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Cytokine-Modulated Dendritic Cell Immunotherapy in Autoimmune Diseases

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Stockholm 2005

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7140-149-0

To my family

ABSTRACT

Autoimmunity is the result of failure of self-tolerance processes. When the immune system fails to differentiate self from foreign, the effector mechanisms are directed towards the individual's own tissues, and pathological autoimmune disease results.

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that regulate immune responses. Mature DCs induce immunity that can be utilized in cancer treatment, whereas immature DCs induce tolerance, a property that can be utilized in the therapy of autoimmune diseases. The function of immature DCs can be further modified to suit the therapy of autoimmune diseases by exposing them to immunomodulatory cytokines.

The aim of this thesis was to study the effects DCs exposed to the cytokines interleukin (IL)-10 (IL-10-DCs) and interferon (IFN)-γ (IFN-γ-DCs), respectively, in the treatment of myasthenia gravis (MG) and multiple sclerosis (MS), two autoimmune diseases with different immunological background. MG is a T cell dependent, antibody-mediated disease, while MS is mainly a T cell-mediated disease. Therefore, it is of interest to analyse the response to DC immunotherapy in both diseases.

Paper I and IV revealed that IL-10-DCs and IFN-γ-DCs, respectively, could efficiently suppress experimental autoimmune myasthenia gravis (EAMG) in Lewis rats, an animal model of MG. Rats with on-going EAMG developed less severe clinical signs when injected intraperitoneally with IL-10-DCs, or with IFN-γ-DCs subcutaneously. Mononuclear cells (MNCs) from draining lymph nodes of rats treated with IL-10-DCs showed reduced proliferation, and reduced IL-10 and IFN-γ production in response to nicotinic acetylcholine receptor (AChR). The proportion of AChR antibody-secreting cells among MNCs, and the affinity of the antibodies produced were reduced. The expression of CD80 and CD86 by MNCs was also reduced after treatment with IL-10-DCs. However, the treatment with IFN-γ-DCs did not alter the proliferation of MNCs, nor the IL-10 or IFN-γ production. There was a low number of AChR antibody-producing cells and plasma cells in rats treated with IFN-γ-DCs, as well as a low number of B cell-activating factor (BAFF)-expressing cells. A specific inhibitor of indoleamine 2,3-dioxygenase (IDO), 1-methyl-DL-tryptophan (1-MT), aggravated the clinical signs of EAMG and abolished the IFN-γ-DC-induced reduction in the number of AChR antibody-secreting cells *in vitro*. The results were suggestive of an 'IDO-BAFF pathway' in the suppression of EAMG by IFN-γ-DCs.

In Paper II and III, DCs generated from peripheral blood MNCs of patients with MG or MS, were modified *in vitro* with IL-10 or LPS, and co-cultured with autologous lymphocytes. DCs from healthy controls (HCs) were used for comparisons. Assessment of phenotype and cytokine secretion pattern of DCs revealed that irrespective of whether the DCs were generated from MG, MS or HCs, they responded to LPS, but not IL-10, with increased expression of HLA-DR, CD80, CD86 and CD83. Similarly, exposure of DCs to LPS, but not IL-10, increased secretion of the proinflammatory cytokines IL-12, IL-6 and TNF-α. IL-10-DCs were found to be resistant to maturation by LPS. In co-cultures, DCs derived from MG patients were more potent in activating T cells than DCs derived from HCs, as determined by the expression of CD25 and CD69 on $CD4^+$ T cells. IL-10-DCs stimulated autologous lymphocytes to produce IL-10 and IL-4 in MS, MG and HCs. This was a Th2 response, indicating the possible benefit of IL-10-DCs in the treatment of MS, but not MG.

Based on the present data and earlier data, IL-10-DCs and IFN-γ-DCs appear as potential candidates for future clinical trials with DC immunotherapy in autoimmune diseases.

LIST OF PUBLICATIONS

This thesis is based on the following publications, referred to by their Roman numerals.

I. Duan RS, **Adikari SB**, Huang YM, Link H, Xiao BG

Protective potential of experimental autoimmune myasthenia gravis in Lewis rats by IL-10-modified dendritic cells

Neurobiol Dis. 2004; 16: 461-467

II. **Adikari SB**, Lefvert AK, Pirskanen R, Press R, Link H, Huang YM

Dendritic cells activate autologous T cells and induce IL-4 and IL-10 production in myasthenia gravis

J Neuroimmunol. 2004; 156: 163-170

III. **Adikari SB**, Pettersson Å, Söderström M, Huang YM, Link H

Interleukin-10-modulated immature dendritic cells control the proinflammatory environment in multiple sclerosis

Scand J Immunol. 2004; 59: 600-606

IV. **Adikari SB**, Lian H, Link H, Huang YM, Xiao BG

Interferon-γ-modified dendritic cells suppress B cell function and ameliorate the development of experimental autoimmune myasthenia gravis

Clin Exp Immunol. 2004; 138: 230-236

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
AChR	Nicotinic acetylcholine receptor
APC	Antigen-presenting cell
BAFF	B cell-activating factor
BBB	Blood-brain barrier
CD	Cluster of differentiation
CNS	Central nervous system
CSF	Cerebro-spinal fluid
CTLA	Cytotoxic T lymphocyte antigen
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EAMG	Experimental autoimmune myasthenia gravis
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot
FACS	Fluorescence-activated cell sorting
FCA	Freund's complete adjuvant
FSC	Forward scatter characteristics
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
Lin	Lineage [T cells (CD3), B cells (CD19/CD20), NK cells (CD56), monocytes (CD14) and macrophages (CD16)]
LPS	Lipopolysaccharide
MBP	Myelin basic protein
MFI	Mean fluorescence intensity
MG	Myasthenia gravis
MHC	Major histocompatibility complex
MLR	Mixed leukocyte reaction
MNC	Mononuclear cell
MOG	Myelin oligodendrocyte protein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
PLP	Proteolipoprotein
SLE	Systemic lupus erythematosus
SSC	Side scatter characteristics
TCR	T cell receptor
TGF	Transforming growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
T_R	Regulatory T cells

1 INTRODUCTION

1.1 The concept of self-tolerance

The adaptive immune system of the body is organized to differentiate self from foreign. This organization is likely to have evolved to protect complex organisms from microbial pathogens, without reacting against tissues and proteins of the organism itself. In the life of an individual, the specific receptors on T cells and B cells adapt to the antigens that are part of that individual (self) by developing tolerance. Therefore, reactivity to self is inactivated. This self-tolerance is critical if the powerful set of effector mechanisms that characterize the immune system are to be directed at virulent foreign invaders and not cause unacceptable damage to the individual (Eisenberg, 2003).

The immunological specificity of the antigen receptors of T cells and B cells is the result of random shuffling of the genes that constitute the DNA code for the antigen-binding site of these receptors (Chien et al., 1984; Delves and Roitt, 2000; Tonegawa, 1983). Theoretically, this process could generate 10^9 different T cell receptors, including some that can bind to autoantigens. Cells with such autoreceptors are often called autoreactive T cells. Tolerance is the process that eliminates or neutralizes such autoreactive cells. Breakdown in this system can cause autoimmunity (Kamradt and Mitchison, 2001).

1.1.1 Central tolerance

The chief mechanism of central T cell tolerance is the deletion of autoreactive T cells in the thymus. Immature T cells migrate from the bone marrow to the thymus and meet peptides derived from endogenous proteins bound to major histocompatibility complex (MHC) molecules on thymic epithelial cells or dendritic cells (DCs). T cells having either very low affinity or very high affinity receptors for these peptide-MHC complexes ultimately undergo apoptosis and are eliminated from the body. T cells with an intermediate affinity for the peptide-MHC complexes mature in the thymus and migrate to the periphery, a process referred to as positive selection. Another reason for escape of T cells from the thymic selection is that the thymus does not express all self-antigens. The peripheral T cell tolerance is the mechanism adopted by nature to neutralize such autoreactive T cells that escape the thymic selection (Kamradt and Mitchison, 2001).

1.1.2 Peripheral tolerance

In the periphery, autoreactive T cells ignore the target cells if the autoantigen is expressed at a level below the threshold level that is required to induce the activation or deletion of T cells. In the absence of help from the $CD4^+$ T cells, autoreactive $CD8^+$ T cells cannot damage tissues (Kurts et al., 1997). Physical separation of the T cells from the antigen, as in the case of the central nervous system (CNS), may be another reason for ignorance.

Deletion of autoreactive T cells in the periphery may occur due to the presentation of self-antigens by MHC molecules in the absence of co-stimulation (Janeway, 1992), or the lack of growth factors for T cells (Kamradt and Mitchison, 2001). Furthermore, the occurrence of both Fas and its ligand on activated T cells, leads to apoptosis following interaction between T cells or between T cells and antigen-presenting cells (APCs) (Brunner et al., 1995; Critchfield et al., 1994). These autoreactive T cells can simply be inhibited through CTLA-4/B7 interaction (Chambers and Allison, 1999). Regulatory T cells can inhibit or suppress other T cells, including autoreactive T cells, through the production of inhibitory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β (Buer et al., 1998; Kamradt and Mitchison, 2001). Thus, autoreactive T cells in the periphery cannot lead to disease as long as they are silenced, by various mechanisms.

1.2 Autoimmune diseases

Autoimmunity is the result of failure of self-tolerance processes. When the specific immune receptors fail to discriminate between self and foreign, the effector mechanisms are directed towards the individual's own tissues, and pathological autoimmune disease results (Eisenberg, 2003). The clinical presentation of the autoimmunity or the autoimmune disease depends on which tissues are targeted. Presently, at least 40 disorders constitute the known autoimmune diseases in humans (Mackay et al., 2000), some of which are summarized in Table 1.

Several mechanisms have been suggested for the initiation of the pathological process in autoimmunity. Infection can lead to autoimmunity by the release of sequestered autoantigens through tissue damage (Kurtzke, 1993), and by induction of inflammatory cytokines and co-stimulatory molecules in response to microbial products (Klinman et al., 1996). Alternatively, a structural similarity between the microbial and

the self-antigens ("molecular mimicry") can lead to activation of T cells with autoreactivity (Albert and Inman, 1999). Defective clearance of apoptotic remnants bearing self-antigens is another mechanism suggested for the induction of autoimmunity. Defects in the normal pathway of clearance in the presence of normal turnover of cells may lead to autoimmunity. This may also be due to inadequate clearance, as a result of increased apoptotic cell numbers following tissue injury (Cline and Radic, 2004). Another suggested mechanism of autoimmunity is an imbalance between T-helper type 1 (Th1) and Th2 cytokines. Diseases such as multiple sclerosis (MS) occur in an environment dominated by Th1 cytokines, while diseases such as myasthenia gravis (MG) and systemic lupus erythematosus (SLE) occur in an environment with predominantly Th2 cytokines (O'Shea et al., 2002).

Disease	Autoantigen/s	Outcome
Myasthenia gravis and experimental autoimmune myasthenia gravis	Nicotinic acetylcholine receptor	Striated muscle weakness
Multiple sclerosis and experimental autoimmune encephalomyelitis	Myelin basic protein, proteolipoprotein, myelin oligodendrocyte glycoprotein	CNS invasion by autoreactive T cells
Rheumatoid arthritis	Rheumatoid factor IgG complexes	Arthritis
Systemic lupus erythematosus	DNA, histones	Generalized (vasculitis, skin rash, arthritis, glomerulonephritis)
Insulin-dependent diabetes mellitus	Pancreatic β-cell antigen	β-cell destruction
Pemphigus vulgaris	Epidermal adherin (desmolein-3)	Blistering of the skin

Table 1. Some common autoimmune diseases that are referred to in this thesis, classified by the autoantigen and the outcome.

The susceptibility to autoimmune disease is undoubtedly influenced by genetic factors. Among the genes involved, those encoding human leukocyte antigen (HLA) molecules, the human counterpart of MHC molecules, have been shown to be the most important for several autoimmune diseases (Cooke et al., 2004). Depending on the genetic make up of the individual, he or she may develop an autoimmune disease

through one or more of the mechanisms mentioned. Nevertheless, the environment in which the individual lives, could be even more important, as suggested by different frequencies of a given autoimmune disease in ethnically similar groups, living in different geographic locations (Mackay et al., 2000).

The studies included in this thesis focus on MG and MS, two autoimmune diseases with different immunological background. MG is a T cell dependent, antibody-mediated disease, while MS is mainly a T cell-mediated disease. Therefore, it is of interest to analyse whether the responses to immunotherapy differ or whether they are similar in the two diseases.

1.2.1 Myasthenia gravis (MG)

MG is an autoimmune disease in which up to 85% of the cases have IgG autoantibodies specific for nicotinic acetylcholine receptor (AChR). AChRs are concentrated in the post-synaptic region of the neuromuscular junction and binding of the autoantibodies leads to reduction in the number of receptors available for neurotransmitter, acetylcholine, resulting in impaired neuromuscular transmission and muscle weakness. MG is a heterogeneous disorder and in about 90% of the patients, no specific cause can be found. There is evidence to suggest that the individual's genetic make-up is an important predisposing factor for the development of the disease. The most important of the known causes is a tumour in the thymus. Thirty to 60% of thymomas are associated with MG, and about 10% of patients with the disease have thymoma (Vincent et al., 2001).

The thymus gland, thought to be necessary for the deletion of autoreactive T cells, seems to have an important role in the pathogenesis of MG. In early-onset patients, the thymus is typically enlarged, and contains many germinal centers with T and B cell areas, similar to those seen in lymph nodes (Kirchner et al., 1986). This, together with the finding that B cells are unable to cause the disease in the absence of T cell help, suggests that MG is a T cell dependent, B cell-mediated disease (Ahlberg et al., 1994; Zhang et al., 1996).

Administration of acetylcholinesterase (AChE) inhibitors is the first line of treatment. When treatment with such inhibitors does not control the symptoms, a regime of immunosuppressive therapy in the form of prednisolone and/or azathioprine is frequently introduced. If this fails, more potent immunosuppressive drugs have to be used. Severe cases can be temporarily improved by plasmapheresis or intravenous immunoglobulin (Ig) therapy (Palace et al., 2001), both of which are quite expensive

treatments. All of these treatment strategies may have significant side effects. Thymectomy is performed particularly in young patients with generalized disease, or when the gland is enlarged. However, the value of thymectomy in patients older than 55 years, and in patients with ocular forms of the disease is controversial. A considerable number of side effects may occur following surgery (Byrne, 2002). DC-based immunotherapy has been proposed as a future alternative (Link and Xiao, 2001; Xiao et al., 2003a).

1.2.2 Experimental autoimmune myasthenia gravis

The animal model of MG, the experimental autoimmune myasthenia gravis (EAMG) can be induced in vertebrates by immunization with AChR from either *Torpedo californica* or electric eel, in adjuvant. EAMG is induced in rabbits, guinea pigs, rats or mice for research purposes. In the studies included in this thesis, EAMG was induced in female Lewis rats by immunization with AChR from *Torpedo californica*.

1.2.3 Multiple sclerosis (MS)

MS is a chronic inflammatory demyelinating disease of the CNS. The clinical and pathological features of MS were first described more than a century ago (Hickey, 1999). Imaging and pathological studies have provided insight into possible disease mechanisms operative in MS pathogenesis. They have clearly demonstrated that in most patients, MS is a disease with an acute onset accompanied by active inflammation and demyelination. Recent studies indicate that there is axonal degeneration even during the early stages of the disease (Ferguson et al., 1997; Giuliani and Yong, 2003; Kurnellas et al., 2004). Despite major research efforts within the past few decades, the etiology of MS is still unclear. Neither, is there a definitive treatment for the disease. In recent years, the possibility that MS is a hereditary disease has been explored. Thus, many studies have shown the association of the HLA-DR2 serotype with MS (Hillert, 1994; Liblau and Gautam, 2000). Epidemiological studies, however, have suggested that environmental factors also play a critical role in MS pathogenesis (Marrie, 2004).

There is evidence that both myelin-reactive $CD4^+$ T cells and $CD8^+$ cytotoxic T cells are responsible for the axonal damage in MS (Huseby et al., 2001; Steinman, 2001). Both of these cell types can cross the blood-brain barrier (BBB) through a tightly controlled process and enter the brain parenchyma (Sellebjerg and Sørensen, 2003). Once inside the CNS, the autoreactive $CD8^+$ T cells can directly damage the myelin sheaths. The $CD4^+$ T cells can recognize myelin antigens within the CNS parenchyma

in the context of MHC class II molecules expressed by APCs (Hohlfeld, 1997), upon which they secrete Th1 cytokines, and drive other immune cells such as macrophages to damage the myelin sheaths, and possibly axons.

The potential pathogenic role of antibodies has been discussed since the discovery of intrathecal Ig production in MS (Archelos et al., 2000; Cross et al., 2001). Antibodies to several CNS antigens, including oligodendroglial/myelin antigens, have been demonstrated in MS (Archelos et al., 2000). Indeed, the occurrence of oligoclonal IgG bands in the cerebro-spinal fluid (CSF), still represents the only valuable immune parameter in the diagnosis of MS. The antibody response in MS seems to be remarkably stable over a long period of time, suggesting that it is focused on a few target antigens, without major changes during the disease course.

1.2.4 Experimental autoimmune encephalomyelitis

Experimental autoimmune encephalomyelitis (EAE) is an experimental model for MS, with inflammatory, autoimmune and demyelinating characteristics. EAE is mediated by $CD4^+$ T cells, and can be induced in a number of experimental laboratory animals, including primates, by administration of a whole-brain homogenate, or purified preparations of myelin basic protein (MBP), myelin oligodendrocyte protein (MOG), or proteolipoprotein (PLP), in adjuvant. Depending on the immunization protocol, acute EAE, or a chronic form of EAE, referred to as chronic relapsing EAE (CR-EAE) can be induced. In the CR-EAE model, animals have multiple episodes of relapses and remissions, a clinical picture that closely mimics MS. The clinical and pathological similarities between EAE and MS make EAE an ideal model for studies of etiology and potential therapies for MS (Bright and Sriram, 2001).

1.3 T-helper (Th) cells

Naïve $CD4^+$ T cells can differentiate into different types of Th cells depending on which cytokines they are exposed to (Figure 1). Exposure of naïve $CD4^+$ T cells to IL-12, IL-18 and interferon (IFN)-γ drives them towards a Th1 phenotype secreting IL-2, IFN-γ and tumour necrosis factor (TNF)-α (Chitnis and Khoury, 2003). Th1-type cytokines activate macrophages and cell-mediated reactions involved in resistance to infection due to intracellular pathogens, and in cytotoxic and delayed-type hypersensitivity reactions (Raghupathy, 2001).

The presence of IL-4 leads to inhibition of Th1 differentiation, and drives naïve $CD4^+$ T cells towards a Th2 phenotype producing IL-4, IL-5, IL-10 and IL-13. Th2-type cytokines encourage vigorous antibody production and are, therefore, commonly found in association with strong antibody responses that are important in combating infections with extracellular organisms (Raghupathy, 2001). Presence of high concentrations of IL-12 inhibits Th2 differentiation.

In the presence of IL-4, IL-10 and TGF-β, the naïve $CD4^+$ T cell differentiation is skewed towards a Th3 phenotype. These Th3 cells produce TGF-β and are important in immune mechanisms involving oral tolerance. TGF-β has suppressive properties for both Th1 and Th2 cells (Weiner, 2001). Similarly, Th1 and Th2 cells inhibit each other. Another type of Th cells, producing both Th1 and Th2 cytokines, are called Th0 cells.

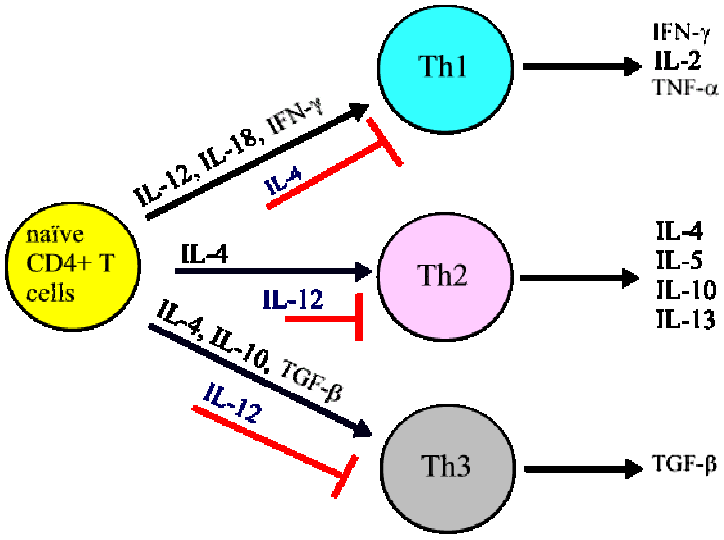

Figure 1. Differentiation of naïve $CD4^+$ T cells into Th1, Th2 and Th3 cells. Cytokines promoting or inhibiting the differentiation, and the main cytokines produced by the differentiated cells, are shown.

1.4 T cells, cytokines and MG

Although the symptoms of MG and EAMG are caused by antibodies, $CD4^+$ T cells specific for the target antigen, AChR, and the cytokines they secrete, have important roles during pathogenesis (Milani et al., 2003; Wang et al., 1999b).

Studies on AChR-specific $CD4^+$ T cells, commonly present in the blood of MG patients, support the role of $CD4^+$ T cells in the development of MG. $CD4^+$ T cells facilitate the synthesis of high-affinity AChR antibodies. Th1 cells are especially important because they drive the synthesis of anti-AChR complement-fixing IgG

subclasses. Binding of the antibodies to the muscle AChRs at the neuromuscular junction will trigger the complement-mediated destruction of the post-synaptic membrane. This explains why a cytokine that is crucial for the differentiation of Th1 cells, such as IL-12, is necessary for the development of EAMG (Milani et al., 2003).

Th2 cells secrete cytokines that have other effects on the pathogenesis of EAMG. Among them, IL-10, a potent growth and differentiation factor for B cells was shown to facilitate the development of EAMG (Poussin et al., 2000). In contrast, IL-4 appears to be involved in the differentiation of AChR-specific regulatory $CD4^+$ T cells, which can prevent the development of EAMG and its progression to a self-maintaining, chronic autoimmune disease (Ostlie et al., 2003). Circumstantial evidence supports a pathogenic role of IL-10 also in human MG. On the other hand, there is no direct or circumstantial evidence, so far, indicating a role of IL-4 in the immunomodulatory or immunosuppressive circuits in MG (Milani et al., 2003).

1.5 Th1/Th2 paradigm in MS

There is evidence that $CD4^+$ T cells play a critical role in the induction of MS (Zhang et al., 1994) and EAE (Chitnis and Khoury, 2003). In EAE, the production of Th1 cytokines by myelin-specific T cells has been shown to correlate with encephalitogenicity (Powell et al., 1990; Zamvil et al., 1985). Increased production of Th1 cytokines precedes clinical attacks of MS (Beck et al., 1988). Treatment of MS patients with recombinant IFN-γ induced exacerbations of the disease (Panitch et al., 1987). On the other hand, Th2 cytokine production has been shown to correlate with disease recovery (Issazadeh et al., 1995). Individual cytokines within these two categories have been shown to exert different effects on EAE. Thus, partial elimination, rather than complete absence of IFN-γ and TNF-α, was found to be beneficial (Chitnis and Khoury, 2003). The administration of IL-4 reduced the disease severity (Racke et al., 1994; Shaw et al., 1997), while absence of IL-4 did not alter the disease course (Liblau et al., 1997). IL-10 has been shown to be associated with disease remission in EAE (Kennedy et al., 1992) and MS (Balashov et al., 2000). The absence of IL-10 consistently resulted in severe EAE. TGF-β was shown to reduce the immune response, but enhanced glial reactivity within CNS, with the risk of increased fibrosis and worsening of the clinical outcome. Furthermore, the proinflammatory cytokine IL-12, known to drive a Th1 response, seemed to aggravate EAE (Chitnis and Khoury, 2003).

These observations suggest that EAE, and possibly MS, is a Th1-mediated disease, and that Th2 cytokines are protective.

1.6 Dendritic cells (DCs)

1.6.1 General characteristics

DCs were first described as Langerhans cells in the skin already in 1868, but the characterization of DCs began only 30 years ago. DCs are the professional APCs in the immune system, with the capacity to regulate immune responses. T and B cells are under the control of DCs (Banchereau and Steinman, 1998). Antigens in the peripheral blood and tissues are taken up and processed by DCs, which subsequently migrate to draining lymph nodes, where they present the antigens to resting lymphocytes. Depending on their state of maturation, DCs can either stimulate or inhibit immune responses (Figdor et al., 2004). Mature, antigen-loaded DCs (mDCs) induce antigen-specific immunity. Immature DCs (imDCs) capture and present self-antigens to T cells, which in the absence of appropriate co-stimulation leads to tolerance (Banchereau et al., 2004). The role played by DCs in central and peripheral tolerance of T cells has been described above (see section 1.1).

In humans, at least two different subsets of DCs are described: lineage-negative (Lin^-) $CD11c^+CD123^-$ myeloid DCs (myDCs), and $Lin^-CD11c^-CD123^+$ plasmacytoid DCs (pDCs) (Banchereau et al., 2000). pDCs constitute <1% of blood mononuclear cells (MNCs). The pDCs constitutively migrate from the blood to T cell areas in secondary lymphoid organs (Grouard et al., 1997). They are virtually absent from normal non-lymphoid tissues, but can be recruited during inflammation (Farkas et al., 2001; Jahnsen et al., 2000). Large amounts of type I IFNs are produced by pDCs, in response to infection with enveloped viruses and certain bacteria (Siegal et al., 1999). Once mature, they can also induce antigen-specific adaptive immune responses against pathogens.

Immature myDCs are located at the sites of potential antigen delivery, namely the skin, interstitial tissues of the respiratory and the gastro-intestinal systems, and the marginal zone of the spleen. Upon the encounter with antigens, myDCs mature and migrate to T cell areas of draining lymph nodes. Plastic-adherent peripheral blood MNCs, *i.e.* monocytes, can be converted to myDC *in vitro* in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (Sallusto and Lanzavecchia, 1994). Alternatively, myDCs can be isolated from plastic-adherent MNCs of rat spleen by overnight culture and subsequent collection of the floating cells. The

papers included in this thesis concern myDCs, and therefore, the focus in this thesis hereafter will be on myDCs, rather than pDCs.

1.6.2 Interactions between DCs and other immune cells

Located in the linings of the body, *i.e.* tissues in direct contact with the outside world, DCs represent one of the first cell types to encounter invading pathogens. Toll-like receptors (TLRs) located on DCs recognize 'pathogen-associated molecular patterns' and the DCs phagocytose such pathogens, digest the proteins and transport antigenic peptides to MHC molecules. The binding of pathogen-associated molecular patterns to TLRs results in activation and partial maturation of DCs, and trigger the migration of DCs to the regional lymphoid organs via the lymphatic system (Reis e Sousa, 2004).

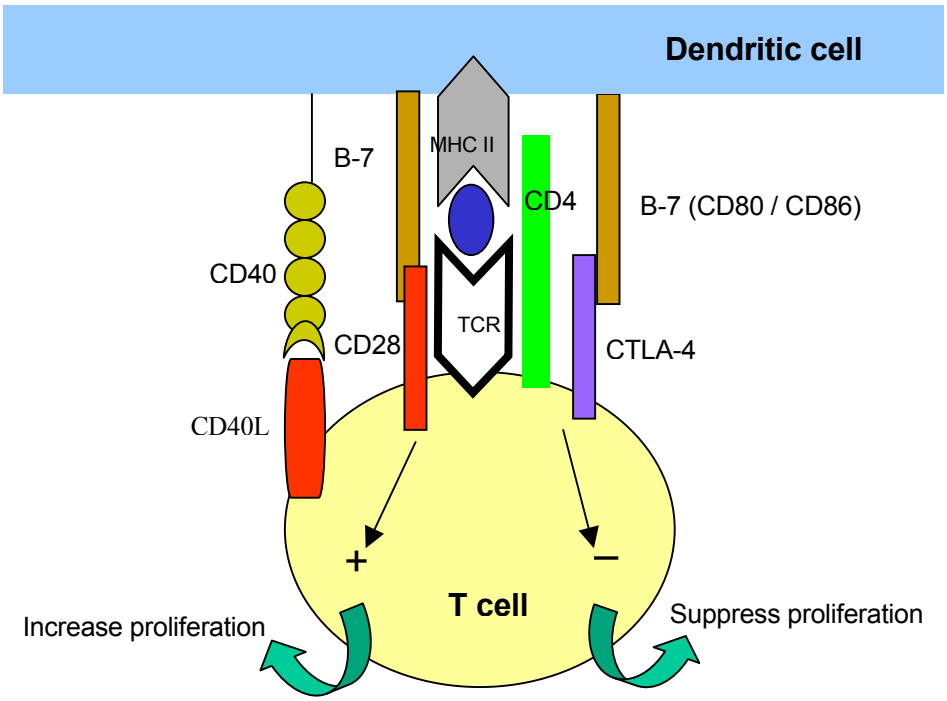

Figure 2. Diagram illustrating the DC - T cell interaction. The main surface molecules involved in the interaction are shown.

In the T cell areas of lymphoid organs, the antigen-bearing DCs present antigenic peptides to T cells through their MHC molecules. $CD4^+$ T cells recognize such antigens presented to them by MHC class II molecules, whereas $CD8^+$ T cells need the antigens to be presented to them by MHC class I molecules. B cells, on the other hand, can

directly identify antigens associated with pathogens by way of the type of Ig on their surface, and do not need DCs to process the antigens. Nevertheless, B cells do require the help from $CD4^+$ T cells, which in turn are activated by DCs for their optimal function (Banchereau and Steinman, 1998).

$CD4^+$ T cells receive two types of signals from DCs. The first signal is antigen-specific and is given by the T cell receptor (TCR) upon its recognition of the antigen-MHC complex, provided that CD4 receptors are present in the vicinity of the antigen-MHC complex (Figure 2). This signal determines the specificity of the T cell response, but in the absence of a second signal it leads to T cell unresponsiveness, *i.e.* T cell anergy (Eagar et al., 2002). The second signal is a co-stimulatory signal, resulting from the interaction of a B-7 molecule (B7.1: CD80; B7.2: CD86) on the surface of DCs, with CD28 on T cells. This is a positive signal (Chambers et al., 2001), that stimulates T cells to proliferate and secrete IL-2 and IFN-γ. Since the CD28 molecules are constitutively expressed on naïve $CD4^+$ T cells, this second signal is an early event in the T cell activation (Chen, 2004). Upon activation, a second co-stimulatory molecule, CTLA-4 (CD152), expressed at very low levels on naïve $CD4^+$ T cells, is translocated to the cell membrane (Leung et al., 1995). CTLA-4 competes with CD28 in the interaction with B-7 molecules. CTLA-4 generates a negative signal, once it engages with B-7, and suppresses T cell proliferation, and reduces IL-2 and IFN-γ secretion by T cells (Figure 2).

The proliferation of $CD8^+$ T cells is due to a similar interaction with DCs, although MHC class I, rather than MHC class II, is the antigen-presenting molecule.

The two signals activating $CD4^+$ T cells are also important for the DCs, with regard to the decision of their fate. If DCs receive only the first signal, through MHC class II, they become tolerogenic (Chen, 2004). If they receive both signals, together with additional co-stimulatory signals, such as that resulting from interaction of CD40 with the CD40 ligand (CD40L) on T cells, the DCs become further activated, fully matured, immunogenic cells.

A third signal, in the form of the cytokines released by DCs and T cells, has also been suggested during the process of DC - T cell interaction. Thus, DCs secreting large amounts of IL-12 and IL-18 drive naïve $CD4^+$ T cells towards a Th1 phenotype, whereas the secretion of small amounts of these cytokines promote the differentiation of the naïve $CD4^+$ T cells towards a Th2 phenotype (Kalinski et al., 1999; Pasare and Medzhitov, 2003) (Figure 1).

1.6.3 IL-10-modified DCs

IL-10 was first described as a 'cytokine synthesis inhibitory factor' (CSIF), an activity shown by Th2 cells, inhibiting the activation of Th1 cells and their cytokine production (Fiorentino et al., 1989). Human IL-10 is a homodimer with a molecular weight of 39 kD. IL-10 is produced by many cell types including T cells, B cells, monocytes, macrophages and DCs (de Waal Malefyt et al., 1992). *In vitro* and *in vivo* studies with recombinant IL-10 and neutralizing antibodies revealed pleiotropic activities on B cells, T cells, natural killer (NK) cells, monocytes, macrophages, mast cells and DCs (de Waal Malefyt et al., 1992; Moore et al., 2001).

The exposure of DCs to IL-10 for 48 h, down-regulates the expression of surface molecules such as MHC class II, CD83 and B-7. Furthermore, IL-10 reduces the release from DCs of a variety of inflammatory cytokines, including IL-1β, IL-6, IL-12 and TNF-α. T cells co-cultured with IL-10-modified DCs (IL-10-DCs) become anergic as indicated by reduced proliferation and reduced secretion of IL-2 and IFN-γ (McBride et al., 2002; Steinbrink et al., 1997). It has been proposed that IL-10-DCs could be beneficial in the treatment of autoimmune diseases because of their ability to suppress autoreactive T cells and to dampen the autoimmune reaction (Link and Xiao, 2001; Steinbrink et al., 1997; Xiao et al., 2003b).

1.6.4 IFN-γ-modified DCs

IFN-γ is a 14 kD polypeptide secreted by NK cells and activated T cells. It is a major cytokine produced as part of the Th1 response. The secretion of IFN-γ is stimulated by proinflammatory cytokines, such as IL-1, IL-12, IL-18 and TNF-α, released by DCs, macrophages and neutrophils (Lieberman and Hunter, 2002; Saunders et al., 1996).

IFN-γ induces the expression of indoleamine 2,3-dioxygenase (IDO), an intracellular enzyme, encountered in a variety of cells including DCs (Robinson et al., 2003; Widner et al., 2000). IDO converts tryptophan, an amino acid that is essential for T cell survival, into N-formylkynurenine, which is then converted to L-kynurenine by the enzyme formamidase (Takikawa et al., 1986).

IDO-induced tryptophan depletion has been demonstrated to play important roles in physiological conditions, such as prevention of delayed-type hypersensitivity responses and maternal tolerance to fetal allografts (Mellor et al., 2002; Mellor et al., 2001; Munn et al., 1998). Expression of IDO by trophoblasts in the human placenta has been shown to affect the contact zone between fetal and maternal immune cells, and thereby prevent

rejection of the fetus due to maternal immune cell activity (Kamimura et al., 1991). Furthermore, IDO expression was shown to prolong the survival of pancreatic islet-cell grafts (Alexander et al., 2002). The immunosuppressive effect of IDO appears to be mediated by suppression of T cell proliferation, possibly by local tryptophan deprivation (Munn et al., 1999). DCs have been shown to suppress T cell proliferation through a rapid and IDO-mediated deprivation of tryptophan (Fallarino et al., 2002; Moffett and Namboodiri, 2003).

Exposure of DCs to IFN-γ for 48 h, and injection of these IFN-γ-modified DCs (IFN-γ-DCs) is a potential way of DC immunotherapy in autoimmune diseases that is explored in Paper IV.

1.6.5 DC immunotherapy in autoimmune diseases

To date, there is no definite treatment for any of the known autoimmune diseases. Even if the presently available immunosuppressive therapy could suppress the disease progression and associated complications, the therapy itself gives rise to considerable side effects. Treatment with DCs is a possible alternative in the search for new treatment strategies for these diseases.

The immune-enhancing or adjuvant actions of DCs are exerted in two phases. In the first immediate phase, DCs capture the antigen, begin to mature in response to stimuli, particularly microbial components, and produce cytokines and chemokines that mobilize and differentiate other cells, including NK cells. In the slower (adaptive) phase, DCs stimulate several components of the T cell response: clonal expansion, differentiation, specifically into Th1 or killer cells, and memory. A large part of current research efforts focus on a different effect of DCs, namely their capacity, when in the immature state, to induce antigen-specific unresponsiveness, or tolerance following antigen capture (Steinman and Pope, 2002).

In the steady state, when there is no acute inflammation or infection, DCs are immature, and may silence immunity in an antigen-specific manner through two mechanisms, *i.e.* deletion of autoreactive T cells, and induction of regulatory T cells (T_Rs) (Hawiger et al., 2001; Steinman and Nussenzweig, 2002). Subcutaneous injection of imDCs, previously pulsed with keyhole-limpet hemocyanin and influenza matrix peptide, into two healthy volunteers that had previously been exposed to influenza, gave rise to suppressed antigen-specific effector T cell function. The effect was seen one week after the injection and lasted for 3-4 months in both individuals. Further analysis revealed that the suppression was due to a $CD8^+$ T cell subset, and was

dependent on cell - cell contact, but not on the production of IL-10 (Dhodapkar and Steinman, 2002). These features were similar to those observed in $CD4^+$ T_Rs induced by imDCs *in vitro* (Jonuleit et al., 2000).

Treatment with imDCs has been shown to suppress EAE in mice, through an increased IL-10 production by $CD8\alpha^-$ DCs (Legge et al., 2002). DCs transduced to secrete a T cell receptor mimicking peptide (TCRpep), and injected into mice with on-going EAE, abrogated the EAE symptoms and prolonged the survival (Mahnke et al., 2003). Administration of IFN-γ-DCs was shown to suppress acute EAE in Lewis rats, and chronic EAE in mice. The treatment also induced an increased T cell apoptosis in the periphery, and a reduced macrophage and $CD4^+$ T cell infiltration within the CNS (Xiao et al., 2004). The injection of DCs exposed to 17β-estradiol (Pettersson et al., 2004), or to TNF-α (Menges et al., 2002), resulted in suppression of on-going EAE in rats and mice, respectively.

The administration of DCs modified with TGF-β (Yarilin et al., 2002) or pulsed with AChR (Xiao et al., 2003a), resulted in suppression of EAMG in Lewis rats.

In a mouse model of SLE, an autostimulatory loop maintaining a cascade of autoimmunity by DCs, presenting self-antigens derived from apoptotic cells, has been described (Kalled et al., 2001). Increased bio-availability of IFN-α and β in SLE was shown to convert imDCs to mDCs, which in turn present self-antigens (including nuclear antigens) derived from apoptotic cells, to autoreactive T cells, and stimulate their proliferation. This creates the initial autoimmune injury, which may be amplified by a vicious cycle of self-antigen capture, and presentation by IFN-matured DCs (Banchereau et al., 2004). These findings suggest that certain alterations in DCs that would keep them immature, and at the same time antigen-specific, would help develop new treatments for this disease.

DCs have been suggested to be involved in the pathogenesis of rheumatoid arthritis, and hence the possibility of using DCs in the treatment of this disease was considered (Pettit and Thomas, 1999; Robbins et al., 2003). DCs that were genetically engineered to express IL-4, were shown to inhibit collagen-induced arthritis in mice (Morita et al., 2001).

DCs have also been tested as a treatment for type I diabetes, a multifactorial autoimmune disease directed against antigens of the insulin-producing β-cells of the pancreatic islets. Non-obese diabetic (NOD) mice develop a spontaneous form of diabetes with many etiological and pathogenic similarities to the human disease

(Young et al., 2004). Adoptive transfer of bone marrow-derived DCs was shown to prevent spontaneous development of diabetes in NOD mice. This treatment appeared to induce the generation or stimulation of T_R cells (Feili-Hariri et al., 1999). DCs with transgenic expression of IL-4 enhanced the efficacy of the DC treatment in NOD mice with more advanced insulitis, and protected these mice from diabetes (Feili-Hariri et al., 2003).

It is clear from these observations, that DCs play a central role, either in the pathogenesis, or in the suppression, of many autoimmune diseases. DCs could be considered as a 'biofactory' producing many different cytokines, and transmitting various signals to other cells through these cytokines, or directly by cell - cell contacts. It would therefore not be surprising if manipulation of these remarkable cells in the appropriate way could ultimately solve the problem of treating autoimmune diseases.

2 AIMS OF THE STUDIES

2.1 General aims

In view of what is known from the literature about DCs, it is clear that DCs have the potential to play a major role in the treatment of autoimmune diseases. IL-10 and IFN-γ seem to be two important immunomodulatory cytokines with the capacity to modify DC function. Therefore, it is of interest to characterize the activities and products of DCs modified with cytokines such as IL-10 and IFN-γ, and study the effects of these cells in the treatment of autoimmune diseases. Human blood samples were collected for analysis of *in vitro* effects, and animal models were used to assess *in vivo* effects of cytokine-modified DCs.

2.2 Specific aims

Paper I: To investigate the therapeutic effects of IL-10-DCs in EAMG

Paper II: To study the properties of IL-10-DCs and assess the effects of IL-10-DCs on autologous lymphocytes from MG patients

Paper III: To study the properties of IL-10-DCs and assess the effects of IL-10-DCs on autologous lymphocytes from MS patients

Paper IV: To investigate the therapeutic effects of IFN-γ-DCs in EAMG

3 MATERIALS AND METHODS

3.1 Studies on clinical material

3.1.1 Patients and healthy controls

Blood samples were obtained from patients with MG, admitted to the neurological clinics at the Karolinska University Hospital at Solna or Huddinge. The diagnosis of MG was based on the presence of the characteristic fluctuating muscle fatigability, serum AChR antibodies, single-fibre electromyography, and the response to treatment with AChE inhibitors.

Blood samples from patients with MS were obtained at the neurological clinic, Karolinska University Hospital at Huddinge. MS patients were diagnosed based on the lesions observed in magnetic resonance imaging (MRI) of the brain, and the oligoclonal IgG bands in the CSF.

Samples from healthy controls (HCs) were obtained from the blood bank at Karolinska University Hospital at Huddinge, and from the staff of the Immunological Research Unit, Department of Medicine, Centre for Molecular Medicine (CMM), Karolinska Institutet.

Venous blood samples were collected in heparin-containing tubes, and were used within 4 h of collection. All studies were approved by the local ethical committees of the Karolinska Institutet and the Karolinska University Hospital at Huddinge.

3.1.2 Generation and modification of human DCs

The heparinized peripheral blood samples were centrifuged over Lymphoprep density gradients in order to isolate MNCs. The MNCs were plated in 6-well plates in incomplete medium (RPMI 1640), and incubated for 2 h at 37ºC, resulting in adherence of monocytes. Lymphocytes remained floating, and were gently washed away, and preserved for later use in co-culture experiments. The adherent monocytes, *i.e.* DC precursors, were cultured for 4 (Paper III) or 5 (Paper II) days in the presence of recombinant human (rh) GM-CSF and rhIL-4 in complete medium, to generate DCs. The DC preparations contained <10% lin^+ cells and >80% $CD11c^+$ cells.

mDCs (LPS-DCs) were obtained by culture of imDCs with 5 μg/ml of LPS for 24 h. IL-10-DCs were obtained by culture of imDCs with 10 ng/ml of rhIL-10 for 48 h.

3.1.3 Lymphocyte cultures and co-cultures

The non-adherent lymphocytes washed off and preserved during DC preparation (see section 3.1.2), were re-suspended in complete medium and cultured in the presence of rhIL-2 for proliferation and differentiation. On day 4 (Paper III) or 5 (Paper II), the lymphocytes were harvested, washed and re-suspended in culture medium. Among the lymphocytes, the percentage of $CD3^+$ T cells was >80% in Paper III, and >85% in Paper II.

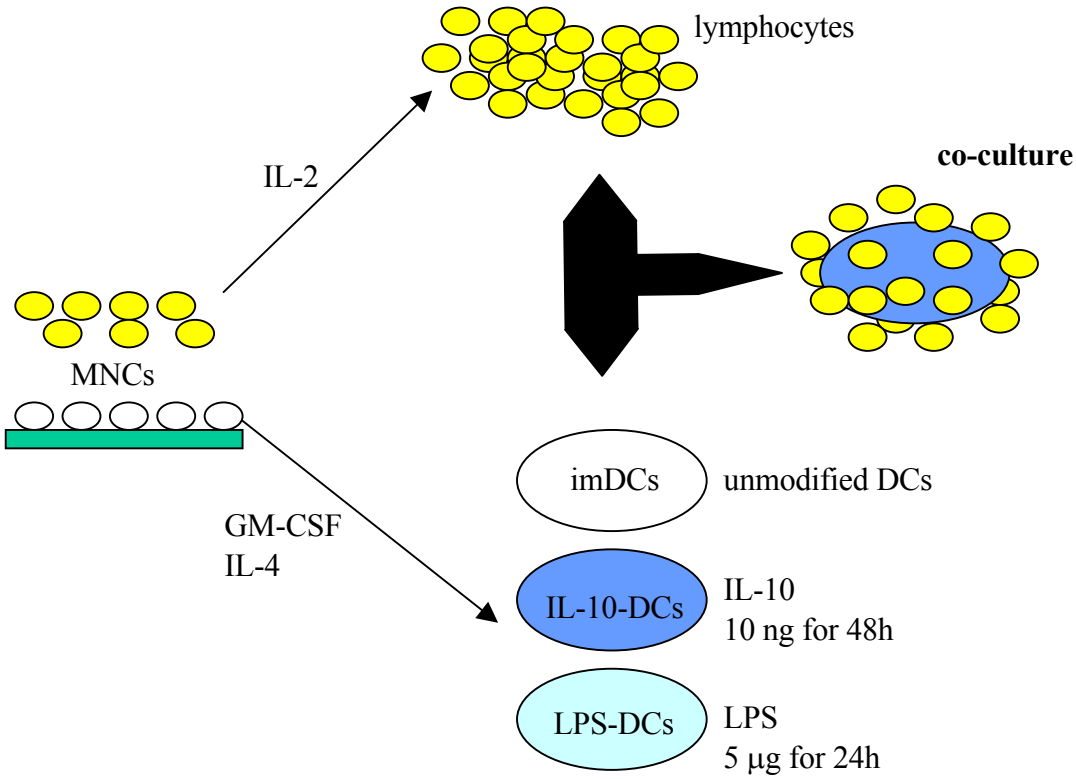

Figure 3. Generation and *in vitro* modulation of monocyte-derived DCs, and co-culture of DCs with autologous lymphocytes.

DCs were co-cultured with autologous lymphocytes in a ratio of 1:20 ($5x10^4$ DCs with $1x10^6$ lymphocytes). For each patient, lymphocytes cultured alone served as control. DCs cultured alone were maintained in order to obtain baseline cytokine secretion by DCs. Cultures were maintained for 3 (Paper III) or 7 (Paper II) days. Figure 3 schematically illustrates the human cell culture system.

3.2 Animal experiments

Female Lewis rats weighing 150-180g were purchased from Zentralinstitut fur Versuchstierzucht, Hannover, Germany. The rats were housed under specific pathogen-free conditions and were used at 6-8 weeks of age.

3.2.1 Induction and evaluation of EAMG

The rats were immunized subcutaneously (s.c.) in the base of the tail, with AChRs emulsified in Freund's complete adjuvant (FCA) containing *Mycobacterium tuberculosis* (strain H37RA). The rats were weighed and evaluated daily with regard to clinical signs in a blinded fashion, by at least two investigators. The clinical symptoms were graded from 0 to 3 according to the following scale: 0, no weakness; 1, mildly decreased activity, weak grip or cry, with fatigability; 2, markedly decreased activity and body weight, hunched posture at rest with the head down and the forelimb-digits flexed, and tremulous ambulation; and 3, severe generalized weakness, no cry or grip, and moribund.

3.2.2 Isolation, modification and injection of DCs in EAMG

MNCs were obtained from the spleen of rats. The spleen was squashed and the erythrocytes were lysed by osmosis. MNCs were plated in serum-free Dulbecco's modification of Eagle's medium containing 50 IU penicillin, 50 μg/ml streptomycin, 1% MEM amino acids and 10 mM Hepes at 37°C and 5% CO_2. After 2 h, non-adherent cells were gently removed by swirling the flasks and aspirating the medium. This procedure was repeated 4 times, and then new medium containing 10% fetal calf serum (FCS) was added to the flasks. After 18 h of culture, re-floating cells were collected as a DC-enriched fraction, while adherent cells mostly consisted of macrophages.

The DCs were exposed to the relevant cytokine (200 ng/ml of rhIL-10 in Paper I, and 100 U/ml of rrIFN-γ in Paper IV) for 48 h before they were finally harvested, washed with serum-free medium and injected into rats with on-going EAMG. The DCs were injected 1 x 10^6/rat intraperitoneally (i.p.) (Paper I) or s.c (Paper IV), in rats that had been immunized 5 days earlier with AChR + FCA. EAMG rats injected with naïve DCs or vehicle, served as two types of controls. Additional control groups were included in Paper IV, in order to investigate the mechanism of action of IFN-γ-DCs. Figure 4 schematically illustrates how the EAMG experiments were carried out.

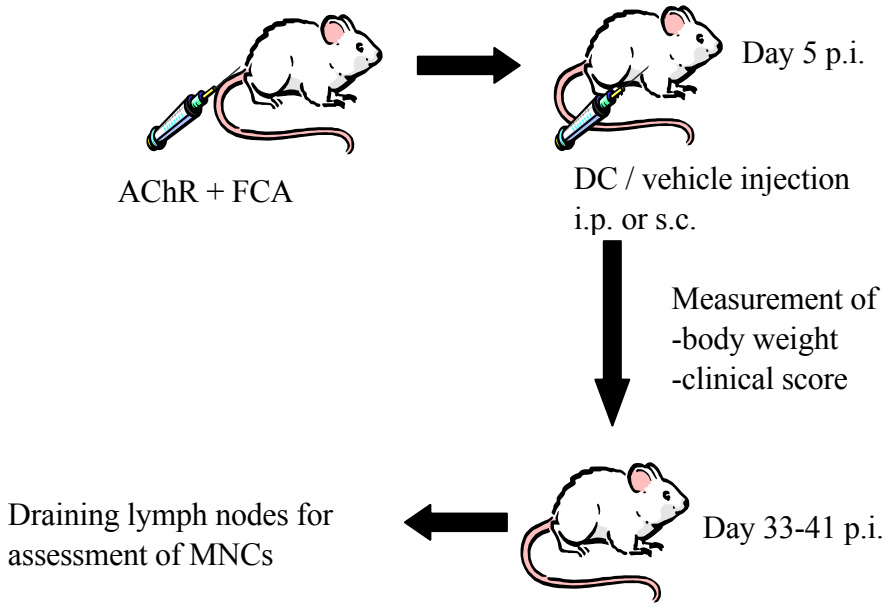

Figure 4. General protocol for the EAMG experiments. Lewis rats were immunized with AChR in FCA. Injections with different types of DCs were given on day 5 post-immunization (p.i.) by the i.p. or s.c. route. Clinical assessment was performed daily till 33-41 days p.i.. The rats were sacrificed and the MNCs were prepared from the draining lymph nodes for analysis of immunological parameters.

3.2.3 Preparation of MNCs for immunological assessment

The draining lymph nodes and the spleen were removed under aseptic conditions from rats with EAMG on day 33-41 post-immunization (p.i.). In Paper I, the mesenteric lymph nodes were used (by mistake indicated in the paper as the inguinal and popliteal lymph nodes). In Paper IV, the inguinal and popliteal lymph nodes as well as the spleen were used. MNC suspensions were obtained by grinding the organs through a 40 μm nylon mesh in culture medium. The splenic erythrocytes were lysed by osmosis and the cells were washed three times and re-suspended in medium. These MNCs were used for proliferation assays, ELISA, ELISPOT and flow cytometry.

3.3 Flow cytometry

In order to analyse the expression of surface molecules and intracellular molecules of interest, the cells were examined by a FACScan and analysed using CellQuest Software (Becton Dickinson).

For cell surface staining, the cells were incubated for 15 min at 4ºC with the relevant antibodies. For intracellular staining, the cells were fixed with 4% paraformaldehyde,

permeabilized, and then stained with the relevant antibodies. The different antibodies used for cell staining are given in the respective papers.

In order to study a specific cell type by flow cytometry, the cells were first gated according to forward scatter (FSC) and side scatter (SSC). A second gate was applied to select cells with specific molecules, in order to study the phenotypic expression of each cell type. For example, when studying $CD4^+CD25^+$ T cells, the first gate was applied to select lymphocytes, according to FSC and SSC (R1 in Figure 5A). Then, the second gate was applied to CD4 and CD25 double-positive cells (R2 in Figure 5A). DCs were analysed by applying a gate with FSC and SSC to suit DCs (R1 in Figure 5B).

In experiments on human cells, the yield of DCs was >80%, as determined by flow cytometry. Cells positive for the 'lineage cocktail': CD3 for T cells, CD19 and CD20 for B cells, CD16 for macrophages, CD14 for monocytes, and CD56 for NK cells, represented less than 10% of the total number of cells analysed.

In experiments on rat cells, the DC fraction showed a purity of >85%, as determined by flow cytometry after staining with OX62 monoclonal antibodies (mAbs), which recognize the αE2 subunit of an integrin specifically expressed on rat DCs. Anti-rat CD3 (T cells), CD45RA (B cells) and CD161 (NK cells), were used to detect contamination by other cells. In the DC-enriched fraction, the contamination was about 2.3% for T cells, 1.9% for B cells, and ≤1% for NK cells.

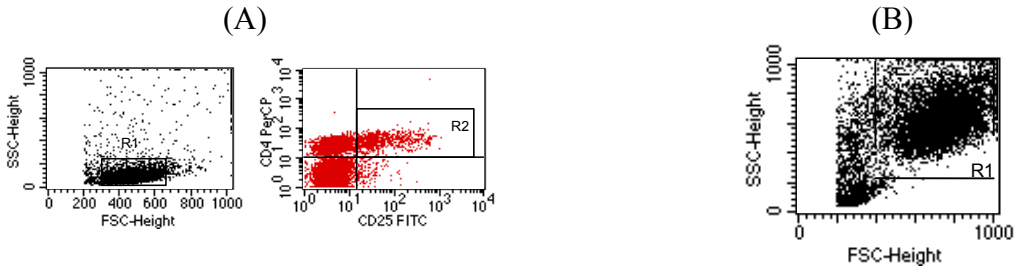

Figure 5. Gating of cells during flow cytometry. (A) Analysis of $CD4^+CD25^+$ T cells (B) Analysis of DCs.

3.4 T cell proliferation assay

3.4.1 Methylthymidine method

In Paper I, MNCs were exposed to AChR in round-bottomed 96-well microtitre plates for 48 h. The cells were then pulsed for 18 h with ^{3}H-methylthymidine and harvested

on to glass fibre filters for measurement of thymidine incorporation. The cell experiments were run in triplicate and results were expressed as cycles per minute (cpm).

3.4.2 AlamarBlue method

AlamarBlue is a dye that is reduced by mitochondrial enzyme activity, resulting in a change of colour. The degree of colour change, which can be determined as change in absorbance, reflects the extent of cellular proliferation. In Paper IV, MNCs were cultured for 72 h in the presence of AChR or an irrelevant antigen, MBP. AlamarBlue was added to the cultures and after incubation for 4 h, the absorbance was measured by a spectrophotometer. The cultures were run in triplicate, and the results were expressed as mean absorbances.

3.5 Enzyme-linked immunosorbent assay (ELISA)

In Paper I and IV, supernatants of MNC cultures, or serum of rats, were analysed for IL-10 and IFN-γ. In Paper II and III, supernatants of DC cultures were analysed for IL-12p40, IL-12p70, IL-6 and TNF-α, while supernatants of co-cultures were analysed for IL-2, IL-4, IL-10 and IFN-γ. Flat-bottomed 96-well plates were used. Optical densities were measured using an ELISA-plate reader. Cytokine concentrations were calculated using standard curves.

3.6 Enzyme-linked immunospot (ELISPOT)

ELISPOT was used to detect AChR antibody-secreting cells in Paper I and IV. Microtitre plates with nitrocellulose bottoms were coated with AChR or an irrelevant antigen, MBP. Aliquots of experimental samples of MNCs were added to the wells and incubated for 24 h. The wells were emptied and secreted antibodies to AChR were detected with rabbit anti-rat IgG followed by immunocytochemistry using peroxidase staining. The resulting red-brown spots, corresponding to cells secreting AChR antibodies, were counted in a blinded fashion by using a dissection microscope. The samples were run in triplicate and the results were expressed as number of antibody-secreting cells per 10^5 MNCs.

3.7 Statistical analysis

Nonparametric tests were used for statistical comparisons in Paper II and III, as suggested by the results of the normality test ($P \geq 0.05$). For multiple comparisons

between imDCs, IL-10-DCs and LPS-DCs, in terms of their cytokine production and expression of surface molecules, the Friedman test followed by Dunns *post hoc* test was used. Similarly, for multiple comparisons between different co-cultures, in terms of cytokine production by lymphocytes and expression of CD69 and CTLA-4 by $CD4^+CD25^+$ T cells, the Friedman test followed by Dunns *post hoc* test was used. For comparisons between patients and HCs, the Mann-Whitney test with two-tailed *P* value was used.

One-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test was used for group comparisons of animal experiments, in Paper I and IV. Differences were considered statistically significant when the *P* value was ≤ 0.05.

4 RESULTS AND DISCUSSION

4.1 IL-10-DCs suppressed EAMG (Paper I)

The exposure of imDCs to IL-10 has been shown to strongly reduce the capacity of DCs to stimulate $CD4^+$ T cell response in an allogeneic mixed leukocyte reaction (MLR) (Steinbrink et al., 1997). DCs pre-cultured with IL-10 induced a state of alloantigen-specific anergy in $CD4^+$ T cells, and peptide-specific anergy in an influenza hemagglutinin-specific T cell clone. Analysis of the supernatants of these anergic T cells revealed a reduced production of IL-2 and IFN-γ as compared with that in control cells. Suppression of T cell proliferation by IL-10-DCs has also been reported *in vivo* in mice (Haase et al., 2002).

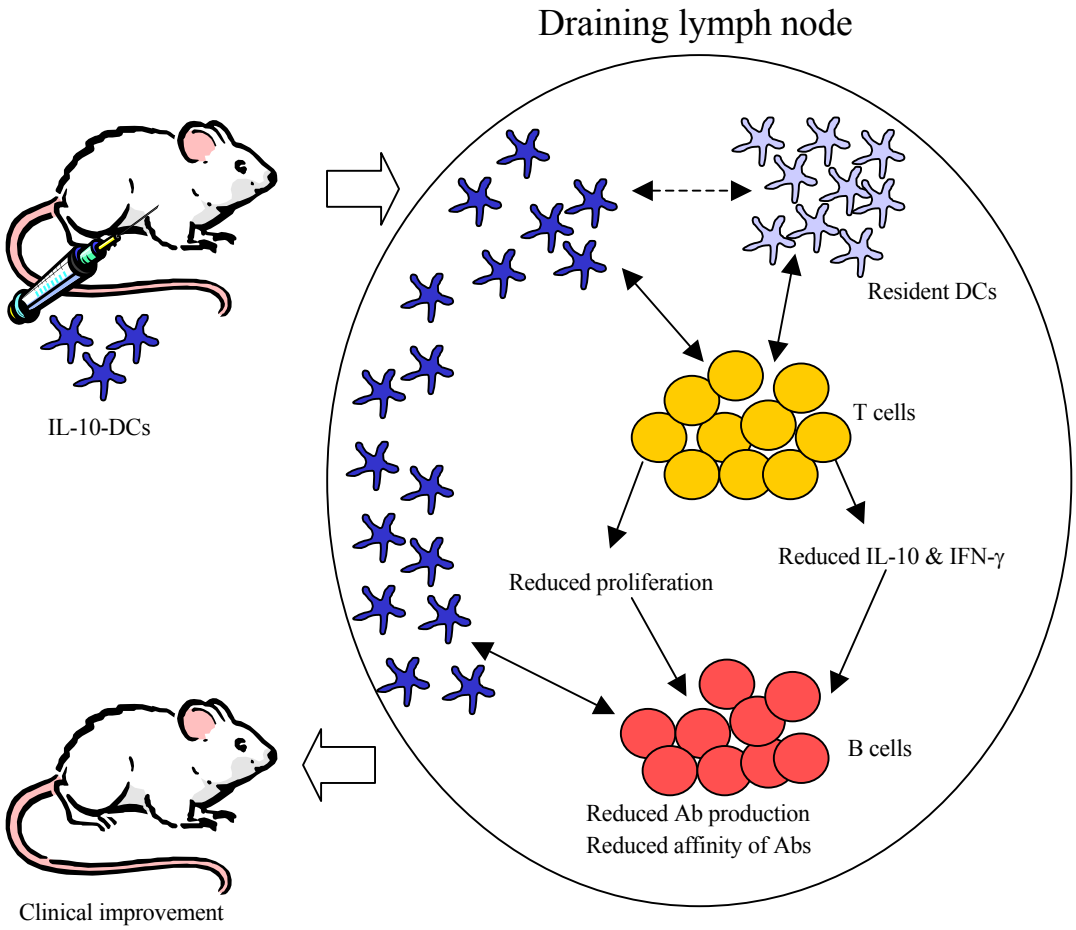

Figure 6. Interaction of IL-10-DCs in the draining lymph node. IL-10-DCs can directly interact with T cells and B cells, and possibly also with resident DCs. Ab = antibody.

We have explored the effects of IL-10-DC immunotherapy in EAMG. Rats with EAMG were injected i.p. on day 5 p.i. with splenic DCs (1×10^6 cells/rat) isolated from rats with on-going EAMG on day 39 p.i. and exposed *in vitro* to IL-10.

The treatment with IL-10-DCs resulted in reduced clinical scores, less body weight loss, lower numbers of AChR antibody-secreting cells, and a lower affinity of the AChR antibodies. In addition, there was a reduced proliferation, and a decreased expression of the co-stimulatory molecules, CD80 and CD86, by MNCs from draining lymph nodes. However, the proportion of $CD4^+CD25^+$ T cells among the MNCs was not altered upon treatment with IL-10-DCs. *In vitro* stimulation of lymph node MNCs with AChRs resulted in a reduced secretion of IL-10 and IFN-γ from cells obtained from rats treated with IL-10-DCs.

DCs, known for their T cell stimulatory properties, also have major effects on B cell growth and Ig secretion. DCs activate and expand $CD4^+$ Th cells, which in turn stimulate B cell growth and antibody production. During this process, there is also a more direct DC - B cell dialogue (Banchereau and Steinman, 1998; Dubois et al., 1997). After injection, the DCs can migrate to the draining lymph nodes via the local lymphatic system. Within the T cell area of the draining lymph nodes, they are able to interact with T and B cells directly. In addition, these DCs can influence T and B cell functions indirectly via the soluble factors that they release (Dubois et al., 1997). The suppressed proliferation of MNCs from draining lymph nodes could therefore be a result of an interaction between T and B cells and the injected IL-10-DCs, expressing reduced numbers of co-stimulatory molecules and proinflammatory cytokines (see section 1.6.3). Reduction in AChR antibodies could be due to direct interaction between DCs and B cells, or to the lack of T cell help, that is secondary to reduced T cell proliferation. Reduction of IL-10, a potent growth and differentiation factor for B cells (Banchereau, 1995), is sufficient to block the maturation of B cells to antibody-secreting plasma cells (Figure 6). In addition to IL-10, also IFN-γ can drive resting B cells towards Ig-secreting cells and affinity maturation (Keller et al., 1990; Rizzo et al., 1992; Sidman et al., 1984). IFN-$\gamma^{-/-}$ mice were shown to develop less severe EAMG, associated with production of low-affinity AChR antibodies (Zhang et al., 1999). Administration of IFN-γ increased the severity of EAMG (Wang et al., 2000). Therefore, it appears that if both IL-10 and IFN-γ are reduced, there will be a potential response consisting of suppression of antibody production, and affinity maturation of B cells.

The markedly reduced levels of IFN-γ, together with the reduced proliferation of MNCs, would seem to indicate (even without data on IL-2) that the T cells, the main producer of this cytokine, have become anergic. Low levels of IL-10 together with unaltered $CD4^+CD25^+$ T cells, suggests that the induction of tolerance was unlikely to be caused by an increase in a subset of T_R, but was more likely due to T cell anergy.

It is possible that the observed reduction in co-stimulatory molecules on MNCs from draining lymph nodes, can be explained by an interaction of migrating IL-10-DCs with resident APCs, such as B cells or DCs (Futagawa et al., 2002; Kleindienst and Brocker, 2003), in the lymph nodes (Figure 6). The co-stimulatory molecules expressed on APCs, which provide the second signal during APC - T cell interaction, are important for the activation of T cells (see section 1.6.2). The absence of a second signal due to low expression of co-stimulatory molecules on APCs, may lead to T cell unresponsiveness.

It has been suggested that the ability of IL-10 to convert imDC into tolerogenic APCs could be useful in the therapy of patients with autoimmune disease (Jonuleit et al., 2001; Steinbrink et al., 1997). The present findings support this view and represent a step forward towards a possible use of IL-10-DCs in the therapy of autoimmune diseases.

4.2 DC responses were similar in MG, MS and HCs (Paper II & III)

It has been shown that the exposure of DCs to IL-10 reduces the expression of surface molecules, such as MHC class II, CD83 and B-7 (de Waal Malefyt et al., 1991b; Willems et al., 1994). Furthermore, IL-10 reduces the release of a variety of inflammatory cytokines, including IL-1, IL-6, IL-12 and TNF-α (de Waal Malefyt et al., 1991a; Steinbrink et al., 1997). In contrast, the exposure of DCs to LPS results in high expression of maturation and co-stimulatory molecules, and an increased production of inflammatory cytokines (Granucci et al., 1999; Nagorsen et al., 2004).

In order to compare the responses of DCs from MG and MS patients with those of DCs from HCs, three different types of DCs were generated from both patients and HCs. Naïve imDCs were defined as monocytes cultured with GM-CSF + IL-4 for 4 (Paper III) or 5 (Paper II) days. IL-10-DCs were obtained by incubation of the DC culture with IL-10 for the last 48 h, and mDCs were obtained by incubation with LPS for the last 24 h. The phenotype of these different DCs was analysed with regard to the expression of HLA-DR, CD80, CD86 and CD83, as well as to the secretion of the

cytokines IL-12, IL-6 and TNF-α. All three types of DCs derived from MS or MG patients were similar to their counterpart derived from HCs, in terms of the expression of surface molecules and the secretion of cytokines.

The exposure of DCs to LPS, but not IL-10, resulted in increased expression of HLA-DR as well as the co-stimulatory molecules, CD80 and CD86, and the maturation molecule, CD83, in both MS and MG patients and in HCs. Similarly, the secretion of the proinflammatory cytokines IL-12p40, IL-12p70, IL-6 and TNF-α, was increased by treatment with LPS, but not IL-10. However, no significant difference was observed between imDCs and IL-10-DCs in these responses.

In the context of DC immunotherapy, the occurrence of an altered DC response in autoimmune disease is probably not of major concern. The most important issue is the possibility to induce tolerance in DCs, and to maintain this *in vivo*, after administration to the patient.

4.3 DCs exposed to IL-10, resisted maturation by LPS (Paper III)

It is of interest to know whether the IL-10-DCs can remain immature in a proinflammatory environment, such as that found in autoimmune diseases. It has been shown that IL-10 could inhibit LPS-induced expression of HLA-DR, CD80, CD86 and CD83, and secretion of IL-12, TNF-α, IL-6 and IL-1β by DCs from healthy donors (McBride et al., 2002). It appeared that changes in cell surface molecules, rather than the inhibition of cytokine production, was the major mechanism of IL-10 modulation of DCs, and DC-induced T cell suppression (McBride et al., 2002). Haase et al. (2002) demonstrated that IL-10 could inhibit the maturation of mouse bone marrow-derived DCs *in vitro* by LPS or a mixture of cytokines (TNF-α, IFN-γ and IL-4). Upon injection into mice, DCs pretreated with IL-10, irrespective of whether they were exposed in parallel to LPS or the cytokine mixture, suppressed antigen-specific T cell proliferation (Haase et al., 2002).

In Paper II, naïve imDCs and IL-10-DCs from MS patients were exposed to LPS, and the expression of HLA-DR, CD80, CD86 and CD83 was measured. Unlike imDCs, the IL-10-DCs did not respond to LPS with increased expression of these surface molecules. This, together with existing reports, suggests that IL-10-DCs have the ability to resist *in vivo* maturation upon injection into patients with an autoimmune disease. However, it should be noted that other stimulatory/maturation factors that could promote maturation of IL-10-DCs may occur in autoimmune diseases.

4.4 DCs activated T cells more in MG than in HCs (Paper II)

The proportion of $CD4^+CD25^+$ T cells and their CD69 co-expression increased upon co-culture of autologous lymphocytes with imDCs, IL-10-DCs and LPS-DCs derived from MG patients. This was not seen in co-cultures with lymphocytes from HCs. Furthermore, all three types of DCs from MG patients increased the proportion of $CD4^+CD25^+$ T cells in the co-culture, as compared to DCs derived from HCs. This indicates that the DCs from MG patients are either more effective in activating T cells than DCs from HCs, or that the T cells of MG patients are more sensitive to signals from DCs.

In vivo, the proportion of $CD4^+CD25^+$ T cells in MG patients have been shown to be increased (Ragheb et al., 1999), or unaltered (Huang et al., 2004) as compared to HCs. More focussed studies taking into consideration the duration and the type of disease will be necessary to clarify this issue.

4.5 IL-10-DCs induced a Th2 response in co-culture (Paper II & III)

IL-10-DCs, irrespective of whether they were generated from MS, MG or HCs, stimulated autologous lymphocytes to produce IL-10 and IL-4, but not IL-2 and IFN-γ to the same extent, in co-cultures. There is evidence for MS to be a Th1-mediated disease, and MG to be Th2-mediated. The role of Th1 and Th2 cytokines has been studied extensively in animal models of these two diseases.

IL-10 has disease-suppressing effects in EAE and disease-promoting effects in EAMG. The absence of IL-10 consistently resulted in more severe forms of EAE, suggesting that its function is unique and cannot be replaced by other Th2 cytokines (Chitnis and Khoury, 2003). On the other hand, IL-10, being an important growth and differentiation factor for B cells, contributes to the pathogenesis of antibody-mediated diseases. Thus, IL-10 deficiency in knock-out (KO) mice resulted in resistance to the induction of EAMG (Poussin et al., 2000), whereas IL-10 over-expression rendered the mice more susceptible to the development of EAMG (Ostlie et al., 2001). The number of circulating AChR-specific cells secreting IL-10 was increased in MG patients (Huang et al., 1999).

The role of IL-4, on the other hand, is not very clear in either of these diseases. The presence of additional IL-4 may reduce the severity of EAE (Racke et al., 1994; Shaw et al., 1997), while the absence of IL-4 generally does not alter the course of disease

(Liblau et al., 1997). This implies that in the absence of IL-4, other Th2 cytokines such as IL-10 may substitute for its function, and contribute to the induction of tolerance in EAE (Bettelli et al., 1998; Chitnis and Khoury, 2003; Inobe et al., 1998; Liblau et al., 1997). IL-4 was not necessary for the development of EAMG (Balasa et al., 1998), but its absence increased the susceptibility to the disease (Milani et al., 2003), and IL-4 deficiency permitted the development of a chronic form of EAMG (Ostlie et al., 2003).

The Th1 cytokines, IFN-γ and TNF-α, have been shown to play dual roles in EAE, *i.e.* by inducing the disease, and later conferring protection from the disease, primarily by limiting the immune response. A complete deficiency of these Th1 cytokines does not induce tolerance to EAE, and may have deleterious effects. However, incomplete elimination or modulation of the expression may be protective (Chitnis and Khoury, 2003). IL-2 may have a similar role, considering the fact that it is an essential cytokine for expansion of $CD8^+$ T cells as well as of $CD4^+$ T cells, irrespective of their skewing towards Th1, Th2 or Th3. Although it is generally considered that Th2 responses predominate in MG, the Th1 cytokine IFN-γ has been shown to be involved in the pathogenesis in animal models of the disease (Balasa et al., 1997; Zhang et al., 1999).

In MS, increased levels of IL-10 and IL-4, together with a reduction, but not absence, of IFN-γ, have shown to be beneficial. Therefore, IL-10-DCs would seem to be a good candidate for future immunotherapy in MS. On the other hand, since IL-10-DCs can enhance the secretion of IL-4 and IL-10 by autologous lymphocytes, this should be taken into consideration when planning human trials with IL-10-DCs in antibody-mediated autoimmune diseases that predominantly involve a Th2 response. In addition, it should be kept in mind that the scope of *in vitro* experiments in predicting *in vivo* outcome in patients is limited.

4.6 IFN-γ-DCs suppressed EAMG (Paper IV)

The Th1 cytokine IFN-γ has been shown to induce the expression of IDO, an enzyme that was demonstrated to be involved in immunotolerance. Thus, the activity of IDO plays an important role *e.g.* in prevention of delayed-type hypersensitivity responses and maternal tolerance to fetal allografts (Mellor et al., 2002; Mellor et al., 2001; Munn et al., 1998). To explore the effects of a cytokine with a different mode of action than IL-10 in the modification of DCs, we studied the responses to IFN-γ-DCs in EAMG. The beneficial effects of IFN-γ-DCs have been demonstrated in other autoimmune disease models such as acute and chronic EAE (Xiao et al., 2004). In Paper IV, splenic

DCs isolated from rats with EAMG were exposed *in vitro* to IFN-γ, and then injected to rats with on-going EAMG. Control groups consisted of rats with EAMG that were injected with naïve DCs, with 1-methyl-DL-tryptophan (1-MT), or with DCs exposed to IFN-γ in the presence of 1-MT.

The treatment with IFN-γ-DCs resulted in lower clinical scores and less body weight loss, as compared to control treatments. The number of AChR antibody-secreting MNCs, measured by ELISPOT, was lower in draining lymph nodes of rats injected with IFN-γ-DCs, than in the control groups. The number of plasma cells among the MNCs of rats treated with IFN-γ-DCs, measured by flow cytometry, was also low as compared to the controls.

We observed a reduced expression of B cell-activating factor (BAFF) by MNCs from the draining lymph nodes of rats treated with IFN-γ-DCs. BAFF is a TNF-family ligand expressed by APCs of myeloid origin, including macrophages and DCs, and is important in B cell activation and in the differentiation of B cells into antibody-secreting plasma cells (Do and Chen-Kiang, 2002). The formation of antibody-secreting plasma cells during the terminal differentiation of B cells generally begins with assistance from $CD4^+$ Th cells. However, some antigens can elicit an antibody response in the absence of T cells, and the expression of BAFF by APCs seems to play an important role in this process (Do and Chen-Kiang, 2002; MacLennan and Vinuesa, 2002). In Paper IV, the treatment with IFN-γ-DCs suppressed neither T cell proliferation, nor the production of IL-10 and IFN-γ. However, the number of AChR antibody-secreting cells and the number of plasma cells were reduced. It is possible that this reduction was a direct result of the decrease in BAFF expression by the APCs.

The clinical signs of EAMG were aggravated by administration of 1-MT, a specific inhibitor of IDO. Furthermore, 1-MT abolished the IFN-γ-induced reduction in the number of AChR antibody-secreting cells *in vitro*. These observations are suggestive of a possible involvement of IDO in suppression of antibody production by B cells, and in the improvement in EAMG. In conclusion, IFN-γ-DCs improved clinical signs of EAMG by suppressing autoantibody production by B cells possibly through an 'IDO-BAFF pathway'.

5 CONCLUDING REMARKS

The results obtained, so far, from DC immunotherapy in autoimmune diseases are encouraging. There are several issues yet to be resolved in this field, limiting these studies to a pre-clinical level (Figdor et al., 2004). One main issue is the type of modulation that is necessary before the DCs can be administered to a patient. A modulation that is beneficial in one disease may not be useful in another. Although the categorization of autoimmune diseases according to the predominance of Th1 or Th2 responses would be a good start, this does not solve the problem, since even within these two categories there is a variation between the diseases, depending on the levels of individual Th1 and Th2 cytokines, and possibly many other unidentified factors, giving rise to the disease. Therefore, it is necessary to identify the most suitable intervention for each individual disease, rather than for the disease category.

The route of administration is important in DC immunotherapy. The route should not only be efficient in suppressing the disease, but also be feasible for the use in patients. Both IL-10 and IFN-γ were potent in modifying DCs in such a way as to suppress EAMG. IL-10-DCs were effective when administered i.p. and IFN-γ-DCs were effective when administered s.c. In preliminary experiments it was found that IL-10-DCs, when administered s.c., did not have a positive effect, whereas IFN-γ-DCs seemed to be beneficial by both routes (Link and Xiao, 2001). With regard to IL-10-DCs, a reduced trafficking to draining lymph nodes, when injected s.c. (Demangel et al., 2002), may be the reason for the lack of effect via this route. The trafficking of DCs from the inflammatory sites to lymphoid tissue seems to be associated with a chemokine receptor switch on the DC surface (Dieu et al., 1998), and this switch is blocked by the exposure of DCs to IL-10 (D'Amico et al., 2000). Consistent with this, migration of Langerhans cells from the skin to the draining lymph nodes is enhanced in IL-$10^{-/-}$ mice (Wang et al., 1999a). However, the reason why the same IL-10-DCs are effective by i.p. administration, is a question that remains to be answered.

The suppression of EAMG by IL-10-DCs was found to be associated with a reduced secretion of IL-10 and IFN-γ, and a reduced proliferation and AChR antibody-production by MNCs from the draining lymph nodes in response to the autoantigen, AChR. These results are promising for future use of IL-10-DCs in MG.

However, upon generation of IL-10-DCs from MG patients and co-culturing with autologous lymphocytes, there was an increased production of IL-10 and IL-4 by lymphocytes in contrast to the response observed in EAMG. Both IL-4 and IL-10 are potent growth and differentiation factors for B cells (Banchereau, 1995), which are the main players in this disease, producing the autoantibodies. However, in addition to analysis of the T cell phenotype and cytokine secretion pattern in response to IL-10-DCs, data on B cell response and T cell proliferation will be important in order to understand the mechanisms at play in the human disease. It should be kept in mind that responses in the human may be quite different from those observed in the animal model, simply due the fact that the age of the patient and the stage of the disease at the time of intervention can alter the outcome. This leads to a potential problem in extrapolating results obtained from animal models to the human diseases.

In Paper III, we demonstrated that IL-10-DCs stimulate autologous lymphocytes to secrete IL-4 and IL-10, skewing the $CD4^+$ Th response towards Th2 in MS. According to the present understanding of MS and its models, this is a protective immune response. Furthermore, we showed that IL-10-DCs have the capacity to resist maturation induced by LPS. Others have shown IL-10-DCs to remain immature upon exposure to different maturation signals, and to be able to suppress T cell proliferation *in vivo* (Haase et al., 2002). Taken together, these findings indicate that IL-10-DCs would represent a good candidate for future immunotherapy in MS. *In vivo* studies of the effects of IL-10-DCs in EAE are warranted for further strengthening of this hypothesis. Studies with IL-10-DCs by the s.c. route have already been shown not to be effective in suppressing EAE (Link and Xiao, 2001), but the effects of IL-10-DCs on EAE, using the i.p. route remains to be explored.

Paper IV assessed the effectiveness of IFN-γ-DCs in the treatment of EAMG, as an alternative to IL-10-DCs. The results were promising in that the disease activity was suppressed and there were favourable immunological responses. It would be valuable to study the effects of repeated injections of IL-10-DCs or IFN-γ-DCs during the course of the disease in animal models, in order to simulate the practical situation, for future clinical trials in patients. It would also be of interest to analyse the effects of combining the treatments with IL-10-DCs and IFN-γ-DCs in the animal model, since this may give better results, due to the different mechanisms of action.

Antigen-specificity is another important issue to consider in DC immunotherapy, in order to avoid a generalized immunosuppression that may result in infection. In the present studies, the DCs were isolated from rats with on-going EAMG, and therefore

assumed to be pulsed with the autoantigens already *in vivo*. However, there are now methods to pulse DCs with the autoantigen *in vivo* or *in vitro* without the risk of maturing the DCs, provided that the autoantigen has been identified in the particular disease (Steinman et al., 2003). One strategy would be to pulse the DCs with the autoantigen and then expose them to the relevant cytokine, before they are injected into the patient. The knowledge regarding DC characteristics have come a long way since they were first visualized as Langerhans cells in the skin in 1868, and since the characterization, 30 years ago. Immunotherapy of cancer, based on treatment with DCs, in which antigen-specific immune activation by mDCs is exploited, has already reached the stage of clinical trials, and has given promising results (Paczesny et al., 2004; Palucka et al., 2003). It is predicted, that it will not be long before the DCs are used in the therapy of autoimmune diseases for the induction of antigen-specific tolerance.

6 ACKNOWLEDGEMENTS

Many people directly or indirectly contributed to this work. I hope those whom I may have forgotten to mention would forgive me for innocent human error. A special thanks goes to the patients and healthy volunteers who contributed with samples to the studies in this thesis.

I am grateful to Professor Hans Link for inviting me to Karolinska Institutet, providing me with funds for the initial period of my studies and guiding me to get my first scientific publication. I will always remember Professor Malini Udupihille, Professor Malkanthi Chandrasekera and Professor Mallika Sabanayagam, from the University of Peradeniya, Sri Lanka, who gave me letters of recommendation, when I was applying for postgraduate positions. Associate Professor Yu-Min Huang for guiding me during the first two years of studies, teaching me how to write papers and getting them published.

I also want to thank Professor Jan Hillert and Ms. Gunnel Larsson for arranging my initial period of research at the Division of Neurology at Huddinge University Hospital. I will remember Professor Jan Hillert as a person who was always there to help students.

Associate Professor Marianne Schultzberg, my main supervisor, for her guidance and valuable advice during the final year of my studies. Without her help I would not be able to achieve the goal of finalizing my Ph.D. studies. My co-supervisors, Dr. Clara Gumpert and Dr. Bao-Guo Xiao, for their advice and support. Dr. Bao-Guo Xiao taught me the basics of animal experiments and was always there to assist me during experiments. He was a person who could answer every question I had during my studies. I was lucky to have him as one of my supervisors.

Professor Åke Seiger, Ms. Siw Lundin, Ms. Maria Roos, Ms. Monica Wikström and all other staff at the Neurotec Department for their assistance. Monica has been like a friend, willing to devote her time answering my questions and solving my problems.

Professor Ann Kari Lefvert and her staff, Dr. Ritva Pirskanen and Dr. Rayomand Press, for providing me with blood samples from MG patients. Professor Ann Kari Lefvert has always been kind and supportive, also in times that were difficult. I wish to extend my sincere thanks also to Rui-Sheng Duan, Åsa Pettersson and Hong Lian, for their fruitful collaborations in papers included in this thesis.

Never to forget all friends at the Division of Neurology, including Rayomand Press, Andreia Gomes, Ajith Sominanda, Thomas Masterman, Kristina Duvefelt, Eva Åkesson, Helena Modin, Mathula Thangarajh, Carolina Ciumas, Leszek Stawiarz, Anna Ljungberg and Cecilia Svarén Quiding. Friends at Experimental Geriatrics, Catharina Lindberg, Åsa Forslin Aronsson, Mircea Oprica, Erik Hjorth, Stefan Spulber and Atiqur Rahman. Rayomand was a valuable family friend, a reliable collaborator and an experienced researcher, who could advice me on many things. Yassir Hussien, Prabhakar Putheti and Mikhail Pashenkov, my senior colleagues for teaching me lab techniques and sharing their experiences with me. Rasheed Khan for his valuable help during the core-course.

All my colleagues back in my home university in Sri Lanka, Devika Gunasinghe, Jayampathi Dissanayake, Lakal Dissabandara, Keerthi Kulasekara, Yasas Jayasinghe, Deepthi Nanayakkara, Sanath Wijeratne, Nirmala Herath and Sujeevi Nawaratne for their valuable friendship. Sanath, whom I used to call 'SR', and had known me for many years, will always be remembered for encouraging me to apply for a Ph.D. position in Sweden. Many thanks to Kumara Kaluarachchi, who inspired me by his own method of applying for Ph.D. positions. Dr. Udaya Dangahadeniya and Dr. Vajira Weerasinghe for not only being my teachers, but also generous friends. Udaya volunteered to be a surety to my bond with the university, in order to get my study leave, which made the task easy.

Mr. and Mrs. Dissanayake for filling the void created by the absence of my parents here in Sweden. The Sri Lankan student community in Stockholm, for breaking the monotony of life through frequent gatherings.

My loving wife, Samanthi, for being my best friend and life companion, supporting my tough decisions in life, accompanying me to Sweden and spending three gloomy Swedish winters at home taking care of our son, while I was busy with the experiments in the lab. My dear son, Hasitha, for repeating everything I did when I was at his age, and embarrassing me in front of others. To come home and play with Hasitha after the day's work, made all obstacles in the lab disappear.

Most of all, my parents, the best parents in the world, who brought me to this world and filled my life with endless love and care. They devoted their lives and sacrificed their comfort to make me comfortable, brought me up and provided me with the best education available in the country. My sisters, Jeevanee and Lalani, and my brother, Mahinda, for their love and care.

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