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Inflammation and cytokine production in experimental neuroinflammatory disorders

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ABSTRACT

Glutamate excitotoxicity is involved in the pathogenesis of a variety of neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). Kainic acid (KA), an analogue of excitotoxic glutamate, can elicit selective neuronal death in the brain of rodents, of which the pathological changes partially mimic the neurodegenerative disorders in humans. Experimental autoimmune neuritis (EAN) is an immune-mediated acute inflammatory disease of peripheral nervous system (PNS) and shares many characteristics of human Guillain-Barré syndrome (GBS). Thus EAN is considered to represent as an animal model to study pathogenesis and therapy of human GBS.

Cytokines play a key role in neuroinflammatory disorders. In our studies, we attempted to explore the possible roles of tumor necrosis factor (TNF)-α and interferon gamma (IFN-γ) in experimental neurodegenerative and neuroinflammatory disorders.

In **Paper I**, the role of TNF-α in KA-induced hippocampal neurodegeneration was studied by comparing TNF-α knockout (KO) mice with C57BL/6 wild type (WT) mice. After KA treatment, TNF-α KO mice showed more sensitivity to KA-induced neurotoxicity than WT mice, as demonstrated by more severe seizures, measurable behavior changes, greater neuronal degeneration and enhanced glial cell activation, as well as nitric oxide (NO) production. Additionally, KA-treatment up-regulated the expression of nuclear factor kappa B (NFκB) in TNF-α KO mice to a greater degree as compared to that in KA-treated WT mice. In **Paper II**, we aimed to further clarify the protective role of TNF-α in KA-induced hippocampal neuronal death in vitro and elucidated the potential signaling pathways. After 24-hours treatment with KA, comparing with WT mice, TNF-α KO mice showed more susceptibility to KA-induced neurotoxicity, as demonstrated by higher expression of lactate dehydrogenase (LDH) and lower neuronal survival rates, as well as elevated NO production. It is also evidenced that pretreated with anti-TNF-α antibody increased the production of LDH and NO, and decreased the neuronal survival rate. In contrast, neurons from WT mice pretreated with recombinant TNF-α were more resistant to KA induced neurotoxicity. TNF-α deficiency induced down-regulation of phospho-IκBα, total AKT and phospho-AKT, as well as up-regulation of phospho-p38 MAPK expressions after KA treatment. The reverse results can be achieved in WT hippocampal neurons with TNF-α treatment, i.e. up-regulation of phospho-IκBα and AKT. In **Paper III,** to further explore the role of TNF-α in the pathogenesis of neuroinflammation, the animal model-EAN was introduced. TNF-α deficiency significantly attenuated the clinical signs of EAN. Further, anti-TNF-α receptor 1 (TNFR1) antibodies markedly suppressed the clinical severity of EAN. TNF-α deficiency down-regulated the production of interleukin (IL)-12 and NO, as well as enhanced the production of IL-10 in macrophages. In **Paper IV,** the role of IFN-γ in the pathogenesis of EAN was investigated. The clinical signs of IFN-γ KO EAN mice were aggravated when compared with WT EAN mice. At the peak of EAN course, the IL-17A expressing cells in cauda equine (CE) and the levels of IL-17A in sera were elevated in IFN-γ KO mice. The proportions of MHC II, macrosialin, and IL-12 expressing cells, relative to total CE infiltrating cells were correspondingly higher in IFN-γ KO than WT mice with EAN.

In summary, TNF-α may play a protective role in KA-induced excitotoxic neurodegeneration, while TNF-α exacerbates EAN via TNFR1 by inducing the proinflammatory phenotype of macrophage. IFN-γ deficiency enhanced the clinical severity of EAN via upregulating of IL-17A and Th2 cytokines production. These findings have relevance for future studies on pathogenesis and treatment of neurodegenerative and neuroinflammatory disorders in humans.

LIST OF PUBLICATIONS

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Paper I- IV

List of abbreviations

AD	Alzheimer's disease
AIDP	acute inflammatory demyelinating polyneuropathy
ANOVA	one-way analysis of variance
BBB	blood brain barrier
BNB	blood nerve barrier
CA	cornu ammonis
CE	cauda equina
CNS	central nervous system
Con A	concanavalin A
COX	Cyclooxygenase
CSF	cerebrospinal fluid
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
ELISA	enzyme-linked immunosorbent assay
GBS	Guillain-Barré syndrome
GFAP	glial fibrillary acidic protein
IFN-γ	interferon-γ
IL	Interleukin
KA	kainic acid
KO	knockout
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
MCP	monocyte chemotatic protein
MHC	major histocompatibility complex
MNC	mononuclear cells
MS	multiple sclerosis
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFκB	nuclear factor kappa B
NO	nitric oxide
NGF	nerve growth factor
NMDA	N-methyl-D-aspartate
PBS	phosphate-buffered saline
PEMs	peritoneal exudates mononuclear cells
PD	Parkinson's disease
p.i.	post immunization
Poly I:C	Polyinosinic-polycytidylic acid
PNS	peripheral nervous system
ROS	reactive oxygen species
ROR	retinoic acid-related orphan receptor
RNS	reactive nitrogen species
TCR	T cell receptor
Th	T helper
TLR	Toll like receptor
TNF	tumor necrosis factor
TNFR	TNF receptor
Treg	regulatory T cells
WT	wild-type

1 INTRODUCTION

1.1 KAINIC ACID INDUCED EXCITOTOXIC NEURODEGENERATIVE ANIMAL MODEL

Excitotoxicity is involved in the pathogenesis of various neurodegenerative disorders in the humans' central nervous system (CNS), including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). Kainic acid (KA), an analog of excitotoxic glutamate, can elicit selective neuronal death in the brain of rodents, of which the pathological changes partially mimic the neurodegenerative disorders of humans in the CNS (Pollard et al. 1994). Systemic (intravenous or intraperitoneal), intranasal, or local administration of KA result in a series of clinical manifestations and pathological changes in rodents, such as recurrent seizures, behavioral changes, oxidative stress production including the generation of reactive oxygen species (ROS), reactive nitrogen species (RNS), hippocampal neuronal death and glial cells activation, etc (Wang et al. 2005b). Thus, KA-induced neurodegeneration in rodents has been used as a model for exploring the pathogenesis of excitotoxicity in neurodegenerative disorders in humans (Zheng 2011).

1.1.1 Generic characteristics of KA and KA receptors

KA ($C_{10}H_{15}NO_4$) was originally isolated from the seaweed called "Kaininsou" or "Makuri". KA has been used as an anthelminthic compound to remove worms from gut. Subsequent studies indicated that KA is a nondegradable analog of glutamate and 30-fold more potency in neurotoxicity than glutamate (Vincent and Mulle 2009). This neuroexcitant can bind to the kainate receptors (KARs). With activation of KARs, KA induces a number of cellular events, including the increase of influx of cellular Ca^{2+}, production of reactive oxygen species (ROS), and mitochondrial dysfunction leading to neuronal apoptosis and necrosis (Wang et al. 2005b). KA has been extensively used as a specific agonist to mimic the effect of glutamate on neurodegenerative models, and KARs mediate most of effects of kainate to induce seizures and cause excitotoxic neuronal death. The KARs family is divided into two subfamilies, including glutamate receptors (GluRs) 5-7 and KAR1-2.

1.1.2 Features of KA-induced neurodegenerative animal model

In rodents, systemic administration of KA leads to a well-characterized seizure syndrome (Ben-Ari 1985; Chen et al. 2002; Mulle et al. 1998). During the first 20-30 minutes after KA administration, the rodents show "staring" spells, followed by head nodding. Then, they stand upright and fall down, with numerous wet-dog shakes for around 30 minutes. One hour after KA treatment, the animals start to present with recurrent limbic motor seizures. In the following 1-2 hours, the limbic seizures develop into status epilepticus (Chuang et al. 2004).

KARs are most abundant in CA3 region of hippocampus and the activation of KARs can increases glutamate efflux (Rodriguez-Moreno and Sihra 2004; Sari E. Lauri 2001). Systemic injection of KA produced extensive neuronal death, primarily within the hippocampus hilus, CA3, and CA1 areas (Lee et al. 2010). However, high dose of KA can also induce neurotoxicity in the medial amygdaloid nuclei. After KA administration, selective damage in the brain of rodents shows behavioral changes in many behavioral tests, including the water maze, the object exploration tasks, Y-maze test, elevated plus maze and open-field test due to spatial learning deficits or depression changes (Gobbo and O'Mara 2005; Groticke et al. 2008; Ratte and Lacaille 2006).

1.1.3 Neurodegenerative animal model induced by intranasal administration of KA: our experience

In the past ten years, our lab developed a model of KA-induced neurodegeneration by intranasal administration of KA into C57BL/6 mice (Chen et al. 2005; Chen et al. 2002). C57BL/6 mice are widely used strain for transgenic studies, while they are resistant to systemic administration of KA (intravenous or intraperitoneal). Our intranasal administration of KA present almost same seizure activity as systemic injection of KA, which provides a valuable tool to explore the role of excitotoxicity in neurodegeneration using transgenic mice with C57BL/6 background. As to behavioral changes, intranasal administration of KA induced elevated levels of spontaneous activity in the open-field test and Y-maze test (Chen et al. 2002; Zhang et al. 2007b). We also found that KA treatment changed the degree of anxiety in the elevated plus maze (Zhang et al. 2013b). We have several speculations to clarify the mechanism as

regards how KA induces excitotoxic neurodegeneration through intranasal administration. Firstly, KA may reach the hippocampus via passive diffusion (Illum 2000). Secondly, protracted postsynaptic stimulation by activating KARs in the olfactory nerves and olfactory bulb can be relayed through projections to the hippocampus and thus result in neuronal damage (Montague and Greer 1999). Thirdly, since the cerebrospinal fluid (CSF) has direct connection with the olfactory bulb, KA may directly enter the CSF via the nasal cavity (Mathison et al. 1998). Additionally, small molecular weight of less than 20 kDa drugs can directly transported from the nasal cavity to the CSF (Sakane et al. 1995). Finally, KA may reach the hippocampus via the blood circulation (Genter et al. 2009).

1.2 INFLAMMATION IN NEURODEGENERATIVE DISORDERS

Neuroinflammatory process is a defense mechanism aimed at protecting the nervous system from infectious insults and injury. Inflammation typically occurs as an acute response to injury and might play a benefit role in tissue repairing during recovery phase, It has been evidenced that inflammation is strongly associated with glial (microglia and astrocyte) activation and expression of proinflammatory mediators in the CNS (Streit 2010). Thus, whether inflammatory reaction is helpful or harmful, which remains largely unknown. Neuroinflammation is involved in a wide variety of neurological diseases, such as PD and AD, and may also associate with stroke (Hirsch et al. 2005; McGeer and McGeer 2003; Zheng et al. 2003). For example, neuroinflammation may accelerate amyloid deposition that can activate microglia, producing a deleterious positive feedback loop in AD patients (von Bernhardi 2007).

Traditionally, CNS was regard as “immune privileged” area. A major reason for this view is due to exist of blood brain barrier (BBB). A decade ago, investigations have been revealed an active interaction of the CNS with the immune system through cytokines and other inflammatory molecular (McGeer and McGeer 2001). Among the resident brain cells, microglia and astrocytes, as well as neurons are able to secrete cytokines that modulate the neurons after activated by various stimuli and insult (Schinder et al. 1996) .

1.2.1 Glial cell activation in inflammation

Glial cells, particularly microglia and astrocytes, produce cytokines and chemokines, as well as express their receptors. The activation of glial cell is accompanied with neuronal death, characterized by clustering of activated microglia and astrocytes in the injured hippocampal lesions (Chen et al. 2005; Ravizza et al. 2005).

1.2.1.1 Microglia

Microglia is the primary immunocompetent cells and main effector cells of the inflammatory responses in the CNS after injury, and interacts with other gliocytes and neurons (Mack et al. 2003). Microglia exert their functions through interacting with extracellular matrix composition under physiological conditions, whereas microglia may become activated and secrete a great amount of proinflammatory cytokines, chemokines, complements, and so forth under pathophysiological conditions (Mack et al. 2003). Microglia acts as phagocyte in response to injury. Some studies suggested that activated microglia may play a neuroprotective role in MS and its animal model, experimental autoimmune encephalomyelitis (EAE) by facilitating reparatory and regenerative processes (Napoli and Neumann 2010). However, in several CNS neurodegenerative diseases, such as AD and PD, microglia may initiate and exacerbate neuropathological changes through secreting proinflammatory and cytotoxic molecules (Marinova-Mutafchieva et al. 2009; Venneti et al. 2009). Activated microglia express major histocompatibility complex (MHC) class I, MHC class II, and costimulatory molecules, produce complements, cytokines (IL-1, IL-6, IL-12, IL-18, TNF-α, etc.), chemokines, ROS, and RNS and secrete proteases and excitatory amino acids, which contribute to the neuronal death (Mack et al. 2003; Penkowa et al. 2001; Walz 2000). The activation of microglia may serve as an “accomplice” in KA-induced excitotoxicity (Giaume et al. 2007).

1.2.1.2 Astrocytes

Astrocytes are the largest glial cell population within the CNS, and were formerly regarded as passive supporters of neurons in the past years. Astrocytes have important physiological properties, maintaining the functional integrity of the synapse and BBB,

as well as contributing to the extracellular matrix proteins. However, recent studies raised a new concept of neuron-glial intercommunication that astrocytes play a dynamic role by integrating neuronal inputs and modulating synaptic activity, whereby contributing to neurodegenerative development (Gill et al. 2008; Vesce et al. 2007). Astrocytes can prevent neurons from death by producing growth factors (Braun et al. 2009; Dakubo et al. 2008; Sandhu et al. 2009). Moreover, astrocytes express most neurotransmitter receptors such as ionotrophic glutamate receptors (iGluRs) (Brand-Schieber and Werner 2003). In pathological conditions, astrocytes may secrete ROS and proinflammatory factors, which contribute to brain damage (Heneka et al. 2010). The expression of glial fibrillary acidic protein (GFAP), a marker for astrogliosis, has been shown to steadily increase from one day up to one month after intra-hippocampal or intraperitoneal injection of KA (Bendotti et al. 2000; Ding et al. 2000). Up-regulated transcription factors in astrocytes, such as nuclear factor kappa B (NFκB) and nuclear factor erythroid-2-related factor 2 (Nrf2) induce the production of neuroprotective molecules and exert its neuroprotective actions on the neighboring neurons (Vargas et al. 2008; Vargas and Johnson 2009).

1.3 INFLAMMATORY MEDIATORS PRODUCTION AFTER KA-INDUCED INJURY

Neurodegenerative disorders are associated with increased expression of inflammatory mediators in the CNS, such as in AD and MS (Rojo et al. 2008). A large number of inflammatory mediators including nitric oxide (NO), IL-6, IL-12 and TNF-α are released by activated microglia and astrocytes after KA treatment, which can exacerbate or reduce neuronal damage and influence the disease process (Figure 1) (Colton and Wilcock 2010; Kerschensteiner et al. 2009; Walz 2000). The inflammatory mediators may affect the pathological process with regard to seizure activity, behavioral changes, as well as the neuronal cell death in KA-induced excitotoxicity (Chen et al. 2004). The inflammatory cytokines can be divided into pro- and anti-inflammatory cytokines, which play the contradictory biological roles in the pathological process.

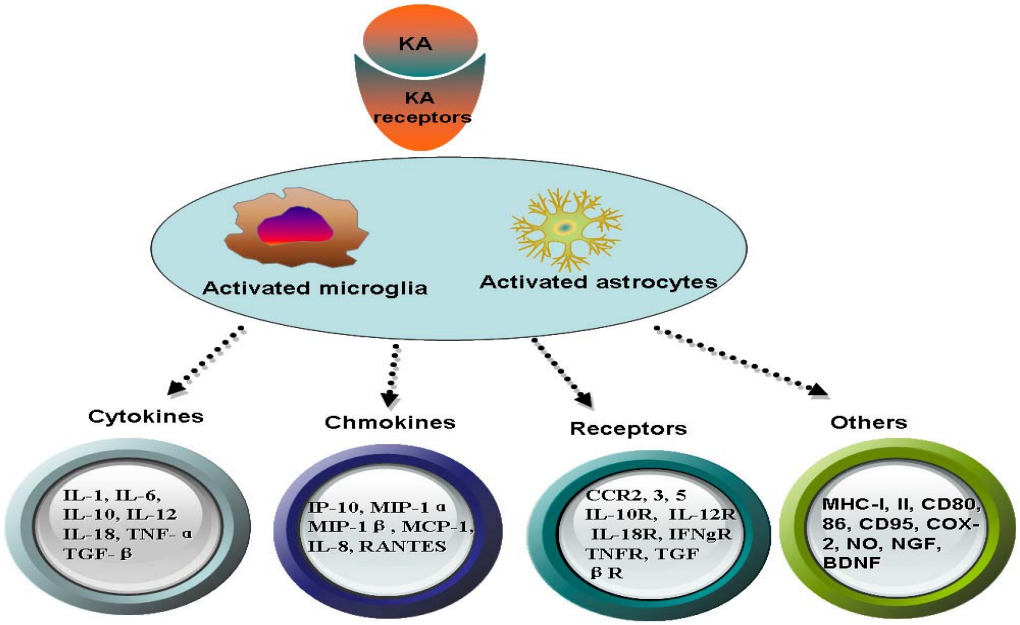


Figure 1. KA-induced glial cell activation and inflammatory molecule production. After KA treatment, activated microglia and astrocytes can induce production of proinflammatory cytokines, such as IL-1, IL-6, IL-12, IL-18, TNF-α and anti-inflammatory cytoknes, such as TGF-β and IL-10, as well as induce chmokines (IP-10, MIP-1α, MIP-1β, MCP-1, IL-8, RANTES), chemokine receptors (CCR2, 3, 5, etc.), and cytokine receptors (IL-10R, IL-12R, IL-18R, IFNgR, TNFR, TGFβR, etc.) expression, as well as express neutrophins (NGF, BDNF, etc.), MHC class I and II, costimulatory molecules (CD80 and CD86), etc., which may either contribute to excitotoxic damage or be protective from KA injury.

IL: interleukin; CCR: C-C chemokine receptor; IFNgR: interferon gamma receptor; TNF: tumor necrosis factor; TGF: transforming growth factor; IP: Interferon gamma-inducible protein; MIP: macrophage inflammatory protein; MCP: monocyte chemotactic protein; RANTES: regulated upon activation, normal T-cell expressed and presumably secreted; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor.

1.3.1 TNF-α

The inflammatory cytokine, tumor necrosis factor-α (TNF-α) is a 17 kDa protein, mainly produced by activated macrophages and T cells in the immune system and by microglia and astrocytes in the CNS. Initially it was characterized as having anti-tumor activity, but it was later found to play pleiotropic roles, often contradictory biological effects (Yang et al. 2002; Yang et al. 2013). Its functions are mediated by binding with two receptors, TNF-α receptor (TNFR) 1 and TNFR2 (Ware et al. 1991). TNF-α binding to it's two receptors can activate three major signaling pathways. First, TNF receptors associate with the TNF receptor-associated death domain (TRADD) .This

results in recruitment of factor associated suicide (Fas), and subsequent activation of caspase-8, which is an apoptotic signaling cascade (Schneider-Brachert et al. 2004). Secondly, NFκB signaling pathway is activated. The process is initiated when phosphorylation of inhibitor of NFκB (IκB) via NFκB essential modulator occurs, which leads to the degradation of IκB. NFκB acts as either a transcriptional activator or repressor by binding to specific DNA sequences (Bonizzi and Karin 2004). The third pathway is the c-Jun N-terminal kinase (JNK) pathway. The activity of transcription factors, such as protein-1 and specificity protein-1 is enhanced after the activation of cascade, via JNK-mediated phosphorylation (Benasciutti et al. 2004). These transcription factors involve in cell differentiation, proliferation, and pro-apoptotic (Park and Bowers 2010).

TNF-α is a key mediator in both acute and chronic systematic inflammatory processes, since it can stimulate the production of several inflammatory cytokines and chemokines and their receptors expression. TNF-α plays a crucial role in the glial–neuronal interactions that influences memory mechanisms and neuropathic pains. TNF-α alters synaptic transmission in rat hippocampal slices and produces a rapid exocytosis of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors in hippocampal pyramidal cells (Stellwagen and Malenka 2006; Tancredi et al. 1992). Additionally, TNF-α can regulate synaptic strength and synaptic scaling (Beattie et al. 2002; Stellwagen and Malenka 2006). TNF-α may be the necessary factor to maintain neuronal function in physiologic conditions. The pro-inflammatory effects of TNF-α are widely recognized to contribute to the pathogenesis of a variety of neurological diseases in humans, especially bacterial meningitis (Grandgirard et al. 2013), MS (Rossi et al. 2013) and cerebral malaria (Zhu et al. 2012), where inflammatory cells contribute significantly to locally elevated TNF-α levels. TNF-α has been proved to mediate the disruption of longterm potentiation and related memory mechanisms produced by amyloid and amyloid oligomers in AD models (Tobinick 2009; Wang et al. 2005a). TNF-α also bears neuroprotective properties in contrast to its well-known deleterious role as a proinflammatory cytokine, which implies an intricate biological effect in immune and inflammatory responses mediated by TNF-α (Lu et al. 2008; Zhang et al. 2013b). For example, TNF-α deficiency worsened Listeria infection (Rothe et al. 1993) and the neuronal damage was enhanced after excitotoxicity in mice lacking the TNF-α receptors (Lu et al. 2008). Inhibition of TNF-α has been proposed as a potential method of modulating glial activation in neuroinflammatory disorders

involving both the brain and the spinal cord (Griffin 2008). Recently, it has been demonstrated that increased brain levels of TNF-α result in significant inhibition of seizures in mice induced by intrahippocampal injection of KA (Balosso et al. 2005). Therefore, it is speculated that TNF-α could play double roles in the CNS, which may depend on different conditions: enhancing NFκB pathway processing or attenuating NFκB activity, while simultaneously promoting other pro-apoptotic TNF-α signals (Kaltschmidt et al. 1999).

Since high dose of local TNF-α can enhance efficacy of chemotherapy, TNF-α has been used for regional treatment of metastatic melanoma (Chu 2013). TNF-α antagonists (Remicade/Infliximab, Adalimumab/Humira and Golimumab) are used as a therapy for inflammatory bowel disease, cancer-related cachexia, leukemia and ovarian cancer (Thalayasingam and Isaacs 2011). The drugs exert their effects by blocking TNF-α signaling either utilize anti-TNF antibodies to neutralize soluble TNF-α or fusing with extracellular domains of TNFR1 or TNFR2 to block the interaction of TNF-α with TNF-α receptors. Overall, even anti-TNF-α therapy has been used in autoimmune disorders; however, some side effects have been reported. For example, anti-TNF-α therapy may increased the risk of developing cancer. Yet, the potential effect of anti-TNF therapy requires further investigations.

1.3.2 NO

NO is an important signaling molecule, and involved in many physiological and pathological processes. NO represents as one of the principle features of activated macrophage/microglia, and is a major effector in the innate immunity. One of the hallmarks of excitotoxic neurodegeneration is the production of NO (Lau and Tymianski 2010). It has been proved that nitric oxide synthase (NOS) inhibitors can prevent cell death in glutamate-mediated neurodegeneration in vitro study (Dawson et al. 1991). KA administration increases the generation of ROS and RNS by neuroglia, and NO can be formed enzymatically from L-arginine by inducible NOS (iNOS) in neuroglia (Wiesinger 2001). Elevated production of NO by increased activity of iNOS is thought to contribute to KA-induced neuronal damage (Amor et al. 2010). Pretreatment with iNOS inhibitor can significantly attenuate KA-induced neuronal

death in the hippocampal CA3 area with concomitant suppress microglial activation (Byun et al. 2009).

1.3.3 Transcription factors production after KA injury

NFκB is a heterodimeric transcription factor that regulates the cell differentiation, proliferation and survival (Yang et al. 2002). Many factors, including pro-inflammatory cytokines, TNF-α, interleukin (IL)-1 and foreign antigens can induce NFκB activation (Goldminz et al. 2013). NFκB signaling is hypothesized to promote pro-survival signal cascades, indicating again the dichotomistic ability of TNF-α to induce both pro-life and pro-death cellular outcomes (Park and Bowers 2010). Activated NFκB proteins have been detected in many chronic inflammatory conditions, including inflammatory bowel disease and rheumatoid arthritis (Kaser et al. 2010; Tak et al. 2001) Although originally NFκB was associated with immune and inflammatory cell function, the realization that such transcription factors also have essential roles in coordinating antimicrobial immunity and maintaining barrier function (Ben-Neriah and Karin 2011; Gewirtz et al. 2000). Inhibition of NFκB was found to increase or even cause inflammation in the mice devoid of NFκB activity (Pasparakis 2009). Further studies are still necessary to clarify the function of NFκB.

The p38 mitogen-activated protein kinase (MAPK) has been considered as a stress-activated protein kinase that responds to external signals, including osmotic shock, inflammatory cytokines, ultraviolet light, and growth factors (Oeztuerk-Winder and Ventura 2012). P38 MAPK signalling pathway is involved in several disease models, including rheumatoid arthritis, psoriasis and AD (Johnson and Bailey 2003). In vitro study, TNF-α enhances the tetrodotoxin-resistant (TTX-R) Na^+ current from wild-type but not from TNFR1 knockout mice, and such current is abolished by a p38 MAPK inhibitor; implying that TNF-α acts via TNFR1 and activates TTX-R Na^+ channels via the p38 MAPK system (Jin and Gereau 2006). Another study by using TNFR1/TNFR2 knockout mice have suggested a neuroprotective role of TNFR2 through p38 MAPK signaling (Yang et al. 2002). In a rat model with spinal nerve ligation enhanced the production of TNF-α and phosphorylation-p38 MAPK, and the TNF-α blockade can in turn suppress p38 MAPK activation (Schafers et al. 2003). Further study by using nerve injury animal models showed induced expression of soluble TNFR2 in dorsal root

ganglia neurons, resulting in decreased phosphorylation of p38 MAPK (Leung and Cahill 2010). The above studies suggested that there is a causal link between TNF-α and the p38-MAPK system.

Protein kinase B (PKB)/AKT pathway is an essential pathway for cell survival since AKT plays a key role in up-regulation of anti-apoptotic proteins. AKT is a serine-threonine kinase that is regulated mainly following activation of the second messenger phospholipid kinase phosphatidylinositol 3-kinase (PI3K) (Zhang et al. 2011). During apoptotic process, the PI3K/AKT pathway can enhance glial cell survival, maintain the integrity of endothelial cells, prevent neurodegeneration and provide tolerance against oxidative stress (Maiese et al. 2012). Experimental model targeted to block the activation of PI3K/AKT pathway can suppress medulloblastoma growth, reduce cancer growth through induction of autophagy and increase radiosensitivity in tumors (Chung et al. 2012; Fokas et al. 2012). In vitro study, cortical neurons after pre-treated with TNF-α were exposured to glutamate, a PI3K-dependent PKB/AKT phosphorylation was ensued by NFκB activation (Marchetti et al. 2004). AMPA receptors stimulation by glutamate can activate AKT-dependent pathway, and this activation can regulate cell survival (Noch and Khalili 2009).

1.4 GUILLAIN-BARRÉ SYNDROME AND ITS ANIMAL MODEL EXPERIMENTAL AUTOIMMUNE NEURITIS

Guillain-Barré syndrome (GBS) is an acute inflammatory demyelination disease in peripheral nervous system (PNS). GBS is the most common cause of the acute flaccid paralysis in developing countries and is characterized by acute progressive and symmetrical motor weakens of the extremities as well as bulbar and facial musculature (Newswanger and Warren 2004; Olive et al. 1997). Georges Charles Guillain and Jean-Alexandre Barré, two French neurologists, who together with the doctor André Strohl firstly described the syndrome in 1916 (Guillain et al. 1916). Experimental autoimmune neuritis (EAN) is a useful animal model for GBS, which is mediated by $CD4^+$ T cell and mirrors many of the clinical, immunologic, electrophysiologic and morphologic aspects of GBS in the human (Rostami 1995; Zou et al. 2000).

1.4.1 Generic features of GBS and classification of GBS

GBS is considered as a clinical syndrome with combination of rapidly progressive symmetric weakness in the limbs, sensory disturbance, hyporeflexia or areflexia, and albuminocytologic dissociation. The progressive phase of GBS usually last from a few days to one month (Newswanger and Warren 2004). About 73% patients reach a nadir of clinical function at one week (Newswanger and Warren 2004). Additionally, around 85% of GBS patients achieve a fully recovery in 6 or 12 months (Kuwabara 2004).

According to the clinical, electrophysiological, and pathological features, GBS can be divided into seven subtypes of acute peripheral neuropathy (Kieseier et al. 2004; Newswanger and Warren 2004), including acute inflammatory demyelinating polyneuropathy (AIDP) (Hughes and Cornblath 2005), acute motor axonal neuropathy (AMAN) (McKhann et al. 1993), acute motor-sensory axonal neuropathy (AMSAN) (Feasby et al. 1993), acute sensory neuronopathy (Sterman et al. 1980), acute pandysautonomia, Miller-Fisher syndrome (MFS) (Fisher 1956) and MFS-GBS overlapping syndrome (Mori et al. 2001). The 90% of all GBS cases in Europe and North America is AIDP (Hughes and Cornblath 2005). However, AMAN and AMSAN are more prevalent in Asia, South and Central America (Drenthen et al. 2011).

1.4.2 Experimental autoimmune neuritis (EAN)

Experimental autoimmune neuritis (EAN) is a $CD4^+$ T cell-mediated acute inflammatory demyelinating disorders of the PNS that can be induced in rats, mice, rabbits and monkeys, which serves as an animal model for AIDP (Lehrich and Arnason 1971). EAN can be induced by active immunization with whole peripheral nerve homogenate, or myelin proteins P0 or P2 or their neuritogenic peptides plus Freund's complete adjuvant (Rostami et al. 1990; Zou et al. 2000) or by passive transfer of P0, P2 as well as their peptide-specific $CD4^+$ T cell lines (Maurer et al. 2002).

The pathological hallmark of EAN is the breakdown of the blood nerve barrier (BNB), demyelination in the PNS and robust accumulation of autoreactive T cells and macrophages (Zhang et al. 2010; Zhang et al. 2013a). The early sign of EAN is the alteration in BNB permeability and the inflammatory cell infiltration in the PNS (Hahn

et al. 1985). A critical step in the immune response of EAN is the blood-derived inflammatory cells across the BNB (Zhang et al. 2013a). Macrophages act as the main antigen presenting cells (APCs) in the PNS by expressing MHC-I, MHC-II, and co-stimulatory molecules (Schmidt et al. 1992; Vass and Lassmann 1990). Macrophages secrete pro-inflammatory cytokines, including IL-1, IL-6, IL-12 and TNF-α, which further promote the T helper (Th)1 prolarization (Kieseier et al. 1999). After that, polarized Th1 cells further stimulate macrophage to express a proinflammatory phenotype (M1) (Heusinkveld et al. 2011). In acute phase, macrophages can directly attack the myelin by releasing of proinflammatory and noxious molecules (Jung et al. 1993; Kiefer et al. 2001; Zhang et al. 2010). During recovery stage, M1 can strip off myelin and phagocytose myelin debri by Fc/complement receptors (Kiefer et al. 2001; Wohlleben et al. 2000). Macrophage, cytokines, chemokines, NO, matrix metalloproteinases (MMPs) and adhesion molecules also contribute to the pathogenesis of EAN.

1.4.3 Cytokines in GBS and EAN

Cytokines are secreted by immune cells and other cells, and exert their functions through binding to specific receptors (Tsang and Valdivieso-Garcia 2003; Zhu et al. 1998). Cytokines involved in the pathogenesis of EAN and GBS include TNF-α, IFN-γ, IL-12 and IL-17, etc. The balance of pro- and anti-inflammatory cytokines will shape the outcome of the response of GBS and EAN (Figure 2).

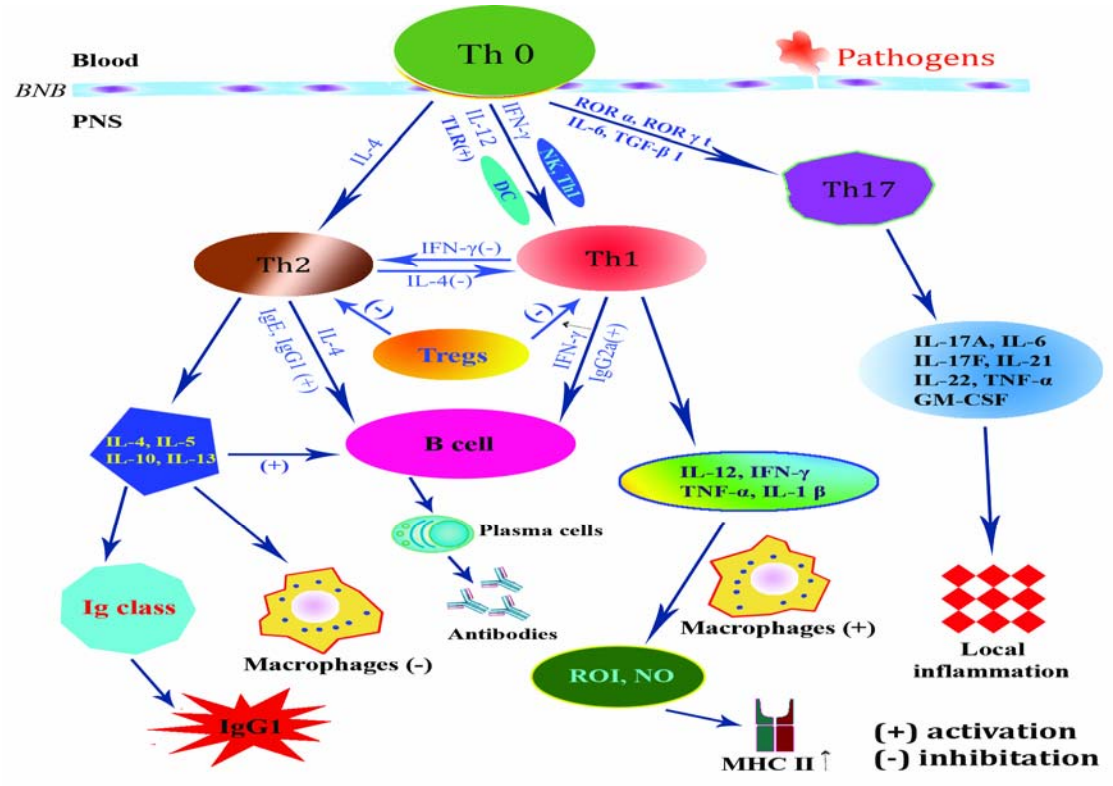


Figure 2. Role of cytokines in the pathogenesis of EAN.

EAN is characterized by breakdown of BNB and accumulation of inflammatory cells in the PNS. Th0 cells can differentiate into Th1 or Th2 cells depending on their milieu. Th1 differentiation is regulated by IFN-γ and IL-12 produced by macrophages, NK cells, Th1 cells per se and DCs. During the acute phase, the production of Th1 cytokines, such as IFN-γ, TNF-α, IL-1β and IL-6 are increased. These cytokines can activate macrophages to produce ROI and NO, and enhance their antigen presenting capacity by upregulating the expression of MHC II. However, Th2 cytokines such as IL-4 and IL-10 suppress the disease by palying an antiinflammatory role during the recover phase. Th2 cytokines including IL-4, IL-5, IL-10 and IL-13, provide potent help for B cell activation, which can produce antibodies, and Th2 cytokines can promote Ig class switching to IgG1, and downregulate proinflammatory macrophage activation. RORα and RORγt are important transcription factors for stimulating the Th0 cells to Th17 cells. The cytokines of Th17 cells can induce local inflammation and autoimmune reaction in the target organ.

BNB: brood-nerve barrier; DCs: dendritic cells; GM-CSF: granulocyte-macrophage colony-stimulating factor; Ig: immunoglobulin; NK: natural killer; NO: nitric oxide; ROI: reactive oxygen intermediates; ROR: retinoic acid-related orphan receptor; TLR: Toll-like receptor.

1.4.3.1 TNF-α

TNF-α is a prototypical inflammatory cytokine and a key regulator in the immune and

inflammatory responses (Radhakrishnan et al. 2004; Zhang et al. 2007a), which is mainly produced by activated macrophages and T cells after tissue injury (Chaparro et al. 2012). TNF-α exerts its functions through binding its two receptors, 1 (P55) and 2 (P75) (Kassiotis and Kollias 2001). Both TNFRs express on various cell types, especially activated $CD4^+$ and $CD8^+$ T cells. TNF-α has direct toxic effects on the Schwann cells (SCs) of rodents, thereby contributes to EAN development (Argall et al. 1992; Duan et al. 2007). Injection of TNF-α into rat sciatic nerve produces inflammatory demyelination (Redford et al. 1995); anti-TNF-α antibodies and inhibitors of TNF-α synthesis play a protective role in EAN (Korn et al. 2001). Increased serum levels of TNF-α correlated directly with disease severity of GBS and the concentrations returned to normal in parallel with clinical recovery (Sharief et al. 1993). The anti-inflammatory compounds down-regulated the production of TNF-α can suppress the clinical signs of EAN (Korn et al. 2001; Zou et al. 2000). However, TNF-α also has a protective role, since TNF-α antagonist exacerbate the symptoms of MS patients (Ghosh 2012).

The protective or deleterious effects of TNF-α may depend on the levels of its two receptors (Smith et al. 1994). In EAN model, TNFR1 deficiency ameliorates clinical syndrome in mice (Mao et al. 2010b). However, in EAE model, TNFR2 knockout mice showed enhanced expression of Th1 cytokines and T cell infiltration (Suvannavejh et al. 2000).

Suppressing TNF-α is considered as a therapeutic target in EAN. In our previous study, treatment with soluble TNFR1 ameliorated EAN (Bao et al. 2003). Moreover, inhibition of TNF-α reduces T-cell apoptosis and prevents liver necrosis in EAN (Mao et al. 2010a). Nevertheless, further studies are still needed in clinical works.

1.4.3.2 IFN-γ

IFN-γ, acts as a typically cytokine for the Th1 response, is produced by T cells, natural killer (NK) cells and professional APC. IFN-γ was originally characterized as a molecule to mediate an immediate defensive response against viral infections (Pestka et al. 1987). IFN-γ exerts its function by activating IFN-γ receptors, 1 (IFNGR1) and IFNGR2 (Pestka et al. 1997). IFN-γ plays a crucial role as a general proinflammatory molecule, including activation of macrophages, increasing the expression of MHC-I

and MHC-II on the APC, enhancing the differentiation of T cells to Th1 phenotype, while suppressing Th2 and Th17 responses, as well as modulating leukocyte trafficking (Lu and Zhu 2011). IFN-γ also can augment the production of other inflammatory cytokines, such as TNF-α, IL-1β, IL-6, and receptor activator of NFκB (Schroder et al. 2004). IFN-γ can induce the expression of cytokines and chemokines required for the recruitment of myeloid cells to the site of inflammation, and increase the expression of Toll like receptors (TLRs), and phagocyte oxidase by macrophages (Hu and Ivashkiv 2009).

The role of IFN-γ in GBS and EAN has been widely investigated. There was elevated IFN-γ expression in serum of GBS patients, however "paradoxical" findings have been indicated that IFN-γ plays the protective role in some models of autoimmune diseases, such as EAE and asthma (Flaishon et al. 2002; Willenborg et al. 1996). It's also reported that the levels of IFN-γ-secreting cell were not increased in the whole course of GBS (Hohnoki et al. 1998; Press et al. 2001). IFN-γ KO mice showed ameliorated EAN severity, furthermore, anti-IFN-γ antibody treatment shortened the EAN course (Elkarim et al. 1998; Zhu et al. 2001). Several studies indicated that IFN-γ function changes may accord to the different stages of autoimmune diseases. Administration of IFN-γ in the early phase of EAE can attenuate disease severity, however, administration of IFN-γ at later stage of EAE can aggravate the clinical signs (Wildbaum et al. 2010). These findings suggest that IFN-γ might have dual roles in the pathogenesis of GBS and EAN.

1.4.3.2 IL-12

IL-12 , mainly produced by macrophages, neutrophils and dendritic cells (DCs), is a heterodimeric cytokines composed of subunits p35 and p40 (Watford et al. 2003). IL-12 mediates cellular immunity by inducing T cell and NK cells proliferation and promoting production of IFN-γ (Del Vecchio et al. 2007). IL-12 promotes the polarization of $CD4^+$ T cell to the Th1 phenotype that plays the major role in the initiation, enhancement and perpetuation in the immune response to pathogens in EAN (Watford et al. 2003). Il-12 mRNA is expressed on the sciatic nerve sections over the course of EAN and was maximally up-modulated at height of clinical EAN (Zhu et al. 1997). The administration of recombinant IL-12 prolonged the course of EAN and

exacerbated chronic EAN (Pelidou et al. 2000a). Moreover, IL-12 deficient mice exhibited attenuated clinical severity through decreasing production of TNF-α and IFN-γ in inflamed nerves (Bao et al. 2002).

1.4.3.2 IL-10

IL-10 is an anti-inflammatory cytokine which primarily produced by monocytes, macrophage, and different T cell subsets (Sabat 2010). IL-10 suppressess proinflammatory cytokines production such as IL-1β and TNF-α and represses expression of MHC and co-stimulatory molecules, and inhibits their antigen presenting functions (Sabat 2010). IL-10 promotes the Th2 polarization through inhibiting IFN-γ, as well as enhances MHC II expression on B cells. The main function of IL-10 in inflammatory and autoimmune is the limitation and termination of inflammatory response and regulation of differentiation and proliferation of immune cells (Bashyam 2007). Low dose of IL-10 administration promoted better regeneration of the damaged axons (Atkins et al. 2007). Maximal expression of IL-10 was observed after clinical recovery from EAN (Zhu et al. 1998). Furthermore, in the progressive phase of GBS, the expressions of IL-10 were significantly upregulated (Nyati et al. 2011).

1.4.3.2 IL-17

IL-17 family includes six members, namely IL-17A-F. IL-17A, the fundamental member of this family, is a signature cytokine which is released by Th17 cells and has been found to play important roles in autoimmune diseases, host defense, allergy, etc (Song and Qian 2013). IL-17A exhibits its inflammatory effects through upregulation of proinflammatory cytokines (IL-6, TNF-α and IL-1), chemokines, antimicrobial peptide and matrix metalloproteinases (MMPs) (Zhu and Qian 2012). IL-17 administration enhanced the clinical signs during acute phase and suppressed the chronic phase of the EAN (Pelidou et al. 2000b). Furthermore, in IFN-γ deficiency EAN mice, significantly worse signs may related to higher percentages of IL-17A-producing cells from cauda equina and the levels of serum IL-17A (Zhang et al. 2012a). Consistently with this, another study in our group found the level of IL-17A in cauda equina-infiltrating cells and spleen were correlated with the clinical severity of EAN, which might imply the underlying involvement of IL-17A in EAN (Zhang et al. 2012b).

The serum level of IL-17A was elevated in GBS patients (Liang et al. 2012). We also reported the same finding that the levels of IL-17A and IL-22 in CSF and plasma of GBS patients in the acute phase was increased (Li et al. 2012). These findings suggest that IL-17A may involve in the pathogenesis of GBS and EAN.

2 AIMS OF THE STUDIES

To identify the roles of TNF-α and IFN-γ in the experimental neurodegenerative and neuroinflammatory disorders.

Specific aims:

Study I: To study the role of TNF-α in KA-induced neurodegeneration in vivo.

Study II: To investigate the role of TNF-α in the excitotoxic neurodegeneration in vitro.

Study III: To explore the role of TNF-α in the pathogenesis of EAN.

Study IV: To clarify the role of IFN-γ in the pathogenesis of EAN.

3. MATERIALS AND METHODS

3.1 ANIMALS (STUDIES I-IV)

TNF-α knockout (KO) as well as C57BL/6 wild-type (WT) male mice were used in Studies I and III. Embryonic day 16 (E16) from TNF-α KO mice as well as C57BL/6 WT mice were used in Study II. IFN-γ KO mice were used in Study IV. The TNF-α KO mice and the IFN-γ KO mice had been generated through targeted disruption of the TNF-α gene and the IFN-γ gene, respectively, and being backcrossed to the C57BL/6 strain. 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.

3.2 KA ADMINISTRATION AND ASSESSMENT OF CLINICAL SIGNS (STUDY I)

Mice were partially anesthetized with Isofluen. KA was slowly and gently dropped by micropipette into the noses of the mice. Doses of KA used were 40 mg per kg body weight in both studies. Age-, sex- and body weight-matched TNF-α KO mice and WT mice, respectively, received the same amount of distilled water intranasally as controls. Mice were monitored continuously for 5 h to register the onset and extent of seizure activity. Seizures were rated as follows: 0, normal; 1, immobilization; 2, rearing and falling; 3, seizures for less than 1 h; 4, seizures for 1-3 h; 5, seizures for more than 3 h; and 6, death.

3.3 BEHAVIORAL TESTS (STUDY I)

3.3.1 Elevated Plus Maze ™

The activities performed in the Elevated Plus Maze (EPM) tasks were measured before and after KA administration. The EPM consisted of two opposite open arms and two opposite closed arms surrounded by Plexiglas™ walls. The testing was carried out in a dimly lit room. The mouse was placed on the central area of the EPM facing an open

arm and tested for a 5 min period. An arm entry was defined as the entry of all four paws into the arm. The time spent, and the numbers of entries in the open and closed arms, respectively, were recorded.

3.3.2 Y-maze

The spontaneous alteration behavior in a Y-maze was performed two days before KA and five days after KA treatment. The Y-maze is used for identification of discrimination learning, spatial alteration tasks, and working memory of rodents. Test subjects were placed in a Y-shaped maze for 6 min. All arm entries were sequentially scored, so that the total number of arm entries and the sequence of entries were recorded. Data were analyzed to determine the number of arm entries without repetition.

3.3.3 Open-field test

Open-field (OF) activity was measured three days before and four days after KA administration. The OF test is used for measuring exploratory behavior and spontaneous motor activity of the animals. The apparatus consisted of a transparent acrylic plastic box in which the floor was subdivided into 64 equal squares. A mouse was gently placed in the center of the box. Locomotor activity and rearing were recorded every 3 min for a period of 9 min. Locomotor activity was evaluated by counting the number of times the mouse crossed the floor squares with both hind paws. Rearing was evaluated by counting the number of times the mouse showed vertical attitude with the forepaws raised or placed on the walls.

3.4 HISTOPATHOLOGICAL ANALYSIS (STUDY I)

All mice were anesthetized with sodium pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) five days after the administration of KA. The left hemisphere of each brain was fixed in 4% paraformaldehyde and kept in 10% sucrose until being frozen. Coronal sections (12-μm slices) from -1.15, -1.94 and -2.80 mm, respectively, relative to the bregma were prepared according to the information in Franklin's brain atlas (Franklin 1997). Sections were stained by Fluoro-Jade B (FJB) to evaluate degenerating neurons.

3.5 IMMUNOHISTOCHEMISTRY OF BRAIN SECTIONS (STUDY I)

Frozen hippocampal sections were prepared as described above for immunohistochemistry. After washes with Tris buffer, the sections were blocked by "protein block" at room temperature (RT) for 30 min. Subsequently, they were exposed to rat antibodies to CD11b and rabbit antibodies to glial fibrillary acidic protein (GFAP), respectively, followed by staining with the avidin-biotin technique. Peroxidase-substrate solution DAB was added until the desired intensity of brown color developed. Omission of primary antibodies served as negative control.

3.6 PREPARATION AND CULTURE OF PRIMARY HIPPOCAMPAL NEURONS (STUDY II)

Primary cultures of hippocampal neurons were isolated at E16 from mice according to the method by Fath et al (Fath et al. 2009). Briefly, the hippocampi were carefully dissected out. Then the hippocampus was moved to the tube with Neurobasal medium including 1% L-glutamine, 2% B27 supplement. Then dissociate tissue fragment mechanically with a Pasteur pipette until have the cell suspension. Count the hippocampal neuronal cells and seed in 6-wells plate coated with 50 ug/ml poly-D-lysine at a density of 2 x 10^5 cells per well. The cells were cultured at 37°C and 5% CO_2 /95% O_2 incubator. On the seventh day, cultures were treated with KA dissolved in PBS. After co-culture with KA 24 h, ice-cold lysis buffer was used to harvest the hippocampal neurons for subsequent experiments.

3.7 MEASUREMENT OF LEVEL OF LACTATE DEHYDROGENASE (LDH) AND NEUROTOXICITY (STUDY II)

LDH production was detected in the culture supernatants 24 hour after KA treatment by using Cytotoxicity Detection Kit and the kit directions were followed. The results were presented as "relative value of LDH production versus total protein".

Twenty-four hours after KA treatment, neuronal viability was determined by the method utilizing metabolic dye 3-(4, 5-Dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide (MTT) as described previously (Mosmann 1983); 1.25 mg/mL MTT solution

was added to each well of a 96-well plate. After 3 h of incubation, cells were lysed by adding 120 μL of isopropyl-HCl solution (37% HCl/isopropyl alcohol: 1/166) for 15 min. The absorbance of each well was recorded at 595 nm with an automated ELISA reader.

3.8 WESTERN BLOTTING (STUDIES I AND II)

The hippocampal supernatants were prepared as described above. 50 μg of each sample were electrophoresed on a 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were then blocked in 5% nonfat dry milk in PBS-Tween 20 for 1 h at RT with gentle agitation. After blocking, the membranes were incubated with appropriate primary antibody rabbit anti-phospho-p38 mitogen-activated protein kinase, phospho- IκBα, rabbit anti-mouse NFκB, rabbit anti-mouse AKT, rabbit anti-mouse phospho-AKT or rabbit anti-mouse β-actin, overnight at 4°C. After extensive washing in PBS-Tween 20, membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at RT. The membranes were rinsed again. Enhanced chemiluminescent Western blotting detection reagents were used for exposure according to the manufacturer's instructions. Densitometric analysis was performed using the Image J program. Results were presented as "relative densities to β-actin".

3.9 ANTIGEN (SUTIES III AND IV)

The neuritogenic peptide 180-199 of the murine PNS myelin P0 protein, was synthesized by the 9-fluorenylmethoxycarbonyl solid-phase synthesis, purified by high-performance liquid chromatography (HPLC) using a Vydac reverse-phase column, and analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

3.10 INDUCTION OF EAN AND ASSESSMENT OF CLINICAL SIGNS (STUDIES III AND IV)

Emulsion of P0 peptide and Freund's complete adjuvant (FCA) was prepared by gently adding P0 peptide solution in 0.9% saline to FCA being vortexed. FCA refers to Mycobacterium tuberculosis plus Freund's incomplete adjuvant (FIA). EAN was

induced by immunizing mice twice (days 0 and 8) via subcutaneous injection of 150 mg P0 peptide 180–199, 0.5 mg Mycobacterium tuberculosis in 25 ml saline, and 25 ml FIA to the back of mice. All mice received 400, 300, and 300 ng pertussis toxin (PTX) by intravenous injection (via tail veins) on days -1, +1 and +3 post immunization (p.i.), respectively. 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; 0.5, intermediate clinical signs.

3.11 LYMPHOCYTE PROLIFERATION TEST (STUDIES III AND IV)

Mice were sacrificed at the height of EAN (day 28 p.i.) after perfusion with PBS. Spleens were removed and single cell suspensions of mononuclear cells (MNCs) in RPMI-1640 were prepared and cultured. Concanavalin A (ConA), P0 peptide 180–199 and IL-23 were used as stimuli. After 60 h of cultivation with respective stimulus, the proliferation was assessed using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay according to the manufacturer's instructions. Briefly, assays were performed by adding a small amount of the reagent directly to culture wells. The CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay 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 bioreduced into a colored formazan product soluble in cell culture medium 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.

3.12 MACROPHAGE CULTURES (STUDIES III AND IV)

Mice were sacrificed and standard lavage of the peritoneal exudates with serum-free culture medium DMEM/F12 was performed. The lavage fluid contained peritoneal exudate mononuclear cells (PEMs), which represented mainly macrophages. The single cell suspension was centrifuged at 300 x g for 10 min. The pellets were resuspended with DMEM/F12 supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and the concentration was adjusted to 2×10^6 /ml. Macrophages were seeded in 5.3 cm Petri-dishes. The cells were stimulated with Lipopolysaccharide (LPS), recombinant IFN-γ, TNF-α, poly I:C and

combinations thereof at 37 °C. After 24 and 48 hours of incubation, respectively, the supernatants were collected for detection of cytokine and NO. After removal of the 48 hours supernatant, 5 ml culture media with Brefeldin A was added. Cells were harvested for subsequent flow cytometric analysis after 6 hours.

3.13 ISOLATION OF INFLITRATING CELLS IN CAUDA EQUINA (CE) (STUDIES III AND IV)

Briefly, cauda equina (CE) fragments of spinal cords were carefully removed from PBS perfused mice, transferred to RPMI-1640, ground and passed through a 70 μm cell nylon mesh. The resultant cells were suspended in 27% percoll in PBS and centrifuged at 1000×*g* for 30 min at 4°C. The pellet was kept for further analysis. .

3.14 ELISA (STUDIES III AND IV)

A standardized procedure for the sandwich ELISA was established after optimization of experimental parameters. Briefly, monoclonal antibodies were coated onto standard ELISA plates in a volume of 100 ml/well overnight at 4°C. After washing three times with PBS, uncoated sites were blocked with 100 ml 10% FBS in PBS for 1 h at RT. Duplicates of samples or of recombinant standards were added and the plates were incubated for 1 h at RT. After washing, the plates were incubated with biotinylated detecting antibody for 2 h at RT. Then avidin-conjugated horseradish peroxidase (HRP) was added for 30 min. Color reaction was performed with 100 ml of tetramethylbenzidine (TMB) for 30 min and the reaction was terminated by adding 2 M sulfuric acid. The plates were immediately read at 450 nm with an ELISA reader. The concentrations of proteins were quantified by extrapolation from the standard curve.

3.15 FLOW CYTOMETRY (STUDIES III-IV)

Cells were harvested and washed with 1% BSA in PBS. For staining of molecules with extracellular expression, cells were incubated with FITC-, PE- and/or APC-conjugated antibodies. For staining of molecules with intracellular expression, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% freshly prepared saponin in PBS containing 1% BSA. The permeabilized cells were incubated with FITC-, PE-

and/or APC-conjugated antibodies for 15 min at RT. FITC-, PE-, APC- and PerCP conjugated isotype antibodies were used as negative controls. After fixation and permeabilization, cells were washed twice, resuspended in 1% paraformaldehyde in PBS and stored at 4°C until flow cytometric analysis by a FACSCantoTM II cytometer using FlowJo or CellQuest software. Surface and intracellular molecule expression were assessed by determining the mean fluorescence intensities or positive cell percentages. Cells from all groups were collected and analyzed at each time point on the same day with the same cytometer settings. Values are presented for at least five mice per group.

3.16 MEASUREMENT OF ANTI-P0 PEPTIDE ANTIBODIES IN SERA (STUDIES III AND IV)

Serum samples were obtained from mice at the peak of EAN (day 28 p.i.). Purified P0 peptide 180-199 was coated at 4 °C overnight by adding 10 μg/ml in 100 μl per well onto ELISA plates. After three washings with PBS, uncoated sites were blocked with 100 μl 10% FBS in PBS for 2 hours at RT. After three washings with PBS, test serum samples were diluted to 1:100 with 1% BSA in PBS, applied to plate wells and incubated for 1.5 hours at RT. Then plates were incubated for 1hour with peroxidase-conjugated affinipure rabbit anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b after dilution to 1:5000 with 1% BSA in PBS (100 μl/well). After three washings, perioxidase substrate TMB was added and 15-30 minutes later the reaction was terminated by adding 1 M sulfuric acid. The plates were read at 450 nm using the ELISA reader.

3.17 TNFR1 BLOCKING (STUDY III)

Anti-TNFR1 monoclonal antibodies were administered intravenously to WT mice with EAN at a dose of 200 mg/(kg body weight) on days -1, +3 and +7 p.i., respectively. Another group of age- and sex-matched WT mice received 0.9% saline as controls. By using a blinded protocol, two examiners assessed clinical signs of EAN mice every two days.

3.18 DETECTION OF NITRIC OXIDE PRODUCTION (STUDIES I-IV)

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

3.19 STATISTICS (STUDIES I-IV)

Each set of data was presented as mean value ± standard deviation (SD). One-way analysis of variance (ANOVA) and Kruskal Wallis test were used to compare values within groups followed by student's t test and Mann-Whitney u-test to compare values between groups. All tests were two-tailed, and the level of significance was set to $p < 0.05$.

4 RESULTS AND DISCUSSION

4.1 STUDY I: TNF-α MAY PLAY A PROTECTIVE ROLE IN KA-INDUCED NEUROTOXICITY VIA THE DOWN-REGULATION OF NFκB SIGNALING PATHWAY

After KA administration, TNF-α deficiency deteriorates KA-induced neurotoxicity, as demonstrated by more severe seizure activity and hippocampal neurodegeneration, changed behaviors, more activated microglia and astrogliosis, and increased NO production. Additionally, KA-treatment up-regulated the expression of NFκB to a greater degree in TNF-α KO mice when compared with KA-treated WT mice.

TNF-α plays pleiotropic, often contradictory biological roles in neurodegenerative and neuroinflammatory diseases. TNF-α treatment in rats increased seizure susceptibility and enhanced level of TNF-α was found in an animal model of inflammatory bowel disease (Riazi et al. 2008). TNF-α derived from KA activated microglia aggravated excitotoxicity of hippocampal neurons, and might induce neuronal apoptosis in vitro and in vivo in rats (Zhu et al. 2010).

KA-induced damage seriously impacted the hippocampus, which is particularly vulnerable to KA-induced neurotoxicity due to the high density of its kainate receptors (Darstein et al. 2003). CA3 region has the highest abundance of kainate receptors, the activation of which can elevate the concentration of reactive oxygen species (ROS) and impair the normal function of mitochondria (Carriedo et al. 2000; Lauri et al. 2001; Reynolds and Hastings 1995). CA3 neurons are directly excited by stimulation of their KA receptors, and indirectly by increased glutamate efflux secondary to KA stimulation of mossy fibers. CA3 synchronization produces spreading epileptiform activity that extends to CA1 and other limbic structures (Bausch and McNamara 2004; Ding et al. 1998). In this study, C57BL/6 WT mice displayed degenerating neurons in CA3 area, while TNF-α KO showed degenerating neurons in both CA3 and CA1 regions.

CA1 pyramidal neurons receive two distinct excitatory inputs that are capable of influencing hippocampal output, and thus involved in spatial memory and memory

consolidation (Iijima et al. 1996; Speed and Dobrunz 2009). Damage in CA1/CA3 regions of hippocampus induced by KA mainly results in spatial learning deficits. We presented mice to a battery of behavioral tasks. Using the elevated plus-maze test, TNF-α KO mice showed fewer counts of head drops in open-arms compared with that of before KA treatment, indicating changed ability of risk assessment. However, we didn't find any significant difference in the number of entries and time spent in each arm between TNF-α KO mice and WT mice before and after KA treatment, suggesting that KA may not influence the anxiety response in these mice (Komada et al. 2008). In the open-field test, KA-treated TNF-α KO mice showed more locomotion activity in the first 6 minutes compared with KA-treated WT mice. And in 6-min Y-maze test, KA-treated TNF-α KO mice showed more total arm entries compared with KA-treated WT mice. However, no difference was obtained for the successful alteration between TNF-α KO and WT mice, either before or after KA treatment. It has been shown that KA-treated Wistar rats are impaired in the water maze and object exploration tasks, while hyperactive in the open field test (Gobbo and O'Mara 2004; Gobbo and O'Mara 2005). Intraperitoneal injection of KA into the developing rat brain induces impaired short-term spatial memory in the radial-arm maze, deficient long-term spatial learning and retrieval in the water maze, and a greater degree of anxiety in the elevated plus maze(Sayin et al. 2004).

KA-induced neuronal death is accompanied by increased activation of microglia and astrocytes. In the present study, TNF-α deficiency enhanced KA-induced microglia activation and astrogliosis in hippocampus. Microglia are the main effector cell type responsible for immune and inflammatory responses in the CNS. The normal role of microglia could be partly connected to neuroprotection, whereas in pathological conditions microglia may become disease-promoting cells. In KA-induced hippocampal injury, microglial activation is generally believed to contribute to neuroinflammation and neurodegeneration. Astrocytes have functional receptors for the excitatory neurotransmitter, glutamate, and respond to physiological concentrations of this substance with oscillations in intracellular Ca^{2+} concentrations and spatially propagating Ca^{2+} signals. Astrogliosis induced by excitotoxicity has been considered as a marker for neurotoxicity. Our results also showed that TNF-α deficiency increased KA-induced NO production in hippocampi. The activation of KA receptors produces membrane depolarization and results in alteration in intracellular calcium

concentrations, which is required to trigger the neuronal death cascade (Brorson et al. 1994).

TNF-α has a complex biological role in modulating immune and inflammatory responses, which functions are mediated through two receptors, TNFR1 and TNFR2. TNF binding with its receptors enables TNFR1-associated death domain protein (TRADD) to bind to the death domain. Following TRADD binding, NF-κB pathway can be activated. NF-κB is a heterodimeric transcription factor that translocates to the nucleus and mediates the transcription of a vast array of proteins involved in cell survival and proliferation, inflammatory response, and anti-apoptotic factors (Wajant et al. 2003). Additionally, the stimulatory effect of TNF-α can be mediated via the AKT signalling pathway. AKT is a serine/threonine protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, cell proliferation, apoptosis, transcription and cell migration. We examined NFκB and AKT expression one and five days after KA treatment. Five days after KA treatment, TNF-α KO mice showed significantly higher NFκB expression than WT mice, and there was no difference for AKT expression among all groups of mice. We document that NFκB pathway, but not AKT, is important for KA-induced neurotoxicity, which agrees with a previous report (Marchetti et al. 2004).

In summary, TNF-α deficiency deteriorates KA-induced neurotoxicity, as demonstrated by more severe seizure activity and hippocampal neurodegeneration, changed behaviors, more activated microglia and astrogliosis, and increased NO production. TNF-α may play a protecting role in KA-induced neurotoxicity via down-regulation of NFκB signaling pathway.

4.2 STUDY II: TNF-α PROTECTS HIPPOCAMPAL NEURONS FROM KAINIC ACID-INDUCED NEURODEGENERATION VIA UP-REGULATION OF AKT AND DOWN-REGULATION OF P38 MAPK SIGNALING PATHWAYS

In the present study, we found that hippocampal neurons from TNF-α deficient mice showed more susceptibility to KA-induced neurotoxicity, as demonstrated by obviously increased LDH and NO production, and decreased neuronal survival in the

primary cultures. Additionally, TNF-α deficiency clearly down-regulated the expression of phospho-IκBα and AKT, and up-regulated phospho-p38 MAPK expression in hippocampal neurons after KA insult. In WT mice, the pretreatment with anti-TNF-α increased LDH and NO expression, and aggravated KA-induced neurotoxicity in hippocampal neurons after KA insult. Furthermore, anti-TNF-α treatment distinctly down-regulated the production of phospho-IκBα,, AKT and phospho-AKT in hippocampcal cultures, as well as up-regulated the expression of phospho-p38 MAPK. However, the pretreatment with TNF-α in WT mice decreased LDH and NO expression, and attenuated KA-induced neurotoxicity in hippocampal neurons after KA insult. Furthermore, TNF-α treatment distinctly up-regulated the production of phospho-IκBα, AKT and phospho-AKT in hippocampcal cultures.

The role of TNF-α in neurodegenerative disorders is diverse and complex. Over-expression of TNF-α in transgenic mice is associated with the occurrence of age-dependent neurodegenerative changes and sporadic spontaneous seizures. Antagonisms of TNF-α using a monoclonal antibody assist neuron survival. However, TNF-α deletion has also shown interesting effects on neuronal maturation and arborization. Golan et al. found that the dendritic arborization complexity of pyramidal neurons residing within the CA1 and CA3 regions of the hippocampus was reduced in the absence of TNF-α expression (Golan et al. 2004).

The activation of KARs can induce the production of ROS and compromise the function of mitochondria in the region. KA stimulated the release of LDH, an indicator for loss of cell membrane integrity, suggesting KA induced damage to mitochondrial function. Hippocampal neurons from TNF-α KO mice displayed higher expression of LDH and lower cell survival than WT mice after 24 h KA treatment, which indicated that hippocampal neurons from TNF-α KO mice are more sensitive to KA induced neurotoxicity. Also after pretreated with anti-TNF-α, the expression of LDH was increased and the cell survival rate was lower compared with WT mice without anti-TNF-α treatment. Pretreatment of TNF-α caused the lower expression of LDH and higher cell survival rate compared with WT mice without TNF-α treatment.

NO is involved in many physiological and pathological processes within the CNS and can be formed enzymatically from L-arginine by inducible NO synthase (iNOS) in

neuroglia (Wiesinger 2001). Elevated production of NO by increased activity of iNOS is thought to participate in KA-induced neurotoxicity. In the present study, we observed that KA is capable of increasing the expression of NO in hippocampal neurons from KO mice or the WT mice pretreatment with anti-TNF-α. However, TNF-α treatment obviously reduced the expression of NO in WT mice after KA insult.

The signaling pathway of p38 MAPK can regulate gene expression and lead to increased production of pro-inflammatory cytokines by a number of different mechanisms. P38 MAPK activity has also been associated with the induction of apoptosis in numerous cell types and in response to a multitude of stimuli, such as osmotic shock, inflammatory cytokines, lipopolysaccharides (LPS). Our present results showed that TNF-α deficiency or pretreatment with anti-TNF-α enhanced the expression of p38 MAPK after KA insult. However, pretreatment with different dose of TNF-α didn't influence the production of p38 MAPK after KA treated.

IκBα is the prime target after AKT phosphorylation in the heterotrimeric IκB kinase complex, resulting in IκBβ activation and subsequent IκBα phosphorylation. AKT is necessary for neuronal survival since AKT plays a key role in up-regulation of anti-apoptotic proteins. An established cell lines showed that PKB/AKT may serve as an IKK kinase in response to TNF-α treatment. In the study, after KA treatment, the hippocampal neurons from KO mice and WT mice with anti-TNF-α antibody treatment showed significantly lower expression of phospho-IκBα, AKT and phospho-AKT than WT mice without treatment, as well as TNF-α treatment in the hippocampal neurons of WT mice increased phospho-IκBα and phospho-AKT production compared with WT mice without TNF-α treatment.

In conclusion, TNF-α may play a protective role in KA-induced neurotoxicity, which is via up-regulation of AKT and down-regulation of p38 MAPK signaling pathways.

4.3 STUDY III: ATTENUATED EAN IN TNF-α DEFICIENT MICE IS ASSOCIATED WITH AN ALTERED BALANCE OF M1/M2 MACROPHAGES

By inducing EAN in TNF-α KO mice, we found that TNF-α deficiency attenuated the clinical severity of EAN. The clinical signs of WT mice treated with TNFR1 antibodies were less severe than of the control WT mice receiving PBS. TNF-α deficiency induced an anti-inflammatory 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-γ in infiltrating cells in CE were found in TNF-α KO mice with EAN.

TNF-α signals through TNFR1 and TNFR2, mainly TNFR1, to elicit partly opposite reactions in multiple cell types. In the present study, TNF-α KO mice were not completely resistant to EAN induction. The reason could be that TNF-α 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-α, 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-α acting via TNFR2. We then blocked the functions of TNFR1 in both TNF-α 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). Proinflammatory Th1 cytokines such as IFN-γ and IL-1β, 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-α KO mice with EAN, indicating an antiinflammatory M2 phenotype of macrophages resulting from TNF-α 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-α KO than in WT mice with EAN. After cultured macrophages with proinflammatory stimulation, reduced levels of IL-12 and IL-6 and increased levels of IL-10 were produced from TNF-α KO mice compared with those from WT mice with EAN. Moreover, reduced production of NO was detected in culture supernatants of macrophages from TNF-α KO mice after proinflammatory challenge. The reduced production of NO in TNF-α KO mice may explain the attenuated clinical severity of EAN.

In summary, TNF-α deficiency and TNFR1 blockade remarkably attenuated the clinical severity of EAN and TNF-α deficiency induced an anti-inflammatory phenotype of macrophages (M2).

4.4 STUDY IV: IFN-γ DEFICIENCY EXACERBATES EAN IN MICE DESPITE MITIGATED SYSTEMIC TH1 IMMUNE RESPONSE

IFN-γ deficiency exacerbates the clinical signs after the acute phase of EAN. The proliferation of splenic MNCs was significantly higher in IFN-γ KO than WT mice with EAN after antigenic stimulation. At the nadir 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-γ 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-γ KO than WT mice with EAN. However, IFN-γ 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-γ 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-γ 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-γ may play a pathogenetic role in EAN.

We then focused on the alteration of IL-17A production and found that IFN-γ deficiency induced increased infiltrating of Th17 cells in CE during EAN. In addition, IFN-γ 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-γ in EAN might be due to its inhibition of Th17 development, since IFN-γ can prevent IL-23 triggered expansion of Th17 cells. Moreover, IFN-γ increases T-bet expression, the overexpression of which may in turn lead to a robust reduction of IL-17 generation. Of note is that in line with the traditional Th1/Th2 paradigm, IFN-γ 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 sysmptoms of EAN in IFN-γ 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-γ 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. By using a synthesized inorganic compound that specifically inhibits RORγt and Th17 cells, we found that EAN was significantly attenuated when the compound was administrated from the immunization day. 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 (Zepp et al. 2011).

In summary, IFN-γ deficiency exacerbated the clinical severity of EAN, concomitant with higher production of IL-17A and Th2 cytokines.

5 CONCLUSIONS

1) TNF-α may play a protective role in KA-induced neurotoxicity via the down-regulation of NFκB signaling pathway.

2) TNF-α may play a protecting role in KA-induced neurotoxicity in vitro, which is via up-regulation of AKT and down-regulation of p38 MAPK signaling pathways

3) Attenuated EAN in TNF-α deficient mice is associated with an altered balance of M1/M2 macrophages.

4) IFN-γ deficiency exacerbates EAN via up-regulating Th17 cells despite a mitigated systemic Th1 immune response.

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