Peripheral immune response in chronic relapsing experimental autoimmune encephalomyelitis in SJL mice

JINGHUI YANG

DEPARTMENT OF BACTERIOLOGY AND IMMUNOLOGY
HAARTMAN INSTITUTE
UNIVERSITY OF HELSINKI

NEUROSCIENCE PROGRAM
BIOMEDICUM HELSINKI

DEPARTMENT OF NEUROLOGY
HELSINKI UNIVERSITY CENTRAL HOSPITAL

HELSINKI, FINLAND

ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Medicine of the University of Helsinki, in the small Lecture Hall of the Haartman Institute, Haartmaninkatu 3, Helsinki, on November 14, 2003, at 12 noon.

Supervisors: Seppo Meri
MD, PhD, Professor
Haartman Institute
Department of Bacteriology and Immunology
University of Helsinki

Perttu Lindsberg
MD, PhD, Docent
Department of Neurology
Helsinki University Central Hospital

Reviewers: Jukka Pelkonen, Professor
Dept. of Clinical Microbiology
University of Kuopio, Finland

and

Olli Lassila, Professor
Dept. of Medical Microbiology
University of Turku, Finland

Opponent: Christian Vedeler, MD, PhD, Professor
Dept. of Neurology
University of Bergen, Norway

ISBN 952-91-6600-1 (print)
ISBN 952-10-1480-6 (PDF)
Helsinki 2003
Yliopistopaino

Peripheral immune response in chronic relapsing experimental autoimmune encephalomyelitis in SJL mice

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles.

I. J. Yang, V. Hukkanen, R. Seljelid and A.A. Salmi. Altered IL1β and IL-2 mRNA expression in murine splenic cells after concomitant stimulation by Semliki Forest virus and lipopolysaccharide. Scand. J. Immunol. 1997. 45, 349-353.

II. J. Yang, S. Lindal, E. Ylinen, N. Setälä, V. Hukkanen and R. Seljelid. A novel and efficient regimen for producing chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE) in SJL mice. APMIS.1999. 107, 800-806.

III. J. Yang, M. Kubera, A. Zelek-Molik, I. Nalepa, V. Hukkanen, P. Lindsberg, S. Meri and R. Seljelid. Splenectomy and adoptive cell transfer reveal a prominent role for splenic memory lymphocytes in the development of chronic relapsing experimental autoimmune encephalomyelitis. Scand. J. Immunol. 2000. 52, 356-361.

IV. J. Yang, P. Lindsberg, V. Hukkanen, R. Seljelid, C.G. Gahmberg and S. Meri. Differential expression of cytokines (IL-2, IFN-γ, IL-10) and adhesion molecules (VCAM-1, LFA-1, CD44) between spleen and lymph nodes associates with remission in chronic relapsing experimental autoimmune encephalomyelitis. Scand. J. Immunol. 2002. 56, 286-293.

V. J. Yang, S. Meri, M.-L. Karjalainen-Lindsberg, V. Hukkanen, R. Seljelid, C.G. Gahmberg and P. Lindsberg. Expression of ICAM-5 in the spleen - a role in experimental autoimmune encephalomyelitis in SJL mice (submitted)

ABBREVIATIONS

APC	Antigen presenting cell
BBB	Blood-brain barrier
CAM	Cellular adhesion molecule
CFA	Complete Freund's adjuvant
CNS	Central nervous system
CR-EAE	Chronic relapsing EAE
CSF	Cerebrospinal fluid
DC	Dendritic cell
DTH	Delayed-type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EAM	Experimental autoimmune myasthenia gravis
EAO	Experimental autoimmune orchitis
EAU	Experimental autoimmune uveoretinitis
ICAM	Intercellular adhesion molecule
IDDM	Insulin dependent diabetes mellitus
IFA	Incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
i.v.	Intravenous
LFA	Lymphocyte function antigen
LN	Lymph node
pLN	Popliteal lymph node
LNC	Lymph node cell
LPS	lipopolysaccharide
MAG	Myelin associated glycoprotein
MBP	Myelin basic protein
M. b.	*Mycobacterium butyricum*
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MHH	Mouse heart homogenate
M.O.I.	Multiplicities of infection
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSCH	Mouse spinal cord homogenate
MOBP	Myelin oligodendrocyte basic protein
MOG	Myelin oligodendrocyte glycoprotein
M. tb.	*Mycobacterium tuberculosis*

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLP	Proteolipid protein
PTX	Pertussis toxin
RRMS	Relapsing-remitting multiple sclerosis
sICAM-5	Soluble ICAM-5
SPMS	Secondary progressive multiple sclerosis
TCR	T cell receptor
Th1	T-helper type-1
Th2	T-helper type-2
TNF	Tumor necrosis factor
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen

ABSTRACT

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS). Experimental autoimmune encephalomyelitis (EAE) induced in rodents has served as an animal model for MS. However, no animal model established so far represents all the facets of human MS. Thus, it is warranted to develop new models that simulate MS more closely.

EAE is mediated by autoimmune $CD4^+$ T cells, which develop first in the peripheral lymphoid organs and migrate into the CNS to cause an autoimmune response. Meanwhile, regulatory T cells may also develop to contain the harmful autoimmune response to the myelin antigens. The development of T cells is controlled largely by the expression of different cytokines as well as of cellular adhesion molecules (CAMs). Thus, for understanding the pathogenesis of the demyelinating disease, it is important to study the immune parameters in the peripheral lymphoid organs.

The methodology for quantification of the expression of molecules involved in the immune system at the mRNA level was established. Although the method was developed in vitro with cultured splenic cells, this technique was utilized throughout this study.

Chronic relapsing EAE (CR-EAE) was established through a novel regimen. In this new model, more mice survived the acute disease attack and the majority of the surviving mice developed CR-EAE that resembled human MS closely. The relapsing-remitting course of the disease in this model is thus useful for the study of the pathogenesis of MS and for therapeutic approaches.

The cellular mechanism underlying the development of CR-EAE was investigated. Through splenectomy and adoptive cell transfer, it was observed that the spleen as well as the memory cells harboured in it played a prominent role in the development of CR-EAE.

The expression profiles of cytokines and CAMs in the lymph nodes (LNs) and spleen were studied. It was found that a stronger expression of the cytokines IL-2, IFN-γ, IL-10 and CAMs VCAM-1, LFA-1, CD44 in the spleen than in the lymph nodes correlated with disease remission in CR-EAE. These results indicated that a polarized expression of different immune molecule in the different peripheral lymphoid organs may determine the outcome of the disease progression.

The expression of a particularly interesting novel adhesion molecule, ICAM-5, was studied. With several techniques, it was demonstrated that the antigen presenting cells: dendritic cells (DCs), macrophages and a small percentage of B cells expressed ICAM-5. Quantitative RT-PCR analysis showed that a higher expression of ICAM-5 in the spleen than in the LNs was associated with disease remission in CR-EAE.

Taken together, these results showed that a new mouse model for MS could be developed with a modified protocol. The study of various parameters in the peripheral lymphoid organs indicated that the activities in the different immune compartments may evolve differentially in remission and relapse. Interactions between the subsets of immune cells which are developed in the different compartments may determine the net outcome of the immune response. The relapsing-remitting pattern of MS is thus regulated by cytokines and adhesion molecules, not only in the central nervous system itself, but in the peripheral immune organs.

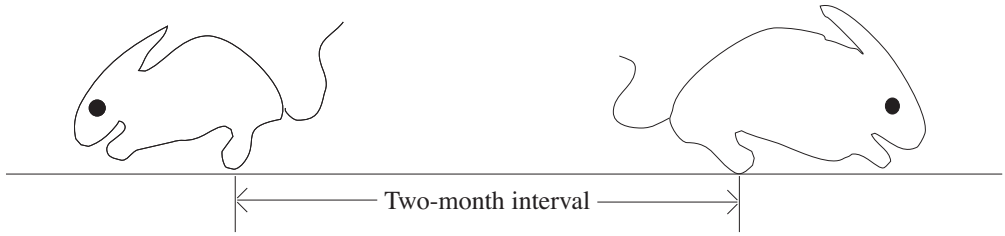
1 INTRODUCTION

Multiple sclerosis (MS) is one of the most common disabling neurological diseases in young adults. The cardinal pathological features are focal areas (plaques) of demyelination and immune-mediated inflammation, usually perivenular in orientation. MS patients often develop first symptoms during their early 20's and the course of the disease is life-long. The pathogenesis of MS is still unclear, although a viral aetiology (possibly through an autoimmune mechanism) has been proposed.

The assumption that MS is an autoimmune-mediated disease is largely based on the findings that its pathological changes in the CNS are similar to those in an experimentally induced autoimmune disease: experimental autoimmune encephalomyelitis (EAE) in rodents. However, the disease course of EAE is mainly monophasic, in contrast to the typical chronic or relapsing-remitting disease course of MS.

With a modified immunization protocol, a novel model of MS-chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE) was established in SJL mice (Fig.1). This new model is characterized by a chronic relapsing-remitting disease course which resembles human MS more closely. In this thesis, I have further investigated the cellular and molecular mechanisms underlying the development of CR-EAE. In particular, the patterns of certain cytokines as well as cellular adhesion molecules (CAMs) in relation to the disease course of CR-EAE were addressed.

Fig. 1. Scheme of the induction of CR-EAE



Pretreatment:

Injection of 3 mg MSCH, 0.2 mg M.tb and 0.025 mg M. b in 0.05 ml PBS into the **left** hind footpad.

Second injection:

Injection of 3mg/1.5 mg MSCH, 0.1 mg M. tb and 0.125 mg M. b in water-oil emulsion into the **right** hind footpad, Followed by an injection of 0.2 mg pertussis toxin i.v.

2 REVIEW OF THE LITERATURE

2.1. Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating disease of the central nervous system (CNS) that affects various parts of the brain and the spinal cord and causes tremendous suffering to the affected individuals (1). It is the most common neurological disturbance among young adults in populations of Northern European origin (2). The chronic disease course of MS is unpredictable and life-long. The patients often lose their ability to work and need considerable extra nursing assistance in the worst cases. The various clinical manifestations of MS, such as paralysis, reduced coordination, speech problems, visual, sensory and mental disturbances, incontinence etc. are direct consequences of the tissue damage in the CNS, but are typically pronounced during a relapse. The tissue damage is due to perivenous demyelination of nervous tissue, mononuclear cell infiltration as well as astrocytic gliosis (3). For unknown reasons, MS affects women more frequently than men. Geographically, there is an increasing gradient for MS from the Equator to the Northern hemisphere (2). However, the commonly accepted belief that MS frequency in Europe is directly related to latitude has been refuted by the many prevalence studies carried out in Southern Europe since 1980, which show that MS prevalence in this area is, in fact, much higher than had been believed (4). The new estimated prevalence rates in continental Italy and Sicily since 1980 range from 32 to 58/100,000, those in Sardinia ranging from 59 to 103/100,000. This is much higher when compared with a rate of 5 to 25/100,000 in European countries from about 36 to 46˚ north latitude, based on prevalence studies carried out in Italy between 1959 and 1975. MS not only causes physical disability but also has a major adverse psychosocial and economic impact on the affected individual, his family, and the society.

Epidemiological data in Finland demonstrated an increased prevalence rate of MS in recent years (5). It was also found that the increased prevalence is mainly due to an increased incidence in western (Seinäjoki) and southern (Uusimaa) Finland (5, 6), pointing to locally acting environmental factors.

Epidemiological studies have suggested an interplay between genetic and environmental factors in the aetiology of MS. A genetic component in MS was indicated by several lines of investigation. First, in studies with monozygotic

twins, if one twin developed MS there was a nearly 30% chance that the other twin would also develop MS (7-9). However, one should keep in mind that monozygotic twins are more likely to share a similar life style, which unavoidably renders them exposed to mutual environmental factors. Second, there is a much higher familial recurrence rate (about 15%) compared to a background age-adjusted risk in the investigated population (about 0.3%) (10-12). However, the possibility that the higher familial recurrence rate is due to an exposure to the same environmental factors within the family can not be totally excluded. Third, there is an HLA association in MS patients with HLA DRw15, DQw6, Dw2 in Caucasian Europeans and North Americans (13, 14) and a secondary association with DRw15, DQw6, Dw2 in Swedish MS patients (15). Fourth, descriptive data since 1980 indicates that ethnicity plays an important role in determining European MS distribution (4). On the other hand, substantial evidence supports an environmental contribution to the pathogenesis of MS. Epidemiological data indicates that there is a latitude distribution in the incidence of MS: The northern latitudes tend to have higher incidence of MS (2). Furthermore, when individual countries were surveyed for the incidence of MS, a clustering of MS cases was reported (6, 16-20). This clustering was also seen when the same regions were resurveyed a generation later. Thus, Kurtzke suggested that "MS is intrinsically related to geography, and therefore MS can be defined as an acquired, exogenous, environmental disease" (21).

Several studies have suggested that infections may be the cause of MS. In particular, a viral aetiology for MS has been proposed for a long time (22-27). Studies in Iceland indicated that there was an increase in the incidence of MS following World Wars I and II when the British, Canadian and American soldiers occupied the country (28). Similarly, epidemiological surveys of the Shetland-Orkney Islands and Faroe Islands demonstrated an increase in MS following the Second World War, and the locations of the British troop encampments strongly correlated with the place of residence of all MS patients (29-35). In fact, there were few, if any, documented cases of MS prior to 1943 in the Faroe Islands. Furthermore, an increase in MS exacerbations has been found in patients experiencing bacterial infections (36, 37). Likewise, it has also been found that viral infections were often temporally related to exacerbations of MS (24, 38-40).

Further evidence supporting an infectious aetiology for MS comes from migration studies. It has been consistently reported that those who migrate before adolescence develop MS at a rate similar to that of natives of their adopted

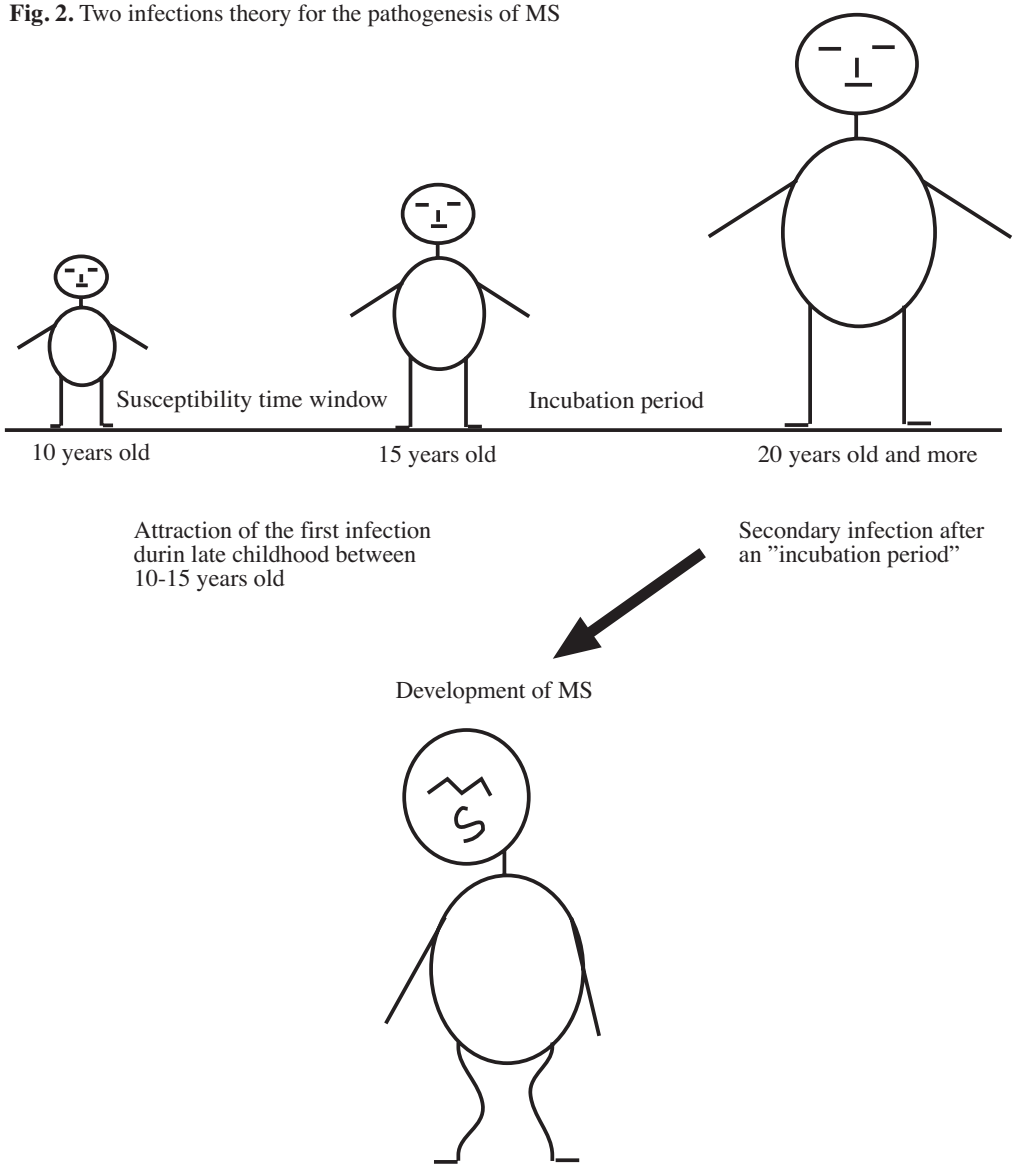
country. Those who migrate after adolescence, on the other hand, seem to retain the risk of their country of origin (41-48). These data indicate that there is a susceptibility "time window" during late childhood for the development of MS later. During this time the individuals are assumed to catch some infectious agents. Intriguingly, MS does not occur immediately after a clinical infection. A latent incubation period of about 15-20 years is needed before the onset of MS (21, 49). The concept of a critical exposure time during late adolescence for conceiving MS is further supported by a cluster study in Hordaland, Norway. This study demonstrated that patients in the same birth cohort had lived closer to each other than would have been expected between the ages of 13 and 20 (50). All in all, these data are consistent with the hypothesis that infections early in life could subclinically prime genetically susceptible individuals for MS (51). Interestingly, this view may also apply to insulin dependent diabetes mellitus (IDDM) (52). Another study on the Orkney islands identified two clusters: the MS patients were found to have clustered in a time period at least 21 years before onset, and again just before onset (53). The authors went on to speculate that this finding may reflect exposure to the same agent at two points in time or exposure to two different agents (Fig. 2).

2.2 Experimental autoimmune encephalomyelitis (EAE)

2.2.1 Background

EAE is induced by immunization of animals with a neuroantigen emulsified in complete Freund's adjuvant (CFA). It is an inflammatory and demyelinating disease of CNS and has served as a prototype animal model for human MS (54). The origin of this model can be traced to efforts to develop a vaccine for the rabies virus. It was found that a small percentage of humans who received Pasteur's rabies vaccine suffered from encephalomyopathy (55), which appeared to be due to the whole CNS homogenates in the vaccine rather than to the rabies virus or infective contaminants (56). Subsequently, Koritschoner and Schweinburg (57), and later Hurst (58), reported of a successful induction of paralytic disease in rabbits by a repeated injection of normal brain tissue. However, no obvious pathological changes were present in the brains or spinal cords of the paralysed rabbits. Rivers et al induced experimental allergic encephalomyelitis (EAE) by multiple intramuscular injections of nonhuman primates with rabbit brain homogenates or the concentrated alcohol-ether extracts (59, 60). The EAE induced in rhesus monkeys by Rivers was charac-

Fig. 2. Two infections theory for the pathogenesis of MS



terized by an inflammatory reaction accompanied by demyelination. Shortly after, Freund and McDermott developed a new technique of adding various adjuvants as aquafor, paraffin oil and heat-killed tubercle bacilli (61) to the antigens. This new immunization technique opened the way for the experimental production of encephalomyelitis with a considerably reduced number of injections. By using such a technique, antibrain antibodies in the monkey

were produced (62). Shortly after, rapid production of acute EAE in Rhesus monkeys (63, 64), guinea pigs and rabbits (65-68), following the injections of brain tissue with adjuvants, was reported. EAE was also induced in other species such as sheep, dogs and chickens (69). In 1949 Olitsky et al induced demyelinating EAE in mice (70), thus establishing murine EAE as a model for a CNS demyelinating disease, such as MS. For the first time, Ferraro reported the production of chronic EAE in Rhesus monkeys with repeated injections (71). Thereafter, a number of different EAE models have been reported (see reviews (72, 73)).

For the past 40 years, rats and mice have proven extremely useful for investigating the pathogenesis of EAE. However, the disease induced in rats of the Lewis strain is mainly acute, and most animals recover spontaneously. Moreover, they are resistant to subsequent active EAE induction. Although there is perivascular mononuclear cell infiltration in the spinal cord and the brain stem, demyelination is minimal. In contrast, EAE induced in mice is characterized by both mononuclear cell inflammation and demyelination, features that resemble human MS (72). Another advantage for the use of mice to study EAE is that there are many inbred and congenic strains. In addition, many mouse-specific reagents are available for study of the genetic aspects and cellular interactions associated with the disease (74). The susceptible strains of mice, such as SJL/J and PL/J, are not resistant to reinduction of the disease. Furthermore, it is possible to induce the chronic relapsing EAE in mice - a model that resembles human MS more closely (75, 76). Based on the above considerations, and because the current phamacotherapy of MS lies on immunomodulation, the current studies were concentrated on analyzing the immunopathogenesis of EAE in SJL/J mice.

EAE is the most thoroughly studied animal model for human autoimmune diseases, and much of our understanding of the pathogenesis of autoimmune diseases have been derived from studies of EAE. Several reasons may account for the popularity of EAE as a model for antigen-induced autoimmunity: (a) EAE has been developed earlier than other models, (b) EAE is easily induced in many species, (c) the clinical manifestations of EAE, namely paralysis and loss of tail tone (rodents), appear apparent to most observers (77) and (d) myelin basic protein (MBP), the primary autoantigen in EAE, abundant in CNS tissues, is easy to extract and purify. In contrast with other autoantigens such as acetylcholine receptor (experimental autoimmune myasthenia gravis) or collagen (collagen induced arthritis), MBP is relatively small in size; murine MBP

has a molecular weight of approximately 17 kDa. The small size of MBP has facilitated the identification of its critical determinants. Recently, a proteolipid protein and myelin oligodendrocyte glycoprotein (MOG) have been shown to induce EAE effectively. Hitherto, a considerable amount of literature has been generated following the first paper on EAE by Rivers (59). In the following, those aspects of EAE that are relevant to this dissertation will be reviewed.

2.2.2 Autoantigens used in the immunization

Initially, brain homogenates or its crude extracts were used as the antigen for immunization (57-59). It has been shown that heterologous brain tissue is less efficacious than homologous brain tissues (78), and that it is possible to induce EAE by endogenous brain tissue (79). These data suggest that the host is not tolerant to certain self CNS antigens. Kabat suggested that the autoantigens were located in the white matter, since injection of fetal CNS tissue, lacking myelin, did not cause EAE (80). MBP, the major protein constituent of myelin (approximately 30% of total protein), was identified as an encephalitogenic antigen in the CNS tissue (81, 82). MBP consists of a series of charge isomers, resulting from posttranslational modifications (e.g., deamidation or phosphorylation) (83). The predominant form of MBP has a molecular weight of 18.5 kDa. In rodents, another MBP molecule of 14-kDa is produced in addition to the 18.5-kDa molecule as a result of differential RNA splicing of the MBP gene (84). The amino acid sequences of MBP from various species have been determined (85).

MBP has been shown to induce acute EAE in mice, rats, guinea pigs, rabbits and monkeys, as well as in humans accidentally injected during the course of laboratory experiments (69). Encephalitogenic epitopes of MBP have been identified by several investigators using different EAE models. The MBP peptide H-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-OH causes EAE in guinea pigs (86). The MBP peptide H-Thr-Thr-His-Tyr-Gly-Ser-Leu-Pro-Gln-Lys-OH causes EAE in rabbits (87). The MBP peptide H-Phe-Lys-Leu-Gly-Gly-Arg-Asp-Ser-Arg-Ser-Gly-Ser-Pro-Met-OH causes EAE in monkeys (88). The MBP peptide H-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg- Ser-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe-OH (89-91) causes EAE in rats. Guinea pig MBP residues 68-86 and its corresponding synthetic peptides (YGSLPQK-SQRSQDENPV) and synthetic peptides 73-86 (QKSQRSQDENPV) are encephalitogenic in Lewis rats (74). Initial studies in mice demonstrated that

MBP 1-37 and C-terminal 89-169 of guinea pig MBP cause EAE in inbred mouse strains (92-94). It has been shown that SJL/J mice responded mainly to the encephalitogenic determinant in peptide 89-169, whilst PL/J mice as well as (SJL/JxPL/J) F1 mice responded primarily to an encephalitogenic determinant within peptide 1-37 of guinea pig MBP (92, 95). By using overlapping synthetic peptides, MBP residues 1-9 (ASQKRPSQR) were identified to be the dominant PL/J epitope (96). The N-terminal alanine in native MBP is N-acetylated, and the synthetic peptide induces EAE only when N-acetylated. The encephalitogenic epitope for SJL/J mice was localized to residues 89-101 (97), corresponding closely to the 87-99 epitope that is encephalitogenic in Lewis and Buffalo rats. In addition, two minor determinants of mouse MBP spanning residues 87-98 and 91-104 were found to induce EAE in SJL/J mice (98).

Another major protein of myelin is proteolipid protein (PLP). PLP is only found in CNS myelin and it represents about 50% of the myelin protein. PLP is capable of inducing EAE in mice (99, 100). However, PLP is a hydrophobic protein that has been difficult to isolate (101). Synthetic peptides of PLP have been broadly used for the induction of EAE. Murine PLP peptide residues 103-116 (YKTTICGKGLSATV), 139-151 (HSLGKWLGHPDKF) and 178-191 (NTWTTCQSIAFPSK) were found to be encephalitogenic in SWR/J (H-2^q) and SJL/J (H-2^s)mice (102, 103). Minimum sequence requirements within these PLP peptides for induction of EAE were also determined (104). PLP 43-64 (EKLIETYFSKNYQDYEYLINVI) was found to be encephalitogenic in PL/J mice and induced a relapsing disease (105). Three overlapping synthetic peptides corresponding to the region of PLP residues 217-240 were encephalitogenic for Lewis rats, provided that the peptides were N-acetylated (106).

Recently, myelin oligodendrocyte glycoprotein (MOG) has received considerable attention from researchers as a prominent autoantigen for induction of EAE (107). MOG is a quantitatively minor oligodendrocyte-specific protein that is preferentially incorporated into the outermost surface of the myelin sheath (108), where it accounts for only 0.01-0.05 wt% of the membrane protein (109). The mature MOG protein is a highly conserved 218-amino acid member of the immunoglobulin superfamily (110). The putative N-terminal Ig domain (MOG^{igd}) is located at the outermost surface of the myelin sheath, making it an ideal target for antibody-mediated demyelination (111). It has been shown that many MOG peptide sequences contain determinants that are

encephalitogenic in several different species/strains (rats, mice and monkeys etc.). Compared with other autoantigens, MOG is unique in that it is the only CNS autoantigen known to induce both an encephalitogenic T-cell response and a demyelinating autoantibody response in EAE. EAE can be induced in many different inbred mouse strains using MOG^{igd} and synthetic MOG peptides. MOG^{igd} induces severe chronic EAE in DBA/1, C57l/6 and NOD/Lt mice, but a somewhat milder disease in SJL/J and Biozzi AB/H mice (112, 113). MOG_{35-55} has been used to induce a severe, chronic EAE in Biozzi AB/H ($H\text{-}2^{dq1}$), PL/J ($H\text{-}2^{u}$), C57Bl/6 ($H\text{-}2^{b}$), C3H.SW ($H\text{-}2^{b}$), and NOD/Lt ($H\text{-}2^{g7}$) mice, while MOG_{92-106} induces EAE in SJL/J and A.SW mouse strains ($H\text{-}2^{s}$) (112, 114-117) ; and MOG 79-96 induces severe EAE in $H\text{-}2^{q}$ mice (113). In SJL/J mice, it has been shown that MOG^{igd} is a far more potent encephalitogen than MOG_{92-106} and $PLP_{130-151}$ (111). EAE induced in SJL/J mice with MOG^{igd} is characterized by large cellular infiltrates reaching deep into the parenchyma associated with large areas of demyelination and extensive axonal damage (111).

In addition to MBP, PLP and MOG, the list of encephalitogenic components of myelin also includes myelin associated glycoprotein (MAG) (118), myelin oligodendrocyte basic protein (MOBP) (119) and S-100β (120).

2.2.3 Role of killed Mycobacterium tuberculosis bacteria

Killed *M. tuberculosis* - a major component of CFA (121) is one of the most potent adjuvants. Lewis and Loomis discovered that living, virulent tubercle bacilli injected into the peritoneal cavities of guinea pigs enhanced antibody formation to various antigens subsequently introduced by the same route (122, 123). This adjuvant activity of *M. tuberculosis* has been confirmed by many other investigators subsequently.

The smallest subunit of the mycobacterial cell wall that retains adjuvant activity is N-acetyl muramyl-L alanyl-D isoglutamine, called muramyl dipeptide (MDP) for short (124). Muramyl dipeptide is able to replace killed mycobacteria in CFA for the induction of both humoral and cellular immunity (124).

The adjuvant activity of mycobacteria could be due to the recognition of defined microbial components by phylogenetically ancient receptors present on accessory cells (125). This recognition signals the danger of infection and elicits

an adaptive immune response (126). When an autoantigen is presented by accessory cells that are simultaneously activated by microbial components, an autoimmune response may be initiated through a bystander mechanism (127) (Fig. 3). Indeed, it has been shown that CFA (killed mycobacteria plus paraffin oil) induced Th1 cytokines whilst paraffin oil-the incomplete Fruend's adjuvant (IFA) alone induced Th2 cytokines (128-130). However, the co-stimulation theory failed to explain why autoimmune diseases are so rare whilst infections are so common. Neither can it explain the chronicity - the hallmark of all the clinical autoimmune diseases.

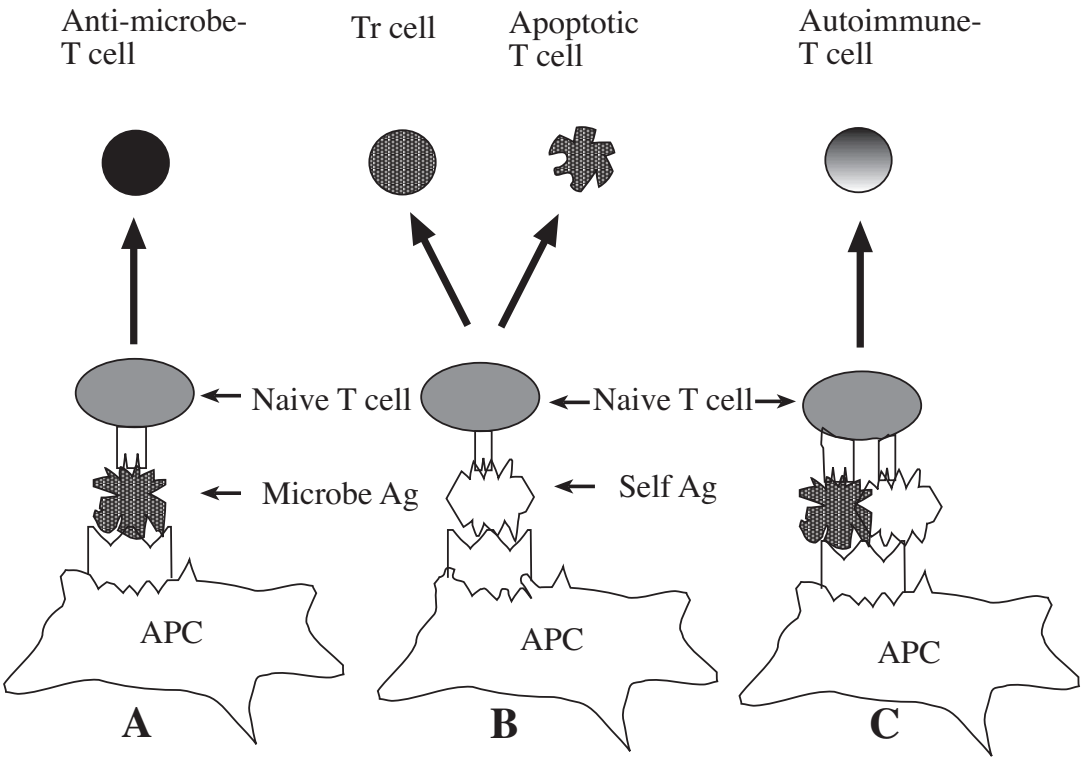


Fig. 3. Schematic illustration of the bystander mechanism for the development of autoimmune T cells. **A:** anti-microbe effector T cells developed after microbial Ag is presented to naive T cells by APC. **B:** Presentation of self-Ag alone by APC to naive T cells resulted in either a development of regulatory T cells (Tr) or apoptosis of self-Ag specific T cells. **C:** when self-Ag is presented together with microbe-Ag by the same APC, autoimmune T cells develops.

In searching for substitutes for *M. tuberculosis*, several Gram-positive and negative organisms have been tested and found to be effective in the induction of EAE (131). It was found that practically all mycobacteria (*M. butyricum, fortuitum, phlei, smegmatis, and tuberculosis H37 Ra*) are equally good as *M. tuberculosis* (131, 132). Nevertheless, killed *M. tuberculosis* has been traditionally used in the induction of EAE. We have used both *M. tuberculosis* and *M. butyricum* as constituents of CFA in our studies (76).

While both neuroantigen and killed mycobacteria are essential for a successful induction of EAE (133), it is of interest to note that their proportion to each other is more important than their absolute quantity. Shaw et al first presented without discussion or comment data indicating a crucial role of the ratio between neural antigen and killed *M. tuberculosis* (134). Production of EAE was affected adversely not only by a reduction in the amount of killed *M. tuberculosis* as adjuvant, but also by its increase beyond a certain critical level. The same held true for neural antigen. In other words, the optimal induction of EAE depends on the optimal proportion between neuroantigen and killed mycobacteria (132, 133). In the induction of EAE in guinea pigs, the threshold amount (minimum amount for effective disease induction) for brain antigen was determined to be around 0.03 to 0.05 mg (dry weight), and about 0.02 mg for *M. butyricum* and about 0.1 mg of *M. tuberculosis*. The threshold amount for the spinal cord antigen is just below 0.06 mg, but about ten times more is required for the development of maximal severe EAE. As little as 0.01 mg of *M. tuberculosis* can serve as an adjuvant when spinal cord is used as an antigen, but at least five times more is required for maximal disease (133).

At the moment, the mechanism underlying this interesting phenomenon is unclear. Further studies are warranted to look into the changes of various immunological parameters caused by changes in the ratio between neuroantigen and killed mycobacteria in the induction of EAE.

2.2.4 Role of paraffin oil

The use of paraffin oil (hydrocarbons, alternatively called mineral oil) as an adjuvant has a long history. In 1899, Grassberger found that the cellular reaction to mycobacteria was enhanced by the addition of butter and even more so by paraffin oil (135). With the aid of emulsifying agents (136), Freund and

McDermott in 1942 immunized Guinea pigs with horse serum emulsified in paraffin oil containing killed tubercle bacilli in a water-in-oil emulsion (61), thus starting the era of complete Freund adjuvant - CFA. Soon after, CFA became popularly used for a rapid induction of EAE (63, 64) as well as other experimental autoimmune diseases.

The mode of action of paraffin oil as an adjuvant is not clear. It has been postulated that the effect of paraffin oil is to localize the antigen in its initial depot, resulting in a slow release of the antigen (137). However, the effect of paraffin oil can not merely be a depot effect. Oil of plant origin can also form stable emulsions with antigen, but it has not been found to substitute paraffin oil, which is resistant to enzymes available in the animal economy (138). On the other hand, it has also been shown that EAE can still be induced when the injection sites are removed only one hour after the injection of neuroantigen emulsified in CFA (139). Freund proposed that the effect of paraffin oil is to bring a relatively large amount of antigen into contact with phagocytic mononuclear cells. Additionally, the creation of numerous foci, remote from the site of injection, in the lymph nodes and lungs that may act as sources of antigenic stimuli, might also be important (139).

2.2.5 Role of Pertussis toxin

Pertussis toxin (PTX) is the major protein toxin produced by virulent strains of *Bordetella pertussis*, the organism that causes whooping cough (140). PTX has multifaceted effects that include induction of lymphocytosis, stimulation of insulin secretion, enhancement of vascular permeability, inhibition of lymphocyte recirculation, mitogenic effects on T and B cells, enhancement of IgE production and sensitization to histamine. It has thus been variously referred to in the literature as lymphocytosis-promoting factor, islet-activating protein, histamine-sensitizing factor and pertussigen (reviewed in (141)).

There has been a long history of using pertussis as an adjuvant in the enhancement of experimental autoimmune diseases elicited by immunization of laboratory animals with the appropriate tissue antigens. Such models include e.g. experimental autoimmune orchitis (EAO) (142), experimental autoimmune uveoretinitis (EAU) (143) and EAE (144). As little as 20 ng of PTX enhanced the induction of EAE in rats (145). In mice, normally 100-200 ng of PTX is injected intravenously and this dose is well tolerated.

The mechanism by which PTX promotes the development of experimental autoimmune diseases is not fully understood. Early studies by Linthicum and Sudweeks (146-148) demonstrated the effects of PTX in the EAE model to histamine sensitization genes and the disruption of the blood-brain barrier (BBB). Based on these observations, it has been widely assumed for over 20 years that the enhancing effect of PTX on the induction of autoimmunity is due to the breakdown of the BBB and a subsequently facilitated infiltration of inflammatory cells into a target organ. However, this dogma can not be conclusive. Two concurrently occurring events (here, the enhancement of EAE and increased BBB permeability by PTX) do not necessarily have a causal relationship. One may well argue that the enhancing effect of PTX on EAE was a result of hypoglycaemia caused by PTX (141). It has been known that the encephalitogenic T cells take about 7 days to develop and migrate into the CNS immediately before the onset of EAE (around 9 days after the immunization) (149). If the enhancement of vascular permeability is indeed a major mechanism, the effect of PTX should be maximal when it is administered at the time of effector cell migration to the CNS. However, it has been shown that PTX was most effective when given i.v. from 1 day before to 5 days after injection of MSCH in CFA, when a uniform and severe disease was induced 11-13 days after immunization of mice (150). Administration of PTX 12 days after immunization was not effective (150). McAllister found that treatment of recipient rats with PTX at the time of uveitogenic cell transfer delayed the onset of EAU (143). Likewise, it has also been shown that administration of PTX to mice concurrently with adoptive transfer of activated uveitogenic T cells or 1 week after immunization with retinal Ag in CFA not only failed to enhance but actually completely blocked the development of EAU (144). These data support the notion that PTX plays its enhancing role in the early phase of the development of autoimmune T cells.

2.2.6 Acute vs chronic relapsing EAE

The most intensively studied EAE models have been established in rodents by a single immunization regimen and the disease course is usually acute. Chronic relapsing EAE (CR-EAE) resembles MS more closely than acute EAE. Spontaneous relapses of EAE (71, 151, 152) have been reported to occur in a small proportion of sensitized animals. Chronic (153) and relapsing (154, 155) forms of EAE induced by single sensitization of immature guinea pigs have also been reported. Similar work has also been done with hamsters (156), Lewis

rats (157) and Biozzi mice (158). Disease relapse was also induced by low-dose cyclosporin-A treatment in a Lewis rat EAE model (159). However, relapses in these models were either rare or late at the onset. Chronic relapsing EAE has been established in mice also by adoptive transfer of myelin basic protein-sensitized T cells (160) or T-cell clones (161). However, the onset of relapse is late (40-50 days) in the former model, and only few mice survive in the latter one, and the frequency of relapse is low. The cell transfer models have limited relevance to the human disease, since it is unlikely that patients have ever been transferred with autoimmune T cells. When the pertussis vaccine was included in the immunization, a delayed form of relapsing EAE was induced (162, 163). However, only few relapses were recorded in a prolonged follow-up. It was also reported that bacterial superantigen SEB induced relapses of paralytic disease in mice that were in clinical remission (164). A. M. Brown established relapsing EAE in SJL/J mice by two injections of neuroantigen emulsified in CFA at 1 week intervals (75). This regimen has been broadly used for the induction of CR-EAE thereafter. However, the first relapse in this model appeared also rather late (30 to 50 days) and only 2 to 3 relapses were recorded during the 6 month follow-up. Occasionally, the majority (up to 90%) of mice died of the first disease attack.

2.3 Cytokine expression in EAE

Cytokines are small protein or glycoprotein messenger molecules that convey information from one cell to another (165), and play an important role in inflammation and autoimmune responses (166). $CD4^+$ T lymphocytes can develop into Th1 or Th2-type cells, which are characterized by their abilities to produce different cytokines (167). Th1 cells produce IL-2, IFN-γ and TNF-β whereas Th2 cells produce IL-4, IL-5 and IL-10. These two groups of cytokines are often antagonistic to each other in their effects (168) (Fig. 4). In EAE - a $CD4^+$ T cell - mediated autoimmune disease (77), Th1-type cytokine expression in CNS as well as in the peripheral lymphoid organs has been reported to be associated with disease progression, whereas the production of Th2-type cytokines has been correlated with disease recovery (169-172). Similarly, oral tolerance to myelin basic protein and a natural recovery from EAE are associated with a down regulated production of inflammatory cytokines and upregulation of transforming growth factor ß and IL-4 (172-174). Both IL-4 and IL-10 are required for the induction of oral tolerance (175). The brains from EAE susceptible SJL mice contain mainly IL-2 and IFN-γ positive cells, whereas the

Fig. 4. Regulation of Th responses

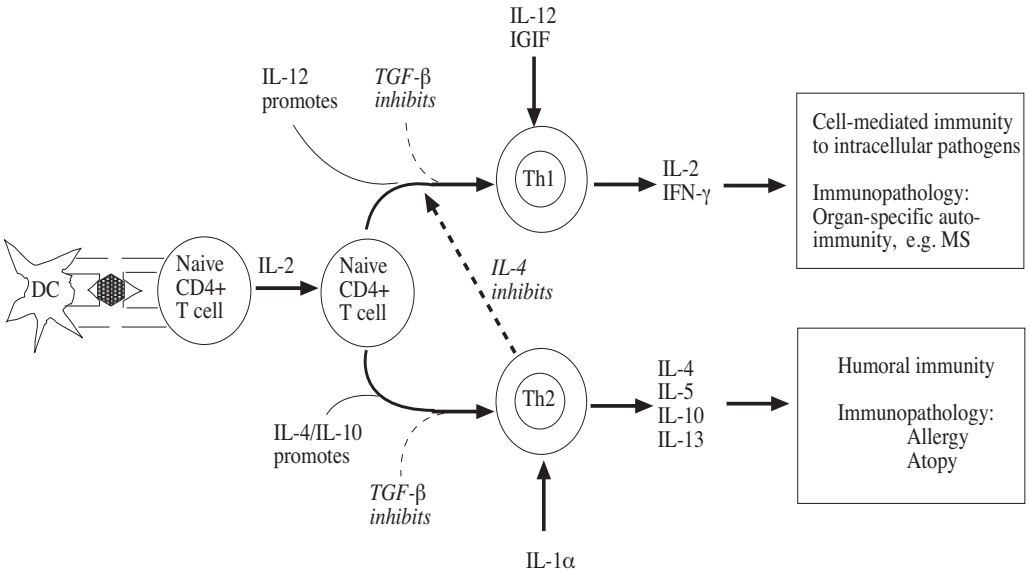


Fig. 4. Naive CD4+ Tcells can develop into Th1 cells responsible for cell-mediated immunity in response to IL-12. Th1 development is dependent on IFN-γ, and maintenance of the phenotype depends on stimulation in the presence of IL-12 and IGIF (IFN-γ inducing factor). Naive CD4+ T cells develop into Th2 cells in the presence of IL-4 and IL-10. It has been suggested that a Th1 to Th2 switch may prevent the development of organ-specific autoimmune diseases.

brains from EAE resistant B.10 S mice have fewer cell infiltrates, which predominantly express IL-4 and IL-10 (176). Similarly, it has also been found that mice with IFN-γ receptor deficiency are less susceptible to experimental autoimmune myasthenia gravis (EAM) (177). The regulatory roles of IL-4 and IL-10 have further been strengthened by findings showing that IL-4 and IL-10-deficient mice have developed a more severe EAE (178, 179), and a local production of IL-4 and IL-10 in the brain prevented the induction of EAE (180, 181). Taken together, these studies suggest that the Th1 cytokines are involved in disease progression, whereas the Th2 cytokines promote disease recovery.

In contrast to the above, there is now ample evidence that challenges the Th1/Th2 paradigm in EAE/MS. For example, myelin basic protein-specific Th2 cells were found to cause EAE in immunodeficient hosts rather than to protect them from the disease (182). Mice rendered deficient for IL-2 by gene targeting developed a generalized autoimmune disease (183). IFN-γ has been

shown to suppress EAE, and IFN-γ-knockout mice had a more severe EAE (184-187). However, these findings were contradictory to demonstrations that the treatment of patients suffering from MS with IFN-γ led to an exacerbation of the disease (188). It has been reported that a local production of IL-10 by islet cells accelerated immune-mediated destruction of β cells in nonobese diabetic mice (189). Also, recombinant IL-4 has been found to aggravate experimental autoimmune uveoretinitis (EAU) in rats (190). Recently, there have been reports showing that both Th1 and Th2 cytokines are produced by the peripheral lymphoid organs, peripheral blood mononuclear cells as well as by immune cells in the CNS in EAE (191, 192). Similarly, both Th1 and Th2 cytokines have been found to be produced in patients suffering from MS (193, 194). However, little information is available on the cytokine mRNA expression profiles in the peripheral lymphoid organs during relapse or remission of MS or EAE.

2.4 Expression of cellular adhesion molecules (CAMs) in EAE

The lymphoid system is functionally compartmentalized into discrete primary (bone marrow and thymus), secondary (lymph nodes, gut-associated lymphoid tissues and the spleen) and tertiary (the remainder of the body's tissues) lymphoid organs (195). Lymphocytes produced in the primary lymphoid organs migrate to the secondary lymphoid organs where antigen presentation takes place. The activated antigen-specific lymphocytes seek out and localize to particular tissues to elicit a local immune response. This process of lymphocyte trafficking is tightly regulated by the differential expression of cellular adhesion molecules (CAMs) (195-198). Furthermore, CAMs such as LFA-1 (by interaction with its ligand-ICAM-1), provide an important costimulatory signal for T cell receptor-mediated activation of resting T cells (199-201). In this part, only a few CAMs that have been studied in this thesis are reviewed.

Among identified CAMs, LFA-1 is one of the most thoroughly studied (202). LFA-1 can mediate the adhesion of leukocytes to endothelial cells through binding to ICAM-1 (203-206). The structure and functions of LFA-1 have been thoroughly studied (202, 207-209). LFA-1 expression becomes upregulated on infiltrating mononuclear cells and some resident cells in the brain of MS patients and in animals with EAE (193, 210-212). Lymphocytes from either cerebrospinal fluid (CSF) or the blood of MS patients express increased levels of CAMs including LFA-1 and CD44 (213). Parallel to the above reports, anti-

LFA-1 therapy has been shown to prevent EAE in mice and rats (214-216). Similarly, treatment with antibodies against LFA-1 and ICAM-1 prevented the adoptive transfer of murine Sjögren's syndrome into severe combined immunodeficient mice (217). In models of organ transplantation, injection of anti-LFA-1 antibody has been shown to dramatically prolong the survival of the grafts (217-219). However, it has also been reported that anti-LFA-1 therapy could actually exacerbate EAE (220, 221).

CD44 is a pro-inflammatory cell surface molecule participating in cell homing into target organs (195). CD44 is expressed by a wide variety of cell types and not just leukocytes (222). Monoclonal antibodies to different epitopes of CD44 interfere with a number of distinct lymphocyte adhesive interactions (223), and polyclonal anti-CD44 antibodies block cell binding to high endothelial venules (HEV) (224). Memory T cells display more CD44 than naive T cells (225). The expression of CD44 is increased on lymphocytes from either the CSF or blood of MS patients (213), suggesting a possible role for CD44 in the autoimmune demyelinating disease. In collagen II-induced arthritis, treatment with anti-CD44 antibodies has been shown to be beneficial (226-229). However, in EAE anti-CD44 therapy has yielded controversial results. While it has been shown that CD44 was involved in selective leukocyte extravasation during the inflammatory central nervous system disease and anti-CD44 treatment proved beneficial in EAE (230, 231), others showed that such a treatment had no effect on lymphocyte binding to blood vessels (232) and even had an exacerbating effect on EAE (233) and experimental thyroiditis (234). At the present time, it is difficult to interpret these controversial results.

ICAMs are members of the immunoglobulin superfamily and serve as cellular ligands for the leukocyte integrins (198). ICAM-1 is present on leukocytes, endothelial cells and many other tissues, and is perhaps the most important binding ligand for LFA-1 (208). Its expression is upregulated on activated cells and inflamed tissues. On the other hand, ICAM-2 expression is relatively stable (198). VCAM-1 is the endothelial ligand for $\alpha 4\beta 1$ integrin (or VLA-4). Its expression is inflammation induced and heterogeneous (235, 236), suggesting the possibility of tissue-selective function (195). So far, most of the studies on the role of ICAMs during MS have relied on immunohistochemical analyses of the expression of ICAMs during the different stages of disease on autopsy CNS material or on blood or CSF-derived lymphocytes from patients with MS (210). Upregulation of ICAM-1 and VCAM-1 in typical MS lesions has been observed (193, 211). The levels of both soluble ICAM-1 and VCAM-1 in CSF

were increased during MS relapse (237, 238). In EAE, upregulation of ICAM-1 and VCAM-1 in the CNS tissue coincided with the immigration of $CD4^+$ lymphocytes, while downregulation coincided with their emigration (239). In relapsing EAE, ICAM-1 is expressed intensely by vascular endothelium during the acute phase, less intensely during relapses, and at diminished levels during remission (240, 241). On the other hand, VCAM-1 mRNA levels were elevated in the spinal cord only at the end of the acute phase of EAE (242). Interestingly, it has been reported that VCAM-1 was expressed at higher levels in chronic MS lesions, while ICAM-1 was more uniformly expressed in lesions of all ages (193, 243). Moreover, increases in soluble VCAM-1 but not in ICAM-1 correlated with a decrease in MRI lesions in MS patients treated with interferon -β1b (244). These findings point to the possibility that VCAM-1 expression is involved in the recovery of the disease. Despite the fact that the expression and role of adhesion molecules have been a subject of studies focusing on the EAE/MS affected CNS, there is little information of the natural expression levels in the peripheral lymphoid tissues modulating the disease process.

2.5 ICAM-5

ICAM-5 (telencephalin) was first identified as a 130-kDa type I integral membrane glycoprotein (245). It has been reported that ICAM-5 is expressed exclusively within the telencephalon of mammalian brains, by certain subsets of neurons (245-248). Its expression is initiated in the newborn, and increases during postnatal CNS maturation in parallel with dendritic elongation and branching (246, 247). Because it is able to induce dendritic outgrowth (249), ICAM-5 has been suggested to function in the synaptogenesis in the developing mammalian brain.

However, the fact that ICAM-5 binds to the integrin receptor CD11a/CD18 (LFA-1) which mediates leukocyte adhesion (249, 250), and that the CSF concentration of sICAM-5, the soluble form of ICAM-5, was significantly increased in encephalitis (251), suggests that ICAM-5 might function as a mediator of communication and adaptation in the neuroendocrine and immune systems (252). The role of ICAM-5 in EAE has not been studied earlier, but the CSF concentration of ICAM-5 in human MS was not different from the control level (251).

2.6. Dendritic cells

Dendritic cells (DCs) are a sparsely distributed, migratory group of bone-marrow-derived leukocytes that are specialized for the uptake, transport, processing and presentation of antigens to T cells (253). The function of DCs, as exemplified by the Langerhans cells of skin, is that the DCs collect foreign antigens in peripheral sites, process the antigens, migrate to the T cell areas of lymph nodes, and then mature into a form competent to stimulate an immune response by those T cells that recognize the MHC-presented foreign antigenic peptides (253).

Distinct subtypes of DCs were initially more evident among mouse DCs than among human DCs, because of the ready availability of different murine markers not present on human DCs. Mouse DCs that are classed as ”mature” expresss CD11c (the integrin-α chain) and the co-stimulator molecules such as CD80, CD86 and CD40, and have moderate to high surface levels of MHC II. The T-cell markers CD4 and CD8 are also expressed on mouse DCs. CD11b (the integrin α_M chain of Mac-1) and the interdigitating DC marker CD205 (originally known as NLDC-145) are also expressed by mouse mature DCs (254). Using these surface markers, five DC subtypes are consistently found in the lymphoid tissues of uninfected laboratory mice (Table 1). Mouse spleen contained predominantly two DC populations in equal proportions (255).

The activation of the adaptive immunity starts when leukocytes recognize antigen peptides presented by the antigen presenting cells (APCs), such as dendritic cells (DCs), macrophages and B cells, in the context of MHC class I or II molecules. DCs express accessory molecules including LFA-3/CD58, ICAM-1/CD54, B7-2/CD86 that interact with receptors on T cells to enhance adhesion and signalling (256). Different co-stimulatory molecules on DCs affect the development of T cells in different ways. For example, microbial products may activate DCs through binding to Toll-like receptors (257, 258), thus resulting in the up-regulation of MHC class II and costimulatory molecules (CD80/CD86), and the development of pathogen-specific T cells. On the other hand, self antigens are usually not able to stimulate DCs to express those molecules that are induced by microbial products. As a result, self-antigen presentation by DCs to T cells in the lack of certain co-stimulatory molecules may actually lead to the development of regulatory T cells that down-regulate the autoimmune response. Studies on the role of DCs in MS are still limited. In particular, DCs in the peripheral immune organs could play a pivotal role in disease modification.

Table 1. Distribution of mouse DC subtypes in Lymphoid tissue (Reference 254)

	$CD4^-CD8^{hi}$ $CD205^{hi}CD11b^-$	$CD4^+CD8^-$ $CD205^-CD11b^+$	$CD4^-CD8^-$ $CD205^-CD11b^+$	$CD4^-CD8^-$ $CD205^+CD11b^+$	$CD4^-CD8^{lo}$ $CD205^{hi}CD11b^+$
Percentage of all DCs in					
Spleen	23	56	19	<4	<1
Thymus	70				
Mesenteric LNs	19	4	37	26	<4
Skin-draining LNs	17	4	17	20	33

2.7 Immunotherapy of MS

Until recently, no definitive therapies aimed at reducing attacks and slowing the disease process of MS have been available. However, among currently available therapies, Interferon-beta and glatiramer acetate (copolymer 1) have proven to have a modest effect on relapsing-remitting MS (RRMS).

β-interferon. In the belief that MS may be caused by a latent viral infection of the CNS, interferons (IFNs) have been tested, and IFN-β was found effective in the treatment of MS when administered intrathecally (259). The development of recombinant IFN-β and the demonstration of efficacy when administered via the subcutaneous route paved the way for large-scale clinical application. Recombinant IFN-β preparations approved for RRMS are interferon beta-1-b (Betaseron®) and interferon beta-1-a (Avonex®, Rebif®). However, the benefits have been only modest. The preparations were applicable only to patients with relapsing-remitting disease, and the safety and efficacy have been demonstrated only over a short period relative to the typical course of MS (260). Besides, neutralizing antibodies against IFN-β develop in some patients and are associated with reduced clinical and magnetic resonance imaging (MRI) efficacy (261). The exact mechanism of the action of IFN-β in MS is unknown. However, it has been reported that IFN-β suppresses T cell proliferation and inhibits T cell migration from the systemic circulation into the CNS, and alters the cytokine profile from proinflammatory Th1 to the relatively anti-inflammatory Th2 response (262). It has also been reported that IFN-β inhibits CD40-induced production of IL-12 by dendritic cells (263).

Glatiramer acetate. Glatiramer acetate was initially synthesized under the name copolymer 1 as a potential encephalitogen for the induction of EAE, but was found to do the opposite - to inhibit the induction of EAE in guinea pigs (264). It was later found to suppress or ameliorate EAE in a number of species, which led to trials in MS. The phase III trial in RRMS showed that glatiramer acetate had a similar efficacy to that of β-interferon in terms of reducing the relapse rate in RRMS (265). In addition, there was evidence for a beneficial effect on disability progression in patients with RRMS. However, no significant benefit was found on sustained disability progression (265). The mechanism of action of glatiramer acetate is also unclear. Thus, its activity has also been attributed to a shift from Th1 to Th2 predominance (266).

In addition to IFN-β and glatiramer acetate, mitoxantrone has been approved for RRMS as well as for secondary progressive MS (SPMS). This was based on positive outcomes from European studies (267), despite equivocal previous trials (268).

3 AIMS OF THE STUDY

To study the pathogenesis of a complicated human illness like MS, proper animal models are needed to represent the multiple facets of the disease. The general aim of this study was to establish a mouse model that resembles human MS more closely than classical EAE. Using different laboratory techniques, the cellular and molecular disease mechanisms in this model were investigated.

Specifically, the aims of the study were the following:

1. To establish the methodology for quantification of mRNA expression for cytokines as well as adhesion molecules (I)

2. To establish a mouse model that can resemble human MS closely (II)

3. To study the effect of memory responses in peripheral immune organs on the development of CR-EAE (III)

4. To study the expression of different cytokines and CAMs in the peripheral lymphoid organs of mice with CR-EAE (IV)

5. To study the expression of ICAM-5, a characteristic neural adhesion molecule, on immune cells and during disease evolution of CR-EAE (V)

4 MATERIALS AND METHODS

4.1 Animals

SJL (SJL/Ola/Hsd) mice studied at the Dept. of Virology, University of Turku, were bred and kept at the animal facilities of the University of Turku. For the experiments performed at the University of Tromsø and the Krakow Institute of Pharmacology, Polish Academy of Sciences, female SJL (SJL/Ola/Hsd) mice were purchased from Harlan UK Ltd. (Bicester, England). Mice were kept at the animal facilities of the University of Tromsø, Tromsø, Norway, and of the Krakow Institute of Pharmacology, Polish Academy of Science, Krakow, Poland. The experiments were carried out in accordance with the ethical guidelines for animal research in the respective countries and institutes.

4.2 Adjuvants

Killed *Mycobacterium tuberculosis* (M. tb), *Mycobacterium butyricum* (M. b) and incomplete Freund's adjuvant (IFA) were obtained from Difco Laboratories (Detroit, MI, USA). Complete Freund's adjuvant (CFA) was generated by adding 80.0 mg of M. tb and 10.0 mg of M. b to 10.0 ml of IFA. Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA, USA).

4.3 Neuroantigen and control antigen

Mouse spinal cord homogenates (MSCH) and mouse heart homogenates (MHH) were produced by homogenizing spinal cords and hearts from 2- to 4- month-old SJL mice in PBS and lyophilized. When used, MSCH was dissolved in PBS and kept on ice for 20 minutes. This MSCH suspension was emulsified with an equal volume of CFA. 25 μl of the antigen emulsion was injected into the hind footpads of mice.

4.4 Semliki Forest virus and reagents for in vitro cell culture

An avirulent mutant strain of Semliki Forest virus (SFV-A7) was obtained from Dr. H.E. Webb (Neurology Unit, Department of Neurology, Rayne Institute, St

Thomas' Hospital, London, UK). The virus was propagated in a mouse brain cell line-MBA-13. Virus stocks were titrated by a standard plaque assay method and aliquots stored at -70˚C. Lipopolysaccharide from *Escherichia coli* was purchased from Sigma (L-8399) (St Louis, MO, USA). The round bottom tissue culture tubes were from Nunc (Roskilde, Denmark).

4.5 Antibodies and agents for immunohistochemistry and flow cytometry

A rabbit polyclonal antiserum against the extracellular part of ICAM-5 was made by using ICAM-5 (D1-D9)-Fc as an immunogen as described previously (Tian et al. 1997). Goat anti-rabbit immunoglobulin-specific polyclonal antibody (FITC), rat anti-mouse CD3 (R-PE) and CD19 (PerCP) monoclonal antibodies, hamster anti-mouse CD11c (APC) and their control monoclonal antibodies were all from BD Biosciences Pharmingen (San Diego, CA, USA). MACS microbeads conjugated with hamster anti-mouse CD11c and rat anti-mouse CD11b monoclonal antibodies were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). DNase I was from Roche Diagnostics GmbH (Mannheim, Germany). Collagenase was purchased from Sigma Chemical Co (St. Louis, MO, USA). The three-step avidin-biotin complex/horseradish peroxidase method was from ABC, Vectastain, Vector Laboratories (Burlingame, CA).

4.6 Reagents for RNA preparation, DNA isolation, PCR reaction and In situ hybridization

Guanidine thiocyanate was purchased from Sigma. Phenol and chloroform were obtained from Amresco (Solon, OH, USA). SuperScript™ choice system for cDNA synthesis was purchased from GIBCO BRL, Life Technologies Inc. (Rockville, MD, USA). AmpliTaq Gold DNA polymerase was from Perkin Elmer (Branchburg, NJ, USA). The QIAGEN plasmid purification kit, QIAquick gel extraction kit and QIAquick PCR purification kit were from QIAGEN, (Valencia, CA, USA). Nylon membrane (Gene Screen Plus) was purchased from NEN® Research Products (Boston, MA, USA). SeaPlaque low gelling temperature agarose was from FMC (Rockland, ME, USA). The [α-^{32}P] dCTP was purchased from NEN Life Science Products (Boston, MA, USA). Agarose and DNA random primed labeling kit were products of Boehringer Mannheim (Mannheim, Germany). The phosphoimaging system

used for radioactivity quantification was Fujifilm BAS-2500, Fuji Photo Film Co., Ltd (Tokyo, Japan). SDS (sodium dodecyl sulphate), EDTA (ethylenediaminetetraacetic acid), Tris-HCl, yeast tRNA, NaAc, HCl, NaCl, paraformaldehyde, proteinase K, TEA (triethanolamine), acetic anhydride, RNase A, Denhardt's solution, dextran sulphate were all from Sigma. 1.4-dithio-DL-threitol (DTT) solution was purchased from Fluka Chemie (GmbH-9471, Buchs, Switzerland). Riboprobe (SP6, T3, T7) RNA labeling kit was from Promega (Madison, WI, USA). NICK™ columns were from Amersham Pharmacia Biotech AB (Uppsala, Sweden). Xylene was from YA-Kemia Oy (Helsinki, Finland) and formamide from Sigma-Aldrich Laborchemikalien GmbH (Seelz, Germany). Kodak NTB-2 emulsion, Kodak developer and fixer was from Eastman Kodak Company (Rochester, NY, USA). Ammonium acetate was from E. Merck (Darmstadt, Germany).

4.7 In vitro stimulation of murine splenic cells with A7-SFV and lipopolysaccharide

4.7.1 Preparation of splenic cells

Six-week-old SJL mice were sacrificed by cervical dislocation and spleens were collected under sterilized conditions. The spleens were minced and squeezed through a sterilized steel mesh. After lysing the red blood cells in a buffer (0.15 M of NH_4Cl, 1.0 M of $KHCO_3$, 0.1 mM of EDTA), the cells were washed twice in RPMI-1640. Twenty million splenic cells in 5 ml of RPMI-1640 supplemented with 10% inactivated fetal calf serum (FCS) and 0.01 mg/ml gentamycin were cultured at 37˚C in 15 ml round bottom tissue culture tubes in a 5% CO_2 atmosphere for 1 h before virus and/or LPS were added.

4.7.2 Stimulation of splenic cells in vitro

For virus treatment, 20 million mouse splenic cells were incubated with SFV-A7 virus at multiplicities of infection (m.o.i.) of 5 in 2 ml of culture medium for 1 h and then washed once in fresh culture medium. For LPS stimulation, 10 ng/ml of LPS was added to the culture. After 1 h, the cells were washed once, centrifuged and the cell pellet was suspended in 5 ml of fresh culture medium containing 10 ng/ml of LPS. For a combined treatment with virus and LPS, the cell preparation was handled as described for LPS treatment except

that SFV-A7 was added at 5 m.o.i. at the beginning and left to absorb for 1 h. Control cells were also washed once after 1 h and re-suspended in 5 ml of fresh medium. Cultured cells were harvested after 1, 3, 6 and 12 h with duplicate samples for each time point. In another experiment, SFV-A7 virus was added at 5 m.o.i. in 3 h before (-3 h) or after (+3 h) LPS and allowed to absorb for 1 h. The cells were then washed and suspended in fresh culture medium containing 10 ng/ml of LPS and cultured for another 3 h before harvesting. At the end of each experiment, the cultured medium was centrifuged and the total RNA was prepared from the cell pellets as described in 4.14.

4.8 Induction of acute EAE

Acute EAE was induced in SJL mice by a single injection regimen. Female SJL /J mice were injected with MSCH at a various doses (from 0.12 - 3.0 mg), together with either 100 μg of M. tb/12.5 μg of M. b or 200 μg of M. tb/25 μg of M. b, emulsified in paraffin oil (water:oil at 1:1 v/v). Immediately after the injection of MSCH emulsion, 200 ng of pertussis toxin was injected i.v. through the tail vein. The mice were checked daily and the disease index was recorded for up to 3 months.

4.9 Induction of chronic relapsing EAE (CR-EAE)

4.9.1 Pre-treatment of SJL mice

Three mg of MSCH, 200 μg of M. tb and 25 μg of M.b in 50 μl of PBS were injected into the left hind footpads of 6-to-8-week old female SJL mice. To control the effect of mineral oil, some mice were injected with the same amount of MSCH, M. tb and M. b in a water:mineral oil (1:1) emulsion (MSCH in CFA). As a control of the effects of mycobacteria, some mice were pre-treated with only 200 μg of M. tb and 25 μg of M.b in 50 μl of PBS (without MSCH).

4.9.2 Induction of CR-EAE in the pre-treated mice

In one experiment, two months after the pre-treatment, either 1.5 or 3 mg of MSCH, plus 100 μg of M. tb and 12.5 μg of M. b in 25 μl of water-oil emul-

sion (1:1) were injected into the right hind footpad (contralateral) of 10 and 5 mice, respectively. Immediately thereafter, the mice received an injection of 200 ng of PTX in 0.1 ml of PBS i.v.. As injection site controls, the second injection involving either 1.5 or 3 mg of MSCH was given into the left hind footpad (homolateral) in two groups of 5 mice. The mice were evaluated daily and disease severity was ranked as follows: 0, normal; 1, fur ruffling; 2, tail atonia, slight hind limb paralysis; 3, severe hind limb paralysis; 4, complete hind limb paralysis; 5, moribund. A clinical relapse was scored when a mouse developed a new neurological deficit (an increase in clinical severity of at least 1 grade) after a period of stabilization or improvement of its clinical score.

In another experiment, the same procedure was followed. However, the MSCH and CFA emulsion was prepared with a much weaker sonicator. One group of ten mice received 3 mg and another group of 9 mice received 1.5 mg of MSCH in the second injection. The mice were checked four times daily and weighed once daily at the same time point. Paralyzed mice were helped to food and water. This experiment was terminated one month after the second injection.

In the two control groups for monitoring the effects of mineral oil and mycobacteria, the second (contralateral) injection containing 1.5 mg of MSCH in CFA was also given two months later.

4.10 Cell isolation, adoptive cell transfer and active EAE induction

Two months after the pre-treatment, the pre-treated mice (injected with MSCH + M. tb + M. b in PBS), mice treated with an unrelated antigen (MHH + M. tb + M. b), and mice injected with MSCH in CFA were killed by cervical dislocation. Spleens and popliteal lymph nodes (pLN) that drain the pre-treatment injection sites were removed under sterile conditions, and squeezed through a sterilized steel mesh. Single cell suspensions were prepared in Eagle's cell culture medium. Lymphocytes were isolated by density gradient centrifugation in a Lymphoprep^{TM} solution (NYCOMED AS, Pharma Diagnostic Division, Oslo, Norway), according to the manufacturer's instructions. The purified lymphocytes were counted, and immediately thereafter 10, 20 or 50 millions of either spleen or lymph node cells were injected in 0.5 ml of Eagle's solution into age-matched mice through the tail vein. One week after the cell transfer, the recipient mice were injected into the hind footpad with 3 mg of MSCH plus 100 μg of M. tb and 12.5 μg of M. b in 25 μl of water-oil emulsion (1:1), followed by administration of 200 ng of Pertussis toxin in 0.1 ml PBS through the tail vein immediately thereafter. The conditions of the mice were assessed daily, and the disease severity was rated as follows: 0, normal; 1, fur ruffling; 2, tail atonia, slight hind limb paralysis; 3, severe hind limb paralysis; 4, complete hind limb paralysis; 5, complete paralysis in the four limbs; 6, moribund. The fatal outcome was also rated as grade 6. A clinical relapse was scored when a mouse developed a new neurological deficit (an increase in the clinical severity by at least one grade) after a period of stabilization or improvement of its clinical score.

4.11 Induction of EAE in splenectomized and lymphadenectomized mice

Splenectomy and lymphadenctomy were carried out on the pre-treated mice. Five of the pre-treated mice, as well as 5 mice from each control group were splenectomized, and another 5 were both splenectomized and lymphadenectomized. The mice were anesthesized by ether inhalation. Following surgery the operation wounds were closed and the mice were allowed to recover. One week after the surgery, the operated mice were injected into the right hind footpad with 3 mg of MSCH plus 100 μg of M. tb and 12.5 μg of M. b in 25 μl of water-oil emulsion (1:1). Thereafter 200 ng of pertussis toxin in 0.1 ml of PBS was administered through the tail vain. The disease severity was scored daily as described above.

4.12 Histological examinations

The mice were sacrificed and perfused with buffered 4% paraformaldehyde through cardiac puncture. Brain tissue was subsequently immersed in the same fixative overnight and embedded in paraffin. Sections were stained with hematoxylin and eosin and Luxol Fast blue (for myelin staining) and examined by light microscopy.

4.13 Gene subcloning

Murine IL-1ß cDNA (1.9 kb) was provided by Dr. J.J. Huang, Sterling Research Group (Malvern, PA, USA). Rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) cDNA was provided by Prof. R. Mäntyjärvi at the University of Kuopio, Kuopio, Finland. Plasmids containing cDNA of murine IL-2 (mIL-2) (0.93 bp), mIL-4 (590 bp), mIL-7 (492 bp), mIL-10 (500 bp), and mIFN-γ (700 bp) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). These cDNA fragments were isolated by either electrophoresis in a low melting point gel followed by agarase digestion or QIAquick gel extraction kit according to the manufacturer's instructions. For subcloning, the plasmid containing a 1.9 kb IL-1ß cDNA was digested by Pst I and a fragment of 103 bp was isolated, and subcloned into the PGEM3Z vector. The orientations of the subcloned plasmids were determined by either enzyme digestion or direct sequencing. Similarly, a 333 bp fragment of mIL-2 cDNA (obtained by digesting the original plamid with Pst I and Hind III) was subcloned into PGEM3Z vector. A 1233 bp fragment of rGAPDH was subcloned into PGEM 3Z vector at Pst I site. mIL-4, mIL-7, mIL-10 and mIFN-γ were subcloned into PGEM 3Z vector. A 607 bp PCR product of murine ICAM-5 (telencephalin) was subcloned into pGEM® vector, and the orientation was determined by sequencing. The plasmid constructs were transformed into JM109 strain of *E. coli*, amplified in Luria broth culture medium and purified with the QIAGEN plasmid purification kit, according to the manufacturer's instructions. The DNA fragment inserts were excised by digestion with DNA restriction enzymes, separated by electrophoresis in agarose gel and purified with the QIAquick gel purification kit. The DNA fragments were used for DNA probe preparation.

4.14 Total RNA extraction

Total RNA extraction was carried out by a slightly modified protocol of Chomczynski and Sacchi (269). Briefly, tissues or cell pellets were homogenized in 4 M guanidinium thiocyanate and extracted in phenol: chloroform. RNA contained in the supernatant after phenol:chloroform extraction was precipitated in cold ethanol.

4.15 Semi-quantitative RT-PCR

Thirty days after the contralateral injection (the second injection), the mice were sacrificed by cervical dislocation. The spleens and popliteal lymph nodes from both sides were immediately excised and frozen in liquid nitrogen. Total RNA was isolated from the tissues by a modification of the method of Chomczynski and Sacchi (269). Three micrograms of total RNA were reverse transcribed with the SuperScript^{TM} choice system for cDNA synthesis (GIBCO BRL, Life Technologies Inc., Rockville, MD, USA) by using an oligo-dT primer. The cDNA solution was further diluted 5 times in water, and 4 μl of it was used for each PCR amplification. The primer sequences used in this study are shown in Table 2. The PCR reactions were performed in 50 mM potassium chloride, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each dATP, dTTP, dGTP and dCTP, 1.25 units of AmpliTaq Gold DNA polymerase (Perkin Elmer, Branchburg, NJ, USA) and 0.4 μM of each forward and reverse primer. To prevent nonspecific amplification, a "hot start" PCR protocol was applied: 94 ˚C incubation for 10 minutes at the beginning followed by three PCR cycles: 30 seconds at 94 ˚C; 1 minute at 60 ˚C; 2 minutes at 72 ˚C. At the end, the reaction was held at 72 ˚C for 7 minutes. In order to keep the PCR reaction in the exponential phase, eighteen and 25 circles of PCR amplification were used for mouse GAPDH and other cytokines, respectively. The rat GAPDH cDNA fragments were isolated with a QIAquick gel extraction kit and the PCR products of mouse cytokines and CAMs were purified with QIAquick PCR purification kit. The sequences were confirmed by direct DNA sequencing (100% match). The [a-^{32}P] dCTP (NEN Life Science Products, Boston, MA., USA) labeled probes were made by random priming (DNA random primed labeling kit, Boehringer Mannheim, Mannheim, Germany) to a specific activity of 0.9 - 2.1x 10^9 cpm/μg. The PCR products were transferred to nylon membranes, hybridized with specific probes labeled with [a-^{32}P] dCTP, and quantified using the Fujifilm BAS-2500 phosphoimaging system. The amount

Table 2. Primers for PCR reactions

GAPDH	sense, 5'-TGA AGG TCG GTG TGA ACG GAT TTG GC-3' antisense, 5'-CAT GTA GGC CAT GAG GTC CAC CAC-3'
IL-1ß	sense, 5'-ATG GCA ACT GTT CCT GAA CTC AAC T-3' antisense, 5'-CAG GAC AGG TAT AGA TTC TTT CCT TT-3'
IL-2	sense, 5'-GAC AGA AGG CTA TCC ATC TCC TCA GAA AGT CC-3' antisense, 5'-GAC AGA AGG CTA TCC ATC TCC TCA GAA AGT CC-3'
IL-4	sense, 5'-ATG GGT CTC AAC CCC CAG CTA GT-3' antisense, 5'-GCT CTT TAG GCT TTC CAG GAA GTC-3'
IL-7	sense, 5'-GCC TGT CAC ATC ATC TGA AGT GCC-3' antisense, 5'-CAG GAG GCA TCC AGG AAC TTC TG-3'
IL-10	sense, 5'-ATG CAG GAC TTT AAG GGT TAC TTG GGT T-3' antisense, 5'-ATT TCG GAG AGA GGT ACA AAC GAG GTT T-3'
IFN-g	sense, 5'-TGC ATC TTG GCT TTG CAG CTC TTC CTC ATG GC-3' antisense, 5'-TGG ACC TGT GGG TTG TTG ACC TCA AAC TTG GC-3'
ICAM-1	sense, 5'-CAA CTG GAA GCT GTT TGA GCT G-3' antisense, 5'-TAG CTG GAA GAT CGA AAG TCC G-3'
ICAM-2	sense, 5'-TGC TGG AGC CTG TCT CTT CTT ATC-3' antisense, 5'-TTC AGA GCT GTG CTG TTG AAC GTG-3'
VCAM-1	sense, 5'-CAA GGG TGA CCA GCT CAT GA-3' antisense, 5'-TGT GCA GCC ACC TGA GAT CC-3'
LFA-1	sense, 5'-CCA CGA CAT AAC CCG CTA CAT CAT C-3' antisense, 5'-TCC CCA CCA AAG TAA GAT CCG ATC-3'
CD44	sense, 5'-AAT TCC GAG GAT TCA TCC CA-3' antisense, 5'-CGC TGC TGA CAT CGT CAT C-3'
ICAM-5	sense, 5'-GCG GTC GCG CTA GAA CCT TTC T-3' antisense, 5'-GAA TGT GGA GGC ATG GCA AAG GTA-3'

of cDNA used for each PCR reaction was normalized with GAPDH, i.e., the relative value of each sample was expressed as a ratio between the numeral value (from phosphoimaging analysis) of the target gene and GAPDH.

4.16 In situ hybridization

4.16.1 Preparation of RNA probes

The following components were sequentially added at RT: 4 μl of 2xTB buffer, 2 μl of 100 mM DTT, 1 μl of rRNasin, 4 μl of r(AGC)TP, 3 μl (1 μg) of linearized DNA, 5 μl of [$^{35}\alpha$-S] rUTP, 1 μl of SP6 and T3 or T7 DNA dependent RNA polymerase. The mixture was incubated for 1 hr. To remove the DNA template, 1 μl of RQ1 RNase-free DNase was added and incubated for 15 min at 37˚C. Unincorporated nucleotides were removed by size chromatography through prepacked G-50 Sephadex column. Labeled RNA probes were precipitated in ethanol and suspended in 50 μl of RNA buffer X (10 mM Tris-HCl, 1mM EDTA + 50 mM DTT).

4.16.2 In situ hybridization of tissue specimens

Five μm sections of paraffin-embedded tissues were placed on silinase-coated slides (super-frost plus), using DEPC-treated water. The sections were prewarmed at 60˚C for 2 hr and dewaxed in xylene (2x10) min, and then hydrated in graded alcohol (absolute ethanol 2x5 min, 94% ethanol 1x3 min, 70% ethanol 1x3 min, 50% ethanol 1x3 min). The sections were rinsed in 1xPBS for 3 min, denatured in 0.2 N HCl at RT for 20 min, washed in 2xSSC twice and heated at 70˚C for 15 min. Following PBS washes the tissue was fixed in fresh 4% paraformaldehyde (PFA) in 1xPBS for 12 min in a fumehood, washed again in PBS and incubated with proteinase K (10 μg/ml in 50 mM Tris-HCl, 5 mM EDTA at pH 8.0) at RT for 15 min. The tissue was washed once in 2xSSC and once in PBS and fixed in 4% PFA for 10 min. Following washes in PBS twice, the tissue was acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine. Following further washes in PBS, the tissue was dehydrated in graded ethanol (50% ethanol: 3 min, 70% ethanol: 3 min, 94% ethanol: 3 min, 100% ethanol: 5 min) and dried in air. The tissue was incubated in the hybridization buffer (50% deionized formamide, 0.3 M 0f NaCl, 10 mM of Tris. Cl, pH 8.0, 1 mM of EDTA, 1XDenhardt's solution, 10% of dextran sulphate, 50 mM of DTT, 0.5

mg/ml of yeast tRNA) containing [^{35}S] UTP labeled sense and anti-sense RNA probes (4 million cpm/100 μl) at 50˚C for 4 hr. The probe was prepared with riboprobe RNA labeling kit, according to the manufacturer's instructions. The slides were covered with parafilm and placed in a box containing the moisture solution (2xSSC and 50% formamide). After the hybridization, the slides were washed once in 2xSSC, 50% formamide containing 10 mM of DTT at 50˚C for 30 min, once in 0.2xSSC containing 10 mM of DTT at 50˚C for 30 min and once in NTE buffer (0.5 M of NaCl, 10 mM of Tris-HCl, 5 mM of EDTA at pH 8.0). After the above washes, the tissues were incubated in an NTE buffer containing RNase A (30 μg/ml) at 37˚C for 15 min. The tissue was further washed once in 2xSSC and 50% formamide containing 10 mM of DTT at 50˚C for 15 min, then in 2xSSC at RT for 5 min. Following these washes, the slides were dehydrated in graded ethanol: 50% ethanol 3min, 70% ethanol 3 min, 94% ethanol 3 min, 100% ethanol 5min twice. The slides were dried in air when covered with a lid to protect them from dust. After this, melted Kodak NTB-2 emulsion was applied on the slides according to the procedure recommended by the manufacturer. After drying in air in the dark room for at least 2 hr, the slides were kept in a sealed light proof box and exposed at 4˚C for about two weeks. The slides were developed with Kodak reagents, according to the manufacturer's instructions and counter stained with hematoxylin. After drying in air, the slides were mounted with glass cover slids by use of permount, and examined under light microscope.

4.17 Immunohistochemistry

Formalin-fixed, paraffin-embedded spleen tissue sections from normal mice and normal human spleen were placed on polylysine-coated glass slides. Sections were permeabilized with 0.3% Triton-X, blocked with 10% normal goat serum and incubated overnight at +4˚C with the primary antibody in dilutions 1:200-1:500, as appropriate. The three-step avidin-biotin complex/horseradish peroxidase method (ABC, Vectastain, Vector Laboratories, Burlingame, CA) was used with diaminobenzidine as the chromogen. Sections were lightly counterstained with Meyer's Hemalum. Adjacent sections incubated with normal rabbit serum in similar dilutions underwent the same staining protocol and were examined as controls. Light microscopy was performed with Leitz Laborlux D (Leitz, Wetzlar, Germany) microscope equipped with Nikon Coolpix 995 (Nikon, Japan) digital camera.

4.18 Flow cytometry

4.18.1 Direct isolation of splenic dendritic cells and macrophages

Spleens from adult male ICR mice were cut into small pieces and incubated at 37˚C in RPMI 1640 supplemented with 0.5% FCS, 0.02 mg/ml DNase I (Roche Diagnostics GmbH, Mannheim, Germany) and 0.1 mg/ml of collagenase (Sigma-Aldrich) that was free of tryptic activity. The cell suspension was filtered through a fine steel mesh and the cells were collected by centrifugation at 200g. Splenic dendritic cells were enriched by MACS with anti-CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany) using positive selection columns. $CD11c^-$ cells were used to isolate $CD11b^+$ splenic macrophages by MACS with anti-CD11b magnetic beads.

4.18.2 FACS analysis

ICAM-5 expression was measured by immunofluorescence. Enriched $CD11c^+$ dendritic cells and $CD11b^+$ macrophages were stained with rabbit anti-human ICAM-5 polyclonal antibody first. After washing in PBS twice, the cells were stained with goat anti-rabbit monoclonal antibody conjugated with FITC. After washing, the cells were stained with APC-conjugated with hamster anti-mouse CD11c monoclonal antibody (clone HL3, BD Biosciences Pharmingen). An aliquot of cells received an APC conjugated irrelevant Ab of the same isotype. As a negative control for the FITC labeling, one aliquot of cells did not receive the primary anti-ICAM-5 but all the other antibodies. Surface fluorescence was assayed by flow cytometry. The eluted cells after the two-round positive selection, which contained T and B cells were stained for ICAM-5 expression as mentioned. After washing, the cells were stained with R-PE-conjugated rat anti-mouse CD3 (clone 17A2, BD Biosciences Pharmingen) and PerCP-CY5.5-conjugated rat anti-mouse CD19 (clone 1D3, BD Biosciences Pharmingen). Triple color FACS analysis was performed for the surface fluorescence thereafter.

5 RESULTS

5.1 Stimulation of murine splenic cells by SFV-A7 and LPS

5.1.1 mRNA expression of IL-1ß and IL-2 induced by LPS and SFV-A7

Cytokines play a pivotal role in both infections and autoimmune diseases. Thus, one could assume that an altered immune response with a co-pathogen infection might be associated with an altered cytokine production. In the first study the methodology for the quantification of cytokine mRNA expression in CR-EAE was established. For this, the effects of LPS and SFV-A7 on cytokine mRNA expression in mouse splenic cells was characterized. LPS at 10 ng/ml and SFV-A7 at 5 m.o.i. was added to the cell culture and IL-1ß and IL-2 mRNA expression was studied by Northern blot hybridization and phosphoimaging analysis. It was found that 10 ng/ml of LPS induced substantial IL-1ß expression. However, IL-2 mRNA expression was not increased by this treatment. SFV-A7 induced neither significant IL-1ß nor IL-2 mRNA expression.

5.1.2 Combined effects of LPS and SFV-A7 on IL-1ß and IL-2 mRNA expression

To study whether or not a combined treatment of SFV-A7 and LPS could result in an enhanced cytokine mRNA expression, SFV-A7 (m.o.i. of 5) and 10 ng/ml of LPS were added to the splenic cell culture at the same time. The mRNA expression of both IL-1ß and IL-2 was increased at 3 and 6 h but not at 1 and 12 h, as compared with LPS or SFV-A7 treatment alone. Further study demonstrated that an addition of SFV-A7 3 h before LPS enhanced IL-1 mRNA expression as compared with a 3 h culture with LPS alone. However, when SFV-A7 was added 3 hours later, the IL-1ß mRNA expression was actually reduced, as compared with a 6 hours culture with LPS alone. On the other hand, this treatment did not affect IL-2 mRNA expression.

5.2 Induction of CR-EAE

5.2.1 Effects of paraffin oil in the pre-treatment

Paraffin oil is usually used for the preparation of the antigen emulsion for the

induction of EAE in mice. To determine if EAE can still be induced without paraffin oil (IFA), MSCH, M. tb and M. b in PBS were injected into the hind footpad of the mice. This treatment proved to be inefficient in inducing EAE: only 24% of the mice in one group developed slight and transient hind limb paralysis, whilst the majority remained normal. In another experiment, all the mice failed to show any clinical sign of illness after the pre-treatment. These results demonstrate that at least paraffin oil is needed for the induction of clinical EAE.

5.2.2 CR-EAE induced in the pre-treated mice after the contralateral injection

Although the sub-optimal pre-treatment failed to induce EAE in SJL mice, it may produce memory responses which in turn may affect the conventional EAE induction. To study the nature of EAE induced in the pre-treated mice, we immunized the mice with a standard acute EAE induction treatment two months after the pre-treatment (Fig. 1). In experiment I, the neuroantigen emulsion was prepared with a stronger sonicator, in contrast to a weaker sonicator used in experiment II. It was found that, in experiment I, all the 10 mice receiving 1.5 mg of MSCH in the second (contralateral) injection demonstrated complete paralysis within 7 days after the injection, as compared with usually 9 days in acute EAE induced with a single injection regimen. Two mice died during the acute attack. One mouse recovered from severe paralysis to mild paralysis (from grade 5 to grade 2). This mouse had one relapse but failed to recover from it and died at grade 4 (30 days after the second injection). All the rest of the mice had a slow recovery (that lasted between 20 to 30 days) before they stabilized at grade 2. In this group of mice, we observed 5 rather clear relapses within the next 11 months. Similarly, the 5 mice which received 3 mg of MSCH in CFA in the contralateral (right) hind footpad also had an early onset of disease (within 7 days), which became chronic thereafter. All the 5 mice demonstrated clear-cut relapses (often with an increase of two grades). However, 4 mice died of the first relapse within one month. Only one mouse showed persistent paralysis until it was sacrificed after 15 weeks.

In experiment II where the neuroantigen emulsion was prepared with a weaker sonicator, the majority of mice survived the acute disease attack and developed either a chronic and/or relapsing EAE. We observed 6 clear-cut relapses in this group (see reference 76 and paper II). The weights of the mice

were measured once a day. It was found that the change in weight paralleled the change in the clinical status of the mice: the mice gained weight when they were recovering and lost weight when they had relapses.

To control the effect of paraffin oil, a group of 6 mice were pre-treated with 3 mg of MSCH in CFA, followed by a second (contralateral injection) two months later. It was observed that some mice developed severe EAE with an earlier disease onset (7 days after the second injection) and died. Some recovered completely and remained normal thereafer. No relapses were recorded. In the control group for the effect of mycobacteria, none of the mice showed any signs of illness at any point during the experiment.

5.2.3 Disease induction by a single injection regimen

It has been reported that CR-EAE could be induced in SJL mice by a single injection of neuroantigen in CFA. To test this, naive SJL/J mice were injected with different doses of MSCH in CFA. All the mice developed a monophasic acute EAE. The disease onset was about 12 days after the injection. All the mice survived the acute disease attack and recovered quickly and remained normal thereafter. If pertussis toxin was injected i.v. after the injection of MSCH in CFA, all mice died of EAE.

5.2.4 Effects of injection sites on the induction of EAE

CR-EAE developed in the pre-treated SJL mice after the contralateral injection of MSCH in CFA as described above. Interestingly, if a homolateral injection (i.e. the first and the second injections into the same footpad) of MSCH in CFA was given to the pre-treated mice, all the mice died of a severe acute disease attack.

5.3 Effect of splenectomy and lymphadenectomy

To analyze the effect of memory cells on the induction of CR-EAE, we conducted splenectomy and lymphadenectomy on the pre-treated mice. Five mice from each group were splenectomized two months after the pre-treatment. These splenectomized mice received a contralateral injection of MSCH in CFA

one week after the operation. It was found that splenectomy per se substantially abolished the development of CR-EAE. Two mice died of an acute attack. Two mice recovered slowly and one remained normal. Not a single relapse was recorded during a follow-up of 41 days. When both splenectomy and lymphadenectomy were performed, not only the CR-EAE was abolished but also the disease was ameliorated dramatically: four out of five mice developed a typical acute EAE with a quick recovery. Only one mouse died after 3 weeks; one mouse made a slow recovery, but no relapse was recorded during the 41-day follow-up period. Thus, it can be concluded that the immune cells harbored in the spleen after the pre-treatment play a pivotal role in the development of CR-EAE.

5.4 Effect of adoptive cell transfer

Next it was asked whether an adoptive transfer of putative memory lymphocytes from the pre-treated mice would affect the development of EAE induced by active immunization. Lymphocytes were isolated from either the spleen or the local draining popliteal lymph nodes of the suboptimally pre-treated mice two months after the pretreatment. Different amounts of cells were transferred to naive syngeneic recipients one week before active immunization. It was found that the transfer of pLN cells exacerbated the disease: all the 10 mice died of an acute attack and their average maximum disease index was significantly higher than in mice that received a spleen cell transfer (Student's t-test, $p<0.02$). On the contrary, a transfer of splenic lymphocytes delayed the onset of the disease to an average 16.3 days after immunization, as compared to an average of 10.7 days after the pLN transfer ($p<0.01$). Furthermore, as many as 50% of mice survived compared to no survivors after the pLN cell transfer.

Most interestingly, CR-EAE developed only in the group of mice with spleen cell transfer. Five clear-cut relapses were recorded in five of the seven surviving mice. This suggested that the spleen cells could harbor memory cells that affect the development of CR-EAE.

5.5 Histological examination

Brains from mice that survived the 11 months' follow-up in the first experiment of CR-EAE showed an inflammatory cell infiltrate in the ventricular areas, especially around the blood vessels and adjacent tissue. There was a more

pronounced mononuclear cell infiltration in mice with severe paralysis than in mice with mild paralysis. It is of interest to note that the inflammatory cell infiltrate in our CR-EAE model is highly disseminated, and without an obvious focus, which is atypical of EAE in SJL mice.

In mice with CR-EAE, extensive demyelination was observed with Luxol fast blue staining.

5.6 Cytokine mRNA expression

Because cytokines play a pivotal role in inflammation and autoimmune responses, we asked whether a specific expression pattern of cytokines would be associated with the disease course in CR-EAE. At the termination of the experiment (30 days after the contralateral injection) cytokine mRNA expression in the pLN and spleen was studied in the two groups of mice with either a severe paralysis (n=5) or complete remission (n=6) by semi-quantitative RT-PCR. In order to keep the PCR reaction in the exponential phase the yield of the PCR reaction was kept under 50 ng.

The tested cytokines included IL-1β, IL-2, IL-4, IL-7, IL-10 and IFN-γ. All of them were found to be expressed at considerable levels in both groups of mice. Surprisingly, the expression levels of the cytokine mRNAs tested between the groups of mice with either relapse or remission was not found to be significantly different. Interestingly, when the cytokine mRNA expression was compared between the LNs and the spleens within the group of mice with remission, the expressions of IL-2, IFN-γ and IL-10 (but not of IL-1β, IL-4 or IL-7) were found to be significantly higher in the LNs than in the spleens ($p<0.01$ for IL-2 and IFN-γ; $p<0.05$ for IL-10; Student's t-test). However, in the group of mice with relapse, although the mRNA expression of IL-2 and IFN-γ was slightly higher in the LNs than in the spleens in this group, no significant differences were found between levels of any cytokine mRNAs tested. Unlike the mice with remission, the mice with relapse showed actually a higher expression of IL-10 mRNA in the spleens than in the LNs. However, this difference was not statistically significant.

5.7 mRNA expression of cellular adhesion molecules (CAMs)

The cellular adhesion molecules (CAMs) are believed to play a role in the traffic of lymphocytes. The immigration and emigration of encephalitogenic T cells to and from the CNS is a hallmark of CR-EAE. Thus, we studied the mRNA expression of ICAM-1 and 2, VCAM-1, LFA-1 and CD44 in the two groups of mice with either relapse or remission by semi-quantitative RT-PCR. All the tested CAMs were found to be expressed in all the samples. The mRNA expressions of CAMs in LNs or spleens between the two groups of mice with either relapse or remission was not significantly different. However, when the mRNA expression was compared between the LNs and the spleens in the group of mice with remission, a significantly higher expression was found for VCAM-1, LFA-1 and CD44 in the spleen than in the LNs ($p<0.01$ for VCAM-1 and LFA-1; $p<0.05$ for CD44; Student's t test). In contrast, no such significant difference was found in the group of mice with relapse. Likewise, mRNA expression of ICAM-5 between mice with either relapse or remission was not significantly different. Interestingly, it was found that ICAM-5 mRNA expression was significantly higher in the spleens than in the pLNs of mice with disease remission, but not of mice with relapse.

5.8 ICAM-5 mRNA expression studied by in situ hybridization

ICAM-5 (telencephalin) is a recently characterized integral membrane glycoprotein of the Ig superfamily. It has been reported that ICAM-5 was expressed exclusively in telencephalic neurons. In order to see whether ICAM-5 was also expressed in the peripheral lymphoid organs, ICAM-5 mRNA expression was studied in the spleen in the naive SJL mice by *in situ* hybridization. It was found that ICAM-5 mRNA was expressed in the red pulp area of the spleen.

5.9 Immunohistochemistry staining of ICAM-5 protein in the spleen

To determine whether ICAM-5 was expressed at the protein level in the secondary lymphoid organs, we performed immunohistochemistry staining of both mouse and human spleens. It was found that the red pulp (r) area of mouse spleen stained positive for ICAM-5 intensely, as compared to the rather scat-

tered positive staining in the white pulp (w) area surrounding the central artery. The cells staining positive for ICAM-5 in the white pulp appeared to be non-lymphocytoid cells with an abundant cytoplasm. Likewise, the human spleen tissue demonstrated a similar scattered staining pattern in the area of white pulp (w) adjacent to a central artery. The ICAM-5 positive cells did not morphologically correspond to monocytoid lymphocytes, which are the predominant cell type found in the B-cell -rich marginal zone of white pulp adjucent to the T-cell -rich periarterial lymphatic sheath (PALS). Similarly to the mouse spleen, more cells in the red pulp of human spleen stained positive for ICAM-5.

5.10 Flow cytometry demonstration of ICAM-5 on dendritic cells

In order to determine the phenotype of cells that expressed ICAM-5 in the mouse spleen, we conducted multi-color-labeling FACS analysis of the enriched $CD11c^+$ dendritic cells and $CD11b^+$ macrophages as well as of T cells and B cells collected after positive selection. It was found that 58% of $CD11c^+$ dendritic cells stained positive for ICAM-5. A similar percentage of $CD11b^+$ macrophages was also found to stain positively for ICAM-5 (data not shown). However, only about 37% of $CD19^+$ B cells and about 10% of $CD3^+$ T cells stained positive for ICAM-5. Interestingly, it appeared that two distinct groups of dendritic cells expressed ICAM-5, one group staining brighter than the other. The $CD11b^+$ macrophages stained to the same extent as the $CD11c^+$ dendritic cells that stained less bright.

6 DISCUSSION

6.1 IL-1ß and IL-2 mRNA expression in murine splenic cells

It has been reported that predisposing C57BL/J mice to SFV-A7 virus facilitated the induction of EAE (270). It has also been found that an infection of BALB/c and SJL/J mice with SFV-A7 virus could either inhibit or facilitate the development of EAE, depending on whether the virus was injected before or after the EAE induction (271). The mechanisms underlying these phenomena remain unknown. EAE is a $CD4^+$ T cell mediated, delayed type hypersensitivity in which cytokines play a central role (77). Therefore, it was speculated that an altered cytokine production due to the interaction between SFV-A7 and mycobacterial components, the major factor in CFA, might be responsible for the altered autoimmune response. Thus, it was tested whether a concomitant stimulation by SFV-A7 and LPS, a complex glycolipid and the major component of the outer membrane of Gram-negative bacteria (272), would result in an altered cytokine expression. In this respect IL-2 and IL-1β are probably important, since IL-2 supports T cell development, and its production is influenced by IL-1, a major pro-inflammatory cytokine with pleiotropic properties (273, 274). In addition, the methods for splenic cell transfer as well as for the quantification of cytokine mRNAs were established for use throughout this work. Infection with the Semliki Forest virus mutant SFV-A7 alone did not activate IL-1ß and IL-2 mRNA expression by mouse splenic cells. However, SFV-A7 enhanced the effect of LPS on the expression of these two cytokines. The enhancing effects were even more pronounced if the SFV-A7 was added 3 h before LPS addition, while adding the virus 3 h after the addition of LPS inhibited the effect of LPS. The results demonstrate that a concomitant stimulation of murine splenic cells with viral and bacterial components can result in an altered cytokine production. It has been speculated that an infection with a co-pathogen might play a role in autoimmunity (275). Since cytokines play a major role in autoimmune diseases, it can be assumed that a concomitant infection could affect disease pathogenesis via an altered cytokine production.

6.2 CR-EAE as an animal model for human MS

As one of the most thoroughly studied animal models for human autoimmune diseases, EAE has served well in elucidating the cellular and molecular mechanisms of autoimmunity (77, 171, 276). However, the often monophasic disease course in acute EAE is only remotely related to human MS (277). Because CR-EAE resembles the unique clinical features of MS, various protocols for the induction of CR-EAE have been described (74, 75, 278). Nevertheless, convincing relapsing EAE has always proved difficult to produce. As a result, the CR-EAE models described so far are either with mild or rare relapses. For these reasons, the establishment of more appropriate animal models for human MS has recently been advocated (3). With a modified protocol, a CR-EAE model which is characterized by a high percentage of survival, frequent and clear-cut relapses was established. This CR-EAE model has proven to be highly reproducible (in three different laboratories) and should facilitate the study of the mechanisms of chronicity and relapse of human demyelinating diseases.

6.3 Biased immune memory precipitates the chronicity of the autoimmunity

As it has been reported and confirmed in this study, a single immunization regimen involving neuroantigen and adjuvants usually induced a monophasic acute EAE (277). Brown et al induced relapsing EAE with two identical injections with a one-week interval (75), indicating the relevance of immune memory to subsequently developing chronic autoimmunity. The results reported in this thesis have demonstrated that a biased immune memory caused by a suboptimal immunization is critical in altering an otherwise acute EAE into a chronic disease. In contrast, a full scale assault of acute EAE tends to cause resistance to later EAE inductions, if the mice survive (279). This phenomenon is of interest, given the fact that the development of human MS has been believed to be preceded by infections in late adolescence (21, 22, 26). It is proposed that the pre-treatment in our protocol for inducing CR-EAE is reminiscent of a late childhood infection which precipitates MS later in the adulthood. Thus, a subclinical neurotropic infection in adolescence could cause a biased immune memory towards certain self neuroantigens. This pathogenic immune memory could interact with immune responses to subsequent infections in the adulthood, and result in the development of MS.

Although the co-stimulation and/or "danger" theories (126, 127) could explain how the autoimmune response can be initiated, they fail to explain why infections are frequent but autoimmune diseases are relatively rare. They also fail to explain the chronicity of virtually all autoimmune diseases. It can be postulated that the development of autoimmune diseases is a slow process of the break-down of the immune homeostasis and tolerance which can not be attributed to a single infection. Rather, the interactions of the immune memories after various infections that occurred during different periods of human life may contribute more to the loss of immune homeostasis. Indeed, these results have demonstrated that the memory cells that developed after a sub-optimal treatment were able to convert an otherwise actively induced acute EAE into a CR-EAE in the naive recipient mice. However, the mechanism underlying the development of chronic autoimmunity in our model remains unknown. Likewise, the impact of the memory from different infections on the development of chronic autoimmune diseases has not been adequately studied.

6.4 Differential immune response within the secondary immune compartments might determine the outcome of autoimmune disease progression

It has been optimistically believed that the two antagonistic patterns of cytokines, i.e., Th1- and Th2-type response (167), could determine the overall immune response in autoimmune demyelinating diseases (171). However, recent data indicates that the Th1/Th2 dogma is oversimplified. In EAE, it has been reported that myelin basic protein-specific Th2 cells actually cause EAE in immunodeficient hosts rather than protect the host from the disease (182). Likewise, it has been shown that IFN-γ, a Th1 cytokine, could suppress EAE and IFN-γ knockout mice had a more severe EAE (184-187). However, the treatment of MS patients with IFN-γ actually exacerbated the disease (188). In our study, we observed that both Th1 and Th2 cytokine mRNAs were expressed in mice with either relapse or remission. Interestingly, we found that a differential expression of certain cytokines (IL-2, IFN-γ, IL-10) between spleens and lymph nodes was associated with disease remission in CR-EAE. The mechanism is unknown. However, it can be speculated that cytokines function in a finely coordinated nonlinear network in which a slight quantitative change of certain cytokines may have a dramatic systemic effect (165). The results indicate that the relative amounts of cytokines produced at

different locations (i.e. LN versus spleen) in the peripheral lymphoid organs may be important in the immune regulation and the development of CR-EAE.

CAMs are molecules that regulate the trafficking of lymphocytes (198). The selected expression of CAMs in certain immune compartments not only targets the effector cells to sites of antigenic or microbial invasion, but also directs lymphocyte subsets to the specialized microenvironments that control their differentiation and regulate their survival (196). It has been reported that the expression levels of LFA-1 and CD44 on infiltrating mononuclear cells and on some resident cells in MS and EAE brains were increased (193, 213, 276). It has also been found that upregulation of ICAM-1 and VCAM-1 in CNS coincided with the immigration of $CD4^+$ lymphocytes, while downregulation coincided with their emigration (239). It was found that disease remission in CR-EAE was associated with a relatively increased mRNA expression of VCAM-1, LFA-1 and CD44 in the spleen and a decreased mRNA expression in pLNs.

Parallel to the above findings, we also found that the memory cells from the spleen were more involved in the development of CR-EAE. Immune response in one compartment might be synergistic or antagonistic to the immune response initiated in another compartment. In this regard, the deviated expression of cytokines and CAMs in LNs versus spleens might favor the development of functionally different T-cell subsets in these two immune compartments. Indeed, it has been reported that the spleen, but not LN, is a major source of suppressor T cell activity (280-283) and is needed for the suppression of EAE (284, 285). It can be speculated that an elevated expression of IL-2 and IFN-γ in LNs may enhance the activation-induced apoptosis of the autoimmune T cells and facilitate disease recovery accordingly. Conversely, the downregulated expression of IL-10 as well as the upregulated expression of VCAM-1, LFA-1 and CD44 in the spleen may favour the development of regulatory T cells which suppress the activity of encephalitogenic T cells. Alternatively, it can be assumed that the increased expression of LFA-1, CD44 and VCAM-1 in the spleen may help to trap the encephalitogenic T cells in the spleen, and thus prevent them from emigrating into the CNS.

6.5 Expression of ICAM-5 outside the CNS and role in CR-EAE

In this study, ICAM-5 was found to be strongly expressed on $CD11c^+$ DCs and its mRNA expression was elevated in the spleen of mice with disease remission, suggesting that ICAM-5 might be such a molecule that suppresses the autoimmune response.

The antigen presenting cells (APCs) express many accessory molecules that interact with receptors on T cells to enhance adhesion and signalling (co-stimulation). These molecules include LFA-3/CD58, ICAM-1/CD54 and B7-2/CD86 (256). The direction of T cell development largely depends on which co-stimulatory molecules are expressed by the APCs. Microbial products may activate DCs through binding to Toll-like receptors (257, 258), and thus result in the up-regulation of MHC class II and costimulatory molecules (CD80/CD86), and the development of pathogen-specific T cells. However, the so called ”tolerogenic” DCs that play a pivotal role in immune homeostasis may express totally different co-suppressor molecules (286). Self-antigen presentation to T cells by the tolerogenic APCs may lead to either a deletion of autoimmune T cells or the development of regulatory T cells (Tr) (287). Indeed, it has been reported that the myelin basic protein (MBP)-pulsed thymic DCs can prevent the development of EAE when injected intravenously into adoptive hosts (288). Thus, expression of ICAM-5 on DCs may facilitate the development of regulatory T cells that suppress the disease progression in CR-EAE.

SUMMARY AND CONCLUSIONS

The hallmark of autoimmune diseases is the chronic tissue damage caused by a persisting or recurrent autoimmune response. Thus, to understand the pathogenesis of autoimmune diseases, it is important to know not only how the autoimmune response is initiated but also why it becomes chronic. Increasing evidence has pointed to the critical involvement of peripheral immune regulation in the development and control of chronic autoimmune demyelinating diseases in the CNS. In the present work, the disease evolution of chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE) in SJL mice was investigated. Attention was directed to studying the expression of various immune parameters in the secondary lymphoid organs of mice with either relapsing or remitting course of the disease.

It was observed that the acute disease course of conventional EAE could be converted into CR-EAE by a pre-treatment which itself was sub-optimal for the induction of EAE. Apparently, the pre-existing immune status resulting from the pre-treatment influenced the subsequent immune activity of a standard acute EAE induction and resulted in a break-down of immune homeostasis in the peripheral immune organs. Further studies using splenectomy, lymphadenectomy as well as adoptive cell transfer indicated that it was the splenic memory T cells developed after the pre-treatment that interacted with a secondary immunization and were responsible for the development of the chronic disease course.

The expression of several immune parameters in the peripheral lymphoid organs, namely, lymph nodes and spleens of mice with either disease relapse or remission were studied. No significant difference in the expression of cytokines (IL-2, IFN-γ, IL-10) or adhesion molecules (VCAM-1, LFA-1, CD44) was found between the mice having either disease relapse or remission. Interestingly, however, a higher mRNA expression of cytokines IL-2, IFN-γ, IL-10 and adhesion molecules VCAM-1, LFA-1, CD44 in the spleens than in the lymph nodes was observed in mice with disease remission but not relapse. These results indicated that an increased expression of certain cytokines as well as adhesion molecules in the spleen might be beneficial to the control of autoimmune response.

Finally, we studied the expression of ICAM-5, an interesting novel adhesion molecule, in the spleens of humans and naive mice as well as in mice with CR-

EAE. For the first time, we identified that ICAM-5 was expressed in the red pulp area of human and mouse spleens. Flow cytometry analysis revealed that ICAM-5 was expressed on the surface of dendritic cells, macrophages and a small percentage of B cells. Furthermore, we observed that a higher expression of ICAM-5 in the spleen than in the lymph nodes was associated with disease remission in CR-EAE.

In the present study, a novel mouse model for human MS was established with a modified protocol. Because this new model resembles human MS closely, it can be used for studying the pathogenesis of MS as well as for testing therapeutic approaches. The role of the spleen in suppressing the autoimmune response and influencing the relapsing-remitting nature of EAE was particularly intriguing. The observation of a differential expression of cytokines as well as CAMs in different peripheral lymphoid organs may also help in the design of therapies by manipulating the expression of various immune parameters in the peripheral lymphoid organs.

ACKNOWLEDGMENTS

This thesis was carried out at 5 different departments in three universities: Department of Virology, University of Turku, Finland; Department of Experimental Pathology, University of Tromsø, Norway; Department of Biochemistry, Viikki Biocentrum; Department of Bacteriology and Immunology and Department of Neurology, Biomedicum Helsinki, Helsinki University Hospital and the University of Helsinki. During all these years of jumping from place to place, I have been supported by many people and I have a long list to thank here:

I want to thank Professor Aimo Salmi, who accepted me as his PhD student in the first place when I desperately wanted to leave China at the end of 1989. Unfortunately, I chose the wrong day to depart from China to Finland, and that was, basically, the reason why my research did not go well during the first five years.

I specially want to thank Docent Veijo Hukkanen, who has been my mentor during my stay at the Department of Virology, University of Turku. I have learned many laboratory skills, such as recombinant DNA technology, which have proven to be very useful for my later research work. Veijo's enthusiasm and understanding always encouraged me to persevere in my struggle. I am grateful to my senior scientist, Docent Ari Hinkkanen, for his technical assistance in my laboratory work and the inspiring scientific discussions. During my five years in Turku, I enjoyed a healthy and unforgettable social life. I am glad that I was able to socialize with colleagues Dr. Simo Nikkari and Kaisa Antilla. My friendship with many people, including quite a few foreigners (you know who you are) in Turku, has greatly contributed to the quality of my life.

I am most grateful to my supervisor, Prof. Rolf Seljelid at the Department of Experimental Pathology, Institute of Biology, University of Tromsø, Norway, for giving me the opportunity to continue my research project in which I was so interested. This thesis would not have been possible without his full support. I have enjoyed my fruitful collaborations with Dr. Roald Bøe, who worked as a postdoc in the same molecular biology laboratory. Roald is a great friend indeed. I could always count on him whenever I needed help. I will never forget those exciting skiing and

fishing trips with Dr. Roald Bøe and Dr. Kjell Lingåd. The Norwegian knife-making course at Roald's home once a week was a most enjoyble social event, even though it took me a whole year to finish making only one knife.

Finally, I want to thank my supervisors, Professor Seppo Meri and Docent Perttu Lindsberg for providing me with the opportunity to finish my dissertation. They kindly gave me much freedom to pursue my own scientific interests while providing me with the necessary guidance. I also want to thank Professor Carl Gahmberg for his assistance in my work during my short stay in his lab. I am indebted to colleagues from the complement team, Sakari Jokiranta, Antti Väkevä, Sami Junnikkala, Juha Hakulinen, Hanna Jarva, Taru Meri, Antti Lavikainen, Antti Harjunpää, Mikko Holmberg and Antti Alitalo for their assistance with my work, and for stimulating discussions.Thanks to Markus Lehtinen and Jasse Tiainen for their assistance in my work and the friendly discussions we have had. I wish to express my sincerest thanks to Monica von Schoultz for performing the FACScan analyses. Special thanks to Dr. Marja-Liisa Karjalainen-Lindsberg for precious support and discussions. I especially want to thank my colleagues and compatriots Dr. Zhu-Zhu Cheng and Dr. Tong Chen for their generous support. Our discussions in Chinese often made me feel at home. I thank Marjatta Ahonen and Tanja Eriksson for helping with the experiments. Many thanks to Marjatta Turunen, Kirsi Mönttinen and Carina Wasström for their daily office help and very friendly attitude.

Helsinki, October 11, 2003

Jinghui Yang, MD.

6. REFERENCES

1. Raine, C. S. 1994. The Dale E. McFarlin memorial lecture: The immunolgy of the multiple sclerosis lesion. *Ann. Neurol. 36:S61-S72.*

2. Kurtzke, J. F. 1985. Epedemiology of multiple sclerosis, in Vinken PJ, Bruyn GW, Klawans HL (eds): Handbook of Clinical Neurology, ed 2. Amsterdam, Elsevier Science Publishers. *38(47).*

3. Hohlfeld, R., M. Londei, L. Massacesi, and M. Salvetti. 1995. T-cell autoimmunity in multiple sclerosis. *Immunol. Today 16:259-261.*

4. Rosati, G. 1994. Descriptive epidemiology of multiple sclerosis in Europe in the 1980s: a critical overview. *Ann. Neurol. 36:164-174.*

5. Sumelahti, M.-L., P. J. Tienari, J. Wikström, J. Palo, and M. Hakama. 2001. Increasing prevalence of multiple sclerosis in Finland. *Acta. Neurol. Scand. 103:153-158.*

6. Sumelahti, M.-L., P. J. Tienari, M. Hakama, and J. Wikström. 2002. Multiple sclerosis in Finland: incidence trends and diferences in relapsing remitting and primary progressive disease courses. *J. Neurol. Neurosurg. Psychiatry. 74:25-28.*

7. Ebers, G. C., D. E. Bulman, A. D. Sadovnick, D. W. Paty, S. Warren, W. Hader, T. J. Murray, T. P. Seland, P. Duquette, T. Grey, R. Nelson, M. Nicolle, and D. Brunet. 1986. A population-based study of multiple sclerosis in twins. *N. Engl. J. Med. 315:1638-1642.*

8. Sadovnick, A. D., H. Armstrong, G. P. A. Rice, D. Bulman, L. Hashimoto, D. W. Paty, S. A. Hashimoto, S. Warren, W. Hader, T. J. Murry, T. P. Seland, L. Metz, R. Bell, P. Duquette, T. Gray, R. Nelson, B. Weinshenker, D. Brunet, and G. C. Ebers. 1993. A population-based study of multiple sclerosis in twins: Update. *Ann. Neurol. 33:281-285.*

9. Mumford, C. J., N. W. Wood, H. Kellar-Wood, J. W. Thorpe, D. H. Miller, and D. A. Compston. 1994. The British isles survey of multiple sclerosis in twins. *Neurology 44:11-15.*

10. Sadovnick, A. D., P. A. Baird, and R. H. Ward. 1988. Multiple sclerosis, updated risks for relatives. *Am. J. Med. Genet. 29:533-541.*

11. Robertson, N. P., M. Fraser, J. Deans, D. Clayton, and D. A. S. Compston. 1996. Age adjusted recurrence risks for relatives of patients with multiple sclerosis. *Brain 119:449-455.*

12. Carton, H., R. Vlietinck, J. Debruyne, J. De Keyser, M.-B. D'Hooghe, R. Loos, R. Medaer, L. Truyer, I. M. L. Yee, and D. Sadovnick. 1997. Recurrence risks of multiple sclerosis in relatives of patients in Flanders, Belgium. *J. Neurol. Neurosurg. Psychiatr. 62:329-333.*

13. Kalman, B., and F. D. Lublin. 1999. The genetics of multiple sclerosis. A review. *Biomed. Pharmacother. 53:358-370.*

14. Martin, R. 1997. Genetics of multiple sclerosis-how could disease-associated HLA-types contribute to pathogenesis? *J. Neural. Transm. Suppl. 49:177-194.*

15. Olerup, O., and J. Hillert. 1991. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. *Tissue Antigens 38:1-5.*

16. Kurtzke, J. F. 1967. On the fine structure of the distribution of multiple sclerosis. *Acta. Neurol. Scand. 43:213-222.*

17. Kurtzke, J. F. 1967. Further considerations on the geographic distribution of multiple sclerosis. *Acta. Neurol. Scand. 43:283-297.*

18. Kurtzke, J. F. 1974. Further features of the Fennoscandian focus of multiple sclerosis. *Acta. Neurol. Scand. 50:478-502.*

19. Larsen, J. P., J. A. Aarli, H. Nyland, and T. Riise. 1984. Western Norway, a high-risk area for multiple sclerosis: a prevalence/incidence study in the county of Hordaland. *Neurology 34:1202-1207.*

20. Larsen, J. P., R. Kvalle, T. Riise, H. Nyland, and J. A. Aarli. 1984. An increase in the incidence of multiple sclerosis in Western Norway. *Acta. Neurol. Scand. 70:96-103.*

21. Kurtzke, J. F. 1993. Epidemiological evidence for MS as an infection. *Clin. Microbiol. Rev. 6:382-427.*

22. Alter, M., and W. Cendrowski. 1976. Multiple sclerosis and childhood infections. *Neurology 26:201-204.*

23. Cook, S. D., and P. C. Dowling. 1980. Multiple sclerosis and viruses: an overview. *Neurology 30(2):80-91.*

24. Sibley, W. A., and C. R. Bamford. 1985. Clinical viral infections and multiple sclerosis. *Lancet 1:1313-1315.*

25. Compston, D. A. S., B. N. Vakarelis, E. Paul, W. I. McDonald, J. R. Batchelor, and C. A. Mims. 1986. Viral infection in patients with multiple sclerosis and HLA-DR matched controls. *Brain 109:325-344.*

26. Alvord, E. C. J., U. Jahnke, E. H. Fischer, M. W. Kies, B. F. Driscoll, and A. S. Compston. 1987. The multiple causes of multiple sclerosis: the importance of age of infections in childhood. *J. Child. Neurol. 2:313-327.*

27. Challoner, P. B., K. T. Smith, and J. D. Parker. 1995. Plaque-associated expression of human herpesvirus 6 in multiple sclerosis. *Proc. Natl. Sci. USA 92:7440-7444.*

28. Kurtzke, J. E. 1982. Multiple sclerosis in Iceland: 1. Evidence of a postwar epidemic. *Neurology 32:143-150.*

29. Sutherland, J. M. 1956. Observations on the prevalence of multiple sclerosis in Northern Scotland. *Brain 79:635-654.*

30. Allison, R. S. 1963. Some neurological aspects of medical geography. *Proc. R. Soc. Med. 56:71-76.*

31. Fog, M., and K. Hyllested. 1966. Prevalence of disseminated sclerosis in the Faroes, the Orkneys and Shetland. *Acta. Neurol. Scand. 42(Suppl. 19):9-11.*

32. Poskanzer, D. C., L. B. Prenney, J. L. Sheridan, and J. Y. Kondy. 1980. Multiple sclerosis in the Orkney and Shetland Islands. I: Epidemiology, clinical factors, and methodology. *J. Epidemiol. Community Health 34:229-239.*

33. Kurtzke, J. F., K. Hyllested, A. Heltberg, and A. Olsen. 1993. Multiple sclerosis in the Faroe Islands. 5. The occurrence of the fourth epidemic as validation of transmission. *Acta. Neurol. Scand. 88:161-173.*

34. Kurtzke, J. F., and K. Hyllested. 1986. Multiple sclerosis in the Faroe Islands. II. Clinical update, transmission, and the nature of MS. *Neurology 36:307-328.*

35. Kurtzke, J. F., and K. Hyllested. 1987. Multiple sclerosis in the Faroe Islands. III. An alternative assessment of the three epidemics. *Acta. Neurol. Scand. 76:317-339.*

36. Rapp, N. S., J. Gilroy, and A. M. Lerner. 1995. Role of bacterial infection in exacerbation of multiple sclerosis. *Am. J. Phys. Med. Rehabil. 74:415-418.*

37. Metz, L. M., S. D. McGuinness, and C. Harris. 1998. Urinary tract infections may trigger relapse in multiple sclerosis. *Axone 19:67-70.*

38. Andersen, O., P.-E. Lygner, T. Bergström, M. Andersson, and A. Vahlne. 1993. Viral infections trigger multiple sclerosis relapses: a prospective seroepidemiological study. *J. Neurol. 240:417-422.*

39. Panitch, H. S. 1994. Influence of infection on exacerbations of multiple sclerosis. *Ann. Neurol. 36(Suppl):S25-S28.*

40. Edwards, S., M. Zvartau, H. Clarke, W. Irving, and L. D. Blumhardt. 1998. Clinical relapse and disease activity on magnetic resonance imaging associated with viral upper respiratory tract infections in multiple sclerosis. *J. Neurol. Neurosurg. Psychiatry 64:736-741.*

41. Alter, M., U. Leibowitz, and J. Speer. 1966. Risk of multiple sclerosis related to age at immigration to Israel. *Arch. Neurol. 15:234-237.*

42. Alter, M., and M. Okihiro. 1971. When is multiple sclerosis acquired? *Neurology 21:1030-1036.*

43. Alter, M., E. Kahana, and R. Loewenson. 1978. Migration and risk of multiple sclerosis. *Neurology 28:1089-1093.*

44. Kurtzke, J. F., G. Dean, and D. P. Botha. 1970. A method for estimating the age at immigration of white immigrants to South Africa, with an example of its importance. *S. Afr. Med. J. 44:663-669.*

45. Kurtzke, J. F., K. T. Kurland, and I. D. Goldberg. 1971. Mortality and migration in multiple sclerosis. *Neurology 21:1186-1197*.

46. Dean, G., and J. F. Kurtzke. 1971. On the risk of multiple sclerosis according to age at immigration to South Africa. *Br. Med. J. 3:725-729*.

47. Detels, R., B. R. Visscher, R. W. Haile, R. M. Malmgren, J. P. Dudley, and A. H. Coulson. 1978. Multiple sclerosis and age at migration. *Am. J. Epidemiol. 108:386-393*.

48. Elian, M., and G. Dean. 1987. Multiple sclerosis among the United Kingdom born children of immigrants from the West Indies. *1987 50:327-332*.

49. Wolfson, C., and D. B. Wolfson. 1993. The latent period of multiple sclerosis: A critical review. *Epidemiology 4:464-470*.

50. Riise, T., M. Gronning, R. Klauber, E. Barrett-Connor, H. Nyland, and G. Albrektsen. 1991. Clustering of residence of multiple sclerosis patients at age 13 to 20 years in Hordaland, Norway. *Am. J. Epidemiol. 133:932-939*.

51. Fujinami, R. 2001. Can virus infection triger autoimmune disease? *J. Autoimmun. 16:229-234*.

52. Leslie, R. D., and R. B. Elliott. 1994. Early environmental events as a cause of IDDM. Evidence and implications. *Diabetes 43:843-853*.

53. Poskanzer, D. C., A. M. Alker, and J. L. Prenney. 1981. The etiology of multiple sclerosis: temporospatial clustering indicating two environmental exposures before onset. *Neurology 31:708-713*.

54. Alvord, E. C., M. W. Kies, and A. J. Suckling. 1984. Experimental allergic encephalomyelitis: A useful model for multiple sclerosis. *Prog. Clin. Biol. Res. 146:1-554*.

55. Stuart, G., and K. S. Krikorian. 1928. The neuro-paralytic accidents of anti-rabies treatment. *Ann. Trop. Med. Parasitol. 22:327-377*.

56. Remlinger, P. 1904. Contribution à l'étude de la toxine rabique: faits cliniques. *C. R. Soc. Biol. 56:349-356*.

57. Koritschoner, R., and F. Schweinburg. 1925. Klinische und experimentelle beobachtungen über lähmungen nach wutschutzimptung. *Z. Immunitätsforsch. 42:217-283*.

58. Hurst, E. W. 1932. Effects of injection of normal brain emulsion into rabbits, with special reference to aetiology of paralytic accidents of antirabic treatment. *J. Hyg. 32:33-44*.

59. Rivers, T. M., D. H. Sprunt, and G. P. Berry. 1933. Observations on attempts to produce disseminated encephalomyelitis in monkeys. *J. Exp. Med. 58:39-52*.

60. Rivers, T. M., and F. F. Schwentker. 1935. Encephalomyelitis accompanied by myelin destruction exprerimentally produced in monkeys. *J. Exp. Med. 61:689-705*.

61. Freund, J., and K. McDermott. 1942. Sensitization to horse serum by means of adjuvants. *Proc. Soc. Exp. Bid. and Med. 49:548-553.*

62. Kopeloff, L. M., and N. Kopeloff. 1943. The production of antibrain antibodies in the monkey. *J. Immunol. 48:297-304.*

63. Morgan, I. M. 1946. Allergic encephalomyelitis in monkeys in response to injection of normal monkey cord. *J. Bacteriol. 5:614-619.*

64. Kabat, E. A., A. Wolf, and A. E. Bezer. 1946. Rapid production of acute disseminated encephalomyelitis in Rhesus monkeys by injection of brain tissue with adjuvants. *Science 104:362-363.*

65. Morrison, L. R. 1947. Disseminated encephalomyelitis experimentally produced by the use of homologous antigen. *Arch. Neurol. & Psychiat. 58:391.*

66. Freund, J., E. R. Stern, and T. M. Pisani. 1947. Isoallergic encephalomyelitis and radiculitis in guinea pigs after one injection of brain and mycobacteria in water-in oil emulsion. *J. Immunol. 57:179-185.*

67. Kopeloff, L. M., and N. Kopeloff. 1947. Neurologic manifestations in laboratory animals produced by organ (adjuvant) emulsions. *J. Immunol. 57:229.*

68. Jervis, G., and H. Koprowsky. 1948. Experimental allergic encephalomyelitis. *J. Neuropath. & Exper. Neurol. 7:309.*

69. Paterson, P. Y. 1976. Experimental autoimmune (allergic) encephalomyelitis: induction, pathogenesis and suppression. In Texbook of Immunopathology, ed. P.A. Mescher, H.S. Mueller-Eberhard, pp. 179-213. New York: Grune & Stratton..

70. Olitsky, P. K., and R. H. Yager. 1949. Experimental disseminated encephalomyelitis in white mice. *J. Exp. Med. 90:213-223.*

71. Ferraro, A. 1948. Chronic experimental allergic encephalomyelitis in monkeys. *J. Neuropath. Exp. Neurol. 7:235-261.*

72. Martin, R., H. F. McFarland, and D. E. McFarlin. 1992. Immunological aspects of demyelinating diseases. *Annu. Rev. Immunol. 10:153-187.*

73. Goverman, J., and T. Brabb. 1996. Rodent models of experimental allergic encephalomyelitis applied to the study of multiple sclerosis. *Lab. Ani. Sci. 46:482-492.*

74. Swanborg, R. H. 1995. Short analytical review: Animal models of human disease: Experimental autoimmune encephalomyelitis in rodents as a model for human demyelinating disease. *Clin. Immunol. Immunopathol. 77:4-13.*

75. Brown, A. M., and D. McFarlin. 1981. Relapsing experimental allergic encephalomyelitis in the SJL/J mouse. *Lab. Invest. 45:278-295.*

76. Yang, J., S. Lindal, E. Ylinen, N. Setälä, V. Hukkanen, and R. Seljelid. 1999. A novel and efficient regimen for producing chronic relapsing experimental autoimmune encephalomyelitis (CR-EAE) in SJL mice. *APMIS 107:800-806*.

77. Zamvil, S. S., and L. Steinman. 1990. The T lymphocyte in experimental allergic encephalomyelitis. *Annu. Rev. Immunol. 8:579-621*.

78. Lumsden, C. E. 1949. Experimental "allergic" encephalomyelitis II. - on the nature of the encephalitogenic agent. *Brain 72:517-537*.

79. Kabat, E. A., A. Wolf, and A. E. Bezer. 1949. Studies on acute disseminated encephalomyelitis produced experimentally in Rhesus monkeys. *J. Exp. Med. 89:395-398*.

80. Kabat, E. A., A. Wolf, and A. E. Bezer. 1947. The rapid production of acute disseminate encephalomyelitis in rhesus monkeys by injection of heterozygous and homologous brain tissue with adjuvants. *J. Exp. Med. 85:117-129*.

81. Einstein, E. R., D. M. Robertson, J. M. DiCarpio, and W. Moore. 1962. The isolation from bovine spinal cord of a homogenous protein with encephalitogenic activity. *J. Neurochem. 9:353-361*.

82. Kies, M. W. 1965. Chemical studies on an encephalitogenic protein from guinea pig brain. *Ann. N. Y. Acad. Sci. 122:161-172*.

83. McLaurin, J., C. A. Ackerley, and M. A. Moscarello. 1993. Localization of basic proteins in human myelin. *J. Neurosci. Res. 35:618-628*.

84. Takahashi, N., A. Roach, D. B. Teplow, S. B. Prusiner, and L. Hood. 1985. Cloning and characterization of the myelin basic protein gene from mouse: One gene can encode both 14 kd and 18.5 kd MBPs by alternate use of exons. *Cell 42:139-148*.

85. Martenson, R. E. 1984. Myelin basic protein speciation. In Experimental allergic encephalomyelitis: A useful model for multiple sclerosis, ed. E.C. Alvord Jr., pp. 146-511. New york: Liss..

86. Eyler, E. H., J. Caccam, J. J. Jackson, F. C. Westall, and R. B. Robertson. 1970. Experimental allergic encephalomyelitis: sythesis of disease inducing site of the basic protein. *Science 168:1220-1223*.

87. Shapira, R., C.-H. F. Chou, S. McKneally, E. Urban, and R. Kibler. 1971. Biological activity and synthesis of an encephalitogenic determinant. *Science 173:736-738*.

88. Karkhanis, Y. D., J. D. Carlo, S. W. Brostoff, and E. H. Eylar. 1975. Allergic encephalomyelitis: isolation of an encephalitogenic peptide in the monkey. *J. Biol. Chem. 250:1718-1722*.

89. McFarlin, D. E., S. E. Blank, R. F. Kibler, S. McKneally, and R. Shapiro. 1973. Experimental allergic encephalomyelitis in the rat: responses to encephalitogenic proteins and peptides. *Science 179:478-480*.

90. Kibler, R. F., R. B. Fritz, F. C. H. Chou, C. H. J. Chou, N. Y. Peacocke, N. M. Brown, and D. E. McFarlin. 1977. Immune response of Lewis rats to peptide C1 (residues 68-88) of guinea pig and rat myelin basic protein. *J. Exp. Med. 146:1323-1331.*

91. Hashim, G. A., E. F. Carvalho, and R. D. Sharpe. 1978. Definition and synthesis of the essential amino acid sequence for experimental allergic encephalomyelitis in Lewis rats. *J. Immunol. 121:665-670.*

92. Fritz, R. B., C. H. J. Chou, and D. E. McFarlin. 1983. Induction of experimental allergic encephalomyelitis in PL/J and (SJL/JxPL/J)F mice by myelin basic protein and its peptides: Localization of a second encephalitogenic determinant. *J. Immunol. 130:191-194.*

93. Fritz, R. B., C. H. J. Chou, and D. E. McFarlin. 1983. Relapsing murine experimental allergic encepohalomyelitis induced by myelin basic protein. *J. Immunol. 130;:1024-1026.*

94. Fritz, R. B., M. J. Skeen, C. H. J. Chou, M. Garcia, and I. K. Egorov. 1985. Major histocompatibility complex-linked control of the murine immune response to myelin basic protein. *J. Immunol. 134:2328-2332.*

95. Pettinelli, C. B., R. B. Fritz, C. H. J. Chou, and D. E. McFarlin. 1982. Encephalitogenic activity of guinea pig myelin basic protein in the SJL mouse. *J. Immunol. 129:1209-1211.*

96. Zamvil, S. S., D. J. Mitchell, A. C. Moore, K. Kitamura, L. Steinman, and J. Rothbard. 1986. T-cell epitope of the autoantigen myelin basic protein that induces encephalomyelitis. *Nature 324:258-260.*

97. Sakai, K., S. S. Zamvil, D. J. Mitchell, M. Lim, J. B. Rothbard, and L. Steinman. 1988. Characterization of a major encephalitogenic T cell epitope in SJL/J mice with synthetic oligopeptides of myelin basic protein. *J. Neuroimmunol. 19:21-32.*

98. Kono, D. H., J. L. Urban, S. J. Horvath, D. G. Ando, R. A. Saavedra, and L. Hood. 1988. Two minor determinants of myelin basic protein induce experimental allergic encephalomyelitis in SJL/J mice. *J. Exp. Med. 168:213-227.*

99. Waksman, B. H., H. Porter, and M. B. Lees. 1954. A study of the chemical nature of components of bovine white matter effective in producing allergic encephalomyelitis in the rabbit. *J. Exp. Med. 100:451-471.*

100. Trotter, J. L., H. B. Clark, K. G. Collins, C. L. Wegeschiede, and J. D. Scarpellini. 1987. Myelin proteolipid protein induces demyelinating disease in mice. *J. Neurol. Sci. 79:173-188.*

101. Tuohy, V. K., R. A. Sobel, and M. B. Lees. 1988. Myelin proteolipid protein-induced experimental allergic encephalomyelitis: Variations of disease expression in different strains of mice. *J. Immunol. 140:1868-1873.*

102. Tuohy, V. K., Z. Lu, R. A. Sobel, R. A. Laursen, and M. B. Lees. 1989. Identification of an encephalitogenic determinant of myelin proteolipid protein for SJL mice. *J. Immunol. 142:1523-1527.*

103. Tuohy, V. K. 1994. Peptide determinants of myelin proteolipid protein (PLP) in autoimmune demyelinating disease: A review. *Neurochem. Res. 19:935-944.*

104. Tuohy, V. K., R. A. Sobel, Z. Lu, R. A. Laursen, and M. B. Lees. 1992. Myelin proteolipid protein: minimum sequence requirements for active induction of autoimmune encephalomyelitis in SWR/J and SJL/J mice. *J. Neuroimmunol. 39:67-74.*

105. Whitham, R. H., R. E. Jones, G. A. Hashim, C. M. Hoy, R. Y. Wang, A. A. Vandenbark, and H. Offner. 1991. Location of a new encephalitogenic epitope (residues 43 to 64) in proteolipid protein that induces relapsing experimental autoimmune encephalomyelitis in PL/J and (SJLxPL)F1 mice. *J. Immunol. 147(11):3803-3808.*

106. Zhao, W., K. W. Wegmann, J. L. Trotter, K. Ueno, and W. F. Hickey. 1994. Identification of an N-terminally acetylated encephalitogenic epitope in myelin proteolipid apoprotein for the Lewis rat. *J. Immunol. 153:901-909.*

107. Lebar, R., C. Lubetzki, C. Vincent, P. Lombrail, and J. M. Boutry. 1986. The M2 autoantigen of central nervous system myelin, a glycoprotein present in oligodendrocyte membrane. *Clin. Exp. Immunol. 66:423-434.*

108. Brunner, C., H. Lassmann, T. V. Waehneldt, J.-M. Matthieu, and C. Linington. 1989. Differential ultrastructural localization of myelin basic protein, myelin/oligodendrocyte glycoprotein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase in the CNS of adult rats. *J. Neurochem. 52:296-304.*

109. Linington, C., M. Webb, and P. L. Woodhams. 1984. A novel myelin-associated glycoprotein defined by a mouse monoclonal antibody. *J. Neuroimmunol. 6:387-396.*

110. Gardinier, M. V., P. Amiguet, C. Linington, and J.-M. Matthier. 1992. Myelin/oligodendrocyte glycoprotein is a unique member of the immunoglobulin superfamily. *J. Neurosci. Res. 33:177-187.*

111. Iglesias, A., J. Bauer, T. Litzenburger, A. Schubart, and C. Linington. 2001. T- and B-cell responses to myelin oligodendrocyte glycoprotein in experimental autoimmune encephalomyelitis and multiple sclerosis. *Glia 36:220-234.*

112. Amor, S., N. Groome, C. Linington, M. M. Morris, K. Dornmair, M. V. Gardinier, J.-M. Matthieu, and D. Baker. 1994. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J. Immunol. 153:4349-4356.*

113. Abdul-Majid, K. B., J. Jirholt, C. Stadelmann, A. Stefferl, P. Kjellen, E. Wallstrom, R. Holmdahl, H. Lassmann, T. Olsson, and R. A. Harris. 2000. Screening of several H-2 congenic mouse strains identified H-2 (q) mice as highly susceptible to MOG-induced EAE with minimal adjuvant requirement. *J. Neuroimmunol. 111:23-33.*

114. Kerlero de Rosbo, N., I. Mendel, and A. Ben-Nun. 1995. Chronic relapsing experimental autoimmune encephalomyelitis with a delayed onset and an atypical clinical course, induced in PL/J mice by myelin oligodendrocyte glycoprotein (MOG)-derived peptide: preliminary analysis of MOG T cell epitopes. *Eur. J. Immunol. 25:985-993.*

115. Mendel, I., N. Kerlero de Rosbo, and A. Ben-Nun. 1995. A myelin oligodendrocyte glycoprotein peptide induces typical chronic experimental autoimmune encephalomyelitis in H-2b mice: fine specificity and T cell receptor V beta expression of encephalitogenic T cells. *Eur. J. Immunol. 25:1951-1959.*

116. Bernard, C. C. A., T. G. Johns, A. Slavin, M. Ichikawa, C. Ewing, J. Liu, and J. Bettadapura. 1997. Myelin oligodendrocyte glycoprotein: a novel candidate autoantigen in multiple sclerosis. *J. Mol. Med. 75:77-88.*

117. Tsunoda, I., L. Q. Kuang, D. J. Theil, and R. S. Fujinami. 2000. Antibody association with a novel model for primary progressive multiple sclerosis: induction of relapsing-remitting and progressive forms of EAE in H2s mouse strains. *Brain Pathol. 10:402-418.*

118. Weerth, S., T. Berger, H. Lassmann, and C. Linington. 1999. Encephalitogenic and neuritogenic T cell responses to the myelin-associated glycoprotein (MAG) in the Lewis rat. *J. Neuroimmunol. 95:157-164.*

119. Kaye, J. F., N. Kerlero de Tosbom, I. Mendel, S. Flechter, M. Hoffman, I. Yust, and A. Ben-Nun. 2000. The central nervous system-specific myelin oligodendrocytic basic protein (MOBP) is encephalitogenic and a potential target antigen in multiple sclerosis (MS). *J. Neuroimmunol. 102:189-198.*

120. Kojima, K., T. Berger, H. Lassmann, D. Hinze-Selch, Y. Zhang, J. Gehrmann, K. Reske, H. Wekerle, and C. Linington. 1994. Experimental autoimmune panencephalitis and uveoretinitis transferred to the Lewis rat by T lymphocytes specific for the S100-beta molecule, a calcium binding protein of astroglia. *J. Exp. Med. 180:817-829.*

121. Freund, J., J. Casals, and E. P. Hismer. 1937. Senstitization and antibody formation after injection of tubercle bacilli and paraffin oil. *Proc. Soc. Exp. Biol. Med. 37:509.*

122. Lewis, P., and D. Loomis. 1925. Allergic irritability. The formation of anti-sheep hemolytic amboceptor in the normal and tuberculous guinea pig. *J. Exp. Med. 40:503-515.*

123. Lewis, P., and D. Loomis. 1925. Allergic irritability. Anaphylaxis in the guinea pig as affected by inheritance. *J. Exp. Med. 41:327-335.*

124. Ellouz, F., A. Adam, R. Ciorbaru, and E. Lederer. 1974. Minimal structural requirements for adjuvant activity of bacterial peptidoglycan derivatives. *Biochem. Biophys., Res. Com. 59:13-17.*

125. Rook, G. A. W. 1993. New meanings for an old word: adjuvanticity, cytokines and T cells. *Immunol. Today 14:95-96.*

126. Janeway, C. A. 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol. Today 13:11-16.*

127. Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol. 12:991-10454.*

128. Audibert, F. M., and L. D. Lise. 1993. Adjuvants: current status, clinical perspectives and future prospects. *Immunol. Today 14:281-283.*

129. Yip, H. C., A. Y. Karulin, M. Tary-Lehmann, M. D. Hesse, H. Radeke, P. S. Heeger, R. P. Trezza, F. P. Heinzel, T. Forsthuber, and P. V. Lehmann. 1999. Adjuvant-guided type-1 and type-2 immunity: infectious/noninfectious dichotomy defines the class of response. *J. Immunol. 162:3942-3949.*

130. Schnare, M., G. M. Barton, A. C. Holt, K. Takeda, S. Akira, and R. Medzhitov. 2001. Toll-like receptors control activation of adaptive immune responses. *Nat. Immunol. 2:947-950.*

131. Alvord, E. C., C. M. Shaw, W. J. Fahlberg, and M. W. Kies. 1964. An analysis of components of Freund´s adjuvants in the production of experimental "allergic" encephalomyelitis in the guinea pig. *Immun. Allergie. Forsch. 126:193-206.*

132. Lee, J. M., and H. A. Schneider. 1962. Critical relationships between constituents of the antigen-adjuvant emulsion affecting experimental allergic encephalomyelitis in a completely susceptible mouse genotype. *J. Exp. Med. 115:157-168.*

133. Shaw, C. M., E. C. Alvord, W. J. Fahlberg, and M. W. Kies. 1962. Adjuvant-antigen relationships in the production of experimental "allergic" encephalomyelitis in the guinea pig. *J.Exp.Med. 115:169-179.*

134. Shaw, C., W. J. Fahlberg, M. W. Kies, and E. C. Alvord. 1960. Suppression of experimental "allergic" excephalomyelitis in guinea pigs by encephalitogenic proteins extracted from homologous brain. *J Exp Med III:171-180.*

135. Grassberger, G. 1899. Weber die nach intraperiton ealer injection von Markbutter bei Meerschweinchen entstehenden Veranderungen. *München. med. Wchnschr. 46:341-344.*

136. Freund, J., K. J. Thomson, H. B. Hough, H. E. Sommer, and T. M. Pisani. 1948. Antibody formation and sensitization with the aid of adjuvants. *J. Immunol. 60:383-398.*

137. Herbert, W. J. 1968. The mode of action of mineral-oil emulsion adjuvants on antibody production in mice. *Immunology 14:301-306.*

138. Freund, J. 1951. The effect of paraffin oil and mycobacteria on antibody formation and sensitization. *Am. J. Clin. Path. 21:645-656.*

139. Freund, J., and M. M. Lipton. 1955. Experimental allergic encephalomyelitis after the excision of the injection site of antigen-adjuvant emulsion. *J. Immunol. 75:454-459.*

140. Pittman, M. 1979. Pertussis toxin: the cause of the harmful effects and prolonged immunity of whooping cough. A hypothesis. *Rev. Infect. Dis. 1:401-412.*

141. Furman, B. L., F. M. Sidey, and M. Smith. 1988. Metabolic disturbances produced by pertussis toxin. *Pathogenesis and Immunity in Pertussis. John Wiley & Sons Ltd:147-193.*

142. Kohno, S., J. J. Munoz, T. M. Williams, C. Teuscher, C. C. A. Bernard, and K. S. K. Tung. 1983. Immunopathology of murine experimental allergic orchitis. *J. Immunol. 130:2675-2682.*

143. McAllister, C. G., B. P. Vistica, R. Sekura, T. Kuwabara, and I. Gery. 1986. The effects of Pertussis toxin on the induction and transfer of experimental autoimmune Uveoretinitis. *Clin. Immunol. Immunopathol. 39:329-336.*

144. Su, S. B., P. B. Silver, M. Zhang, C. C. Chan, and R. R. Caspi. 2001. Pertussis toxin inhibit induction of tissue-specific autoimmune disease by disrupting G protein-coupled signals. *J. Immunol. 167:250-256.*

145. Munoz, J. J., H. Arai, R. K. Bergman, and P. L. Sadowski. 1981. Biological activities of crystalline pertussigen from Bordetella pertussis. *Infect. Immun. 33:820-826.*

146. Linthicum, D. S., J. J. Munoz, and A. Blaskett. 1982. Acute experimental autoimmune encephalomyelitis in mice. I. Adjuvant action of Bordetella pertussis is due to vasoactive amine sensitization and increased vascular permeability of the central nervous system. *Cell. Immunol. 73:299-310.*

147. Linthicum, D. S., and J. A. Frelinger. 1982. Acute autoimmune encephalomyelitis in mice. II. Susceptibility is controlled by the combination of H-2 and histamine sensitization genes. *J. Exp. Med. 155:31-40.*

148. Sudweeks, J. D., J. Todd, E. P. Blankenhorn, B. B. Wardell, S. R. Woodward, N. D. Meeker, S. S. Estes, and C. Teuscher. 1993. Locus controlling Bordetella pertussis-induced histamine sensization (Bphs), an autoimmune disease-susceptibility gene maps distal to T-cell receptor β-chain gene on mouse chromosome 6. *Proc. Natl, Acad. Sci. USA 90:3700-3704.*

149. Flügel, A., T. Berkowicz, T. Ritter, M. Labeur, D. E. Jenne, Z. Li, J. W. Ellwart, M. Willem, H. Lassmann, and H. Wekerle. 2001. Migratory activity and functional changes of green fluorescent effector cells before and during experimental autoimmune encephalomyelitis. *Immunity 14:547-560.*

150. Munoz, J. J., C. C. A. Bernard, and I. R. Mackay. 1984. Elicitation of experimental allergic encephalomyelitis (EAE) in mice with the aid of pertussigen. *Cell. Immunol. 83:92-100.*

151. Raine, C. S., H. M. Wisniewski, and P. C. Dowling. 1971. An ultrastructural study of experimental demyelination and remyelinatinon: IV. Recurrent episodes and peripheral nervous system plaque formation in experimental allergic encephalomyelitis. *Lab. Invest. 25:28-34.*

152. McFarlin, D. E., S. E. Blank, and R. F. Kibler. 1974. Recurrent experimental allergic encephalomyelitis in the Lewis rat. *J. Immunol. 113:712-715.*

153. Stone, S. H., and E. M. I. Lerner. 1965. Chronic disseminated allergic encephalomyelitis in guinea pigs. *Ann. NY. Acad. Sci. 122:227-241.*

154. Wisniewski, H. M., and A. B. Keith. 1977. Chronic relapsing experimental allergic encephalomyelitis:an experimental model of multiple sclerosis. *Ann. Neurol. 1:144-148.*

155. Keith, A. B., and J. R. McDermott. 1980. Optimum conditions for inducing chronic relapsing experimental allergic encephalomyelitis in guinea pigs. *J. Neurol. Sci. 46:353-364.*

156. Massanari, R. M. 1980. A latent-relapsing neuroautoimmune disease in syrian hamsters. *Clin. Immunol. Immunopath. 16:211-220.*

157. Panitch, H., and C. Ciccone. 1981. Induction of recurrent experimental allergic encephalomyelitis with myelin basic protein. *Ann. Neurol. 9:433-438.*

158. Baker, D., J. K. O'Neill, S. E. Gschmeissner, C. E. Wilcox, C. Butter, and J. L. Turk. 1990. Induction of chronic relapsing experimental allergic encephalomyelitis in Biozzi mice. *J. Neuroimmunol. 28:261-270.*

159. Mustafa, M., P. Diener, J. B. Sun, H. Link, and T. Olsson. 1993. Immunopharmacologic modulation of experimental allergic encephalomyelitis: low-dose cyclosporin-A treatment causes disease relapse and increased systemic T and B cell-mediated myelin-directed autoimmunity. *Scand. J. Immunol. 38:499-507.*

160. Mokhtarian, F., D. E. McFarlin, and C. S. Raine. 1984. Adoptive transfer of myelin basic protein-sensitized T cells produces chronic relapsing demyelinating disease in mice. *Nature 309:356-358.*

161. Zamvil, S., P. Nelson, J. Trotter, D. Mitchell, R. Knobler, R. Fritz, and L. Steinman. 1985. T-cell clones specific for myelin basic protein induce chronic relapsing paralysis and demyelination. *Nature 317:355-358.*

162. Lublin, F. D., P. H. Maurer, R. G. Berry, and D. Tippett. 1981. Delayed, relapsing experimental allergic encephalomyelitis in mice. *J. Immunol. 126:819-822.*

163. Lublin, F. D. 1982. Delayed, relapsing experimental allergic encephalomyelitis in mice. *J. Neurol. Sci. 57:105-110.*

164. Brocke, S., A. Gaur, C. Piercy, A. Gautam, K. Gijbels, C. G. Fathman, and L. Steinman. 1993. Induction of relapsing paralysis in experimental autoimmune encephalomyelitis by bacterial superantigen. *Nature 365:642-644.*

165. Callard, R., A. J. T. George, and J. Stark. 1999. Cytokines, chaos, and complexity. *Immunity 11:507-513.*

166. Gianani, R., and N. Sarvetnick. 1996. Viruses, cytokines, antigens, and autoimmunity. *Proc. Natl. Acad. Sci. USA 93:2257-2259.*

167. Mosmann, T. R., and R. L. Coffman. 1987. Two types of mouse helper T-cell clone. *Immunol. Today 8:223-227.*

168. O'Garra, A. 1998. Cytokines induces the development of functionally heterogeneous T helper cell subsets. *Immunity 8:275-283.*

169. Kennedy, M. K., D. S. Torrance, K. S. Picha, and K. M. Mohler. 1992. Analysis of cytokine mRNA expression in the central nervous system of mice with experimental autoimmune encephalomyelitis reveals that IL-10 mRNA expression correlates with recovery. *J. Immunol. 149:2496-2505.*

170. Racke, M. K., D. Burnett, S.-H. Pak, P. S. Albert, B. Cannella, C. S. Raine, D. E. McFarlin, and D. E. Scott. 1995. Retinoid treatment of experimental allergic encephalomyelitis: IL-4 production correlates with improved disease course. *J. Immunol. 154:450-458.*

171. Olsson, T. 1995. Critical influence of cytokine orchestration on the outcome of myelin antigen-specific T cell autoimmunity in experimental autoimmune encephalomyelitis and multiple sclerosis. *Immunol. Rev. 144:245-268.*

172. Chen, Y., W. W. Hancock, R. Marks, P. Gonnella, and H. L. Weiner. 1998. Mechanisms of recovery from experimental autoimmune encephalomyelitis: T cell deletion and immune deviation in myelin basic protein T cell receptor transgenic mice. *J. Neuroimmunol. 82:149-159.*

173. Khoury, S. J., W. W. Hancock, and H. Weiner. 1992. Oral tolerance to myelin basic protein and natural recovery from experimental autoimmune encephalomyelitis are associated with downregulation of inflammatory cytokines and differential upregulation of transforming growth factor β, interleukin 4, and prostaglandin E expression in the brain. *J. Exp. Med. 176:1355-1364.*

174. Jensen, M. A., A. Dayal, and G. W. Arnason. 1999. Cytokine secretion by γδ and αβ T cells in monophasic experimental autoimmune encephalomyelitis. *J. Autoimmun. 12:73-80.*

175. Rizzo, L. V., R. A. Morawetz, N. E. Miller-Rivero, R. Choi, B. Wiggert, C. Chan, H. C. Morse III, R. B. Nussenblatt, and R. R. Caspi. 1999. IL-4 and IL-10 are both required for the induction of oral tolerance. *J. Immunol. 162:2613-2622.*

176. Maron, R., W. W. Hancock, A. Slavin, M. Hattori, V. Kuchroo, and H. L. Weiner. 1999. Genetic susceptibility or resistance to autoimmune encephalomyelitis in MHC congenic mice is associated with differential production of pro- and anti-inflammatory cytokines. *Int. Immunol. 11:1573-1580.*

177. Zhang, G., B. Xiao, X. Bai, P. van der Meide, A. Orn, and H. Link. 1999. Mice with IFN-gamma receptor deficiency are less susceptible to experimental autoimmune myasthenia gravis. *J. Immunol. 162:3775-3781.*

178. Falcone, M., A. J. Rajan, B. R. Bloom, and C. F. Brosnan. 1998. A critical role for IL-4 in regulating disease severity in experimental allergic encephalomyelitis as demonstrated in IL-4 deficient C57BL/6 mice and BALB/c mice. *J. Immunol. 160:4822-4830.*

179. Bettelli, E., M. P. Das, E. D. Howard, H. L. Weiner, R. A. Sobel, and V. K. Kuchroo. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. *J. Immunol. 161:3299-3306.*

180. Shaw, M. K., J. B. Lorens, A. Dhawan, R. DalCanto, H. Y. Tse, A. B. Tran, C. Bonpane, S. L. Eswaran, S. Brocke, N. Sarvetnick, L. Steinman, G. P. Nolan, and C. G. Fathman. 1997. Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. *J. Exp. Med. 185:1711-1714.*

181. Cua, D. J., H. Groux, D. R. Hinton, S. A. Stohlman, and R. L. Coffman. 1999. Transgenic interleukin 10 prevents induction of experimental autoimmune encephalomyelitis. *J. Exp. Med. 189:1005-1010.*

182. Lafaille, J. J., F. Van de Keere, A. L. Hsu, J. L. Baron, W. Haas, C. S. Raine, and S. Tonegawa. 1997. Myelin basic protein-specific T helper 2 (Th2) cells cause experimental autoimmune encephalomyelitis in immunodeficient hosts rather than protect them from the disease. *J. Exp. Med. 186:307-312.*

183. Sadlack, B., J. Löhler, H. Schorle, G. Klebb, H. Haber, E. Sickel, R. J. Noelle, and I. Horak. 1995. Generalized autoimmune disease in interleukin-2-deficient mice is triggered by an uncontrolled activation and proliferation of $CD4^+$ T cells. *Eur. J. Immunol. 25:3053-3059.*

184. Billiau, A., H. Heremans, F. Vandekerckhove, R. Dijkmans, H. Sobis, E. Meulepas, and H. Garton. 1988. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-γ. *J. Immunol. 140:1506-1510.*

185. Krakowski, M., and T. Owens. 1996. Interferon-γ confers resistance to experimental allergic encephalomyelitis. *Eur. J. Immunol. 26:1641-1646.*

186. Heremans, H., C. Dillen, M. Groenen, E. Martens, and A. Billiau. 1996. Chronic relapsing experimental autoimmune encephalomyelitis (CREAE) in mice: enhancement by monoclonal antibodies against interferon-γ. *Eur. J. Immunol. 26:2393-2398.*

187. Ferber, I. A., S. Brocke, C. Toylor-Edwords, W. Ridgwoy, C. Dinisco, L. Steinman, D. Dalton, and C. G. Fathman. 1996. Mice with a disrupted IFN-γ gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). *J. Immunol. 156:5-7.*

188. Panitch, H. S., R. L. Hirsch, J. Schindler, and K. P. Johnson. 1987. Treatment of multiple sclerosis with gamma-interferon: exacerbations associated with activation of the immune system. *Neurology 37:1097-1102.*

189. Wogensen, L., M.-S. Lee, and N. Sarvetnick. 1994. Production of interlerkin 10 by islet cells accelerates immune-mediated destruction of β cells in nonobese diabetic mice. *J. Exp. Med. 179:1379-1384*.

190. Ramanathan, S., Y. D. Kozak, A. Saoudi, O. Goureau, P. H. V. D. Meide, P. Druet, and B. Bellon. 1996. Recombinant IL-4 aggravates experimental autoimmune uveoretinitis in rats. *J. Immunol. 157:2209-2215*.

191. Okuda, Y., S. Sakoda, and T. Yanagihara. 1998. The pattern of cytokine gene expression in lymphoid organs and peripheral blood mononuclear cells of mice with experimental allergic encephalomyelitis. *J. Neuroimmunol. 87:147-155*.

192. McCombe, P. A., I. Nickson, and M. P. Pender. 1998. Cytokine expression by inflammatory cells obtained from the spinal cords of Lews rats with experimental autoimmune encephalomyelitis induced by inoculation with myelin basic protein and adjuvants. *J. Neuroimmunol. 88:30-38*.

193. Cannella, B., and C. S. Raine. 1995. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann. Neurol. 37:424-435*.

194. Inogés, S., J. Merino, E. Bandrés, P. De Castro, M. L. Subira, and A. Sanchez-Ibarrola. 1999. Cytokine flow cytometry differentiate the clinical status of multiple sclerosis (MS) patients. *Clin. Exp. Immunol. 115:521-525*.

195. Picker, L. J., and E. C. Butcher. 1992. Physiological and molecular mechanisms of lymphocyte homing. *Annu. Rev. Immunol. 10:561-591*.

196. Butcher, E. C., and L. J. Picker. 1996. Lymphocyte homing and homeostasis. *Science 272:60-66*.

197. Archelos, J. J., and H.-P. Hartung. 1997. The role of adhesion molecules in multiple sclerosis: biology, pathogenesis and therapeutic implications. *Mol. Med. Today 3(7):310-321*.

198. Gahmberg, C. G., L. Valmu, S. Fagerholm, P. Kotovuori, E. Ihanus, L. Tian, and T. Pessa-Morikawa. 1998. Leukocyte integrins and inflammation. *Cell. Mol. Life Sci. 54:549-555*.

199. Van Seventer, G. A., Y. Shimizu, K. J. Horgan, and S. Shaw. 1990. The LFA-1 ligand ICAM-1 provides an important costimulatory signal for T cell receptor-mediated activation of resting T cells. *J. Immunol. 144:4579-4586*.

200. Moy, V. T., and A. A. Brian. 1992. Signaling by lymphocyte function-associated antigen 1 (LFA-1) in B cells: Enhanced antigen presentation after stimulation through LFA-1. *J. Exp. Med. 175:1-7*.

201. Lub, M., Y. van Kooyk, and C. G. Figdor. 1995. Ins and outs of LFA-1. *Immunol. Today 16:479-483*.

202. Gahmberg, C. G., M. Tolvanen, and P. Kotovuori. 1997. Leukocyte adhesion: Structure and function of human leukocyte β_2-integrins and their cellular ligands. *Eur. J. Biochem. 245:215-232*.

203. Hynes, R. O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell 69:11-25*.

204. Pardi, R., L. Inverardi, and J. R. Bender. 1992. Regulatory mechanisms in leukocyte adhesion receptors: flexible receptors for sophisticated travelers. *Immunol. Today 13:224-230*.

205. Springer, T. A., M. L. Dustin, T. K. Kishimoto, and S. D. Marlin. 1987. The lymphocyte function-associated LFA-1, CD2 and LFA-3 molecules: cell adhesion receptors of the immune system. *Ann. Rev. Immunol. 5:223-252*.

206. Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature 346:425-434*.

207. Valmu, L., and C. G. Gahmberg. 1995. Treatment with okadaic acid reveals strong threonine phosphorylation of CD18 after activation of CD11/CD18 leukocyte integrins with phorbol esters or CD3 antibodies. *J. Immunol. 155:1175-1183*.

208. Gahmberg, C. G. 1997. Leukocyte adhesion: CD11/CD18 integrins and intercellular adhesion molecules. *Curr. Opin. Cell Biol. 9:643-650*.

209. Valmu, L., S. Fagerholm, H. Suila, and C. G. Gahmberg. 1999. The cytoskeletal association of CD11/CD18 leukocyte integrins in phorbol ester-activated cells correlates with CD18 phosphorylation. *Eur. J. Immunol. 29:2107-2118*.

210. Raine, C. S. 1994. Multiple sclerosis: immune system molecule expresssion in the central nervous system. *J. Neuropathol. Exp. Neurol. 53:328-337*.

211. Bö, L., J. W. Peterson, S. Mork, P. A. Hoffman, W. M. Gallatin, R. M. Ransohoff, and B. D. Trapp. 1996. Distribution of immunoglobulin superfamily members ICAM-1, -2, -3, and the β2 integrin LFA-1 in multiple sclerosis. *J. Neuropathol. Exp. Neurol. 55:1060-1070*.

212. Tsunoda, I., and R. S. Fujinami. 1996. Two models for multiple sclerosis: experimental allergic encephalomyelitis and Theiler's murine encephalomyelitis virus. *J. Neuropathol. Exp. Neurol. 55:673-686*.

213. Svenningsson, A., G. K. Hansson, O. Andersen, R. Andersson, M. Patarroyo, and S. Stemme. 1993. Adhesion molecule expression on cerebrospinal fluid T lymphocytes: evidence for common recruitment mechanisms in multiple sclerosis, aseptic meningitis, and normal controls. *Ann. Neurol. 34:155-161*.

214. Gordon, E. J., K. J. Myers, J. P. Dougherty, H. Rosen, and Y. Ron. 1995. Both anti-CD11a (LFA-1) and anti-CD11b (MAC-1) therapy delay the onset and diminish the severity of experimental autoimmune encephalomyelitis. *J. Neuroimmunol. 62:153-160*.

215. Willenborg, D. O., M. A. Staykova, and M. Miyasaka. 1996. Short term treatment with soluble neuroantigen and anti-CD11a (LFA-1) protects rats against autoimmune encephalomyelitis: treatment abrogates autoimmune disease but not autoimmunity. *J. Immunol. 157:1973-1980*.

216. Kawai, K., Y. Kobayashi, M. Shiratori, G. Sobue, T. Tamatani, M. Miyasaka, and Y. Yoshikai. 1996. Intrathecal administration of antibodies against LFA-1 and against ICAM-1 suppresses experimental allergic encephalomyelitis in rats. *Cell. Immunol. 171:262-268.*

217. Hayashi, Y., H. Yanagi, H. Higashiyama, H. Yagita, and H. Hamano. 1995. Prevention of adoptive transfer of murine Sjögren´s syndrome into severe combined immunodeficient (SCID) mice by antibodies against intercellular adhesion molecule-1 (ICAM) and lymphocyte function-associated antigen-1 (LFA-1). *Clin. Exp. Immunol. 102:360-367.*

218. Talento, A., M. Nguyen, T. Blake, A. Siorotina, C. Fioravanti, D. Burkholder, R. Gibson, N. H. Sigal, M. Springer, and G. Koo. 1993. A single administration of LFA-1 antibody confers prolonged allograft survival. *Transplantation 55:418-422.*

219. van Dijken, P. J., T. Ghayur, P. Mauch, J. Down, S. J. Burakoff, and J. Ferrara. 1990. Evidence that anti-LFA-1 in vivo improves engraftment and survival after allogenic bone marrow transplantation. *Transplantation 49:882-886.*

220. Cannella, B., A. H. Cross, and C. S. Raine. 1993. Anti-adhesion molecules therapy in experimental autoimmune encephalomyelitis. *J. Neuroimmunol. 46:43-56.*

221. Welsh, C. T., J. W. Rose, K. E. Hill, and J. J. Townsend. 1993. Augmentation of adoptively transferred experimental allergic encephalomyelitis by administration of a monoclonal antibody specific for LFA-1α. *J. Neuroimmunol. 43:161-168.*

222. Picker, L., M. Nakache, and E. C. Butcher. 1989. Monoclonal antibodies to human lymphocyte homing receptors define a novel class of adhesion molecules on diverse cell types. *J. Cell Biol. 109:927-937.*

223. Haynes, B. F., M. J. Telen, L. P. Hale, and S. M. Denning. 1989. CD44-a molecule involved in leukocyte adherence and T cell activation. *Immunol. Today 10:423-428.*

224. Jalkanen, S., R. F. Bargatze, J. M. de los Toyos, and E. C. Butcher. 1987. Lymphocyte recognition of high endothelium: Antibodies to distinct epitopes of an 85-95 kd glycoprotein antigen differentially inhibit lymphocyte binding to lymph node, mucosal, or synovial endothelial cells. *J. Cell Biol. 195:983-990.*

225. Picker, L. J., L. W. M. M. Terstappen, L. S. Rott, P. R. Streeter, H. Stein, and E. C. Butcher. 1990. Differential expression of homing-associated adhesion molecules by T-cell subsets in man. *J. Immunol. 145:3247-3255.*

226. Zeidler, A., R. Bräuer, K. Thoss, J. Bahnsen, V. Heinrichs, D. Jablonski-Westrich, M. Wroblewski, S. Rebstock, and A. Hamann. 1995. Therapeutic effects of antibodies against adhesion molecules in murine collagen type II-induced arthritis. *Autoimmunity 21:245-252.*

227. Mikecz, K., F. R. Brennan, J. H. Kim, and T. T. Glant. 1995. Anti-CD44 treatment abrogates tissue edema and leukocyte infiltration in murine arthritis. *Nat. Med. 1:558-563.*

228. Verdrengh, M., R. Holmdahl, and A. Tarkowski. 1995. Administration of antibodies to hyaluronanreceptor (CD44) delays the start and ameliorates the severity of collagen II arthritis. *Scand. J. Immunol. 42:353-358.*

229. Nedvetzki, S., M. Walmsley, E. Alpert, R. O. Williams, M. Feldmann, and D. Naor. 1999. CD44 involvement in experimental collagen-induced arthritis (CIA). *J. Autoimmun. 13:39-47.*

230. Brennan, F. R., J. K. O'neill, S. J. Allen, C. Butter, G. Nuki, and D. Baker. 1999. CD44 is involved in selective leukocyte extravasation during inflammatory central nervous system disease. *Immunology 98:427-435.*

231. Brocke, S., C. Piercy, L. Steinman, I. L. Weissman, and T. Veromaa. 1999. Antibodies to CD44 and integrin α4, but not L-selectin, prevent central nervous system inflammation and experimental encephalomyelitis by blocking secondary leukocyte recruitment. *Proc. Natl. Acad. Sci. USA 96:5896-6901.*

232. Yednock, T. A., C. Cannon, L. C. Fritz, F. Sanchez-Madrid, L. Steinman, and N. Karin. 1992. Prevention of experimental autoimmune encephalomyelitis by antibodies against α4β1 integrin. *Nature 356:63-66.*

233. Baron, J. L., J. A. Madri, N. H. Ruddle, G. Hashim, and C. A. J. Janeway. 1993. Surface expression of α4 integrin by CD4 T cells is required for their entry into brain parenchyma. *J. Exp. Med. 177:57-68.*

234. Parish, N. M., F. R. Brennan, and A. Cooke. 1999. Anti-CD44 treatment does not prevent the extravasation of autopathogenic T cells to the thyroid in experimental autoimmune thyroiditis. *Immunology 97:533-539.*

235. Briscoe, D. M., R. S. Cotran, and J. S. Prober. 1992. Effects of tumor necrosis factor, lipopolysaccharide, and IL-4 on the expression of vascular cell adhesion molecule-1 *in vivo*. Correlation with CD3+ T cell infiltration. *J. Immunol. 149:2954-2960.*

236. Rice, G. E., J. M. Munro, C. Corless, and M. P. Bevilacqua. 1991. Vascular and nonvascular expression of INCAM-110. A target for mononuclear leukocyte adhesion in normal and inflamed human tissue. *Am. J. Pathol. 138:385-393.*

237. Rieckmann, P., B. Altenhofen, A. Riegel, J. Baudewig, and K. Felgenhauer. 1997. Soluble adhesion molecules (sVCAM-1 and sICAM-1) in cerebrospinal fluid and serum correlate with MRI activity in multiple sclerosis. *Ann. Neurol. 41:326-333.*

238. McDonnell, C. V., S. A. McMillan, J. P. Douglas, A. G. Droogan, and S. A. Hawkins. 1998. Raised CSF levels of soluble adhesion molecules across the clinical spectrum of multiple sclerosis. *J. Neuroimmunol. 1998:186-192.*

239. Dopp, J. M., S. M. Breneman, and J. A. Olschowka. 1994. Expression of ICAM-1, VCAM-1, L-selectin, and leukosialin in the mouse central nervous system during the induction and remission stages of experimental allergic encephalomyelitis. *J. Neuroimmunol. 54:129-144.*

240. Cannella, B., A. H. Cross, and C. S. Raine. 1991. Adhesion-ralated molecules in the central nervous system. Upregulation correlates with inflammatory cell influx during relapsing experimental autoimmune encephalomyelitis. *Lab. Invest. 65:23-31.*

241. Wilcox, C., A. Ward, A. Evans, D. Baker, R. Rothlein, and J. Turk. 1990. Endothelial cell expression of the intercellular adhesion molecule-1 (ICAM-1) in the central nervous system of guinea pigs during acute and chronic relapsing experimental allergic encephalomyelitis. *J. Neuroimmunol. 30:43-51.*

242. Lindsey, J. W., and L. Steinman. 1993. Competitive PCR quantification of CD4, CD8, ICAM-1, VCAM-1, and MHC class II mRNA in the central nervous system during development and resolution of experimental allergic encephalomyelitis. *J. Neuroimmunol. 48:227-234.*

243. Sobel, R. A., M. E. Mitchell, and G. Fondren. 1990. Intercellular adhesion molecule-1 (ICAM-1) in cellular immune reactions in the human central nervous system. *Am. J. Pathol. 136:1309-1316.*

244. Galabresi, R. A., L. R. Tranquill, J. M. Dambrosia, L. A. Stone, H. Maloni, C. N. Bash, J. A. Frank, and H. F. McFarland. 1997. Increases in soluble VCAM-1 correlate with a decrease in MRI lesions in multiple sclerosis treated with interferon -β1b. *Ann. Neurol. 41:669-674.*

245. Yoshihara, Y., S. Oka, Y. Nemoto, Y. Watanabe, S. Nagata, H. Kagamiyama, and K. Mori. 1994. An ICAM-related neuronal glycoprotein, telencephalin, with brain segment-specific expression. *Neuron 12:541-553.*

246. Mori, K., C. Fujita, Y. Watanabe, K. Obata, and O. Hayaishi. 1987. Telencephalon-specific antigen identified by monoclonal antibody. *Proc. Natl. Acad. Sci. USA 84:3921-3925.*

247. Oka, S., K. Mori, and Y. Watanabe. 1990. Mammalian telencephalic neurons express a segment-specific membrane glycoprotein, telencephalin. *Neuroscience 35:93-103.*

248. Yoshihara, Y., and K. Mori. 1994. Telencephalin: a neuronal area code molecule? *Neurosci. Res. 21:119-124.*

249. Tian, L., P. Kilgannon, Y. Yoshihara, K. Mori, W. M. Gallatin, O. Carpén, and C. G. Gahmberg. 2000. Binding of T lymphocytes to hippocampal neurons through ICAM-5 (telencephalin) and characterization of its interaction with the leukocyte integrin CD11a/CD18. *Eur. J. Immunol. 30:810-818.*

250. Tian, L., Y. Yoshihara, T. Mizuno, K. Mori, and C. G. Gahmberg. 1997. The neuronal glycoprotein telencephalin is a cellular ligand for the CD11a/CD18 leukocyte integrin. *J. Immunol. 158:928-936.*

251. Lindsberg, P. J., J. Launes, L. Tian, H. Välimaa, V. Subramanian, J. Sirén, L. Hokkanen, T. C. Hyypiä, O. Carpen, and C. G. Gahmberg. 2002. Regulation of ICAM-5, a neuronal adhesion molecule, in acute encephalitis. *Neurology 58:446-451.*

252. Goetzl, E. J., and S. P. Sreedharan. 1992. Mediators of communication and adaptation in the neuroendocrine and immune systems. *FASEB 6:2646-2652.*

253. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol. 9:271-296.*

254. Shortman, K., and Y.-J. Liu. 2002. Mouse and human dendritic cell subtypes. *Nature 2:151-161*.

255. Vremec, D., and K. Shorman. 1997. Dendritic cell subtypes in mouse lymphoid organs. *J. Immunol. 159:565-573*.

256. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245-251*.

257. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature 388:394-397*.

258. Pasare, C., and R. Medzhitov. 2003. Toll pathway-dependent blockade of $CD4^{+}CD25^{+}$ T cell-mediated suppression by dendritic cells. *Science 299:1033-1036*.

259. Jacobs, L., and K. P. Johnson. 1994. A brief history of the use of interferons as treatment of multiple sclerosis. *Arch. Neurol. 51:1245-1252*.

260. Pender, M. P., and N. P. Wolfe. 2002. Prevention of autoimmune attack and disease progression in multiple sclerosis: current therapies and future prospects. *Internal Medicine Journal 32:554-563*.

261. The PRISMS study group, t. u. o. B. c. M. M. a. g. 2001. PRISMS-4: long-term efficacy of interferon-beta-1a in relapsing MS. *Neurology 56:1628-1636*.

262. Yong, V. W., S. Chabot, O. Stuve, and G. Williams. 1998. Interferon beta in the treatment of multiple sclerosis: mechanisms of action. *Neurology 51:682-689*.

263. McRae, B. L., B. A. Beilfuss, and G. A. van Sevener. 2000. IFN-beta differentially regulates CD40-induced cytokine secretion by human dentritic cells. *J. Immunol. 164:23-28*.

264. Teitelbaum, D., A. Meshorer, T. Hirshfeld, R. Amon, and M. Sela. 1971. Suppression of experimental allergic encephalomyelitis by a synthetic polypeptide. *Eur. J. Immunol. 1:242-248*.

265. Johnson, K. P., B. R. Brooks, J. A. Cohn, C. C. Ford, J. Goldstein, and R. P. Lisak. 1995. The copolymer 1 multiple sclerosis study group. Copolymer 1 reduces relapse rate and improves disability in relapsing-remitting multiple sclerosis: results of a phase II multicenter, double-blind, placebo-controlled trial. *Neurology 45:1268-1276*.

266. Neuhaus, O., C. Farina, H. Wekerle, and Hohlfeld. 2001. Mechanisms of action of glatiramer acetate in multiple sclerosis. *Neurology 56:702-708*.

267. Krapf, H., S. Morrissey, and O. Zenker. 1999. Mitoxantrone in progressive multiple sclerosis: MRI results of the European phase II trial. *Neurology 52:A495*.

268. Noseworthy, J. H., M. B. Hopkins, and M. K. Vandervoort. 1993. An open-trial evaluation of mitoxantrone in the treatment of progressive MS. *Neurology 43:1401-1406*.

269. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem. 162:156-159*.

270. Mokhtarian, F., and P. Swoveland. 1987. Predisposition to EAE induction in resistant mice by prior infection with semliki forest virus. *J. Immunol. 138:3264-3268.*

271. Wu, L., M. J. Mäkelä, M. Royttä, and A. Salmi. 1988. Effect of viral infection on experimental allergic encephalomyelitis in mice. *J. Neuroimmunol. 18:139-153.*

272. Morrison, D. C., and J. L. Ryan. 1987. Endotoxins and disease mechanisms. *Annu. Rev. Med. 38:417-432.*

273. Dinarello, C. 1984. Interleukin-1. *Rev. Infect. Dis. 6:51-95.*

274. Dinarello, C. A. 1993. Modalities for reducing interleukin 1 activity in disease. *Immunol. Today 14:260-264.*

275. Kotb, M. 1995. Short analytical review: Infection and autoimmunity: a story of the host, the pathogen and the copathogen. *Clin. Immunol. Immunopath. 74:10-22.*

276. Kuchroo, V. K., C. A. Martin, J. M. Greer, S. T. Ju, R. A. Sobel, and M. E. Dorf. 1993. Cytokines and adhesion molecules contribute to the ability of myelin proteolipid protein-specific T cell clones to mediate experimental allergic encephalomyelitis. *J. Immunol. 151:4371-4382.*

277. Wekerle, H., K. Kojima, J. Lannes-Vieira, H. Lassmann, and C. Linington. 1994. Animal models. *Ann. Neurol. 36:S47-S53.*

278. McRae, B. L., M. Kennedy, L.-J. Tan, M. C. D. Canto, K. S. Picha, and S. D. Miller. 1992. Induction of active and adoptive relapsing experimental autoimmune encephalomyelitis (EAE) using an encephalitogenic epitope of proteolipid protein. *J. Neuroimmunol. 38:229-240.*

279. Jiang, H., S. Zhang, and B. Pernis. 1992. Role of CD8+ T cells in murine experimental allergic encephalomyelitis. *Science 256:1213-1215.*

280. Coons, T. A., and E. H. Goldberg. 1978. Rejection of male skin grafts by splenectomized female mice. *Science 200:320-321.*

281. Jacobson, E. B., L. A. Herzenberg, R. Riblet, and L. A. Herzenberg. 1972. Active suppression of immunoglobulin allotype synthesis. II. Transfer of suppressing factor with spleen cells. *J. Exp. Med. 135:1163-1176.*

282. Moorhead, J. W. 1978. Subpopulations of mouse T lymphocytes. II. Suppression of graft-vs-host reactions by naturally proliferating splenic T cells. *Eur. J. Immunol. 8:163-167.*

283. Sampson, D., H. Kaufman, C. Grotelenescher, and J. Metzig. 1976. Suppressor activity of the human spleen and thymus. *Surgery 79:393-397.*

284. Adda, D. H., E. Beraud, and R. Depieds. 1977. Evidence for suppressor cells in Lewis rats´experimental allergic encephalomyelitis. *Eur. J. Immunol. 7:620-623.*

285. Mertin, J., and A. Stackpoole. 1979. The spleen is required for the suppression of experimental allergic encephalomyelitis by prostaglandin precursors. *Clin. Exp. Immunol. 36:449-455.*

286. Steinman, R. M., and M. C. Nussenzweig. 2002. Avoiding horror autotoxicus: The importance of dendritic cells in peripheral T cell tolerance. *PNAS 99:351-358.*

287. Goldscheneider, I., and R. E. Cone. 2003. A central role for peripheral dendritic cells in the induction of acquired thymic tolerance. *Trends Immunol. 24:77-81.*

288. Khoury, S. J., L. Gallon, W. Chen, K. Betres, M. E. Russell, W. W. Hancock, C. B. Carpenter, M. H. Sayegh, and H. L. Weiner. 1995. Mechanisms of acquired thymic tolerance in experimental autoimmune encephalomyelitis: Thymic dendritic-enriched cells induce specific peripheral T cell unresponsiveness in vivo. *J. Exp. Med. 182:357-366.*