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**ROLE OF APOLIPOPROTEIN E ISOFORMS  
AND CYTOKINES IN IMMUNE RESPONSES  
AND INFLAMMATION OF THE MOUSE  
PERIPHERAL NERVOUS SYSTEM**

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Cover picture: cultured Schwann cells under the inverted microscope.  
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## ABSTRACT

Experimental autoimmune neuritis (EAN) as an animal model for Guillain-Barré syndrome (GBS) in humans is an immune-mediated disorder affecting the peripheral nervous system (PNS). Apolipoprotein E (apoE) is a glycosylated protein characterized by its wide tissue distribution and multiple biological functions. ApoE can suppress proinflammatory signalings, and vice versa, indicating an intricate apoE-mediated feedback regulation of inflammatory and immune responses. Immune cells together with cytokines produced by various cells contribute to the inflammatory process of EAN by acting as mediators or effectors.

In **Paper I**, the effects of apoE isoforms on the functions of immune cells were investigated. Clinical signs of EAN were most severe in wild type (WT) C57BL/6 mice and apoE4 transgenic (Tg) mice, followed by apoE2 Tg mice and apoE3 Tg mice (WT  $\approx$  E4 > E2 > E3). Proliferation tests of purified T cells from naïve mice stimulated with phytohemagglutinin or interleukin (IL)-12 showed isoform-specific differences (WT  $\approx$  E4 > E3  $\approx$  E2). Macrophages from both naïve and EAN mice produced nitric oxide (NO) upon inflammatory stimulation in an isoform-dependent manner (WT  $\approx$  E4 > E2 > E3). During the recovery stage of disease, the highest expression of CD178 (FasL) on Schwann cells (SCs) was found in apoE3 Tg mice. In **Paper II**, the effects of different isoforms of apoE on SCs in response to inflammatory stimulation (lipopolysaccharide plus interferon gamma (IFN- $\gamma$ )) were studied. Upon stimulation, a change in the morphology of cultured SCs was observed. Pronounced production of IL-6 and IL-10 within SCs, and increased levels of IL-6 and NO in culture supernatants were found in an isoform-dependent manner (apoE3 > apoE2  $\approx$  apoE4). Further results indicated that both nuclear factor kappa B (NF $\kappa$ B) and Akt signaling pathways were involved in the process by the same isoform-dependent pattern. In **Paper III**, the role of IFN- $\gamma$ , a signature T helper (Th)1 cytokine, in the pathogenesis of EAN was investigated. The clinical signs of EAN in IFN- $\gamma$  knockout (KO) mice were evidently aggravated. At the peak of EAN, the proportion of IL-17A expressing cells in cauda equina (CE) infiltrating cells, and the serum levels of IL-17A were elevated in IFN- $\gamma$  KO mice. The proportions of MHC II, macrosialin, and IL-12/IL-23p40 expressing cells, relative to total CE infiltrating cells were correspondingly higher in IFN- $\gamma$  KO than WT mice with EAN. In **Paper IV**, the role of tumor necrosis factor alpha (TNF- $\alpha$ ), another Th1 cytokine, in the pathogenesis of EAN was studied. TNF- $\alpha$  deficiency significantly attenuated EAN. Furthermore, TNF- $\alpha$  deficiency induced an antiinflammatory phenotype of macrophages (M2) characterized by reduced production of IL-12 and NO, and enhanced production of IL-10. Moreover, TNF receptor (TNFR)1 monoclonal antibodies markedly suppressed the severity of EAN when they were administered from the beginning of immunization for EAN induction.

In summary, our data support an isoform-dependent effect of apoE on EAN. This might be due to the isoform-specific effects of apoE on functions of T cells, macrophages and SCs, which contribute to the distinct clinical severity of EAN. SCs from apoE2 and apoE4 Tg mice bear some dysfunction in producing cytokines (IL-6 and IL-10) and NO as compared with their apoE3 counterparts, probably resulting from their insufficiency to suppress the activation of NF $\kappa$ B and Akt pathways. IFN- $\gamma$  deficiency exacerbates EAN via upregulating Th17 cells despite a mitigated systemic Th1 immune response. TNF- $\alpha$  exacerbates EAN via TNFR1 by inducing the proinflammatory phenotype of macrophage (classically activated macrophage, M1).

## LIST OF PUBLICATIONS

- I. **Hong-Liang Zhang**, Xi-Jing Mao, Xing-Mei Zhang, Hai-Feng Li, Xiang-Yu Zheng, Abdu Adem, Eilhard Mix, Jie Zhu. APOE  $\epsilon$ 3 attenuates experimental autoimmune neuritis by modulating T cell, macrophage and Schwann cell functions.

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- II. Ke-Jia Zhang\*, **Hong-Liang Zhang\***, Xing-Mei Zhang, Xiang-Yu Zheng, Hernan Concha Quezada, Duo Zhang, Jie Zhu. Apolipoprotein E isoform-specific effects on cytokine and nitric oxide production from mouse Schwann cells after inflammatory stimulation.

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- IV. **Hong-Liang Zhang**, Mohammed Y Hassan, Xiang-Yu Zheng, Sheikh Azimullah, Hernan Concha Quezada, Naheed Amir, Mohamed Elwasila, Eilhard Mix, Abdu Adem, Jie Zhu. Attenuated EAN in TNF- $\alpha$  deficient mice is associated with an altered balance of M1/M2 macrophages.

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## LIST OF ABBREVIATIONS

AIDP	Acute inflammatory demyelinating polyneuropathy
ANOVA	One-way analysis of variance
ApoE	Apolipoprotein E
BNB	Blood-nerve barrier
CE	Cauda equina
CNS	Central nervous system
ConA	Concanavalin A
CSF	Cerebrospinal fluid
EAE	Experimental autoimmune encephalomyelitis
EAN	Experimental autoimmune neuritis
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
Foxp3	Forkhead box P3
GBS	Guillain-Barré syndrome
GFAP	Glial fibrillary acidic protein
IFN- $\gamma$	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KO	Knockout
LPS	Lipopolysaccharide
MCP	Monocyte chemotactic protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MNC	Mononuclear cell
NF $\kappa$ B	Nuclear factor kappa B
NO	Nitric oxide
PBS	Phosphate-buffered saline
PEMs	Peritoneal exudate mononuclear cells
PHA	Phytohemagglutinin
p.i.	post immunization
Poly I:C	Polyinosinic-polycytidylic acid
PNS	Peripheral nervous system
ROR	Retinoic acid-related orphan receptor
SCs	Schwann cells
TCR	T cell receptor
Tg	Transgenic
Th	T helper
TLR	Toll-like receptor
TNF- $\alpha$	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor alpha receptor
Treg	Regulatory T cells
WT	Wild type

# 1 INTRODUCTION

## 1.1 GUILLAIN-BARRÉ SYNDROME AND ITS ANIMAL MODEL

Guillain-Barré syndrome (GBS) was firstly documented as early as 1916, when three French neurologists, Drs Guillain, Barré, and Strohl described two soldiers who developed acute flaccid paralysis with spontaneous recovery (Guillain et al., 1916). As a clinical syndrome, GBS was initially a descriptive diagnosis of a combination of rapidly progressive symmetric weakness in the limbs usually with sensory disturbance, hyporeflexia or areflexia, and albuminocytologic dissociation, i.e. increased protein concentration with a normal cell count in the cerebrospinal fluid (CSF) (van Doorn et al., 2008). Although the pathogenesis of GBS remains largely unclear, GBS is generally defined as an immune-mediated disorder in the peripheral nervous system (PNS).

### 1.1.1 Classification of GBS

GBS consists of several subtypes of acute peripheral neuropathy. Classification of GBS into subtypes depends on an understanding of the involved nerve fiber types (sensory, motor, and autonomic) and the predominant nature of nerve or nerve root injury, e.g. demyelination versus axonal degeneration. The prototype of GBS, which accounts for 90% of all GBS cases in Europe and North America, is acute inflammatory demyelinating polyneuropathy (AIDP) (Haymaker and Kernohan 1949; Asbury et al., 1969). Acute motor axonal neuropathy (AMAN) (McKhann et al., 1991; 1993) and acute motor-sensory axonal neuropathy (AMSAN) (Feasby et al., 1986; 1993), however, are more prevalent in Asia, South and Central America (Vucic et al., 2009; Drenthen et al., 2011). The seven most common clinical subtypes of GBS are illustrated in Table 1 (Hughes and Cornblath, 2005). The different patterns of GBS are probably due to the diverse interplay between antibodies and T cells of divergent specificities (Hughes et al., 1999). The axonal variants of GBS (AMAN and AMSAN) and Miller-Fisher syndrome (MFS) (Fisher, 1956) are more related to autoantibodies against ganglioside GQ1b (Sekiguchi et al., 2012), while AIDP mainly involves CD4<sup>+</sup>-T-cell-induced macrophage- and complement-associated demyelination (Hughes et al.,

1999). Anti-GQ1b antibodies are present in approximately 95% of patients with MFS (Chiba et al., 1992).

**Table 1 Clinical subtypes of Guillain-Barré syndrome (GBS)**

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Acute inflammatory demyelinating polyneuropathy (AIDP) (Haymaker and Kernohan, 1949; Asbury et al., 1969)
Acute motor axonal neuropathy (AMAN) (McKhann et al., 1991; 1993)
Acute motor-sensory axonal neuropathy (AMSAN) (Feasby et al., 1986; 1993)
Acute sensory neuronopathy (Sterman et al., 1980)
Acute pandysautonomia (Young et al., 1969; Suarez et al., 1994)
Miller-Fisher syndrome (MFS) (Fisher, 1956)
MFS-GBS overlapping syndrome (Mori et al., 2001)

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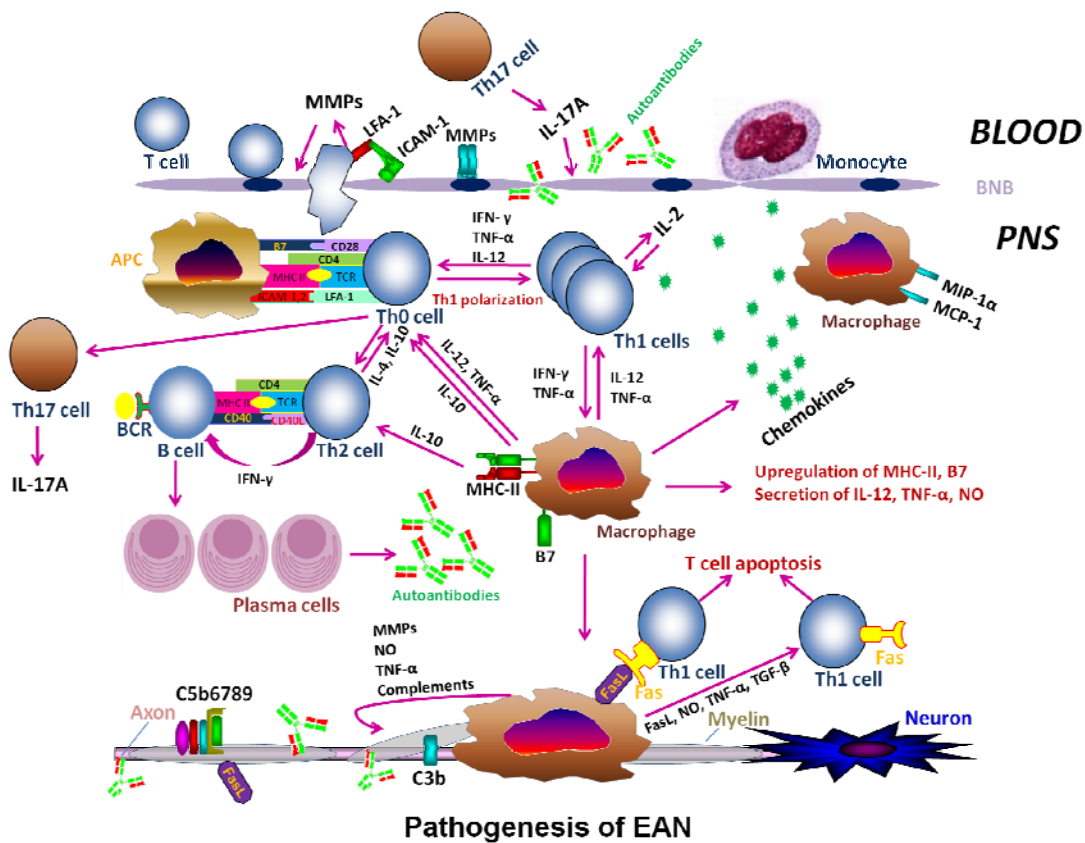
### **1.1.2 Experimental autoimmune neuritis**

Experimental autoimmune neuritis (EAN) is an immune-mediated inflammatory disorder of the PNS that serves as an animal model for AIDP. EAN can be induced in susceptible animal strains including mouse, rat, sheep, chicken, and monkey (Lehrich and Arnason, 1971) by active immunization with whole peripheral nerve homogenates, myelin proteins P0 or P2, or their neuritogenic peptides plus Freund's complete adjuvant (Waksman and Adams, 1955; Milner et al., 1987; Kadlubowski and Hughes, 1979; Rostami et al., 1990; Zou et al., 2000b), or by passive transfer of P0, P2, or their peptide-specific CD4<sup>+</sup> T cell lines (Gold et al., 2000; Maurer et al., 2000).

Pathophysiologically, EAN is characterized by breakdown of the blood-nerve barrier (BNB), robust accumulation of autoreactive T cells and macrophages in the PNS, and demyelination (Figure 1) (Zhang et al., 2008; Zhang et al., 2010b). An alteration in BNB permeability occurs early in EAN, coincident with inflammatory cell infiltration (Hahn et al., 1985). The migration of blood-derived inflammatory cells across the BNB or the transduction of chemotactic signals for migration is a critical step in the initiation of immune responses in EAN. Cytokines, chemokines, adhesion molecules, nitric oxide (NO), and matrix metalloproteinases (MMPs) contribute to this process (Kieseier et al., 1999). Autoreactive inflammatory cells penetrate the compromised BNB, accumulate in the PNS, and give rise to the effector phase of the immune response in EAN (Hartung and Toyka, 1990). Locally, macrophages serve as the main antigen presenting



cells (APCs), thereby promoting the T helper (Th)1 polarization (Kieseier et al., 1999). Polarized Th1 cells in turn activate macrophages to express a proinflammatory phenotype (M1) (Gratchev et al., 2006; Heusinkveld et al., 2011). M1 macrophages are the key effector cells in EAN (Kiefer et al., 2001). The pivotal detrimental role of macrophages in the acute phase of immune-mediated nerve damage consists of a direct phagocytotic attack on myelin, as well as release of proinflammatory cytokines including tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL)-1 and IL-6 and other noxious molecules (Zhang et al., 2010b; Jung et al., 1993; Kiefer et al., 2001). Proinflammatory cytokines and other toxic mediators (NO, MMPs, etc) released by activated macrophages further promote T cell activation and induce inflammation (Kiefer et al., 2001). Schwann cells (SCs) can function as facultative APCs in certain conditions by expressing major histocompatibility complex (MHC) class II molecules (MHC II) and costimulatory molecules (Argall et al., 1992; Duan et al., 2007). Anti-myelin autoantibodies, crossing the BNB (Hadden et al., 2002) or locally produced by B cells, can also mediate demyelination by antibody-dependent cellular cytotoxicity (ADCC) or activating the complement system, and can block functionally relevant epitopes for nerve conduction (Kiefer et al., 2001). Although the mechanism of action of IL-17 in EAN remains largely unclear, it presumably acts as a proinflammatory effector that directly stimulates endothelial cells and fibroblasts to produce proinflammatory cytokines and chemokines, further aiding in the recruitment of neutrophils and macrophages (Shen and Gaffen, 2008; Zepp et al., 2011). During the recovery phase, Fas ligand (FasL, CD95) expressed by SCs and macrophages is important for inducing the apoptosis of effector T cells and for terminating the local immune responses (Wohlleben et al., 2000; Kiefer et al., 2001). Macrophages can phagocytose myelin debris by Fc/complement receptors through ADCC or complement mediated mechanism (Vriesendorp et al., 1998; Stoll et al., 1991). Yet macrophages may also contribute to tissue repair and promotion of SCs proliferation and remyelination, through secretion of antiinflammatory cytokines, such as IL-10 and transforming growth factor beta (TGF- $\beta$ ) (Kiefer et al., 2001).



**Figure 1.** The pathogenetic scenario of EAN is illustrated. EAN is characterized by breakdown of the BNB, robust accumulation of inflammatory cells and demyelination in the PNS. The migration of blood-derived inflammatory cells across the BNB is a critical step in the initiation of immune responses in EAN. Cytokines, chemokines, adhesion molecules, NO, and other molecules contribute to this process. In the PNS, macrophages serve as the main APCs, thereby promoting the Th1 polarization. Polarized Th1 cells in turn activate macrophages to express a proinflammatory phenotype (M1). M1 macrophages play a key role by directly attacking myelin and releasing proinflammatory cytokines and other toxic mediators (NO, MMPs, etc). SCs may function as facultative APCs in certain conditions by expressing MHC II and costimulatory molecules. Anti-myelin autoantibodies also mediate demyelination by ADCC or activating the complement system, and can block functionally relevant epitopes for nerve conduction. Presumably IL-17 acts as a proinflammatory effector that directly stimulates endothelial cells and fibroblasts to produce proinflammatory cytokines and chemokines. During the recovery phase, macrophages phagocytose myelin debris by Fc/complement receptors; FasL expressed by macrophages and SCs induces the apoptosis of effector T cells and terminates the local immune responses. Macrophages also promote proliferation of SCs and remyelination through secreting antiinflammatory cytokines, such as IL-10 and TGF- $\beta$ .

BCR: B cell receptor; CD: cluster of differentiation; ICAM: intercellular adhesion molecule; LFA-1: lymphocyte function-associated antigen 1; MCP-1: monocyte chemotactic protein 1, i.e. chemokine

(C-C motif) ligand 2 (CCL2); MIP-1 $\alpha$ : macrophage inflammatory protein 1 alpha, i.e. chemokine (C-C motif) ligand 3 (CCL3).

## 1.2 APOLIPOPROTEIN E AND ITS IMMUNOMODULATORY ROLE IN EAN

Apolipoprotein E (ApoE) is a 34.2 kDa glycosylated protein with 299 amino acid residues. The gene encoding apoE, namely *APOE*, is located on chromosome 19q13.2, consisting of four exons and three introns and spanning 3597 nucleotides (Rall Jr. et al., 1982). There are three isoforms in humans (apoE2, apoE3, and apoE4) due to different amino acid residues at positions 112 and 158 (Weisgraber, 1994). However, there is only one isoform in rodents, which resembles human apoE3 in terms of lipoprotein binding and metabolism (Raffai et al., 2001; Li et al., 2008).

ApoE is synthesized predominantly in the liver, but also in the spleen, brain, lung, kidney, ovary, adrenal, and muscle tissues. Hepatic parenchyma cells are the main apoE-producing cells in mammals, accounting for 2/3 to 3/4 of the plasma apoE (Mahley, 1988). In the nervous system, apoE mRNA is present in neurons, astrocytes, ependymal cells, and nonmyelinating SCs, etc (Boyles et al., 1985; Beffert et al., 1998; Refolo and Fillit, 2004).

ApoE has been widely studied in lipid metabolism, cardiovascular diseases (Knopman et al., 2009), multiple sclerosis (MS) (Julian et al., 2009) and Alzheimer's disease (Kim et al., 2009), etc. The immunoregulatory role of apoE was originally described to be due to suppression of lymphocyte activation (Macy et al., 1983; Pepe and Curtiss, 1986). Later, with the application of genetic engineering techniques, studies on the mechanisms of apoE in immunomodulation were greatly deepened by using apoE deficient and transgenic (Tg) mice. The immunomodulatory properties of apoE are extensive, including suppression of T cell proliferation, stimulation of cultured neutrophils, regulation of macrophage functions, facilitation of lipid antigen presentation by CD1 molecules to natural killer T (NKT) cells, and modulation of inflammation and oxidation, and so forth (Zhang et al., 2010b).

Proinflammatory cytokines such as interferon gamma (IFN- $\gamma$ ) and TNF- $\alpha$  inhibit the production of apoE (Brand et al., 1993; Starck et al., 2000). Conversely, apoE

suppresses the production of proinflammatory cytokines and induces an antiinflammatory phenotype of macrophages (Baitsch et al., 2011; Zhang et al., 2011). ApoE can suppress proinflammatory signalings, and vice versa, indicating an intricate apoE-mediated feedback regulation of inflammatory and immune responses (Zhang et al., 2011). In GBS patients, the concentration of apoE in the CSF is downregulated (Jin et al., 2007). Although the exact role of apoE in GBS has not been fully addressed, by using apoE KO mice, Yu and colleagues found that apoE mainly serves as a suppressor for EAN by shifting the Th1/Th2 balance to the Th1 direction and by inhibiting P0-specific antibody production (2004). Moreover, the antigen presenting capacity of SCs in apoE deficient mice was enhanced via downregulated intracellular production of IL-6 (Duan et al., 2007). Collectively, apoE may modulate immune responses in EAN by altering the functions of macrophages, T cells, SCs and BNB, and shifting the Th1/Th2 balance (see a systemic review by Zhang et al., 2010b). However, the isoform-dependent effect of apoE on GBS and EAN has not been studied.

### **1.3 TH CELLS, TREGS CELLS AND CYTOKINES IN GBS AND EAN**

#### **1.3.1 The Th1/Th2 paradigm and M1/M2 balance in GBS and EAN**

The Th1/Th2 paradigm of Th cell differentiation, which was initially introduced by Mosmann and Coffman (Mosmann et al., 1986; 1987; Coffman, 2006), has aided in explaining many observed phenomena in the dynamic immune responses against infections. A Th1-oriented response is related to an acute-phase reaction to pathogens, while a Th2 response is related to the elimination of antigens and recovery of diseases (Gigi et al., 2008). This paradigm has also been utilized to explain the adaptive immunity and autoimmune diseases.

Th0 cells can differentiate into Th1 or Th2 cells depending on the exogenously or endogenously provided cytokines in their milieu. Th1 differentiation is regulated by IFN- $\gamma$  produced mainly by natural killer (NK) cells and Th1 cells per se (Martín-Fontecha et al., 2004) and by IL-12 produced by dendritic cells (DCs) after Toll-like receptor (TLR) activation (Trinchieri, 2003). IL-4 is essential for Th2 cell differentiation (Mowen and Glimcher, 2004). IFN- $\gamma$  and IL-4, respectively, can act as autocrine growth factors for themselves as well as inhibitory factors for the opposite

subset. Th1 cells potentiate immunoglobulin (Ig)G2a synthesis by B cells through IFN- $\gamma$ , whereas Th2 cells induce B cell IgE and IgG1 production through IL-4 (Liew, 2002). Th1 cytokines include IL-12, IFN- $\gamma$ , TNF- $\alpha$  and IL-1 $\beta$ . These cytokines can activate macrophages to produce reactive oxygen intermediates and NO, stimulate their phagocytotic functions and enhance their antigen presenting capacity by upregulating the expression of MHC II (Zhang et al., 2011). Th2 cytokines include IL-4, IL-5, IL-10 and IL-13, which provide potent help for B cell activation, Ig class switching to IgE and IgG1, and downregulation of proinflammatory macrophage activation (Zhang et al., 2011).

The balance of functionally distinct T cell subsets between Th1 and Th2 cells has a direct relevance to autoimmune responses such as those that have been observed in GBS. Nyati and colleagues reported characteristic cytokine expression profiles of peripheral blood mononuclear cells (PBMCs) from patients with GBS in response to the *C jejuni* outer membrane protein (OMP) (Nyati et al., 2011). During the acute phase, a Th1 response predominated, characterized by upregulated levels of IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$  and IL-6, and low levels of IL-4; during the recovery phase, however, the cytokine profile switched to low levels of IFN- $\gamma$  and high levels of TGF- $\beta$ 1 and IL-4, indicative of a Th2 predominance (Nyati et al., 2011). These findings follow the Th1/Th2 paradigm, indicating that Th1 cells are disease promoting, while Th2 cells serve as a countermeasure to limit the Th1 response (Lambracht-Washington and Wolfe, 2011).

In EAN, similarly, Th1 cytokines predominate in sciatic nerves and lymphoid organs during the acute phase and mediate inflammatory damage to the peripheral nerves, whereas Th2 cytokines are associated with recovery from the disease (Zhu et al., 1998; 1997). Th1 cytokines including IL-12, IFN- $\gamma$  and TNF- $\alpha$  contribute to disease development by recruiting inflammatory cells to the PNS and by enabling in situ release of other inflammatory products such as free radicals, oxygen intermediates and NO, leading to damage of SCs and myelin (Bao et al., 2002; Hartung et al., 1990; Said and Hontebeyrie-Joskowicz, 1992). Th2 cytokines such as IL-4 and IL-10 suppress the disease by playing an antiinflammatory role (Deretzi et al., 1999; Bai et al., 1997).

However, the Th1/Th2 paradigm in autoimmune diseases has been greatly challenged in recent years, with the identification of another important Th cell population, namely

Th17 cells. Studies on the association between Th17 cells and GBS/EAN are reviewed in the following section.

Macrophages are the principal APCs and effector cells in the pathogenesis of EAN (Kieseier et al., 1999). In demyelinated peripheral nerves, the MHC II positive cells are mainly macrophages (Schmidt et al., 1990). The pivotal role of macrophages in immune-mediated nerve damage in EAN is direct phagocytotic attack on myelin, and release of proinflammatory cytokines including TNF- $\alpha$ , IL-1 and IL-6 and other noxious molecules (Zhang et al., 2010b; Jung et al., 1993; Kiefer et al., 2001). Activated macrophages can be divided into two distinct subsets: classically activated macrophages (M1) and alternatively activated macrophages (M2) (Martinez et al., 2008). Proinflammatory Th1 cytokines such as IFN- $\gamma$  and IL-1 $\beta$ , and TLR agonists such as lipopolysaccharide (LPS) induce the M1 phenotype, which is characterized by increased production of proinflammatory cytokines, e.g. IL-12, upregulated expression of MHC II, and enhanced generation of free radicals including NO (Gordon, 2003). M1 macrophages are considered to mediate host defence to infections, but also to cause autoimmune tissue damage (Martinez et al., 2008). M2 macrophages are induced by Th2 cytokines and express high levels of antiinflammatory molecules, e.g. IL-10, which underlines their role in antiinflammation and tissue repair (Martinez et al., 2008). A switch of macrophage phenotype from M1 to M2 by compound A, a plant origin ligand of glucocorticoid receptors, has been shown to attenuate EAN (Zhang et al., 2009a).

### **1.3.2 Th17 cells and Tregs in GBS and EAN**

#### **1.3.2.1 Th17 cells in GBS and EAN**

As a distinct Th cell population, Th17 cells have been shown to mediate inflammatory and autoimmune responses in both animal models and human diseases (Infante-Duarte et al., 2000). Th17 cells can induce local inflammation in the target organs and also aid B cells in production of antibodies (Doreau et al., 2009). Th17 cells mainly produce IL-17A, whose secretion is further stimulated by IL-23 in vitro (Aggarwal et al., 2003) and in vivo (Langrish et al., 2005). IL-6 in the presence of TGF- $\beta$ 1 can advance the differentiation of Th17 cells and enhance the secretion of IL-17 (Bettelli et al., 2006).

Retinoic acid-related orphan receptor (ROR) $\alpha$  and ROR $\gamma$ t (called RORC in humans) are crucial transcription factors for stimulating the differentiation of Th0 cells to Th17 cells (Hwang, 2010; Yang et al., 2007; 2008). However, IL-2, IL-4, IL-27, IL-35 and IFN- $\gamma$  suppress the differentiation of Th17 cells (Yang et al., 2008). In addition to IL-17A, activated Th17 cells secrete IL-6, IL-17F, IL-21, IL-22, TNF- $\alpha$  and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Yang et al., 2008; Duvallet et al., 2011), which induce massive tissue reactions by promoting the recruitment of inflammatory cells (Miossec et al., 2009; Leipe et al., 2010; Trifari et al., 2009).

In experimental autoimmune encephalomyelitis (EAE), an analogous experimental disorder of EAN in the central nervous system (CNS), Th17 cells, independent of Th1 cells, play an important pathological role in the progression of disease (Jadidi-Niaragh and Mirshafiey, 2011). More recently, we found a significantly increased proportion of Th17 cells in the peripheral blood and an elevated level of IL-17A in the plasma of patients with GBS during the acute phase (1-14 days after the onset of disease) (data have been submitted for publication). Moreover, the levels of IL-17A were correlated with the GBS disability scale scores. Furthermore, circulating Th17 cells and IL-17A concentrations were downregulated after intravenous Ig (IVIg) treatment (unpublished data). By using a synthesized inorganic compound that inhibits the production of Th17 cells, we found that EAN was attenuated when the compound was daily administered from the immunization day (unpublished data). Collectively, these findings suggest that Th17 cells and their effector cytokines are implicated in the pathogenesis of GBS and EAN.

### **1.3.2.2 Regulatory T cells in GBS and EAN**

Regulatory T cells (Tregs) are a subset of CD4<sup>+</sup> T cells expressing high levels of CD25 and the transcription factor forkhead box P3 (Foxp3). Tregs are crucial in maintaining immunological homeostasis and preventing autoimmunity by suppressing self-reactive T cells (Sakaguchi et al., 2006). Also Tregs abolish antigen-specific T cell proliferation and suppress secretion of Th1 and Th2 cytokines. In addition, increasing evidence suggests that Tregs are implicated in chronicity development of inflammatory diseases (Braga et al., 2011).

Tregs strongly control the T cell repertoire of healthy individuals that harbors self-reactive lymphocytes with a potential to cause autoimmune diseases (Hori et al., 2003b; Akdis 2006). Naturally occurring thymus-derived Tregs play a central role in the control of immune responses in autoimmune diseases, allergic disorders, infections, transplantation and cancer (Hori et al., 2003a; Sakaguchi, 2000; Takahashi et al., 2000). In addition to development in the thymus, Tregs cells can be differentiated or induced in vitro from CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>-</sup> T cells in the periphery with the aid of TGF-β supplementation (Chen et al., 2003).

In GBS, the number and the proportion of CD4<sup>+</sup>CD25<sup>+</sup> cells were decreased (Harness and McCombe, 2008; Pritchard et al., 2007). However, this decrease was reversible; presumably contributing to the monophasic self-limited course in GBS (Chi et al., 2007). A novel strain of P0-specific T cell receptor (TCR) Tg mice developed fulminant autoimmune polyneuropathy, associated with production of IFN-γ from P0-specific T cells and a lack of Tregs (Louvet et al., 2009). Li and colleagues found that atorvastatin, a lipid-lowering drug that bears antiinflammatory properties, ameliorated the clinical signs of EAN by increasing the number of Tregs in the mononuclear cell (MNC) population, and decreasing the levels of IFN-γ in MNC culture supernatants (Li et al., 2011).

### **1.3.3 Cytokines in GBS and EAN**

#### **1.3.3.1 IFN-γ**

As a signature cytokine for the Th1 response, IFN-γ is produced by NK cells and NKT cells as part of the innate immune response, and by effector Th1 cells and CD8<sup>+</sup> T cells once antigen-specific immunity develops (Schoenborn and Wilson, 2007). Cellular responses to IFN-γ are activated through its interaction with the IFN-γ receptor consisting of IFN-γ receptor 1 (IFNGR1) and IFN-γ receptor 2 (IFNGR2) (Pestka et al., 1997). IFN-γ exerts its proinflammatory role by activating endothelial cells, macrophages, T cells, and SCs, etc (Lu and Zhu, 2011). IFN-γ increases the expression of MHC II on macrophages and SCs (Schroder et al., 2004; Duan et al., 2007). The potent proinflammatory activities of IFN-γ combined with its inhibitory potential for the development of Th2 cells make IFN-γ a crucial mediator of Th1 mediated



autoimmune disorders by deflecting the immune response toward a Th1 phenotype. In addition, IFN- $\gamma$  induces B cell class switching, apoptosis of T cell, and enhancement of production of other proinflammatory cytokines such as TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 (Schroder et al., 2004).

The proinflammatory role of IFN- $\gamma$  in GBS and EAN has been extensively investigated (Nyati et al., 2011; Lu and Zhu, 2011). The serum level of IFN- $\gamma$  was elevated during the acute phase of GBS (Hohnoki et al., 1998). PBMCs spontaneously secrete IFN- $\gamma$  in 25% of patients with GBS, providing further evidence for a role of IFN- $\gamma$  in the immunopathogenesis of GBS (Csruhes et al., 2005). Treatment with antibodies against IFN- $\gamma$  was correlated with improved clinical outcome in patients with GBS (Elkarim et al., 1998). Administration of recombinant IFN- $\gamma$  markedly augmented both myelin-induced and T cell line-mediated EAN in rats; application of a monoclonal antibody to IFN- $\gamma$  suppressed the disease (Hartung et al., 1990; Hartung and Toyka, 1990). Moreover, IFN- $\gamma$  receptor deficient mice showed milder signs of EAN than wild type (WT) mice (Zhu et al., 2001). However, it has also been reported from other studies that there was no increase in IFN- $\gamma$  secreting PBMCs in GBS (Dahle et al., 2003). Press and colleagues studied the temporal profiles of IFN- $\gamma$ -secreting PBMCs in GBS and also found that the levels of IFN- $\gamma$ -secreting cells were not increased over the course of GBS (2001). This might be due to different pathomechanisms that contribute to pathologically heterogeneous GBS. Nevertheless, IFN- $\gamma$  has been found to be protective in EAE (Chu et al., 2000; Billiau et al., 1988; Ferber et al., 1996). Collectively, IFN- $\gamma$  in the pathogenesis of GBS and EAN should probably be considered to play an immunoregulatory role.

As mentioned above, the Th1/Th2 paradigm in the explanation of autoimmune disorders has been challenged with the discovery of Th17 cells. Functions of Th17 cells are inhibited by both Th1 and Th2 cytokines (Harrington et al., 2005). The protective or detrimental role of IFN- $\gamma$  is probably dependent on the balance among Th1, Th2 and Th17 cells as well as Tregs. Therefore the exact role of IFN- $\gamma$  in the pathogenesis of EAN requires reexamination in the context of Th17 cells.

### 1.3.3.2 TNF- $\alpha$

TNF- $\alpha$  is a Th1 cytokine that plays an important role in many aspects of immune system development, immunoregulation, and T cell mediated tissue injury (Mao et al., 2010a; Stübgen, 2008). TNF- $\alpha$  is generated as a transmembrane type II polypeptide precursor (tmTNF) that is expressed on activated macrophages, T cells, NK cells, and, to a lesser extent, on tissue cells, such as endothelial cells, smooth muscle cells, fibroblasts, astrocytes, neurons and SCs (Chaparro et al., 2012; Mao et al., 2010a). Soluble TNF- $\alpha$  (sTNF- $\alpha$ ) is released after cleavage of tmTNF- $\alpha$  by TNF- $\alpha$  converting enzyme (Mao et al., 2010a). Depending on binding to different TNF- $\alpha$  receptors (TNFRs), i.e. TNFR1 or TNFR2, TNF- $\alpha$  bears proinflammatory or antiinflammatory properties (Kassiotis and Kollias, 2001).

TNF- $\alpha$  has been identified as a key mediator in the pathogenesis of GBS and EAN (Stübgen, 2008). Polymorphism of TNF- $\alpha$  and its promoter has been associated with susceptibility to GBS (Prasad et al., 2010; Zhang et al., 2007; Wu et al., 2012; Jiao et al., 2012). Clinically, an increased level of TNF- $\alpha$  in serum has been correlated with the disease severity of GBS (Créange et al., 1996; Radhakrishnan et al., 2004; Deng et al., 2008). Moreover, serum levels of TNF- $\alpha$  decrease after immunomodulatory treatment and are in parallel with the clinical recovery of GBS (Sharief et al., 1993). TNF- $\alpha$  positive macrophages appear in peripheral nerves around the onset of EAN (Oka et al., 1998); expression of TNF- $\alpha$  mRNA in the PNS is upregulated at the peak of clinical EAN (Zhu et al., 1997). Injection of TNF- $\alpha$  into rat sciatic nerves resulted in inflammatory vascular changes within the endoneurium along with demyelination and axonal degeneration (Redford et al., 1995). Moreover, systemic administration of TNF- $\alpha$  markedly worsened EAN (Said and Hontebeyrie-Joskowicz, 1992). Conversely, treatment with monoclonal antibodies (mAb) against TNF- $\alpha$  or soluble TNFR1 ameliorated EAN (Stoll et al., 1993; Bao et al., 2003). Antiinflammatory compounds such as rolipram, linomide and leflunomide markedly inhibited cellular infiltration and downregulated production of TNF- $\alpha$  thereby suppressing the clinical signs of EAN (Korn et al., 2001; Zou et al., 2000a; Zhu et al., 1999).

The protective and deleterious effects of TNF- $\alpha$  may segregate at the level of its two receptors (Smith et al., 1994). TNFR1 KO mice were totally resistant to EAE,

exhibiting reduced antigen-specific proliferative responses and production of Th1 cytokines, whereas TNFR2 KO mice exhibited exacerbated EAE, enhanced production of Th1 cytokines, and enhanced macrophage and T cell infiltration (Suvannavejh et al., 2000). Previously we reported conflicting findings with regard to the role of TNFR1 in EAN by using TNFR1 KO mice (Lu et al., 2007; Mao et al., 2010b). The discrepancy has been discussed in the latter article (Mao et al., 2010b). Moreover, TNFR2 may compensate for the loss of TNFR1 in TNFR1 KO mice, since TNFR1 and TNFR2 are known to trigger overlapping intracellular signaling events, e.g. nuclear factor kappa B (NF $\kappa$ B) (Mao et al., 2010a). Nevertheless, further studies are still needed to clarify the roles of TNFR1 and TNFR2 in the pathogenesis of EAN.

TNF- $\alpha$  antagonists (infliximab, etanercept and adalimumab) are indicated for treatment of inflammatory rheumatic and bowel diseases (Thalayasingam and Isaacs, 2011; Peyrin-Biroulet, 2010). However, these drugs can induce a range of autoimmune diseases that attack the CNS and the PNS (Lozeron et al., 2009). The association between anti-TNF- $\alpha$  treatment and various disorders in the PNS such as GBS and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) has been reported in case histories and series (Stübgen, 2008; Shin et al., 2006). The neuropathy usually occurs after exposure to the anti-TNF- $\alpha$  agent and improves after withdrawal of medication (Hanaoka et al., 2008; Stübgen, 2008; Eguren et al., 2009). Although the underlying mechanism still remains to be investigated, it is postulated that chronic exposure of T cells to TNF- $\alpha$  in chronic inflammatory diseases uncouples TCR signal transduction pathways, leading to hyporesponsiveness of T cells (Cope, 2002). When exogenous TNF- $\alpha$  antagonists are administered, T cell proliferative responses and cytokine production might be enhanced (Stübgen, 2008). The prolonged administration of TNF- $\alpha$  antagonists is therefore thought to enhance autoimmune responses by altering antigen presentation by APCs, potentiating TCR signaling, and decreasing apoptosis of autoreactive T cells (Stübgen, 2008; Cope et al., 1997). Functions of Th17 cells are inhibited by both Th1 and Th2 cytokines (Harrington et al., 2005). TNF- $\alpha$  antagonists may lead to a rebound of Th17 cells, as seen in the treatment of collagen-induced arthritis with TNF- $\alpha$  and TNFR1 neutralizing antibodies (Notley et al., 2005). With emerging evidence pointing to a role of Th17 cells and IL-17 in the pathogenesis of GBS/EAN, the above mentioned rebound of Th17 cells after use of TNF- $\alpha$  antagonists may subsequently lead to an uncontrollable proinflammatory cascade, which may trigger the onset of GBS or CIDP.

### 1.3.3.3 IL-12 and IL-23

IL-12, mainly produced by macrophages and DCs (Xu et al., 2010), is critical for the differentiation of Th1 cells (Watford et al., 2003). IL-12 is a disulfide-linked heterodimer p70 complex composed of the p40 and p35 subunits. By promoting production of IFN- $\gamma$  and proliferation of NK and T cells, IL-12 mediates cellular immunity (Del Vecchio et al., 2007).

Enhanced expression of IL-12 and the IL-12 receptor was found on PBMCs during the acute phase of AIDP (Deng et al., 2008). IL-12 has a major role in the initiation, enhancement and perpetuation of pathogenic events in EAN by promoting a Th1 cell-mediated immune response and suppressing the Th2 response (Bao et al., 2002). The additional treatment of EAN in rats with recombinant IL-12 prolonged the course of EAN characterized by earlier onset and delayed recovery of the disease (Pelidou et al., 2000a). Moreover, IL-12 significantly increased the proliferation of lymph node MNCs in response to P0 peptide 180-199 stimulation and IFN- $\gamma$  production in the sciatic nerves of EAN (Pelidou et al., 2000a).

IL-23, a heterodimeric cytokine comprising the p40 subunit of IL-12 but a different p19 subunit (Oppmann et al., 2000), is involved in the differentiation of Th17 cells especially in the presence of TGF- $\beta$  and IL-6 (Korn et al., 2009). IL-23 preferentially acts on memory T cells (Lankford and Frucht, 2003). Activated macrophages express the IL-23 receptor and can be stimulated by IL-23 to produce IL-1 and TNF- $\alpha$ , as well as IL-23 per se (Duvallet et al., 2011). These effects identify IL-23 as a central cytokine in autoimmunity and a highly promising therapeutic target for inflammatory diseases (Duvallet et al., 2011). IL-23 rather than IL-12 is critically involved in various immune-mediated disorders (Cua et al., 2003; Coriely et al., 2008). IL-23 induces a population of IL-17 producing cells that are critically involved in the pathogenesis of EAE (Touil et al., 2006). Both IL-23p19 and IL-23 receptor KO mice are resistant to EAE (Cua et al., 2003).

IL-23p19 is detectable in CSF of GBS patients, and endoneurial macrophages are identified as the cellular source of IL-23p19 in sural nerve biopsies (Hu et al., 2006). IL-23p19 RNA was found to be upregulated in sciatic nerves from rats with EAN prior

to the onset of clinical symptoms, with peak expression levels preceding maximum disease severity, indicating that IL-23 is more important during the early effector phase of EAN (Hu et al., 2006).

#### **1.3.3.4 IL-10**

IL-10 is primarily produced by monocytes, macrophages, and different T cell subsets (Sabat et al., 2010a). Besides, DCs, B cells, NK cells, mast cells, neutrophils, eosinophils and SCs synthesize IL-10 (Sabat, 2010). As an antiinflammatory cytokine, IL-10 modulates macrophage and DC functions by suppressing production of cytokines such as IL-1 $\beta$  and TNF- $\alpha$ , repressing expression of costimulatory and MHC molecules, and inhibiting their antigen presenting functions (Sabat et al., 2010b). IL-10 can also promote MHC II expression on B cells and Ig production including IgA (O'Garra et al., 2008). Furthermore, IL-10 plays a crucial role for the development of Tregs (O'Garra et al., 2008).

In inflammatory and autoimmune diseases, IL-10 functions as limiting and terminating inflammatory responses and regulating the differentiation and proliferation of immune cells (Bashyam et al., 2007). IL-10 inhibits the proliferative responses of T cells in PBMCs to various antigens; however, it does not suppress the proliferative responses of T cells stimulated by anti-CD3 antibodies (Akdis et al., 2000). IL-10 exerts its inhibitory effect through blocking CD2 and CD28 in a rapid signal transduction cascade (Taylor et al., 2007).

IL-10 promoter polymorphism is associated with GBS, and IL-10 may promote disease, probably by increasing the ganglioside antibody response (Myhr et al., 2003). The number of PBMCs spontaneously secreting IL-10 is elevated during the acute phase of GBS (Press et al., 2001). Moreover, IL-10 responses to peripheral nerve antigens occur significantly more often in GBS patients (Makowska et al., 2008). Upregulation of IL-10 mRNA was noted in lymph node MNCs and sciatic nerves at the onset of EAN, which peaked at plateau (Zhu et al., 1997). This increase may account for the self-limited clinical course of GBS and EAN (Press et al., 2002). Furthermore, administration of a low dose of IL-10 permitted better regeneration of damaged axons

(Atkins et al., 2007) and treatment with recombinant IL-10 ameliorated the inflammatory responses in EAN (Bai et al., 1997).

#### **1.3.3.5 IL-4**

IL-4 is a typical antiinflammatory cytokine related to Th2 cells, basophils, mast cells, and NKT cells, with pleiotropic actions on different cell types (Sholl-Franco et al., 2009; Paul and Zhu, 2010). IL-4 exerts its biological activities through interaction with its cell surface receptor (Nelms et al., 1999). IL-4 inhibits the activation of Th1 cells, whereby decreasing the production of IFN- $\gamma$ , IL-1 $\beta$  and TNF- $\alpha$  (Wurtz et al., 2004). In addition to being an important effector cytokine in Th2 responses, IL-4 has a crucial role in the differentiation of Th2 cells in vitro (Le Gros et al., 2008; Swain et al., 1990). Through its action on STAT6, IL-4 can upregulate the expression of GATA-binding protein 3 (GATA3), the master regulator for Th2 cell differentiation (Zheng and Flavell, 1997; Zhang et al., 1997); STAT6 activation is necessary and sufficient for IL-4-mediated GATA3 upregulation and Th2 cell differentiation (Zhu et al., 2001; Kaplan et al., 1996).

IL-4 responses were observed during the plateau and recovery phases of GBS, suggesting a beneficial role of IL-4 in GBS, and that IL-4 may have a role in terminating the disease process in this self-limited inflammatory disease (Dahle et al., 1997). Hohnoki and colleagues investigated the levels of IL-4 in the sera of GBS patients and found elevated levels of IL-4 at the acute stage of GBS (1998). An increase of IL-4 during the recovery phase of EAN was shown by detection of cytokine mRNA expression in the PNS (Zhu et al., 1996). Intranasal administration of recombinant IL-4 ameliorated demyelination and progression of EAN (Deretzi et al., 1999). Rolipram, a phosphodiesterase type 4 inhibitor, was reported to have antiinflammatory effects; protective effect of rolipram in EAN was associated with downregulation of IFN- $\gamma$  and proinflammatory chemokines as well as upregulation of IL-4 in the PNS (Abbas et al., 2000).

### 1.3.3.6 IL-17

Thus far, six IL-17 family members have been revealed, namely IL-17A-F. IL-17A, which is mainly produced by Th17 cells, is the prototypic IL-17 family member. IL-17A exerts its actions as a homodimer or a heterodimer with IL-17F (Gaffen, 2008). IL-17A signals through IL-17 receptors (mainly IL-17RA and IL-17RC), whereby coordinating local tissue inflammation through upregulation of proinflammatory cytokines (IL-6, GM-CSF, TNF- $\alpha$  and IL-1), chemokines (CXCL1, CCL2 (MCP-1), CCL7 (MCP-3), CXCL2 (MIP-2), and CCL20 (MIP-3A)), and matrix metalloproteases (MMPs) (Ruddy et al., 2004; Zepp et al., 2011; Shen et al., 2008).

The blood level of IL-17A has been found elevated in multiple inflammatory and autoimmune diseases (Chen et al., 2012; Arican et al., 2005). IL-17A gene expression is significantly increased in inflammatory bowel disease and IL-17A positive cells are increased in the lamina propria and epithelium of patients with inflammatory bowel disease (Olsen et al., 2011). The frequency of Th17 cells is higher in CSF of patients with relapsing-remitting MS during the relapse phase (Brucklacher-Waldert et al., 2009). IL-17 was found in T cells (both CD4<sup>+</sup> and CD8<sup>+</sup>) and astrocytes of MS lesions (Tzartos et al., 2008). Treatment with anti-IL-17A antibodies attenuated EAE (Hofstetter et al., 2005). IL-17A KO mice (with normal level of IL-17F) showed milder disease of EAE than WT mice (Komiyama et al., 2006).

Plasma IL-17A and IL-22 levels were markedly elevated during the acute phase of GBS and IVIg therapy reduced IL-17A concentrations [unpublished data in our group]. A German group has reported a compound, FTY720 to attenuate EAN via reducing Th17 cells in the PNS (Zhang et al., 2009b). IL-17A has been observed to decrease with the ameliorated clinical severity of EAN when atorvastatin, a lipid-lowering drug with antiinflammatory properties is administered (Li et al., 2011). IL-17A is expressed in sciatic nerves of EAN and its expression is significantly decreased by AUY954 treatment (Zhang et al., 2009c). Both ROR $\alpha$  and ROR $\gamma$ t are key transcription factors for regulating Th0 cells to differentiate into Th17 cells (Yang et al., 2007; 2008). By using a synthesized inorganic compound that specifically inhibits ROR $\gamma$ t, we found that EAN was evidently attenuated (unpublished data in our group). Pelidou and colleagues in our group reported exacerbated acute phase of EAN via intranasal administration of

recombinant IL-17A, along with increased infiltration of inflammatory cells into the sciatic nerves and more severe demyelination (2000b).



## 2 AIMS OF THE STUDIES

To investigate the roles of apoE and proinflammatory cytokines in EAN.

### **Specific aims:**

**Study I:** To study the role of apoE isoforms in the pathogenesis of EAN;

**Study II:** To identify the effects of apoE isoforms on the functions of SCs in vitro;

**Study III:** To investigate the role of IFN- $\gamma$  in the pathogenesis of EAN;

**Study IV:** To explore the role of TNF- $\alpha$  in the pathogenesis of EAN.

## **3 MATERIALS AND METHODS**

### **3.1 ANIMALS**

ApoE 2, 3, and 4 Tg mice, IFN- $\gamma$  KO mice and TNF- $\alpha$  KO mice were purchased from Taconic (Taconic, Hudson, NY, USA, or Taconic, Ry, Demark). The apoE Tg mice had been generated to express human apoE2, apoE3 and apoE4, respectively, under the control of glial fibrillary acidic protein (GFAP) promoter, on apoE KO mice with the genetic background of C57BL/6 (Sun et al., 1998). The TNF- $\alpha$  KO mice and the IFN- $\gamma$  KO mice had been generated through targeted disruption of the TNF- $\alpha$  gene and the IFN- $\gamma$  gene, respectively, and being backcrossed to the C57BL/6 strain. Control animals were age- and sex-matched C57BL/6 mice (Scanbur, Karlslunde, Denmark). All mice were housed on a 12/12 light-dark schedule with water and food available ad libitum. Detailed accommodation and care complied with local legislations. Male mice, 5-6 weeks old, were used for induction of the EAN model.

### **3.2 ANTIGEN (PAPERS I, III AND IV)**

The neuritogenic peptide 180-199 (SSKRGRQTPVLYAMLDHSRS-amide) of the murine PNS myelin P0 protein, was synthesized by the 9-fluorenylmethoxycarbonyl solid-phase synthesis (Chan and White, 2000), purified by high-performance liquid chromatography (HPLC) using a Vydac reverse-phase column (Grace Vydac, Hesperia, CA, USA), and analyzed by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (Cambridge Research Biochemicals, Billingham, UK).

### **3.3 INDUCTION OF EAN AND OBSERVATION OF THE CLINICAL COURSE (PAPERS I, III AND IV)**

Mice were immunized twice (days 0 and 8) by subcutaneous injection of 150  $\mu$ g of P0 peptide 180-199 and 0.5 mg of Mycobacterium tuberculosis (strain H37 RA; Difco, Franklin Lakes, NJ, USA) in 25  $\mu$ l saline and 25  $\mu$ l Freund's incomplete adjuvant (ICN Biomedicals, Costa Mesa, CA, USA). Mycobacterium tuberculosis

plus Freund's incomplete adjuvant is referred to as Freund's complete adjuvant. Mice received 400, 300, and 300 ng pertussis toxin (PTX) (Merck, Whitehouse Station, NJ, USA) by intravenous injection on days -1, +1 and +3 post immunization (p.i.), respectively. Using a blinded protocol, two examiners assessed the clinical signs of EAN mice immediately before immunization (day 0) and thereafter every two or three days until day 60 p.i. The severity of EAN was scored as follows: 0, normal; 1, less lively, reduced tone of the tail; 2, flaccid tail; 3, abnormal gait; 4, ataxia; 5, mild paraparesis; 6, moderate paraparesis; 7, severe paraparesis; 8, paraplegia. The EAN model on mice was approved by the South Stockholm Research Animal Ethics Committee, Huddinge County Court, Stockholm, and the Animal Research Ethics Committee, Faculty of Medicine & Health Sciences, United Arab Emirates (UAE) University, Al Ain, UAE.

### **3.4 LYMPHOCYTE PROLIFERATION TEST (PAPERS I, III AND IV)**

Mice were sacrificed after perfused with phosphate-buffered saline (PBS) and spleens were removed. Single cell suspensions of MNCs were prepared and incubated in 25 cm<sup>2</sup> Falcon culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) with serum-free RPMI-1640 (Invitrogen, Carlsbad, CA, USA) for 1.5 h at 37 °C. To isolate purified T cells, non-adherent cells were collected and passed through a nylon wool column (Kisker, Steinfurt, Germany). T cells were obtained via depletion of nylon wool adherent cells. Phytohemagglutinin (PHA) (Gibco-Invitrogen, Grand Island, NY, USA), P0 peptide 180-199, anti-mouse-CD3 antibody (AbD Serotec, Kidlington, UK), IL-12 (eBioscience, San Diego, CA, USA) and IL-23 (eBioscience) were used as stimulants. The optimal concentrations were assessed in pilot experiments. After 60 h incubation, cells were pulsed with 1 µCi/well <sup>3</sup>H-methylthymidine (Amersham, Buckinghamshire, UK) and cultured for an additional 12 h. Cells were harvested onto glass fiber filters (Titertek, Lierbyen, Norway) by a cell harvester (Tomtec, Unterschleissheim, Germany). The incorporation of <sup>3</sup>H-thymidine was measured with a β-scintillation counter (Wallac, Turku, Finland) and results were expressed as counts per minute (cpm). The proliferation was alternatively assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Briefly, assays were performed by adding a small amount of the reagent directly to culture wells. The

solution contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)]. The MTS tetrazolium compound is bio-reduced into a colored formazan product soluble in cell culture supernatants by NADPH or NADH produced by dehydrogenase in metabolically active cells. After 4 h incubation, the absorbance at 490 nm was recorded with an ELISA reader (Tecan, Männedorf, Switzerland).

### **3.5 MACROPHAGE CULTURES (PAPERS I, III AND IV)**

Thioglycollate (Fluka, Milwaukee, WI, USA) elicited mononuclear cells (PEMs) were harvested for cultivation by standard lavage of peritoneal exudates with 10 ml serum-free culture medium DMEM/F12 (Invitrogen). PEMs were centrifuged at 300 x g for 10 min. The pellets were resuspended with DMEM/F12 supplemented with 5% (v/v) fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (both from Gibco-Invitrogen), and the cell concentration was adjusted to  $2 \times 10^6$  /ml. Macrophages were seeded into 5.3 cm Petri-dishes (Becton Dickinson) and stimulated with LPS (Sigma-Aldrich), recombinant mouse IFN- $\gamma$  (Hycult Biotechnology, Uden, The Netherlands), TNF- $\alpha$  (Sigma-Aldrich), polyinosinic-polycytidylic acid (poly I:C) (Sigma-Aldrich) or combinations thereof. After 24 h and 48 h incubation, the supernatants were collected and snap-frozen for cytokine and NO detection. After removal of the 48 h supernatant, 5 ml culture medium with Brefeldin A (3 µg/ml, eBioscience) was added. Six hours later cells were harvested for subsequent flow cytometric analysis.

### **3.6 SC CULTURES (PAPERS I AND II)**

Sciatic nerves were excised under aseptic conditions and put into ice-cold RPMI-1640 (Invitrogen). The epineurium was gently stripped off. Minced pieces were cultured in RPMI-1640 (Invitrogen) supplemented with 10% (v/v) FBS, 10 µg/ml transferrin, 10 µg/ml insulin, 0.01 µmol/ml non-essential amino acid (MEM), 0.2 µg/ml sodium selenite, 0.05 mg/ml L-ascorbic acid, 6 mg/ml glucose (all from Sigma-Aldrich) and 50 µg/ml gentamycin (Gibco-Invitrogen) for 5-6 days. Subsequently, the tissue fragments were digested with 1.25 IU/ml dispase, 0.05% (w/v) collagenase and 0.1% (w/v) hyaluronidase (all from Sigma-Aldrich). Cells were

then cultured in the aforementioned incomplete culture medium additionally supplemented with 2  $\mu$ M forskolin (Calbiochem, San Diego, USA) and cultured in poly-L-lysine (Sigma-Aldrich) and laminin (Invitrogen) coated Petri-dishes at 37 °C. The coating procedure was as previously described (Duan et al., 2007). After 5-6 days' culture, when approximately 80% cells were confluent, 100 ng/ml LPS plus 100 IU/ml recombinant mouse IFN- $\gamma$  were added to challenge the cells. After 72 h stimulation, SCs were harvested for subsequent experiments.

### **3.7 ISOLATION OF INFILTRATING CELLS IN CAUDA EQUINA (CE) (PAPERS III AND IV)**

Infiltrating cells in CE were isolated according to the previously described method (Duan et al., 2004). Briefly, CE fragments were carefully removed from PBS perfused mice, transferred to RPMI-1640, ground and passed through a 70  $\mu$ m cell nylon mesh (Becton Dickinson). The collected cells were suspended with 27% Percoll (Amersham Biosciences AB, Uppsala, Sweden) in PBS and centrifuged at 1000 x g for 30 min at 4 °C. The cell pellets were harvested for further analysis.

### **3.8 FLOW CYTOMETRY (PAPERS I-IV)**

FITC-, PE-, APC- and PerCP-conjugated antibodies were purchased from commercial suppliers. Cells (CE infiltrating cells, SCs, macrophages, splenic MNCs, or PEMs) were harvested and washed with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS. For staining of molecules with extracellular expression, cells were incubated with fluorochrome-conjugated antibodies for 15 min at room temperature (RT). For staining of molecules with intracellular expression, cells were fixed with 2% paraformaldehyde (Merck) for 15 min at RT and permeabilized with 0.5% freshly prepared saponin (Sigma-Aldrich) in PBS containing 1% BSA. The permeabilized cells were incubated with fluorochrome-conjugated antibodies for 15 min at RT. FITC-, PE-, APC- and PerCP-conjugated isotype antibodies were used as controls. Cells were washed twice, resuspended in 1% paraformaldehyde in PBS and stored at 4°C until flow cytometric analysis with an FACSCalibur or FACSCanto<sup>TM</sup> II cytometer (Becton Dickinson). Surface and intracellular molecule expressions were assessed by determining the positive cell percentage or the mean fluorescence intensity (MFI). Flow cytometric data

were analyzed with the CellQuest Pro software (Becton Dickinson) or FlowJo (TreeStar, Ashland, OR, USA).

### **3.9 ELISA (PAPERS I-IV)**

Standardized procedure for the sandwich ELISA was established after optimization of experimental parameters. Antibodies against mouse IL-4, IL-6, IL-10, IL-12, IL-17A, TNF- $\alpha$  and IFN- $\gamma$ , and recombinant mouse IL-4, IL-6, IL-10, IL-12, IL-17A, TNF- $\alpha$  and IFN- $\gamma$  as standards were purchased from eBioscience. Anti-human pan-apoE ELISA kit was purchased from Medical Biological Laboratories, Naka-ku Nagoya, Japan. Briefly, antibodies were coated onto standard ELISA plates (Nalge Nunc, Roskilde, Denmark) in a volume of 100  $\mu$ l/well overnight at 4°C. After three washings with PBS, uncoated sites were blocked with 200  $\mu$ l 10% FBS in PBS for 1 h at RT. Duplicates or triplicates of samples and recombinant standards were added and the plates were incubated for 1 h at RT. After washing, the plates were incubated with biotinylated detecting antibody (eBioscience) for 2 h at RT. Then avidin-conjugated horseradish peroxidase (HRP) (eBioscience) was added for 30 min. Color reaction was performed with 100  $\mu$ l of tetramethylbenzidine (TMB) (Sigma-Aldrich) for 30 min and the reaction was terminated by adding 2 M sulfuric acid (Sigma-Aldrich). The plates were immediately read at 450 nm with an ELISA reader (Tecan). The concentrations of proteins were quantified by extrapolation from the standard curve.

### **3.10 MEASUREMENT OF ANTI-P0 PEPTIDE ANTIBODIES IN SERA (PAPERS I, III AND IV)**

Sera were obtained from mice at the peak of EAN (day 28 p.i.). Samples of 8-10 mice in each group were pooled. P0 peptide 180-199 was coated onto standard ELISA plates (10  $\mu$ g/ml, 100  $\mu$ l per well) overnight at 4 °C. After three washings with PBS, uncoated sites were blocked with 200  $\mu$ l 10% FBS in PBS for 2 h at RT. After three washings, serum samples were diluted at 1:100 with 1% BSA in PBS, applied to plate wells and incubated for 1.5 h at RT. Then plates were incubated for 1 h with peroxidase-conjugated affinipure rabbit anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) after dilution at 1:5000 with 1% BSA in PBS. After three washings, the peroxidase substrate TMB

(Sigma-Aldrich) was added and 15-30 min later the reaction was terminated by adding 2 M sulfuric acid (Sigma-Aldrich). The plates were read at 450 nm using an ELISA reader (Tecan).

### **3.11 NO DETECTION (PAPERS I-IV)**

NO production was measured by detecting the supernatant levels of nitrite, the stable biological oxidation product of NO, by using the modified Griess reagent (Sigma-Aldrich). The detecting procedure was performed according to the manufacturer's instructions. The standard curve was obtained by using sodium nitrite (Sigma-Aldrich) solutions at concentrations of 9, 3, 1, 0.33, 0.11, 0.033, and 0 µg/ml.

### **3.12 WESTERN BLOTTING (PAPER II)**

Cultured SCs after 72 h stimulation were harvested and proteins were obtained. The protein samples were boiled at 100 °C for 10 min and centrifuged at 20000 x g for 10 min. Protein concentrations were quantified by using the bicinchoninic acid (BCA) protein assay kit (Bio-Rad Laboratories AB, Sundbyberg, Sweden). For protein separation, each sample was electrophoresed through a 12% polyacrylamide gel and transferred to nitrocellulose membranes. After blocking with 5% non-fat dry milk, the membranes were incubated with primary antibodies (rabbit anti-mouse NFκB, or rabbit anti-mouse Akt, BD Biosciences) (1:100) and then peroxidase-conjugated goat anti-rabbit antibodies (BD Biosciences). Enhanced chemiluminescent (ECL) Western blotting detection reagents (Bio-Rad Laboratories AB) were used for exposure according to the manufacturer's instructions. Densitometric analysis was performed using the ImageJ program (NIH, Bethesda, MD, USA).

### **3.13 INOS BLOCKING (PAPER I)**

1400W (Sigma-Aldrich), a specific inducible nitric oxide synthase (iNOS) inhibitor was dissolved in PBS and administered intraperitoneally at a dose of 3 mg/(kg body weight) every third day from day 1 p.i. till day 53 p.i. Five mice from each strain received 1400W treatment, and 5 mice from the same strain received PBS as controls.

### **3.14 TNFR1 BLOCKING (PAPER IV)**

Anti-TNFR1 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were administered intravenously to WT mice ( $n = 5$ ) with EAN at a dose of 200  $\mu\text{g}/(\text{kg}$  body weight) on days -1, +3 and +7 p.i., respectively. Another group of age- and sex-matched WT mice ( $n = 5$ ) as controls received 0.9% saline. In another independent experiment, the TNFR1 blocking was performed in both TNF- $\alpha$  KO and WT mice according to the same protocol ( $n = 5$  in both groups).

### **3.15 STATISTICS (PAPERS I-IV)**

Data have been expressed as mean value  $\pm$  standard deviation (SD). The Statistical Package for the Social Sciences 14.0 (SPSS, IBM, West Grove, PA, USA) was used for analyzing all data. One-way analysis of variance (ANOVA) and Kruskal-Wallis test were used to compare values among groups followed by Student's t-test or Mann-Whitney u-test to compare values between groups. All tests were two-tailed, with the level of significance set to  $p < 0.05$ .



## 4 RESULTS AND DISCUSSION

### 4.1 STUDY I: APOE E3 ATTENUATES EAN BY MODULATING T CELL, MACROPHAGE AND SCS FUNCTIONS

Clinical signs of EAN were most severe in C57BL/6 mice and apoE4 Tg mice, followed by apoE2 Tg mice, with the least severe EAN in apoE3 Tg mice ( $WT \approx E4 > E2 > E3$ ). At nadir of EAN, spleen weight and lymphocyte proliferation were in line with the clinical severity of disease. The proliferation of purified T cells from naïve mice stimulated with PHA or IL-12 showed isoform-specific differences ( $WT \approx E4 > E3 \approx E2$ ). Macrophages from both naïve and EAN mice produced NO upon inflammatory stimulation with LPS, IFN- $\gamma$ , poly I:C or combinations thereof, in an isoform-dependent manner ( $WT \approx E4 > E2 > E3$ ). Generalized intervention with 1400W, a specific iNOS inhibitor, significantly suppressed the clinical course of EAN in apoE2, E3 and E4 Tg mice, as well as in WT mice. During the recovery stage, the expression of CD178 (FasL) was found the highest on SCs of apoE3 Tg mice.

In vitro, apoE can inhibit the proliferation of antigen- and mitogen-stimulated T cells, which is probably mediated by the modification of IL-2 receptor or modification of intracellular signaling pathways (Kelly et al., 1994; Bocksch et al., 2001; Getz and Reardon, 2009). Our data suggest an isoform-dependent suppressive effect of apoE on T cell proliferation, stimulated either unspecifically by antibodies against CD3, or specifically by P0 peptide 180-199, with apoE3 being the most potent suppressor. Since EAN is mainly a T cell mediated experimental disorder of the PNS, the differential proliferation capacity as determined by apoE genotype may well explain the distinct clinical severity of EAN in different stains of mice.

Our findings regarding NO production by macrophages upon inflammatory stimulation in naïve apoE Tg mice are in accordance with a report of Colton and co-workers (Colton et al., 2002) and point to an immunoregulatory dysfunction in apoE4 carriers. ApoE4 Tg mice appear to deal with inflammatory insults in an overcompensatory manner (Zhang et al., 2010b). This in our present study, is evidenced by increased production of both NO and IL-10. The enzyme iNOS is pivotal for NO production by

macrophages in response to inflammatory insults. The expression of iNOS by PEMs was lower in apoE3 and apoE2 Tg EAN mice as compared with apoE4 Tg and WT EAN mice, which were correlated well with the clinical severity in each mouse strain. This suggests that apoE3 can alleviate EAN by inhibiting the upregulation of iNOS expression. Additionally, SCs from apoE3 Tg EAN mice expressed lower levels of iNOS upon inflammatory stimulation than apoE2 Tg EAN mice, which seems to reflect the severity of local immune responses and peripheral nerve damage. Innate immunity such as NO production by macrophages might play a role in the isoform-dependent effects of apoE on the pathogenesis of EAN. In order to address whether the differential expression of iNOS and production of NO can explain the apoE-isoform-dependent clinical severity of EAN, we used 1400W to specifically suppress the function of iNOS and the production of NO in EAN. Systemic administration of 1400W before the onset of EAN markedly suppressed the clinical severity of EAN in all the four strains of mice. This indicates that innate immunity such as NO production might not play a key role in the isoform-dependent effects of apoE on the pathogenesis of EAN.

ApoE may modulate inflammatory responses in a dose-dependent manner (Riddell et al., 2008; Vitek et al., 2009). We found that intracellular production and extracellular levels of apoE by SCs and PEMs, as well as serum levels of apoE were comparable among the three strains of mice. Thus the possibility of a confounding dose-effect relationship has been ruled out.

In summary, we explored the isoform-specific effects of apoE on the pathogenesis of EAN and found that apoE3 can inhibit the onset, suppress the clinical severity and promote the recovery of EAN.

#### **4.2 STUDY II: APOE ISOFORM-SPECIFIC EFFECTS ON CYTOKINE AND NO PRODUCTION FROM MOUSE SCS AFTER INFLAMMATORY STIMULATION**

Upon stimulation with LPS plus IFN- $\gamma$ , a change in the morphology of cultured SCs was observed. Pronounced production of IL-6 and IL-10 within SCs, and increased levels of IL-6 and NO in the culture supernatants were found in an isoform-dependent manner (apoE3 > apoE2  $\approx$  apoE4). Further results indicated that both NF $\kappa$ B and Akt

signaling pathways were involved in the process by the same isoform-dependent pattern. However, the expression of costimulatory molecules that reflects the antigen presenting capacity of SCs was not significantly different among these groups. Taken together, SCs respond to inflammatory stimuli by increasing the production of IL-6, IL-10 and NO in an apoE-isoform-dependent manner. SCs from apoE2 and apoE4 Tg mice seem to bear some dysfunction in producing cytokines (IL-6 and IL-10) and NO as compared with their apoE3 counterparts, probably resulting from their insufficiency to suppress the activation of NF $\kappa$ B and Akt pathways.

IL-6/STAT3 signaling pathway targets GFAP, which is pivotal for proper regeneration of peripheral nerves (Lee et al., 2009). Although the positive percentage of IL-6 expressing SCs was low, the results from ELISA detecting the proteins concentrations in the culture supernatants were confirmatory. ApoE4 Tg mice resemble apoE-deficient mice with regard to an insufficiency to deal with inflammatory insults (Zhang et al., 2010b). Since there is only one isoform of apoE in rodents, which resembles human apoE3 (Vitek et al., 2009), our findings that apoE3 can upregulate the production of IL-6, along with the previous findings that apoE deficiency results in downregulated expression of intracellular IL-6 (Duan et al., 2007), further imply an isoform-dependent effect of apoE on SCs in terms of modulating local inflammatory responses, in which IL-6 may play a pivotal role.

As an antiinflammatory cytokine, IL-10 functions as regulating the differentiation and proliferation of immune cells and limiting/terminating inflammatory responses (Bashyam et al., 2007). The enhanced production of IL-10 by SCs may arise from their protective responses to local inflammation, possibly leading to the limitation and termination of inflammatory responses by regulating the differentiation and proliferation of Th2 cells.

The downstream gene expression induced by TLR4, such as NF $\kappa$ B, is the typical signaling pathway during LPS treatment. ApoE can regulate inflammatory responses by preventing the activation of NF $\kappa$ B (Singh et al., 2008). Our findings, along with previous ones, indicate that SCs from apoE2 and apoE4 Tg mice bear some insufficiency to suppress the activation of the NF $\kappa$ B pathway whereby modulating inflammatory responses less sufficiently than their apoE3 counterparts. The Akt

pathway was suggested to negatively regulate LPS stimulation in macrophages (Luyendyk et al., 2008). The higher levels of Akt expression by SCs in apoE2 Tg mice may probably reflect a late-onset approach to compensate the insufficiency in dealing with proinflammatory challenges. Alternatively, apoE3 may increase the production of cytokines by SCs via suppressing the Akt pathway.

In summary, SCs respond to inflammatory stimuli in an apoE-isoform-dependent fashion. ApoE3 may inhibit inflammation in the PNS via modulating cytokine (especially IL-6 and IL-10) and NO production in SCs, whereas SCs from apoE2 and apoE4 Tg mice seem to bear a certain insufficiency in producing cytokines and NO in response to inflammatory stimuli.

#### **4.3 STUDY III: IFN- $\gamma$ DEFICIENCY EXACERBATES EAN IN MICE DESPITE A MITIGATED SYSTEMIC TH1 IMMUNE RESPONSE**

The clinical signs of EAN in IFN- $\gamma$  KO mice were significantly more severe than those of the WT controls. The proliferation of splenic MNCs was significantly higher in IFN- $\gamma$  KO than WT mice with EAN after antigenic stimulation. At the peak of EAN, the proportion of IL-17A expressing cells in CE infiltrating cells, and the levels of IL-17A in sera were elevated in IFN- $\gamma$  KO mice when compared with their WT counterparts. The proportions of MHC II, macrosialin, and IL-12/IL-23p40 expressing cells, relative to total CE infiltrating cells were correspondingly higher in IFN- $\gamma$  KO than WT mice with EAN. However, IFN- $\gamma$  deficiency reduced the production of NO by cultured macrophages in response to proinflammatory stimuli and induced a systemic Th2-oriented immune response.

We initially hypothesized that IFN- $\gamma$  deficiency might lead to an amelioration of the Th1 immune response, thereby attenuating the clinical severity of EAN. However, neither was the clinical course of EAN shortened, nor was the severity of EAN attenuated, although a mitigated Th1 immune response was remarkable in IFN- $\gamma$  KO mice, evidenced by a reduced production of NO by cultured macrophages, and lower production of Th1 cytokines including IL-6 and IL-12, as well as reduced levels of anti-P0 peptide 180-199 IgG2a in sera. We thus postulated that the IL-17A and Th17 axis, in addition to IFN- $\gamma$  may play a pathogenetic role in EAN.

We then focused on the alteration of IL-17A production and found that IFN- $\gamma$  deficiency induced increased infiltrating of Th17 cells in CE during EAN. In addition, IFN- $\gamma$  deficiency enhanced the proportions of MHC II and macrosialin expressing cells in CE of mice with EAN. Functions of Th17 are inhibited by both Th1 and Th2 cytokines (Harrington et al., 2005). Here, the protective role of IFN- $\gamma$  in EAN might be due to its inhibition of Th17 development, since IFN- $\gamma$  can prevent IL-23 triggered expansion of Th17 cells (Harrington et al., 2005). Moreover, IFN- $\gamma$  increases T-bet expression, the overexpression of which may in turn lead to a robust reduction of IL-17 generation (Mathur et al., 2006). Of note is that in line with the traditional Th1/Th2 paradigm, IFN- $\gamma$  deficiency induced a systemic Th2-oriented immune response, which may be beneficial to EAN. However, the pathophysiological effects resulting from the upregulated levels of IL-17A may outweigh the effects of the Th2-oriented immune response, and thus lead to the more severe clinical symptoms of EAN in IFN- $\gamma$  deficient mice. An alternative explanation is that the exacerbated Th2 response may result in the generation of a pathogenic humoral response, as seen in many antibody-mediated autoimmune disorders (Sheikh et al., 2004). We excluded this possibility on the one hand because EAN is a well-accepted Th1-mediated disease, and on the other hand because an aberrant level of Th2-associated IgG1 antibody was absent in IFN- $\gamma$  deficient mice with EAN.

More recently, we found a significantly increased proportion of Th17 cells in the peripheral blood and a higher level of IL-17 in the plasma of GBS in the acute phase (1-14 days after the onset of disease); the level of IL-17 was correlated with the GBS disability scale score (data submitted for publication). Moreover, circulating Th17 cells and IL-17 concentrations were downregulated after IVIg treatments. By using a synthesized inorganic compound that specifically inhibits ROR $\gamma$ t and Th17 cells, we found that EAN was significantly attenuated when the compound was administered from the immunization day (our unpublished data). These findings suggest that Th17 cells and their effector cytokines may be involved in the pathogenesis of GBS and EAN. Although the mechanism of action of IL-17A in EAN remains unclear, IL-17A mainly acts as a proinflammatory cytokine that upregulates the expression of inflammatory genes including proinflammatory chemokines, hematopoietic cytokines, acute phase response genes and antimicrobial substances in neutrophils, macrophages and endothelial cells (Shen et al., 2008; Zepp et al., 2011).

In summary, IFN- $\gamma$  deficiency exacerbated the clinical severity of EAN, concomitant with higher production of IL-17A and Th2 cytokines. Our results point to a modulatory role of IFN- $\gamma$  in EAN.

#### **4.4 STUDY IV: ATTENUATED EAN IN TNF-A DEFICIENT MICE IS ASSOCIATED WITH AN ALTERED BALANCE OF M1/M2 MACROPHAGES**

The onset of EAN in TNF- $\alpha$  KO mice was markedly delayed compared to WT mice. From day 14 p.i., the clinical signs of TNF- $\alpha$  KO mice were significantly milder than those of their WT counterparts. The clinical signs of WT mice treated with TNFR1 antibodies was less severe than of the control WT mice receiving PBS. TNF- $\alpha$  deficiency induced an antiinflammatory phenotype of macrophages (M2) characterized by reduced production of IL-12 and NO, and enhanced production of IL-10. Increased ratio of Tregs and reduced production of IFN- $\gamma$  in infiltrating cells in CE were found in TNF- $\alpha$  KO mice with EAN.

TNF- $\alpha$  signals via TNFR1 and TNFR2 to elicit a wide variety of partly opposite reactions in multiple cell types (Smith et al., 1994). In the present study, TNF- $\alpha$  KO mice were not completely resistant to EAN induction. The reason could be that TNF- $\alpha$  is not a uniquely necessary inflammatory molecule for the induction of EAN. To further elucidate which receptor is responsible for the detrimental effect of TNF- $\alpha$ , we blocked the functions of TNFR1 in WT mice with EAN by using anti-TNFR1 monoclonal antibodies. The clinical severity of EAN was markedly mitigated after administration of the TNFR1 antagonist. However, this might also be due to the beneficial role of TNF- $\alpha$  acting via TNFR2. We then blocked the functions of TNFR1 in both TNF- $\alpha$  KO and WT mice with EAN and corroborated the detrimental role of TNFR1 in EAN.

Activated macrophages can be divided into two distinct subsets: classically activated macrophages (M1) and alternatively activated macrophages (M2) (Martinez et al., 2008). Proinflammatory Th1 cytokines such as IFN- $\gamma$  and IL-1 $\beta$ , and TLR agonist such as LPS and poly I:C induce the M1 phenotype, which is characterized by increased production of proinflammatory cytokines, e.g. IL-12, upregulated expression of MHC

II, and enhanced generation of free radicals including NO (Gordon, 2003). We found a reduced ratio of IL-12/IL-10 in CE infiltrating cells and a lower level of IL-12 in unstimulated PEMs from TNF- $\alpha$  KO mice with EAN, indicating an antiinflammatory M2 phenotype of macrophages resulting from TNF- $\alpha$  deficiency.

We purified and cultivated macrophages in vitro and manipulated the culture milieu by adding various inflammatory stimuli. The proportion of more activated macrophages (higher levels of IL-6 and IL-12 expression) was lower in naïve than in EAN mice and lower in TNF- $\alpha$  KO than in WT mice with EAN. After in vitro cultivation with proinflammatory stimulation, reduced levels of IL-12 and IL-6 and increased levels of IL-10 were produced by macrophages from TNF- $\alpha$  KO mice compared with those from WT mice with EAN. Moreover, reduced production of NO was detected in culture supernatants of macrophages from TNF- $\alpha$  KO mice after proinflammatory challenge. The reduced production of NO in TNF- $\alpha$  KO mice may explain the attenuated clinical severity of EAN (Zhang et al., 2010a).

In summary, TNF- $\alpha$  deficiency and TNFR1 blockade remarkably attenuated the clinical severity of EAN and TNF- $\alpha$  deficiency induced an antiinflammatory phenotype of macrophages (M2).

## 5 CONCLUSIONS

- 1) ApoE3 attenuates EAN by modulating T cell, macrophage and Schwann cell functions.
- 2) Schwann cells respond to inflammatory stimuli in vitro by increasing production of IL-6, IL-10 and NO in an apoE-isoform-dependent manner.
- 3) IFN- $\gamma$  deficiency exacerbates EAN via upregulating Th17 cells despite a mitigated systemic Th1 immune response.
- 4) Attenuated EAN in TNF- $\alpha$  deficient mice is associated with an altered balance of M1/M2 macrophages.



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