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Immune Invasion and Glial Activation in Experimental Autoimmune Encephalomyelitis

Elise H. Tran

Department of Microbiology and Immunology,
McGill University

March 2000

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of
DOCTOR OF PHILOSOPHY

In the memory of my father.

The most exciting phrase to hear in science,
the one that heralds the most discoveries
is not "Eureka!" but "That's funny..."

Isaac Asimov

I dedicate this thesis to

my mother, my sister, and my three brothers.

ACKNOWLEDGEMENTS

I am forever indebted to my supervisor, Dr. Trevor Owens, for his teaching, guidance, countless hours of discussions, endless encouragement to taking up challenge after challenge, for giving me the total freedom to pursue new ideas, and for always being there to advise and to share his optimism.

I am grateful to Dr. Josephine Nalbantoglu for her comments on the introduction of my thesis, and for her support. I would like to thank Drs. Jack P. Antel, Wee Yong, Neil R. Cashman for their input on my projects in unit lab meetings. I am thankful to the members of my committee, Drs. Gitte Jensen and Edith Hamel, for their feedback and advice. I am also thankful to Drs Christine D. Dijkstra (Amsterdam), Nico van Rooijen (Amsterdam), and Williams Kuziel (Texas), for numerous e-mail discussions and encouragement.

My heartfelt thanks to members of the Owens' lab past and present: Lyne Boubonnière for being always present for me, and for proofreading my French abstract; Dr. Mina Hassan-Zahraee for inviting me to collaborate on various projects, and for sharing her philosophy of life; Gail Verge for training me in immunohistochemistry; Drs. Michelle Krakowski, Helene Hardin-Pouzet, Veronique Taupin, Roberta De Simone, Michael Jensen, and Joanne Poudrier, for sharing their knowledge and enthusiasm; Lawrence Lai for helping in initiating the iNOS project; Julie Mercure for initiating the liposome project; Esther N. Prince for assisting in the IFNγ project; Grace Chan for her help in my initial adoptive transfer experiments, and for hours of sanity in the gym; Maria Caruso, Qiaoke 'Jack' Gong, Joanna Holland, Dewen Kong, and Alicia Babcok for their encouragement; Dr. C. Annette Hollmann for proofreading the abstract and the introduction of my thesis. To all of you, thank you very much for your endless support, help, and time, for listening to me, even when I did not make much sense!

I wish to thank members of the Neuroimmunology Unit past and present for discussions, ideas, protocols, reagents, advice, and support: Dr. Sandrine Pouly, Manon Blain, Orce Jovsceski, Ellie McCrea, Drs. Burkhard Becher, Alexandre Prat, Sonia Jung, Vincent Dodelet, and Rosanne Séguin. I am also grateful to Liliana Cetola for her help and laughters!

I am grateful to Jim Dixon for his expertise in electron microscopy imaging, Fiona Yong for her expertise on confocal microscopy imaging, Lily Li and Lixia Zhu for advice and assistance in immunohistochemistry.

I wish to thank my roomate Betty Tam for her encouragement during the preparation of my thesis, for commenting on my introduction, feeding me, listening to my whining, and for asking her friend Elise Jalil to proofread my French abstract. I am also grateful to Nina D. Lomholt for always 'e-cheering' me up, and to Kathy Fu for dragging me to the tennis and squash courts. J'aimerais également remercier Geneviève Grenier Denis et Priscilla N. Lam pour leur amitié, affection, aide à faire face à la musique, et d'avoir toujours été là pour moi!

Hugs, hugs, hugs!
I am forever grateful to all of you.
I could not have done it without you all.
Thank you again and again!
Merci encore et encore!

ABSTRACT

Leukocyte recruitment into tissues in response to infection or injury is a crucial event for the elimination of pathogens to protect the host. However, when leukocytes invade the central nervous system (CNS) and neuroinflammatory disorders result, neurological function may be compromised. Infiltration of the CNS, predominantly by T cells and macrophages, characterizes Multiple Sclerosis and its animal counterpart, Experimental Autoimmune Encephalomyelitis (EAE).

Autoreactive T cells that initiate EAE produce Th1 cytokines (e.g., IFNγ, TNFα). Nevertheless, previous studies also indicated an unnecessary or even protective role for IFNγ in EAE. I have identified a novel role for IFNγ in my studies using IFNγ- or IFNγR-knockout mice. IFNγ promotes the expression of the chemokines RANTES, MIP-1α, and MCP-1, which recruit mononuclear cells in the CNS to induce a non-lethal remitting EAE. Without IFNγ, the chemokines MIP-2 and TCA-3, and polymorphonuclear leukocytes prevail, producing an unusually lethal EAE. MIP-1α is, however, dispensable in recruiting mononuclear cells, as EAE could still be induced in mice deficient in MIP-1α or its CCR5 receptor.

To examine how much T cells depend on the cooperation with macrophages in the CNS to induce EAE, selective depletion of peripheral macrophages in mice was achieved by intravenous administration of clodronate-loaded liposomes. Treated mice showed no clinical signs of EAE following adoptive transfer of myelin-reactive T cells, but an altered distribution of leukocytes. These leukocytes were confined within the perivascular or meningeal space, not invading the CNS parenchyma. Levels of TNFα and inducible nitric oxide synthase (iNOS) in the CNS were reduced in these asymptomatic macrophage-depleted mice compared to untreated mice with EAE. In these asymptomatic mice, iNOS expression was restricted to parenchymal astrocytes. In mice with EAE, however, both macrophages/microglia and astrocytes in infiltrates expressed iNOS. Surprisingly, some astrocytes that were distant from infiltrates also expressed iNOS, thus suggesting that astrocytes may modulate leukocyte infiltration via release of NO through their foot processes in the blood-brain barrier. Collectively, my data propose a model of a dynamic network in which the interplay among cytokines, chemokines and nitric oxide, may determine the magnitude, the composition, or the resolution of inflammatory infiltrates, as well as the clinical outcome of EAE.

RÉSUMÉ

Le recrutement des leukocytes dans un tissu en réponse à une infection ou une blessure est un événement essentiel pour l'élimination des pathogènes afin de protéger l'hôte. Cependant, quand les leukocytes envahissent le système nerveux central (SNC) et des désordres neuroinflammatoires s'ensuivent, les fonctions neurologiques peuvent être endommagées. La Sclérose en Plaques et son modèle animal, l'Encephalomyélite Autoimmunitaire Expérimentale (EAE), sont characterisés par une accummulation préférentielle de lymphocytes T et de macrophages dans le SNC.

Les lymphocytes T autoimmunitaires qui engendrent l'EAE produisent des cytokines Th1, telles que l'IFNγ et le TNFα. Néanmoins, les études précédentes ont démontré que l'IFNγ n'est pas nécessaire, ou peut même protéger contre l'EAE. En utilisant les souris knock-out pour les gènes d'IFNγ ou de son récepteur, j'ai identifié un nouveau rôle de l'IFNγ. L'IFNγ favorise la production des chimokines RANTES, MIP-1α et MCP-1 qui recrutent des lymphocytes T et des macrophages, pour induire un épisode non-fatal de l'EAE. En revanche, en absence de l'IFNγ, les chimokines MIP-2 et TCA-3, ainsi que les neutrophiles dominent et provoquent l'EAE fatale. Par contre, j'ai démontré que la chimokine MIP-1α ne joue pas un rôle dans le recrutement des lymphocytes et des macrophages, parce que l'EAE pouvait être induite dans les souris knock-out pour les gènes de MIP-1α ou de son récepteur CCR5.

Dans le but d'examiner la mesure par laquelle les lymphocytes dépendent de la coopération avec les macrophages dans le SNC pour induire l'EAE, les souris ont été traitées avec des liposomes contenant du clodronate pour éliminer spécifiquement les macrophages périphériques. Les souris ainsi traitées n'ont pas démontré de symptômes de l'EAE après le transfer adoptif de lymphocytes T réagissant contre la myéline. Cependant, la distribution des leukocytes dans le SNC a été perturbée. Les leukocytes sont demeurés confinés dans l'espace périvasculaire ou méningiale; ils n'ont jamais envahi le parenchyme du SNC. Le niveau de l'expression de TNFα et de l'oxide nitrique synthase inductible (iNOS) dans le SNC est réduit dans les souris traitées comparativement aux souris non-traitées atteintes de l'EAE. Dans ces souris

asymptomatiques, seuls les astrocytes exprimaient l'iNOS. En revanche, dans les souris atteintes de l'EAE, en plus des astrocytes, les macrophages et les microglies dans les infiltrats produisaient l'iNOS. Quelques astrocytes qui sont éloignés des infiltrats exprimaient aussi l'iNOS, suggérant que les astrocytes peuvent moduler l'infiltration des leukocytes par l'oxide nitrique le long de leurs projections dans la barrière hématoencéphalique. Collectivement, mes résultats me permettent de proposer un modèle de réseau dynamique dans lequel les intéractions entre les cytokines, les chimokines et l'oxide nitrique peuvent déterminer la magnitude, la composition ou la résolution des infiltrats inflammatoires.

CONTRIBUTION OF AUTHORS

This thesis consists of a collection of four original papers, of which three have been published and one has been accepted for publication. Each paper constitutes a chapter (II-V) which contains an Abstract, Introduction, Materials and Methods, Discussion, Acknowledgements, and References section. I have included prefaces as the connecting texts that bridge between the different papers.

I am the first author in all four papers. I analyzed and interpreted all my data, and wrote all the manuscripts with my supervisor, Dr. Trevor Owens, who is the senior author.

I was responsible for the research in all papers, with the except of one paper in which Esther N. Prince, as specified in details below, assisted me.

Chapter II

Astrocytes and microglia express inducible nitric oxide synthase in experimental autoimmune encephalomyelitis.

J. Neuroimmunol. 74:121-129

E. H. Tran, H. Hardin-Pouzet, G. Verge, and T. Owens.

Dr. Helene Hardin-Pouzet was a post-doctoral fellow in the lab and a collaborator, and reviewed the manuscript.

Gail Verge assisted with the initial histology.

Chapter III

Immune invasion of the central nervous system parenchyma and experimental autoimmune encephalomyelitis, but not leukocyte extravasation from blood, was prevented in macrophage-depleted mice.

J. Immunol. 161:3767-75

E. H. Tran, K. Hoekstra, N. van Rooijen, C. D. Dijkstra, and T. Owens.

Karin Hoekstra, Vrije Universiteit, Amsterdam, The Netherlands, prepared the clodronate-loaded liposomes which I used to deplete macrophages in mice.

Drs. Nico van Rooijen and Christine D. Dijkstra, Vrije Universiteit, Amsterdam, The Netherlands, were collaborators and reviewed the manuscript.

Chapter IV

IFNγ shapes immune invasion of the central nervous system via regulation of chemokines.

J. Immunol. 164:2759-68.

E. H. Tran, E. N. Prince, and T. Owens.

Esther N. Prince performed immunohistochemical stainings for Figure 1 (C, D, E, F, G), and 3/6 [^{3}H]-thymidine incorporation experiments described in the paper.

Chapter V

Induction of EAE in C57BL/6 mice deficient in either the chemokine MIP-1α or its CCR5 receptor.

Eur. J. Immunol. (in press)

E. H. Tran, W. A. Kuziel, and T. Owens.

Dr. William A. Kuziel, University of Texas, Austin, was a collaborator who provided the CCR5-/- mice and reviewed the manuscript.

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Date: Wed, 12 Jul 2000 21:08:37 +0200
From: Helene Pouzet <Helene.Pouzet@snv.jussieu.fr>
X-Sender: hpouzet@hall.snv.jussieu.fr
To: Elise Tran <elise@med.mcgill.ca>
MIME-version: 1.0

Elise:
You may have my permission to include in your thesis our publication in J. Neuroimmunol.

Helene Hardin-Pouzet

Helene Hardin-Pouzet, Ph.D.
Neurobiologie des Signaux Intercellulaires
Universite Paris 6 - CNRS UMR 7624
7 quai Saint Bernard, 75252 Paris Cedex 05, France
tel : (33) 1 44 27 33 95 - fax : (33) 1 44 27 25 08

Verge, Gail,7/12/00 12:14 PM -0700,RE: permission 1

Date: Wed, 12 Jul 2000 12:14:41 -0700
From: "Verge, Gail" <GVerge@neurocrine.com>
Subject: RE: permission
To: 'Elise Tran' <elise@med.mcgill.ca>
MIME-version: 1.0

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Here is the permission for including the paper in your thesis (I am not quite sure what they want):

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Tran, E.H., Hardin-Pouzet, H., Verge, G. & Owens,T. (1997): Astrocytes and Microglia express inducible nitric oxide synthase in mice with experimental allergic encephalomyelitis. Journal of Neuroimmunology 74(1-2):121-9.

If there are any questions concerning this matter please do not hesitate to contact me at (858)658-7771.

Sincerely,

Gail Verge

Date: Wed, 12 Jul 2000 10:23:46 -0500
From: "William A. Kuziel" <wakuziel@mail.utexas.edu>
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Dear Elise,

You have my permission to include in your thesis our recent publication entitled "Induction of experimental autoimmune encephalomyelitis in C57BL/6 mice deficient in either the chemokine macrophage inflammatory protein 1alpha or its CCR5 receptor" which appeared in the European Journal of Immunology, vol. 30, pp. 1410-1415.

Best regards,

William A. Kuziel, Ph.D.
Assistant Professor
Section of Molecular Genetics and Microbiology
The University of Texas at Austin
Austin, Texas

TEL: 512-471-5358
FAX: 512-471-7088
e-mail: wakuziel@mail.utexas.edu

ABBREVIATIONS

APC	antigen-presenting cell
BBB	blood-brain barrier
Cl_2MDP-mnL	dichloromethylene diphosphonate (or clodronate)-containing mannosylated liposomes
CNS	central nervous system
CSF	cerebrospinal fluid
EAE	experimental autoimmune/allergic encephalomyelitis
ECM	extracellular matrix
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
H&E	hematoxylin and eosin
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IP-10	interferon-inducible protein-10
LNC	lymph node cells
LTα	lymphotoxin alpha
mAb	monoclonal antibody
MBP	myelin basic protein
MCP-1	macrophage chemoattractant protein
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MMP	metalloproteinase
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
NO	nitric oxide
PECAM	platelet endothelial cell adhesion molecule
PLP	proteolipid protein
PMN	polymorphonuclear leukocyte
RANTES	regulated upon activation, normal T cell expressed and secreted
ROI	reactive oxygen intermediates
RPA	RNase protection assay
RT-PCR	reverse transcriptase polymerase chain reaction
TCA-3	T cell activation gene-3
TGF	transforming growth factor
TNFα	tumor necrosis factor-alpha

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CHAPTER I

GENERAL INTRODUCTION

The immune system operates physiologically to defend the body against a myriad of environmental microorganisms, which potentially threaten the survival of the individual. T and B lymphocytes can identify pathogens and generate specific responses. In most instances, the initiation of such a response has been proposed to be through the action of antigen-presenting cells (APC), such as macrophages, which present pathogens or antigens to T lymphocytes. The CD4+ T subset has specific T cell receptors that recognize foreign antigens presented by class II major histocompatibility complex (MHC) molecules on APC, while the receptors of the CD8+ T subset recognizes antigens bound to class I MHC molecules. The release of soluble factors, such as cytokines, which relay signals among cells, orchestrates the immune response. Activated B lymphocytes secrete specific antibodies to neutralize pathogens. Macrophages can also phagocytose and destroy microorganisms via free radical nitric oxide (NO) and superoxide cytotoxicity, as do neutrophils. Without such immunity, the host ultimately succumbs to infection. Yet, the immune system, like many cellular systems, can be implicated in disease. This can become particularly devastating and difficult to control when it occurs in the central nervous system (CNS).

1. Multiple Sclerosis: an autoimmune disorder of CNS myelin

Multiple Sclerosis (MS) is currently the most prominent disabling disease of the CNS that affects young adults in North America and Europe. Myelin ensheaths axons and enhances neural transmission. Although the etiology of MS remains elusive, the classical consensus is that MS arises when the immune system attacks myelin components within the white matter of the CNS (Steinman, 1996). Oligodendrocytes, the myelin-producing cells of the CNS, and myelin are destroyed in this disease process. When axons lose myelin, they can no longer conduct efficiently. In addition to myelin damage, there is also clear evidence for axonal loss and dysfunction (Trapp et al., 1999). Since myelinated axons anywhere in the CNS can be targeted, neurological deficits vary widely. Nevertheless, the most common

clinical manifestations include tremor, paralysis, incontinence, and visual-sensory dysfunction. The course of disease is highly variable, but commonly presents as frequent episodes of clinical relapses and remissions, and progression to chronicity. Although disease activity may respond to immunosuppressive or immunomodulatory therapies such as systemic interferon (IFN)-β (Yong et al., 1998a; Karp et al., 2000), no definitive treatment has yet emerged from this approach, and MS remains the major disabling neurological illness of young adults. Women are twice as susceptible as men. Both genetic and environmental factors are suspected to influence susceptibility to disease expression.

2. Experimental Autoimmune Encephalomyelitis: a model of MS

This concept of autoimmune-mediated inflammation and destruction of the white matter within the CNS has been extensively studied using the animal model, Experimental Autoimmune Encephalomyelitis (EAE). EAE is a T cell-mediated disease that is provoked in a variety of susceptible animal species and strains, by peripheral immunization with CNS myelin proteins or their peptide derivatives (Martin and McFarland, 1995; Zamvil and Steinman, 1990).

Generally, there are 2 major courses of EAE: (1) acute and monophasic paralysis followed by recovery or steady state of disease; and (2) chronic relapsing or relapsing-remitting, characterized by a series of remissions and exacerbations of paralysis (Zamvil and Steinman, 1990). Clinical signs are characterized by ascending paralysis, initially tail floppiness, followed by incomplete or complete limb paralysis. The topography of the leukocyte infiltrates may be influenced by the distribution and the nature of the neuroantigen against which the animals are immmunized (Berger et al., 1997). Usually, histopathological alterations of the CNS include mononuclear cell infiltrates comprising predominantly T cells and monocytes/macropages, with a few B cells (Raine et al., 1980). Polymorphonuclear leukocytes (PMN) become as prominent as monocytes/macrophages in a rare hyperacute

(rapid onset followed by paralysis and death) variant of EAE (Levine, 1974; Raine et al., 1980). Infiltrating leukocytes typically rim or cuff around venules mostly within the white matter, and rarely in the gray matter (Figure 1A). Infiltrating leukocytes can also accumulate in the meninges that surround the CNS, or at subpial areas within the CNS parenchyma (Figure 1A). Many different EAE models have been reported, and each model represents an aspect of the human disease, thus making it a valuable tool for study.

In most rodent EAE models, inflammatory infiltrates spontaneously resolve, resulting in a natural recovery, and may start again, in relapse. Curiously, many other neurological disorders, such as Alzheimer's disease, Parkinson's disease, Prion disease, AIDS dementia syndrome, stroke and epilepsy, involve in part, leukocyte infiltration and aberrant glial reactivity in the brain (Price, 1999). Regulatory mechanisms at leukocyte entry and retention potentially represent a critical checkpoint for the initiation or progression of neuroinflammatory diseases. *In this thesis, I have exploited murine EAE models to define the cellular and molecular pathways involved in controlling immune cell entry into the CNS.*

3. Immunopathogenesis of EAE: an overview

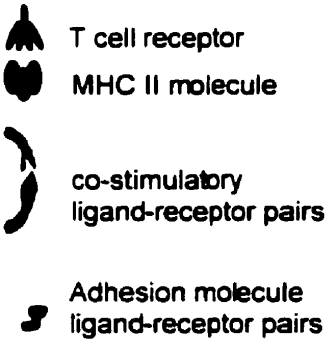
The classical view of the immunopathogenesis of EAE has focused on myelin reactive CD4+ T cells (Zamvil and Steinman, 1990; Owens et al., 1994; Steinman, 1996) (Figure 1B). The development of T cell-mediated autoimmune diseases, such as EAE, is a complex process that requires several different events, including activation of autoreactive T cells, their entry to the target tissue, local immune reactivity and tissue injury.

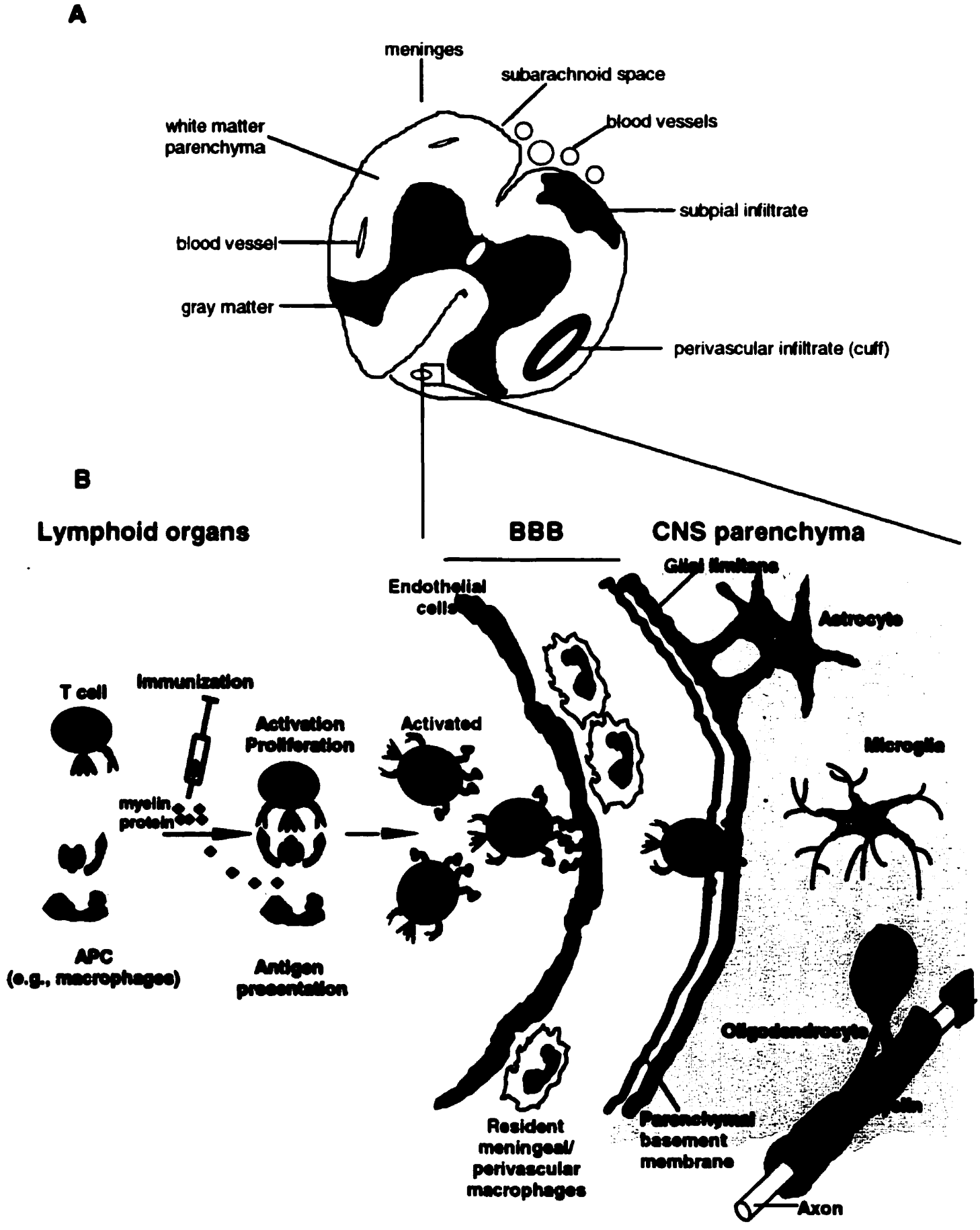
3.1 Activation of autoreactive CD4+ T cells in the periphery

Autoreactive CD4+ T cells against CNS myelin do exist in the circulation, but remain innocuous in normal healthy individuals and animals (Wekerle, 1992). What, then, changes the quiescent behavior of these cells and converts them into aggressive cells that attack the

Figure 1
(**A**) A schematic representation of a cross-section of the spinal cord, showing typical distribution of infiltrates in EAE.

(**B**) A model for the initiation of EAE (*see text*). Myelin-specific T cells are activated in the periphery. Activated T cells enter the CNS, and trigger a cascade of inflammatory events upon local recognition of their myelin antigens. Resident cells, such as local macrophages, astrocytes and microglia, become activated. Oligodendrocytes, and myelin, can be damaged during the inflammatory process.




A
meninges
subarachnoid space
white matter parenchyma
blood vessels
subpial infiltrate
blood vessel
gray matter
perivascular infiltrate (cuff)
B
Lymphoid organs
BBB
CNS parenchyma
Endothelial cells
Glial limitans
Astrocyte
T cell
Immunization
Activation Proliferation
Activated
Microglia
myelin protein
APC (e.g., macrophages)
Antigen presentation
Oligodendrocyte
Resident meningeal/ perivascular macrophages
Parenchymal basement membrane
Axon

CNS in disease? One possible scenario is that the induction of a peripheral immune response is required to initially activate them. This is demonstrated in EAE which is provoked by peripheral immunization with myelin autoantigens, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or proteolipid protein (PLP), along with adjuvant (Zamvil and Steinman, 1990; Martin et al., 1992; Bernard et al., 1997). In response to this, CD4+ T cells are activated in the peripheral lymphoid organs by APC's through presentation of myelin autoantigens complexed with class II MHC molecules. Additional interactions between surface receptors on APC's and their ligands on T cells (e.g., B7-CD28, CD40-CD40L) provides co-stimulatory signals that help to drive antigen-specific T cell activation and clonal expansion (Lenschow et al., 1996). The activated autoreactive T cells then circulate in blood and migrate to the CNS. Alternatively, adoptive transfer of myelin-reactive CD4+ T cells that have been previously activated and expanded *in vitro* can also induce EAE. This approach avoids the potential systemic influence of adjuvants and immunogen (as in the 'active' immunization model) on the recipient animals, and allows the characterization of the effector functions of the activated autoreactive T cells (Zamvil and Steinman, 1990).

How, when and where circulating myelin-reactive T cells become activated in MS remains enigmatic, but microbial infection is often invoked as the initial trigger (Noseworthy, 1999). For example, self-reactive T cells can be activated through molecular mimicry, whereby infectious agents that share antigenic determinants with the host's tissue can induce cross-reactive activation of autoantigen-specific T cells (Steinman and Oldstone, 1997). Activated T cells are normally subjected to a variety of immunoregulatory mechanisms that attenuate their responses *in vivo*. It may be that immunoregulatory mechanisms fail to eliminate activated autoreactive T cells or render them unresponsive, in the development of autoimmune disease.

3.2 Immune cell entry to the CNS

The blood-brain barrier (BBB) is constituted of specialized endothelial cells with tight intercellular junctions, and of the glial limitans that is formed by astrocyte foot processes. Although the intact BBB has been considered impermeable to macromolecules (e.g., antibodies) and to most circulating cells, there is compelling evidence to support that activated T cells can enter the CNS (Hickey et al., 1991). Considerable evidence suggests that non-reactive astrocytes are normally involved in the maintenance of the BBB (Kettenmann and Ransom, 1995), but to what degree such function is sustained or compromised by astrocytes that become reactive in neuroinflammatory disease is uncertain. The perivascular space between the endothelium and the glial limitans that encircles deep cerebral vessels, or the subarachnoid space around blood vessels in the meninges that surround the CNS, contains local macrophages. They are thought to be ideally positioned to contact T cells entering the brain. These perivascular cells or meningeal macrophages are in constant turnover with peripheral monocytes under normal conditions (Hickey et al., 1992). *I examined how astrocytes, as well as local and peripheral macrophages may influence leukocyte migration into the CNS parenchyma in EAE, in Chapter II and III.*

Activated T cells express numerous adhesion molecule receptors, and this allows them to attach to the endothelium of the BBB expressing the corresponding ligands. The most critical receptor-ligand pair that has been identified thus far is the very late antigen-4α (VLA-4α) and the vascular cell adhesion molecule-1 (VCAM-1), up-regulated on activated T cells and the endothelium, respectively (Weller et al., 1996; Sung and Benveniste, 1999).

Infiltrating CD4+ T cells are reactivated locally in the CNS upon recognition of their antigens on resident APC, which are most likely perivascular macrophages and microglia (Krakowski and Owens, 1997; Katz-Levy et al., 1999). Such T-cell autoreactivity is thought to result in the release of various inflammatory cytokines, chemokines and other mediators.

These factors alter the local homeostasis of the CNS and the integrity of the BBB, leading to further activation of microglia and astrocytes as well as additional recruitment and activation of T cells and other leukocytes. Interestingly, despite a breach in the BBB during EAE, leukocyte entry does not appear to be random, but rather selective (Weller et al., 1996). Macrophages and T lymphocytes predominate over B cells and PMN in infiltrates during an episode of EAE in mice (Raine et al., 1980).

3.2.1 Chemokines

Chemokines (chemotactic cytokines) are responsible for selective recruitment and activation of leukocyte subsets *in vitro* and *in vivo*. In general, the CC chemokines (e.g., macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation, normal T cell expressed and secreted (RANTES)) attract lymphocytes and monocytes/macrophages (Fuentes et al., 1995; Asensio and Campbell, 1999a). The chemokines IFN-inducible protein-10 (IP-10) and monokine induced by IFNγ (Mig) attract activated T lymphocytes but not neutrophils (Zlotnik et al., 1999). In contrast, the murine CXC chemokines, MIP-2 and KC, are considered as functional homologues to the human interleukin (IL)-8 and growth related oncogene-alpha (GROα) which attract primarily neutrophils (Tani et al., 1996; Asensio and Campbell, 1999a).

Table I (*see next page*) summarizes the expression of chemokines and chemokine receptors in EAE. Chemokines are clearly over-expressed in the CNS during acute disease. During recovery from EAE, however, most chemokines are significantly down-regulated (Hulkower et al., 1993; Berman et al., 1996; Adamus et al., 1997; Youssef et al., 1998; Asensio et al., 1999; Glabinski et al., 1995). De novo up-regulation of chemokines correlates with appearance of clinical relapses (Godiska et al., 1995; Glabinski et al., 1997). Whether similar chemokine patterns are expressed among the first acute attack and subsequent

Table I Expression of chemokine and chemokine receptors in the CNS during EAE.

A. Chemokines[1]	Target cells[2]	EAE models	References
MCP-1	Monocytes/macrophages T cells	MBP/CFA, Lewis rats	Hulkower et al., 1993 Ransohoff et al., 1993
		PLP/CFA, SJL/J mice	Godiska et al., 1994
		PLP-T cells, SJL/J mice	Karpus et al., 1995
		MBP-T cells, Lewis rats	Youssef et al., 1998
		PLP/CFA, (SWRxSJL/J)F1 mice	Glabinski et al., 1997
		$MOG_{35-56+92-106}$/CFA, (C57BL/6xSJL/J)F1 mice	Asensio et al., 1999
		MBP-T cells, SJL/J mice	Rajan et al., 2000
		MOG_{35-56}/CFA, C57BL/6 mice	Juedes et al., 2000
RANTES	T cells (Th1>Th2, memory T) Monocytes/macrophages NK cells	PLP/CFA,SJL/J mice	Godiska et al., 1994
		PLP-T cells, SJL/J mice	Karpus et al., 1995
		MBP-T cells, Lewis rats	Youssef et al., 1998
		PLP/CFA, (SWRxSJL/J)F1	Glabinski et al., 1997
		$MOG_{35-56+92-106}$/CFA, (C57BL/6xSJL/J)F1 mice	Asensio et al., 1999
		MBP-T cells, SJL/J mice	Rajan et al., 2000
		MOG_{35-56}/CFA, C57BL/6	Juedes et al., 2000
IP-10	Activated T cells (Th1>Th2)		
MIP-1α	T cells (Th1>Th2) Monocytes/macrophages		
MIP-1β	T cells (Th1>Th2) Monocytes/macrophages NK cells	MBP/CFA, Lewis rats	Miyagishi et al., 1997
		MBP-T cells, Lewis rats	Youssef et al., 1998
		$MOG_{35-56+92-106}$/CFA, (C57BL/6xSJL/J)F1 mice	Asensio et al., 1999
C10	Monocytes/macrophages	$MOG_{35-56+92-106}$/CFA, (C57BL/6xSJL/J)F1 mice	Asensio et al., 1999
		MBP/CFA, SWR/J mice	
Fractalkine	Activated T cells NK cells Monocytes	PLP/CFA, SJL/J mice	Pan et al., 1997
B. Receptors	**Established ligands**	**EAE models**	**References**
CCR2	MCP-1-5	MBP/CFA, Lewis rats	Jiang et al., 1998
		MBP-T cells, SJL/J mice	Rajan et al., 2000
CCR5	MIP-1α, MIP-1β and RANTES		
CX3CR1	Fractalkine	MBP/CFA, Lewis rats	Jiang et al., 1998

[1]The traditional nomenclature is used in this thesis. Zlotnik and Yoshie (2000) have recently proposed a new classification for chemokines and chemokine receptors.
[2]As described by Zlotnik et al. (1999).

relapses remains debatable (Godiska et al., 1995; Glabinski et al., 1997; Kennedy et al., 1998). Are chemokines expressed prior to the onset of clinical signs of EAE? Since chemokine mRNA's were not detectable prior to the onset of clinical or histopathological signs of murine PLP-induced EAE, it was suggested that chemokines serve to amplify rather than to initiate leukocyte recruitment into the CNS (Glabinski et al., 1995). However, other studies, using various EAE models (e.g., passive or active; MBP, PLP or MOG; mouse or rat) and various methods of detection for mRNA or proteins, all showed a small but consistent increase in chemokines at the preclinical stage 1-2 days before onset of clinical signs (Godiska et al., 1995; Berman et al., 1996; Adamus et al., 1997; Asensio et al., 1999; Youssef et al., 1999; Rajan et al., 2000). One explanation for these disparate findings may relate to the presence of clinically silent infiltrates in presymptomatic animals in latter studies but not in the former.

As shown in Table I, chemokines associated with the recruitment of mononuclear cells are up-regulated in EAE. The critical question is whether such over-expression is responsible for the pathogenesis of the disease. Thus far, studies have relied on the use of antibodies or naked DNA vaccines to block the action of individual chemokines, and have implicated MIP-1α, but not RANTES, as crucial in the development of acute EAE following adoptive transfer of myelin-reactive T cells into mice or rats (Karpus et al., 1995; Youssef et al., 1998). The role of MCP-1 in acute EAE has remained elusive (Karpus et al., 1995; Youssef et al., 1998). MCP-1, rather than MIP-1α, has nevertheless been shown to play an essential role in relapses of EAE (Kennedy et al., 1998). In contrast, the neutrophil chemoattractant MIP-2 is either not or barely detectable in most EAE models (Godiska et al., 1995; Karpus et al., 1995; Adamus et al., 1997; Asensio et al., 1999; Rajan et al., 2000). Blocking MIP-2 by antibodies did not affect the course of EAE, suggesting that MIP-2 is dispensable. Surprisingly, administration of MIP-1α naked DNA vaccines induced endogenous anti-MIP-1α antibodies and exacerbated EAE (Youssef et al., 1998). Taken together, these findings

suggest that chemokines, when over-expressed during inflammation, are not necessarily pathogenic.

Chemokines have various other functions that extend beyond the regulation of leukocyte trafficking (Asensio and Campbell, 1999b). Glial cells and neurons constitutively express various chemokine receptors, of which CCR5 and CXCR4 have been identified as co-receptors by which HIV infect the brain (Miller and Meucci, 1999). Activation of these receptors has been shown to promote glial and neuronal apoptosis *in vitro* (Miller and Meucci, 1999). The fact that CNS cells, especially glial cells, have multiple chemokine receptors raises questions regarding their responses to elevations of chemokines in pathological settings.

Recently, chemokines have been shown to modulate expression of metalloproteinases (MMP) and tissue inhibitors of metalloproteinases (TIMP). (Cross and Woodroofe, 1999). MMP's are a family of zinc-containing proteolytic enzymes that are involved in the degradation of proteins in the extracellular matrix (ECM). MMP activity is tightly controlled by specific TIMP's. The balance between MMP and TIMP secretion is perturbed in the CNS during EAE and MS (Yong et al., 1998b). MMP are thought to be needed for facilitating immune cell penetration and migration through the CNS ECM, and for the release of soluble tumor necrosis factor-alpha (TNFα) and adhesion molecules. Treatment with MMP inhibitors blocked degradation of the ECM, reduced T cell entry into the CNS and suppressed EAE (Liedtke et al., 1998a). One mechanism of beneficial action of IFNβ in MS may be the inactivation of MMP-9 activity of T lymphocytes (Stuve et al., 1996). MMP's also degrade myelin *in vitro* (Chandler et al., 1995), suggesting that they may contribute directly to myelin destruction in EAE or MS.

3.3. Mechanisms of tissue injury

In addition to MMP's, activated macrophages and microglia can phagocytose myelin, and release a variety of other potentially cytopathic mediators, such as superoxide and other free oxygen radicals (e.g., hydroxyl ions), complement components, and proteases (Sriram and Rodriguez, 1997; Benveniste, 1997). Autoantibodies directed against myelin proteins, MOG and MBP, are also involved in myelin damage (Van der Goes et al., 1999; Genain et al., 1999; Raine et al., 1999). Furthemore, nitric oxide (NO), when produced by inducible nitric oxide synthase, can destroy oligodendrocytes *in vitro* (Merrill et al., 1993).

3.3.1 Inducible nitric oxide synthase

Three isoforms of NO synthase (NOS) catalyse the production of NO: NOS1 or neuronal NOS (nNOS), NOS2 or inducible NOS (iNOS), and NOS3 or endothelial NOS (eNOS). Generally, nNOS and eNOS are both constitutively expressed and sustain low output of NO in response to elevation in the intracellular Ca^{+2} level (MacMicking et al., 1997). These constitutive nNOS and eNOS are involved in physiological functions, such as neurotransmission and vasodilation, respectively. In contrast, iNOS is primarily transcriptionally regulated. When expressed, it produces substantial amounts of NO for long periods of time and independently of the intracellular Ca^{+2} concentration (MacMicking et al., 1997). Modulation of iNOS at the level of mRNA and protein stability might provide a powerful mechanism for down-regulation of its activity (Nathan and Xie, 1994). iNOS mediates microbial cytotoxicity via the formation of multiple free radicals. NO can rapidly decompose to nitrate which, in the presence of superoxide, forms two other free radicals, peroxynitrite and hydroxyl ions. Uncontrolled over-production of iNOS can cause oxidative damage and protein nitration in the tissues.

iNOS, originally cloned from activated macrophages, is the most widely distributed isoform among cell types. Both *in vitro* and *in vivo* experiments indicate that numerous cytokines (e.g., IFNγ, TNFα, IL-1) and microbial products (e.g., lipopolysaccharide), acting alone or in synergy, can enhance expression of the iNOS gene (MacMicking et al., 1997). The interaction between CD40 on macrophages and CD40L on T-cells appears to serve as a co-stimulus to cytokines for iNOS induction (Stout et al., 1996). Other cytokines (e.g., IL-4, IL-10, IL-13 and transforming growth factor-beta1 (TGF-β1)) and steroids (e.g., glucocorticoids) have all been described to suppress iNOS expression and activity *in vitro* (MacMicking et al., 1997). The physiological importance of the down-regulating action of TGF-β1 is further suggested by the observation that TGF-β1-/- mice spontaneously express iNOS (Vodovotz et al., 1996).

In the brain, iNOS is normally not (Lowenstein et al., 1992) or is minimally (Keilhoff et al., 1996) expressed. Astrocytes have the highest intracellular concentration of the NO precursor L-arginine within the CNS (Aoki et al., 1991). These cells are therefore well equipped to induce iNOS in response to changes in the CNS microenvironment. Indeed, reactive astrocytes have been reported to express iNOS in transient global ischemia injury (Endoh et al., 1994), scrapies-infected mice (Ju et al., 1998), and in amyotrophic lateral sclerosis (Almer et al., 1999). *In vitro* activated microglia can produce sufficient NO to destroy oligodendrocytes and neurons (Merrill et al., 1993). Oligodendrocytes are more vulnerable to NO-mediated injury than are astrocytes or microglia (Mitrovic et al., 1994). NO has also been demonstrated to block conduction in axons *in vitro* and *in vivo* (Redford et al., 1997). Demyelinated axons are especially vulnerable to NO-mediated block, and this effect was observed at concentrations of NO anticipated at sites of inflammation (Redford et al., 1997). NO can also increase vascular permeability (Cho et al., 1999). *In Chapter II, I examined whether the cytokine change in the CNS during EAE induces glial production of NO which may affect demyelination and immune cell entry.*

3.4 Th1/Th2 cytokines: Peripheral and local regulators

Cytokines are small polypeptides that are usually secreted, and some can also be expressed as a biologically active form on a cell surface. Cytokines bind to specific receptors expressed on the surface of the target cell, thereby triggering complex intracellular signaling cascades, which ultimately turn on or off expression and activity of many genes, including cytokines themselves, chemokines, iNOS, adhesion molecules, and MMP. In many instances, the outcome of cytokine signaling can be shaped by negative or positive feedback from these molecules that cytokines induce. In EAE, alteration in cytokine production occurs in peripheral lymphoid organs and locally in the CNS (Owens et al., 1994; Merrill and Murphy, 1997; Okuda et al., 1998). Can cytokines circulating in the blood cross the intact BBB in enough amounts to affect CNS function? Although the means by which circulating cytokines communicate with the CNS is not yet clear, circulating levels of some cytokines, such as IL-1 and IL-6, are elevated in response to systemic injury or infection, and can affect CNS-controlled responses, such as fever and neuroendocrine changes.

T lymphocytes are critical in orchestrating an immune response, and are a major source of cytokines for activation of macrophages, glial and endothelial cells. The understanding of cytokine regulation in inflammatory diseases has been strikingly influenced by the Th1/Th2 paradigm. CD4+ T lymphocytes can be classified into two distinct subsets of effector cells. The Th1 subset of CD4+ T cells secretes cytokines (e.g., IFNγ, lymphotoxin alpha (LTα), TNFα), induces cell-mediated immune responses and activates macrophages (O'Garra et al., 1997). The Th2 subset of CD4+T cells produces IL-4, IL-5, IL-10, and IL-13, some of which help B cells to proliferate and differentiate. Th2 cytokines have also been implicated in allergic inflammation because of their ability to induce IgE secretion and activate mast cells and eosinophils (Romagnani, 1998). Individual cytokines can produce opposing effects depending upon levels and timing of their participation in the immune response. Cytokines

are also produced by cells other than lymphocytes, and can have different effects depending on cell types, and stages of cellular proliferation, maturation, and activation. To what extent Th1 and Th2 cells dominate in vivo responses is as yet unclear, but their ability to influence disease evolution by their production of high levels of cytokines seems clear.

3.4.1 The Th1 inducing cytokine IL-12 in EAE

IL-12 is composed of two covalently associated chains, a constitutive p35 and an inducible p40 subunit. IL-12 produced by activated APC is crucial for the development of Th1 responses in most experimental systems, leading secondarily to increased in IFNγ production and macrophage activation (Gately et al., 1998). Other known effects of IL-12 on T cell function include: T-cell growth and anti-apoptotic activities, suppression of cross-regulatory Th2 cytokines (e.g., IL-4 and IL-10), and lymphocyte trafficking (Gately et al., 1998). Intervention that favored the production of IL-12 was shown to convert quiescent MBP-specific T cells into effector cells capable of transferring or inducing EAE (Segal et al., 1997; Smith et al., 1997). IL-12 appears to be necessary to generate encephalitogenic effector Th1 cells and to enhance pathology in EAE, and this strongly supports a Th1 response in this disease (O'Garra et al., 1997; Leonard et al., 1995; Segal et al., 1998a).

Blocking the production of endogenous IL-12 has proven to be effective at preventing or ameliorating EAE. For example, when interaction between CD40 and CD40L, known to induce IL-12 production by APC (Balashov et al., 1997), was hindered by anti-CD40L blocking mAb (Constantinescu et al., 1999; Samoilova et al., 1997) or was absent in CD40L-/- mice (Grewal et al., 1996), EAE could not develop. Such ability of anti-CD40L mAb to prevent EAE was abolished by *in vivo* administration of IL-12 (Constantinescu et al., 1999). Many other therapies, such as IL-4, IL-10, IL-13 or IFNβ, that have been beneficial in EAE or MS have the common feature of suppressing IL-12 (Karp et al., 2000).

3.4.2 Are Th1 pro-inflammatory and Th2 anti-inflammatory in EAE?

Much evidence supports that Th1 cytokines are pathogenic while Th2 cytokines are protective in EAE and many other organ-specific autoimmune diseases (King and Sarvetnick, 1997; Merrill and Murphy, 1997; O'Garra et al., 1997; Feldmann and Maini, 1999). Immune deviation therapies that shift a Th1 to a Th2 response have been beneficial in both EAE and MS (Jewell et al., 1998; Ruuls and Sedgwick, 1998; Miller et al., 1998a; Miller et al., 1998a; Waisman et al., 1996; Brocke et al., 1996). Nevertheless, other evidence has emerged to caution that such a definition of Th1 cytokines as pathogenic and Th2 cytokines as regulatory in various models of autoimmune disease is perhaps not as defined as originally proposed. Lack of Th2 cytokine up-regulation in the CNS during natural recovery of EAE has recently been reported (Di Rosa et al., 1998). IL-10 was also found to worsen EAE (Cannella et al., 1996), although numerous studies indicated a protective function for IL-10 in the disease (Mathisen et al., 1997; Bettelli et al., 1998; Croxford et al., 1998; Cua et al., 1999; Samoilova et al., 1998; Xiao et al., 1998). The Th2 cytokine IL-4 exacerbated EAE or played no down-regulatory role (Libleau et al., 1997; Nagelkerken et al., 1997; Zhao et al., 1998; Croxford et al., 1998; Segal et al., 1998b;), although it may also protect from EAE (Shaw et al., 1997; Croxford et al., 1998; Dal Canto et al., 1998; Falcone et al., 1998; Furlan et al., 1998; Piccirillo and Prud'homme, 1999). Immune deviation therapy was found to increase production of pathogenic autoantibodies and exacerbated EAE in marmosets (Genain et al., 1996). Furthermore, Th2 cells can transfer autoimmune disease in immunocompromised hosts (Lafaille et al., 1997; Pakala et al., 1997). Surprisingly, if high enough numbers were transferred, neuroantigen-specific Th2 cells could induce EAE even in immunocompetent SJL/J mice (Khoruts et al., 1995). Target tissues of such Th2-induced autommunity were, however, characterized predominantly by neutrophilic and eosinophilic infiltration (Khoruts et al., 1995; Lafaille et al., 1997; Pakala et al., 1997), a feature reminiscent of allergic inflammation.

The involvement of individual Th1 cytokines, such as IFNγ, TNFα or LTα, in EAE has been extensively studied. Nevertheless, the precise role of each Th1 cytokine in the initiation or progression of disease has remained debatable. For example, TNFα or LTα blockade using antibodies or soluble receptors prevented or ameliorated disease (Ruddle et al., 1990; Korner et al., 1997), although studies using mice with gene-targeted deletion have yielded conflicting conclusions (Suen et al., 1997; Korner et al., 1997b; Liu et al., 1998; Riminton et al., 1998; Eugster et al., 1999). However, high-level production of IFNγ is the most popular criterion to categorize a response as Th1-type.

3.4.3 The prototypic Th1 cytokine IFNγ in EAE

IFNγ acts on cells that possess the IFNγR by inducing increased expression of several genes, including those encoding for MHC, co-stimulatory molecules B7, cytokines (e.g., IL-1, IL-12, TNFα), chemokines and adhesion molecules (Boehm et al., 1997; Bach et al., 1997). Receptors for IFNγ are present on virtually all cells of the body. Therefore, many organs and systems can respond to IFNγ.

Evidence for the pro-inflammatory role of IFNγ in the CNS includes: (1) CNS-specific overexpression of IFNγ in transgenic mice promoted or enhanced microglial reactivity and demyelination (Corbin et al., 1996; Horwitz et al., 1997; Renno et al., 1998); (2) IFNγ production by infiltrating T cells in the CNS correlated with EAE development (Olsson, 1992); (3) IFNγ-secreting T cells isolated from animals experiencing EAE transferred the disease to naïve recipients (Zamvil and Steinman, 1990); (4) the CNS of mice with severe EAE contained IFNγ mRNA at higher levels than those in mice with milder disease (Renno et al., 1994); (5) many pathological changes observed in MS and EAE, such as increased MHC II expression, macrophage activation, and reactive gliosis are consistent with the known effects of IFNγ (Olsson, 1992; Balasingam et al., 1994); (6) systemic administration of IFNγ worsened MS (Panitch et al., 1987). Furthermore, IL-18, an IFNγ-inducing

cytokine, was highly transcribed in the brain at the onset and during EAE (Wildbaum et al., 1998). Blocking the action of IL-18 by antibodies suppressed the production of IFNγ and TNFα, and ameliorated EAE (Wildbaum et al., 1998).

Yet, evidence for the anti-inflammatory role of IFNγ in EAE also exists. Injection of a non-coding plasmid DNA (a plasmid vector carrying no insert) induced IFNγ production in both peripheral lymph nodes and the CNS and suppressed EAE in Lewis rats (Boccaccio et al., 1999). IFNγ, given systemically or intraventricularly, suppressed EAE (Billiau et al., 1988; Voorthuis et al., 1990). Conversely, systemic administration of anti-IFNγ antibodies had no effect, or even enhanced EAE (Billiau et al., 1988; Voorthuis et al., 1990; Duong et al., 1992; Heremans et al., 1996). Mice deficient in either IFNγ or its receptor developed EAE with high mortality following immunization with MBP or MOG (Krakowski and Owens, 1996; Ferber et al., 1996; Willenborg et al., 1996; Zhang et al., 1997; Segal et al., 1998b). More importantly, targeted disruption of the IFNγ or its receptor gene, or systemic administration of anti-IFNγ mAb in otherwise resistant mice rendered them highly susceptible to EAE (Billiau et al., 1988; Krakowski and Owens, 1996; Willenborg et al., 1996). Furthermore, MOG-reactive T cells isolated from IFNγR-/- mice with EAE produced high levels of IFNγ and TNFα but no IL-4 in a recall response to MOG *in vitro* (Willenborg et al., 1996). Upon transfer into IFNγ-unresponsive recipient mice, these cells induced lethal EAE. In contrast, transfer of these cells into IFNγ-responsive wild-type recipients induced EAE from which the animals recovered. Recent studies using bone marrow chimeras indicate that it is the IFNγ response from not only the hematopoeitically derived cells, but also from the CNS parenchymal resident cells, that down-regulate a T-cell mediated autoimmune response (Willenborg et al., 1999a). Collectively, this set of data implies that IFNγ down-modulates EAE not only systemically, but also locally in the CNS.

It is difficult to reconcile the contrasting observations on the role of IFNγ in EAE. Factors such as the concentration, the duration of IFNγ, the type of target cell populations involved, and the simultaneous presence of molecules such as other cytokines and chemokines, may influence the outcome of an IFNγ response.

3.5 Interrelationships between Th1/Th2 cytokines and chemokines

Chemokines can influence the outcome of an immune response by altering the profile of cytokines that are produced. CC chemokines have the capacity to drive differentiation of T lymphocytes toward a Th1 or Th2 response (Kennedy and Karpus, 1999). In particular, MCP-1 can drive Th2 differentiation, whereas MIP-1α appears to promote a Th1-type response by enhancing IFNγ and decreasing IL-4 production (Karpus and Kennedy, 1997). To what extent chemokine modulation of Th1/Th2 differentiation affects the development of EAE is uncertain.

Th1 and Th2 cells can be distinguished by their differential expression of chemokine receptors. Th1 cells express CCR5 and CXCR3 whereas Th2 cells express CCR3, CCR4 and CCR8 (Sallusto et al., 1998; Syrbe et al., 1999). Despite the redundancy in the activities of both chemokines and their receptors, several chemokines can exert selective effects on subsets of CD4+ T cells and other leukocytes. Th1 and Th2 cytokines may induce distinctive profiles of chemokines that determine the cellular composition and activation of infiltrates in inflammation (O'Garra et al., 1998; Bradley et al., 1999).

IFNγ is the prototypic Th1 cytokine, and myelin-reactive CD4+ T cells secreting IFNγ are encephalitogenic (Zamvil and Steinman, 1990). Surprisingly, CD4+ T cells that do not secrete IFNγ induce an even more severe EAE in mice lacking IFNγ (Ferber et al., 1996; Krakowski and Owens, 1996; Zhang et al., 1997; Segal et al., 1998b). Myelin-reactive CD4+ T cells secreting the Th2 cytokines IL-4 and IL-10 do not normally induce EAE, but

become encephalitogenic under some conditions (Khoruts et al., 1995; Lafaille et al., 1997). *How the interplay between cytokines and chemokines may dynamically direct the formation of distinctive inflammatory infiltrates and the clinical outcome of EAE will be addressed in Chapter IV. The availability of mice deficient in the Th1-associated chemokine MIP-1α and its CCR5 receptor has allowed me to assess their roles in the induction of EAE in Chapter V.*

CHAPTER II

Astrocytes and microglia express inducible nitric oxide synthase in experimental autoimmune encephalomyelitis

Abstract

Nitric oxide (NO), produced by inducible NO synthase (iNOS), may play a role in inflammatory demyelinating diseases of the central nervous system (CNS). We show up-regulation of iNOS mRNA in CNS of SJL/J mice with experimental allergic encephalomyelitis (EAE). Using antibodies against mouse iNOS, GFAP (a marker for astrocytes) and Mac-1/CD11b (a marker for macrophages/microglia), both astrocytes and macrophages/microglia were identified as iNOS-expressing cells *in situ* in EAE lesions. $GFAP^+$ astrocytes not associated with inflammatory infiltrates were also found to express iNOS. Because microglia rather than astrocytes are implicated in demyelinating pathology, we propose that microglial NO may be cytopathic whereas astrocyte-derived NO may be protective in EAE.

Introduction

Experimental allergic encephalomyelitis (EAE) is an autoimmune inflammatory disease of the central nervous system (CNS) that can be induced by immunization with myelin proteins (e.g., myelin basic protein, MBP) or by adoptive transfer of myelin-reactive CD4+ T cells. In mice, the relapsing-remitting progression and pathology of EAE resemble those of the human disorder multiple sclerosis (MS) (Raine, 1984; Raine et al., 1984). Like MS, EAE is characterized by blood-brain barrier breakdown, infiltration of the CNS by active CD4+ T cells and macrophages, activation of adjacent microglial and astroglial cells, and demyelination (Raine, 1984; McFarlin et al., 1982). Although precise pathogenetic mechanisms underlying EAE are still not fully understood, complex interactions between infiltrating and resident cells of the CNS are thought to result in the formation and release of mediators that contribute to tissue damage.

The free radical nitric oxide (NO) is implicated in neurotransmission, vasorelaxation and host defense against microbial infections of neural tissues (Paakkari and Lindsberg, 1995; Bi et al.,1995; Schmidt and Walter, 1994). NO has been suggested to mediate tissue injury in demyelinating diseases. Activated glial cells have been shown *in vitro* to express the enzyme inducible NO synthase (iNOS or type 2 NOS) that catalyzes a high output of NO to which cultured neurons and oligodendrocytes are vulnerable (Skaper et al., 1995; Dawson et al., 1994; Merrill et al., 1993; Boje and Arora, 1992; Chao et al., 1992). Leukocytes isolated from the CNS during hyperacute EAE secrete substantially increased amounts of reactive nitrogen intermediates (MacMicking et al., 1992). Furthermore, induction of iNOS mRNA occurs in spinal cord of mice (Okuda et al., 1995), in brain of rats (Koprowski et al., 1993) with EAE and in MS brain (Bö et al., 1994). In mice, this expression of iNOS mRNA correlates with severity of EAE (Okuda et al., 1995) and levels of NO rise significantly in spinal cord during an episode of EAE (Lin et al., 1993). Consistent with this, a selective inhibitor of iNOS can prevent adoptive transfer of EAE in mice (Cross et al., 1994).

There has been much interest in discerning the primary cell type(s) responsible for such *in vivo* overproduction. Astrocytes are the major glial population in the CNS. In both MS and EAE lesions, astrocytic activation is prominent. In MS lesions, astrocytes have been suggested as the main source of excess NO (Bö et al., 1994; Brosnan et al., 1994). Yet, comparative immunohistochemical analyses of adjacent tissue sections showed localization of the iNOS protein to be restricted to invading macrophages and/or activated microglial cells within inflammatory infiltrates in brain or spinal cord of rodents with EAE (Ruuls et al., 1996; Van Dam et al., 1995; Okuda et al., 1995). This apparent divergence in the identity of cellular sources of a potential cytopathic mediator, NO, between MS and EAE, could reflect different pathogenetic mechanisms underlying the two diseases.

We report here not only that astrocytes express iNOS in SJL/J mice with EAE, but that that astrocytes distal from the inflammatory infiltrates do so, as well. These findings may offer new insights into intercellular communication in the CNS, as well as resolving an apparent paradox.

Materials and methods

Induction of EAE and tissue collection

Animal maintenance and experimental protocols were in accordance with guidelines set forth by the McGill University Animal Care Committee. Pathogen-free 6-8-week-old female SJL/J mice (Harlan-Sprague Dawley) were immunized s.c. at the base of tail and boosted s.c. in the flanks 7 days later with 400 μg of bovine MBP (prepared as described by Cheifetz et al.,1984) emulsified in complete Freund's adjuvant containing 50 μg of *Mycobacterium tuberculosis* H37 Ra (Difco). Symptoms were first observed 14 days after the initial immunization. Mice with severe EAE (loss of tail tone, and partial or complete hind limb paralysis corresponding to grades 3 to 4 as defined in Renno et al. 1995) were anesthetized prior to intracardial perfusion with ice-cold sterile phosphate-buffered saline. Brains and spinal cords were rapidly removed, frozen in liquid nitrogen for RNA extraction, or snap-frozen in 2-methylbutane in liquid nitrogen for immunohistochemistry.

ANA-1 cell culture and stimulation

The murine macrophage cell line ANA-1 (Blasi et al., 1985), was maintained (37°C, 5% CO_2) with RPMI 1640 supplemented with 10% fetal bovine serum (ICN Biochemicals), 50 mM 2-Mercaptoethanol (Sigma), 2mM L-glutamine (Gibco), 100 U/ml penicilin (Gibco), and 100 mg/ml streptomycin (Gibco). ANA-1 cells ($1x10^6$/ml) were sitmulated with 5μg/ml LPS (from *Escherichia coli* 011:B4, Sigma) and 10 U/ml IFNγ (contained in supernatant from activated T cell line E9D4 [Owens, 1988]) for 24 h. ANA-1 has been previously demonstrated to be capable of expressing iNOS mRNA, protein and secreting NO after exposure to cytokines and microbial products (Sheffler et al., 1995; Melillo et al., 1993). We, therefore, used stimulated ANA-1 as a positive control in our RT-PCR analysis.

Isolation of RNA

Total RNA was purified from homogenized CNS or from ANA-1 cells using TriZOL RNA isolation Reagent (Gibco) according to the manufacturer's protocol.

Preparation of cDNA probes and Northern blot analysis

The iNOS probe used was a 1.4 Kb NotI-StuI fragment from plasmid CL-BS-macNOS containing a mouse iNOS cDNA insert (Lowenstein et al., 1992). The plasmids were kindly provided by Dr. Kris Chadee (McGill, Montreal). iNOS and β-actin cDNA probes were labelled with α^{32}PdCTP (Dupont) using a random priming kit (Strategene).

40 μg of RNA was electrophoresed in a 1.2% agarose gel containing 18% formaldehyde, blotted onto a Hybond-N membrane (Amersham), cross-linked by UV irradiation, and hybridized with ^{32}P-labelled mouse iNOS or β-actin cDNA probes overnight at 42°C in 50% formamide, 1M NaCl, 1% SDS and 10% dextran sulphate. After hybridization, the membrane was washed in 1X SSC (twice 15 min) and in 0.1X SSC/0.1% SDS (twice 15 min) at room temperature, finally once for 40 min at 65°C. The membrane was exposed overnight against a PhosphorImager screen (Molecular Dynamics) and analyzed by a PhosphorImager computing system (Molecular Dynamics).

RT-PCR

RNA (1 μg) was reverse-transcribed using Superscript Pre-amplification system (Gibco). PCR conditions used were previously optimized for linear amplification to allow direct comparison between samples. Equal amounts of the cDNA products were amplified using 2.5 U Taq DNA polymerase (Gibco), 10 mM of each dNTP, 50 pmol of each primers and a 10X PCR buffer mixture containing 500 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$ and 0.1% gelatin. iNOS primers were selected from the cDNA sequence of murine macrophage iNOS (Xie et al., 1992; Lyons et al., 1992) as follows: sense, 5'-TGCTCCCTTCCGAAGTTTCTGGCAGCAGCG-3'; antisense, 5'-CTGTCAGAGCCTC

GTGGCTTTGGGCTCCTC-3'. The primers for β-actin as an internal control were:sense, 5'-TGGGTCAGAAGGACTCCTATC-3'; antisense, 5'CAGGCAGCTCATAGCTCTTCT-3' (Renno et al., 1995). PCR was performed in a Perkin-Elmer Cetus Thermal cycler for 30 cycles (denaturation 30 sec 94°C, annealing 30 sec 60°C, extension 30 sec 72°C). PCR products (500bp for iNOS and 650bp for β-actin) were separated on a 1% agarose gel and visualized by ethidium bromide staining.

Immunohistochemistry

Frozen sagittal sections (10 μm) of brain or transverse sections of spinal cord were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min, rinsed in Tris-buffered saline (TBS) pH 7.5, treated with 0.3% H_2O_2/TBS for 20 min, then incubated with 2% fetal bovine serum for 30 min.

For immunoperoxidase single-labeling, sections were incubated with anti-mouse iNOS (1:500, rabbit polyclonal, Transduction laboratories), anti-Mac-1/CD11b (1:10, rat monoclonal, Boehringer Mannheim) or anti-GFAP (1:100, rabbit polyclonal, DAKO) overnight at 4°C. Antibody binding was revealed by the biotin-avidin-peroxidase method using the ABC kit (Vectastain) and AEC substrate (Dimension laboratories) as chromagen. Sections were counterstained in Hematoxylin, then mounted in aqueous mounting medium (Lipshaw Immunon) and examined under a light microscope. Control sections were incubated with normal rabbit IgG or in TBS without primary antibodies.

For immunofluorescent double-labeling, sections were incubated first with anti-iNOS (1:500) then with anti-GFAP (1:100, mouse monoclonal, Boehringer Mannheim). Anti-iNOS was visualized using biotinylated anti-rabbit IgG antibody (1:200, Vectastain) and streptavidin-Texas Red (1:1000, DAKO) while anti-GFAP by anti-mouse IgG-FITC (1:100,

DAKO). Sections were mounted with a drop of aqueous mounting medium (Shandon) and analyzed by confocal microscopy imaging.

Results

iNOS gene expression in CNS during EAE

We analyzed the brains and spinal cords of SJL/J mice by Northern blot and RT-PCR to confirm iNOS gene expression in EAE. As shown in Fig. 1A, up-regulation of iNOS mRNA occurred at levels detectable by Northern blot in EAE CNS, as compared to normal controls. Indeed, EAE brains or spinal cords contained four- to fivefold (after normalization with β-actin signal) increases in iNOS transcripts. That expression was low in normal tissues was suggested by use of a more sensitive assay, RT-PCR (Fig. 1B).

Distribution and identification of cells expressing iNOS in CNS during EAE

Demonstration of the production of iNOS protein in the CNS in EAE, and determination of its distribution and cellular localization was obtained by immunostaining. At least 3 mice were examined for each treatment and staining.

In sagittal sections of mouse brains with EAE examined at low magnification, strong iNOS immunoreactivity was observed within inflammatory lesions in the white matter of the cerebellum (Fig. 2A and B) and of the brainstem (data not shown). By contrast, iNOS immunostaining was not apparent in CNS tissue sections from normal mice (data not shown), nor was there any staining of control sections that were incubated with normal rabbit IgG (Fig. 2D) or without primary antibodies (data not shown). The possibility that the anti-iNOS antibody which we used may cross-react with neuronal NOS (type 1 NOS) was excluded by the fact that neuronal-NOS enriched regions such as the molecular layer of the cerebellum (Bredt et al., 1990a; 1990b) were not immunoreactive in our staining of normal mouse brain (data not shown).

Examination of perivascular infiltrates at higher magnification permitted cellular localization of iNOS. In Fig. 2 (C and E), two morphologically different cell types in cerebellar white

matter were intensely stained for iNOS: (1) large round- to oval-shaped cells and (2) proccess-bearing cells. On the basis of their morphology, these iNOS positive cell types likely represent macrophages/activated microglia, and astrocytes. To verify this, adjacent tissue sections were stained for iNOS or the prototypic markers for macrophages/microglia (Mac-1/CD11b) or for astrocytes (GFAP). Both of these markers have elevated levels of staining in activated compared to quiescent cells. Comparison between Fig. 2E and F reveals that most iNOS+ cells, having a round to oval appearance, corresponded in adjacent sections to cells of identical morphology that were stained by Mac-1/CD11b (i.e., invading macrophages and/or activated microglia). The iNOS+ star-shaped cells were most likely astrocytes since there was correspondence between iNOS and GFAP staining of cells with this morphology in adjacent sections (Fig. 2E vs G: arrows).

Similar to the findings in brain, enhanced iNOS immunoreactivity in transverse sections of spinal cords from mice with EAE occurred within perivascular (Fig. 3A and D) and subpial (Fig. 3E) infiltrates in the white matter. Again, the same two morphological subpopulations of cells expressing iNOS were detected: stellate-shaped cells (Fig. 3E: arrow) and round/oval cells (Fig. 3D and E: arrowheads) that were morphologically reminiscent of Mac-1/CD11b+ cells (Fig. 3G). Unlike Mac-1/CD11b staining (Fig. 3B), iNOS immunoreactivity was not confined to inflammatory cuffs (Fig. 3A); some cells at varying distances from infiltrates were also found to express iNOS (Fig. 3A, curved arrows). These isolated iNOS+ parenchymal cells also corresponded in adjacent sections to cells stained by GFAP (Fig. 3A vs C). When viewed at higher magnification (Fig. 3D and F), these iNOS+ cells that were not in association with infiltrated vessels, resembled astrocytes.

Co-localization of iNOS with GFAP in reactive astrocytes in EAE

To confirm astrocytic localization of iNOS in EAE, immunofluorescent double-labeling for iNOS and GFAP was performed. Several cells doubly labeled for iNOS and GFAP were

evident in spinal cord sections of mice with EAE. Examples of such double-labelled cells are shown in Fig. 4. Fig. 4A shows numerous red fluorescent (iNOS+) cells in an infiltrate, in which astrocytes (arrows) as identified with a green fluorescence indicating GFAP staining (Fig. 4B) were also found. When the two colors were superimposed, doubly labelled cells appeared yellow (Fig. 4C and D), indicating iNOS-expressing astrocytes.

This definitively establishes that iNOS+ cells, which corresponded in adjacent sections to cells with astrocytic morphology, were indeed astrocytes. The identity of reactive astrocytes in EAE tissue sections was therefore established by their characteristic morphology (larger cell body and thickened stellar processes) and increased GFAP staining, compared to the morphology of astrocytes, which stain with GFAP in normal CNS tissue sections.

Discussion

We have examined expression of iNOS mRNA and protein in SJL/J mice with EAE. We have confirmed expression of iNOS mRNA in both the brain and spinal cord during an episode of EAE, and we have unequivocally localized enhanced iNOS expression to reactive astrocytes (as judged by their characteristic morphology and increased GFAP immunoreactivity) in EAE CNS. Interestingly, we find astrocytes expressing iNOS not only in association with inflammatory lesions, but also in the parenchyma at varying distances from the lesions. This is the first report to demonstrate the ability of adult astrocytes to express iNOS *in situ* in EAE, and identifies astrocytes as being at least partially responsible for the release of excess NO during EAE.

Others have failed to detect astrocytic iNOS in rats (Ruuls et al., 1996;Van Dam et al., 1995) or in mice (Okuda et al., 1995) with EAE and have concluded that iNOS is restricted to infiltrating macrophages and activated microglia. However, astrocytes have been shown to be the primary source of NO in MS brains (Bö et al., 1994; Brosnan et al., 1994). The discrepancy between previous results and our findings in EAE may relate to the use by others of a more intense fixation and/or of antibodies directed against iNOS of one species to detect iNOS in tissues of another species.

As iNOS is not normally detected in CNS tissues, the nature of the stimulus that induces astrocytes to express iNOS in EAE must then be considered. *In vitro*, inflammatory cytokines (e.g., TNFα, IL-1, IFNγ) can synergize in stimulating astrocytes to express iNOS and to release high titers of NO into the medium (Skaper et al., 1995; Brosnan et al., 1994; Galea et al., 1994; Lee et al., 1993; Simmons and Murphy, 1992). Production of these cytokines is up-regulated in EAE (Owens et al., 1994) and is preferentially confined to lesioned sites (Villarroya et al., 1996; Merrill et al., 1992; Baker et al., 1991). The recent demonstration that levels of inflammatory cytokine mRNA correlate temporally with

expression of iNOS mRNA (Okuda et al., 1995) further suggests a relationship between cytokines and iNOS gene expression *in vivo*. Moreover, Hewett et al. (1996) have shown that conditioned medium from MBP-sensitized lymphoid cells can induce iNOS expression in cultured murine astrocytes. It is likely that inflammatory cytokines act as potent transcriptional inducers of iNOS expression in adjacent astrocytes *in vivo*.

However, the observation that astrocytes distal from inflammatory infiltrates also expressed iNOS is surprising. Although not previously detected (Villarroya et al., 1996; Merrill et al., 1992; Baker et al., 1991), cytokines might diffuse away from inflammatory infiltrates and activate iNOS expression in distal astrocytes. If so, a gradient of iNOS staining intensity (i.e. the farther the astrocytes are away from the infiltrates the more weakly they stain) would be expected. Such a gradient was not observed in our staining, making it unlikely that diffusing cytokines directly stimulate iNOS expression in distal astrocytes. Alternatively, astrocytes are known to form a network via gap junctions through which information can flow (Jensen et al., 1993), so that cytokine-mediated activation of astrocytes that are proximal to infiltrates could result in induction of iNOS in distal astrocytes. The fact that not all astrocytes are interconnected (Jensen et al., 1993) could explain the fact that iNOS was only induced in a subpopulation of parenchymal astrocytes. Moreover, considerable *in vitro* and *in vivo* evidence supports functional heterogeneity of astrocytes (Wilkin et al., 1990); this may extend to enzyme expression.

The functional consequences of NO production via astrocytic iNOS in EAE are a matter of speculation. With their end-feet ensheathing microvessels, astrocytes contribute to the integrity of the blood-brain barrier and seem strategically well-positioned to influence the entry of hematogenous cells into the CNS. NO has been implicated in both regulation of cerebral blood flow and in causing cell death (Moncada et al., 1991; Nathan and Xie, 1994). There is a potential role for astrocyte-derived NO in the blood-brain barrier leakage that

occurs in both MS and EAE. For instance, high levels of NO, released within an infiltrate, could disrupt the permeability of the infiltrated vessel, thereby contributing to edema and promoting recruitment of further inflammatory mononuclear cells into the parenchyma. Inhibition of such destructive effect would prevent EAE (Cross et al., 1994).

On the other hand, our finding of iNOS+ astrocytes not associated with infiltrated vessels suggests an alternative role for NO. NO has been shown to inhibit adhesion of leukocytes to activated endothelium (Kubes et al., 1991; Moncada et al., 1991) and may exert anti-inflammatory activities. Such a beneficial effect of NO is consistent with the findings that selective iNOS inhibitors worsened EAE in rats (Ruuls et al., 1996; Zielasek et al., 1996). Effects of NO are numerous (Nathan and Xie, 1994; Moncada et al., 1991), and whether it is detrimental or protective presumably depends on a multitude of factors, such as the cellular source, amount produced, and prevailing conditions (e.g. redox status at the sites of productions) (Lipton et al.,1993). Therefore, interference with its production by selective iNOS inhibitors can either ameliorate (Cross et al., 1994) or aggravate (Ruuls et al., 1996; Zielasek et al., 1996) the disease.

Macrophages/microglia presumably play a cytopathic role in both MS and EAE (Merrill et al., 1993). In contrast, astrocytes have not been implicated in demyelinating pathology (Mucke and Eddleston, 1993). Instead, evidence suggest that astrocytes are likely to play a crucial role in providing support to neurons and other CNS resident cells through the production of, for example, various growth factors in response to injuries (Mucke and Eddleston, 1993; Louis et al., 1993; Barres et al., 1992; Franklin et al., 1991). Yet, astrocytes appear to produce the majority of NO in MS (Bö et al., 1994; Brosnan et al., 1994). The dichotomy between microglia and astrocytes with respect to their involvement in demyelinating pathology suggests that astrocyte-derived NO might be protective, whereas microglial NO is involved in cytotoxicity. Resolving the issue of whether astrocytic NO has

cytopathic effects or not in MS will be important for understanding demyelinating pathology and for designing therapeutic intervention against this disease. Our finding that astrocytes are also a source of NO in EAE, further supports the similarities between EAE and MS, and hence reinforces EAE as a useful model for such purposes.

Acknowledgements

We thank Lawrence Lai for contributions to the initiation of this project and Michelle Krakowski for review of this manuscript. We are also grateful to Dr. Wee Yong for excellent advice in histology, Fiona Yong for her expertise on confocal microscope imaging, and Grace Chan for MBP preparation.

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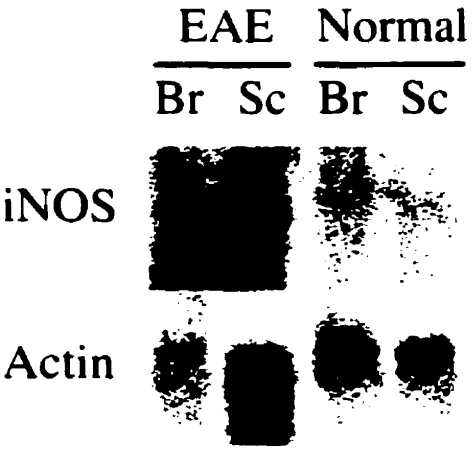
Fig. 1. Up-regulation of iNOS mRNA in the CNS of SJL/J mice with grade 3-4 EAE at day 16 post-immunization. **[A]** Northern blot analysis of RNA from brain (Br) and spinal cord (Sc) of normal controls or mice with EAE. **[B]** RT-PCR analysis of RNA from spinal cord of normal (N) controls or mice with EAE. Positive control: murine macrophage cell line ANA-1 activated with LPS (5μg/ml) and IFNγ (10 U/ml) for 24 h. Negative control: unstimulated ANA-1.

A



B

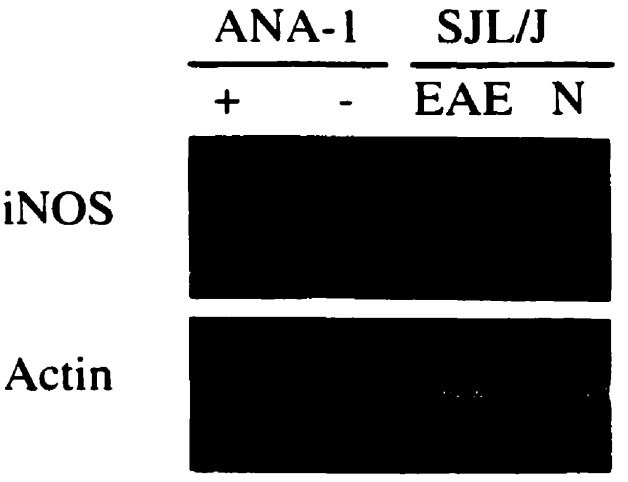


Fig. 2. Cellular localization of iNOS, Mac-1/CD11b and GFAP in perivascular infiltrates within cerebellar white matter of mice with grade 4 EAE at day 16 post-immunization. Stainings were as follows: (**A,B,C and E**) anti-iNOS, (**D**) normal rabbit IgG, (**F**) anti-Mac-1/CD11b, (**G**) anti-GFAP. **A,B**: original magnification, 90x. **C,E**: high magnification (900x) of the area contained in the frame in **A** and **B** respectively. **D-G**: adjacent sections. Arrowheads: round/oval iNOS$^+$ cells, morphologically reminiscent of Mac-1/CD11b$^+$ macrophages/microglia. Arrows: iNOS$^+$ process-bearing cells resembling GFAP$^+$ astrocytes. (*): lumen of an infiltrated vessel.

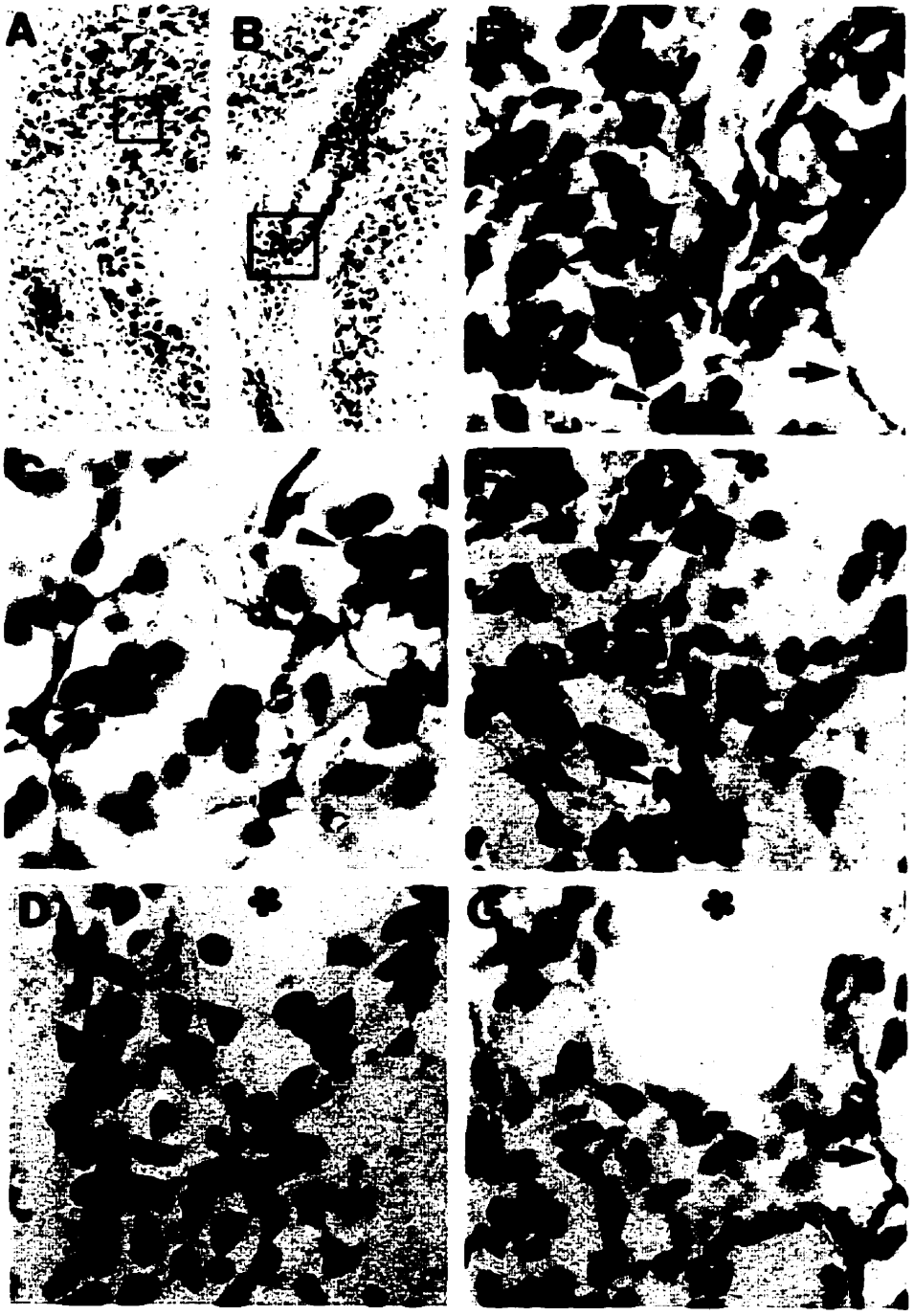

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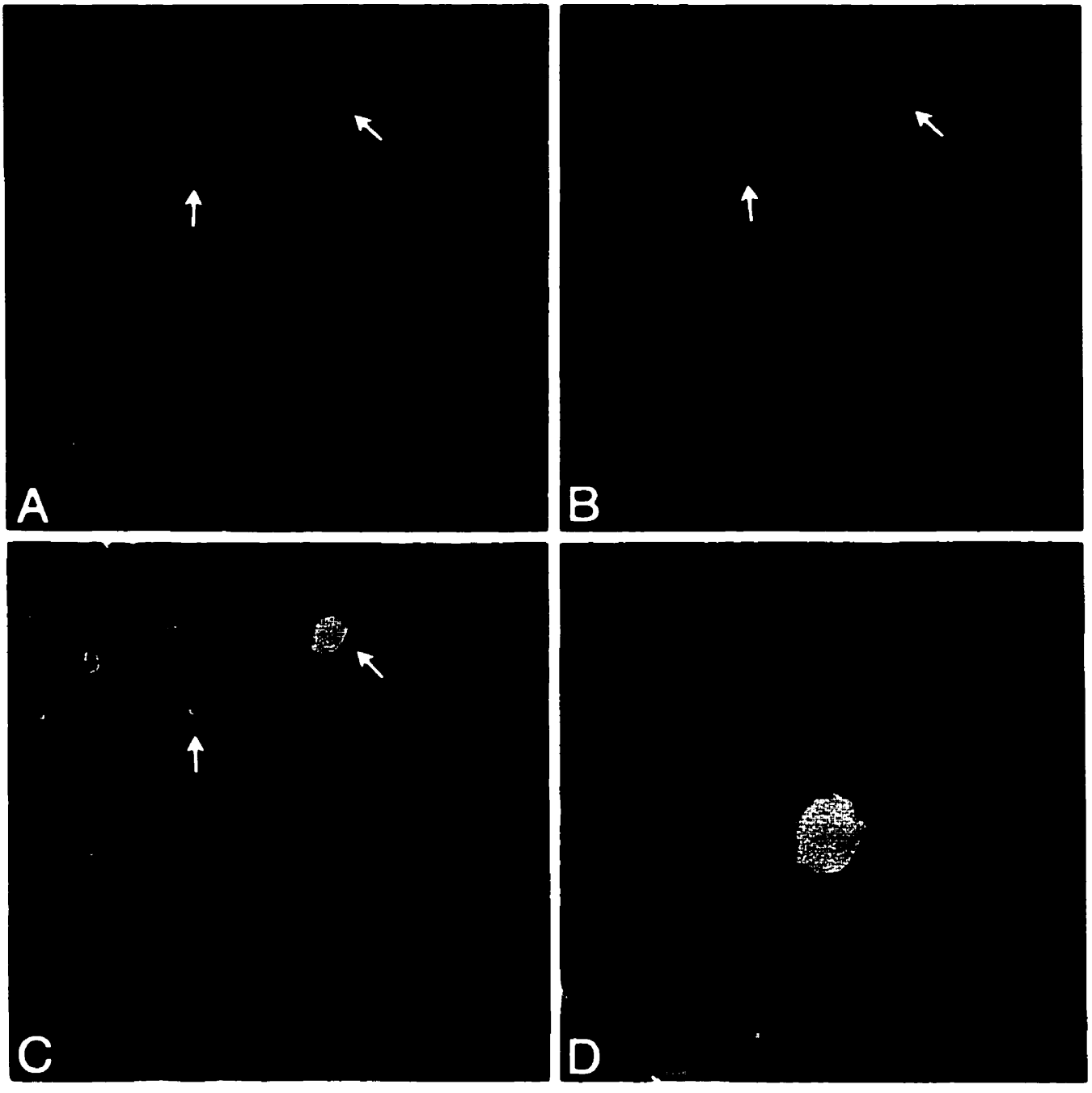
Fig. 3. Cellular localization of iNOS, Mac-1/CD11b, and GFAP in the lumbar spinal cord of mice with grade 4 EAE at day 16 post-immunization. Stainings were as follows: (**A,D-F**) anti-iNOS, (**B,G**) anti-Mac-1/CD11b, and (**C**) anti-GFAP. **A-C**: adjacent sections. Original magnification: (**A-C**) 90x, (**D-G**) 900x. **A:** shows iNOS immunoreactivity in inflammatory cuffs (e.g., black frame) and in parenchymal cells (curved arrows). **B**: Mac-1/CD11b$^+$ cells were preferentially localized within infiltrates (black frame). **C**: GFAP immunoreactivity was not restricted to sites of inflammation (black frame) but was seen throughout the white matter (curved arrows); with a pattern similar to iNOS staining (**A**). Perivascular infiltrate (**D**) and parenchymal astrocyte-like cells (arrows) containing iNOS. **E**: subpial infiltrate in which astrocyte-like cells (arrow) and macrophage/microglia-like cells (arrowheads; compared to cells in **G**) expressed iNOS. **G**: Mac-1/CD11b$^+$ cells in the subpial infiltrate adjacent to **E**.

Fig. 4. Confocal microscope images of a section from spinal cord of mice with grade 4 EAE at day 16 post-immunization, stained with antibodies to iNOS (red) and GFAP (green). The images (**A, B, C**: original magnification, 700x) were obtained from the same field: each individual staining, iNOS (**A**), GFAP (**B**), and their overlap (**C**). In **C**, iNOS-expressing astrocytes (doubly stained) appear in yellow (arrows). **D**: high magnification (1200x) of an $iNOS^+$ reactive astrocyte in **C**. The overall pattern was reproduced in 2 separate experiments.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CHAPTER III

Immune invasion of the central nervous system parenchyma and experimental autoimmune encephalomyelitis, but not leukocyte extravasation from blood, was prevented in macrophage-depleted mice

Preface

In Chapter II, I demonstrated that infiltrating macrophages, resident microglia and astrocytes expressed iNOS in inflammatory foci during EAE. Surprisingly, some astrocytes, but not microglia, that were distant from infiltrates, also expressed iNOS, suggesting that astrocytes perhaps through their foot processes that surround blood vessels may down-regulate further leukocyte entry via release of iNOS-derived NO. In this chapter, I investigate the role of macrophages in lesion formation and in the regulation of glial iNOS expression within the CNS parenchyma.

Note added in proof:

The following chapter includes description of cells expressing B220 in perivascular locations in macrophage-depleted mice. These were assumed to be B lymphocytes. Subsequent to publication of this work, immunohistochemical staining showed absence of CD19+ expression on these cells. CD19 is a B-cell lineage differentiation antigen. This calls into question the B–cell nature of these B220+ cells. Their definitive identification remains uncertain.

Abstract

Organ-specific autoimmune diseases are characterized by infiltrates including T lymphocytes and activated macrophages. Macrophages and secondarily activated tissue resident counterparts can both present antigen to and contribute to cytokine secretion by T lymphocytes. We have previously shown a crucial role of peripheral macrophages in experimental allergic encephalomyelitis (EAE), a Th1-mediated demyelinating disease that serves as an animal model for multiple sclerosis (MS), by their depletion using mannosylated liposome-encapsulated clodronate or dichloromethylene diphosphonate (Cl_2MDP). Here we describe studies to investigate the mechanisms by which macrophages contribute to the lesion formation in EAE, by studying the effect of Cl_2MDP-containing mannosylated liposomes (Cl_2MDP-mnL) on adoptively transferred EAE in SJL/J mice. Adoptive transfer of EAE with myelin basic protein-reactive $CD4^+$ T cells to SJL/J mice was abrogated by Cl_2MDP-mnL treatment. $CD4^+$ T cell and MHC II^+ $B220^+$ B cell extravasation from blood vessels, and Th1 cytokine production were not inhibited. However, invasion of the central nervous system intraparenchymal tissues by lymphocytes, $F4/80^+$, $Mac\text{-}1^+$, and $MOMA\text{-}1^+$ macrophages was almost completely blocked after treatment with Cl_2MDP-mnL. Furthermore, in Cl_2MDP-mnL-treated mice the myelin sheaths appeared completely normal, whereas in the control groups marked demyelination occurred. Production of TNF-α and inducible nitric oxide synthase, both associated with macrophage/microglial activation, was inhibited. This intervention reveals a role for macrophages in regulating the invasion of autoreactive T cells and secondary glial recruitment that ordinarily lead to demyelinating pathology in EAE and MS.

Introduction

Experimental allergic encephalomyelitis (EAE) is a T cell-mediated autoimmune demyelinating disease of the central nervous system (CNS) that can be induced in rodents by immunization with myelin proteins or by adoptive transfer of myelin-reactive $CD4^+$ T blasts (1). In mice, the relapsing-remitting course of paralysis in EAE and its histopathology mimic those seen in the human disease multiple sclerosis (MS) (2,3). Studies using the adoptively transferred EAE model strongly support the view that activated neuroantigen-specific $CD4^+$ T cells cross the blood-brain barrier (BBB), infiltrate the CNS parenchyma, interact with glial cells and initiate an inflammatory response (4,5). Inflammatory infiltrates in acute EAE and MS contain predominantly a diverse accumulation of T cells, macrophages and only a few B cells (1-3). These macrophages include monocyte-derived macrophages that had trafficked to the CNS from the blood circulation and perivascular macrophages. These latter represent a subpopulation of monocytic cells in adult CNS that are continuously renewed by bone-marrow-derived precursors (6), and hence are considered functionally equivalent to peripheral macrophages. Microglia, a more stable and irradiation-resistant subpopulation of monocytic cells in the CNS, acquire similar functions as blood-derived macrophages during inflammation (7).

Many lines of evidence point to a critical role for macrophages/microglia in EAE. Major histocompatibility complex (MHC) class II is rarely expressed in the normal mouse CNS, but is up-regulated on macrophages/microglia in EAE and MS (1,2). Activated macrophages/microglia in CNS also up-regulate B7 molecules, which are critical for antigen presentation (8,9), so these cells have the potential of stimulating T cell responses. Activated macrophages/microglia are also major sources of inflammatory and potentially cytopathic mediators such as TNFα (10), IL-1 (11), reactive oxygen species (12), and nitric oxide (13,14), in the CNS during EAE. Involvement of macrophages/microglia in the demyelination process is supported by ultrastructural studies showing phagocytic cells

engulfing myelin debris in close apposition to naked axons in EAE and MS lesions (3) and by the observation that antibodies against complement receptor type 3 blocked phagocytosis of myelin (15,16). Furthermore, interactions between CD40 and its ligand (CD40L) on $CD4^+$ T cells are implicated in the activation of macrophages/microglia, and blocking these interactions with anti-CD40L antibody treatment was shown to completely prevent EAE (17).

Sedgwick et al. (18) passively transferred EAE with IL-2R/$CD25^+$ $CD4^+$ T cells to recipient rats that were rendered leukopenic and suggested that coinfiltrating non-T-cell leukocytes in the CNS are superfluous to the induction of disease. Berger et al. (19) showed disease severity to correlate with the absolute number of macrophages invading the CNS parenchyma, but not with the number of infiltrating T cells. We demonstrated that selective depletion of peripheral macrophages by intravenous administration of mannosylated liposomes containing dichloromethylene diphosphonate (Cl_2MDP-mnL) abrogated EAE in Lewis rats (20-22). This treatment did not seem to deplete parenchymal microglia, though their activation was impaired (22). Thus, although it is undisputed that autoreactive $CD4^+$ T cells trigger EAE, it remains unclear how much they depend on cooperation with macrophages. In our previous studies, the effect of macrophage depletion on the number and functional characteristics of encephalitogenic T cells was not evaluated, raising the possibility that the macrophage depletion may result in suppression of EAE by indirectly affecting migratory properties and/or function of encephalitogenic T cells. Further studies on this matter are essential to determine which cells are the most important targets for development of new therapies. We have now approached these questions in an adoptively transferred myelin basic protein (MBP)-induced EAE in SJL/J mice. We find that depletion of peripheral macrophages prevented EAE and demyelination. Leukocytes, including T cells, monocytes/macrophages and an unusually high proportion of MHC II^+ $B220^+$ B cells, accumulated in subarachnoid spaces of the leptomeninges around spinal cord, but did not

infiltrate the CNS parenchyma. Although T-cell effector function as evidenced by Th1 cytokine production was unimpaired, production of TNFα and iNOS mediators associated with glial cell activation was significantly reduced. These results show a critical role for macrophage infiltration in inducing CNS inflammation and underscore their importance in organ-specific autoimmune diseases.

Materials and Methods

Induction of EAE

Specific pathogen-free female SJL/J mice (8-10 week old) were purchased from Charles River Canada (St-Constant, Canada). EAE was elicited by passive transfer of MBP-reactive T cells. Donor mice were immunized subcutaneously at the base of the tail with an emulsion containing 400 μg of bovine MBP (prepared as described by Cheifetz et al. (23) and 50 μg of Mycobacterium tuberculosis H37 RA (Difco, Detroit, MI) in complete Freund's adjuvant (Difco); and boosted in the flanks 7 days later with the same amount. A single cell suspension was prepared from the draining lymph nodes 14 days after the first immunization, and cells ($4x10^6$/ml) were cultured in the presence of 50 μg/ml MBP in RPMI 1640 supplemented (Gibco/BRL, Burlington, Canada) with 10% fetal calf serum (UBI, Lake Placid, NY), 50 mM 2-mercaptoethanol (Sigma, Montreal, Canada), 2 mM L-glutamine (Gibco/BRL), 100 U/ml penicillin (Gibco/BRL), and 100 μg/ml streptomycin (Gibco/BRL). After 4 days of culture, non-adherent cells were collected by centrifugation on Ficoll Hypaque (Pharmacia, Montreal, Canada) and $8\text{-}10x10^6$ blasts were injected per mouse i.v. into naive mice. MBP reactivity of the lymph node cells was measured by [^{3}H]thymidine (ICN Biochemicals, Mississauga, Canada) incorporation assay of parallel microplate cultures. Animal maintenance and experimental protocols were approved by McGill University animal care committee.

Treatment with liposomes

Mannosylated liposomes containing dichloromethylene diphosphonate (Cl_2MDP, a gift of Boehringer Mannheim GmbH, Mannheim, Germany) or PBS were prepared as described by Huitinga et al. (20). PBS, PBS-containing mannosylated liposomes (PBS-mnL) or Cl_2MDP-containing mannosylated liposomes (Cl_2MDP-mnL) (200 μl/mouse) were administered by i.v. injection at days 3, 5 and 7 after T cell transfer. Clinical signs of EAE were scored as follows: 1-flaccid tail, 2-poor righting ability, 3-limb weakness/loss of righting ability, 4-

limb paralysis, 5-moribund. At the peak of disease observed in PBS- or PBS-mnL-treated groups, usually 10-12 days after T cell transfer, all mice were sacrified for flow cytometric, histological, and RT-PCR analyses.

Immunohistology

Mice were anesthesized with Somnotol (4.45 ml/kg body weight) (MT Pharmaceutical, Cambridge, Canada) and perfused intracardially through left ventricle with 25ml of ice-cold PBS. Tissues were snap-frozen in 2-methylbutane (Fisher, Montreal, Canada) pre-chilled in liquid nitrogen. Cryostat sections (12 or 16 μm) were blocked in 3% ovalbumin (Sigma) in PBS for 30 min at room temperature (RT), incubated with primary rat monoclonal antibodies (mAb) overnight at 4°C, then with biotinylated rabbit anti-rat Ig for 1 h at RT. Sections were treated with 0.3% H_2O_2 to quench endogenous peroxidase activity, then incubated with an avidin-horseperoxidase complex (Vectastain ABC kit, Vector Labs, Mississauga, Canada) following the manufacturer's instructions. Biotin-avidin complex binding was detected by the use of 3-amino-9-ethylcarbazole (Dako Labs) as chromagen. The mAbs used were: GK1.5 (CD4) (ATCC), F4/80 (24), MOMA-1 (25) (kindly provided by Dr. Georg Kraal), ER-TR9 (26), M1/70 (Mac-1/CD11b) (ATCC), YN1/1.7.4 (ICAM-1/CD54) (ATCC), P7/7.1 (MHC II) (ATCC), and clone 14.8 (B220) (27) (kindly provided by Dr. Dennis Osmond). Control sections were incubated with isotype-matched primary antibodies or with secondary antibodies alone. Staining for iNOS using polyclonal anti-mouse iNOS (Transduction laboratories) was performed as previously described (14).

Neuropathological assessment for demyelination

Mice were perfused with PBS, followed by 0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.4. Tissues were post-fixed in 2% osmium tetroxide, dehydrated in graded concentrations of methanol, cleared in propylene oxide, and embedded in Epon. Transverse sections of spinal cord (1 μm) were stained with toluidine

blue and examined by light microscope. Ultrathin sections of spinal cord were mounted on nickel grids, stained with uranyl acetate and lead citrate, and examined by electron microscope.

Flow cytometry analysis

After perfusion with ice-cold PBS, brains were removed and spinal cords were dissected from the vertebral canal, taking care to collect the meninges. Isolation of CNS mononuclear cells was performed as previoulsy described (28). Briefly, tissues were dissociated in RPMI/10%FCS by passing through a metal sieve, then centrifuged at 400 x g for 10 min at 4°C. The pellet was resuspended in 70% isotonic Percoll (Pharmacia), overlaid with equal volumes of 37% and 30% isotonic Percoll, and centrifuged at 500 x g for 20 min at RT. Cells were collected from the 37%:70% interface and washed with RPMI/10%FCS. Cells were first incubated at RT for 10 min with 100 μg/ml normal rat Ig to block Fc receptors and avoid nonspecific staining, then double stained either with phycoerythrin-conjugated anti-CD4 (PE-CD4, Becton Dickinson, Mississauga, Canada) and fluorescein-conjugated anti-CD3 (FITC-145.2C11), or with FITC-conjugated anti-CD11b/Mac-1 (FITC-M1/70) and biotinylated anti-CD45 (M1/89) which was visualized by PE-coupled streptavidin. Cells were analyzed using a FACScan® (Becton Dickinson). Propidium iodide staining, and forward/side scatter gating were used to exclude dead cells.

RT-PCR

Total RNA was purified from homogenized PBS-perfused CNS using TriZol (Gibco/BRL) following the manufacturer's instructions. RNA was reverse-transcribed with 10 μM random hexamer primers (Boehringer Mannheim, Montreal, Canada), 0.5 mM each dNTPs (Pharmacia), 3.3 mM DTT (Gibco/BRL) and 400 U MMLV-RT (Gibco/BRL) at 42°C for 1 h, terminated by heating at 75°C for 10 min. PCR conditions were optimized for linear amplification to allow direct comparison between samples. Equal amounts of cDNA were

amplified using 1X PCR buffer (Sigma), 2 mM $MgCl_2$ (Sigma), 80 μM each dNTPs, 2 U Taq polymerase (Sigma), and 50 pmol of each primer. The PCR reaction was performed with a Perkin-Elmer thermocycler for 30 cycles (denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C, extension for 30 sec at 72°C) for CD3γ, TNFα, IFNγ and IL-4 and GAPDH or (40 sec at 94°C, 40 sec at 63°C, 40 sec at 72°C) for IL-12p40 and iNOS, then followed by a final extension for 5 min at 72°C. The primer sequences for TNFα were: sense 5'-CGGGGCAGCCTTGTCCCTTG-3', antisense 5'-GGGGTGATCGGTCCCCAAAGG-3'. The other primer sequences were described elsewhere: CD3γ and IFNγ (10); IL-12p40 and IL-4 (29); GAPDH (30); iNOS (14). PCR products were electrophoresed in a 1.8% agarose gel, visualized by SYBR Green (Molecular Probes Inc., Eugene, OR) staining, and analyzed by PhosphoImager (Molecular Dynamics, Sunnyvale, CA).

Results

Cl_2MDP-mnL treatment inhibits clinical signs of acute EAE.

Cl_2MDP-mnL have been shown to transiently eliminate phagocytic macrophages from spleen, bone marrow, liver and peripheral circulation as soon as 24 hours after intravenous administration (31). The number and function of other immune cells including dendritic cells, B cells, and T cells, are not affected after treatment with Cl_2MDP-mnL (32,33). Although different subsets of splenic macrophages require varying periods of time to repopulate, repopulation was not evidenced until 1-2 weeks after one single administration or until 1 month after sequential administrations (31). To examine how peripheral macrophages influence the course of adoptively-transferred EAE in SJL/J mice, Cl_2MDP-mnL were administered intravenously to mice after transfer of MBP-reactive T blasts and before the anticipated onset of EAE. We chose an injection schedule and a dosage such that a macrophage-depleted environment could be created as soon as 4 days after T cell transfer and could be maintained for at least 14 days after T cell transfer. As expected, mice receiving PBS or PBS-mnL developed severe symptoms of EAE (ranging from grades 3-5) (Table I). Since no difference was noted in the course of the disease whether the mice were treated with empty-liposomes (PBS-mnL) or PBS alone, PBS was used as control in most experiments. In contrast to PBS-treated mice, Cl_2MDP-mnL-treated mice developed only mild or no signs of disease. Neither progression to paralysis nor delayed onset of disease was observed, even when treated mice were followed for at least 2 months after T cell transfer (not shown).

At the peak disease severity in the PBS-treated group, usually 10-12 days after T cell transfer, all mice were sacrificed for analysis. To verify depletion of peripheral macrophages, we performed immunostaining with monoclonal antibodies that recognized murine macrophage markers, F4/80, ER-TR9 and MOMA-1, on frozen sections of spleen. The major splenic macrophage populations ($F4/80^+$ red pulp macrophages, $ER\text{-}TR9^+$

marginal zone macrophages and MOMA-1$^+$ marginal metallophilic macrophages) were depleted in mice treated with Cl_2MDP-mnL but not in mice treated with either PBS alone (Table I and Fig. 1) or PBS-mnL (not shown). Consistent with a previous report (31), lymph node macrophages were not greatly influenced by this treatment in which Cl_2MDP-mL was administered intravenously. MOMA-1$^+$ macrophages lining the subcapsular sinus and the medullary sinuses could still be seen in lymph nodes of mice treated with Cl_2MDP-mnL (not shown). Nevertheless, the selective depletion of peripheral macrophages was sufficient to suppress development of clinical signs of acute EAE.

Recruitment of CD4$^+$ T cells to the CNS is not compromised in Cl_2MDP-mnL-treated mice. In EAE, T cells and monocytes/macrophages infiltrate the CNS. These extravasated T cells and monocytes/macrophages can be recovered from perfused CNS tissues by discontinuous density gradient centrifugation, and quantified by flow cytometry (28). We analyzed mononuclear cell isolates from CNS of both PBS-mnL- and Cl_2MDP-mnL-treated mice for their expression of T cell surface markers, CD4 and CD3, and of the leukocyte markers, CD45 and Mac-1 (CD11b) (Fig 2). Expression level of CD45 allows distinction between blood-derived macrophages (CD45^{hi}) and parenchymal microglia (CD45^{lo}) (34). Unexpectedly, similar proportions of CD4$^+$CD3$^+$ T cells were isolated from CNS of both PBS-mnL- and Cl_2MDP-mnL-treated mice (Fig. 2 *upper panels*) in spite of the profound difference in disease severity (Table I). Neither was any appreciable change observed in the percentages of CD45^{hi}Mac-1$^-$ leukocytes (Fig. 2 *lower panels*, R3), or of CD45^{lo}Mac-1$^+$ microglia (Fig 2 *lower panels*, R2) following Cl_2MDP-mnL treatment. Although CNS content of T cells was not diminished by this treatment, recruitment of CD45^{hi}Mac-1^{hi} macrophages to the CNS was considerably reduced (Fig 2 *lower panels*, R4).

Cl_2MDP-mnL treatment inhibits massive leukocytic invasion of the CNS parenchyma.

We conducted histological examination of spinal cord to determine whether distribution of leukocytes within the CNS had altered to modulate their ability to induce EAE. In contrast to the spinal cords of PBS-treated mice, which showed diffuse parenchymal-invading subpial leukocyte infiltrates (Fig. 3A), a pathological feature characteristic of EAE, Cl_2MDP-mnL-treated mice showed tightly constrained accumulation of leukocytes in the leptomeninges that did not invade the parenchyma (Fig. 3C). Perivascular cuffs of inflammatory cells were seen throughout white matter in EAE (not shown), as well as large diffuse subpial infiltrates (Fig. 3A). ICAM-1 is not detectable in normal CNS tissue but is up-regulated on many cells, such as monocytes/macrophages, lymphocytes, and endothelial cells during inflammation. Despite the lack of clinical signs of EAE, spinal cord-associated leukocytes of Cl_2MDP-mnL-treated mice showed ICAM-1 immunoreactivity which, apart from distribution, was not appreciably different from PBS-treated mice (Fig. 3A,C). ICAM-1 was also up-regulated on blood vessels, indicating that the lack of leukocyte migration into the CNS parenchyma after Cl_2MDP-mnL treatment was not a consequence of defective vascular activation.

The distribution of inflammatory cells was further examined by immunostaining for various cell surface markers. In PBS-treated mice with severe EAE, there was a typically strong up-regulation of both F4/80 (Fig. 4A) and Mac-1 (not shown) reactivity in subpial lesions and throughout the white matter of the spinal cord from lumbar to cervical levels. In Cl_2MDP-mnL-treated mice, however, large round F4/80$^+$ and Mac-1$^+$ cells were mostly restricted to the leptomeninges, and there was a clear reduction in the proportions of F4/80$^+$ (Fig. 4E) and Mac-1$^+$ (not shown) macrophages/microglia. A few occasional Mac-1$^+$ and F4/80$^+$ cells were found scattered in the parenchyma, but they were not likely activated as judged by their more ramified morphology and/or weaker staining intensity for Mac-1. Thus, Cl_2MDP-mnL treatment not only prevented infiltration of leukocytes into the parenchyma but also modulated macrophage/microglial response.

The CNS of healthy unmanipulated SJL/J mouse occasionally contains MOMA-1^+ cells with thin and fusiform morphology in the mesothelial lining of the leptomeninges and around blood vessels (not shown). This contrasted with spinal cord of mice with EAE in which numerous strongly-stained large MOMA-1^+ cells (with a hypertrophied morphology) were seen in subpial lesions (Fig. 4B). Some of these were associated with the inflamed leptomeninges, and some were also identified in the underlying parenchyma. Hypertrophied MOMA-1^+ cells were also conspicuous in leptomeningeal infiltrates in brain of mice with severe EAE (not shown). In CNS of Cl_2MDP-mnL-treated mice, these large MOMA-1^+ cells were not detected at all in the subarachnoid leukocytes of these mice, and were absent from the parenchyma (Fig. 4F).

As expected by flow cytometric analysis (Fig. 2), $CD4^+$ T cells were equivalently represented in spinal cord leukocytes in PBS- (not shown) and Cl_2MDP-mnL-treated mice (Fig. 4C). We observed expression of MHC II on macrophages/microglia in parenchymal infiltrates in the spinal cord of PBS-treated mice (not shown). Surprisingly, expression of MHC II not only was detectable in leukocytic accumulations in spinal cord of asymptomatic Cl_2MDP-mnL-treated mice but also was strongly enhanced (Fig. 4G). The distribution was also different. MHC II was strongly expressed by a great majority of the leukocytes clustering in subarachnoid or perivascular spaces, but there was no parenchymal MHC II expression. Given the relative paucity of macrophages in CNS of Cl_2MDP-mnL-treated mice, and the fact that murine T cells do not express MHC II, this unusual prominence of MHC II immunoreactivity suggested the presence of B cells. This was confirmed by immunostaining for B220, which was widespread in extraparenchymal leukocytes in Cl_2MDP-mnL-treated mice (Fig. 3D). Whether these perivascular B cells in Cl_2MDP-mnL-treated mice produced anti-myelin was not determined.

Cl_2MDP-mnL treatment prevents demyelination.

Spinal cord of mice with severe paralytic EAE showed extensive demyelination (Fig. 5A). In these demyelinated areas, lipid-laden macrophages were noted (Fig. 5B). Neither demyelination nor phagocytic cells engulfing myelin debris were seen in Cl_2MDP-mnL-treated mice (Fig. 5, C and D). Also evident in Fig. 5 (C and D) are fully extravasated cells in perivascular spaces including cells with nuclear morphology typical of lymphocytes.

Cl_2MDP-mnL treatment does not inhibit Th1 cytokine mRNAs but represses TNFα and iNOS expression.

Th1 cytokines are selectively up-regulated in the CNS during EAE (2,35). There is also a significant increase in IL-12p40 mRNA expression in the CNS of mice with EAE (29,36). Fig. 6 shows comparative RT-PCR values for CD3γ and cytokine mRNA from the perfused CNS of individual mice. Despite the absence of clinical disease, the CNS of Cl_2MDP-mnL-treated mice contained mRNA for CD3γ, IL-12 p40 and IFNγ. Levels were in the same range as those seen in the PBS-treated mice, and the distributions overlapped (Fig. 6). IL-4 mRNA was not detectable in CNS of either Cl_2MDP-mnL- or PBS-treated mice. Interestingly, TNFα mRNA levels not only were reduced but also did not overlap with those from PBS-treated mice (Fig. 6). Expression of iNOS (type II NOS) mRNA was also very much reduced in the Cl_2MDP-mnL-treated group (Fig. 6), being almost ablated in 3 mice. This reduction of iNOS mRNA was consistent with the reduced iNOS immunostaining in both extraparenchymal leukocytes and glial cells, some of which had the morphological appearance of astrocytes in spinal cord (Fig. 4, D and H). Expression levels of CD44 and CD45RB on $CD4^+$ T cells were equivalent in Cl_2MDP-mnL- and PBS-treated mice (not shown). Thus, the near-abrogation of clinical disease in Cl_2MDP-mnL-treated mice cannot be attributed to impaired lymphocyte recruitment, an inhibition of pro-inflammatory cytokine expression, or switching from a Th1 to Th2 response in the CNS but is associated with reduced production of nitric oxide and TNFα in the CNS parenchyma.

Discussion

In this study, we depleted peripheral macrophages in SJL/J mice by intravenous injections of Cl_2MDP-mnL and showed that adoptive transfer of EAE was inhibited. Surprisingly, this inhibition was not due to the absence of either inflammatory cells or Th1 cytokine production in the CNS, but rather to the failure of leukocytes to migrate across blood-brain barrier and/or subsequently accumulate in the CNS parenchyma. Leukocytes, however, extravasated from blood across the endothelium in the CNS of Cl_2MDP-mnL-treated mice, aggregated in subarachnoid and perivascular spaces, and were recoverable from perfused tissues. It appears that the movement of leukocytes in Cl_2MDP-mnL-treated mice is not impeded at the level of transendothelial migration but rather at the level of movement through the parenchymal basement membrane, the glia limitans and the extracellular matrix. Our data support an important role for macrophages in regulating leukocyte infiltration into the CNS parenchyma.

In adult mouse, most macrophages can be detected by mAbs M1/70 (anti-Mac-1) and F4/80 (24) while the mAb MOMA-1 labels a particular subset of macrophages that is F4/80$^-$ and Mac-1$^-$ in the lymphoid organs (25). Microglia also express F4/80 and Mac-1, but not MOMA-1 (37). Here we have used these mAbs to show for the first time that parenchymal spinal cord lesions in mice with EAE contain hypertrophied MOMA-1$^+$ macrophages, and F4/80$^+$, Mac-1$^+$ macrophages/microglia. However, after Cl_2MDP-mnL treatment many fewer F4/80$^+$ and Mac-1$^+$ macrophages were detected while the hypertrophied MOMA-1$^+$ macrophages were undetectable in the extraparenchymal leukocyte accumulations around spinal cord. Our data suggest that the extent of and/or the heterogeneity in macrophage infiltration of the mouse CNS parenchyma determine the clinical outcome of a leukocytic aggregate. Hypertrophied MOMA-1$^+$ macrophages in infiltrates in EAE must derive either from blood-derived monocytes or from CNS-endogenous precursors, responding to intraparenchymal cytokines and/or cellular interactions. The absence of MOMA-1$^+$ cells in

extraparenchymal populations may reflect a lack of inducing stimulus, the nature of which, and whether it acts on MOMA-1$^-$ or MOMA-1$^+$ precursors, remain to be determined. The few F4/80$^+$/Mac-1$^+$ cells in extraparenchymal populations in Cl_2MDP-mnL-treated mice likely derived from monocytes recruited from bone marrow, which are known to be increased following macrophage depletion (20,38). Regardless of their sources, the residual monocytes/macrophages clustering in subarachnoid spaces around spinal cord of Cl_2MDP-mnL-treated mice were clearly not sufficient to bring about neurological deficit. We did not find T cells infiltrating the spinal cord parenchyma in CL_2MDP-mnL-treated mice, whereas parenchymal T cells were noted in CL_2MDP-mnL-treated rats in our previous study (20). This may relate to differences between mice and rats, supported by our observation of a shift from spinal cord to brain infiltrates in CL_2MDP-mnL-treated Lewis rats (Bauer J., I. Huitinga, K. Wegmann, C. D. Dijkstra, and W. F. Hickey, unpublished), which we did not see in this study.

The inefficient migration of leukocytes into the CNS parenchyma of Cl_2MDP-mnL-treated mice could reflect an imbalance in matrix metalloproteinase and/or chemokine production (39,40,41,42,43). A chemokine imbalance might also include overproduction of a B cell-specific chemokine such as B-lymphocyte chemoattractant (44) or the murine homolog of B-cell-attracting chemokine 1 (45). Chemokine/matrix metalloproteinase imbalance could relate to the absence of MOMA-1$^+$ macrophages, or to another functional subset. Whether such macrophages were depleted from perivascular locations or peripherally is unknown. Our previous studies showed that non-mannosylated liposomes did not inhibit EAE in rats (20), although splenic macrophages were depleted. This would argue that effects on mannose-receptor expressing cells were critical, and these were likely to be in CNS perivascular space.

The presence of IL-12p40 mRNA in the CNS of Cl_2MDP-mnL-treated mice suggests the presence of activated microglia or monocytes/macrophages. Although IL-12 production by B cells and MOMA-1$^-$ dendritic cells has been reported (46,47), mouse B cells may not share this capability (48) but cannot be ruled out as one source of IL-12. Other potential sources in perivascular populations include perivascular microglia and macrophages. Whether IL-12 protein was produced by these leukocytes has not been determined, although the presence of IFNγ mRNA indicates a Th1 response, which is usually associated with IL-12. On the other hand, transcripts for TNFα were expressed at reduced levels in CNS of mice treated with Cl_2MDP-mnL. If parenchymal microglial cells, which can produce this mediator (10,29,36), were not induced to up-regulate TNFα in Cl_2MDP-mnL-treated mice, it would account for reduced TNFα levels. The absence of iNOS message is consistent with this and further argues that glial cells are normally the predominant source of iNOS. This would mean that cytokines diffusing from perivascular or subarachnoid spaces were not sufficient stimuli for glial activation and that infiltration was also required. Our data could also argue for reduced activation/function of those macrophages that did extravasate.

That MBP-reactive T cells did not transfer EAE in Cl_2MDP-mnL-treated mice could also relate to lack of restimulation by macrophages. The possibility that adoptively-transferred MBP-reactive T blasts require restimulation in the spleen of recipient mice in order to cause disease is considered unlikely, because EAE can be adoptively transferred in splenectomized rats (49) and, in our experiments, we applied a long-term depletion protocol that depletes both splenic macrophages as well as all of the macrophages that would traffic to spleen (31). The fact that Th1 cytokine mRNAs were detected in the CNS of Cl_2MDP-mnL-treated mice supports that effector functions of transferred CD4$^+$ T cells were not absolutely dependent on splenic macrophages. Furthermore, IL-4 mRNA was not up-regulated, arguing against immune deviation from a pathogenic Th1 response to a nonpathogenic Th2 response as a protective mechanism in Cl_2MDP-mnL-treated mice. Yet, despite activation to Th1 effector

function, MBP-reactive Th1 $CD4^+$ T cells did not induce EAE in Cl_2MDP-mnL-treated mice.

Activated T cells are considered to enter the CNS irrespective of antigen specificity (4,5). Thus, T cells might have entered but not persisted in the CNS of Cl_2MDP-mnL-treated mice, due to lack of restimulation *in situ*, which would support that coinfiltrating macrophages are the critical APC in EAE. Failure to invade CNS parenchyma might also reflect lack of restimulation extraparenchymally, although this would conflict with evidence that T cells of any specificity can enter the CNS (4,5). If perivascular macrophages/microglia do contribute to antigen presentation function, then their impairment after Cl_2MDP-mnL treatment (22) might account for the slight reduction in levels of IFNγ mRNA. Nevertheless, T cell activation phenotypes and numbers were essentially normal, so deficiency in antigen presentation alone cannot account for our observations.

Our results show that extravasation of autoreactive effector T cells from blood to the target tissue is not sufficient by itself to lead to autoimmune pathology. Similarly, EAE was inhibited after treatment by leukotriene B_4 receptor antagonists (50) or a TNF receptor-IgG fusion protein (51), despite unaltered influx of autoreactive T cells to CNS in both studies. In mice that overexpressed the chemokine MCP-1 in the CNS, macrophages extravasated to perivascular or subarachnoid spaces outside the CNS parenchymal tissue without any overt neurological deficit (52). Parallel findings in other tissues include the observation of peri-islet infiltrates but no diabetes in mice treated with mAbs against complement receptor type 3 (53) and non-diabetogenic infiltrates in pancreata of TNF transgenic mice (54). These findings underline the importance of macrophages and their products in regulating both the infiltration of leukocytes into the target tissue and the subsequent activation of inflammatory events that lead to tissue damage in organ-specific autoimmune diseases.

We therefore show there to be a role for peripheral macrophages, including MOMA-1$^+$ cells in regulating entry of leukocytes to the CNS. Th1 cytokine production was not inhibited by macrophage depletion, whereas activation of cytokine production by glial cells within the CNS was abrogated, with resultant inhibition of disease symptoms and pathology. These findings focus renewed attention on macrophages as potential targets for immunotherapy in CNS inflammatory diseases such as MS.

Acknowledgements

We thank Julie Mercure for contributions to the initiation of this project; Lyne Bourbonnière, Gail Verge and Grace Chan for technical assistance; and Jim Dixon for electron microscope imaging.

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Table I. *Attenuation of passively transferred EAE in SJL/J mice by Cl_2MDP-mnL treatment*

Treatment	Incidence (%)	Mean Clinical Score ± SD (/total mice)	Mean Day of Onset ± SD	Peripheral Macrophages (spleen)[b]		
				F4/80	ER-TR9	MOMA-1
PBS[a]	40/42 (95%)	3.4 ± 1.1	7.5 ± 0.82	+	+	+
Cl_2MDP-mnL	20/38 (53%)	0.80 ± 0.85	7.8 ± 0.60	-	-	-
PBS-mnL	4/4 (100%)	4.3 ± 0.50	6.0 ± 0.0	+	+	+

[a]In five separate experiments (data are pooled here), each mouse received 200 µl PBS or Cl_2MDP-mnL by intravenous injection at day 3, 5, and 7 after T cell transfer. At the peak of clinical disease in the control group, 10 to 12 days after T cell transfer, all mice were sacrificed for analysis.

[b]Frozen sections of spleen from mice sacrificed for histologic analysis at day 10 to 12 after T cell transfer were stained for F4/80, MOMA-1 (also see Fig. 1) and ER-TR9 (data not shown). Immunoreactivity for murine macrophage markers was evaluated as (+) for normal numbers of immunoreactive cells and (-) for no immunoreactive cells.

FIGURE 1. Loss of splenic macrophages in Cl_2MDP-mnL-treated mice.

Frozen sections of spleen from PBS-treated (**A, B**) and Cl_2MDP-mnL-treated (**C, D**) mice mice 12 days after transfer of MBP-reactive T cells were immunostained with F4/80 (**A, C**) and MOMA-1 (**B, D**) monoclonal antibodies, which identify red pulp macrophages and marginal metallophilic macrophages, respectively. Sections were counterstained with Mayer's hematoxylin. Residual isolated staining deposits in C and D were attributable to macrophage remnants, being morphologically distinguishable from normal healthy cells. r, red pulp; m, marginal zone; w, white pulp. Scale bar = 100 μm.

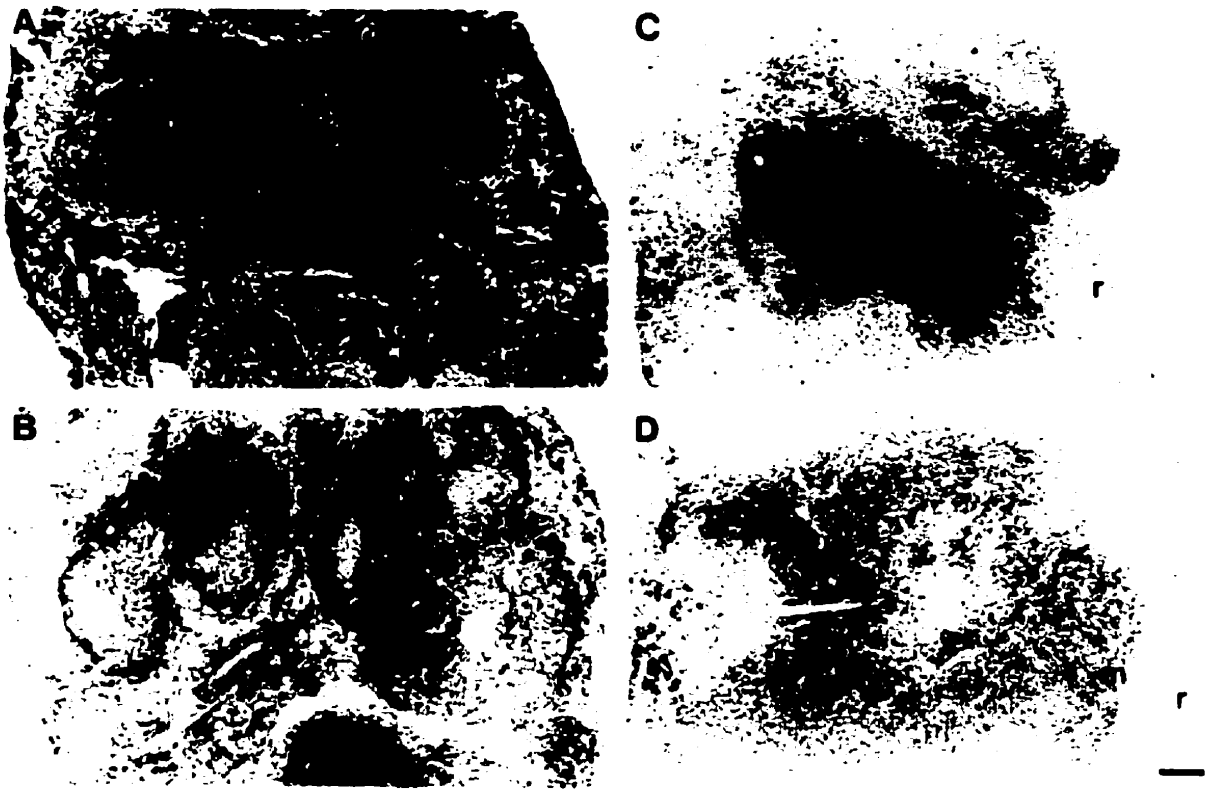

A
C
r
B
D
r

FIGURE 2. Cl_2MDP-mnL treatment reduces influx of macrophages but not that of T lymphocytes to the CNS.

Mononuclear cells isolated from perfused CNS of PBS-mnL- or Cl_2MDP-mnL-treated mice 12 days after transfer of MBP-reactive T cells were stained for CD4 and CD3 (upper pannels), CD45 and Mac-1/CD11b (lower pannels), and analyzed by flow cytometry. Dead cells were excluded using propidium iodide staining and forward/side scatter gating. Analysis quadrants for CD4 versus CD3 (upper pannels) were drawn on the basis of an unstained profile. Elliptical regions in lower pannels were drawn to demarcate 3 subpopulations differing in their expression levels of CD45 and Mac-1/CD11b. Proportions of subpopulations in PBS-mnL- vs Cl_2MDP-mnL-treated CNS were as follows: $CD45^{lo}Mac\text{-}1^{+}$ (ellipse R2), 19% vs 16%; $CD45^{hi}$ $Mac\text{-}1^{-}$ (ellipse R3), 39% vs 32%; and $CD45^{hi}Mac\text{-}1^{hi}$ (ellipse R4), 5% vs 1%. $CD45^{-}Mac\text{-}1^{-}$ cells have not been defined but could represent nonhematopoietically derived CNS resident cells, e.g., oligodendrocytes, neurons, endothelial cells.

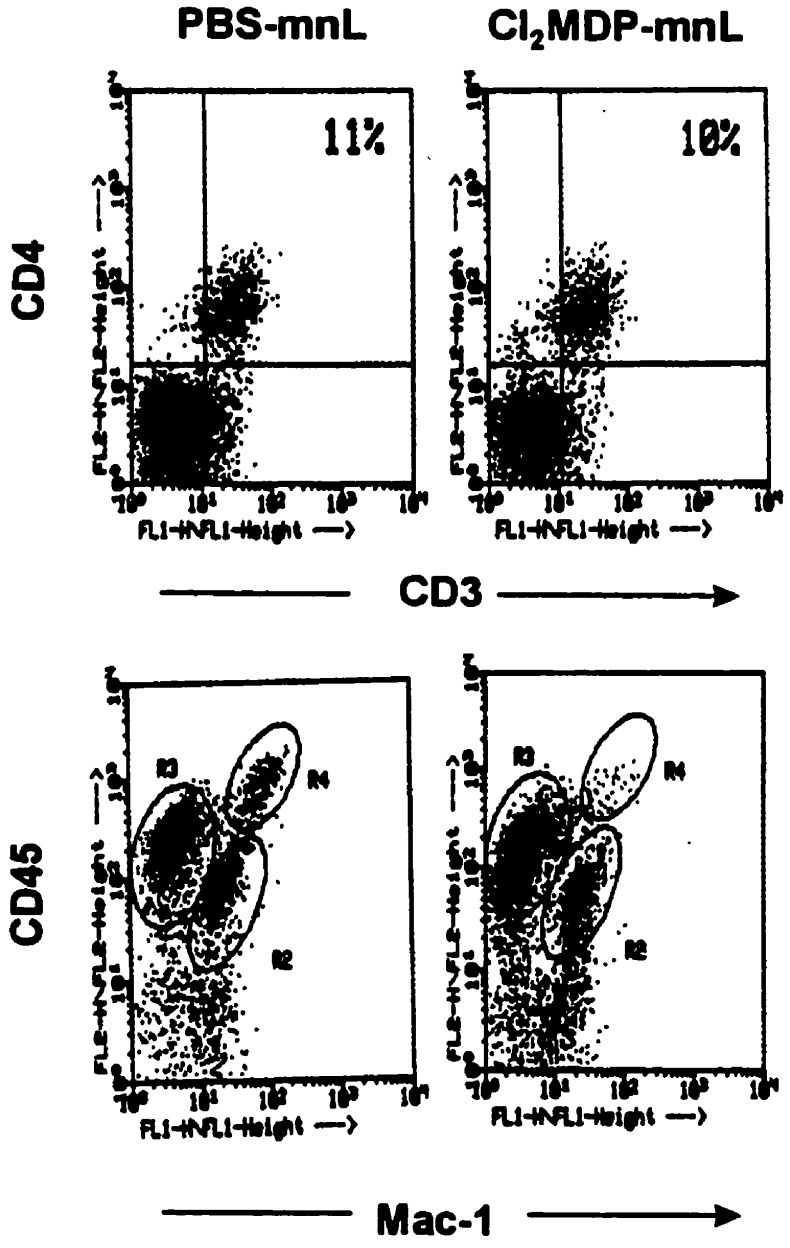

PBS-mnL
Cl2MDP-mnL
11%
10%
CD4
CD3
CD45
Mac-1
R2
R3
R4
FL1-H/FL1-Height

FIGURE 3. Asymptomatic accumulation of inflammatory cells in leptomeninges around spinal cord of Cl_2MDP-mnL-treated mice.

Lumbar spinal cord cryostat sections from PBS-treated mice with paralytic (grade 4) EAE (**A, B**) and Cl_2MDP-mnL-treated mice (**C, D**) that did not show signs of clinical disease were immunostained for ICAM-1 (A, C: scale bar = 100 μm) or B220 (B, D: scale bar = 10 μm). No significant immunoreactivity for B220 was noted in infiltrates in mice with EAE (**B**) while B220+ cells were clearly abundant in Cl_2MDP-mnL-treated mice (**D**). A and C were counterstained with Mayer's hematoxylin.

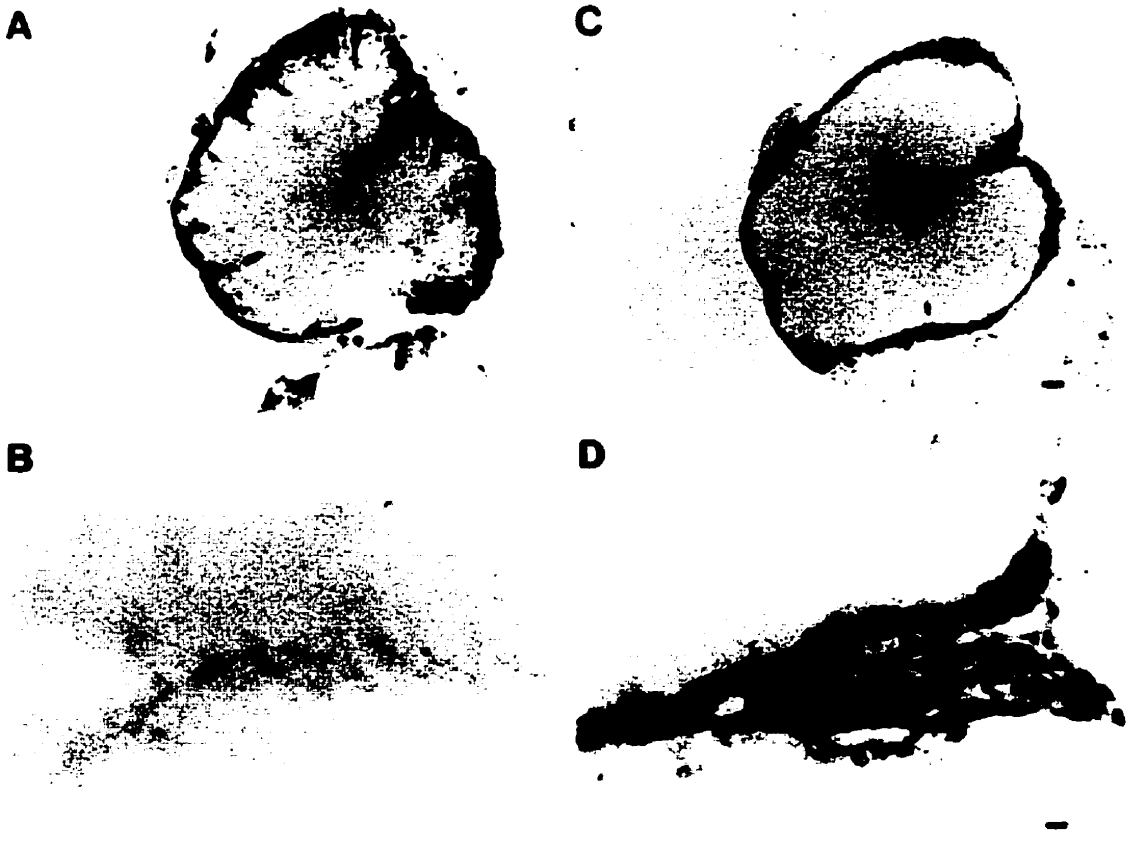

A
C
B
D

FIGURE 4. Accumulation of $CD4^+$ T lymphocytes and MHC II^+ cells in leptomeninges around spinal cord of Cl_2MDP-mnL-treated mice.

Cryostat sections of lumbar spinal cord from PBS-treated mice with paralytic (grade 4) EAE (**A, B, D**) and asymptomatic Cl_2MDP-mnL-treated mice (**C, E-H**) were immunostained for F4/80 (**A, E**), MOMA-1 (**B, F**), CD4 (**C**), MHC II (**G**) and iNOS (**D, H**) and counterstained with Mayer's hematoxylin. Scale bar = 10 μm.

FIGURE 5. Absence of demyelination in spinal cord of Cl_2MDP-mnL-treated mice. **A, C,** One micron-thick epoxy sections of lumbar spinal cord were stained with toluidine blue. **B, D,** Ultrathin sections of lumbar spinal cord were viewed by electron microscopy. **A,** Shows a large subpial lesion with mononuclear cell infiltration and demyelination in a PBS-treated mouse with paralytic (grade 4) EAE. **B,** Electron micrograph showing lipid-laden macrophages in the same PBS-treated mouse. **C,** Normal myelin and perivascular leukocytic accumulation in a Cl_2MDP-mnL-treated mouse that did not exhibit clinical signs of EAE. **D,** Electron micrograph showing accumulation of leukocytes in perivascular spaces, with no parenchymal invasion nor disruption of the parenchymal basement membrane. Magnification: 700x (A, C), 3600x (B), and 2400x (D). v = blood vessel.

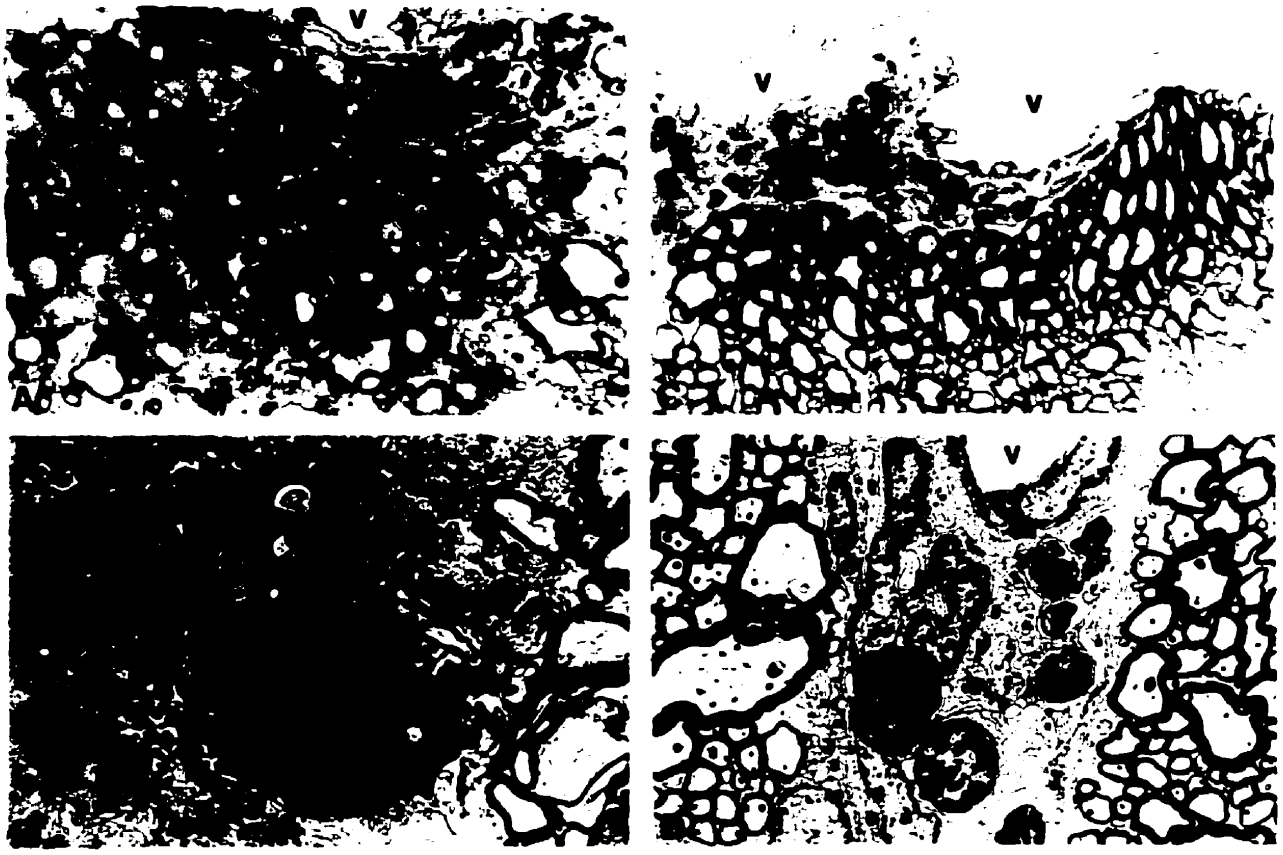
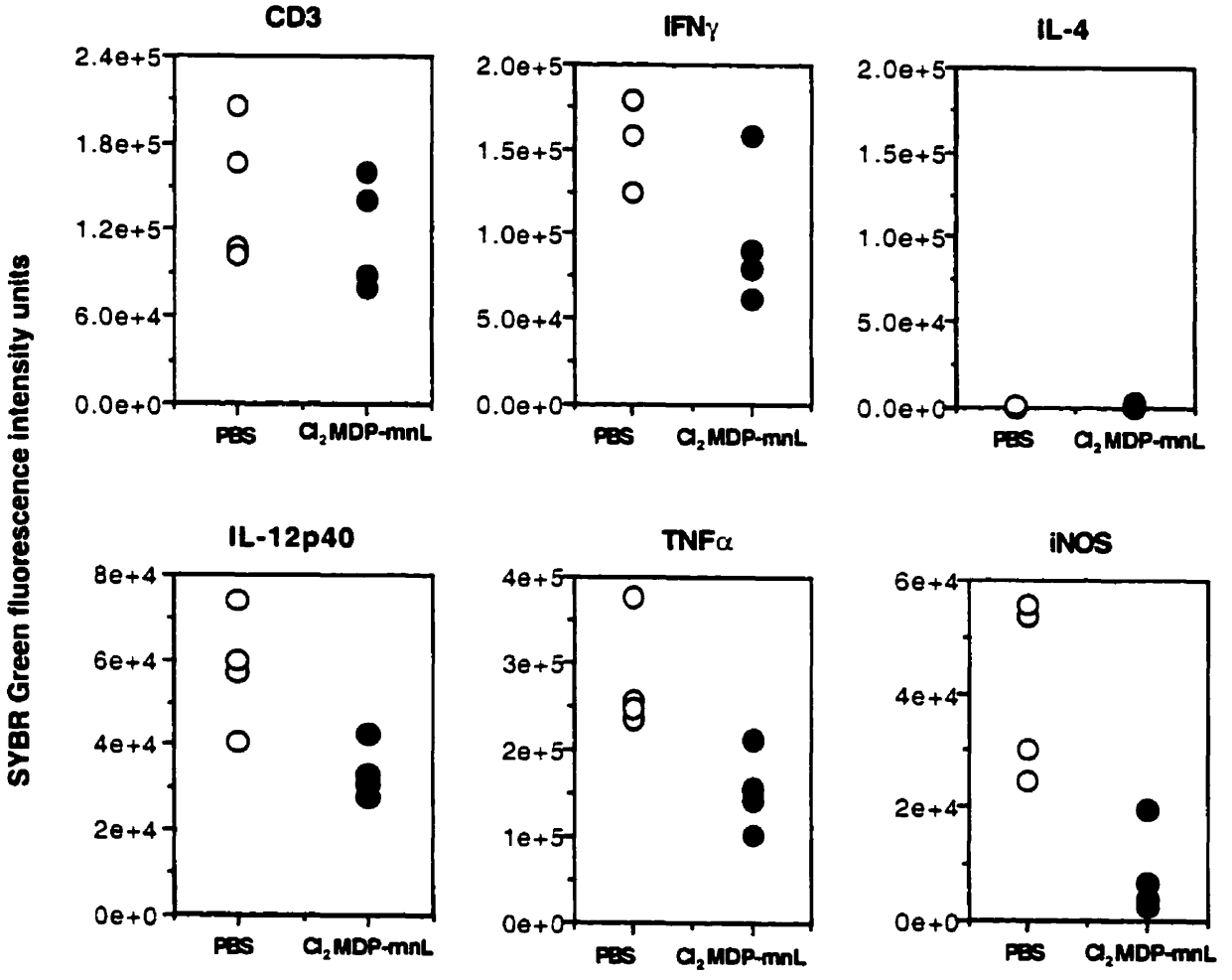

V
V
V
V

FIGURE 6. Levels of CD3γ, cytokine and iNOS mRNA in spinal cord of mice treated with PBS or Cl_2MDP-mnL.

Semiquantitative RT-PCR analysis for CD3γ, cytokine, iNOS and GAPDH was performed on RNA isolated from perfused CNS of PBS- and Cl_2MDP-mnL-treated mice 10 days after transfer of MBP-reactive T cells. PCR products were separated in a 1.8% agarose gel and visualized by SYBR Green staining. Fluorescence intensity for CD3γ, cytokine and iNOS signals were quantified and normalized to GAPDH. Each point represents results from an individual mouse. Three to four mice were examined per group.


SYBR Green fluorescence intensity units
CD3
2.4e+5
1.8e+5
1.2e+5
6.0e+4
0.0e+0
PBS
Cl2MDP-mnL
IFNγ
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0e+0
PBS
Cl2MDP-mnL
IL-4
2.0e+5
1.5e+5
1.0e+5
5.0e+4
0.0e+0
PBS
Cl2MDP-mnL
IL-12p40
8e+4
6e+4
4e+4
2e+4
0e+0
PBS
Cl2MDP-mnL
TNFα
4e+5
3e+5
2e+5
1e+5
0e+0
PBS
Cl2MDP-mnL
iNOS
6e+4
4e+4
2e+4
0e+0
PBS
Cl2MDP-mnL

CHAPTER IV

IFNγ shapes immune invasion of the central nervous system via regulation by chemokines.

Preface

As shown in previous chapters, the initial trafficking of IFNγ-secreting myelin-reactive T cells to the CNS is not sufficient to induce clinical manifestation of EAE as long as macrophage recruitment and function are impaired. It was previously shown that IFNγ was unnecessary for but even seemed protective against EAE. These observations raised questions regarding leukocyte subsets and immune invasion to the CNS. Here, I examine how cytokines and chemokines coordinate to recruit and activate selective leukocyte subpopulations into the CNS parenchyma, and how such dynamics influence the clinical outcome of EAE in the presence or absence of IFNγ.

Abstract

Dynamic interplay between cytokines and chemokines directs trafficking of leukocyte subpopulations to tissues in autoimmune inflammation. We have examined the role of IFNγ in directing chemokine production and leukocyte infiltration to the central nervous system (CNS) in experimental autoimmune encephalomyelitis (EAE). BALB/c and C57BL/6 mice are resistant to induction of EAE by immunization with myelin basic protein. However, IFNγ-deficient (BALB/c) and IFNγR-deficient (C57BL/6) mice developed rapidly progressing lethal disease. Widespread demyelination and disseminated leukocytic infiltration of spinal cord were seen, unlike the focal perivascular infiltrates in SJL/J mice. Gr-1$^+$ neutrophils predominated in CNS, and CD4$^+$ T cells with an activated (CD69$^+$, CD25$^+$) phenotype, and eosinophils were also present. RANTES and MCP-1, normally up-regulated in EAE, were undetectable in IFNγ- and IFNγR-deficient mice. MIP-2 and TCA-3, both neutrophil-attracting chemokines, were strongly up-regulated. There was no induction of the Th2 cytokines, IL-4, IL-10 or IL-13. RNase protection assays and RT-PCR showed prevalence of IL-2, IL-3 and IL-15, but no increase in IL-12p40 mRNA levels in IFNγ- or IFNγR-deficient mice with EAE. Lymph node cells (LNC) from IFNγ-deficient mice proliferated in response to MBP, whereas BALB/c LNC did not. These findings show a regulatory role for IFNγ in EAE, acting on T cell proliferation and directing chemokine production, with profound implications for the onset and progression of disease.

Introduction

Entry of immune cells into, and their retention and activation within tissues are crucial features of host immune response against pathogens, and of autoimmune pathogenesis. A complex interplay among cytokines, chemokines and adhesion molecules orchestrates these cellular events and shapes the outcome of an inflammatory reaction. In general, the CXC or α-chemokines, including IL-8, KC, and MIP-2, act primarly on neutrophils, while the CC or β-chemokines (e.g., MCP-1, MIP-1α, MIP-1β, and RANTES) act mainly on monocytes/macrophages and lymphocytes (1, 2). IFNγ, the prototypic Th1 cytokine, plays an important role in protective, cell-mediated immunity (1, 3). In many organ-specific autoimmune diseases, however, IFNγ is implicated in pathology (1-3). Th1 cytokine-producing $CD4^+$ T cells induce organ-specific autoimmune responses characterized by mononuclear infiltrates in the target tissue. Recently, several chemokines have been shown to have selective effects on subsets of $CD4^+$ T cells. Th1 $CD4^+$ T cells respond preferentially to RANTES, MIP-1α, and MIP-1β, while Th2 cells respond to TCA-3 (4-6). By contrast, Th2-cytokines, such as IL-4, IL-5, IL-10 and IL-13, are implicated in pathologic allergic responses that are dominated by eosinophilia or neutrophilia (1, 7). These cytokines have been implicated in amelioration of autoimmune disease, or remission (1, 8-11). In immunocompromised hosts, Th2 $CD4^+$ T cells can induce autoimmunity (12, 13). Infiltrates in the affected tissues of Th2-induced recombinase-activating gene-deficient mice with autoimmune disease included many granulocytes (12, 13).

In multiple sclerosis (MS) and its animal model, experimental autoimmune encephalomyelitis (EAE), Th1 $CD4^+$ T cells infiltrate the central nervous system (CNS) (14). Chemokines, including RANTES, MIP-1α, MCP-1 and IP-10, and chemokine receptors are up-regulated in MS and EAE, and their expression has been shown to correlate with the distribution of CNS inflammatory infiltrates and clinical disease activity (15-22). Anti-MIP-1α mAb treatment and vaccination with naked DNA encoding MIP-1α and

MCP-1 prevented EAE in rodents (23, 24), and anti-MCP-1 mAb treatment attenuated relapses of EAE (25). EAE can be induced by various myelin components, such as myelin basic protein (MBP), and by adoptive transfer of T cells reactive to these components. SJL/J ($H\text{-}2^s$) is a widely used susceptible strain to MBP-induced EAE, while strains such as BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) are resistant to induction of EAE by immunization with MBP/CFA (14). However, IFNγ-deficient mice on resistant BALB/c and C57BL/6 background are susceptible to MBP-induced EAE (26, 27). Similarly, Willenborg et al. (28) reported that $MOG_{35\text{-}55}$ induced EAE with high mortality in otherwise resistant 129sv mice lacking IFNγR. Neutrophilia was noted in the CNS of IFNγR-/- mice with MOG-induced EAE (28).

Dynamic interplay between cytokines and chemokines may direct the trafficking and recruitment of selective leukocyte subpopulations to the tissue sites of inflammation. In this study, we explored the cellular and molecular mechanisms underlying the CNS autoimmune disease in the absence of the prototypic Th1 cytokine IFNγ or its receptor.

Materials and Methods

Mice

Specific pathogen-free female SJL/J, and BALB/c mice (8–10 wk old) were purchased from Charles River Canada (St-Constant, Canada). Heterozygous BALB/c-backcrossed mice with the disrupted IFNγ gene were obtained from Genentech (South San Franscisco, CA) (29). These heterozygotes were intercrossed in our animal facility, and the progeny were genotyped by PCR amplification of tail DNA according to the method of Goes et al. (30). Only homozygotes (IFNγ-/-) were used in this study. C57BL/6-backcrossed mice with the disrupted IFNγR were obtained from Dr Michel Aguet (Institute of Molecular Biology I, Zürich, Switzerland) (31). They were further crossed to the C57BL/6 backgound in our animal facility, and screened by PCR of genomic DNA as previously described (31). The heterozygotes were interbred to yield homozygous (IFNγR-/-) mice. All mice with disrupted IFNγR used in this study were homozygous (IFNγR-/-), and wild-type littermates (IFNγR+/+) were used as controls.

Induction of EAE

EAE was elicited by s.c. immunization (base of tail) with an emulsion containing 400 μg of bovine MBP (prepared as described by Cheifetz et al. (32) or purchased from Sigma (Montreal, Canada)) and 50 μg of Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) in CFA (Difco) and boosted in the flanks 7 days later with the same amount. Mice were monitored daily for clinical signs of EAE that was scored as: 1, flaccid tail; 2, hindlimb weakness and poor righting ability; 3, inability to right and one hindlimb paralyzed; 4, both hindlimbs paralyzed with or without forelimb paralysis and incontinence; and 5, moribund. All mice were kept in specific pathogen-free environment. Animal breeding and maintenance, and all experimental protocols were in accordance with the Canadian Council for Animal Care guidelines and approved by McGill University animal care committee.

In vitro proliferation assay of lymph node cells (LNC)

A single cell suspension was prepared from the draining lymph nodes 14 days after the first immunization, and cells (4 x 10^6/ml) were cultured for 4 days in 200 µl/well with or without 50 µg/ml MBP in RPMI 1640 (Life Technologies, Burlington, Canada) supplemented with 10% FCS (Upstate Biotechnology, Lake Placid, NY), 50 mM 2-ME (Sigma), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies), and 100 µg/ml streptomycin (Life Technologies). Cultures were pulsed with 0.5 µCi [^{3}H]thymidine/well (ICN Biochemicals, Mississauga, Canada) during the last 18h of incubation. [^{3}H]thymidine uptake was measured as counts per minutes (cpm).

Histology and immunohistochemistry

Mice were anesthetized with sodium pentobarbital (MT Pharmaceutical, Cambridge, Canada) and perfused intracardially through the left ventricle with ice-cold PBS for OCT-embedded tissues, or followed by 10% buffered formalin for paraffin-embedded tissues. 1µm paraffin sections were stained with hematoxylin and eosin (H&E), or Luxol Fast Blue to assess demyelination. Immunohistochemical staining was performed on 10 µm cryostat sections. Frozen sections were blocked in 5% normal rabbit serum (Vector, Mississauga, Canada) in PBS for 30 min at room temperature, incubated with primary rat mAbs (mAb) 1 h at room temperature or overnight at 4°C, then with biotinylated rabbit anti-rat Ig (Vector) for 1 h at room temperature. Sections were treated with 0.3% H_2O_2 to quench endogenous peroxidase activity, then incubated with an avidin-horse peroxidase complex (Vectastain ABC kit, Vector Labs), following the manufacturer's instructions. Biotin-avidin complex binding was detected by the use of diaminobenzidene (Medicorp, Montreal, Canada) as chromagen. The mAbs used were: GK1.5 (CD4, American Type Culture Collection, Manassas, VA), F4/80 (kindly provided by Dr. Georg Kraal, Vrije Universiteit, Amsterdam), M1/70 (Mac-1/CD11b, American Type Culture Collection), P7/7.1 (MHC II, American Type Culture Collection), RB6-8C5 (Gr-1/Ly6G, PharMingen, Mississauga, Canada), and

MEC 13.3 (CD31/PECAM-1, PharMingen). Control sections were incubated with isotype-matched primary Abs or with secondary Abs alone. Staining for iNOS using polyclonal anti-mouse iNOS (Transduction Laboratories, Lexington, KY) was performed as previously described (33).

Electron microscopy

Mice were perfused with PBS, followed by 0.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Tissues were postfixed in 2% osmium tetroxide, dehydrated in graded concentrations of methanol, cleared in propylene oxide, and embedded in Epon. Ultrathin sections of spinal cord were mounted on nickel grids, stained with uranyl acetate and lead citrate, and examined by electron microscope.

Flow cytometry analysis

After perfusion with ice-cold PBS, brains were removed and spinal cords were dissected from the vertebral canal, taking care to collect the meninges. Isolation of cells from the CNS was performed as previously described (34). Briefly, tissues were dissociated in RPMI 1640/10% FCS by passing through a metal sieve, then centrifuged at 400 x g for 10 min at 4°C. The pellet was resuspended in 70% isotonic Percoll (Pharmacia), overlaid with equal volumes of 37% and 30% isotonic Percoll, and centrifuged at 500 x g for 20 min at room temperature. Cells were collected from the 37%:70% interface and washed with RPMI 1640/10% FCS. Cells were first incubated on ice for 30 min with 100 μg/ml normal rat Ig in 2.4G2 (anti-FcγRIIb/III) supernatant to block Fc receptors and avoid nonspecific staining, then double stained with phycoerythrin-conjugated anti-CD4 (PE-CD4, Becton Dickinson, Mississauga, Canada) and fluorescein-conjugated anti-CD3 (FITC-145.2C11), FITC-conjugated anti-CD69 (PharMingen) or biotinylated anti-CD25 (PC61, American Type Culture Collection) mAbs, which were visualized by FITC-coupled streptavidin. Cells were also double stained with fluorescein-conjugated anti-Mac-1/CD11b (M1/70) and biotinylated

anti-B7.2/CD86 (GL1, PharMingen), which were visualized by phycoerythrin-coupled streptavidin. Cells were analyzed using a FACScan (Becton Dickinson). Propidium iodide staining and forward/side scatter gating were used to exclude dead cells.

RNase Protection Assay (RPA)

Total RNA was purified from homogenized PBS-perfused CNS using TriZol (Life Technologies) following the manufacturer's instructions. Multiprobe DNA templates for chemokines (lymphotactin, RANTES, eotaxin, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1, and TCA-3), and for cytokines (IFNγ, IL-2, IL-3, IL-4, IL-5, IL-9, IL-10, IL-13, and IL-15), and for the housekeeping genes, L32 and GAPDH, were all purchased from PharMingen. RPA was performed according to the manufacturer's protocol. Briefly, the DNA templates were used to synthesize antisense riboprobes, which were labeled with α-[^{32}P]UTP (Dupont NEN Research Products, Guelph, Canada) using T7 polymerase. Labeled probes were hybridized with 20 μg of total RNA at 56°C for 16h. Samples were then digested with RNase A and T1, and treated with proteinase K. The remaining RNase-protected RNA duplexes were extracted with phenol/cholorofom/isoamyl alcohol (Gibco), and resolved on 5% denaturing polyacrylamide gels. Undigested labeled probes were loaded in the gels to serve as size markers. Dried gels were visualized by autoradiography or PhosphorImager (Molecular Dynamics, Sunnyvale, CA) after an exposure of 12-48 hours for chemokines and 4-7 days for cytokines.

RT-PCR

RNA was reverse-transcribed with 10 μM random hexamer primers (Boehringer Mannheim), 0.5 mM each dNTPs (Pharmacia), 3.3 mM DTT (Life Technologies), and 400 U Moloney murine leukemia virus-RT (Life Technologies) at 42°C for 1 h; this was terminated by heating at 75°C for 10 min.

PCR conditions were optimized for linear amplification to allow direct comparison between samples. Equal amounts of cDNA were amplified using 1x PCR buffer (Sigma), 2 mM $MgCl_2$ (Sigma), 80 μM each dNTPs, 2 U Taq polymerase (Sigma), and 50 pmol of each primer. The PCR reaction was performed with a Perkin-Elmer (Mississanga, Canada) thermocycler for 30 cycles with denaturation/annealing/extension conditions optimal to each primer sets: IL-10 (94°C 45sec, 58°C 45sec, 72°C 1min); IFNγ (94°C 30sec, 65°C 30sec, 72°C 1min), IL-12p40 (94°C 45sec, 65°C 45sec, 72°C 1min) and β-actin. The primer sequences for IL-10 were: sense, 5'-TGGCTCAGCACTGCTATGCT-3', and antisense, 5'-ATGGCCTTGTAGACACCTTG-3'. The other primer sequences were described elsewhere as follows: IFNγ and β-actin (35); IL-12p40 and IL-4 (36). For IL-4 and β-actin duplex RT-PCR, cDNA were amplified with 1x PCR buffer (Sigma), 4 mM $MgCl_2$ (Sigma), 120 μM each dNTPs, 4 U Taq polymerase (Sigma), and 50 pmol of each IL-4 primer and 10 pmol of each β-actin for 30 cycles (94°C 45sec, 56°C 45sec, 72°C 1min). PCR products were electrophoresed in a 1.8% agarose gel, visualized by ethydium bromide or SYBR Green staining (Molecular Probes, Eugene, OR), and analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

IFNγ-/- and IFNγR-/- mice develop lethal EAE following immunization with MBP

Immunization with MBP induces an MS-like relapsing-remitting course of disease in SJL/J (H-2^s) mice. BALB/c (H-2^d) and C57BL/6 (H-2^b) mice are resistant to MBP-induced EAE (14, 37). However, whether on a susceptible or a resistant background, mice lacking IFNγ have been shown to develop EAE with unsually high mortality following immunization with MBP (26, 27, 38). In Table 1, we show that MBP also induced lethal EAE in mice deficient in IFNγR on a C57BL/6 background, while their wild-type littermates were resistant. All IFNγ-/- and IFNγR-/- mice exhibited complete paralysis of both fore- and hindlimbs and immobility, and they died or were euthanized within 24-48h after onset. By contrast, SJL/J mice showed a wide range of severity ranging from mild to severe disease, but all of them remitted. As we have consistently observed (39, 40), some of the SJL/J mice that remitted showed a relapsing progression. IFNγR-/- mice immunized with CFA alone did not develop signs of neurological deficit (Table 1). The rapidly progressive course of disease and lethality observed in both IFNγ-/- and IFNγR-/- mice are not features of typical relapsing-remitting EAE, suggesting unique CNS pathology related to the lack of IFNγ response.

Disseminated neutrophil invasion and demyelination in IFNγ-/- and IFNγR-/- mice

To understand how IFNγ influences histopathology, we compared mice that lack IFNγ response (IFNγ-/- and IFNγR-/- mice) to SJL/J mice in which IFNγ was up-regulated during EAE and its receptor was intact. Neither BALB/c nor C57BL/6 mice developed EAE following MBP/CFA immunization; therefore, they could not be used in our comparative study of CNS pathology. To characterize the acute lethal disease provoked in IFNγ-/- or IFNγR-/- mice, CNS inflammation and demyelination were assessed. Findings in IFNγ-/- and IFNγR-/- mice were indistinguishable from each other with respect to all parameters examined. H&E staining of spinal cord and brainstem from IFNγ-/- mice with fulminant clinical signs of EAE revealed extensive infiltration by cells morphologically identifiable as

polymorphonuclear leukocytes (PMN; Fig. 1A). The cerebellum was not affected. This contrasts with acute non-lethal, EAE, whether mild or severe, in SJL/J mice. Infiltration in SJL/J mice was characterized by perivascular cuffs containing predominantly mononuclear cells (Fig. 1B), and occuring in spinal cord, brainstem and cerebellum.

The presence of PMN's was further confirmed by immunohistochemical staining using the RB6-8C5 antibody that recognizes Gr-1/Ly6G on neutrophils (41). Fig. 1D and F shows widespread, disseminated infiltration by Gr-1^+ neutrophils that formed confluent hypercellular areas in the meninges, white matter, and extending into gray matter of IFNγ-/- and IFNγR-/- mice with fulminant EAE. Although Gr-1^+ cells could be identified within the vascular lumen and perivascularly, they did not appear to be restrained within defined cuffs around PECAM-1^+ activated endothelial cells (Fig. 1L). Staining for Mac-1 was also widely disseminated in IFNγ-/- spinal cord (Fig. 1C). In SJL/J mice with paralytic EAE on day 16 post-immunization, neutrophils were also detectable in infiltrates but were less numerous and were confined to perivascular locations in the meninges and subpial areas, and did not spread into gray matter (compare Fig. 1D vs E). Also, they were less numerous in infiltrates than Mac-1^+ cells (Fig. 1J vs G). Even fewer neutrophils were noted in SJL/J mice examined at earlier time points or with lesser disease severity. Infiltrating leukocytes in SJL/J mice formed perivascular cuffs around PECAM-1^+ endothelial cells (Fig. 1I). ICAM-1 was also strongly up-regulated in SJL/J, IFNγ-/- and IFNγR-/- mice (not shown). Thus, lethality was associated with marked neutrophilia in the absence of IFNγ or its receptor.

Neutrophil infiltration in IFNγ-/- and IFNγR-/- mice was accompanied by extensive demyelination. Luxol Fast Blue was used to stain myelin in Fig 2A,C. Loss of myelin was localized to areas of spinal cord dominated by PMN infiltration (Fig. 2A), so that some sections at other levels showed no evidence of pathology (Fig. 2C). As expected, in SJL/J mice, focal zones of demyelination surrounded infiltrated vessels often in subpial infiltrates

(not shown). Widespread neutrophil infiltration and demyelination in IFNγ-/- mice were further confirmed on epoxy sections stained with toluidine blue (Fig. 2E). Moreover, electron micrographs revealed the presence of macrophages and PMN's phagocytosing myelin residues (Fig. 2B,D). Neutrophils with the ultrastructure of condensed nuclear chromatin consistent with apoptotic cell death were not observed (Fig. 2B,D). Reactive astrocytes, identified by their hypertrophied appearance, were also prominent in inflammatory and demyelinating regions (not shown). Eosinophils could be identified among the PMN's in knockout mice but were not detectable in SJL/J mice. Fig. 2F shows an electron micrograph of cells with characteristic bilobed nuclear morphology and large ovoid granules in a demyelinated region of spinal cord of IFNγ-/- mice. These were a minority compared to neutrophils.

Macrophage/microglial reactivity

To assess the presence and reactivity of monocytes/macrophages and microglia, we stained for F4/80. Unlike CD11b/Mac-1 which is up-regulated on macrophages/microglia and neutrophils after activation, the expression of the F4/80 glycoprotein is restricted to macrophages, and becomes detectable on activated microglia. The F4/80 molecule was not detected in CNS tissues of a naive mouse (not shown), but was strongly up-regulated on macrophages/microglia in the spinal cords of SJL/J mice with EAE (Fig. 1H). $F4/80^+$ cells with amoeboid/round morphology were intermingled with infiltrating lymphocytes within inflammatory foci, and some $F4/80^+$ reactive microglia of 'dendritic' morphology were also dispersed in the parenchyma (Fig. 1H). Mac-1 staining paralleled that of F4/80 in SJL/J mice. F4/80 staining in CNS of IFNγ-/- or IFNγR-/- mice differed from that in SJL/J mice in both intensity and distribution. The spinal cords of IFNγ-/- or IFNγR-/- mice with acute lethal EAE contained weakly stained $F4/80^+$ cells. These were found girdling the neutrophil-dominated infiltrates (Fig. 1K). No $F4/80^+$ cells with 'dendritic' microglia-like morphology were found scattered in the parenchyma of mice deficient in IFNγ response. As expected,

Mac-1 staining followed the same pattern as Gr-1+ neutrophils (compare Fig. 1C vs D), consistent with neutrophil activation. The iNOS immunoreactivity in the CNS of IFNγ-/- or IFNγR-/- mice with EAE was barely detectable, whereas in SJL/J mice (not shown), infiltrates were strongly stained for iNOS during EAE.

$CD4^+$ T cell infiltration and lack of MHC II induction

Disseminated infiltrates in IFNγ-/- mice contained $CD4^+$ T cells that were interspersed with $Gr\text{-}1^+$ cells (Fig. 3A, top panels). $CD4^+$ cells had the appearance of lymphocytes with scanty cytoplasm and were discretely stained. By contrast, $CD4^-$ cells frequently showed PMN phenotype, having larger cell bodies that were intensively stained for Gr-1. Of note is that Gr-1 staining appeared to extend beyond the cell bodies of PMN, possibly reflecting involvement of extracellular matrix, and formed a densely packed network. Strikingly, no MHC II immunoreactivity was observed in the CNS of IFNγ- or IFNγR-knockout mice with EAE (Fig. 3A, bottom panels). However, flow cytometric analysis of cells recovered from the CNS of IFNγ-/- mice with EAE showed that up-regulation of B7.2 occurred (Fig. 3B), and that both CD69 and CD25 were expressed on infiltrating $CD4^+$ T cells (Fig. 3D,E), consistent with recent T cell activation.

Up-regulation of MIP-2 and TCA-3 mRNA in CNS

Chemokines influence leukocyte invasion by virtue of their ability to selectively attract and activate subsets of leukocytes. To assess whether the unusual infiltration of the CNS in mice with defective IFNγ response reflected a chemokine imbalance, we performed RPA to study chemokine gene expression. As expected, no chemokine mRNA was observed in perfused CNS of naive mice, while multiple chemokine mRNA transcripts were up-regulated during EAE in all mice (Fig. 4). In SJL/J mice with MBP/CFA-induced EAE, induction of RANTES and MCP-1 predominated over that of other chemokines, including lymphotactin, eotaxin, IP-10, MIP-1α, MIP-1β, and MIP-2 transcripts, and TCA-3 was not detectable (Fig.

4A,B,C). Similar chemokine expression profiles were observed in spinal cords of SJL/J mice with passively transferred EAE (Fig. 4A), suggesting that chemokine gene expression was not influenced by adjuvant. Mice with impaired IFNγ response, however, displayed a chemokine gene expression profile distinct from that of wild-type mice. MIP-2 and TCA-3 mRNA were markedly up-regulated while RANTES and MCP-1 mRNA were barely detectable in spinal cord of IFNγ-/- (Fig. 4B) and IFNγR-/- mice (Fig. 4C) with fulminant EAE. Quantitative analysis revealed a 10-fold increase in MIP-2 transcripts levels in IFNγ-/- mice compared with that in SJL/J mice with EAE. Conversely, RANTES transcript levels were 20-fold more abundant in SJL/J mice than in IFNγ-/- mice with EAE.

No Th2 cytokine switch

IFNγ promotes Th1 responses and suppresses development of Th2 responses. In the absence of IFNγ, it was possible that Th2 cytokine production would predominate. We therefore examined whether the lack of IFNγ or IFNγR led to a Th2 cytokine response in the CNS.

RPA analysis of RNA from spinal cord of IFNγ-/- mice with EAE did not detect mRNA transcripts for the Th2 cytokines, IL-4, IL-5, IL-9, IL-10 and/or IL-13 (Fig. 5A). The absence of IL-4 mRNA in the CNS of both IFNγ-/- and SJL/J mice with EAE was further confirmed by a more sensitive RT-PCR assay (Fig. 5B). Interestingly, IL-10 mRNA was not expressed in the absence of IFNγ, although it was detectable in SJL/J mice with EAE (Fig. 5B).

As expected, expression of IFNγ mRNA was not detected by RPA (Fig. 5) or RT-PCR (not shown) in CNS of IFNγ-/- mice, while it was up-regulated in SJL/J mice with EAE. IFNγR-/- mice up-regulated IFNγ mRNA to lower levels than those in SJL/J mice (not shown). The cytokines IL-2 and IL-12 are also hallmarks of Th1 responses. Comparable levels of IL-2 mRNA transcripts were noted in spinal cords of both IFNγ-/- and SJL/J mice with EAE (Fig.

5A). Consistent with previous studies (36), IL-12p40 mRNA was only weakly detected in spinal cords of unimmunized SJL/J mice, and levels were strongly elevated in EAE (Fig. 5C). In contrast, levels of IL-12p40 mRNA in spinal cords of IFNγ-/- or IFNγR-/- mice with EAE were indistinguishable from those in unimmunized BALB/c, IFNγ-/-, and IFNγR-/- mice (Fig. 5C). Taken together, these data make it unlikely that a Th2 cytokine bias was responsible for the PMN-dominated CNS pathology seen in mice lacking an IFNγ response.

IFNγ disruption in BALB/c enhances T cell response to MBP

Finally, we were interested in how deficiency in IFNγ response overcame resistance to MBP-induced EAE in BALB/c mice. To test whether disease induction was associated with enhanced T cell reactivity against MBP, we assessed the proliferation of draining LNC from MBP/CFA-primed IFNγ-/- mice, wild-type BALB/c and SJL/J controls in a recall response to MBP in vitro. Fig. 6 shows proliferative responses of LNC, 14 days after priming with MBP/CFA. LNC from IFNγ-/- mice on a BALB/c background that become EAE-susceptible proliferated as strongly as SJL/J LNC in response to MBP, despite the higher background response that is characteristic of GKO mice (29). Proliferative responses increased as the cell density in the culture increased, and consistently paralleled those of SJL/J mice (not shown). BALB/c LNC did not incorporate thymidine in response to MBP to a greater extent than background, at any cell density (Fig. 6).

Discussion

Evidence to date has pointed to a detrimental role for IFNγ in the CNS (35, 42, 43). We have now shown that in the presence of an IFNγ response, the typical T cell and macrophage chemoattratants, RANTES and MCP-1, and infiltrating mononuclear cells predominate in CNS to produce a non-lethal remitting EAE, while in its absence, MIP-2, TCA-3 and PMN prevail, producing an acute, lethal EAE. Our data thus cast new light on the role of IFNγ in autoimmune CNS inflammation, as a primary regulator of chemokine profiles. CNS-infiltrating $CD4^+$ T cells are the major sources of local IFNγ (44), which now appears essential not in the induction but, rather, in the evolution of the autoimmune attack. Our data further suggest that IFNγ plays an anti-proliferative role in EAE-resistant BALB/c mice, such that T cell responses to MBP become detectable in IFNγ-/- animals. We propose that IFNγ shapes an autoimmune inflammatory response, and exerts a protective role by restricting neutrophil infiltration, activation and proliferation.

The potential for neutrophil infiltration to the CNS probably exists to mediate host resistance to infection (45). CNS neutrophilia is also associated with acute stroke, traumatic brain injury, fatal hemorrhagic leukoencephalitis, and acute primary progressive forms of EAE and MS (46-49). These acute reactions often lead to rapid death. Hence, it is important to unravel mechanisms regulating immune cell invasion and activation in inflamed tissues to provide insights into efficient therapeutic intervention. McColl et al. (49) reported that neutrophil depletion inhibits MBP-induced EAE in SJL/J mice and MOG-induced EAE in IFNγR-/- mice, suggesting a crucial role for PMN in the pathogenesis of this inflammatory disease. In their study, EAE was induced by immunization with MBP or MOG using pertussis toxin as an adjuvant. Pertussis toxin increased blood neutrophilia and vascular permeability (49), making it difficult to dissect mechanisms governing immune cell infiltration of the CNS by IFNγ, and those pertaining to T-cell mediated pathogenesis.

We describe an IFNγ-regulated neutrophilia in the CNS induced without pertussis toxin or other systemic immunomodulation. IFNγ-/- or IFNγR-/- mice may be predisposed to enhanced myelopoeisis and granulocytosis in the blood and spleen (50, 51), but we found selective recruitment to the CNS. Organs that were not targets of neuroantigen-reactive $CD4^+$ T cells, such as kidneys, were not affected by neutrophilia in IFNγ-/- mice. The presence of activated $CD4^+$ T cells in the CNS argues for antigen-directed infiltration and focuses attention on the T cells as regulators of immune invasion. IL-3, a growth factor for granulocytes, was up-regulated in the CNS of IFNγ-/- mice, and may have contributed to neutrophil accumulation. Local expansion of neutrophils in CNS may also be dysregulated in the absence of an IFNγ response. IFNγ is therefore a key cytokine in coordinating regulation of local autoimmune response. These data further point to chemokines, MIP-2 and TCA-3, as inducers of neutrophil invasion.

In rodents, MIP-2 acts preferentially on neutrophils and is functionally analogous to human IL-8. Overproduction of MIP-2 using recombinant human adenovirus induced prolonged PMN recruitment to the murine brain (52). MIP-2 is implicated in neutrophil recruitment in bacterial meningitis (53). Anti-MIP-2 treatment reduced neutrophil infiltration and improved survival (53). In the mouse, TCA-3 also acts on neutrophils (54). Our study is the first to show a correlation between MIP-2 and TCA-3 up-regulation, enhanced neutrophilia in a T cell-mediated autoimmune disease.

Downregulation of MIP-2 expression by IFNγ might be direct (as in ref. 55) or could be via the action of IL-10. IL-10 was not detectable in the CNS of IFNγ-/- mice while it was consistently up-regulated in IFNγ-intact SJL/J mice. Significantly reduced production of IL-10 was reported from IFNγR-/- mice in response to viral antigen (31). Inhibition of IL-10 bioactivity in vivo resulted in a sustained increase in MIP-2 levels (56). IL-10 has been shown to suppress macrophage and neutrophil activities, including cytokine, chemokine, and

superoxide production (57). Our finding that mice lacking IFNγ did not express detectable IL-10 is consistent with neutrophil activation and unopposed MIP-2 up-regulation, and supports a disease-modulating role of IL-10 (27, 58, 59).

TNFα, superoxide radicals and NO are implicated as mediators of demyelinating pathology (14, 60). In IFNγ-/- or IFNγR-/- mice, demyelination probably involved reactive oxygen intermediates (ROI) and/or TNFα rather than NO, as very little iNOS immunoreactivity was evidenced in areas of infiltration and demyelination in the spinal cords. IFNγ appears as a crucial stimulator of iNOS protein expression as lack of iNOS protein has consistently been reported in IFNγ-/- or IFNγR-/- mice in other infectious or inflammatory diseases (61). Whether reactive oxygen intermediates and/or TNFα are the mediators, activated neutrophils were clearly implicated in the demyelination process.

While IFNγ abrogates MIP-2 production, it promotes RANTES and MCP-1 expression, probably via the synergistic action of TNFα. IFNγ was shown to synergize with TNFα to induce human glial cells to increase RANTES production in vitro (62). In mice with intact IFNγ response, there is a strong up-regulation of RANTES and MCP-1 in the CNS during EAE (4, 20, 63, 64). The fact that such up-regulation did not occur in IFNγ-/- and IFNγR-/- mice suggests that TNFα, known to be present in IFNγ-/- mice (26), is insufficient for response. Our finding that IFNγ is an important stimulator of RANTES production in EAE is consistent with previous data for other inflammatory diseases in the CNS, such as lymphocytic choriomeningitis (65). That MCP-1 expression was barely detectable in our IFNγ-/- mice with EAE is also in agreement with the in vitro evidence that IFNγ up-regulates MCP-1 gene transcription (66).

One of the effects of IFNγ is to suppress the development of Th2 cytokines. Experimental autoimmune thyroiditis and uveitis, both Th1-mediated diseases, were reported to become

biased to Th2-type response in IFNγ-/- mice (67, 68). Th2-induced pathologies in these diseases were dominated by granulocyte infiltration. Myelin-reactive Th2 cells do not induce EAE in immunocompetent animals, but could transfer disease in immunocompromised mice, and the resulting CNS pathology was again dominated by PMN (13). Similar observations were made in a diabetes model (12). These findings suggest that Th2 cytokines might prevail if EAE were induced in IFNγ response-deficient mice. Furthemore, in CNS of mice lacking IFNγ response there was an up-regulation of TCA-3 which, like its human homologue I-309, is a potent chemoattractant for Th2-polarized cells (5). However, we found no evidence for a Th2 cytokine switch in IFNγ-/- mice during EAE. This argues that PMN-dominated autoimmune pathology is more likely related to lack of IFNγ regulation of chemokine profiles, rather than to effects of Th2 cytokines.

IL-12 favors the development of a Th1 response, and is crucial in the pathogenesis of EAE (27, 69). Systemic administration of anti-IL-12 mAb starting at the time of immunization blocked EAE in IFNγ-intact or -deficient mice (27, 69). Development of pathogenic autoreactive T cells was abrogated after such systemic IL-12 neutralization or in IL-12-/- mice (27, 69). We did not detect an enhanced induction of IL-12p40 mRNA in the CNS of IFNγ-/- mice, unlike the case in SJL/J mice. One interpretation of our data could be that disease induction or progression does not require the up-regulation of IL-12 in the local CNS microenvironment, and that the endogenous baseline level of IL-12 is sufficient for the generation of a local immune response.

Interestingly, there was no detectable MHC II staining in infiltrated CNS in IFNγ-/- or IFNγR-/- mice with EAE. This contrasts with IFNγ-intact mice in which MHC II is strongly up-regulated, both on infiltrating macrophages and reactive glial cells. Although MHC II induction in the CNS was IFNγ-dependent, B7.2 up-regulation did not depend on IFNγ function. The fact that the T cells in CNS were $CD69^+$ and $CD25^+$ suggests that basal levels

of MHC II expression were sufficient for disease induction, although such levels were below the limit of detection by immunohistochemical staining.

It is noteworthy that activated Th1 cells were found in the CNS of IFNγ-/- mice in the absence of CC chemokines (RANTES, MIP-1α and MCP-1) usually associated with a Th1 response. We have shown elsewhere that MIP-1α is dispensable for Th1 infiltration in EAE (Tran, E.H., Kuziel, W.A., and Owens T., Eur. J. Immunol. *In press*), and RANTES and MCP-1 may be similarly dispensable. The chemokine IP-10 has been associated with Th1 responses in the CNS (21,22), and it is possible that, despite the low residual levels, it promoted Th1 recruitment in absence of IFNγ. Other chemokines, not assayed in our experiments, such as Mig, neurotactin, and TCA-4, might also serve as IFNγ-independent recruitment stimuli for Th1 cells to the CNS. Alternatively, activated T cells might not be dependent on specific chemokines to traffic to the CNS, but may act to induce chemokine production by glial cells. Thus, IFNγ-deficient T cells, or Th1's in an IFNγ-unresponsive CNS, might promote a chemokine profile that induces neutrophil infiltration rather than macrophages.

The mice that we studied were on a variety of strain backgrounds. It was not possible to match backgrounds, due to the inherent resistance of wild-type mice to MBP-induced EAE. However, the fact that IFNγ-/- and IFNγR-/- mice, on different strain backgrounds showed identical patterns of chemokine expression, infiltration and disease progression suggests that the influence of IFNγ response overrides other potential influences. Likewise, patterns of infiltration and cytokine and chemokine production are similar in SJL/J mice with MBP-induced EAE and in C57BL/6 mice with MOG-induced EAE (63; E. H. Tran, V. Asensio, T. Owens, and I. Campbell, unpublished observations; and M. Hassan-Zahraee, E.H. Tran, and T. Owens, manuscript in preparation). Whether susceptibility and neutrophil pathology could reflect enhanced pathogenic activity of T cells is not supported by the fact that proliferative

responses of IFNγ-/- LN T cells to MBP were never greater than those of SJL/J mice. Furthermore, although there was a high basal proliferative activity of LN T cells, immunization of IFNγ-/- or IFNγR-/- mice with PBS/CFA (Table 1) or OVA/CFA (not shown) did not induce disease, and maximization of EAE symptoms and penetration in SJL/J mice using adoptive transfers with high T cell numbers does not induce an IFNγ$^{-/-}$-like pathology. Thus, the simplest and most plausible interpretation of our findings is that the susceptibility of IFNγ-/- or IFNγR-/- mice to EAE can be attributed to the absence of IFNγ regulation, leading to chemokine imbalance and lethal neutrophil invasion.

Our findings suggest a model for events leading to induction of EAE, and MS. IFNγ is an inhibitor of cellular proliferation, and our data show that IFNγ deficiency overcame EAE resistance in BALB/c mice, by overriding the inability of their LNC to proliferate in response to MBP. For initiation of disease, a supra-threshold frequency of autoantigen-specific CD4$^+$ T cells is required. This entails clonal expansion through proliferation. Strains of animals in which T cell proliferation is curtailed, such as by the action of IFNγ, will not initiate disease. Our experiments, as well as those of Yoshizawa et al (70), confirm the presence of potentially encephalitogenic T cells in BALB/c. Work by Yoshizawa et al (70) supports the idea that a high frequency of these T cells, as obtained via in vitro expansion, can transfer disease in BALB/c. Once sufficient numbers of T cells are activated, they migrate to the CNS. Although migration may itself depend on macrophages (71), whether macrophages or neutrophils are finally recruited to the CNS is dependent on whether or not the CD4$^+$ T cells secrete IFNγ. This model places IFNγ in the pivotal role of directing eventual disease outcome, via its primary regulation of chemokine secretion. Our model makes no prediction as to the cellular source of chemokines, although CNS glia and leukocytes are plausible candidates. One intriguing possibility that is suggested from this model is that the usual lack of eosinophils in inflamed CNS may result from the autocrine action of CD28-triggered IFNγ (72). This further predicts that eosinophils in CNS in IFNγ-

intact animals, when present, are not triggered to secrete IFNγ. Analogous considerations may apply to other potential cell sources of IFNγ, so that the interplay between IFNγ secretion, and the chemokines it regulates, directs leukocyte population dynamics in inflamed tissues such as the CNS.

Acknowledgements

We thank Dr Alexandre Prat for comments on the manuscript. We are grateful to Lyne Bourbonnière and Maria Caruso for breeding and screening of knockout mice, Jim Dixon for his expertise in electron microscopy, and Lily Li and Lixia Zhu for advice in histology and technical assistance.

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Table I. MBP-induced EAE in IFNγ-/-, IFNγR-/- mice, wild-type littermates, and SJL/J mice

Mouse strain	Immunization	Incidence of EAE	Day of onset (mean±SD)	Clinical severity (mean±SD)	Remission
SJL/J	MBP/CFA	8/11	14.1±0.5	2.6±1.1	yes
BALB/c	MBP/CFA	0/9	-	-	-
IFNγ-/-	MBP/CFA	7/7	19.8±2.3	4.7±0.5[a]	no
IFNγR-/-	MBP/CFA	15/16[b]	16.8±1.7	4.9±0.5[a]	no
	PBS/CFA	0/5	-	-	-
IFNγR+/+[c]	MBP/CFA	0/5	-	-	-

[a]Mean clinical scores were less than 5 due to the fact that mice were euthanized both for ethical reasons (grade 5) as well as for histology and RNA analyses, which often involved termination before maximal disease.

[b]Data were pooled from three separate experiments.

[c]Littermate controls for IFNγR-/- mice.

FIGURE 1. Spinal cord histopathology of IFNγ-/-, IFNγR-/- and SJL/J mice with EAE at day 16-17 post-immunization. A, B: H&E staining of paraffin sections from an IFNγ-/- (A) and a SJL/J mouse (B) with EAE. Note the disseminated infiltration of PMN (arrows) into the parenchyma away from the inflamed vessel in IFNγ-/- mice (A), and perivascular cuffs of mononuclear cells in SJL/J mice (B). **C, G:** Immunohistochemical staining using anti-Mac-1/CD11b mAb on frozen sections of an IFNγ-/- (C) and a SJL/J (G) mouse. **D, E, F, J:** Anti-Gr-1 staining on frozen sections of IFNγ-/- (D), SJL/J (E) and IFNγR-/- mice (F), and higher magnification of an infiltrate stained for Gr-1 in a SJL/J mouse (J). Note that infiltrates in SJL/J mice contained many Mac-1^+ cells but fewer Gr-1^+ cells (G vs J). **H, K:** Anti-F4/80 staining in a SJL/J (H) and an IFNγ-/- (K) mouse. **I, L:** Anti-CD31/PECAM-1 in a SJL/J (I) and IFNγ-/- (L) mouse. All immunohistochemical stainings were revealed using DAB as chromagen and counterstained with Harris' Hematoxylin, except (I, L) in which AEC was used as chromagen and counterstained with Mayer's Hematoxylin. Original magnification: A,B,H,K (180x); C,D,E,F (20x); G,I,J,L (200x).


A
B
C
D
E
F
G
H
I
J
K
L

FIGURE 2. Extensive demyelination in CNS in mice lacking IFNγ response.

Sections were derived from spinal cord of mice at day 17 post-immunization. **A, C:** Luxol fast blue staining of paraffin-embedded sections, with (A) and without (C) evidence of infiltration, from the spinal cord of an IFNγ-/- mouse with grade 5 EAE. **E:** Toluidine blue staining of an epoxy section of spinal cord of IFNγ-/- mice shows neutrophil infiltration and associated demyelination. **B, D, F:** Electron micrographs of spinal cord from an IFNγ-/- mouse with EAE showing a neutrophil (arrow) engulfing myelin residues (B), and macrophages (ma) phagocytozing myelin residues (D) among other neutrophils (n) and eosinophils (e) in a infiltrated and demyelinated region. A,C (25x); B (1050x); D (2000x); E (200x); F (2400x).

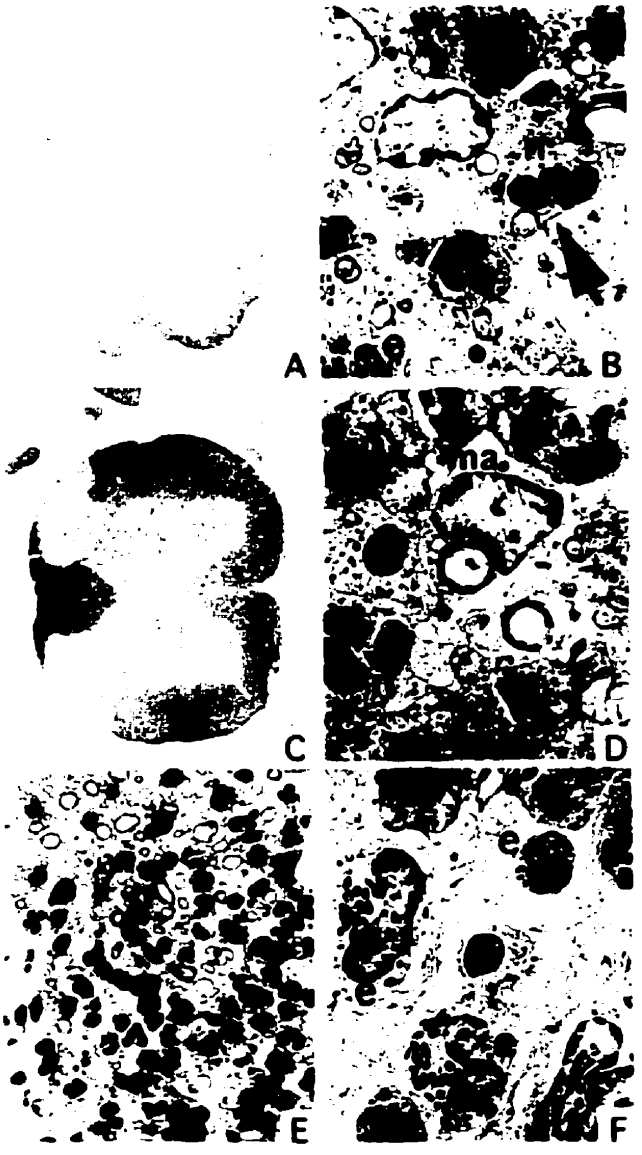
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FIGURE 3. Infiltration of activated CD4$^+$ T cells to CNS of IFNγ-/- mice.

A: Immunohistochemical staining for CD4 and MHC II on spinal cord sections of IFNγ-/- and SJL/J mice with grade 4 EAE at day 17 post-immunization. **B-E**: FACS analysis of cells recovered from the CNS of IFNγ-/- mice with EAE. **B**: Dot plot showing expression of B7.2 and Mac-1/CD11b on live-gated cells. **C-E:** Histograms showing surface staining for CD3 (C), CD69 (D) or CD25 (E) on CD4+ gated cells from the CNS of IFNγ-/- mice with fulminant EAE. Shaded curves represent controls for nonspecific staining.

FIGURE 4. RPA analysis of chemokine mRNA expression in spinal cords of IFNγ-/-, IFNγR-/- and SJL/J mice with EAE. A: Chemokine mRNA expression in a naive (N) SJL/J mouse, or in a SJL/J mouse with active (A) or passive (P) EAE. This was representative of 5 mice. **B:** Chemokine gene expression in SJL/J and IFNγ-/- mice, naive (N) or with EAE. 3 other IFNγ-/- mice with EAE showed the same pattern. **C:** Chemokine gene expression in IFNγR-/-, and SJL/J mice, naive (N) or with EAE (E). This was representative of 2 mice.

Figure 3

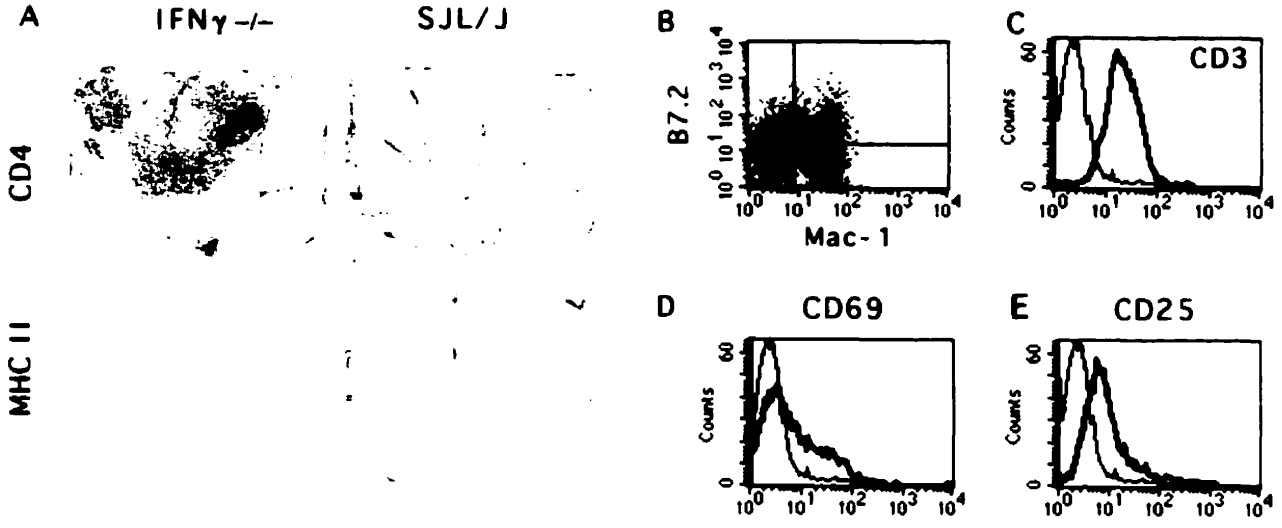


Figure 4

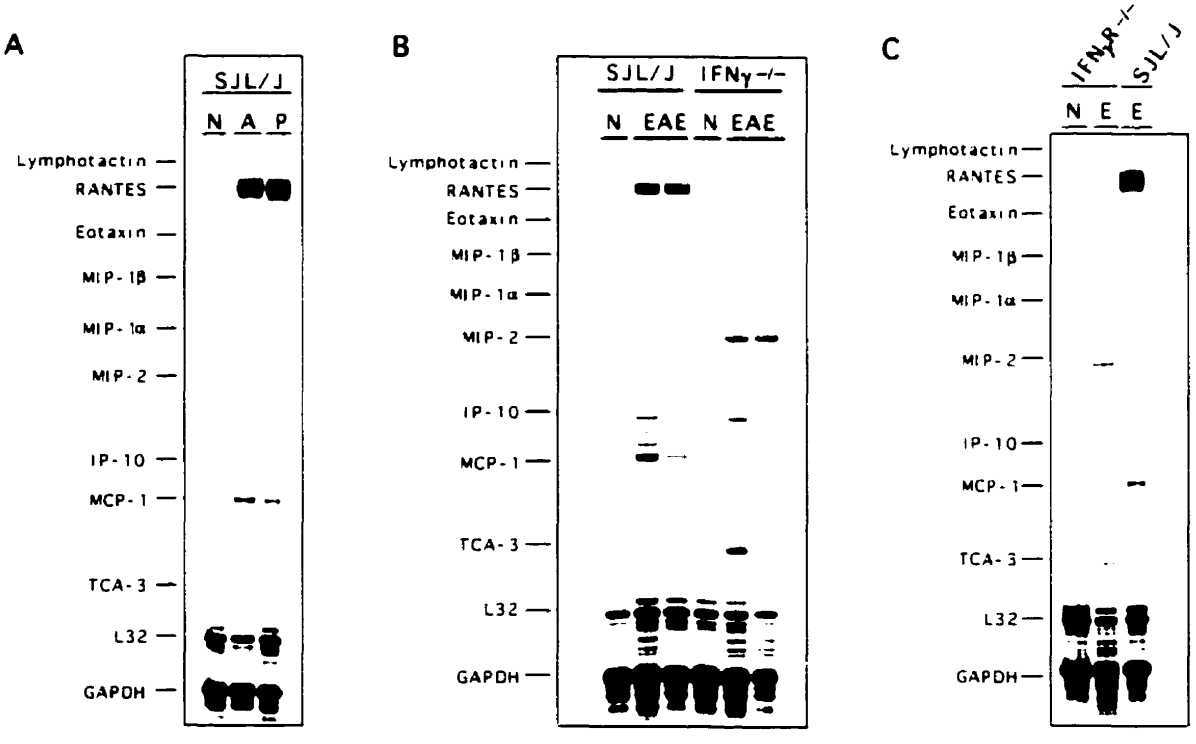


FIGURE 5. Cytokine mRNA expression in spinal cords of IFNγ-/-, IFNγR-/- and SJL/J mice with EAE.

A: RPA analysis of cytokine gene expression in SJL/J and IFNγ-/- mice with (E) and without (N) EAE. **B:** Duplex RT-PCR analysis for IL-4 mRNA and the housekeeping gene, β-actin, and RT-PCR for IL-10 mRNA. +control represents mouse RNA control (PharMingen) extracted from cells in which housekeeping and specific cytokine genes, including IL-4 and IL-10, were expressed using the baculovirus expression system. **C:** Levels of IL-12p40 mRNA were detected by RT-PCR and expressed as arbitrary fluorImager units after normalization to β-actin signals. Bars represent mean ratio (IL-12p40/β-actin) of 3-4 mice/group ± SD in a representative experiment. Unimmunized (-) and immunized (+) with MBP/CFA. All of the immunized mice had developed EAE at the time of analysis, except BALB/c mice.

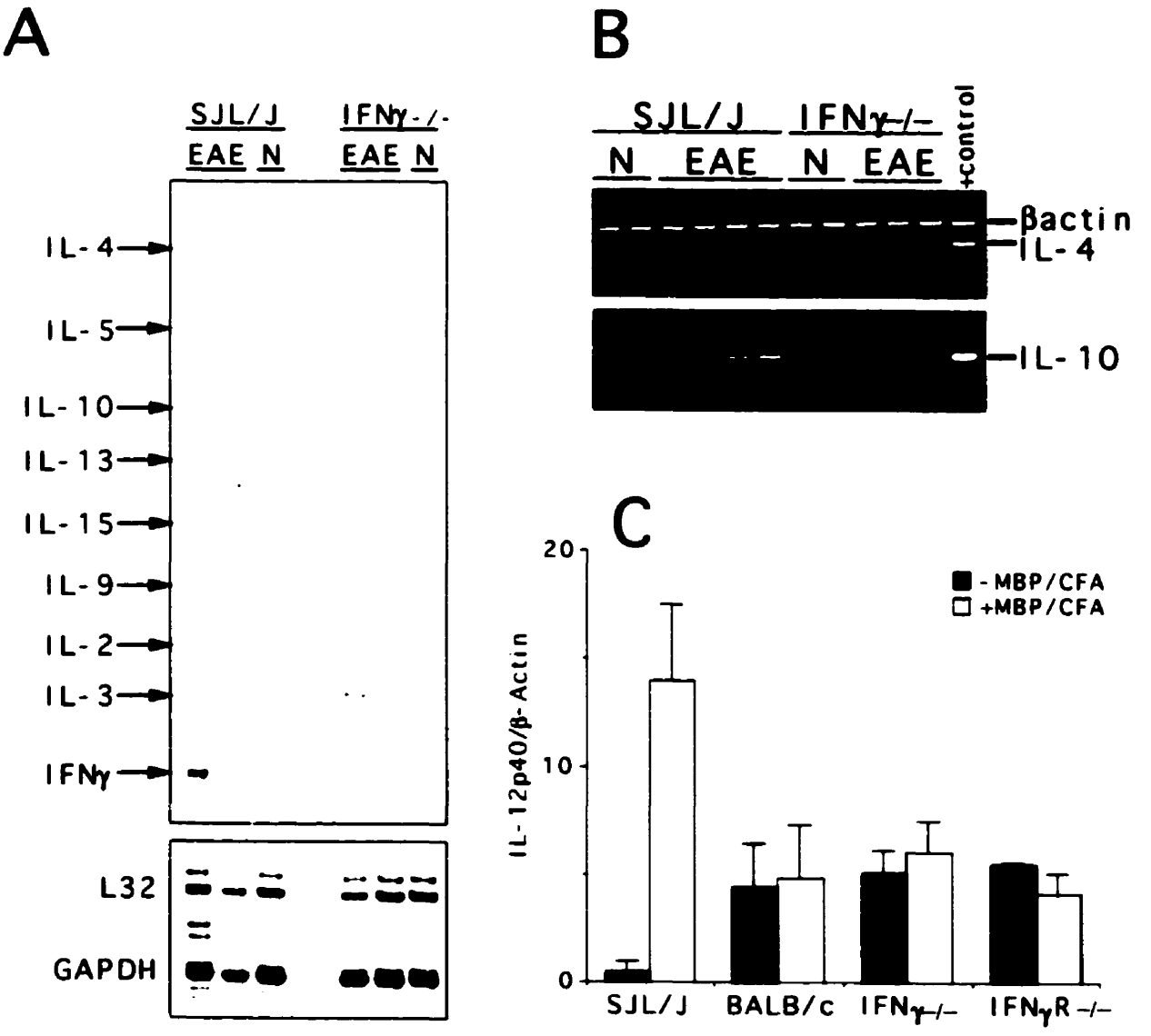
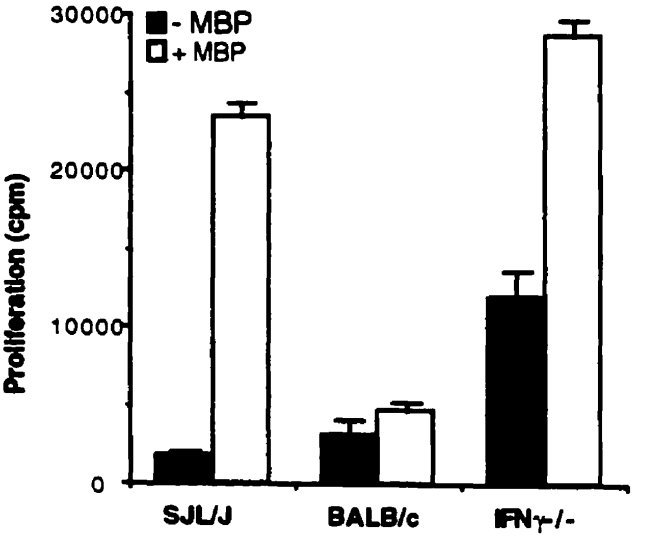

A
SJL/J
IFNγ-/-
EAE N
EAE N
IL-4
IL-5
IL-10
IL-13
IL-15
IL-9
IL-2
IL-3
IFNγ
L32
GAPDH
B
SJL/J
IFNγ-/-
N EAE N EAE
+control
βactin
IL-4
IL-10
C
20
10
0
■ -MBP/CFA
□ +MBP/CFA
IL-12p40/β-Actin
SJL/J
BALB/c
IFNγ-/-
IFNγR-/-

FIGURE 6. Enhanced proliferation of LNC from IFNγ-/- mice in response to MBP. 14 days post-immunization with MBP/CFA, LNC ($8x10^5$/well) from IFNγ-/-, BALB/c and SJL/J mice, were incubated with or without MBP (50 μg/ml) for 4 days, and pulsed with [^{3}H]-Thymidine during the last 18h of incubation. Bars show mean cpm of triplicate cultures with SEM. These data are representative of 6 other experiments. BALB/c LNC responded to PPD (24223 ± 860 cpm). [^{3}H]-Thymidine uptake of stimulated LNC from SJL/J or IFNγ-/- mice was significantly different ($P<0.001$) from their respective backgrounds, while that of stimulated LNC from BALB/c mice was not ($P>0.05$), as determined by one-way ANOVA.


30000
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Proliferation (cpm)
SJL/J
BALB/c
IFNγ-/-

Chapter V

Induction of EAE in C57BL/6 mice deficient in either the chemokine MIP-1α or its CCR5 receptor

Preface

My previous findings suggest that activated myelin-reactive Th1 cells that secrete IFNγ are important in inducing chemokines such as RANTES, MIP-1α and MCP-1 to recruit mononuclear cells in EAE. The relative importance of these chemokines was of interest, especially whether any one of them could be identified as critical to the disease process. To initiate such analysis, I asked whether MIP-1α was necessary for EAE.

Abstract

Macrophage inflammatory protein-1α (MIP-1α) is a chemokine that acts on T lymphocytes and monocytes/macrophages and is associated with Th1 cytokine responses. Expression and antibody blocking studies have strongly implicated MIP-1α in the effector phase of human neuroinflammatory disease multiple sclerosis (MS) and in the corresponding animal model experimental autoimmune encephalomyelitis (EAE) in SJL/J mice. We examined the role of MIP-1α and its CCR5 receptor in the induction of EAE by immunizing C57BL/6 mice deficient in either MIP-1α or CCR5 with myelin oligodendrocyte glycoprotein (MOG). We found that MIP-1α-deficient mice were fully susceptible to MOG-induced EAE. These knockout animals were indistinguishable from wild-type mice in Th1 cytokine gene expression, the kinetics and severity of disease, and infiltration of the central nervous system by lymphocytes, macrophages and granulocytes. RNase protection assays showed comparable accumulation of mRNA for the chemokines IP-10, RANTES, MCP-1, MIP-1β, MIP-2, lymphotactin and TCA-3 during the course of the disease. CCR5-deficient mice were also susceptible to disease induction by MOG. Resistance to MBP-induced EAE, a characteristic of C57BL/6 genetic background, was not affected by lack of MIP-1α or CCR5. The apparent dispensability of MIP-1α and CCR5 for MOG-induced EAE in C57BL/6 mice supports the idea that differential chemokine expression patterns represent a significant difference in disease mechanism that underlies various models of EAE, and possibly distinct patterns of pathology seen in MS.

Introduction

Chemokines are low molecular weight proteins that attract and activate leukocytes. MIP-1α and other chemokines, such as MIP-1β, RANTES, MCP-1 and IP-10, act mainly on lymphocytes and/or monocytes/macrophages. Chemokines bind to specific receptors which, despite some redundancy nevertheless show specificity for individual chemokines. For example, MIP-1α binds and signals through three known CC chemokine receptors, including CCR5 [1].

Many cell types, including lymphocytes, macrophages and central nervous system (CNS) glial cells, can be induced to produce MIP-1α [2-5]. In MS, an inflammatory disorder of the CNS, the expression of MIP-1α and a plethora of other chemokines (e.g., IP-10, MCP-1) are upregulated [6-9]. The frequency of $CCR5^+$ and $CXCR3^+$ T cells is elevated in MS [8-9]. In the rodent model for MS, EAE, there is a tight correlation between expression of these chemokines, their receptors, and the distribution of inflammatory cells infiltrating the CNS as well as the development of clinical disease [10-17].

EAE is a $CD4^+$ Th1-mediated autoimmune inflammatory disease of the CNS. Various myelin proteins/peptides, or $CD4^+$ T cells reactive to these autoantigens, can induce EAE. Susceptibility and disease progression may vary from species to species, and strain to strain. For example, EAE induced in SJL/J mice by MBP or PLP peptides exhibits a relapsing-remitting progression, while a monophasic remitting/nonrelapsing form of disease is usually seen in Lewis rats following immunization with MBP. C57BL/6 mice are resistant to MBP- or PLP-induced EAE, but MOG provokes a chronic disease in this mouse strain.

The importance of MIP-1α in the effector phase of EAE was suggested by the ability of anti-MIP-1α Ab to prevent PLP-specific T cell transfer of EAE in SJL/J mice [18] and by the ability of vaccination with naked DNA encoding MIP-1α to protect Lewis rats from MBP-

induced EAE [19]. In the present study, we directly examined whether C57BL/6 mice deficient in either MIP-1α or its CCR5 receptor are resistant to induction of EAE after immunization with the MOG35-55 peptide. Surprisingly, we found that the genetic absence of MIP-1α or CCR5 did not block induction of EAE in this model. These results strongly support the emerging view that the pathogenic mechanisms behind the various EAE models, and perhaps the distinct patterns of MS pathology, involve differential chemokine regulation.

Materials and Methods

Mice and induction of EAE

MIP-1α-deficient mice [30] backcrossed to the C57BL/6 genetic background for 7 generations and wild-type C57BL/6J females were purchased from the Jackson Laboratory (Bar Harbor, ME). CCR5-deficient mice were generated on a 129/Ola x C57BL/6J genetic background (Kuziel et al., manuscript submitted for publication) and were subsequently backcrossed for 8 generations to C57BL/6J. Female mice at 8-10 weeks of age were used in these experiments. Although MIP-1α-deficient and CCR5-deficient mice have been shown to be deficient in immune response to specific pathogens [30, 31], MIP-1α-/- and CCR5-/- mice were healthy in our colony and showed no signs of pathologic or behavioural defects that would compromise our study. EAE was elicited by s.c. immunization (base of tail) with an emulsion containing 50 μg of MOG_{35-55} peptide (Sheldon Biotechnology Centre, Montreal, Canada) and 1 mg of *Mycobacterium tuberculosis* H37RA (Difco, Detroit, MI) in CFA (Difco). Pertussis toxin (200 ng/mouse, Research Biochemicals International, Natick, MA) was injected i.v. on the day of immunization and again 2 days later. Mice were monitored daily for clinical signs of EAE, scored as: 1, flaccid tail; 2, hindlimb weakness and poor righting ability; 3, inability to right and one hindlimb paralyzed; 4, both hindlimbs paralyzed with or without forelimb paralysis and incontinence; 5, moribund. Mice were kept in a specific pathogen-free environment. Animal breeding and maintenance, and all experimental protocols were in accordance with the Canadian council for animal care guidelines and approved by McGill University animal care committee.

Immunohistochemistry

Mice were anesthetized with sodium pentobarbital (MT Pharmaceutical, Cambridge, Canada) and perfused intracardially through the left ventricle with ice-cold PBS. 10 μm frozen sections were fixed with 4% paraformaldehyde, blocked in 5% Normal Rabbit Serum (Vector Labs, Mississauga, Canada) in PBS for 30 min at room temperature, then incubated

with primary rat mAbs overnight at 4°C, followed with biotinylated rabbit anti-rat Ig (Vector Labs) for 1 h at room temperature. Sections were treated with 0.3% H_2O_2 to quench endogenous peroxidase activity, then incubated with an avidin-horseradish peroxidase complex (Vectastain ABC Elite kit, Vector Labs), following the manufacturer's instructions. Biotin-avidin complex binding was detected by the use of DAB (Medicorp Inc., Montreal, Canada) as chromagen. The mAbs used were: GK1.5 (CD4, American Type Culture Collection (ATCC), Manassas, VA), F4/80 (kindly provided by Dr. Georg Kraal, Vrije Universiteit, Amsterdam), M1/70 (Mac-1/CD11b, ATCC), RB6-8C5 (Gr-1/Ly6G, PharMingen, Mississauga, Canada). Control sections were incubated with isotype-matched primary Abs or with secondary Abs alone.

RNase Protection Assay (RPA)

RNA was purified from PBS-perfused CNS using TriZol (Life Technologies, Burlington, Canada) following the manufacturer's instructions. Multiprobe DNA templates for chemokines (Lymphotactin, RANTES, eotaxin, MIP-1α, MIP-1β, MIP-2, IP-10, MCP-1, and TCA3) and for the housekeeping genes, L32 and GAPDH, were purchased from PharMingen. RPA was performed according to the manufacturer's protocol using 20 μg of total RNA.

RT-PCR

RNA was reverse-transcribed as previously described (32). PCR conditions were optimized for linear amplification to allow direct comparison between samples. Equal amounts of cDNA were amplified using 1x PCR buffer (Sigma, Montreal, Canada), 2 mM $MgCl_2$ (Sigma), 80 μM each dNTPs, 2 U Taq polymerase (Sigma), and 50 pmol of each primer. The PCR reaction was performed with a Perkin-Elmer (Mississauga, Canada) thermocycler for 30-35 cycles (denaturation: 94°C 30sec, annealing: 65°C (IFNγ), 58°C (MIP-1α) or 56°C

(IL-4) 30sec, extension: 72°C 1min). The primer sequences are described elsewhere: IFNγ and β-actin [33], IL-4 [34] and MIP-1α [26].

Results and discussion

Fig. 1A shows upregulation of MIP-1α in spinal cord of wild-type C57BL/6 mice with MOG-induced EAE. This is consistent with previous reports showing induction of MIP-1α in EAE and MS [7, 12, 15, 16]. As expected, MIP-1α transcripts were not detected in MIP-1α-deficient mice (Fig. 1A). Despite the lack of MIP-1α, knockout mice developed clinical signs of EAE with kinetics and severity indistinguishable from C57BL/6 wild-type controls (Table 1). EAE was also induced in mice deficient for CCR5, one of the three known receptors for MIP-1α. The incidence of disease in CCR5-/- mice was 80%, consistent with that in wild-type controls (70-100%). These disease phenotypes held for knockouts on the C57BL/6 background; whether knockouts on other strain backgrounds would exhibit similar results is unknown. Resistance to disease induction by immunization with MBP, a characteristic of C57BL/6 mice, was not affected by MIP-1α or CCR5 deficiency (Table 1). Immunohistological examination of spinal cord from MIP-1α-/- (Fig. 2) or CCR5-/- mice (not shown) with hindlimb paralysis revealed parenchymal infiltration by $CD4^+$ T cells, macrophages and granulocytes. Comparable Th1 responses were seen in CNS of both knockout and wild-type mice (Fig. 1B), consistent with unaltered disease phenotype. Thus, the ablation of the genes for MIP-1α or its CCR5 receptor did not perturb the activation, trafficking and recruitment of specific leukocyte subpopulations to the CNS or their subsequent accumulation, all of which are key events to the development of this demyelinating disease.

Our finding that EAE can be induced in MIP-1α-/- mice seems at odds with previous reports which showed reduced lymphocyte infiltration of the CNS and inhibition of EAE following administration of anti-MIP-1α Ab [18-21]. It should be noted that in two of these studies, administration of anti-MIP-1α Ab during remission did not inhibit progression to the relapsing phase of PLP-induced EAE in SJL/J mice [20, 21]. Redundancy in the chemokine system might explain disparities between findings from different groups. As shown in Fig.

3, the overall expression pattern of other chemokines in the CNS was comparable between MIP-1α-/- and wild-type mice, with a slight increase (2-3 fold) in the chemokines, IP-10, TCA-3 and lymphotactin in MIP-1α-/- mice. Chronic intrathecal administration of antisense oligonucleotide against IP-10 has been shown to suppress IP-10 expression and to attenuate EAE [22], so it is possible that IP-10 functionally compensated for MIP-1α activity in our study. Activated encephalitogenic T cells express MIP-1α, TCA3, and RANTES [12], and whether myelin-specific T cell lines/clones can transfer EAE has been shown to depend on their ability to express MIP-1α and TCA3 [23]. The chemokines, IP-10, TCA3 and RANTES, share functions with MIP-1α by virtue of their ability to act on T lymphocytes, and may contribute to disease induction in MIP-1α-/- mice.

It might be argued that neutralization of MIP-1α by Ab [18, 19] was incomplete and residual low levels of MIP-1α were protective, so accounting for disease blockade. The complete removal of MIP-1α by gene ablation would then be expected to remove such protection. However, EAE was not more severe in MIP-1α-/- mice than in wild-type controls (Table 1) following our immunization protocol, making this possibility unlikely. Furthermore, reported levels of MIP-1α vary widely in EAE, without any evidence that low levels are disease-inhibiting.

We found predominance of the chemokines, IP-10, MCP-1, and RANTES over MIP-1α, MIP-1β and MIP-2 in CNS of C57BL/6 mice with active EAE (Fig. 3). This agrees with previous findings in SJL/J mice with active, MBP- or PLP-induced EAE [16, 24]. In other studies [18, 21], MIP-1α was shown to predominate over MCP-1 and MIP-2 in passive, PLP-reactive T cell-induced EAE. However, we and others have demonstrated indistinguishable profiles of chemokine mRNA accumulation in the CNS during active and passive EAE [19, 25] and evidence has been reported that chemokine protein levels correlate with levels of the corresponding mRNAs in EAE [26]. It is unlikely that variation in relative

levels of chemokines contributes to the disparity between our current findings and those of others regarding the importance of MIP-1α in EAE. In those studies, levels of RANTES and MIP-1α were comparable in passive EAE, but unlike the blocking effect of anti-MIP-1α antibodies, anti-RANTES antibodies did not suppress EAE [20, 21]. Consistent with this observation, vaccination with DNA encoding RANTES induced endogenous anti-RANTES antibodies but these did not prevent EAE [19].

Disparity in chemokine levels was also noted in patients with MS. While some investigators observed upregulation of MIP-1α in MS plaques [7], others have found prevalence of IP-10, RANTES and MCP-1 in brain lesions and cerebrospinal fluid (CSF) of MS patients [8,9]. Levels of CCR5 were increased in the CNS and CSF of MS patients, but the IP-10 receptor CXCR3 was more abundant [9]. Our finding that CCR5 is not essential for MOG-induced EAE in C57BL/6 mice is consistent with the report of Bennetts and colleagues of two patients with relapsing remitting MS who are naturally deficient in CCR5 [27]. Thus, the genetic absence of CCR5 from humans does not appear to be protective against MS attacks. However, this finding must be confirmed by analysis of a larger, genetically diverse MS patient population to rigorously exclude the possibility that absence of CCR5 might be protective only in the proper genetic context or only in certain types of MS pathogenesis [28]. As reported here, the absence of MIP-1α or CCR5 in the context of a predominantly C57BL/6 genetic background is not protective against MOG-induced EAE. However, MIP-1α or CCR5 deficiency on a different genetic background might block CNS inflammation and disease development, as suggested by the ability of anti-MIP-1α Ab [18, 20, 21] or MIP-1α naked DNA vaccines [19] to block PLP-specific T cell or MBP-specific T cell transfer of EAE in SJL/J mice and Lewis rats. Different rodent strains may invoke significantly different mechanisms involving distinct arrays of chemokines that lead ultimately to EAE [29]. Such diversity in animals might reflect the heterogeneity of pathogenesis in MS patients [28].

Acknowledgements

We thank Drs. Jack P. Antel and Rosanne Séguin for comments on this manuscript, Tracey Dawson for help with backcrossing the CCR5 mutation to C57BL/6, and Lyne Bourbonnière and Maria Caruso for maintenance and breeding of CCR5-/- mice in the Montreal Neurological Institute's animal facility. This work was supported by grants to T.O. from the Multiple Sclerosis Society of Canada and the Medical Research Council of Canada . E.H.T. was supported by a Multiple Sclerosis Society of Canada studentship.

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Table 1. MOG-induced EAE in MIP-1α$^{-/-}$, CCR5$^{-/-}$ and wild-type C57BL/6 mice

Mice	Immunized with	Incidence of EAE	Mean clinical score ± SD	Mean day of onset ± SD
C57BL/6	MOG	5/5	4.0 ± 0.0	12.0 ±1.4
MIP-1α-/-	MOG	4/4	3.5 ± 1.0	15.5 ± 4.7
	MBP	0/3	-	-
C57BL/6	MOG	3/4	3.7 ± 0.6	19.7 ± 5.5
CCR5-/-	MOG	4/5	3.5 ± 0.6	17.0 ± 2.5
	MBP	0/5	-	-

Figure 1. Expression of MIP-1α, IFNγ and IL-4 in CNS.

(A) RNA from PBS-perfused spinal cords of wild-type mice (WT), either naive or with EAE following immunization with MOG, and MIP-1α-/- mice, either naive or with EAE, was analyzed by RT-PCR for expression of MIP-1α and β-actin mRNA. (B) RNA from PBS-perfused spinal cords of wild-type mice, either naive or with EAE following immunization with MOG, and MIP-1α-/- mice with EAE was analyzed for expression of IFNγ, IL-4 and β-actin mRNA. PCR products are visualized by ethidium bromide staining.

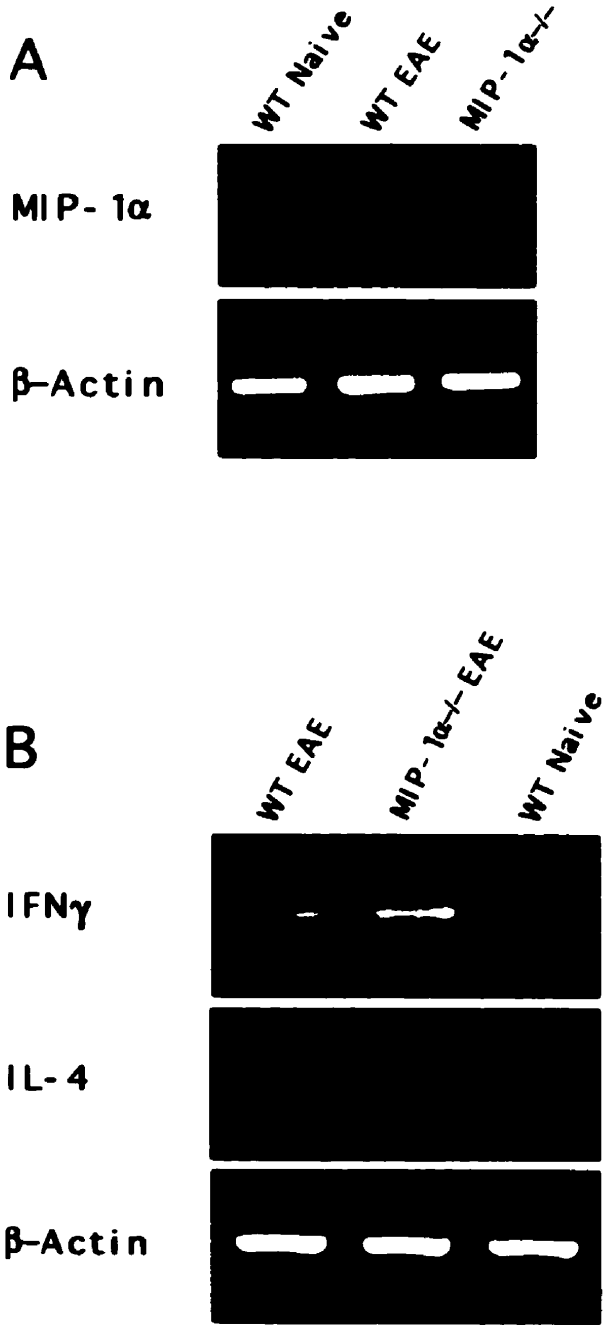
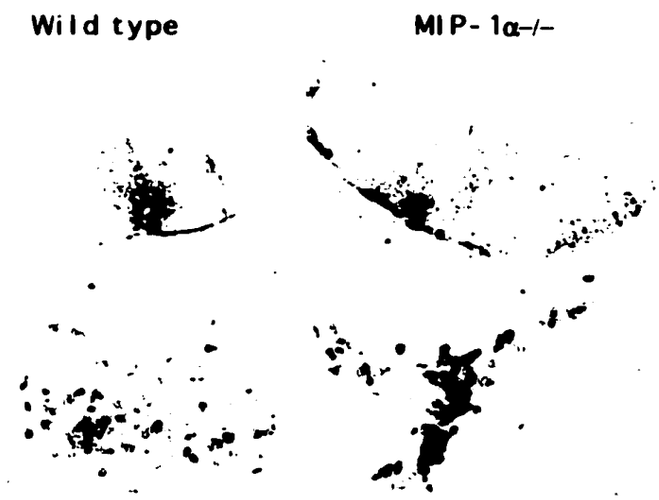

A
WT Naive
WT EAE
MIP-1α-/-
MIP-1α
β-Actin
B
WT EAE
MIP-1α-/- EAE
WT Naive
IFNγ
IL-4
β-Actin

Figure 2. Infiltration of CNS by lymphocytes and macrophages in EAE.

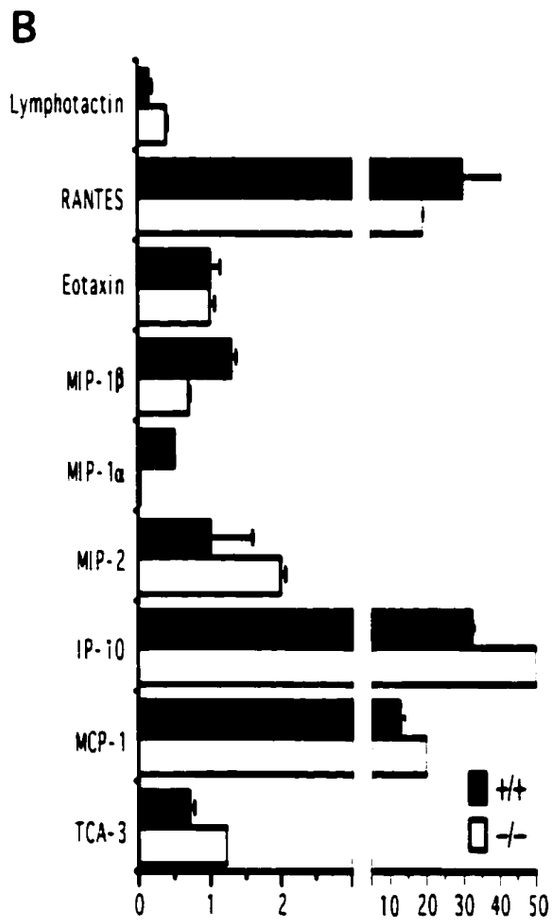
Spinal cord sections from wild-type and MIP-1α-/- mice with EAE were stained for Mac-1 and CD4. Comparable staining in spinal cord of both knockout and wild-type mice was also noted for F4/80 and Gr-1 (not shown). Original magnification: Mac-1 (35x) and CD4 (140x).

Figure 3. RNase protection assay analysis of chemokine mRNA expression in spinal cords of MIP-1α-/- and wild-type mice.

(A) RNA from perfused spinal cords of wild-type (+/+) mice, either naive (N) or with EAE, and of MIP-1α-/- (-/-) mice with EAE was analyzed for expression of chemokines by RPA. (B) Quantitative analysis of chemokine gene expression. Levels of chemokine mRNA were analyzed by ImageQuant, and expressed as arbritrary units after normalization to GAPDH.


Wild type
MIP-1α−/−


+/+
−/−
EAE
EAE


B
Lymphotactin
RANTES
Eotaxin
MIP-1β
MIP-1α
MIP-2
IP-10
MCP-1
TCA-3
+/+
−/−
0
1
2
10
20
30
40
50

CHAPTER VI

CONCLUSION & SUMMARY

My findings allow me to delineate roles for IFNγ and macrophages at 3 critical checkpoints in the development of EAE. Checkpoint 1 controls the number of activated autoreactive Th1 cells. Checkpoint 2 controls the entry and retention of autoreactive T cells and other leukocytes within the CNS parenchyma. Checkpoint 3 controls the selective recruitment of leukocyte subsets. My findings can be integrated to form a model of cellular and molecular mechanisms that underlie these checkpoints (*see the schematic representation of the model on the next page*).

Checkpoint 1

Does IFNγ play a role in shaping the precursor frequency of circulating autoreactive T cells or regulatory T cells?

A high precursor frequency of autoreactive myelin-specific T cells in the peripheral pool of naïve mice can enhance susceptibility to or severity of EAE (Huseby and Goverman, 2000). Thus, in this view, the precursor frequency of MBP-specific T cells may be higher in the highly susceptible SJL/J mice than in the essentially resistant BALB/c mice. Nevertheless, a recent study by Anderson et al. (2000) has indicated that SJL/J mice, although having a high precursor frequency of PLP 139-151-specific T cells, do not possess a high precursor frequency of MBP-specific T cells. In the thymus, developing T cells are being educated to distinguish self-molecules from foreign intruders. Many of the myelin components, including MBP and PLP, are expressed in the thymus (Huseby and Goverman, 2000). Thymic expression of these molecules helps to maintain immunological tolerance (Huseby and Goverman, 2000). For example, unlike T cells from wild-type BALB/c mice, those from MBP-deficient mice on the BALB/c background proliferated well in response to MBP (Yoshizawa et al., 1998). Transfer of MBP-reactive T cells from MBP-deficient donors into naïve BALB/c recipients induced EAE (Yoshizawa et al., 1998). These findings suggest that the expression of MBP promotes tolerance to MBP in wild-type BALB/c mice. The maintenance of self-tolerance guards against autoimmunity. However, mice with a high

precursor frequency of non-tolerant autoreactive myelin-specific CD4+ T cells do not necessarily develop spontaneous EAE; activation of these autoreactive CD4+ T cells, their access to the CNS, or loss of regulatory CD4+ T cells is required for disease development (Brabb et al., 1997;Van de Keere and Tonegawa, 1998; Olivares-Villagomez et al., 1998; Anderson et al., 2000). Regulatory CD4+ T cells, producing TGFβ and/or IL-10, can control activation of autoreactive T cells to protect from EAE or other autoimmune diseases (Chen et al., 1994; Groux et al., 1997; Mason and Powrie, 1998). However, not much is known about the precise antigenic specificities and effector mechanisms of regulatory T cells as well as which cytokines play a role in the differentiation of regulatory T cells (Mason and Powrie, 1998). Several regulatory T cell populations, including the natural killer T cell subset, appear to require IFNγ secretion to exert down-regulation of T-cell mediated autoimmune disease (Kumar and Sercarz, 1998; Falcone et al., 1999). It is tempting to speculate that IFNγ may also affect the delicate balance between autoaggressive T cells and regulatory T cells, such that IL-10-secreting regulatory T cells, for example, could not be generated in IFNγ-/- mice to down-regulate the autoimmune response once triggered. Alternatively, it is possible that cytokine imbalance in the thymic microenvironment may result in higher precursor frequency of autoreactive MBP-specific T cells in IFNγ-/- mice than in wild-type resistant BALB/c mice. Higher precursor frequency of autoreactive myelin-specific T cells would increase the chances of activating these autoreactive T cells after immunization with the corresponding autoantigen, thereby enhancing disease susceptibility in otherwise resistant animals.

IFNγ suppresses the generation of activated encephalitogenic T cells

Autoreactive CD4+ T cells that initiate EAE generally produce Th1-type cytokines (e.g., IFNγ, LTα, TNFα) and express Th1-type chemokine receptors (e.g., CCR5, CXCR3). An increase in the frequency of such activated autoreactive CD4+ T cells is required for the initiation of EAE. Certain cytokines and chemokines in the microenvironment may adjust

the threshold of T cell activation during the encounter of autoantigen. IL-12 is critical in the development of EAE, and can even promote the activation and proliferation of encephalitogenic Th1 cells, independently of IFNγ (Segal et al., 1998b). The IL-12Rβ2 subunit is the critical molecule involved in maintaining IL-12 responsiveness on activated encephalitogenic Th1 cells (Chang et al., 1999a). My data point to a down-modulatory role for IFNγ/IFNγR and no role for MIP-1α/CCR5 in the generation of activated encephalitogenic CD4+ Th1 cells (Chapter IV and V). Protection from EAE in mice deficient in both co-stimulatory molecules, B7.1 and B7.2, has also been associated with higher levels of IFNγ and a reduced proliferation of autoreactive T cells when compared to wild-type mice with disease (Chang et al., 1999b). IFNγ also thwarts the peripheral generation of pathogenic autoreactive T cells to protect from other Th1-cell mediated autoimmune diseases (Tarrant et al., 1999; Meyers and Zhang, 1999). IFNγ acts on macrophages to up-regulate iNOS-derived NO, reactive oxygen species, or TNFα, which in turn induce apoptosis of Th1 cells (Liu et al., 1998; Allione et al., 1999; Mix et al., 1999; Tarrant et al., 1999; Willenborg et al., 1999a). IFNγ may also promote FAS-mediated apoptosis of activated encephalitogenic T cells (Zhou et al., 1999).

Thus, endogenous IFNγ and IL-12, despite their known concordant action, may counteract each other: IL-12 promotes the generation of encephalitogenic Th1 effector cells, while IFNγ may have, as suggested above, a way to exert a negative feedback regulation on IL-12 activity. This would predict that in EAE resistant strains of animals, such as BALB/c mice, there might be a MBP-specific defect in IL-12-induced activation (Chang et al, 1999a), or the immunomodulatory effect of IFNγ may dominate to curtail the clonal expansion of encephalitogenic T cells (Chapter IV). Loss of such IFNγ immunoregulatory function, as in IFNγ-/- BALB/c mice, or exogenous IL-12, would lead to massive numbers of activated autoreactive T cells circulating in the blood and trafficking to the CNS. Of note, susceptible SJL/J or PL/J mice that lack IFNγ also developed a more severe EAE than their wild-type

counterparts (Krakowski and Owens, 1996; Feber et al., 1996). Whether such exacerbation was associated with a more prolific proliferation of MBP-reactive T cells has not been documented. Nevertheless, the immunomodulatory effect of IFNγ on autoreactive T cells is not as dominant in susceptible strains, since EAE can readily be initiated. This may relate to the balance between expression levels of IFNγ and IFNγR among animal strains. High levels of IFNγR have been associated with apoptotic response while low-level expression with a proliferative one (Novelli et al., 1994). The IFNγR is composed of two chains, α and β. Recently, IFNγRβ-deficient mice were found to differ from IFNγRα-deficient mice in their ability to develop Th1 responses (Lu et al., 1998). EAE developed in mice deficient in IFNγRα (Willenborg et al., 1996) (Chapter IV), but whether it is also the case for IFNγRβ-deficient mice is not known. Another possibility is the propensity of susceptible strains to produce and respond to IL-12, which then counteract IFNγ, thereby facilitating the development of pathogenic autoreactive T cells. Optimization of the immunization protocols to enhance IL-12 production and responsiveness, overcame EAE resistance in BALB/c mice (Määttä et al., 1998; Falcone et al., 1998), thus suggesting that IL-12 can override the immunomodulatory effect of IFNγ.

Checkpoint 2

Regulation of invasion at T-cell entry by local macrophages

For immune cells to invade the CNS parenchyma, they must cross many barriers of the BBB: the endothelial and its vascular basal lamina barriers, the parenchymal basement membrane barrier, and the glia limitans that are formed by astrocytic processes. My findings suggest that a macrophage-independent pathway regulates the transendothelial migration of T cells, whereas further migration is likely to depend on the function of local macrophages (Chapter III).

The subarachnoid space of the meninges and the perivascular (Virchow-Robin) space of deep cerebral vessels, together represent a compartment which immune cells must enter, after extravasation from blood. Resident macrophages in this compartment are replenished by blood monocytes slowly under normal conditions, but rapidly under pathological conditions (Lassmann et al., 1993). My findings suggest that when the function of these meningeal/perivascular macrophages is altered and the peripheral pool of healthy monocytes/macrophages is depleted, the migration of extravasated neuroantigen-reactive Th1 cells halts at the meningeal/perivascular compartment, leaving the parenchyma unharmed (Chapter III). Meningeal retention of T cells was also noted in Lewis rats with asymptomatic MOG-induced EAE, and was associated with a reduction in CNS macrophages (Linington et al., 1993; Berger et al., 1997). Leukocytes were also constrained within the perivascular space in TNFα-/- mice with a delayed onset of clinical signs of EAE (Korner et al., 1997). It has recently been postulated that macrophages are a major source of TNFα which induces glial production of chemokines and MMP's to facilitate leukocyte movement from the perivascular space into the CNS parenchyma (Sedgwick et al., 2000).

The CNS parenchymal environment tends to inhibit T-cell activation and cause apoptosis of infiltrating T cells. Ceramides, TGFβ production, gangliosides, and antigen-specific interactions between T cells and astrocytes have all been suggested to induce T-cell death in the CNS parenchyma (Irani et al., 1996; Bauer et al., 1998; Gold et al., 1996; Gold et al., 1997). Disease severity correlates with the numbers of myelin-reactive T cells transferred (Zamvil and Steinman, 1990). I envision that one way to perturb such an intrinsic CNS defense for initiation of an autoimmune response is the maintenance of an enhanced influx of activated encephalitogenic T cells and macrophages into the parenchyma.

Does T cell entry to the CNS parenchyma require an antigen-dependent reactivation event?

The subarachnoid and perivascular compartment, instead, appears as a site more auspicious for T cell proliferation and effector T cell selection in EAE (Shin et al., 1995; Bauer et al., 1998). Bone marrow chimera studies have indicated that local meningeal/perivascular macrophages are important in presenting antigen to neuroantigen-specific CD4+ T cells to induce EAE (Hickey and Kimura, 1988). Meningeal/perivascular macrophages may reactivate and expand neuroantigen-specific T cells to increase the influx of these into the CNS parenchyma. Adoptively transferred encephalitogenic T cells, but not transferred non-CNS antigen-reactive T cells, accumulate in the CNS parenchyma for longer periods of time (Lannes-Vieira et al., 1994). Transferred non-encephalitogenic T cells transiently accumulate in the subarachnoid/perivascular space (Lannes-Vieira et al., 1994). My findings demonstrate that class II MHC molecules are not necessarily required to be up-regulated for antigen-dependent reactivation of T cells to trigger an autoimmune response in the CNS (Chapter IV). Surprisingly, enhanced class II MHC up-regulation is, unexpectedly, associated with protection (Chapter III). I also found an unusual population of B220+ B cells in association with such enhanced MHC up-regulation (Chapter III). Whether this B cell population also modulates the migratory properties of encephalitogenic T cells to confer protection is not known. Up-regulation of B7 molecules, however, appears crucial in local T cell reactivity. Myelin-reactive wild-type T cells transferred minimal EAE in mice lacking both B7.1 and B7.2, and infiltrates were again restricted to the meninges (Chang et al., 1999b). Thus, local macrophages may regulate T cell invasion into the CNS parenchyma via antigen-specific reactivation.

Does T cell entry requires specific adhesion molecules and MMP?

The composition of the ECM in the CNS has been shown to differ significantly from non-CNS tissues (Sobel, 1998), suggesting that encephalitogenic T cells may need to up-regulate a specific set of adhesion molecules and MMP to move across parenchymal barriers and within the CNS parenchyma (Engelhardt et al., 1998). Different adhesion molecules are likely involved in migration across the endothelial barrier and through the parenchymal ECM (Engelhardt et al., 1995; Romanic et al., 1997). It is possible that such up-regulation of adhesion molecules and MMP activities was defective in macrophage-deficient mice, despite evidence of IFNγ and certain activation surface markers (Chapter III). Full neuroantigen-specific activation of T cells in the subarachnoid/perivascular space may entail not only cytokine secretion but also expression of multiple adhesion molecules and MMP. In addition to T cells, activated macrophages and microglia produce substantial amounts of various MMP (Yong et al., 1998b).

Regulation of invasion at the level of leukocyte retention

Although the interpretation of meningeal/perivascular macrophages as gatekeepers of the CNS is an attractive one, whether the function of meningeal/perivascular macrophages is indeed affected by systemic administration of clodronate-containing liposomes has recently been questioned (Xue et al., 1999). Moreover, it is difficult to conceive how peripheral and local macrophages in our study were to affect the initial migration of adoptively transferred myelin-reactive T cells, as treatment with liposomes was only administered 3 days later (Chapter III). As early as 2-24 hours after transfer, activated T cells can infiltrate various tissues, including the CNS (Lannes-Vieira et al., 1994; Bauer et al., 1998). However, transferred non-CNS specific T cells disappear at later times, while transferred encephalitogenic T cells are retained in the CNS parenchyma (Lannes-Vieira et al., 1994). Regulation of parenchymal invasion may occur at the level of cell retention. In this regard,

activated T cells of any specificity may enter the CNS parenchyma (Hickey et al., 1991), but only those that recognize their antigen are retained. In support of this, injection of a non-CNS antigen, ovalbumin, directly into the dorsal column of the spinal cord caused adoptively transferred ovalbumin-reactive T cells to accumulate there, and when high numbers of these T cells were transferred, neural conduction impairment and demyelination were evidenced (Westland et al., 1999). Without prior intrathecal injection of ovalbumin, transferred ovalbumin-reactive T cells did not accumulate in the parenchyma (Westland et al., 1999). Which cells in the CNS can, then, act as APC to reactivate infiltrating T cells? Although microglia might not be as efficient as local macrophages in inducing antigen-specific T cell proliferation, they have recently been shown to be as effective as local macrophages in reactivating T cells to produce cytokines in an antigen-dependent manner (Ford et al., 1996; Krakowski and Owens, 1997; Katz-Levy et al., 1999).

IFNγ-inducible TNFα and NO: mediators and modulators of leukocyte trafficking

IFNγ can induce glial production of TNFα and iNOS. TNFα is involved in facilitating the initial movement of leukocyte within the CNS (Akassoglou et al., 1997; Korner et al., 1997b; Sedgwick et al., 2000). iNOS-derived NO, on the other hand, may inhibit leukocyte trafficking depending on the cellular sources (Chapter II). Inhibition of iNOS can have deleterious or protective effects in EAE (Willenborg et al., 1999b). I propose that astrocytic iNOS is protective. This is also consistent with a crucial role of reactive astrocytes in restricting leukocyte infiltration into the inflamed CNS (Bush et al., 1999; Liedtke et al., 1998b), although astrocytic MCP-1 has been shown to direct leukocyte transmigration across the BBB (Weiss et al., 1998). Cell-type specific controls of iNOS expression for astrocytes and microglia have also been demonstrated (Hellendall and Ting, 1997; Pahan et al, 1998). To further define the contribution of astrocytic iNOS in EAE, I have collaborated with Dr. Mina Hassan-Zahraee in generating bone marrow chimeras in which leukocytes and

meningeal/perivascular macrophages cannot express iNOS, but parenchymal microglia and astrocytes can. We found that in these chimeras, EAE was delayed, and astrocytes but not parenchymal microglia expressed iNOS (M. Hassan-Zahraee, E. H. Tran, and T. Owens, manuscript in preparation). Up-regulation of the other isoforms, nNOS and eNOS, has also been reported in EAE (De Groot et al., 1997; Shin, 1999); and surprisingly, this was also localized to astrocytes.

Checkpoint 3

IFNγ and associated chemokines recruit and activate macrophages

High levels of IFNγ, perhaps in combination with TNFα, favor the recruitment and activation of macrophages over neutrophils via induction of specific chemokines (e.g., MCP-1 and RANTES) (Chapter IV). Activated T cells and glial cells could be the sources of chemokines that direct recruitment of more T cells and macrophages (Berman et al., 1996; Ransohoff et al., 1997). The invasion of the CNS parenchyma by macrophages themselves also appears critical for activation of microglial cells (Chapter III). The number of activated macrophages, rather than the number of neuroantigen-specific T cells, in the CNS parenchyma has been shown to correlate best with neurological dysfunction (Berger et al., 1997). Entry of encephalitogenic T cells into the CNS is not sufficient for the initiation of pathological or clinical manifestation of EAE as long as peripheral macrophage recruitment and activation is impaired. Increasing death by apoptosis of macrophages by dexamethasone (Nguyen et al., 1997) or 1,25-dyhydroxyvitamin D3 (Nashold et al., 2000) treatment, or deactivating macrophages by IL-13 (Cash et al., 1994) or the drug CNI-1493 (Martiney et al., 1998), all suppressed both clinical and histopathological manifestation EAE.

Accumulation of activated macrophages in the CNS parenchyma can cause demyelination even in the absence of T cells or autoantibodies (Chiang et al., 1996), although activated

macrophages have also been shown to promote CNS remyelination and regeneration (David et al., 1990; Lazarov-Spiegler et al., 1996; Diemel et al., 1998; Rapalino et al, 1998). Mice over-expressing MCP-1 in the CNS showed pronounced meningeal/perivacular macrophage accumulation that did not cause overt clinical symptoms or parenchymal inflammation or demyelination (Fuentes et al., 1995). Systemic administration of lipopolysaccharide into these MCP-1 transgenic mice caused only minimal parenchymal infiltration. This further supports that additional intraparenchymal events, such as T-microglial interaction or cytokine overproduction, are required to recruit, activate, and retain macrophages to the CNS parenchyma. Over-expression of TNFα in the CNS, even in mice lacking CD4 or B cells, is sufficient to induce chronic glial activation and demyelination (Kassiotis et al., 1999). We also showed that if IFNγ or TNFα production is maintained in the CNS, as in IFNγ- or TNFα–transgenic mice (Taupin et al., 1997; Renno et al., 1998), macrophage infiltration is no longer required for perpetuating glial activation and demyelination (Owens et al., 1999).

IFNγ and IL-10 restrict lethal neutrophil invasion and function

In the absence of IFNγ response, macrophages/microglia are not activated, but neutrophils become the predominant effector cells to mediate EAE (McColl et al., 1998) (Chapter IV). Recruited neutrophils can self-sustain through additional production of selective chemokines. Neutrophils are predominant in MOG-induced CNS pathology in CD28-/- mice and are a major source of MIP-2 (Perrin et al., 2000), although in two other studies CD28-/- mice were resistant to EAE (Girvin et al., 2000; Chang et al., 1999b). CNS-specific over-expression of another neutrophil-selective chemokine KC produced exuberant neutrophil infiltration of the parenchyma but no demyelination (Tani et al., 1996). Lack of demyelination may relate to the quiescent or apoptotic state of the recruited neutrophils (Tani et al., 1996) or to the ability of KC to promote remyelination (Robinson et al., 1998). Florid microglial activation and neurological dysfunction without demyelination were

observed in older KC-transgenic mice, suggesting that glial cells can react to neutrophil invasion or chemokines (Tani et al., 1996). In our model, MIP-1α is dispensable (Chapter V), and this is also consistent with the other recent findings that inhibition of EAE with a myelin basic peptide analog was not associated with a decrease in the expression of MIP-1α, but rather with MCP-1 and MIP-2 (Reiseter et al., 1998).

Apoptosis of effector cells, such as T cells, macrophages and neutrophils, in the CNS has often been associated with recovery from EAE in IFNγ-intact mice or rats (Reiseter et al., 1998; Bonetti et al., 1999), but neutrophil apoptosis was not evidenced in IFNγ-/- or IFNγR-/-mice with rapidly progressing lethal EAE (Chapter IV). Although neutrophils and eosinophils can also be found in the CNS of IFNγ-intact animals (Määttä, et al., 1998; Gladue et al., 1996; McColl et al., 1998; Milici et al., 1998; Reiseter et al., 1998), their invasion was not as extensive and lethal as in IFNγ-/- or IFNγR-/- mice (Chapter IV). IL-10 was up-regulated in the CNS of IFNγ-intact mice with non-lethal EAE, but was absent in IFNγ-/- mice with lethal EAE (Chapter IV). IL-10-/- mice were also more susceptible to EAE (Segal et al., 1998b); however, the histopathology of the CNS has not been studied in detail. Recent studies using bone marrow chimeras have indicated that IFNγ can also down-regulate EAE by iNOS-derived NO within the CNS (Willenborg et al., 1999a). Thus, IFNγ, perhaps in synergy with IL-10, acting locally in the CNS restricts the recruitment, activation, expansion, and survival of PMN in EAE to protect hosts from death.

Ongoing regulation of local autoimmune response by spleen

In the spleen of IFNγ-/- mice with EAE, there was an unusual over-expansion of Mac-1+ cells, that are likely neutrophils and macrophages (my unpublished observation). How this population affects the course of EAE in IFNγ-/- mice is not known. These splenic Mac-1+ myeloid cells may represent an additional source of IL-12 or TNFα that is otherwise not

available in resistant strains, to enhance differentiation of MBP-reactive T cells following immunization with MBP/CFA. Thus, IFNγ appears as a key cytokine in coordinating regulation of autoimmune responses locally in the inflamed CNS and of myeloproliferative response in peripheral hematopoeitic tissue, such as the spleen. The spleen, rather than lymph nodes, has been suggested to be involved in the ongoing regulation of immune response (Karandikar et al., 1998).

Relevance to MS

It is difficult to reconcile the immunoregulatory role of endogenous IFNγ in animal models with the detrimental effect of exogenous IFNγ in MS (Panitch et al., 1987). IFNγ may have different effects at different stages of the disease depending on the concentration of IFNγ or stages of cellular differentiation/activation. One general limitation of the knockout and transgenic technologies is that the deletion of a gene throughout development may lead to functional compensation by other genes in the mouse adult life, and that it is difficult to control the appropriate expression levels and timing of the added transgene. This could therefore mask the physiological activity of the desired gene. The emerging creation of inducible or conditional knockout or transgenic mice in which there are means to turn on or off the desired gene at opportune time in the desired discrete cell-types during the adult life may help to further clarify the role of IFNγ in multiple stages of CNS autoimmune disease.

It is unclear how, when and where circulating autoreactive T cells become activated in MS. These questions are particularly difficult to address in animal models in which the disease is experimentally provoked. Instead, regulatory mechanisms that underlie immune cell entry could represent a critical step in shaping the outcome of activating these autoreactive T cells. EAE is valuable for dissecting such regulatory mechanisms, but in the end it is only a model. Nevertheless, my data suggest that macrophage-dependent events at the subarachnoid and perivascular compartments may determine, by as yet undefined mechanisms, the fate of

migrating autoreactive T cells in the CNS. My data also support a beneficial role for IL-10 in keeping the inflammatory effects of Th1 cytokines (e.g., IFNγ, TNFα, LTα) in control. Moreover, my data suggest that differences in the recruitment of Th1, Th2 cells, regulatory T cells (including TGFβ-secreting Th3 cells), or other leukocytes by specific chemokines may represent a crucial checkpoint for regulation of MS. Recruitment of activated autoreactive T cells can also protect neurons after CNS damage, presumably through the release of neurotrophins (Moalem et al., 1999). Our lab showed that astrocytes lose their capacity to metabolise glutamate in EAE (Hardin-Pouzet et al., 1997), possibly leading to enhanced glutamate excitotoxicity which promotes damage of neurons and oligodendrocytes directly or through involvement of NO (Almeida et al, 1998; Urushitani et al., 1998). Recently, blockade of glutamate excitotoxicity has been demonstrated to protect both neurons and oligodendrocytes from immune-mediated damage, without reducing the lesion size or the degree of inflammation (Pitt et al., 2000; Smith et al., 2000). Thus, at later stages of the disease process in MS or EAE, influx of a subset of autoreactive T cells into the CNS may promote recovery. It may be possible that exogenous IFNγ worsened the ongoing CNS destruction in MS patients (Panitch et al., 1987) through suppression of 'neuroprotective' autoreactive T cells.

Although the Th1/Th2 dichotomy has at times been challenged, it has proven extremely useful and will continue to be so as it describes a clearly observable phenotype with a particular set of cytokines. Perhaps, understanding how Th1/Th2 cytokines or other molecular mechanisms influence the balance between autoaggressive T cells and regulatory T cells will also be of interest in the prevention of autoimmunity. Copaxone, a random copolymer of alanine, glutamic acid, tyrosine, and lysine, is the most recent immunomodulatory drug approved for the treatment of MS. A recent report has indicated that Copaxone causes a shift from Th1 to Th2/Th3 response (Miller et al., 1998). Clearly, deciphering the complex cytokine network and how chemokines selectively guide Th1, Th2,

or Th3-producing cells into the inflamed CNS, is not just of academic interest, since therapeutic intervention against neuroinflammatory diseases may stem from influencing these pathways.

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