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Statin-Mediated Attenuation of Chemokine Expression in Peripheral Nerve Vascular Endothelial Cells

Kelly Ann Langert
Loyola University Chicago

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STATIN-MEDIATED ATTENUATION OF CHEMOKINE EXPRESSION IN PERIPHERAL NERVE VASCULAR ENDOTHELIAL CELLS

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN NEUROSCIENCE

BY

KELLY ANN LANGERT

CHICAGO, ILLINOIS
AUGUST 2012
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To Dad
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<tr>
<td>AIDP</td>
<td>acute inflammatory demyelinating polyneuropathy</td>
</tr>
<tr>
<td>AMAN</td>
<td>acute motor axonal neuropathy</td>
</tr>
<tr>
<td>AMSAM</td>
<td>acute motor and sensory axonal neuropathy</td>
</tr>
<tr>
<td>BNB</td>
<td>blood-nerve barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAM</td>
<td>cell adhesion molecule</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>EAN</td>
<td>experimental autoimmune neuritis</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FPP</td>
<td>farnesyl pyrophosphate</td>
</tr>
<tr>
<td>FTase</td>
<td>farnesyl transferase</td>
</tr>
<tr>
<td>FTI</td>
<td>farnesyl transferase inhibitor</td>
</tr>
<tr>
<td>GBS</td>
<td>Guillain-Barré syndrome</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDI</td>
<td>guanine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factors</td>
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<tr>
<td>GGPP</td>
<td>geranylgeranyl pyrophosphate</td>
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GGTase  geranylgeranyl transferase
GGTI  geranylgeranyl transferase inhibitor
GTP  guanine nucleoside triphosphate
GTPases  GTP-binding proteins or G-proteins
HMG  3-hydroxy-3-methylglutaryl
HMG-CoA  3-hydroxy-3-methylglutaryl coenzyme A
HUVEC  human umbilical vein endothelial cell
ICAM-1  intercellular adhesion molecule 1 (CD54)
IL-8  interleukin 8
JAM  junctional adhesion molecule
LFA-1  leukocyte function antigen 1
MCP-1  monocyte chemoattractant protein 1 (CCL2)
MFS  Miller-Fisher Syndrome
MIP-1α  macrophage inflammatory protein-1 alpha
NFκB  nuclear factor kappa-B
OPD  o-phenylenediamine dihydrochloride
PECAM  platelet endothelial cell adhesion molecule 1
p.i.  post-immunization
PNS  peripheral nervous system
PNVEC  peripheral nerve vascular endothelial cell
RIP kinase  receptor-interacting protein kinase
RT-PCR  reverse-transcriptase polymerase chain reaction
qRT-PCR  real time RT-PCR
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>SODD</td>
<td>silencer of death domain</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF-α receptor</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF-α receptor associated factor-2</td>
</tr>
<tr>
<td>VLA-4</td>
<td>very late antigen 4</td>
</tr>
<tr>
<td>VWF</td>
<td>von Willebrand factor</td>
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ABSTRACT

Pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), play a pivotal role in the pathogenesis of Guillain-Barré Syndrome (GBS), a debilitating autoimmune disorder that affects the peripheral nervous system. By up-regulating endothelial expression of chemokines and cell adhesion molecules (CAMs), TNF-α facilitates the recruitment and trafficking of autoreactive leukocytes across the blood-nerve barrier and into peripheral nerves, an early pathological hallmark of GBS. Literature indicates that TNF-α, monocyte chemoattractant protein-1 (MCP-1), and intercellular adhesion molecule-1 (ICAM-1) are locally increased in patients with GBS, correlating with disease severity. Similar findings have been demonstrated in an animal model of GBS, experimental autoimmune neuritis (EAN).

Treatment options for patients with GBS are limited to nonspecific, immune-modulating strategies, including plasmapheresis and IVIg. Half of GBS patients fail to respond, and many remain severely disabled despite receiving treatment. Despite advancements, there remains a pressing need for the development of effective and specific immune-modulating therapeutic strategies for the care and management of GBS.

Our group previously reported that statins therapeutically attenuate the course of EAN by inhibiting leukocyte migration. This dissertation project was designed to
determine the mechanism(s) by which statins attenuate pathological transendothelial migration. We hypothesize that statins limit transendothelial migration of leukocytes into peripheral nerves by attenuating TNF-α mediated, RhoGTPase-facilitated secretion of chemokines by the peripheral nerve vascular endothelial cells (PNVECs) that form the blood-nerve barrier. Experiments using PNVECs are lacking, and there is no commercially available cell line. We formed a novel, immortalized cell line of PNVECs by SV40 large T antigen transduction. We report in these cells that TNF-α increases mRNA and protein expression of ICAM-1 and MCP-1, as well as the secretion of MCP-1. Simvastatin pretreatment attenuates TNF-α mediated MCP-1 secretion, but does not alter intracellular levels of ICAM-1 or MCP-1 mRNA or protein. This effect is mimicked by a specific inhibitor of protein geranylgeranylation.

Our novel findings suggest that TNF-α mediated migration of autoreactive leukocytes into peripheral nerves during EAN proceeds, in part, by a mechanism that involves GGPP-dependent secretion of MCP-1. We argue that identifying this precise geranylgeranylated target that mediates chemokine secretion will significantly advance the development of novel treatment options for patients with GBS.
CHAPTER 1
INTRODUCTION

Pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), play a pivotal role in the pathogenesis of Guillain-Barré Syndrome (GBS), an aggressively debilitating autoimmune disorder that affects peripheral nerves and is a leading cause of autoimmune neuromuscular paralysis (Hughes & Cornblath 2005). By up-regulating endothelial cell expression of chemotactic cytokines (chemokines) and cell adhesion molecules (CAMs), TNF-α facilitates the recruitment and trafficking of autoreactive leukocytes into peripheral nerves, an early pathological hallmark of GBS (Exley et al. 1994). Serum plasma levels of TNF-α are elevated in GBS patients and correlate with disease severity (Zhu et al. 1998). Likewise, monocyte chemoattractant protein-1 (MCP-1) is expressed within vascular endothelial cells of the epineurial and endoneurial blood vessels of GBS patients, and its expression correlates well with disease severity (Orlikowski et al. 2003). Furthermore, intercellular adhesion molecule-1 (ICAM-1) has been detected on the endothelial cells and macrophages in sural nerve samples from patients with GBS, which corresponds to involvement of ICAM-1 in the early phase of disease development (Putzu et al. 2000). Similar findings have been demonstrated in a well-characterized animal model of GBS, experimental autoimmune neuritis (EAN)

Due to the aggressive rapid onset of GBS, affected patients often require life-saving respiratory supportive care and must be monitored closely for development of secondary complications. Current treatment options for patients with GBS are limited to nonspecific and often cost prohibitive immune-modulating therapies, such as plasmapheresis and IVIg therapy (Hartung et al. 1995). These are transiently effective immune-modulating strategies, and they do not address the causality of the aberrant autoimmune responses in affected patients. Furthermore, approximately half of GBS patients fail to respond to plasmapheresis or IVIg therapy. Of those patients that respond favorably, a portion remains severely disabled (Hughes & Cornblath 2005). Therefore, despite clinical advancements, there remains a pressing need for the development of more effective and specific immune-modulating therapeutic strategies for the care and management of GBS patients.

Statins are a group of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors that are clinically approved for the treatment of hypercholesterolemia. However, increasing clinical and experimental evidence demonstrates a variety of beneficial effects beyond what can be reasonably attributed to the reduction in serum cholesterol (Goldstein 2007, Halcox & Deanfield 2004, Undas et al. 2002). These pleiotropic effects are mediated by depletion of small isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesyl pyrophosphate.
(FPP) and geranylgeranyl pyrophosphate (GGPP). These isoprenoids serve as lipid attachment anchors for monomeric GTPases (Zhang & Casey 1996), including members of the Rho family, facilitating their activity in cell cycle pathways and gene expression, including NFκB activation (Mackay & Hall 1998, Ridley 2006). By altering Rho activation, statins may influence the expression of NFκB-dependent components of the inflammatory cascade.

It has been previously reported that statins therapeutically attenuate the development and progression of EAN by inhibiting leukocyte migration (Sarkey et al. 2007). This dissertation project was designed to determine the mechanism(s) by which statins attenuate EAN. This will be accomplished by elucidating the mechanism(s) by which FPP and GGPP isoprenoids facilitate transendothelial migration during inflammation. It is hypothesized that statins, and other inhibitors of isoprenylation, limit transendothelial migration of autoreactive leukocytes into peripheral nerves by selectively attenuating TNF-α mediated, RhoGTPase-facilitated expression of chemokines and CAMs by the peripheral nerve vascular endothelial cells (PNVECs) that form the blood-nerve barrier. Experiments using these PNVECs are lacking, and there is no commercially available cell line. To contribute to this void in the literature, and efficiently test the hypothesis, a novel, stably transduced immortalized cell line of PNVECs was created using the method of SV40 large T antigen transduction.
Hypothesis

Statins limit the migration of autoreactive leukocytes across the blood-nerve barrier during EAN by attenuating the expression of inflammatory mediators.

Specific Aims

This hypothesis was tested using three, in vitro, specific research aims, designed to:

I. Determine the effect of TNF-α on MCP-1 and ICAM-1 expression using primary and transformed rat peripheral nerve endothelial cell cultures.

II. Determine the effect of statins, or other inhibitors of isoprenylation, on TNF-α mediated MCP-1 and ICAM-1 expression using primary and transformed rat peripheral nerve endothelial cell cultures.

III. Determine the functional consequence of statin-treatment on transendothelial migration of monocytes using an established transwell migration assay.

In this dissertation, it is demonstrated that TNF-α treatment of both primary and transformed PNVECs rapidly and robustly increases mRNA and protein expression of ICAM-1 and MCP-1, as well as the secretion of MCP-1 into the culture media.

Pretreatment overnight with simvastatin attenuates TNF-α mediated MCP-1 secretion, but does not alter intracellular levels of ICAM-1 or MCP-1 mRNA or protein. This effect is mimicked by a specific inhibitor of protein geranylgeranylation, but not farnesylation. Pretreatment with geranylgeranyltransferase inhibitor I also attenuates the chemotactic
properties of media harvested from TNF-α treated PNVECs and decreases transendothelial migration of CCR2-expressing monocytes.

These novel findings suggest that TNF-α mediated migration of autoreactive leukocytes into peripheral nerves during EAN proceeds, in part, by a mechanism that involves GGPP-dependent secretion of MCP-1. It is argued that identification of the precise geranylgeranylated target (Rac1, Cdc42, RalA) that mediates chemokine secretion has the potential to significantly advance the development of novel treatment options for patients with GBS.
CHAPTER 2

LITERATURE REVIEW

Guillain-Barré Syndrome

1. Clinical Classification

With the successful eradication of Polio, Guillain-Barré syndrome (GBS) is now considered the leading cause of autoimmune neuromuscular paralysis in the United States and Europe. Clinically characterized by a monophasic course of rapidly ascending limb weakness, GBS typically peaks within 4 weeks of disease onset. Approximately twenty percent of GBS-affected patients develop paralysis of the respiratory muscles (diaphragm), requiring life-saving mechanical ventilation and prolonged supportive clinical care. Over 2000-2004, the incidence of GBS in the USA has remained stable at approximately 2 cases per 100,000 persons. The likelihood of an individual acquiring GBS in their lifetime is estimated at approximately 1:1000 (van Doorn 2009, Willison 2005). Unlike many neurodegenerative disorders, GBS is not a disease of the aged, although incidence does increase slightly with advancing age. Elderly patients do
experience a poorer prognosis (Hughes & Cornblath 2005). Interestingly, in contrast with other autoimmune diseases, men are 1.5 times more likely to be affected than women (Hughes & Cornblath 2005).

Guillain-Barré syndrome is not a single disease, but rather encompasses a spectrum of clinical subtypes, each eliciting uniquely different subtypes (Fig. 1). In some GBS patients the immune target is axons of the ventral root, resulting in neurological deficits that are purely motor. This subtype of GBS is referred to as acute motor axonal neuropathy (AMAN). However, when both motor and sensory axons are involved, the

**Figure 1** - An operational classification of Guillain-Barré syndrome. Adapted from (Ho et al. 1998).
GBS subtype is referred to as acute motor sensory axonal neuropathy (AMSAN). Both AMAN and AMSAN are pathologically characterized by axonal damage. Interestingly, these GBS subtypes most commonly affect patients of Asian descent and are most prevalent in China and Japan (Hughes & Cornblath 2005).

Whereas AMAN and AMSAN result in immune-mediated damage to the axons, the acute inflammatory demyelinating polyneuropathy (AIDP) subtype of GBS is characterized pathologically by immune-mediated selective demyelination of the nerve fibers. Similar to axonal forms of GBS, AIDP presents with a geographical distribution and largely affects patients of European descent. The AIDP subtype of GBS accounts for up to 95% of the underlying GBS subtype observed in North America and Europe (Hughes & Cornblath 2005). All subtypes of GBS typically affect the limbs and often involve cranial nerves and nerves affecting respiration. However, involvement of autonomic nerves is more commonly observed in patients with AIDP. Another, less common, variation of GBS is known as Miller-Fisher Syndrome (MFS). Patients with MFS exhibit paralysis of the extraocular muscles (ophthalmoplegia), which is thought to result from autoantibody-mediated selective injury of the cranial nerves (primarily III, VI, and VII) (Hughes & Cornblath 2005, Willison 2005). Involvement of the cranial nerves is a unique characteristic of MFS and is not observed in patients with AIDP and AMAN/AMSAN.

2. Neuropathology of Guillain-Barré Syndrome

Whereas the etiology of GBS remains unclear, aberrant (pathological) localized activation of the blood-nerve barrier (BNB) with subsequent infiltration of autoreactive
mononuclear cells is an early pathological hallmark of this disorder (Hartung et al. 1995). In axonal GBS subtypes, auto-antibodies directed against axolemma gangliosides GM₁ and GD₁a are observed decorating motor nerve fibers and are thought to play a major role in eliciting peripheral nerve injury (Hartung et al. 1995). GM₁ gangliosides are particularly concentrated within the nodes of Ranvier. In these patients, GM₁ antibody-mediated attack of affected nerve fibers results primarily in axonal damage, typically sparing the myelin sheath (Hartung et al. 1995). By comparison, auto-antibodies directed against specific gangliosides (GD₁b) are also observed in patients with AMSAN, where these antibodies decorate both sensory (dorsal) as well as motor (ventral) nerve roots (Hughes & Cornblath 2005). GD₁b gangliosides are uniquely enriched in dorsal root ganglia and sympathetic ganglia (Hartung et al. 1995). In the Miller-Fisher variant of GBS, affected patients have serum antibodies that largely react with GQ₁b gangliosides, which are enriched in cranial nerves that innervate the extraocular muscles (Hartung et al. 1995).

While an antibody-mediated mechanism is considered penultimate to the pathogenesis of AMAN, both humoral and cellular (T cell) immune responses are involved in the pathogenesis of AIDP (Kuwabara 2004). In patients with AIDP, cellular immunity against the peripheral nervous system (PNS) is principally directed against Schwann cells and the intact myelin sheath. While the exact antigenic targets on the myelin sheath remain unknown, it is established that autoreactive leukocyte infiltration into affected peripheral nerves is mediated, in part, by antibody and complement deposition on Schwann cells and on myelin membranes (Willison 2005). Alternatively, resident macrophages aberrantly targeting myelinating Schwann cells may release
inflammatory mediators, eliciting recruitment of autoreactive T cells (Hughes & Cornblath 2005). Elevated levels of proinflammatory cytokines including IFN-γ, IL-1β, and TNF-α have been reported in nerves from GBS patients (Putzu et al. 2000) and are thought to play a pivotal role in the pathogenesis of GBS (Zhu et al. 1998). Of these cytokines, plasma concentrations of TNF-α in particular have been shown to strongly correlate with disease severity, reaching 200 pg/ml in most severe cases (Exley et al. 1994, Radhakrishnan et al. 2004, Reuben et al. 2002).

3. **Experimental Autoimmune Neuritis**

Experimental autoimmune neuritis (EAN) is a well-characterized T cell-mediated animal model of AIDP. Clinical, electrophysiological, morphological, and immunological parameters of EAN have been characterized and previously documented (Hahn 1996). EAN is actively induced in susceptible animals (Lewis rats) by immunization with peripheral nerve myelin or with purified peptide fragments of specific myelin proteins emulsified in the presence of complete Freund’s adjuvant (Zhu et al. 1998). Within 14 days, immunized rats develop a reproducible, acute, monophasic course of hind limb paraparesis that peaks near day 19 with complete clinical recovery after four weeks.

The neuropathology of EAN closely resembles that of human AIDP. Similar to AIDP, the immune response precipitating EAN is driven by CD4+ T cell-mediated immunity involving the aberrant activation of the BNB and subsequent infiltration of the PNS by auto-activated mononuclear cells (Hartung et al. 1995). Observations in EAN provided the initial evidence that demyelinating forms of GBS were, in part, a result of T-cell mediated immune responses (Hartung et al. 1995). As with AIDP, pro-inflammatory
cytokines play a central role in disease development, with IFNγ, TNF-α, and IL-1β acting synergistically during the early stages of EAN (Zhu et al. 1998).

Early in vitro studies investigating the role of cytokines in EAN used primary Schwann cells in culture (Argall & Armati 1994, Argall et al. 1992, Tsai et al. 1991). In vivo studies of EAN, by comparison, focused initially on the activation of the peripheral nerve vascular endothelium as an early pathological event strongly associated with EAN (Powell et al. 1983), thus directing interest towards the endothelial cells themselves.

Currently, no studies to date have addressed the role of cytokines and the vascular endothelium in the development and progression of GBS or that of the corresponding animal model EAN. Of particular relevance to this dissertation are the observations that vascular endothelial cells harvested from the brain and peripheral nerve are uniquely distinct from vascular endothelial cells isolated from the aorta, kidney, or other peripheral organs (Sano et al. 2007). Brain-derived vascular endothelial cells maintained in culture markedly upregulate specific chemokines in direct response to inflammatory cytokines (Chui & Dorovini-Zis 2010, Harkness et al. 2003). Our understanding of how peripheral nerve vascular endothelial cells respond to inflammatory mediators and their role in the development of GBS/EAN remain to be fully elucidated (Argall et al. 1994, Sano et al. 2007, Yosef et al. 2010).

4. Clinical Management of GBS

Due to the aggressive rapid onset of GBS, affected patients often require life-saving respiratory supportive care and must be monitored closely for development of secondary complications including infections and thromboembolic events (Kuwabara
Current treatment options for patients with GBS are limited to nonspecific and often cost prohibitive immune-modulating therapies. These include extracorporeal therapy such as plasmapheresis (or plasma exchange) or courses of high-dose intravenous immunoglobulin (IVIg) (Hartung et al. 1995, Kuwabara 2004, Lindenbaum et al. 2001, Meena et al. 2011).

Plasmapheresis and IVIg therapy are transiently effective nonspecific immune-modulating strategies. As such, these clinical strategies have limited efficacy - they do not address the causality of the aberrant autoimmune responses in affected patients. Furthermore, approximately half of GBS patients fail to respond to plasmapheresis or IVIg therapy. Even of those patients that respond favorably, approximately 20% remain severely disabled (van Doorn 2009). Despite clinical advancements, there remains a pressing need for the development of more effective and specific immune-modulating therapeutic strategies for the care and management of GBS patients. To address this concern, novel therapeutic adjunctive strategies are currently under investigation for the management of GBS. These include phosphodiesterase inhibitors (Zou et al. 2000), sphingosine-1-phosphate receptor antagonists (Zhang et al. 2009), and statins (Li et al. 2011, Sarkey et al. 2007).

**Statins**

Statins are a group of naturally occurring fungal metabolites that potently inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA), the rate-limiting enzyme of the cholesterol biosynthetic pathway. Despite varying physiochemical and pharmacokinetic properties, all statins (whether natural or synthetic) inhibit HMG-CoA reductase. HMG-
CoA reductase catalyzes the reduction of HMG-CoA to mevalonate (Fig. 2). A series of subsequent enzymatic steps mediate the conversion of mevalonate to the 15-carbon isoprenoid moiety, farnesyl pyrophosphate (FPP). Here, squalene synthetase catalyzes the condensation of two FPP molecules to form squalene. Alternatively, FPP may be siphoned from the pathway for use as a critically important post-translational modifier of the Ras superfamily of small monomeric GTPases (Fig. 2).

Table 1- Pharmacologic properties of selected statins

<table>
<thead>
<tr>
<th>Name</th>
<th>Mevastatin</th>
<th>Lovastatin</th>
<th>Simvastatin</th>
<th>Pravastatin</th>
<th>Atorvastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brand name</td>
<td>Compactin</td>
<td>Mevacor</td>
<td>Zocor</td>
<td>Pravachol</td>
<td>Lipitor</td>
</tr>
<tr>
<td>Structure</td>
<td><img src="image1" alt="Mevastatin" /></td>
<td><img src="image2" alt="Lovastatin" /></td>
<td><img src="image3" alt="Simvastatin" /></td>
<td><img src="image4" alt="Pravastatin" /></td>
<td><img src="image5" alt="Atorvastatin" /></td>
</tr>
<tr>
<td>Solubility</td>
<td>L</td>
<td>L</td>
<td>L</td>
<td>H</td>
<td>L</td>
</tr>
<tr>
<td>Metabolism</td>
<td>CYP3A4</td>
<td>CYP3A4</td>
<td>CYP3A4</td>
<td>Stomach</td>
<td>CYP3A4</td>
</tr>
<tr>
<td>Origin</td>
<td><em>Penicillium citrinum</em></td>
<td><em>Aspergillus terreus</em></td>
<td><em>Penicillium citrinum</em></td>
<td><em>Nocardia autotrophica</em></td>
<td>Synthetic</td>
</tr>
</tbody>
</table>

Adapted from Kajinami K et al, 2003

Abbreviations: L, Lipophilic; H, Hydrophilic; CYP, Cytochrome P450 (isoform)
The statins used in this dissertation include the lactones lovastatin (Mevacor) and simvastatin (Zocor). These compounds are produced naturally by *Aspergillus terreus* and *Penicillium citrinum* fungi, respectively, as inactive lipophilic prodrugs that are metabolized into the active hydroxyacid form by cytochrome P450 (CYP) in the liver. By comparison, the popular statin atorvastatin (Lipitor) is a synthetically produced active hydroxyacid (Garcia et al. 2003). Pravastatin (Prevachol) remains as the only naturally occurring hydrophilic statin. As such, pravastatin has unique pharmacokinetic properties including very rapid elimination. The pharmacological properties of these statins are summarized in Table 1.

Widely accepted as the first-line therapy for the management of hypercholesterolemia, statins are now recognized as exhibiting a variety of beneficial pleiotropic effects beyond what can be reasonably attributed to the reduction in serum cholesterol. Initial observations in hypercholesterolemic patients include improvement of endothelial dysfunction, increased nitric oxide bioavailability, antioxidant effects, stabilization of atherosclerotic plaques, and anti-thrombotic and anti-inflammatory properties, all occurring largely independently of reduction in lipid levels (Goldstein 2007, Halcox & Deanfield 2004). *In vitro* studies demonstrate that statins markedly decrease levels of inflammatory mediators including C-reactive protein, TNF-α, and other pro-inflammatory cytokines (Undas et al. 2002). Because of these pleiotropic effects, the therapeutic potential of statins is now being intensively investigated for the management of a number of inflammatory diseases, including multiple sclerosis, Alzheimer’s disease, stroke, and more recently, acute inflammatory demyelinating polyneuropathy (Bifulco et al. 2008, Kandiah & Feldman 2009, Sarkey et al. 2007). The
mechanisms by which statins exert these immune modulating and anti-inflammatory effects are believed to involve inhibition of isoprenoid availability (Greenwood et al. 2006).

In addition to its role as an intermediate in the canonical cholesterol biosynthetic pathway, geranylgeranyl-PP synthetase catalyzes the condensation of FPP with isopentenyl-PP to form a key 20-carbon (diterpene) isoprenoid protein modifier, geranylgeranyl pyrophosphate (GGPP). This isoprenoid is responsible for the isoprenylation of small monomeric GTPases of the Rho subfamily including Rho, Rac-1, Cdc42. Alternatively, FPP itself can post-translationally modify other members of the Ras superfamily of GTPases (Fig. 2). Currently, isoprenylation is thought to facilitate targeting and anchoring of GTPases to the cell membrane, which is required for their functional activation (Zhang & Casey 1996). By limiting isoprenoid synthesis and availability, statins indirectly alter the activation of Rho GTPases (Mackay & Hall 1998), thereby disrupting a variety of essential cell signaling pathways.
Figure 2. **Cholesterol and isoprenoid biosynthetic pathway.** The isoprenoid metabolic intermediates farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are essential post-translational modifiers of many small monomeric GTPases, including those belonging to the Rho subfamily of GTPase.
Transendothelial Migration

1. Peripheral Nerve Anatomy

Peripheral nerves are composed of bundles of fascicles that are ensheathed by a connective tissue matrix, the epineurium. Individual fascicles are encased by perineurial tissue. These perineurium-bound fasicles contain axons, Schwann cells, fibroblasts, and small blood vessels, all contained in another connective tissue matrix. This inner connective tissue matrix within each fascicle that supports the Schwann cells and axons is

Figure 3. Peripheral nerve trunks are comprised of axons traversing in fascicles within the body of a nerve. The perineurium is vascularized via microvasculature. ("Nerve Anatomy." A.D.A.M Anatomy. 2009)
referred to as endoneurium. Blood is supplied to these peripheral nerve bundles by a longitudinal anastomotic network of blood vessels in the epineurium. The vasculature passes through the perineurium to a plexus of microvessels in the endoneurium. Within the endoneurium, the endothelial cells that line the vessel walls are sealed together by tight junctions, forming a blood-nerve barrier that protects the endoneurial compartment. Other cells contained within the fascicles include fibroblasts, which produce the fibrous collagen lattice, and resident, resting tissue macrophages.

2. Cell Adhesion Molecules

Under healthy resting conditions, quiescent leukocytes traffic into and out of the endoneurial compartment by a highly regulated process referred to as transendothelial migration that provides peripheral immune surveillance (Hordijk 2006). However, under pathological conditions, such as encountered in GBS/EAN, aberrant or excessive transendothelial migration is observed (Greenwood & Mason 2007).

Transendothelial migration occurs by sequential interactions between adhesion molecules expressed on the surface of activated leukocytes and on activated vascular endothelial cells (Fig. 3). Within minutes to hours of cytokine-mediated activation, the normally quiescent peripheral nerve vascular endothelium begins to upregulate the expression of the cell adhesion molecules (CAMS), including both E-selectins and P-selectins (Springer 1990). Leukocytes, by comparison, constitutively express E- and P-selectin binding proteins, such as P-selectin glycoprotein ligand-1 (PSGL-1), which begin to interact weakly with the local activated vascular endothelium, resulting in leukocyte tethering and rolling.
Chemokines, released in response to inflammatory cytokines, elicit the expression of the heterodimeric α,β integrins leukocyte function antigen-1 (LFA-1 or CD11a/CD18) and very late antigen-4 (VLA-4 or CD49d/CD29) on the surface of rolling leukocytes. These leukocyte-expressed integrins recognize binding partners present on activated vascular endothelial cells - the immunoglobulin-like adhesion molecules intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule (VCAM-1, CD106), respectively. ICAM-1 and VCAM are later responders that facilitate firm adhesion, with time frames on the order of hours, and expression sustained through 24 hours. Paracellular extravasation of the firmly adhered activated leukocyte proceeds via homophilic binding of junctional proteins including junctional adhesion molecule (JAM)

**Figure 4. Transendothelial migration of autoreactive leukocytes.** E- and P-selectins expressed on the surface of cytokine-activated endothelial cells initiate tethering and rolling of autoreactive leukocytes. Subsequent expression of ICAM-1 and VCAM mediate firm adhesion of autoreactive leukocyte resulting in paracellular extravasation through tight junctions. Alternatively, transcellular diapedesis may also occur.

CAMs are involved in the pathogenesis of both GBS and EAN. ICAM-1 has been detected on the endothelial cells and macrophages in sural nerve samples from patients with GBS, which corresponds to involvement of ICAM-1 in the early phase of disease development (Putzu et al. 2000). Furthermore, an antibody to ICAM-1 has been demonstrated to suppress adoptive transfer-induced EAN in Lewis rats (Archelos et al. 1993). VCAM was detected in sciatic nerves from Lewis rats with EAN, while none was expressed in nerves harvested from control rats. Monoclonal antibody blockade of VCAM, as well as its ligand VLA-4, attenuated the course of EAN, when administered prophylactically (Enders et al. 1998).

Transendothelial migration of activated leukocytes into the target tissue initiates a escalating cascade of events exacerbated by an increased localized production of pro-inflammatory cytokines, recruitment of monocytes, and their localized differentiation into phagocytic macrophages, resulting in an ultimate compromise of the blood-nerve barrier.

3. Chemotactic Cytokines

The exact mechanism regulating the homing and extravasation of autoreactive leukocytes into localized inflamed areas of affected peripheral nerves in GBS/EAN remains unclear, but it most likely involves increased localized vascular endothelial cell expression of the chemotactic cytokines (chemokines) interleukin-8 (IL-8), macrophage inflammatory protein-1α (MIP-1α), and, in particular, monocyte chemoattractant protein-1 (MCP-1 or CCL2) (Zou et al. 1999). Chemokines are a class of small (8-15 kDa)
peptides that regulate cell trafficking (Miller & Krangel 1992). Classified into four distinct structural families based on the presence and location of highly conserved N-terminal cysteine residues (C, CC, CXC, and CX₃C (Table 2)), over 50 distinct chemokines and 20 unique chemokine receptors have been reported (Deshmane et al. 2009, Rollins 1997). The groups of chemokines differ in the cell types they recruit: CC chemokines recruit predominantly monocytes and CXC chemokines neutrophils. As implied, inflammatory chemokines such as MCP-1 function to recruit activated leukocytes to sites of inflammatory nerve injury (Deshmane et al. 2009, Rollins 1997).

The localized synthesis and release of MCP-1 during peripheral nerve inflammation has largely been associated with infiltrating monocytes (macrophages) (Deshmane et al. 2009). However, they are not the only cellular source of MCP-1, since smooth muscle cells (Cushing et al. 1990), Schwann cells (Orlikowski et al. 2003), and vascular endothelial cells (Cushing et al. 1990) have been reported to produce and release MCP-1 in response to inflammatory cytokines. Within human vascular endothelial cells (HUVECs), MCP-1 has been localized to small “type 2” storage granules that are rapidly mobilized in a regulated manner in response to inflammatory stimuli (Oynebraten et al. 2004). These storage granules are uniquely distinct from the more common Weibel-Palade bodies, which are known to store von Willebrand Factor (Knipe et al. 2010, Oynebraten et al. 2005). The exact mechanism by which chemokines, such as MCP-1, are concentrated in storage granules and subsequently released into the extracellular compartment by regulated exocytosis remains unclear.
Table 2. Selected chemokines and their receptors. Adapted from Deshmane et al, 2009; Abbreviations: I, inflammatory; H, homeostatic; D, dual.

<table>
<thead>
<tr>
<th>Family</th>
<th>Systematic name</th>
<th>Alternative name</th>
<th>Receptor(s)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>C Chemokine (γ chemokine)</td>
<td>XCL1</td>
<td>Lymphotactin α, ATAC, SCM-1α</td>
<td>XCR1</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>XCL2</td>
<td>SCM-1 β</td>
<td>XCR1</td>
<td>D</td>
</tr>
<tr>
<td>CC Chemokine (β chemokine)</td>
<td>CCL2</td>
<td>MCP-1, MCAF, TDCF</td>
<td>CCR2</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL3</td>
<td>MIP-1 α, LD78 α</td>
<td>CCR1, CCR5</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL4</td>
<td>MIP-1b</td>
<td>CCR5</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL5</td>
<td>RANTES</td>
<td>CCR1, CCR3, CCR5</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL7</td>
<td>MCP-3</td>
<td>CCR1, CCR2, CCR3</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL8</td>
<td>MCP-2</td>
<td>CCR1, CCR2, CCR3, CCR5</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL11</td>
<td>Eotaxin</td>
<td>CCR3</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL13</td>
<td>MCP-4</td>
<td>CCR1, CCR2, CCR3</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CCL17</td>
<td>TARC</td>
<td>CCR4</td>
<td>D</td>
</tr>
<tr>
<td>CXC Chemokine (α chemokine)</td>
<td>CXCL8</td>
<td>IL-8</td>
<td>CXCR1, CXCR2</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CXCL10</td>
<td>IP-10</td>
<td>CXCR3-A, CXCR3-B</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>CXCL12</td>
<td>SDF-1a/b</td>
<td>CXCR4, CXCR7</td>
<td>H</td>
</tr>
<tr>
<td>CX₃C Chemokine (δ chemokine)</td>
<td>CX₃CL1</td>
<td>Fractalkine</td>
<td>CX₃CR1</td>
<td>I</td>
</tr>
</tbody>
</table>
MCP-1 is also implicated in the pathogenesis of many inflammatory and autoimmune diseases, including GBS. In GBS patients, MCP-1 immunoreactivity is found localized to vascular endothelial cells within peripheral nerves, and the degree of its expression is more pronounced in the most severe cases (Orlikowski et al. 2003). Experimentally, MCP-1 expression precedes the onset of EAN and persists through the peak of disease (Fujioka et al. 1999, Kieseier et al. 2000). Of particular relevance to this dissertation is the report that MCP-1 neutralizing antibodies ameliorate the course of EAN (Zou et al. 1999).

**The Inflammatory Cascade**

1. **Tumor Necrosis Factor-α**

   TNF-α plays a major role in the pathogenesis of GBS and EAN. In GBS patients, plasma concentrations of TNF-α strongly correlate with disease severity (Exley et al. 1994). In EAN, the number of TNF-α expressing macrophages localized to peripheral nerve roots closely mimics the course of the disease (Stoll et al. 1993). Systemic administration of TNF-α neutralizing antibodies (Stoll et al. 1993) or soluble TNF-α receptor (Bao et al. 2003) ameliorates the course of EAN. Interestingly, TNF-α receptor knockout mice develop a less severe form of EAN (Mao et al. 2010).

   Tumor necrosis factor-alpha (TNF-α) is a 51-kD homotrimeric pro-inflammatory cytokine that is produced as part of the inflammatory cascade by activated leukocytes, macrophages, fibroblasts, and vascular endothelial cells (Wajant et al. 2003). This trimeric cytokine binds to and activates two distinct receptors referred to as TNF-α
receptor 1 (TNFR1) and TNF-α receptor 2 (TNFR2). When activated by soluble TNF-α, TNFR1, which is constitutively expressed on the surface of vascular endothelial cells, initiates a cascade of intracellular signaling events that ultimately results in up-regulation of the inflammatory response (Wajant et al. 2003). In contrast, TNFR2 is selectively expressed by cells of the immune system and is highly regulated and is only activated by membrane-associated (not soluble) TNF-α trimers (Wajant et al. 2003). Germane to this dissertation is the action of TNF-α through TNFR1, as related to inflammation.

Activation of TNFR1 by TNF-α may stimulate either gene expression or induce apoptosis. These distinct effects are both initiated by the dissociation of an intracellular-associated silencer of death domain (SODD) protein from the receptor complex and subsequent binding of the TNF-α receptor-associated death domain adaptor protein TRADD. Following TRADD binding, activated TNFR1 may recruit Fas-associated death domain adaptor protein FADD, initiating apoptosis (Wajant et al. 2003). Alternatively, activated TNFR1 may recruit TRAF2 and the receptor interacting protein RIP kinase. This leads to SAP kinase- or IκB kinase-mediated gene transcription of inflammatory mediators via NFκB, a penultimate nuclear transcription factor that drives the inflammatory response (Baldwin 1996, Wajant et al. 2003). It remains unclear as to what determines whether TNF-α mediated activation of TNFR1 will activate the apoptotic or inflammatory signaling pathways.

2. Nuclear Factor Kappa B

Nuclear factor kappa B (NFκB), first described as a B cell nuclear transcription factor (Sen & Baltimore 1986), is an inducible activator of various chemokine and CAM
genes involved in the initial stages of inflammatory immune response (Baldwin 1996, Ueda et al. 1997). Active, DNA-binding NFκB is a dimer, classically containing both p65 and p50 subunits. NFκB activation is regulated by the inhibitory cytoplasmic protein IκB. Upon release from IκB, NFκB rapidly translocates to the nucleus; NFκB regulated genes can be transcriptionally activated within minutes of exposure to an inducer (primarily immunological stimuli). As reviewed by Baldwin in 1996, regulation of NFκB activation is well established, but is growing in complexity.

Aberrant activation of NFκB is considered to play a key role in initiating/mediating numerous vascular-related autoimmune disorders including GBS/AIDP (Hume & Fairlie 2005, Kuldo et al. 2005, Kulkarni et al. 2006, Mazzeo et al. 2004, Monaco & Paleolog 2004, Tas et al. 2005). Sural nerves from patients with GBS express activated NFκB (Andorfer et al. 2001). Similarly, activated NFκB is upregulated in sciatic nerves harvested from Lewis rats with EAN, with highest levels occurring at the peak of disease (Laura et al. 2006).

3. TNF-α, Rho GTPase, and NFκB Activation

In addition to activating distinct intracellular kinase cascades, TNF-α signaling has also been reported to activate small monomeric GTPases of the Rho subfamily (McKenzie & Ridley 2007, Papaharalambus et al. 2005, Ridley 2006). Moreover, NIH-3T3 fibroblasts expressing dominant-negative RhoA GTPases exhibit a significant reduction in TNF-α mediated activation of NFκB (Perona et al. 1997), suggesting a role for Rho GTPases in NFκB activation. Inhibition of Rho GTPases in HeLa cells impaired NFκB DNA-binding activity and decreased expression of an NFκB-dependent reporter
gene (Gnad et al. 2001). Rho GTPase inhibition impaired NFκB activation that was evoked by UV light or doxorubicin in HeLa cells; however, it did not alter TNF-α mediated activation of NFκB. By comparison, HUVECs require activation of Rho GTPases for thrombin-mediated NFκB activation, but not TNF-α mediated NFκB activation (Anwar et al. 2004). These findings indicate that the role of Rho GTPases in NFκB activation may be cell-type specific.

Using dominant negative RhoA or inhibitors of Rho GTPases, Xu et al. (2006) report in synoviocytes that Rho GTPases do regulate TNF-α mediated NFκB activation (Xu et al. 2006). Similarly, Hippenstiel et al. (2002) demonstrated in HUVECs that Rho GTPases are indeed necessary for translocation of NFκB (p65/RelA) to the nucleus, as well as expression of an NFκB-dependent reporter gene. This is in direct contrast with findings from the Rahman group in HUVECs.

To date, no studies have evaluated a role for TNF-α mediated Rho GTPase activation of NFκB in the development and progression of EAN. Given the apparent association of Rho GTPases with TNF-α mediated signaling pathways in some cell types, this dissertation was designed to determine the role of Rho GTPases in promoting TNF-α mediated inflammatory activation of peripheral nerve vascular endothelial cells.

Small Monomeric GTPases

1. GTPase Classification

Small monomeric guanosine nucleoside triphosphatas (GTPases) are classified as a superfamily of Ras-related GTPases that are further organized into five distinct families: Rho, Rab, Ras, Arf, and Ran (Wennerberg et al. 2005), outlined in Table 3.
Each family is further divided into subfamily members which collectively function to regulate a diverse array of intracellular signaling pathways affecting vesicle transport/trafficking, endocytosis, cell cycle progression, cell contractility, and formation of stress fiber formation or focal adhesions (Wennerberg et al. 2005). Functioning as “molecular switches”, these GTPases control different processes within the cell by cycling between active (GTP-bound) and inactive (GDP-bound) states (Wennerberg et al. 2005, Bishop & Hall 2000).

Small monomeric GTPases bind guanosine nucleotides with high affinity while possessing low intrinsic GDP/GTP exchange activities and intrinsic GTPase activities. Activation of Ras GTPases is facilitated by specific guanosine nucleotide exchange factors (GEFs), which function to enhance intrinsic GDP/GTP exchange activities. Inactivation of the GTP-bound active GTPase is enhanced by the action of specific GTPase-activating proteins (GAPs), which increase the intrinsic rate of GTP hydrolysis. Another key factor involved in the regulation of specific GTPases is the cytosolic protein guanosine nucleotide dissociation inhibitor (GDI), which sequesters GDP-bound G-proteins in the cytosol, thereby minimizing GEF catalyzed activation of these proteins (Bishop & Hall 2000, Mackay & Hall 1998).

An essential functional property shared among the Ras superfamily of GTPases is their ability to be post-translationally modified with sesquiterpene or diterpene cholesterol biosynthetic metabolic intermediates. Specifically, conserved C-terminal amino acid recognition sequences (the CAAX motif, where C=Cys, A=aliphatic, X=any amino acid) can be covalently modified by selective isoprenylation (Rab, Ras, Rho families) or myristoylation (Arf family). Prenylation or myristoylation is thought to
enhance functional activation of GTPases by targeting these proteins to the plasma membrane (Wennerberg et al. 2005). Ran family of GTPases are an exception in that they are not post-translationally prenylated or modified with fatty acids and thus do not readily associate with membranes.

Most relevant to this dissertation is the Ras homologous (Rho) family of monomeric GTPases. The Rho family of monomeric GTPases contains up to 20 members, of which RhoA, Rac1, and Cdc42 are the best understood. Given their role in regulating vascular endothelial permeability, cell adhesion molecule expression, and their effects on transcription factor activation (Mackay & Hall 1998), the Rho subfamily (RhoA, RhoB, and RhoC) is of particular relevance to this dissertation.

2. GTPases in Vesicle Trafficking and Exocytosis

The Rab family of GTPases are considered part of the conserved core machinery for regulated storage vesicle budding, trafficking, fusion, and fission (Burgoyne & Morgan 2003). Collectively, the Rab family is a large group of G-proteins (61 in humans) that function to facilitate different aspects of storage vesicle dynamics. Although not typically considered as participants in vesicle dynamics, members of the Rho family of GTPases have more recently emerged as additional regulators of these processes (Melendez et al. 2011, Ridley 2006). In this regard, Rho GTPases, in particular Cdc42, have been reported to be involved in thrombin-mediated secretion of von Willebrand Factor from vascular endothelial cells (Fish et al. 2007). Interestingly, pretreatment of HUVECs with fluvastatin or geranylgeranyl transferase inhibitor-I (GGTI-298) significantly attenuated von Willebrand Factor secretion, indicating that a geranylgeranylated GTPase is indeed
involved in regulated exocytosis. Another candidate GTPase with an emerging role in secretion is RalA. RalA is a member of the Ras family and, unlike other members of the family, is geranylgeranylated. Multiple groups have demonstrated a role of RalA in regulated exocytosis (de Leeuw et al. 2001, Rondaij et al. 2004, van Dam & Robinson 2006). To date, no studies have addressed the role of GTPases, including Rho family members, in cytokine-mediated vesicle dynamics in peripheral nerve vascular endothelial cells.
Table 3. Ras superfamily of small monomeric GTPases.

<table>
<thead>
<tr>
<th>Family</th>
<th># of Human isoforms</th>
<th>Notable members</th>
<th>Function</th>
<th>C-terminal consensus sequence</th>
<th>Post-translational modification</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rho</td>
<td>22</td>
<td>RhoA, RhoB, Rac1, Cdc42</td>
<td>Actin organization, Cell cycle progression, Gene expression</td>
<td>CAAX</td>
<td>GG, F</td>
<td>GGTase I, FTase</td>
</tr>
<tr>
<td>Rab</td>
<td>63</td>
<td>Ras, Ral</td>
<td>Vesicle transport, Vesicle trafficking</td>
<td>CC, CXC, CCX, CCXX, CCXXX</td>
<td>GG</td>
<td>GGTase II</td>
</tr>
<tr>
<td>Ras</td>
<td>39</td>
<td>Ras, Ral</td>
<td>Cell proliferation, Cell survival, Oncogenesis</td>
<td>CAAX</td>
<td>GG, F</td>
<td>GGTase I, FTase</td>
</tr>
<tr>
<td>Ran</td>
<td>1</td>
<td></td>
<td>Nuclear import, Nuclear export</td>
<td></td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>Arf</td>
<td>30</td>
<td></td>
<td>Vesicle trafficking</td>
<td></td>
<td>Myristoylated</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from (Wennerberg et al. 2005); Abbreviations: C=Cys, A=aliphatic, X=any amino acid; GG: geranylgeranylated, F: farnesylated; GGTase: geranylgeranyl transferase, FTase: farnesyl transferase.
CHAPTER 3

TUMOR NECROSIS FACTOR-α ENHANCES EXPRESSION OF MCP-1 AND ICAM-1 IN PERIPHERAL NERVE VASCULAR ENDOTHELIAL CELLS

Abstract

Pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), play a pivotal role in the pathogenesis of Guillain-Barré Syndrome (GBS), an aggressively debilitating autoimmune disorder affecting peripheral nerves and a leading cause of autoimmune neuromuscular paralysis. Recruitment and trafficking of autoreactive leukocytes across the blood-nerve barrier and into peripheral nerves is an early pathological hallmark of GBS. Whereas the mechanism by which cytokines contribute to peripheral nerve injury in GBS remains unclear, localized activation of the peripheral nerve vascular endothelium has been suggested as an initiating pathological insult. In this study, peripheral nerve vascular endothelial cells (PNVECs) harvested from sciatic nerves of naïve Lewis rats were immortalized by SV40 large T antigen transduction using a producer cell line (SVU19.5) expressing a replication-deficient SV40 retrovirus with a neomycin resistant gene. Resistant cell lines were isolated by single colony selection. SV40 large T antigen transduction of rat primary PNVECs yielded immortalized clones...
that retain many phenotypic and morphological characteristics of primary vascular endothelial cells, including monolayer formation, a cobblestone-like morphology, and localized expression of von Willebrand Factor/Factor VIII (vWF) in Weibel-Palade bodies. A clone (4.3) retaining these key morphological and immunocytochemical characteristics exhibited by primary PNVEC cultures was expanded, and TNF-α mediated changes in intercellular adhesion molecule-1 and MCP-1 chemokine expression were determined. TNF-α (0.1-100 ng/ml, 0-24h) elicited a robust dose- and time-dependent increase in both ICAM-1 and MCP-1 mRNA and protein expression while enhancing secretion of functional MCP-1 into the culture media. Marked increases in ICAM-1 and MCP-1 expression and release in response to TNF-α may facilitate recruitment and trafficking of autoreactive leukocytes across the blood-nerve barrier in autoimmune disorders, including GBS.

**Introduction**

Proinflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), play a pivotal role in the pathogenesis of Guillain-Barré syndrome (GBS) and its North American and European variant, acute inflammatory demyelinating polyneuropathy (AIDP) (Zhu *et al.* 1998). GBS/AIDP is an aggressively debilitating autoimmune disorder that affects peripheral nerves and remains a leading cause of autoimmune neuromuscular paralysis. Although the etiology of GBS remains unknown, the

Cytokine-mediated recruitment and trafficking of autoreactive leukocytes across the blood-nerve barrier (BNB) and into peripheral nerves is a well established early pathological hallmark of this disorder (Hartung et al. 1995). A strong correlation between elevated plasma concentrations of TNF-α and clinical severity of GBS has been described (Exley et al. 1994). More recently, Putzu et al. documented an increased presence of proinflammatory cytokines, including TNF-α, within affected peripheral nerves of GBS patients (Putzu et al. 2000). Stoll et al. (1993) showed an enhanced presence of TNF-α expressing macrophage infiltrates within nerve roots harvested from Lewis rats with experimental autoimmune neuritis (EAN), a well-characterized animal model of GBS that shares many pathological characteristics with AIDP (Hahn 1996). Neutralizing TNF-α, or its homotrimeric cell surface receptor TNFR1, ameliorates the course of EAN (Bao et al. 2003, Stoll et al. 1993). Knocking out the TNF-α receptor results in a less severe form of EAN induced in mice (Mao et al. 2010). Moreover, marked expression of the chemokine monocyte chemoattractant protein-1 (MCP-1) is reported to precede the onset of EAN-induced clinical deficits (Fujioka et al. 1999, Kieseier et al. 2000). Antibody-mediated neutralization of MCP-1, in vivo, ameliorates the course of EAN (Zou et al. 1999). In GBS patients, MCP-1 is expressed within vascular endothelial cells of the epineurial and endoneurial blood vessels, and its expression correlates well with disease severity (Orlikowski et al. 2003).
The mechanism by which TNF-α contributes to peripheral nerve injury in GBS remains unclear. Localized activation of the peripheral nerve vascular endothelium in response to proinflammatory cytokines may represent an initiating pathological insult. Emerging evidence, however, supports both phenotypic and functional differences between vascular endothelium from different tissues (Aird 2007b, Aird 2007a, Bell & Weddell 1984), emphasizing the importance of evaluating the appropriate vascular bed tissue. Given that recruitment and trafficking of leukocytes across activated vascular endothelial barriers is most likely governed by changes in expression of specific CAMs and chemokines unique to the localized vascular bed (Chui & Dorovini-Zis 2010, Harkness et al. 2003, Springer 1994), this study focuses on cytokine-mediated responses of vascular endothelial cells harvested from peripheral nerve.

Earlier studies characterizing the development and permeability of the BNB using vascular tissue harvested from rats support the presence of a relatively specialized restrictive barrier (Smith et al. 2001). The blood supply of peripheral nerves consists of a longitudinal anastomotic network of vessels present in the epineurium that connect through the perineurium to a plexus of microvessels traversing the endoneurium, a connective tissue matrix which supports the axons and Schwann cells within each nerve fascicle (Flores et al. 2000). Macro-permeability studies demonstrate qualitatively similar properties of the BNB to that of the blood-brain barrier, showing restricted passage of IgG antibodies and of albumin (the major protein of endoneurial fluid) and selective transport of insulin, nerve growth factor, and transferrin (Olsson 1966, Poduslo et al. 1994). Although advancements have been made toward the purification and culture of vascular endothelial cells derived from peripheral nerve (Argall et al. 1994, Sano et al. 1994),
2007, Yosef et al. 2010), no studies to date have addressed how peripheral nerve vascular endothelial cells respond to a localized inflammatory challenge such as that experienced during GBS/EAN.

To address this concern, peripheral nerve vascular endothelial cells (PNVECs) were harvested from sciatic nerves of naïve Lewis rats and immortalized by SV40 large T antigen transduction using a producer cell line (SVU19.5) expressing a replication-deficient SV40 retrovirus with a neomycin resistant gene. The successful and stable immortalization of rat PNVECs and retention of key phenotypic, morphologic, and biochemical characteristics are demonstrated. Activation of primary or transformed PNVEC cultures with TNF-α elicited marked dose- and time-dependent changes in MCP-1 and ICAM-1 expression and MCP-1 release. This secreted MCP-1 promotes the chemotaxis and transendothelial migration of CCR2-expressing monocytes. Immortalized rat PNVEC cultures provide a novel and physiologically relevant in vitro tool for the functional assessment of pathological alterations in peripheral nerve endoneurial homeostasis during an inflammatory challenge.

Materials and Methods

Peripheral Nerve Vascular Endothelial Cell Culture

Primary cultures of peripheral nerve vascular endothelial cells (PNVECs) were prepared from sciatic nerves of naïve adult male Lewis rats as previously described (Sarkey et al. 2007). Primary PNVEC cultures were cleared of epitheliod myofibroblasts,
the major cell contaminant, by Thy-1.1 antibody-mediated complement-driven cell lysis (Argall et al. 1994). This technique exploits the fact that the Thy-1.1 surface glycoprotein is selectively expressed on fibroblasts but notably absent from vascular endothelial cells (Kisselbach et al. 2009). Primary PNVEC preparations were cultured at 37°C (95% air, 5% CO₂) on T25 tissue culture flasks for one week prior to fibroblast elimination. Established mixed population cultures were harvested by gentle scraping and suspended in undiluted tissue culture media containing Thy-1.1 IgM monoclonal antibodies collected from cultured TIB-103 hybridoma cells (ATCC) and 1% fresh rabbit complement for 3 h at 37°C (95% air, 5% CO₂). Tissue culture media was removed, adherent cells were washed once with Ham’s F10 basal media, and surviving PNVECs were allowed to proliferate in complete PNVEC media containing Ham’s F10 basal media (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 50 µg/ml endothelial cell growth supplement (ECGS; BD Bioscience, San Jose, CA), 0.4 µg/ml heparin (Sigma-Aldrich, St. Louis, MO), 5.6 µg/ml amphotericin B, 100 units/ml penicillin, and 100 µg/ml streptomycin). Purified PNVEC cultures were treated with Thy 1.1 antibody/ complement mixture twice prior to use. The efficacy of the fibroblast removal was routinely monitored by immunocytochemistry. Purified PNVEC cultures were passaged onto collagen-coated (rat tail collagen type I, 5 µg/cm²) coverslips and allowed to grow to 50% confluency. PNVEC monolayers were fixed for 5 min (phosphate buffered pH 7.4 4% paraformaldehyde, PFA), blocked with 1% normal goat serum and permeabilized (1% normal goat serum and 0.1% Triton X-100 in phosphate buffered saline (PBS)) for 30 min at 37°C. Treated PNVECs were incubated overnight at 4°C in the presence of a 1:50 dilution of mouse anti-rat Thy-1.1 monoclonal antibody.
Washed immunostained cells were visualized with a 1:1000 dilution of AlexaFluor 488 conjugated goat anti-mouse IgG secondary antibody. Coverslips were inverted and mounted onto slides with Fluoroshield containing DAPI. When analyzed in this manner, the PNVEC population was > 95% (95±7.4%, N=3) pure. The presence of contaminating Thy1.1 positive fibroblasts in these cultures was routinely sparse, making up less than 5% of the total cell population and in good agreement with published methodology (Argall et al. 1994).

**SV40 Large T antigen transduction**

Given the difficulties in preparing needed quantities of purified primary PNVEC cultures for experimentation, a stably transformed cell line was produced from these highly (>95%) purified primary PNVEC culture preparations using a replication-deficient SV40 retrovirus encoding a temperature sensitive, non-SV40-origin binding mutant of the large T antigen and a selectable neomycin resistance gene (Greenwood et al. 1996, Jat et al. 1986, Jat & Sharp 1986). Currently, no commercial or private source of rat PNVECs exists. This group is the first to successfully immortalize rat PNVECs. Semi-confluent cultures of highly purified primary PNVECs were incubated for 36h at 37°C in the presence of 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) with undiluted filtered particle-free viral supernatant collected from SVU19.5 producer cells. Media was replaced and treated cells were allowed to reach confluency. Selection was achieved by passage into complete PNVEC culture media containing 200 µg/ml G418 (Invitrogen, Carlsbad, CA). Single antibiotic resistant clones were isolated by dilution into 96-well plates at a theoretical density of 0.33 cells/well. Several clones exhibited morphologic
characteristics of primary endothelial cells including a cobblestone-like appearance in monolayer. These clones were further evaluated.

Successful transduction of the large T antigen was confirmed by Western immunoblot. Primary and transformed PNVECs were cultured to confluency onto collagen-coated T75 tissue culture flasks. Cells were harvested by gentle scraping, washed twice in ice-cold PBS supplemented with a commercial cocktail of protease inhibitors (Roche Applied Science, Indianapolis, IN) and resuspended in 100 µl dH2O containing protease inhibitors. Whole-cell lysates were prepared by probe-sonication, and protein concentrations were determined using a BCA protein assay kit (Pierce). Proteins in cell lysates were resolved by SDS-PAGE gel electrophoresis using 4-20% polyacrylamide gels (BioRad) and transferred onto nitrocellulose membranes (Sigma-Aldrich) as previously described (Stubbs & Von Zee 2012). Membranes were blocked for 30 minutes at 37 ºC in PBS containing 0.05% Tween-20 and 5% non-fat dried milk (Carnation®). Membranes were incubated overnight at 4 ºC in a 1:10 dilution of mouse anti-rat large T antigen primary monoclonal antibody (kindly provided by Dr. P. Jat) followed by an incubation with 1:2500 dilution of horseradish peroxidase (HRP)-conjugated anti-mouse-IgG secondary antibody. Lysates prepared from an established, commercially available SV40-transduced cell-line (trabecular meshwork GTM3 cells) were run as a positive control.

Factor VIII/von Willebrand Factor (vWF) and platelet endothelial cell adhesion molecule (PECAM, CD31) are both proteins that are constitutively expressed by primary vascular endothelial cells. They are routinely used as established markers of vascular endothelial cells. Primary or transformed PNVECs were passaged onto collagen coated
coverslips. Semi-confluent PNVEC monolayers were fixed for 5 min (phosphate buffered pH 7.4, 4% PFA), blocked with 1% normal goat serum and permeabilized (1% normal goat serum and 0.1% Triton X-100 in PBS) for 30 min at 37°C and incubated overnight (4°C) in the presence of a 1:50 dilution of mouse anti-human polyclonal antibody against vWF or a 1:50 dilution of rabbit anti-rat monoclonal antibody against CD31. Washed immunostained cells were incubated with a 1:1000 dilution of an AlexaFluor488 conjugated goat anti-mouse IgG secondary antibody or a FITC-conjugated goat anti-rabbit IgG secondary antibody. Coverslips were inverted and mounted onto slides with Fluoroshield containing DAPI. vWF expression was also confirmed by RT-PCR.

**Real Time RT-PCR**

Primary and transformed PNVECs were cultured on collagen-coated T25 tissue culture flasks to near confluency and treated with TNF-α (0-100 ng/ml) for 0-6h in fresh complete culture media. A stock solution (0.1 mg/ml) of lyophilized rat recombinant TNF-α (Sigma) was prepared using sterile dH₂O and a working solution (5.0 µg/ml) was brought up in fresh tissue culture media. Total RNA was extracted from PNVECs using TRIzol reagent (Invitrogen, Carlsbad, CA) and 5 µg was reverse-transcribed using Super Script III First Strand Synthesis system (Invitrogen, Carlsbad, CA) as previously described (Von Zee et al. 2009). MCP-1 and ICAM-1 specific cDNA sequences were amplified by real-time PCR using a Mini-Opticon PCR detection system and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The following primer pairs were used: **MCP-1**, forward, 5’-ATGCAGGTCTCTGTCACG; reverse, 5’-CTAGTTCTCTGTCACTACT;
ICAM-1, forward, 5'-CTGCAGAGCACAAACAGCAGAG; reverse, 5'-AAGGCGCGAGCAAAAGAAGC. For each sample, the housekeeping gene GAPDH (forward, 5'-TCCCTCAAGATTGTCAGCAA; reverse, 5'-AGATCCACAACGGATACATT) was found not to change under the experimental conditions and therefore used as an appropriate reference control. Optimized amplification steps used were: 94°C x 5 minutes; 94°C x 15s, specifically tailored annealing temperature x 30s, 72°C x 1 minute for 40 cycles. Specific annealing temperature for MCP-1 and GAPDH reactions was 55°C; annealing temperature for ICAM-1 reactions was 60°C. Reaction efficiencies for each product were typically >90%.

For each sample, the specificity of the real-time reaction product was determined using the melt-curve function analysis. In some cases, amplified products were captured during the linear phase, resolved on a 1.2% agarose E-gels, and visualized with a FluorChem SP fluorescence image analyzer. GAPDH normalized relative fold-changes in message expression was determined using the $2^{\Delta\Delta CT}$ method of Livak (Livak & Schmittgen 2001).

**Cell-based ELISA**

Relative changes in intracellular MCP-1 and ICAM-1 protein expression were quantified using cell-based ELISA methodology. Primary and transformed PNVECs were cultured onto collagen-coated 96-well plates and confluent cultures were treated with TNF-α (0-100 ng/ml) for 0-24h in fresh complete culture media. Treated cells were fixed with phosphate buffered (pH 7.4) 4% PFA for 10 min at 23°C, washed, and permeabilized with 0.1% Triton X-100. Permeabilized cells were blocked for 1h at 23°C
with 1% BSA and incubated with a 1:2000 dilution of rabbit anti-rat MCP-1 polyclonal antibody or a 1:500 dilution of mouse anti-rat ICAM-1 monoclonal antibody (Serotec, Raleigh, NC) overnight at 4°C. Immunostained cells were washed and incubated for 1h at 23°C in the presence of a 1:5000 dilution of HRP-conjugated goat anti-rabbit (MCP-1) or goat anti-mouse (ICAM-1) secondary antibody (Invitrogen). Washed cells were incubated with SigmaFast OPD® substrate for 30 min at 23°C. Color development was stopped by the addition of 50 µl 3N HCl per well, and samples were read at 492 nm. Nonspecific binding (secondary only control) was subtracted from each reading and data reported as a percent increase compared with un-stimulated cells.

**Western Immunoblot**

Specificity of the primary antibodies used in the cell-based ELISA was confirmed by Western immunoblot. PNVECs were cultured onto collagen-coated T75 tissue culture flasks and semi-confluent cells were treated with 10 ng/ml rat recombinant TNF-α for 0-24h. Cells were harvested by gentle scraping, washed, and whole-cell lysates were prepared. Lysates were probe-sonicated, and protein concentration was determined using a BCA protein assay kit. Proteins in cell lysates were resolved by SDS-PAGE as described above and transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBS-T) for 30 minutes at 37 °C. Blocked membranes were incubated overnight at 4 °C in a 1:4000 dilution of rabbit anti-rat MCP-1 polyclonal antibody or a 1:100 dilution of mouse anti-rat ICAM-1 monoclonal antibody. Immunostained membranes were washed and incubated for 1h at 23°C with a 1:10,000 dilution of HRP-conjugated goat anti-rabbit
(MCP-1) or a 1:2,500 dilution of an HRP-conjugated goat anti-mouse (ICAM-1) secondary antibody. Equal protein loading was confirmed by re-probing the same blot with primary rabbit anti-GAPDH antibody (1:10,000), followed by anti-rabbit IgG (1:10,000) secondary antibody. Immunostained proteins were visualized by enhanced chemiluminescence (ECL).

**Enzyme Linked Immunosorbent Assay**

The content of secreted MCP-1 protein was determined by quantifying MCP-1 protein levels in cell media using a commercially available ELISA kit (Thermo Scientific, Rockford, IL). PNVECs were cultured at a density 2x10^5 cells per well onto collagen-coated 24-well culture plates in a total volume of 300 µl. Semi-confluent monolayers were treated with TNF-α (0-100 ng/ml) for 0-8h. Culture media was collected, centrifuged at 700g to remove any displaced cells, and stored at -80°C until use. Samples were diluted 1:200 and processed according to manufacturer’s instructions. A standard curve (range: 0-1500 pg/ml) was used to quantify the amount of secreted MCP-1 protein.

**THP-1 monocyte culture**

The THP-1 monocytic cell line was a generous gift from Dr. E. Kovacs, Loyola University Chicago. THP-1 cells express the G-protein coupled receptor, CCR2, which is the receptor for MCP-1 (also known as CCL2). Because human CCR2 has been shown to interact with rodent MCP-1 (Matoba et al. 2010), this cell line was used as a responder cell in the chemotaxis assay. THP-1 cells were maintained in RPMI 1640 containing 10%
FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen), and 5 mM 2-
Mercaptoethanol (Sigma-Aldrich) at 37°C under an atmosphere of (95% air, 5% CO₂).
Cells were maintained at a density of 4x10⁵ to 1x10⁶ cells/ml and brought to the
appropriate density before use by centrifugation at 700 g x 5 minutes.

**Chemotaxis assay**

PNVECs (quiescent or pretreated) were treated with TNF-α (0-100 ng/ml in 300
µl) for 0-8h. Culture media from treated cells was pooled and transferred to the lower
chamber of a fresh 24-well plate. Transwell inserts that were previously populated with
immortalized PNVECs were gently rinsed to remove any constitutively secreted
chemokines and transferred to the fresh 24-well plate containing conditioned media. An
aliquot (125 µl) of THP-1 monocytic cell suspension (8x10⁵ cells/ml) will be added to
the upper compartment. After 0-6h, the Transwell inserts were rinsed to dislodge
adherent cells and combined with the cells in the lower chamber. The pooled migrated
cells were collected by centrifugation (700g x 5 min) and resuspended in 100 µl. The
number of cells in a 10 µl aliquot was counted, and the total number of migrated cells
was calculated. Fresh, complete culture media served as a negative control. To assess
MCP-1-specific chemotaxis, anti-rat MCP-1 polyclonal antibody (10 µg/mL, Serotec)
was added to neutralize the secreted MCP-1. Normal rabbit IgG was used as a negative
control for MCP-1 neutralization study.
**Statistical Analysis**

Data are expressed as the mean ± SEM of \( N \) observations unless noted otherwise. Statistical significance between multiple experimental groups was determined by one-way or two-way ANOVA with a Bonferroni or Newman Keul’s *post-hoc* analysis, or by a Student’s *t-test* where appropriate. In each case, \( p < 0.05 \) was considered statistically significant.

**Results**

**Rat Peripheral Nerve Vascular Endothelial Cells**

Initial cultures of primary cells prepared from rat peripheral nerves consist of a mixed population of vascular endothelial cells (PNVECs), collagen-producing perineurial epitheliod myofibroblasts, and Schwann cells (Yosef *et al.* 2010). Under the culture conditions employed here, proliferation of Schwann cells was minimal. In contrast, epitheliod myofibroblasts proliferated quite well along side vascular endothelial cells. Unwelcome fibroblasts were routinely eliminated in this study from freshly prepared primary PNVEC cultures using a Thy 1.1 antibody-mediated complement-driven cell lysis strategy (Argall *et al.* 1994). Cultures prepared in this manner were greater than 95 ± 7.5 % (\( N = 3 \)) free of contaminating fibroblasts and consistently established contact-inhibited monolayers with a distinctive cobblestone-like morphology with localized expression of von Willebrand Factor/Factor VIII (vWF), a highly characteristic quintessential marker of vascular endothelial cells (*Figs. 5A, B; 6A, B*). Cell viability of
purified PNVEC cultures was typically greater than 96 ± 5% (N =12) as routinely monitored by Trypan Blue vital dye exclusion. Despite efforts to obtain highly purified cultures, Thy-1.1 immunoreactive particles were observed in some primary PNVEC cultures (Fig. 5C & 5D), suggesting the presence of contaminating fibroblasts. However, these particles did not co-localize with DAPI-stained nuclei and therefore most likely represent remnants of lysed fibroblasts.

By comparison, primary PNVEC cultures immortalized with large T antigen retained many of the phenotypic (contact-inhibition) and morphologic (cobblestone-like) characteristics of primary PNVEC cultures (Fig. 6) while completely devoid of contaminating fibroblasts. Similar to primary cells, monolayers of immortalized PNVEC cultures stained positive for vWF (Fig. 6D) and for PECAM-1 (Fig. 6F), a constitutively expressed adhesion molecule considered as a selective vascular endothelial cell marker (Weksler et al. 2005). Immortalized cells, but not primary cells, express the large T antigen protein (Fig. 6G), consistent with stable transformation. The successful and stable immortalization of rat PNVECs with demonstrated retention of key phenotypic and morphologic characteristics expected of vascular endothelial cells was encouraging and provided opportunities not possible with primary cells for detailed assessment of pathological alterations in peripheral nerve endoneurial homeostasis during an inflammatory challenge.
Figure 5. Rat Primary Peripheral Nerve Vascular Endothelial Cell Cultures. Representative phase-contrast (A) and fluorescent (B-D) images of primary PNVECs. Cultures were immunostained for von Willebrand Factor (vWF) or Thy-1.1 antigen as indicated and counterstained with DAPI. Arrows indicate Thy-1.1 positive fragments. Scale bars, 50 µm.
Figure 6. Immortalized PNVEC cultures retain their primary characteristics. Phase-contrast (A, B) and immunofluorescent images (C-F) of primary or immortalized PNVECs that were immunostained for expression of von Willebrand Factor (vWF) or PECAM-1, as indicated. The image in panel A is the same as used in Figure 5A. (G) Western immunoblot of large T antigen protein expressed in lysates prepared from primary or transformed PNVECs, as indicated. Large T antigen immortalized human trabecular meshwork cells (GTM3) are shown for comparison as a positive control. Scale bar, 100 µm.
**TNF-α induction of MCP-1 mRNA expression**

Quiescent immortalized PNVEC cultures respond in a dose- and time-dependent manner to the proinflammatory cytokine TNF-α by markedly increasing mRNA content of the chemokine MCP-1 (Fig. 7A). To verify the specificity of the real-time reaction, amplified qRT-PCR MCP-1 product was captured during the linear phase of the reaction and resolved on a 1.2% agarose E-gel followed by visualization with a FluorChem SP fluorescence image analyzer (Fig. 7A). In good agreement with the expected amplicon size of 447 base pairs, a single product migrating between 400 and 500 base pairs was amplified from TNF-α treated cells. By comparison, only a marginally faint product was amplified from vehicle-treated cells, indicating that expression of MCP-1 by quiescent PNVEC cultures was largely negligible. Importantly, no qualitative differences were observed between vehicle- and TNF-α treated cells with respect to amplification of the housekeeping gene GAPDH, demonstrating the use of this gene as an appropriate reference control (Fig. 7A). The response to TNF-α (10 ng/ml) was rapid (<1h), robust (60-fold, Fig. 7B), and dose-dependent (0-100 ng/ml, Fig. 7C), with immortalized PNVECs expressing MCP-1 mRNA for up to 6h (maximal time assayed). In contrast, vehicle-treated cells did not express MCP-1 mRNA (Fig. 7B). Importantly, primary PNVEC cultures responded similarly to TNF-α (10 ng/ml, 3h), exhibiting significant increases in MCP-1 mRNA content (Fig. 8).
Figure 7. TNF-α induces MCP-1 mRNA expression in immortalized PNVEC cultures. Cells were treated without (media) or with TNF-α (0-6h, 0-100 ng/ml) and relative changes in MCP-1 mRNA content were quantified by qRT-PCR. Data normalized to GAPDH expression. (A) Agarose gel of amplified qRT-PCR products. (B) Time- and (C) dose (2h)-dependent fold-changes in MCP-1 mRNA expression. Data shown are the means ± SEM (B, N = 4-9; C, N = 5). #, p<0.05; *, p<0.01 compared to 0h; one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 8. TNF-α induces MCP-1 mRNA expression in primary PNVECs. Cells were treated without (media) or with TNF-α (3h, 10 ng/ml) and relative changes in MCP-1 mRNA content were quantified by qRT-PCR. Data normalized to GAPDH expression. Data shown are the means ± SEM (N = 5-6). *, p<0.05 compared to vehicle-treated; unpaired Student’s t-test.
TNF-α increases intracellular MCP-1 protein expression

Changes in MCP-1 mRNA expression elicited by TNF-α do not necessarily represent or reflect changes in MCP-1 protein expression. A cell-based ELISA method was employed to determine if TNF-α similarly elicits changes in intracellular MCP-1 protein expression within primary and immortalized PNVEC cultures. The expression of MCP-1 protein content in quiescent immortalized PNVEC cultures was negligible and did not change in response to vehicle-treatment over the 24h time span assayed (Fig. 9). In contrast, quiescent cells markedly responded to TNF-α by increasing the expression of a single 30-kDa protein identified by Western immunoblot as MCP-1 (Fig. 9A). The molecular mass of this protein was, however, approximately twice that of the mass reported for human isoforms of MCP-1. The observed difference in protein mass most likely is due to heavy glycosylation of the rodent isoform of MCP-1 at the n-terminus (Liu et al. 1996, Ruggiero et al. 2003). In a time- (Fig. 9B) and dose-dependent (Fig. 9C) manner, TNF-α elicited maximal changes in MCP-1 protein expression within 4h of stimulation and at a maximal concentration of 10 ng/ml. Primary PNVEC cultures responded similarly to TNF-α stimulation by markedly increasing MCP-1 protein expression (Fig. 10). From these collective data, not only are marked, TNF-α mediated increases in MCP-1 mRNA and protein observed, but the continued presence of TNF-α apparently sustains expression of MCP-1 (Figs. 7 & 9). Clearly, immortalized PNVEC cultures do not readily desensitize or down-regulate their response to the continued presence of TNF-α. Whether this is a result of large T antigen transformation or is a physiologic responses of primary PNVECs remains to be determined, but is consistent with that previously reported (Rollins et al. 1990).
Figure 9. TNF-α increases MCP-1 protein content in immortalized PNVECs. Cells were treated without (media, veh) or with TNF-α (0-24h, 0-100 ng/ml), as indicated, and MCP-1 protein expression was quantified by (A) immunoblot or by (B, C) cell-based ELISA. (B) Time- and (C) dose (4h)-dependent changes in MCP-1 protein expression. Data shown are the means ± SEM. B: *, p<0.01 vs. vehicle-treated control, two-way ANOVA with Bonferroni’s post-hoc analysis (N = 6). C: *, p<0.01 vs. 0h, one-way ANOVA with Bonferroni’s post-hoc analysis (N=10).
Figure 10. TNF-α increases MCP-1 protein content in primary PNVECs. Cells were treated (4h) without (media, 0) or with TNF-α (10 or 50 ng/ml), as indicated, and MCP-1 protein expression quantified by cell-based ELISA. Data shown are the means ± SEM (N = 9-12). *, p<0.01 compared to 0h; one-way ANOVA with Bonferroni’s post-hoc analysis.
**TNF-α enhances ICAM-1 mRNA expression**

Temporal changes in cell adhesion molecule (CAM) expression are a well-established response by cytokine-activated vascular endothelial cells (Raab et al. 2002). Expression of E-selectin (CD62E) occurs early (<1 hr) in the inflammatory response and participates in activated leukocyte slowing or rolling (Eccles et al. 2008). At later time points, and coincident with chemokine expression, is the expression and surface appearance of intercellular CAMs. To determine if rat PNVEC cultures similarly respond to proinflammatory mediators, primary or immortalized PNVEC cultures were exposed to TNF-α and relative changes in the content of ICAM-1 mRNA was quantified. Compared to vehicle controls, TNF-α (10 ng/ml) elicited a time-dependent increase in ICAM-1 mRNA expression in immortalized PNVECs that was maximal at 2h and sustained for up to 6h assayed (Fig. 11A). The response to TNF-α was dose-dependent with near-maximal changes in ICAM-1 mRNA expression seen at 10 ng/ml (Fig. 11B). Primary PNVEC cultures similarly responded to TNF-α treatment (10 ng/ml, 3h), significantly increasing ICAM-1 mRNA content (Fig. 12).
Figure 11. TNF-α increases ICAM-1 mRNA content in immortalized PNVECs. Cells were treated without (media) or with TNF-α (0-6h, 0-100 ng/ml) and relative changes in ICAM-1 mRNA content were quantified by qRT-PCR. Data normalized to GAPDH expression. (A) Time- and (B) dose (2h)-dependent fold-changes in ICAM-1 mRNA expression. Data shown are the means ± SEM (A, N = 5; B, N = 4). #, p<0.05; *, p<0.01; **, p<0.001 vs. 0h; one-way ANOVA with Newman-Keuls’ post-hoc analysis.
Figure 12. TNF-α increases ICAM-1 mRNA content in primary PNVECs. Cells were treated without (media) or with TNF-α (3h, 10 ng/ml) and relative changes in ICAM-1 mRNA content were quantified by qRT-PCR. Data normalized to GAPDH expression. Data shown are the means ± SEM (N = 5-6). *, p<0.01 vs. vehicle-treated; unpaired Student’s t-test.
**TNF-α increases intracellular ICAM-1 protein expression**

As with MCP-1, TNF-α mediated changes in ICAM-1 mRNA expression do not necessarily mirror changes in ICAM-1 protein expression. A cell-based ELISA method was used to determine if TNF-α similarly elicits changes in surface ICAM-1 protein expression by primary and immortalized PNVEC cultures. The expression of ICAM-1 protein content in quiescent immortalized PNVEC cultures was negligible. In contrast to MCP-1, however, small fluctuations in constitutive ICAM-1 protein expression were noted within the first 8h of vehicle-treatment. This change most likely represents random noise and was not statistically relevant. In contrast, quiescent cells markedly responded to TNF-α by increasing the expression of a single 90-kDa protein identified by Western immunoblot as ICAM-1 (Fig. 13A). In a time- (Fig. 13B) and dose-dependent (Fig. 13C) manner, TNF-α elicited maximal changes in ICAM-1 protein expression within 8h of stimulation, slightly later than seen with MCP-1 protein expression (4h, Fig.7). The dose response to TNF-α was quite different that that observed for MCP-1 protein expression. A clear biphasic response was noted, with maximal responses seen at 0.1 ng/ml and again at 10 ng/ml (Fig. 13). The functional significance of this biphasic response remains unclear. Primary PNVEC cultures responded similarly to TNF-α stimulation by markedly increasing ICAM-1 protein expression (Fig. 14).
Figure 13. TNF-α increases ICAM-1 protein content in immortalized PNVECs.
Cells were treated without (media, veh) or with TNF-α (0-24h, 0-20 ng/ml), as indicated, and ICAM-1 protein expression quantified by (A) immunoblot or by (B, C) cell-based ELISA (B) Time- and (C) dose (16h)-dependent changes in ICAM-1 protein expression. Data shown are the means ± SEM (B, N = 6; C, N = 6). B: *, p<0.01 vs. vehicle-treated control; two-way ANOVA with Bonferroni’s post-hoc analysis. C: *, p<0.01 vs. 0h; one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 14. TNF-α increases ICAM-1 protein content in primary PNVECs. Cells were treated (4h) without (media, 0) or with TNF-α (10 or 50 ng/ml), as indicated, and ICAM-1 protein expression quantified by cell-based ELISA. Data shown are the means ± SEM (N = 3). *, p<0.001 vs. 0h; one-way ANOVA with Bonferroni’s post-hoc analysis.
**TNF-α promotes secretion of MCP-1 protein**

Chemokines promote the chemotaxis of immune cells into privileged tissue compartments, such as peripheral nerves, by establishing localized gradients (Yadav *et al.* 2010). Whereas quantifying TNF-α mediated changes in MCP-1 mRNA and intracellular protein expression in PNVEC cultures represent an important advancement, recruitment and extravasation of activated leukocytes across the BNB is ultimately governed by the levels of chemokines released into the localized vascular endothelial interstitial milieu. Here, the functional consequence of TNF-α activation was addressed by determining the ability of PNVEC cultures to release synthesized MCP-1.

Immortalized PNVECs passaged onto collagen-coated 24-well culture plate responded to TNF-α activation by significantly enhancing the release of MCP-1 protein into the tissue culture media (**Fig. 15**). Quiescent immortalized PNVEC cultures release a measurable amount (~50 ng/ml) of MCP-1 into the culture media. Within 1h of TNF-α treatment (10 ng/ml), however, a marked increase in MCP-1 protein was released into the tissue culture media and continued to increase in concentration up to 8h assayed (**Fig. 15A**). The effect of TNF-α on MCP-1 release was dose-dependent and near maximal at 10 ng/ml (**Fig. 15B**). Importantly, TNF-α (10 ng/ml, 4h) similarly elicited a near 4-fold increase in the release of MCP-1 protein from primary PNVEC cultures (**Fig. 15C**). This release elicited by TNF-α, however, was ~5-fold less than that observed from immortalized PNVEC cultures, emphasizing unique quantitative differences between these two cell types. The mechanism by which PNVECs release MCP-1 into the
interstitial milieu is not immediately clear, but most likely involves G-protein regulated vesicular release processes (Knipe et al. 2010).
Figure 15. TNF-α promotes secretion of MCP-1 protein. (A, B) Immortalized or (C) primary PNVEC cultures were treated without (media, 0 or vehicle) or with TNF-α (10 ng/ml, 4h), as indicated, and secreted MCP-1 protein was quantified by ELISA. (A) Time- and (B) dose (4h)-dependent changes in MCP-1 protein content. Data shown are the means ± SEM (N = 3-6). A, B: #, p<0.05; *, p<0.001, one-way ANOVA with Bonferroni’s post-hoc analysis. C: *, p<0.01, Student’s t-test (N = 3-6).
MCP-1 containing conditioned media promotes transendothelial migration of THP-1 monocytes

Functional MCP-1 promotes chemotaxis and transendothelial migration of monocytes that express the cognate G-protein coupled receptor, CCR2 (Deshmane et al. 2009). To assess the functional properties of MCP-1 secreted by PNVECs, a Transwell migration assay was used, with THP-1 monocytes as the responder cells. THP-1 is a human, monocytic cell line that expresses CCR2 and has been demonstrated to respond to rodent MCP-1 (Matoba et al. 2010). PNVECs were passaged onto collagen-coated Transwell inserts (8 µm pore size) and cultured until confluent monolayers were formed. In parallel, PNVECs were cultured on 24-well plates. These cultures were treated with TNF-α (10 ng/ml, 4h) on the day of the assay to produce MCP-1-containing conditioned media. Migration of THP-1 monocytes through an endothelial monolayer and into fresh, MCP-1-containing conditioned media was significantly increased after 4h (Fig. 16A). This transendothelial migration was indeed MCP-1-dependent, as it was prevented by a blocking antibody to MCP-1 (Fig. 16B). This effect was not seen when the antibody was heat-denatured prior to incubation (Fig. 16B).
Figure 16. MCP-1 containing conditioned media promotes transendothelial migration of THP-1 monocytes. Transendothelial migration of CCR2-expressing THP-1 monocytes in the absence (A) or presence (B) of a specific MCP-1-blocking antibody. Data shown are the means ± SEM (A, N=3; B, N=6) of total cells migrated. A: *, p<0.01 vs. vehicle control, two-way ANOVA with Bonferroni’s post-hoc analysis. B: *, p<0.01; **, p<0.001; one-way ANOVA with Bonferroni’s post-hoc analysis.
**TNF-α facilitates NFκB p65 translocation to the nucleus and activates RhoA**

It is well established that proinflammatory cytokines, including TNF-α, mediate their effects in part by activation of the nuclear transcription factor NFκB (Baldwin 1996). To determine if TNF-α similarly elicits changes in NFκB activation within PNVEC cultures, treated cultures were immunostained for the presence of the p65 subunit of NFκB (Fig. 17). Quiescent immortalized PNVEC cultures expressed measurable levels of the NFκB p65 subunit diffusely distributed throughout the cell cytosol (Fig. 17). In response to TNF-α (10 ng/ml) treatment, NFκB p65 subunit rapidly (within 20 minutes) translocated to DAPI-stained cell nuclei. These findings support the presence of NFκB in PNVECs and further suggest its role in regulating TNF-α mediated activation of rat peripheral nerve vascular endothelial cells. Whether activation of NFκB is involved in TNF-α mediated changes in MCP-1 and/or ICAM-1 expression within rat PNVECs remains to be established.

The precise mechanisms by which TNF-α activates NFκB remain undefined. However, multiple groups have suggested active Rho GTPases as a necessary intermediate (Hippenstiel et al. 2002, Williams et al. 2008, Zhao & Pothoulakis 2003), and several labs have reported that TNF-α treatment leads to an increase in GTP-bound, active RhoA (Hume & Fairlie 2005, Mong et al. 2008, Papaharalambus et al. 2005, McKenzie & Ridley 2007). In PNVECs, it is demonstrated that TNF-α treatment (10 ng/ml) leads to an apparent increase in GTP-bound RhoA after 10 minutes, and this increase is significant after 2h (Fig. 18). While these findings do not implicate the necessity of active RhoA in TNF-α mediated gene expression, they do bolster this novel cell line as a useful tool for further elucidation of these mechanisms.
Figure 17. TNF-α facilitates NFκB p65 translocation to the nucleus.
Immunocytochemical analysis of NFκB p65 expression in vehicle- (media) and TNF-α (10 ng/ml, 20 min)-treated transformed PNVECs, Scale bar, 100 µm.

Figure 18. TNF-α activates RhoA in immortalized PNVECs. GTP-bound ELISA analysis of TNF-α mediated (10, 120 min; 10 ng/ml) activation of RhoA. Data shown are the means ± SEM (n=12), #, p<0.05; *, p<0.01, compared with baseline; one-way ANOVA with Newman-Keuls’ post-hoc analysis.
Discussion

In this study, vascular endothelial cells harvested and purified from rat sciatic nerve were immortalized by SV40 large T antigen transduction. Transformed cells were found to retain key phenotypic (contact-inhibited monolayers), morphologic (distinctive cobblestone-like appearance), and biochemical (localized expression of vascular endothelial cell marker von Willebrand Factor/Factor VIII) characteristics exhibited by primary rat peripheral nerve vascular endothelial cells (PNVECs). Activation of primary or transformed PNVEC cultures with TNF-α elicited marked dose- and time-dependent changes in chemokine (MCP-1) and cell adhesion molecule (ICAM-1) expression and MCP-1 release. This is the first group to successfully immortalize rat PNVECs that retain properties highly characteristic of primary vascular endothelial cells. Immortalized rat PNVEC cultures provide a novel and physiologically relevant in vitro tool for the functional assessment of pathological alterations in peripheral nerve endoneurial homeostasis during an inflammatory challenge.

Although advancements have been made toward the purification and culture of vascular endothelial cells derived from peripheral nerve (Argall et al. 1994, Sano et al. 2007, Yosef et al. 2010), no studies to date have addressed how peripheral nerve vascular endothelial cells respond to a localized inflammatory challenge such as that experienced during GBS/EAN. In this study, viable cultures of primary vascular endothelial cells from rat sciatic nerve were routinely prepared at > 95% purity. However, the mass quantity of cells required to statistically test the proposed hypothesis required the procurement of a stable cell line, that retained properties characteristic of primary cells, with which large
quantities of vascular endothelial cells may be experimentally manipulated. Here, a stably transformed rat PNVEC clones was produced using a replication-deficient SV40 retrovirus encoding a temperature sensitive, non-SV40-origin binding mutant of the large T antigen. This method has been previously used to immortalize primary vascular endothelial cells harvested from rat brain and rat aorta with retention of their primary characteristics (Greenwood et al. 1996, Jat et al. 1986, Jat & Sharp 1986). Rat primary PNVEC cultures immortalized in this manner consistently formed contact-inhibited monolayers with a cobblestone-like morphology and constitutively expressed von Willebrand Factor/Factor VIII and PECAM-1, cell markers highly characteristic of differentiated vascular endothelial cells. A single isolated rat PNVEC clone (designated here as 4.3) exhibiting stable characteristics of primary cells was subsequently used throughout this study to functionally assess how peripheral nerve endoneurial homeostasis may be altered in response to an inflammatory challenge such as that encountered during acute GBS/EAN.

Recruitment of autoreactive leukocytes across an activated endothelial barrier and into peripheral nerves initially involves localized increases in chemotactic cytokines, including MCP-1 (Yadav et al. 2010). Consistent with this thesis, a rapid (within 1h) and marked (~50-fold) increase in MCP-1 mRNA content was observed in TNF-α challenged immortalized PNVEC cultures. Importantly, TNF-α also elicited significant increases in the content of intracellular and functional, secreted MCP-1 protein. Activation of primary rat PNVEC cultures with TNF-α resulted in similar increases in MCP-1 mRNA and intracellular protein expression and protein secretion.
The mechanism by which autoreactive leukocytes migrate across an activated endothelial barrier is well established (Springer 1994), and involves temporal changes in endothelial cell adhesion molecule expression resulting in the tethering, slowing, firm adhesion, and ultimate diapedesis (Raab et al. 2002). Selectins facilitate initial events (tethering and slow rolling) and are upregulated by activated endothelium on a time scale of minutes to hours (Eccles et al. 2008). By comparison, the surface expression of ICAM-1 on activated endothelial cells is reported to peak within 8-16 hours of activation. Given that ICAM-1 is directly involved in leukocyte trafficking (Greenwood et al. 2002), this study focused on TNF-α mediated changes in ICAM-1 expression. The content of endogenously expressed ICAM-1 in rat PNVEC cultures was below the level of detection. This was unexpected, as previous studies reported constitutive ICAM-1 expression in vascular endothelial cells (Rahman & Fazal 2009). TNF-α, however, induced ICAM-1 mRNA and protein expression, enabling these cells capable of firm adhesion. Importantly, the effect of TNF-α on ICAM-1 expression was similarly observed in rat primary PNVEC cultures.

Given that primary and transformed PNVECs responded similarly to a TNF-α challenge, these findings are interpreted to represent a physiological response, rather than an artifact of cell transformation. The physiological concentration of TNF-α measured in plasma of patients with GBS is reported to exceed 0.1 ng/ml (Radhakrishnan et al. 2004, Reuben et al. 2002). Concentrations of TNF-α needed to elicit measurable changes in MCP-1 and ICAM-1 mRNA and protein expression within PNVECs were, however, 10-100 fold higher. While this may appear non-physiological, it can be argued that the
localized concentration of TNF-α within peripheral nerves during an immune challenge may very well exceed concentrations used in this study.

The mechanism by which TNF-α elicits changes in MCP-1 and ICAM-1 mRNA content may involve NFκB-dependent increases in transcription of the MCP-1 and ICAM-1 genes (Deshmane et al. 2009, Xing & Remick 2007). Consistent with this thesis, TNF-α was capable of inducing translocation of NFκB p65 to the nucleus in immortalized PNVECs. The precise mechanisms by which TNF-α binding to its receptor leads to an increase in NFκB activation remain unknown, but it has been suggested that Rho GTPases may be involved (Williams et al. 2008, Zhao & Pothoulakis 2003).

TNF-α treatment of transformed PNVECs leads to an increase in active, GTP-bound RhoA. TNF-α has been shown to elicit increases in active RhoA in pulmonary endothelial cells and HUVECs (Mong et al. 2008, Papaharalambus et al. 2005). Hippenstiel et al. demonstrated that active RhoA is necessary for the TNF-α mediated activation of NFκB (Hippenstiel et al. 2002). Additional studies are currently being conducted to determine the mechanism by which TNF-α elicits changes in MCP-1 and ICAM-1 gene and protein expression. However, the similarities between primary and transformed PNVEC cultures further validates the use of immortalized rat PNVECs as a novel and physiologically relevant in vitro tool for the functional assessment of pathological alterations in peripheral nerve endoneurial homeostasis during an inflammatory challenge.
CHAPTER 4
THE ROLE OF PROTEIN GERANYLGERANYLATION IN TNF-α mediated SECRETION OF MCP-1 FROM PERIPHERAL NERVE VASCULAR ENDOTHELIAL CELLS

Abstract

Recruitment and trafficking of autoreactive leukocytes across the blood-nerve barrier and into peripheral nerves is an early pathological hallmark of Guillain-Barré Syndrome (GBS), a debilitating inflammatory demyelinating disorder. Whereas the mechanism by which autoreactive leukocytes home to and extravasate across select areas of the blood-nerve barrier in GBS remains unclear, localized cytokine-mediated activation of the peripheral nerve vascular endothelium has been suggested as an initiating pathological insult. TNF-α is believed to facilitate transendothelial migration during GBS by up-regulating endothelial cell expression of chemokines and cell adhesion molecules (CAMs). Of these chemokines and CAMs, monocyte chemoattractant-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) in particular have been identified as key regulators of GBS disease progression.
Despite clinical advancements, there remains a pressing need for the development of more effective and specific immune-modulating therapeutic strategies for the care and management of GBS. Previous *in vivo* experiments demonstrate that statins therapeutically attenuate the development and progression of a well-characterized animal model of GBS by inhibiting leukocyte migration. It has also recently been demonstrated *in vitro* that TNF-α treatment rapidly and robustly increases MCP-1 and ICAM-1 mRNA and protein expression in peripheral nerve vascular endothelial cells. **This in vitro study is designed to determine the mechanism(s) by which TNF-α increases the expression of these inflammatory mediators.** In turn, the mechanisms by which statins attenuate pathological transendothelial migration will be elucidated. Here, it is demonstrated that pretreatment with simvastatin attenuates TNF-α mediated MCP-1 secretion, but does not alter intracellular levels of ICAM-1 or MCP-1 mRNA or protein. This effect is mimicked by a specific inhibitor of protein geranylgeranylation, but not farnesylation. These novel findings suggest that TNF-α mediated migration of autoreactive leukocytes into peripheral nerves during EAN proceeds, in part, by a mechanism that involves GGPP-dependent secretion of MCP-1. It is argued that identifying this precise geranylgeranylated target (Rac1, Cdc42, RalA) that mediates chemokine secretion has the potential to significantly advance the development of novel treatment options for patients with GBS.
Guillain-Barré Syndrome (GBS) is a debilitating inflammatory demyelinating disorder that is currently the leading cause of autoimmune neuromuscular paralysis (Hughes & Cornblath 2005). Due to the aggressive, rapid onset of GBS, patients often require life-saving respiratory supportive care and must be monitored closely for development of secondary complications (Hartung et al. 1995). Current treatment options for the GBS patient are limited to nonspecific immune-modulating therapies, such as plasmapheresis and intravenous immunoglobulin (IVIg). These palliative strategies are transiently effective, and they do not address the causality of the aberrant autoimmune responses seen in affected patients. Worse, approximately half of GBS patients fail to respond to either plasmapheresis or IVIg therapy. Of those patients that do respond favorably to palliative therapy, a significant number remain severely disabled (Hughes & Cornblath 2005). Advancement of care for these affected patients is clearly dependent on the development of new selective immune-modulating agents or the novel application of existing therapeutic strategies.

While the etiology of GBS remains unknown, cytokine-mediated activation of the blood-nerve barrier (BNB) is widely believed to be among the initiating pathological events. In support of this thesis, pro-inflammatory cytokines, in particular TNF-α, are elevated in sera of patients with GBS and play a pivotal role in the early activation of the BNB (Zhu et al. 1998, Exley et al. 1994). By up-regulating endothelial cell expression of chemotactic cytokines (chemokines) and cell adhesion molecules (CAMs), TNF-α
facilitates the recruitment and trafficking of autoreactive leukocytes across the BNB and into peripheral nerves. Of these chemokines and CAMs, monocyte chemoattractant-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) have been identified as key participants in GBS disease progression (Orlikowski et al. 2003, Putzu et al. 2000). It has recently been demonstrated that activation of peripheral nerve vascular endothelial cell (PNVEC) cultures with TNF-α elicits marked increases in MCP-1 and ICAM-1 expression and MCP-1 release. Elucidating the mechanism by which TNF-α induces expression of these inflammatory mediators may provide insight into the development of selective therapeutic strategies for the management of GBS and related autoimmune inflammatory peripheral nerve disorders.

Statins have emerged as the leading therapeutic option for treating hypercholesterolemia and are prescribed to more than 25 million people worldwide. Increasing clinical and experimental evidence demonstrates a variety of beneficial effects of statins beyond what can be reasonably attributed to the reduction in serum cholesterol (Bifulco et al. 2008, Undas et al. 2002), including important anti-inflammatory (Katznelson & Kobashigawa 1995, Kobashigawa et al. 1995) and neuroprotective properties (Stuve et al. 2003, Stanislaus et al. 1999, Paintlia et al. 2005). As a result, statins are now being considered as potential therapeutic agents for the management of a wide variety of inflammatory diseases, including ischemic stroke, Alzheimer’s disease, multiple sclerosis, and more recently inflammatory demyelinating peripheral neuropathies (Menge et al. 2005, Greenwood et al. 2006), (Sarkey et al. 2007).

Statins reduce endogenous cholesterol levels in patients by competitive inhibition
of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis. By inhibiting this enzyme, statins limit the synthesis of the 15-carbon farnesyl pyrophosphate (FPP) and the 20-carbon geranylgeranyl pyrophosphate (GGPP) isoprenoids. These isoprenoids serve as lipid attachment anchors for small monomeric GTPases (Zhang & Casey 1996), including members of the Rho family, facilitating their activity in cell cycle pathways and gene expression (Mackay & Hall 1998, Ridley 2006). Early in vivo studies have focused on the repurposing of statins as a novel adjunctive therapeutic strategy for the management of GBS. It was found that statins therapeutically attenuate the development and progression of experimental autoimmune neuritis (EAN, a well-characterized animal model of GBS) by inhibiting leukocyte migration into the peripheral nerves (Sarkey et al. 2007).

Here, the role of small monomeric GTPases in the TNF-α mediated inflammatory responses are elucidated in purified rat primary and immortalized PNVEC cultures. These novel findings suggest that proinflammatory cytokines that are elevated in GBS mediate the migration of autoreactive leukocytes into peripheral nerves, in part, by a mechanism that involves GTPase-dependent secretion of MCP-1 from the blood-nerve barrier. It is argued that identifying the precise geranylgeranylated GTPase (Rac1, Cdc42, RalA) that mediates chemokine secretion has the potential to significantly advance the development of novel treatment options for patients with GBS.
Materials and Methods

Peripheral Nerve Vascular Endothelial Cell Culture

Primary cultures of peripheral nerve vascular endothelial cells (PNVECs) were prepared from sciatic nerves of naïve adult male Lewis rats as previously described (Sarkey et al. 2007). Primary PNVEC cultures were cleared of fibroblasts, the major cell contaminant, by Thy-1.1 antibody-mediated complement-driven cell lysis. This technique exploits the fact that the Thy-1.1 surface glycoprotein is selectively expressed on fibroblasts but notably absent from vascular endothelial cells (Kisselbach et al. 2009). Primary PNVEC preparations were cultured at 37°C (95% air, 5% CO₂) on T25 tissue culture flasks for one week prior to fibroblast elimination. Established mixed population cultures were harvested by gentle scraping and suspended in undiluted tissue culture media containing Thy1.1 IgM monoclonal antibodies collected from cultured TIB-103 hybridoma cells (ATCC) and 1% fresh rabbit complement for 3 h at 37°C (95% air, 5% CO₂). Tissue culture media was removed, adherent cells were washed once with Ham’s F10 basal media, and surviving PNVECs were allowed to proliferate in complete PNVEC media containing Ham’s F10 basal media (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 50 µg/ml endothelial cell growth supplement (ECGS; BD Bioscience, San Jose, CA), 0.4 µg/ml heparin (Sigma-Aldrich, St. Louis, MO), 5.6 µg/ml amphotericin B, 100 units/ml penicillin, and 100 µg/ml streptomycin). Purified PNVEC cultures were treated with Thy 1.1 antibody/ complement mixture twice prior to use. The efficacy of the fibroblast removal was routinely monitored by immunocytochemistry and consistently
demonstrated to be >95% pure, in agreement with published methodology (Argall et al. 1994).

**Transformed Human THP-1 Monocytes**

Transformed human THP-1 monocytes expressing MCP-1 receptors (CCR2) were a generous gift from Dr. E. Kovacs, Loyola University Chicago. THP-1 cells migrate robustly in response to rodent MCP-1 (Matoba et al. 2010) and therefore were used throughout this study as primary responder cells for all chemotaxis assays. THP-1 cell cultures were maintained at a density of 4x10^5 to 1x10^6 cells/ml in RPMI 1640 containing 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen), and 5 mM 2-Mercaptoethanol (Sigma-Aldrich) at 37°C under an atmosphere of (95% air, 5% CO₂).

**SV40 Large T antigen transduction**

Given the difficulties in preparing needed quantities of purified primary PNVEC cultures for experimentation, a stably transformed cell line was produced from highly (>99%) purified primary PNVEC culture preparations using a replication deficient SV40 retrovirus encoding a temperature sensitive, non-SV40-origin binding mutant of the large T antigen and a selectable neomycin resistance gene (generous gift from Dr. P. Jat, University College of London, London, UK). Currently, no commercial or private source of rat PNVECs exists. We are the first group to successfully immortalize rat PNVECs. Semi-confluent cultures of highly purified primary PNVECs were incubated for 36h at 37°C in the presence of 8 µg/ml polybrene (Sigma-Aldrich, St. Louis, MO) with
undiluted filtered particle-free viral supernatant collected from SVU19.5 producer cells. Media was replaced and treated cells were allowed to reach confluency. Selection was achieved by passage into complete PNVEC culture media containing 200 µg/ml G418 (Invitrogen, Carlsbad, CA). Single antibiotic resistant clones were isolated by dilution into 96-well plates at a theoretical density of 0.33 cells/well. Several clones exhibited morphologic characteristics of primary endothelial cells including a cobblestone-like appearance in monolayer. These clones were further characterized (as described in Chapter 3). Clone 4.3 was used for all transformed PNVEC experiments in this chapter.

**Inhibitors**

Confluent cultures of purified rat primary or immortalized PNVEC cultures were pretreated overnight at 37°C (95% air, 5% CO₂) without (vehicle, 0.01% ethanol) or with 0.01-20 µM lovastatin, simvastatin, or pravastatin (Calbiochem). Lovastatin and simvastatin are inactive lactone prodrugs that are converted to the active hydroxy acid form by first pass through the liver. For use in tissue culture, however, these lipophillic agents are first chemically activated by alkaline hydrolysis and stored as an aqueous pH neutralized 10 mM stock solution at -20°C until use. All pretreatments were performed in complete PNVEC tissue culture media (as described in Chapter 3). To determine HMG-CoA reductase specificity, some statin-treated PNVEC cultures were co-incubated with geranylgeranyl pyrophosphate (10 µM). The role of isoprenoid-dependent protein modification on TNF-α mediated MCP-1 chemokine and ICAM-1 expression and MCP-1 release was determined using farnesyl transferase inhibitor (FTI-277; 10 µM, 16h) or
geranylgeranyl transferase inhibitor-I (GGTI-298; 1-20 µM, 16h). The protein specificity of these transferase inhibitors was determined using C3 exoenzyme transferase (2 µg/ml, 4h), a membrane permeable inhibitor of the Rho subfamily of small monomeric GTPases. In each case, pretreated PNVEC cultures were subsequently challenged with TNF-α to stimulate MCP-1 and ICAM-1 expression and MCP-1 release.

**Real Time RT-PCR**

Primary and immortalized PNVECs were cultured on collagen-coated T25 tissue culture flasks to near confluence and pretreated with a statin or inhibitor of isoprenylation, as described above. Pretreated vascular endothelial cells were challenged with rat recombinant TNF-α (10 ng/ml, 2h) to stimulate MCP-1 and ICAM-1 expression (optimized in Chapter 3). Total RNA was extracted from treated PNVEC cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) and 5 µg was reverse-transcribed using SuperScript III First Strand Synthesis system (Invitrogen, Carlsbad, CA) as previously described (Von Zee et al. 2009). MCP-1 and ICAM-1 specific cDNA sequences were amplified by real-time PCR using a Mini-Opticon PCR detection system and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The following primer pairs were used: **MCP-1**, forward, 5'-ATGCAGGTCTCTGTCACG; reverse, 5'-CTAGTTCTCTGTCATACT; **ICAM-1**, forward, 5'-CTGCAGAGCACAAACAGCAGAG; reverse, 5'-AAGGCCGCAGAGCAAAAGAAGC. For each sample, the housekeeping gene **GAPDH** (forward, 5'-TCCCTCAAGATTGCAGACAG; reverse, 5'-AGATCCACAACGGGATACTT) was found not to change under these experimental
conditions and therefore used as an appropriate reference control. Optimized amplification steps used were: 94°C x 5 minutes; 94°C x 15s, specifically tailored annealing temperature x 30s, 72°C x 1 minute for 40 cycles. Specific annealing temperature for MCP-1 and GAPDH reactions was 55°C; annealing temperature for ICAM-1 reactions was 60°C. Reaction efficiencies for each product were typically >90%.

For each sample, the specificity of the real-time reaction product was determined using the melt-curve function analysis. GAPDH normalized relative fold-changes in message expression was determined using the $2^{\Delta\Delta CT}$ method of Livak (Livak & Schmittgen 2001).

**Cell-Based ELISA**

The effect of statins on TNF-α mediated changes in intracellular MCP-1 protein expression was quantified using cell-based ELISA methodology. Semiconfluent immortalized rat PNVEC cultures seeded onto collagen-coated 96-well plates were pretreated overnight without (vehicle, 0.01% ethanol) or with lovastatin (10 µM, 16h) followed by TNF-α (4h, 10 ng/ml) in fresh complete culture media. Treated cells were fixed with phosphate buffered (pH 7.4) 4% PFA for 10 min at 23°C, washed, and permeabilized with 0.1% Triton X-100. Permeabilized cells were blocked for 1h at 23°C with 1% BSA and incubated with a 1:2000 dilution of rabbit anti-rat MCP-1 polyclonal antibody (Serotec, Raleigh, NC) overnight at 4°C. Immunostained cells were washed and incubated for 1h at 23°C in the presence of a 1:5000 dilution of HRP-conjugated goat anti-rabbit secondary antibody (Invitrogen). Washed cells were incubated with SigmaFast
OPD® substrate for 30 min at 23°C. Color development was stopped by the addition of 50 µl 3N HCl per well, and samples were read at 492 nm. Nonspecific binding (secondary only control) was subtracted from each reading and data expressed as a percentage of un-stimulated control cultures.

**Western Immunoblot**

The effect of statins on TNF-α mediated intracellular ICAM-1 protein expression was determined by Western immunoblot. Semiconfluent immortalized PNVEC cultures were seeded on collagen-coated T75 tissue culture flasks and were pretreated overnight without (vehicle, 0.01% ethanol) or with lovastatin (10 µM), lovastatin + GGPP (10 µM), GGTI-298 (10 µM) followed by TNF-α (16h, 10 ng/ml) in fresh complete culture media. Cells were harvested by gentle scraping, washed, and whole-cell lysates were prepared. Soluble (cytosolic) and particulate (crude membranes) subcellular fractions from whole-cell lysates were prepared by centrifuging at 100,000 g x 1h. Lysates and subcellular fractions were probe-sonicated and protein concentrations were determined using a BCA protein assay kit. Proteins in prepared lysates or subcellular fractions were resolved on 4-20% polyacrylamide gels and electro-transferred onto nitrocellulose membranes. Membranes were blocked with 5% milk in PBS (pH 7.6) containing 0.05% Tween-20 (PBS-T) for 30 min at 37 °C and incubated overnight at 4 °C in the presence of a 1:100 dilution of rabbit anti-mouse MCP-1 or ICAM-1 monoclonal antibody. Immunostained and washed blots were subsequently incubated with horseradish peroxidase-conjugated anti-mouse-IgG (1:2,500) secondary antibody x 1h at 37 °C.
Immunostained proteins were visualized by enhanced chemiluminescence (ECL). In all cases, equal protein loading was confirmed by probing the same blot with a 1:10,000 dilution of rabbit anti-GAPDH primary antibody followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000) secondary antibody.

**MCP-1 Immunocytochemistry**

Changes in the intracellular distribution of MCP-1 protein were evaluated with immunocytochemistry. Semi-confluent PNVEC cultures were seeded onto collagen-coated coverslips and monolayers were stimulated with vehicle (media) or rat recombinant TNF-α (10 ng/ml) for 4h to stimulate MCP-1 chemokine expression. Following TNF-α treatment, monolayers were fixed for 5 min phosphate buffered (pH 7.4) 4% PFA, blocked, and incubated for 30 min at 37°C by immersion in a solution of 1% normal goat serum and 0.1% Triton X-100 in PBS. Treated monolayers were incubated overnight (4°C) in the presence of a 1:200 dilution of rabbit anti-MCP-1 polyclonal antibody (Serotec). Immunostained monolayers were incubated with a 1:1000 dilution of FITC-conjugated goat anti-rabbit IgG secondary antibody. Coverslips were inverted, mounted onto slides with Fluoroshield containing DAPI, and visualized with confocal microscopy.

**Quantification of Secreted MCP-1**

The amount of MCP-1 protein secreted by treated PNVEC cultures was determined using a commercially available ELISA kit (Thermo Scientific, Rockford, IL).
Semi-confluent PNVECs were seeded onto collagen-coated 24-well culture plates at a density 2x10^5 cells per well in a total volume of 300 µl. Semi-confluent PNVEC monolayers were pretreated overnight at 37°C (95% air, 5% CO_2) without (vehicle, 0.01% ethanol) or with 0.01-20 µM of lovastatin, simvastatin, or pravastatin or with 10 µM FTI-277 or GGTI-298. Pretreated cultures were challenged with TNF-α (0-100 ng/ml) for 4h, culture media was collected, centrifuged at 700g to remove any displaced cells, and stored at -80°C until use. Collected media samples were diluted 1:200 and assayed for MCP-1 content by ELISA according to manufacturer’s instructions. A standard curve (range: 0-1500 pg/ml) was used to quantify the amount of MCP-1 protein released into the media.

Transendothelial Chemotaxis Assay

Quiescent or pre-treated PNVEC cultures were incubated with TNF-α (10 ng/ml in 300 µl) for 4h, and the conditioned media was transferred to the lower chambers of 24-well Transwell plate. Transwell inserts (8 µm pore size) that were previously populated with immortalized PNVECs were gently rinsed to remove any constitutively expressed chemokines and placed within the lower chamber containing conditioned media. Migration was initiated by adding an aliquot (125 µl) of THP-1 monocytic cells (8 x 10^5 cells/ml) to the upper compartment. After 0-6h, the undersides of the Transwell inserts were rinsed to dislodge adherent cells and combined with cells in the lower chamber. The pooled migrated cells were collected by centrifugation (700g x 5 min) and resuspended in 100 µl. The total number of cells in a 10 µl aliquot was counted, and the number of
migrated cells was calculated. Fresh, complete (un-conditioned) culture media added to the lower chamber served as a negative control. To assess MCP-1-specific chemotaxis, neutralizing anti-rat MCP-1 polyclonal antibody (10 µg/mL, Serotec) was co-incubated with conditioned media in some experiments. In parallel, heat-denatured (95°C, 5 min.) MCP-1 antibody was used as a negative control.

**Statistical Analysis**

Data are expressed as the mean ± SEM of N observations unless noted otherwise. Statistical significance between multiple experimental groups was determined by one-way or two-way ANOVA with a Bonferroni or Newman-Keuls’ *post-hoc* analyses, or by a Student’s *t*-test for analyses of two independent groups. In each case, p < 0.05 was considered statistically significant.

**Results**

**TNF-α mediated induction of MCP-1 mRNA expression proceeds independently of GTPase protein prenylation**

To assess whether small monomeric GTPases play a role in facilitating TNF-α induced increases in MCP-1 mRNA expression, immortalized PNVEC cultures were pre-treated overnight with a maximal dose (10 µM) of lovastatin, simvastatin, or pravastatin. Statins have previously been reported to indirectly inhibit activation of small monomeric
GTPases by limiting essential posttranslational isoprenylation of these proteins (Von Zee et al. 2009). Statin pre-treated PNVEC cultures responded to TNF-α (10 ng/ml, 2h) stimulation by exhibiting a robust increase in MCP-1 mRNA content that was statistically indistinguishable from vehicle-pretreated TNF-α stimulated control cultures (Fig. 19). Lovastatin-pretreated cultures co-incubated with the isoprenoid geranylgeranyl pyrophosphate (GGPP, 10 µM) responded similarly to TNF-α stimulation, exhibiting marked increases in MCP-1 mRNA content (Fig. 19B). Moreover, overnight pretreatment with selective inhibitors of geranylgeranyl transferase I (10 µM GGTI-298) or farnesyl transferase (10 µM, FTI-277) had no effect on TNF-α induced increases in MCP-1 mRNA content, as compared to levels seen in vehicle (DMSO, 0.6%)-pretreated cells (Fig. 20). Interestingly, DMSO (0.6%) pretreatment alone significantly attenuated TNF-α induced increases in MCP-1 mRNA content compared ethanol (0.01%) pretreatment (Fig. 21). These findings are expected, and agree well with previous studies demonstrating a modest attenuation by DMSO of stimulated MCP-1 expression (Xing & Remick 2007).
Figure 19. Statin pretreatment does not alter TNF-α mediated increases in MCP-1 mRNA content. Immortalized PNVEC cultures were pretreated overnight without (ethanol, 0.01%) or with (10 µM each) simvastatin, lovastatin ± 10 µM GGPP, or pravastatin, followed by incubation with TNF-α (10 ng/ml, 2h) as indicated. Shown are the means ± SEM (A: n=3-9, B: n=3-6). *, p<0.01 compared with unstimulated control, one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 20. Inhibition of isoprenylation does not alter TNF-α mediated increases in MCP-1 mRNA content. Immortalized PNVECs were pretreated overnight without (DMSO, 0.6%) or with (10 µM each) GGTI-298 or FTI-277, followed by 2h TNF-α (10 ng/ml) as indicated. Shown are the means ± SEM (n=3-6). *, p<0.01 compared with unstimulated control, one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 21. DMSO inhibits MCP-1 mRNA expression in transformed PNVECs. Immortalized PNVECs were pretreated overnight with 0.6% DMSO or 0.01% ethanol, followed by 2h TNF-α (10 ng/ml). Shown are the means ± SEM (n=3). *, p<0.01 compared with ethanol pre-treated; two-way ANOVA with Bonferroni’s post-hoc analysis.
TNF-α mediated increases in ICAM-1 mRNA content proceeds independently of GTPase protein prenylation

It has been demonstrated that Rho GTPases are involved in the cytokine-mediated mRNA expression of ICAM-1, VCAM and e-selectin (Greenwood & Mason 2007, Takeuchi et al. 2000, Zapolska-Downar et al. 2004). To assess whether small monomeric GTPases play a role in facilitating TNF-α induced increases in ICAM-1 mRNA content in PNVECs, immortalized PNVEC cultures were pre-treated overnight with a maximal dose (10 µM) of lovastatin, simvastatin, or pravastatin. Statin pre-treated PNVEC cultures responded to TNF-α (10 ng/ml, 2h) stimulation by exhibiting a robust increase in ICAM-1 mRNA content that was statistically indistinguishable from vehicle-pretreated TNF-α stimulated control cultures (Fig. 22). Overnight pretreatment with inhibitors of geranylgeranyl transferase I (10 µM GGTI-298) or farnesyl transferase (10 µM, FTI-277) had no effect on TNF-α induced increases in ICAM-1 mRNA content, as compared to levels seen in vehicle (DMSO, 0.6%)-pretreated cells (Fig. 23).
Figure 22. Statin pretreatment does not alter TNF-α mediated increases in ICAM-1 mRNA content. Immortalized PNVECs were pretreated overnight without (ethanol, 0.01%) or with (10 µM each) simvastatin, lovastatin, or pravastatin, followed by incubation with TNF-α (10 ng/ml, 2h) as indicated. Shown are the means ± SEM (n=3-9). *, p<0.01 compared with unstimulated control, one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 23. **Inhibition of isoprenylation does not alter TNF-α mediated increases in ICAM-1 mRNA content.** Immortalized PNVECs were pretreated overnight without (DMSO, 0.6%) or with (10 µM each) GGTI-298 or FTI-277, followed by 2h TNF-α (10 ng/ml) as indicated. Shown are the means ± SEM (n=3-6). *, p<0.01 compared with unstimulated control, one-way ANOVA with Bonferroni’s post-hoc analysis.
TNF-α mediated increases in intracellular MCP-1 or ICAM-1 protein proceeds independently of GTPase protein prenylation

Disrupting protein prenylation had no apparent effect on TNF-α induced increases in MCP-1 mRNA expression (Fig. 19). However, translational regulation of protein expression may also involve prenylated proteins/GTPases (Xiao et al. 2008). To address this possibility, a cell based ELISA approach was used to quantify relative changes in intracellular MCP-1 protein expression. Immortalized PNVEC cultures were pre-treated overnight (16h) with a maximal dose (10 µM) of lovastatin. Pre-treated PNVEC cultures responded to TNF-α (10 ng/ml, 4h) stimulation by exhibiting a significant increase in intracellular MCP-1 protein expression (Fig. 11 & Fig. 24). TNF-α stimulated changes in MCP-1 protein content was statistically indistinguishable from vehicle-pretreated TNF-α stimulated control cultures (Fig. 24). Given that lovastatin did not affect TNF-α stimulated changes in MCP-1 protein content, studies using geranylgeranyl transferase I or farnesyl transferase inhibitors were not pursued.

To determine whether protein prenylation affects TNF-α stimulated changes in ICAM-1 protein expression, immortalized PNVEC cultures were treated as described above and ICAM-1 protein levels in whole cell lysates or crude membrane fractions were measured by Western immunoblot. TNF-α (10 ng/ml, 4h) elicited a significant increase in intracellular ICAM-1 protein expression (Fig. 13). Similar to MCP-1, pre-treatment with lovastatin (10 µM, 16h) did not alter TNF-α mediated ICAM-1 protein expression (Fig. 25).
Figure 24. Lovastatin does not attenuate TNF-α mediated increases in intracellular MCP-1 protein in immortalized PNVECs. Immortalized PNVECs were pretreated (16h) without (ethanol, 0.01%) or with (10 µM) lovastatin, followed by TNF-α (10 ng/ml, 4h) and changes in intracellular MCP-1 were quantified by cell-based ELISA. Data shown are the means ± SEM (n=8). *, p<0.01; one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 25. Lovastatin does not attenuate TNF-α mediated increases in intracellular ICAM-1 protein expression. Semiconfluent cultures of immortalized PNVECs were pretreated overnight without (ethanol, 0.01%) or with (10 µM) lovastatin ±10 µM GGPP, followed by incubation with TNF-α (10 ng/ml, 16h). Data shown are Western immunoblots, representative of 5 separate experiments.
Statins attenuate TNF-α mediated secretion of MCP-1

As shown in Fig. 15, quiescent PNVEC cultures respond to TNF-α by releasing MCP-1 into the culture medium. The mechanism by which this occurs is unclear, but may involve a GTPase-regulated vesicular release process (Knipe et al. 2010, Oynebraten et al. 2004, Oynebraten et al. 2005). Immortalized PNVEC cultures were pre-treated overnight with a maximal dose (10 µM) of lovastatin, simvastatin, or pravastatin. TNF-α (10 ng/ml, 4h) robustly enhanced MCP-1 protein secretion from vehicle-pretreated cultures (Fig. 26). By comparison, lovastatin (10 µM) modestly (~20%), but significantly, reduced TNF-α mediated MCP-1 secretion (Fig. 26). Pretreatment with simvastatin (10 µM) attenuated TNF-α mediated MCP-1 secretion by nearly 50% whereas pravastatin (10 µM), a hydrophilic statin, was without effect (Fig. 26). Interestingly, the structures of lovastatin and simvastatin are quite similar (Table 1, Chapter 2), differing by one methyl group. This highlights the differing pharmacological properties of statins and may warrant further investigation. The ability of simvastatin to attenuate TNF-α mediated MCP-1 secretion was dose-dependent, with 20 µM simvastatin reducing stimulated MCP-1 release by nearly 75% (Fig. 27A). Interestingly, the inhibitory effect of simvastatin on TNF-α mediated MCP-1 secretion was completely prevented by co-incubation with GGPP (Fig. 27). Of particular relevance, simvastatin similarly attenuated by approximately 40% TNF-α mediated MCP-1 secretion from primary PNVEC cultures, an effect that was also prevented by co-incubation with GGPP (Fig. 28). As indicated in Chapter 3, immortalized PNVEC cultures respond rather robustly to TNF-α, releasing MCP-1 nearly an order of magnitude greater than seen with
primary PNVEC cultures (Fig. 15). Whereas the quantity of MCP-1 released in response to TNF-α is clearly enhanced by cell transformation, the inhibitory effect of simvastatin on this mechanism remains quantitatively evident in primary cell cultures (Fig. 28). Collectively, these findings strongly support a role of post-translational geranylgeranylation of proteins, possibly small monomeric GTPases, in TNF-α mediated MCP-1 secretion from rat PNVEC cultures.
Figure 26. Statin mediated attenuation of TNF-α mediated increases in MCP-1 protein secretion. Immortalized PNVECs were pretreated (16h) without (ethanol, 0.01%) or with (10 µM each) lovastatin (Lov), simvastatin (Simv), or pravastatin (Prav), followed by TNF-α (10 ng/ml, 4h). Changes in secreted MCP-1 were quantified by ELISA. Data shown are the means ± SEM (n=3). #, p<0.05; *, p<0.01; one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 27. Simvastatin attenuates TNF-α mediated MCP-1 secretion. Immortalized PNVECs were pretreated (16h) without (ethanol, 0.01%) or with simvastatin (1-20 µM) ± GGPP (10 µM), followed by TNF-α (10 ng/ml, 4h) as indicated. Changes in secreted MCP-1 were quantified by ELISA. Data shown are the means ± SEM (A: n=3-6, B: n=3-6). *, p<0.01; one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 28. Simvastatin attenuates TNF-α mediated MCP-1 secretion in primary PNVECs. Primary PNVECs were pretreated without (ethanol, 0.01%) or with simvastatin (Sim, 10 µM) ± GGPP (10 µM) followed by TNF-α (10 ng/ml, 4h). Changes in secreted MCP-1 were quantified by ELISA. Data shown are the means ± SEM (n=3). #, p<0.05; *, p<0.01; one-way ANOVA with Newman-Keuls’ post-hoc analysis.
Protein geranylgeranylation facilitates TNF-α mediated secretion of MCP-1

Statins may inhibit TNF-α mediated increases in MCP-1 secretion by limiting endogenous isoprenoid availability. Whereas studies are currently underway in our lab to quantify the effect of statins on isoprenoid absolute content in PNVEC cultures (data not shown), the effect of isoprenylation on TNF-α mediated MCP-1 secretion was determined by ELISA. Pre-treating immortalized PNVEC cultures with an inhibitor of geranylgeranyl transferase I (10 μM GGTI-298), but not with an inhibitor of farnesyl transferase (10 μM, FTI-277), markedly attenuated TNF-α mediated MCP-1 secretion by approximately 70%, compared with vehicle (DMSO) pre-treated controls (Fig. 29A). The effect of GGTI-298 was dose-dependent (Fig. 29B). As observed above for statins, TNF-α mediated MCP-1 secretion from primary PNVEC cultures was similarly reduced by ~50% following selective inhibition of geranylgeranyl transferase I (Fig. 30), an effect nearly identical to that seen in transformed cells (Fig. 29). It is important to emphasize, the inhibitory effects of simvastatin and GGTI-298 were not mimicked by pretreatment with FTI-277 (Fig. 30). These data strongly implicate the selective participation of a geranylgeranylated protein in TNF-α mediated MCP-1 secretion from peripheral nerve vascular endothelial cells.

To begin to investigate what geranylgeranylated protein facilitates MCP-1 secretion in PNVECs, the exoenzyme C3 transferase was used. C3 exoenzyme inhibits specific Rho isoforms (RhoA, RhoB, and RhoC) by ADP-ribosylation in the effector
binding domain. Other Rho family members, Cdc42 and Rac1, are not inactivated by C3 exoenzyme. Pre-treating immortalized cultures with C3 exoenzyme (4ug/ml, 6h) did not attenuate TNF-α mediated MCP-1 secretion (Fig. 31), indicating that Rho subfamily proteins are not involved in TNF-α mediated MCP-1 secretion in PNVECs. Other possible candidates include Rho family members Cdc42 and Rac1, and the geranylgeranylated Ras family GTPase, RalA.
**Figure 29. Inhibition of protein geranylgeranylation prevents TNF-α mediated MCP-1 secretion by transformed PNVECs.** Immortalized PNVECs were pretreated (16h) without (DMSO, 0.6%) or with GGTI-298 (1-20 µM) or FTI-277 (10 µM), followed by TNF-α (10 ng/ml, 4h), as indicated. Changes in secreted MCP-1 were quantified by ELISA. Shown are the means ± SEM (A: n=5-8, B: n=3-8). *, p<0.01; one-way ANOVA with Bonferroni’s post-test.
Figure 30. Inhibition of protein geranylgeranylation attenuates TNF-α mediated MCP-1 secretion in primary PNVECs. Primary PNVECs were pretreated without (DMSO, 0.6%) or with (10 µM each) GGTI-298 or FTI-277, followed by TNF-α (10 ng/ml, 4h). Changes in secreted MCP-1 were quantified by ELISA. Data shown are the means ± SEM (n=3). *, p<0.001; one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 31. C3 exoenzyme does not attenuate TNF-α mediated MCP-1 secretion in immortalized PNVECs. Immortalized PNVECs were pretreated (6h) without (glycerol, 0.5%) or with C3 exoenzyme (4 µg/ml), followed by TNF-α (10 ng/ml, 4h), as indicated. Changes in secreted MCP-1 were quantified by ELISA. Shown are the means ± SEM (n=5-8). *, p<0.01; one-way ANOVA with Bonferroni’s post-test.
Protein geranylgeranylation facilitates MCP-1-dependent transendothelial migration of human THP-1 monocytes

Disrupting posttranslational geranylgeranylation of intracellular proteins significantly attenuates the ability of TNF-α to promote MCP-1 secretion without affecting net changes in MCP-1 mRNA or protein expression. The functional consequence of this measure was next assessed using a Transwell migration assay. Immortalized PNVEC cultures were seeded onto Transwell permeable inserts at a density (1 x 10^5/insert) sufficient to establish a confluent monolayer. Separate confluent parallel PNVEC cultures established on 24-well plates were pre-treated overnight (16h) with vehicle (DMSO) or with (10 µM each) GGTI-298 or FTI-277, followed by activation with TNF-α (10 ng/ml, 4h). Conditioned media was collected from pre-treated cultures and an aliquot added to the lower chamber of a Transwell plate. Migration across the established endothelial monolayers was initiated by the addition of CCR2-expressing human THP-1 monocytes. As shown in Figs. 16 & 32, conditioned media promotes transendothelial migration of human THP-1 monocytes. In contrast, conditioned media collected from GGTI-298, but not FTI-277, pre-treated PNVEC cultures exhibited a marked reduction in chemotactic properties (Fig. 32).

Geranylgeranylation promotes intracellular trafficking of MCP-1 protein

Monomeric GTPases play a key role in the regulation of intracellular vesicle transport and are similarly post-translationally isoprenylated prior to functional activation (Burgoyne & Morgan 2003, de Leeuw et al. 2001, Fish et al. 2007). Thus, the effect of
disrupting protein geranylgeranylation on intracellular trafficking of MCP-1-containing vesicles (Knipe et al. 2010) within PNVEC cultures was investigated. Whereas disrupting protein prenylation had no effect on TNF-α mediated MCP-1 protein expression (Fig. 24), the qualitative distribution of MCP-1 protein-containing vesicles was markedly altered when immortalized PNVEC cultures were pre-treated overnight with GGTI-298 (10 µM) followed by stimulation with TNF-α (10 ng/ml, 4h; Fig. 33). In vehicle-pretreated cells, punctate, vesicle-like structures and a diffuse distribution can be appreciated (arrows). In cells that were pretreated with GGTI-298, MCP-1 exhibits a restricted distribution and appears to be sequestered within an intracellular compartment resembling the golgi (Oynebraten et al. 2004, Oynebraten et al. 2005). These results indicate that a geranylgeranylated GTPase is involved in the trafficking and localization of MCP-1 in PNVECs.
**Figure 32. Disruption of geranylgeranylation attenuates transendothelial migration.** Immortalized PNVECs were treated without (DMSO, 0.6%) or with (10 µM each) GGTI-298 or FTI-277, followed by TNF-α (10 ng/ml, 4h). MCP-1-containing conditioned media was transferred to the lower chamber of a Transwell plate. THP-1 monocytes were allowed to migrate (4h) into the conditioned media across a monolayer of PNVECs that had previously been seeded on a permeable Transwell insert. Number of migrated cells was counted with a hemocytometer. Data shown are the means ± SEM (n=6). *, p<0.01; one-way ANOVA with Bonferroni’s post-hoc analysis.
Figure 3. Geranylgeranyl transferase inhibitor disrupts intracellular MCP-1 protein distribution in immortalized PNVECs. Effect of GGtI-298 pretreatment (10 µM, 16h) on TNF-α (10 ng/ml, 4h)-mediated expression of intracellular MCP-1 protein. Arrows indicate punctate, vesicle-like structures.
Discussion

Vascular endothelial cells harvested and purified from rat sciatic nerve were used in this study as an in vitro model of a localized vascular bed to further investigate the mechanism by which tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine implicated in the early pathogenesis of Guillain-Barré syndrome (GBS), mediates robust migration (trafficking) of autoreactive leukocytes into peripheral nerves. It was previously shown that TNF-α elicits a marked dose- and time-dependent increase in chemokine monocyte chemotactic protein-1 (MCP-1) and intercellular cell adhesion molecule-1 (ICAM-1) expression and MCP-1 release (Chapter 3). Here, these observations are extended by addressing the mechanism by which TNF-α induces MCP-1 expression and release from rat primary and immortalized PNVEC cultures. For the first time, secretion of functional MCP-1 and subsequent trafficking of CCR2-expressing monocytes is demonstrated to be regulated by a mechanism that involves the post-translational geranylgeranylation of an, as yet, undefined protein. Geranylgeranylation was found to facilitate the intracellular distribution of expressed MCP-1 protein, strongly implicating the participation of a geranylgeranylated monomeric GTPase in intracellular trafficking or vesicle release of MCP-1. Whereas the identity of this putative monomeric GTPase remains unclear, preliminary studies performed here have ruled out expected regulators of intracellular vesicular trafficking, including Rho subfamily (Rho A, B, C) and Rab family of GTPases. It is argued that identifying the geranylgeranylated protein
target (Rac1, Cdc42, RalA) that mediates chemokine secretion will significantly advance
the development of novel treatment options for patients with GBS.

TNF-α is thought to play a pivotal role in the early pathogenesis of GBS,
preumably by promoting the recruitment and trafficking of autoreactive leukocytes
across the blood-nerve barrier and into peripheral nerves (Hartung et al. 1995, Hughes &
Cornblath 2005). Whereas the mechanism by which pro-inflammatory cytokines like
TNF-α contribute to peripheral nerve injury in GBS remains unclear, localized activation
of the peripheral nerve vascular endothelium has been suggested as an initiating
pathological insult (Hartung et al. 1995, Hughes & Cornblath 2005). One way TNF-α
may activate the peripheral nerve vascular endothelial barrier may involve increased
localized expression and release of chemoattractants such as MCP-1. Indeed, studies in
Chapter 3 clearly demonstrate robust and rapid increases in MCP-1 expression and
release from TNF-α treated peripheral nerve vascular endothelial cell (PNVEC) cultures.
Also observed, subsequent to MCP-1 expression and release, was a marked increase in
ICAM-1 expression. These findings agree with current literature (Orlikowski et al. 2003,
Putzu et al. 2000, Stoll et al. 1993) and strongly support a major role of TNF-α, MCP-1,
and ICAM-1 as initiators of localized immune responses in the early pathogenesis of
GBS. The mechanism by which TNF-α elicits changes in MCP-1 and ICAM-1 expression
and MCP-1 release from peripheral nerve vascular endothelial cells has not been
addressed, and was focus of this study.

TNF-α elicits changes in gene expression, in part, by a mechanism involving Rho
this occurs is not yet clear. Here, TNF-α treatment led to an increase in active RhoA (Fig. 18). It follows that disrupting the endogenous activation of Rho GTPases may compromise the ability of TNF-α to subsequently facilitate NFκB-dependent expression of MCP-1 and ICAM-1 (Gawaz et al. 1998). However, in stark contrast with what has been demonstrated in the literature (Hippenstiel et al. 2002, Perona et al. 1997, Xu et al. 2006), limiting the activation of Rho GTPases (Von Zee et al. 2009) in PNVEC cultures with isoprenylation inhibitors did not alter TNF-α induced increases in MCP-1 or ICAM-1 mRNA content in this study. These findings are not without precedence, as previous studies by multiple groups have demonstrated either no inhibitory effect or a potentiation of cytokine-mediated CAM expression with isoprenylation inhibitors (Eccles et al. 2008, Sawa et al. 2007, Schmidt et al. 2002). These findings may best be explained by cell-type specific mechanisms governing cytokine-mediated changes in MCP-1 or CAM expression during an inflammatory response.

In contrast to a lack of effect on induced mRNA or protein expression, disrupting endogenous isoprenylation within PNVEC cultures with lovastatin, simvastatin, or GGTI-298 (but not with FTI-277) elicited a statistically significant reduction in TNF-α mediated MCP-1 protein secretion. In vascular endothelial cells, chemokines including MCP-1 are reportedly stored in novel regulated secretory granules that are distinct from the Weibel-Palade body (Knipe et al. 2010). Upon exposure to an inflammatory agonist, these vesicles rapidly translocate to the cell surface where they release their chemotactic contents into the extracellular space (Deshmane et al. 2009, Knipe et al. 2010, Oynebraten et al. 2004, Oynebraten et al. 2005). ICAM-1 proteins are, of course, integral
constituents of plasma membranes and are not stored in regulated intracellular vesicular compartments. It is tempting to consider that MCP-1 protein released from TNF-α treated PNVEC cultures may act in an autocrine manner to promote ICAM-1 expression. This, however, does not appear to be of relevance in PNVEC cultures. Disrupting isoprenylation with GGTI-298 clearly attenuates TNF-α mediated MCP-1 release from PNVEC cultures without effecting ICAM-1 expression (Fig. 29 and 30).

It is well established that select members of the Ras superfamily of small monomeric GTPases intimately regulate intracellular vesicle trafficking and regulated exocytosis (Burgoyne & Morgan 2003, Wennerberg et al. 2005). Members of the Rab family are best considered part of the conserved machinery involved in vesicle docking and release (Burgoyne & Morgan 2003). Moreover, Rab GTPases are geranylgeranylated. However, the enzyme responsible for catalyzing geranylgeranylation of Rab GTPases (geranylgeranyl transferases II) is not inhibited by GGTI-298 (a selective inhibitor of geranylgeranyl transferase I). This suggests, to us, that a geranylgeranylated protein other than Rab is responsible for facilitating TNF-α mediated MCP-1 release from PNVEC cultures.

Alternatively, geranylgeranyl transferase I catalyzes the functional activation of a number of other Ras related GTPases, including members of the Rho family (Rho, Rac, and Cdc42). However, pre-treating PNVEC cultures with C3 exoenzyme, a specific inhibitor of the Rho subfamily isoforms (RhoA, RhoB, and RhoC), did not attenuate TNF-α mediated MCP-1 secretion. Rac1 or Cdc42 Rho GTPases remain possible candidates. A precedence for Cdc42 as a facilitator of cytokine-mediated secretion of von
Willebrand Factor in HUVEC cultures has been established (Fish et al. 2007).

Transfecting HUVEC cells with a dominant negative mutant of Cdc42 impaired cytokine-mediated MCP-1 secretion. Interestingly, dominant negative mutants of Rho or Rac GTPase had no effect (Fish et al. 2007).

RalA is another Ras related GTPase that is functionally activated by geranylgeranyl transferase I and may be involved in cytokine regulated vesicle secretion. RalA GTPases are generally not considered mediators of inflammation but rather are involved in cell cycle progression. Of interest, RalA has been recently demonstrated to be involved in regulated secretion (de Leeuw et al. 2001, Falsetti et al. 2007, Rondaij et al. 2004, van Dam & Robinson 2006). It is argued that identifying this precise geranylgeranylated target (Rac1, Cdc42, RalA) that facilitates TNF-α mediated increases in MCP-1 secretion will significantly advance the development of novel treatment options for patients with GBS.
CHAPTER 5
DISCUSSION

Summary of the main findings

1. Peripheral nerve vascular endothelial cells (PNVECs) immortalized by the method of SV40 large T antigen transduction retain their primary characteristics. These include monolayer formation, a cobblestone-like morphology, contact-dependent inhibition, and von Willebrand Factor and PECAM-1 expression.

2. Primary and transformed peripheral nerve vascular endothelial cells upregulate monocyte chemoattractant-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) expression and functional MCP-1 secretion in response to treatment with the proinflammatory cytokine tumor necrosis factor alpha (TNF-α).

3. Statins and prenyltransferase inhibitors have no effect on TNF-α mediated MCP-1 mRNA and intracellular protein expression, or on ICAM-1 mRNA and intracellular protein expression.

4. Lovastatin and simvastatin, but not pravastatin, significantly attenuate TNF-α mediated MCP-1 secretion and transendothelial migration in primary and transformed PNVECs. This effect is mimicked by an inhibitor of protein geranylgeranylation (GGTI-298) but not farnesylation (FTI-277).
5. GGTI-298 appears to disrupt the intracellular distribution of MCP-1 protein in TNF-α treated PNVECs. This implicates a geranylgeranylated GTPase in vesicle localization and intracellular trafficking of MCP-1.

**Revised hypothesis, based on these main findings:**

Statins limit the migration of autoreactive leukocytes across the blood-nerve barrier during EAN by inhibiting cytokine-mediated, GTPase-dependent MCP-1 release from peripheral nerve vascular endothelial cells.

**Discussion**

These *in vitro* studies were designed to elucidate the mechanisms that facilitate the pathological expression of inflammatory mediators by the blood-nerve barrier. Aberrant transendothelial migration of autoreactive leukocytes across the blood-nerve barrier and into the endoneurial compartment is a key feature in the pathogenesis of GBS/AIDP (Hartung *et al.* 1995). Defining these mechanisms may lead to the identification of new therapeutic targets, and this ultimately may provide patients who are struggling with GBS/AIDP with specific, novel therapeutic options which they so direly need.

Experiments were performed using the rat peripheral nerve endothelial cells (PNVECs) that form the blood-nerve barrier. There exists a handful of published and well-characterized blood-brain barrier endothelial cell lines (RBE4 (Roux *et al.* 1994), GP8/3.9 (Greenwood *et al.* 1996), GPNT (Regina *et al.* 1999), RBEC1 (Ishiguro *et al.*
2004), TR-BBBs (Terasaki & Hosoya 2001) and rBCEC4 (Blasig et al. 2001)). While advancements have been made toward the purification and culture of primary vascular endothelial cells derived from peripheral nerve (Argall et al. 1994, Sano et al. 2007, Yosef et al. 2010), experimental data using these cells is lacking. To date, no studies have addressed how peripheral nerve vascular endothelial cells respond to a localized inflammatory challenge such as that experienced during GBS/AIDP.

In these studies, viable cultures of primary vascular endothelial cells from rat sciatic nerve were routinely prepared at > 95% purity, in accordance with previous reports (Argall et al. 1994). However, primary cells bear many limitations. Indeed, the limited life span and the sensitivity to the complement-mediated cell lysis procedure used to purify cultures proved to be insurmountable technical hurdles. The lack of a commercially available cell line required the exploration of methods of immortalization. The method of SV40 large T antigen transduction facilitated the production of stably transformed rat PNVEC clones using a replication-deficient SV40 retrovirus that encodes a temperature sensitive, non-SV40-origin binding mutant of the large T antigen. This method has been successfully used to immortalize primary vascular endothelial cells harvested from rat brain and rat aorta with retention of their primary characteristics (Greenwood et al. 1996, Jat et al. 1986, Jat & Sharp 1986). Rat primary PNVEC cultures immortalized in this manner consistently formed contact-inhibited monolayers with a cobblestone-like morphology and constitutively expressed von Willebrand Factor/Factor VIII and PECAM-1, cell markers highly characteristic of differentiated vascular endothelial cells. A single isolated rat PNVEC clone (designated here as 4.3) exhibiting
stable characteristics of primary cells was subsequently used throughout the three specific aims to functionally assess how peripheral nerve endoneurial homeostasis may be altered during an acute inflammatory episode.

In GBS/EAN, proinflammatory cytokines, in particular TNF-α, facilitate the aberrant trafficking of autoreactive leukocytes across the blood-nerve barrier and into peripheral nerves. This involves localized, temporal increases in chemotactic cytokines, including MCP-1 (Yadav et al. 2010), followed by adhesion molecules, including ICAM-1 (Putzu et al. 2000). Consistent with this thesis, in Chapter 3 it was demonstrated that TNF-α rapidly (within 1h) and markedly (~50-fold) increases MCP-1 mRNA content in immortalized PNVECs. A similar, although slightly less robust, increase was seen in primary PNVECs. Importantly, TNF-α also subsequently (4h) elicited significant increases in the content of intracellular MCP-1 protein and release of functional MCP-1 into the culture media. A similar time-dependent enhancement of ICAM-1 mRNA (2h) and protein (16h) expression was observed in response to TNF-α treatment in both primary and immortalized cells. The skewed time course reflects the different temporal dynamics of CAM expression as compared to chemokine expression that have been reported in the literature (Springer 1994, Raab et al. 2002). Concentrations of TNF-α required to elicit measurable changes in MCP-1 and ICAM-1 mRNA and protein expression within PNVECs were 10-100 fold higher than reported physiological levels detected in plasma of patients with GBS (Radhakrishnan et al. 2004, Reuben et al. 2002). However, it can be argued that the localized concentration of TNF-α within peripheral
nerves during an immune challenge may very well exceed concentrations used in this study.

The experiments described in Chapter 4 were designed to investigate the mechanisms that facilitate TNF-α mediated MCP-1 release and ICAM-1 expression that was observed in Chapter 3. Multiple groups have suggested active monomeric GTPases as a necessary intermediate in the cytokine-mediated expression of these inflammatory mediators (Hippenstiel et al. 2002, Williams et al. 2008, Zhao & Pothoulakis 2003). It is well-documented that statins, by depleting the availability of isoprenoids, disrupt the activation of monomeric GTPases (Zhang & Casey 1996). Thus, to test this thesis, primary and immortalized PNVECs were pretreated with statins (lovastatin, simvastatin, or pravastatin) prior to TNF-α challenge. Both lovastatin and simvastatin significantly attenuated TNF-α mediated secretion of functional MCP-1 protein in both primary and transformed PNVECs, while exhibiting no effect on MCP-1 mRNA or intracellular protein expression, or ICAM-1 mRNA or intracellular protein expression. The current state of the literature is contradictory (Greenwood & Mason 2007). Some groups have demonstrated that statins attenuate chemokine (Veillard et al. 2006) and CAM expression (Zapolska-Downar et al. 2004), while others have demonstrated no effect (Eccles et al. 2008, Nubel et al. 2004), or even a potentiating effect of statins (Dimitrova et al. 2003, Schmidt et al. 2002).

The use of statins as novel therapeutic agents has been explored in a number of other disease models, including experimental autoimmune encephalitis (Stanislaus et al. 2002, Stanislaus et al. 1999), adjuvant-induced arthritis (Leung et al. 2003), experimental
ocular inflammation (Kohno et al. 2007), and experimental colitis (Jahovic et al. 2006). It has been demonstrated that a short-term, high dose course of lovastatin attenuates the development and progression of experimental autoimmune neuritis EAN, when administered prophylactically or therapeutically (Sarkey et al. 2007). Generally, statins have been demonstrated to reduce transendothelial migration in vivo in these experimental models of inflammatory diseases, although the mechanisms by which this occurs remain poorly defined. These in vivo experiments have led to a number of studies examining the efficacy of statins in managing some corresponding human conditions, including multiple sclerosis (Paul et al. 2008, Sorensen et al. 2011), rheumatoid arthritis (Okamoto et al. 2007, Goto 2010, Leung et al. 2003), and normal tension glaucoma (Leung et al. 2010). However, more research is needed for statins to become FDA-approved for these uses.

A number of hurdles exist in terms of statins being repurposed as novel treatments for disease conditions other than hyperlipidemia. While generally well-tolerated and safe with normal use (Guthrie 2006, Escobar et al. 2008), statins have been reported to cause myopathy (Tomaszewski et al. 2011) and rhabdomyolysis (Tiwari et al. 2006). Although rare, these side effects can be fatal (Baek et al. 2011, Federman et al. 2001). In addition to rare but well-documented myotoxic effects, a number of recent correlational studies have implicated statin use with additional unwanted side effects. Quite recently, meta-analyses revealed an association between statin-use and the development of diabetes (Preiss & Sattar 2011, Preiss et al. 2011, Preiss 2012). Despite the lack of any demonstrated causal association, statins now carry a black box warning from the FDA. In addition to these
reports of diabetes, correlative studies have also implicated statin-use with memory loss and cognitive impairment (Healy et al. 2009, Rojas-Fernandez & Cameron 2012). These side effects of statins appear to be dose-dependent, with higher doses of statins associated with higher risk (Baek et al. 2011, Preiss 2012, Kaski 2011, Mitka 2009). Despite these recent associations, it stands that the cardiovascular benefits of statins vastly outweigh any rare possible side-effect.

Due to these established and recent reports of side effects, especially associated with higher doses of statins, the focus has shifted from the repurposing of statins to the implementation of them as a valuable research tool, with the ultimate goal of identifying more specific interventions. As mentioned above, it has been demonstrated that statin-treatment attenuates the development and progression of EAN, in part by decreasing the transendothelial migration of autoreactive leukocytes (Sarkey et al. 2007). As described in chapter 4, it appears that statins may do so by attenuating cytokine-mediated secretion of MCP-1 by the blood-nerve barrier. These findings indicate that MCP-1 secretion, but not mRNA or protein expression, is dependent on protein isoprenylation. To determine the specific isoprenoid involved, PNVECs were pretreated with inhibitors of geranylgeranyl transferase (GGTI-298) or farnesyl transferase (FTI-277). GGTI, but not FTI, mimicked the inhibitory effects of simvastatin, indicating that a geranylgeranylated protein is necessary for TNF-α mediated MCP-1 secretion from PNVECs.

Unexpectedly, geranylgeranylation appears to facilitate to intracellular distribution of MCP-1 protein. GGTI-298 pretreatment resulted in a disrupted intracellular distribution of MCP-1 in TNF-α treated PNVECs, as compared to vehicle-
pretreated PNVECs (Fig. 32). We demonstrate earlier that inhibiting isoprenylation does not affect the overall level of MCP-1 protein in the cell (Fig. 24). This suggests that a geranylgeranylated protein may be involved in the trafficking or vesicle release of MCP-1. MCP-1 has in fact been demonstrated to localize to novel storage granules, and released by regulated exocytosis (Knipe et al. 2010, Oynebraten et al. 2004, Oynebraten et al. 2005). Interestingly, a few geranylgeranylated GTPases have been demonstrated to be involved in regulated exocytosis in endothelial cells, in particular Cdc42 (Fish et al. 2007) and RalA (de Leeuw et al. 2001, Falsetti et al. 2007, Rondaij et al. 2004, van Dam & Robinson 2006). Proposed future studies will use dominant negative (-CaaX mutant) Cdc42 and RalA GTPases to continue to elucidate the mechanisms that regulate MCP-1 secretion. It is argued that identifying this geranylgeranylated protein has the potential to advance the development of novel, specific treatment options for patients suffering from Guillain-Barre syndrome and other autoimmune, inflammatory neuropathies.
**Figure 34. Putative mechanism.** In the left panel, TNF-α leads to an increase in MCP-1 mRNA content, intracellular protein content, and, in a process that depends on an active, geranylgeranylated GTPase, trafficking and release. The right panel represents this process in the presence of the geranylgeranyltransferase inhibitor GGTI-298. TNFα increases mRNA and intracellular protein content of MCP-1; however, this intracellular MCP-1 protein is sequestered within the cell and is not released.
REFERENCES


VITA

The author, Kelly Ann Langert (nee Clinkenbeard), was born on July 4, 1984 in Racine, WI to Dave Clinkenbeard and Peggy Smithana. She graduated from Washington Park High School in Racine in June 2002 and enrolled at the University of Wisconsin-Madison in Madison, Wisconsin in August 2002. In 2006, she received her Bachelor of Science degree from the University of Wisconsin with majors in neurobiology and psychology.

In August of 2006, Kelly entered the graduate program at Loyola University Chicago as a pre-doctoral candidate in the interdepartmental Neuroscience Program. She joined the laboratory of Dr. Evan B. Stubbs in the summer of 2007 and began researching methods of statin-dependent neuroprotection in experimental autoimmune neuritis. Between 2008 and 2012, Kelly focused on the mechanisms by which statins attenuate the expression of inflammatory mediators at the blood-nerve barrier, \textit{in vitro}. During this time, she has won awards for her research presentations at local scientific conferences, and has presented posters at international scientific meetings. She also applied for, and received, a pre-doctoral health and rehabilitation research fellowship from the Department of Veterans Affairs in 2010, and numerous travel grants for meeting attendance. Kelly is a student member of the American Society for Neurochemistry, and the Society for Neuroscience.