Myelin regulates immune cell adhesion and motility

Madeline Pool [a], Masaaki Niino [a,b,1], Isabel Rambaldi [a], Kristin Robson [a], Amit Bar-Or [a,b,*], Alyson E. Fournier [a,*]

[a] Department of Neurology and Neurosurgery, Montreal Neurological Institute, 3801 Rue University, Montreal, Quebec, Canada H3A 2B4
[b] Experimental Therapeutics Program, Montreal Neurological Institute, 3801 Rue University, Montreal, Quebec, Canada H3A 2B4

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ABSTRACT

The etiology of multiple sclerosis (MS) has not been fully elucidated, however evidence supports an autoimmune disease model notable for the infiltration of pro-inflammatory immune cells into sites of active demyelination and axonal injury. Previous findings demonstrate that neutralization of Nogo, a protein originally identified as a myelin-associated inhibitor (MAI) of axon regeneration, ameliorates experimental autoimmune encephalomyelitis (EAE), a commonly used animal model of MS. More efficient axonal regeneration was suggested as a mechanism underlying the improved EAE outcome. However, neutralization of Nogo also led to an anti-inflammatory shift of T cell cytokines during EAE suggesting that another therapeutic mechanism may involve regulation of immune cell responses. Here we report that human immune cells from healthy individuals and MS patients express Nogo receptor1 (NgR1) indicating that they may be subject to regulation by MAIs. B cells, T cells and monocytes express NgR1 in a regulated fashion upon activation. While direct stimulation of human immune cells with an inhibitory fragment of Nogo does not impact their *in vitro* proliferation or cytokine production, the immune cells display reduced adhesion and enhanced motility in response to myelin, effects that are in part attenuated by antagonizing NgR1 signaling. We conclude that NgR1 alters the motility of immune cells exposed to myelin and may thus impact their behaviour within the CNS, particularly under conditions when immune cell activation is heightened.

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Introduction

Multiple sclerosis (MS) is a progressive inflammatory disease of the central nervous system (CNS) resulting in myelin breakdown, axonal damage and neurological disability. Evidence from patients as well as studies in the commonly used animal model experimental autoimmune encephalomyelitis (EAE), point to an autoimmune process in which peripherally activated immune cells infiltrate the CNS and contribute to both myelin and axonal injury (Keegan and Noseworthy, 2002). How immune cells interact with myelin in the CNS is an important question when considering mechanisms of demyelination and repair.

CNS myelin contains Myelin-Associated Inhibitors (MAIs) including Nogo-A, Myelin-Associated Glycoprotein (MAG) and Oligodendrocyte Myelin Glycoprotein (OMgp). MAIs were originally identified for their ability to restrict axonal repair following nerve transection (David and Lacroix, 2003) but novel roles for these proteins in a variety of neurological conditions and diseases are now emerging (David et al., 2008, Strittmatter, 2002). Blocking Nogo signaling in the context of MOG-induced EAE improves clinical outcome (Fontoura et al., 2004, Karnezis et al., 2004). While a reasonable prediction would be that the mechanism underlying this benefit is related to enhanced axonal regeneration within MS lesions, it was also reported that Nogo neutralization impacted T cell cytokine responses. Specifically, splenocyte cytokine profiles from Nogo-vaccinated mice shifted from a pathogenic Th1 response to a protective Th2/3 response. Further, the mice had fewer overall inflammatory lesions and reduced axonal damage during MOG-induced EAE (Karnezis et al., 2004). This suggests that Nogo signaling may directly impact immune cell responses in addition to its well-characterized role in neurite outgrowth inhibition.

The MAIs bind and signal through multiple receptors on the neuronal cell surface (Atwal et al., 2008, Fournier et al., 2001, Goh et al., 2008, Hu and Strittmatter, 2008, Liu et al., 2002, Venkatesh et al., 2005). Among these receptors, Nogo, MAG and OMgp all bind and signal through a common tripartite receptor complex on the neuronal cell surface and signal through RhoA and Rho Kinase to inhibit outgrowth (Alabed et al., 2006, Dergham et al., 2002, Fournier et al., 2003). The binding subunit of this receptor complex, NgR1 (Nogo receptor1; (Fournier et al., 2001)), is a glycosylphosphatidylinositol (GPI)-linked protein that complexes with p75[NTR] (Wang et al., 2002, Wong et al., 2002) or TROY (Park et al., 2005, Shao et al., 2005) and LINGO (Mi et al., 2004) to mediate inhibitory signals in response to Nogo, MAG and OMgp. We hypothesized that human immune cells may express NgR1,

* Corresponding authors. A. Fournier is to be contacted at Montreal Neurological Institute, BT-109, Montreal, PQ, Canada H3A 2B4. Fax: +1 514 398 6547. A. Bar-Or, Montreal Neurological Institute, Rm 111, Montreal, PQ, H3A 2B4, Canada.
E-mail addresses: amit.bar-or@mcgill.ca (A. Bar-Or), alyson.fournier@mcgill.ca (A.E. Fournier).
[1] Current Address: Department of Neurology, Hokkaido University Graduate School of Medicine, Kita-15 Nishi-7, Kita-ku, Sapporo 060-8638 Japan.

rendering these cells responsive to Nogo. Here, we report that NgR1 and its co-receptors are indeed expressed in freshly isolated human peripheral blood mononuclear cells (PBMCs). Moreover, B cell, T cell and monocyte subsets express NgR1 and such expression is upregulated upon immune cell activation. Direct stimulation with Nogo does not affect *in vitro* human immune cell proliferation or cytokine expression; however, these cells do display reduced adhesion and enhanced motility on myelin substrates. These effects are attenuated by removing NgR1 from the cell surface with phosphatidylinositol specific phospholipase C (PI-PLC) and by treatment with an inhibitor of Rho Kinase, a downstream effector of NgR1 signaling. Further, treatment with a specific NgR1 antagonist peptide, NEP1–40, abolishes the myelin-dependent enhancement of T cell motility. These findings suggest that NgR1 signaling alters the motility of immune cells and may therefore impact immune cell infiltration or migration within the CNS.

Methods

Cell isolation

For all studies on human samples, the protocols and the informed consent documents were approved by the Ethics Review Board of the Montreal Neurological Institute and Hospital. Venous blood samples were obtained from consenting healthy donors and patients with
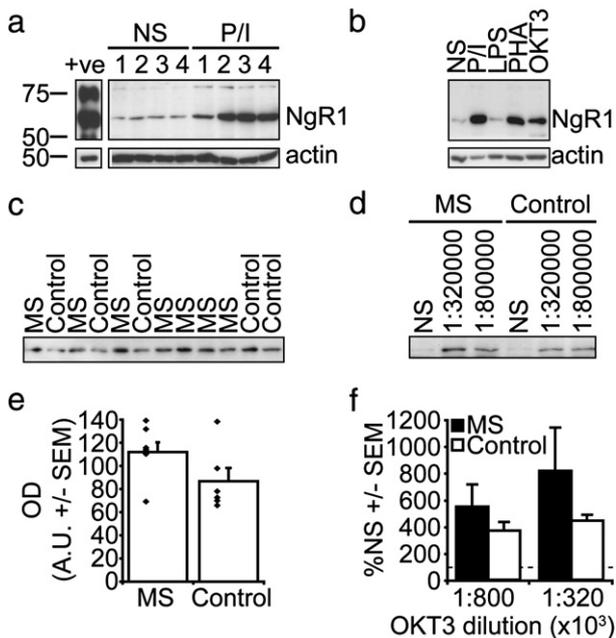

Fig. 1. NgR1 is expressed in human PBMCs and is regulated by cellular activation. (a, b, c, d) PBMC lysates were separated by SDS-PAGE and Western blotted with an anti-NgR1 antibody or anti-actin antibody to control for even protein loading. An NgR1-positive control lysate (+ve) is from 293T cells transfected with a full-length cDNA for NgR1. Representative blots of at least 4 experiments are shown. PBMCs were maintained in culture for 1, 2, 3 or 4 days without stimulation (NS) or in the presence of 20 ng/ml PMA and 1 µg/ml Iono (P/I) (a) or for 3 days with P/I, 100 ng/ml LPS, 5 µg/ml PHA or OKT3 stimulation (b). *Ex vivo* (c) or OKT3-stimulated (d) PBMCs from healthy controls or MS patients were compared directly on the same Western blot. OKT3 hybridoma supernatant was applied at the indicated dilutions (d). (e, f) Quantification of NgR1 expression levels by densitometry. Optical density (OD) as arbitrary units (A.U.) +/− SEM are indicated. Determinations are from 7 MS patients and 5 control patients (e). Individual data points (♦) are indicated on the graph. Statistical analysis by unpaired *t*-test indicates no significant difference between control and MS populations ($p = 0.0805$, two-tailed). For OKT3 stimulation (f), levels were normalized to unstimulated (100%) for each set of PBMCs. OKT3 dilutions of 1:800,000 or 1:320,000 are indicated. Determinations are from 9 control and 9 MS patients. Statistical analysis by two-way repeated measures ANOVA indicates no significant change in the response to OKT3 stimulation between PBMCs from control and MS patients ($p = 0.2829$).
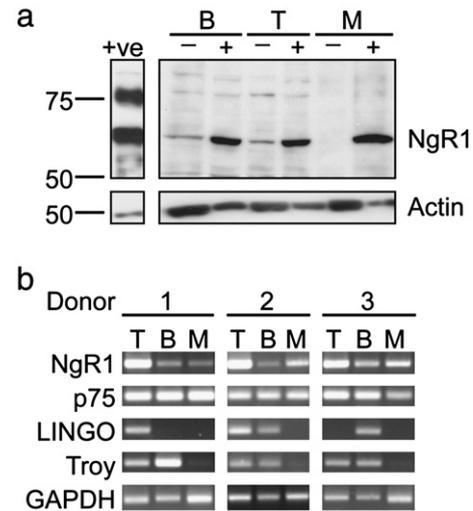

Fig. 2. NgR1 and NgR1 co-receptors are expressed in immune cell subsets. (a) Lysates from CD19+ B cells (B), CD3+ T cells (T) or CD14+ monocytes (M) cultured for 3 days in the presence (+) or absence (−) of P/I were separated by SDS-PAGE and Western blotted with anti-NgR1 or anti-actin antibody. Positive control lysate (+ve) is as per Fig. 1. Data is representative of 3 experiments. (b) Detection of NgR1, p75[NTR], LINGO, Troy and GAPDH transcripts in freshly isolated CD19+ B cells (B), CD3+ T cells (T) or CD14+ monocytes (M) by RT-PCR. GAPDH was detected as a control.

untreated, clinically definite MS (Poser criteria). PBMCs were isolated by Ficoll density centrifugation and cultured *in vitro* as previously described (Alter et al., 2003). PBMCs were then stimulated with 20 ng/ml Phorbol Myristate-13-Acetate and 1 µg/ml Ionomycin (P/I), 100 ng/ml lipopolysaccharide (LPS), 5 µg/ml phytohemagglutinin (PHA) or anti-CD3 antibody (OKT3 at indicated dilutions of hybridoma supernatants) for 1 to 4 days. For cell subset experiments, immune cell subsets (CD19+ B cells, CD3+ T cells and CD14+ monocytes) were isolated from PBMCs prior to activation, using MACS (Miltenyi Biotec, Toronto, ON). Immune cell subset purities of >95% were routinely achieved and confirmed by flow cytometry as previously described (Alter et al., 2003).

Western blotting

Cells were lysed in RIPA buffer (20 mM Hepes, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100) with a protease inhibitor cocktail (Roche, Mannheim, Germany), separated by SDS-PAGE, transferred to Polyvinylidene Difluoride membrane and analyzed with antibodies recognizing NgR1 (generously provided by Dr. Stephen Strittmatter, Yale University). Films were scanned and densitometry was performed using Adobe Photoshop.

RT-PCR

Total RNA from MACS separated cells was prepared using the RNeasy kit (Qiagen, Mississauga Ontario). The RNA was treated with DNaseI (Amersham Biosciences, Baie d'Urfè, Quebec) for 1 h at 30 °C (50 mM Tris acetate (pH 8.4), 75 mM potassium acetate, 8 mM magnesium acetate), phenol/chloroform extracted and ethanol precipitated. cDNA was prepared using the Thermoscript RT-PCR System (Invitrogen, Burlington Ontario) using oligo dT primers. All primers for PCR detection were designed to span an intron boundary except for Lingo-1, which does not contain any introns. Primer sequences were as follows: NgR1 F 5′ATGAAGAGGGCGTCCGCT, NgR1 R 5′GGAGCTGTGCATTATCGCTGA; Troy F 5′AAACTGTGTTCCCTGCAACC, Troy R 5′TCTCCACAAGGCACACACTC; p75 F 5′CGACAACCTCATCCC-TGTCT, p75 R 5′TCGCTGTGGAGTTTTTCTCC; Lingo F 5′ATCTCCCACTGG-CCCTACTT, Lingo R 5′TTGCCAGAGACATTGAGCAC; Product sizes were as follows: NgR1, 354 bp; Troy, 288 bp; p75, 168 bp and Lingo, 299 bp.
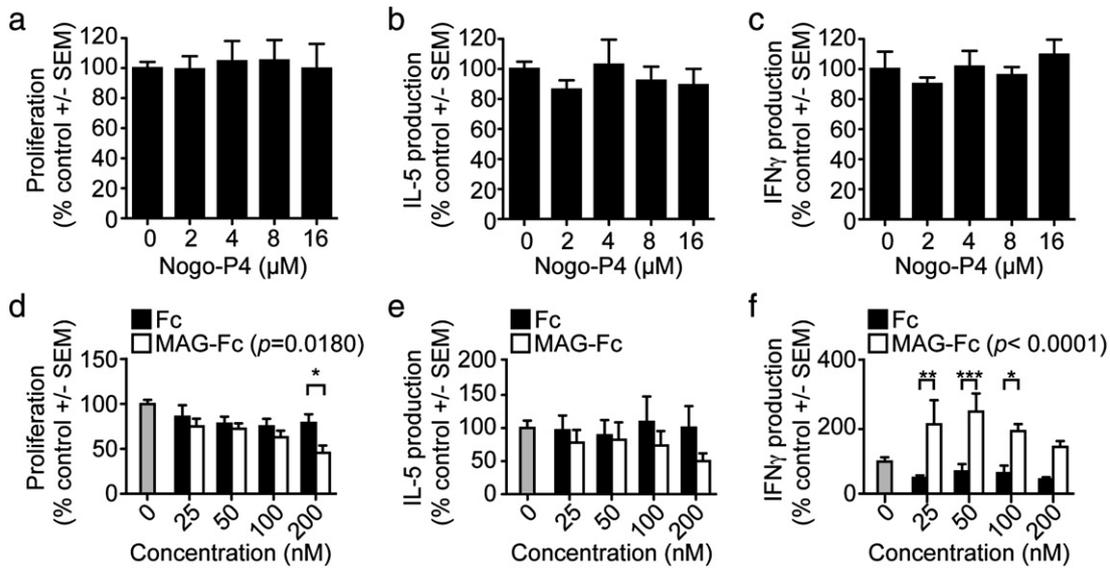
Fig. 3. Nogo-P4 and MAG-Fc have differential effects on *in vitro* PBMC proliferation and cytokine production. (a–f) PBMCs were stimulated for 3 days with anti-CD3 supernatant (1:128,000) in the presence of Nogo-P4 peptide, MAG-Fc, or Fc at the indicated concentrations and assayed for proliferation (a, d), IL-5 cytokine production (b, e), or IFNγ cytokine production (c, f). The experiment was repeated twice in triplicate for Nogo-P4 treatment and three times in triplicate for MAG and Fc treatment. One-way repeated measures ANOVA indicates no statistically significant differences with Nogo-P4 treatment ($p = 0.7602$, 0.8052, 0.6626 for proliferation, IL-5 and IFNγ, respectively). For MAG and Fc treatment, two-way repeated measures ANOVA indicates a statistically significant decrease in proliferation, no significant difference in IL-5 production, and an significant increase in IFNγ production ($p = 0.0004$, $p < 0.0001$, $p = 0.1296$ for proliferation, IL-5 and IFNγ, respectively).

PBMC proliferation and cytokine production

For proliferation and cytokine assays, PBMC supernatants were collected after three days in culture and replaced with culture medium containing 1 μCi ^{3}H-thymidine (ICN Biochemicals, Mississauga, ON). Uptake of ^{3}H-thymidine over the subsequent 18-hour period was measured using a beta scintillation counter. IL-5 and IFNγ concentrations in the cell supernatants were measured using BD OptEIA Sets (BD Biosciences, Mississauga, ON) according to the manufacturer's protocol.

Adhesion assays

Nitrocellulose spots were dried in 96-well plates for 30 min. For experiments with myelin substrates, the nitrocellulose spots were coated with poly-L-lysine for 1 h at room temperature followed by the indicated concentrations of myelin isolated from bovine brain as previous described (Hsieh et al., 2006) in HBSS at 4 °C overnight. For heat inactivation, diluted myelin was heated at 95 °C for 20 min prior to coating. PBMCs were washed with PBS and resuspended in RPMI1640 (Invitrogen). For assays with NEP1–40 peptide, CD14+ cells were depleted by MACS separation and the assays performed with the depleted fraction to avoid non-specific effects of peptide presentation by monocytes. For assays in the presence of Y-27632 (EMD Biosciences, Gibbstown, NJ), PI-PLC (Invitrogen), or NEP1–40 (Alpha Diagnostic, San Antonio, TX), cells were incubated for 1 h with 5 μM Y-27632, 0.5 U/ml PI-PLC or 1 μM NEP1–40 and 100,000 cells were plated in each well and allowed to adhere for 2 h in a humidified incubator at 37 °C with 5% CO_2. For subset staining, adherent cells were washed once with ice-cold PBS and incubated on ice for 15 min with 10% human A/B serum (Sigma-Aldrich Canada, Oakville, ON) in PBS followed by PE-conjugated anti-CD3, anti-CD14 or anti-CD19 (BD Biosciences) in 10% human A/B serum in PBS for 30 min. The plates were then washed with ice-cold PBS and the adherent cells fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 30 min. The nuclei were stained with 10 μg/ml Hoechst 33342 (Sigma-Aldrich Canada) for 30 min. After washing off the excess dye with PBS, images were captured on an ImageXpress Micro instrument (Molecular Devices, Sunnyvale, CA) and cell counts obtained using the Multiwavelength Cell Scoring Module of MetaXpress (Molecular Devices).
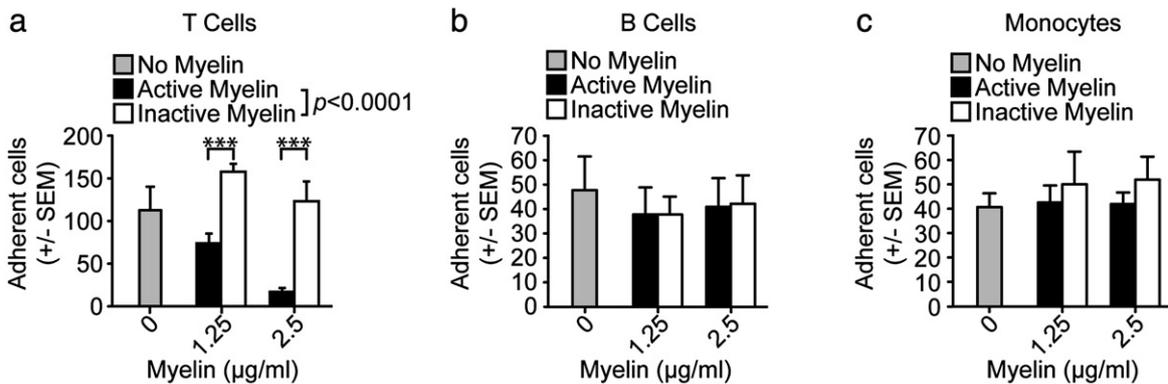

Fig. 4. Myelin reduces T cell adhesion. (a–c) Freshly isolated PBMCs were seeded on myelin substrates, and the number of adherent CD3+ T cells (a), CD19+ B cells (b) and CD14+ monocytes (c) was counted. Values were averaged from 5 independent experiments. Statistical analysis by two-way repeated measures ANOVA indicates a dose-response of T cells to active myelin ($p = 0.0132$) that is significantly different from heat-inactivated myelin ($p < 0.0001$) whereas B cells and monocytes demonstrate no significant response to active myelin ($p = 0.8291$ and $p = 0.1539$, respectively).
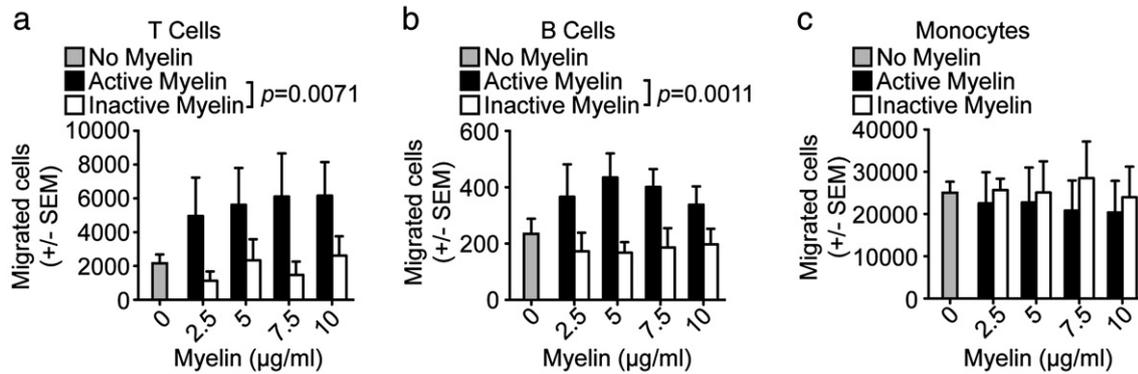
Fig. 5. Myelin promotes immune cell motility. (a–c) Freshly isolated PBMCs were allowed to migrate along a serum gradient through transwells coated with myelin. FACS was used to count the number of CD3+ T cells (a), CD19+ B cells (b) and CD14+ monocytes (c) that migrated through the transwell. Values were averaged from 4 independent experiments. Statistical analysis by two-way ANOVA indicates significant differences in the responses of both T and B cells to active versus heat-inactivated myelin ($p = 0.0071$ and $p = 0.0011$, respectively) whereas monocytes demonstrate no significant response ($p = 0.4349$).

Migration assays

5 μm pore-size polycarbonate transwell chambers (Corning Incorporated Life Sciences, Lowell, MA) were coated overnight with myelin diluted in 0.1 M NaHCO$_3$, pH 8.0. The transwells were rinsed with HBSS and blocked with 2% human serum albumin (EMD Biosciences) for 30 min at room temperature. 1×10^6 freshly isolated PBMCs were allowed to migrate from the top chamber to the bottom chamber along a serum gradient (2.5% FBS – 10%FBS/20% human A/B serum in RPMI1640) for 4 h. For assays in the presence of Y-27632, PI-PLC or NEP1–40, cells were pretreated for 1 h with 5 μM Y-27632, 0.5 U/ml PI-PLC or 1 μM NEP1–40 and these concentrations were maintained throughout the migration period. The cells in the bottom chamber were collected and stained with FITC-conjugated anti-CD3, PE-conjugated anti-CD14 and APC-conjugated anti-CD19 (BD Biosciences). The number of migrated cells from each subset was then counted using a FACSCalibur.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. The specific tests used, the *p*-values obtained, and the number of independent experiments analyzed, are reported in the figure legends. For ANOVA analyses, Bonferroni post-tests were performed and the significant comparisons are indicated by * ($p<0.05$), ** ($p<0.005$), or *** ($p<0.0005$). Data were considered statistically significant if $p<0.05$.

Results

NgR1 is expressed in human immune cells and is upregulated upon activation

To investigate the expression of NgR1 in the immune system, we used a Western blotting approach to analyze protein expression in human PBMCs cultured in the presence or absence of P/I for 1 to 4 days (Fig. 1a). *Ex vivo* PBMCs express NgR1 and stimulation with P/I results in a strong upregulation of NgR1 expression. Upregulation is detectable by 18 h following stimulation.

The effects of additional modes of stimulation, at levels that induce typical *in vitro* proliferative responses, were also assessed (Fig. 1b). OKT3 strongly enhances expression of NgR1. OKT3 is known to primarily activate T cells through direct cross-linking of the T cell receptor; the activated T cells can secondarily activate antigen-presenting cells (APC). PHA stimulation, which triggers activation of T cells in the presence of APC, also stimulates NgR1 expression. Stimulation with LPS, representing a partial activator of APC, has no effect on NgR1 expression. Stimulation of PBMCs with myelin extracts fails to alter NgR1 expression indicating that MAIs do not directly regulate expression levels of their receptors (data not shown). Together these findings demonstrate that human PBMCs, activated by a variety of mechanisms, exhibit regulated expression of NgR1.
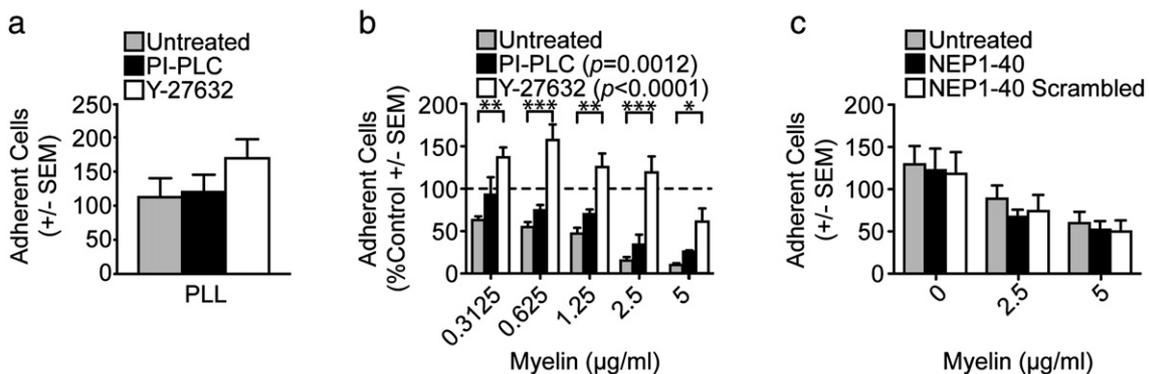

Fig. 6. PI-PLC and Y-27632 attenuate myelin-mediated inhibition of T cell adhesion. (a–c) Adhesion assays were performed in the presence of PI-PLC, Y-27632, or NEP1–40 to block NgR1 signaling. (a) The number of cells adhering to control substrates in the presence of Y-27632 or PI-PLC was averaged for 5 experiments. Statistical analysis by paired *t*-tests demonstrates no significant change in adhesion to control substrates in the presence of either agent ($p = 0.0550$ for Y-27632 and $p = 0.0862$ for PI-PLC, two-tailed). (b) The number of adherent cells is expressed as a percentage of the number of cells adhering to heat-inactivated myelin substrates for 5 experiments. Statistical analysis by two-way repeated measures ANOVA indicates a significant effect of both Y-27632 and PI-PLC on myelin-mediated inhibition of adhesion of T Cells ($p<0.0001$ and $p = 0.0012$ for treatment with Y-26732 and PI-PLC, respectively). (c) The number of cells adhering to myelin substrates in the presence of NEP1–40 was averaged for 5 experiments. Statistical analysis by two-way repeated measures ANOVA demonstrates no significant change in adhesion to myelin substrates in the presence of NEP1–40 peptide ($p = 0.1223$).

Expression of Nogo and NgR1 by immune cells from patients with MS

Regulated expression of NgR1 on immune cells could be relevant in the context of MS as indicated by the finding that neutralization of Nogo attenuates the clinical course of EAE (Fontoura et al., 2004, Karnezis et al., 2004). We therefore examined the expression of NgR1 in PBMCs isolated from healthy individuals or untreated patients with MS. Baseline expression of NgR1 from unstimulated PBMCs overlaps in MS patients and healthy volunteers, however there is an interesting although not statistically significant trend towards stronger NgR1 expression in the MS population compared to the healthy control population in this small cohort (Figs. 1c, e). We also examined PBMC responses to OKT3 stimulation and found similar response profiles in PBMCs from MS patients and healthy volunteers (Figs. 1 d, f).

Components of the Nogo receptor complex are expressed in T cells, B cells and monocytes

To investigate which immune cell subsets can upregulate NgR1 expression, we examined CD19+ B cells, CD3+ T cells and CD14+ monocytes isolated from human PBMCs and cultured for 3 days in the presence or absence of P/I (Fig. 2a). NgR1 is expressed by unstimulated B cells and T cells and is strikingly absent from non-stimulated monocytes. Stimulation with P/I leads to enhanced expression of NgR1 in all three cell subsets. Other members of the NgR1 signaling complex, LINGO, p75[NTR] and TROY, are also expressed by freshly isolated immune cells as indicated by RT-PCR (Fig. 2b). In all cases examined, expression of NgR1 is most robust in *ex vivo* T cells followed by monocytes and B cells as assessed by semi-quantitative RT-PCR (Fig. 2b). p75[NTR] is strongly expressed in T cells, B cells and monocytes. Both LINGO and TROY are detected in T and B cells. In monocytes, Troy is weakly expressed, while expression of LINGO is undetectable in most cases.

Nogo fails to affect PBMC proliferation or cytokine production

The shift in cytokine profiles in Nogo-vaccinated EAE mice from a pathogenic Th1 response to a protective Th2/3 response together with our data demonstrating NgR1 expression in human PBMCs raised the possibility that Nogo may signal to NgR1-expressing PBMCs to regulate their proliferation and/or cytokine profiles. To test this hypothesis we treated PBMCs with Nogo-P4 peptide, the minimal sequence of Nogo-66 required for binding and signaling through NgR1 (GrandPre et al., 2000). PBMCs were stimulated for 3 days with OKT3 to upregulate NgR1 expression in the presence or absence of Nogo-P4 peptide. The activity of Nogo-P4 was validated in neuronal signaling assays (data not shown). Stimulation with Nogo-P4 fails to significantly modify PBMC proliferation or production of either IL-5 or IFNγ (Figs. 3a–c).

To determine whether other myelin components could alter immune cell proliferation or cytokine production, we treated PBMCs with recombinant MAG-Fc or Fc control protein. Intriguingly, high doses of MAG inhibit PBMC proliferation (Fig. 3d). MAG-Fc does not have a significant effect on IL-5 production (Fig. 3e) but does enhance IFNγ production (Fig. 3f). Common receptors for Nogo-P4 and MAG include NgR1 and PirB; however, MAG also signals through additional cell surface receptors such as NgR2 (Venkatesh et al., 2005), gangliosides (Kelm et al., 1994, Strenge et al., 1999, Vyas and Schnaar, 2001), and integrins (Goh et al., 2008). The differential immune cell responses to MAG-Fc and Nogo-P4 indicate that the MAG-Fc effects on proliferation and IFNγ production are likely to be independent of NgR1.

Myelin alters the adhesive properties of immune cells

To test the possibility that NgR1 signaling could alter immune cell behaviour within the CNS environment, adhesion and migration assays were performed to examine the ability of immune cells to attach to myelin substrates that contain multiple NgR1 ligands (MAG, Nogo, OMgp). We find that *ex vivo* T cell adhesion is significantly diminished by myelin in a dose-dependent manner and that this activity is absent in heat-inactivated myelin (Fig. 4a). Unlike T cells, B cells and monocytes did not undergo regulated adhesion (Figs. 4b, c). These differential responses correlate with the relatively strong NgR1 expression levels in *ex vivo* T cells compared to B cells and monocytes (Fig. 2b).
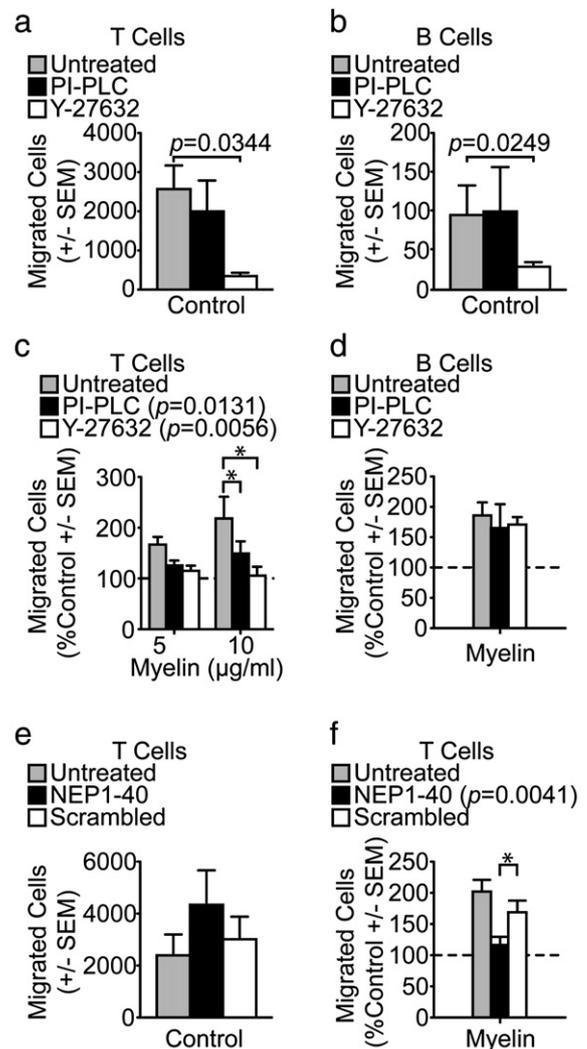

Fig. 7. Myelin-dependent enhancement of T cell motility is reduced in the presence of PI-PLC, Y-27632 or NEP1–40. (a–f) Migration assays were performed in the presence of Y-27632, PI-PLC or NEP1–40. The number of T cells (a, e) or B cells (b) migrating though control transwells in the presence of PI-PLC, Y-27632 (a, b), or NEP1–40 (e) was averaged from 3 (PI-PLC) or 4 (Y-27632 and NEP1–40) independent experiments. Statistical analysis indicates no significant effect of PI-PLC (by paired *t*-tests, $p = 0.2310$ and $p = 0.8729$ for T and B cells, respectively, two-tailed) or NEP1–40 (by one-way ANOVA, $p = 0.4180$) but a significant effect of Y-27632 ($p = 0.0344$ and $p = 0.0249$ for T and B cells, respectively, two-tailed). (c, d) The number of cells that migrated through transwells coated with 5 and 10 µg/ml myelin (c) or 5 µg/ml myelin (d) is expressed as a percentage of the number of cells that migrated through transwells coated with the same concentration of heat-inactivated myelin in the presence of PI-PLC or Y-27632 averaged from 4 independent experiments. Statistical analysis by two-way repeated measures ANOVA indicates a significant effect of both PI-PLC and Y-27632 on myelin-mediated promotion of T cell motility ($p = 0.0131$ for PI-PLC and $p = 0.0056$ for Y-27632). Statistical analysis by paired *t*-tests indicates no significant effect of either PI-PLC or Y-27632 on myelin-mediated promotion of B cell motility ($p = 0.5386$ for PI-PLC and $p = 0.7415$ for Y-27632). (f) The number of T cells that migrated through transwells coated with 10 µg/ml myelin is expressed as a percentage of the number of cells that migrated through control transwells in the presence of NEP1–40, averaged from 4 independent experiments. Statistical analysis by two-way ANOVA indicates a significant effect of NEP1–40 on myelin-mediated promotion of T cell motility ($p = 0.0041$).

Myelin enhances immune cell motility

Previous studies have demonstrated that macrophages isolated from lesioned peripheral nerve fail to migrate onto a myelin substrate and that this activity is dependent on NgR1 expression (Fry et al., 2007). We assessed the impact of myelin on immune cell motility using a modified Boyden chamber assay system. PBMCs were seeded in the upper chamber on polycarbonate filters coated with active or heat-inactivated myelin and their migration in response to a serum gradient was assessed by counting the number of cells migrating into the lower chamber over a fixed time interval. We find that myelin significantly promotes the motility of both T and B cells in response to a serum gradient (Figs. 5a, b) while monocytes are not affected (Fig. 5c).

Myelin effects on immune cell motility are attenuated by antagonizing NgR1

To investigate the contribution of NgR1 to immune cell responses to myelin, PBMCs were treated with PI-PLC to remove GPI-anchored proteins including NgR1 from the cell surface (Fournier et al., 2001), Y-27632 an inhibitor of the NgR1 downstream effector Rho Kinase (Alabed et al., 2006, Fournier et al., 2003), or NEP1–40 an NgR1 antagonist (GrandPré et al., 2002). Treatment with Y-27632 or PI-PLC had no significant impact on T cell adhesion to a permissive poly-L-lysine substrate (Fig. 6a). However, when cells were plated on myelin, PI-PLC and Y-27632 but not NEP1–40 significantly attenuated the anti-adhesive effect of myelin (Figs. 6b, c).

In migration assays, Y-27632 significantly diminished T and B cell migration in response to a serum gradient across a non-coated filter consistent with previous studies (Figs. 7a, b; (Vicente-Manzanares et al., 2002)). The myelin-dependent increase in T cell (Fig. 7c) but not B cell (Fig. 7d) motility is significantly attenuated by treatment with PI-PLC or Y-27632. NEP1–40 does not significantly affect T cell migration across a non-coated filter (Fig. 7e) but does antagonize the myelin-dependent increase in T cell migration (Fig. 7f). Together, these results demonstrate that myelin reduces adhesion and promotes motility of immune cell subsets and that the change in T cell motility is mediated via an NgR1-dependent mechanism.

Discussion

We find that NgR1 and components of the NgR1 receptor complex are expressed in *ex vivo* human T cells, B cells and monocytes. Expression of NgR1 is markedly upregulated by various modes of immune cell activation and there is a trend towards increased NgR1 expression in immune cells of MS patients compared to matched controls. Consistent with our expression data, we find that *ex vivo* T cells respond directly to myelin in both adhesion and migration assays. The pro-migratory activity of myelin is NgR1-dependent as indicated by attenuation of the responses upon either removal of GPI-anchored proteins, inhibition of Rho Kinase or antagonism of NgR1 with NEP1–

40. These findings suggest that NgR1 expression may impact immune cell infiltration and/or motility within the CNS (Table 1).

The NgR1 receptor complex is expressed in immune cells

NgR1 is expressed in *ex vivo* T cells and B cells, is absent in *ex vivo* monocytes, and is upregulated upon activation of all three cell subsets *in vitro*. Our finding that NgR1 can be upregulated by activated human monocytes is consistent with the report by Satoh et al. that myeloid cells within MS lesions express NgR1 (Satoh et al., 2005) and extends a recent report of NgR1 expression by rat macrophages at the site of sciatic nerve lesion (Fry et al., 2007). There is also a trend towards increased NgR1 expression in immune cells of MS patients compared to matched controls, perhaps reflecting several prior reports of increased immune cell activation in patients (Bar-Or, 2008) and, more recently, the observation that T cell quiescence is abrogated in circulating MS immune cells (Corvol et al., 2008).

Human immune cells respond to myelin in an NgR1-dependent fashion

While neutralization of Nogo in the context of MOG-induced EAE has been associated with an anti-inflammatory (TH1 towards TH2) cytokine shift (Fontoura et al., 2004, Karnezis et al., 2004), we observed no change in T cell proliferation or in the profile of cytokine production by human T cells stimulated *in vitro* in the presence of Nogo-P4 peptide. However, our findings are from short-term stimulation assays and do not preclude a potential *in vivo* role of NgR1 signaling in modulating T cell effector cytokine profiles, since such differentiation is known to be influenced by multiple factors including the profile of antigen-presenting cells and repeated rounds of stimulation. Intriguingly we find that stimulation with MAG-Fc affects PBMCs by reducing their proliferation and enhancing IFNγ production. We believe that these MAG-Fc effects are NgR1-independent since Nogo-P4 does not have similar effects. The mechanism through which MAG-Fc may impact immune cell behaviour remains to be explored.

For adhesion and migration assays, we restricted our analysis to *ex vivo* cells since *in vitro* activation of immune cells in culture leads to cellular clumping that can artifactually impact these assays. We find that T cells respond to myelin in both adhesion and migration assays and that both effects are dependent on the expression of GPI-anchored proteins and Rho Kinase activity. The anti-adhesive effects of myelin were insensitive to treatment with the NgR1 antagonists NEP1–40. This suggests that the anti-adhesive activity of myelin is NgR1-independent, however it is possible that multiple receptors are engaged making it difficult to tease out the contribution of NgR1 in this type of *in vitro* assay. The pro-migratory effect of myelin was abolished by treatment with NEP1–40 indicating a clear NgR1-dependence.

Ex vivo B cells express NgR1 and respond to the motility-promoting myelin activity but surprisingly not to the anti-adhesive properties of myelin. Intriguingly, enhanced B cell motility on myelin does not

Table 1
Summary of NgR1 expression analysis and myelin effects on adhesion and migration.

		T cells	B cells	Monocytes
NgR1 expression	Resting	+	+	−
	Activated (P/I)	+++	+++	+++
Myelin-mediated adhesion		Decrease	No effect	No effect
		Y-27632 dependent		
		PI-PLC dependent		
		NEP1–40 independent		
Myelin-mediated migration		Increase	Increase	No effect
		Y-27632 dependent	Y-27632 independent	
		PI-PLC dependent	PI-PLC independent	
		NEP1–40 dependent		

−, +, and +++ signify the relative level of expression of NgR1 in indicated immune cell subsets without stimulation (resting) and following activation with PMA/Ionomycin (P/I).

appear to be NgR1-, Rho Kinase- or GPI-anchored protein-dependent, pointing to further differences in the mechanisms underlying myelin-dependent effects on immune cells. While our RT-PCR results suggest that B cells can express all components of the NgR1 complex it is possible that cell surface levels of individual receptor components are insufficient to mediate a full range of responses to MAIs or that the intracellular milieu of the B cell affects NgR1-dependent signaling. For example, intracellular levels of cyclic nucleotides can regulate the response of an injured neuron to inhibitory cues (Cai et al., 2001). This leaves open the possibility that B cells may respond to Nogo in an *in vivo* context.

The failure of *ex vivo* monocytes to respond to myelin in our migration and adhesion assay is consistent with the lack of NgR1 protein expression prior to their activation (See Fig. 2a). However, we postulate that in conditions of immune cell activation and subsequent to NgR1 upregulation, monocytes have the capacity to respond to myelin in a similar fashion to T cells. A previous study has demonstrated that NgR1 is expressed by macrophages infiltrating the site of a sciatic nerve lesion and that macrophages undergo an NgR1-dependent efflux from a peripheral nerve lesion site upon nerve remyelination (Fry et al., 2007). The same authors have further advanced the idea that NgR1 expression may regulate macrophage efflux under a variety of inflammatory conditions (David et al., 2008). Our *in vitro* data demonstrating diminished adhesion of NgR1-expressing T cells to myelin, and enhanced motility of T cells in the Boyden chamber are consistent with such a model, and suggest that NgR1 signaling may play a more generalizable role in regulating the motility of different immune cell subsets in the context of immune-neural tissue interactions, such as in the inflamed CNS of MS patients.

Together this data suggests a new avenue of investigation towards our understanding of the Nogo signaling axis in the context of neuroinflammation. The expression of NgR1 on human immune cells and its upregulation following immune cell stimulation is of particular interest in the context of CNS inflammatory conditions such as MS, where activated immune cells are found at sites of myelin and axonal injury. Further studies may provide important insights into the roles that Nogo signaling may play during the MS disease process, thereby guiding the development of novel therapies aimed at beneficially resolving inflammation while also optimizing axonal repair and functional recovery.

Acknowledgments

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