IMMUNOLOGICAL EFFECTS OF INTERFERON-β AND GLATIRAMER ACETATE IN MULTIPLE SCLEROSIS

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Stockholm 2004

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This thesis is dedicated to the memory of my father and Omar Elnour

To my mother
&
to Rihab, Mohammed and Sarah

ABSTRACT

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system, which results in demyelination, axonal loss and neurological deficits. The aetiology is unknown, however, immunopathological events, which are believed to be of an autoimmune nature, are considered to be responsible for the development of the disease. Interferon-β (IFN-β) and glatiramer acetate (GA; Copaxone) are disease-modifying therapeutic drugs for MS, and their efficacy is attributed to their immune regulatory properties. Studying their immunological effects in MS may lead to a better understanding of the pathogenesis of MS, and more importantly, may result in more efficient therapies.

The aims of this thesis were to examine the possible involvement of blood dendritic cells (DCs) in MS, and to investigate *in vitro* responses of DCs to IFN-β and GA separately, as well as in combination, and the possible synergistic effects between IFN-β and GA (papers I-II). Another aim was to examine responses of MS patients' blood mononuclear cells (MNCs) during combined treatment with IFN-β and GA, and their effects on the immunological markers under investigation in MS (papers III-IV).

Using flow cytometry and enzyme-linked immunosorbent assay (ELISA), we found that DCs from MS patients expressed higher levels of CD1a and produced lower amounts of interleukin-10 (IL-10) than DCs from healthy controls (HCs). IFN-β or GA upregulated HLA-DR and CD86, and reduced CD1a expression on DCs from both MS patients and HCs. IFN-β induced Th2-driving DCs, which produced increased amounts of IL-10 and reduced amounts of IL-12 as compared to control DCs. Accordingly, IFN-β-treated DCs, when co-cultured with MNCs from HCs, reduced the production of IFN-γ, but enhanced the production of IL-10 by MNCs. GA reduced IL-12 production by DCs in both MS patients and HCs. GA and IFN-β synergistically suppressed CD1a and enhanced CD86 expression on DCs. IFN-β induced Th2 immune responses via the DCs pathway. This may represent one mechanism for the action of IFN-β in MS. We assume that increased CD1a expression on DCs is relevant to autoaggressive immunity against myelin autoantigens in MS and that CD86 promotes Th2 responses, and that suppression of CD1a and up-regulation of CD86 by DCs could be beneficial in MS.

Cross-sectional comparisons revealed that the proportion of $CD1a^{+}$ $HLA\text{-}DR^{+}$ MNCs was higher and the production of IL-10 was lower in untreated MS patients as compared to patients treated with IFN-β or IFN-β and GA, or HCs. MNCs from untreated MS patients also had higher IFN-γ and IL-12 p70 production as compared to HCs or patients treated with IFN-β and GA, but not when compared to patients on monotherapy with IFN-β. The proportion of $CD1a^{+}$ $HLA\text{-}DR^{+}$ MNCs and the production of IL-12 p70 and IFN-γ were even lower in patients treated with IFN-β and GA in combinaton. Follow-up studies revealed that treatment with IFN-β and GA in combination, but not with IFN-β alone, resulted in normalization of $CD1a^{+}$ $HLA\text{-}DR^{+}$ MNCs levels, and of IL-12p70 and IFN-γ production by MNCs.

Using an automated computer-assisted enzyme-linked immunoadsorbent spot (ELISPOT) assay, we demonstrated that IL-12 and IFN-γ secreting MNCs were higher, and IL-10 were fewer in untreated MS patients than in patients treated with IFN-β or GA or IFN-β and GA, or in HCs. By contrast, MS patients treated with IFN-β or GA or IFN-β and GA in combination had similar numbers of IL-12, IFN-γ and IL-10 secreting MNCs compared to HCs. MS patients treated with IFN-β and GA in combination had similar numbers of IL-12, IFN-γ and IL-10 secreting MNCs compared to patients treated with IFN-β or GA. No differences were observed between MS patients treated with IFN-β, or GA and MS patients treated with IFN-β and GA, nor between any of these groups and HCs, for numbers of IL-4 secreting MNCs. The same trends for the IFN-γ, IL-10 and IL-4 responses to *in vitro* exposure to GA were found in all groups of MS. Suppression of IL-12 and IFN-γ, and promotion of IL-10 secretion by MNCs by combination therapy with IFN-β and GA was similar as monotherapy with IFN-β or GA. Treatment with IFN-β and GA in combination may not alter cytokine profiles by MNCs in response to GA.

In conclusion, both IFN-β and GA act in MS through distinct immunomodulatory effects and the immunological effects of IFN-β and GA in combination are complex. However, whether or not these immunological changes imply clinical benefits in MS, remains to be investigated in clinical trials.

CONTENTS

ABSTRACT 4

PREFACE 7

ABBREVIATIONS 8

1. INTRODUCTION 9
   1.1 Multiple sclerosis: General aspects 9
   1.2 Immunology of MS 11
      1.2.1 Initiation of immune responses 11
      1.2.2 Targeted antigens 13
      1.2.3 Th1 and Th2 concept in MS 14
   1.3 Are DCs important in the pathogenesis of MS? 15
   1.4 DCs and induction of tolerance to autoreactive T cells 16
   1.5 Treatment of MS 17
      1.5.1 IFN-β 17
      1.5.2 GA 18
      1.5.3 Mechanisms of action of IFN-β and GA in MS 18
      1.5.3 Combination of IFN-β and GA in MS 21

2. GENERAL AIM OF THE THESIS 22

3. MATERIALS AND METHODS 23
   3.1 Materials 23
      3.1.1 Patients with MS 23
      3.1.2 HCs 24
      3.1.3 Ethical committee approval 24
   3.2 Methods 24
      3.2.1 Media, monoclonal antibodies, recombinant human cytokines and GA 24
      3.2.2 Preparation of MNCs from blood 25
      3.2.3 Preparation of blood monocytes 25
      3.2.4 Generation of immature monocytes-derived DCs 25
      3.2.5 Flow cytometric analysis 25
      3.2.6 Allogeneic and autologous mixed leukocyte reactions 26
      3.2.7 Enzyme-linked immunosorbent assay (ELISA) 26
      3.2.8 An automated computer-assisted enzyme-linked immunoadsorbent spot assay (ELISPOT) 27
   3.3 Statistics 27

4. RESULTS 28
   4.1 Effects of IFN-β on morphology, phenotype and function of DCs (Paper I) 28
   4.2 Comparison of the effects of IFN-β and GA on DCs (Paper II) 29

4.3 Effects of IFN-β and GA in combination on blood MNCs in MS (Paper III and IV) 30
4.3.1 Surface markers on blood MNCs 30
4.3.2 Cytokine production by blood MNCs 32
4.3.3 Cytokine secretion by blood MNCs 32
4.3.4 Cytokine secretion by blood MNCs in response to *in vitro* exposure to GA 33
4.3.5 Cytokine secretion by GA-reactive blood MNCs 34

5. DISCUSSION 35
5.1 DCs: possible role in MS 35
5.2 IFN-β and GA and surface marker expression 36
5.2.1 IFN-β and GA separately or in combination - DCs 36
5.2.2 IFN-β alone or in combination with GA - Blood MNCs 37
5.3 cytokines in MS 38
5.4 IFN-β and GA - cytokine production and release 39
5.4.1 IFN-β and GA - DCs 39
5.4.2 IFN-β - Blood monocytes: Differentiation towards Th2-driving DCs 39
5.4.3 IFN-β alone or in combination with GA - Blood MNCs: Th1 cytokines 40
5.4.4 IFN-β alone or in combination with GA – Blood MNCs: Th2 cytokines 41
5.4.5 IFN-β and GA in combination – Blood MNCs: Cytokine profiles 41
5.5 Synergism of IFN-β and GA in MS? 42

6. CONCLUSIONS AND FUTURE PERSPECTIVES 44

7. ACKNOWLEDGEMENTS 45

8. REFERENCES 47

PREFACE

This thesis is based on the following articles and a manuscript, which will be referred to in the text by their Roman numerals:

I. Huang Y-M, **Hussien Y**, Yarilin D, Xiao B-G, Liu Y-J, Link H. Interferon-beta induces the development of type 2 dendritic cells. Cytokine 2001; 13: 264-271.

II. **Hussien Y**, Sanna A, Söderström M, Link H, Huang YM. Glatiramer acetate and IFN-beta act on dendritic cells in multiple sclerosis. J Neuroimmunol 2001; 121: 102-110.

III. **Hussien Y**, Sanna A, Söderström M, Link H and Huang Y-M. Multiple sclerosis: expression of CD1a and production of IL-12 p70 and IFN-γ by blood mononuclear cells in patients on combination therapy with IFN-β and glatiramer acetate compared to monotherapy with IFN-β. Multiple sclerosis 2004; 1: 16-25

IV. **Hussien Y**, Giannna A, Putheti P, Söderström M, Link H, and Huang Y-M. Multiple sclerosis: Effects of combination therapy with IFN-β and glatiramer acetate on cytokine profiles. Manuscript.

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ABBREVIATIONS

APCs	Antigen-presenting cells
BBB	Blood-brain barrier
CD	Cluster of differentiation
CNS	Central nervous system
CSF	Cerebrospinal fluid
DCs	Dendritic cells
EDSS	Expanded disability status scale
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunoadsorbent spot
GA	Glatiramer acetate
HCs	Healthy controls
IFN	Interferon
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MNCs	Mononuclear cells
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
PPD	Purified protein derivative
RR	Relapsing remitting
TCR	T cell receptor
Th	T helper
TNF	Tumour necrosis factor

1. INTRODUCTION

1.1 MULTIPLE SCLEROSIS: GENERAL ASPECTS

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS), characterized by focal demyelination, axonal loss and progressive neurological dysfunction (Filippi, 2001; Bjartmar et al., 2003; Rammohan, 2003). MS exclusively involves the CNS and is not associated with any other disorder. The prevalence of MS is highest among populations in Northern and Western Europe, approximately 1 in 1,000 (Kurtzke, 2000). MS typically begins in early adulthood and remains a major cause of disability in both the young and older population. The disease is about twice as common in women as in men (Runmarker and Andersen, 1993). Although more than 150 years have elapsed since the first description of MS, its aetiology has remained elusive. No definitive therapies to eliminate attacks and effectively stop the disease process are available.

The pathological hallmark of MS is the demyelinated plaque, which is characterized by loss of myelin, relative preservation of axons and formation of astrocytic scars (Ferguson et al., 1997; Bitsch et al., 2000; Paty and Arnold, 2002). MS lesions may occur anywhere within the white and grey matter, with a predilection for the highly myelinated areas. The infiltration of inflammatory cells is a characteristic pathological feature of the MS lesion (Hauser et al., 1986; van der Valk and De Groot, 2000), indicating the immune-mediated character of the demyelination. Distinct pathological subtypes may exist characterized by the degree of humoral contribution to the lesion, suspected target of the immune-mediated attack, and potential for remyelination (Lucchinetti et al., 1998; Bruck et al., 2003). This may have profound consequences in clinical practice, since distinct subtypes may require distinct therapeutic interventions (Kornek and Lassmann, 2003). Although MS predominantly affects the CNS myelin, studies indicate that irreversible axonal loss is a feature of the disease and contributes to long-term disability in MS (Trapp et al., 1998; Rammohan, 2003).

The starting point for diagnosis of MS is the clinical picture and, when necessary, the diagnosis is facilitated by supportive laboratory and radiological investigations (Poser et al., 1983; McDonald et al., 2001). A purely clinical diagnosis of MS is made upon the occurrence of two or more distinct attacks and clinical evidence of

two or more lesions in separate locations within the CNS. The diagnosis of MS is also made if a single clinical attack is accompanied by additional lesions discovered by paraclinical evidence, e.g. magnetic resonance imaging (MRI) abnormalities, cerebrospinal fluid (CSF) changes and abnormal evoked potentials. The lesions must be clearly disseminated in time and space. Besides meeting the criteria for MS, a diagnosis of MS requires that no better explanation can be found for alternative diagnoses (Gasperini, 2001).

The clinical course of MS is variable regarding development, fluctuation, remission and progression of symptoms. Eighty to eighty five percent of patients with MS initially have a relapsing-remitting (RR) course, which starts with a series of exacerbations and remissions over time. Years after onset, the number of relapses decreases, but a majority of these patients will have a secondary progressive phase when the neurological deficit increases between acute bouts. A minority (15%) of patients with MS have a primary phase characterized by a progressive course from the onset without clinically evident relapses, affects middle-aged people, more often men, and less marked lesions in the brain as determined by MRI (Revesz et al., 1994; Stevenson et al., 1999). The long-term clinical feature of MS is an increased disability: about 50% of MS patients will need walking assistance within 15 years after onset.

The aetiology of MS is unknown. However, circumstantial evidence suggests that a combination of environmental factors and genetic predisposition influences susceptibility and the course of MS (Hillert and Olerup, 1993; Ebers and Sadovnick, 1994; Willer and Ebers, 2000). The prevalence of MS varies strongly, depending on the genetic background (Rosati, 2001). Prevalence of MS is high in Caucasians and rare in Asians and Africans. Family members of MS patients are at greater risk, with the highest risk in monozygotic twins, but not increased in spouses and adopted children (Ebers and Dyment, 1998; Mumford et al., 1994; Ebers et al., 1995). Studies indicate that MS follows a polygenetic trait, which involves a large number of genes each with only a small contribution to the overall risk (Ebers and Dyment, 1998; Oksenberg et al., 2001; Compston and Coles, 2002). The role of genetic factors is even more complex, since they appear also to have impact on the disease course. Positional cloning and candidate gene approaches have largely yielded inconclusive results (Oksenberg et al., 2001). Only the human leukocyte antigen has consistently been associated with MS in Caucasians (Hillert and Olerup, 1993). Studies on migration (Dean and Kurtzke, 1971; Gale and Martyn, 1995; Dean and Elian, 1997), geographical

variations in the prevalence of MS (Kurtzke, 1980; Kurtzke, 2000), clusters (Dean, 1985) and epidemics of MS (Kurtzke et al., 1995) have provided evidence for certain environmental factors as possible risk factors (Rosati, 2001). Viral infections have long been suspected to be associated with MS (Fazakerley and Buchmeier, 1993; Buljevac et al., 2002; Tejada-Simon et al., 2003). The prevailing hypothesis of today is focusing on the immune system, suggesting that MS result from an interplay between genetic and environmental factors, culminating in autoimmune responses, which are confined to the CNS. However, it remains an open question whether autoimmune responses represent the original cause of MS, or are secondary phenomena, that occur at a certain stage of the disease.

1. 2 IMMUNOLOGY OF MS

Indirect evidence suggests that MS is an autoimmune disease, in which the immune system attacks self antigens of the CNS (see figure 1). Thus, there is an intense inflammatory reaction in the MS lesions, an association of susceptibility to MS with immune-regulating genes, alterations in the level of immune-mediating factors, and partial success of therapies that suppress or modulate the immune responses.

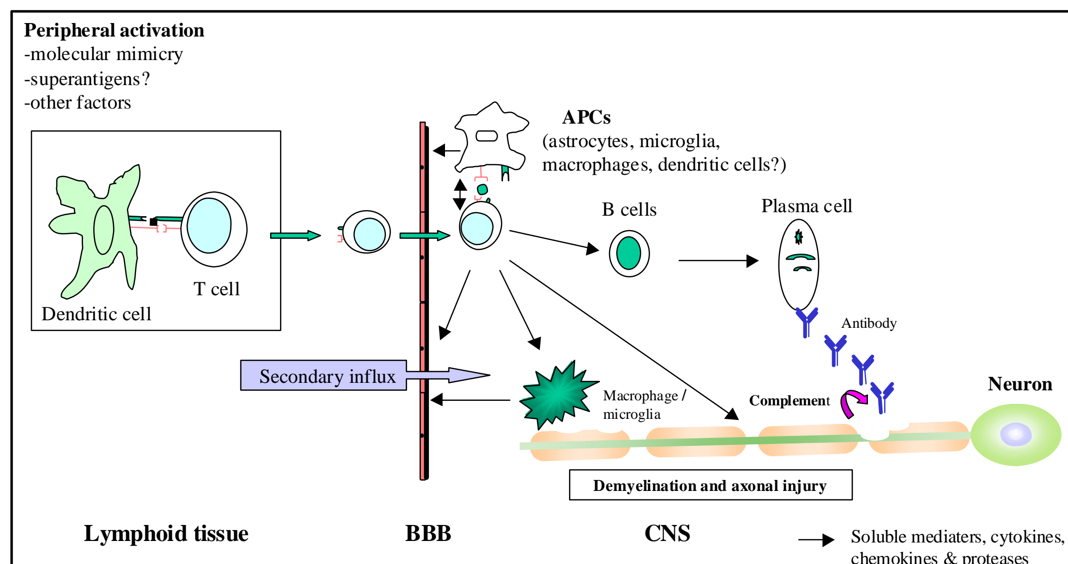

Fig. 1. Putative immunological steps involved in multiple sclerosis pathogenesis. BBB = blood-brain barrier; APCs = antigen presenting cells; CNS = central nervous system.

1.2.1 Initiation of immune responses

Findings in experimental autoimmune encephalomyelitis (EAE), an animal model of MS, and observations in human MS have led to the hypothesis that MS is an

autoimmune disease mediated by CNS antigen-specific T cells (Pettinelli and McFarlin, 1981; Hohlfeld, 1997; Hafler, 2004). These cells are directed to CNS antigens, and irrespective of their antigen specificity, they pass through the blood-brain barrier (BBB). The passing of T cells through BBB is mediated by the complex interplay of cellular adhesion molecules, chemokines, and metalloproteinases (Kieseier et al., 1999). Initially, only activated T cells are able to cross the BBB (Persidsky, 1999) after following their activation in the periphery (Hickey, 1991).

Co-stimulatory signals, together with the signal generated by formation of the trimolecular complex (MHC/peptide/T cell receptor (TCR)), are necessary for the activation of T cells. Recognition of the MHC-peptide complex by the TCR induces the expression of the cluster of differentiation 40 ligand (CD40L) on T cells. Engagement of the CD40L on T cells by CD40 on antigen presenting cells (APCs) leads to the upregulation of the CD80 and CD86 on APCs. Subsequently, CD80 or CD86 bind to CD28 on T cells which leads to activation of T cells (Grewal and Flavell, 1996). Association of MS with the MHC class II (Hillert and Olerup, 1993; Jersild et al., 1993) suggest that APCs may have role in MS. Dendritic cells (DCs) are the only APCs that express MHC class II that occupy the T cell areas of the lymph nodes, process antigens efficiently, and have high mobility (Steinman et al., 1997). DCs are probably professional APCs that activate naïve T cells in the secondary lymphoid organs. Whether DCs acquire antigens in the periphery or in the CNS, as suggested by their presence in human CSF (Pashenkov et al., 2001) needs to be clarified.

The initial activation process is necessary for the subsequent trafficking of immune cells into the CNS. Within the CNS T cells encounter their target antigens again through APCs. $CD4^{+}$ T cells recognize their antigens as peptides bound to MHC class II molecules, and respond by the production of inflammatory cytokines and chemokines. This results in the attraction of bystander immune cells from the blood, activation of microglia and astrocytes, production of myelinotoxic substances, and finally, in demyelination (Hohlfeld, 1997). $CD8^{+}$ T cells respond to antigen presented by MHC class I-expressing CNS cells, including neurons and glial cells (Hoftberger et al., 2004), resulting in the release of cytokines and direct damage of these cells (Hemmer et al., 2003).

1.2.2 Targeted antigens

In MS, T cells are essential for the initiation of a specific immune response and therefore, it is important to identify the initial antigen. In addition demyelinating factors are required (Lucchinetti et al., 2000). Although the immune system seems to play an important role in the pathogenesis of MS, the identify of target antigen is still uncertain and pathways leading to tissue destruction are not known (Hemmer et al., 2003).

Several putative self myelin antigens, including myelin basic protein (MBP) (Martin et al., 1991), proteolipid protein (Sun et al., 1991; Markovic-Plese et al., 1995), myelin oligodendrocyte glycoprotein (Sun et al., 1991; Kerlero de Rosbo et al., 1997), myelin oligodendrocyte basic protein (Holz et al., 2000), myelin associated glycoprotein (Link et al., 1992), and non-myelin antigens, including S100 (Schmidt et al., 1997) and B-crystallin (van Noort et al., 1995) have been proposed to be involved in the pathogenesis of MS. Moreover, some data suggest that autoantibodies (Link et al., 1990; Archelos et al., 2000) and cytokines (Navikas and Link, 1996) are important in the pathogenesis of MS.

Since the first description of MS, axonal pathology has been demonstrated to occur (Ludwin, 1981) and it has reported that axonal injury may occur early. Axonal injury may result from the primary attacks, or it can be secondary to demyelination (Owens, 2003). An immune attack that is directed primarily towards axonal, rather than myelin antigens may explain the progressive course of this form of MS. The inherent capacity for axonal regeneration in the CNS is much more limited, than the capacity for CNS remyelination, that is likely to contribute to the clinical recovery from attacks of RRMS. It is also possible that the transition from RR to secondary progressive MS, may involve spreading of the immune response from myelin to axonal antigens (Pender, 1998).

Gangliosides constitute an important group of axonal antigens and are also minor constituents of myelin. They are particularly enriched in the plasma membrane of axons and neuronal cell bodies, as compared to the plasma membrane of most other cell types (Kracun et al., 1984). Several studies have demonstrated increased responses of peripheral blood T lymphocytes to gangliosides in patients with MS (Beraud et al., 1990; Shamshiev et al., 1999; Pender et al., 2003) as well as increased levels of antibodies to gangliosides in serum and/or CSF samples from MS patients (Stevens et al., 1992; Sadatipour et al., 1998; Mata et al., 1999). The increased immune reactivity to gangliosides in MS may be pathogenic, and may contribute or be secondary to

axonal damage observed. The fact that the levels of antibodies to gangliosides are higher in MS than in patients with CNS axonal damage due to other neurological diseases suggests that the immunoreactivity is not secondary to the axonal damage, and but rather a possible pathogenic factor.

1.2.3 Th1 and Th2 concept in MS

The activation and clonal expansion of T cells require the recognition of an antigen presented by MHC class I and II molecules on APCs (eg, DCs, macrophages, monocytes and B cells), and the simultaneous delivery of a co-stimulatory signal (Delves and Roitt, 2000a; 2000b). This signal is transduced by discrete pairs of molecular markers on DCs and T cells, and is essential for optimal initiation and maintenance of T cell proliferation, trafficking, and cytokine production (Schoenberger et al., 1998). DCs, which also have the capacities to secrete cytokines such as interleukin (IL)-12 (Becher et al., 1999), may carry a third signal that contributes to T cell differentiation (Reid et al., 2000), and that may polarize naïve T cells to T helper (h)1 or 2 (Banchereau et al., 2000).

The concept of Th1 and Th2 was introduced in 1986 (Mosmann et al., 1986). Based on their distinctive cytokine secretion pattern, $CD4^+$ T cells are classified into Th1 and Th2 cells (Mosmann and Coffman, 1989). Th1 cells predominantly secrete IL-2, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ, and promote macrophage activation, antibody-dependent cell cytotoxicity, and delayed hypersensitivity and are associated with a switch to the IgG2a isotype. Th2 cells mainly produce IL-4, IL-5, IL-6, IL-10 and IL-13, promote humoral responses against extracellular pathogens, and are associated with a switch to IgG1 and IgE isotypies (Paul and Seder, 1994; Romagnani, 1996). Accordingly, cytokines can be classified as Th1 and Th2 type cytokines. There is a reciprocal antagonism between Th1 and Th2 responses.

Many studies suggest a role for cytokines in MS. MS is considered to be an autoimmune disease in which Th1 cells recognize components of CNS antigens and, in consequence, initiate a self-propagating autodestructive process within the CNS (Merrill and Benveniste, 1996; Link, 1998). In contrast, the Th2 response is considered beneficial in MS, as it is anti-inflammatory. Therefore, it has been suggested that an imbalance between Th1 and Th2 type cytokines in favour of the Th1 cytokines may result in an immune response that detrimental (Navikas and Link, 1996) (see figure 2).

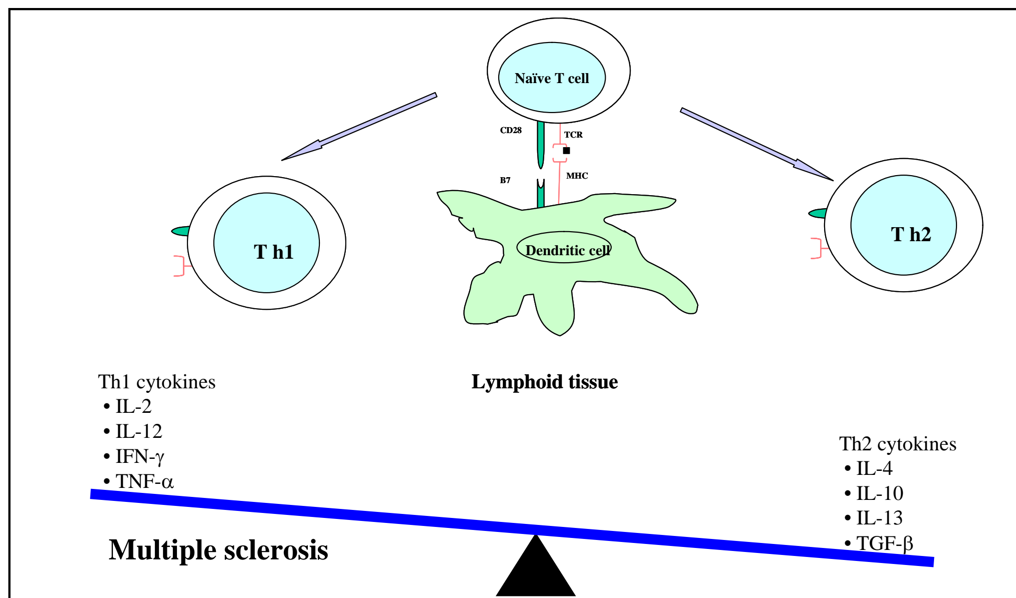

Fig. 2. Naïve T cells differentiate into T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells are pro-inflammatory, whereas Th2 cells are anti-inflammatory or regulatory. Evidence suggests that imbalance between Th1 and Th2 cytokines in favour of the Th1 may be detrimental in multiple sclerosis (Navikas and Link, 1999).

1.3 ARE DCs IMPORTANT IN THE PATHOGENESIS OF MS?

DCs are APCs that play a pivotal role in the initiation and regulation of adaptive immunity against pathogens and tumours, as well as in the triggering of autoimmunity. Any circulating T cells that have "autoreactive potential", presumably have T cell receptors with low affinity for autoantigens. It is thus likely, that DCs are involved in the priming and/or boosting of these T cells through various mechanisms. In the T cell areas of lymphoid organs, mature DCs not only present pathogen-derived peptides to T cells, but also adapt the class of immune response.

In MS, the dissemination of inflammation indicates that peripheral T cell activation is continuous. The cause of this is still unclear. Aberrations in the expression of surface markers and in the cytokines secretion by DCs could contribute to an initiation of aberrant immune responses in MS (Link et al., 1999). The presence of DCs in the CNS may represent one of the factors contributing to the chronicity in MS (Pashenkov et al., 2003b).

The proportion of DCs expressing CD1a, a molecule important for the presentation of lipid antigens to T cells, was found to be higher in MS patients as compared to healthy subjects (Huang et al., 1999). The activation of autoreactive T cells requires presentation by APCs, and therefore, defective co-stimulatory signalling has been implicated in the development of MS. For example, the co-stimulatory pathway has been shown to be involved in the development of EAE (Kuchroo et al.,

1995). It was demonstrated that a reduced proportion of the DCs in MS patients express the co-stimulatory molecule CD86, which is assumed to promote Th2 differentiation (Huang et al., 1999). IL-12 is important for the control of Th1/Th2 responses, e.g. for Th1 differentiation (Heufler et al., 1996). Interestingly, high levels of circulating DCs secreting pro-inflammatory cytokines, are associated with MS (Huang et al., 1999). Recently, it was reported that human CSF contains DCs, and that there are elevated numbers in early and in clinically definite MS (Pashenkov et al., 2001; Pashenkov et al., 2002). Moreover, DCs have been detected in MS lesions (Plumb et al., 2003). The migration of DCs to the CNS may be mediated by signals in the shape of CNS-produced chemokines (Pashenkov et al., 2002). These findings suggest that DCs are likely to have a role in the pathogenesis of MS (Link et al., 1999; Pashenkov et al., 2003b).

1.4 DCs AND INDUCTION OF TOLERANCE TO AUTOREACTIVE T CELLS

DCs are specialized to present pathogens to T cells and to initiate primary immune responses (Schuler and Steinman, 1997; Banchereau and Steinman, 1998). However, mature DCs are also capable of presenting self antigens (Guery and Adorini, 1995). Protection from autoimmunity is thought to be due to removed of T cell clones that are directed to self antigens by the occurrence of self tolerance mechanisms. These include negative selection in the thymus, which is driven by thymic DCs (Ardavin, 1997) and peripheral tolerance, which is incompletely understood. Clearly, if either thymic or peripheral tolerance mechanisms fail, autoreactive T cells against self antigens will survive, and may potentially cause an autoimmune attack.

The role of DCs in maintaining peripheral tolerance has been investigated. Repetitive stimulation of naïve T cells with immature DCs generates regulatory T cells (Jonuleit et al., 2000). In addition, immature DCs capture apoptotic bodies (Huang et al., 2000), which do not induce DCs maturation (Gallucci et al., 1999). Immature DCs presenting self antigens in the absence of the co-stimulatory signals, may induce T cell non-responsiveness (anergy) or regulatory T cells. Pretreatment of DCs with IL-10 induced a state of alloantigen-specific anergy in $CD4^+$ and $CD8^+$ T cells, due to conversion into tolerogenic APCs (Steinbrink et al., 1997; Steinbrink et al., 1999). Apoptosis has been implicated in peripheral tolerance. Repeated activation of T cells by DCs was shown to trigger T cell death (Suss and Shortman, 1996). This effect may be mediated by different molecular mechanisms (Link et al., 2001).

Further studies are needed to investigate the role of DCs in the induction of tolerance to autoreactive T cells.

1.5 TREATMENT OF MS

The accumulated knowledge regarding a possible immunopathogensis in MS has lead to many potential targets and modes of therapy for MS. Therapies in MS are directed at resolving acute attacks, reducing the number of exacerbations, treating the sequelae of previous attacks, and preventing progression of disability. Symptomatic treatments include measures against problems with ambulation, spasticity, fatigue, neurogenic sphincter dysfunction, and cognitive and affective disorders.

Corticosteroids are often used to treat clinically significant relapses in an attempt to fasten recovery. Advances in the MS treatments with immunomodulatory therapies include interferon-beta (IFN-β)-1a, 1b, and glatiramer acetate (GA). However, there are some limitations, including cost, inconvenience, adverse effects, long-term efficacy, and importantly these drugs are not curative (Forbes et al., 1999; Noseworthy, 1999). Each of these agents has a number of immune-mediating activities that decrease CNS inflammation. However, their specific mechanisms of action in MS are incompletely understood. More detailed knowledge on how currently available, but only partly effective, treatment strategies regulate the immune system may lead to a better understanding of the disease mechanism. In the long term, this could lead to development of more potent drugs or drug combinations for the treatment of MS.

This thesis is focused on the possible immunological effects of IFN-β and GA in MS.

1.5.1 IFN-β

IFNs are cytokines that mediate anti-viral, anti-proliferative and immunomodulatory activities in response to viral infection and other pathogens. The initial rationale for applying IFN-β in MS was based on the theory that recurrent or continued disease activity may be related to viral persistence or latency in the CNS. At the outset, IFN-α (Knobler et al., 1984), IFN-γ (Panitch et al., 1987) and IFN-β (Jacobs et al., 1981) were assessed in MS. Currently, two forms of recombinant IFN-β (IFN-β1a and IFN-β1b) have been approved for the treatment of MS. IFN-β1a (Avonex, Rebif) is a glycosylated, recombinant product from mammalian cells, with an amino acid sequence identical to that of natural IFN-β. IFN-β1b (Betaferon) is a non-

glycosylated recombinant product from bacterial cells in which serine is substituted for cysteine at position 17.

All three drugs have been studied in large double-blind placebo-controlled randomised clinical trials, which show reduction in frequency of exacerbation by about one third (The IFNB Study Group, 1993; Jacobs et al., 1996; PRISMS, 1998), and these findings were supported by MRI data showing reduction of active lesions and lesion load in the brain (Paty and Li, 1993; Li and Paty, 1999; Simon et al., 1998).

1.5.2 GA

GA (Copolymer 1, Copaxone) is a mixture of synthetic polypeptides composed of L-glutamic acid, L-lysine, L-alanine and L-tyrosine, with molar residue ratio of 0.14:0.34:0.43:0.09 and an average molecular mass of 4.7 - 11.0 kDa. GA was originally developed to mimic MBP for the induction of EAE (Teitelbaum et al., 1971). Surprisingly, however, it turned out that GA inhibits MBP-induced EAE (Arnon et al., 1996). It has been shown that GA has beneficial effect on clinical symptoms of MS (Bornstein et al., 1987; Johnson et al., 1995; Johnson et al., 1998) and reduces the pathological changes observed by MRI (Mancardi et al., 1998; Comi et al., 2001; Filippi et al., 2001).

1.5.3 Mechanisms of action of IFN-β and GA in MS

In spite of the clinical advances, the mechanisms behind the beneficial effects of IFN-β and GA in MS are incompletely known. IFN-β and GA have immunological effects, which probably are the most relevant to their clinical efficacy in MS (Yong et al., 1998; Yong, 2002; Zhang et al., 2002). It is possible that the efficacy of these drugs is mediated by different mechanisms that regulate various components of the inflammatory process in MS (see figure 3).

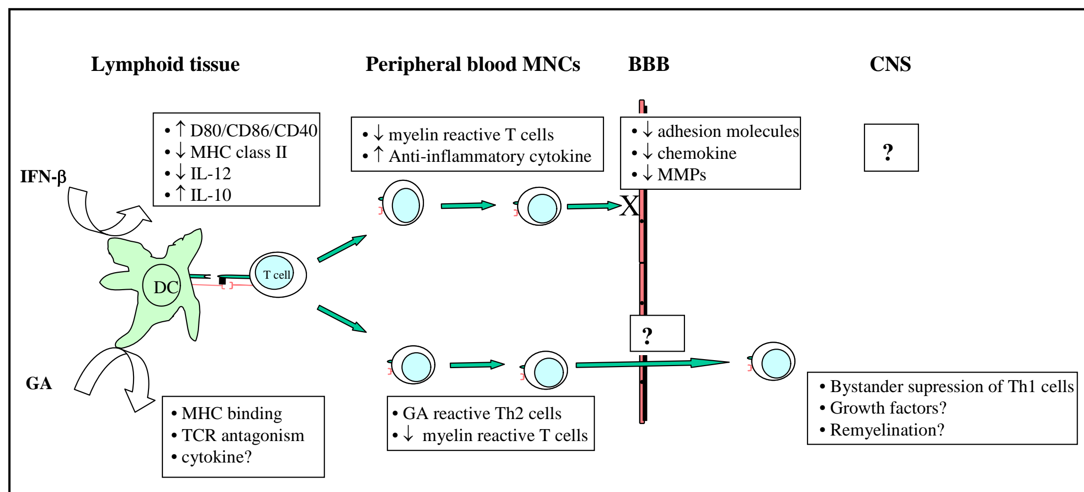

Fig. 3. Putative mechanisms of interferon-β (IFN-β) and glatiramer acetate (GA) in MS, by reduction of central nervous system (CNS) inflammation. MHC = major histocomatibility complex; DC = dendritic cell; TCR = T cell receptor; MNCs = mononuclear cells; BBB = blood-brain barrier; MMPs = matrix metalloproteinases; ↓ decrease; ↑ increase; ? Not clear.

In the periphery, IFN-β and GA affect antigen presentation and cytokine milieu by different mechanisms (Yong, 2002). IFN-β affects antigen presentation by modulating the expression of molecules that are necessary for this process. IFN-β blocks the IFN-γ-induced expression of MHC class II on APCs (Arnason et al., 1996) and has been shown to reverse the increased expression of the co-stimulatory molecule CD80 on B lymphocytes derived from patients with MS (Genc et al., 1997). IFN-β also interrupts T cell activation by altering the co-stimulation pathway at the level of DCs (Bartholome et al., 1999a; 1999b; Huang et al., 2001b).

GA seems to preferentially affect immune cells specific for MBP and perhaps other myelin antigens (Arnon et al., 1996; Teitelbaum et al., 1997). *In vitro* studies suggest that GA competes with MBP for binding to MHC molecules in the peptide-binding groove (Webb et al., 1973; Teitelbaum et al., 1988; Teitelbaum et al., 1992), and the complex of GA bound to MHC molecule competes with the complex of MBP bound to MHC, for binding to the TCR (Aharoni et al., 1999). This will lead to the generation of GA-specific T cells, which are regulatory T cells of the Th2 type, as has been demonstrated in animal studies (Aharoni et al., 1997; 1999), and in leukocytes from MS patients treated with GA (Sela and Teitelbaum, 2001).

IFN-β has shown to reduce the proliferation of T cells (Noronha et al., 1993) and to inhibits the expression of Fas-associated death domain-like interleukin-1beta-converting enzyme inhibitory protein (FLIP), an anti apoptotic protein, resulting in the death of T cells (Sharief et al., 2001). In contrast, GA was shown to induce proliferation

of blood lymphocytes (Burns et al., 1986; Qin et al., 2000). Both IFN-β and GA may reduce the frequency of MBP-reactive T cells. Thus, treatment with IFN-β resulted in reduction of MBP-reactive T cells in MS patients (Zang et al., 2000). GA was found to inhibit MBP-induced proliferation of human MBP-specific T-cell lines and their secretion of IL-12 (Teitelbaum et al., 1992). Furthermore, GA was shown to induce a state of non-responsiveness (anergy) in MBP-specific T-cell clones (Gran et al., 2000).

Both IFN-β and GA modulate cytokine production. IFN-β was shown to inhibit IL-12p40 expression by DCs (McRae et al., 1998; Karp et al., 2000; Huang et al., 2001b) and to decrease the T cells production of pro-inflammatory mediators such as IFN-γ and TNF-α, which are considered detrimental in MS (Noronha et al., 1993; McRae et al., 1997). In addition, IFN-β treatment was observed to stimulate the production of the anti-inflammatory cytokines IL-10 and IL-4 (Rudick et al., 1998). GA induces a shift from Th1 to Th2 (Miller et al., 1998; Neuhaus et al., 2000). Only recently, it was reported that GA also promotes the development of Th2 cells and increases IL-10 production through modulation of DCs (Vieira et al., 2003).

In the CNS, IFN-β was shown to reduce the passage of immune cells across the BBB by reducing the expression of adhesion molecules, chemokines, chemokine receptors and matrix metalloproteinases, which are important for migration of inflammatory cells into the CNS (Iarlori et al., 2000; Avolio et al., 2003; Nelissen et al., 2003; Sellebjerg and Sorensen, 2003; Waubant et al., 2003). In contrast, GA does not appear to affect the transmigration of immune cells into the CNS (Yong, 2002). GA-reactive Th2 cells readily cross the BBB (Aharoni et al., 2000), and within the CNS they believed to decrease CNS inflammation by a phenomenon described as bystander suppression. Some form of partial cross-reaction or competition between MBP and GA has been considered crucial to the mechanism of action of GA. Most investigators of the human immune response to GA found that GA and MBP cross-stimulate T cells more profoundly at the level of cytokine production (Gran et al., 2000; Neuhaus et al., 2000; Qin et al., 2000), which is not restricted to MBP (Neuhaus et al., 2000). The expansion of GA-reactive Th2 cells within the CNS result in the release of anti-inflammatory cytokines, which can impair the expansion of myelin autoreactive T cells. Moreover, GA-specific T-cell lines can produce brain-derived neurotrophic factors (Ziemssen et al., 2002), which may attenuate injury or reduce the inflammation through their immunoregulatory effects (Hohlfeld et al., 2000).

1.5.3 Combination of IFN-β and GA in MS

Although IFN-β and GA have provided a new approach to the treatment of MS (The IFNB Study Group, 1993; Johnson et al., 1995; Jacobs et al., 1996; PRISMS, 1998; Johnson et al., 1998), patients may or may not respond beneficially to either IFN-β or GA. Since IFN-β and GA act through different immunomodulatroy mechanisms in MS (Yong, 2002) (see above) the combination of these drugs may be considered for MS treatment.

Based on the current understanding of the mechanisms of action of IFN-β and GA, their interaction may be highly complex. On one hand, a combination of IFN-β and GA may create a synergy to effectively regulate the immune system. This combination could lead to a more profound regulatory effect on the cytokine profile, on autoreactive T cells, and on other autoimmune inflammatory components that may be of importance in the disease process. On the other hand, IFN-β and GA have opposite effects in proliferation thus, the beneficial effect of GA on the activation of Th2 cells may be blocked by the addition of IFN-β. In support of this possibility, a recent study demonstrated that IFN-β antagonises the *in vitro* stimulatory effect of GA on Th2cells and on their cytokine production (Zang et al., 2003). Furthermore, the combination of IFN-β and GA did not produce a synergistic effect on the inhibition of T-cell responses to MBP, as compared to the effect by each agent alone. In EAE, IFN and GA were effective in the treatment when used alone. However, the effects disappeared when IFN and GA were combined (Brod et al., 2000).

It has been shown that treatment with IFN-β results in the production of neutralizing antibodies to IFN-β and this may affect its clinical efficacy (Sorensen et al., 2003). The titers of antibodies correlated with the dosage of IFN-β (Giovannoni et al., 2002). When combining IFN-β with GA, reduction of the IFN-β dose may decrease foundation of IFN-β antibodies.

The immunological effects of combining IFN-β and GA on MS patients are unclear. Both IFN-β and GA are potent drugs that affect several stages of the process that contributes to MS pathology. While both drugs ultimately lead to reduction of a pro-inflammatory response in the periphery and within the CNS, the mechanisms by which they do so are remarkably different.

2. GENERAL AIM OF THE THESIS

The aim of is thesis was to further explore the immunological changes induced by IFN-β and GA separately, as well as in combination. Specifically, we investigated cytokine production and surface molecule expression by DCs and mononuclear cells (MNCs).

Specific aims

Paper I. to examine the effects of IFN-β on DCs from healthy subjects by examining the expression of HLA-DR, CD1a, CD11c, CD123, CD40, CD80 and CD86 and the production of IL-10 and IL-12p40 *in vitro*;

Paper II. to investigate *in vitro* the immunological effects of IFN-β and GA separately, as well as in combination, on DCs from MS patients vs. healthy controls (HCs), with regard to the expression of HLA-DR, CD1a, CD11c, CD54, CD123, CD80 and CD86, and the production of IL-10, IL-12p40, and to examine the involvement of DCs in MS;

Paper III. to compare the effects of IFN-β alone with the effects of the combined treatment with IFN-β and GA with respect to the expression of HLA-DR by blood MNC and CD1a, CD80 and CD86 on HLA-DR$^+$ MNC, and the production of IL-4, IL-10, IL-12 p40, IL-12 p70 and IFN-γ by blood MNC, and to examine the involvement of these immunological markers in MS;

Paper IV. to analyse the effects of IFN-β or GA, and of the combined treatment with IFN-β and GA with respect to the secretion of IL-12, IFN-γ, IL-10 and IL-4 by blood MNC, and to examine the involvement of these cytokines in MS.

3. MATERIALS AND METHODS

3.1 MATERIALS

These studies were performed on peripheral blood MNCs and blood monocytes-derived dendritic cells (DCs) from HCs and MS patients. Some of the studies are carried out directly on MNCs, and some on DCs.

3.1.1 Patients with MS

Only patients with clinically definite MS were included (Poser et al., 1983) in these studies. In all patients, the score of the expanded disability status scale (EDSS) was determined (Kurtzke, 1983).

The groups of MS patients included in the present studies are: MS patients treated with IFN-β or GA, or IFN-β in combination with GA, as well as untreated MS patients.

Untreated MS patients: Fifty one patients had never been treated with immunomodulatory drugs including corticosteroids and IFN-β.

IFN-β treated MS patients: Seventy eight patients had been treated IFN-β; Fifty nine patients had been treated for a minimum of 6 months with IFN-β1a (58 patients received Avonex 30 μg in 1 ml by the intramuscular route once weekly, and one patient received Rebif 22 μg in 1 ml by the subcutaneous route 3 times/week); Nineteen patients were enrolled in a follow-up study after receiving IFN-β1a (Avonex) administered as monotherapy at a recommended dose for >1 year, then the patients followed over 6 months after shifting to treatment with IFN-β1a (Avonex) as recommended doses plus GA (Copaxone) administered at dose 20 mg subcutaneously daily.

GA treated MS patients: Fifteen patients had been treated with GA (Copaxone) administered at recommended doses.

IFN-β combined with GA treated patients: Thirty-nine patients had been treated with a combination of IFN-β1a (Avonex) in combination with GA (Copaxone) at recommended doses for at least 4 months when included.

All patients receiving combination therapy with IFN-β and GA had deteriorated clinically during ongoing therapy with IFN-β as reflected by the occurrence of ≥2 exacerbations or worsening of the EDSS by 1.0 or more over one year. Alternatively they experienced two or more of T1 lesions on MRI of the brain and / or spinal cord

compared to corresponding MRI performed earlier during ongoing therapy with IFN-β1a. After adding GA administered at a recommended dose, the patients were followed and re-examined after 1-3 and 4-6 months. Blood sampling was not standardized for interval from the last injection of IFN-β1a.

3.1.2 HCs

Blood samples from HCs were obtained from the staff of the Department and from blood donors**.**

3.1.3 Ethical committee approval

Studies were approved by the Ethical Committee of the Karolinska Institute at Huddinge University Hospital. Numbers of the ethical permission are 335/98, 133/99, and 285/03.

3.2 METHODS

3.2.1 Media, monoclonal antibodies, recombinant human cytokines and GA

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 monoclonal antibodies (mAbs) were purchased: phycoerythrin (PE)-conjugated CD123, CD80, CD56, CD40, CD14, and CD11c, fluorescein isothiocyanate (FITC)-conjugated CD86, CD83, CD19, and CD1a from Pharmingen (San Diego, CA); FITC-conjugated lineage cocktail 1 (CD3, CD14, CD16, CD19, CD20, CD56) and CD54 (ICAM-1) from Serotec (Oxford, UK), and peridin chlorophyll protein (PerCP) conjugated CD3 and HLA-DR from Becton Dickinson (San Jose, CA). Isotype control mAbs were FITC-, PE- or PerCP-conjugated IgG1 or IgG2a (Becton Dickinson). Recombinant human (rh) GM-CSF (Leucomax; Novartis, Basel, Switzerland) and rhIL-4 (Genzyme, Cambridge, MA or R&D System, Minneapolis, MN) were used. IFN-β-1b (Betaferon; Schering AG, Berlin, Germany) and glatiramer acetate (Copaxone; Teva, Petah Tiqva, Israel) prepared for therapeutic purposes were used.

3.2.2 Preparation of MNCs from blood

Blood MNCs were isolated by density gradient centrifugation of heparinised blood on Lymphoprep (Nycomed, Oslo, Norway). Cells from the interphase were collected, washed three times with RPMI 1640 medium (Gibco, Paisley, Scotland) and resuspended at 1 x 10^6 cells/ml in RPMI and used for the generation of DCs (Paper I and II) or cultured for 48 h in complete medium (Paper III and IV).

3.2.3 Preparation of blood monocytes

Isolated MNCs were resuspended again at 5 x 10^6 cells/ml in RPMI, plated in 12-well plastic plates (1 x 10^7 cells/well) and incubated for 2 h at 37°C in a humidified atmosphere with 5% CO_2. Non-adherent cells were removed. The adherent cells (monocytes) were cultured in 2 ml of RPMI 1640 medium, supplemented with 2 mM L-glutamine, 1% non-essential amino acids, 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin (all from Gibco).

3.2.4 Generation of immature monocytes-derived DCs

To generate immature monocyte-derived DCs, monocytes were prepared as described above (3.2.3). Adherent monocytes were cultured in 2 ml of culture medium with or without test substance in addition of 500 U/ml GM-CSF and 250 U/ml IL-4. On day 3, 1 ml of medium was removed and replaced with the same volume of medium containing 1,000 U/ml of GM-CSF and 500 U/ml of IL-4. On day 7, DC were harvested, washed, counted, and analysed.

3.2.5 Flow cytometric analysis

Cell surface molecules were examined in a FACScan flow cytometer with CellQuest software (Becton Dickinson) using two or three colour staining. Cells at a concentration of 1 x 10^6/ml in PBS pH 7.4, were incubated for 20 min at 4^{0}C in the dark with specific or control antibodies. After staining, the cells were washed and resuspended in 200 μl of PBS. Non-viable cells were gated out by forward and side light scatter. FACS data were presented as percentage of positive cells calculated by gating against samples labelled with irrelevant control isotype-matched mAb.

3.2.6 Allogeneic and autologous mixed leukocyte reactions

To characterize the DCs, mixed leukocyte reactions (MLRs) were performed by co-culturing DCs with MNCs from HCs (Bartholome et al., 1999a). Allogeneic or autologous MNCs were used as responders at a concentration of 10^5 cells per well were co-cultured with 1 x 10^3 or 1 x 10^4 cells per well of irradiated (1,500 rad) DCs as stimulators, for three days in 96-well round-bottom mictrotitre plates in triplicates (Costar, Cambridge, MA). Cultures were maintained in a humidified atmosphere at 37°C with 5% CO_2 for 72 h and then pulsed with 1 μCi/well [^{3}H]-thymidine (Amersham Life Science, Buckinghamshire, UK) for 18 h. [^{3}H]-thymidine incorporation was measured using a beta-scintillation counter (Beckman, Fullerton, CA, USA). The results were expressed as counts per minute (cpm).

3.2.7 Enzyme-linked immunosorbent assay (ELISA)

Cytokine production by DCs was determined in supernatants obtained after 7 days culture of the DCs (Paper I and II). Determination of cytokine production by MNCs co-cultured with DCs was performed in supernatants collected after 72 h (Paper II). Cytokine production by MNCs cultured in complete medium, was determined in supernatants collected after 48 h (Paper III). The supernatants were stored at -20°C until assayed for cytokines. Production of cytokine was measured by enzyme-linked immunosorbent assay (ELISA) (kits from PharMingen) following the manufacturer's protocols. Briefly, 96-well plates (Costar) were coated with capture mAb overnight at 4°C. The following day, the plates were washed with 0.05% Tween 20 in filtered phosphate-buffered saline (PBS) and blocked with 200 μl/well of 10% FCS in PBS. The plates were washed 3 times with PBS containing 0.05% Tween. Cytokine standards and samples were diluted in PBS containing 0.05% Tween and 2% FCS, and 100 μl were added to each well and incubated for 2 h at room temperature. Both samples and standards were run in duplicates. After washing, biotinylated secondary mAb with avidin-horseradish perxidase at 1:250 dilution was added and incubated for 1 h at room temperature. After washing, tetramethylbenzidine/hydrogen peroxide (PharMingen) was used as a substrate and colour development was stopped using 1 M H_2SO_4. Optical densities were measured using an ELISA reader. Cytokine concentrations were calculated using standard curves that were performed for each ELISA plate.

3.2.8 An automated computer-assisted enzyme-linked immunoadsorbent spot assay (ELISPOT)

An automated computer-assisted enzyme-linked immunoadsorbent spot (ELISPOT) is a technique that allows detection and enumeration of individual protein-secreting cells. Solid-phase ELISPOT assays as described by (Czerkinsky et al., 1988) was adopted to detect and enumerate IL-12, IFN-γ, IL-10 and IL-4 secreting MNCs (Paper IV). Microtitre plates with nitrocellulose bottoms (Multiscreen-HA plates; Millipore, Mulsheim, France) were coated overnight at 4°C with 100 μl/well of monoclonal anti-human IL-12 (IL12-I), IFN-γ (1-D1K), IL-10 (9D7) or IL-4 (IL4-I) antibody (Mabtech, Stockholm, Sweden) diluted in filtered PBS (pH 7.4) to a concentration of 10 μg/ml. After removal of the coating solutions the plates were washed with PBS, and then 200 μl of each MNCs suspension containing 2×10^5 blood MNCs was assayed. The samples were assayed in duplicate. The plates were incubated at 37°C for 48 h in humidified air containing 5% CO2, emptied and washed before addition of monoclonal biotinylated anti-human IL-12 (IL12-II), IFN-γ (7-B6-1), IL-10 (12G8), or IL-4 (IL4-II) (Mabtech) diluted in filtered PBS to a concentration of 1 μg/ml. After incubation the wells were washed and 100 μl of streptavidin-alkaline phosphatase (Mabtech) diluted 1:1,000 was added. After 1.5 h incubation at room temperature, the plates were emptied, washed and stained with BCIP-NBT (Mabtech). Blue spots were seen after 5 - 15 min and the reaction was allowed to proceed for another 5 min before the wells were rinsed, emptied and dried over night. Each spot was considered to represent a cell secreting IL-12, IFN-γ, IL-10 or IL-4. The number of cytokine secreting MNCs was analysed with an automated imaging system and appropriate computer software (Ks ELISPOT automated image system; Zeiss, Jena, Germany).

3.3 STATISTICS

The Student's t-test or Mann-Whitney U-test was used for two group comparisons. For multiple comparisons, non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) test or non-parametric Friedman ANOVA test was used followed by Dunn's test and Bonferroni test, respectively. $P < 0.05$ was considered statistically significant. Wilcoxon signed rank test was used for comparing two time-points from paired samples from the same individuals.

4. RESULTS

4.1 EFFECTS OF IFN-β ON MORPHOLOGY, PHENOTYPE AND FUNCTION OF DCs (PAPER I)

IFN-β is one of the few treatments approved for MS, but the mechanisms of action of IFN-β in MS are not fully understood. Elucidation of the effects of IFN-β on DCs would increase our understanding of its immunoregulatory mechanisms and, indirectly, the pathogenesis of MS. Results from such studies could also promote the search for more effective therapies. Therefore, the *in vitro* effects of IFN-β on DCs were studied.

Exposure of DCs to IFN-β *in vitro*, resulted in lower degree of clustering of DCs, which became more irregular in shape and smaller in size, as compared to DCs cultured without IFN-β. These effects became increasingly obvious with higher concentrations of IFN-β (1000 and 10 000 U/ml).

Increasing the IFN-β concentrations resulted in higher expression of CD123 and HLA-DR, and lower expression of CD1a on the DCs. Furthermore, the increase in IFN-β concentrations, gave rise to an induction of the co-stimulatory molecules CD80 and CD86 on the DCs. The increase in IFN-β levels to 1 000 and 10 000 U/ml, gave rise to upregulation of the co-stimulatory molecule CD40 (see table 1).

Table 1. Phenotypic analysis of dendritic cells (DCs) derived from healthy controls. The DCs were cultivated without IFN-β (control DCs) or with IFN-β at different concentrations for 7 days. Results are shown as the mean values of percentages of 1×10^4 cells ± SD.

IFN-β (U/ml)	CD123	CD1a	CD40	CD80	CD86	HLA-DR
0 (control DCs)	1 ± 0.5	56 ± 16	4 ± 3	7 ± 3	30 ± 10	27 ± 13
10	5 ± 2	45 ± 14	11 ± 4	26 ± 13	45 ± 16	47 ± 18
100	22 ± 8	33 ± 12	10 ±3	27 ± 15	63 ± 18	56 ± 15
1 000	35 ± 10	23 ± 9	16 ± 7	21 ± 9	67 ± 12	55 ± 13
10 000	44 ± 15	16 ± 7	17 ± 12	20 ± 14	69 ± 11	65 ± 10

To investigate whether the changes in surface markers are relevant to altered DCs function, IFN-β-treated DCs were compared to control DCs with regard to their cytokine profile. The levels of IL-10 were increased, whereas the IL-12p40 production was suppressed in IFN-β-treated DCs.

Analysis of MLR with blood MNCs as responders showed that IFN-β-treated DCs stimulated the IL-10 production, but reduced IFN-γ production by blood MNCs, suggesting IFN-β skew the immune response from Th1 towards Th2 via a DC pathway.

4.2 COMPARISON OF THE EFFECTS OF IFN-β AND GA ON DCs (PAPER II)

Several studies have implicated the involvement of DCs in MS (Pashenkov et al., 2001; Pashenkov et al., 2003b; Plumb et al., 2003). It has been proposed, that the beneficial effects of IFN-β in MS patients may depend, at least in part, on its modulation of DCs functions (Karp et al., 2000; Huang et al., 2001b). However, the effects of GA on DCs have received little attention. Since a combined treatment with IFN-β and GA is considered for MS patients, we have analysed the effects of these agents in combination on DCs.

We found that GA and IFN-β, alone or together, when added at the initiation of DCs culture, stimulated (within 3 days) the development of adherent MNCs into non-adherent, floating DCs. DCs cultured in the presence of IFN-β, or GA plus IFN-β were less clustered, more irregularly shaped and smaller, as compared to DCs cultured with only or in the absence of these substances.

Both GA and IFN-β induced up-regulation of HLA-DR on DCs derived from HCs and IFN-β induced the up-regulation of HLA-DR and CD123 on DCs derived from MS patients. However, GA did not further increase this effect by IFN-β (see table 2).

Table 2. Phenotypic analysis of dendritic cells (DCs) derived from patients with multiple sclerosis (MS) and healthy controls (HCs). The DCs were cultivated in the absence of GA and IFN-β (control DCs) or in the presence of GA or IFN-β or IFN-β+GA for 7 days. Results are shown as the mean values of percentages of 1 x 10^4 cells ± SD.

		HLA-DR	**CD1a**	**CD86**
Control (DCs)	MS	49 (24)	50 (20)	42 (15)
	HCs	40 (21)	29 (20)	44 (19)
GA-DCs	MS	57 (24)	30 (17)	64 (20)
	HCs	51 (18)	16 (13)	66 (19)
IFN-β-DCs	MS	66 (23)	32 (19)	79 (21)
	HCs	62 (21)	11 (8)	79 (18)
(GA+IFN-β)-DCs	MS	62 (24)	12 (11)	89 (10)
	HCs	57 (20)	8 (5)	78 (20)

The expression of CD1a was found to be higher in DCs derived from MS patients than in DCs from HCs. Treatment with either GA or IFN-β suppressed the CD1a expression on DCs irrespective of whether they were derived from MS patients or HCs. The treatment with GA and IFN-β in combination resulted in a synergistic effect on MS DCs (see table 2).

Both GA and IFN-β increased CD86 expression on DCs from HCs and MS patients. GA and IFN-β in combination resulted in a synergistic effect on DCs from MS patients (see table 2).

The spontaneous production of IL-10 was lower in DCs from MS patients than from HCs, and the increase in IL-10 production by treatment with IFN-β was also lower in DCs from MS patients. The addition of GA to the cultures did not affect IL-10 production by DCs from either HCs or MS patients.

Spontaneous IL-12 p40 production by DCs did not differ between MS patients and HCs. Both IFN-β and GA reduced IL-12 p40 production by DCs from MS patients as well as HCs. There was no synergistic effect of culturing with IFN-β and GA in combination.

4.3 EFFECTS OF IFN-β AND GA IN COMBINATION ON BLOOD MNCs (PAPER III AND IV)

In view of varying responsiveness in MS patients to IFN-β and GA, the combination of IFN-β and GA may be considered, and it is therefore important to analyse the effects in MNCs in MS patients. Both cross sectional and longitudinal studies were performed with this purpose.

4.3.1 Surface markers on blood MNCs

In the cross-sectional study we found that untreated MS patients had higher number of $CD1a^+$ $HLA\text{-}DR^+$ MNCs than the HCs (see table 3). Patients treated with IFN-β alone had lower $CD1a^+$ $HLA\text{-}DR^+$ MNCs as compared to untreated MS patients, and patients treated with IFN-β and GA in combination had even lower $CD1a^+$ $HLA\text{-}DR^+$ MNCs (see table 3). The MS patients examined during monotherapy with IFN-β, selected for the follow-up study, had higher levels of $CD1a^+$ $HLA\text{-}DR^+$ MNCs as compared to HCs or during treatment with IFN-β and GA in combination for 1 – 3 or 4

– 6 months. The levels of $CD1a^+$ $HLA\text{-}DR^+$ MNCs did not differ between MS patients treated with IFN-β and GA for 1 – 3 or 4 – 6 months (see table 3).

In the cross-sectional study, untreated MS patients had higher number of $CD80^+$ $HLA\text{-}DR^+$ MNCs than HCs. However, patients treated with IFN-β alone, or with IFN-β in combination with GA, showed no difference in the number of $CD80^+$ $HLA\text{-}DR^+$ MNCs as compared to HCs. In the follow-up study, there was no difference in the proportion of $CD80^+$ $HLA\text{-}DR^+$ MNCs between MS patients treated with IFN-β and HCs. MS patients examined during treatment with IFN-β alone, had fewer $CD80^+$ $HLA\text{-}DR^+$ MNCs as compared during treatment with IFN-β and GA for 1 – 3 and for 4 – 6 months. The MS patients that received IFN-β and GA in combination had a larger proportion of $CD80^+$ $HLA\text{-}DR^+$ MNCs than that in HCs, both after 1 – 3 and after 4 – 6 months of therapy.

In the cross-sectional study, untreated MS patients had a lower number of $CD86^+$ $HLA\text{-}DR^+$ MNCs than HCs. However, after treatment with IFN-β or with IFN-β and GA in combination, there was no difference between MS patients and HCs. In the follow-up study, the proportion of $CD86^+$ $HLA\text{-}DR^+$ MNCs did not differ between MS patients and HCs at any time i.e., during the monotherapy with IFN-β, followed by the combined therapy of IFN-β and GA for 1 – 3 and 4 – 6 months.

Table 3. Expression of CD1a, CD80 and CD86 on $HLA\text{-}DR^+$ on blood mononuclear cells (MNCs) form patients with multiple sclerosis (MS) and healthy controls (HCs). Both the cross-sectional and the follow-up studies revealed that the reduction of $CD1a^+$ $HLA\text{-}DR^+$ MNCs was more pronounced in MS patients treated with interferon-β(IFN-β) and glatiramer acetate (GA) in combination (IFN-β+GA) than in MS patients treated with IFN-β alone. The Results are shown as the median values of 1 x 10^4 cells.

	Cross-sectional study				**follow-up study**			
	Untreated	**IFN-β**	**IFN-β+GA**	**HCs**	**IFN-β**	**IFN-β+GA** *1–3 months*	**IFN-β+GA** *4–6 months*	**HCs**
$CD1a^+$ $HLA\text{-}DR^+$ MNCs	12%	6%	3%	5%	8%	4%	4%	3%
$CD80^+$ $HLA\text{-}DR^+$ MNCs	24%	8%	11%	5%	17%	28%	34%	16%
$CD86^+$ $HLA\text{-}DR^+$ MNCs	11%	20%	22%	22%	23%	28%	25%	25%

4.3.2 Cytokine production by blood MNCs

The overall production of IL-12, IFN-γ, IL-10 and IL-4 by MNCs was analysed by ELISA in cells from untreated MS patients, MS patients treated with IFN-β alone followed by IFN-β+GA, and from HCs.

In the cross-sectional study, MNCs from untreated MS patients had lower IL-10 production than patients treated with IFN-β or IFN-β and GA in combination, and HCs. No difference for IL-10 production was observed between any treatment groups of the MS patients, nor between any of these groups and HCs. The follow up study revealed that the MS patients examined during monotherapy with IFN-β or during treatment with IFN-β and GA in combination for 1 – 3 or 4 – 6 months continued to have lower IL-10 production compared to HCs. There was no difference between MS patients during monotherapy with IFN-β and IFN-β and GA in combination for 1 – 3 or 4 – 6 months regarding IL-10 production.

The production of IL-12p70 was higher in untreated MS patients than in patients treated with IFN-β and GA in combination but not in those treated with IFN-β alone. The treatment with IFN-β and GA in combination further decreased the production of IL-12p70 observed after treatment with IFN-β alone. The same observation was made in the follow-up study, and after treatment with a combination of IFN-β and GA for 1 – 3 or for 4 – 6 months, the production of IL-12p70 had decreased to the same level as in the HCs.

The cross-sectional study showed that the untreated MS patients had higher IFN-γ production as compared to the HCs. Treatment with IFN-β alone or with GA in combination reduced the IFN-γ production in MS patients. In the follow-up study, patients on monotherapy with IFN-β had higher IFN-γ production as compared to HCs. This difference was not seen when comparing the patients after 1 – 3 or 4 – 6 months of combined treatment with IFN-β and GA with the HCs.

There were no differences between MS patients in the cross-sectional and follow-up studies and the HCs for IL-4 and IL-12p40 production.

4.3.3 Cytokine secretion by blood MNCs

In view of the high sensitivity and specificity of ELISPOT assay, we also examined individual MNCs with regard to their secretion of IL-12, IFN-γ, IL-10 and

IL-4 in MS patients, and the effects of treatment with IFN-β or GA, or with both IFN-β and GA on the secretion of these cytokines.

The numbers of IL-12 secreting MNCs were higher in patients with untreated MS than in HCs. Treatment with IFN-β or GA or IFN-β and GA in combination reduced the numbers of IL-12 secreting MNCs to similar levels as in the HCs.

The numbers of IFN-γ secreting MNCs were higher in patients with untreated MS than in HCs. Treatment with IFN-β or GA, or IFN-β and GA in combination, resulted in reduction of the numbers of IFN-γ secreting MNCs to levels similar to those of HCs.

The numbers of IL-10 secreting MNCs was lower in patients with untreated MS than in HCs. Treatment with IFN-β, or GA, or IFN-β and GA in combination, resulted in induction of IL-10 secreting MNCs to the level observed in HCs.

There was no difference in the numbers of IL-4 secreting MNCs between any of the groups examined.

4.3.4 Cytokine secretion by blood MNCs in response to *in vitro* exposure to GA

In view of the anti-proliferative effects of IFNs (Yong et al., 1998), it may hypothesized that IFN-β in combination with GA, could interfere with the lymphoproliferative and Th2 responses to GA.

We investigated how MNCs from HCs, untreated MS patients and patients treated with IFN-β or GA, or IFN-β and GA in combination were affected by incubation with GA, with regard to the number of IL-12, IFN-γ, IL-10 and IL-4 secreting MNCs.

We found that GA induced IL-12 secretion by MNCs in MS patients treated with GA, as well as those treated with IFN-β and GA in combination, but not in HCs, in untreated patients or in patients treated with IFN-β. Cultures with the control antigen purified protein derivative (PPD) induced IL-12 secretion by MNCs in all groups of MS patients and in HCs.

We found that GA induced IFN-γ secretion by MNCs in all groups of MS patients examined, but not in HCs, whereas PPD induced IFN-γ secretion by MNCs in all groups of MS patients examined, and in HCs.

GA induced IL-4 secreting MNCs in untreated MS patients and in patients treated with IFN-β, GA, or IFN-β and GA in combination, but not in HCs. Upon exposure of

MNCs to PPD did not induced IL-4 secretion by MNCs in any of the groups of MS or in HCs.

4.3.5 Cytokine secretion by GA-reactive blood MNCs

Assessment of the numbers of GA- or PPD-reactive MNCs secreting IL-12, IFN-γ, IL-10 and IL-4, were carried out in the ELISPOT assay, by subtraction from the number of spots after GA or PPD stimulation of the number of spots detected without stimulation. These cells were considered to represent GA- or PPD-reactive MNCs secreting the cytokine in question.

The numbers of GA or PPD-reactive MNCs secreting IL-12, were similar in the all groups examined.

Comparison of the numbers of GA- and PPD-reactive MNCs secreting IFN-γ showed that the numbers of GA-reactive, but not PPD-reactive MNCs secreting IFN-γ, were increased in untreated MS patients. The treatment with IFN-β or GA, or IFN-β and GA in combination did not reduce the levels of IFN-γ. Similar numbers of GA-reactive MNCs secreting IFN-γ were found in untreated MS, MS patients treated with IFN-β and GA, whereas MS patients treated with IFN-β and GA in combination, however, had higher numbers of GA-reactive MNCs secreting IFN-γ than in untreated patients or patients treated with IFN-β or GA, or HCs. The numbers of PPD-reactive MNCs secreting IFN-γ did not differ between the groups examined.

Comparison of the numbers of GA- or PPD-reactive MNCs secreting IL-10 or IL-4 showed no differences between any of the groups examined.

5. DISCUSSION

5.1 DCs: POSSIBLE ROLE IN MS

DCs are the most potent APCs and probably the most essential APCs in activating naïve T cells in secondary lymphoid organs (Steinman et al., 1997). It is thus likely that DCs are involved in the priming and /or boosting of these T cells through various mechanisms. It has been hypothesised that DCs are involved in the pathogenesis of MS and that aberrations in the expression of surface markers and in cytokine secretion may contribute to the initiation of an aberrant immune response in MS (Link et al., 1999).

Our studies have shown in MS patients, a high proportion of DCs are of $CD1a^+$ cells as compared to HCs (Paper II). This finding in line with a previous report (Huang et al., 2001a). We also observed that in untreated MS patients there is a higher proportion of the $HLA\text{-}DR^+$ MNCs that are $CD1a^+$, than in HCs (Paper II).

CD1a is an HLA-DR-like molecule, that is expressed independently of HLA-DR. Consequently, DCs may use CD1a as a major pathway for presentation of lipid antigens to T cells (Porcelli et al., 1998).

Immune cells in the blood including T cells and DCs, are assumed to enter the CNS (Hohlfeld, 1997; Pashenkov et al., 2001). Interestingly, as reported recently, DCs are present in MS lesions, and may migrate to the CNS from the peripheral circulation in response to signals such as CNS-produced chemokines (Pashenkov et al., 2003a; Plumb et al., 2003).

The present finding of high CD1a expression on MS DCs, would seem to indicate that initiation of autoaggressive immunity against myelin autoantigens is more likely to occur in MS, and that suppression of CD1a expression could be beneficial in MS.

The proportion of $CD80^+$ and $CD86^+$ DCs, derived from MS was similar to the proportion in DCs derived from HCs. CD80 and CD86 expressed on APCs bind to CD28 on T cells, which leads to activation of the Th1 and Th2 pathways, respectively (Kuchroo et al., 1995; Tseng et al., 2001). Our finding may reflect similarity with regard to the proportion of Th1 and Th2 responses in MS patients and HCs. However, the involvement of CD80 and CD86 in Th1/Th2 polarisation is a complex process, that also engages other factors (Tseng et al., 2001).

With regard to the cytokine production by DCs, we found that the spontaneous production of IL-12 did not differ between MS patients and HCs (Paper II). IL-12 is a key cytokine produced by DCs, which is important for Th1 differentiation (Heufler et

al., 1996). Again our finding may reflect similarity regarding the Th1 vs. Th2 responses in MS patients and HCs in response to IL-12 from DCs.

In contrast, the spontaneous production of IL-10 by DCs from MS patients, was lower than by DCs from HCs (Paper II). IL-10 is an anti-maturation factor for DCs and can convert immature cells into tolerogenic cells (Steinbrink et al., 1999; Steinbrink et al., 1997), which drive the differentiation of Th2 cells (Liu et al., 1998). The present data of a decreased spontaneous production of IL-10 by DCs, would seem to favour a Th1 response differentiation in MS.

In summary, the demonstration that blood DCs from MS patients expressing CD1a are more numerous and that their production of IL-12 is increased, and the finding that of IL-10 is decreased, suggest that the disease may be associated with functionally important immunological variables of DCs, and these may represent potential therapeutic targets in MS.

5.2 IFN-β AND GA AND SURFACE MARKER EXPRESSION

5.2.1 IFN-β and GA separately or in combination - DCs

IFN-β and GA are approved treatments for MS. However, their mechanisms of action in MS are not fully understood (Yong, 2002). Elucidation of the action of IFN-β and GA at the level of DCs in MS would increase our understanding of their immunoregulatory mechanisms and, indirectly, of the pathogenesis of MS. The results from such studies would also promote the search for more effective therapies.

Type 1 IFNs are known to cause maturation of DCs from $CD34^+$ progenitors (Luft et al., 1998). We found that human blood monocytes, upon culture in the presence of IFN-β together with GM-CSF and IL-4, differentiated into a population expressing $CD14^-CD1a^-HLA\text{-}DR^+$ (Paper I). This population also expressed CD123. IFN-β was found to induce the maturation of these cells by upregulation of the co-stimulatory molecules CD40, CD80 and, in particular, CD86 (Paper I). Almost all $CD123^+$ cells expressed HLA-DR.

GA and IFN-β have similar effects in suppressing the development of $CD1a^+$ DCs generated from blood monocytes and in promoting the maturation of myeloid DC as reflected by up-regulation of HLA-DR and CD86 expression on DCs surface (Paper II). Effects of IFN-β on DCs maturation have been observed in other studies showing enhanced expression of HLA-DR and co-stimulatory molecules (Luft et al., 1998;

Bartholome et al., 1999a; Santini et al., 2000). IFN-β was shown to induce the expression of CD123 on DCs and this was consistently associated with an increased expression of CD80, CD86 and HLA-DR (Paper I and II).

The present results show that both GA and IFN-β suppress $CD1a^+$ DCs differentiation, and when GA and IFN-β are used in combination, they have synergistic suppressive effects on CD1a expression by blood DCs from MS patients (Paper II). In view of possible harmful effects of high CD1a expression on blood DCs it is conceivable that suppression of CD1a expression may be beneficial in MS. As a result, T cells that are auto-reactive against myelin antigens would die. However, both GA and IFN-β alone markedly increased CD86 expression on blood DCs. When combined, GA and IFN-β synergistically enhanced the expression of CD86 by blood DCs from MS patients (Paper II). It is possible that this synergistic effect may result in a predominance of Th2 cell responses, that is not obtained by GA or IFN-β alone in MS.

5.2.2 IFN-β alone or in combination with GA – Blood MNCs

$HLA\text{-}DR^+$ MNCs in the blood represent APCs. In order to study the effects of IFN-β and GA in combination as compared to IFN-β alone on these cells, both cross-sectional and longitudinal studies were performed.

The results showed that MS patients treated with IFN-β alone or, in particular, with IFN-β and GA in combination had lower levels of $CD1a^+$ $HLA\text{-}DR^+$ MNCs as compared to untreated MS patients (Paper III). In the follow-up study, the patients treated with IFN-β and GA in combination had lower levels of $CD1a^+$ $HLA\text{-}DR^+$ MNCs as compared to patients treated with IFN-β alone (Paper III). This observation is in line with our *in vitro* study showing that IFN-β and GA in combination had synergistic suppressive effects on CD1a expression by blood DCs from MS patients (Paper II).

In a cross-sectional study, untreated MS patients had higher $CD80^+$, as well as lower $CD86^+$ $HLA\text{-}DR^+$ MNCs, compared to HCs (Paper III). In the view of the role of CD80 and CD86 in determining the direction towards a Th1 or Th2 pathway (see above), our findings may thus reflect a dominance of Th1 vs. Th2 responses in the periphery in MS. The treatment of MS patients with IFN-β alone or IFN-β and GA in combination resulted in reduced levels of $CD80^+$, and increased levels of $CD86^+$ $HLA\text{-}DR^+$ MNCs, as compared to untreated MS patients, although none of the alterations

reached statistical significance. In the follow-up study, higher levels of $CD80^+$ HLA-DR^+ MNCs were found in MS patients after 4 – 6 months of treatment with IFN-β and GA in combination, which is consistent with an augmented Th1 response. Whether this observation affects the clinical outcome in this combined therapy group, needs to be investigated further.

5.3 CYTOKINES IN MS

Cytokines are soluble proteins or glycoproteins that play role in the regulation of immune responses (Belardelli, 1995). Alterations in cytokines have been proposed to be important in MS (Navikas and Link, 1996). Therefore, studying the cytokine profile is important for understanding of the pathological mechanisms involved in MS (Ozenci et al., 2002). Many studies, using different methods have addressed the question of cytokine levels in MS. These methods are based on the different stages of cytokine production and secretion (Ozenci et al., 2002). Northern blot, semiquantitative reverse transcription polymerase chain reaction (RT-PCR) or *in situ* hybridization histochemistry are used to study mRNA cytokine levels. These methods are sensitive enough to detect mRNA in the absence of stimulant, yet mRNA levels do not necessarily reflect the rate of protein production and secretion. Immunohistochemistry detects the occurrence of cytokines in the cells. However, the sensitivity of this method in the detection of cytokines is low, and generally requires *in vitro* stimulation. ELISA and ELISPOT are used to study cytokine protein levels in extracellular fluids and in the culture medium cells actively secreting cytokines, respectively. The overall concentration of a cytokine may not reflect its rate of secretion. Thus, detection of a cytokine at the individual cell level may by more relevant. The difficulty to establish an assay for a new cytokine, the inability to identify the phenotype of the cytokine-secreting cell and, the amount of the cytokine secreted by individual cells are challenges for using ELISPOT.

In fact, ELISA and ELISPOT were used in this thesis to measure the level of secreted cytokines. The contrasting results in this thesis, may reflect methodological differences, different subtypes of MS studied and the complex biology of cytokines (Ozenci et al., 2002).

5.4 IFN-β AND GA - CYTOKINE PRODUCTION AND RELEASE

5.4.1 IFN-β and GA - DCs

Hypothetically, shifting the immune response from pro-inflammatory (Th1) towards an anti-inflammatory (Th2) pattern, may have beneficial effects in MS (Navikas and Link, 1996).

DCs may be important in differentiation of naïve T cells to Th1 or Th2. The induction of Th1 or Th2 cells by DCs can by determined by the level of IL-12 production (Buelens et al., 1995). Since the capacity of DCs to produce IL-12 can be modulated by microenvironmental factors, we studied the effects of IFN-β and GA separately, as well as in combination, with regard to the production of IL-10 and IL-12 by blood DCs.

The results showed that *in vitro* exposure of blood DCs cultures to GA or IFN-β, or both affect the differentiation of DCs and their cytokine production. The production of IL-12 was similar in DCs form MS patients and HCs. GA and IFN-β alone reduced the IL-12 production, but there was no synergistic effect by GA and IFN-β in combination (Paper II).

The spontaneous production of IL-10 was reduced in DCs from MS patients, as compared to DCs from HCs. GA alone did not affect IL-10 production, while IFN-β alone resulted in a significant increase, both in MS patients and HCs. There was no synergistic effect by GA and IFN-β in combination (Paper II).

Taken together, these results indicate that GA and IFN-β affect the production of IL-12 and IL-10 by DCs. GA did not affect the production of IL-10, whereas IFN-β resulted in increased production. The treatment with GA or IFN-β resulted in decreased production of IL-12, providing a permissive environment for Th2 responses, that probably is beneficial in MS.

5.4.2 IFN-β - Blood monocytes: Differentiation towards Th2-driving DCs

As an immunomodulator, IFN-β has both anti- and pro-inflammatory properties. The balance between these two functions of IFN-β may determine its therapeutic effects in MS. Suppression of IL-12 production by DCs has been hypothesized to be a principal mechanism underlying the biological action of IFN-β in MS (McRae et al., 1998; Karp et al., 2000; McRae et al., 2000; Huang et al., 2001b;).

In the present studies, IFN-β-treated DCs had a decreased release of IL-12 and an increased release of IL-10 (Paper II). It is conceivable that IFN-β may produce clinically relevant changes in IL-12 and IL-10 production by DCs. Thus, an environment created by IFN-β-treated DC would favour Th2 cytokine production, resulting in a negative feedback on Th1 differentiation. Furthermore, IFN-β markedly reduced the IFN-γ production but enhanced the IL-10 production by MNCs in HCs (Paper I). These results indicate that, in the presence of IFN-β, DCs derived from blood monocytes induce Th2 differentiation in HCs. Thus, generation of these DCs could be one of the immunomodulatory effects of IFN-β, being relevant for its clinical effects in MS.

5.4.3 IFN-β alone or in combination with GA - Blood MNCs: Th1 cytokines

Pro-inflammatory cytokine are believed to be key mediators of the inflammatory process in MS. In these studies, we found that untreated MS patients had higher IFN-γ and IL-12p70 production by MNCs than in HCs (Paper III). Untreated MS patients also had higher numbers of IL-12 and IFN-γ secreting MNCs than in HCs (Paper IV). Both IL-12 and IFN-γ may have harmful effects in MS (Panitch et al., 1987; van Boxel-Dezaire et al., 1999).

One of proposed mechanisms of IFN-β and GA in MS is reduction of the production of Th1 cytokines (Miller et al., 1998; Zang et al., 2000). Our results showed that treatment with IFN-β and GA together, reduced the production of IFN-γ and IL-12p70 by MNCs, whereas the reduction was less pronounced in MS patients on monotherapy with IFN-β (Paper III). These results were confirmed in the follow-up study.

With regard to IL-12 and IFN-γ secreting MNCs, we found that IFN-β and GA in combination had the same effects as IFN-β or GA alone, in the reduction of IL-12 and IFN-γ secreting MNCs (Paper IV). These data may indicate beneficial effects on the course of MS and that the combination of IFN-β and GA may result in the similar responses in MS as the responses of IFN-β or GA monotherapy in MS in terms of cytokine profiles.

5.4.4 IFN-β alone or in combination with GA – Blood MNCs: Th2 cytokines

Anti-inflammatory cytokines are known to have beneficial effects on the course of MS (Navikas and Link, 1996). We found that untreated MS patients had lower IL-10 overall production by MNCs than in HCs (Paper III). Untreated MS patients also had higher numbers of IL-10 secreting MNCs than in HCs (Paper IV).

One of the proposed mechanisms of IFN-β and GA in MS is the induction of the Th2 cytokine profile. Accordingly, we found that treatment with IFN-β or GA resulted in the promotion of the anti-inflammatory cytokine IL-10, while the production of IL-4 was not affected by IFN-β or GA (Paper III).

Our results demonstrated that the combination treatment with IFN-β and GA had the same effect as the monotherapy with IFN-β or GA, with regard to the stimulation of IL-10 secretion by MNCs, indicating a potential beneficial effect similarly to the effects of IFN-β or GA alone in MS, in terms of cytokine profiles.

5.4.5 IFN-β and GA in combination - Blood MNCs: Cytokine profiles

The therapeutic effects of GA are partly attributed to its ability to induce GA-reactive T cells with a Th2 cytokine profile (Miller et al., 1998; Neuhaus et al., 2000). IFNs are known to have anti-proliferative effects (Yong et al., 1998). Therefore, IFN-β in combination with GA could interfere with the lymphoproliferative and cytokine responses to GA. However, IFN-β is unlikely to inhibit the cellular immune responses to GA (Dhib-Jalbut et al., 2002). In order to investigate whether IFN-β interferes with the cytokine responses to GA, we cultured MNCs from HCs, untreated MS patients, and from patients treated with IFN-β or GA, or both IFN-β and GA, in presence or absence of GA or PPD as a control. The effects of GA on the cytokine profiles were examined.

MS patients treated with IFN-β and GA in combination was found to have similar trends of induction of IFN-γ or IL-4, and no effect on IL-10 secretion by MNCs, as in MS patients treated with IFN-β or GA alone, as well as in untreated patients, but not in HCs (Paper IV). No induction of IL-4 was seen in the presence of PPD by MNCs from all groups of MS patients, and HCs, but there was an induction of IFN-γ by MNCs from the groups examined. It is clear that the addition of GA did not alter the effects of IFN-β treatment with regard to IFN-γ, IL-4 and IL-10 (Paper IV). The induction of IL-4 was

seen in all MS patients (treated and untreated) after GA exposure *in vitro*, but did not occur in HCs. These findings indicate that the effect of GA is not entirely specific for Th2 responses since it also induced the production of IFN-γ in MNCs from all groups of MS patients.

The *in vivo* relevance of these findings in MS is not understood at this time. However, it may be speculated that treatment with IFN-β and GA in combination *in vivo* may not alter the pattern of cytokine profiles by MNCs in response to GA, which may have important clinical implications.

5.5 SYNERGISM OF IFN-β AND GA IN MS?

In this thesis, the effects of combining IFN-β and GA *in vitro* and *in vivo* with regard to immunological parameters were analysed (see fig. 4).

In vitro, IFN-β acts synergistically with GA to suppress CD1a and enhance CD86 expression on DCs from MS patients. There was no effect on the CD80 expression. *In vivo,* the treatment with IFN-β and GA was more potent than the treatment with IFN-β alone in reducing CD1a, enhancing CD80 expression by DCs from MS patients. These findings may have implications for the clinical relevance of the combined treatment and for synergistic therapeutic effects in MS.

With regard to cytokines, the levels of IL-12p70 and IFN-γ were reduced more in supernatants form MNCs, as measured by ELISA, of patients treated with IFN-β and GA in combination, as compared to IFN-β monotherapy. ELISPOT analysis indicated, however, that there were no additive effects with regard to numbers of IL-12 and IFN-γ secreting MNCs.

IFN-β and GA may thus have synergistic beneficial effects preferentially on the surface markers on APCs, particularly DCs, but not on cytokine profiles.

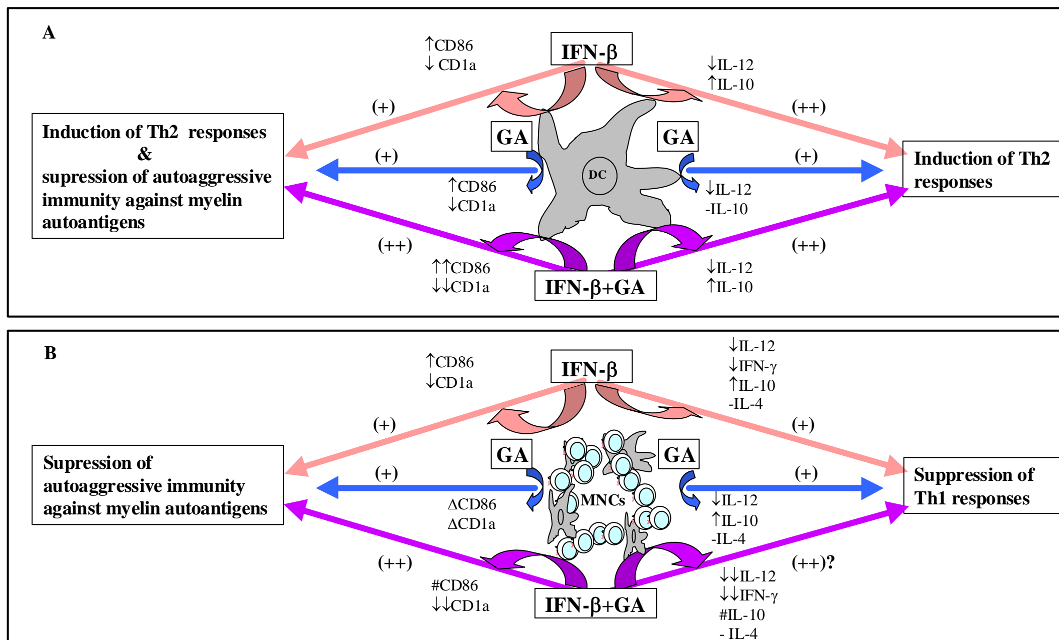

Fig. 4. Effects of interferon-beta (IFN-β), glatiramer acetate (GA) or IFN-β and GA in combination in multiple sclerosis. (A) *in vitro,* IFN-β or GA increase CD86 and reduce CD1a expression on dendritic cells (DCs). IFN-β and GA in combination synergistically suppress CD1a and enhance CD86 expression on DCs. (B) *in vivo*, IFN-β and GA in combination more potent than IFN-β alone in reducing CD1a expression by DCs, and in reducing IL-12 and IFN-γ production by mononuclear cells (MNCs).↑ = increase; ↓ = decrease; # controversial effect; Δ = not examined ; - = no effects; + = moderate outcome; ++ strong outcome; ? not clear.

6. CONCLUSIONS AND FUTURE PERSPECTIVES

Complex alterations in surface molecule expression and in cytokine production by DCs form MS patients raise the possibility that these cells are involved in the pathogenesis of MS. Therefore, DCs may also be relevant as potential therapeutic targets in MS. Additional studies are needed to delineate the roles of these immunological parameters in the pathogenesis of MS.

DCs are critically involved in the mechanisms underlying the immunomodulatory effects of IFN-β and GA in MS. These effects may be important for the clinical consequences of these substances. The immunomodulatory effects have been studied on two levels, as surface marker expression and as cytokine profiles. The molecular mechanisms by which IFN-β and GA modulate DCs remain elusive and further investigations are necessary.

The combination of IFN-β and GA may result in synergistic immunological effects preferentially on the surface markers on APCs, particularly DCs. These findings may, have synergistic impact on the effects in clinical use in MS. Further studies are warranted to investigate this possibility.

The combination of IFN-β and GA treatment in MS may have similar effects as the treatment of IFN-β or GA alone, on cytokine profiles from DCs and MNCs, as determined in the present experimental studies. Whether, or not, there are synergistic or additive effects of IFN-β and GA on cytokine profiles needs further analysis. The duration of treatment, methodological differences and the complex biology of cytokines should also be considered. More sensitive methods are required for studying the effects of different drugs on certain cytokines.

It is also important to emphasize that the clinical effects in patients after treatments with various drugs may be impossible to predict from laboratory studies *in vitro*, but can only be evaluated in carefully designed clinical trials.

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