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Article

A Role for Nogo Receptor in Macrophage Clearance from Injured Peripheral Nerve

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SUMMARY

We report a role for Nogo receptors (NgRs) in macrophage efflux from sites of inflammation in peripheral nerve. Increasing numbers of macrophages in crushed rat sciatic nerves express NgR1 and NgR2 on the cell surface in the first week after injury. These macrophages show reduced binding to myelin and MAG in vitro, which is reversed by NgR siRNA knockdown and by inhibiting Rho-associated kinase. Fourteen days after sciatic nerve crush, regenerating nerves with newly synthesized myelin have fewer macrophages than cut/ligated nerves that lack axons and myelin. Almost all macrophages in the cut/ligated nerves lie within the Schwann cell basal lamina, while in the crushed regenerating nerves the majority migrate out. Furthermore, crush-injured nerves of NgR1- and MAG-deficient mice and Y-27632-treated rats show impaired macrophage efflux from Schwann cell basal lamina containing myelinated axons. These data have implications for the resolution of inflammation in peripheral nerve and CNS pathologies.

INTRODUCTION

Macrophages respond rapidly to injury and other insults to the nervous system and play an important role in tissue repair. Successful repair also requires that these cells be signaled to leave the site of injury appropriately and thus prevent chronic inflammation. Macrophages, primarily of hematogenous origin, are recruited into the degenerating distal segment of the injured nerve between 2 and 3 days after injury (Bendszus and Stoll, 2003; Bruck, 1997; Mueller et al., 2003; Perry et al., 1987) and increase in number in the first 2 weeks. The initial breakdown of the axon itself occurs rapidly within the first day, undergoing granular degeneration of cytoskeletal structures via the action of calcium-dependent endogenous proteolytic activity and does not require the presence of macrophages (Coleman and Perry, 2002; George et al., 1995). Macrophages phagocytose axonal and myelin debris particularly in the first 2 weeks and decrease in number thereafter (Bendszus and Stoll, 2003; Mueller et al., 2003). The factors that regulate the recruitment and activation of macrophages during Wallerian degeneration have been intensively studied (De et al., 2003; Rotshenker, 2001; Stoll and Jander, 1999). However, little is known of the mechanisms that signal the clearance or elimination of macrophages from the nerve at the end of the period of Wallerian degeneration. Apoptosis has been reported to account for the loss of only a very small number of macrophages (Kuhlmann et al., 2001; Mueller et al., 2003). The available evidence suggests that the majority of these macrophages leave the nerve via the circulation to the spleen and draining lymph nodes (Kuhlmann et al., 2001). The signals that trigger the end-stage phagocytic macrophages to migrate out of the nerve are still not known.

We show here that macrophage clearance from lesioned peripheral nerves is mediated in part by Nogo receptors (NgRs). Three members of the NgR family, NgR1, NgR2, and NgR3, have recently been identified (Venkatesh et al., 2005). Of these, NgR1 has been extensively studied in neurons and shown to induce repulsive effects on the growth cone leading to its collapse, and possibly to inhibition of axon regeneration (Filbin, 2003; Fournier and Strittmatter, 2001; McKerracher and David, 2004). NgR1 binds to three axon growth inhibitory molecules in myelin: Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), while NgR2 binds selectively to MAG but not Nogo-A and OMgp (Filbin, 2003; Venkatesh et al., 2005). We now provide evidence that NgR1 and NgR2 are expressed on the surface of phagocytic macrophages and that interaction between these receptors and their ligands such as MAG, associated with newly synthesized myelin wrapping around regenerated axons, mediates the efflux of macrophages out of the Schwann cell basal lamina. The efflux of macrophages into the endoneurial space appears to be an important first step in their eventual migration out of the injured nerve, resulting in the resolution of the inflammatory response after peripheral nerve injury. Our current studies therefore reveal a novel role (to our knowledge) for NgRs in the immune cell response in injured peripheral nerves and may also have implications for other nervous system pathologies.

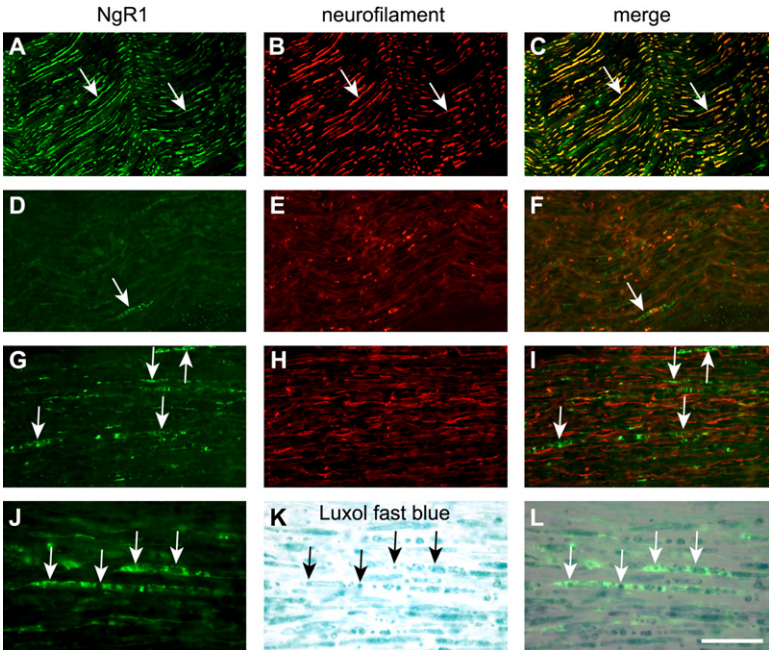

Figure 1. NgR1 Expression Is Lost in Axons after Crush Injury

Double immunofluorescence labeling of NgR1 (A) and neurofilament (B) in a longitudinal section of uninjured rat sciatic nerve shows colocalization of NgR1 in axons ([A–C], arrows). At 3 days after crush injury, NgR1 expression is not seen in degenerating axons (D–F) but is observed in a few cells containing neurofilament debris (arrows in [D] and [F]). These NgR1$^+$ cells increase in number at 5 days post-injury ([G–I], arrows), but the newly regenerated neurofilament$^+$ axons do not express NgR1 (I). Luxol fast blue staining for myelin (K) of 5 day post-crush nerve shows that NgR1$^+$ cells contain myelin debris ([J–L], arrows), indicating that these cells are macrophages. Micrographs are of longitudinal sections of injured nerve, 2 mm distal to the crush site. Scale bar, 100 μm.

RESULTS

Loss of NgR1 Expression in Degenerating Axons

Robust expression of NgR1 is detected in intact axons of the normal adult rat sciatic nerve (Figures 1A–1C). The axonal labeling with the anti-NgR1 antibody was confirmed by its colocalization with profiles containing neurofilament (Figure 1C). Within 3 days after a sciatic nerve crush injury in adult rats, all the axons degenerate. This is evident from the lack of neurofilament immunoreactivity (Figure 1E) and is in keeping with previous reports of rapid calcium-dependent degeneration of axons that does not require the presence of macrophages (George et al., 1995). The labeling with the anti-neurofilament antibody is confined to small globular structures that are likely to be the remnants of degenerated neurofilaments (Figure 1E). The NgR1 immunoreactivity that is normally associated with axons is therefore completely lost at 3 days post-crush injury (Figures 1D and 1F). The loss of neurofilament and NgR1 is not due to phagocytosis by macrophages because at 3 days post-injury they are in the initial stages of recruitment, and there are only a few macrophages in the degenerating nerve (Bruck, 1997; Mueller et al., 2003).

NgR Expression in Macrophages in the Degenerating Nerve

Regenerating axons are present in the distal nerve segment by 5 days after crush injury, but these axons do not express NgR1 (Figures 1G–1I). This immunoreactivity returns several weeks later when the axons have reinnervated their targets (data not shown). However, a small number of NgR1$^+$ cells appear in the degenerating nerve at day 3 (Figure 1D) and increase in number thereafter (Figure 1G). At 3 days, these NgR1$^+$ cells contain neurofilament debris (Figure 1F), but this is not evident at day 5 (Figure 1I). Furthermore, double labeling tissue sections with Luxol fast blue, a histological stain for myelin, reveals that the NgR1$^+$ cells colocalize with degenerating myelin ovoids (Figures 1J–1L), suggesting that they may be macrophages that have phagocytosed myelin debris. Additional evidence that these NgR1$^+$ cells are also OX-42$^+$ confirm that they are macrophages (Figures 2A–2F). Macrophages in the degenerating crush-injured nerves almost double in number between days 3 and 5 post-injury and remain elevated at day 7 (Figure 2G). The percentage of these OX-42$^+$ macrophages that express NgR1 increases from approximately 30% at day 3 to 75% at day 7 post-lesion (Figure 2H). An increase in NgR1 mRNA is detected in 3 and 7 day lesioned sciatic nerve (Figure 2I), providing evidence that the NgR1 detected in macrophages is due to de novo synthesis after injury. Furthermore, the NgR1 immunoreactivity in tissue sections appears to be present around the periphery of these cells, especially by day 7 post-crush (Figures 2D–2F), suggesting a cell surface localization.

To confirm that NgR1 is on the cell surface of the macrophages, we dissociated macrophages from 3 and 7 day post-crush rat sciatic nerves by mild trypsinization, plated the cells onto poly-L-lysine (PLL) coated round glass coverslips for 2 hr, and incubated the live cells with NgR1 antibodies to detect cell surface labeling. These experiments show punctate cell surface localization of NgR1 (Figures 3A–3F). The percentage of OX-42$^+$ macrophages that express NgR1 on the cell surface almost doubles between days 3 and 7 post-crush (Figure 3G), similar to that seen in vivo, as noted above. Furthermore, NgR1 immunoreactivity is detected in 70.6% ± 2.0% and 81.5% ± 10.5% of very strongly OX-42-immunostained macrophages at

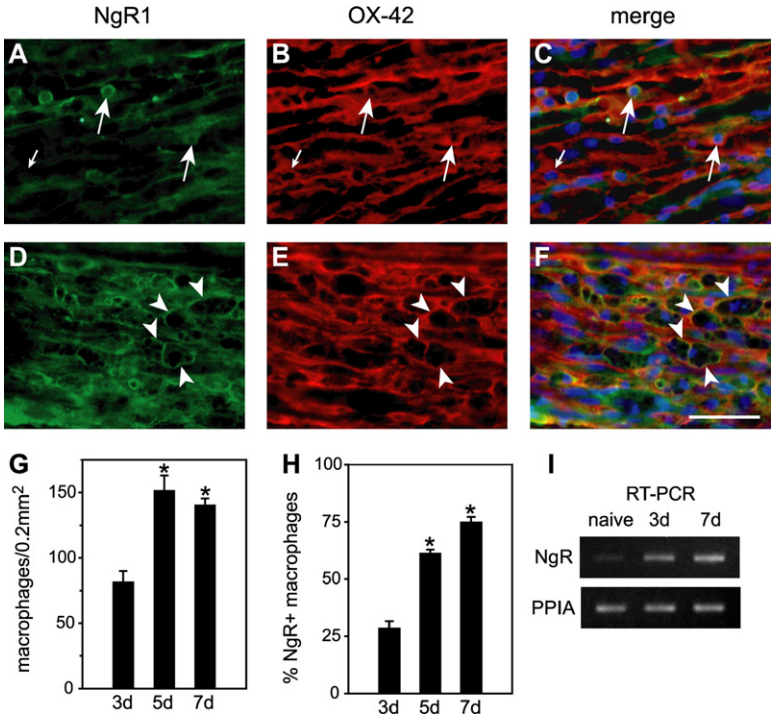

Figure 2. Macrophages in the Degenerating Nerve Express NgR1

Double immunofluorescence labeling of injured rat sciatic nerve at 5 days post-crush (A–C) shows that OX-42$^+$ macrophages (B) express NgR1 (large arrows in [A]–[C]). OX-42$^+$ macrophages that do not express NgR are also seen (small arrow in [A]–[C]). Tissue section of a 7 day crush-injured nerve (D–F) shows that the NgR1$^+$ immunoreactivity (D) of OX-42$^+$ macrophages (E) appears to be largely localized to the cell surface of these large phagocytic cells (arrowheads in [D]–[F]). Micrographs are of longitudinal frozen sections at 3 mm distal to the injury site; (C) and (F) show triple labeling for NgR1, OX-42, and DAPI. Scale bar, 50 μm. Quantification of the number of OX-42$^+$ macrophages (G), and the percentage of OX-42$^+$ macrophages that express NgR1 (H) in the distal segment of 3, 5, and 7 day post-crush nerves reveal an increase in macrophage number and the proportion that express NgR1 between 3 and 7 days. Data represent mean ± SEM; *$p < 0.001$. n = 3 for each age group. (I) RT-PCR analysis of NgR1 expression in sciatic nerve shows increasing upregulation of NgR1 mRNA in the distal nerve segment at 3 and 7 days after crush injury. PPIA is used as a control (see Experimental Procedures for more details).

3 and 7 days, respectively, indicating that NgR1 is mainly expressed by more activated macrophages (arrows in Figures 3A–3F), as increased expression of the $\alpha_M\beta_2$ integrin recognized by the OX-42 antibody is an indicator of a greater activation state. Since macrophages in the lesioned nerve are largely derived from blood monocytes that enter the nerve during the first week after injury (Bendszus and Stoll, 2003), we assessed the percentage of blood monocytes that express NgR1. Only 17.2% ± 4.3% of the monocytes show NgR1 immunoreactivity on the cell surface (Figure 3G). This includes cells that show relatively low as well as higher levels of OX-42 staining,

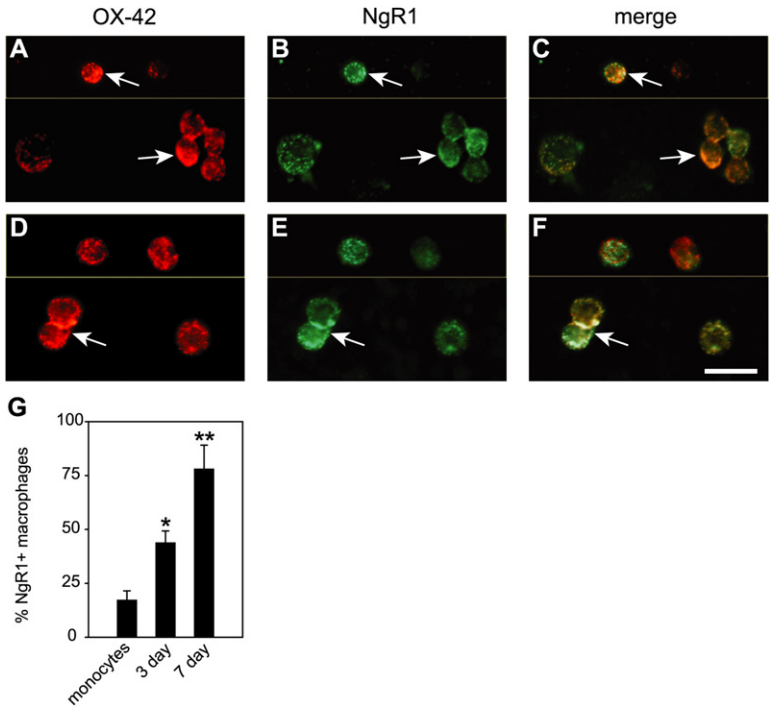

Figure 3. Cell Surface Expression of NgR1 by Macrophages

Montages showing immunofluorescence labeling of live cultures of macrophages harvested from 3 (A–C) and 7 day (D–F) crush-injured nerves reveal that OX-42$^+$ macrophages (A and D) show punctate cell surface labeling for NgR1 (B and E). Note that the more activated macrophages (i.e., stronger OX-42 immunoreactivity) express more NgR1 (arrows). Scale bar, 20 μm. (G) Only a small percentage of OX-42$^+$ blood monocytes express NgR1. The percentage of OX-42$^+$ macrophages in the lesioned nerves that show cell surface NgR1$^+$ labeling increases significantly from monocytes and between 3 and 7 days post-crush injury. Data represent mean ± SEM; *$p = 0.003$, **$p < 0.001$. n = 3 for each group.

suggesting that the latter may be cells that were activated by the purification process. Only 11.5% ± 3.1% of monocytes that express low levels of OX-42 are NgR1$^+$, indicating that only a small number of monocytes express NgR1 in the resting state. These data suggest that increasing numbers of macrophages express NgR1 after they enter the degenerating nerve and become activated, i.e., after they phagocytose axonal and myelin debris. The delayed expression of NgR1 in macrophages after they enter the nerve also suggests that it is unlikely to have a role in the recruitment of these cells into the damaged nerve.

We also assessed the cell surface expression of NgR2 in cells dissociated from the lesioned nerves as described above. NgR2 is expressed on the cell surface of 53.4% ± 1.8% of the macrophages from 3 day and 74.1% ± 1.9% of 7 day post-lesioned nerve (Figures 4A–4C and 4J). As with NgR1, only 16.5% ± 3.3% of monocytes purified from the blood were NgR2$^+$, of which only 12.3% that were weakly OX-42$^+$ express NgR2. Since both NgR1 and NgR2 are GPI-anchored proteins, we examined whether their transmembrane binding partners, p75^{NTR} and TROY (a member of the TNF receptor family) (Filbin, 2003; Park et al., 2005; Shao et al., 2005; Yamashita et al., 2002), are expressed by these macrophages. p75^{NTR} was not expressed by macrophages at any time points (Figures 4D–4F); however, TROY was expressed by 65.7% ± 4.7% and 69% ± 5% of OX-42$^+$ macrophages from 3 day and 7 day lesioned nerves, respectively (Figures 4G–4I and 4K). Although there was no significant difference in the numbers of cells expressing TROY at 3 and 7 days post-injury, only 20.8% of blood monocytes express TROY (Figure 4K), of which only 15.8% that were weakly OX-42$^+$ express TROY. TROY, like NgR1 and NgR2, is therefore expressed in monocytes/macrophages largely after they enter the lesioned nerve and become activated.

NgR/Ligand Interactions Mediate Reduced Binding and Repulsion of Macrophages to Myelin

Since NgR1 is known to mediate the inhibitory or repulsive effects of several myelin proteins (Nogo-A, MAG, and OMgp) to cause collapse of axonal growth cones and NgR2 mediates MAG-induced neurite growth inhibition, we first assessed whether macrophages that express these receptors show repulsion to myelin, i.e., show reduced binding to a myelin substrate in vitro. Macrophages were dissociated from the distal segment of 3 and 7 day crushed nerves and plated onto myelin-coated tissue culture wells for 3 hr. The number of macrophages from 7 day post-crush nerves that bind to the myelin substrate is reduced by 35% as compared to macrophages obtained from 3 day crushed nerves (Figure 5A), which correlates well with the increase in the percentage of macrophages that express NgR1 and NgR2 at 7 days post-injury.

To obtain direct evidence for the role of NgRs in reducing the binding of 7 day post-injury macrophages to myelin, we assessed the effect of selective gene silencing (knockdown) of NgR1 and NgR2 by small interfering RNA (siRNA). For these experiments, macrophages from

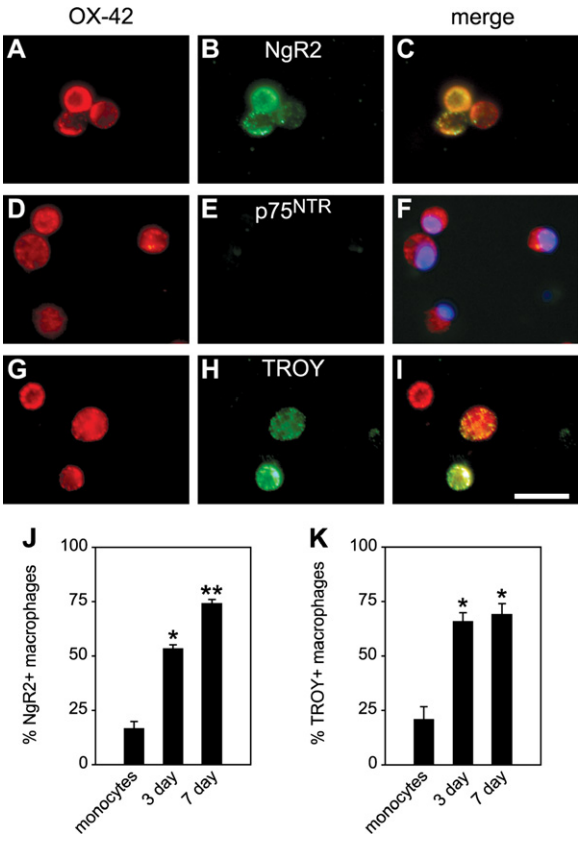

Figure 4. Cell Surface Expression of NgR2 and TROY by Macrophages

Micrographs showing macrophages dissociated from the distal stumps of 7 day crushed adult rat sciatic nerves. Macrophages were identified by immunofluorescence staining with the OX-42 antibody. (A), (D), and (G) show cell surface labeling for NgR2 (B), and TROY (H) but not for p75NTR (E). Panels (C) and (I) are merged images of panels (A) and (B) and (G) and (H), respectively, and panel (F) is a merge of panels (D) and (E) plus the nuclear DAPI stain. Scale bar, 20 μm. (J) Only 16.5% of OX-42+ monocytes express NgR2 on the cell surface. In contrast, about 53% of the macrophages from nerves 3 days after injury express NgR2 (*p<0.001) and this number increases further to about 74% at 7 days post-injury (**p = 0.002). (K) TROY is expressed on the cell surface of about 20% of blood monocytes, and this number increases rapidly to between 65% and 70% in macrophages obtained from 3 and 7 day crushed nerves. The values at 3 and 7 days are significantly higher than for blood monocytes (*p = 0.003). Data represent mean ± SEM; n = 3 for each group.

7 day crushed sciatic nerves were harvested and transfected in vitro with either a cocktail of NgR1 and NgR2 siRNA or negative control siRNA containing a nonspecific sequence. Cells were first monitored daily by immunofluorescence labeling, which showed a sustained knockdown of NgR1 and NgR2 cell surface protein expression for up to 4 days in culture (see Figures S1A and S1B in the Supplemental Data available online). The greatest knockdown efficiency was achieved 2 days after transfection, with a 69% reduction of NgR1$^+$ cells and 72% reduction of NgR2$^+$ cells. The percentage of NgR1$^+$ and NgR2$^+$

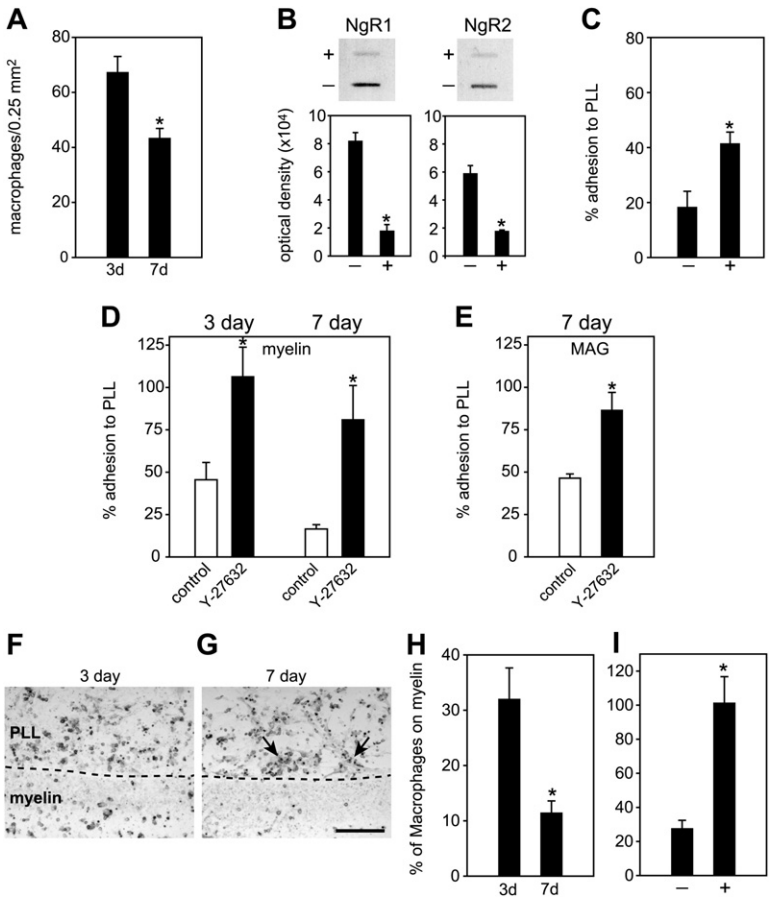

Figure 5. NgR/Ligand Interactions and RhoA Activation Mediate Reduced Binding and Repulsion of Macrophages to Myelin

(A) Graph showing the binding of macrophages obtained from 3 and 7 day crush-injured nerves plated on myelin. The binding of these cells is significantly reduced at 7 days post-injury (*p = 0.025).

(B) Slot blots show a reduction in NgR1 and NgR2 protein expression in macrophages from 7 day lesioned nerves, 2 days after transfection with siRNA specific for NgR1 and NgR2 (+) as compared to macrophages treated with nonspecific siRNA (–). Quantification of the slot blot data (graphs) indicates a knockdown in NgR1 and NgR2 expression of approximately 70%, 2 days after transfection with specific siRNA (+, *p < 0.001).

(C) Graph of macrophage binding to myelin substrate 2 days after transfection with specific (+) or nonspecific (–) siRNA. Binding is significantly increased after NgR1 and NgR2 siRNA knockdown (+) (*p = 0.03). The values were normalized to that obtained from poly-L-lysine (PLL) coated areas of the same wells (see Experimental Procedures).

(D) Treatment with Y-27632 (10 μM) of macrophages harvested from nerves 3 and 7 days after injury increases binding of these cells to myelin-coated substrates (*p = 0.03). Controls were treated with vehicle.

(E) A similar reversal of the decrease in binding to recombinant MAG-coated substrates is also detected when cultures were treated with Y-27632 (*p = 0.028).

(F and G) Micrographs showing cultures of the migration assay with macrophages from 3 and 7 day lesioned nerves. Note that the majority of 7 day macrophages are repulsed at myelin interface ([G], arrows), while a greater number of 3 day macrophages migrate across the myelin interface (F). The dashed lines indicate the myelin interface. Scale bar, 200 μm.

(H) Quantification of the migration assay shows a marked reduction in migration of macrophages from 7 day lesioned nerves versus 3 day lesioned nerves across the myelin interface (*p = 0.004).

(I) Quantification of the migration assay using macrophages from 7 day crushed nerves, 2 days after transfection with NgR1/NgR2-specific (+) or nonspecific control (–) siRNA. The inhibition of migration seen with cells treated with control siRNA (–) was completely reversed with cells after NgR1/NgR2 siRNA knockdown (+) (*p < 0.001).

All data represent mean ± SEM; n = 3.

macrophages transfected with negative control siRNA was not significantly different from nontransfected macrophages over 4 days in culture (p = 0.959). The knockdown at 2 days in the NgR-specific siRNA-treated macrophages was further confirmed by slot blot analysis that showed a 78% and 70% reduction in NgR1 and NgR2 protein expression, respectively, as compared to control siRNA-treated cells (Figure 5B). We then assessed the ability of macrophages from 7 day lesioned nerves, 2 days after transfection with NgR1/NgR2 siRNA, to bind to myelin. The assay used was similar to the assay described above. The NgR1/NgR2 siRNA-transfected macrophages showed a 55% increase in binding to the myelin-coated substrate as compared to control siRNA-transfected macrophages (Figure 5C). The binding to myelin by control (nonspecific) siRNA-transfected macrophages was similar to that observed with nontransfected macrophages from 7 day lesioned nerves. These results therefore provide direct evidence that NgR1 and NgR2 on macrophages mediate the reduced binding of these cells to myelin.

NgR1, with its coreceptors, has been shown to mediate growth cone collapse and axon growth inhibition via activation of RhoA and downstream molecules such as Rho-associated kinase (ROK) (Filbin, 2003; Fournier et al., 2003; McKerracher and David, 2004). We therefore examined whether RhoA plays a role in the reduction of macrophage binding to myelin by treating dissociated cultures of macrophages obtained from the distal segment of 3 and 7 day crushed nerves with Y-27632, an inhibitor of ROK. Blocking ROK increased binding of macrophages from 3 and 7 day crushed nerves to myelin up to 100% and 81% of PLL-control values, respectively (Figure 5D). These results suggest that the expression of NgR1 and NgR2 by phagocytic macrophages reduces their binding to myelin via a RhoA-dependent mechanism. To further

assess whether these effects are mediated via MAG, one of the axon growth inhibitors found in myelin, we carried out similar in vitro assays with recombinant MAG-coated wells. MAG was chosen because (1) it is a ligand for both NgR1 and NgR2; (2) NgR2 selectively binds only MAG and not Nogo-A and OMgp; and (3) MAG is expressed in peripheral nerve myelin but Nogo-A is not. We found that, like the effects of myelin substrates, the binding of macrophages was also inhibited by recombinant MAG-coated substrates, and this effect could also be reversed by treatment with the ROK inhibitor Y-27632. Blocking ROK increased binding of macrophages obtained from 7 day lesioned nerves to MAG-coated substrates up to 86% of the PLL-control value (Figure 5E). This reduction in binding to myelin and MAG may reflect a repulsive effect of myelin proteins on macrophage behavior.

To further assess whether this is a repulsive interaction, we designed a migration assay in which macrophages from 3 and 7 day lesioned nerves were plated on the poly-L-lysine-coated portion of the culture substrate and allowed to migrate toward an adjacent myelin-coated region. The behavior of the cells after they contact the myelin interface was assessed 24 hr after plating. Interestingly, macrophages from the 3 day lesioned nerves migrated across the interface on to the myelin substrate (Figure 5F). In contrast, the majority of the macrophages from the 7 day lesioned nerves appeared to be arrested at the myelin interface (Figure 5G). Quantification of the macrophages that migrated on to myelin showed a 65% reduction between cells from 3 and 7 day lesioned nerves (Figure 5H). To obtain direct evidence that this reduction in the migration of macrophages from 7 day lesioned nerves on to myelin is mediated by NgRs, we did the migration assay using these cells after siRNA knockdown of NgR1 and NgR2. Only 27% of the control siRNA-treated macrophages from 7 day lesioned nerves migrated across the myelin interface on to myelin. This inhibition was completely reversed in the NgR1/NgR2 siRNA-transfected macrophages, which showed migration of up to 100% of PLL-control values (Figure 5I). Taken together, these results indicate that NgR expressed on macrophages mediates the repulsion of these cells by myelin.

Macrophage Efflux from the Lesioned Sciatic Nerve Is Controlled by the Presence of Myelin around Remyelinated Axons

We next assessed whether the reduced binding of macrophages to myelin and the repulsion of macrophages by myelin in vitro is reflected in vivo by increased macrophage clearance from injured nerves in the presence of myelin. To do this, we compared the number of macrophages in the adult rat sciatic nerve 14 days post-lesion in two injury models: (1) sciatic nerve crush injury in which axons regenerate into the distal nerve segment and become remyelinated, and (2) sciatic nerve cut and ligation in which axons are prevented from regenerating, and thus the distal nerve segment lacks newly synthesized myelin. The number of macrophages in the distal segment

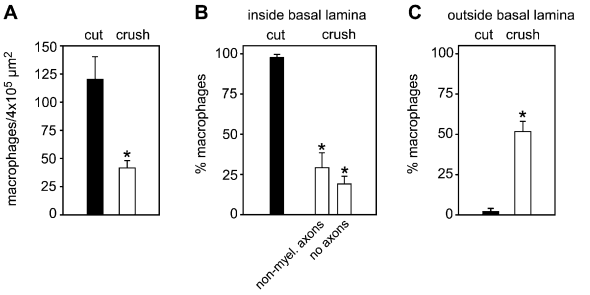

Figure 6. Macrophage Clearance from Lesioned Sciatic Nerve Is Controlled by the Presence of Newly Synthesized Myelin around Regenerated Axons

(A) Quantitative EM analysis of the number of macrophages in 14 day injured nerves indicates that their number is significantly greater in cut/ligated nerves than in crush-injured nerves (*p = 0.003).

(B) The percentage of macrophages located within the Schwann cell basal lamina. Almost all the macrophages in cut/ligated nerves remain inside the basal lamina (97.8%), while in crush-injured nerves that regenerate and remyelinate, only 48.3% are within the Schwann cell basal lamina, of which 29% are associated with axons that have not yet become remyelinated, and the remaining 19% are in basal lamina tubes that lack regenerated axons (*p = 0.001).

(C) The percentage of macrophages located outside the Schwann cell basal lamina. About 52% of the macrophages in the regenerating crush-injured nerves lie outside the Schwann cell basal lamina as compared to only about 2% in the cut/ligated nerves (*p < 0.001).

All data represent mean ± SD; n = 3.

of the cut and crushed nerves 14 days after lesion, quantified from longitudinal frozen sections labeled by immunofluorescence for OX-42, show that there is a significant increase in the number of macrophages in the cut/ligated nerves (201.8 ± 14.8/0.25 mm^2; p = 0.003) as compared to the crushed nerves (132.1 ± 14.6/0.25 mm^2). These data suggest that in the absence of newly synthesized myelin the clearance of macrophages from the injured nerve is impaired.

Macrophages penetrate the Schwann cell basal lamina to reach and phagocytose the myelin that is shed by Schwann cells after nerve injury. In crush-injured nerves, axons regenerate through Schwann cell basal lamina tubes that contain macrophages. If macrophages that express NgR1 were repulsed by myelin, we would predict that they would leave the Schwann cell basal lamina tube when the newly regenerated axons become remyelinated. On the other hand, in the cut/ligated nerves in which axon regeneration is prevented, the macrophages that have entered the Schwann cell basal lamina to phagocytose debris would not be signaled to leave because of the lack of newly synthesized myelin. To assess whether this is the case, we examined cross-sections of 14 day cut/ligated or crush-injured nerves by electron microscopy. Quantification of cross-sections of the nerve by electron microscopy revealed a 65% increase in the total number of macrophages in the cut/ligated nerves as compared to crush-injured nerves (Figure 6A). Furthermore, the number of macrophages within the Schwann cell basal lamina in the cut/ligated nerves is 5.8-fold greater than in

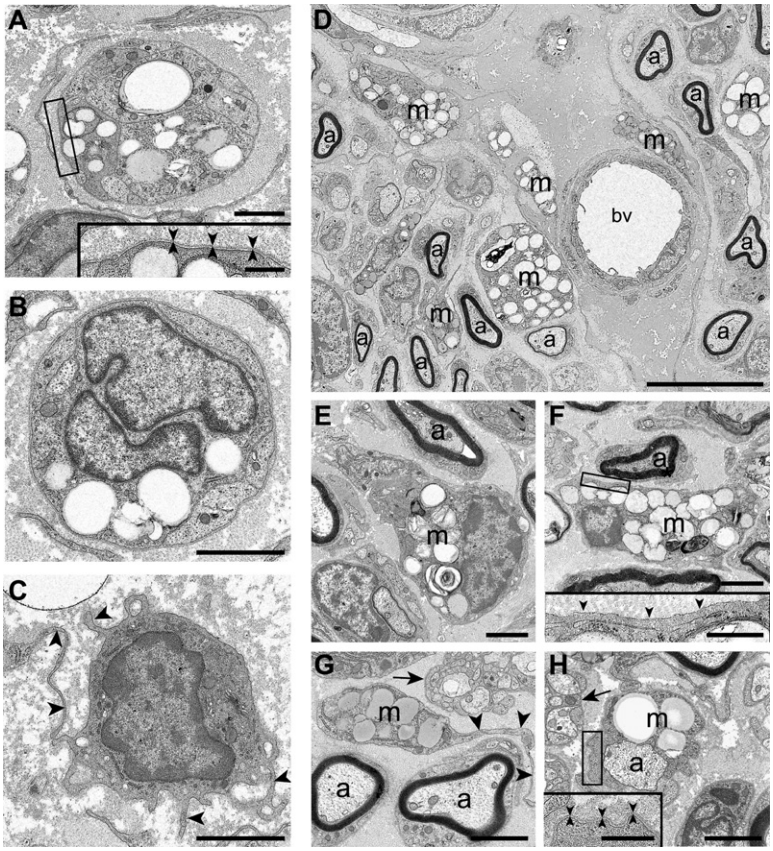

Figure 7. Presence of Newly Synthesized Myelin Influences the Efflux of Macrophages from the Schwann Cell Basal Lamina

Electron micrographs taken from cross-sections of the sciatic nerve, 2 mm distal to the site of cut/ligation (A–C) and crush injury (D–H) taken 14 days after lesioning.

(A and B) Macrophages containing clear and light-density vacuoles that are end-state products of myelin degradation are located within the Schwann cell basal lamina in the nonregenerating cut/ligated nerve. The "insert" in panel (A) is a higher magnification of the boxed area that illustrates the basal lamina (arrowheads) surrounding the cell membrane.

(C) A motile macrophage that lacks phagocytosed material or secondary lysosomes but has numerous cytoplasmic processes (arrowheads) and is located outside of the basal lamina suggests that it might be a newly recruited cell.

(D) In contrast, after crush injury, numerous axons (a) in various stages of remyelination are visible in the nerve. In addition, many foamy phagocytic macrophages (m) located outside the Schwann cell basal lamina are seen close to the blood vessel (bv). Note that none of the myelinated axons have macrophages within their basal lamina.

(E and F) Additional examples of macrophages (m) with phagocytosed material and end-state products of myelin-degradation. These macrophages are located outside the Schwann cell basal lamina as evident by the absence of basal lamina around them. The absence of the basal lamina is illustrated in the higher magnification shown in the "insert" (arrowheads) in panel (F), which is taken from the boxed area. Note that the basal lamina sheaths of myelinated axons ("a" in [E] and [F]) are devoid of macrophages.

(G) A macrophage (m) with an elongated cytoplasmic process (arrowheads) that suggests that this is a motile cell. It is located outside the Schwann basal lamina in the endoneurial space. The Schwann cell basal laminae of the two myelinated axons (a) are devoid of macrophages. Arrow indicates small, unmyelinated axons.

(H) An example of a macrophage (m) that lies within a basal lamina that contains an axon (a) which lacks myelin. The "insert" at the bottom is a higher magnification of the boxed area that illustrates the basal lamina (arrowheads). Note the larger size of this axon (a) as compared to the typical unmyelinated axons (arrows in [G] and [H]), which indicates that it has not yet been remyelinated.

Scale bar in (A)–(C) and (E)–(H), 2 μm; (D), 10 μm; insert in (A), 1 μm; insert in (F) and (H), 0.5 μm.

crushed/regenerated nerves. The cell counts for the two groups are 118 ± 21.5 cells/4 × 10^5 μm^2 in cut/ligated nerves versus 19.3 ± 5.1 cells in crush-injured nerves in the same area ($p < 0.001$). In the cut/ligated nerves, this represents 97.8% ± 1.8% of the total number of macrophages in the distal nerve segment (Figure 6B). Examples of macrophages within the Schwann cell basal lamina in cut/ligated nerves are shown in Figures 7A and 7B. In contrast, of the small number of macrophages present in crushed/regenerated nerves, only 48.3% ± 6.3% are within Schwann cell basal lamina, of which 29% are associated with nonmyelinated axons (Figure 6B). Based on the size of these axons, they are likely to be axons that have not yet become remyelinated (Figure 7H). The remainder of the macrophages within the Schwann cell basal lamina in the crushed/regenerated nerves are within basal lamina tubes that lack regenerated axons (Figure 6B). Importantly, none of the large number of myelinated axons in the crushed/regenerated nerves contain macrophages within their basal lamina (Figures 7D–7G), including those that have just begun to myelinate (Figure 7D and Figures S2A and S2B). Additionally, 51.7% ± 6.3% of the total number of macrophages in crushed/regenerated nerves that contain newly synthesized myelin lie in the endoneurial space outside the Schwann cell basal lamina, as compared to only 2.2% ± 1.8% in the cut nerves lacking myelin (Figures 6C and 7D–7G). In the crushed nerves, macrophages located outside Schwann cell basal lamina were detected in close proximity to blood vessels, suggesting that these cells may be migrating toward these vessels (Figure 7D). Macrophages were easily identified by their foamy appearance due to the presence of numerous light-density vacuoles and lysosomes containing the end products of phagocytosis, and when located outside the Schwann cell basal lamina in crush-injured nerves, they possess an irregular

shape with cytoplasmic processes indicative of motile cells (Figures 7E and 7G). Small-diameter, unmyelinated axons ensheathed by Schwann cell were not associated with macrophages (arrows in Figures 7G and 7H). This is in keeping with previous studies showing that, following injury, macrophages are not recruited into purely unmyelinated peripheral nerves to clear the minimal debris that is present (Bray et al., 1972). Interestingly, macrophages lacking phagocytosed material and with morphological features of highly motile cells were also occasionally detected in cut/ligated nerves, suggesting that they continue to be recruited into nerves lacking myelin (Figure 7C). The 14 day time point was chosen for these studies because, at the earlier time point of 7 days, the majority of the macrophages (71.6% ± 3.6%) in crush-injured nerves were still contained within Schwann cell basal lamina (Figure S2C). Furthermore, 96% of these macrophages within the Schwann cell basal lamina were associated with large nonmyelinated axons, indicating that remyelination had not yet occurred. Only a small proportion (13.6% ± 5.7%) of end-stage macrophages filled with phagocytosed debris were found outside the basal lamina.

Macrophage Efflux from Schwann Cell Basal Lamina Is Impaired by Disrupting NgR/Ligand Interactions

We next carried out experiments to obtain direct evidence whether NgR/ligand interactions influence macrophage efflux out of the Schwann cell basal lamina after nerve-crush injury by assessing this in two different strains of gene knockout mice. Previous studies have reported that NgR1 is a higher-affinity receptor for Nogo-A than for MAG (Fournier and Strittmatter, 2001; Liu et al., 2002), while NgR2 is a high-affinity receptor only for MAG (Venkatesh et al., 2005). Since peripheral nerve myelin expresses high amounts of MAG but not Nogo-A, it would have been preferable for us to use NgR2-deficient mice. However, at present only NgR1-deficient mice are available, which might be expected to yield only a moderate effect on macrophage behavior after injury. On the other hand, MAG-deficient mice provide the best opportunity at present to disrupt the ligand/receptor interactions of both NgR1 and NgR2, as peripheral myelin as mentioned above has MAG, the sole known ligand for NgR2, but does not contain Nogo-A (GrandPre et al., 2000) and has lower levels of OMgp (Huang et al., 2005). If our hypothesis is correct, we would predict that macrophage efflux from the Schwann cell basal lamina would be impaired in the presence of newly synthesized myelin surrounding the regenerated axons in NgR1- and MAG-deficient mice.

We therefore quantified by electron microscopy the number of macrophages containing phagocytosed debris that are found together with myelinated axons within the same Schwann cell basal lamina tube. This analysis was carried out on cross-sections of the nerve 14 days after crush injury in NgR1-deficient (Zheng et al., 2005) and MAG-deficient mice (Li et al., 1994) and their respective wild-type controls. Interestingly, in NgR1-deficient mice, ~16% of the macrophages contained within the Schwann cell basal lamina were associated with well myelinated axons (Table 1). Their containment within the same basal lamina and association with myelinated axons are clearly visible by electron microscopy (Figures 8A and 8B). In contrast, in crush-injured wild-type mice, no macrophages were present within the Schwann cell basal lamina that contained myelinated axons (Table 1). The partial effect seen in the NgR1-deficient mice is not surprising given the lower affinity of NgR1 for MAG (Liu et al., 2002; Venkatesh et al., 2005). As predicted, in MAG-deficient mice, double the numbers of macrophages (~30%, $p = 0.01$) in the basal lamina were associated with myelinated axons as compared to NgR1 null mice (Table 1 and Figure 8C and 8D). Importantly, this was never observed in the wild-type mice 14 days after crush injury (Table 1). It is possible that OMgp, which is localized to the region of the paranodal loops in peripheral nerve myelin (Huang et al., 2005), may also exert some effect on macrophage responses in the MAG null mice. These data, however, provide strong evidence that interactions between NgRs and their ligands may provide one of the initial signals for macrophage repulsion by newly synthesized myelin, resulting in the efflux of these cells from the Schwann cell basal lamina.

Table 1. Disruption of NgR/Ligand Interactions Impairs Macrophage Efflux from Schwann Cell Basal Lamina

	Null/Treated (%)	Wild-Type/Vehicle (%)
NgR1	16.3 ± 1.1	0
MAG	29.1 ± 5.1	0
Y-27632	36.3 ± 7.1	0

The percentage of macrophages located within the Schwann cell basal lamina that also contained well myelinated axons, 14 days after sciatic nerve crush injury in NgR1 null and MAG null mice, and rat sciatic nerves treated with Y-27632 compound, and their respective controls. The mean values for the MAG null and the Y-27632 groups are significantly different from the NgR1 null mice ($p = 0.01$ and 0.02, respectively). The MAG null and the Y-27632 groups are not significantly different from each other. Data represent mean ± SD; $n = 3$.

As NgR/ligand interactions in neurons have been shown to be mediated by Rho activation, and as our in vitro studies detailed above also indicate that macrophage responses to myelin are mediated via Rho activation, we next assessed the effects of treating crush-injured rat sciatic nerve with the ROK inhibitor Y-27632. By blocking Rho activation in these nerves, ~36% of the macrophages remaining inside the Schwann cell basal lamina were associated with well myelinated axons at 14 days after sciatic nerve crush injury in rats (Table 1 and Figures 8E and 8F). Macrophages associated with myelinated axons were not present in vehicle-treated control injured nerves (Table 1). Taken together, these results further suggest that NgR/ligand interactions via Rho-A-mediated signaling is likely

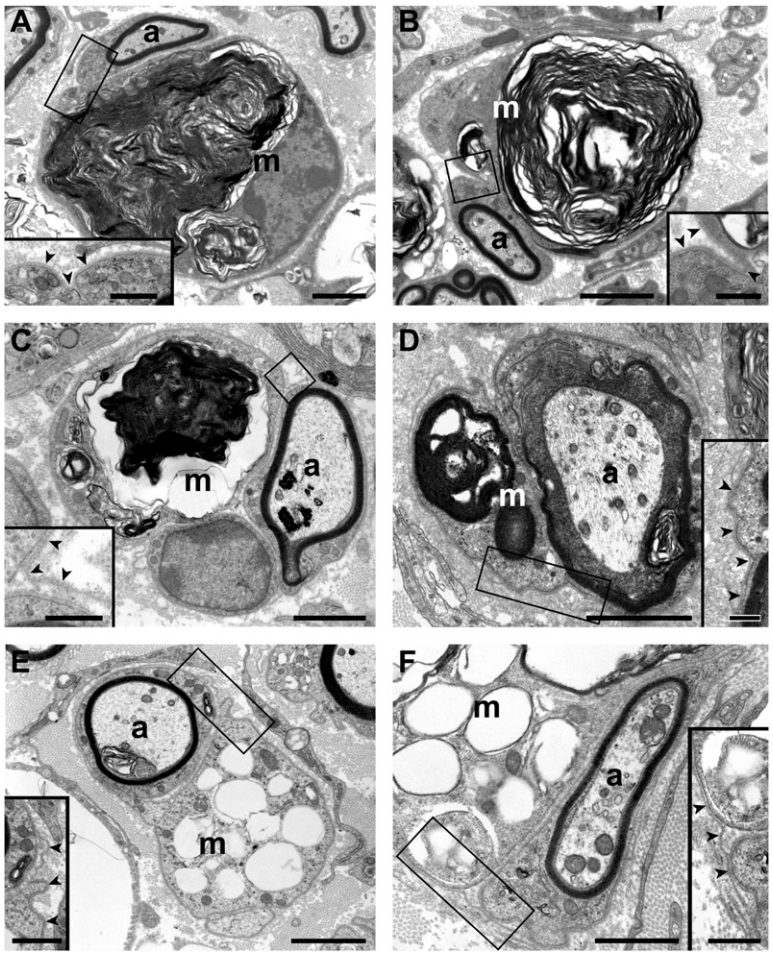

Figure 8. Disruption of NgR/Ligand Interactions Impairs Macrophage Efflux from Schwann Cell Basal Lamina in the Presence of Myelinated Axons

Electron micrographs taken from cross-sections of 14 day crushed sciatic nerve, 2 mm distal to injury from NgR1-deficient (A and B) and MAG-deficient (C and D) mice and Y-27632-treated rats (E and F). Note the presence of macrophages (m) laden with phagocytosed myelin debris that are contained along with fully myelinated axons (a) within the same Schwann cell basal lamina. The "inserts" in each panel are higher magnification of boxed areas that show where the basal lamina (arrowheads) is continuous around the macrophage and myelinating Schwann cell. Panel (D) shows a region of the paranodal loop of myelin that is less compact than the myelin sheaths in the other examples. Panels (E) and (F) illustrate examples of Y-27632-treated rats showing end-stage phagocytic macrophages (m) with clear vacuoles of myelin breakdown products located in the same Schwann cell basal lamina as the myelinated axons (a). The macrophage (m) in (F) is extremely large, and the electron micrograph shows only a portion of the cell.

Scale bars in (A)–(E), 2 μm; (F), 1 μm. Higher magnification "inserts" in (A) and (E), 1 μm, (B)–(D) and (F), 0.5 μm.

to initiate the repulsion of macrophages by newly synthesized myelin and their subsequent efflux from the Schwann cell basal lamina into the endoneurial space. This being one of the first steps in the clearance of macrophages from injured nerves and the termination of inflammatory responses at the end of the period of Wallerian degeneration.

DISCUSSION

The clearance of immune cells from the site of inflammation that ultimately results in the termination of the inflammatory response is essential for affected tissues to return to their normal basal state. If immune cells, such as macrophages, persist for long periods beyond the time required for tissue repair, these cells may contribute to tissue damage through the release of toxic mediators (Kiefer et al., 2001; Prineas and McLeod, 1976). In injured peripheral nerves, the entry of macrophages of hematogenous origin and their activation leading to phagocytosis of debris, followed by their clearance from the nerve, is precisely timed (Bruck, 1997; Leskovar et al., 2000; Mueller et al., 2003). This well-coordinated sequence of macrophage responses serves to rid the distal nerve segment of its myelin and axonal debris and prepare the distal segment to receive regenerating axon sprouts. Much of the work in the field has focused on the mechanisms that underlie macrophage recruitment and activation (De et al., 2003; Kuhlmann and Bruck, 1999; Rotshenker, 2001, 2003; Rotshenker et al., 1992; Saada et al., 1996; Shamash et al., 2002; Siebert and Bruck, 2003; Siebert et al., 2001, 2000; Stoll et al., 1993). The termination of this macrophage response is thought to involve, in part, the downregulation of proinflammatory cytokines and the upregulation of anti-inflammatory ones (George et al., 2004; Perrin et al., 2005; Rotshenker, 2001; Shamash et al., 2002; Stoll et al., 2004). Previous studies have reported that only a small number of macrophages (2%–4%) show signs of apoptosis at day 10 post-injury (Kuhlmann et al., 2001), while other studies failed to detect apoptotic macrophages after sciatic nerve crush injury (Mueller et al., 2003). The majority of the macrophages are thought to leave the nerve via the circulation into the regional lymph nodes and spleen (Kuhlmann et al., 2001). Interestingly, we observed examples by electron microscopy in which macrophages located outside the Schwann cell basal lamina appear to migrate toward blood vessels.

When macrophages enter the lesioned nerve after injury, they need to penetrate into the Schwann cell basal lamina tube to be able to phagocytose the myelin that is shed by Schwann cells. Therefore, before macrophages can migrate out of the lesioned nerve after phagocytosis is completed, they will first need to migrate out of the Schwann cell basal lamina into the endoneurial space. Our work suggests that this initial step is mediated via repulsive interactions between NgRs expressed by macrophages and NgR ligands such as MAG present in newly forming myelin around regenerated axons. NgR1 and NgR2 are known to mediate inhibitory signaling that causes collapse of axonal growth cones (Domeniconi et al., 2002; Filbin, 2003; Fournier et al., 2001; Venkatesh et al., 2005). NgR1 is also expressed by glioma cells and mediates reduced adhesion and migration of these cells in response to MAG (Liao et al., 2004). We show here that the majority of highly activated phagocytic macrophages from the 7 day crush-injured nerve express NgR1 and NgR2 and exhibit reduced binding to myelin and recombinant MAG in vitro, which can be neutralized by siRNA knockdown of NgR1 and NgR2. Additionally, our in vitro migration assay also indicates that macrophages from 7 day lesioned nerves are repulsed by myelin via interactions with NgR. The NgR ligand MAG is rapidly re-expressed in remyelinating peripheral nerve after crush injury (LeBlanc and Poduslo, 1990) and is therefore available to interact with NgR1 and NgR2 expressed on macrophages located within the Schwann cell basal lamina. This repulsive interaction between NgRs expressed on macrophages and the NgR ligands expressed on Schwann cell membranes occurs as the Schwann cells begin to remyelinate because axons with even the first few turns of myelin are devoid of macrophages within their basal lamina (Figures S2A and S2B). This inhibitory effect may reflect a repulsive interaction with myelin as has been reported for neurons and glioma cells. As with neurons (Filbin, 2003; McKerracher and David, 2004), this macrophage effect could also be neutralized by inactivating ROK, a downstream target of RhoA. NgR1 is a GPI-anchored protein that has been reported to act via its transmembrane coreceptors, $p75^{NTR}$ (Filbin, 2003; Yamashita et al., 2002) or TROY (Park et al., 2005; Shao et al., 2005), to activate RhoA (Fournier et al., 2003; Winton et al., 2002; Yamashita et al., 2002, 1999). Our data indicate that none of the macrophages express $p75^{NTR}$ but about 65%–70% of macrophages in the lesioned sciatic nerve express TROY at 3 and 7 days after nerve injury. It is possible that a receptor complex of TROY with NgR1 and NgR2 may mediate the macrophage efflux response after nerve injury, although the coreceptors for NgR2 have yet to be identified. Rho activation reduces adhesion and increases migration of monocytes, and these responses can be reversed by inhibiting RhoA with C3 transferase (Aepfelbacher et al., 1996; Pomorski et al., 2004). Interaction of NgR on macrophages with its ligands in newly synthesized myelin, therefore, has the potential to mediate repulsive effects in vivo and convert them from adherent cells to motile ones that are stimulated to migrate out of the basal lamina. In the nerve, the activation of RhoA is likely to occur on the side of the macrophage that is in contact with newly synthesized myelin and thus constitutes the retracting portion of the cell, while the migrating front would be located on the opposite side that is associated with the Schwann cell basal lamina. RhoA activation in Schwann cells reduces their migration and promotes normal myelination (Melendez-Vasquez et al., 2004; Yamauchi et al., 2004), mediated by neurotrophins acting via $p75^{NTR}$ (Yamauchi et al., 2004). RhoA activation can therefore affect multiple cell types and influence different aspects of the response of the nerve to injury, including macrophage efflux, axon regeneration, and remyelination.

We provide strong in vivo evidence that the presence of myelin stimulates macrophage efflux from the Schwann cell basal lamina and thus influences their eventual clearance from the lesioned sciatic nerve. In the model in which the sciatic nerve was cut and ligated to prevent axon regeneration and therefore lacks newly synthesized myelin in the distal nerve segment, almost 98% of the macrophages were still within the Schwann cell basal lamina into which they had migrated to phagocytose the myelin debris. In contrast, in crush-injured nerves in which axons are permitted to regenerate and remyelinate, macrophages were not found in the Schwann cell basal lamina of any of the remyelinated axons, including those in the early stages of remyelination. Another noteworthy finding is that the efflux of macrophages out of the Schwann cell basal lamina results in a greater number of them leaving the nerve, as the crushed nerves had 65% fewer macrophages than cut nerves as estimated by electron microscopy. This suggests that the efflux of macrophages out of the Schwann cell basal lamina is a first step in their clearance from the regenerating nerve. Once macrophages have effluxed out of the Schwann cell basal lamina into the endoneurial space, they will not be in direct contact with myelin and thus not be influenced by it. Other factors then play a role in their eventual clearance from the endoneurial space.

Additionally, unlike wild-type mice, macrophage efflux from the Schwann cell basal lamina containing well myelinated axons is impaired in NgR1-deficient mice and MAG-deficient mice 14 days after crush injury. These data provide direct evidence that interaction between NgR on macrophages and their ligands such as MAG in Schwann cell membranes that are beginning to form myelin around regenerated axons signal macrophages to leave the Schwann cell basal lamina. Furthermore, the effect of the ROK inhibitor in vivo indicates that these NgR/ligand interactions mediating macrophage responses in lesioned peripheral nerve may occur via Rho activation. These results provide in vivo evidence that NgR-mediated interactions with their ligands at the very early stages of myelination contribute to the efflux of macrophages from Schwann cell basal lamina. Interestingly, activated microglia/macrophages in cerebral ischemic lesions and multiple sclerosis lesions also express NgR1 (Satoh et al., 2005), as do

macrophage/microglia after spinal cord injury (data not shown). Our discovery of this novel (to our knowledge) role for NgRs in mediating the efflux of macrophages from inflamed neural tissue via interactions with myelin could therefore have broader implications for the regulation of inflammatory responses not only in other peripheral nerve pathologies but also in CNS inflammation such as in spinal cord injuries, stroke, and multiple sclerosis.

EXPERIMENTAL PROCEDURES

Sciatic Nerve Lesions

Adult female Sprague-Dawley rats (180–200 g, Charles River Laboratories) and adult MAG null (Mag < tm1Rod > /J) (Li et al., 1994), and wild-type mice (6–8 weeks of age, obtained from Jackson Laboratories) were deeply anesthetized with ketamine:xylazine:acepromazine (50/5/1 mg/kg). Adult NgR null and wild-type mice on a (C57BL/6x129S7) N2 background (Zheng et al., 2005) were deeply anesthetized with Avertin (15 μl of 2.5% solution/g). A unilateral sciatic nerve crush was performed in the region of the upper thigh using a laminectomy forceps chilled in liquid nitrogen and held for 30 s. In other animals, the sciatic nerve was ligated at the same region and cut with a pair of microscissors. This ligation prevented growth of axons into the distal nerve segment. All procedures were approved by the McGill University Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care or were approved by the Genentech and Stanford Animal Care Committees.

Animals were euthanized by perfusion under deep anesthesia at post-surgical survival times of 3, 5, 7, or 14 days with 0.1 M phosphate buffer, followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The distal segment of the lesioned sciatic nerves and uninjured control sciatic nerves were collected and longitudinal cryostat sections (14 μm) picked up on gelatin-coated glass slides.

Immunofluorescence

Double or single immunofluorescence labeling of the sciatic nerves was carried out using standard protocols (De et al., 2003). The following primary antibodies were used: mouse monoclonal antibody SMI-312 for neurofilaments (Sternberger Monoclonals; 1:5000); rabbit polyclonal anti-NgR1 (1:100; Alpha Diagnostic International [ADI]); mouse monoclonal antibody OX-42 to detect macrophages (Serotec, 1:100). Primary monoclonal and polyclonal antibodies were detected with rhodamine-conjugated rabbit anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG (Jackson Laboratories, 1:200), respectively. NgR antibody specificity was assessed by immunofluorescence staining of tissue sections from NgR1-deficient mice and Western blotting (Figure S3). The specificity of the NgR1 antibody was determined by the lack of immunofluorescence staining of tissue sections of sciatic nerve from NgR1-deficient mice (Figures S3A–S3C) as compared to wild-type nerve (Figures S3D–S3F). The tissue sections from NgR1-deficient mice, however, stained with the NgR2 antibody (data not shown), indicating that the NgR2 antibody does not cross-react with NgR1.

For quantification of macrophage numbers, digital images of longitudinal sections of the sciatic nerve for each fluorophore (fluorescein, rhodamine, and DAPI) were captured at a magnification of 20× using a Retiga digital camera with Qcapture software. Images were taken at 1–3 mm distal to the injury site. A minimum of three sections from each animal (n = 3 for each group) was analyzed to obtain the means. The data are presented as the mean ± standard error of the mean (SEM). Statistical significance was determined using the two-tailed student's t test. The alpha value was set to $p < 0.05$.

Cell Culture Studies

Adult rat sciatic nerve segments distal to the site of crush or cut injury were obtained 3 and 7 days after lesion, cut into small pieces, and dissociated by incubation with 0.125% trypsin for 20 min. For immunofluorescence staining, cells were plated on poly-L-lysine-coated 13 mm round glass coverslips at a density of 1×10^5 and allowed to adhere for 2 hr. To demonstrate cell surface localization of NgR1 and -2, TROY and $p75^{NTR}$ live cells were incubated for 30 min at room temperature with a mouse monoclonal antibody OX-42 (to label macrophages; 1:100, Serotec) and one of either polyclonal goat anti-NgR1 antibody (1:100; R & D Systems) or rabbit anti-NgR1 (1:100; ADI), goat anti-NgR2 (1:100; R & D Systems), or rabbit anti-NgR2 (1:100, ADI), goat anti-TROY (1:100 R & D Systems) or $p75^{NTR}$ (1:100, provided by Dr Phil Barker [McGill University]) in Hanks balanced salt solution (HBSS), followed by a 30 min incubation with a goat anti-rabbit or donkey anti-goat secondary antibody conjugated to fluorescein, and a goat or rabbit anti-mouse secondary antibody conjugated to rhodamine (1:200, Jackson Laboratories). Cells were then fixed in acetic acid:ethanol (1:9) for 20 min at −20°C. Coverslips were then mounted on glass slides in an aqueous nonfade mounting medium containing the nuclear marker DAPI (Vector Laboratories). The majority of the cells that are obtained under these conditions are macrophages. A minimum of three coverslips was prepared for each time point and the experiments repeated three times. Cells were examined with a Zeiss Axioskop fluorescence microscope, and at least 100 $OX\text{-}42^+$ cells were counted on each coverslip, and the proportion that was NgR1, NgR2, $p75^{NTR}$, and TROY positive recorded. Data are presented as a mean ± SEM.

For the cell adhesion assay, dissociated cells were plated in 96-well plates coated with CNS myelin or recombinant MAG as described previously (Huang et al., 1999). Briefly, the wells were coated with nitrocellulose solubilized in ethanol. After drying the wells, they were coated with poly-L-lysine (5 μg/ml) overnight. 0.4 μg of bovine myelin or recombinant MAG in a 2 μl drop in HBSS was placed in the center of the wells and incubated for 3 hr at 37°C. Myelin and recombinant MAG were purified as previously reported (McKerracher et al., 1994). The myelin or recombinant MAG drop was then removed, the dissociated cells plated in the wells at a density of 1×10^5 cells per well, and placed in a tissue culture incubator at 37°C and 5% CO_2 for 2 hr. The wells were washed with HBSS to remove unattached cells and then fixed with 4% paraformaldehyde. Macrophages were easily identified by their large size. The number of these large round cells was counted in an area of 0.25 mm^2. Additional experiments were performed by incubating the macrophage cultures plated on myelin or MAG with the ROK inhibitor (Y-27632, 10 μM; CalBioChem) or vehicle (PBS) for 30 min during the preplating stage and for the entire culture period of 3 hr. A minimum of four wells were plated with cells for each experimental condition (myelin substrate, myelin with Y-27632, myelin with PBS, MAG with Y-27632, MAG with PBS). Experiments were repeated three times (n = 3). The number of macrophages bound to the various substrates was counted using an ocular grid in four consistent places in each well within an area of 0.25 mm^2. In addition, the number of macrophages bound to the poly-L-lysine substrate around the edge of the central myelin- or MAG-coated region within the same well was also counted as an internal control. Data are presented as a mean ± SEM. Statistical analysis was performed as described above.

Separation of Blood Monocytes

Blood monocytes were obtained by Percoll density centrifugation. Cells were plated on poly-L-lysine-coated 13 mm round glass coverslips at a density of 2.5×10^5 cells and allowed to adhere for 3 hr. Cells were then labeled by double immunofluorescence with the mouse monoclonal antibody OX-42 and rabbit polyclonal anti-NgR1, NgR2, $p75^{NTR}$, and TROY as described above. Counts were made from three coverslips prepared from each of three animals (n = 3), and the proportions of NgR1, NgR2, $p75^{NTR}$, and TROY positive monocytes were calculated. Data are presented as mean ± SEM.

siRNA Knockdown of NgR1 and NgR2

The following siRNA sequences for rat NgR1 and NgR2 were designed according to criteria stipulated by Elbashir et al. (2001). NgR1,

5′-AATGACTCTCCATTTGGGACT-3′; NgR2, 5′-AAACAGCTCTTCCAACCACCT-3′. Oligonucleotide sequences were synthesized by Ambion. Macrophages plated at a density of 1 × 10^6 in 24-well tissue culture plates were incubated with 10 nM of fluorocein (FAM) labeled NgR1 and NgR2 siRNA or FAM-labeled negative control siRNA (nonspecific Silencer negative control #1, Ambion) in OPTI-MEM medium containing 2 μl of Lipofectamine 2000 (Invitrogen) transfection agent. Cells were incubated for 24 hr, after which the transfection medium was replaced by DMEM culture medium containing 10% fetal bovine serum and the mitotic inhibitor cytosine arabinoside to prevent proliferation of any fibroblasts, and cultured for up to 4 days. siRNA knockdown was first assessed by slot blot analysis (see below) and immunofluorescence staining for cell surface NgR1 and NgR2 on OX42-positive macrophages at 1, 2, and 4 days after transfection. The 2 day time point was used for the myelin-binding and migration assays. Experiments were repeated three times (n = 3).

Slot Blots

Slot blots were used to detect the level of NgR1 and NgR2 protein in macrophages purified from 7 day lesioned nerves, 2 days after transfection with siRNA specific for NgR1 and NgR2 or negative control siRNA. 10 μg of cell extract was blotted onto Protan Pure nitrocellulose membrane (Schleicher and Schueil). Membranes were incubated with goat anti-NgR1 and NgR2 antibodies (R & D systems; 1:2500, 1:2000, respectively) overnight at 4°C. After washing, the membranes were incubated with donkey anti-goat secondary antibody conjugated to horseradish peroxidase (HRP, 1:400,000) for 1 hr at RT, and visualized using enhanced chemiluminescence (Perkin-Elmer Life Science, Boston, MA). Densitometry was done using ImageQuant (Amersham Biosciences, Piscataway, NJ).

Macrophage Migration Assay

Macrophages from 3 day and 7 day crushed rat sciatic nerves were dissociated as described above and were purified on a Percoll gradient. The macrophages were further purified by immunopanning for 30 min in Petri dishes coated with anti-OX-42 antibodies (1:500, Serotec). 24-well tissue culture plates were coated with poly-L-lysine, and one side of each well was coated with myelin (10 μl of 0.2 mg/ml). Macrophages were plated at a density of 1 × 10^5 in a 50 μl droplet on the side coated only with poly-L-lysine and allowed to adhere for 4 hr before adding culture medium to the well. After 24 hr, the cultures were fixed with 4% paraformaldehyde. To visualize the macrophages for analysis, cells were stained with anti-OX-42 antibody and a biotinylated horse anti-mouse secondary antibody conjugated to HRP (1:200, Jackson Laboratories). Immunoreactivity was visualized with diaminobenzidine. The number of macrophages that had migrated past the poly-L-lysine/myelin interface was quantified. Three to four wells per group were quantified per experiment, and the experiments were repeated three times (n = 3).

In additional experiments, the migration assay was carried out with macrophages from 7 day crushed rat sciatic nerves, 2 days after transfection with NgR1 and NgR2 siRNA or nonspecific negative control siRNA. The dissociation method used and the siRNA transfection protocol was similar to that described above. The number of macrophages that migrated past the poly-L-lysine/myelin interface was quantified. Three wells were quantified per experiment and the experiments repeated three times (n = 3).

Histochemical Staining

Luxol fast blue staining for myelin was done as follows: sections were washed in PBS, dehydrated in increasing concentrations of ethanol (50%–95%), and incubated in 0.1% Luxol fast blue (diluted in 95% ethanol and 10% acetic acid) overnight at 37°C. Sections were then differentiated in 0.05% lithium carbonate and 70% ethanol. Luxol fast blue staining was combined with immunofluorescence and the sections mounted in aqueous nonfade mounting medium containing the nuclear marker DAPI (Vector Laboratories).

Electron Microscopy

Rat sciatic nerve injury groups included 7 day crush, 14 day crush and cut, 14 day crush treated with Y-27632 or vehicle treatment. Mouse sciatic nerve injury groups included 14 day crush MAG null and wild-type, 14 day crush NgR1 null and wild-type mice. Y-27632 mixed in a thrombin/fibrin gel (Tisseel; Baxter) at a concentration of 1 mM Y-27632 (CalBioChem) was placed directly on the nerve over the crush-injury site. In addition, daily injections of Y27632 (75 μl of 1 mM in 300 μl of PBS) were administered intraperitoneally. Rats in the control group were treated with the vehicle.

Rats and mice were deeply anesthetized 7 or 14 days after sciatic nerve injury and perfused with 0.1 M phosphate buffer followed by 3% glutaraldehyde, 0.5% paraformaldehyde in 0.1 M phosphate buffer. The distal segment of sciatic nerve was post-fixed in 2% osmium tetroxide for 2 hr and processed for embedding in Epon. Thin cross-sections of the nerves (n = 3 for each group) at a 2 mm distance from the lesion site were stained with lead citrate and viewed with a Philips CM 10 electron microscope. Macrophages were counted in an area of 4 × 10^5 μm^2, and their position within or outside the Schwann cell basal lamina were recorded. Data are presented as the mean ± SD. Statistical analysis was performed as described above.

RT-PCR

RNA was isolated from adult rat sciatic nerves using the RNeasy Lipid Tissue Minikit (QIAGEN) and reverse transcribed to cDNA with the Omniscript Reverse Transcriptase Kit (QIAGEN). RT-PCR was performed using the HotStarTaq PCR Kit (QIAGEN). Primers used were as follows: NgR1, F-5′ AATGAGCCCAAGGTCACAACAAGC-3′, R-5′GTTGCCATGCAGAAAGAGATGCGT-3′; Peptidylprolyl Isomerase A (PPIA), F-5′ATTCCAGGATTCATGTGCCAGGT-3′, R-5′-CAAAGACCACATGCTTGCCATCCA-3′. PCR was performed as described previously (Jeong and David, 2003) with annealing temperatures of 61°C for 30 cycles (NgR1) and 62.5°C for 23 cycles (PPIA). PPIA, also known as cyclophilin A, was used as the internal control, since it shows less variability in expression than GAPDH in inflamed tissue (Feroze-Merzoug et al., 2002).

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/53/5/649/DC1/.

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