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Immunoregulation of experimental autoimmune neuritis focuses on cell immunity

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Stockholm 2003

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

ISBN 91-7349-446-1

To my parents
To Tao and Shuo

Contents

Abstract

Experimental autoimmune neuritis (EAN) is a CD4+ T cell mediated autoimmune disease of peripheral nervous system (PNS). EAN can be induced in susceptible animal strains by active immunization with peripheral nerve tissue, the purified peripheral nerve myelin components P2 and P0, or synthetic P2 and P0 peptides. EAN shares many of clinical, immunological, electrophysiological and morphological characteristics of Guillain-Barré syndrome (GBS), a major inflammatory demyelinating disease of the PNS in human and allows detailed study of the various effector pathways and tests novel therapeutic strategies in vivo. Therefore, EAN serves as a useful model for exploring the pathogenesis and immunotherapy of GBS.

The effects of distinct T cell subtype (CD4+ and CD8+) and B cell in EAN were examined in $CD4^{-/-}$, $CD8^{-/-}$, $CD4^{-}8^{-}$, B cell knockout (μMT) and wild type mice. The clinical sign, T cell response specific to P0180-199 and histopathological changes in $CD4^{-/-}$ mice were significantly lower than in wild type mice; $CD8^{-/-}$ mice also appeared slighter clinical course, less T cell response specific to P0180-199 and slight hitopathological changes compared with wild type mice, but no significant difference between them except for the severity of disease. $CD4^{-}8^{-}$, μMT and wild type mice appeared similar in clinical courses, specific T cell response and histopathological changes. Similar levels of IgG production were found in sera from $CD4^{-/-}$, $CD8^{-/-}$, $CD4^{-}8^{-}$ and wild type mice except for μMT mice, from which no IgG production was detectable in sera.

The roles of costimulatory molecule CD28 in EAN have been investigated in $CD28^{-/-}$ B6 mice and wild-type mice. All the wild-type mice developed severe EAN. None of the $CD28^{-/-}$ mice manifested clinical signs of disease. This was related to fewer IL-12 producing cells in sciatic nerve sections and fewer IFN-γ secreting spleen cells in $CD28^{-/-}$ mice. Meanwhile, milder infiltration of such inflammatory cells into sciatic nerve tissues and less demyelination as well as lower production of specific anti-P0 peptide 180-199 antibodies were found in $CD28^{-/-}$ mice.

To define the effects of IFN-γ on EAN, clinical, pathological, immunological changes were evaluated in IFN-γ receptor-deficient mutant ($IFN\text{-}\gamma R^{-/-}$) and wild-type mice immunized with P0 peptide 180-199. $IFN\text{-}\gamma R^{-/-}$ mice exhibited later onset, less severity of clinical disease accompanying with less infiltration of inflammatory cells and demyelination compared with wild type mice. Fewer IL-12 producing but more IL-4 producing cells were found in sciatic nerve sections from $IFN\text{-}\gamma R^{-/-}$ mice. However, IFN-γR deficiency did not affect the production of specific anti-P0 peptide 180-199 antibody.

Bowman-Birk inhibitor Concentrate (BBIC) is an extract of soybeans enriched in Bowman-Birk inhibitor (BBI) capable of inhibiting the catalytic activities of several serine proteases. The immunoregulatory effects of BBIC have been evaluated in EAN. Administration of BBIC suppressed the development of clinical symptoms, and decreased inflammatory infiltrates in sciatic nerves, inhibited P2-specific T cell proliferation and IFN-γ synthesis by lymphocytes in lymph nodes from EAN rats. In vitro BBIC significantly suppressed B7.2 expression on macrophages from EAN rats, whereas B7.1 expression was slightly increased at peak of disease.

We conclude that 1) Both CD4+ T cells and CD8+ T cells are involved in the pathogenesis of EAN as helper or effector cells through their interaction, although autoreactive CD4+ T cells remain the main effectors of this disease. B cells may less contribute to perpetuating the related inflammatory demyelination. 2) CD28/B7 costimulation plays a pivotal role in either the initiation and/or effector phases of Th1-mediated EAN through both the activation of P0 peptide-specific T cells and of Th-induced differentiation of B cells into Ig-secreting cells. 3) IFN-γ contributes to the pathogenesis of EAN by promoting a Th1 cell-medicated immune response and suppressing a Th2 response. 4) BBIC has a strong ability to inhibit T cell response in EAN

List of original papers

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

I. **Yu Zhu**, Lei Bao, Shunwei Zhu, Zhiguo Chen, Peter van der Meide, Inger Nennesmo, Bengt Winblad, Hans-Gustaf Ljunggren, Jie Zhu. CD4 and CD8 T cells, but not B cells, are critical to the control of murine experimental autoimmune neuritis. Exp. Neurology 2002, 177, 2314-320.

II. **Yu Zhu**, Hans-Gustaf Ljunggren, Eilhard Mix, Hu-Lun Li, Peter van der Meide, Adlan M Elhassan, Bengt Winblad, Jie Zhu. CD28/B7 costimulation: a critical role for initiation and development of experimental autoimmune neuritis in C57BL/6 mice. J. Neuroimmunol. 2001, 114: 114-121.

III. **Yu Zhu,** Hans-Gustaf Ljunggren, Eilhard Mix, Hu-Lun Li, Peter van der Meide, Adlan M Elhassan, Bengt Winblad, Jie Zhu. Suppression of autoimmune neuritis in IFN-γ receptor-deficient mice. Exp. Neurology 2001, 169, 472- 478.

IV. **Yu Zhu**, Guang-Xian Zhang, Jeffery Ware, Elvira Ventura, Jie Zhu, Abdolmohamad Rostami. Bowman-Birk inhibitor Concentrate suppresses ongoing experimental autoimmune neuritis in Lewis rats by inhibiting Th1 cell mediated immunity in the peripheral nerve tissue. Manuscript.

Abbreviations

aa	amino acid
Ab	antibody
ADCC	antibody-dependent cellular cytotoxocity
Ag	antigen
APC	antigen presenting cells
C57BL/6	B6 (a strain of inbred mice)
BBIC	Bowman-Birk Inhibitor Concentrate
BNB	blood-nerve barrier
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosort
C. jejuni	Campylobacter *jejuni*
CFA	complete Freund's adjuvant
Con A	concanavalin A
CNS	center nervous system
CSF	cerebrospinal fluid
CTLA-4	cytotoxic T lymphocyte associated antigen-4
GBS	Guillain-Barré syndrome
IFN	interferon
Ig	immunoglobulin
IgSC	Ig-secreting cells
IL	interleukin
mAb	monoclonal antibody
MBP	myelin basic protein
MHC	major histocomaptibility complex
MNC	mononuclear cells
MOG	myelin oligodendrocyte glycoprotein
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
PBMC	blood mononuclear cell
PBS	phosphate buffered saline
PHA	phytohemagglutinin
p.i.	post immunization
PNS	peripheral nervous system
TGF	transforming growth factor
SD	standard deviation
Th	T helper
TNF	tumour necrosis factor

Introduction

Clinical aspects of Guillain-Barré syndrome (GBS)

Guillain-Barré syndrome (GBS) is an acute inflammatory autoimmune disease affecting myelin and axons of the peripheral nervous system (PNS), which is the most common acute paralytic disease that still produces major mortality and morbidity (Hahn, 1998). It was originally described by Landry in 1859 as a subacute symmetrical ascending paralysis (Hauser and Kernohan, 1949). In 1916, Guillain, Barré and Strohl described albuminocytological dissociation. i.e., increased albumin with very few or no cells, in the cerebrospinal fluid (CSF) of patients with ascending paralysis and helped to distinguish the disorder from other paralytic disorders especially poliomyelitis (Guillain and Barré, 1916).

The disease is characterized by acute progressive and symmetrical motor weakness of the extremities and of bulbar and facial musculature. Sensory symptoms are a reduction or absence of mild and deep tendon reflexes (Rostami, 1995; Bansal et al., 2001). The clinical course is usually favorable, with most cases showing a spontaneous and complete recovery. However, some patients are left with a residual deficit of various severities. The median time of recovery of independent walking is about 85 days and for respiration is 169 days (Rostami, 1995).

GBS has features of demyelination in electrophysiological studies, with the pathological findings of mononuclear cell (MNC) infiltration and segmental demyelination. The pathological changes, including early lymphocytic infiltrates in spinal roots and peripheral nerves, and the subsequent macrophage-mediated segmental stripping of myelin are known to cause profound defects in the propagation of electrical nerve impulses, resulting eventually in conduction block and in the functional modification correlated to flaccid paralysis (Brown et al., 1984).

Etiology and pathogenesis

The average annual incidence of GBS is 1.5 per 100 000 (Raphael and Sharshar, 2000). Most surveys show a slight peak in late adolescence and young adulthood, coinciding with an increased risk of infections with cytomegalovirus and *Campylobacter jejuni* (*C jejuni*), and a second peak in the elderly (Blaser, 1997). Mortality was about 5% in a recent clinical trial (Raphael and Sharshar, 2000). GBS has become the leading cause of acute flaccid paralysis in western (Olive et al., 1997). Children and adults of both sexes are affected with males

slightly more often affected (1.25:1) than females (Schonberger et al., 1981; Hurwitz et al., 1983), though it is rare in infancy (Hahn, 1998). In about two thirds of GBS patients, a history of a proceeding acute infection can be found (Hartung et al., 1995). Most commonly a respiratory-tract infection or gastroenteritis has resolved by the time neuropathic symptoms begin. The interval between the prodromal infection and the onset of GBS symptoms varies between 1 week and 3 weeks, occasionally longer; it averaged 11 days in several large series (Rostamin, 1995). The infecting agents reported are various, including common viruses as cytomegalovirus and Epstei-Barr virus, but lately much interest has centered around the enteropathogenic bacteria *C jejuni* as the most frequent antecedent pathogen for GBS. Antecedent *C. jejuni* infection was reported in 38% of a group of the GBS patients in Australia (Kaldor and Speed, 1984). In China, *C jejuni* may be main cause of GBS (Li et al., 1999; Watanabe et al., 2001). Vaccination has also been associated with GBS, as well as other more rare antecedent events (Arnason and Soliven, 1993), for example, preceding surgery, Hodgkin's disease, lymphoma, and lupus erythematosus. Indeed, partial homology of amino acid sequences of myelin protein P0 has been discovered with cytomegalovirus and varicella zoster virus. Several groups have provided convergent evidence to indicate that certain *C. Jejuni* serotypes bear in their lipopolysaccharide fraction epitopes shared with peripheral nerve glucoconjugates. *C.jejuni* can synthesize sialic acid and have sialylated carbohydrate residues identical to human gangliosides GM1, GD1a, GD3, GT1a, or GQ1b (Hartung et al., 2001). A cross-reactive epitope has also been identified in *C. jejuni* and certain gangliosides found in peripheral nerve myelin. Molecules mimicry has been suggested as a pathogenic mechanism that links the preceding microbial infection with the subsequent immune-mediated nerve damage in GBS.

The immunopathogenesis of GBS is still a matter of debate, but it is generally believed to be an autoimmune disease (Hartung et al., 1995; Shoenfeld e al., 1996). Cellular and humoral immune responses have been suggested to be involved in the pathogenesis of GBS. Activated autoreactive T cells and antibodies cross the blood nerve barrier (BNB), and initiate an inflammatory reaction, which results in demyelination and axonal damage. Proinflammatory cytokines such as IFN-γ, IL-12, TNF-α and IL-1 appear pivotal in the inflammatory reaction of GBS (Mainone et al., 1993; Dahle et al., 1997; Elkarim et al., 1998). However, T helper 2 (Th2) cytokine IL-4 response is of benefit in GBS (Dahle et al., 1997).

Experimental autoimmune neuritis (EAN)

The pathogenesis of the GBS remains poorly understood, but there is evidence that abnormal immune responses may be implicated. The earliest evidence to suggest an autoimmune basis for demyelination in GBS came from the work of Waksman and Adams in 1955. They described EAN, a paralytic illness in rodents induced by immunization with peripheral nerve tissue in complete Freund's adjuvant (CFA), which shows very striking clinical, neurophysiological and histological similarities to GBS (Waksman and Adams, 1955). EAN allows detailed study of the various effector pathways and tests novel therapeutic strategies in vivo. Therefore, EAN serves as a useful model for exploring the pathogenesis and immunotherapy of GBS.

EAN can be also induced by injection of peripheral nerve myelin components P2 or P0 proteins (Kadlubowski and Hughes, 1979; Milner et al., 1987) and their neuritogenic peptides together with CFA in susceptible species e.g. rat, sheep, chicken, mouse and monkey (Arnason 1971; Rostami et al., 1995; Zou et al., 2000a; Zhu et al., 2001a). EAN has been adoptively transferred from one animal to another by intravenous injection of lymphocytes obtained from animals with EAN (Astrom and Waksman, 1962; Zhu et al, 1999a), and in naïve Lewis rats by syngeneic P2-specfic CD4+ T cells (Linington et al., 1984; 1992a).

Peripheral nerve autoantigens

Assuming that EAN is a disease primarily affecting myelin, most works have concentrated on the myelin-specific proteins as putative autoantigens. The PNS antigens normally originate from peripheral nerve myelin sheaths. The myelin sheath is composed of three major proteins (P0, P1, and P2) with molecular weights of 14–30 kDa, which make up more than 70% of the total protein content. In human PNS myelin, P0 accounts for more than half of the total membrane protein, it does not present in center nervous system (CNS). P0 is a 29-kDa, 219-amino acid transmembrane glycoprotein with a larger extracellular domain, a highly hydrophobic membrane spanning portion, and a basic cytoplasmic domain. P0 is thought to be involved in starting and stabilizing the compaction of the extracellular apposition of the myelin membrane in the PNS. P1 is identical to myelin basic protein (MBP) in the CNS, and the 14-kDa P2 protein containing 131 amino acids is common to both CNS and PNS but predominates in the latter (Hartung et al., 1995). It has been important to find out the PNS proteins and protein fragments that can be neuritogenic enable studies of their relevance in human disease. Circulating antibodies to P0 and P2 have been detected in Lewis rat with EAN and in GBS patients (Archelos et al., 1993; Khalili-Shirazi et al., 1993). P2

represents an important neuritogen in EAN (Kadlubowski et al., 1979). P0 protein as a second neuritogen is involved in the pathogenesis of EAN (Milner et al., 1987; Linington et al., 1992). Also gangliosides have been considered as peripheral nerve antigens for the expression of EAN, although this has been controversial (Takeda et al., 1980; Ponzin et al., 1991). Other PNS antigens have not yet been demonstrated to provoke EAN. It is clear that not a single, but several PNS proteins may produce EAN.

The immunopathogenesis of EAN

EAN that occurs following immunization with the different myelin components (termed active immunization) has been proposed to be due to an aberrant immune responses. Auto-immune CD4+ T cells seem to play a pivotal role in initiation of the disease. However, the function of B cells in EAN is still unclear. The mechanisms proposed in the immunopathogenesis of EAN are presented in Fig. 1

Fig. 1.

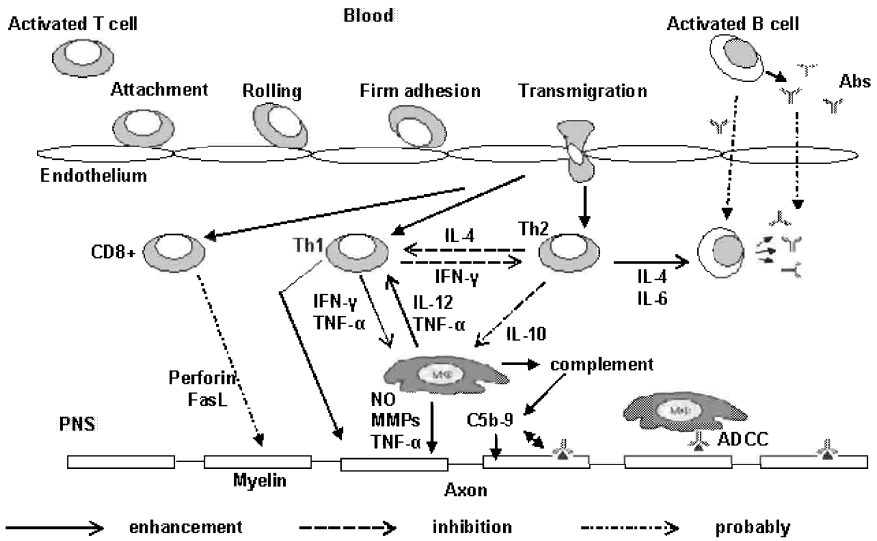

Fig. 1. Schematic illustration of immune responses in blood and within the blood nerve barrier in immune-mediated demyelination of the peripheral nervous system (PNS). MΦ = macrophage; NO = nitric oxide; MMP = metalloproteinase; ADCC = antibody-dependent cellular toxicity

T Lymphocytes in EAN and GBS

T cells express one of the co-receptors CD4 or CD8. CD4+ T cells regulate immune responses by giving 'help' in form of secretion of soluble mediators, like cytokines and chemokines to phagocytes, NK cells, T cells and B cells (Mosmann and Coffman, 1989). After activation, CD4+ helper T cells differentiate, according to their distinct functional responses and the capacity to release different profiles of cytokines, into Th1 or Th2 effector cell type. T cells expressing CD8 act as cytotoxic cells after recognition of antigenic peptides exposed on MHC class I on the surface of the target cells. The evidence implicating cellular immune responses in the pathogenesis of EAN and GBS has been reviewed (Hodgkinson et al., 1994). T lymphocytes are probably involved in the pathogenesis of EAN and GBS. Activated myelin-specific T cells cross the BNB and interact with resident endoneural macrophages and Schwann cells. It has been reported that peripheral blood mononuclear cell (PBMC) from patients with GBS responses to myelin proteins/peptides to a significantly larger extent than PBMC from healthy controls (Khalili-Shrazu et al., 1992). The activation of T cells in GBS is evident from the presence of increased numbers of circulating activated T cells bearing activated markers and of increased concentrations of soluble IL-2 receptor and transferrin receptor, and decreased levels of naïve T cells in the circulation (Koski, 1998). The predominant cells in the endoneurium of GBS are T cells bearing $\alpha\beta$ receptors with CD4 or CD8 cells in proportion to their counterparts in the blood and small numbers of T cells bearing $\gamma\delta$ receptor (Hughes et al., 1992; Schmidt et al., 1996). The findings from a sural nerve biopsy indicated that the particular phenotype of $\gamma\delta$ T cell is common in the human gut and may be stimulated by *C jejuni* to invade the PNS (Cooper et al., 2000). One character of the pathological lesions in EAN is the perivascular infiltration of T cells and macrophages in the PNS. In addition, EAN has been adoptively transferred to naïve Lewis rats by syngeneic P2-spicific CD4+ T cell line (Linington et al., 1984; 1992b) and to naïve BALB/c mice by MBP-specific CD4+ T helper cell clones (Abromson-Leeman et al., 1995), even in Sprague-Dawley and Brown Norway rats that are resistant to actively induced EAN (Hoffmann et al., 1980). The findings implicate that EAN is a CD4+ T cell-mediated autoimmune disorder of the PNS. Several studies have revealed that macrophages are the most numerous cell populations in infiltrating cells of the PNS throughout the course of EAN. Th1-like cell responses to peripheral nerve myelin components were found over the course of EAN in Lewis rats (Zhu et al., 1994a). Apoptosis of autoreactive myelin specific T cells may be an important mechanism to limit the immunoinflammatory response and to abort

disease (Okuda et al., 2002; Van Den Brande et al., 2002). Furthermore, therapy using P2 antigen in EAN animals was associated with an augmentation of T cell apoptosis (Weishaupt et al., 2001). IFN-β suppressed EAN in Lewis rats also by inhibiting T cell responses towards peripheral myelin (Zou et al., 1999a). Clinical symptoms of EAN were strongly suppressed by the anti-inflammatory compounds, such as Linomede, Rolipram and Leflunomide, administration with down-regulated myelin antigen-induced T cell responses, inhibited inflammatory cell infiltration and down-regulated IFN-γ and TNF-α production (Zhu et al., 1999b; Zou et al., 2000b; Korn et al., 2001). These evidences demonstrated that T cell mediated immune response contributes to the pathogenesis of EAN.

Costimulatory molecules

Mounting an appropriate immune response depends on the careful regulation of lymphocyte activation. Two signals are required for antigen (Ag)-specific activation of T lymphocytes. One is provided by the recognition of the antigenic peptide presented in the context of self-MHC molecules by the TCR/CD3 complex; the second signal is provided by the interaction of an accessory receptor on the T cell with its ligand on the antigen presenting cells (APCs) (Fig. 2.) and referred to as costimulation that is independent on the antigen receptor and is critical to allow full activation, sustain cell proliferation, prevent anergy and/or apoptosis, induce differentiation to effector and memory status, and allow cell-cell cooperation (Kenneth et al., 2002). Increasing evidence suggests that interactions of T cell surface receptors CD28 and CD40L (CD154L), with their respective ligands B7-1 (CD80)/B7-2 (CD86) and CD40 on APCs are critical for T and B cell activation, respectively (Grewal and Flavell, 1998; Rademaekers et al., 2001). CD80/CD86 overexpression in peripheral organs can mediate autoimmune diseases (Harlan et al., 1994). CD28 signaling is capable of providing costimulation to T cells for T cell survival and long-term proliferation and IL-2 production after antigenic challenge in vivo (Boise et al., 1995). Current evidence suggests that CD28 is coupled to an intracellular pathway which is distinct form that initiated via the TCR/CD3 complex (Shahinian et al., 1993). Failure to activate the costimulatory signal results in T cell unresponsiveness or clonal anergy (Harding et al., 1992), and, therefore, peripheral tolerance and control of autoimmunity are thought to rely on the absence of costimulatory signaling (Oliveira-dos-Santos et al., 1999). Following activation, T cells up-regulate surface expression of cytotoxic T lymphocyte associated antigen-4 (CTLA-4), a homologue of CD28, which binds the same ligands with higher affinity (Linsley et al., 1991) and serves as a negative regulator of T cell activation (Krummel and Allison 1995). Optimal

activation of B lymphocytes and their subsequent differentiation into Ig-secreting cells (IgSC) is critically dependent on the helper effects of CD4+ Th cells. This process is mediated via both direct (cognate) Th-B cell intercellular contact-mediated interactions and the elaboration of Ag-nonspecific cytokines (Kupfer et al., 1986; Brain and Natl, 1998). Ligation of CD28 molecule on the surface of CD4+Th cells and B7 on B cells during the cognate Th-B interaction leads to B cell differentiation into IgSC (Damle et al., 1991).

Fig. 2.

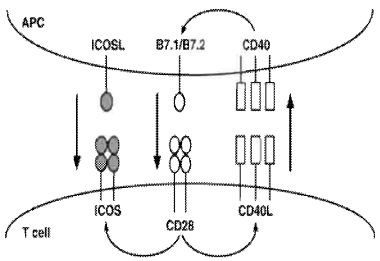

Figure 2. Costimulation involves reciprocal and sequential signals between cells. A T cell–APC interaction begins when the T cell antigen receptor is stimulated by a specific peptide/MHC complex on the surface of the APC (not shown). Low constitutive levels of B7.1 and/or B7.2 on the APC activate CD28 on the T cell, inducing upregulation of CD40L. CD40L in turn binds to CD40 on the APC, enhancing B7.1/B7.2 expression and reinforcing the CD28/CD40 positive feedback loop. CD28 costimulation also induces T cell expression of ICOS, allowing a second level of costimulation by APC-expressed ICOSL (from Frauwirth and Thompson, 2002)

Costimulatory molecules in EAN and GBS

Costimulatory molecules play a crucial role in autoimmune disease. Altered expression of costimulatory molecules is found in a body of human autoimmune diseases (Liossis et al. 1998). Conspicuous expression of B7.1 immunoreactivity was observed on putative macrophages in several biopsies from patients with GBS, particularly within the endoneurium (Kiefer et al., 2000). CD28 expression on activated T cells was downregulated in myasthenia gravis patients. This has been attributed to the sustained upregulation of CD80 and CD86 expression on activated T cells and monocytes (Teleshova et al., 2000). In animals, prior reports have shown that the relapsing-remitting experimental autoimmune encephalomyelitis (EAE) and the cascade of epitope spreading can be inhibited by blockade of B7.1 through inducing tolerance (Lehmann et al., 1992; Miller et al., 1995; McRae et al., 1995). Administration of CTLA-4Ig, a soluble CD28 antagonist that binds to both B7-1 and B7-2, prevented rat cardiac allograft rejection and pancreatic islet cell xenograft rejection in mice (Lenschow et al., 1992; Li et al., 2001a). CTLA-4 blockade augmented cellular infiltration and demyelination in the sciatic nerves and increased T cell proliferation in lymph node cells in EAN (Zhu et al., 2001b). Treatment with anti-CTLA-4 provoked rapid onset and increased severity of experimental autoimmune myasthenia gravis (EAMG) in B6 mice (Wang et al.,

2000). As $CD28^{-/-}$ T cells are unable to trigger an autoimmune response and cannot activate the cytokine circuits responsible for the attraction of MNCs and the establishment of an inflammatory response (Oliveira-dos-Santos et al., 1999). Mice lacking CD28 showed no or minimal clinical signs of EAE and markedly reduced inflammatory infiltrates in the brain and spinal cord (Chang et al., 1999). As the B7/CD28: CTLA-4 costimulatory system plays a vital role in determining the fate of immune responses, it is a highly promising therapeutic target for regulation of immune responses.

Cytokines in EAN and GBS

Cytokines are polypeptides that act as signal molecules in a paracrine or autocrine fashion between the cells of the immune system. Cytokines can be produced by various cell types including CD4+ and CD8+ T cells, innate immune system cells (e.g. NK and mast cells) and APCs (Abbas et al., 1996). T cells respond to antigenic stimulation with a transient burst of cytokine production. Cytokines play a key role in initiating, propagating, and regulating tissue-specific autoimmune injury. The analysis of cytokine production is increasingly important in defining the mechanisms of autoimmune responses and in evaluating specific therapies of autoimmune diseases. As in other T cell-mediated autoimmune diseases, the CD4+ T cells involved in the pathogenesis of GBS and EAN are of the Th1 type. Proinflammatory cytokines secreted from Th1 type cells significantly contribute to the tissue damage in GBS and EAN, by changing the Th1/Th2 cell balance in favor of Th1. Proinflammatory cytokines such as IFN-γ, TNF-α, IL-2, IL-6 and IL-1 appear pivotal in the inflammatory reaction of GBS (Maimone et al., 1993; Dahle et al., 1997; Elkarim et al., 1998; Zhu et al., 1998a). IL-18 is also an inflammatory cytokine and was found significantly higher in GBS patient serum than in controls (Jander and Stoll, 2001). There was positive correlation between CSF IL-6 and clinical severity of GBS (Zhang et al., 2000). Another study showed higher IL-4 production during the plateau- or recovery-phase as compared to controls, suggesting the IL-4 responses are beneficial in GBS, and a possible role in terminating the disease process in this self-limiting inflammatory disease (Dahle et al., 1997). The levels of IL-10 secreted from blood MNC spontaneously were higher in the acute phase of GBS than in control patients (Press et al., 2001a). In EAN, IFN-γ and IL-1β mRNA was peaked at the onset and acute phase of clinical disease. High-dose recombinant mouse (rm) IL-12 increased significantly the lymph node MNC proliferation in response to P0 peptide and IFN-γ production in the sciatic nerves of rats with chronic EAN (Pelidou et al., 2000a). IL-12p40 deficient B6 mice showed reduced incidence and severity of EAN (Bao et al.,

2002). Inhibition of IL-18 effectively ameliorates the clinical manifestations and pathological changes in mice with EAN (Yu et al., 2002). Neutralization of endogenously produced TNF-α by a monoclonal antibody (mAb) partially suppressed EAN (Stoll et al., 1993). RmIL-17 treated rats showed increased infiltration of inflammatory cells into the sciatic nerve, more severe demyelination, augmented proliferation of regional lymph node cells, and increased serum levels of TNF-α (Pellidou et al., 2000b). However, IL-4 peaked late in the course of EAN (Zhu et al., 1994a). This pattern was similar to that of IL-10 mRNA positive cells in lymph node cells (Zhu et al., 1997). Transfer of myelin-specific cells deviated in vitro towards IL-4 production ameliorates ongoing EAN (Ekerfelt et al., 2001). Therefore, IL-4 seems to play an important role in modulating the severity of the disease process (Falcone et al., 1998).

IFN-γ in EAN and GBS

IFN-γ is a cytokine that plays an important role in host defense mechanisms by exerting pleiotropic activities on a wide range of cell types. Cellular responses to IFN-γ are mediated by its heterodimeric cell surface receptor (IFN-γR α and β), which activates downstream signal transduction cascades, ultimately leading to the regulation of gene expression. Within the cytokine network, IFN-γ promotes the differentiation of precursors of T cells into Th1 cells. Other numerous effects of IFN-γ in promoting inflammation have been described, such as, activation of macrophage; up-regulation of both MHC class I and class II molecules necessary for (self) Ag presentation; induction of adhesion molecules, such as, ICAM-1 and VCAM, perhaps promoting homing of inflammatory cells; and induction of expression of receptors for other cytokines, in particular TNF receptors (Olsson, 1995). The main cells producing IFN-γ are activated Th1 cells, activated CD8+ cytotoxic cells of the cytotoxic T cell 1(Tc1) phenotype, and activated NK cells. It is one of the principal activating cytokines for macrophage, and mice with disrupted IFN-γ or IFN-γ receptor (IFN-γR) genes show increased susceptibility to mycobacteria and certain viruses, e.g. vaccinia virus. IFN-γ may influence Th1 development by of both indirect and direct pathways. IFN-γ enhances macrophage production of IL-12 in response to pathogens (Murphy et al., 1995), indirectly promoting Th1 development. Elevated levels of neopterin, a biosynthetic product generated in response to IFN-γ, and IFN-γ derived from Th1 cells were found in serum of patients with GBS (Hohnoki et al., 1998). However, Press et al reported that levels of IFN-γ secreting blood MNC were not increased over the course of GBS (Press et al., 2001a). In EAN, there is

convincing evidence that the level of IFN-γ producing cells in blood, lymph nodes and the PNS tissue roughly parallels clinical EAN, consistent with an inflammatory role of Th1 related cytokines in the pathogenesis of EAN (Zhu et al., 1998a; Fujioka et al., 1998). IFN-γ positive cells were focused in nerve roots in EAN rats from days 11 to 13 post-immunization (p.i.) and correlated with levels of Ia-positive macrophages over the EAN course (Schmidt et al., 1992). Treatment with antibody to IFN-γ after clinical onset of EAN ameliorated the disease (Hartung and Toyka, 1990). Several investigators have reported that an injection of IFN-γ increased the incidence and accelerated the onset of collagen inducted arthritis (CIA) in mice (Boissier et al., 1995). Evidence suggests that IFN-γ plays a deleterious role in immune-mediated demyelination disorders, such as multiple sclerosis (MS) and its animal model EAE (Popko et al., 1997). However, the role of IFN-γ in autoimmune diseases is complex. Treatment with anti-IFN-γ mAb worsened the disease of EAE (Espejo et al., 2001). Mice deficient in IFN-γ or IFN-γ receptor developed progressive and fatal EAE (Willenborg et al., 1996; 1999). Thus, IFN-γ is also one of the factors inhibiting the disease in a mouse strain resistant to EAE, indicating that IFN-γ also exerts immunosuppressive activity (Ferber et al., 1996).

B cells in EAN and GBS

The role of B cells and antibodies for development of EAN has been more controversial than the role of autoreactive T cells. Many patients with GBS have serum antibodies to peripheral nerve myelin constituents (Hughes et al., 1999). Plasma exchange removes antibodies and is an effective therapy, but it remains unclear whether antibodies are primarily responsible for causing damage to nerves, an additive factor or an unrelated epiphenomenon. Most of the research into the pathogenesis of this disorder continues to focus on aberrant B cell immune responses. B cells may be involved in the pathogenesis of EAN and peripheral neuropathies in at least two different ways (Hays et al., 1987; Hughes et al., 1985). Specific antibodies to surface myelin components may initiate either complement or antibody-dependent cellular cytotoxocity (ADCC) mediating demyelination (Williams et al., 1980), or they could affect the course of disease as a consequence of their ability to act as APC (Lanzavecchia, 1987). On the one hand, although EAN is predominantly a T cell mediated disease, autoantibodies to a variety of myelin-antigens have abundantly been found in EAN and GBS (Shoendeld et al., 1996; Zhu et al., 1994b). Serum from EAN animals caused demyelination on mouse dorsal root nerve cell cultures (Yonezawa and Ishihara, 1968; Raine

and Bornstein, 1979) and conduction block (Sumner et al., 1982; Harrison et, al., 1984) and focal demyelination followed injection of most GBS sera into rat sciatic nerve (Saida et al., 1978; Feasby et al., 1982). Systemic injection of antibodies to galactocerebroside, in addition to reactive T cells into the recipient Lewis rats, produces more severe demyelination than the transfer of reactive T cell alone (Hahn et al., 1993), suggesting specific autoreactive T cells and antibodies may act synergistically in the demyelinating process. However, on the other hand, only passive transfer of serum from one EAN animal into another healthy animal does not provoke disease (Toyka and Heininger, 1987). Although a few reports have described antibodies to a bewildering range of glycolipids and to myelin proteins P0 and P2 in some GBS patients (Hughes et al., 1999; Hartung et al., 2001), most of them are also found in other neuropathies and some healthy controls (Vrethem, et al., 1991; Khalili-Shirazi et al., 1993). *C. Jejuni* isolated from patients with GBS and from non-GBS patients with enteritis alone, show similar ganglioside-like epitopes, but anti-ganglioside antibodies are predominantly seen in GBS patients after infections of *Campylobacter* (Sheikh et al., 1998). However, both of GBS serum and monoclonal antibody to *C. jejuni* as well as gangliosides did not exacerbate disease which dose not support an antibody-mediated mechanism in GBS (Hadden et al., 2001). Moreover, intravenous injection of antibody to galactocerebroside alone can not induce EAN in Lewis rats (Hahn et al., 1993). Meanwhile, B cells were almost negligible in the cauda equina of Lewis rats at the active stage of EAN (Fujioka et al., 2000). Therefore, the role of B cells in EAN is still unclear.

Treatments in GBS and EAN

Plasma exchange was found to be effective in decreasing morbidity and shortening course of GBS, also reduced the number of days on the respirator (Raphael et al., 1999). Plasma exchange is reasonably safe, but not totally free of risk, particularly in haemodynamically unstable GBS patients. Such risks, the high cost, and the limited availability of plasma exchange facilities prompted the search for alternative treatments (Govoni and Granieri, 2001). Intravenous IgG (IVIg) treatment is a promising therapy in various disorders with a presumed autoimmune basis, and as the advantage of low risk and ease of application (Thornton et al., 1994). This therapy was therefore introduced as an alternative to plasma exchange. However, the infusion of IVIg should not become routine therapy, unless contraindications, such as renal failure, are carefully checked, and economic constraints should be mentioned. Plasma exchange and IVIg had equivalent efficacy and the combination of the two treatments did not confer a significant advantage (Plasma exchange/sandoflobulin

GBS trial group, 1997). With currently available therapies, plasma exchange or IVIG, only some 60% of patients received benefit (Hartung et al., 2001). Contrary to expectation, corticosteroid therapy is not of benefit in GBS (Rapheal et al., 1999). Further research examining potentially more effective anti-inflammatory or immunomodulatory treatments is urgently needed.

Newer approaches to therapy of autoimmune PNS disease have been tried in EAN. The studies are designed mainly to abrogate the immune attack against self antigens by inductions of immunological tolerance or suppression of abnormally heightened immune response. The predictions for therapeutical strategies derived from these studies are now being successfully tested in EAN (Table 1). Some of these approaches to therapy may become applicable to human GBS in the future.

Table 1. Treatment strategies that aim to inhibit cellular immunity in EAN

Inhibition of T cell activation or functions:	
Anti-T cell receptor antibodies	(Jung et al., 1992)
Induction of antigen-specific T cell tolerance and anergy	
Oral administration of antigens	(Jung et al., 2001)
Nasal administration of antigens	(Zhu et al., 1998b; Zou et al., 1998; 1999c)
Intravenous administration of antigen	(Stienekemeier et al., 2001)
Immunomodulators: Linomide and ABR-215062	(Zhu et al., 1999b; Zou et al., 2002)
Immunosuppressive drug: Leflunomide	(Korn et al., 2001)
Antibodies to ICAM	(Archelos et al., 1993)
Suppression of proinflammatory cytokines	
Antibodies to IFN-γ	(Strigard et al., 1989)
Inhibitors of TNF-α: pentoxifylline	(Constantinescu et al., 1996)
Rolipram	(Zou et al., 2000b)
Inhibitors of IFN-γ	(Tsai et al., 1991)
Antibody to IL-18	(Yu et al., 2002)
Immunosuppressive cytokines	
IL-10	(Bai et al., 1997a)
IL-4	(Deretzi et al., 1999)
TGF-β	(Jung et al., 1994)
IFN-β	(Zou et al., 1999a)
Down-regulation of macrophage functions	
NO synthesis inhibitors	(Zielasek et al., 1995)
Deletion of macrophages	(Jung et al., 1993)
Immunomodulatory compound: Linomide	(Bai et al., 1997b)
Anti-MIP-1α antibodies	(Zou et al., 1999b)
Pharmacological compound: Silica	(Craggs et al., 1984)
Others	
Antidepressants (monoamine reuptake inhibiting antidepressants)	(Bengtsson et al., 1992; Zhu et al., 1994c)

Aims of the studies

1. To define the effects of distinct T cell subtype (CD4+ and CD8+) and B cell in EAN.
2. To investigate the immunoregulatory roles of the costimulatory molecule CD28 in T cell and B cell activation in induction of EAN.
3. To address the functions of cytokine IFN-γ in EAN.
4. To evaluate the role of immunomodulation of BBIC in EAN.

Materials and methods

Animals

Male, 6-8 weeks old, gene knockout mutants: $CD4^{-/-}$, $CD8^{-/-}$, $CD4^{-}8^{-}$ and B cell mutant (uMT) mice (Paper I), $CD28^{-/-}$ mice (paper II) and IFN-$\gamma R^{-/-}$ mice (Paper III) and their corresponding wild type mice as well as Female, 6-8 weeks old, Lewis rats (Paper IV) were housed in the animal facilities of Microbiology and Tumor Biology Center, Karolinska Institute, Sweden and at animal facility of the Medical School, University of Pennsylvania, USA, respectively.

$CD4^{-/-}$ B6 mice by disrupting the CD4 gene in embryonic stem cells by homologous recombination do not express CD4 on the T cell surface and unaltered development of CD8+ T cells and myeloid components (Rahemtulla et al., 1991). A mutant mouse strain without CD8 expression on the cell surface by disruption of encoding sequence of the murine Lyt-2 gene by homologous recombination shows CD8+ T cells are not present in peripheral lymphoid organs, but the CD4+ T cell population seems to be unaltered (Fung-Leung et al., 1991). $CD4^{-}8^{-}$ B6 mice lack surface expression of CD4 and CD8 on T cells (Schilham et al., 1993). μ MT B6 mice lack functional B cells, since B cell differentiation is blocked at the pre-B cell stage due to the disruption of one of the membrane exons of the gene encoding the μ -chain constant region (Kitamura et al., 1991). $CD28^{-/-}$ B6 mice lack surface expression of CD28 on T cells (Shahinian et al., 1993). IFN receptor knockout (IFN-$\gamma R^{-/-}$) mice showed the disruption in the cytoplasmic domain of the IFN-γ receptor and develop a normal immune system (Huang et al., 1993).

Antigen preparation

The P0 protein peptides, corresponding to the amino acid (aa) 180-199 of rat PNS myelin P0 protein, and SP26, corresponding to the aa 53-78 of rat PNS myelin P2 protein were synthesized by solid-phase stepwise elongation using a Tedansyro peptide synthesizer (Multisyntech, Bochum, Germany).

Compound

Bowman-Birk Inhibitor Concentrate (BBIC) is a soybean extract enriched in Bowman-Birk protease inhibitor (BBI). BBIC was provided by Central Soya, Inc., Ft. Wayne, IN.

Induction of EAN and assessment of clinical signs

Mice were immunized twice (designated as days 0 and 7 p.i.) by subcutaneous injection into the back with 60 μg of P0 peptide 180-199 and 0.5 mg Mycobacterium tuberculosis (strain H 37 RA; Difco, Detroit, USA) emulsified in 25 μl saline and 25 μl incomplete Freund's adjuvant. All mice received 400 ng, 200 ng and 200 ng pertussis toxin (Sigma, St. Louis, USA) by intravenous injection on days -1, 0, 3, p.i., respectively (Zou et al., 2000c). Lewis rats were immunized subcutaneously by injection of 400 μg SP26 emulsified in 250 μl CFA as reported previously (Rostami et al., 1990). Clinical scores were assessed blindly before immunization and thereafter every second or third day. The severity of paresis was graded as follows: 0, normal; 1, flaccid tail; 2, moderate paraparesis; 3, severe paraparesis; 4, tetraparesis; 5, death; 0.5; intermediate clinical signs.

BBIC Therapy

Five rats were treated with oral BBIC (200 mg in PBS) daily from the day of beginning on the onset of disease, i.e. on day 9 p.i. to day 18 p.i. 5 rats which received oral PBS daily served as a sham-treated control group. Other four immunized rats were used for in vitro study. All rats were sacrificed on peak of disease, i.e. on day 18 p.i.

Histopathological assessment

Mice and rats were sacrificed and used as the source of sciatic nerve segments excised close to the lumbar spinal cord. These segments were dissected, fixed in 4% formaldehyde before embedded in paraffin. Multiple longitudinal sections (5-6 μm slices) of sciatic nerves were stained with haematoxylin-eosin, and replicate sections of mice were stained with luxol fast blue violet for evaluation of the extent of MNC infiltration and of demyelination. Tissue areas were measured by image analysis, and the numbers of inflammatory cells were counted at x 20 magnification. The results were averaged and expressed as cells per mm^2 tissue section. To assess the severity and extent of demyelination, peripheral nerve sections were scored using a semiquantitative grading system: 0, normal; 1, less than 25% demyelinated fibers; 2, 25-50% demyelinated fibers; 3, 50-75% demyelinated fibers; 4, more than 75% demyelinated fibers.

Immunohistochemistry

Segments of sciatic nerves were dissected and snap-frozen in liquid nitrogen. Cryostat sections (10 μm) were exposed to the rat mAbs, ED1 (anti-mouse macrophage), anti-mouse

CD4 (T helper cells and monocytes), anti-mouse CD8 (HanLan Sera-Lab limited, England), anti-rat IL-4 (Innogenetics, Ghent, Belgium), as well as the anti-mouse IL-12 (Biosource, California, USA). Sections were stained according to the avidin-biotin technique (Vectastain Elite Kit; Vector Labs, Burlingame, CA, USA). Omission of primary antibodies served as negative control. The tissue areas were measured by image analysis, and the numbers of stained cells and infiltrates were counted at x 40 magnification in the entire section area. The results were averaged and expressed as cells per mm^2 or per 100 mm^2 tissue section.

Isolation of mononuclear cells (MNCs) from spleen and lymph nodes

The spleens from mice and the popliteal and inguinal lymph nodes from rats were removed under aseptic conditions. Single cell suspensions of MNC from individual mice were prepared. The cells were washed three times in culture medium before being suspended to 2 x 10^6 MNC/ml. The culture medium consisted of Iscove's modification of Dulbecco's medium (Flow Lab., Irvine, UK) supplemented with 1% (v/v) minimum essential medium (MEM) (Flow), 50 IU/ml penicillin, 60 μg/ml streptomycin (Gibco, Paisley, UK), 2 mM glutamine (Flow) and 3% normal human AB^+ serum without mercaptoethanol. Lymph nodes from rats were pooled into ice-cooled RPMI-1640 containing 2% fetal calf serum (FCS). Single cells were prepared by grinding through a wire mesh. Cells were washed three times, suspended in ice-cooled RPMI-1640 supplemented with 1%(v/v) minimum essential medium, 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% FCS, then re-diluted to a cell concentration of 2.5 x 10^6/ml for analysis of T cell responses.

Lymphocyte proliferation assay

200 μl aliquots of lymph node MNC suspensions containing 5 x 10^5 cells and spleen MNC suspensions at a cell density of 2 ×10^6 cells/ml were applied in triplicate in 96 well microtiter plates (Nunc, Coenhagen, Denmark). For lymphocyte stimulation, 10 μl of SP26 (final concentration 10 μg/ml) was added to the culture of lymph node MNC and either 10 μl aliquots of P0 180-199 peptide at a final concentration of 10 μg/ml or phytohemagglutinin (PHA) (Difco, Detroit, MI) at a final concentration of 20 μg/ml were added to the culture of spleen MNC in triplicate into appropriate wells. After 60 hrs incubation at 37 ^{0}C in a humid condition with 5 - 10% CO_2, the cells were pulsed with 10 μl aliquots containing 1 μCi of [^{3}H] methylthymidine for 12 hrs. Cells were harvested onto glass fiber filters (Titertek, Skatron, Lierbyen, Norway) and levels of thymidine incorporated radioactivity were

measured by liquid β-scintillation counter. The results were expressed as counts per minute (cpm) per culture.

Lymphocyte cell cultures

2 ml aliquots of MNC suspensions containing 5 x10^6 cells were cultured in 24 well microtiter plates (Nunc, Copenhagen, Denmark) in present or absent of SP26 (final concentration 10 μg/ml). After 48 hrs culture at 37 ^{0}C under humid condition with 10% CO_2, supernatants were collected and stored at –70 ^{0}C until detection of cytokine productions by ELISA.

ELISA for measurement of cytokine profiles in cell culture supernatants

IFN-γ and IL-4 production in lymphocyte cell culture supernatants were measured by ELISA. Briefly, monoclonal anti-rat IFN-γ and anti-rat IL-4 antibodies were coated onto 96 well ELISA plates at 2 μg/ml in a volume of 100 μl/well. After 3 times washing, duplicates of serially supernatant samples or of recombinant standard rat IFN-γ and IL-4 were added and the plates were incubated overnight at 4^{0}C. After washes, biotinylated anti-rat IFN-γ and IL-4 antibodies (100 μl of 2 μg/ml) were applied for 2 hr at room temperature (RT), followed by six washed. 100 μl of avidin-peroxidise conjugate (1:10000) were pulsed to wells for 30 minutes at RT. Color reaction was performed with 100 μl of TMB for 30 minutes and then added 50 μl of stop solution (KPL, USA). The plates were read at 450 nm with an ELISA reader (MCC/340 microplate reader, Fisher Scientific, USA) immediately.

Stimulation of MNCs from spleens and enumeration of IFN-γ secreting cells

An enzyme-linked immunospot (ELISPOT) assay was used to detect single cells that secrete IFN-γ upon antigen stimulation. Briefly, nitrocellulose-bottom plates (Microtiter-HAM, Millipore, Bedford, UK) were coated overnight at 4°C with 100 μl (15 μg/ml) of a mouse monoclonal anti-rat IFN-γ antibody (DB1) (Biomedical Primate Research Centre, Rijswijk, The Netherlands). After washing, 200 μl aliquots containing 4 x 10^5 MNC were added in duplicate with either medium alone (control cultures without antigen) or 10 μl aliquots of P0 peptide 180-199 (final concentration of 10 μg/ml) or PHA (diluted 1:1000). After 48 h cultures, the plates were washed with PBS and incubated with rabbit polyclonal anti-rat IFN-γ (Biomedical Primate Research Centre). After washing, the plates were incubated for 2 h with biotinylated swine anti-rabbit IgG (diluted 1:1000; Sigma, St. Louis, USA). After another washing, incubation with avidin-biotin peroxidase complex (diluted

1:400), unbound ABC was removed by immersion with PBS, and 100 μl/well of peroxidase-substrate solution was added. The developed red-brown spots were enumerated in a dissection microscope at low magnification (x 25). Results were expressed as numbers of spots per 10^5 splenic MNC.

Macrophage preparation

Rats with EAN (18 days p.i.) without any treatment were injected intraperitoneally with 20 ml ice cold PBS. After message for 1 min, the cell suspensions were collected from the peritoneal cavities, and rinsed and centrifuged for 10 min with RPMI-1640 containing 2% FCS. The cell number of the suspension was adjusted to 0.5 x 10^6/ml in culture medium ice-cooled RPMI-1640 supplemented with 1% (v/v) minimum essential medium, 2 mM glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin and 10% FCS. 2 ml aliquots of macrophage suspension containing 1 x 10^6 cells were cultured in 24 well microtiter plates. Ten microliter aliquots of BBIC (5 μM) and PBS were added, respectively. After 48 hrs culture at 37 ^{0}C under humid condition with 10% CO_2, cells were collected for Fluorescence active cell assay (FACS) assay.

Flow cytometry analysis of macrophage

Cultured macrophages were washed by PBS containing 2% FCS and 0.1% NaN_3. 1 x 10^6 macrophages were incubated with 50 μl normal sheep serum for half an hour for blocking nonspecific responses, then stained by 1 μg of FITC-labeled anti-rat CD11b and PE-labeled anti-rat B7.2 (CD86) or Biotinylated anti-rat B7.1 (CD80) for half an hour, washing the cells with PBS containing 2% FCS and 0.1%NaN_3. Biotinylated first Abs were detected by streptavidin-peridinin chlorophyll protein for half an hour, washing the cells. Labeled cells gated and fluorescence was analyzed directly using CellQuest (Beckson-Dickson, Mountain View, CA) software. Data represent 10,000 events.

ELISA for measurements of anti-P0 180-199 antibody in serum

Serum from mice was obtained from blood samples at peak clinical sign of EAN (day 24 p.i.). Purified P0 peptide 180-199 was coated onto ELISA plates at 10 μg/ml. After three washings, test samples were diluted with PBS to 1:100, applied to plate wells and incubated for 2 h at RT. After another three washings, plates were incubated for 2 h with rat anti-mouse IgG (Sigma) for 1 h and then with alkaline phosphatase-conjugated AB complex (Vector) for

30 min. The reaction was visualized with p-nitrophenyl phosphate substrate (Sigma) and read at 405 nm using an ELISA reader

Statistics

Differences between pairs of groups were tested by Student's t-test and Mann-Whitney's U-test (Paper I, II and IV) and differences between five groups were evaluated by the Kruskal-Wallis one-way analysis of variance (Paper III). All tests of significance were two-side. The level of significance was set to $p < 0.05$.

Ethics

The EAN model in Lewis rats, B6 mice and transgenic mice were approved by the South Stockholm Research Animal Ethics Committee, Huddinge County Court, Stockholm, Sweden.

Results

CD4 and CD8 T cells, but not B cells: critical to the control of murine EAN

In this study, EAN was induced by P0 peptide 180-199 in $CD4^{-/-}$, $CD8^{-/-}$, $CD4^{-}8^{-}$, B cell knockout μMT mice and wild type mice to further investigate the roles of CD4 and CD8+ T cells, and B cell in the pathogenesis of EAN. The clinical signs (Fig 3), T cell response specific to P0 180-199 and histopathological changes in $CD4^{-/-}$ mice were significantly lower than in wild type mice; $CD8^{-/-}$ mice also appeared slighter clinical course, less T cell response specific to P0180-199 and mild hitopathological changes compared with wild type mice, but no marked difference between them except for the severity of disease. Mice in $CD4^{-}8^{-}$, μMT and wild type groups had similar clinical courses, T cell response to P0180-199 and histopathological changes. Similar levels of IgG production were found in sera from $CD4^{-/-}$, $CD8^{-/-}$, $CD4^{-}8^{-}$ and wild type mice, and IgG production was undetectable in sera from μMT mice, suggesting that induction of EAN is dependent on both CD4+ and CD8+ T cells and may not depend on the B cell response.

Fig. 3.

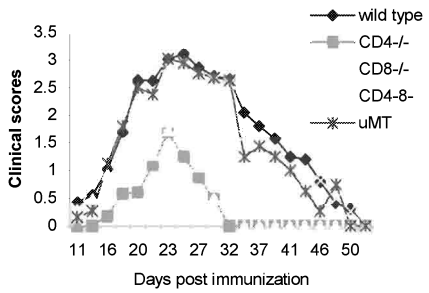

Fig. 3. Clinical course of EAN in $CD4^{-/-}$ (n=10), $CD8^{-/-}$ (n=11), $CD4^{-}8^{-}$ (n=9), μMT (n=8) and wild-type B6 mice (n=11). Mice were immunized with P0 peptide 180-199 plus CFA twice on days 0 and 7 p.i. Symbols refer to mean values and bars to SD.

CD28: a required costimulatory molecule in EAN

Both initial and ongoing autoimmunity to P0 peptide 180-199 is inhibited in $CD28^{-/-}$ mice. All wild type mice sustained clearcut EAN, these clinical signs began on day 9 p.i.,

peaked around 24 days p.i., and recovered on day 44 p.i., thereby, lasting a total 36 days. However, CD28$^{-/-}$ mice did not develop any clinical sign of EAN at all. Resistance to develop clinical signs of EAN in CD28$^{-/-}$ mice is associated with impaired P0 peptide specific T cell responses in terms of decreased T cell proliferation (Fig.4) and reduced IFN-γ production. Histopathological evaluation revealed that fewer inflammatory cells, i.e., macrophages and CD4+ cells; and milder regional demyelination in the sciatic tissue samples from CD28$^{-/-}$ mice than in wild type mice, indicating less inflammatory cell infiltration leads to reduced regional demyelination in sciatic nerves. CD28/B7 interaction is essential for T cell activation which results in inflammatory cell infiltration in sciatic nerve in EAN. IL-12 secreted by APCs is a major inducer of T cell differentiation into Th1 subset and enhances Th1 cells to produce IFN-γ. Lower numbers of IL-12 secreting cells in sciatic nerves of CD28$^{-/-}$ mice reflected less Th1 response to P0 peptide 180-199 in vivo. Additionally, the levels of IgG in sera of CD28-/- mice were significantly lower than of wild type mice (Fig. 5), suggesting that CD28/B7 costimulatory interaction is also required to B cell activation in EAN.

Fig. 4.

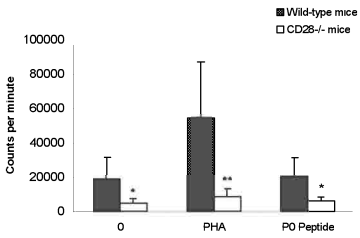

Fig. 4. Proliferation of spleenic mononuclear cells from CD28$^{-/-}$ mice and wild-type mice on day 24 p.i. with P0 peptide 180-199 plus FCA. MNC were cultured in the presence of P0 180-199 peptide or PHA. Mean values and SD are indicated. P values refer to comparison between CD28$^{-/-}$ mice vs wild-type controls. * $p<0.05$; ** $p<0.01$.

Fig. 5.

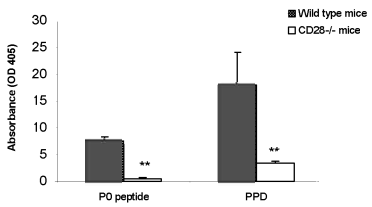

Fig. 5. IgG anti-P0 peptide and anti-PPD antibody levels in sera from CD28$^{-/-}$ mice and wild-type mice were measured on day 24 p.i. with P0 peptide 180-199 plus FCA by ELISA. Amounts of both antibodies were markedly lower in CD28$^{-/-}$ mice than in wild-type mice. Mean values and SD are indicated. P values refer to comparison between CD28$^{-/-}$ mice vs wild-type controls. ** $p<0.01$.

Suppression of EAN in IFN-γR$^{-/-}$ mice

In the present study, deficiency of IFN-γ receptor suppressed the development of EAN in mice. IFN-γR$^{-/-}$ mice manifested less severe disease and delayed onset compared with wild type mice (table 2). Histopathological investigation exhibited less infiltrate and demyelination in sciatic nerves of IFN-γR$^{-/-}$ mice than of wild type mice, which is consistent with clinical symptoms of EAN. IL-12 contributes to optimal IFN-γ production and proliferation of differentiated Th1 cells in response to P0 peptide. IL-4 inhibits the induction and effector functions of Th1 cells. Fewer IL-12-producing but more IL-4-producing cells in sciatic nerves of IFN-γR$^{-/-}$ mice (Fig. 6) were found than of wild type mice on day 24 p.i., i.e., at the peak of clinical EAN, which may lead to mild clinical symptoms of EAN in IFN-γ R$^{-/-}$ mice. However, IFN-γ-secreting cells from the spleen of IFN-γR$^{-/-}$ mice were significantly augmented at day 24 p.i., which might be a result of negative reflection of dysfunction of IFN-γR. The similar levels of anti-P0 peptide 180-199 antibody in sera were found in both IFN-γR$^{-/-}$ and wild type mice.

Fig. 6.

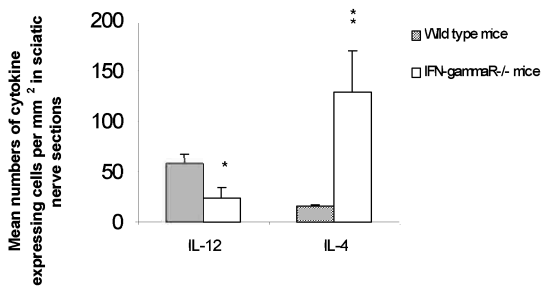

Fig. 6. Numbers of IL-12 and IL-4 positive cells per mm^2 sciatic nerve sections from IFN-γR$^{-/-}$ mice and wild-type mice on day 24 after immunization with P0 peptide 180-199 plus FCA. Detection was by immunohistochemistry. Mean values and SD are indicated. P-values refer to comparison between IFN-γR$^{-/-}$ mice vs wild type controls done by the Student's t-test of discrete values. * $p<0.05$; ** $p<0.01$.

Table 2. Development of EAN in IFN-γ receptor-deficient mice

Clinical Variables	IFN-γR$^{-/-}$	Wild type
Maximum clinical score	1.6 ± 0.6[a]	2.5 ± 0.8
Day of onset	13.6 ± 2.6	6.8 ± 1.7
Average number of days with disease	21.3 ± 3.5	29.6 ± 4.

[a]Means values ± SD

BBIC inhibition of Th1 cell-mediated autoimmunity in the peripheral nerve tissue

EAN induced in Lewis rats by inoculation with peripheral nerve myelin P2 protein peptide 53-78 (SP26) and CFA was suppressed by BBIC administered daily orally from the day 9 p.i. i.e. after onset of clinical EAN. Rats treated with BBIC exhibited the same clinical signs as those with treatment of PBS during the first several days post immunization until day 14 p.i. Rats receiving BBIC started to stop developing disease on day 14 p.i. and went into a plateau phase of disease of EAN. Suppression of EAN was associated with inhibited SP26-specific T cell proliferation and IFN-γ synthesis by lymphocytes in lymph nodes from rats treated with BBIC leading to marked decreased inflammatory infiltrates in sciatic nerves (Fig. 7). Moreover, in vitro BBIC significantly suppressed B7.2 expression, whereas B7.1 expression (Fig. 8) on macrophages from EAN rats was slightly increased on day 18 p.i. which may influence the autoreactive T cell responses.

Fig. 7A.

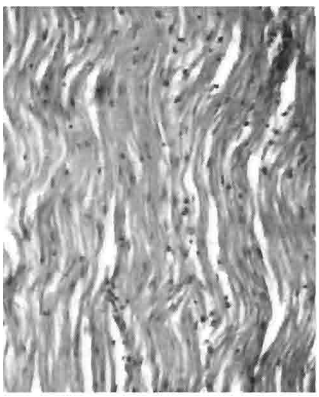

Fig. 7B.

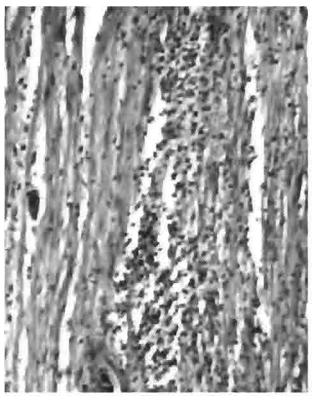

Fig. 7. Photomicrographs of sections of the sciatic nerve from EAN rats treated with BBIC (A) or PBS (B) on day 18 p.i. stained with haematoxylin and eosin.

Fig. 8.

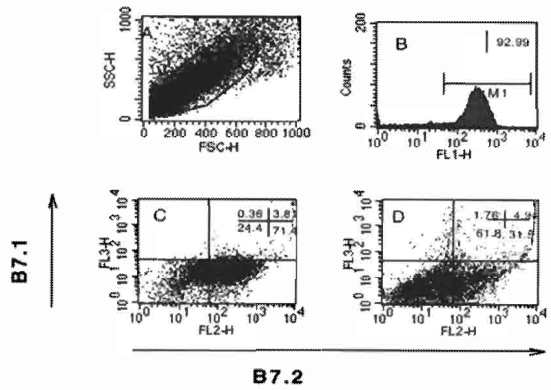

Fig. 8. FACS analyzed B7.1 and B7.2 expression on macrophages in EAN rats. CD11b+ cells (92.99%) (B), without (C) or with BBIC (D) treatment in vitro. Macrophages collected from 5 EAN rats were cultured in the presence or absence of BBIC (5 μM) for 48 h. B7.1 and B7.2 expression on macrophages were detected by FACS using FITC-labeled CD11b, PE-labeled CD86 and Percp-labeled CD80 staining.

General discussion

CD4 and CD8 T cells, not B cells, are necessary for development of EAN (Paper I)

EAN is a T cell-mediated autoimmune disease (Lassmann er al., 1988, Li et al., 2001b), which has been adoptively transferred to naïve Lewis rats by syngeneic P2-spicific CD4+ T cell line (Linington et al., 1984; 1992) and to naïve BALB/c mice by MBP-specific CD4+ T helper cell clones (Abromson-Leeman et al., 1995). CD4 T cells were, until recently, considered the primary effector cells in rodents with the autoimmune demyelinating disease EAN and in patients with GBS. The CD4+ T cells involved in the pathogenesis of EAN are of the Th1 type. Th1 cell-related IFN-γ seems to be vital for perpetuating the inflammatory demyelination of EAN, since the level of IFN-γ producing cells in blood, lymph nodes and the PNS tissue roughly parallels the progress of clinical EAN (Fujioka et al., 1998, Zhu et al., 1996, 1998a). IFN-γ can activate macrophages that directly attack the myelin sheath through phagocytosis and release inflammatory cytokines, such as TNF-α (Zhu et al., 1998a).

More recently, studies showed that myelin-specific CD8 T cells were able to induce a severe and progressive form of EAE (Huseby et al., 2001, Sun et al., 2001). CD8+ T cells may play a more active role in the regulation of the immune response than previously thought. Most CD8+ T cells produce IFN-γ and, in the rat spleen, are the major source of this cytokine (Kemeny et al., 1993), indicating that the participation of this cell subset in local cytokine production has been underestimated (Berner et al., 2000). Recruitment of CD8+ T cells into the target tissue may be mediated by inflammatory cytokines and chemokines produced by the CD4+T cells and aided by activated vascular endothelium. Possibly chemokines secreted by CD8+ T cells also affect the homing behavior of other lymphocyte populations (Kiefer et al., 2001). Furthermore, CD8+ T cells can differentiate into $CD4^-8^-$ cells that secrete IL-4, IL-5 and IL-6, and provide a help to B cell (Erard et al., 1993). In human diseases, such as autoimmune diabetes and MS, indirect evidence also suggests a role for CD8+ T cells in tissue damage, although their antigen specificity is unknown (Vizler et al., 1999). In the acute phase of EAN, the percentages of MHC-specific CD4 and CD8 cells in the lymph nodes have been described as significantly higher than in control rats (Takai et al., 1995). Finally, the phenotype of T cells infiltrating the cauda equina of EAN were characterized as CD45RC+CD8+ (activated cytotoxic lymphocytes) and CD45RC-CD4+ (memory Th cells) at the active stage of the disease (Fujioka et al., 2000). In our study, CD4

T cells are not the only inducers of EAN and lacking CD8 T cells ameliorated the disease, demonstrating that both CD4+ T cells and CD8+ T cells are involved in the pathogenesis of EAN as helper or effector cells through their interaction, despite autoreactive CD4+ T cells remain the main effectors of this disease.

The $CD4^-8^-$ TCR α/β+ T cells had normal levels of TCR $\alpha\beta^+$ T cells, but not $\gamma\delta^+$ T cell, are most likely the inducers of EAN in $CD4^-8^-$ mice, since they comprise higher proportions of MNC of lymph node and spleen in $CD4^-8^-$ than in wild type mice after transfer of P2 peptide-sensitized donor lymph node MNC and injection of bovine peripheral myelin into recipient mice (Zhu et al., 1999a). The lack of CD4 and CD8 expression has no effect on the mature TCR repertoire (Penninger et al., 1995) and is not absolutely necessary for effector function of class II MHC-restricted helper T cells and class I MHC-restricted cytotoxic T cells (Schilham eet al., 1993; Rahemtulla et al., 1994). Since $CD4^-8^-$ cells can be found in a variety of autoimmune disease and appear to mediate autoimmunity in vivo (Penninger et al., 1993). These findings could explain the reasons that $CD4^-8^-$ mice developed EAN similar to that of their wild-type partners.

Neurological deficit in EAN and GBS is probably a consequence of synergy between T and B cell responses to PNS antigens. On the one hand, B cells as APCs may be involved in the pathogensis of autoimmune disease. In EAN, no B cell infiltrates were found in target tissue (Fujioka et al., 1998). However, macrophages were likely to act as local main APCs by using their ability to express MHC antigens and costimulatory molecules (Kiefer et al., 2001). On the other hand, B cells may contribute to disease by producing antibodies that damage their specific target tissues during the autoimmune process. Numerous small series or single case reports have described antibodies to a bewildering range of glycolipids in GBS. The appearance of anti-GD1 and anti-GD1a IgG peaks in sera only after acute phase of GBS suggests that these antibodies are produced secondary to nerve damage (Press et al., 2001b). In addition, detection of anti-GD1a antibodies also indicates axonal damage, suggesting a worse prognosis of disease (Gallardo et al., 2001). However, the GQ1b antibody has been thought of as one of the key factors in the pathogenesis of Miller Fisher syndrome (MFS), a variant of GBS, especially with ophthalmoplegia (Kusunoki and Kanazawa, 1997). Therefore anti-GQ1b antibodies are useful markers for the differential diagnosis of MFS (Kusunoki and Kanazawa, 1997; Rojas-Garcia et al., 2001). Furthermore, anti-GM1 IgM antibodies are only found in multifocal motor neuropathy. These findings may confirm a disease especial correlation between specific neuropathies and anti-ganglioside antibodies clinically useful

(Gallardo et al., 2001). Antibodies to myelin and the myelin proteins P0 and P2 have been reported in some GBS patients, but also in other diseases and some healthy controls (Vrethem et al., 1991; Khalili-Shirazi et al., 1993). Although circulating autoantibodies to peripheral nerve myelin have consistently been identified in a large proportion of GBS patients (Koski, 1990; Ilyas et al., 1992), contradictory results have failed to correlate antibody titers with the severity of GBS (Shoenfeld et al., 1996).

In EAN, the synergy between antibody and P2-reactive T cells has been demonstrated (Spies et al., 1995). Conversely, adoptive transfer of serum from GBS patients intraperitoneally failed to induce EAN in rats and murine monoclonal antibody to *C jejuni* and gangliosides also did not exacerbate disease (Hadden et al., 2001). In EAE, a high analogous disease of the CNS, a series of works indicated that the formation of large, persistently, demyelinated lesions in the Lewis rat was dependent on the presence of an appropriate anti-myelin autoantibody response (Linington et al., 1992b). Antibodies augmented cell-mediated demyelination in EAE (Fierz et al., 1988, Lassmann et al., 1988, Linington et al., 1988). The rats prevented B cell functions were resistant to EAE induction by immunization with MBP (Gausas et al., 1982). Further study showed that B cells are not critical for the development of myelin oligodendrocyte glycoprotein (MOG)-induced EAE but contribute to the severity of disease (Svensson L et al., 2002). However, conflicting results showed that B cells and Ab are unable to induce EAE in susceptible animal strains. No direct correlation has been found between Ab titers and disease severity of EAE (Paterson et al., 1981). Studies of EAE induction in mice genetically deficient in B cells found no requirement for B cells or Ab in disease induction or demyelination in models induced using peptides of MBP and MOG (Hjelmstrom et al., 1998; Dittel et al., 2000). The studies from Lyons et al addressed these discrepancies that B cells and Ab are important to the disease process when a more complex (protein) form of the antigen is used for EAE initiation, but play little role in EAE induced by a short encephalitogenic peptide (Lyons et al., 1999). Our results are coincident with Lyons' study that antibodies specific to P0180-199 peptide may take less of a part in this disease process than previously thought.

T cell costimulation is a precondition in initiation of EAN (Paper II and Paper IV)

CD4+ cells potentially autoreactive for autoantigens are consistently presented in healthy subjects (Conti-Fine et al., 1998). Once CD4 T cells activated in vivo by a suitable stimulus, they might cause an autoimmune response and result in destruction of the self tissue. One mechanism designed to maintain the fidelity of the immune response is the requirement of

two distinct signals for effective activation of antigen-specific T cells: an antigen-specific signal via the T cell receptor (Signal 1) and a noncognate cositmulatory signal (Signal 2) that is provided by soluble factors or cell-surface molecules on the APC. CD28/B7 pathway has been identified as a prominent costimulatory pathway for T cells (Jenkins et al., 1991; Harding et al., 1992). CD28 costimulation is thought to help in the amplification of T cell signals, especially when Ag concentrations are low. The CD28/B7 interaction functions to lower the threshold of T cell activation (Viola and Lanzavecchia, 1996), clonal expansion of T cells, and the differentiation of T cells into effector cells (Lenschow et al., 1996). CD28 is necessary for the induction of various cytokine genes, and the stability of mRNA transcripts, especially that of IL-2 (Jenkins et al., 1991), which is responsible for the clonal expansion of T cells; also stability of IFN-γ mRNA, which enhances production of IFN-γ protein (Lindsten et al., 1989). CD28 also provides an anti-apoptotic signal by up-regulating Bcl-xl (Kearney et al., 1995; Vella et al., 1997). CD28/B7 interaction has been found to be important for the up-regulation and stabilization of CD40L expression on the T cell (Johnson et al., 1998). In the absence of CD28 costimulation, T cell responses are reduced with typically poor proliferation and cytokine responses (Viola et al., 1996; Judge et al., 1999; Howland et al., 2000) and low levels of Ab production. This is also reflected in our system, where the $CD28^{-/-}$ mice completely resist in developing EAN with the minimal proliferation of T cells and low level IgG production.

The overexpression of costimulatory molecules B7.1/B7.2 in peripheral organs can mediate autoimmune diseases (Harlan et al., 1994; Guerder et al., 1994). The ligation of costimulatory B7 molecules on APCs with CD28 on T cells is thought to be crucial to the onset and cause human MS and its animal model, EAE (Wolf et al., 2001). Mice lacking both B7.1 and B7.2 or with anti-CTLA-4 antibody treatment resulted in the minimal clinical signs in EAE, and resistance to induction of EAN with markedly reduced inflammatory infiltrates in the target tissues (Chang et al, 1999; Zhu et al., 2001b).

In most instances, B7.2 is a dominant costimulatory ligand. For example, anti-B7.2 mAbs can inhibite collagen-induced arthritis, EAMG, EAE, and autoimmune uveitis (Salomon and Bluestone, 2001). B7.2 knockout NOD mice do not develop spontaneous diabetes. Similarly, anti-B7.2 mAb treatment inhibited the development of the diabetes in NOD as well as disease progression in autoimmune diabetes (Chakrabarti et al., 1996; Salomon et al., 2000). Although B7.2 is a predominant costimulatory molecule, the combination of B7.1 and B7.2 blockade is most effective in blocking animal models of autoimmunity and transplant

rejection (Finck et al., 1994; Kinoshita et al., 2000). However, unlike B7.2 blockade, the consequence of B7.1 blockade is not always predictable. The expression of B7.1 down-regulates responses mediated by CTLA-4 expressing activated T cells (Bluestone, 1995). For instance, treatment of NOD mice with anti-B7.1 mAb resulted in the exacerbation of diabetes. Anti-B7.1 mAb therapy can exacerbate relapse EAE in a temporal pattern that is identical to the one observed following anti-CTLA-4 mAb treatment (Ikemizu et al., 2000).

B7.1 and B7.2 may have a differential role in setting Th1 and Th2 responses (Kucheroo et al., 1995; Perrin et al., 1996). But, the clear differential effects have been unable to demonstrate in vivo and in vitro (Levine et al., 1995; Schweitzer et al., 1997; MacPhee et al., 2001). Our results showed that BBIC treatment significantly inhibited clonal expansion of SP26 specific T cell in rats with EAN, it may result from lowered expression of B7.2 on macrophages, which is a factor that contributed to the suppression of EAN in BBIC treated rats.

IFN-γ plays an important role in the pathogenesis of EAN (Paper III and Paper IV).

The effector functions of activated T cells are reflected by their phenotype and by the mediators they release. Generally accepted concept is based on the assumption that an adequate balance between pro-inflammatory and anti-inflammatory cytokines, i.e., the Th1 and Th2 balance, is necessary for a physiological immune response, whereas a disturbed balance may lead to disease. Th1 cells are considered to play an important role in inducing cell-mediated autoimmune disease, whereas Th2 cell responses suppress such disease. The Th1 subset of CD4+ T cells is able to orchestrate both cell- and Ab-mediated responses. However, the role of Th1/Th2-related cytokines in immune regulation and autoimmune disease is more complex than a sample Th1-Th2 dichotomy would suggest. The effect of cytokines on immune response depends on the phases of an immune response, the patterns of synthesis, and the cell populations they interact with. Thus cytokines may mediate variable, even contradictory effects on autoimmune diseases.

IFN-γ has been demonstrated to contribute to the pathogenesis of EAN. There is convincing evidence that the level of IFN-γ producing cells in blood, lymph nodes and the PNS tissue roughly parallels clinical EAN, consistent with an inflammatory role of Th1 related cytokines in the pathogenesis of EAN (Zhu et al., 1998a; Fujioka et al., 1998). High doses of IFN-γ induce Schwann cell apoptosis in vitro (Conti et al., 2002). Furthermore, IFN-γ within the inflammatory lesions of the PNS is capable of inhibiting Schwann cell differentiation (Lisak et al., 2001). Stimulation with IFN-γ significantly reduced the

production of IL-6 but increased TNF-α production (Murwani and Armati, 1998) which can activate the vascular endothelium and other cells important to the breakdown of the blood brain barrier or BNB (Lisak et al., 1997 review). IFN-γ can induce the expression of MHC class II molecules on Schwann cell (Olsson, 1995), which allow these cells, at least in vitro, to present antigen to CD4+ antigen-specific T cells (Gold et al., 1995). In addition, IFN-γ also up-regulates MHC class I antigens (Olsson, 1995), which allows Schwann cells to be targets of cytotoxic specific T cell reactions, either to a self-antigen or a microbial antigen infecting Schwann cell (Steinhoff and Kaufman, 1988). Furthermore, IFN-γ can induce adhesion molecules on endothelial cells that are critical in recruitment of inflammatory cells to cross the BNB and trigger immune-mediated demyelinating diseases (Lisak et al., 1997 review). All of above may interpret, at least partly, the experimental results that significantly decreased production of IFN-γ has been found in overt suppressed ongoing EAN by BBIC treatment.

IFN-γ may influence Th1 development by both indirect and direct pathways. Although, IFN-γ enhances macrophages production of IL-12 in response to pathogen (Mosmann and Sad, 1996; Gollob and Coffman, 1994), thus indirectly promoting Th1 development, this event alone may not be sufficient for directing Th1 development. IFN-γ may also act directly on naïve T cells to allow IL-12-induced Th1 responses (Trinchieri, 1995). On the other hand, IL-12 contributes to optimal IFN-γ production and proliferation of differentiated Th1 cells in response to antigen. IL-12 and IL-12-induced IFN-γ favor Th1 cell differentiation by priming $CD4^+$ T cells to increase IFN-γ production. The early preference expressed in the immune response depends on the balance between IL-12, which favors Th1 responses, and IL-4, which favors Th2 responses (Weinberg et al., 1990). Neutralization of IFN-γ blocked IL-12-induced Th1 development in APC-dependent as well as APC-independent systems (Trinchieri, 1995), and IL-12 and IL-4 critically influenced the development of naive T cells toward Th1 and Th2 subsets, respectively (Hsieh et al., 1993, Seder et al., 1994; Adams et al., 1987). Our results in study III resemble the findings that experimental autoimmune thyroiditis and uveitis, both Th1-mediated diseases involve a bias toward Th2-type response in IFN-$\gamma R^{-/-}$ mice (Collart et al., 1986; Toyka et al., 1987). We found that decreased number of IL-12 secreted cells and increased number of IL-4-secreted cells in sciatic nerve sections from IFN-$\gamma R^{-/-}$ mice may reflect the predominance of Th2-over-Th1-type immune reactivity in the mutants but not in the wild-type mice.

Conclusions

1. Both CD4+ and CD8+ T cells are involved in the pathogenesis of EAN as helper or effector cells through their interaction, although autoreactive CD4+ T cells remain the main effectors of this disease. B cells seem less to contribute to perpetuating the related inflammatory demyelination in PNS.
2. CD28/B7 costimulation plays a pivotal role in either the initiation and/or effector phases of Th1-mediated EAN through both the activation of P0 peptide-specific T cells and Th-induced differentiation of B cells into Ig-secreting cells.
3. IFN-γ contributes to the pathogenesis of EAN by promoting a Th1 cell-medicated immune response and suppressing a Th2 response.
4. BBIC can suppress ongoing EAN with reduced inflammatory cell infiltrates in the PNS, which is related to down-regulation of Th1 response to SP26.

Acknowledgements

This work has mainly been carried out at Neurotec, the Division of Experimental Geriatrics, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden. Many people have supported and contributed to this work during past years and I want to express my sincere gratitude to you all. In particular I want to thank:

Associate prof. Jie Zhu, my supervisor, for accepting me as a Ph.D. student and introducing me into immunology research area; for sharing your vast knowledge and for excellent guidance in the field of neuroimmunology; for your belief in me as well as for support, encouragement in my scientific work; for great help whenever needed.

Prof. Bengt Winblad, my co-supervisor, for giving me the opportunity to study at your Department, for generous support, hospitality and kindness.

Co-author, Prof. Hans-Gustaf Ljunggren for kindly providing the all kinds of knockout mice in MTC, Prof. Mix Eilhard, for providing P0 peptide, both of you for all related valuable suggestions, constructive opinions and critical evaluations on manuscripts. Prof. Peter H.van der Meide for providing ELISPOT reagents. Other co-authors and colleagues, for good collaboration and unforgettable moments.

Prof. Abdolmohamad Rostami, and scientist Guangxian Zhang for nice collaboration.

Nagat Abbas for nice talking.

Inga Volkmann for skillful technical assistance

Maria Roos and Agneta Lindahl for excellent secretarial help and kindness.

Rui Liu for unconditional support on computer technology.

My Chinese friends in Sweden, Lei Bao, Shunwei Zhu, Chaorui Huang, Ling Xia, Xupeng Ge, Qingyang Wu, Wenlin An, Zhiguo Chen, Ruisheng Duan, Wenfeng Yu, Wengpeng Niu, Hongmei Li, Mingjie Sun, Tiehua Sun, Jinghua Piao, Wei Ren, for their friendship and invaluable help, especially in my difficult time when I suffered from the arm fracture. Hulun Li, Hongwei Xu ,Yamei Dai and Baoguo Xiao for much-needed help and friendship. Qing Lu, Yu Shi, Wei Jia, Yajuan Liu, Huabing Wang, Yongmei Guan, Zhen Huang, Gang Li, Xingyu Zheng, Jining Liu, Lili Mo, Jinjing Pei, Liyue Huang for friendship.

Professors Mingli Rao, Qun Liu, Jiang Wu, Dihiu Ma, Jiachun Feng, Yu Zhang and my teachers and colleagues at JiLin University for their supports, encouragements and friendship. Thanks extend to the First Hospital of JiLin University for training me to be a qualified physician.

Above all I want to thank my beloved parents professors Shuqin Zhang and Dezhi Zhu for their constant support, encouragement and always being there for me; my parents in law for care, support and taking care of my daughter; my sister, my brother in law and my brother for

their unconditional support and for doing a lot for our parents over these years; my husband Tao Liang for love, concern, support and encouragement in thick and thin; my lovely daughter Shuo Liang for bringing me a lot of joy, you are so much precious to me.

This study was supported by grants from the Swedish Medical Research Council (K1999-71X-013133-01A, K1999-99P-012720-02B, K2000-71X-13133-02B, K2000-99P-12720-03A, K2001-16X-13133-03A, K2001-99PU-12720-04B, K2002-16X-13133-04B and K2002-99PU-12720-05A) and funds from the Åke Wibergs foundation, Kapten Artur Erikssons foundation, Loo och Hans Ostermans foundation, the Konung Gustaf V:s och Drottning Victorias foundation, Gamla Tjänarinnor Foundation, the Swedish Medical Association, and the Karolinska Institute.

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