimmune disease in IL-2 deficient mice (Wolf et al., 2001). The $CD25^+$ T cell population might control autoimmunity at the level of activation of effectors, whereas $CD25^-$ T cells produce IL-2, which mediates the activation-induced cell death of autoreactive T cells. The $CD4^+CD25^-$ T cells can also be converted to FoxP3 expressing $CD4^+CD25^+$ Tr cells in a cytokine-mediated pathway, by $CD4^+CD25^+$ Tr cells (Zheng et al., 2004). However, it is not yet clearly understood if these suppressor $CD4^+CD25^-$ T cells originate in the thymus or if they acquire their suppressor function in the periphery, or elsewhere.

2 AIMS OF THE STUDY

The aim of this thesis was to explore if circulating Tr cells are involved in MS. We have examined the frequencies of $CD4^+CD25^+$ Tr cells and their surface and intracellular molecular expression profiles in MS patients in an untreated stage or during treatments with either IFN-β-1a or GA or IFN-β-1a+GA in combination, and compared with that in healthy controls (HC). We have also examined whether the total lesion load (TLL), BBB leakage or GA stimulation *in vitro* has any influence on $CD4^+CD25^+$ Tr cells.

Specific aims

Paper-I: To identify and quantify the levels of $CD4^+CD25^+$ Tr cells and $CD4^+CD25^{high}$ Tr cells among blood $CD4^+$ T cells, and examine the expression of the cell surface molecules CD45RO, CD69, CD95, HLA-DR, and intracellular CTLA-4 and IL-10 production by $CD4^+CD25^{high}$ Tr cells in patients with untreated MS *vs.* MS treated with either IFN-β-1a, GA or a combination of IFN-β-1a +GA and *vs*. HC.

Paper-II: To examine the correlation of TLL or Gd enhancement detected on MRI of the brain with expression of peripheral blood $CD4^+CD25^+$ Tr cell markers, including HLA-DR, CD45RO, CD95 and intracellular CTLA-4 and IL-10 in untreated MS patients.

Paper-III: To analyse *in-vitro* influence of GA upon the $CD4^+CD25^{high}$ Tr cell surface molecules CD45RO, CD69, CD95, HLA-DR, and the intracellular CTLA-4 and IL-10 production in patients with untreated MS vs. MS treated with either IFN-β-1a, GA or IFN-β-1a +GA in combination, vs. HC.

Paper-IV: To examine whether a functional deficit inherent to $CD4^+CD25^+$ Tr cells occurred in MS patients vs HC, in terms of the ability of Tr cells to suppress the production of pro-inflammatory cytokines by blood mononuclear cells (MNC) upon antigenic stimulation.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Patients with multiple sclerosis and healthy subjects

Some clinical characteristics of the 160 patients with clinically definite MS (Poser et al., 1983) included in Paper-I are presented in Table 1. A cross-sectional study was performed on these 160 patients. Fortyseven of the patients had never been treated with any immunomodulatory drugs including corticosteroids, IFN-β or GA (Copaxone; Teva, Petah Tiqva, Israel). Fifty patients were examined during ongoing treatment with IFN-β-1a (Avonex; Biogen, Cambridge, MA), administered at a dose of 30 μg intramuscularly once per week (mean duration of treatment was 10±5 months). Twentynine patients were examined during treatment with GA (mean duration of treatment was 9±7 months) administered at a dose of 20 mg subcutaneously daily. Thirtyfour patients were examined during ongoing treatment with IFN-β-1a+GA in combination (mean duration of treatment was 8±5 months). This combined treatment was offered to patients who, despite monotherapy with IFN-β, deteriorated clinically by at least 0.5 on the Expanded Disability Status Scale (EDSS, Kurtzke, 1983; 2000), or showed new and/or Gd enhancing lesions on MRI of the brain and/or the spinal cord. This was performed on all MS patients on an irregular basis. A longitudinal study was performed on 13 of the 47 untreated MS patients who started receiving IFN-β-1a treatment. Blood specimens for this study were taken within one week before the first IFN- β-1a injection and after 3-5 months of treatment at a standard dose.

Fiftyone MS patients were enrolled and examined in Paper-II. The median disease duration was 7.5 (0–50) yrs, and median disability score according to the EDSS was 2 (0–7). None of the patients had ever been treated with immunosuppressive or immunomodulatory drugs including steroids and IFN-β.

Table 1. Clinical data of patients with multiple sclerosis (MS) included in Paper-I. MS patients are subgrouped according to treatment: untreated patients; IFN-β-1a-treated patients, GA-treated patients, and patients treated with IFN-β-1a+GA in combination. EDSS: expanded disability status scale; PCP: primary chronic progressive phase of MS; RR: relapsing remitting phase of MS; SCP: secondary chronic progressive phase of MS. The data includes patients enrolled for studying both $CD4^+CD25^+$ Tr cells and $CD4^+CD25^{high}$ Tr cells.

	Numbers of subjects	Sex F/M	Age (mean±SD)	MS phase			MS duration In years		EDSS		
				RR	SCP	PCP	<10	>10	<3	3-6	>6
MS	160	95/65	45±13	77	74	9	95	65	78	56	26
Untreated MS	47	29/18	45±13	27	16	4	31	16	25	13	9
IFN-β-1a treated MS	50	29/21	46±14	26	19	5	32	18	31	16	3
GA treated MS	29	19/10	38±13	18	11	-	11	18	14	11	4
IFN-β-1a GA treated MS	34	18/16	47±9	6	28	-	21	13	8	16	10

Of the 60 patients enrolled in the Paper-III (Table 2), 15 had never been treated with any immunosuppressive or immunomodulatory drugs. Fifteen of the patients were examined during ongoing treatment with IFN-β-1a, administered at a dose of 30 μg intramuscularly once per week (mean duration of treatment 10±6 months). Fifteen patients were examined during treatment with GA (mean duration of treatment 9±8 months). The remaining 15 patients were examined during ongoing treatment with IFN-β-1a+GA in combination (mean duration of treatment 8±5 months).

Table 2. Clinical data of patients with multiple sclerosis (MS) included in Paper-III. MS patients are sub-grouped according to treatment: untreated patients, IFN-β-treated patients, GA-treated patients and IFN-β+GA in combination treated MS patients. EDSS: expanded disability status scale; PCP: primary chronic progressive phase of MS; RR: relapsing remitting phase of MS; SCP: secondary chronic progressive phase of MS.

	Number of subjects	Sex F/M	Age (mean±SD)	MS phase			MS duration		EDSS		
				RR	SCP	PCP	<10	>10	<3	3-6	>6
MS	60	35/25	42±13	28	28	4	32	28	26	22	12
Untreated MS	15	8/7	44±12	8	5	2	9	6	7	5	3
IFN-β1a treated MS	15	9/6	46±13	7	6	2	9	6	9	4	2
GA treated MS	15	10/5	39±12	10	5	-	5	10	7	5	3
IFN-β1a+ GA treated MS	15	8/7	42±11	3	12	-	9	6	3	8	4

Ten patients with clinically definite MS (all females) were included in Paper-IV. Their age range was 27-61 years (median 45 years). The duration of MS was 2-38 years (median 12 years). Three patients were examined during the secondary progressive phase of MS and seven patients had relapsing-remitting MS. Six patients had no or slight disability, defined as an expanded disability status scale (EDSS) score < 3, while two patients had moderate symptoms defined as an EDSS 3-6, and two patients had severe disability defined as an EDSS score >6. The duration of MS was < 10 years in 5 patients. In parallel, ten HC were examined (4 females). Their age range was 27-37 years (median 32 years).

In all of these studies, HC were recruited from staff of the department and blood donors with no history of neurological disorders. The Ethical Committee of the Karolinska Institute at Huddinge University Hospital approved all of the studies, and informed consent was obtained from all MS patients and HC.

3.2 Methods

3.2.1 MRI protocol

Brain MRI was performed on a 1.5 T Siemens Magnetom Vision whole-body scanner (Siemens, Erlangen, Germany) with a standard head coil. Two sagittal and three axial oblique scans of 19 non-contiguous, 5-mm slices with a 1.5-mm gap were acquired. Axial slices were aligned on the inferior borders of the genu and splenium of corpus callosum. A T2W sagittal pulse sequence (TE/TR= 96/3500ms), a dual-echo PD/T2W axial scan (TE1/TE2/TR= 20/120/3500ms) and a turbo FLAIR axial scans (effTE/TI/TR = 110/2500/9000ms) were chosen to detect the lesion load. The final axial and sagittal T1W scans (TE/TR=14/570ms) were performed 15-20 minutes after i.v. administration of a single dose (0.1 mmol/kg body weight) of Gd-DTPA contrast agent (Magnevist®, Schering AG, Berlin, Germany) to evaluate the permeability of the BBB. The following parameters were assessed: (1) Lesion load (LL) on T2-weighted images, used as a semi-quantitative measure of overall brain damage; (2) presence of Gd-enhancing lesions of T1-weighted images, used as a marker of BBB breakdown and hence active inflammation. The LL was classified as either low or high, the latter defined as 10 or more lesions greater than 10 mm in diameter.

3.2.2 Media, monoclonal antibodies, and glatiramer acetate

RPMI 1640 (Gibco, Paisley, Scotland) was used as culture medium, supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin (all from Gibco). The following anti-human monoclonal antibodies (mAbs) were purchased from Becton & Dickinson (Mountain View, CA): Peridinin chlorophyll protein (PerCP, red fluorescence)-labeled anti-CD4, anti-CD3; phycoerythrin (PE, orange fluorescence)-labeled anti-CTLA-4, anti-CD95, anti-CD40L, anti-CD45RO, anti CD-69, anti-HLA-DR, anti-IL-10, anti-CCR5, anti-CXCR4, CD8, anti-CD19, and anti-CD25; fluorescence isothiocyanate, (FITC, green fluorescence)-labelled anti-CD25, anti-CD14, and anti-CD56. Irrelevant isotype-matched mouse mAbs were: FITC-IgG1 and

PE-IgG2a, and irrelevant isotype-matched rat mAb was PE-IgG2a. PE-labelled anti-CCR1 and anti-CCR2; unlabelled anti-CXCR3 mAbs, anti-CD3 and anti-CD28 antibodies were purchased, from R&D Systems Inc. (R&D Systems, Abingdon, UK) and FITC-labelled anti-CD14 mAbs and PE-labelled goat-anti-mouse Ig Fab_2 fragments were purchased from Dako (Copenhagen, Denmark).

3.2.3 Preparation of mononuclear cells from peripheral blood

Blood MNC were separated by density gradient centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway), washed three times with RPMI 1640 medium (Gibco, Paisley, Scotland), and counted. Cell viability measured by trypan blue exclusion always exceeded 95%.

In Paper-II, 30 ml of blood were obtained in heparinized tubes, during MRI examination, immediately prior to the i.v. administration of Gd-DTPA. After isolation of MNC, the cells were spun down, resuspended at 10^7 cells/ml in freezing medium consisting of RPMI with 10% dimethylsulfoxide (Sigma) and 10% fetal calf serum, slowly cooled to –80°C and kept at –80°C until use.

3.2.4 Three-color fluorescent immunostaining and flow cytometry

The frozen blood-MNC were thawed rapidly at 37°C, washed once with washing buffer (PBS with 0.5% BSA) and resuspended at 10^7 cells/ml, whereas the freshly prepared blood MNC were used directly. To examine the expression of chemokine receptors simultaneously by $CD4^+$ T cells and monocytes, the MNC were stained with FITC-labelled anti-CD14, PerCP-labelled anti-CD4, and PE-labelled anti-CCR1, anti-CCR2, anti-CCR5 or anti-CXCR4 mAbs (15 min, 4°C). In case of CXCR3, the MNC were first stained with an unlabelled anti-CXCR3 mAb, washed, incubated with PE-labelled goat-anti-mouse Ig Fab_2 fragments, again washed and, finally, stained with PerCP-anti-CD4. After a final wash, the cells were resuspended in 200 µl of washing buffer for the analysis. To examine the expression of surface molecules by $CD4^+CD25^+$ Tr cells and $CD4^+CD25^{high}$ Tr cells, the MNC were labelled with FITC-anti-CD25, PerCP-anti-CD4, and PE-labelled anti-CD40L, anti-CD45RO,

anti-CD69, anti-CD95, or anti-HLA-DR (15 min, 4°C). To examine intracellular expression of CTLA-4 and IL-10 by $CD4^{+}CD25^{+}$ Tr cells, the MNC were first surface-stained with FITC-anti-CD25 and PerCP-anti-CD4, washed, fixed with 4% ice-cold paraformaldehyde (20 min), permeabilized with washing buffer containing 0.1% saponin, and incubated with PE-anti-CTLA-4 or -anti-IL-10 (25 min, RT). The cells were then washed once with washing buffer/saponin and once with washing buffer only, and resuspended for the analysis. Control samples were stained with isotype-matched irrelevant mAbs.

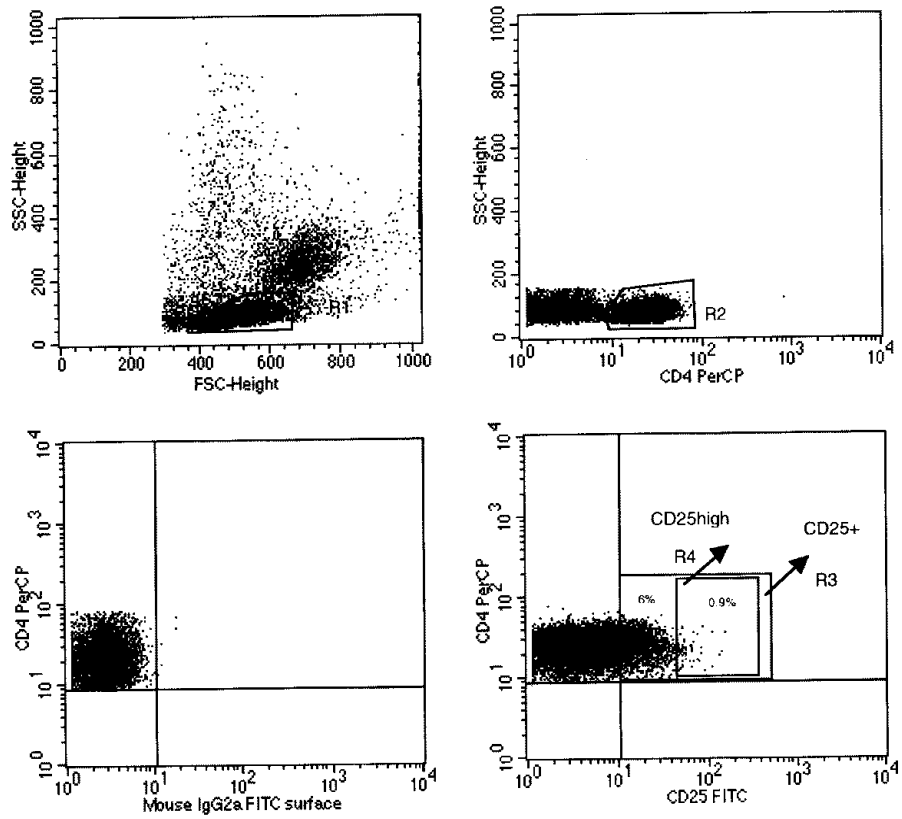

Fig. 2. Representative example of flow cytometry plots showing $CD4^{+}$ $CD25^{+}$ T cells and $CD4^{+}$ $CD25^{high}$ T cells. Region 1 (R1) is selected to set the lymphocyte gate according to forward (FSC) and side light scatter (SSC) properties. Region 2 (R2) gate is selected to separate the $CD4^{+}$ T cells from rest of the lymphocytes, and region 3 (R3) and region 4 (R4) are used to set the third and fourth gates to select the $CD4^{+}$ $CD25^{+}$ T cells and $CD4^{+}$ $CD25^{high}$ T cells from rest of the $CD4^{+}$ T cells. Region 4 (R4) is used to analyse the $CD4^{+}$ $CD25^{high}$ T cells expressing.

Stained cells were analyzed using FACScan flow cytometer and CellQuest software (both from Becton Dickinson), and 50,000 events per test were acquired. The MNC were distinguished from debris by forward and side light scatter properties. The FITC- and PerCP-channels were used to gate cell subpopulations, while the PE-channel was used to analyze the expression of specific molecules by the gated subpopulations. $CD4^+$ T cells were gated as $CD4^+$ MNC, i.e. with low side scatter; and the monocytes as $CD14^+$ MNC, i.e. with high side scatter; Tr cells as $CD4^+$ T cells co-expressing CD25; and non-Tr cells as $CD4^+$ T cells negative for CD25. The signal detected in the PE-channel was then plotted as a histogram for each cell subpopulation. The percent values presented below are fractions of the cell subpopulations that stained positively for the particular molecule in the PE-channel. The threshold for specific staining was set so that <1% of the cells stained with isotype control antibody were positive. In addition, the percentage of $CD4^+CD25^+$ Tr cells and $CD4^+CD25^{high}$ Tr cells among all $CD4^+$ T cells was calculated (Figure 2).

3.2.5 Depletion of CD25$^+$ cells

The MNC were labelled with anti-CD25-coupled magnetic beads, and $CD25^+$ cells were depleted using MACS columns according to the manufacturer's instructions (Miltenyi Biotec). The proportion of $CD25^+$ cells among the CD25-depleted (flow-through) fraction was routinely checked by flow cytometry and was <2% in relation to CD4 T cells and <1% in relation to all MNC. The CD25-enriched cell fraction consisted by >90% of $CD4^+$ T cells, with $CD8^+$ T cells constituting <5%, B cells <1.5%, NK cells <1.5%, and monocytes <0.5%. We therefore concluded that, by our procedure, we removed almost exclusively the $CD4^+CD25^+$ Tr cells. In order to control for possible activation of MNC during the depletion process, whole MNC (which were not to be depleted of Tr cells) were incubated in a labelling buffer not containing CD25 microbeads, and subsequently also passed through the depletion column. Cell viability after depletion always exceeded 95%. Cells were washed and adjusted to 10^6/ml in culture medium for subsequent ELISPOT assays.

3.2.6 ELISPOT assays for detection of cytokine-secreting cells

To detect and enumerate IFN-γ, IL-2, IL-4 and IL-13 secreting MNC, ELISPOT assays described by Czerkinsky et al., (1988) were adopted (Link et al., 1991, Gabrielsson et al., 1998). Microtitre plates with nitrocellulose bottoms (Multiscreen-HA; Millipore, Mulsheim, France) were used for detection of IFN-γ- and IL-2- secreting cells, and plates with poly(vinylidene difluoride) (PVDF) membrane bottoms (Multiscreen-IP; Millipore) were used for detecting IL-4- and IL-13- secreting cells. Plates were coated overnight at 4°C with 100 µl/well of mAbs against human IL-2, IFN-γ, IL-4, or IL-13 diluted in sterile PBS (pH 7.4) to a concentration of 10 µg/ml. After removal of coating solutions and washes with PBS, 200 µl aliquots containing 2×10^5 blood MNC in culture medium were applied to each well. To the wells, 50 µg/ml human MBP, or 10 µg/ml PPD, or 8 µg/ml anti-CD3 plus 2 µg/ml anti-CD28 antibody were added. All stimulations were set up in duplicates. Control cultures did not receive any stimulus. As a negative control of staining, culture medium was added to wells in the absence of MNC. The plates were incubated at 37°C for 48 h in humidified air containing 5% CO_2, then emptied and washed. Then, biotinylated mAbs against respective cytokines (100 µl/well, 2 µg/ml in PBS) were added to the wells and incubated overnight at 4°C. The wells were washed, and 100 µl of streptavidin-alkaline phosphatase (Mabtech, Stockholm, Sweden) diluted 1:1000 were added, and stored for 1.5 h at room temperature. After incubation, the plates were emptied, washed with Tris buffer (pH 9.5) and stained with BCIP-NBT (Mabtech). Blue immunospots, each considered to represent a cell secreting IFN-γ, IL-2, IL-4 or IL-13, were counted with AID ELISPOT Reader System using AID ELISPOT-2.9 software (Autoimmun Diagnostika GmbH, Strassberg, Germany). The mean values of duplicates were calculated. Since IL-4-secreting MNC were very low in numbers, this cytokine was excluded from following experiments. Results are presented as numbers of IFN-γ-, IL-2-, or IL-13-secreting cells per 10^5 blood MNC. Variation of duplicates was generally < 10%.

To rule out possible non-specific activation of MNC by FCS present in the culture medium, preliminary experiments were performed with MNC from three healthy donors, in which FCS-containing medium was compared to the one containing autologous donor's serum. No differences in numbers of cytokine-secreting cells were observed. Subsequently, FCS was chosen to be used in the culture media.

As per testing by the manufacturers, all media and chemicals used in the ELISPOT assays were either endotoxin-free or contained very low levels of endotoxin.

3.3 Statistical analysis

Multiple groups were compared by the non-parametric Kruskal-Wallis ANOVA; in case $p < 0.05$ was obtained, differences between pairs of groups were further compared by the Mann-Whitney U-test or Dunn's test. Differences were considered statistically significant if $p < 0.05$. Throughout the text, data are presented as median (range). Wilcoxon signed rank test was used when comparing paired samples.

4 RESULTS

4.1 The frequency of CD4$^+$CD25$^+$ Tr cells, CD4$^+$CD25^{high} Tr cells, and subpopulations of CD4$^+$CD25^{high} Tr cell in MS patients and healthy controls (Paper-I)

Median percentages of CD4$^+$CD25$^+$ Tr cells among blood CD4$^+$ T cells in MS patients examined were 6 % and in HC 7 %. The levels of CD4$^+$CD25$^+$ Tr cells in untreated, IFN-β-1a-, GA- and IFN-β-1a+GA-treated MS patients were also similar. Thirteen of the untreated MS patients were re-sampled 3-5 months after onset of IFN-β-1a treatment, but no significant difference was observed in the median percentages of CD4$^+$CD25$^+$ Tr cells among blood CD4$^+$ T cells, compared to pre-treatment levels. The frequency of CD4$^+$CD25^{high} Tr cells among CD4$^+$ T cells did not differ between MS patients, irrespective of treatment, and HC, and was about 0.6 –1 %.

Percentages of CD4$^+$CD25^{high} Tr cells expressing CD40L and the early activation marker CD69 were low (<1%) when analysed in MS patients and HC. CD4$^+$CD25^{high} Tr cells showed high expression of the cell surface molecules CD45RO (~90%) and CD95 (~70%), high intracellular expression of CTLA-4 (~80%), and low expression of the cell surface molecule HLA-DR (~30%). Levels of CD4$^+$CD25^{high} Tr cells producing IL-10 were very low (~1%). When comparing the four different groups of MS patients and HC, there were no differences regarding levels CD4$^+$CD25^{high} Tr cells expressing CD45RO, CD95 and HLA-DR, or intracellular CTLA-4 or IL-10.

Thus, in this study, we did not obtain evidence for quantitative and phenotypic alterations of CD4$^+$CD25$^+$ and CD4$^+$CD25^{high} Tr cells in MS.

4.2 Correlation of lesion load and blood brain barrier damage with the frequencies of CD4$^+$CD25$^+$ Tr cells and their phenotype, in untreated MS patients (Paper-II)

On the basis of MRI, untreated MS patients were divided into four subgroups (Table 3). Percentage of CD4$^+$CD25$^+$ Tr cells expressing IL-10 intracellularly was increased in the patients with high LL and Gd-enhancing lesions (24%) compared to patients with low LL (9%), but were decreased in patients with high LL and no Gd-enhancing lesions (2.5%). The

expression of other Tr cell markers, such as HLA-DR, CD45RO, and CD95, and intracellular CTLA-4, was not influenced by LL or by the presence of Gd enhancement.

$CD4^+CD25^-$ (non-regulatory) T cells expressed lower levels of CD45RO, CD95, intracellular CTLA-4 and HLA-DR as compared to Tr cells, which agrees with published data (Dieckmann et al, 2001; Jonuleit et al, 2001). Interestingly, $CD4^+CD25^-$ T cells showed same pattern of IL-10 expression as Tr cells.

The percentage of $CD4^+CD25^+$ Tr cells among all $CD4^+$ T cells was elevated in the high LL, Gd-enhancing subgroup (9%) when compared to the low LL and no Gd-enhancing subgroup (4%; $p<0.05$).

Table 3. *Four groups of patients according to routine MRI parameters.*

	Gd^-	Gd^+
Low LL	19	3*
High LL	15	14

*Excluded from further analysis due to low patient number.

4.3 Correlation of lesion load and blood brain barrier damage with expression of chemokine receptors by $CD4^+$ T cells and monocytes, in untreated MS patients (Paper-II)

As CXCR3 is an inflammatory/inducible type of chemokine receptor expressed on activated T cells (Rabin et al., 1999), it was included in Paper-II to examine if lesion load and blood brain barrier damage affects expression of CXCR3. Our data showed decreased surface levels of CXCR3 in the high LL, Gd-enhancing subgroup, whereas percentages of $CD4^+$ T cell expressing CCR1, CCR2, CCR5 and CXCR4 were similar in all three subgroups of patients.

Percentages of monocytes positive for CCR1, CCR2, CCR5 and CXCR4 were similar in all subgroups, although there was a trend (not reaching statistical significance) towards a decrease of CCR1 and CCR2 on monocytes in the patients with a high LL and Gd-enhancing lesions.

4.4 Effect of glatiramer acetate on $CD4^+CD25^{high}$ Tr cells (Paper-III)

The percentages of $CD4^+CD25^{high}$ Tr cells expressing HLA-DR were statistically similar in untreated cells from HC and all MS patient groups. *In vitro* stimulation with GA did not change the percentages of $CD4^+CD25^{high}$ Tr cells expressing HLA-DR, neither in untreated and IFN-β+GA-treated MS patients nor in HC. However, the percentages of $CD4^+CD25^{high}$ Tr cells expressing HLA-DR were higher in MS patients treated with IFN-β or GA, in comparison to untreated MS patients. Upon $MBP_{(83-99)}$-stimulation, the percentages of $CD4^+CD25^{high}$ Tr cells expressing HLA-DR were reduced in untreated MS patients and HC, as compared to IFN-β- and GA-treated MS patients.

There was no effect of GA or $MBP_{(83-99)}$ stimulation on the percentages of $CD4^+CD25^{high}$ Tr cells expressing CD45RO or CD95, nor on the percentages of $CD4^+CD25^{high}$ Tr cells expressing CD69 (~2%), and intracellular CTLA-4 (80%) in MS patients, and in HC.

The percentages of untreated $CD4^+CD25^{high}$ Tr cells producing IL-10 were similar in HC and all MS patient groups. Upon GA-stimulation, there was an increase in the percentages of $CD4^+CD25^{high}$ Tr cells producing IL-10 in HC and in untreated, or GA- or IFN-β+GA-treated MS patients. $MBP_{(83-99)}$ stimulation did not affect the percentages of $CD4^+CD25^{high}$ Tr cells producing IL-10 in any of the groups. CD3/CD28 ab stimulation significantly upregulated the levels of $CD4^+CD25^{high}$ Tr cells producing IL-10 in all groups.

These results indicate that GA upregulates levels of $CD4^+CD25^{high}$ Tr cells expressing HLA-DR and IL-10, irrespective of whether they are obtained from HC or anyone of the MS treatments groups.

4.5 Influence of $CD4^+CD25^+$ Tr cells on the secretion of Th1 and Th2 cytokines (Paper-IV)

As indicated in Methods, the majority (>90%) of the $CD25^+$ cells removed from blood MNC by the use of anti-CD25-coupled microbeads and MACS procedure, were the $CD4^+CD25^+$ Tr cells. Removal of Tr cells resulted in an increased number of IFN-γ–secreting cells even in the absence of antigens such as human-MBP or PPD in the culture medium. Thus, in MS patients, we observed 9 (0–38) IFN-γ secreting cells per 10^5 wMNC vs. 16 (2–48) per 10^5 $CD25^-$MNC ($p < 0.01$); and in HC, 6 (0–26) IFN-γ secreting cells per 10^5 wMNC vs. 9 (2–28) per 10^5 $CD25^-$MNC ($p < 0.01$). This could not be explained simply by physical removal of the IFN-γ- and IL-2-non-secreting Tr cells, since they constituted <5% of all

MNC. Tr cell depletion did not affect spontaneous secretion of IL-2 in MS patients [4 (0–20) IL-2 secreting cells per 10^5 wMNC vs. 4 (2–25) per 10^5 $CD25^-$MNC], while in HC it caused an increase in frequencies of IL-2 secreting cells [4 (0–7) per 10^5 wMNC vs. 6 (2–15) per 10^5 $CD25^-$MNC ($p < 0.01$)]. Spontaneous secretion of IL-13 was not affected by Tr cell depletion: in MS patients, 9 (3–29) IL-13 secreting cells per 10^5 wMNC vs. 6 (3–22) per 10^5 $CD25^-$MNC; in HC, 6 (2–15) cells per 10^5 wMNC vs. 4 (1–15) cells per 10^5 $CD25^-$MNC.

To estimate the effect of (auto)antigenic stimulation on wMNC and $CD25^-$MNC, we subtracted the number of spots obtained in the absence of stimulation with a particular antigen from the number of spots in the corresponding cell preparation in the presence of that antigenic stimulation. IFN-γ and IL-2 responses to MBP, virtually undetectable in a majority of wMNC samples from MS patients, were significantly augmented in the corresponding $CD25^-$MNC samples. A similar effect was observed in HC. In contrast, frequencies of cells secreting IL-13 in response to MBP was not affected or even slightly decreased in $CD25^-$ MNC, as compared to the corresponding wMNC.

IFN-γ and IL-2 responses to a foreign antigen, PPD, were also increased in the $CD25^-$ MNC as compared to wMNC, although it was only in the case of IL-2, that the difference reached a statistical significance. Depletion of $CD4^+CD25^+$ Tr cells did not affect IL-13 secretion in response to PPD.

Depletion of $CD4^+CD25^+$ Tr cells did not affect IFN-γ secretion in response to a polyclonal stimulation with anti-CD3 and anti-CD28 mAbs, neither in MS patients nor in HC; however, it did augment IL-2 and IL-13 secretion.

Depletion of $CD4^+CD25^+$Tr cells equally affected cytokine responses in MS patients and HC, both to antigenic and polyclonal stimulation. In MS patients, IFN-γ and IL-2 responses to MBP appeared to be affected by Tr cell depletion even somewhat sharper than in HC. However, we did not find any statistically significant differences in antigen-specific cytokine responses between MS patients and HC, neither in wMNC nor in $CD25^-$MNC.

5 DISCUSSION

5.1 Circulating $CD4^+CD25^+$ Tr cells are not altered in multiple sclerosis and are unaffected by disease-modulating drugs

Deletion of $CD4^+CD25^+$ Tr cells results in histologically and serologically evident autoimmune diseases in mice and rat models, whereas, reconstitution of $CD4^+CD25^+$ Tr cells prevents the same autoimmune developments (Fowell et al., 1993; Sakaguchi et al., 1995). It has been illustrated that chronic graft-versus-host disease is associated with increased numbers of peripheral blood $CD4^+CD25^{high}$ regulatory T cells (Clark et al., 2004). It is of importance to determine whether alterations of the $CD25^+$ population of $CD4^+$ T cells are involved in diseases in the humans with a proposed autoimmune background like MS. In Paper-I, we examined the frequencies of $CD4^+CD25^+$ Tr cells in MS in comparison to HC, and during MS treatment with IFN-β, GA and IFN-β+GA in combination. Our data show that, quantitatively, circulating $CD4^+CD25^+$ Tr cells and $CD4^+CD25^{high}$ Tr cell populations do not differ in patients affected with MS compared to HC (Figure 3). Frequencies of $CD4^+CD25^+$ and $CD4^+CD25^{high}$ Tr cells are also unaffected during ongoing treatment with IFN-β-1a or GA or IFN-β-1a+GA. This observation is further strengthened by the follow-up of MS patients before and during IFN-β-1a treatment where there were no changes in the proportions of $CD4^+CD25^+$ and $CD4^+CD25^{high}$ Tr cells.

In view of the findings that suppressive activity of $CD4^+CD25^+$ Tr cells is cell contact dependent (Thornton et al., 1998), we analysed the expression by $CD4^+CD25^{high}$ Tr cells of the functionally important cell surface molecules CD45RO, CD69, CD95 and HLA-DR, and of intracellular CTLA-4 and IL-10 among $CD4^+CD25^{high}$ Tr cells. $CD4^+$ $CD25^{high}$ Tr cells differ from $CD4^+CD25^+$ T regulatory cells in their expression levels of CD45RO and HLA-DR (Baecher-Allan et al, 2001). Our studies (Paper-I) demonstrated that the proportions of the subpopulations of $CD4^+CD25^{high}$ Tr cells, *i.e.* expressing CD45RO, HLA-DR, CD95, CTLA-4 and IL-10, were similar in MS patients and HC. A recent report (Viglietta et al., 2004), supports our results. Our data also yield evidence that *ex vivo* $CD4^+CD25^{high}$ Tr cells do not express CD40L and CD69 molecules.

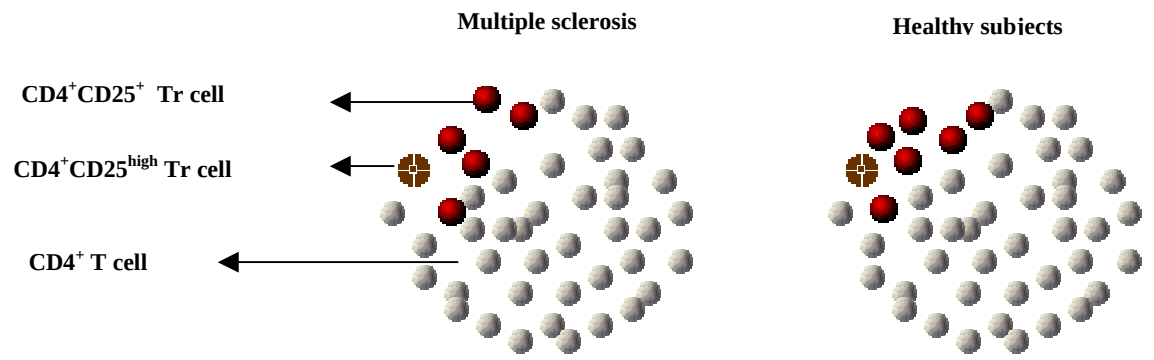

Fig. 3. Proportion of CD4+CD25+ Tr cells among CD4+ T cells in multiple scleorsis (MS) patients and healthy controls (HC) are 5% and 6%. Whereas proportion of CD4+CD25+ Tr cells among CD4+ T cells in MS patients and HC are ~1% (Paper-I).

5.2 Lesion load and blood brain barrier damage correlates the frequencies of CD4+CD25+ Tr cells and their phenotype

Although the relative proportions of the subpopulations of CD4+CD25+ Tr cells are unaffected during treatment with IFN-β-1a or GA or the combination of IFN-β-1a+GA (Paper-I), we found that MS patients with high LL and Gd-enhancing lesions had an increased relative proportion of CD4+CD25+ Tr cells of the total number of CD4+ cells. This may represent a compensatory anti-inflammatory response to a co-existing pro-inflammatory environment (Figure 4).

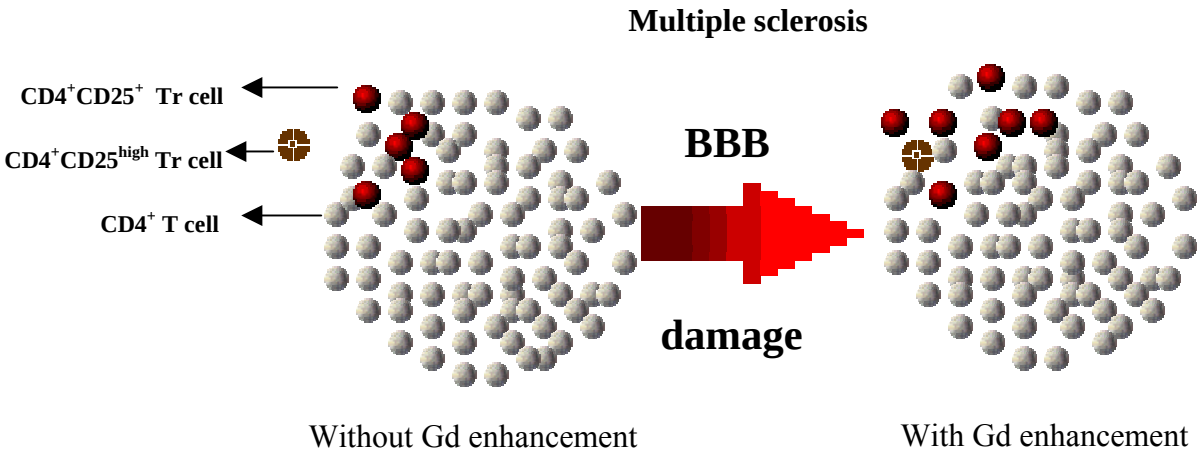

Fig. 4. Proportions of blood CD4+CD25+ Tr cell among CD4+ T cells elevate in multiple sclerosis patients, presenting with Gd enhancing lesions (Paper-II)

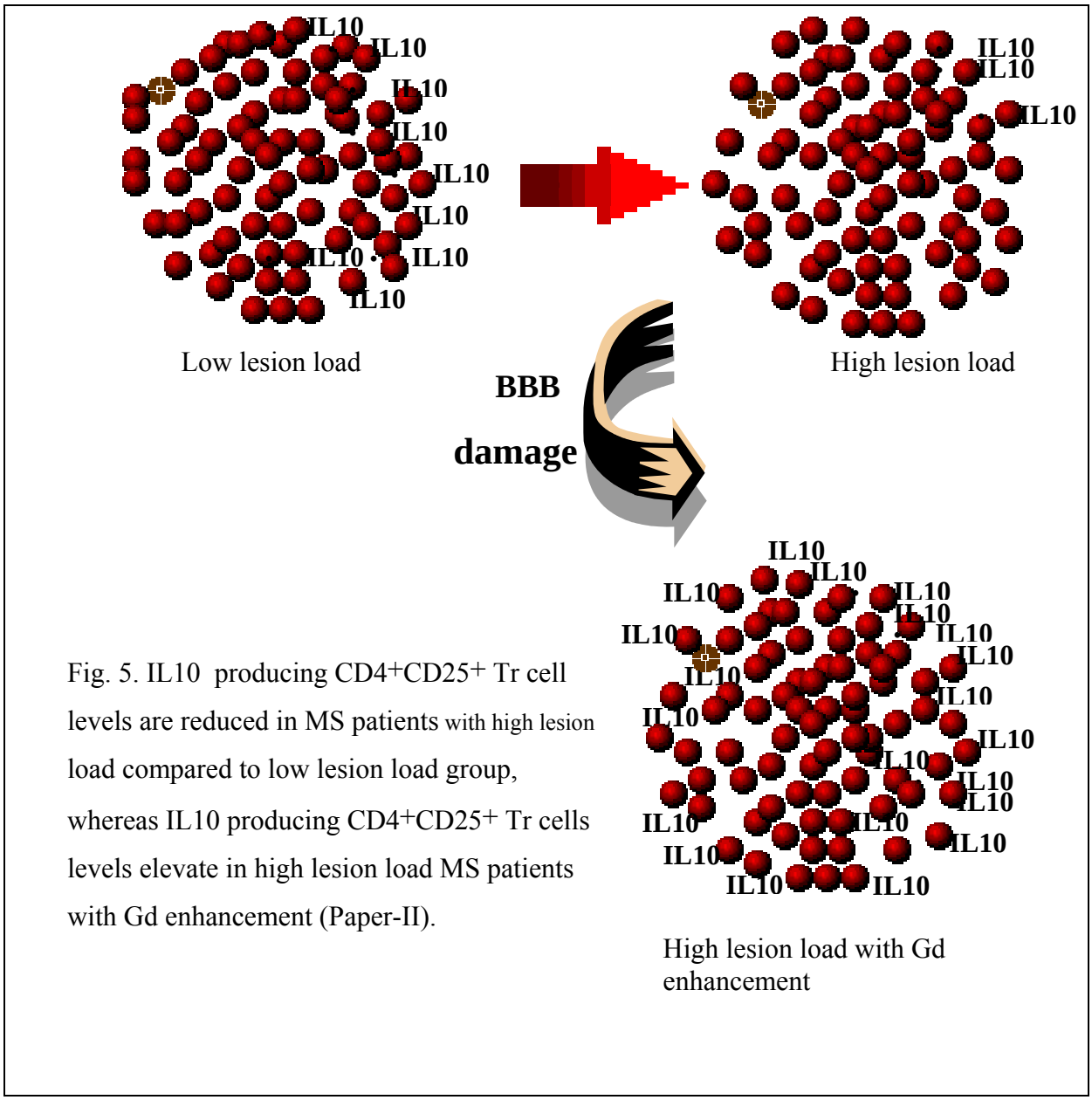

Fig. 5. IL10 producing $CD4^+CD25^+$ Tr cell levels are reduced in MS patients with high lesion load compared to low lesion load group, whereas IL10 producing $CD4^+CD25^+$ Tr cells levels elevate in high lesion load MS patients with Gd enhancement (Paper-II).

Analysis of the expression of the anti-inflammatory cytokine IL-10, showed increased intracellular levels in MS patients with active lesions (Figure 5). Our data are consistent with the observation that serum IL-10 levels were increased during the month that Gd-enhancing lesions resolved (Waubant et al, 2001), and the hypothesis that IL-10 down-regulates inflammation in MS (van Boxel-Dezaire, 1999).

Together, our findings suggest activation of anti-inflammatory mechanisms during periods of active CNS inflammation (Figure 6).

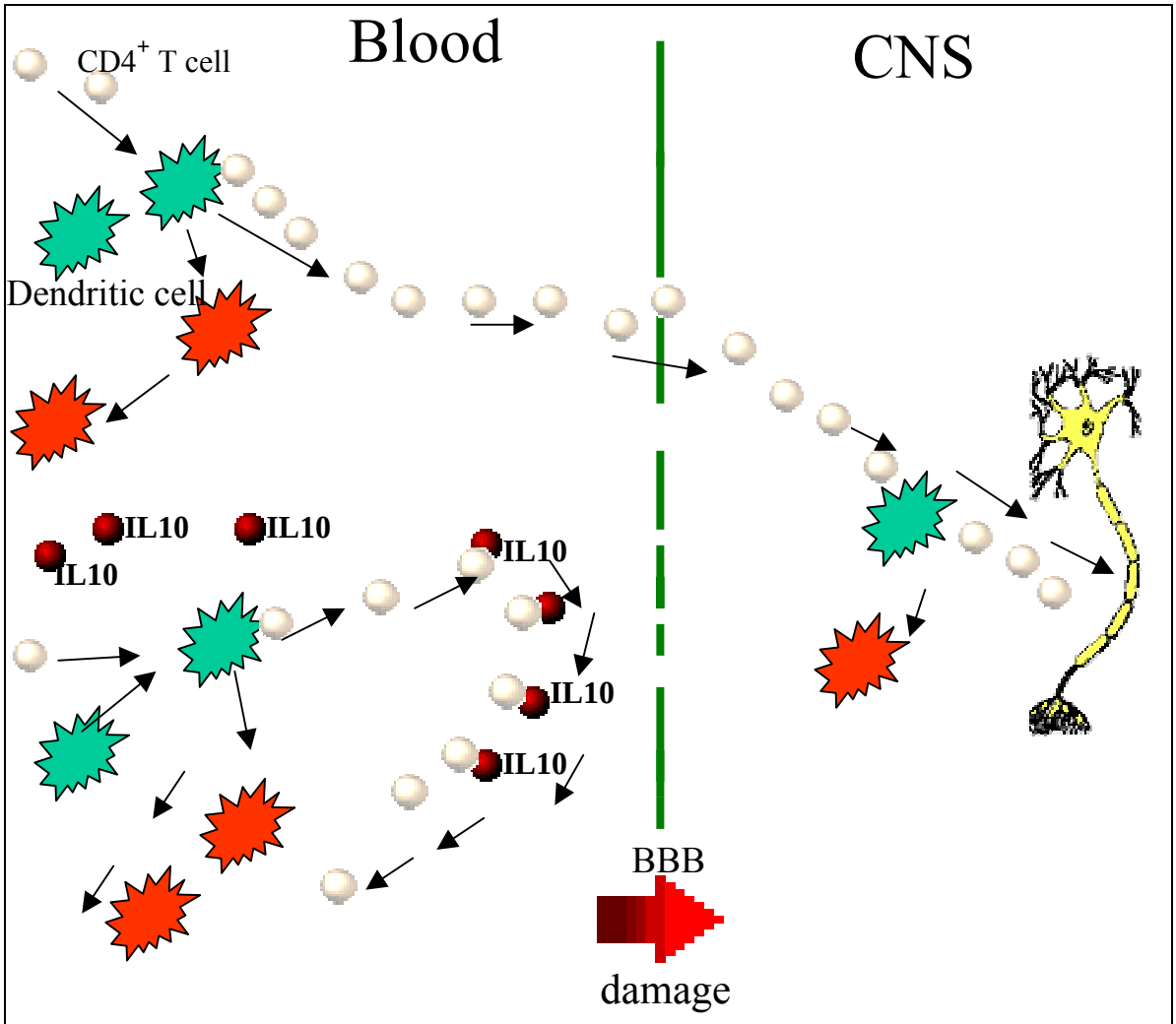

Fig. 6. Diagrammatic representation of autoreactive T cells infiltrating into central nervous system (CNS), in presence of blood brain barrier damage in multiple sclerosis. Peripheral blood $CD4^{+}CD25^{+}$ Tr cells producing IL-10 are assumed to attempt to suppress infiltration of the autoreactive T cells from the periphery into CNS (Paper-II).

5.3 Lesion load and blood brain barrier damage correlates expression of chemokine receptors by $CD4^{+}$ T cells and monocytes

Expression of chemokine receptors by $CD4^{+}$ T cells and monocytes in MS patients is of interest for several reasons: (1) these two cell types are considered to be a primary engine driving inflammation in MS; (2) chemokine receptors participate in the recruitment of inflammatory cells to MS lesions (Sorensen et al, 1999); (3) selected targeting of chemokine receptors by low molecular weight antagonists is of potential therapeutic value (Mackay,

2001). Previous studies have reported elevated expression of CCR5 and CXCR3 by total blood T cells in MS compared to HC (Zang et al, 2000; Teleshova et al, 2002); however, the chemokine receptor expression was not analysed in relation to MRI data. In the present studies, expression of CCR1, CCR2, CCR4 and CCR5 by $CD4^+$ T cells or monocytes, was found not to depend on LL or the presence of Gd-enhancement.

Somewhat unexpectedly, and in contrast to an earlier report (Sindern et al., 2002), the expression of CXCR3 by $CD4^+$ T cells was decreased in patients with high LL and Gd-enhancing brain lesions (A similar trend, not reaching the statistical significance, was observed for CCR1 and CCR2 expression on monocytes). The decrease in CXCR3 may be due to release of the chemokines from active MS lesions into the circulation and subsequent internalization of the respective chemokine receptors on blood MNC. Chemokines like IP-10 (a CXCR3 ligand) are abundantly produced in active MS lesions and increased in CSF (Sorensen et al, 1999; Sorensen et al., 2001). It is conceivable that increased concentrations of chemokines in plasma may result from leakage of chemokines into the blood stream through the disrupted BBB (Franciotta et al., 2001). In addition, active recruitment of CXCR3-positive T cells in Gd-enhancing MS lesions may lead to a deficit of these cells in the circulation.

5.4 Influence of Glatiramer acetate on $CD4^+CD25^{high}$ Tr cells

In vivo and *in vitro* studies using both murine and human cell cultures suggest that the mechanism for GA activity in EAE and MS is involved in induction of Th2 or regulatory T cells which enter the CNS, and get activated by myelin antigens (Arnon *et al.,* 1996; Duda *et al.,* 2000). Such GA-reactive Th2 or regulatory T cells are supposed to suppress Th1 responses (Gran *et al.,* 2000). In Paper-III, we investigated whether the proportion of $CD4^+CD25^{high}$ Tr cells that are activated by GA, is larger in GA- or GA+IFN-β-treated MS patients, than in untreated, IFN-treated MS patients and HC. As functionally important molecules expressed by Tr cells like CD45RO, CTLA-4, HLA-DR (Baecher-Allan *et al.,* 2001) and the death receptor CD95 (Wing *et al.,* 2002), and cytokine IL-10, play important roles in their suppressive actions, we compared effects of GA stimulation on $CD4^+CD25^{high}$ Tr cell surface molecule expression and intracellular CTLA-4 and IL-10 production in GA- and GA+IFN-β-treated MS patients compared to untreated and IFN-β-treated MS patients.

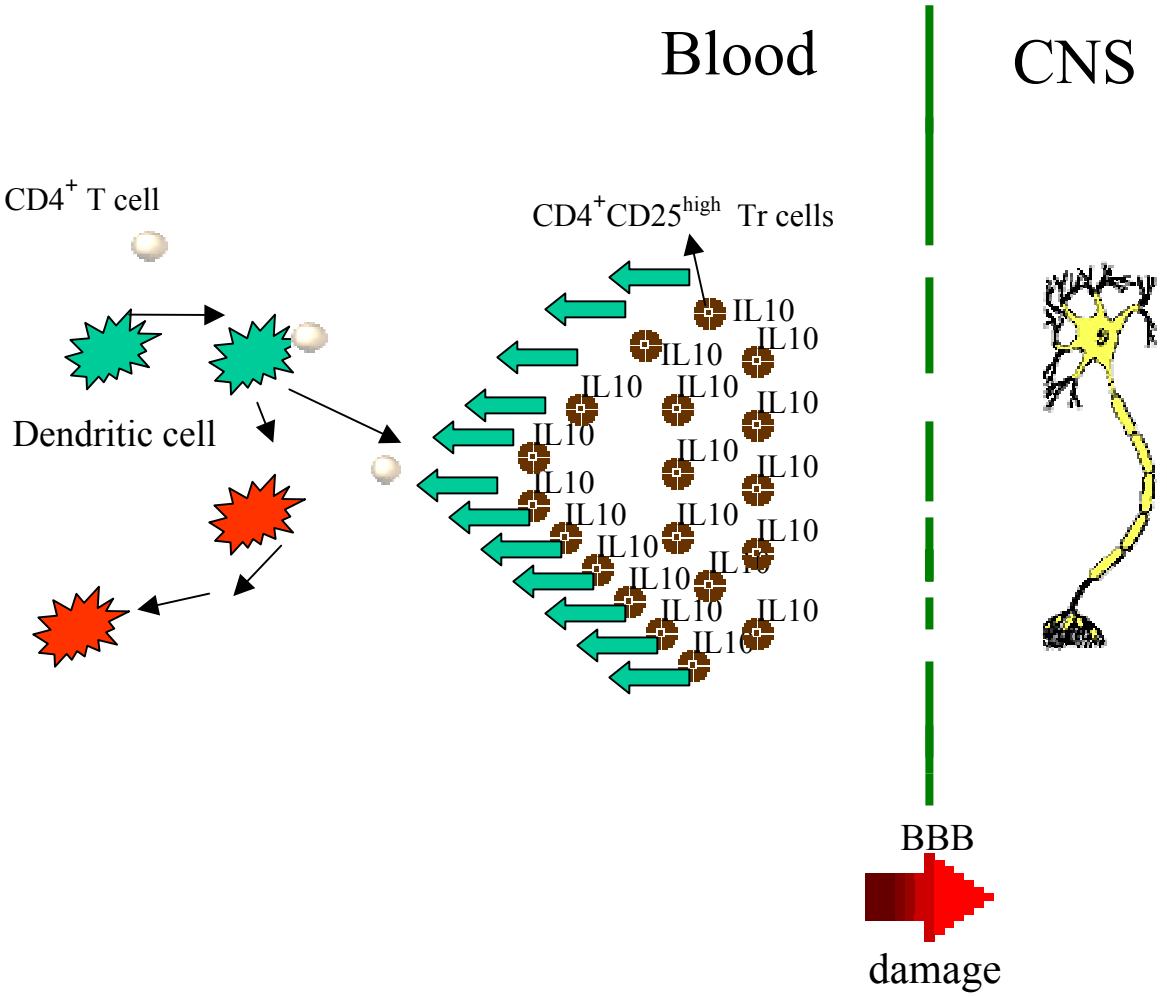

Fig. 7. Glatiramer acetate (GA) *in vitro* elevates levels of $CD4^{+}CD25^{high}$ Tr cells producing IL-10 among multiple sclerosis patients (Paper-III). Possibly GA might elevate *in vivo* levels of $CD4^{+}CD25^{high}$ Tr cells producing IL-10, which might suppress the autoreactive T cells in the periphery from infiltrating the central nervous system (CNS)

The median percentage of $CD4^{+}CD25^{high}$ Tr cells expressing HLA-DR was lower among untreated MS compared to IFN-β- and GA- treated MS patients. Upon *in vitro* stimulation with GA, the levels of Tr cells expressing HLA-DR remained significantly low in untreated MS patients and HC compared to other MS treatment groups. Among GA-treated patients, we assume pre-sensitization of $CD4^{+}CD25^{high}$ Tr cells to GA, which has resulted into elevated levels of $CD4^{+}CD25^{high}$ Tr cells expressing HLA-DR upon additional *in vitro* GA stimulation. Similarly, it may be that IFN-β treatment activated $CD4^{+}CD25^{high}$ Tr cells, and hence resulted in stronger response upon additional *in vitro* GA stimulation. Functional study by blocking HLA-DR molecule on $CD4^{+}CD25^{high}$ Tr cells should be warranted to investigate suppressive functions of $CD4^{+}CD25^{high}$ Tr cells in presence of GA or IFN-β. Upon stimulation with GA or

immunodominant $MBP_{(83-99)}$, the levels of Tr cells expressing CD45RO (90%), CD95 (70%) and CTLA-4 (~ 80%) did not differ in untreated MS patients and HC compared to other MS treatment groups.

IL-10 is immune-suppressive in activity and the mode of action is likely to be through inhibition of APC function (Ding *et al.,* 1992; Fiorentino *et al.,* 1991) and anti-proliferative effects on T cells (Moore *et al.,* 2001). $CD4^+CD25^+$ Tr cells have been reported to inhibit immune pathology by controlling the expansion of other T cell populations through IL-10 (Annacker *et al.,* 2001). The GA-specific T cells are predominantly Th2 biased, as it was demonstrated in animal studies and in leukocytes derived from individuals with MS treated with GA (Neuhaus *et al.,* 2001; Sela *et al.,* 2001). The serum levels of IL-10 were reported to be elevated in GA-treated MS patients (Miller *et al.,* 1998). Similarly, but in relation to $CD4^+CD25^{high}$ Tr cells our data shows that the levels of $CD4^+CD25^{high}$ Tr cells producing IL-10, elevate upon *in vitro* GA stimulation compared to $MBP_{(83-99)}$ and non-stimulation in untreated, IFN-β-, GA-, IFN-β+GA-treated MS patients and in HC. This further supports the earlier reports suggesting the positive role of GA treatment among MS patients. On the basis of our findings, we assume that, probably GA treatment in MS patients could elevate levels of IL-10 producing $CD4^+CD25^{high}$ Tr cells, which in turn might suppress the autoreactive T cell proliferation. IL-10 producing $CD4^+CD25^{high}$ Tr cells may also possibly prevent the activated autoreactive $CD4^+$ T cells from infiltrating the BBB (Figure 7).

5.5 Influence of $CD4^+CD25^+$ Tr cells on the secretion of Th1 and Th2 cytokines

MS is characterized by a breakdown of tolerance to myelin autoantigens Hence, we asked whether Tr cells in MS are functionally deficient and thus unable to prevent autoimmune responses to the most common myelin antigen, MBP. We addressed this question by depleting Tr cells from blood MNC and analysing cytokine responses to MBP and control antigens in the presence and in the absence of Tr cells. We found that (i) depletion of Tr cells augments the secretion of Th1 cytokines IFN-γ and IL-2, but not of Th2 cytokine IL-13 in response to MBP; (ii) this effect of Tr cell depletion is observed not only in case of MBP, but also in case of a foreign antigen, PPD; (iii) the effect of Tr cell depletion is similar in MS patients and HC, suggesting that the functional activity of Tr cells is not significantly altered in MS, as compared to HC. Our data are in line with two of the recent studies: Cosmi et al (2004), who showed that $CD4^+CD25^+$ thymocytes efficiently suppress TH1 cell proliferation, but not TH2

cell proliferation; Wing et al (2003), who showed that in healthy donors, *in vitro* depletion of $CD4^+CD25^+$ Tr cells significantly augments proliferative T cell responses to MOG, another myelin antigen. We expand these observations by showing that a similar phenomenon is present in MS, where the anti-myelin responses are assumed to produce a clinical disease. In addition, we show that Tr cells do not inhibit MBP-induced secretion of IL-13, a Th2 cytokine that is considered beneficial in MS (Merrill and Benveniste, 1996).

In Paper-I (Putheti et al., 2004), we have demonstrated that the frequencies of circulating $CD4^+CD25^+$ Tr cells in MS patients are the same as in non-inflammatory controls, suggesting that the generation of Tr cells in MS is not impaired. Later published report (Viglietta et al., 2004), is in line with our results of Paper-I.

In Paper-IV, we showed that there is also no obvious functional defect of Tr cells in MS, at least with regard to responses to MBP. It remains to be established whether the same is true for other myelin antigens. However, it should be noted that these data are obtained from the experiments on cells *ex vivo,* and is possible that Tr cells are influenced by other regulatory signals *in vivo.* Conceivably, strong antigen-specific and co-stimulatory signals provided e.g. by mature DCs to naïve T cells may override the inhibitory effects of Tr cells. Furthermore, IL-6 produced by mature DCs can temporarily abrogate Tr cell-mediated suppression of T cell responses (Pasare and Medzhitov, 2003). Immunohistochemical staining of deep cervical lymph nodes from MS patients revealed the occurrence of mature DCs containing myelin antigens, in close contact with T cells (de Vos et al., 2002). Additional activation of such DCs, e.g. during common viral infections, may lead to an efficient priming of myelin antigen-reactive T cells. Comparably strong signals for T cell activation can be provided e.g. by IFN-γ-activated brain microglia (Aloisi et al., 1999). In these settings, the apparently "normal" activity of Tr cells, observed in this study, sufficient to inhibit autoimmunity in healthy individuals, may not suffice to suppress the autoaggressive immune response in MS patients. Further studies addressing this issue will clarify the role of $CD4^+CD25^+$ Tr cells in MS pathogenesis as well as define their significance as therapeutic targets in this disease.

6 GENERAL CONCLUSIONS

1. Levels of circulating $CD4^+CD25^+$ Tr cells and $CD4^+CD25^{high}$ Tr cells are not varied in MS and are unaffected by the disease-modulating drugs IFN-β-1a, GA and IFN-β-1a+GA.

2. Although the levels of circulating $CD4^+CD25^+$ T cells in MS are unaltered by the disease-modulating drugs IFN-β-1a, GA and IFN-β-1a+GA, they are increased in presence of Gd-enhancing lesions.

3. Levels of $CD4^+CD25^+$ Tr cells expressing IL-10 intracellularly are increased in the patients with Gd-enhancing lesions compared to patients with no Gd-enhancing lesions.

4. Percentages of $CD4^+$ T cells expressing surface levels of CXCR3 are significantly decreased in the patients with high LL and Gd-enhancement.

5. GA stimulation *in vitro* elevates levels of $CD4^+CD25^{high}$ Tr cells producing IL-10 among MS patients irrespective of the treatments IFN-β, GA, IFN-β+GA in combination and HC. GA modulates the $CD4^+CD25^{high}$ Tr cells among MS patients not only at the intracellular cytokine level but also at the surface molecular level as it modulated surface HLA-DR, the activation marker.

6. Depletion of $CD4^+CD25^+$ Tr cells augment the secretion of Th1 cytokines IFN-γ and IL-2, but not of Th2 cytokine IL-13 in response to MBP; this effect of Tr cell depletion is observed not only in case of MBP, but also in case of a foreign antigen, PPD.

7. With the approach used, the functional activity of Tr cells is not significantly altered in MS, as compared to HC.

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