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Die zelluläre Immunantwort im entzündeten peripheren Nervensystem – Bedeutung von Kostimulation und Cyclooxygenasen

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CONTENT

ABBREVIATIONS

Ab	antibody
AIDP	acute inflammatory demyelinating polyradiculoneuropathy
AMAN	acute motor axonal neuropathy
AMSAN	acute motor and sensory axonal neuropathy
APC	antigen-presenting cells
APS	ammonium persulfate
ATP	adenosine triphosphate
BNB	blood-nerve barrier
BPB	bromophenol blue
BSA	bovine serum albumin
CAMs	cellular adhesion molecules
CD	cluster of differentiation
CIDP	chronic inflammatory demyelinating polyradiculoneuropathy
Cj	campylobacter jejuni
CNS	central nervous system
COX	cyclooxygenase
CSF	cerebral spinal fluid
CTP	cyclophosphamide
cDNA	complementary DNA
DAB	3,3‘-diaminobenzidine
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
dNTP	desoxynucleoside triphosphate
DTT	dithiothreitol (2-ethansulfonic acid)
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
EDTA	ethylene diamintetraacetic acid
ELISA	enzyme-linked immunoabsorbent assay
EMG	electromyographic
FACS	fluorescence-activated cell sorting
Fig	figure
g	gram

GAPDH	glyceraldehyde phosphate dehydrogenase
GBS	Guillain-Barré Syndrome
GTP	guanosine triphosphate
hr	hour
HEPES	N-2-hydroxyethylpiperazine-
ICOS	inducible costimulator
ICOS-L	inducible costimulator ligand
IFN	interferon
IL	interleukin
IVIg	intravenous immunoglobulin
kb	kilobase
kDa	kiloDalton
LPS	lipopolysaccharide
mg	milligram
MHC	major histocompatibility complex
mg	microgram
ml	milliliter
µl	microliter
mM	millimolar
MMPs	matrix metalloproteinases
mRNA	messenger RNA
MS	multiple sclerosis
NF-κB	nuclear factor-[kappa] B
ng	nanogram
NIN	non-inflammatory neuropathy
nm	nanometer
NO	nitric oxide
OD	optical density
oligo	oligonucleotide
PAGE	polyacrylamid gel eletrophoresis
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PE	plasmapheresis
PG	prostaglandin

pmol	picomol
PNS	peripheral nervous system
RT	room temperature
SDS	sodium dodecyl sulfate
Tab	table
TBE	tris-borate-EDTA
TCR	T cell receptor
TEMED	N,N,N',N'-tetramethyl- ethylendiamine
Th	T helper cell
TGF	transforming growth factor
TNF	tumour necrosis factor
Tris	tris(hydroxymethyl)- aminomethane
TTP	thymidine triphosphate
U	unit
UV	ultraviolet
V	volt
w/v	weight/vol
v/v	vol/vol

ABSTRACT

Das Guillain-Barré-Syndrom (GBS) und die chronisch-entzündliche demyelinisierende Polyradikuloneuropathie (CIDP) repräsentieren prototypische entzündliche demyelinisierende Erkrankungen des peripheren Nervensystems (PNS). Obwohl die diesen Erkrankungen zugrundeliegenden immunpathogenetischen Mechanismen bisher nur unvollständig verstanden sind, besteht allgemeiner Konsens darin, dass eine gestörte zelluläre Immunantwort relevant für die Aufrechterhaltung der entzündlichen Reaktion im Rahmen dieser Erkrankungen wichtig erscheint.

Die vorliegende Arbeit untersuchte in N. suralis-Biopsien und Liquor von Patienten mit GBS, CIDP und, zum Vergleich, mit nicht-entzündlichen Neuropathien (NIN) das Expressions- und Verteilungsmuster von Cyclooxygenasen (COX), Schlüsselenzymen bei der Entstehung und Perpetuierung einer entzündlichen Antwort durch die Umwandlung von Arachidonsäure in Prostaglandine (PGs), einschließlich PGE_2 und $PGF_{2\alpha}$. Ferner wurde der entzündliche Co-Stimulator (ICOS), ein unlängst identifiziertes co-stimulatorisches Molekül, das für die Aktivierung von T-Zellen relevant erscheint sowie sein einzigartiger Ligand (ICOS-L) untersucht. Mittels RT-PCR zeigte sich eine signifikante Aufregulation von COX-2 mRNA im N. suralis von Patienten mit GBS und CIDP, jedoch nicht in der NIN-Gruppe, wohingegen die Expression von COX-1 mRNA in allen untersuchten Gruppen unverändert erschien. Mittels Immunhistochemie konnten Makrophagen als zelluläre Quelle identifiziert werden. Erhöhte COX-2-Proteinspiegel konnten mittels Immunoblot im Liquor von Patienten mit GBS und CIDP, nicht jedoch innerhalb der NIN-Gruppe, detektiert werden. Um die funktionelle Aktivität von COX-2 zu bestätigen, wurde die Expression von PGE_2 und $PGF_{2\alpha}$ mittels ELISA gemessen und im Serum von Patienten mit GBS und CIDP sowie in Zellkulturüberständen von *in vitro* stimulierten Makrophagen detektiert. ICOS und ICOS-L mRNAs konnten mittels RT-PCR in Proben von GBS- und CIDP-Patienten im Vergleich zu NIN-Kontrollen gefunden werden. T-Lymphozyten konnten als Ursprungszelle von ICOS identifiziert werden, wohingegen Makrophagen den korrespondierenden Liganden ICOS-L exprimierten, wie mittels Immunhistochemie dargestellt.

Die hier erhobenen Befunde legen nahe, dass Makrophagen als antigenpräsentiernde Zellen agieren und durch die Expression von ICOS-L, das an ICOS bindet und somit aktivierten T-Zellen ein co-stimulatorisches Signal bietet, im entzündeten PNS wichtig bei der Amplifikations- und Effektorphase immunvermittelter Demyelinisierung involviert sind, darüber hinaus COX-2 aufregulieren, PGE_2 und $PGF_{2\alpha}$ sezernieren, und somit weiterhin zum Prozess der entzündlichen Demyelinisierung im peripheren Nervensystem beitragen können.

1 INTRODUCTION

Guillain-Barré Syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) represent prototypic inflammatory, demyelinating disorders of the peripheral nervous system (PNS), characterized by multifocal demyelination and mononuclear cellular infiltration (Ho et al., 1998).

1.1 Clinical spectrum

GBS is the most common cause of acute flaccid paralysis in the western hemisphere and probably world-wide with an average incidence rate of about 1.5/100.000, affecting patients of all ages and both sexes (Govoni and Granieri, 2001; Hartung et al., 1995). Clinically, GBS, termed as a rapidly progressive ascending polyradiculoneuropathy, is viewed as a monophasic and self-limited autoimmune disease triggered by a preceding bacterial, such as Campylobacter jejuni (Cj), or viral infection and characterized by symmetrical ascending flaccid paralysis, reduced or absent reflexes, mild sensory signs, variable autonomic dysfunctions, and sometimes with involvement of cranial nerves (Hahn, 1998). Typically, GBS begins with muscle weakness and parenthesis (abnormal sensations), such as tingling or numbness. Classically, these symptoms initially affect the legs first and progress within days to involve the upper limbs and facial muscles in an ascending fashion. The severity and distribution of weakness may vary from mild lower limb involvement to total, including respiratory, paralysis. Therefore, mechanical ventilation may be required in up to 20% of patients (Hartung et al., 1998; Joseph and Tsao, 2002). The laboratory findings include cerebral spinal fluid (CSF) feature albuminocytological dissociation and electrophysiological evidence of conduction slowing, block, prolonged distal latency, or F-wave latencies (Hahn, 1998). The nadir, by arbitrary definition, is reached within four weeks followed by a plateau of variable duration and eventually, by gradual recovery (Kieseier and Hartung, 2002). Recovery takes place over weeks, months or years and in the majority of cases is virtually

complete. Neurological deficits may remain in 15-20% of cases. The mortality rate in developed countries has been reduced to 2-3% (Dyck et al., 2002). During these years, according to its specific clinical, electrophysiological and pathological features, it has been recognized that GBS can mainly be subdivided into 5 distinct forms (Hughes, 1995; Van der Meche et al., 2001): (A) the classical acute demyelinating type, acute inflammatory demyelinating polyradiculoneuropathy (AIDP) representing the great majority of cases in Europe and North America; (B) a pure motor axonal type, called acute motor axonal neuropathy (AMAN), the most prevalent form in China where it occurs in children and young adults; (C) an axonal variant involving both motor and sensory fibers, called acute motor and sensory axonal neuropathy (AMSAN), which is distinguished by significant sensory involvement and associated with a more severe course and poorer prognosis; (D) Miller Fisher syndrome (MFS), characterized by ophthalmoplegia, ataxia, and areflexia, a proportion of which develop into generalized GBS; and (E) acute pandysautonomia, characterized by the rapid onset of combined sympathetic and parasympathetic failure without sensory and motor involvement (Kieseier and Hartung, 2002). Moreover, a number of other well-characterized but uncommon regional variants occur, including pure ataxic GBS, pharyngeal–cervical–brachial GBS, and isolated bulbar palsy (Kieseier et al., 2004). Up to now, plasmapheresis (PE) and high-does intravenous immunoglobulin (IVIg) are the mainstay of immunomodulatory treatment for GBS (Kieseier and Hartung, 2003; van Doorn and van Koningsveld, 2004).

Whereas GBS is acute and monophasic, CIDP, as its name suggest, being a chronic condition, is a symmetric polyradiculoneuropathy that affects both motor and sensory fibres and begins insidiously and evolves slowly in either steadily or stepwise progressive manner over a period of greater than 8 weeks (Latov, 2002). Clinically, CIDP is distinguished from GBS by its relatively symmetric motor and sensory functions of both proximal and distal extremities, associated with areflexia and its favourable responsiveness to steroids. Sometimes, distal

muscles are more affected and peripheral nerves may be enlarged (Dyck et al., 1993). However, recent observations underline that many patients do not conform to these classic features of the disease, exhibiting predominantly distal distribution, pure sensory neuropathy, marked asymmetries, associated demyelinating disease, or predominant cranial nerve involvement (Kieseier et al., 2004; Rotta et al., 2000). Laboratory findings suggest that CSF protein is elevated, usually above 60 mg/dl and electromyographic (EMG) studies show features of multifocal demyelination. Moreover, recent surveys reported the CIDP prevalence of 1 to 7.7 per 100,000 population (Lunn et al., 1999; McLeod et al., 1999; Mygland and Monstad, 2001), which is likely an underestimate, probably due to underreporting and uncertainty in making the diagnosis (Latov, 2002). For treatment of CIDP, corticosteroid, PE, IVIg, and immunosuppressive drugs, such as azathioprine and cyclosporine, are clinically available, but none of them treat the disease completely (Ropper, 2003; Toyka and Gold, 2003).

1.2 Morphology

The pathologic hallmark of classical GBS is multifocal inflammatory demyelination of the PNS. However, the spectrum of pathologic changes can range from focal or extensive demyelination in the presence of absence of cellular infiltration, to axonal degeneration with or without demyelination or inflammatory infiltrates. Pathological alterations can be found throughout the cranial nerves, ventral and dorsal roots, and dorsal root ganglion and along the entire length of the peripheral nerves. Inflammatory cell infiltration and oedema were considered regular initial events in the spinal nerve roots and the PNS. Typically, demyelination can primarily be found at the nodes of Ranvier where macrophages cluster, cells known to strip off and phagocytes compact myelin, thus participate in the removal of damaged myelin (Dyck et al., 2002; Schröder, 2001). Moreover, the varied histopathological features mirror the clinical diversity of GBS: whereas AIDP is characterized by macrophage-

mediated demyelination and intense T-cell infiltration, AMAN and AMSAN exhibit signs of a macrophage-mediated axonal neuropathy, and lymphocytic infiltrates are scarce (Griffin et al., 1996; Griffin et al., 1995).

CIDP is a multifocal, predominantly proximal, inflammatory demyelinating disorder that chiefly affects spinal nerve roots, spinal nerves, major plexuses, or proximal nerve trunks. The presence of "onion bulb" formations, perivascular lymphocytic infiltrates, segmental demyelination and remyelination, axonal degeneration and a variable degree of hypertrophic neuritis are the typical features of CIDP, although they may not always be found on limited biopsies. The inflammatory infiltrates are seen in roots, segmental nerves, plexuses and different levels of peripheral nerves. They are epineurial, perineurial or endoneurial. The events of demyelination and remyelination and axonal degeneration and regeneration are concurrent (Dyck et al., 2002; Prineas, 1994; Schröder, 2001). With time, affected nerves develop increased endoneurial collagen and "onion bulbs". "Onion bulb" formation is a manifestation of excessive proliferation of Schwann cell processes and is often produced by segmental repetitive demyelination and remyelination. As the disease progresses, secondary axon degeneration becomes more established, but it also may occur early in the disease course (Toyka and Gold, 2003). The results of electron microscopic studies revealed a distinctive pattern of myelin breakdown and phagocytosis by macrophages of the myelin portion of the Schwann cell plasma membrane. Lymphocytes are present in the endoneurium and in the basement membrane of fibres that are in contact with macrophages (Dyck et al., 2002; Griffin et al., 1993).

Taken together, the morphologic observations of GBS and CIDP underline the key role of the immune system, particularly inflammatory mononuclear infiltrates and macrophages, in the pathogenesis of inflamed PNS.

1.3 Immunopathogenesis - cellular immune response

So far, our knowledge of the precise mechanisms involved in these disorders is still incomplete, however, there is consensus that both GBS and CIDP result from aberrant humeral and cellular immune responses directed to peripheral nerve antigens (Hartung et al., 1995). One hypothesis of special relevance to autoimmune neuropathies is molecular mimicry. This term refers to a process in which the host generates an immune response to an inciting factor, usually an infectious organism that shares epitopes with the host's affected tissue. In at least some forms of GBS researchers have identified epitopes that are shared among *Cj, Haemophilus influenzae, Cytomegalovirus*, and human nerve fibres and are targets for aberrant cross reactive immune responses (Willison and Yuki, 2002). However, researchers have not yet found such a specific correlation for CIDP (Kieseier et al., 2002a). On the other hand, histopathological studies on nerve biopsy and autopsy material, studies in experimental autoimmune neuritis (EAN), the animal model of GBS that follows sensitization of the animal to whole peripheral nerve myelin or to specific components of peripheral nerve myelin, such as P2 protein of myelin or P0 glycoprotein, and more recently, the demonstration of cellular infiltrates in sural nerve biopsies from patients with GBS and CIDP support the notion that disordered cellular immune response to peripheral nerve is of critical importance in the pathogenesis of these inflammatory peripheral nervous disorders. The majority of cells infiltrating the nerves in classical GBS and CIDP are macrophages, whereas, CD4 T –helper/inducer cells and CD8 suppressor/ cytotoxic T cells can also be demonstrated in sural nerve biopsies (Arnason and Soliven, 1993; Hartung et al., 1995). The cellular immune response within the PNS is tightly regulated at the transcriptional level. Study results showed that one of its key regulators, the transcription factor nuclear factor-[kappa] B (NF-κB), is up-regulated in nerve sample from patients with GBS and CIDP. In contrast, its controlling inhibitory molecule I-κB is not up-regulated. Macrophages were determined to be the major

cell type expressing NF-κB in chronic inflammatory demyelination. This points to a key role for this transcription factor in the signalling cascade for various pro-inflammatory mediators during ongoing disease of PNS (Andorfer et al., 2001).

1.3.1 The role of macrophages

Macrophage-mediated segmental demyelination is the pathological hallmark of autoimmune demyelinating polyneuropathies, including GBS and CIDP. Whereas axons and Schwann cells form the main cellular constituents of the PNS, a considerable number of local macrophages reside within the endoneurium of peripheral nerve (Arnason and Soliven, 1993). First recognized by Arvidsson (Arvidson, 1977) due to their phagocytosing capacity, resident macrophages are known to comprise 2–4% of total cells in peripheral nerves (Arnason and Soliven, 1993) or , probable, even up to 9% of the cellular components of normal peripheral nerve (Griffin et al., 1993). Resident endoneurial macrophages of normal nerve are elongated cells stretching along the longitudinal axis of peripheral nerves and show small ramifications with two or three terminal branches at their ends (Griffin et al., 1993; Monaco et al., 1992). They are found close to endoneurial blood vessels but also scattered throughout the endoneurium. Moreover, these cells usually lie outside the basal lamina of the blood vessels and have a dendritic appearance (Kiefer et al., 2001). Their perivascular distribution at the blood-nerve interface makes them uniquely suited to act as antigen-presenting cells (APC) in the PNS (Hartung et al., 1995). Many of these resident macrophages constitutively express major histocompatibility complex molecules (MHC) class II molecules, complement receptor type 3(CR3), and CD4, but the level of expression of these molecules can be enhanced greatly in inflammatory conditions (Kiefer et al., 2001).

Macrophages are putatively involved in virtually all steps of the pathogenetic process, from early immune surveillance, antigen presentation and activation of the cellular

immune cascade throughout the disease to antigen-specific demyelination and axonal damage, non-specific secondary tissue destruction, removal of debris and regeneration (Griffin et al., 1993; Kiefer et al., 2001).

It has been well established that macrophage-mediated segmental demyelination is the pathological hallmark of autoimmune demyelinating polyneuropathies, including the classical GBS and CIDP (Hartung et al., 1995; Ho et al., 1998). Histopathologically macrophages invade the Schwann cell basal lamina, penetrate myelin lamellae, strip myelin lamellae and phagocytose both damaged and apparently intact myelin (Arnason and Soliven, 1993; Ballin and Thomas, 1969), which is considered an active immunological process. Macrophages serve a multitude of functions throughout the entire pathogenetic process of autoimmune neuropathy. They may be derived from circulating monocytes that invade the PNS or they may already reside in the PNS. Resident endoneurial macrophages are likely to act as local APC by their capability to express MHC antigens and costimulatory B7-molecules, and may thus be critical in triggering the autoimmune process. In GBS and CIDP, constitutively expressed MHC class I and II molecules are found to be strongly up-regulated on macrophages in sural nerve. Whereas, the costimulatory B7 molecules are undetectable by immunocytochemistry in normal nerves but may be induced on endoneurial as well as epineurial macrophages during infectious or autoimmune inflammatory neuropathies (Kiefer et al., 2001). Therefore, macrophages within the PNS, presumably both resident endoneurial macrophages as well as those of hematogenous origin, are thus equipped with several tools enabling them to present antigen. Hematogenous infiltrating macrophages then find their way into the peripheral nerve together with T cells by the concerted action of adhesion molecules (Hartung et al., 1994; Oka et al., 1994), matrix metalloproteinases (MMPs) (Hughes et al., 1998; Kieseier et al., 1998) as well as chemotactic signals (Zou et al., 1999) and through several consecutive and coactive steps — (i) rolling and loose contact between circulating

monocytes and vascular endothelium; (ii) establishing a firm adhesion between monocytes and endothelial cells; (iii) perception of chemoattractive signals; (iv) penetration of endothelium and the underlying basal lamina; and (v) differentiation into tissue macrophages (Kiefer et al., 2001). The immunological mechanisms leading to macrophage-mediated segmental demyelination have only partially been explored. Macrophages, in contrast to T cells, do not act in a cognizant antigen specific manner and need to be targeted by additional mechanisms. There is good evidence that antibodies may direct macrophages towards their myelin or axonal targets in autoimmune neuropathies and that, macrophages attack sites of antigen binding, in a complement dependent manner (Kiefer et al., 2001). In addition, Macrophages are capable of releasing toxic mediators (oxygen radicals, nitric oxide metabolites, and proteases), targeting the peripheral neural tissue and actively phagocytosing myelin thus responsible for non-specific tissue destruction in inflamed PNS (Kieseier et al., 2002a; Kieseier and Hartung, 2002). The role of macrophages in demyelination was further emphasized from the electron microscopy studies in EAN, which have shown that macrophages strip myelin lamellae and phagocytose off both damaged and apparently intact myelin. Once within the nerve, macrophages promote inflammation by releasing pro-inflammatory cytokines including interleukin (IL)-1, IL-6, as well as tumour necrosis factor alpha (TNF)-α (Maurer et al., 2002). They, thus, not only act as chief effector cells in demyelination and tissue destruction but are also intimately involved in the control of the pathogenetic process.

Collective data here suggest that macrophages act as APC and are of critical importance in the amplification and effector phases of immune-mediated demyelination by the secretion of proinflammatory cytokines, the release of active mediators, and enhanced phagocytosis (Hartung et al., 1995; Kieseier et al., 2002a). Moreover, macrophages also contribute to the termination of inflammation through the induction of T cell apoptosis (Gold et al., 1997) and the release of anti-inflammatory cytokines including transforming growth factor

(TGF)-β1 and IL-10 during the recovery of disorders (Hartung et al., 1988a). As a conclusion, it can be speculated that macrophages support the normal intact microenvironment of the PNS, but when disturbed by autoimmunity, they loose their normal control and develop their destructive potential due to the distorted specific immune response following, for example, a preceding infection as in GBS.

APC in the peripheral nervous system include activated immigrating and resident macrophages or activated Schwann cells. The debate continues as to which of these cells are of greatest importance in this regard. Schwann cells, in contrast to macrophages, do not express MHC II molecules in EAN although in some studies, MHC II positive Schwann cells were described in sural nerve biopsies from patients with inflammatory neuropathies (Pollard et al., 1987). In addition, cultured Schwann cells can readily be induced to express MHC antigens after treatment with interferon (IFN)-γ and TNF-α or present antigen to activated T cells *in vitro* (Maurer et al., 2002). It is thus conceivable that Schwann cells may contribute to antigen presentation in autoimmune neuropathies upon very strong stimulation and under selected conditions, but resident and infiltrating endoneurial macrophages are clearly the professional antigen presenting cells within the PNS (Hartung et al., 1995; Kiefer et al., 2001).

1.3.2 The role of T cell

Besides macrophages, other immunocompetent cells appear critical in the pathogenesis of immune-mediated inflammation of the PNS: one of these is T lymphocytes, which clearly represent another pathogenetically relevant cell population in GBS and CIDP.

The central role of T cells in disease pathogenesis was established by demonstrating that the transfer of autoreactive P2, P0, or P2/P0 peptide-specific T-cell lines can induce the clinical, electrophysiological, and morphological features of classical EAN in naïve recipient rats (Hartung and Toyka, 1990; Ho et al., 1998). The findings that predominantly

demyelinative changes occur with low cell dose while the addition of higher cell numbers produce axonal damage and marked endoneurial oedema were made in actively induced EAN depending on the dose of the immunogen, indicating that an immune response to the same antigen can give rise to a different clinical course and pathology. Moreover, the pivotal role of T cells is also underscored by the preventive and suppressive effects of manipulations that eliminate or silence T lymphocytes in EAN (Hartung et al., 1993).

T-cell immune responses are initiated and activated in the systemic immune compartment by the tri-molecular interaction between APC, MHC, and a specific T cell receptor (TCR) and simultaneously deliver co-stimulatory signals, like B7 to the cell surface of APC, such as macrophages. A bacterial or viral infection might trigger this event by inducing molecular mimicry (Hartung et al., 1995). Circulating T cells that carry on their surface the activation markers HLA-DR, the IL-2 receptor (IL-2R) were shown to be increased in number in both GBS and CIDP patients (Taylor and Hughes, 1989). Likewise, higher concentrations of soluble IL-2R shed from activated T cells were measured in the serum from patients and correlated with disease activity. Moreover, IL-2, the signal required to drive T cells into clonal proliferation, circulates in increased amounts in GBS and CIDP, further evidence for T cell activation within periphery of inflammatory PNS (Hartung et al., 1995; Kieseier et al., 2002a).

In order to generate inflammatory lesions in the peripheral nerve, activated T cells need to cross the blood-nerve barrier (BNB) and enter the PNS. The complex process of its homing, adhesion, and transmigration has been studied in EAN in great details. Although breakdown of the BNB is one of the earliest morphologically demonstrable events in lesion development, a major disruption of the BNB may not be necessary. Thus, it has been postulated that activated T cells can traverse a structurally intact barrier to execute immune surveillance irrespective of their antigenic specificity. Chemokines (Kieseier et al., 2002b), cellular adhesion molecules (CAMs) (Previtali et al., 2001), and MMPs (Kieseier et al.,

1998) have been substantially demonstrated to be critically involved in T-cell trafficking to peripheral nerve. In GBS and CIDP patients, increased serum and/or CSF levels of chemokine, CAMs and MMPs can be detected, reflective active T cell migration across the BNB. Recently, a selective expression pattern of specific chemokines and chemokine receptors has been described in sural nerve biopsies from patients with classical GBS, points to a pathogenic role for CXCL-10 in the genesis of inflammatory demyelinating neuropathies (Kieseier et al., 2002b). Also a differential expression pattern of MMPs, specifically MMP-7 and MMP-9, can be depicted in the endo- and epineurium in sural nerves of affected patients (Hartung et al., 2002; Kieseier et al., 2002a).

Within the PNS, T lymphocytes expand clonally and encounter their target antigen, recognize appropriate MHC molecules, and perceive additional co-stimulatory signals (Gold et al., 1999). These locally expanded T lymphocytes exert various effects in peripheral nerves. Firstly, CD4 T cells of the Th1 inflammatory phenotype (synthesizing TNF-α, IFN-γ and IL-2 could damage myelin by secreting pro-inflammatory and myelin toxic cytokines and operate by recruiting and activating macrophages to produce and release an array of toxic molecules or to engage in increased phagocytotic activity. Secondly, CD4 helper T cells of the Th2 phenotype (synthesizing IL-4, IL-5 and IL-6) may cause B cell proliferation and transformation into plasma cells that manufacture antibodies against peripheral myelin components. Finally, activated CD8 or perhaps a subset of CD4 T lymphocytes may be directly cytotoxic to Schwann cells. In a recent study on archival autopsy material, large numbers of CD8 T lymphocytes were found in patients with an acute course of the disease, implicate an important role of cytotoxic T cell responses for myelin damage in subacute stages of GBS (Wanschitz et al., 2003). On the other hand, specialized subpopulations of T cells may terminate the acute immunoinflammatory process by the secretion of anti inflammatory cytokines, such as IL-10, and TGF-β or other molecules (Hartung et al., 1995).

Taken together, in inflammatory PNS, the pathogenetic sequence of T-cell-mediated responses to nerve antigens comprises a number of distinct steps: homing to and crossing of the BNB, endoneurial activation, clonally expansion, release of injurious molecules, recruitment of other inflammatory effector cells, like macrophages, thus, eventually contribute to the inflammatory demyelinating process of PNS (Fig 1).

In summary, we hereby address the crucial role of both T cells and macrophages during inflammatory demyelination process of autoimmune neuropathies. Although, in recent years, observations of the immunopathology of patients' nerves, serologic studies, transfer experiments, and experimental animal model have advanced our understanding of the cellular immune responses in the immune-mediated demyelination that affects the PNS, the precise etiology of these disorders continues to be elusive in the future.

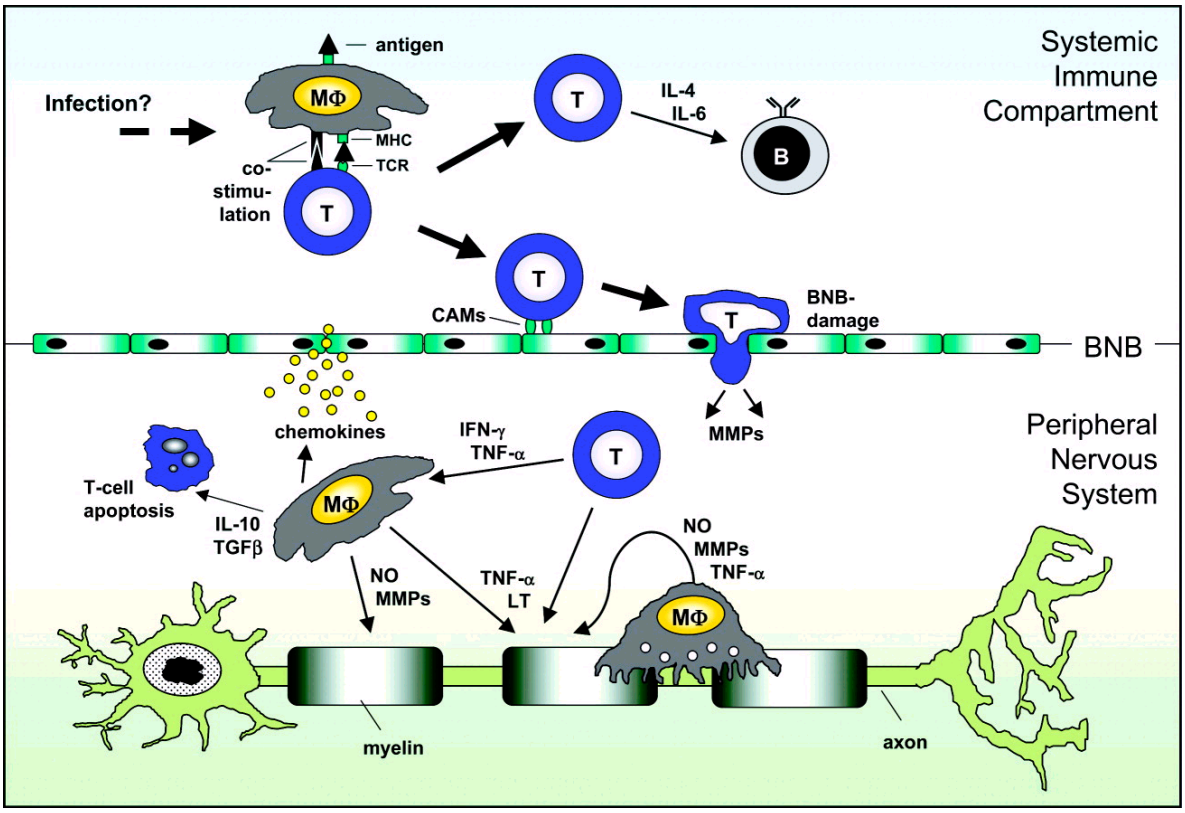


Figure 1. Schematic illustration of the immune responses in the inflamed peripheral nervous system. Basic principles of the cellular immune responses: autoreactive T-cells (T) recognize a specific autoantigen presented by major histocompatibility complex (MHC) class II molecules and the simultaneous delivery of costimulatory signals on the cell surface of antigen-presenting cells, such as macrophages (MΦ), in the systemic immune compartment. Activated T-lymphocytes can cross the blood-nerve barrier (BNB) in order to enter the peripheral nervous system (PNS). Within the PNS, T-cells activate macrophages that enhance phagocytic activity, production of cytokines, and the release of toxic mediators, such as nitric oxide (NO), MMPs, and proinflammatory cytokines, propagating demyelination and axonal loss. The termination of the inflammatory response is mediated, in part, by macrophages by the induction of T-cell apoptosis and the release of anti-inflammatory Th2/Th3 cytokines, such as interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). (Kieseier et al., 2004)

1.4 Cyclooxygenases and prostaglandins

Cyclooxygenase (COX), a rate-limiting enzyme catalyzing the synthesis of prostaglandins (PGs), including prostaglandin E2 (PGE_2), prostaglandin F2α($PGF_{2\alpha}$), prostaglandin D2 (PGD_2), prostacyclin (PGI_2), and thromboxane (TxA_2) from arachidonic acid, was first identified over 20 years ago. In the past decade, more progress has been made in understanding the role of COX enzymes in biology and disease than at any other time in history (Dubois et al., 1998). Two isoforms of the membrane protein COX have been described in mammalian cells so far (Smith et al., 1996): (a) the constitutively expressed isoform COX-1 is expressed throughout the body and considered an important enzyme for the synthesis of homeostatic prostaglandins responsible for the regulation of various physiological processes, including gastric mucosal protection, maintenance of renal blood flow, regulation of platelet activation and aggregation, and (b) the inducible enzyme COX-2, up-regulated after stimulation with certain cytokines, hormones, and lipid mediators, and responsible for the enhanced production of prostaglandins, finally involved in inflammatory reactions and contributed to inflammatory signs like fever, pain, capillary oedema, and vasodilatation (Fig 2) (Parente and Perretti, 2003). The strongest arguments supporting a role for COX-2 in inflammation are, first, that COX-2 expression can be induced by endotoxins, such as lipopolysaccharide (LPS), and pro-inflammatory cytokine, in most cell types involved in inflammation. There include macrophages and monocytes, endothelial cells and synovial cells. Second, COX-2 expression is down-regulated in these cells by anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13, as well as by glucocorticoids. Moreover, in animal models of acute and chronic inflammation, the observations that COX-2 but not COX-1 expression is induced and the symptoms of inflammation parallel COX-2 induction, strongly support a predominant role for COX-2 in inflammatory process. Finally, the role of COX-2 in

patients of inflammatory disorders, such as rheumatoid arthritis and osteoarthritis, has also been further confirmed by different research groups (Pairet et al., 1999).

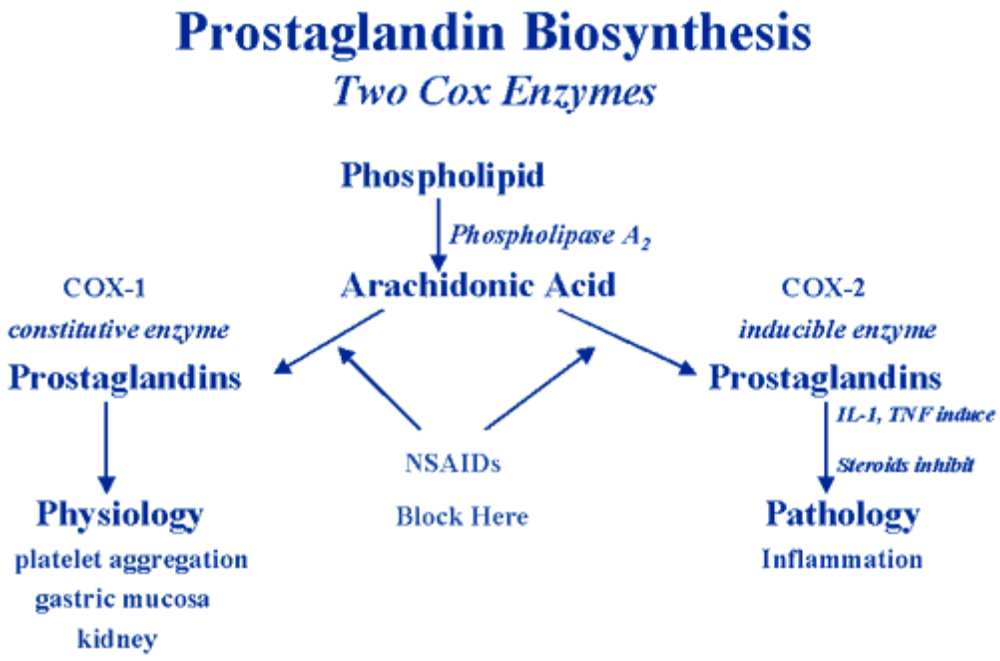


Figure 2. Synthesis of prostaglandins from arachidonic acid catalysed by one of two isoforms of cyclooxygenase: COX-1 was constitutively expressed and responsible for basal PGs production, while COX-2 was induced by inflammatory stimuli such as interleukin-1 and suppressed by glucocorticoids. Available non-steroidal anti-inflammatory drugs (NSAIDs) non-specifically inhibit both the COX-1 and COX-2 enzymes. (From http://www.hopkins-arthritis.som.jhmi.edu/osteo/cox2_ppt_2.html)

Prostaglandins, products of the COX pathway of arachidonic acid metabolism, are important lipid mediators that regulate numerous processes in the body, including kidney function, platelet aggregation, neurotransmitter release and modulation of immune function. One of the best known and most well studied PGs is PGE_2. PGE_2, produced by many cells, including fibroblasts and macrophages, exerts its actions by binding to its specific receptor and intimately involve in regulating the inflammatory response by enhancing vascular permeability, providing chemotactic signalling, and modulating inflammatory cell activities (Harris et al., 2002).

Whereas COX-1 seems to be ubiquitously expressed in most tissues and organs, COX-2 is primarily localized to inflammatory cells and tissues. Evidence is mounting that COX-2 and its product, the prostaglandin PGE_2, are involved in the pathogenesis of inflammatory demyelination of the central nervous system (CNS). In experimental autoimmune encephalomyelitis (EAE), a model disease for multiple sclerosis (MS), a positive correlation between an increase in PGE_2 and the clinical severity of EAE have been found, and both the application of neutralizing antibodies to PGE and COX inhibitors was sufficient to prevent and attenuate clinical active EAE, pointing to an important role of COX in the pathogenesis of autoimmune inflammation within the CNS (Kieseier and Hartung, 1999). Furthermore, In clinical MS patients, increased PGE_2 production by peripheral blood monocytes *ex vivo* has been noted and, whereas, COX-2 was also found to be mainly expressed by microglia near damaged oligodendrocytes in the demyelinating MS lesions (Rose et al., 2004). On the other hand, another interesting prostaglandin- $PGF_{2\alpha}$, partly derives as a product from the activity of the inducible isoform COX-2 in human monocytes (Patrignani et al., 1996), is reported to be a stable lipid peroxidation product used as an index of oxidative stress *in vivo*. It has been found that the CSF level of $PGF_{2\alpha}$ was three times higher in subjects with definite MS than in a benchmark group of subjects with other neurological disorders. The levels of $PGF_{2\alpha}$ were also moderately correlated with the degree of disability, thus, suggesting that $PGF_{2\alpha}$ may be a

reliable marker of lipid peroxidation and oxidative stress with potential relevance to human demyelinating disorders like MS (Greco et al., 1999). As such, collective data here indicate that COX-2 may act as a key player in the generation of various prostaglandins including PGE_2 and $PGF_{2\alpha}$ during inflammatory demyelination of CNS.

Information about the role of COX in the inflamed PNS is limited, although mostly from the experimental models. In EAN, an animal model for GBS, COX-2 was significantly increased in the sciatic nerve at the peak stage of EAN and declined during the recovery phase (Shin et al., 2003). By immunohistochemistry, endoneurial macrophages of EAN have been proved to be the main cellular source of COX-2 (Miyamoto et al., 1999). In addition, the administration of the COX-2 selective inhibitors, like celecoxib and meloxicam, significantly abrogated clinical, neurophysiological, and histomorphological signs of the disease by lower the PGE_2 concentration in EAN (Miyamoto et al., 2002; Miyamoto et al., 1999). Therefore, all the data from the animal models here indicate that COX and prostaglandins represent important factors in the regulation of the immune response in inflammatory demyelination of the PNS. However, data on the presence of COX and their products in human GBS and CIDP patients have not been reported to date.

1.5 Inducible costimulator (ICOS) and its ligand (ICOS-L)

It is widely accepted that optimal T cell activation and differentiation requires at least two distinct signals delivered during interaction with an APC; these include antigen-specific signalling through the TCR and signalling through costimulatory molecules. If the T cell does not receive adequate costimulation, the cell is rendered anergic or undergoes apoptosis. Thus, costimulation is central to T cell activation and survival (Coyle and Gutierrez-Ramos, 2001; Sharpe and Freeman, 2002).

A primary costimulatory signal is delivered through the CD28 receptor constitutively expressed mainly on naïve and resting T cells after engagement of its ligands B7-1 and B7-

2 on APCs. CD28-mediated costimulation plays a critical role in normal T cell activation. However, although CD28-mediated costimulation appears to be essential for initial T cell priming, secondary or memory responses are CD28-independent, which suggest the presence of alternative costimulatory pathways (Rottman et al., 2001; Sharpe and Freeman, 2002).

One such alternative T cell costimulatory pathway involves the recently discovered inducible costimulator protein (ICOS), a glycosylated , disulfide-linked homodimer of 55–60 kDa that shares approximately 20% homology with CD28 encoded in a gene cluster of chromosome 2q33 adjacent to the CD28 gene (Carreno and Collins, 2002). ICOS was first identified in a screen for unique molecules expressed on human peripheral blood T cells following activation (Hutloff et al., 1999). Compared with CD28, ICOS has a distinct expression pattern and is not expressed constitutively on naïve T cell but is induced on CD4 T helper cells and CD8 cytotoxic T cells following cell activation. Increased ICOS mRNA levels can be detected as early as 1h following TCR cross linking, and cell surface expression is detected 12 h after activation, reaching maximum levels at 48h, followed by a slow decline. The fact that ICOS, undetectable in naïve T cell, can be rapidly induced on T cells after TCR engagement indicates that ICOS might provide costimulatory signals to activated T cells (Sharpe and Freeman, 2002). Much progress has been made in a short time and, although many questions remain, it is clear that ICOS ligation can enhance T-cell proliferation and influence T-cell effector function. Moreover, based on the findings that the ability of ICOS to stimulate cytokine production in recently activated T cells, the induction of ICOS expression upon cell activation and the unique expression pattern of its ligand (see below), it appears that ICOS performs a distinct costimulatory function compared with CD28, and primary involves in effector phase of inflammatory responses (Chambers et al., 2001).

On the other hand, the unique ligand for ICOS (ICOS –L), identified as being a new member of the B7 family and shares approximately 20% amino acid identity with B7-1/2. However, it does not contain the SQDX'ELY domain possessed by B7-1/2 and does not bind to CD28. In contrast to B7-1/2, ICOS-L is expressed not only on haematopoietic cells but also on other cell types. Mounting evidence suggest that ICOS-L is expressed constitutively at low levels on APC, including monocytes/macrophages and dendritic cells, and resting B cells. IFN-γ induces ICOS-L expression on monocytes and dendritic cells. B7-1/2 can also be up-regulated by IFN-γ, but the mechanisms seem to be distinct: B7-1/2 induction by IFN-γ depends on NF-κB, whereas ICOS-L induction is NF-κB independent. Moreover, ICOS-L production can be regulated by inflammatory signals in peripheral tissue, like brain, heart, liver, kidney and endothelial cells (Carreno and Collins, 2002; Sharpe and Freeman, 2002).

Recently, it has been well established that ICOS and its specific ligand ICOS-L participate primarily in memory and effector T-cell activation. The effector phase of the immune response occurs after initial T cell activation and differentiation and includes the production of effector cytokines, as well as the expansion and migration of differentiated Th cells to the site of inflammation. The functional importance of ICOS and ICOS-L co-stimulation in autoimmunity has been shown in experimental models of allergic airway disease and transplantation, where blocking of ICOS signalling during the effector phase, but not during the initial priming, ameliorates the course of the disease. Moreover, signals through ICOS appears to be more and more important for regulating cytokine production by recently activated and effector T cells and also participating in Th1 and Th2 effector responses (Sharpe and Freeman, 2002).

Recently, emerging evidence suggest that costimulation mediated through ICOS and its ligand may be more involved in the maintenance of effector immune responses than CD28 costimulation in immune-mediated disorders of the CNS (Rottman et al., 2001; Wiendl et al., 2003). Studies of EAE, a Th1 disease mediated by myelin-specific CD4+ T cells, further

show the influence of ICOS on Th1 responses and indicate that the outcome of ICOS blockade might be distinct when ICOS co-stimulation is blocked during T cell priming than during the effector phase of EAE. Rottman et al found that, after the induction of EAE, brain ICOS mRNA and protein level were upregulated on infiltrating CD3 + T cells before disease onset. ICOS blockade during the efferent immune response (9–20 days after immunization) abrogated disease (Rottman et al., 2001). Moreover, the other study regarding the role of ICOS during the effector phase has shown that ICOS blockade at the peak severity of EAE dramatically inhibits EAE progression and improved the resolution of clinical symptoms (Sporici et al., 2001), indicating that ICOS costimulation has a key role in the maintenance of effector immune responses in inflammatory disorders of the CNS.

On the other hand, the role of CD28 and B-7 mediated costimulation in initiation and development of the inflammatory peripheral nervous disorders have been discerned in the animal models of EAN (Zhu et al., 2001). CD28 mRNA level and its immunoreactivity were also observed on the few endoneurial T cells in CIDP patients (Murata and Dalakas, 2000). Moreover, investigators have found B7 molecules expressed by macrophages in sural nerve biopsy from CIDP patients (Kiefer et al., 2000). However, data on the presence of ICOS and ICOS-L in GBS and CIDP patients have not been reported so far.

As such, my present study focuses on the expression and distribution pattern of COX-1, COX-2, ICOS and ICOS-L in sural nerve biopsies from patients with classical GBS (AIDP), CIDP, and, for comparison, with non-inflammatory neuropathies (NIN). Moreover, COX-2 protein level in the serum and CSF samples from GBS, CIDP patients and controls was also been measured. To confirm functional COX-2 activity, expression and regulation of the prostaglandins PGE_2 and $PGF_{2\alpha}$ were evaluated *ex vivo* and in macrophages after *in vitro* stimulation, respectively.

2. MATERIALS and METHODS

2.1 Human nerve biopsies

Sural nerve biopsies (*n=15*) were obtained with informed consent from patients admitted to the Departments of Neurology at the Heinrich Heine University. Three groups of patients were studied (Tab 1). The first (*n=6*) was diagnosed as GBS (AIDP) according to the criteria of Asbury and Cornblath (Asbury and Cornblath, 1990). The second (*n=6*) involved patients classified as CIDP based on accepted research criteria (1991). The third group (*n=3*) served as control and included NIN. The characteristics of these patients were summarized in detail as below (Tab 1). None of the patients with GBS and CIDP studied had received any immunomodulatory or immunosuppressive treatment within 3 months before biopsy.

2.2 Serum and cerebrospinal fluid (CSF) samples

To measure expression of COX-2 protein in serum as well as CSF, matched samples were obtained with informed consent from patients diagnosed as GBS (*n=10*) and CIDP (*n=10*) at the Department of Neurology. Samples from patients with NIN (*n=10*) served as controls.

2.3 RNA extraction and measurement

Total cellular RNA was extracted from frozen nerves according to the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Afterwards, the concentration and quality were measured by biophotometer (Eppendorf AG, Hamberg).

2.4 Semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The presence of COX-1, COX-2, ICOS and ICOS-L mRNA in human sural nerve biopsies was determined by semiquantitative RT-PCR.

A 20µl reaction by adding the following reagents in the order listed: Reverse Transcription 10X Buffer 2µl; $MgCl_2$, 25mM 4.4µl; dNTP Mixture, 10mM 4µl;Random Primers 1.0µl; RNase-free 0.1% DEPC-H2 O 5.7µl; RNA 2µl ; RNase Inhibitor 0.4µl; Multi Scribe™ Reverse Transcriptase 0.5 µl (Gibco/Invitrogen, Karlsruhe, Germany). Afterwards, the program was carried out (25 ºC, 10 minutes / 48 ºC, 30 minutes / 95 ºC, 5 minutes) in a Perkin Elmer 9700 thermal cycler, then 4 degrees Celsius until ready for down stream application. PCR was performed in 50 µl reactions containing 5µl GeneAmp® 10X PCR Buffers (500mM KCl, 100mM Tris-HCl, pH 8.3; 15mM $MgCl_2$ and 0.01% (w/v) gelatin) (Applied Biosystems, Darmstadt, Germany) in the presence of 4,0µl dNTP (Amersham Biosciences_Freiburg, Germany), 25µl 50 pmoles sense and antisense primers (Invitrogen, Karlsruhe, Germany), 1 U AmpliTaq® DNA Polymerase (Applied Biosystems, Darmstadt, Germany) and 1µl cDNA. Amplification was carried out using 35 cycles (94 ºC, 60 secs / 53 ºC, 60 secs / 72 ºC, 120 secs) in a Perkin Elmer 9700 thermal cycler. Ten µl of the reaction products were electrophoresed on a 6 % polyacrylamide gel, which were read, analysed and quantitated by the software of TINA Version 2.09g (Raytest Isotopenmessgeräte, Straubenhardt, Germany). COX, ICOS and ICOS-L mRNA levels were determined by correlating the amount of signal intensity to the house keeping gene GAPDH.

2.5 Immunohistochemistry

Frozen or formalin fixed and paraffin embedded sural nerve biopsies were cut at 6 µm and 10 µm, respectively, and sections were deparaffinized in xylenes and rehydrated through graded ethanol, when applicable. If necessary, sections were pretreated with protease XXIV (Sigma-Aldrich Chemie, Munich, Germany) or microwaved in 0.1 M citrate buffer for 3 min at 680 W. All primary antibodies were incubated at 4°C overnight after blocking the sections with 10% bovine serum albumin (Sigma-Aldrich Chemie, Munich, Germany) for 30 min. Endogenous peroxidase was blocked with 30% hydrogen peroxide in methanol. Biotinylated

secondary antibodies and avidin-biotin-horseradish peroxidase complex (DAKO, Hamburg, Germany) were applied with DAB (DAKO, Hamburg, Germany) as peroxidase substrate according to the manufacturer's instructions. After development with DAB substrate, sections were counterstained with hematoxylin (Sigma-Aldrich Chemie, Munich, Germany), and mounted in entellan (Merck, Darmstadt, Germany).

Immunoreactivity within the endo- and epineurium was evaluated across an entire transverse section of each biopsy using an Axioplan 2 microscope (Carl Zeiss, Jena, Germany). Only immunoreactivity associated with a cell nucleus was accepted.

2.6 Cell culture

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by Ficoll-Hypaque (Pharmacia, Karlsruhe, Germany) density gradient centrifugation. T cells, B cells, NK cells and granulocytes, if present, were depleted using a Dynabead monocyte negative isolation kit (Dynal Biotech, Hamburg, Germany). Isolated monocytes were resuspended ($2x10^6$/ml) in complete culture media consisting of RPMI 1640 medium supplemented with 10% human serum, 2 mM glutamine (Sigma-Aldrich Chemie, Munich, Germany), 100 U/ml penicillin, and 100 IE/ml streptomycin (Invitrogen, Karlsruhe, Germany), and cultured for 48 h at 37°C and 5% CO_2 under the following conditions: in complete culture medium alone, and stimulated with LPS (Pharmingen, San Diego, CA, USA) for stimulation of macrophages. Purity of the cell population was determined by FACS analysis. Cell culture supernatants were used for further analysis.

2.7 Western blotting

Human serum samples and CSF were subjected to electrophoresis on SDS/PAGE under non reducing conditions. Samples were then electro blotted onto nitrocellulose (Schleicher and Schuell, Darmstadt, Germany) at 100 mA constant power using a semi-dry blotter (Bio-Rad

Laboratories, Munich, Germany). Thereafter, nitrocellulose was saturated with 2.5 % dry milk, washed, and incubated with a murine anti-COX-2 antibody (Santa Cruz, California, USA) overnight at room temperature. Murine anti-mouse IgG (DAKO, Hamburg, Germany) conjugated to horseradish peroxidase was used as secondary antibody, and the reaction was developed using diaminobenzene substrate.

2.8 Enzyme immunoassay

The concentration of PGE_2 and $PGF_{2\alpha}$ in serum of patients as well as in cell culture supernatants from *in vitro* stimulated monocytes was determined by ELISA according to the instructions of the manufacturer (R&D Systems, Abingdon, UK) using an automated ELISA reader (Canberra Packard). The interassay variability was less than 10 % and the lower detection limits were 36.2 pg/ml for PGE_2 and 6.78 pg/ml for $PGF_{2\alpha}$.

2.9 Quantitative assessment

Analysis of positive immunoreactivity was performed by an observer blinded to the patient group studied. Endo-, peri- and epineurial immunoreactivity was evaluated across an entire transverse section of each biopsy, and numbers of positive cells were related to the total area of each section as determined on digitised images using an Axiophot 2 microscope (Carl Zeiss, Jena, Germany). Only immunoreactivity associated with a cell nucleus was accepted.

2.10 Statistical analysis

One-way analysis of variance and Student-Newman-Keuls multiple comparisons were used as principal statistical tests. A P value ‹ 0.05 was considered significant. Data are given as mean ± standard error (SE).

3. RESULTS

3.1 Expression of inflammatory infiltrates

Inflammatory infiltrates were observed in both GBS and CIDP. Increased numbers of CD68 positive cells, indicative of macrophages, could primarily be detected in the endoneurium and in perivascular cuffs in the epineurium. CD3 positive T lymphocytes could be localized to perivascular infiltrates in the epi- and perineurium, while they were diffusely distributed within the endoneurium (Fig 3). In the NIN control group only rarely were few macrophages and T cells found in the epineurium and endoneurium.

3.2 COX-1 and COX-2 mRNA expression

In patients diagnosed as GBS and CIDP a significant up-regulation of mRNAs for COX-2 was found in the biopsy material. Expression in GBS was even higher compared to CIDP but did not reach statistical significance. In contrast, mRNA for COX-1 was detected in equivalent amounts in all patient groups investigated (Fig 4).

3.3 Expression and cellular localization of COX-2 in the PNS

In acute and chronic inflammatory demyelination COX-2 immunoreactivity was localised to mononuclear cells, and was highly expressed within the endoneurium. Immunoreactive cells were only rarely detectable in the epi- and perineurium. The distribution of the COX-2^{+} cells was similar to the pattern obtained with the macrophage marker anti-CD68 (Fig 5).

3.4 COX-2 protein expression in sera and CSF of affected patients

To examine whether increased expression of COX-2 in the inflamed PNS is mirrored in peripheral venous blood and the CSF of affected patients we studied the protein expression of this enzyme in sera and CSF samples from patients with GBS, CIDP, and non-inflammatory

controls by immunoblotting. In sera from all GBS (10/10) and most of the CIDP (6/10) samples investigated a positive signal at the molecular size of 73 kD mirroring protein expression of COX-2 was observed. A similar pattern was obtained when studying CSF samples: all GBS (10/10) and half of the CIDP (5/10) samples studied expressed COX-2 protein. In the NIN group immunoreactivity was absent in all of the serum and CSF samples studied (Fig 6).

3.5 PGE_2 and $PGF_{2\alpha}$ in sera and cell culture supernatants

Monocytes stimulated *in vitro* were studied in order to corroborate our findings in the PNS tissue and to determine their functional significance. In the LPS-stimulated cell population heightened levels of PGE_2 and $PGF_{2\alpha}$ were detectable. Moreover, in sera from patients with GBS and CIDP, but not in non-inflammatory controls, expression levels of these prostanoids were raised but did not differ significantly between the three inflammatory groups (Fig 7).

3.6 ICOS and ICOS-L mRNA expression

In patients diagnosed as GBS and CIDP a significant up-regulation of mRNAs for ICOS as well as ICOS-L was found in the biopsy material. Expression in GBS was even higher compared to CIDP but did not reach statistical significance (Fig 8).

3.7 Expression and cellular localization of ICOS in the PNS

Analysis of sections from inflammatory demyelinating neuropathies stained with anti-ICOS antibody revealed positive small, round immunoreactive cells (Fig. 7A). Such ICOS-positive infiltrates were primarily located in the epi- and perineurium and often clustered around arterioles or capillaries. Serial sections demonstrated that these cells, morphologically consistent with lymphocytes, were similar in appearance and distribution to cells detected with the pan-T cell marker anti-CD3 (Fig 9A). Within the endoneurium ICOS-positive cells

were found to be diffusely distributed, similar to the pattern of T lymphocytes, as described above. Of note, in the NIN group no immunoreactivity for ICOS could be discerned (data not shown).

3.8 Expression and cellular localization of ICOS-L in the PNS

In acute and chronic inflammatory demyelination ICOS-L immunoreactivity was exclusively localised to mononuclear cells, and was found to be expressed within the epi- as well as endoneurium (Fig 9B). The distribution of the ICOS-L positive cells was similar to the pattern found with the macrophage marker anti-CD68 (analysis with serial sections; Fig. 7B). In contrast, Schwann cells did non stain positive for ICOS-L (data not shown). Of note, we did not find any immunoreactivity for ICOS-L in the non-inflammatory controls.

3.9 Quantitative analysis

To obtain insight into the frequency of the expression pattern of ICOS and its ligand in the various disease groups, labelled mononuclear cells were quantified in each nerve and the results are expressed as cells/mm^2. For both costimulatory molecules studied increased numbers were noted in inflammatory demyelinating disease. Numbers counted for ICOS as well as ICOS-L were statistically significantly different when comparing the acute and chronic inflammatory groups against the non-inflammatory controls. On the other hand, there was no statistical difference in the numbers of immunoreactive cells when comparing AIDP versus CIDP (Fig 10).

We further investigated the cellular expression of costimulatory molecules as a fraction of the total number of the respective cellular source. This analysis appeared extremely relevant, since the total number of ICOS immunoreactive cells was higher in the epi- and perineurium, whereas ICOS-L expressing macrophages, implicated in demyelination, were located primarily, based on total numbers, within the endoneurium. Our analysis revealed that the

percentage of ICOS-expressing T lymphocytes in the endoneurium as well as in the epi- and perineurium is significantly higher in AIDP and CIDP compared to the NIN group. More importantly, there was no statistically significant difference in the percentage of ICOS-immunoreactive T cells between those localized to the endoneurium and those found within the epi-/ perineurium. The ratio of ICOS-expressing T lymphocytes did not differ between the acute and chronic inflamed group (Fig 11).

Similarly, the percentage of macrophages expressing the ligand ICOS-L within the endoneurium as well as within the epi- and perineurium was significantly higher in the acute and the chronic inflammatory cases when compared to the non-inflammatory controls. Again, there was no difference in the percentage of ICOS-L expressing cells between the endoneurium and the epi-/ perineurium, and the ratio of ICOS-L positive macrophages did not differ between AIDP and CIDP (Fig 11).

4 DISCUSSION

GBS and CIDP represent prototypic inflammatory, demyelinating disorders of the PNS, characterized by multifocal demyelination and mononuclear cellular infiltration. By immunohistochemistry, we confirm the cellular immune responses in these disorders by observations that the macrophages mainly in the endoneurium, whereas T cell expression in the epi- and perineurium in the affected nerves from both GBS and CIDP patients.

4.1 COX and PGs

Ubiquitous COX and prostaglandins may have a key role in the pathogenesis of immune-mediated demyelination in the PNS. The inducible activity of COX-2 increases the production of specific prostaglandins, such as PGE_2, that collectively orchestrate the ongoing immunological inflammatory process within peripheral nerve. In the present study the expression of the two isoforms of COX, COX-1 and COX-2, as well as of the prostanoids PGE_2 and $PGF_{2\alpha}$ have been investigated. We found consistent alterations in sural nerve biopsies, sera, and CSF samples obtained from patients with acute and chronic inflammatory demyelinating neuropathies of the PNS that were absent in NIN. Studying expression of COX-1 in the inflamed PNS, no differences on the mRNA level could be discerned in any of the three groups investigated. Thus, COX-1, although known to produce pro-inflammatory prostanoids, such as PGE_2 (Smith, 1992), appears to be constitutively expressed in the PNS without being affected in its expression pattern during inflammation. Our observation is in line with studies in other inflammatory disorders, such as rheumatoid arthritis and osteoarthritis, in which COX-1 has also been reported to be constitutively expressed (Vane et al., 1994).

In contrast, COX-2 was found to be up-regulated on the mRNA as well as protein level in sural nerve biopsies from patients with GBS and CIDP in comparison to NIN. The mRNA levels did not differ significantly between acute and chronic inflammatory cases, whereas no

signal was detected in the NIN group, suggesting that COX-2 responds to inflammatory stimuli in an all-or-none fashion rather than being differentially regulated during the disease process. COX-2 immunoreactivity could be localized to mononuclear cells primarily discerned within the endoneurium. On serial sections these cells were identified as $CD68^{+}$ macrophages. The expression of COX-2 by macrophages has been described in other inflammatory disorders, such as rheumatoid arthritis (Crofford et al., 1994). Our observations find further support in a previous investigation demonstrating COX-2 to be expressed by endoneurial macrophages in chronic inflammation of the PNS (Kawasaki et al., 2001). However, our present data indicate that COX-2 is already expressed during the acute phase of inflammatory demyelination of the peripheral nerve. Western blot analysis of sera as well as CSF samples from GBS and CIDP patients underlines this assumption: COX-2 protein was identified in sera and CSF samples in all (10 out of 10) acute cases studied. In contrast, in chronic cases of PNS inflammation only in 6 out of 10 serum samples and 5 out of 10 CSF samples investigated COX-2 protein expression was discernable. Thus, our data indicate that COX-2 protein is more likely found to be expressed during the acute phase of inflammatory demyelination of the PNS. Therefore, expression of COX-2 protein in serum and CSF is not specific for inflammatory demyelination of the PNS, however, in acute GBS the probability to measure COX-2 protein is much higher compared to chronic inflammation within the PNS. To which extent COX-2 protein can be used as a diagnostic surrogate marker needs further investigation and a much larger sample size.

COX-2 is not routinely present in most mammalian cells, but, it can be rapidly induced in various cell types participate in inflammatory responses, including monocytes, macrophages, and endothelial cells, through multiple signalling pathways that involve bacterial endotoxins, such as LPS, or pro-inflammatory cytokines, e.g. IFN-γ or TNF-α (Mitchell et al., 1995). Key requisite in the transcriptional activation of COX-2 is the binding of the activated transcription factor NF-κB to the COX-2 promoter region (Newton et al., 1997; Yamamoto et

al., 1995). In macrophages exposed to LPS, NF-κB has been shown to be a positive regulator of COX-2 expression, whereas the constitutive expression of COX-1 was not altered by NF-κB in these studies (D'Acquisto et al., 1997; Surh et al., 2001). Along this line, we recently reported increased levels of NF-κB in macrophages in the same selection of sural nerve biopsies from patients with GBS and CIDP as in the present study (Andorfer et al., 2001). Thus, it is reasonable to assume that the increased expression of COX-2 in our investigation may be mediated through pro-inflammatory cytokines, known to be critically involved in the pathogenesis of inflammatory neuropathies (Hughes et al., 1999), causing activation of NF-κB in macrophages within the inflamed PNS, and consequently precipitating the release of large amounts of prostaglandins.

We found increased levels of PGE_2 in serum samples from GBS and CIDP patients, whereas no protein could be measured in the NIN group. Prostaglandins, including PGE_2, are intimately involved in regulating the inflammatory response by enhancing vascular permeability, providing chemotactic signalling, and modulating inflammatory cell activities (Hartung et al., 1992). PGE_2, produced by macrophages and other cell types and catalyzed by both isoforms of COX, modulates functional activities of T and B lymphocytes as well as of macrophages by binding to cell-surface receptors via a cyclic AMP dependent pathway (Harris et al., 2002). Since the expression of COX-1 was found to be unaltered in the inflamed PNS, it appears plausible that raised levels of PGE_2 in the serum and CSF samples studied are primarily the result of an induced transcription of the COX-2 gene and enhanced production of the COX-2 enzyme in macrophages. We corroborated this assumption by studying macrophages *in vitro*, which upon stimulation with LPS secreted large amounts of PGE_2. These emphasize the important role of macrophages in the production of prostaglandins, and confirm previous reports (Wadleigh et al., 2000).

It is of note that macrophage-derived prostaglandins have been implicated to significantly contribute to clinical deficits and tissue damage in EAN (Hartung et al., 1988b), and that a

relationship between PGE_2 and lipid peroxidation has been established (Bilodeau et al., 1995), Thus, PGE_2 may be involved in the pathogenesis of demyelinating neuropathy by inducing lipid peroxidation of myelin.

Another reliable marker of lipid peroxidation and oxidative stress with potential relevance to human demyelinating disorders is $PGF_{2\alpha}$ (Greco et al., 1999), which partly derives as a product from the activity of the inducible isoform COX-2 in human monocytes (Patrignani et al., 1996). The elevated *ex vivo* levels of $PGF_{2\alpha}$ in serum samples from GBS and CIDP patients studied as well as the enhanced expression found in the supernatants from LPS-stimulated macrophages *in vitro* point to COX-2 as an important enzyme in the generation of this prostaglandin.

Thus, our present data collectively suggest that COX-2 may act as a key player in the generation of various prostaglandins during PNS inflammation, especially in acute inflammatory demyelination of the peripheral nerve, and as such might be a suitable target for therapeutic intervention. Various selective COX-2 inhibitors have been developed on the assumption that inhibition of this enzyme could provide all the benefits of current nonsteroidal anti-inflammatory drugs, but without their major side-effects on the gastrointestinal system, which are due to inhibition of COX-1 (Willoughby et al., 2000). Such inhibitors have been successfully applied in EAN (Miyamoto et al., 2002; Miyamoto et al., 1998; Miyamoto et al., 1999). However, an emerging body of evidence indicates that the time window of successful application for COX-2 inhibitors is limited to the early phase of inflammation (Gilroy et al., 1999). In a carrageenin-induced pleurisy rat model to study inflammatory responses, Willoughby and colleagues demonstrated that selective COX-2 inhibitors exhibited anti-inflammatory activity early in the onset of the inflammatory response, coincident with the expression of COX-2 protein. However, by 6 hours the COX-2 inhibitors were without effect, at a point where COX-2 protein was no longer present, as shown by western blotting (Gilroy et al., 1998). Thus, if selective COX-2 inhibitors were to

be considered for treatment of inflammatory demyelination of the PNS these drugs need to be applied early in the disease course. One could speculate that in those CIDP cases of our study, in which COX-2 protein was not identifiable in serum or CSF, application of a COX-2 inhibitor would not be beneficial. Well-designed clinical trials are warranted to further define the feasibility and the most appropriate time window for the application of COX-2 inhibitors in the treatment of the inflamed peripheral nerve, either as monotherapy or as an adjunctive treatment strategy. It is hoped that in the future these drugs may enlarge our still restricted therapeutic armamentarium in fighting these disabling disorders.

4.2 ICOS and ICOS-L

Costimulatory signals play a key role in regulating T-cell activation and are believed to have decisive influence in the inciting and perpetuating cellular effector mechanisms in autoimmune diseases. ICOS is critically involved in the differentiation of T helper cells after primary activation and modulates the immune response of effector/memory T cells. In order to delineate the relevance of the ICOS pathway in the immunopathogenesis of inflammatory neuropathies, the expression of the inducible molecule ICOS was investigated in GBS and CIDP. ICOS mRNA was found to be up-regulated in sural nerve biopsies from patients with GBS and CIDP, in comparison to NIN. No ICOS mRNA signal was detectable in NIN. ICOS mRNA levels did not differ significantly between acute and chronic inflammatory cases, indicating that ICOS is involved in the acute as well as the chronic effector phase of immune-mediated inflammatory diseases of the PNS. ICOS immunoreactivity could be localized to $CD3^+$ cells primarily discerned within the epi- and perineurium, confirming T lymphocytes as the cellular source. Our observation is in line with findings from studies in other inflammatory disorders, such as inflammatory myopathies (Wiendl et al., 2003) and rheumatoid arthritis (Okamoto et al., 2003).

ICOS, a novel member of the CD28 family of costimulatory and regulatory receptors on T cells, however, unlike CD28, is virtually absent on naïve T cells, instead it is up-regulated after T cell activation and retained on memory T cells. In contrast to CD28, ICOS is therefore considered as an essential costimulatory molecule modulating the effector function of both CD4 and CD8 T cells. The demonstration of ICOS positive T cells in the target organs of inflammatory disorders such as myositis, rheumatoid arthritis, or here inflammatory neuropathies substantiates the putative *in vivo* relevance of ICOS in the immune response in inflamed tissues. Costimulation mediated via ICOS is involved in both Th1 and Th2 responses and known to up-regulate IFN-γ as well as TNF-α (Carreno and Collins, 2002). Both appear to be particularly important cytokines inducing a plethora of pro-inflammatory effects and mediating myelin damage through activation of macrophages in the pathogenesis of immune-mediated peripheral nervous disorders (Kieseier et al., 2002a; Kieseier and Hartung, 2002). As such, T lymphocytes exhibiting positive immunoreactivity for ICOS, as found in our samples, might represent activated T-cells, not only being critically relevant in inducing an acute immune response in the excruciating inflamed PNS, as in GBS, but also being key effector cells in perpetuating the persistency of inflammation in chronically immune-mediated disorders of the PNS, such as CIDP.

Recent data indicate that the amplification of cytokine production by effector T cells is related to their level of ICOS expression and that this level varies with a changing cytokine environment, underlining the importance of the cytokine production potential of tissue-infiltrating effector cells (Wassink et al., 2004). Although we are not able to define the expression level of ICOS on a single cell stage it is noticeable that the percentage of ICOS expressing T lymphocytes did not differ between acute and chronic PNS inflammation, providing further support for the notion that ICOS positive effector cells may be critically relevant in perpetuating an ongoing immune response.

We also found increased mRNA levels of ICOS-L in the sural nerve samples from GBS and CIDP patients, whereas no ICOS-L mRNA could be measured in the NIN group. ICOS-L, identified as the exclusive B7-ligand binding to ICOS, is constitutively expressed at a low level on APCs including monocytes and macrophages (Sharpe and Freeman, 2002). Its expression can be up-regulated or induced by inflammatory signals such as TNF-α in peripheral sites. The immunoreactivity of ICOS-L was localized to macrophages within the epineurium of the inflamed peripheral nerve samples, in those areas where also ICOS expressing T cells were detectable. This finding is not surprising given the function of macrophages of professional APCs. Similarly, other members of the B-7 family, B7-1 and B7-2, ligands of the CD28 costimulator, were also found to be expressed primarily by macrophages in the inflamed PNS (Kiefer et al., 2000). In contrast, one report describes B7 molecules to be expressed on Schwann cells in CIDP and suggests Schwann cells to act as APCs during inflammation (Murata and Dalakas, 2000). Of note, we could not detect any ICOS-L expression on Schwann cells. Therefore our data support the view that macrophages rather than Schwann cells are the main APCs during the effector phase of immune-mediated inflammation within the PNS and, in addition to executing cytotoxicity towards myelin sheaths, they may be critical components in maintaining the autoimmune response, such as in GBS and CIDP. Especially, since the expression and distribution pattern of ICOS-L did not differ significantly between acute and chronic inflammatory cases, the role of ICOS-L in sustaining an immune response within the PNS finds further support.

Thus, our present data collectively suggest that the new member of costimulatory family, ICOS and its specific ligand ICOS-L, play an important role during PNS inflammation, and we also emphasize the role of cellular immune response in the inflammatory PNS. Therefore, Manipulate the ICOS and ICOS-L interaction can modulate the secondary immune response, and thus may be a new point of immunotherapeutic intervention. Moreover, it has been reported that antagonism of ICOS is more effective late in an immune response(Carreno and

Collins, 2002). ICOS costimulation in EAE does appear to be critical at the time that encephalitogenic T cells begin to migrate into the CNS (Sperling, 2001), and blockade of ICOS only during the effector phase of EAE ameliorated the disease (Rottman et al., 2001). Studies in experimental models have led to the proposal of ICOS as a potential target for selective immune intervention aimed at selectively inhibiting T-cell activation. Our data suggest that such a T-cell specific and selective intervention may also be beneficial in inflammatory neuropathies.

4.3 The role of macrophages

It has been well established that, within the PNS, endoneurial macrophages are able to express MHC antigens and costimulatory B7-molecules, which is engaged to the TCR and CD28 on naïve and resting T cells, respectively, and thus act as local APC and may be critical in T cell activation and triggering the autoimmune process. In our experiments, ICOS-L and ICOS mRNAs was significantly up-regulated in samples from GBS and CIDP patients compared to NIN. By immunohistochemistry, we identified macrophages as the cellular source of ICOS-L expression, whereas, T lymphocytes expressed the corresponding molecule ICOS. As such, during the effector phase of inflammatory PNS, macrophages is also function as main APC by express the ICOS-L, binding to ICOS and delivering a costimulatory signal to the activated T cells in PNS, thus, involved in the cellular immune responses of these disorders. Moreover, according to our results, during inflammatory demyelination of PNS, these macrophages also release a very important proinflammatory mediator, COX-2, which acts as a key player in the generation of various prostaglandins, including PGE_2 and $PGF_{2\alpha}$, thus actively participated in the inflammatory demyelinating process of PNS. Therefore, our collective data here profoundly emphasize the critical role of macrophages in the amplification and effector phase of immune-mediated demyelination in PNS.

5 SUMMARY

The GBS and CIDP represent prototypic immune-mediated disorders of the PNS. Although the precise immunopathogenetic mechanisms underlying these disorders are still incompletely understood, there is consensus that a disordered cellular immunity is of critical importance in disease propagation. It has been suggested that macrophages, appear to be pivotal in the process of demyelination, act as antigen presenters and are of critical importance in the amplification and effector phase of immune-mediated demyelination by the secretion of pro-inflammatory cytokines, the release of active mediators, and enhanced phagocytosis. In addition, in the animal model of GBS, EAN, the disease can be induced by the injection of neuritogenic T cells into naïve animals, furthermore pointing to a central role of activated T lymphocytes in the cellular response arm of inflammatory diseases of the PNS.

In this study, firstly, we analyzed the expression and distribution pattern of the COX, key enzymes in propagating inflammatory responses by converting arachidonic acid to prostaglandins including PGE_2 and $PGF_{2\alpha}$, and ICOS, a recently identified costimulatory molecule implicated in T cell activation, and its unique ligand (ICOS-L), in sural nerve biopsies from patients with GBS, CIDP and, for comparison, with NIN by RT-PCR and immunohistochemistry. Secondly, with immunoblotting, the protein level of COX-2 was measured in the CSF sample from GBS, CIDP, as well as the control patients. Finally, to confirm functional COX-2 activity, expression and regulation of its products, prostaglandins PGE_2 and $PGF_{2\alpha}$, were evaluated in sera and CSF of patients as well as in cell culture supernatants from *in vitro* stimulated monocytes by ELISA.

We found that a significant up-regulation of COX-2 mRNA was detected in sural nerves from patients with GBS and CIDP but not in control subjects with NIN, whereas COX-1 mRNA expression was unaltered in all investigated groups. Macrophages were identified as its primary cellular source. Increased COX-2 protein levels were detectable in CSF from all patients with GBS and, in smaller numbers only, in samples from patients with CIDP but not

from the NIN group studied. Moreover, increased levels of PGE_2 and $PGF_{2\alpha}$ were measurable in sera from patients with GBS, CIDP and in cell culture supernatants from *in vitro* stimulated macrophages, indicative of COX-2 activity. Therefore, these results suggest that COX-2, primarily expressed by macrophages, acts as a key player in the generation of various prostaglandins like PGE_2 and $PGF_{2\alpha}$ during PNS inflammation and especially during acute inflammatory demyelination of the peripheral nerve.

We also found a significantly up-regulated ICOS and ICOS-L mRNA level in samples from GBS and CIDP patients compared to NIN and further identified T lymphocytes as the cellular source of ICOS, whereas macrophages as antigen presenting cells expressed the corresponding ligand ICOS-L.

Thus, we emphasis the antigen presenting function of macrophages by express the ICOS-L, which binds to ICOS and delivering a costimulatory signal to the activated T cells in PNS during the effector phase of these disorders. Furthermore, we also address that macrophages are critical importance in the amplification and effector phase of immune-mediated demyelination by their secretion of the COX-2, which generate prostaglandins like PGE_2 and $PGF_{2\alpha}$, that contribute to the process of inflammatory demyelination in GBS and CIDP. As such, our results indicate that macrophages are critical pathogenetically relevant cell population during the effector phase of these disorders.

Up to now, the immune-based therapies that have been proven to shorten the course of illness in GBS only include PE and IVIg, whereas, for treatment of CIDP, corticosteroid, PE, IVIg, and immunosuppressive drug, like cyclosporine, are clinically available, but none of these treat the disease completely. Only some 60% of GBS patients receive benefit from current therapy. While, the long term outcome of CIDP patients has generally been poor, involving decades of disability and dependence on medication. Moreover, each treatment has some disadvantages: IVIg dosing is required approximately every 2 to 8 weeks in most CIDP patients to maintain improvement. It is expensive, time-consuming to administer, and

availability can be a problem. Furthermore, IVIg is a blood product that is associated with rare thromboembolic events. Corticosteroids and cyclosporine have poor safety and tolerability profiles, and PE is invasive, time-consuming, expensive, and can be performed only at specialized centres (Kieseier and Hartung, 2002; Ropper, 2003). Therefore, alternative therapy regimen is needed. Based on our results, the new COX-2 inhibitors, celecoxib and meloxicam, may become additionally combinational therapeutic agents for GBS and CIDP. However, the cardiovascular safety of selective COX-2 inhibitors has just been questioned because recent clinical trials indicated that these inhibitors could increase the risk of arterial thrombosis, such as myocardial infarction and stroke, by inhibiting the formation of PGI_2, which is a potent vasodilator, and inhibitor of smooth muscle cell proliferation and platelet aggregation. In addition, selective COX-2 inhibitors fail to inhibit the formation, via COX-1 isoenzymes in platelets, of TXA_2, which facilitates vasoconstriction, platelet activation, and smooth muscle cell proliferation (Hankey and Eikelboom, 2003; Mukherjee et al., 2001). As such, the clinical trial of the COX-2 inhibitors in GBS and CIDP should not commenced until the definitive conclusion of such adverse effect is made from the further studies. In addition, our data also suggest manipulation of the ICOS pathway may be effective for therapeutic intervention during the effector phase of these disorders. Thus, a further clinical trial should be investigated to confirm the result here.

6 FIGURE LEGENDS

Table 1 Summary of patient characteristics

Diagnosis	No. of cases	Age (yr)	Sex ratio (f : m)	Duration of disease*
Guillain-Barré syndrome (AIDP)	6	53 (41-62)	2 : 4	12.5 weeks (3-25)
Chronic inflammatory demyelinating polyradiculoneuropathy	6	64 (58-68)	3 : 3	19 months (3-42)
Non-inflammatory neuropathies	3	44 (25-64)	1 : 2	n.a.
HNPP I	2			
HMSN I	1			

f = female; m = male; AIDP = acute inflammatorydemyelinating polyradiculoneuropathy; HNPP I = hereditary neuropathy with liability to pressure palsy; HMSN I, = hereditary motor and sensory neuropathies type I; n.a. = not applicable. * = before biopsy.

Figures denote median values and ranges.

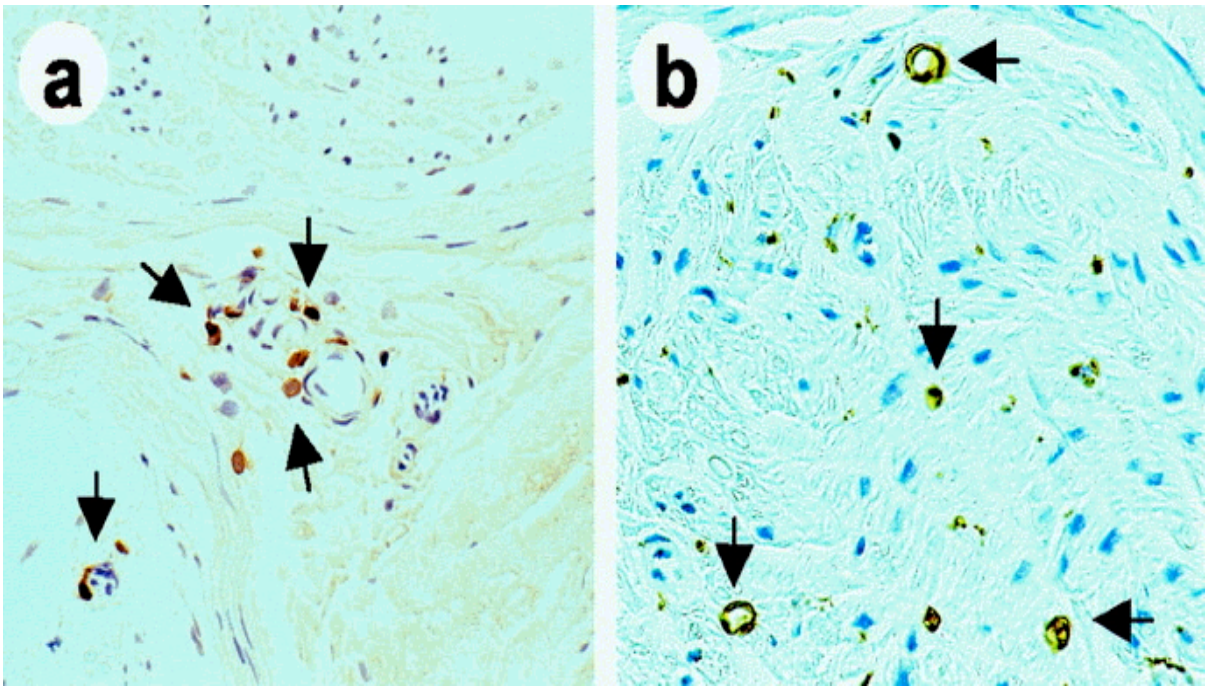


Figure 3 Immunohistochemical stains mirror the distribution pattern of T lymphocytes and macrophages in a sural nerve biopsy from a patient with CIDP. In these sections, invading $CD3^+$ T cells are primarily localized to perivascular infiltrates (arrows) in the epineurium and perineurium (a). $CD68^+$ immunoreactive cells, indicative of macrophages (arrows), are observed within the endoneurium (b) Original magnifications _ 120 (a) and _80 (b). *From:* (Kieseier et al., 2002a)

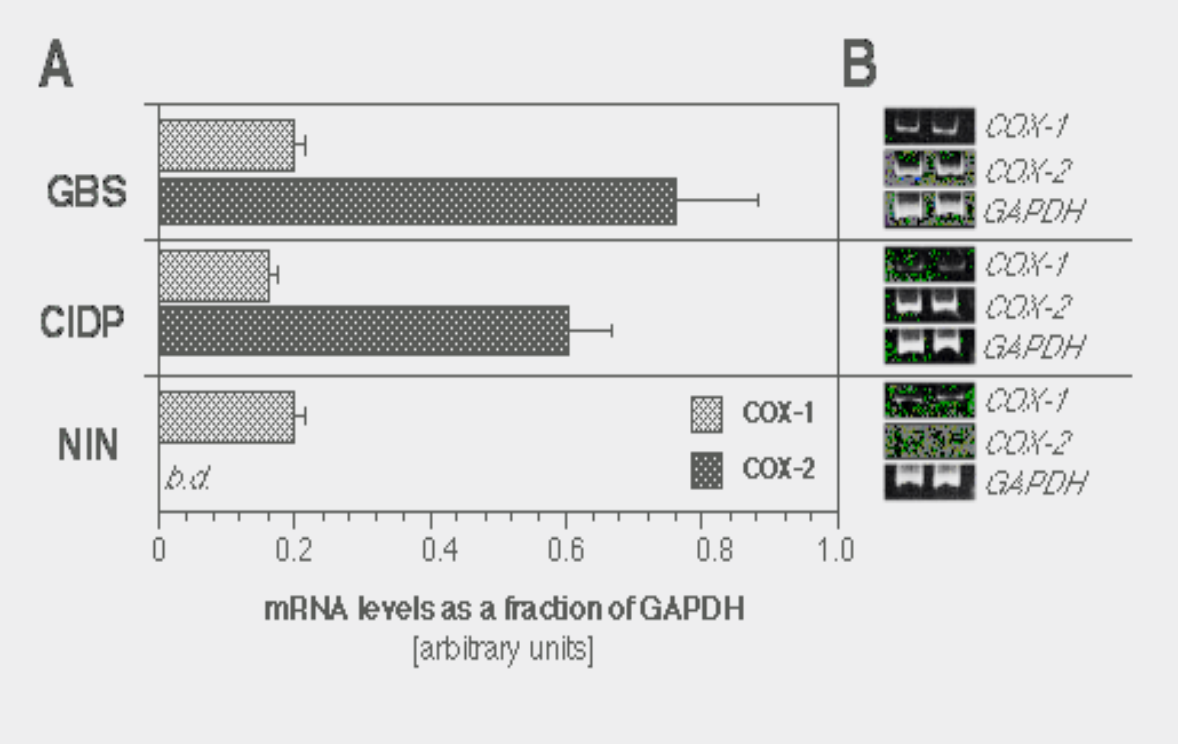


Figure 4 Expression of cyclooxygenase (COX)-1 and COX-2 mRNA in sural nerve biopsies from patients diagnosed as classical Guillain-Barré Syndrome (GBS), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and non-inflammatory neuropathies (NIN). The GBS group comprises 6, the CIDP group 6 and the NIN group 3 cases each. Whereas COX-1 mRNA was found to be present in all four groups studied in similar amounts, mRNA expression for COX-2 was depictable in GBS and CIDP. mRNA levels were not statistically significantly different between GBS and CIDP. GAPDH was used as a house-keeping gene. (A) mRNA levels are expressed as a fraction of mRNA for GAPDH and given as means ± standard error. (B) PCR products as seen on a polyacrylamide gel, stained with SYBR® Gold Nucleic Acid Gel Stain. *b.d.*, below detection limit

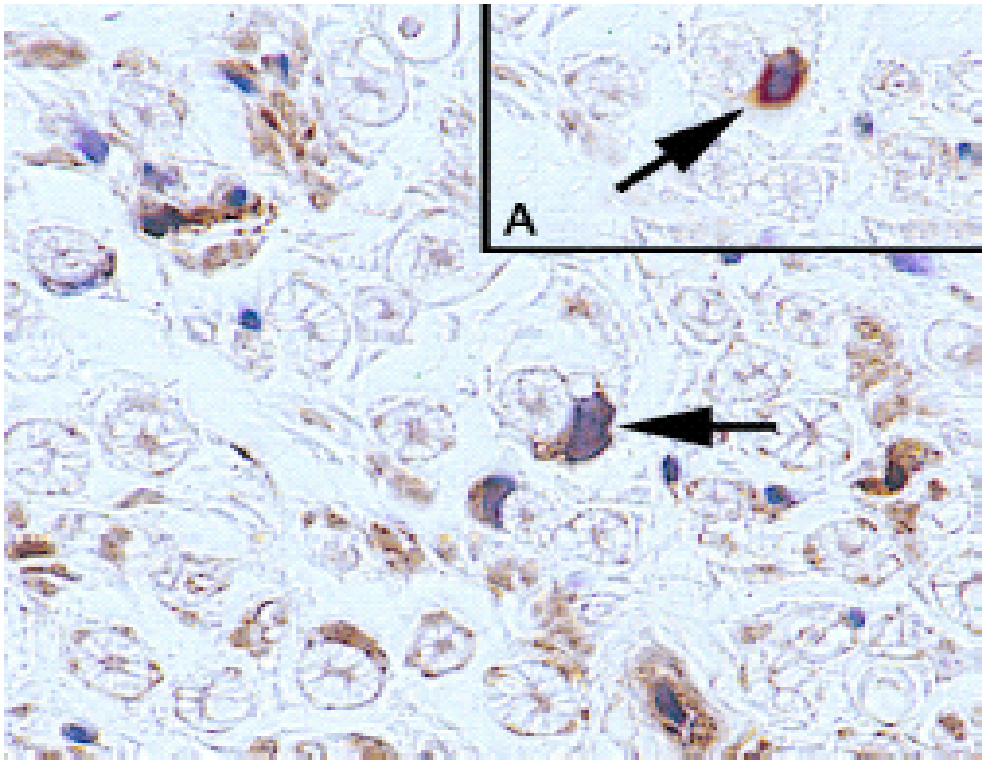
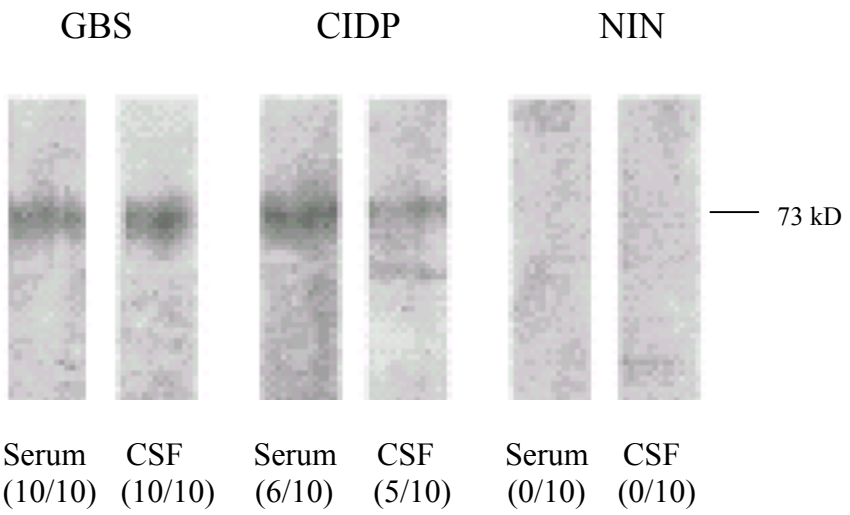


Figure 5 Immunohistochemistry for cyclooxygenase-2 (COX-2) in a sural nerve biopsy from a patient with Guillain-Barré syndrome (GBS). Within the endoneurium immunoreactive cells staining for COX-2 could be detected (arrow), similar in pattern and distribution to CD68 positive macrophages, as shown on serial sections. Original magnification: x200 and x600



	serum	*CSF*
GBS	10/10	10/10
CIDP	6/10	5/10

Figure 6 Western blot of cyclooxygenase-2 (COX-2) in serum (Srm) and cerebrospinal fluid (CSF) from patients with Guillain-Barré Syndrome (GBS), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and non-inflammatory neuropathies (NIN). In sera from all GBS (10/10) and most of the CIDP (6/10) samples investigated a positive signal at the molecular size of 73 kD mirroring expression of COX-2 protein was detectable. A similar pattern was obtained when studying CSF samples: all GBS (10/10) and half of the CIDP (5/10) samples studied expressed COX-2 protein. In none of the serum or CSF samples from the NIN group studied was immunoreactivity detected (0/10).

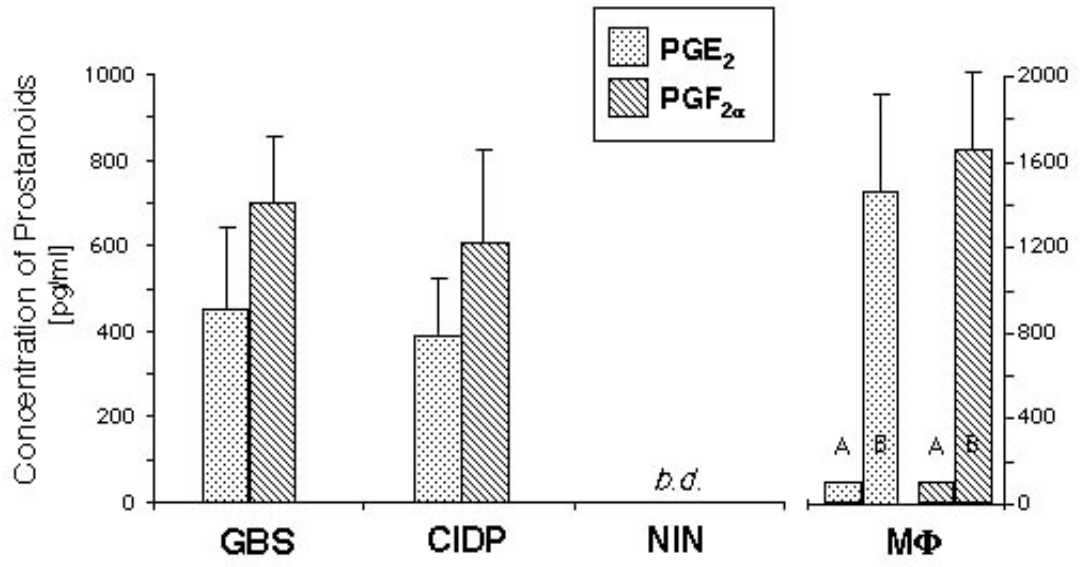


Figure 7 Expression of the prostanoids PGE_2 and $PGF_{2\alpha}$ in the serum from patients with Guillain-Barré Syndrome (GBS), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and non-inflammatory neuropathies (NIN). Whereas in the NIN group both prostanoids were below the detection limit (*b.d.*) of the ELISA, similar expression levels of PGE_2 and $PGF_{2\alpha}$ were measurable in GBS and CIDP samples. In monocytes (MΦ) studied before (A) and after (B) *in vitro* stimulation with LPS a significant increase of PGE_2 and $PGF_{2\alpha}$ expression was found in the supernatants. Concentrations given in pg/ml as means ± standard error. Note differences in the ordinate scale.

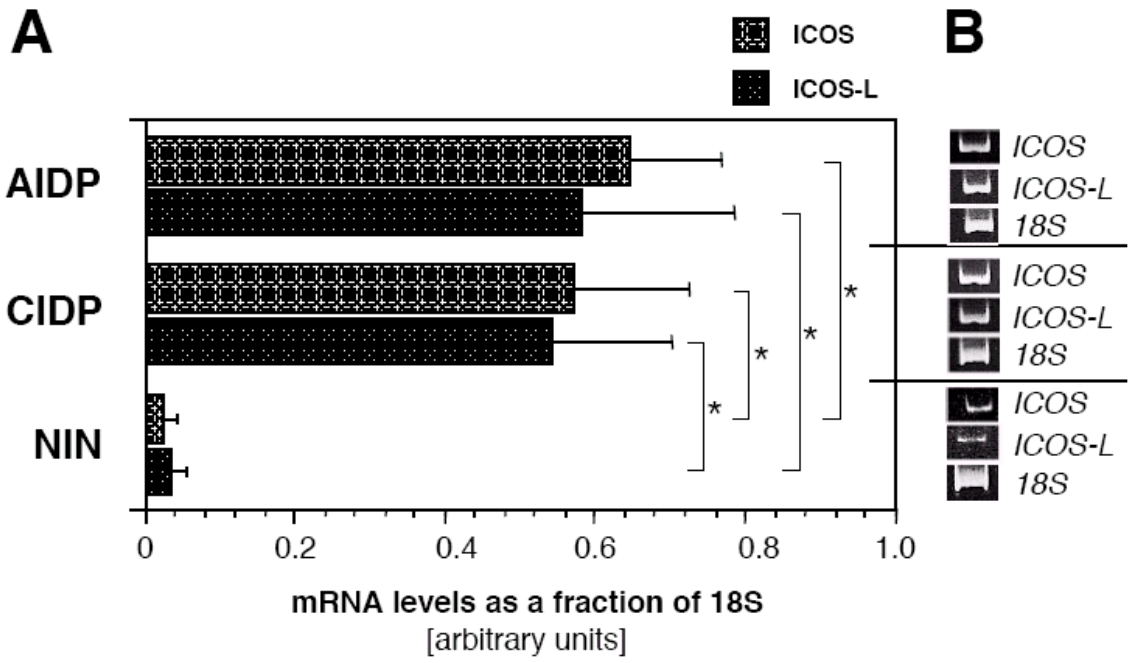


Figure 8 Expression of ICOS and ICOS-L mRNA in sural nerve biopsies from patients diagnosed as classical Guillain-Barré Syndrome (acute inflammatory demyelinating polyradiculoneuropathy, AIDP), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), and non-inflammatory neuropathies (NIN). The GBS group comprises 6, the CIDP group 6, and the NIN group 3 patients each. In patients diagnosed as GBS and CIDP a significant upregulation of the mRNAs for ICOS as well as ICOS-L was detected in the biopsy material compared to the NIN group (*, $p < 0,05$). mRNA levels were statistically not significant between GBS and CIDP biopsies. 18S was used as a house-keeping gene. (A) mRNA levels expressed as a fraction of mRNA for 18S and given as means ± standard error. (B) PCR products as seen on a polyacrylamide gel, stained with ethidium bromide.

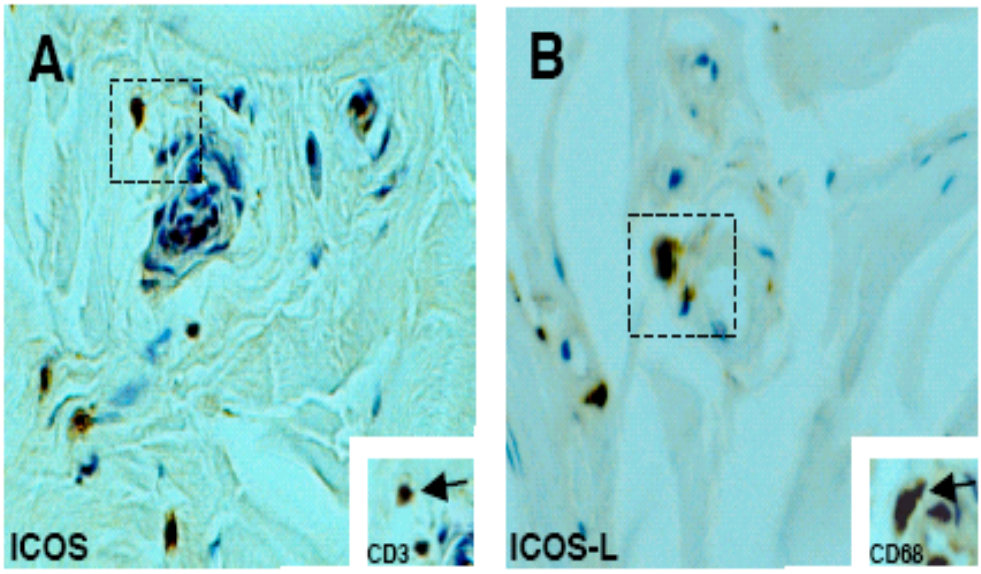


Figure 9 Immunohistochemistry for ICOS and ICOS-L in a sural nerve biopsy from a patient with Guillain-Barré syndrome (GBS). Within the inflamed peripheral nerve immunoreactive cells staining for ICOS could be detected (A), similar in pattern and distribution to CD3 positive T lymphocytes (arrow, insert). Within the epi- and endoneurium ICOS-L immunoreactivity was exclusively localised to mononuclear cells (B), which on serial sections was similar to the pattern found with the macrophage marker anti-CD68 (arrow, insert). Original magnification: x200.

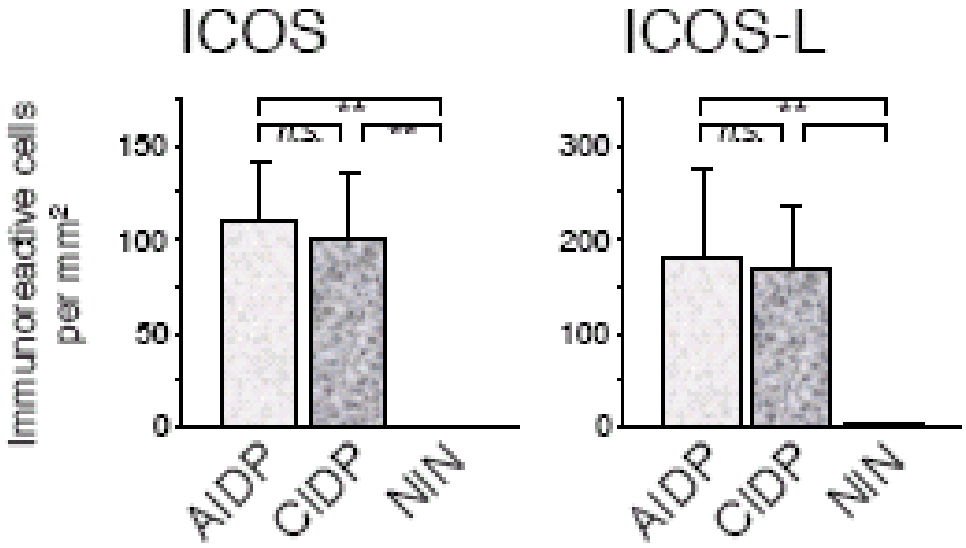


Figure 10 Total number of immunoreactive cells within entire transverse sections of each biopsy expressed as immunoreactive cells / mm^2. Values within each group of patients studied are given as mean ± SD. Differences in the expression of costimulator molecules are statistically significant ($P < 0.05$) when analysed with the Kruskal-Wallis test and the Mann-Whitney U test. AIDP = acute inflammatory demyelinating polyradiculoneuropathy (Guillain-Barré syndrome; $n = 6$); CIDP = chronic inflammatory demyelinating polyradiculoneuropathy ($n = 6$); NIN = non-inflammatory control ($n = 3$); *$P < 0.05$, n.s. = not significant. Note different ordinate scale.

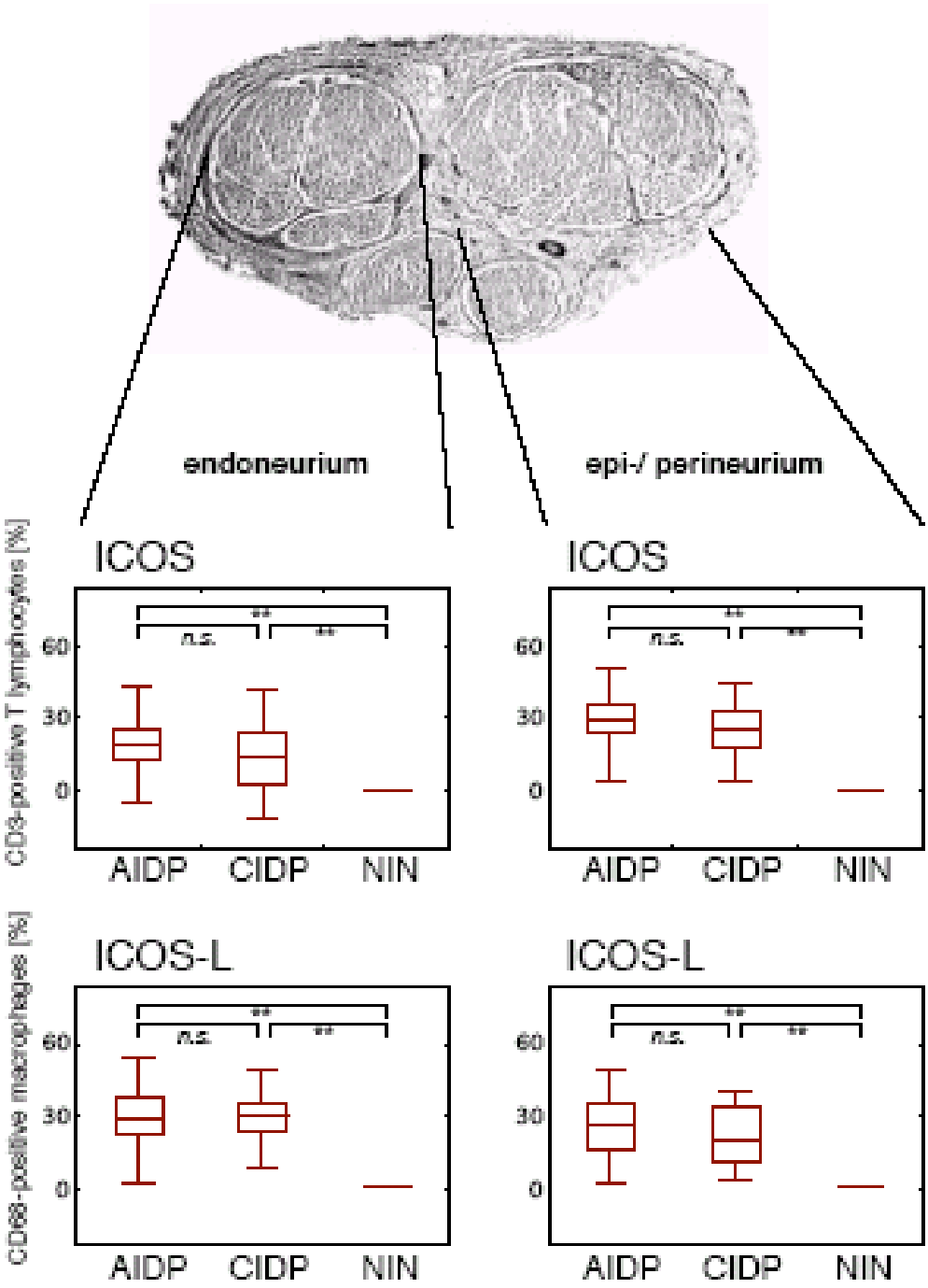

endoneurium
epi-/ perineurium
ICOS
ICOS
CD3-positive T lymphocytes [%]
60
30
0
n.s.
**
**
AIDP
CIDP
NIN
ICOS-L
ICOS-L
CD68-positive macrophages [%]

Figure 11 Density of CD3 positive T lymphocytes expressing the inducible costimulator protein (ICOS) and CD68 positive macrophages carrying its ligand ICOS-L in biopsies from patients with Guillain-Barré syndrome (acute inflammatory demyelinating polyradiculoneuropathy, AIDP; n = 6), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP; n = 6), and non-inflammatory neuropathies (NIN; n = 3). Box and whisker plot. The central horizontal bar denotes the median value, the top and bottom lines of the box denote the 75th and 25th percentiles, respectively, the top and bottom ends of the error bar denote the 95th and 5th percentiles. $^{**}P < 0.01$, n.s. = not significant, when the respective disease groups were analysed with the Kruskal-Wallis test and the Mann-Whitney U test.

7 LITERATURE

Research criteria for diagnosis of chronic inflammatory demyelinating polyneuropathy (CIDP). Report from an Ad Hoc Subcommittee of the American Academy of Neurology AIDS Task Force. Neurology 1991; 41: 617-8.

Andorfer B, Kieseier BC, Mathey E, Armati P, Pollard J, Oka N, et al. Expression and distribution of transcription factor NF-kappaB and inhibitor IkappaB in the inflamed peripheral nervous system. J Neuroimmunol 2001; 116: 226-232.

Arnason BGW, Soliven B. Acute inflammatory demyelinating polyradiculoneuropathy. In: Dyck PJ, Thomas PK, Griffin JW, Low PA and Poduslo J, editors. Peripheral Neuropathy. Vol 3. Philadelphia: Saunders, 1993: 1437-1497.

Arvidson B. Cellular uptake of exogenous horseradish peroxidase in mouse peripheral nerve. Acta Neuropathol (Berl) 1977; 37: 35-41.

Asbury AK, Cornblath DR. Assessment of current diagnostic criteria for Guillain-Barré syndrome. Ann Neurol 1990; 27: S21-S24.

Ballin RH, Thomas PK. Electron microscope observations on demyelination and remyelination in experimental allergic neuritis. I. Demyelination. J Neurol Sci 1969; 8: 1-18.

Bilodeau JF, Wang M, Chung FL, Castonguay A. Effects of nonsteroidal antiinflammatory drugs on oxidative pathways in A/J mice. Free Radic Biol Med 1995; 18: 47-54.

Carreno BM, Collins M. The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol 2002; 20: 29-53.

Chambers CA, Kuhns MS, Egen JG, Allison JP. CTLA-4-mediated inhibition in regulation of T cell responses: mechanisms and manipulation in tumor immunotherapy. Annu Rev Immunol 2001; 19: 565-94.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-9.

Coyle AJ, Gutierrez-Ramos JC. The expanding B7 superfamily: increasing complexity in costimulatory signals regulating T cell function. Nat Immunol 2001; 2: 203-9.

Crofford LJ, Wilder RL, Ristimaki AP, Sano H, Remmers EF, Epps HR, et al. Cyclooxygenase-1 and -2 expression in rheumatoid synovial tissues. Effects of interleukin-1 beta, phorbol ester, and corticosteroids. J Clin Invest 1994; 93: 1095-101.

D'Acquisto F, Iuvone T, Rombola L, Sautebin L, Di Rosa M, Carnuccio R. Involvement of NF-kappaB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. FEBS Lett 1997; 418: 175-8.

Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, et al. Cyclooxygenase in biology and disease. Faseb J 1998; 12: 1063-73.

Dyck PJ, Dyck PJB, Giannini C, Sahenk Z, Windebank AJ, Engelstad J. Peripheral nerves. In: Graham DI and Lantos PL, editors. Greenfield's Neuropathology. London: Arnold; 7th edition, 2002.

Dyck PJ, Prineas J, Pollard J. Chronic inflammatory demyelinating polyradiculoneuropathy. In: Dyck PJ, Thomas PK, Griffin JW, Low PA and Poduslo JF, editors. Peripheral neuropathy. Philadelphia: WB Saunders, 1993: 1498-1517.

Gilroy DW, Colville-Nash PR, Willis D, Chivers J, Paul-Clark MJ, Willoughby DA. Inducible cyclooxygenase may have anti-inflammatory properties. Nat Med 1999; 5: 698-701.

Gilroy DW, Tomlinson A, Willoughby DA. Differential effects of inhibitors of cyclooxygenase (cyclooxygenase 1 and cyclooxygenase 2) in acute inflammation. Eur J Pharmacol 1998; 355: 211-217.

Gold R, Archelos JJ, Hartung HP. Mechanisms of immune regulation in the peripheral nervous system. Brain Pathol 1999; 9: 343-60.

Gold R, Hartung HP, Lassmann H. T-cell apoptosis in autoimmune diseases: termination of inflammation in the nervous system and other sites with specialized immune-defense mechanisms. Trends Neurosci 1997; 20: 399-404.

Govoni V, Granieri E. Epidemiology of the Guillain-Barre syndrome. Curr Opin Neurol 2001; 14: 605-13.

Greco A, Minghetti L, Sette G, Fieschi C, Levi G. Cerebrospinal fluid isoprostane shows oxidative stress in patients with multiple sclerosis. Neurology 1999; 53: 1876-9.

Griffin JW, George R, Ho T. Macrophage system in peripheral nerves. A review. J Neuropathol Exp Neurol 1993; 52: 553-560.

Griffin JW, Li CY, Ho TW, Tian M, Gao CY, Xue P, et al. Pathology of the motor-sensory axonal Guillain-Barre syndrome. Ann Neurol 1996; 39: 17-28.

Griffin JW, Li CY, Ho TW, Xue P, Macko C, Gao CY, et al. Guillain-Barre syndrome in northern China. The spectrum of neuropathological changes in clinically defined cases. Brain 1995; 118 (Pt 3): 577-95.

Hahn AF. Guillain-Barre syndrome. Lancet 1998; 352: 635-41.

Hankey GJ, Eikelboom JW. Cyclooxygenase-2 inhibitors: are they really atherothrombotic, and if not, why not? Stroke 2003; 34: 2736-40.

Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. Trends Immunol 2002; 23: 144-50.

Hartung H-P, Heininger K, Schäfer B, Fierz W, Toyka KV. Immune mechanisms in inflammatory polyneuropathy. Ann N Y Acad Sci 1988a; 545: 122-161.

Hartung HP, Jung S, Stoll G, Zielasek J, Schmidt B, Archelos JJ, et al. Inflammatory mediators in demyelinating disorders of the CNS and PNS. J Neuroimmunol 1992; 40: 197-210.

Hartung HP, Pollard JD, Harvey GK, Toyka KV. Immunopathogenesis and treatment of the Guillain-Barre syndrome--Part I. Muscle Nerve 1995; 18: 137-53.

Hartung HP, Reiners K, Michels M, Hughes RA, Heidenreich F, Zielasek J, et al. Serum levels of soluble E-selectin (ELAM-1) in immune-mediated neuropathies. Neurology 1994; 44: 1153-8.

Hartung H-P, Schafer B, Heininger K, Stoll G, Toyka KV. The role of macrophages and eicosanoids in the pathogenesis of experimental allergic neuritis. Serial clinical, electrophysiological, biochemical and morphological observations. Brain 1988b; 111: 1039-1059.

Hartung H-P, Stoll G, Toyka KV. Immune reactions in the peripheral nervous system. In: Dyck PJ, Thomas PK, Griffin JW, Low PA and Poduslo J, editors. Peripheral Neuropathies. Vol 3. Philadelphia: Saunders, 1993: 418-444.

Hartung HP, Toyka KV. T-cell and macrophage activation in experimental autoimmune neuritis and Guillain-Barre syndrome. Ann Neurol 1990; 27 Suppl: S57-63.

Hartung H-P, Toyka KV, Griffin JW. Guillain-Barré syndrome and chronic inflammatory demyelinating polyradiculoneuropathy. In: Antel J, Birnbaum G and Hartung H-P, editors. Clinical Neuroimmunology. Malden, USA: Blackwell Science, 1998: 294-306.

Hartung HP, Willison HJ, Kieseier BC. Acute immunoinflammatory neuropathy: update on Guillain-Barre syndrome. Curr Opin Neurol 2002; 15: 571-7.

Ho TW, McKhann GM, Griffin JW. Human autoimmune neuropathies. Annu Rev Neurosci 1998; 21: 187-226.

Hughes PM, Wells GM, Clements JM, Gearing AJ, Redford EJ, Davies M, et al. Matrix metalloproteinase expression during experimental autoimmune neuritis. Brain 1998; 121 (Pt 3): 481-94.

Hughes RA. The concept and classification of Guillain-Barre syndrome and related disorders. Rev Neurol (Paris) 1995; 151: 291-4.

Hughes RA, Hadden RD, Gregson NA, Smith KJ. Pathogenesis of Guillain-Barre syndrome. J Neuroimmunol 1999; 100: 74-97.

Hutloff A, Dittrich AM, Beier KC, Eljaschewitsch B, Kraft R, Anagnostopoulos I, et al. ICOS is an inducible T-cell co-stimulator structurally and functionally related to CD28. Nature 1999; 397: 263-6.

Joseph SA, Tsao CY. Guillain-Barre syndrome. Adolesc Med 2002; 13: 487-94.

Kawasaki T, Oka N, Akiguchi I, Miyamoto K. Up-regulation of cyclooxygenase-2 in inflammatory demyelinating neuropathy. Acta Neuropathol 2001; 101: 154-158.

Kiefer R, Dangond F, Mueller M, Toyka KV, Hafler DA, Hartung HP. Enhanced B7 costimulatory molecule expression in inflammatory human sural nerve biopsies. J Neurol Neurosurg Psychiatry 2000; 69: 362-8.

Kiefer R, Kieseier BC, Stoll G, Hartung HP. The role of macrophages in immune-mediated damage to the peripheral nervous system. Prog Neurobiol 2001; 64: 109-27.

Kieseier BC, Clements JM, Pischel HB, Wells GM, Miller K, Gearing AJ, et al. Matrix metalloproteinases MMP-9 and MMP-7 are expressed in experimental autoimmune neuritis and the Guillain-Barre syndrome. Ann Neurol 1998; 43: 427-34.

Kieseier BC, Dalakas MC, Hartung HP. Immune mechanisms in chronic inflammatory demyelinating neuropathy. Neurology 2002a; 59: S7-12.

Kieseier BC, Hartung HP. Guillain -Barre syndrome. Adv clinical Neuroscience 2002; 12: 317-341.

Kieseier BC, Hartung HP. Therapeutic strategies in the Guillain-Barre syndrome. Semin Neurol 2003; 23: 159-68.

Kieseier BC, Hartung H-P. Neuroinflammation. In: Willoughby DA and Tomlinson A, editors. Inducible Enzymes in the inflammatory Response. Basel, Switzerland: Birkhäuser, 1999: 169-186.

Kieseier BC, Kiefer R, Gold R, Hemmer B, Willison HJ, Hartung HP. Advances in understanding and treatment of immune-mediated disorders of the peripheral nervous system. Muscle Nerve 2004; 30: 131-56.

Kieseier BC, Tani M, Mahad D, Oka N, Ho T, Woodroofe N, et al. Chemokines and chemokine receptors in inflammatory demyelinating neuropathies: a central role for IP-10. Brain 2002b; 125: 823-34.

Latov N. Diagnosis of CIDP. Neurology 2002; 59: S2-6.

Lunn MP, Manji H, Choudhary PP, Hughes RA, Thomas PK. Chronic inflammatory demyelinating polyradiculoneuropathy: a prevalence study in south east England. J Neurol Neurosurg Psychiatry 1999; 66: 677-80.

Maurer M, Toyka KV, Gold R. Cellular immunity in inflammatory autoimmune neuropathies. Rev Neurol (Paris) 2002; 158: S7-15.

McLeod JG, Pollard JD, Macaskill P, Mohamed A, Spring P, Khurana V. Prevalence of chronic inflammatory demyelinating polyneuropathy in New South Wales, Australia. Ann Neurol 1999; 46: 910-3.

Mitchell JA, Larkin S, Williams TJ. Cyclooxygenase-2: regulation and relevance in inflammation. Biochem Pharmacol 1995; 50: 1535-42.

Miyamoto K, Oka N, Kawasaki T, Miyake S. New cyclooxygenase-2 inhibitors for treatment of experimental autoimmune neuritis. Muscle Nerve 2002; 25: 280-282.

Miyamoto K, Oka N, Kawasaki T, Satoi H, Akiguchi I, Kimura J. The effect of cyclooxygenase-2 inhibitor on experimental allergic neuritis. Neuroreport 1998; 9: 2331-4.

Miyamoto K, Oka N, Kawasaki T, Satoi H, Matsuo A, Akiguchi I. The action mechanism of cyclooxygenase-2 inhibitor for treatment of experimental allergic neuritis. Muscle Nerve 1999; 22: 1704-9.

Monaco S, Gehrmann J, Raivich G, Kreutzberg GW. MHC-positive, ramified macrophages in the normal and injured rat peripheral nervous system. J Neurocytol 1992; 21: 623-34.

Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA 2001; 286: 954-9.

Murata K, Dalakas MC. Expression of the co-stimulatory molecule BB-1, the ligands CTLA-4 and CD28 and their mRNAs in chronic inflammatory demyelinating polyneuropathy. Brain 2000; 123 (Pt 8): 1660-6.

Mygland A, Monstad P. Chronic polyneuropathies in Vest-Agder, Norway. Eur J Neurol 2001; 8: 157-65.

Newton R, Kuitert LM, Bergmann M, Adcock IM, Barnes PJ. Evidence for involvement of NF-kappaB in the transcriptional control of COX-2 gene expression by IL-1beta. Biochem Biophys Res Commun 1997; 237: 28-32.

Oka N, Akiguchi I, Kawasaki T, Ohnishi K, Kimura J. Elevated serum levels of endothelial leukocyte adhesion molecules in Guillain-Barre syndrome and chronic inflammatory demyelinating polyneuropathy. Ann Neurol 1994; 35: 621-4.

Okamoto T, Saito S, Yamanaka H, Tomatsu T, Kamatani N, Ogiuchi H, et al. Expression and function of the co-stimulator H4/ICOS on activated T cells of patients with rheumatoid arthritis. J Rheumatol 2003; 30: 1157-63.

Pairet M, van Ryn J, Distel M. Overview of COX-2 in inflammation: from the biology to the clinic. In: Willoughby DA and Tomlinson A, editors. Inducible Enzymes in the Inflammatory Response. Basel, Switzerland: Birkhäuser, 1999: 1-30.

Parente L, Perretti M. Advances in the pathophysiology of constitutive and inducible cyclooxygenases: two enzymes in the spotlight. Biochem Pharmacol 2003; 65: 153-9.

Patrignani P, Santini G, Panara MR, Sciulli MG, Greco A, Rotondo MT, et al. Induction of prostaglandin endoperoxide synthase-2 in human monocytes associated with cyclo-oxygenase-dependent F2-isoprostane formation. Br J Pharmacol 1996; 118: 1285-93.

Pollard JD, Baverstock J, McLeod JG. Class II antigen expression and inflammatory cells in the Guillain-Barre syndrome. Ann Neurol 1987; 21: 337-41.

Previtali SC, Feltri ML, Archelos JJ, Quattrini A, Wrabetz L, Hartung H. Role of integrins in the peripheral nervous system. Prog Neurobiol 2001; 64: 35-49.

Prineas JW. Pathology of inflammatory demyelinating neuropathies. Baillieres Clin Neurol 1994; 3: 1-24.

Ropper AH. Current treatments for CIDP. Neurology 2003; 60: S16-22.

Rose JW, Hill KE, Watt HE, Carlson NG. Inflammatory cell expression of cyclooxygenase-2 in the multiple sclerosis lesion. J Neuroimmunol 2004; 149: 40-9.

Rotta FT, Sussman AT, Bradley WG, Ram Ayyar D, Sharma KR, Shebert RT. The spectrum of chronic inflammatory demyelinating polyneuropathy. J Neurol Sci 2000; 173: 129-39.

Rottman JB, Smith T, Tonra JR, Ganley K, Bloom T, Silva R, et al. The costimulatory molecule ICOS plays an important role in the immunopathogenesis of EAE. Nat Immunol 2001; 2: 605-11.

Schröder JM. Pathology of Peripheral Nerves. Vol 9. Berlin: Springer-Verlag, 2001.

Sharpe AH, Freeman GJ. The B7-CD28 superfamily. Nat Rev Immunol 2002; 2: 116-26.

Shin T, Lee Y, Sim KB. Involvement of cyclooxygenase-1 and -2 in the sciatic nerve of rats with experimental autoimmune neuritis. Immunol Invest 2003; 32: 123-30.

Smith WL. Prostanoid biosynthesis and mechanisms of action. Am J Physiol 1992; 263: F181-91.

Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem 1996; 271: 33157-60.

Sperling AI. ICOS costimulation: is it the key to selective immunotherapy? Clin Immunol 2001; 100: 261-2.

Sporici RA, Beswick RL, von Allmen C, Rumbley CA, Hayden-Ledbetter M, Ledbetter JA, et al. ICOS ligand costimulation is required for T-cell encephalitogenicity. Clin Immunol 2001; 100: 277-88.

Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutat Res 2001; 480-481: 243-68.

Taylor WA, Hughes RA. T lymphocyte activation antigens in Guillain-Barre syndrome and chronic idiopathic demyelinating polyradiculoneuropathy. J Neuroimmunol 1989; 24: 33-9.

Toyka KV, Gold R. The pathogenesis of CIDP: rationale for treatment with immunomodulatory agents. Neurology 2003; 60: S2-7.

Van der Meche FG, Van Doorn PA, Meulstee J, Jennekens FG. Diagnostic and classification criteria for the Guillain-Barre syndrome. Eur Neurol 2001; 45: 133-9.

van Doorn PA, van Koningsveld R. Immunotherapy for Guillain-Barre syndrome. Lancet Neurol 2004; 3: 84.

Vane JR, Mitchell JA, Appleton I, Tomlinson A, Bishop-Bailey D, Croxtall J, et al. Inducible isoforms of cyclooxygenase and nitric-oxide synthase in inflammation. Proc Natl Acad Sci U S A 1994; 91: 2046-2050.

Wadleigh DJ, Reddy ST, Kopp E, Ghosh S, Herschman HR. Transcriptional activation of the cyclooxygenase-2 gene in endotoxin-treated RAW 264.7 macrophages. J Biol Chem 2000; 275: 6259-6266.

Wanschitz J, Maier H, Lassmann H, Budka H, Berger T. Distinct time pattern of complement activation and cytotoxic T cell response in Guillain-Barre syndrome. Brain 2003; 126: 2034-42.

Wassink L, Vieira PL, Smits HH, Kingsbury GA, Coyle AJ, Kapsenberg ML, et al. ICOS expression by activated human Th cells is enhanced by IL-12 and IL-23: increased ICOS expression enhances the effector function of both Th1 and Th2 cells. J Immunol 2004; 173: 1779-86.

Wiendl H, Neuhaus O, Mehling M, Wintterle S, Schreiner B, Mitsdoerffer M, et al. The CD28 related molecule ICOS: T cell modulation in the presence and absence of B7.1/2 and regulational expression in multiple sclerosis. J Neuroimmunol 2003; 140: 177-87.

Willison HJ, Yuki N. Peripheral neuropathies and anti-glycolipid antibodies. Brain 2002; 125: 2591-625.

Willoughby DA, Moore AR, Colville-Nash PR. COX-1, COX-2, and COX-3 and the future treatment of chronic inflammatory disease. Lancet 2000; 355: 646-648.

Yamamoto K, Arakawa T, Ueda N, Yamamoto S. Transcriptional roles of nuclear factor kappa B and nuclear factor-interleukin-6 in the tumor necrosis factor alpha-dependent induction of cyclooxygenase-2 in MC3T3-E1 cells. J Biol Chem 1995; 270: 31315-20.

Zhu Y, Ljunggren H, Mix E, Li HL, van der Meide P, Elhassan AM, et al. CD28-B7 costimulation: a critical role for initiation and development of experimental autoimmune neuritis in C57BL/6 mice. J Neuroimmunol 2001; 114: 114-21.

Zou LP, Pelidou SH, Abbas N, Deretzi G, Mix E, Schaltzbeerg M, et al. Dynamics of production of MIP-1alpha, MCP-1 and MIP-2 and potential role of neutralization of these chemokines in the regulation of immune responses during experimental autoimmune neuritis in Lewis rats. J Neuroimmunol 1999; 98: 168-75.

8 APPENDIX

8.1 Materials

8.1.1 Chemical reagent

Amersham Biosciences_Freiburg

DNTP set, 100mM Solutions(dATP, dCTP, dGTP, dTTP), 4X25 µmol

Bio-Rad Laboratories, Munich

30% Acrylamide/ Bis solutions 19:1 (5%C); BIO-RAD-Protein assay; MiniProtean II Protein standard; MiniProtean II semi-dry blotter; nitrocellulose Transfer Membrane

Greiner, Nürtingen

Monolayer flasks for cell culture; Plastic Petri-dishes, ø 9 cm and 14 cm; Plastic test tubes 10 and 50 ml

Invitrogen_Karlsruhe

DNase I, Amplification Grade; 1 Kb DNA Ladder; 123Kb DNA Ladder; TRIzol® Reagents

Merck AG, Darmstadt

Acetic acid; Acrylamide; Ammonium persulfate (APS); B-Mercaptoethanol;Boric acid; Bromophenol blue; Calcium acetate; Calcium chloride; Calcium hydroxide; Chloroform; Dimethyl sulfoxide (DMSO); Dimethylformamid; Dithiothreitol ; Entellan; Ethylene diamintetraacetic acid (EDTA); Ethanol p.A.; Ethidiumbromid; Formamid; Formaldehyd; Glucose; Glycin; Hydrochloric acid ; Isopropanol; Magnesium acetate; Magnesium chloride; Magnesium sulfate; Methanol p.A.; N,N,N',N'-tetra-methyl-ethylendiamine (TEMED); Phenol; Sodium acetate; Sodium chloride; Sodium hydroxide;Sodium dodecyl sulfate (SDS); Saccharose; Tris ;Triton X-100; Urea; Xylen cyande

Molecular Probes Europe, Leiden, The Netherlands

SYBR® Gold Nucleic Acid Gel Stain

Sigma-Aldrich Chemie, Munich

Bovine serum albumin (BSA); Diaminobenzene; Diethylpyrocarbonate (DEPC); Ethidium Bromide; Glutamine; Glycerol; Hematoxylin; Hydrogen peroxide; Protease XXIV

Schleicher and Schuell, Darmstadt

Nitrocellulose filter BA 85, pore size 0.45 µm

8.1.2 Enzymes

Applied Biosystems, Darmstadt

AmpliTaq® DNA Polymerase ; GeneAmp® 10X PCR Buffers: 500mM KCl, 100mM Tris-HCl, pH 8.3; 15mM $MgCl_2$ and 0.01% (w/v) gelatin; TaqMan® MuLV Reverse Transcription Reagents: MultiScribe™ Reverse Transcriptase, RNase Inhibitor, dNTP Mixture, Random Hexamers, 10X RT Buffer, $MgCl_2$ Solution

8.1.3 Primers

Invitrogen, Karlsruhe

human ICOS: sense, 5' – GCAACCAGCTTTGGAGAAAG – 3';

(GeneID: 29851) antisense, 5' – TGCTTTGCAGATTCAGTACCC – 3';

human ICOS-L: sense, 5' – CTT GTG GTC GTG GCG GTG – 3';

(GeneID: 23308) antisense, 5' – TCA CGA GAG CAG AAG GAG CAG GTT CC– 3';

human COX-1: sense, 5' – TGC CCA GCT CCT GGC CCG CCG CTT – 3';

(GeneID: 5742) antisense, 5' – GTG CAT CAA CAC AGG CGC CTC TTC – 3';

human COX-2: sense, 5' – TTC AAA TGA GAT TGT GGG AAA ATT GCT – 3';

(GeneID: 5743) antisense, 5' – TCT AGA GGG CAG GTC AGG TCC ACC – 3';

human GAPDH, serving as a house keeping gene:

(GeneID: 2597) sense, 5' – CCA CCC ATG GCA AAT TCC ATG GCA – 3';

antisense, 5' – TCT AGA GGG CAG GTC AGG TCC ACC – 3'.

8.1.4 Antibodies

DAKO, Hamburg

anti-human ICOS and anti-human ICOS-L were generously provided by R.A. Kroczek, Robert-Koch-Insitut, Berlin, Germany

3,3‘-diaminobenzidine (DAB); murine monoclonal anti-CD68; rabbit anti-mouse IgG; avidin-biotin-horseradish peroxidase complex; Biotinylated secondary antibodies

Santa Cruz, California, USA

murine monoclonal anti-COX-2

Serotec, Oxford, England

murine monoclonal anti-CD3

8.1.5 Cell culture

Dynal Biotech, Hamburg

Dynabead monocyte negative isolation kit

Invitrogen , Karlsruhe

RPMI 1640 medium supplemented with 10% human serum, 2 mM glutamine, 100 U/ml penicillin, and 100 IE/ml streptomycin

Pharmacia, Karlsruhe

Ficoll-Hypaque

Pharmingen, San Diego, CA, USA

lipopolysaccharide (LPS)

SIGMA, Munich

DMSO (Dimethyl Sulfoxide); b-mercaptoethanol

8.1.6 ELISA

R&D Systems, Abingdon, UK

Prostaglandin E_2 Immunoassay kit; Prostaglandin $F_{2\alpha}$ Immunoassay kit

8.2 Methods

8.2.1 RNA extraction

Frozen sural nerve tissues were homogenize in 1 ml of TRIZOL reagent per 50-100 mg of tissue and incubated 5 min. at room temperature (RT), afterward, incubated with 100 µl chloroform 2-3 min. and centrifuged at 10.000 rpm x 15min. at 4 °C. There are three phases: an upper colourless aqueous one (RNA), a thin white-turbid interphase (DNA), and a pink organic one (protein). With a sterile pipette, recover the colourless phase into new tube with 250 µl isopropanol and vortex slightly and incubate overnight at –70 °C. Afterwards, without defrosting, the sample centrifuge 10.000 rpm x 15min. at 4 °C. During this time, take out alcohol to warm it to 4 °C. Then, Discard supernatant with sterilized Pasteur pipettes and clean with 500 µl RNase-free 75% alcohol (Centrifuge 7.000 rpm x5 min.) at 4 °C. Discard alcohol as much as possible. Let evaporate the reminding one under a hood for10-20 min. Finally, add 20 µl RNase free (DEPC-treated) water to each sample and store at – 80 °C.

8.2.2 Measurement of RNA concentration

The concentration of RNA solution can be calculated through measuring RNA at wavelength of OD260 by biophotometer (Eppendorf AG, Hamberg). DEPC-water was used as the blank value.

8.2.3 Reverse Transcription

Prepare a 20µl reaction by adding the following reagents in the order listed: Reverse Transcription 10X Buffer 2µl; MgCl2, 25mM 4.4µl; dNTP Mixture, 10mM 4µl; Random Primers 1.0µl; RNase-free 0.1% DEPC-H_2O 5.7µl; RNA sample 2µl; RNase Inhibitor 0.4µl; Multi Scribe™ Reverse Transcriptase 0.5 µl. afterwards, the program was carried out (25 ºC, 10 minutes / 48 ºC, 30 minutes / 95 ºC, 5 minutes) in a Perkin Elmer 9700 thermal cycler, then 4 degrees Celsius until ready for down stream application.

8.2.4 Polymerase Chain Reaction (PCR)

In a sterile 0.5-ml microfuge tube, mix in the following order:

l GeneAmp® 10X PCR Buffers (500mM KCl, 100mM Tris-HCl, pH 8.3; 15mM MgCl2 and 0.01% (w/v) gelatin)	5 µl
dNTP	4 µl
50 pmoles sense and antisense primers	25 µl
1 U AmpliTaq® DNA Polymerase	0.2µl
cDNA	1µl
DEPC-H_2O	16µl
Total volume	50 µl

Amplification was carried out using 35 cycles (denaturation temperature 94 ºC, 60 secs / annealing temperature 53 ºC, 60 secs / polymerization temperature 72 ºC, 120 secs) in a Perkin Elmer 9700 thermal cycler.

8.2.5 Immunohistochemistry

Frozen or formalin fixed and paraffin embedded sural nerve biopsies were cut at 6 µm and 10 µm, respectively, and sections were deparaffinized in xylenes and rehydrated through graded ethanol, when applicable. If necessary, sections were pretreated with protease XXIV (Sigma Chemical Co., St. Louis, MO, USA) or microwaved in 0.1 M citrate buffer for 3 min at 680 W. the samples were rinsed with distilled water, placed in PBS for 5 minutes. All optimally diluted primary antibodies were incubated at 4°C overnight after blocking the sections with 10% bovine serum albumin for 30 min. Endogenous peroxidase was blocked with 30% hydrogen peroxide in methanol. After the membrane was washed for 3 times with PBS/0,05% Tween20, the secondary antibody, peroxidase conjugated anti-IgG diluted to 1:5000-1:10000 in PBS/0,05% Tween20 was given and incubated for 2 hours with gentle shaking. The blot was washed with PBS/0.05% Tween20 for 3 times and Stained with diaminobenzidin (DAB) (Dakocytomation, Glostruo Denmark) solution (0.1% DAB in PBS and 0.01% H_20_2 freshly prepared) 10 minutes at room temperature. After development with DAB substrate, sections were counterstained with hematoxylin (Sigma), and mounted in entellan (Merck, Darmstadt, Germany).

8.2.6 Monocyte isolation

PBMC from healthy donors were isolated by Ficoll-Hypaque (Pharmacia, Karlsruhe, Germany) density gradient centrifugation, a simple and rapid method of isolating PBMC that takes advantage of the density differences between mononuclear cells and other elements found in the blood sample. Mononuclear cells and platelets collect on top of the Ficoll-Hypaque layer because they have a lower density: in contrast, red blood cells and granulocytes have a higher density than Ficoll-Hypaque and collect at the bottom of the layer. Platelets are separated from mononuclear cells by subsequent washing of through centrifugation through a foetal-calf serum cushioned gradient which allows penetration of

mononuclear cells but not platelets. T cells, B cells, NK cells and granulocytes, if present, were depleted using a Dynabead monocyte negative isolation kit (Dynal Biotech, Hamburg, Germany).

For each 1 x 10^7 mononuclear cells, use 20 µl Blocking Reagent, 20 µl Antibody Mix (Monocyte Kit) and 100 µl Depletion Dynabeads® as described below.

1. Resuspend prepared MNC at 5-10 x 10^7/ml in 0.1% BSA.
2. Add 20 µl Blocking Reagent per 1 x 10^7 MNC.
3. Add 20 µl Antibody Mix (Monocyte Kit) per 1 x 10^7 MNC.
4. Incubate for 10 minutes at 2-8°C (incubation on ice requires longer incubation).
5. Wash cells by adding 1 ml of PBS/0.1% BSA per 1-5 x 10^7 MNC and centrifuge for 8 minutes at 500 g. and remove supernatant with a pipette.
6. Resuspend cells in 0.9 ml of PBS / 0.1% BSA per 1 x 10^7 MNC.
7. Add washed beads to the cells. Use 100 µl Depletion Dynabeads® per 1 x 10^7 MNC. Total volume for cell and bead incubation should be 1 ml per 1 x 10^7MNC.
8. Incubate for 15 minutes at 2-8°C with gentle tilting and rotation.
9. Resuspend rosettes by careful pipetting 5-6 times before increasing the volume by adding 1-2 ml of PBS / 0.1% BSA per 1 x 10^7 MNC.
10. Place in the Dynal MPC for 2 minutes and pipette supernatant (negatively isolated monocytes) to a fresh tube.

8.2.7 Cell culture

Isolated monocytes were resuspended (2x10^6/ml) in complete culture media consisting of RPMI 1640 medium supplemented with 10% human serum, 2 mM glutamine (Sigma, St Louis, MI, USA), 100 U/ml penicillin, and 100 IE/ml streptomycin (Invitrogen, Karlsruhe, Germany), and cultured for 48 h at 37°C and 5% CO_2 under the following conditions: in complete culture medium alone, and stimulated with lipopolysaccharide (LPS) (Pharmingen,

San Diego, CA, USA) for stimulation of macrophages. Purity of the cell population was determined by FACS analysis. Cell culture supernatants were used for further analysis.

8.2.8 Polyacrylamid gel electrophoresis for DNA (PCR products)

8.2.8.1 Gel preparation

Glass plates must be clean and free of dried gel and soap residues. To remove residues, both plates were cleaned with ethanol. The solution was poured between the plates of the gel apparatus and a 1.0 mm comb was inserted between the plates. The gel was left to polymerize for 1 hr. The comb was removed and the wells and top surface were rinsed with TBE buffer.

6% polyacrylamide gel electropherosis solution

30% Acrylamide/ Bis solutions 19:1 (5%C)	4.5 ml
Water (distilled)	15.75ml
TBE bufferX10	2.25 ml
Ammonium peroxydisulphate 25%	30 µl
TEMED (added last)	30 µl

Tris-Borate-EDTA (TBE) electrophoresis buffer

Tris	108g
Boric acid	55g
EDTA 0,25M (PH 8.0)	80ml
Distilled water	1L

8.2.8.2 Electrophoresis

The anodal and cathodal chambers were filled with 1 x TBE buffer. The comb was taken out from the plates and the 10 µl PCR products samples and 3.5 µl 6XDNA sample loading buffer were loaded onto the gel. With the power supply, the samples run at 90V for 1 hr. After the

run was finished, the gel was immediately immersed in SYBR® Gold Nucleic Acid Gel Stain for 10-30 minutes and then read and quantitated by the software of TINA version 2.09g.

6 X DNA sample loading buffer

0,25% Bromophenol blue 125mg

0.25% Xylene cyande FF 125mg

30% Glycerol in H_2 O 15ml

0.5M EDTA pH 8.0 100µl

H_2 O 35ml

8.2.9 SDS-polyacrylamid gel eletrophoresis (SDS-PAGE)

This gel system uses the method described by Laemmli (1970). In this technique, The SDS coating gives the protein a high net negative charge that is proportional to the length of the polypeptide chain. The sample is loaded on a polyacrylamid gel and high voltage is applied, causing the protein components to migrate toward the positive electrode (anode). Since all of the proteins have a net negative charge that is in proportion to their size, the proteins are separated solely on the basis of their molecular mass, a result of sieving effect of the gel matrix. The molecular mass of the protein can be estimated by comparing the mobility of a band with protein standards. Shape banding of the protein components is achieved by using a discontinuous gel system, which is composed of stacking and separating gel layers that differ in either salt concentration or pH or both. Resolving and stacking mini-gel solutions for the preparation of two 7 x 10 cm gels with a thickness of about 0.75-1.00 mm were prepared.

8.2.9.1 Pouring SDS-polyacrylamide Gel

Assemble the glass plates according to the manufacturer's instructions. In a disposable plastic tube, prepare the 10% resolving SDS-Gel (size 10x10cm) solution, mix the components in the

order shown. Polymerisation will begin as soon as the TEMED has been added. Without delay, swirl the mixture rapidly and pour the solution into the gap between the glass plates, leave sufficient space for the stacking gel. Use a Pasteur pipette to carefully overlay the acrylamide solution with isobutanol and place the gel in a vertical position at room temperature. The gel was allowed to polymerize for at least 15-30 min. After polymerization, the isopropanol layer was removed and the surface of the resolving gel was rinsed with water. The remaining space was filled with the stacking gel solution and the comb was inserted immediately. After the stacking gel had polymerized, the comb was removed and the wells were rinsed with water to remove unpolymerized acrylamid. At least 1 cm of stacking gel should be present between the bottom of the loading wells and the resolving gel. Mount the gel in the electrophoresis apparatus. Add tris-glycine electrophoresis buffer to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates with a bent hypodermic needle attached to a syringe. Load up to 15µl of each of protein sample with 1X SDS loading buffer (Invitrogen, Karlsruhe) and protein standard marker -Rainbow™ Molecular Weight Markers (Amersham Biosciences, Freiburg, Germany) in a predetermined order into the bottom of the wells with a micro-pipette equipped with gel-loading tips that is washed with buffer from the bottom reservoir after each sample is loaded. Attach the electrophoresis apparatus to an electric power supply. The gel was run in SDS gel running buffer at 120V for 1 hour. Afterwards, remove the glass plates from the electrophoresis apparatus and place them on a paper towel. Use an extra gel spacer to carefully pry the plates apart. At this stage, the gel can be fixed, used to establish an immunoblot.

<u>10% resolving SDS-Gel</u>

4 ml Aqua dest

3.3 ml 30% Acrylamide/ Bis solutions 19:1 (5%C) (37.5:1)

2.5 ml 1,5 M Tris PH 8,8

100 µl 10% SDS(w/v)

100 µl 10% APS

4 µl TEMED

5% stacking Gel:

2.1 ml Aqua dest

0.5 ml 30% Acrylamide/ Bis solutions 19:1 (5%C) (37.5 :1)

380 µl 1 M Tris PH 6.8

30 µl 10% SDS(w/v)

30 µl 10% APS

3 µl TEMED

SDS gel running buffer:

25 mM Tris 10% SDS

192 mM glycin

0.1% SDS

8.2.10 Coomassie blue staining

Detection of protein bands in a gel by Coomassie Blue staining can be achieved due to nonspecific binding of a dye, coomassie Brilliant Blue R, to the proteins. The detection limit is 20 ng to 1 mg/protein band.

After the SDS gel was run, the gel was shaken in Coomassie Blue staining solution for 15 min. Coomassie Blue turned the entire gel blue and the gel should be destained by detaining solution. After destaining, the blue protein bands appeared against a clear background. The gel could be stored in acetic acid or water or was photographed or dried to maintain a permanent record.

Coomassie Blue staining solution:

50% (v/v) methanol

0.05% (v/v) Coomassie brilliant blue R-250

10% acetic acid

40% H_2O

Destaining solution:

5% methanol

7% acetic acid

88% H_2O

8.2.11 Western-blot (Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell)

Western-blots can be used to separate 0.0005-25 mg of complex mixtures of proteins. The protocol for Semi-Dry electrophoretic transfer listed as below.

1, Cut the Nitrocellulose membrane to the dimensions of the gel. Wet the membrane by slowly sliding it at a 45° angle into transfer buffer and allowing it to soak for 15–30 minutes. Complete wetting of the membrane is important to insure proper binding. To avoid membrane contamination, always use forceps or wear gloves when handling membranes.

2, Cut filter paper to the dimensions of the gel. Two pieces of extra thick filter paper per gel are needed for each gel/membrane sandwich. Completely saturate the filter paper by soaking in transfer buffer.

Remove the safety cover and the stainless steel cathode assembly. Place a pre-soaked sheet of extra thick filter paper onto the platinum anode. Roll a pipet or test tube over the surface of the filter paper (like a rolling pin) to exclude all air bubbles. If thick or thin filter paper is used, repeat with one or two more sheets of buffer soaked filter paper.

3. Place the pre-wetted blotting media on top of the filter paper. Roll out all air bubbles.

4. Carefully place the equilibrated gel on top of the transfer membrane, aligning the gel on the center of the membrane. Roll out all air bubbles.

5. Place the other sheet of pre-soaked filter paper on top of the gel, carefully removing air

bubbles from between the gel and filter paper. Place two sheets on top of the gel, and remove bubbles from between each layer.

6. Carefully place the cathode onto the stack. Press to engage the latches with the guide posts without disturbing the filter paper stack.

8. Place the safety cover on the unit. Plug the unit into the power supply. Normal transfer polarity is cathode to anode, turn on the power supply. Transfer mini gels for 15–30 minutes at 10–15 V.

Following transfer, turn the power supply off, and disconnect the unit from the power supply. Remove the safety cover and the cathode assembly. Discard the filter paper (and dialysis membrane, if used). The transfer efficiency can be monitored by staining the gel and the membrane with Coomassie blue R-250 protein stain and Ponceau S for 5 minutes, respectively. The membrane was washed by distilled water for at least 20 minutes and incubated in PBS buffer containing 0,05% Tween20 and 2,5% milk powder for 2 hours with gentle shaking to block unspecific bindings of antibody. Specific primary antibody was given to the blot and incubated overnight with gentle shaking. Depending on the antibody's specificity and affinity, the antibody was diluted to 1:100 to 1:250 in PBS containing 0,05% Tween20. After the membrane was washed for 3 times with PBS/0,05% Tween20, the secondary antibody, peroxidase conjugated anti-IgG diluted to 1:5000-1:10000 in PBS/0,05% Tween20 was given and incubated for 2 hours with gentle shaking. The blot was washed with PBS/0.05% Tween20 for 3 times and Stained with diaminobenzidin (DAB) solution (0.1% DAB in PBS and 0.01% H_20_2 freshly prepared) 10 min. at room temperature.

<u>Transfer buffer for semi-dry electrophoretic transfer</u>

10X transfer buffer stock - store at 4° C

30.3 g Tris base

144.1 g glycine

water to 1 liter

1X transfer buffer - store at 4° C

100 ml 10X stock

500 ml H_2O

200 ml methanol

water to 1 L

8.2.12 ELISA (Enzyme-Linked Immunosorbent Assay)

8.2.12.1 Reagent preparation

Bring all reagents to room temperature before use.

Wash Buffer - Dilute 30 mL of Wash Buffer Concentrate into distilled water to prepare 300 mL of Wash Buffer (1X).

PGE_2 and $PGF_{2\alpha}$ Standard - Pipette 900 µL of Assay Buffer ED1 into the 50,000 pg/mL tube. Pipette 750 L of Assay Buffer ED1 into the remaining tubes. When pipetting standards, it is important to pre-rinse the pipette tip. Use the 500,000 pg/mL standard stock to produce a dilution series. Mix each tube thoroughly and change pipette tips between each transfer. The 50,000 pg/mL standard serves as the high standard and the Assay Buffer ED1 serves as the zero standard (B0) (0 pg/mL).

8.2.12.2 Assay procedure

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards are assayed in duplicate.

Total Activity (TA), Non-Specific Binding (NSB), Maximum Binding (B0), and Substrate Blank wells should be run in the assay as a means of quality control for each assay.

Afterwards follow the protocol as below:

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.

3. Reserve wells for TA and Substrate Blank.

4. Add 150 L of Assay Buffer to the NSB wells.

5. Add 100 L of Assay Buffer to the zero standard (B0) wells.

6. Add 100 L of Standard or sample to the remaining wells.

7. Add 50 L of PGE_2 or $PGF_{2\alpha}$ Conjugate to each well (excluding the TA and Substrate Blank wells).

8. Add 50 L of PGE_2 or $PGF_{2\alpha}$ Antibody Solution to each well (excluding the NSB, TA, and Substrate Blank wells). Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 50 rpm.

9. Aspirate or decant each well and wash, repeating the process twice for a total of three washes. Wash by filling each well with Wash Buffer (400 L) using a multi-channel pipette. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

10. Add 5 L of PGE_2 or $PGF_{2\alpha}$ Conjugate to the TA wells.

11. Add 200 L Substrate to all wells. Incubate for 1 hour at room temperature on the benchtop.

12. Add 50 L of Stop Solution to each well.

13. Determine the optical density of each well immediately using a microplate reader set to 405 nm with wavelength correction set between 570 nm and 590 nm.

PUBLICATION

1. Hu W, Mathey E, Hartung HP, Kieseier BC. Cyclo-oxygenases and prostaglandins in acute inflammatory demyelination of the peripheral nerve. Neurology 2003 Dec 23;61(12):1774-9.
2. Hu W, Janke A, Hartung HP, Kieseier BC, Wiendl H. The CD28 related costimulatory molecule ICOS and its ligand are expressed in acute and chronic immune-mediated neuropathies. Submitted

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