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Studies on dendritic cells and cytokines in inflammatory diseases of the central nervous system

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To all my friends

Abstract

Multiple sclerosis (MS) is characterised by chronic inflammation in the central nervous system (CNS), which results in primary demyelination, axonal loss and neurological deficit. MS is believed to be mediated by myelin antigen-reactive T cells, which after priming in the periphery enter the CNS and initiate demyelination. The cause of the autoaggressive T cell response in MS remains unclear. Consequently, there is no cure in this disease. In most neuroinfections, on the other hand, the CNS inflammation resolves either spontaneously, or after appropriate antimicrobial therapy.

Dendritic cells (DCs) are key players in induction and regulation of T cell responses. DCs capture antigens in non-lymphoid tissues and, subsequently, migrate to regional lymphoid nodes, in order to activate naïve T cells. Up to recently, this paradigm appeared not to be applicable to the CNS, due to presumed absence of DCs. However, recent animal studies have shown that DCs normally occur in the meninges and choroid plexus, and infiltrate inflamed brain parenchyma. DC migration from the CNS to deep cervical lymph nodes has also been demonstrated. These findings have made it likely that DCs are directly involved in T cell activation in MS as well as in neuroinfections.

The aim of this thesis was to explore the presence of DCs in human cerebrospinal fluid (CSF) and possible involvement of CSF DCs in regulation of immune responses in MS and bacterial neuroinfections (studies 3–6). Expression of pro-inflammatory cytokines (interferon (IFN)-γ, interleukin (IL)-12, IL-15) by blood DCs and mononuclear cells (MNCs) was also studied in MS patients and controls (studies 1–2).

Numbers of IL-15–expressing blood MNCs, presumably monocytes or DCs, were elevated in MS compared to healthy donors, as detected by immunocytochemistry. This elevation was confined to patients with primary and secondary progressive MS, and was not observed in the subgroup of relapsing-remitting MS. Thus, IL-15 might be of importance in clinically more advanced forms of MS.

Spontaneous production of cytokines by enriched blood DCs was analysed by flow cytometry. Cells expressing IFN-γ, IL-12 and IL-15 but not IL-10 were more frequent among enriched DC than among corresponding MNC samples.

Human blood contains two DC subsets, namely myeloid ($CD11c^+$) DC, which are potent antigen-presenting cells, and plasmacytoid ($CD11c^-$) DC, which can produce large amounts of type I IFNs upon infection with certain bacteria and enveloped viruses. Using flow cytometry, we found the same two DC subsets in the CSF. DCs were present in minute numbers in CSF from patients with non-inflammatory neurological diseases. In MS, numbers of myeloid and plasmacytoid CSF DCs were increased, in particular during the early stage of the disease, inversely correlated with MS duration. The highest levels were observed in acute monosymptomatic optic neuritis (ON) with oligoclonal IgG in CSF, which usually represents the first bout of MS. In bacterial meningitis (BM), only numbers of myeloid DCs were consistently increased in CSF. In Lyme meningoencephalitis (LM), there was a striking accumulation of myeloid and especially of plasmacytoid DCs in CSF. Myeloid DCs in CSF expressed higher levels of HLA-DR, CD40, CD80 and CD86 than in blood and were thus more mature, while plasmacytoid DCs in CSF and in blood were equally immature.

In BM and LM, DCs appeared to be recruited to the CSF compartment by chemokines present in CSF, like SDF-1α, MCP-1 and C5a. MS patients had increased expression of CCR5 by myeloid blood DCs, which could facilitate their recruitment in response to CCR5 ligands expressed in MS brain (MIP-1α/β, RANTES). CSF supernatants from BM patients inhibited the T cell-stimulatory and Th1-deviating capacities of myeloid DCs *in vitro*, likely due to the presence of IL-10, whereas CSF from LM and MS patients augmented these capacities of DCs.

Based on the present data and those of others, we suggest that CSF DCs participate in immune surveillance in the CNS and can activate T cells locally and/or in regional lymph nodes. The character of the T cell response induced by CSF DCs may depend on DC numbers, cytokine milieu and, in MS, on inherited properties of DCs. Further studies are warranted to investigate, whether repeated presentation of myelin antigens by CSF DC triggers the chronic autoimmune response in MS.

CONTENTS

ABBREVIATIONS

ADEM	Acute disseminated encephalomyelitis
APC	Antigen-presenting cell
BBB	Blood-brain barrier
BCG	Bacillus Calmette-Guerin
BM	Bacterial meningitis
C	Complement component
CCR	CC-chemokine receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCR	CXC-chemokine receptor
DC	Dendritic cell
DTH	Delayed type hypersensitivity
EAE	Experimental allergic encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
fMLP	Formyl-methionine-leucine peptide
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
IP-10	Interferon-γ-inducible protein of 10 kDa
Lin	A collective term for lineage markers of T cells, B cells, NK cells and monocytes/macrophages (CD3, CD14, CD16, CD19, CD20 and CD56)
LPS	Lipopolysaccharide
LM	Lyme meningoencephalitis
MBP	Myelin basic protein
MCP	Monocyte chemoattractant protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
MLR	Mixed leukocyte reaction
MNC	Mononuclear cell
MoDC	Monocyte-derived dendritic cell
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NIND	Non-inflammatory neurological diseases
OIND	Other inflammatory neurological diseases
ON	Acute monosymptomatic optic neuritis
RANTES	Regulated on activation, normal T cell expressed and secreted
SDF	Stromal cell-derived factor
TCR	T cell receptor
Th	T helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor

PREFACE

This thesis is based on the following articles and manuscripts, referred to by arabic numbers:

1. M. Pashenkov, M. Mustafa, P. Kivisäkk and H. Link. Levels of IL-15–expressing blood mononuclear cells are elevated in multiple sclerosis. Scand J Immunol 1999; 50: 302-308.
2. M. Pashenkov, M. Kouwenhoven, V. Özenci and Y.-M. Huang. Phenotypes and cytokine profiles of enriched blood dendritic cells in healthy individuals. Eur Cytokine Network 2000; 11: 456-463.
3. M. Pashenkov, Y.-M. Huang, V. Kostulas, M. Söderström and H. Link. Two subsets of dendritic cells are present in human cerebrospinal fluid. Brain 2001; 124: 480-492.
4. M. Pashenkov, N. Teleshova, T. Smirnova, M. Kouwenhoven, V. Kostulas, Y.-M. Huang, B. Pinegin, A. Boiko, H. Link. Recruitment of dendritic cells to the cerebrospinal fluid in bacterial neuroinfections. Submitted.
5. M. Pashenkov, N. Teleshova, M. Kouwenhoven, V. Kostulas, M. Söderström, H. Link, Y.-M. Huang. Elevated expression of CCR5 by myeloid ($CD11c^+$) blood dendritic cells in multiple sclerosis and acute optic neuritis. Clin Exp Immunol, accepted subject to minor revisions.
6. M. Pashenkov, M. Söderström, Y.-M. Huang, H. Link. Cerebrospinal fluid affects phenotype and functions of myeloid dendritic cells. Manuscript.

1. INTRODUCTION

1.1 Multiple sclerosis

1.1.1 General characteristics

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS). MS affects mainly young adults, more often females than males. Incidence and prevalence of MS rise from the equator to the poles; estimated prevalence in the Western Europe is 1 per 1000 (Lauer, 1994). MS almost inevitably leads to disability and, in severe cases, to death of the patient.

Pathologically, MS is characterised by multiple, sharply demarcated foci of demyelination and mononuclear leukocyte infiltration (Weller, 1985). The latter indicates the immune-mediated character of demyelination. MS lesions can be located throughout the CNS white and grey matter, but more often in highly myelinated areas, such as periventricular areas of brain hemispheres, corpus callosum, optic nerves, cerebellum and its connexions, myelinated pathways of brainstem and of spinal cord. Cellular components of the actively demyelinating lesions are T cells, monocytes/ macrophages, plasma cells, activated microglia and astrocytes (Brosnan and Raine, 1996). Depositions of IgG and complement may occur, myelin and sometimes oligodendrocytes are lost (Lucchinetti et al, 2000). Axonal damage mediated by activated macrophages occurs in the lesions and is considered as the cause of irreversible neurological deficit (Ferguson et al, 1997; Trapp et al, 1998). In some lesions, the inflammation fades with time, resulting in astroglial scar (chronic silent lesions). Certain degree of remyelination seems to take place frequently. Other lesions remain inflammatory on their border and grow in size (chronic active lesions) (Weller, 1985).

MS is a heterogenous disease with regard to its course. Most frequently, MS starts with a series of exacerbations and complete remissions (relapsing-remitting MS), followed after various time by a secondary progressive phase, when the neurological deficit increases steadily. MRI examinations, however, show that the inflammatory process is rather continuous even during the relapsing-remitting phase, since subclinical exacerbations revealed only by MRI are several times more frequent than clinical ones (Thorpe et al, 1996). The secondary progressive phase probably begins at the point when the magnitude of brain damage exceeds compensatory capacities of the CNS. Relapsing-remitting MS often starts as a clinically isolated

syndrome, e.g. as an episode of acute unilateral optic neuritis (ON) (Matthews, 1985). Other clinical syndromes seen at onset or during the course of MS signs of pyramidal tract involvement, disturbances of coordination, sensory dysfunction, affection of cranial nerves, and sphincter disturbances. Intellectual impairment is often seen in advanced MS and is ascribed to lesions in corpus callosum and to diffuse brain atrophy (Matthews, 1985; Huber et al, 1987; Rovaris and Filippi, 2000).

Primary progressive MS (about 15% of all cases) is a partly distinct form of MS. It affects middle-aged people, more often males, and is from the onset characterised by a steady increase of neurological deficit. The lesions are located mainly in the spinal cord and subtentorial stuctures of the brain, and are characterised by smaller size, less pronounced leukocyte infiltration and less pronounced blood-brain barrier (BBB) breakdown (Thompson et al, 1991; Revesz et al, 1994; Stevenson et al, 1999).

The diagnosis of MS requires demonstration of dissemination of lesions in space and time, using clinical, MRI and electrophysiological examinations (Poser et al, 1983; McDonald et al, 2001). Important signs supporting a diagnosis of MS are found in the cerebrospinal fluid (CSF): (1) increased CSF IgG index in about two-thirds of patients (Link and Tibbling, 1977) and (2) the presence of oligoclonal IgG in practically all patients with clinical MS (Kostulas et al, 1987). Both abnormalities are indicative of intrathecal IgG synthesis, and their absence should lead to a re-evaluation of the diagnosis of MS (Kostulas et al, 1987). The finding of oligoclonal IgG in CSF in a patient with monosymptomatic ON indicates a 60–70% risk of developing MS, while ON patients without this sign develop MS in <10% of cases (Söderström et al, 1998).

The etiology of MS is unknown. A combination of complex genetic predisposition with environmental factors (first of all infections) is generally assumed to cause the disease (Hillert and Olerup, 1993; Ebers and Sadovnick, 1994; Willer and Ebers, 2000). MS is an inflammatory disorder with clear autoimmune traits, such as (1) organ specificity; (2) increased frequencies of myelin antigen-reactive T and B cells in blood and CSF (Link et al, 1990; Söderström et al, 1993); (3) association with certain HLA haplotypes, such as HLA-DR15 (Jersild et al, 1993; Hillert and Olerup, 1993); (4) similarity with experimental allergic encephalomyelits (EAE), an experimental autoimmune disease used as a model of MS (Wekerle et al, 1994). However, it remains an open quesiton whether autoimmune response are the original

cause of MS, or are secondary phenomena that occur at a certain stage of the disease. In the former case, myelin-reactive T cells may be activated systemically by cross-reacting microbial peptides, not necessarily derived from neurotropic pathogens. There is clear evidence that systemic, e.g. respiratory, infections can cause MS exacerbations, and molecular mimicry between myelin and microbial antigens has been shown by many studies (Fujinami and Oldstone, 1985; Andersen et al, 1993; Wucherpfennig and Strominger, 1995); however, the role of molecular mimicry in MS pathogenesis has not been directly demonstrated. Alternatively, the initial response may be directed against a subclinical (e.g., viral) infection persisting in the CNS, the myelin damage being a bystander effect at this stage. Continuous release of myelin antigens and inflammatory mediators may trigger a true autoimmune response. However, no pathogen has been directly demonstrated to cause MS through the persistence in the CNS, although many have been suggested (Cermelli and Jacobson, 2000; Yucesan and Sriram, 2001). More promising evidence has been obtained for human herpes virus-6 (HHV-6): (1) there may be reactivation of the latent HHV-6 infection in oligodendrocytes in MS but not in control brains (Challoner et al, 1995); (2) humoral and cellular responses against HHV-6 antigens are augmented in MS (Sola et al, 1993; Soldan et al, 1997; Soldan et al, 2000).

1.1.2 The Th1/Th2/Th3 concept and MS

This concept was first introduced in mice, in order to describe functional heterogeneity of T helper (Th) cell clones (Mosmann et al, 1986; Mosmann and Coffman, 1989; Mosmann and Sad, 1996). Th1 cells produce IFN-γ, IL-2 and TNF-β (called Th1 cytokines) and promote cell-mediated immunity. Th2 cells produce IL-4, IL-5 and IL-13 (called Th2 cytokines) and promote humoral immunity. There is a reciprocal antagonism between Th1 and Th2 branches of the immune response. Th3 cells produce TGF-β and downregulate functions of Th1 cells (Chen et al, 1994). This concept has been firmly validated in humans, although the distinction between human Th subsets is less stringent, as Th cells with transitional cytokine profiles exist.

The Th1 response against myelin antigens is assumed to at least partly mediate the myelin damage in MS (Merill and Benveniste, 1996; Link, 1998). IFN-γ produced by Th1 cells activates macrophages and microglia, which are considered as main effectors of demyelination and axonal destruction (Huitinga et al, 1900; Trapp et al, 1998). Frequencies of IFN-γ–secreting myelin antigen-reactive T cells are increased

in blood and especially in the CSF of MS patients, compared to controls (Sun et al, 1991a; Sun et al, 1991b; Söderström et al, 1993). Finally, adminstration of IFN-γ to MS patients exacerbate the disease (Panitch et al, 1987). In contrast, the Th2 response is considered anti-inflammatory, beneficial in MS, as it inhibits activation of mononuclear phagocytes.

Based on experiments in EAE, the following sequence of events is assumed to happen during MS exacerbations. Activated T cells (Th1 cells), irrespectively of their antigen specificity, can pass through the intact BBB (Hickey, 1991). More recently, it was demonstrated that naïve T cells can also cross the BBB, although at a much lower rate (Brabb et al, 2000). If such T cells recognize their antigen within the CNS (e.g., a myelin or a viral antigen) presented to them in the context of HLA and appropriate costimulatory signals, they home to the CNS and respond by production of inflammatory cytokines and chemokines. This results in attraction of bystander T cells and monocytes from blood ("second wave"), activation of microglia and astrocytes, production of myelinotoxic substances (NO, TNF-α, complement components and proteases) and, finally, in demyelination (Cross et al, 1991; Brosnan and Raine, 1996).

Several principal questions remain unresolved in MS immunology, in particular: (1) Why and how is the anti-myelin Th1 response is initiated? (2) Which are the antigen-presenting cells that reactivate Th1 cells entering CNS early in MS plaque development? (3) Why does the anti-myelin T cell response become chronic. Therefore, events upstream of T cell IFN-γ secretion have attracted attention of MS researchers. Association of MS and MS-type ON with the HLA-DR15 haplotype suggests involvement of antigen presenting cells (APCs). HLA-DR15 can present an immunodominant peptide of MBP, a putative target of the autoimmune attack in MS (Vogt et al, 1994). Recently, IL-12, the principal APC-derived IFN-γ–inducing cytokine (Kobayashi et al, 1989; Manetti et al, 1993), has been implicated in MS pathogenesis (Segal et al, 1998; Karp et al, 2000). Several groups have demonstrated increased expression of IL-12 in MS (Ferrante et al, 1998; Matusevicius et al, 1998; van Boxel-Dezaire et al, 1999). Blocking of IL-12 by antibodies ameliorates EAE and prevents relapses (Leonard et al, 1995; Constantinescu et al, 1998; Ichikawa et al, 2000), whereas administration of IL-12 worsens EAE and provokes relapses (Leonard et al, 1995; Smith et al, 1997).

Major producers of IL-12 are dendritic cells (DCs). They are also the only known APC type that can efficiently induce primary immune responses. DC-derived IL-12 is critical for Th1 cell differentiation (Macatonia et al, 1995; Heufler et al, 1996). It is therefore likely that DCs participate in the pathogenesis of MS (Link et al, 1999).

1.2 Infectious diseases of the CNS

The pathogenesis of neuroinfections is better understood than that of MS, because etiological agents are known. Neuroinfections can be broadly divided into viral, bacterial, protozoan, fungal, and prional. In bacterial meningitis (BM), where the pathogen grows rapidly and causes massive tissue destruction, the innate (granulocytic) response predominates, and the adaptive immune system takes over only at recovery, when the final clearance of the pathogen and establishment of immunological memory are needed. If the pathogen is less cytopathic, like in Lyme neuroborreliosis, then the host response is mounted almost exclusively by the adaptive immunity (Sigal et al, 1997).

As excessive inflammatory responses in the CNS are life-threatening, the CNS has evolved mechanisms limiting the inflammation. They are partly mediated by high production of anti-inflammatory cytokines such as IL-10 (Frei et al, 1993). Consequently, the host response in acute CNS infections is inefficient and, in the absence of antimicrobial treatment, is unable to eliminate the pathogen. On the other hand, failure of the anti-inflammatory mechanisms is likely to be fatal for the host due to excessive brain edema and irreversible damage of respiratory centers.

In spite of pronounced BBB breakdown, massive leukocyte entry into the CNS as well as massive release of CNS antigens, acute neuroinfections are rarely followed by a chronic demyelinating disease. This is even the case in acute disseminated encephalomyelitis (ADEM), a para-infectious or postvaccination disease characterised by acute development of perivascular inflammatory demyelination: the disease is self-limiting and rarely transforms into MS (Allen and Kirk, 1992; Olek and Dawson, 2000). The factors preventing chronic autoimmunity after acute neuroinfections or ADEM are not fully understood. It is not unlikely that the same anti-inflammatory mechanisms determine both the inefficiency of host defense in acute neuroinfections and the absence of autoimmunity afterwards.

The mechanisms minimizing the tissue damage may be partly compromised in subacute or chronic neuroinfections caused by persistent, low-cytopathic pathogens. This is especially relevant to Lyme borreliosis, an endemic tick-born disease caused by *Borrelia burgdorferi* (Burgdorfer et al, 1982). The early stage of the disease is characterized by skin pathology (*erythema migrans*) and general infectious symptoms. It is followed by a disseminated stage, which may manifest itself by arthritis, carditis, and/or various neurological signs. The CNS pathology is represented by lymphocytic meningoencephaloradiculitis, which, if left untreated, is followed by chronic encephalopathy or by multifocal encephalomyelitis that clinically and pathologically resembles MS (Garcia-Monco and Benach, 1995). An immunological hallmark of the disseminated Lyme disease is a suppression of systemic production of IL-4 (Oksi et al, 1996; Ekerfelt et al, 1999). In case of CNS involvement, there is a prominent and long-lasting intrathecal IFN-γ response against *Borrelia* (Ekerfelt et al, 1997).

Animal work in disseminated Lyme disease has mainly focused on arthritis, due to the lack of appropriate models of neuroborreliosis. It has shown that administration of IFN-γ worsens the disease, whereas IL-4 ameliorates it, probably through an antibody-mediated clearance of *Borrelia* (Keane-Myers et al, 1995; Matyniak and Reiner, 1995; Anguita et al, 1996). Splenocytes from C3H mice, which are susceptible to Lyme arthritis, produce more IFN-γ and less IL-4 than those from resistant BALB/c mice (Keane-Myers and Nickell, 1995). Even more intriguing, B6 mice, which after inoculation with *Borrelia* have the same pathogen load in joints as C3H mice, remain healthy (Ma et al, 1998). This has been linked to high production of IL-10 by the B6 strain (Brown et al, 1999). One may conclude that (1) eradication of *Borrelia* may not be necessary for the host; (2) the host's attempt to eliminate *Borrelia* through a Th1 response is probably not efficient, but leads to the tissue damage. Data in human are quite in line with this view: in symptomatic Lyme patients, the inflammation is more prominent than could be expected from the relatively low pathogen load (Goronzy et al, 1992), and it is difficult to isolate *Borrelia* from blood or CSF even in active neuroborreliosis (Nadelman et al, 1990; Garcia-Monco and Benach, 1995); at the same time, *Borrelia burgdorferi* can be isolated from the CSF of repeatedly infected but clinically healthy individuals showing only increased anti-*Borrelia* IgG antibody titers (Pfister et al, 1989).

The situation in the CNS in Lyme neuroborreliosis may be similar to that in MS. Activation of macrophages and microglia by IFN-γ and directly by borrelial lipoproteins (Radolf et al, 1995) may lead to tissue damage. Tissue destruction may be augmented by anti-*Borrelia* antibodies and T cells cross-reacting with neural antigens (Sigal and Tatum, 1988; Aberer et al, 1989). Release of myelin antigens in the context of the ongoing Th1 response against *Borrelia* may trigger a true autoimmune response. Indeed, frequencies of myelin antigen-reactive Th1 cells and B cells are increased in neuroborreliosis, especially in the CSF (Baig et al, 1991; Wang et al, 1996). Yet, the inflammation in most cases of the disease can be cured by antibiotic treatment, suggesting that other factors are necessary for transition into MS.

DCs, as the main professional APC type, by large determine the nature of the immune response in infections. The role of DCs in neuroinfections, however, has received little attention. The following questions can be asked: Are DCs involved in induction of the adaptive immune responses in neuroinfections? Where do DCs first encounter the neurotropic pathogen: in the CNS or in peripheral tissues? What is the role of DCs in determining the type and the course of immune response in different CNS infections? Can DCs provide a link between neuroinfections and autoimmunity in the CNS?

The studies presented in this thesis where undertaken in order to better understand the putative role of DCs in human neuroinflammatory diseases, such as MS, bacterial meningitis (BM) and Lyme meningoencephalitis (LM).

1.3 Dendritic cells

1.3.1 Definition and functions

DCs are professional APCs that are capable of activating naïve T cells, have a branched or veiled morphology, and do not express lineage markers of T cells (CD3), B cells (CD19/CD20), NK cells (CD56) and monocytes/macrophages (CD14/CD16). DCs were first described in epidermis by Langerhans in 1868. DCs of secondary lymphoid organs were discovered and characterised in 1970s by R. Steinman and colleagues (Steinman et al, 1973; Steinman et al, 1979).

In human, three principal DC subsets, or lineages have been identified: myeloid DCs, plasmacytoid DCs and Langerhans cells (Banchereau et al, 2000). Each subset has distinct surface phenotype, developmental pathways, tissue distribution and

functions. However, all DCs develop from the hematopoietic stem cell and have a similar scheme of differentiation, which can roughly be divided into four stages: (1) proliferating progenitors in the bone marrow; (2) proliferating and non-proliferating precursors in blood; (3) immature DCs in tissues; (4) mature DCs in secondary lymphoid organs (Banchereau and Steinman, 1998; Banchereau et al., 2000).

DCs, with some exceptions, play an active role in immunity during the last two stages of their development (Fig. 1). Immature DCs are located at the sites of potential antigen invasion (Banchereau and Steinman, 1998). In case of inflammation, they can be additionally recruited from blood precursors (McWilliam et al, 1996; Robert et al, 1999). Immature DCs take up antigens, but are yet unable to present them. DCs recognise antigens by the innate receptor repertoire: (1) pattern recognition receptors (PRR), which bind conserved molecular structures shared by large groups of

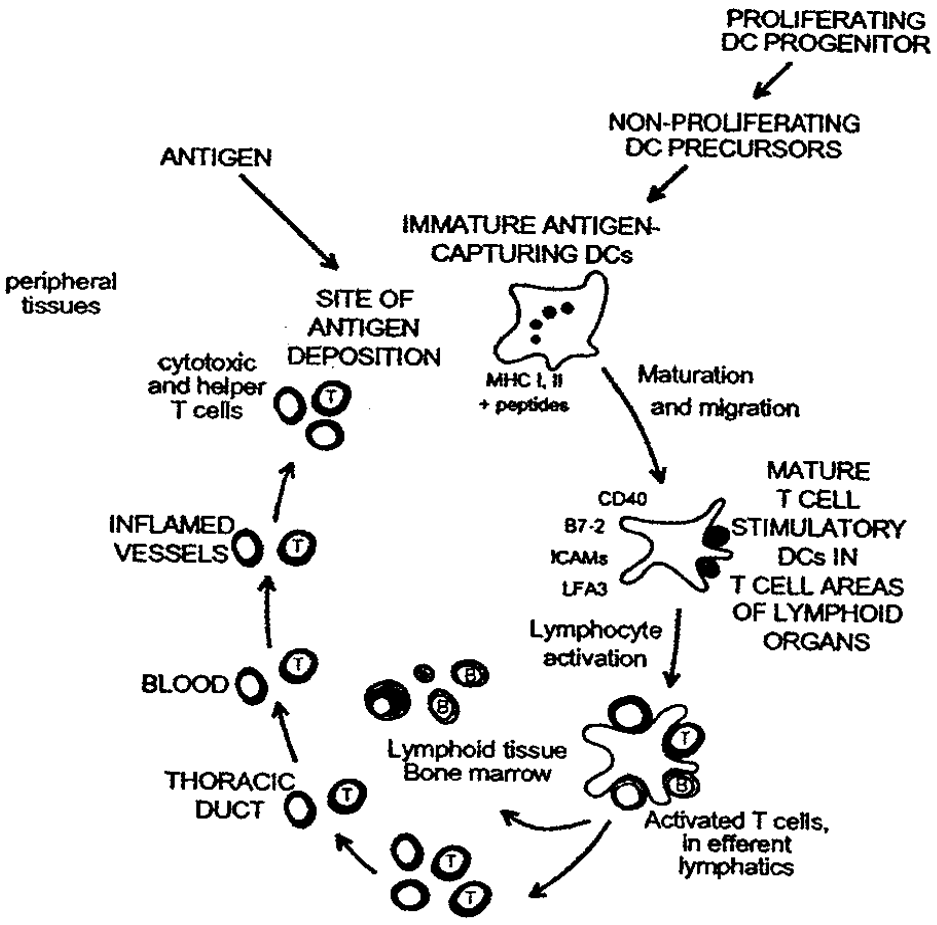

Figure 1. DCs in induction of the adaptive immune response (from Banchereau and Steinman, Nature 1998; 392: 245-252).

microorganisms (Toll-like receptors [TLR], mannose receptors), and (2) receptors of endogenous opsonins (Fc receptors, complement receptors, CD36) (Medzhitov and Janeway, 1997; Albert et al, 1998; Medzhitov and Janeway, 2000; Imler and Hoffmann, 2001). Inflammation or tissue necrosis trigger maturation of DCs. Upon maturation, DCs lose the ability to take up antigens, but strongly upregulate surface expression of MHC and costimulatory molecules (Sallusto and Lanzavecchia, 1994; Steinman et al, 1997b), which enables efficient presentation of previously captured antigens. Mature DCs migrate from inflammatory lesions to T cell areas of secondary lymphoid organs (Xia et al, 1995), where they become interdigitating DCs (Steinman et al, 1997a) and interact with naïve T helper (Th) cells. Mature DCs provide naïve Th cells with two obligatory activation signals: (1) an antigen-specific signal (interaction MHC:peptide:TCR); (2) a costimulatory signal necessary for T cell survival and proliferation (interaction B7:CD28). There is strong evidence for a third signal that is transmitted from mature DCs to naïve Th cells and drives Th1 or Th2 polarization (Kalinski et al, 1999). Besides activating Th cells, DCs prime naïve cytotoxic ($CD8^+$) T cells (Specht et al, 1997; Dhodapkar et al, 1999) and NKT cells (Kadowaki et al, 2001a), contribute to B cell activation (Dubois et al, 1999), and probably support the long-term T cell memory (Ludewig et al, 1999).

Thus, the role of DCs is "bridging" the innate and adaptive immunity: DCs recognise antigens by low-diversity, low-specificity innate receptor machinery, but turn on the highly diverse and specific T and B cell responses. Likely, DCs also participate in maintenance of the immune tolerance.

This thesis will focus on myeloid and plasmacytoid DCs.

1.3.2 Myeloid DCs

Immature myeloid DCs are located in the derma, interstitial tissues, and in the marginal zone of the spleen (i.e., at the sites of potential antigen delivery) (Table 1). Mature myeloid DCs are found in T cell areas of secondary lymphoid organs as interdigitating DCs (Steinman et al, 1997a). Germinal center DCs probably also belong to the myeloid subset (Dubois et al, 1999). Peripheral blood contains two types of myeloid DC precursors: more numerous monocytes, which constitute up to 30 % of blood MNCs, and more rare $lin^{dim/-}$HLA-$DR^{++}CD11c^+$ cells, which are usually called myeloid ($CD11c^+$) blood DCs and constitute <1% of blood MNCs (O'Doherty et al, 1994; Kohrgruber et al, 1999; Robinson et al, 1999). $CD11c^+$ blood DCs are potent

Table 1. Characteristics of myeloid and plasmacytoid DCs

	Myeloid DCs	Plasmacytoid DCs
Tissue localization	Derma, interstitial tissues. Mature DC: T cell areas of secondary lymphoid organs	T cell areas of secondary lymphoid organs, peripheral tissues (in inflammation)
Morphological features	Pseudopods, vacuolized cytoplasm	Smooth plasma membrane, abundant endoplasmic reticulum (disappears with maturation)
Markers		
Myeloid (CD11c, CD13, CD33)	+	–
CD1a	+	–
CD2	+	–
CD4	+	+
CD9	+	–
CD36	+	++
CD45RA	–	+
CD45RO	+	–
CD68	+	+
CD83	+*	+*
CD123 (IL-3Rα)	Dim	High
pre-α chain of TCR	–	+
Growth factor	GM-CSF	IL-3
Phagocytosis	+	–
Differentiation into macrophages	+	–
Blood precursors	$CD11c^+$ myeloid blood DCs, monocytes	$CD11c^-$ plasmacytoid blood DCs
Special functions		Production of type I IFNs

* Expressed only by mature DCs

professional APCs. They can be recruited to tissues during inflammation (Robert et al, 1999), but probably also under normal conditions, giving rise to resident (dermal or interstitial) DCs. Monocytes can be converted into immature myeloid DCs *in vitro* by culture with GM-CSF and IL-4 (Sallusto and Lanzavecchia, 1994). The existence of this pathway *in vivo* has not yet been demonstrated; presumably it functions in acute inflammation, when the rare $CD11c^+$ blood DCs cannot fully cover the needs of the immune system. Antigen-loaded monocytes/macrophages, however, do become DCs

in vivo as they cross the walls of afferent lymphatics (Randolph et al, 1999). Importantly, both monocytes and $CD11c^+$ blood DCs can turn into macrophages, i.e. an effector (scavenger) cell type with low antigen-presenting capacity (Robinson et al, 1999), while macrophages can turn into DCs (Zou et al, 2000). A dynamic balance between myeloid DCs and macrophages may exist in tissues, depending on specific requirements of the immune system.

In inflammatory lesions, myeloid DCs undergo initial maturation, which can be induced by two groups of factors: (1) pathogen-derived factors interacting with TLR, such as whole bacteria, LPS, double-stranded RNA and CpG DNA (Larrick et al, 1989; Heufler et al., 1996; Hartmann et al, 1999; Cella et al, 1999b); (2) inflammatory cytokines such as TNF-α and IL-1β (Sallusto and Lanzavecchia, 1994). At this stage, DC maturation can be blocked by IL-10 (Buelens et al, 1997).

Initially matured myeloid DCs migrate to T cell areas of secondary lymphoid organs and interact with naïve Th cells. This cross-talk leads both to activation of the T cells and to final maturation of the DCs. Initial interaction between the DC and the T cell is promoted by adhesion molecules (Gejtenbeek et al, 2000). Then, MHC:peptide:TCR interaction triggers expression of CD40L on the T cell (Jaiswal et al, 1996). CD40L interacts with CD40 on the DC and triggers final DC maturation, which is manifested by a further increase of CD80 and CD86 expression, as well as by induction of IL-12 (Caux et al, 1994; Cella et al, 1996). Other T cell molecules, such as OX-40 and TRANCE interacting with OX-40L and RANK on DC, can synergize with CD40L (Andersson et al, 1997; Ohshima et al, 1997). In turn, the co-stimulatory molecules CD80 and CD86 interact with the T cell antigen CD28, triggering production of IL-2 by T helper cells and preventing their apoptosis (Schwartz, 1992). CD40-activated myeloid DCs can prime naïve CD8 T cells (Ridge et al, 1998).

1.3.3 Plasmacytoid DCs

Plasmacytoid DCs, previously known as plasmacytoid monocytes or plasmacytoid T cells (Facchetti et al, 1988), may be a human counterpart of mouse lymphoid DCs. Although their development from a lymphoid progenitor in human has not been finally proven (Olweus et al, 1997; Ito et al, 1999), a number of features distinguish plasmacytoid DCs from cells of myeloid lineage and are in common with

lymphocytes (Table 1). Plasmacytoid DCs express high levels of CD123 (α-chain of the IL-3 receptor) (Kohrgruber et al, 1999), not observed on any other type of leukocytes except for basophils (Olweus et al, 1997).

Plasmacytoid DCs are present in blood (<1% of blood MNCs). Unlike $CD11c^+$ myeloid DCs, freshly isolated plasmacytoid blood DCs do not capture antigens and poorly stimulate T cell responses; moreover, they can proliferate in the presence of IL-3 (Grouard et al, 1997; Kohrgruber et al, 1999). Therefore, plasmacytoid blood DCs should be regarded as proliferating DC precursors. They constitutively migrate from blood to T cell areas of secondary lymphoid organs (Grouard et al, 1997; Olweus et al, 1997). In case of lymphadenitis, this process may be enhanced (Cella et al, 2000). Plasmacytoid DCs appear to be virtually absent from normal non-lymphoid tissues, but can be recruited there during inflammation (Jahnsen et al, 2000; Farkas et al, 2001). When isolated from blood and tonsils, they rapidly apoptose, unless IL-3 is present in the culture (Grouard et al, 1997). IL-4 and IL-10 induce apoptosis of plasmacytoid DCs even in the presence of IL-3 (Rissoan et al, 1999).

The major function of plasmacytoid DCs at the precursor stage appears to be the production of large amounts of type I interferons (5–6 U/cell/day) in response to infection with enveloped viruses and certain bacteria (Siegal et al, 1999). Plasmacytoid DCs are thus identical to the previously described natural interferon-producing cells (NIPC) (Sandberg et al, 1991; Fitzgerald-Bocarsly, 1993). Production of type I IFNs by plasmacytoid DCs is triggered via mannose receptor (Milone and Fitzgerald-Bocarsly, 1994).

In vitro, development of these cells into classical DCs can be triggered by (1) IL-3 (addition of TNF-α or CD40L induces maturation); (2) enveloped viruses; (3) CpG DNA (Grouard et al, 1997; Rissoan et al, 1999; Cella et al, 2000; Kadowaki et al, 2000; Bauer et al, 2001). Upon culture with IL-3, plasmacytoid DCs acquire a dendritic morphology. On day 2 of culture, they become immature DCs, as manifested by increased antigen uptake (Kohrgruber et al, 1999). Upon further culture with IL-3 in combination with TNF-α or CD40L, plasmacytoid DCs mature, their antigen-capturing capacity drops, while T cell-stimulatory capacity rises (Grouard et al, 1997; Kohrgruber et al, 1999). Virus-induced maturation of plasmacytoid DCs is mediated by autocrinely produced type I IFNs and TNF-α (Kadowaki et al, 2000). A

similar maturation process may occur in inflamed lymph nodes (Cella et al, 2000). Plasmacytoid DCs do not respond to LPS, due to the lack of specific receptor.

Thus, the plasmacytoid DCs play a dual role: at the precursor and immature stage, they act as early non-specific effectors of antiviral immunity, and at the mature stage, they can induce adaptive responses against pathogens.

1.3.4 Migration of DCs

Antigen uptake by DCs usually occurs in non-lymphoid tissues, and antigen presentation in T cell areas of secondary lymphoid organs. This separation in space is made possible by migration of DCs, which is governed by rapid and strictly regulated changes of chemokine receptor expression by DCs upon maturation.

Chemokines, or chemoattractant cytokines, can be divided into two functional groups, inflammatory and constitutive (Sallusto et al, 1998a). The former are induced in peripheral tissues upon inflammation (Table 2). The latter are constantly produced in secondary lymphoid organs. Although many chemokine receptors can bind several ligands, the spectra of receptors for inflammatory and constitutive chemokines in general do not overlap (Table 2).

Immature monocyte-derived DCs (moDCs) express receptors for inflammatory chemokines (CCR1, CCR2, CCR5, CXCR1) (Lin et al, 1998; Sallusto et al, 1998b). They can be chemoattracted by ligands of CCR1 and CCR5 (MCP-3, MIP-1α, MIP-1β, RANTES) and to a lesser extent by ligands of CXCR4 (SDF-1α/β) (Sozzani et al, 1995; Lin et al, 1998; Sallusto et al, 1998b), while CCR2 and CXCR1 do not transmit a chemotactic signal in moDCs. Myeloid ($CD11c^+$) blood DCs express the same chemokine receptors as immature moDC and respond to the same chemokines, as well as to ligands of CCR2 (MCP-1) (Lee et al, 1999; Caux et al, 2000). A recent paper reported myeloid blood DCs responding to CXCR3 ligands (Penna et al, 2001), which disagrees with results of another group (Caux et al, 2000). Inflammatory chemokines as well as other host- and pathogen-derived chemoattractants (C5a, platelet-activating factor, fMLP) direct immature myeloid DCs to the sites of inflammation. Upon maturation, myeloid DCs lose CCR1, CCR2, CCR5 and CXCR1 from their surface, but upregulate expression of CCR7 and CXCR4 (Dieu et al, 1998; Lin et al, 1998; Sallusto et al, 1998b). Such DCs are no longer retained by inflammatory chemokines at sites of inflammation and migrate to secondary lymphoid

Table 2. Chemokine receptors, their ligands and expression on DC subsets

Receptor	Ligands	MoDCs		Blood MDCs		Blood PDCs	
		Imm.	Mat.	Imm.	Mat.	Imm.	Mat.
Receptors for inflammatory chemokines							
CCR1	MIP-1α, RANTES, MCP-2, -3	+	–	+/–	–	+/–*	?
CCR2	MCP-1, -2, -3, -4	+*	–	+	–	+	?
CCR5	RANTES, MIP-1α, MIP-1β	+	–	+	–	+*	–
CXCR1	IL-8	Low*	–	–	–	–	?
CXCR3	IP-10, Mig	–	–	+/–**	–	+*	–
Receptors for constitutive chemokines							
CCR7	MIP-3β, 6Ckine	–	+	–	+	–	+
CXCR4	SDF-1α, SDF-1β	Low	+	+	+	+	–

+/–, Controversial reports. * Do not induce chemotaxis. ** May induce chemotaxis

organs in response to the constitutive chemokines MIP-3β and 6Ckine (ligands for CCR7), as well as SDF-1α/β (ligands for CXCR4). CXCR4 is the only chemokine receptor expressed by both immature and mature myeloid DCs (Table 2).

Plasmacytoid blood DCs express the same spectrum of chemokine receptors as myeloid DCs, plus CXCR3 (Cella et al, 1999a; Lee et al, 1999; Caux et al, 2000; Penna et al, 2001), but respond only to the CXCR4 ligand SDF-1 and weakly to MCP-1 (Caux et al, 2000; Penna et al, 2001). Responsiveness to SDF-1 may explain their migration to secondary lymphoid organs in the absence of inflammation. Upon maturation, plasmacytoid DCs downregulate the expression of all chemokine receptors including CXCR4 and, similarly with myeloid DCs, become $CCR7^+$ and respond to MIP-3β (Cella et al, 2000; Penna et al, 2001).

1.3.5 DCs and T helper cell differentiation

Depending on maturation conditions, both myeloid and plasmacytoid DCs can induce Th1, Th2 or Th0 responses. In case of moDCs (i.e., myeloid DCs), shorter

maturation time, high DC:T ratios and high antigen doses favor a Th1 response, while the opposite conditions favor a Th2 response (Langenkamp et al, 2001). Furthermore, presence of IFN-γ, poly-I:C or pertussis toxin during myeloid DC maturation leads to development of myeloid DC1 (Vieira et al, 2000), which express high levels of IL-12 and ICAM-1 (Kalinski et al, 1999), and induce Th1 responses. Presence of PGE_2, cholerotoxin or extracellular parasites favors development of DC2 (Kalinski et al, 1997; Vieira et al, 2000), which lack IL-12 production, express moderate levels of ICAM-1 but, instead, express OX-40L (Kalinski et al, 1999). IL-12/ICAM-1 and OX-40L, appear to represent, respectvely, Th1- and Th2-deviating signals displayed by myeloid DCs.

Plasmacytoid DCs matured with IL-3 and CD40L do not express IL-12 and induce Th2 polarization (Rissoan et al, 1999). In contrast, plasmacytoid DCs matured with enveloped viruses induce IFN-γ and IL-10 production from naïve T cells (Kadowaki et al, 2000). IFN-γ production is in this case driven by type I IFNs rather than by IL-12, while IL-10 production is type I IFN-independent (Kadowaki et al, 2000). The cytokine profile of T cells induced by virally matured plasmacytoid DC ($IFN\text{-}\gamma^{+}IL\text{-}10^{+}$) reminds that of recently described 'Tr1 cells' with regulatory properties (Levings et al, 2001a). It appears from the above reports that plasmacytoid DCs, irrespectively of how they have matured, are able to prime IL-10 production by T cells.

Altogether, both myeloid and plasmacytoid DC possess functional plasticity. Their ability to induce Th1 and Th2 responses is modulated depending on the pathogen and on microenvironment. In addition, there are complex cytokine-mediated reciprocal interactions between DC subsets, Th1 and Th2 cells, which probably keep a necessary balance between Th1 and Th2 cytokine production (Rissoan et al, 1999).

1.3.6 DCs and infections

One may assume that early stages of anti-infectious adaptive immunity are driven mainly by myeloid DCs, which reside in peripheral tissues, readily take up foreign antigens and mature more rapidly than plasmacytoid DCs. There may be certain specialization of DC subsets: myeloid DCs may respond preferentially to bacterial lipoproteins (via TLR2) and LPS (via TLR4) (Muzio et al, 2000; Hertz et al, 2001), while plasmacytoid DCs to CpG-DNA (via TLR9) (Hemmi et al, 2000;

Kadowaki et al, 2001b) and to enveloped viruses (via mannose receptors) (Milone and Fitzgerald-Bocarsly, 1998). Plasmacytoid DCs are more resistant to cytopathic viruses than myeloid DCs, and are thus more efficient in settings of viral infections (Cella et al, 2000). Both myeloid and plasmacytoid DCs, depending on the factors described above, can induce Th1 or Th2 responses. An intriguing but not yet proven possibility is that Th1 or Th2 deviation of the immune response in infections depends on triggering of specific PRR on DC subsets (Medzhitov and Janeway, 1997). Plasmacytoid DCs, in addition, produce type I IFNs, the cytokines which suppress viral replication and modulate the immune response by: (1) enhancing expression of MHC class I molecules and improving recognition of viral antigens by cytotoxic T cells (Biron, 1998); (2) activating NK cells (Biron, 1998); (3) inducing moderate proliferation of cytotoxic T cells and keep activated T cells viable (Biron, 1998; Marrack et al, 1999); (4) inducing maturation of myeloid and plasmacytoid DCs (Luft et al, 1998; Kadowaki et al, 2000); (5) when present during differentiation of myeloid DCs from monocytes, driving development of IL-12-deficient $CD123^{high}CD11c^{+}$ DCs that induce production of IL-10 from naïve T cells (McRae et al, 1998; Bartholome et al, 1999; Huang et al 2001); (6) when present at priming of naïve T cells, inducing differentiation of Th1 cells or, more likely, Tr1 cells expressing IFN-γ, IL-10 and the functional IL-12R (IL-12Rβ1/β2) (Rogge et al, 1999; Levings et al, 2001a).

As for *Borrelia burgdorferi*, its lipoproteins are recognised by myeloid DCs via TLR2 (Hirschfeld et al, 1999), which leads to phagocytosis of the microorganism and induction of IL-12 secretion by DC (Filgueira et al, 1996).

1.3.7 DCs, immune tolerance and autoimmune diseases

Since the initial TCR repertoire allows T cells to recognize in principle any antigen, the immune system must prevent responses to autoantigens. T cell clones with high affinity TCR directed against self antigens are to be deleted in the thymus. This process of negative selection is driven by thymic DCs (Ardavin, 1997). The output from the thymus, in terms of autoreactivity, consists of low-affinity T cells directed against intrathymic antigens, and of T cells directed against tissue antigens that are absent from the thymus during embryonic development (Janeway et al, 1999). In the periphery, T cells recognising tissue autoantigens in the absence of costimulatory signals probably undergo clonal deletion or anergy (Buer et al, 1998),

similarly to what happens after administration of high doses of antigens to animals (Chen et al, 1995). Likely, the process of clonal deletion/anergy will be the more complete, the higher is expression of autoantigens and the higher is TCR affinity of specific clones. The resulting autoreactive TCR repertoire in the periphery is thus made up mostly by low-affinity TCR, and by TCR of varying affinity against low-expressed autoantigens (Janeway et al, 1999). These autoreactive T cells can be temporarily activated, e.g. in infections, but normally they do not induce chronic autoimmunity, due to several putative mechanisms: (1) antigens with low expression are not recognized by T cells (immunological ignorance); (2) tissue cells do not express costimulatory molecules necessary for T cell activation; (3) regulatory T cells actively suppress autoimmune responses (Powrie et al, 1993; Sakaguchi et al, 1995).

The role of DCs in maintaining peripheral tolerance has been extensively investigated. In mice, spleen $CD8\alpha^+$ DCs, which constitutively present high levels of autoantigens (Steinman et al, 1997a), can suppress proliferation and IL-2 production of T cells (Kronin et al, 1996; Suss et al, 1996). These DCs constitutively express FasL and probably kill activated autoreactive T cells by Fas-dependent apoptosis. In human, DCs with such functions have not been identified.

Several groups have demonstrated the ability of non-proliferating $CD4^+CD25^+$ regulatory T cells to suppress T cell responses in human (*in vitro*), as well as T cell-mediated autoimmune diseases in experimental animals (Powrie et al, 1993; Salomon et al, 2000; Dieckmann et al, 2001; Jonuleit et al, 2001; Levings et al, 2001b). It has been suggested that $CD4^+CD25^+$ T cells develop as an independent lineage in the thymus. Presumably, they bear autoreactive TCRs with intermediate affinity, which is high enough to induce intrathymic activation and subsequent anergy, but not sufficient to induce deletion (Shevach, 2001). At the same time, Jonuleit and colleagues (2000) showed that repetitive stimulation of naïve T cells with immature allogeneic moDCs induces development of non-proliferating T cells that share many properties with natural $CD4^+CD25^+$ T cells (anergic state, suppression of T cell proliferation by a contact-dependent, antigen-independent mechanism, expression of IL-10 and CTLA-4). This implied that induction of peripheral anergy may in fact mean generation of regulatory T cells (Buer et al, 1998), and that regulatory T cells can be generated outside of the thymus. How can naïve T cells, which do not recirculate through peripheral tissues, encounter immature DCs *in vivo*? One proposed

mechanism is that, in the absence of inflammation, immature tissue DCs capture apoptotic bodies and continuously traffick to lymph nodes (Huang et al, 2000). Apoptotic cells, in contrast to necrotic ones, do not induce DC maturation (Galucci et al, 1999). These immature DCs can probably present certain levels of autoantigens from apoptotic bodies, which in the absence of costimulatory signals will induce T cell anergy or regulatory T cells. Future studies will determine whether immature DCs help maintain peripheral tolerance by this mechanism.

At the same time, it has been clearly documented that DCs can *induce* autoimmune diseases. Injections of mature DCs loaded with an encephalitogenic peptide induce EAE (Dittel et al, 1999). DCs loaded with thyroglobulin induce experimental autoimmune thyroiditis (Watanabe et al, 1999). In melanoma patients, vitiligo (i.e., autoimmune damage to melanocytes) can develop after treatment with DCs loaded with melanoma lysates (Mackensen et al, 2000). There is also ample indirect evidence for DC involvement in human autoimmune diseases: (1) myeloid DCs accumulate in target organs of the autoimmune attack, for instance in synovium and synovial fluid in rheumatoid arthritis (de Vere Tyndall et al, 1983; Thomas et al, 1994), in thyroid gland in Hashimoto's disease (Kabel et al, 1988), and in the gut wall in Crohn's disease (Seldenrijk et al, 1989); (2) myeloid DCs isolated from the sites of autoimmune inflammation are more mature than their blood counterparts (Thomas and Quinn, 1996); (3) myeloid DCs from inflamed tissues in psoriasis and rheumatoid arthritis are more potent stimulators of autologous MLR than blood DCs, although both blood and tissue DCs equally well stimulate allogeneic MLR, suggesting that DCs from autoimmune lesions carry (auto)antigen(s) recognised by T cells in context of self HLA (Nestle et al, 1994; Thomas et al, 1994). It has been hypothesised that, in rheumatoid arthritis, antigen-loaded DCs migrate from inflamed joints to regional lymph nodes and activate autoreactive T cells (Thomas and Lipsky, 1996).

Many autoimmune diseases are triggered or exacerbated by infections. Most infections are accompanied by tissue necrosis and release of autoantigens and inflammatory mediators. These events, regarded as tissue stress or "danger", induce maturation of resident DCs (Gallucci and Matzinger, 2001), which may take up autoantigens and, potentially, present them to autoreactive T cells. Since mature DCs possess high co-stimulatory activity, they may efficiently activate the autoreactive T cells with low-affinity TCR. Mature DCs may also cause bystander (non-specific) activation of T cells, including autoreactive ones. This effect can be mediated by

certain combinations of DC-derived cytokines, e.g. IL-12 + IL-15 (Avice et al, 1998). Finally, T cells initially directed against pathogens may cross-react with autoantigens due to molecular mimicry. The affinity of such cross-interactions should be low; however, again, this may be compensated by the magnitude of T cell activation provided by mature DCs. As can be seen, molecular mimicry may not always required for the autoimmune response to begin, as autosensitization may occur simply due to release of autoantigens and their presentation by such a potent APC as the mature DC. DCs are also able to induce responses against cryptic determinants, triggering the epitope spreading (Drakesmith et al, 1998).

Apparently, the immune system must possess mechanisms that prevent DC-triggered autoimmunity. One possible mechanism has been highlighted by Steinbrink et al (1997) and de Jong et al (1999), who demonstrated that tolerogenic DCs can be generated *in vitro* by culturing immature moDCs with IL-10 or corticosteroids: such DCs express low levels of CD80/CD86 and, therefore, induce T cell anergy. A role of this mechanism in preventing autoimmunity remains to be established. Another mechanism is probably mediated by pre-existing $CD4^{+}CD25^{+}$ regulatory T cells (Jonuleit et al, 2001).

Summarizing, two conditions should probably be fulfilled in order for DCs to induce autoimmunity: (1) autoantigenic peptides must be able to bind to HLA molecules of a particular patient, which may explain associations of autoimmune diseases with specific HLA haplotypes; (2) the immune system in general and DCs in particular must be "tuned" to induce a *pathogenic* autoimmune response (for example, Th1 response in MS or Th2 response in *Myasthenia gravis*), which may be due to genetic traits, such as cytokine gene polymorphisms, and due to environmental factors.

1.3.8 DCs and the CNS

The classical view of the CNS immune privilege was based on several postulates: (1) the intact BBB is not permeable for immunocompetent cells; (2) there are no professional APCs in the CNS; (3) there are no conventional lymphatics draining the CNS; (4) high levels of anti-inflammatory cytokines such as TGF-β are constitutively produced in the CNS (Wilbanks and Streilein, 1992). Indeed, foreign antigens inoculated into brain parenchyma of experimental animals are not rejected by

the immune system (Matyszak and Perry, 1998). However, inflammation can still develop in the CNS.

Studies of the past decade have shown that activated T cells, as well as naïve T cells, do pass through the intact BBB independently of their antigen specificity (Hickey, 1991; Brabb et al, 2000). However, in the perivascular space, such T cells need to be reactivated, otherwise they probably die by apoptosis (Bauer et al, 1998). Thus, professional CNS APCs are required for the inflammation to begin. One such APC type may be perivascular macrophages ("perivascular microglia") (Hickey and Kimura, 1988). However, for their optimal function they need to be primed by IFN-γ (Matyszak et al, 1999), which is itself produced by activated T cells.

It was therefore a key observation that DCs are also present in the CNS. Normally, DCs are only found in meninges and choroid plexuses (Hanly and Petito, 1998; McMenamin, 1999). However, in case of inflammation, DCs appear in the perivascular spaces and parenchyma of the brain. Brain DCs have been observed at least in three murine models of human inflammatory diseases: in DTH against BCG sequestered in the brain parenchyma (Matyszak and Perry, 1996a), in EAE (Suter et al, 2000; Serafini et al, 2000; Fischer and Reichmann, 2001), and in experimental toxoplasmic encephalitis (Fischer et al, 2000). In acute EAE, DCs are localized mainly in perivascular infiltrates in the brain and spinal cord, as well as in meningeal infiltrates (Serafini et al, 2000). The appearance of DCs in perivascular spaces coincides with the development of clinical signs of EAE (Suter et al, 2000), and it has been proposed that DCs constitute the most early, IFN-γ-independent pool of brain APCs (Fischer et al, 2001). In chronic EAE and in toxoplasmic encephalitis, DCs are located both perivascularly and throughout brain parenchyma (Fischer et al, 2000; Serafini et al, 2000). Brain DCs mature over the course of inflammation and become able to produce IL-12 and to activate naïve T cells *in vitro* (Fischer et al, 2000).

The subset affiliation and origin of brain DCs has been controversial. Fischer et al (2000, 2001) described $CD11b^{+}CD11c^{low/+}DEC\text{-}205^{-}$ DCs, and suggested their microglial origin. In contrast, the DCs described by Serafini et al (2000) are $CD11b^{-}$ $CD11c^{+}DEC\text{-}205^{+}$ and appear to originate either from blood precursors, or, in the spinal cord, from meningeal DCs that migrate into the spinal cord tissue along Virchow—Robin spaces.

1.4 Cerebrospinal fluid and its role in intrathecal immune responses

The CSF, together with the interstitial fluid of brain and spinal cord, constitutes the extracellular fluid compartment of the CNS. The CSF is produced by choroid plexus of the lateral, III and IV ventricles of the brain. The choroid plexus, which represent the blood-CSF barrier, contain fenestrated microvascular endothelium and the ependyma, which at these sites has features of secretory cells (Davson and Segal, 1996). From the brain ventricles, the CSF gets into the subarachnoid space and is resorbed therefrom. Much of water and low molecular weight substances are resorbed through Pacchionian granulations to the venous blood. However, a substantial part of the CSF, including proteins and cells, drains by the so-called olfactory pathway (Bradbury et al, 1981; Cserr et al, 1992; Kida et al, 1993; Lowhagen et al, 1994; Seabrook et al, 1998), which conducts the CSF along perineural spaces of the olfactory nerves towards mucosa of the upper nasal ducts. There, the CSF mixes up with mucosal interstitial fluid and is directed to deep cervical lymph nodes by conventional afferent lymphatics. Interstitial fluid of the brain drains partly into CSF (from convexital areas), partly directly along olfactory nerves (from basal structures) (Harling-Berg et al, 1999). Existence of the olfactory pathway undermines the postulate that the CNS lacks the afferent branch of immune response. Importantly, DCs injected into CSF appear to use the olfactory pathway to migrate to deep cervical lymph nodes (Carson et al, 1999).

Although proteins inoculated into CSF and into brain parenchyma equally well reach the deep cervical lymph nodes (Bradbury et al, 1981), their immunogenicity differs. BCG injected into CSF induces DTH, whereas BCG injected into brain parenchyma is not recognised, unless there is peripheral rechallenge with the same pathogen (Matyszak and Perry, 1995, 1996b). Influenza virus injected into CSF of previously immunised mice induces lymphocyte proliferation in deep cervical lymph nodes and a cytotoxic T cell response, whereas similar injections into brain parenchyma do not (Stevenson et al, 1997). The cause of this difference is unclear. In any case, the position of CSF is ambiguous from the immunological point of view. On one hand, CSF freely communicates with interstitial fluid of the brain and may contain all the same soluble factors, including (auto)antigens. On the other hand, CSF lacks the immune privilege of the brain, because immune responses can be elicited from the CSF as from non-privileged sites. This “ambiguity” of the CSF may have profound implications for immune regulation in the CNS.

2. AIMS OF THE STUDY

The literature reviewed above indicates a potentially important and so far insufficiently understood role of DCs in the pathogenesis of inflammatory diseases of the CNS. The present thesis focuses on two questions in this context: (1) expression of a DC-associated pro-inflammatory cytokine IL-15 in MS and controls, and expression of pro- and anti-inflammatory cytokines by freshly isolated blood DCs; (2) presence of DCs in the CSF in different inflammatory and non-inflammatory CNS diseases in human, mechanisms of DC recruitment to CSF, and involvement of CSF DCs in immune responses within the CNS.

Specific aims

1. To analyse expression of IL-15 by blood and CSF MNCs in MS compared to control groups (Study 1).
2. To study spontaneous expression of pro-inflammatory (IL-12, IL-15, IFN-γ) and anti-inflammatory (IL-10) cytokines by freshly isolated blood DCs from healthy individuals (Study 2).
3. To investigate whether myeloid and plasmacytoid DCs are present in CSF (Study 3).
4. To quantify myeloid and plasmacytoid DCs in blood and CSF in non-inflammatory neurological diseases (NIND), multiple sclerosis (MS), acute monosymptomatic optic neuritis (ON), bacterial meningitis (BM), and Lyme meningoencephalitis (LM) (Studies 3, 4).
5. To analyse the phenotype of CSF DCs compared to blood DCs (Study 3).
6. To investigate the involvement of chemokines and chemokine receptors in recruitment of DCs to CSF in bacterial neuroinfections (Study 4) and MS (Study 5).
7. To investigate the influence of soluble factors present in the CSF from patients with neuroinflammatory diseases on phenotype and functions of myeloid (monocyte-derived) DCs (Study 6).

3. MATERIALS AND METHODS

3.1 Patients and healthy subjects

All studies were approved by the Ethical Committee of the Karolinska Institute at Huddinge University Hospital.

Patients with MS were from the MS Center of the Division of Neurology, KI, Huddinge University Hospital. MS was diagnosed according to established criteria (Poser et al., 1983). In all patients, expanded disability status scale (EDSS) score was determined (Kurtzke et al., 1983). Duration of MS was 0–43 years. All patients had oligoclonal IgG in the CSF, revealed by isoelectric focusing on agarose gel (Kostulas and Link, 1982). MRI examination revealed ≥ 4 MS-like lesions in the brain and spinal cord.

Patients with ON were from the Eye Clinic at Huddinge University Hospital. ON was diagnosed by an experienced ophthalmologist (Dr. Mats Söderström) using established criteria (Perkin and Rose, 1979). Patients were included 1–4 weeks after disease onset. All ON patients included in the study had oligoclonal IgG in the CSF, and were therefore considered as those with early MS. None of the ON patients had received a corticosteroid therapy prior to inclusion into the studies.

Patients with LM were from the Neurological Clinic, Huddinge University Hospital. Blood and CSF samples were obtained before antibiotic treatment, 1–10 weeks after disease onset. One patient was also sampled twice during the treatment. LM was diagnosed on the basis of the clinical picture and CSF findings (lymphocytic pleocytosis, oligoclonal IgG bands and presence of IgG against the flagellar antigen of *Borrelia burgdorferi*).

Patients with BM were from the Neurological Clinic, Huddinge University Hospital, and from the Department of Neuroinfection, 3rd Infectious Hospital, Moscow. BM was diagnosed on the basis of the clinical picture and neutrophilic pleocytosis in CSF, and was caused by *N. meningitidis*, *S. pneumoniae*, *H. influenzae* and unestablished pathogens. Patients were sampled twice: at acute stage (≤3 d after disease onset, before treatment) and at recovery (7–9 d after onset, during treatment).

Patients with other inflammatory neurological diseases (OIND) and non-inflammatory neurological diseases (NIND) were from Neurological Clinic, Huddinge University Hospital. The OIND group included patients with aseptic meningitis/encephalitis of unidentified etiology and Behçet syndrome. The NIND

patients had no clinical signs of inflammation and no pleocytosis in the CSF (< 5 cells per 1 μl). The majority of these patients had minor strokes, transient ischemic attacks, tension headache, or pseudotumor cerebri.

Healthy donors were from the staff of the Division of Neurology, Karolinska Institute, as well as from the Bloodbank at Södertälje Hospital.

Venous blood samples (20–30 ml) were obtained from all subjects. From some patients, CSF samples (10–20 ml) were obtained at the same time as blood. CSF was subject to total and differential cell counting, determination of albumin and IgG, as well as isoelectric focusing on agarose gel in order to identify oligoclonal IgG bands (Kostulas and Link, 1982). The CSF/plasma albumin ratio was used as a measure of BBB permeability, and the CSF IgG index as a measure of intrathecal IgG synthesis (Link and Tibbling, 1977).

Buffy coats were obtained from South Hospital, Stockholm.

3.2 Cell isolation

3.2.1 Isolation of blood and CSF MNCs (Studies 1–6)

Blood MNCs were isolated by centrifugation over Lymphoprep density gradient (Nycomed, Oslo, Norway). CSF samples were centrifuged at 100×g. Supernatants were collected, and cells were resuspended in culture medium or 1% BSA, depending on upcoming experiment. CSF supernatants were frozen at –70°C until use.

3.2.2 Isolation of DCs by fluorescence-activated cell sorting (Studies 3, 4)

Plasmacytoid DCs were sorted from CSF of two patients with LM as $lin^{-}HLA\text{-}DR^{+}$ cells, using FACSVantage cell sorter (Becton Dickinson, Mountain View, CA).

3.2.3 Isolation of naïve T cells (Study 6)

Naïve T cells were negatively isolated from donor blood MNCs using magnetic beads (Dynal, Oslo, Norway). The purity of resulting $CD45RA^{+}$ T cells was >90%, with B cells, NK cells, monocytes and $CD45RO^{high}$ T cells constituting <1% each, and red blood cells <5–7%.

3.2.4 Enrichment of DCs from peripheral blood (Study 2)

A method described by Steinman et al (1979) was used, with modifications. DCs were isolated based on their capacity to adhere in a serum-free medium and to refloat in a complete medium. In some experiments, contaminating T cells were further depleted using neuraminidase-treated sheep erythrocytes.

3.3 Cell cultures

3.3.1 Generation and culture of moDCs (Studies 4–6)

MoDCs were generated from adherent blood MNCs by 7-day culture with 800 U/ml of rhGM-CSF (Novartis, Basel, Switzerland) and 500 U/ml of rhIL-4 (R&D Systems, Minneapolis, MA). By flow cytometry, immature DC preparations contained <15% lin^+ cells and >70% $CD1a^+$ cells. Mature DCs were obtained by culture of immature DCs with 50–100 ng/ml of rhTNF-α (R&D Systems) for 24–48 h. For culture with CSF supernatants (Study 6), moDCs were plated in 24-well plates (10^5/well), and equal volumes of CSF supernatants were added. Before addition to cultures, CSF supernatants were centrifuged at 3000 × g to remove any particles. Wells with medium only and with rhTNF-α (100 ng/ml) served as negative and positive controls, respectively. In neutralisation experiments, CSF was pretreated with anti-TNF-α mAb (R&D Systems, 10 μg/ml, 1 h, 37°C), and then added to cultures. rhIL-10 (1 ng/ml) was added directly to moDC cultures. After 24 h, moDCs were harvested, washed, counted, and used for immunophenotyping and for co-cultures with naïve T cells.

3.3.2 Mixed leucocyte reaction (MLR)

3.3.2.1 MLR with CSF-treated moDCs (Study 6)

MoDCs were irradiated (30 Gy) and co-cultured in triplicates with 5×10^4/well of allogeneic naïve T cells (in 96-well U-bottomed plates). After 5 days, co-cultures were pulsed with 0.5 μCi of ^{3}H-thymidine (Amersham, UK) for another 18 h. ^{3}H-thymidine incorporation was measured by a β-counter. Results were expressed as per cent cpm induced by moDCs cultured with CSF or TNF-α in relation to cpm induced by moDCs cultured with medium alone.

3.3.2.2 MLR with *ex vivo* CSF and blood MNCs (Study 4)

To evaluate the T cell-stimulatory capacity of DCs in CSF, irradiated (30 Gy) blood or CSF MNCs (5×10^4/well) were co-cultured for 72 h in triplicates with 5×10^4 allogeneic T cells isolated from donor blood MNCs using nylon wool columns. Co-cultures were pulsed with 0.5 μCi/well of ^{3}H-thymidine for another 18 h. ^{3}H-thymidine uptake was analysed by a β-counter. In each case, the degree of HLA mismatch between stimulators and responders was estimated as a ratio between cpm of the T cell/blood MNCs co-culture and cpm of T cells alone (stimulation index).

Then, cpm of the T cell/CSF MNC co-cultures were divided by this stimulation index, and these corrected cpm values were used for analysis.

3.3.3 T cell differentiation assay (Study 6)

2×10^5 naïve T cells were co-cultured in 96-well flat-bottomed plates with 2×10^4 untreated, or CSF-treated, or rhTNF-α–treated allogeneic moDCs. After 5 days, T cells were transferred into 24-well plates and expanded with 10 U/ml of rhIL-2. After another 6 days, T cells were harvested, washed, and restimulated with 10^{-7} M PMA and 2×10^{-6} M ionomycin during 5 h. 2×10^{-6} M monensin was added for the last 3 h of stimulation. Expression of IFN-γ, IL-4 and IL-10 by T cells was analysed by flow cytometry.

To study IL-12p40 production by moDCs, separate co-cultures of DCs with naïve T cells were set up in parallel under the same conditions. Supernatants were collected after 60 h and frozen at –70°C until use.

3.4 Flow cytometry

3.4.1 Detection, enumeration and immunophenotyping of DCs in blood and CSF (Studies 3–5)

Flow cytometric identification of DCs in blood and CSF was based on their lack of markers for T cells, B cells, NK cells, or monocytes (CD3, CD14, CD16, CD19, CD20 and CD56), positivity for HLA-DR and expression of CD123 (for plasmacytoid DCs) or CD11c (for myeloid DCs) (Olweus et al., 1997; Kohrgruber et al., 1999). A representative analysis of CSF cells is shown in Fig. 2. To calculate numbers of DC subtypes per 1 ml of blood, total leukocyte counts were multiplied by percentages of MNCs among leukocytes and then by percentages of myeloid or plasmacytoid DCs

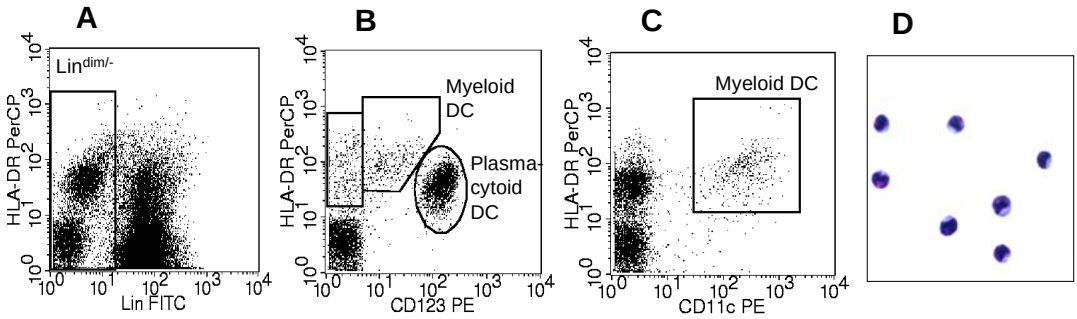

Figure 2. Detection of myeloid and plasmacytoid DCs in CSF (a patient with LM as an example). $Lin^{dim/-}$ CSF MNCs are gated (A) and plotted against HLA-DR and CD123 (B) or HLA-DR and CD11c (C). D, sorted plasmacytoid DCs from CSF of this patient (Giemsa staining, ×400).

among MNCs as determined by flow cytometry. To calculate numbers of DC subtypes per 1 ml of CSF, total CSF cell counts were multiplied by percentages of myeloid or plasmacytoid DCs determined by flow cytometry. Expression of co-stimulatory molecules and chemokine receptors was studied separately on DC subsets in blood and CSF essentially as described by Robinson et al (1999).

3.4.2 Immunophenotyping of moDCs cultured with CSF (Study 6)

Cultured moDCs ($2–4\times10^4$ per test) were analysed for the expression of CD1a, CD14, CD80, CD83, CD86 and HLA-DR (MFI). All data were expressed as per cent MFI after culture with CSF or rhTNF-α in relation to MFI after culture with medium alone (100%).

3.4.3 Detection of intracellular cytokines (Studies 2, 3, 6)

Flow cytometry was used to detect (1) IFN-γ–, IL-10–, IL-12– and IL-15–expressing cells among enriched blood DCs (Study 2); (2) IFN-α-expressing cells among *ex vivo* CSF and blood MNCs (Study 3); (3) IFN-γ–, IL-4– and IL-10–expressing T cells after co-culture with moDCs (Study 6). Paraformaldehyde-saponin procedure was used in all cases. Appropriate isotype controls and, in case of IFN-α, a preabsorption control were performed.

3.5 Immunocytochemistry (Studies 1, 3)

This technique was used to detect IL-15–expressing blood and CSF MNCs (Study 1), as well as IFN-α expressing cells among sorted CSF DCs and non-DCs (Study 3). A paraformaldehyde-saponin procedure described in details by Litton et al (1997) was adopted. A representative staining for IL-15 is shown in Fig. 3.

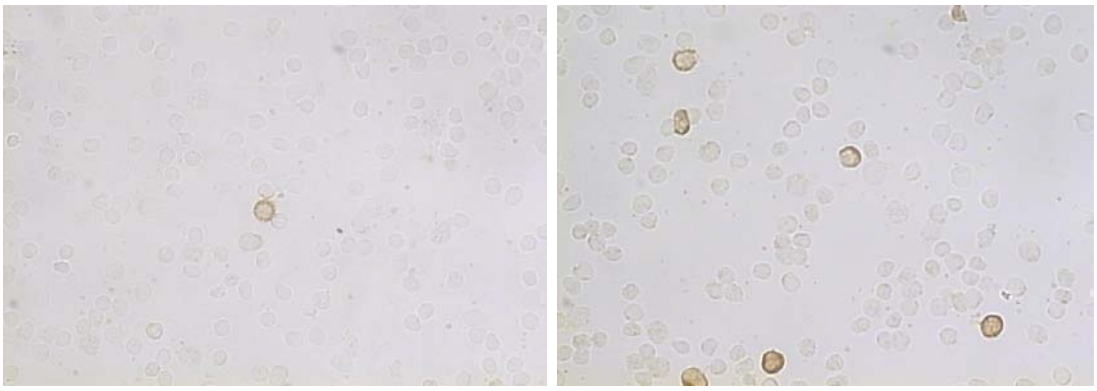

Figure 3. Immunocytochemical detection of IL-15 in *ex vivo* blood MNCs (left panel) and in MNCs stimulated overnight with 1 μg/ml LPS (positive control, right panel).

3.6 Cytokine and chemokine ELISAs (Studies 1, 4–6)

Concentrations of IFN-γ, IL-10 and IL-15 in CSF supernatants, and of IL-12p40 in MLR supernatants were determined by OPTEIA kits (Becton Dickinson). Concentrations of IP-10, MCP-1, MCP-3, MIP-1β, RANTES and SDF-1α in plasma and CSF were determined by sandwich ELISA using antibody pairs from R&D Systems. All measurements were done in duplicates. Optical densities were converted into concentrations using calibration curves generated in each experiment. Lower detection limit was 2 pg/ml for RANTES, MCP-1 and MCP-3, 4 pg/ml for IFN-γ, IL-10 and MIP-1β, 16 pg/ml for IL-12p40, IL-15 and IP-10, and 125 pg/ml for SDF-1α.

3.7 Transwell chemotaxis assay (Studies 4, 5)

3.7.1 moDCs

CSF- and chemokine-induced chemotaxis of "indicator" moDCs from a healthy donor were assessed using 24-well Transwell plates with porous (5 μm) inserts (Corning Costar Corporation, Cambridge, MA), essentially as described for DCs (Sallusto et al, 1998b) and for CSF (Lahrtz et al, 1997). moDCs were washed, resuspended at 5×10^5/ml in medium (RPMI with 1% FCS) and placed into upper chambers of transwells (100 μl/well), while lower chambers contained 600 μl of either medium alone, recombinant chemokines diluted in the medium, or CSF supernatants diluted 1/2 (v/v) in the same medium (Fig. 4). After a 90-min incubation at 37°C, inserts were lifted, washed on their upper surface with PBS, and Giemsa-stained on their lower surface, so as to visualize migrated DCs. Migrated cells in 10 consecutive high-power (×250) fields along the diameter of each insert were counted. Chemotactic effect of chemokines or CSF supernatants was presented as chemotactic factors (CF):

$$CF_{moDC} = \frac{\text{Number of moDCs migrated in response to a chemokine or CSF}}{\text{Number of moDCs migrated in response to medium}}$$

In some experiments, mAbs against CCR5, CXCR4 or CD88, or irrelevant IgG2a (final concentrations 10 μg/ml), or rhSDF-1α (10 or 50 ng/ml) or CSF supernatants (1/2, v/v) were added to upper chambers at the beginning of the assay.

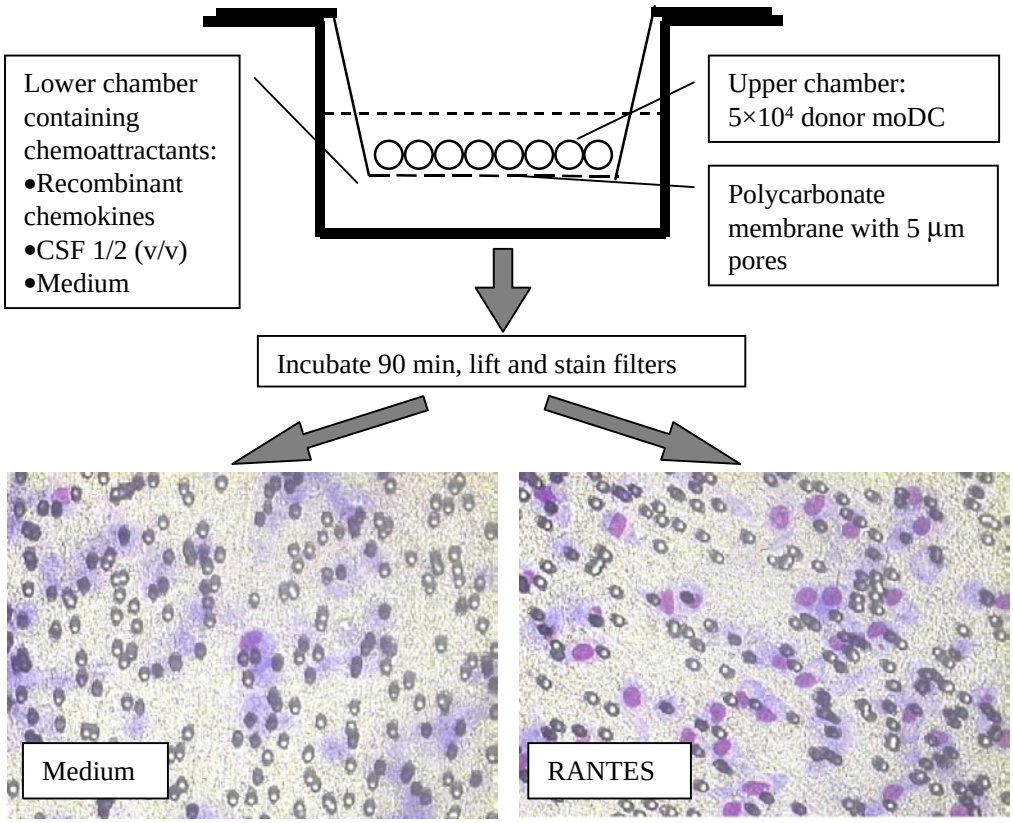

Figure 4. Principle of Transwell chemotaxis assay for moDCs. Cells migrated to the undersurface of filters have clearly stained nuclei, while cells on the opposite (upper) surface are vaguely stained.

3.7.2 Blood DCs

First, blood DCs were enriched by centrifugation of blood MNCs over a discontinuous gradient of Percoll (34% : 48.5% : 60% in complete culture medium) (Pharmacia, Uppsala, Sweden). Low density MNCs, containing DCs and monocytes, and depleted of lymphocytes, were collected from the 34% : 48.5% interphase, resuspended at 3×10^6 cells/ml in medium (RPMI with 2% heat-inactivated human AB serum), and placed in upper chambers of transwells in triplicates (100 µl/well). Lower chambers contained 600 µl of medium or diluted chemokines. After a 2 h incubation at 37°C, inserts were lifted, and cells that had migrated to their lower surface were washed off into the lower chamber by 2 ml cold PBS, collected (triplicate wells were pooled), spun down, resuspended in 100 µl PBS, counted and stained with FITC-lin, PE-anti-CD123 and PerCP-anti-HLA-DR. Myeloid and plasmacytoid DCs among migrated cells were determined by flow cytometry (Fig. 5A). Numbers of migrated DCs were calculated, and chemotactic factors were determined as for moDCs (Fig. 5B).

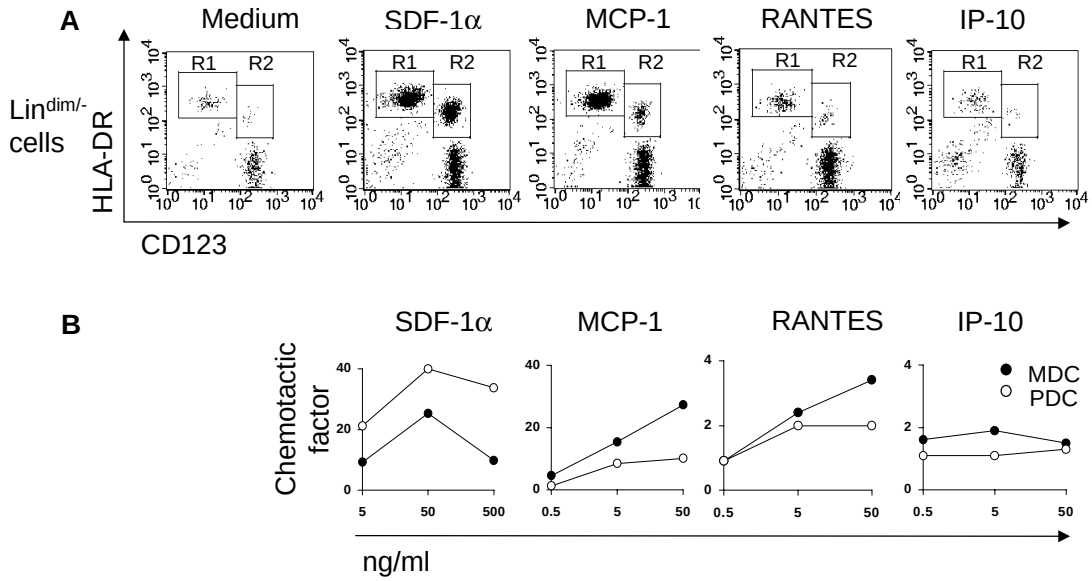

Figure 5. Principle of Transwell chemotaxis assay for blood DCs. Low density blood MNCs, containing monocytes and DCs, were enriched by Percoll density gradients and placed in the upper chambers of Transwells (3×10^5/well). After 120 min, instead of staining filters, migrated cells were washed off into lower chambers, collected, stained by fluorochrome-labelled mAbs and analysed by flow cytometry (A). Myeloid DCs are in R1, plasmacytoid DCs in R2. Chemotactic factors were calculated separately for myeloid and plasmacytoid DCs for concentrations of chemokines encompassing levels in the CSF (B).

3.8 Statistics

Multiple groups were compared by non-parametric ANOVA (Kruskal-Wallis test). If $p < 0.05$ was obtained, pairs of groups were further compared by non-parametric Mann-Whitney U-test. Paired comparisons were done by non-parametric Wilcoxon signed rank test. Correlations between two variables were tested by Spearman rank correlation test. The differences or correlations were considered significant if $p < 0.05$.

4. RESULTS

4.1 Elevated expression of IL-15 in MS (Study 1)

IL-15, a relatively recently cloned proinflammatory cytokine, is produced by DCs, monocytes/macrophages, astrocytes, microglia and epithelial cells (Grabstein et al, 1994; Doherty et al, 1996; Lee et al, 1996; Jonuleit et al, 1997). Similarly with IL-2, the main effect of IL-15 is induction of T and NK cell proliferation (Carson et al, 1994; Grabstein et al, 1994). IL-15 also augments cytotoxicity of, and IFN-γ production by T and NK cells (Carson et al, 1995), induces T cell chemotaxis (Wilkinson et al, 1995), and causes isotype switching of B cells (Armitage et al, 1995). A combination of IL-12 and IL-15 can induce a full-blown bystander activation of T cells (Avice et al, 1998). Compared with IL-2, IL-15 has a more pronounced effect on γδ T cells (Leclerq et al, 1996; Inagaki-Ohara et al, 1997), which accumulate in chronic active MS plaques (Selmaj et al, 1991). IL-15 may thus play a role in the pathogenesis of MS. A previous study from our group showed elevated numbers of IL-15 mRNA-expressing blood MNCs in MS patients compared to controls (Kivisäkk et al, 1998). However, expression of IL-15 is regulated at the translation level: despite the presence of IL-15 mRNA in many cell types, production of IL-15 protein is much more restricted (Andersson et al, 1995). Therefore, immunocytochemistry was adopted to study spontaneous expression of IL-15 at the protein level by blood and CSF MNCs.

Numbers of IL-15–expressing blood MNCs were elevated in patients with MS compared with healthy controls. Increased numbers of IL-15–positive blood MNCs were confined to the subgroup of chronic progressive MS and were especially high in primary progressive MS. Numbers of IL-15–expressing blood MNCs correlated positively with duration of MS and EDSS score. These correlations, however, could have been secondary to MS course, since patients with primary progressive MS had longer disease duration and more severe disability. Elevated numbers of IL-15–expressing blood MNCs were also observed in patients with OND (mainly stroke), compared with healthy subjects.

IL-15–expressing cells were not detected in CSF. Accordingly, levels of IL-15 measured by ELISA were usually lower in CSF than in plasma. However, CSF levels of IL-15 were higher in chronic progressive MS than in relapsing-remitting. There

was no correlation between expression of IL-15 and numbers of γδ T cells neither in blood nor in CSF.

Thus, expression of IL-15 is increased in chronic forms of MS, suggesting a role for IL-15 in chronicity of inflammation.

4.2 Freshly isolated blood DCs spontaneously produce pro- and anti-inflammatory cytokines (Study 2)

DCs produce a wide variety of immunoregulatory cytokines, including pro-inflammatory (IL-1β, IL-12, IL-15, IFN-γ, TNF-α) and anti-inflammatory (IL-10, TGF-β) (Zhou and Tedder, 1995; de Saint-Vis et al, 1998; Ohteki et al, 1999). However, spontaneous expression of cytokines by *ex vivo* blood DCs has received relatively little attention. DCs were enriched from blood of healthy donors using adherence properties. By flow cytometry, cells expressing IFN-γ, IL-12 and IL-15 but not IL-10 were more frequent among enriched blood DCs than among corresponding MNC samples. The expression of IFN-γ and IL-12 was confined to $CD83^+$ cells, presumably mature DC. Expression of IL-12 correlated positively with that of HLA-DR, CD80 and CD86. DCs from females showed higher levels of CD80, IL-10 and IL-15 than DCs from males, which may contribute to modulation of immune responses in males and females. These data indicate that blood DCs can spontaneously express pro- and anti-inflammatory cytokines. Their ability to produce IL-12 and IFN-γ depends on the maturation status.

4.3 Two subsets of DCs are present in human CSF (Study 3)

Several groups have reported on the occurrence and distribution of DCs in normal and inflamed murine CNS. In human, the occurrence of DC in the CSF, which mirrors the CNS inflammation, has not been reported. Therefore, we investigated whether myeloid and plasmacytoid DCs are present in CSF in non-inflammatory and inflammatory conditions. By flow cytometry, the majority of CSF samples contained myeloid and plasmacytoid DCs, which were phenotypically similar to corresponding blood DCs. Thus, myeloid DCs were $CD4^+$, $CD11c^+$, $CD45RO^+$, $CD83^-$, $CD123^{dim}$, $CCR5^+$, HLA-DR^{++}, plasmacytoid DCs were $CD4^+$, $CD11c^-$, $CD45RO^-$, $CD83^-$, $CD123^{high}$, $CCR5^+$, HLA-DR^+. A few DCs of either subset were present in non-inflammatory CSF (≤1% of CSF MNCs, ≤60 cells/ml). Both DC subsets were

elevated in CSF in acute monosymptomatic ON — early MS, as well as in clinically definite MS. In MS, however, numbers of DCs in CSF inversely correlated with the disease duration. Numbers of CSF DCs were especially high in LM, where plasmacytoid DCs reached up to 18% of CSF cells (up to 133,000 cells/ml). Numbers of CSF DCs correlated with common indicators of CNS inflammation, such as total CSF cell counts and CSF IgG index. The myeloid DCs in CSF were more mature than in blood, as they expressed higher HLA-DR, CD40, CD80 and CD86, whereas plamacytoid DCs in CSF remained as immature as in blood. Plasmacytoid DCs were sorted from CSF MNCs of one LM patient, and their morphology was confirmed by Giemsa staining. These DCs did not induce allogeneic MLR. On flow cytometry, there was no clear indication of plasmacytoid CSF DCs expressing more intracellular IFN-α than the rest of CSF MNCs. Rather, all CSF MNCs expressed more IFN-α than blood MNCs. This study for the first time demonstrated the presence of DCs in human CSF and suggested that accumulation of DCs in CSF is a hallmark of CNS inflammation.

4.4 Recruitment of DCs from blood to CSF in neuroinfections (Study 4)

This study was performed in order to quantify DCs in blood and CSF in BM and to understand what chemokines are involved in recruitment of myeloid and plasmacytoid DCs to CSF in BM and LM. Numbers of myeloid DCs in CSF were increased both in BM and LM compared to NIND. In BM, they were higher at the acute phase (≤3d after onset, before antibiotic treatment) and tended to normalize at recovery (7–9 d after onset, during treatment). Numbers of myeloid DCs in blood of BM patients showed reciprocal changes, i.e. a decrease at the acute stage, tendency to normalization at recovery. In LM, numbers of myeloid blood DCs were not changed compared to NIND. Chemotactic activity of CSF for immature myeloid (monocyte-derived) DCs was increased in LM, at the acute phase of BM, and to a lesser extent at recovery of BM, and correlated with numbers of myeloid DCs per 1 ml CSF. Checkerboard assay showed that the migratory responses of moDCs induced by CSF were truly chemotactic but not chemokinetic. Altogether, these data indicated that there is recruitment of myeloid DCs from blood to CSF, mediated by chemotactic factors present in CSF.

Similarly to myeloid DCs, numbers of plasmacytoid DCs in blood were decreased at the acute phase of BM and tended to normalize at recovery. However, there was no clear accumulation of plasmacytoid DCs in CSF of the BM patients. There was a pronounced accumulation of plasmacytoid DCs in LM patients' CSF.

To study mechanisms of DC recruitment, levels of six different chemokines (IP-10, MCP-1, MCP-3, MIP-1β, RANTES, SDF-1α) were studied in plasma and CSF by ELISA. All these chemokines have been suggested to be chemoattractants of blood DCs. In BM, all six chemokines were elevated in CSF compared to NIND. In LM CSF, only IP-10, MIP-1β, RANTES and SDF-1α were increased.

Surprisingly, SDF-1α which is usually regarded as a secondary lymphoid organ chemokine, was present at relatively high levels in NIND CSF. In BM, SDF-1α levels in CSF were higher at recovery than at the acute phase, in contrast to the other five chemokines. Among the six chemokines studied, levels of SDF-1α most strongly correlated with numbers of myeloid DCs in CSF. Numbers of plasmacytoid DCs in CSF correlated only with levels of SDF-1α. Concentrations of SDF-1α in NIND CSF were lower than in NIND plasma, whereas the opposite was observed in the majority of samples from patients with infectious CNS diseases. SDF-1α mediated up to 50% of the observed chemotactic activity of CSF for immature moDCs at recovery of BM and in LM.

We have directly examined chemokine responsiveness of myeloid and plasmacytoid blood DCs, as these data were not available from the literature at the time of this study. SDF-1α induced strong chemotaxis of both DC subsets, MCP-1, MIP-1β and RANTES chemoattracted mainly myeloid DCs (MCP-1 > MIP-1β = RANTES), while IP-10 was inactive towards both DC subsets (Fig. 5, page 36). These data are in line with those recently published by Caux et al (2000). High responsiveness of blood DCs to SDF-1α fitted well with the strong correlation between CSF DC numbers and CSF levels of this chemokine.

Altogether, these data indicate a role for SDF-1α in recruitment of DCs to CSF in the two neuroinfections under study. However, other chemoattractants, such as C5a, MCP-1, MIP-1α/β, are probably also involved in recrtuitment of DCs to CSF.

SDF-1α present in the CSF also attracted TNF-matured moDCs *vitro* and inhibited their migration towards MIP-3β. *In vivo,* this could correspond to a

diminished migration of antigen-bearing DCs from CSF to secondary lymphoid organs.

4.5 Elevated CCR5 on myeloid blood DCs in MS (Study 5)

In MS, chemotactic activity of the CSF was not changed compared to NIND. However, expression of CCR5, a chemokine receptor for RANTES, MIP-1α and MIP-1β was elevated on blood myeloid DCs in MS and ON (early MS) compared to non-inflammatory controls, and correlated with numbers of myeloid DCs per 1 ml of CSF. Myeloid blood DCs responded chemotactically to RANTES and MIP-1β, which are expressed in MS plaques (Hvas et al, 1997; Boven et al, 2000). Thus, CCR5 may be involved in recruitment of myeloid DCs to CNS in MS and ON.

Expression of CCR5 by plasmacytoid DCs in blood was not increased in MS and ON, and did not correlate with numbers of plasmacytoid DCs in CSF. Plasmacytoid DCs did not respond to MIP-1β and very weakly to RANTES. This indicates that CCR5 on plasmacytoid DCs may not be coupled with intracellular signaling pathways.

Expression of CXCR4, the receptor for SDF-1α/β, by myeloid and plasmacytoid DCs in blood was equal in all groups and did not correlate with numbers of corresponding DC subsets in CSF.

Since chemotactic activity of CSF was not increased in MS and ON, the elevated numbers of CSF DCs observed are more likely a result of shedding of DCs from inflammatory lesions located close to the CSF space (e.g., in optic nerves or corpus callosum), or from meningeal infiltrates, which are typical of early MS and probably represent the same inflammatory process as that seen in MS plaques.

In addition, we showed that percentages of myeloid DCs in CSF, but not percentages of monocytes, plasmacytoid DCs, or all HLA-DR$^+$ cells, correlate with the ability of CSF MNCs to induce allogeneic MLR.

4.6 CSF environment affects phenotype and function of moDCs (Study 6)

Functional studies of CSF DCs are hindered by paucity of these cells. Nevertheless, even small numbers of DCs in CSF could be of importance: for instance, one myeloid DCs can activate more than 100 T cells (Banchereau and Steinman, 1998). To overcome the problem of low cell numbers, we decided to

"model" the *in vivo* situation by studying how the CSF environment influences phenotype and function of myeloid (monocyte-derived) DCs from healthy donors. A number of molecules are present in CSF that may affect the behavior of DCs. CSF has been reported to contain low levels of GM-CSF (Leonardi et al, 1999), the key growth factor for myeloid DCs. TGF-β, which is constitutively present in CSF (Tailor and Streilein, 1993), can divert development of myeloid DC towards Langerhans cells (Jaksits et al, 1997; Geissmann et al, 1998) and prevent LPS- or TNF-induced DC maturation (Geissmann et al, 1999). In addition, TGF-β partly mediates the ability of human CSF to suppress the DTH-inducing potential of macrophages (Wilbanks and Streilein, 1992). On the other hand, vasoactive intestinal peptide, which also constitutively occurs in CSF (Taylor and Streilein, 1993), can induce DC maturation and potentiate the maturation induced by TNF-α (Delneste et al, 1999). TNF-α itself is present in MS patients' CSF and correlates with disease activity (Sharief and Hentges, 1991). Multiple maturation factors for DCs (TNF-α, IL-1β and LPS) are present in CSF in bacterial meningitis (Waage et al, 1989). However, CSF from these patients also contains potent DC-downregulating factors, such as IL-10 (Frei et al, 1993), which prevents LPS-induced DC maturation (Buelens et al, 1997) and converts DCs into macrophage-like cells (Fortsch et al, 2000). In different neuroinflammatory diseases, all these factors may have different net effects on functions of DCs, which in turn may influence the character of the intrathecal immune response.

NIND CSF did not suppress the antigen-presenting function of DCs, in contrast to what was previously reported for macrophages (Wilbanks and Streilein, 1992). MS CSF and LM CSF, and to a lesser extent NIND CSF induced maturation of moDCs, reflected by a decrease of CD1a expression, increase of HLA-DR, CD80, CD86, and in rare cases increase of CD83. This agreed with our previous observation that myeloid DCs in CSF are more mature than in blood (Study 3). In contrast, CSF from BM patients did not induce moDC maturation or even converted moDCs into macrophage-like $CD14^+$ cells. Accordingly, moDCs treated with BM CSF were deficient in inducing proliferation and IFN-γ production by naïve T cells, whereas moDCs treated with MS CSF and especially with LM CSF induced a remarkable production of IFN-γ. Furthermore, moDCs treated with LM CSF produced high levels of IL-12 upon coculture with allogeneic naïve T cells.

ELISA revealed that both LM and BM CSF contained IFN-γ, a cytokine that favors development of myeloid DC1 (Kalinski et al, 1999). However, BM CSF contained significantly higher amounts of IL-10, a cytokine that suppresses DC functions. These differences in cytokine levels could explain the different effects of BM and LM CSF on DCs. In addition, moDC maturation induced by MS and LM CSF could be blocked by pre-treatment of CSF with anti-TNF-α mAbs or by addition of rhIL-10 (1 ng/ml). Thus, the cytokine milieu may modulate properties of myeloid DCs once they enter the CSF.

5. DISCUSSION

5.1 IL-15 and its possible role in MS

In study 1, increased numbers of IL-15–expressing blood MNCs were found in patients with primary progressive and to a lesser extent with secondary progressive MS, as compared to relapsing-remitting MS and healthy controls, suggesting that (1) there is activation of IL-15–producing cells (presumably monocytes or DCs) in chronic forms of MS, and (2) IL-15 may play a role in chronic forms of MS. The function of IL-15 in MS could be the activation of T cells, including bystander activation, as well as their chemoattraction to MS lesions. Involvement of IL-15 has been demonstrated in other cell-mediated immunopathological conditions, such as rheumatoid arthritis and sarcoidosis (Agostini et al, 1996; McInnes et al, 1996; McInnes et al, 1997). Interestingly, HHV-6 that has been implicated in MS pathogenesis, is a strong inducer of IL-15 production by MNCs (Flamand et al, 1996).

The higher expression of IL-15 by blood MNCs in primary progressive MS than in relapsing-remitting MS adds one more point to the list of clinical, pathological and immunological dissimilarities between these two types of MS (Thompson et al, 1991; Revesz et al, 1994; Pirttilä and Nurmikko, 1995; Giovanoni et al, 1996; Thompson et al, 1997; McDonnell et al, 1998).

Expression of IL-15 by CSF MNCs was not detectable by immunocytochemistry, and ELISA, showed lower levels of IL-15 in CSF compared to plasma. This somewhat contradicts to our later finding that DCs, one possible source of IL-15, are present in CSF. Following explanations can be provided: (1) cells that produce IL-15 are not DCs and are not recruited to CSF; (2) IL-15 production is specifically downregulated in CSF cells.

5.2 DCs: Methodological questions

5.2.1 Detection of DCs in blood and CSF

Three-color flow cytometry is a simple tool for detection of DCs in blood and CSF on the basis of the following criteria: (1) dim or negative staining for lineage markers; (2) positivity for HLA-DR; (3) high expression of either CD123 (plasmacytoid DCs), or CD11c (myeloid DCs). Both DC subpopulations were minor, each making up <1% of blood MNCs and <3% of CSF MNCs in the majority of samples. To reliably enumerate DCs, large volumes of CSF were obtained, and a total

of at least 5,000 events per test in the case of CSF and 50,000–100,000 events in the case of blood were acquired. It was possible to perform a more detailed phenotyping of DCs in selected CSF samples, which revealed a greater maturity of myeloid DCs in CSF than in blood. However, all these CSF samples had to have pleocytosis, so as to obtain sufficient amounts of CSF cells. Therefore, this finding may not be extrapolated to non-inflammatory conditions.

5.2.2 Functional studies of DCs from CSF

These are hindered by the low numbers of CSF DCs. We have FACS-sorted plasmacytoid DCs from two LM CSF samples that contained large proportions of these cells. Sorted DCs were used for Giemsa staining (n = 2), phenotyping (n = 1), cytokine staining (n = 1) and MLR (n = 1). Otherwise, indirect approaches to evaluate CSF DC functions had to be used: (1) immunophenotyping (Study 3); (2) analysis of correlation between the ability of unseparated CSF MNC samples to induce allogeneic MLR and percentages of DCs in these samples (Study 5); and (3) modelling the *in vivo* situation by culturing donor moDCs with CSF from different patient groups (Study 6).

5.2.3 Chemotactic assays

Transwell system used here is a rather simple and reliable tool to study DC chemotaxis (Lin et al, 1998). In case of a homogenous cell population, such as moDCs, migrated cells were simply counted on the lower surface of filters after Giemsa staining (Fig. 4, page 37). In case of a heterogeneous population, such as low density blood MNCs consisting of DCs and monocytes, migrated cells were washed off into lower chambers, collected, counted, stained by fluorochrome-labelled mAbs and analysed by flow cytometry (Fig. 5, page 37). The majority of experiments were done with moDCs, for two interrelated reasons: (1) it was not technically possible to obtain pure populations of myeloid and plasmacytoid DCs from blood, whereas pure populations of moDC could be obtained in large numbers; (2) the approach with moDCs, i.e. staining filters, was more reliable, as it did not include centrifugations and subsequent cell losses. Checkerboard assay was used to confirm that CSF-induced migration of moDCs was a result of chemotaxis but not chemokinesis.

5.3 DCs in the CSF: Possible role in the neuroinflammation

5.3.1 DC infiltration in the CSF is associated with CNS inflammation

In the present studies, myeloid and plasmacytoid DCs were identified in human CSF. We observed that (1) myeloid and plamsacytoid DCs are present in minute numbers in non-inflammatory CSF; (2) both DC subsets accumulate in CSF in different neuroinflammatory conditions, such as MS, BM and LM, and the numbers of CSF DCs correlate with common indicators of CNS inflammation (CSF IgG index, total CSF cell count); (3) in MS, numbers of myeloid and plasmacytoid CSF DCs are increased mainly at an early stage and inversely correlate with disease duration; (4) in BM, only numbers of myeloid DCs are consistently increased in CSF, in parallel with their decrease in blood; (5) in LM, there is a striking accumulation of myeloid and especially plasmacytoid DCs in CSF; (6) myeloid DCs in CSF are more mature than in blood, while plasmacytoid CSF DCs are as immature as their blood counterparts; (7) in neuroinfections, DCs appear to be recruited to CSF by chemokines that are present in the CSF, such as SDF-1α, MCP-1 and C5a; (8) in MS, increased expression of CCR5 by blood myeloid DCs may contribute to their recruitment to CNS/CSF in response to CCR5 ligands; (9) cytokines present in CSF can either inhibit or enhance the T cell-stimulatory capacity of myeloid DCs.

Accumulation of DCs in CSF was thus directly related to the CNS inflammation. This is in line with recent reports from several groups, observing DCs in inflamed brain tissue in murine models of CNS inflammation (Matyszak and Perry, 1996a; Fischer et al, 2000, 2001; Serafini et al, 2000; Suter et al, 2000). In these models, brain DCs probably play a pro-inflammatory role, as they are absent from the normal CNS and appear at early stages of inflammation, produce IL-12, induce proliferation of, and IFN-γ production by naïve T cells. Importantly, other types of CNS APCs, such as macrophages, microglia and astrocytes, in order to activate T cells, need to be primed by IFN-γ, which is itself produced by activated T cells. The existence of CNS DCs, which do not depend on IFN-γ may obviate this problem. DCs may therefore represent the earliest pool of CNS APCs at the initial stage of inflammation (Fischer et al, 2001). In established chronic inflammation, brain DCs may continue to act as APCs, sustaining the intrathecal Th1 response and contributing to the epitope spreading (Drakesmith et al, 1998).

Our data favor a role of CSF DCs early in neuroinflammation. In MS, numbers of CSF DCs are increased in early disease and inversely correlate with disease duration. In 5 out of 6 BM patients examined, numbers of myeloid CSF DCs were increased already in the first few days of the disease. However, in BM patients as well as in the only prospectively examined LM patient, numbers of CSF DCs dropped several-fold after antibiotic treatment, whereas in a total of four followed-up patients with early MS (two with clinically definite MS and two with MS-type ON), numbers of CSF DCs remained elevated for at least several months. Whether this is the cause or consequence of MS chronicity remains to be established. Our data on myeloid CSF DCs in MS and ON remind findings on synovial myeloid DCs in rheumatoid arthritis (Thomas et al, 1999): in both diseases, myeloid DCs are more frequent at the site of inflammation (in synovial fluid or CSF) than in blood; both synovial and CSF DCs are more mature than blood DCs.

5.3.2 Recruitment of DCs to CSF

Based on perivascular localization of brain DCs in EAE, their phenotype ($CD11b^{-}CD11c^{+}DEC\text{-}205^{+}$) and colocalization with MIP-3α^{+} cells, Serafini et al (2000) suggested that brain DCs are recruited from the circulation and are not derived from glial precursors. On the other hand, Fischer and colleagues described $CD11b^{+}CD11c^{low/+}$ $DEC\text{-}205^{-}$ brain DCs and generated similar cells from murine glial cultures stimulated with GM-CSF or *Toxoplasma gondii* (Fischer and Bielinski, 1999; Fischer et al, 2000), suggesting DC development from microglia or another co-isolating DC precursor. This option was confirmed by another group (Santambrogio et al, 2001). Two different types of brain DCs may have been described in these reports. At early stages of EAE, activation of the BBB by systemically produced cytokines may trigger recruitment of blood DCs to CNS in parallel with, or even before the recruitment of T cells. At later stages, intrathecally produced cytokines and chemokines may recruit more DCs from the circulation and induce DC development from microglia. Inflammatory cytokines (TNF-α) and infiltrating $CD40L^{+}$ T cells (Gerritse et al, 1996) may trigger maturation of brain DCs.

There is indirect evidence that human CSF DCs are recruited from circulating blood DCs: (1) myeloid and plasmacytoid DCs in CSF are phenotypically similar to their blood counterparts; (2) numbers of CSF DCs in neuroinfections correlate with

levels of specific chemokines (such as SDF-1α); (3) numbers of myeloid CSF DCs in MS correlate with expression of CCR5 by myeloid DCs in blood. In non-inflammatory conditions, DCs may be predominantly recruited at the blood-CSF barrier, i.e. the choroid plexus. Non-inflammatory CSF contains constitutive chemotactic activity for immature moDCs, which is not mediated by IP-10, MCP-1, MCP-3, MIP-1α/β, RANTES or SDF-1α (our observations). Candidate factors are neuropeptides, such as vasoactive intestinal peptide, which is chemotactic for immature moDCs at the concentrations present in the CSF (Dunzendorfer et al, 2001). In BM and LM, DC recruitment at the choroid plexus may be augmented by increased levels of chemokines in the CSF; DCs may also be shed to CSF from inflamed meninges. In MS and ON, where the chemotactic activity of CSF is the same as in NIND, DCs may be shed into CSF from juxta-CSF lesions, where chemokines are expressed, and from meningeal infiltrates, which are typical for early MS (Guseo and Jellinger, 1975) and probably represent the same inflammatory process as that in MS plaques. A number of DC chemoattractants are expressed in MS plaques, including MCP-1–3, MIP-1α/β and RANTES (Hvas et al, 1997; McManus et al, 1998; Balashov et al, 1999; Boven et al, 2000).

In BM, we observed a prominent reduction of both myeloid and plasmacytoid DCs in peripheral blood at the acute stage, with partial restoration at recovery. Possible reasons are recruitment of these cells to CSF and/or to draining lymph nodes (Cella et al, 1999a, 2000). In less acute conditions, such as LM and MS, DC populations in blood were not changed compared to NIND, perhaps due to a lower recruitment rate and compensatory release from the bone marrow.

Our results highlighted a role for SDF-1α in recruitment of DCs to CSF in neuroinfections (study 4): (1) CSF levels of SDF-1α were increased in BM and LM and strongly correlated with myeloid and plasmacytoid DC infiltration; (2) SDF-1α induced potent chemotaxis of myeloid and plasmacytoid blood DCs; (3) SDF-1α mediated up to 50% of CSF chemotactic activity for immature moDCs in LM and at recovery from BM. SDF-1α appears to be constitutively produced in the CNS by yet unidentified cell type(s). In infections, SDF-1 may be produced by cortical astrocytes, which secrete this chemokine after LPS stimulation *in vitro* (Bajetto et al, 1999). In BM, CSF levels of SDF-1α were higher at recovery than in the acute phase, unlike the five other chemokines studied, suggesting distinct regulation mechanisms.

Importantly, in all NIND cases, levels of SDF-1α in CSF were 2–3 times lower than in plasma, while in patients with neuroinfections SDF-1α was higher in CSF than in plasma. Given its potent chemotactic effect on DCs, SDF-1α may be a "switch" allowing DCs to enter the CSF in infections. SDF-1α was apparently not the only factor mediating DC recruitment to CSF, and other factors, such as C5a, MCP-1, MIP-1α/β and fMLP are probably of importance.

Although in all patients with LM and those recovering from BM the levels of SDF-1α in CSF were higher than in plasma, the massive accumulation of plasmacytoid DCs occurred only in LM. One reason could be the low numbers of plasmacytoid DCs in blood of BM patients, which, in turn, may have resulted from recruitment of plasmacytoid DCs to other sites, such as lymph nodes. Second, the blood-CSF barrier may influence the entry of DCs into CSF. It has been reported that myeloid DCs pass through endothelium irrespectively of its activation state, while plasmacytoid DCs can pass only through activated endothelium (Pettit and Thomas, 1999). Thus, recruitment of myeloid DCs to CSF may depend merely on chemokine gradients, while plasmacytoid DCs may need additional signals, e.g. L-selectin ligands (Cella et al, 1999; Jahnsen et al, 2000). Ligands for L-selectin may be induced on CNS endothelium in LM but not in BM. Finally, IL-10, which is highly expressed in BM CSF, can induce apoptosis of plasmacytoid DCs (Rissoan et al, 1999) and convert myeloid DCs into $CD14^{+}$ macrophage-like cells with increased bactericidal but low antigen-presenting capacity (Fortsch et al, 2000). We cultured immature moDCs with five different BM CSFs, and in two cases observed a transformation of $CD14^{dim/-}CD1a^{high}$ moDCs into $CD14^{+}CD1a^{dim}$ cells. By ELISA, these two CSFs contained the highest levels of IL-10. In one of these patients, CSF cells were analysed by flow cytometry, which revealed complete absence of DCs. In LM, CSF levels of IL-10 were three-fold lower than in BM and were less likely to influence DC phenotype or survival.

As for MS, expression of CCR5 by blood myeloid DCs was increased compared to non-inflammatory controls. This could contribute to recruitment of these cells to MS lesions, where CCR5 ligands are expressed (Hvas et al, 1997; Boven et al, 2000). Elevated CCR5 expression by T cells in MS has been reported (Zang et el, 2000). Such concordant aberrations of CCR5 expression can make it easier for DCs and T cells to interact within MS lesions. The presence of DCs in MS lesions has not been

demonstrated so far. However, perivascular $CD68^+CCR5^+$ cells found in active lesions and usually regarded as monocytes or macrophages (Simpson et al, 2000) are phenotypically consistent with DCs as well. Mechanisms of plasmacytoid DCs recruitment to MS CSF are less clear; they appear to be CCR5-independent.

5.3.3 Migration of DCs from CSF to deep cervical lymph nodes

A possibility for mature DCs to migrate from the CNS to deep cervical lymph nodes via the olfactory pathway (see 1.4) has been demonstrated by Carson et al (1999). They injected mature fluorochrome-labelled DCs into brain cortex in mice, and after 48 h observed some labelled DCs in T cell areas of deep cervical lymph nodes. The role of this pathway in the natural course of neuroinflammation remains to be clarified. Homing of DCs to T cell areas of secondary lymphoid organs is directed by chemokines such as MIP-3β and 6Ckine, which interact with CCR7, the receptor expressed by DCs only at the mature stage (Dieu et al., 1998; Sallusto et al., 1998). Myeloid DCs in CSF are phenotypically more mature than in blood and may be more capable of homing to T cell areas of deep cervical lymph nodes. If these DCs have captured antigens in the CSF, they may activate naïve antigen-specific T cells.

Harling-Berg et al (1989) suggested that the microenvironment in the cervical lymph nodes is such that antigens injected into CSF can induce only a B cell and a limited cytotoxic response. However, these authors experimented only with soluble antigens, such as albumin. When an appropriate corpuscular antigen is injected, such as BCG, it induces DTH, i.e. a Th1 response (Matyszak and Perry, 1996b). If migration of CSF DCs along the olfactory pathway does occur naturally, it may explain several phenomena: (1) the possibility to induce DTH by injecting BCG into CSF but not into brain parenchyma (Matyszak and Perry, 1996b), where DCs are normally absent; (2) similarly, the possibility to induce lymphocyte proliferation in deep cervical lymph nodes by injecting antigens into CSF, but not into brain parenchyma (Stevenson et al, 1997); (3) precipitation of EAE by brain cryotrauma and prevention of such precipitation by removal of deep cervical lymph nodes (Phillips et al, 1997); (4) efficiency of nasal IL-10 treatment in EAE: cytokines such as IL-10 may block maturation of DCs migrating via the olfactory pathway (Xiao et al, 1998; Xu et al, 2000); (5) exacerbations of MS after acute respiratory infections, which affect upper nasal ducts and may cause additional maturation of DCs migrating from CNS/CSF (Edwards et al, 1998); (6) association of MS with chronic tonsillitis

(Gusev et al, 1996): lymph from the tonsils drains to deep cervical lymph nodes (Koornstra et al, 1991; Belz and Heath, 1995) and, in case of tonsillitis, may augment maturation of DCs arriving from CNS/CSF.

CXCR4, the receptor for SDF-1α/β, is expressed both by immature and mature DCs, being upregulated upon maturation (Lin et al, 1998; Sallusto et al, 1998b; Caux et al, 2000). If SDF-1 is released at the sites of inflammation, it may attract immature myeloid DCs via CXCR4 and then trap them upon maturation, preventing migration to secondary lymphoid organs. Our data in neuroinfections (study 4) favor this hypothesis. CSF levels of SDF-1α in BM and LM are high enough to attract mature moDCs and to decrease migration of mature moDCs from CSF supernatants towards MIP-3β. This mechanism may not function at the acute stage of BM, as LPS present in CSF of these patients (Waage et al, 1989) shuts down surface expression of CXCR4 by DCs (Sallusto et al., 1998b). SDF-1α may play its "retaining" role at recovery of BM and in LM. At recovery of BM, retention of DCs in the CNS/CSF may downregulate systemic activation of T cells when it is no longer needed. On the other hand, it has recently been hypothesized that mature DCs retained in peripheral tissues attract T cells and set on chronic inflammation (Sallusto and Lanzavecchia, 1999). It is tempting to speculate that this mechanism works in LM, where numbers of DCs in CSF are high and inflammation is subacute or chronic. Expression of SDF-1α in MS plaques has not been studied. However, CSF levels of SDF-1α in MS equal to those in NIND and do not prevent DC migration towards MIP-3β *in vitro* (our observations).

5.3.4 Possible roles of CSF DCs in neuroinflammation

What is the role of CSF DCs? Do they merely reflect DC infiltration in meninges or brain parenchyma, or do they play an independent role in neuroinflammation?

Plasmacytoid DCs. When comparing different diseases, numbers of plasmacytoid DCs in CSF can be ranked as LM >> ON > MS > NIND; an inconsistent elevation is observed in BM. As mentioned in the introduction, plasmacytoid DCs play a dual role: (1) at the precursor stage, they produce large amounts of type I IFNs (Siegal et al, 1999); (2) after maturation, they act as APCs (Rissoan et al, 1999; Kadowaki et al, 2000). Plasmacytoid DCs in CSF are as immature as in blood, even in inflammatory conditions. When isolated from CSF of

an LM patient, plasmacytoid DCs did not induce allogeneic MLR. One could guess that their function is to produce type I IFNs. We could not demonstrate this in CSF. However, plasmacytoid DCs may still produce type I IFNs in inflamed meninges or brain tissue, upon direct contact with the pathogen. Considering a beneficial effect of type I IFNs in MS, one may wonder whether plasmacytoid DCs play any anti-inflammatory role in this disease.

Myeloid DCs. Numbers of myeloid DCs in CSF could be ranked as LM > BM > ON > MS > NIND. Already in blood, these cells are potent APCs (O'Doherty et al, 1994). In CSF, they are more mature, suggesting an even higher T cell-stimulatory capacity. The ability of CSF MNCs to stimulate allogeneic T cells correlates with percentages of myeloid DCs among CSF MNCs. It is not known, where these DCs present antigens *in vivo*. Low cell density in the CSF will in most cases hinder effective interactions between DCs and T cells, although the situation may be different in the meninges or in the brain tissue. Existence of the olfactory pathway connecting the CSF with conventional lymphatics, as well as the key observation by Carson et al (1999) regarding DC migration from the CNS/CSF to deep cervical lymph nodes allow the proposal that CSF DCs can present CNS-derived antigens to naïve T cells in deep cervical lymph nodes. A following model could be suggested (Fig. 6).

In the absence of inflammation, small numbers of myeloid DCs are recruited to the CSF (Fig. 6). These DCs, together with DCs of the choroid plexus and meninges, survey the CNS environment for the presence of antigens, including those released from brain parenchyma. CSF DCs may normally migrate to deep cervical lymph nodes, but as their numbers and antigen load are low, they will cause little or no T cell activation. Antigens that reach the deep cervical lymph nodes on their own and not in the context of DCs may not be efficiently presented to T cells.

As soon as an infection occurs, CSF DCs would take up foreign antigens, and new myeloid DCs would be recruited from the circulation by released chemoattractants. The fate of these DCs would depend on the balance between pro- and anti-maturation factors. In BM CSF, high levels of IL-10 may prevent DC maturation. If such DCs migrate to deep cervical lymph nodes, they would cause T cell anergy or induce regulatory T cells. On the other hand, IL-10 present in BM CSF can convert DCs into macrophages (Fortsch et al, 2000), thereby augmenting the innate response against pyogenic infections. IL-10 may be absent from CSF during

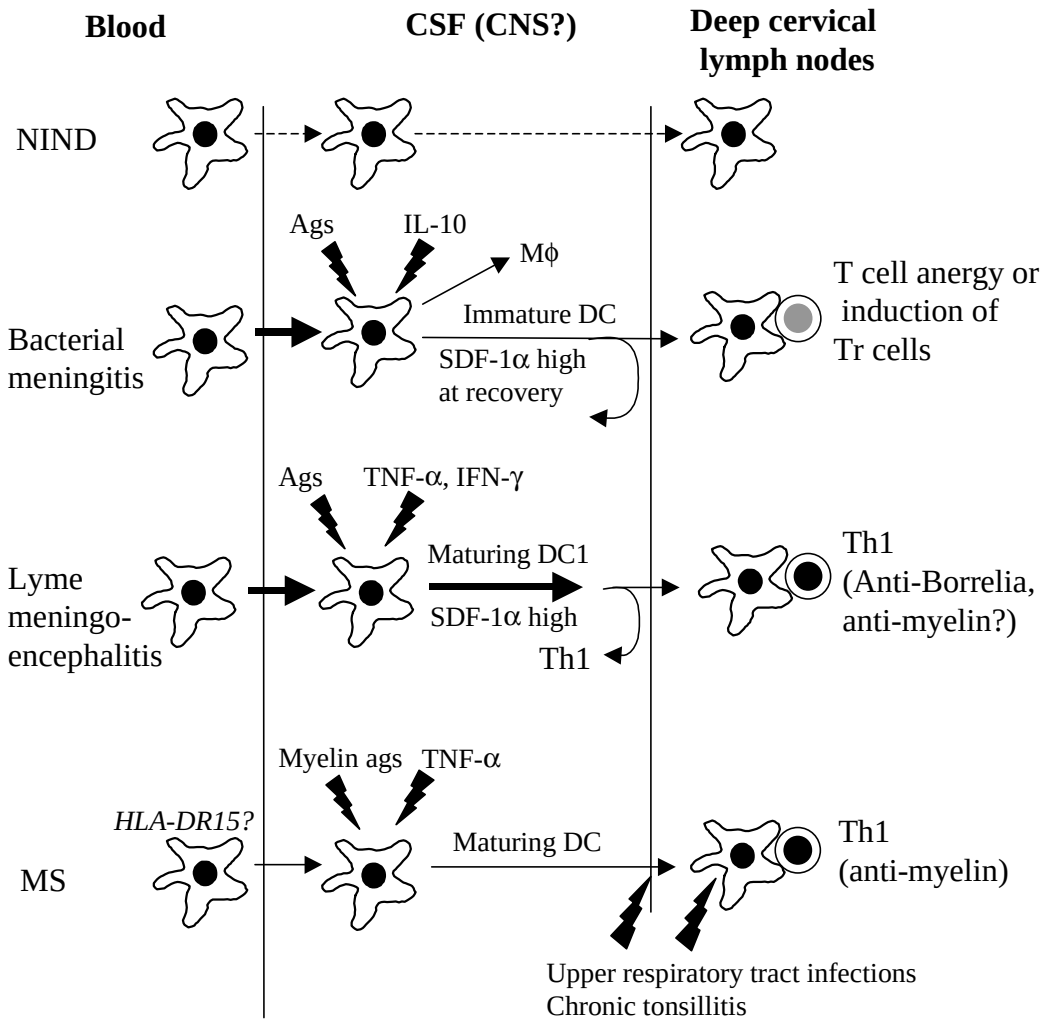

Figure 6. Putative involvement of myeloid CSF DCs in immune regulation within the CNS.

the very first hours of BM (Frei et al, 1993). Within this time window, there may be an increased traffick of maturing DCs from CSF to the lymph nodes, allowing initiation of the adaptive immune response. At recovery from BM, increased levels of SDF-1α in CSF may, presumably, prevent migration of DCs to deep cervical lymph nodes.

In LM CSF, myeloid DCs are more abundant (up to 5000 cells/ml), levels of IL-10 are relatively low, and IFN-γ is present, which altogether favors a Th1 response. Furthermore, even in the absence of IFN-γ, *Borrelia burgdorferi* can induce IL-12 production by myeloid DCs (Filgueira et al, 1996). *Borrelia*-loaded myeloid DCs may migrate from the CSF to deep cervical lymph nodes and induce new pools of Th1 cells. High SDF-1α in LM CSF suggests that a proportion of DCs may be retained in the CNS/CSF and activate T cells intrathecally. Altogether, this may contribute to the

strong and sustained intrathecal Th1 response seen in Lyme neuroborreliosis (Ekerfelt et al, 1997).

In MS, recruitment of DCs to CSF is also augmented. In early MS, myeloid CSF DCs reach up to 3–4% of CSF cells (up to 500 cells/ml) and can remain elevated over several months; thus, relatively high DC:T cell ratios can be maintained during long time, favouring the Th1 deviation. The "target" antigen in MS remains unknown. However, any such antigen is likely to be released into CSF and taken up by CSF DCs, as MS lesions are often located periventricularly. A candidate antigen is myelin basic protein-like material (MBPLM), which is released into CSF during MS exacerbations (Whitaker, 1998). This event is non-specific and occurs in other pathological processes, such as trauma and stroke (Whitaker, 1998). However, in MS, presentation of MBPLM or other (auto)antigens by more numerous CSF DCs, in the context of appropriate HLA molecules and other putative genetic factors, and in the presence of pro-inflammatory cytokines such as TNF-α may induce new pools of autoreactive Th1 cells in deep cervical lymph nodes. CSF levels of SDF-1α in MS equal to those in NIND and may not prevent DC migration to lymph nodes. Upper respiratory tract infections and tonsillitis will boost maturation of DCs migrating via the olfactory pathway. The Th1 cells generated in deep cervical lymph nodes may then migrate via blood back to the CNS and incite new inflammatory lesions.

Summarizing literature and our own data, the following hypothesis may be proposed: (1) any CNS-derived antigen (auto- or exo-), in order to become immunogenic, must reach the CSF and be taken up by CSF DCs; (2) CSF DCs, like DCs of other organs, can transport antigens to regional (deep cervical) lymph nodes; (3) in inflammation, the pool of CSF DCs is increased due to their recruitment from the periphery; (4) the character of T cell response induced by CSF DCs in deep cervical lymph nodes will depend on the number of DCs migrating and on properties of these DCs; (5) properties of CSF DCs may depend on the patient's genetic background and can be modulated by the local milieu present in the CSF and along the olfactory pathway. This hypothesis thus assumes an independent role of CSF DCs in neuroinflammation, but does not exclude a similar role of DCs from choroid the plexus, the meninges, or the brain. It may explain MS as a self-perpetuating disease, caused by continuous traffick of antigen-bearing DCs from the CSF to deep cervical

lymph nodes and subsequent recirculation of activated autoreactive T cells to the CNS.

Following experiments will probably be required to test this hypothesis: (1) to demonstrate migration of DCs from CNS to deep cervical lymph nodes in a model of chronic EAE, as well as in an acute and a chronic experimental CNS infection; (2) to demonstrate that CSF DCs express CCR7, a receptor that regulates homing of DCs to T cells areas of secondary lymphoid organs; (3) to demonstrate that CSF DCs from MS patients, or moDCs pulsed with auto-CSF, induce proliferation of, and IFN-γ production by autologous T cells, provided that this does not happen in non-inflammatory controls. Other experiments are also needed to elucidate other aspects of DC involvement in MS and neuroinfections, including: (1) investigation of molecular mechanisms of DC recruitment at the blood-brain barrier; (2) identification of myeloid and plasmacytoid DCs in inflammatory lesions in MS and neuroinfections; (3) investigation of molecular mechanisms of plasmacytoid and myeloid DC interactions with *Borrelia burgdorferi*. If the above hypothesis is true, then interfering with CNS/CSF DC migration and maturation may be a therapeutic tool to control inflammation in MS and Lyme neuroborreliosis.

6. GENERAL CONCLUSIONS

1. IL-15-expressing MNCs, presumably DCs or monocytes, are elevated in blood of primary progressive and chronic progressive MS patients.
2. Blood DCs are a source of the proinflammatory cytokines IL-12, IL-15 and IFN-γ.
3. Myeloid and plasmacytoid DCs are present in non-inflammatory CSF and are additionally recruited in MS and neuroinfections.
4. The cytokine milieu in the CSF influences DC phenotype and functions.
5. CSF DCs may induce and regulate T cell responses intrathecally and in regional lymph nodes. Interfering with CNS/CSF DC functions may be a therapeutic tool of controlling CNS inflammation.

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