

# Tumour necrosis factor $\alpha$ enhances CCL2 and ICAM-1 expression in peripheral nerve microvascular endoneurial endothelial cells

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## ABSTRACT

Recruitment and trafficking of autoreactive leucocytes across the BNB (blood–nerve barrier) is an early pathological insult in GBS (Guillain-Barré syndrome), an aggressive autoimmune disorder of the PNS (peripheral nervous system). Whereas the aetiology and pathogenesis of GBS remain unclear, pro-inflammatory cytokines, including TNF $\alpha$  (tumour necrosis factor  $\alpha$ ), are reported to be elevated early in the course of GBS and may initiate nerve injury by activating the BNB. Previously, we reported that disrupting leucocyte trafficking *in vivo* therapeutically attenuates the course of an established animal model of GBS. Here, PNMECs (peripheral nerve microvascular endothelial cells) that form the BNB were harvested from rat sciatic nerves, immortalized by SV40 (simian virus 40) large T antigen transduction and subsequently challenged with TNF $\alpha$ . Relative changes in CCL2 (chemokine ligand 2) and ICAM-1 (intercellular adhesion molecule 1) expression were determined. We report that TNF $\alpha$  elicits marked dose- and time-dependent increases in CCL2 and ICAM-1 mRNA and protein content and promotes secretion of functional CCL2 from immortalized and primary PNMEC cultures. TNF $\alpha$ -mediated secretion of CCL2 promotes, *in vitro*, the transendothelial migration of CCR2-expressing THP-1 monocytes. Increased CCL2 and ICAM-1 expression in response to TNF $\alpha$  may facilitate recruitment and trafficking of autore-

active leucocytes across the BNB in autoimmune disorders, including GBS.

Key words: cytokine, trafficking, endothelial cell, peripheral nerve

## INTRODUCTION

AIDP (acute inflammatory demyelinating polyneuropathy), the most prevalent North American and European variant of GBS (Guillain-Barré syndrome), is an aggressively debilitating autoimmune disorder that affects the PNS (peripheral nervous system) and remains a leading cause of autoimmune neuromuscular paralysis (Zhu et al., 1998). Although the aetiology of GBS remains unknown, the pathogenesis of GBS probably involves both humoral and cell-mediated immune responses (Quarles and Weiss, 1999; Hartung et al., 2001; Kiefer et al., 2001; Maurer et al., 2002; Ariga and Yu, 2005). Enhanced cytokine-mediated recruitment and trafficking of autoreactive leucocytes across the BNB (blood–nerve barrier) is an established early pathological hallmark of GBS (Hartung et al., 1995; Hughes and Cornblath, 2005) and EAN (experimental autoimmune neuritis), a well-characterized animal model that shares many pathological characteristics with AIDP (Spies et al., 1995; Hahn, 1996; Sarkey et al., 2007).

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**Abbreviations:** AIDP, acute inflammatory demyelinating polyneuropathy; BNB, blood–nerve barrier; CAM, cell adhesion molecule; CCL2, CC chemokine ligand 2; CSF, cerebrospinal fluid; DAPI, 4',6-diamidino-2-phenylindole; EAN, experimental autoimmune neuritis; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GBS, Guillain-Barré syndrome; HRP, horseradish peroxidase; ICAM-1, intercellular adhesion molecule 1; JAM1, junctional adhesion molecule 1; MEC, microvascular endothelial cell; NF- $\kappa$ B, nuclear factor  $\kappa$ B; PECAM-1, platelet endothelial cell adhesion molecule 1; PFA, paraformaldehyde; PNMEC, peripheral nerve microvascular endothelial cell; PNS, peripheral nervous system; RT-PCR, reverse transcription-PCR; SV40, simian virus 40; TEER, transendothelial electrical resistance; TNF $\alpha$ , tumour necrosis factor  $\alpha$ ; vWF, von Willebrand factor; ZO-1, zona occludens 1.

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Circulating autoreactive leucocytes may initially home to peripheral nerves by a mechanism that involves enhanced local gradients of chemokine expression (Zou et al., 1999; Kieseier et al., 2000). At these sites, proinflammatory cytokines, in particular TNF $\alpha$  (tumour necrosis factor  $\alpha$ ), play a pivotal role in the pathogenesis of both GBS and EAN, as recently reviewed by Lu and Zhu (Lu and Zhu, 2011). An increased presence of TNF $\alpha$  has been detected within affected peripheral nerves of GBS patients (Putzu et al., 2000). Others have demonstrated a strong correlation between elevated plasma concentrations of TNF $\alpha$  and clinical severity of GBS (Exley et al., 1994). Similarly, previous studies have demonstrated an enhanced presence of TNF $\alpha$  expressing macrophage infiltrates within nerve roots from rats with EAN (Stoll et al., 1993). Therapeutically, neutralizing TNF $\alpha$  or its homotrimeric cell surface receptor TNFR1 has been demonstrated to ameliorate the course of EAN (Stoll et al., 1993; Bao et al., 2003). Similarly, TNF $\alpha$  receptor knockout mice exhibit an attenuated course of EAN (Mao et al., 2010).

Chemotactic cytokines and immunoglobulin-like CAMs (cell adhesion molecules) are inflammatory mediators that are highly involved in leucocyte trafficking and transendothelial migration. In patients with GBS, disease severity correlates with enhanced expression of CCL2 (chemokine ligand 2) in CSF (cerebrospinal fluid), as well as by epineurial and endoneurial vascular endothelial cells (Orlikowski et al. 2003, Press et al. 2003). In EAN, marked enhancement of CCL2 expression is reported to preclude the onset of clinical deficits (Fujioka et al., 1999; Kieseier et al., 2000). Antibody-mediated neutralization of CCL2 *in vivo* ameliorates the course of EAN in rats (Zou et al., 1999). By comparison, expression of ICAM-1 (intercellular adhesion molecule 1), a CAM involved in the firm adhesion stage of leucocyte migration, is elevated in the CSF and by sural nerve endothelial cells in patients at the peak of GBS (Putzu et al., 2000; Sainaghi et al., 2010). In animal studies, similar increases in ICAM-1 mRNA and protein were detected in cauda equina harvested from Lewis rats with EAN (Stoll et al., 1993; Tran et al., 2010), and antibody-mediated neutralization of ICAM-1 attenuates the course of EAN (Archelos et al., 1993).

The mechanism by which inflammatory mediators contribute to peripheral nerve injury in GBS remains unclear. Given that (i) there are clear phenotypic and functional differences between vascular endothelium from different tissues (Yosef et al., 2010) and that (ii) recruitment and trafficking of leucocytes across the activated vascular endothelial barriers is most probably governed by changes in the expression of specific inflammatory mediators unique to the localized vascular bed (Springer, 1994; Harkness et al., 2003; Chui and Dorovini-Zis, 2010), we chose in this study to focus on TNF $\alpha$ -mediated activation of MECs (microvascular endothelial cells) harvested from the rat peripheral nerve. Although advancements have been made towards the purification and culture of MECs derived from peripheral nerve (Argall et al., 1994; Sano et al., 2007; Yosef et al., 2010), no studies to date have addressed how PNMEC (peripheral nerve microvascular

endothelial cell) cultures respond to a localized inflammatory challenge such as that experienced during AIDP/EAN. To address this concern, we harvested PNMECs from sciatic nerves of naïve Lewis rats and immortalized them by SV40 (simian virus 40) large T antigen transduction.

Here, we report the stable immortalization of rat PNMEC cultures and demonstrate their retention of key phenotypic, morphologic and biochemical characteristics expected of MECs from the endoneurium of rat peripheral nerve. Activation of primary or immortalized PNMEC cultures with TNF $\alpha$  elicited marked dose- and time-dependent changes in CCL2 and ICAM-1 expression and CCL2 release. Enhanced TNF $\alpha$ -mediated CCL2 secretion also facilitated chemotaxis of CCR2-expressing human THP-1 monocytes in an *in vitro* migration assay. We argue that increased CCL2 and ICAM-1 expression in response to TNF $\alpha$  may facilitate recruitment and trafficking of autoreactive leucocytes across the BNB in autoimmune disorders, including GBS. Collectively, these findings support the application of PNMEC cultures as a novel *in vitro* resource for the functional assessment of pathological alterations in endoneurial homeostasis during an inflammatory challenge, such as that encountered during GBS/EAN.

## MATERIALS AND METHODS

### PNMEC

This study was conducted using protocols approved by the Edward Hines Jr. VA Hospital Institutional Animal Care and Use Committee in accordance with the principles of laboratory animal care. Primary cultures of MECs were prepared from sciatic nerves of naïve Lewis rats as described previously (Argall et al., 1994; Sarkey et al., 2007). Briefly, harvested sciatic nerves were desheathed to remove the epineurium, perineurium and macrovascular endothelium. Remaining endoneurium was minced and digested with 0.05% (w/v) trypsin and 0.25% (w/v) collagenase at 37 °C for 2 h. After incubation, cells were collected, filtered and sedimented at 700 g for 5 min. The cell pellet was resuspended in *Endo media* [Ham's F10 basal media (Life Technologies) supplemented with 10% (v/v) FBS (foetal bovine serum), 50  $\mu$ g/ml ECGS (endothelial cell growth supplement; BD Biosciences), 0.4  $\mu$ g/ml heparin, 5.6  $\mu$ g/ml amphotericin B (Sigma-Aldrich), 100 unit/ml penicillin, and 100  $\mu$ g/ml streptomycin (Life Technologies)] and plated on a collagen-coated (6.0  $\mu$ g/cm<sup>2</sup>, rat tail collagen type I, Millipore) tissue culture flask under an atmosphere of 5% CO<sub>2</sub>/95% air. Initial cultures consisted of a mixed population of microvascular endoneurial endothelial cells, perineurial epithelioid myofibroblasts and Schwann cells (Yosef et al., 2010). Myofibroblasts were selectively eliminated by Thy1.1 antibody-mediated complement-dependent cell lysis (Argall et al., 1994). Cultures prepared in this manner

were  $95 \pm 7.5\%$  ( $n = 3$ ) pure,  $96 \pm 5\%$  ( $n = 12$ ; Trypan Blue dye exclusion) viable (means  $\pm$  S.D.), and consistently established contact-inhibited monolayers with a distinctive cobblestone-like morphology. Primary PNMEC cultures were restricted to six passages. Under these culture conditions, proliferation of microvascular endoneurial endothelial cells was optimal, whereas Schwann cells did not appreciably proliferate (Argall et al., 1994).

A stably immortalized PNMEC cell line was produced from purified rat primary PNMEC cultures 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 (gift from Dr. P. Jat, University College of London, London, U.K.). Briefly, semi-confluent primary PNMEC cultures were incubated for 36 h at 37 °C in the presence of 8  $\mu$ g/ml polybrene (Sigma-Aldrich) with undiluted filtered viral supernatant collected from SVU19.5 producer cells (Jat et al., 1986; Jat and Sharp, 1986). Selection was achieved by passage into *Endo media* containing 200  $\mu$ g/ml gentamycin (G418; Life Technologies). Antibiotic resistant clones were isolated by dilution into 96-well plates at a theoretical density of 0.33 cells/well. Several clones exhibiting phenotypic (contact-inhibition), morphologic (cobblestone-like appearance) and biochemical [expression of vWF (von Willebrand Factor)/Factor VIII and PECAM-1 (platelet endothelial cell adhesion molecule 1)] properties characteristic of primary MECs were expanded, and a single clone (clone 4.3) was arbitrarily selected for use throughout this study. Immortalized PNMEC cultures were maintained on collagen-coated flasks in *Endo media* at 37 °C under an atmosphere of 5% CO<sub>2</sub>/95% air.

For migration experiments, a CCR2 expressing human acute monocytic leukaemia cell line (THP-1; a gift from Dr. E. Kovacs, Loyola University Chicago) was maintained in RPMI 1640 containing 10% FBS, 100 unit/ml penicillin, 100  $\mu$ g/ml streptomycin and 5 mM 2-mercaptoethanol (Sigma-Aldrich).

### TEER (transendothelial electrical resistance)

Immortalized PNMEC cultures ( $1 \times 10^5$  cells/insert) were passaged onto collagen-coated Transwell<sup>®</sup> microporous (5.0  $\mu$ m pore size, 0.33 cm<sup>2</sup> growth area) inserts in 24-well tissue culture plates. TEER was measured with a DT92 Advanced Series Digital Multimeter (World Precision Instruments) at 24 h intervals after passaging. Collagen coated inserts without cultured cells served as negative controls, and these resistance values were subtracted from experimental resistance values. Measurements were taken in triplicate for each insert and averaged to ensure uniformity.

### Immunocytochemistry

Semi-confluent rat primary or immortalized PNMEC cultures grown on collagen-coated coverslips were fixed for 5 min at 23 °C by immersion in phosphate-buffered (pH 7.4) 4% PFA (paraformaldehyde), washed and blocked with 1% (v/v) normal goat serum and 0.1% (v/v) Triton X-100. Coverslips

were incubated overnight (4 °C) in the presence of a 1:50 dilution of mouse anti-human Factor VIII/vWF (Dako) or a 1:50 dilution of rabbit anti-rat PECAM-1 (Serotec) monoclonal antibody. Washed coverslips were incubated with a 1:1000 dilution of either AlexaFluor488-conjugated goat anti-mouse IgG secondary antibody (Life Technologies) or FITC-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Labs, Inc.), respectively. Coverslips were mounted onto slides using Fluoroshield containing DAPI (4',6-diamidino-2-phenylindole, Sigma-Aldrich) and visualized with a Leica DMLB upright fluorescent microscope. Images were captured using QCapture Pro software (Q Imaging, Inc.).

### Immunoblot analysis

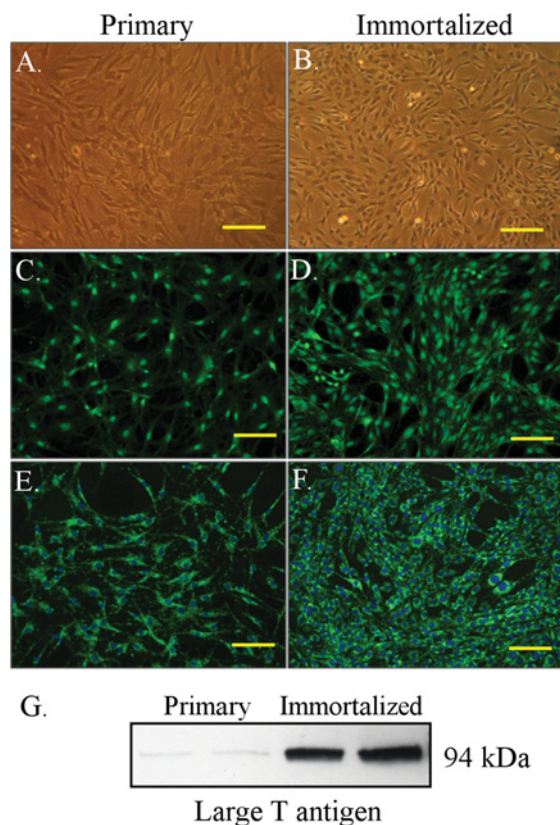
PNMEC cultures were harvested by gentle scraping and lysates were prepared in deionized water supplemented with a commercial cocktail of protease inhibitors (Roche Applied Science). Protein concentrations in cell lysates were quantified by the BCA method (ThermoScientific) using BSA as the standard. Proteins in cell lysates were resolved by SDS/PAGE and transferred onto nitrocellulose membranes as previously described (Von Zee and Stubbs, 2011). Washed membranes were blocked and incubated overnight at 4 °C in the presence of primary antibody (1:10 dilution of mouse anti-rat large T antigen (provided by Dr. P. Jat), 1:4000 dilution of rabbit anti-rat CCL2 polyclonal, or 1:100 dilution of mouse anti-rat ICAM-1 monoclonal antibody (Serotec). Washed membranes were incubated for 1 h at 23 °C in the presence of HRP (horseradish peroxidase)-conjugated goat anti-mouse IgG (1:2500 dilution) or goat anti-rabbit IgG (1:10000 dilution) secondary antibody (Jackson ImmunoResearch). Immunostained proteins were visualized by ECL (enhanced chemiluminescence; ThermoScientific). To confirm equal protein loading, immunoblots were separately incubated overnight at 4 °C in the presence of a 1:10000 dilution of rabbit anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) polyclonal antibody (Trevigen) followed by washing, incubation with HRP-conjugated goat anti-rabbit IgG (1:10000 dilution) secondary antibody and ECL detection.

### Real-time RT-PCR (reverse transcription-PCR)

Total RNA was extracted from rat primary and immortalized PNMEC cultures with TRIzol reagent, and 5  $\mu$ g was reverse-transcribed using Super Script III First Strand Synthesis System (Life Technologies) as previously described (Von Zee et al., 2009). CCL2, ICAM-1, and tight junction associated specific cDNA sequences were amplified by real-time PCR on a Mini-Opticon PCR detection system using iQ SYBR Green Supermix (Bio-Rad) with the rat-specific primer pairs listed in Table 1. For each sample, GAPDH (Table 1) was used as a reference control. Optimized amplification steps of 94 °C for 5 min; 94 °C for 15 s, annealing at 55–60 °C for 30 s,

**Table 1** Rat primer pairs used in RT-PCR analysis

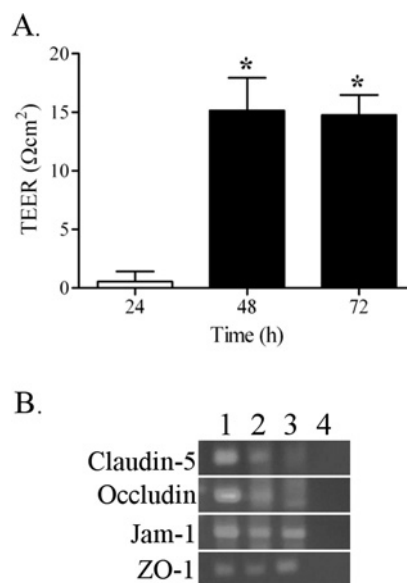
| Molecule  | Sense (5'→3')          | Antisense (5'→3')        | Amplicon size (bp) | Annealing temperature (°) |
|-----------|------------------------|--------------------------|--------------------|---------------------------|
| ICAM-1    | CTGCAGAGCACAACAGCAGAG  | AAGCCGCAGAGCAAAAAGAAGC   | 332                | 60                        |
| CCL2      | ATGCAGGTCTGTGTCACG     | CTAGTCTCTGTGTCATACT      | 447                | 55                        |
| Claudin-5 | GCAGAGCACCGGGCACATGC   | TAGTTCCTCTGTGCGTAATCGCC  | 483                | 55                        |
| Occludin  | GCCTTTTGCTTCATCGCTTCC  | AACAATGATTAAGCAAAAAGCCAC | 351                | 55                        |
| JAM-1     | ACAGCCATGAGGTCAGAGGCT  | ACCTAGAAGACATTGAAGGCATC  | 348                | 55                        |
| ZO-1      | GCGAGGCATCGTTCCTAATAAG | TCGCCACCTGCTGTCTTTG      | 81                 | 60                        |
| GAPDH     | TCCTCAAGATTGTCAGCAA    | AGATCCACAACGGATACATT     | 308                | 55                        |



**Figure 1** Immortalized PNMEC cultures retain their primary characteristics

(A, B) Phase-contrast or (C–F) immunofluorescent photomicrographs of primary or immortalized PNMEC cultures, as indicated, immunostained for expression of (C, D) vWF or (E, F) PECAM-1 (CD31). Data shown are photomicrographs of stained cells representative of six separate cultures. Nuclei are counterstained with DAPI. Bar, 100  $\mu$ m. (G) Western immunoblot of large T antigen protein expressed in lysates prepared from primary or immortalized PNMEC cultures, as indicated. Data shown are from a single experiment performed in duplicate.

72 °C for 1 min were used. Reaction efficiencies were typically >90%. For each sample, the specificity of the real-time reaction product was determined by melting curve analysis. In some cases, amplified products were visualized by resolution on a 2% (w/v) agarose gel impregnated with ethidium bromide. The size of each amplified product was compared with a 0–500-bp ladder. In all cases, the amplified product



**Figure 2** Barrier properties of immortalized PNMEC cultures

(A) TEER values of immortalized PNMECs cultured on collagen-coated inserts. Data shown are results from a single experiment ( $n = 5$ ). \*  $P < 0.01$ ; one-way ANOVA with Dunnett's *post-hoc* analysis. (B) Qualitative comparison of tight junction associated molecules claudin-5, occludin, JAM1 and ZO-1 expressed in (1) primary PNMEC cultures, (2) immortalized PNMEC cultures and (3) rat brain as determined by RT-PCR. Lane (4), no template control.

corresponded with the predicted amplicon size. Endogenous expression of GAPDH was unaltered by TNF $\alpha$  treatment. Relative fold-changes in CCL2 and ICAM-1 mRNA content in each sample were therefore normalized to expressed levels of GAPDH.

**ELISA**

Relative changes in intracellular CCL2 or ICAM-1 protein content were quantified using a cell-based ELISA. Briefly, confluent rat primary or immortalized PNMECs were cultured on collagen-coated 96-well plates and fixed with phosphate-buffered (pH 7.4) 4% PFA for 10 min at 23 °C. Fixed cells were permeabilized with 0.1% (v/v) Triton X-100 and blocked for 1 h at 23 °C with 1% (w/v) BSA. Fixed PNMEC cultures were incubated overnight at 4 °C in the presence of either a 1:2000 dilution of rabbit anti-rat CCL2 polyclonal antibody

or a 1:500 dilution of mouse anti-rat ICAM-1 monoclonal antibody (Serotec). Immunostained cells were washed and incubated for 1 h at 23 °C in the presence of a 1:5000 dilution of HRP-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (Jackson ImmunoResearch), respectively. Colour development was initiated with SigmaFast OPD substrate (Sigma-Aldrich) for 30 min at 23 °C, and stopped with 3 M HCl. Samples were read at 492 nm. Non-specific binding (secondary only control) was subtracted and data reported as percentage increase over quiescent vehicle-treated cells.

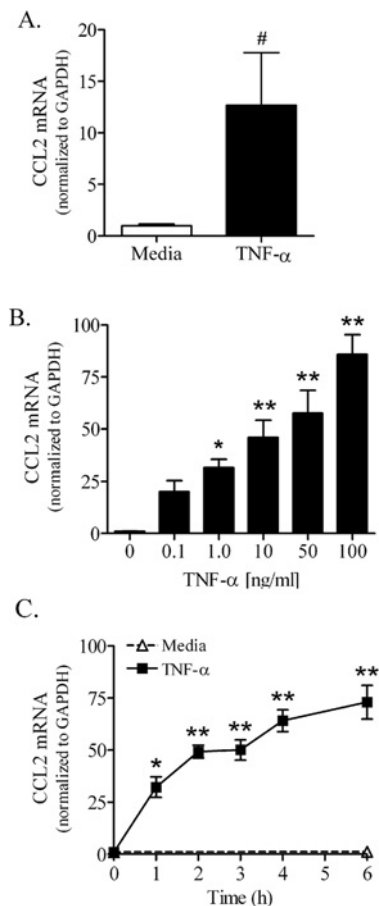
The content of CCL2 released from treated PNMEC cultures was determined using a commercially available ELISA kit (ThermoScientific). The working range for this rat-specific CCL2 ELISA kit is 38–1500 pg/ml. Briefly, PNMECs were cultured at a density of  $2 \times 10^5$  cells per well (24-well collagen-coated) and media from treated cultures were collected, clarified by centrifugation (700g), and stored at  $-80^\circ\text{C}$  until use. Samples were diluted with *Endo media* (1:50 for primary PNMEC cultures, 1:200 for immortalized PNMEC cultures), and aliquots (50  $\mu\text{l}$ ) were assayed according to the manufacturer's instructions. Samples were read at 450 nm with a 550 nm correction and results expressed as ng/ml of undiluted CCL2 released.

### Transendothelial migration assay

PNMEC cultures were grown to confluency on Transwell<sup>®</sup> microporous inserts as described above, creating a physiological barrier separating upper and lower compartments. An aliquot ( $1 \times 10^5$  cells) of CCR2-expressing human THP-1 monocytes was added to the upper compartment, while conditioned medium harvested from the separate vehicle- or TNF $\alpha$ -treated immortalized PNMEC cultures was simultaneously added to the bottom compartment. Vehicle- or TNF $\alpha$ -treated cultures were discarded after the collection of conditioned media. After 4 h at 37 °C, cells that migrated into the lower chamber were collected and counted using a haemocytometer. To assess the specific role of CCL2 in THP-1 cell chemotaxis, conditioned medium was pre-treated (30 min, 37 °C) with a neutralizing polyclonal antibody directed against rat CCL2 (5  $\mu\text{g}/\text{ml}$ ; Serotec). As an additional control, the neutralizing CCL2 antibody was denatured (95 °C, 10 min) in some experiments prior to addition to conditioned medium.

### Statistical analysis

Data are expressed as the mean  $\pm$  SEM of  $n$  observations unless noted otherwise. Statistical significance between two groups was determined by unpaired Student's *t*-test. Significance between multiple experimental groups was determined by one-way or two-way ANOVA with a Bonferroni or Dunnett's *post-hoc* analysis. In each case,  $P < 0.05$  was considered statistically significant.



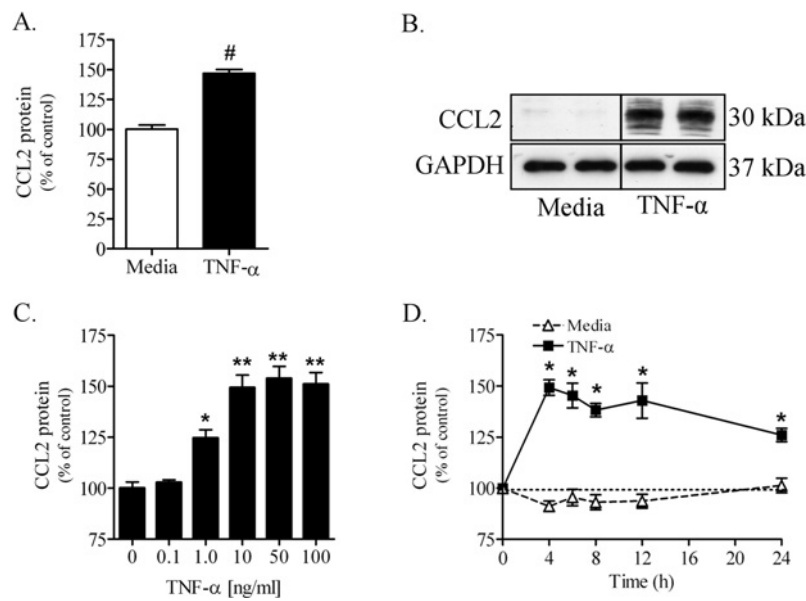
**Figure 3** TNF $\alpha$  increases CCL2 mRNA content in primary and immortalized PNMEC cultures

(A) Primary PNMEC cultures were incubated in the absence (media) or presence of TNF $\alpha$  (10 ng/ml) for 3 h. (B, C) Immortalized PNMEC cultures were treated with (B) 0–100 ng/ml TNF $\alpha$  for 2 h or (C) 0–6 h with 10 ng/ml TNF $\alpha$ , as indicated. Relative changes in CCL2 mRNA content were quantified by qRT-PCR (quantitative real-time PCR). Data shown are the GAPDH-normalized fold changes from two separate experiments ( $n = 4$ –9 cultures) and expressed as means  $\pm$  S.E.M. #,  $P < 0.05$ ; unpaired Student's *t*-test. \*,  $P < 0.05$ ; \*\*  $P < 0.01$ ; one-way ANOVA with Dunnett's *post-hoc* analysis.

## RESULTS

### PNMEC

Primary MECs were freshly harvested from rat sciatic nerve using a modification of the procedure used by Argall et al. (1994). Epithelioid myofibroblasts, the major cell contaminant (Yosef et al., 2010), were routinely eliminated from prepared cultures by Thy 1.1 antibody-mediated complement-driven cell lysis (Argall et al., 1994). PNMEC cultures purified in this manner were found to (i) be  $>95 \pm 7.5\%$  ( $n = 3$ ) free of Thy-1.1 expressing fibroblasts, (ii) consistently establish contact-inhibited monolayers with a distinctive cobblestone-like morphology (Figure 1A) and (iii)



**Figure 4** **TNF $\alpha$  increases CCL2 protein content in primary and immortalized PNMEC cultures**  
 Intracellular CCL2 content in (A) primary or (B) immortalized PNMEC cultures incubated in the absence (media) or presence of TNF $\alpha$  (10 ng/ml) for 4 h was quantified by cell-based ELISA or immunoblot, respectively. (C, D) Immortalized PNMEC cultures were cultured with (C) 0–100 ng/ml TNF $\alpha$  for 4 h or (D) 0–24 h with 10 ng/ml TNF $\alpha$ , as indicated, and intracellular CCL2 protein was quantified by cell-based ELISA. Data shown are results from 2–3 separate experiments (A,  $n = 12$ –14 cultures; C, D,  $n = 6$ –10 cultures). Immunoblot data shown in (B) are from a single experiment performed in duplicate. (A) #,  $P < 0.0001$  unpaired Student's  $t$ -test; (C) \*,  $P < 0.05$ ; \*\*  $P < 0.01$ ; one-way ANOVA with Dunnett's *post-hoc* analysis. (D) \*,  $P < 0.001$ ; two-way ANOVA with Bonferroni's *post-hoc* analysis.

markedly express vWF/Factor VIII (Figure 1C) and PECAM-1 (CD31, Figure 1E), cell markers highly characteristic of MECs. Cell viability of prepared primary cultures was typically  $96 \pm 5\%$  ( $n = 12$ ), as routinely monitored by Trypan Blue dye exclusion. SV40 large T antigen transduction of rat primary PNMECs was found to yield stable immortalized clones that uniquely retain these phenotypic and morphologic characteristics of primary MECs (Figures 1B, 1D and 1F). Expression of large T antigen was specific to immortalized clones, as primary PNMEC cultures did not express measurable quantities of this protein (Figure 1G).

To confirm barrier formation and function in PNMEC cultures, we measured TEER and determined expression of tight junction-associated molecules. Twenty-four hours after passaging immortalized cells onto collagen-coated Transwell inserts, TEER values did not significantly differ from negative control (insert only) (Figure 2A). However, 48–72 h after passaging, an increase ( $15 \Omega \text{ cm}^2$ ) in TEER values was observed across monolayers of immortalized PNMEC cultures (Figure 2A), in good agreement with previously reported TEER values of endothelial cells that form the highly restrictive blood–brain barrier (Davidson et al., 2009).

Both primary and immortalized PNMECs express measurable levels of tight junction associated molecules including claudin-5, occludin, JAM1 (junctional adhesion molecule 1) and ZO-1 (zona occludens 1), as qualitatively demonstrated by RT-PCR (Figure 2B). In each case, the observed amplicon size of amplified products agreed well with predicted values.

For comparison, we demonstrate that these molecules are also measurably expressed in brain, where they are known to participate in establishing tight junctions of the blood–brain barrier (Brown et al., 2007).

### TNF $\alpha$ induces CCL2 mRNA and protein expression

Confluent PNMEC cultures responded robustly to TNF $\alpha$  treatment by markedly increasing CCL2 mRNA. In primary PNMEC cultures, TNF $\alpha$  (10 ng/ml, 3 h) elicited a marked (>12-fold) increase in the content of CCL2 mRNA compared with quiescent (media-treated) cells (Figure 3A). Immortalized PNMEC cultures responded similarly to TNF $\alpha$  stimulation, with both a dose- (Figure 3B) and time- (Figure 3C) dependent increase (>75-fold) in CCL2 mRNA content. In contrast, the content of CCL2 mRNA in quiescent (media-treated) immortalized PNMEC cultures was below the level of detection (Figure 3C).

Treating primary PNMEC cultures with TNF $\alpha$  (10 ng/ml, 4 h) increased, by approximately 50%, the content of intracellular CCL2 protein as measured by cell-based ELISA (Figure 4A). Consistent with this observation was a robust TNF $\alpha$  (10 ng/ml, 4 h)-mediated increase of a 30 kDa protein identified by immunoblot as CCL2 in lysates of treated immortalized PNMEC cultures (Figure 4B). The higher molecular mass isoform of this chemokine is in agreement with the previous reports of N-terminal glycosylation of CCL2 in rodents

(Liu et al., 1996; Ruggiero et al., 2003). By comparison, the content of CCL2 protein in lysates of quiescent (media-treated) PNMEC cultures was below the detectable levels (Figure 4B). TNF $\alpha$  elicited both dose- (Figure 4C) and time- (Figure 4D) dependent increases in CCL2 protein content in immortalized PNMEC cultures. No significant changes in intracellular CCL2 protein content were observed over the course of 24 h in media-treated immortalized PNMEC cultures (Figure 4D), consistent with minimal constitutive expression of this chemokine.

### TNF $\alpha$ induces ICAM-1 mRNA and protein expression

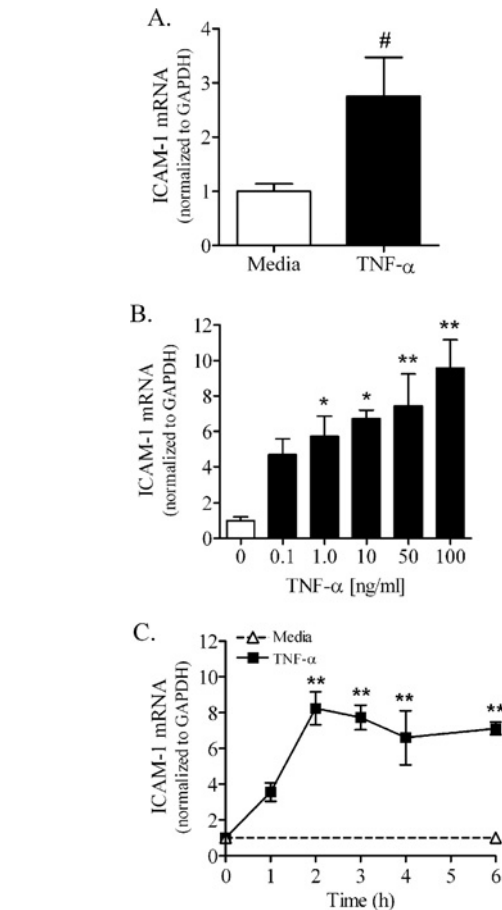
PNMEC cultures treated with TNF $\alpha$  exhibited marked increases in ICAM-1 mRNA and protein expression (Figures 5 and 6). TNF $\alpha$  (10 ng/ml, 3 h) elicited in primary PNMEC cultures a modest but significant (>2-fold) increase in ICAM-1 mRNA content (Figure 5A). Immortalized PNMEC cultures similarly responded to TNF $\alpha$  treatment by up-regulating ICAM-1 mRNA content nearly 10-fold in a manner that was both dose- and time-dependent (Figures 5B and 5C). By comparison, constitutive ICAM-1 mRNA expression was unchanged in quiescent (media-treated) PNMEC cultures over the course of the 6 h assay (Figure 5C).

Constitutive expression of ICAM-1 protein in quiescent primary or immortalized PNMEC cultures was below the level of detection (Figure 6). Primary PNMEC cultures treated with TNF $\alpha$ , however, exhibited a 50% increase in ICAM-1 protein content as quantified by cell-based ELISA (Figure 6A). Lysates of TNF $\alpha$ -treated immortalized PNMEC cultures contained a marked increase in a 90 kDa protein identified by immunoblot as ICAM-1 (Figure 6B). TNF $\alpha$  elicited increases in ICAM-1 protein content within immortalized PNMEC cultures were dose- (Figure 6C) and time- (Figure 6D) dependent. By comparison, constitutive ICAM-1 protein expression was unchanged in quiescent (media-treated) PNMEC cultures over the course of 24 h assayed (Figure 6D).

### TNF $\alpha$ promotes CCL2-mediated chemotaxis

In addition to inducing quantifiable increases in CCL2 mRNA and protein expression, TNF $\alpha$  significantly enhanced the release of CCL2 protein into the culture media from primary and immortalized PNMEC cultures (Figure 7). The absolute content of CCL2 protein constitutively secreted from quiescent (media-treated) PNMEC cultures was within detectable limits (10–50 ng/ml), and was significantly enhanced by TNF $\alpha$  treatment (Figures 7A and 7B). TNF $\alpha$  (10 ng/ml, 4 h) enhanced CCL2 secretion >4-fold from primary PNMEC cultures (Figure 7A). By comparison, TNF $\alpha$  treatment elicited a dose- (Figure 7B) and time- (Figure 7C) dependent effect on CCL2 release from immortalized PNMEC cultures.

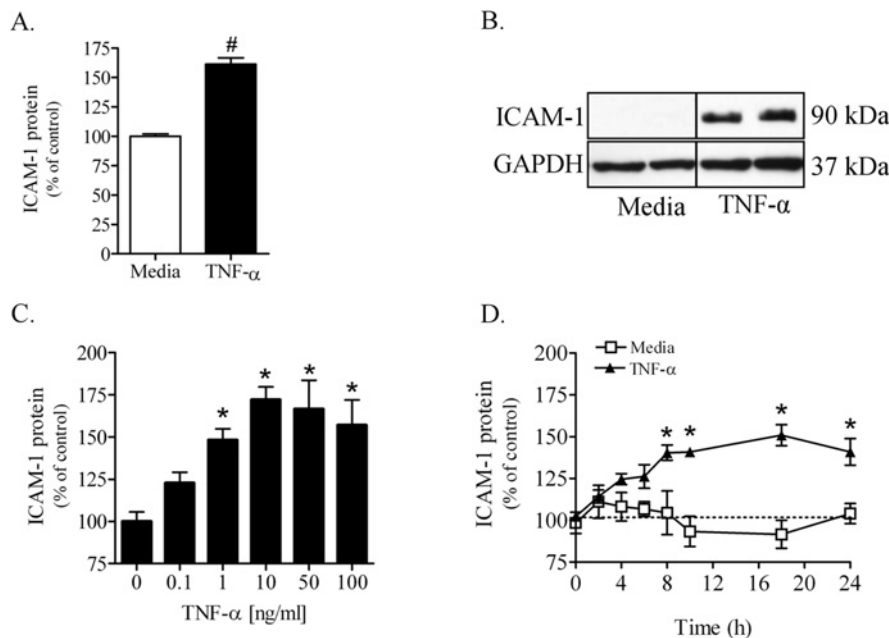
The functional consequence of TNF $\alpha$ -mediated enhancement of CCL2 secretion was determined *in vitro* using CCR2-expressing human THP-1 monocytes (Figure 8). Human CCR2



**Figure 5** TNF $\alpha$  increases ICAM-1 mRNA content in primary or immortalized PNMEC cultures

(A) Primary PNMEC cultures were incubated in the absence (media) or presence of TNF $\alpha$  (10 ng/ml) for 3 h. (B, C) Immortalized PNMEC cultures were treated with (B) 0–100 ng/ml TNF $\alpha$  for 2 h or (C) 0–6 h with 10 ng/ml TNF $\alpha$ , as indicated. Relative changes in ICAM-1 mRNA content were quantified by qRT-PCR (quantitative RT-PCR). Data shown are the GAPDH-normalized fold changes from two separate experiments ( $n = 4$ –9 cultures) and expressed as means  $\pm$  S.E.M. #,  $P < 0.05$ ; unpaired Student's *t*-test. \*,  $P < 0.05$ ; \*\*  $P < 0.01$ ; one-way ANOVA with Dunnett's *post-hoc* analysis.

is reported to recognize rodent CCL2 (Sarafi et al., 1997). In addition, CCR2-expressing human THP-1 cells respond to rodent CCL2 (Neumark et al., 1999). Human THP-1 monocytes have been used in *in vitro* assays with mouse or rat CCL2 as a chemoattractant (Lynn et al., 2001; Matoba et al., 2010). Chemotaxis of THP-1 monocytes through monolayers of quiescent immortalized PNMEC cultures in response to control media was minimal (Figure 8). In contrast, addition of conditioned media harvested from TNF $\alpha$  (10 ng/ml, 4 h) treated immortalized PNMEC cultures significantly promoted THP-1 monocyte chemotaxis (Figure 8A). The presence of a CCL2-neutralizing antibody significantly attenuated THP-1 monocyte chemotaxis. Heat-denatured CCL2-neutralizing antibody was, however, ineffective at preventing THP-1 monocyte chemotaxis in response to TNF $\alpha$ -conditioned media (Figure 8B).



**Figure 6** TNF $\alpha$  increases ICAM-1 protein content in primary and immortalized PNMEC cultures  
 ICAM-1 content in (A) primary or (B) immortalized PNMEC cultures incubated in the absence (media) or presence of TNF $\alpha$  (10 ng/ml) for 16 h was quantified by cell-based ELISA or immunoblot, respectively. (C, D) Immortalized PNMEC cultures were cultured with (C) 0–100 ng/ml TNF $\alpha$  for 16 h or (D) 0–24 h with 10 ng/ml TNF $\alpha$ , as indicated, and ICAM-1 protein was quantified by cell-based ELISA. Data shown are results from two separate experiments (A,  $n = 12$ –14 cultures; C, D,  $n = 6$  cultures). Immunoblot data shown in (B) are from a single experiment performed in duplicate, representative of three separate experiments. (A) #,  $P < 0.0001$  unpaired Student's  $t$ -test; (C) \*,  $P < 0.01$ ; one-way ANOVA with Dunnett's *post-hoc* analysis. (D) \*,  $P < 0.01$ ; two-way ANOVA with Bonferroni's *post-hoc* analysis.

## DISCUSSION

To our knowledge, this is the first report demonstrating stable SV40 large T antigen immortalization and partial characterization of purified microvascular endoneurial endothelial cells harvested from sciatic nerves of naïve Lewis rats. Immortalized PNMEC cultures are shown to retain key phenotypic, morphologic and biochemical properties highly characteristic of primary microvascular endoneurial endothelial cells. In addition to exhibiting contact-inhibited monolayers with a cobblestone-like morphology and marked expression of endothelial cell and barrier markers, immortalized PNMEC cultures responded robustly to a proinflammatory cytokine (TNF $\alpha$ ) challenge by increasing the expression of inflammatory mediators CCL2 and ICAM-1. The functional consequence of a TNF $\alpha$  challenge on localized up-regulation of inflammatory mediators is underscored by a marked increase in release of CCL2 from the activated peripheral nerve microvascular endothelium. Here, we propose that increased CCL2 and ICAM-1 expression in response to TNF $\alpha$  may facilitate recruitment and trafficking of autoreactive leucocytes across the BNB in autoimmune disorders, including GBS.

Advancements have been made towards the purification and culture of MECs derived from peripheral nerve (Argall et al., 1994; Sano et al., 2007; Yosef et al., 2010). Vascular

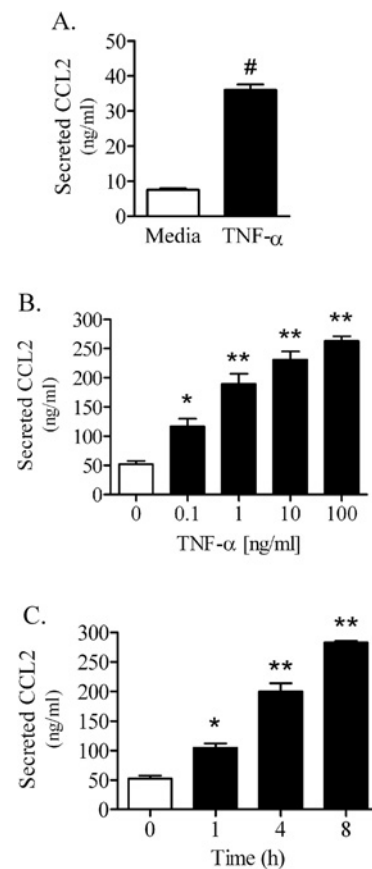
endothelial cell characteristics vary according to tissue origin and species (Bell and Weddell, 1984a, 1984b) and, to our knowledge, no studies to date have addressed how PNMECs respond to a localized inflammatory challenge such as that experienced during GBS/EAN. In this study, viable cultures of primary microvascular endoneurial endothelial cells from rat sciatic nerve were routinely prepared at >95% purity. We used a replication-deficient SV40 retrovirus encoding a temperature sensitive non-SV40-origin binding mutant of the large T antigen (Jat et al., 1986; Jat and Sharp, 1986; Greenwood et al., 1996) to produce from these primary PNMEC cultures a stably immortalized clone. Immortalized PNMECs were found to retain key phenotypic (contact-inhibited monolayers), morphologic (distinctive cobblestone-like appearance), biochemical (localized expression of vascular endothelial cell markers vWF/Factor VIII and PECAM-1) and barrier-related (expression of tight junction associated molecules) characteristics exhibited by primary rat PNMEC cultures. Immortalized PNMEC monolayers also exhibited increased TEER, an established measure of barrier function.

Recruitment and extravasation of autoreactive leucocytes into privileged tissue compartments, such as peripheral nerves, is thought to initially involve establishment of localized chemotactic cytokine gradients, including CCL2 (Yadav et al., 2010). Consistent with this thesis, we observed a rapid and marked increase in CCL2 mRNA and protein expression in



PNMEC cultures responding to a TNF $\alpha$  challenge. The physiological concentration of TNF $\alpha$  in plasma of patients with GBS is reported to exceed 0.1 ng/ml (Reuben et al., 2002; Radhakrishnan et al., 2004). At this low concentration, TNF $\alpha$  elicited measurable increases in CCL2 mRNA content in immortalized PNMEC cultures. Higher concentrations of TNF $\alpha$  (10–100 fold) were found to elicit measurable changes in both CCL2 mRNA and protein expression within PNMECs. Localized concentrations of TNF $\alpha$  within peripheral nerves during an immune challenge remain to be determined, and may very well mimic or exceed concentrations used in this study. In vascular endothelial cells, chemokines (including CCL2) are reportedly stored in novel regulated secretory granules that are distinct from vWF-containing Weibel-Palade bodies (Knipe et al., 2010). Exposure of endothelial cells to an inflammatory mediator elicits rapid translocation to the cell surface of stored vesicles where they release their chemotactic contents into the extracellular space (Oyenebraten et al., 2004, 2005; Deshmane et al., 2009; Knipe et al., 2010). Consistent with these observations, primary and immortalized PNMEC cultures used in this study similarly responded to a TNF $\alpha$  challenge by releasing into the culture medium measurable quantities of functionally active CCL2 protein. Antibody-mediated neutralization of released CCL2 protein attenuated migration of CCR2-expressing human THP-1 monocytes in an *in vitro* migration assay. A modest, although statistically insignificant, increase in migrated monocytes over baseline was observed with antibody neutralization. This discrepancy may be attributed to the presence of other C–C chemokines [MIP-1 $\alpha$  (macrophage inflammatory protein 1 $\alpha$ ) and RANTES (regulated upon activation, normal T-cell expressed and secreted)] in the TNF $\alpha$ -conditioned media.

Responding leucocytes migrate through the endothelium into peripheral nerves, in part, by a process that first involves their engagement of temporally expressed endothelial CAMs precipitating tethering, slow rolling, firm adhesion and ultimate diapedesis (Springer, 1994; Raab et al., 2002). Cytokine-mediated activation of the endothelium facilitates migration of autoreactive leucocytes into peripheral nerves in EAN (Stoll et al., 1993). Given that ICAM-1 is directly involved in leucocyte trafficking (Greenwood et al., 2002), we determined the effects of a TNF $\alpha$  challenge on eliciting changes in ICAM-1 expression. Previous studies have reported constitutive ICAM-1 expression in vascular endothelial cells (Rahman and Fazal, 2009). However, endogenous expression of ICAM-1 by primary and immortalized rat PNMEC cultures was below the level of detection. Challenging immortalized PNMEC cultures with TNF $\alpha$  induced a dose- and time-dependent increase in ICAM-1 mRNA and protein expression, rendering these cells capable of firm adhesion with autoreactive leucocytes. Importantly, the effect of TNF $\alpha$  on ICAM-1 expression was similarly observed in primary PNMEC cultures, in strong support of a physiological response rather than an artefact of cell transformation. Compared with expression of CCL2, the time course by which TNF $\alpha$  elicited an increase in ICAM-1



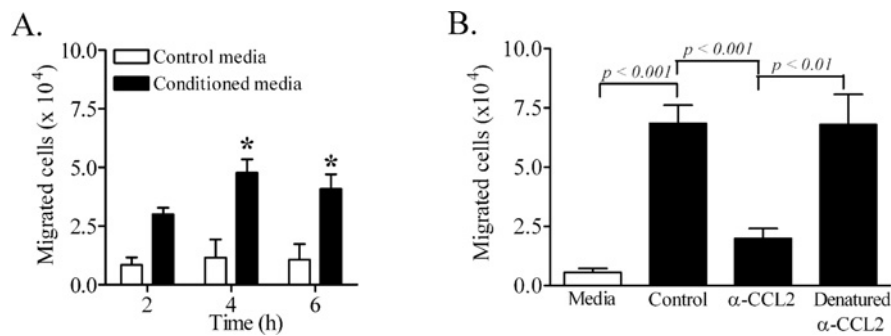
**Figure 7** TNF $\alpha$  promotes secretion of CCL2 from primary and immortalized PNMEC cultures

(A) Content of CCL2 secreted from primary PNMEC cultures incubated in the absence (media) or presence of TNF $\alpha$  (10 ng/ml) for 4 h. (B, C) Immortalized PNMEC cultures were treated with (B) 0–100 ng/ml TNF $\alpha$  for 4 h or (C) 0–8 h with 10 ng/ml TNF $\alpha$ , as indicated, and the content of CCL2 released into the cell culture media was quantified by ELISA. Data shown are the means  $\pm$  S.E.M. ( $n = 3$ –6 cultures). (A) #,  $P < 0.0001$ , Student's *t*-test. (B, C) \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , one-way ANOVA with Dunnett's *post-hoc* analysis.

expression was slightly delayed, reflecting differential temporal dynamics consistent with their respective physiological roles in leucocyte recruitment and extravasation (Springer, 1994; Raab et al., 2002).

The mechanism by which a TNF $\alpha$  challenge elicits increases in CCL2 and ICAM-1 mRNA content in PNMEC cultures may involve NF- $\kappa$ B (nuclear factor  $\kappa$ B)-dependent transcription of the CCL2 and ICAM-1 genes (Xing and Remick, 2007; Deshmane et al., 2009). Consistent with this thesis, TNF $\alpha$  was capable of inducing translocation of NF- $\kappa$ B p65 to the nucleus in immortalized PNMECs (results not shown). The precise mechanisms by which TNF $\alpha$  binding to its receptor leads to an increase in NF- $\kappa$ B activation remain unknown, but a role for Rho GTPase activation in this process has been previously suggested (Zhao and Pothoulakis, 2003; Williams et al., 2008).

Expression of CCL2 and ICAM-1 protein content by TNF $\alpha$  in PNMEC cultures may result from an increase in *de novo* synthesis, reduced proteasomal-, autophagosomal- or



**Figure 8** Secreted CCL2 promotes migration of THP-1 monocytes across PNMEC culture monolayers. Immortalized PNMEC cultures were incubated in the absence (media) or presence of TNF $\alpha$  (10 ng/ml) for 4 h, and conditioned cell-culture medium was used to assay for THP-1 monocyte chemotaxis across PNMEC monolayers. (A) Total migrated cells in response to 2–6 h treatment with medium or TNF $\alpha$ -conditioned medium. (B) Total migrated cells in response to 4 h treatment with medium or TNF $\alpha$ -conditioned medium supplemented without (control) or with an CCL2 neutralizing antibody ( $\alpha$ -CCL2) or with heat-denatured anti-CCL2 antibody. Data shown are the means  $\pm$  S.E.M. ( $n = 3$ –7 cultures). (A) \*,  $P < 0.01$ , two-way ANOVA with Bonferroni's *post-hoc* analysis. (B) One-way ANOVA with Bonferroni's *post-hoc* analysis.

lysosomal-dependent degradation of existing protein or from decreased release (exocytosis) of stored chemokine (Knipe et al., 2010). While these various mechanisms have not yet been elucidated in these PNMEC cultures, the observed rapid increase in CCL2 protein release from TNF $\alpha$ -challenged PNMEC cultures suggests, to us, that exocytosis of CCL2 is not impaired in these cells. Additional studies are currently being conducted to determine the mechanism by which TNF $\alpha$  elicits changes in CCL2 and ICAM-1 gene and protein expression.

In conclusion, we report the stable immortalization of rat PNMECs, which retain key phenotypic, morphological, biochemical characteristics and barrier-forming properties expected of primary microvascular endoneurial endothelium. We demonstrate that TNF $\alpha$  elicits robust increases in CCL2 and ICAM-1 mRNA and protein content, while significantly promoting CCL2 release, in both primary and immortalized PNMEC cultures. TNF $\alpha$ -mediated enhancement of CCL2 secretion facilitated migration of human THP-1 monocytes *in vitro*. TNF $\alpha$  facilitated alterations in other inflammatory mediators in PNMEC cultures are anticipated and remain to be established. Increased TEER, coupled with the expression of tight junction associated molecules, represent unique characteristics of specialized barrier-forming endothelial cells. Collectively, these findings support the application of PNMEC cultures as a novel *in vitro* resource for the functional assessment of pathological alterations in endoneurial homeostasis during an inflammatory challenge, such as that encountered during GBS/EAN.

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