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A role for galectin-1 in the immune response to peripheral nerve injury

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ABSTRACT

Galectin-1 (Gal1) is a multi-functional protein that has key roles in organismal growth and survival. In the adult nervous system, Gal1 promotes axonal regeneration following peripheral nerve injury. Although the mechanism by which Gal1 promotes regeneration is unclear, previous reports suggested that Gal1 acts indirectly by activating macrophages. An appropriate response of macrophages is crucial for repair of injured nerves: these immune cells remove obstructive axon and myelin debris in the distal nerve. Here we establish a role for Gal1 in the accumulation of immune cells following peripheral axotomy. We used immunohistochemistry to visualize macrophages (F4/80) in wild-type (*Lgals1*$^{+/+}$) and knockout (*Lgals1*$^{-/-}$) mouse sciatic nerves following injury and/or manipulation of Gal1 levels. Density of F4/80 immunoreactivity, which peaks around 3 days post-injury, was decreased in *Lgals1*$^{+/+}$ nerves injected with Gal1 antibody. The typical injury-induced peak of macrophage/microglial density was delayed in the sciatic nerves and fifth lumbar dorsal root ganglia of *Lgals1*$^{-/-}$ mice relative to control mice. Injection of oxidized Gal1 into uninjured sciatic nerve promoted the accumulation of macrophages in *Lgals1*$^{+/+}$ nerves. Finally, we used transplants of sciatic nerve to uncover a compensatory mechanism in *Lgals1*$^{-/-}$ mice that allows for macrophage accumulation (albeit delayed and diminished) following axotomy. We conclude that Gal1 is necessary to direct the typical accumulation of macrophages in the injured peripheral nerve, and that Gal1 is sufficient to promote macrophage accumulation in the uninjured nerve of wild-type mice.

Introduction

Lectins are proteins that have the ability to bind specific carbohydrate motifs. Galectin-1 (Gal1) is a 14 kDa lectin whose structure and function depend on its oxidation state: reduced Gal1 is a homodimer that binds β-galactosides, whereas oxidized Gal1 (Gal1/Ox) is monomeric and lacks lectin activity (Inagaki et al., 2000). Gal1 is expressed in various tissues throughout development and into adulthood in mammals, and has been implicated in numerous fundamental cellular processes, including apoptosis, proliferation, and cell adhesion. Gal1 is found both in the peripheral and central nervous systems (PNS and CNS) during rodent development, but its expression is restricted mainly to the PNS in the adult (Dodd and Jessell, 1986; Regan et al., 1986; Hynes et al., 1990). In the nervous system, Gal1 has roles in axonal pathfinding during development (Puche et al., 1996; McGraw et al., 2005b), in neuropathic pain (Imbe et al., 2003), and in the regeneration of axons following injury (Horie et al., 1999) (see Gaudet et al. (2005) for review). Gal1 is expressed by axons and nonneuronal cells in peripheral nerves (Sango et al., 2004), and its expression is increased following injury. Extracellular Gal1 in the nerve is thought to act exclusively on macrophages (Horie et al., 2004).

Damage to any type of tissue initiates a characteristic response from immune cells that assist with and may regulate the repair of the wound (Martin and Leibovich, 2005). Although cells resident to the injured tissue contribute to the repair process, the vast majority of effective immune cells emigrate from nearby blood vessels. Neutrophils are usually the first immune cells to invade the injury site, followed by macrophages and mast cells. Macrophages play an important role in phagocytosis upon arrival, and provide multiple cytokines and growth factors to the surrounding tissue. In the injured PNS, macrophages cooperate with Schwann cells to break down detached myelin and axonal debris (Wallerian degeneration) and initiate nerve repair (Hirata and Kawabuchi, 2002). Several soluble

Abbreviations: CNS, central nervous system; dpi, days post-injury; DRG, dorsal root ganglion; Gal1, galectin-1; Gal1-Ab, antibody specific for galectin-1; Gal1/Ox, oxidized galectin-1; HPLC, high-performance liquid chromatography; IgG, immunoglobulin G; *Lgals1*$^{+/+}$, galectin-1 wild-type; *Lgals1*$^{-/-}$, galectin-1 null mutant; PNS, peripheral nervous system.

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factors thought to be secreted by Schwann cells following injury are involved in the recruitment of hematogenous macrophages to the injured nerve, including monocyte chemoattractant protein-1 (CCL2) (Toews et al., 1998; Perrin et al., 2005), leukemia inhibitory factor (Tofaris et al., 2002), and pancreatitis-associated protein-III (Namikawa et al., 2006) (for review, see Martini et al. (2008)).

Gal1 is also involved in the macrophage response to peripheral nerve injury. Recent studies have shown that extracellular Gal1/Ox may expedite repair of injured nerves by binding to macrophages and activating intracellular signaling pathways leading to the release of an unidentified factor that promotes Schwann cell migration and axonal regeneration (Horie et al., 2004; Okada et al., 2005). Since Gal1 is upregulated in the injured peripheral nerve following axotomy, and since Gal1/Ox binds a receptor on macrophages, we hypothesized that Gal1 may have an effect on the injury-induced accumulation of macrophages. Here, we show that exogenous Gal1/Ox is sufficient to facilitate accumulation of macrophages in uninjured sciatic nerve, and that Gal1 is necessary for the normal accumulation of macrophages following peripheral axotomy.

Materials and methods

Preparation of oxidized galectin-1

Production of oxidized recombinant human galectin-1 (Gal1/Ox) has been described previously (Inagaki et al., 2000). Briefly, Gal1 was expressed by *Escherichia coli*, and was isolated from the supernatant by diethylaminoethyl high-performance liquid chromatography (HPLC). Purified Gal1 underwent air oxidation in the presence of $CuSO_4$: Ga1l was diluted 20× with 20 mM Tris–HCl (pH 8.0), $CuSO_4$ was added (final concentration: 0.0001%, w/v), and the solution remained at 4 °C overnight to promote disulphide bond formation. Gal1/Ox was then purified using reverse-phase HPLC (YMC-Pack Protein RP column; YMC, Kyoto, Japan) with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. This Gal1/Ox did not degenerate after a 10-day incubation at 37 °C in PBS (5 μg/mL), as determined by SDS–PAGE and HPLC.

Animals and surgery

A total of 181 adult (2–4 months old) 129P3/J (*Lgals1*$^{+/+}$, Jackson Labs, Maine) and 171 adult 129P3/J *Lgals1*null mutant (*Lgals1*$^{-/-}$ (Poirier and Robertson, 1993); *Lgals1* is the gene that encodes galectin-1) mice were used for these experiments. All experiments were performed in accordance with the Canadian Council for Animal Care and approved by the University of British Columbia Animal Care Committee. Mice were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (80 mg/kg; Bimeda-MTC, Cambridge, ON) and xylazine hydrochloride (40 mg/kg; Bayer, Inc., Etobicoke, ON), and surgeries were carried out under sterile conditions. Lacri-Lube opthalmic ointment was used to lubricate the animals' eyes while under anesthesia. Mice were treated with buprenorphine (0.05–0.10 mg/kg; Animal Resources Centre, McGill University, Montreal, QC) intramuscularly to relieve pain and administered 1.5 mL Ringer's solution subcutaneously to rehydrate following surgery. Daily monitoring was carried out thereafter.

All surgeries involved exposure of the sciatic nerve by blunt dissection of the overlying thigh muscle. To test the effect of injection of oxidized Gal1 (Gal1/Ox), sciatic nerves of *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ mice were exposed just distal to the greater sciatic notch at the hip. In most mice, the sciatic was divided into two branches at this point: one branch was injected with 0.2 μL of either human immunoglobulin G (IgG), zymosan, or Gal1/Ox (all 5 μg/mL) using a fine-tipped Hamilton (Reno, NV) syringe, while the other branch was tied tightly with a 7-0 silk suture to mark the site of injection. Animals in this group ($n=$ 6 per treatment, per strain) were killed 3 days later. Other surgeries involved tight ligation of the entire sciatic nerve just distal to the greater sciatic notch using 7-0 silk suture. To study the effect of Gal1-specific function-blocking antibody (Gal1-Ab; host goat) on injury-induced macrophage accumulation, we injected 1.5 μL of human IgG or Gal1-Ab into the nerve just distal to ligation, immediately following injury ($n=5$ *Lgals1*$^{+/+}$ mice per treatment). These mice survived for 3 days after axotomy. To determine whether macrophage accumulation is altered in mice lacking Gal1, we harvested *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ tissue from animals that were uninjured, and those that had received tight sciatic nerve ligation 1, 3, 7, 14, 21, and 28 days previously ($n=$ 5 per group, per strain). In sciatic nerve transplant experiments, we excised 1-cm-long pieces of sciatic nerve from *Lgals1*$^{+/+}$ or *Lgals1*$^{-/-}$ mice. Excised nerves were washed and maintained briefly in phosphate-buffered saline (PBS) to prevent them from drying out during the procedure. We grafted nerve explants into gaps created by resecting the sciatic nerve in host mice, so that both ends of the donor nerve abutted and stuck to the transected proximal and distal ends of the recipient nerve. Animals survived for 3 or 7 days following transplant ($n=$ 6 per treatment, per strain). At the end of the experiment, sciatic nerves, fifth lumbar segment (L5) dorsal root ganglia (DRGs), and spinal cord were harvested (see below).

Dorsal rhizotomy was performed unilaterally from the fourth cervical level (C4) to the second thoracic level (T2). Dorsal rhizotomy was performed as described by Ramer et al. (2001). Briefly, the dorsal roots of C3-T2 segments were exposed by removing small pieces of vertebrae, and the roots were transected midway between the DRG and dorsal root entry zone. Cervical spinal cords from rhizotomized *Lgals1*$^{+/+}$ and $^{-/-}$ mice were collected at 7, 14, and 21 days post-injury (dpi).

Tissue processing/immunohistochemistry

At the appropriate time, animals were injected with a lethal dose of chloral hydrate. Upon the loss of nociceptive reflexes, animals were perfused transcardially with 0.1 M PBS (pH 7.4) followed by cold 4% paraformaldehyde in 0.1 M phosphate buffer. Collected tissue was placed in 4% paraformaldehyde at 4 °C overnight. The tissue was then cryoprotected for at least 1 day in 20% sucrose in PBS. Tissue was cut into longitudinal (sciatic nerve and DRG, 10 μm) or transverse (spinal cord, 16 μm) sections, thaw-mounted onto glass slides (Superfrost Plus; VWR, Mississauga, ON), and stored at −80 °C. After blocking in 10% normal donkey serum, slides were incubated with rat anti-F4/80 (MCAP497, 1:200; Serotec, Raleigh, NC), and/or rabbit anti-galectin-1 (1:400; Kirin Breweries, Gunma, Japan) primary antibodies overnight. The next day, slides were washed three times in PBS and were incubated with Alexa 488-conjugated donkey anti-rat (A21208, 1:250; Molecular Probes, Carlsbad, CA) and/or Cy3-conjugated donkey anti-rabbit (711-165-152, 1:400; Jackson ImmunoResearch, West Grove, PA) secondary antibodies for 2 h. All antibodies were diluted with PBS, 0.2% Triton X-100, and 0.1% sodium azide.

Image analysis

Images were captured using an Axioplan 2 microscope (Zeiss, Jena, Germany), QImaging digital camera (Burnaby, BC), and Northern Eclipse 6.0 software (Empix Imaging, Mississauga, ON). All images for a given marker were captured under the same exposure settings. Images were analyzed using SigmaScan Pro 5 software (SPSS, Chicago, IL). We estimated the density of macrophages in a particular section of tissue by using an intensity threshold to analyze the area of the tissue occupied by F4/80 immunoreactivity. The area of interest was identified and an intensity threshold was applied on the entire image (the threshold was consistent for all images). For injured or injected sciatic nerves, we analyzed areas that were 3–6 mm distal to treatment, and for DRGs, we analyzed the entire area bounded by the

DRG capsule. The 'overlay math' function allowed us to determine the density of F4/80-immunoreactive cells and their processes within these areas of interest. Using SigmaPlot 2001 (SPSS) we calculated macrophage density by dividing the total area of F4/80 immunoreactivity in a given image by the total area of interest. Thresholds defining F4/80-positive areas were used for analyzing the density of macrophages, since F4/80-immunoreactive cells were often grouped together and were therefore impossible to count reliably. For all analyses, four images per animal were analyzed and averaged. Data are expressed as mean proportion ± standard error of the mean. Data were analyzed using GraphPad InStat (San Diego, CA): Tukey–Kramer multiple comparisons tests were used to calculate significance ($p < 0.05$).

Results

The density of Gal1-immunoreactive cells is increased in distal nerve following sciatic nerve injury

Although we have shown previously that Gal1 mRNA is upregulated in the peripheral portion of the dorsal root following dorsal rhizotomy (McGraw et al., 2005a), the up-regulation of Gal1 protein following peripheral nerve injury has never been confirmed immunohistochemically. We characterized Gal1 immunoreactivity at various timepoints after tight sciatic nerve ligation in the distal nerve of *Lgals1*$^{+/+}$ mice (Fig. 1). The density of Gal1 immunoreactive cells was higher in distal nerve at 3 dpi compared to uninjured control nerve ($p<0.05$). Gal1 immunoreactivity partially co-localized with F4/80-positive macrophages in both uninjured and axotomized nerve (arrowheads, Fig. 1), and the intensity of Gal1 immunoreactivity in these cells was enhanced following injury. This suggests that macrophages, in addition to Schwann cells and neurons, may contribute to the expansion of the Gal1 pool in the injured peripheral nerve.

Endogenous Gal1 is necessary for the typical injury-induced response of macrophages during Wallerian degeneration

To establish whether extracellular Gal1 expressed within the sciatic nerve has a role in the accumulation of macrophages following peripheral axotomy, we compared macrophage density in sciatic nerves distal to ligation 3 days post-operation. *Lgals1*$^{+/+}$ mice were divided into three groups: injury only, injury plus IgG injection, and injury plus injection of Gal1-Ab (Fig. 2). Macrophage density was very low in the unmanipulated contralateral sciatic nerve (Fig. 2; cont.). Sciatic nerve ligation led to a massive accumulation of macrophages by 3 dpi (Fig. 2; ispi.). Injection of IgG solution significantly increased injury-induced macrophage accumulation ($p< 0.001$; Fig. 2, +IgG) (cf. Li et al. (2007)). In contrast, injection of Gal1-Ab into the nerve just distal to ligation at the time of injury attenuated the axotomy-induced increase in the density of F4/80 immunoreactivity by more than 50% ($p< 0.001$; Fig. 2, +Gal1-Ab), which is especially remarkable given the increase in macrophage density induced by injection of non-specific IgG. Therefore, extracellular Gal1 is required for the normal macrophage response to nerve injury in wild-type mice.

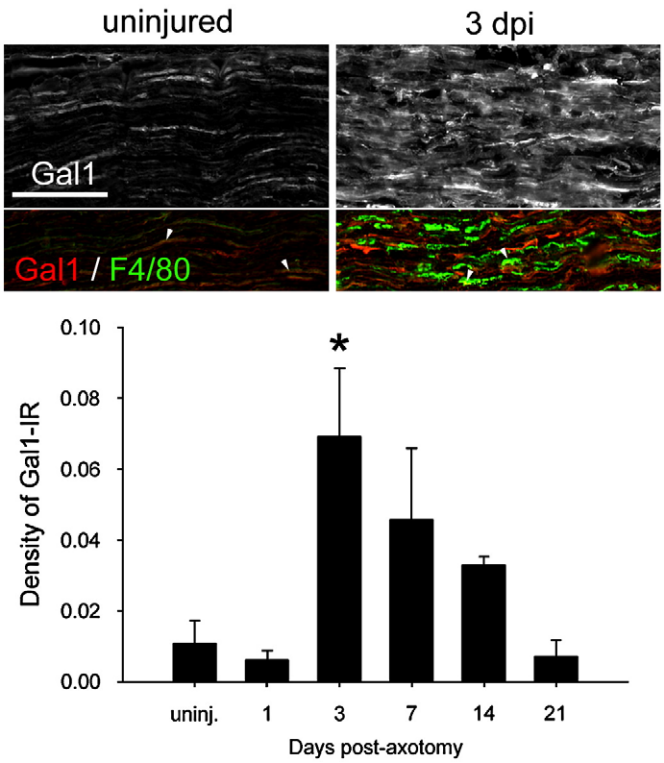

Fig. 1. The density of galectin-1 (Gal1) immunoreactive cells is increased in the distal nerve following peripheral axotomy. The density of Gal1 immunoreactive cells was examined in the uninjured *Lgals1*$^{+/+}$ sciatic nerve, and at various timepoints following injury in the distal nerve. The peak density of Gal1 immunoreactivity was observed at 3 days post-injury (dpi). Gal1 immunoreactivity (red) partially co-localized with F4/80 immunoreactivity (green) at all timepoints (arrowheads), and intensity and density of both proteins was increased in the distal nerve by axotomy. Scale bar, 200 μm.

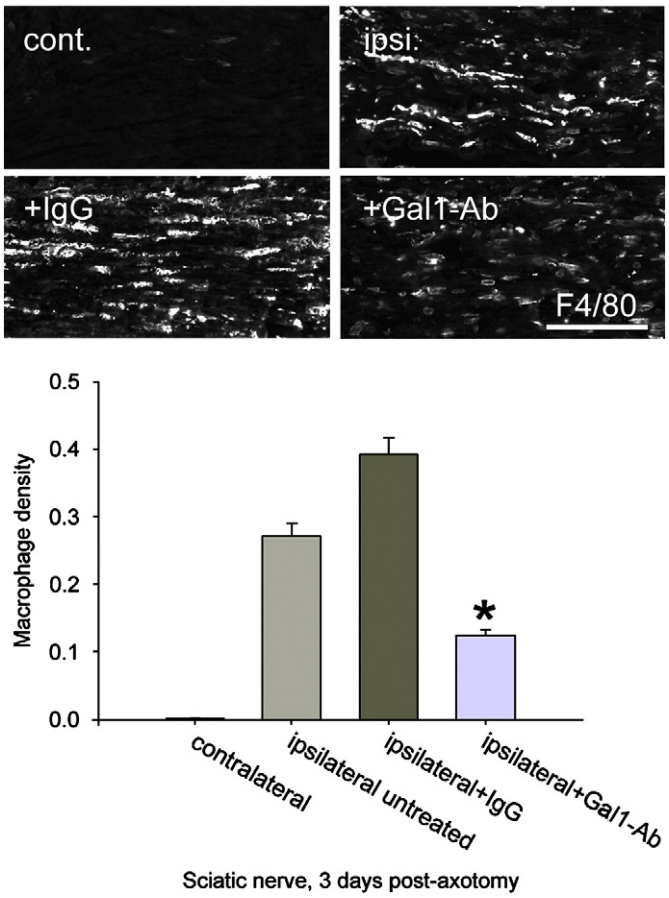

Fig. 2. The axotomy-induced increase in macrophage density in the *Lgals1*$^{+/+}$ sciatic nerve distal to ligation is attenuated by injection of Gal1-specific function-blocking antibody (Gal1-Ab) at 3 days post-injury. Photomicrographs show F4/80 immunoreactivity in sciatic nerve contralateral to injury (cont.); ligated, untreated sciatic nerve (uninj.); ligated, IgG-treated sciatic nerve (+IgG); and ligated, Gal1-Ab-treated sciatic nerve (+Gal1-Ab). Axotomy-induced macrophage accumulation in the Gal1-Ab-treated nerve is significantly less than both positive controls. ‡, significant difference between IgG-injected and ipsilateral untreated groups; *, significant difference between Gal1-Ab-treated group and all other groups. Scale bar, 200 μm.

Mice lacking Gal1 exhibit impaired macrophage responses following peripheral axotomy

Since injection of Gal1-Ab had a significant effect on the accumulation of macrophages following sciatic nerve ligation, we were interested in whether the response of macrophages is altered in *Lgals1*$^{-/-}$ mice following sciatic nerve ligation. We completed a timecourse of injury-induced macrophage accumulation in the sciatic nerve, L5 DRG, and L5 ventral horn of *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ mice. In the sciatic nerve distal to ligation (Fig. 3), macrophage density had increased significantly in both strains by 1 dpi, and density remained elevated for at least 21 dpi. Macrophage accumulation in the nerve distal to axotomy peaked at 3 dpi in *Lgals1*$^{+/+}$ mice ($p< 0.05$). This was significantly different from the highest density in *Lgals1*$^{-/-}$ mice ($p< 0.001$), which occurred around 14 dpi. Density of F4/80 immunoreactivity in the nerve distal to injury differed in *Lgals1*$^{-/-}$ compared to *Lgals1*$^{+/+}$ mice at various timepoints: at 1 dpi ($p< 0.05$), at 3 dpi ($p< 0.001$), at 14 dpi ($p< 0.01$), and at 21 dpi ($p< 0.01$). Thus, injury-induced macrophage accumulation in the distal sciatic nerve is both delayed and diminished in *Lgals1*$^{-/-}$ mice.

A similar but less pronounced accumulation of phagocytic cells occurs in the L5 DRG following sciatic nerve ligation (Lu and Richardson, 1993; Hu and McLachlan, 2002, 2003). The L5 DRG contains a large sensory neuron population that projects to peripheral targets via the sciatic nerve. In the L5 DRG (Fig. 4), the density of F4/80 immunoreactivity had increased significantly by 7 dpi in *Lgals1*$^{+/+}$ mice ($p< 0.001$). This was also the timepoint at which macrophage density peaked in these mice ($p< 0.005$), and this density differed significantly from that in *Lgals1*$^{-/-}$ mice ($p< 0.001$). Macrophage accumulation peaked at 14 dpi in *Lgals1*$^{-/-}$ DRGs ipsilateral to injury ($p< 0.05$); this peak was not significantly different from that induced by injury in *Lgals1*$^{+/+}$ mice. Therefore, the induction of macrophage accumulation by sciatic nerve injury was delayed (but not diminished) in the L5 DRG of mice lacking Gal1.

Immune cells also accumulate in regions associated with degeneration following dorsal root injury. We found that the density of F4/80 immunoreactivity in the dorsal horn and dorsal column ipsilateral to septuple cervical dorsal rhizotomy was significantly lower in *Lgals1*$^{-/-}$ compared to *Lgals1*$^{+/+}$ mice at 21 dpi ($p< 0.05$; Supplementary Fig. 1 and data not shown). There was no difference between genotypes in the uninjured dorsal horns and columns, nor was there a difference between genotypes on the ipsilateral side at 7 or 14 dpi. The density of F4/80 immunoreactivity in the dorsal horn in *Lgals1*$^{+/+}$ mice was increased after both sciatic nerve ligation and dorsal rhizotomy, but the increase induced by sciatic nerve injury was more pronounced (Supplementary Fig. 2).

Exogenous Gal1/Ox facilitates accumulation of macrophages in uninjured Lgals1$^{+/+}$*, but not Lgals1*$^{-/-}$*, sciatic nerve*

In order to determine whether Gal1/Ox has an effect on the accumulation of macrophages, we injected 1 ng of either human IgG (negative control), the yeast particulate zymosan (positive control), or Gal1/Ox into one uninjured branch of the sciatic nerve of *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ mice (Fig. 5). Injection of human IgG had little effect on the density of F4/80 immunoreactivity compared to zymosan 3 days after injection. The effect of Gal1/Ox on macrophage accumulation differed between strains: Gal1/Ox injection caused significantly more accumulation in *Lgals1*$^{+/+}$ nerves than did IgG ($p< 0.001$); however, exogenous Gal1/Ox did not increase macrophage density higher than control levels in *Lgals1*$^{-/-}$ nerves. Whereas the density of macrophages in zymosan-treated *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ nerves was not significantly different, macrophage density was significantly higher in Gal1/Ox-treated *Lgals1*$^{+/+}$ nerves than in *Lgals1*$^{-/-}$ nerves ($p< 0.01$). In addition, macrophage density in Gal1/Ox-treated *Lgals1*$^{+/+}$ nerves did not differ significantly from zymosan-treated

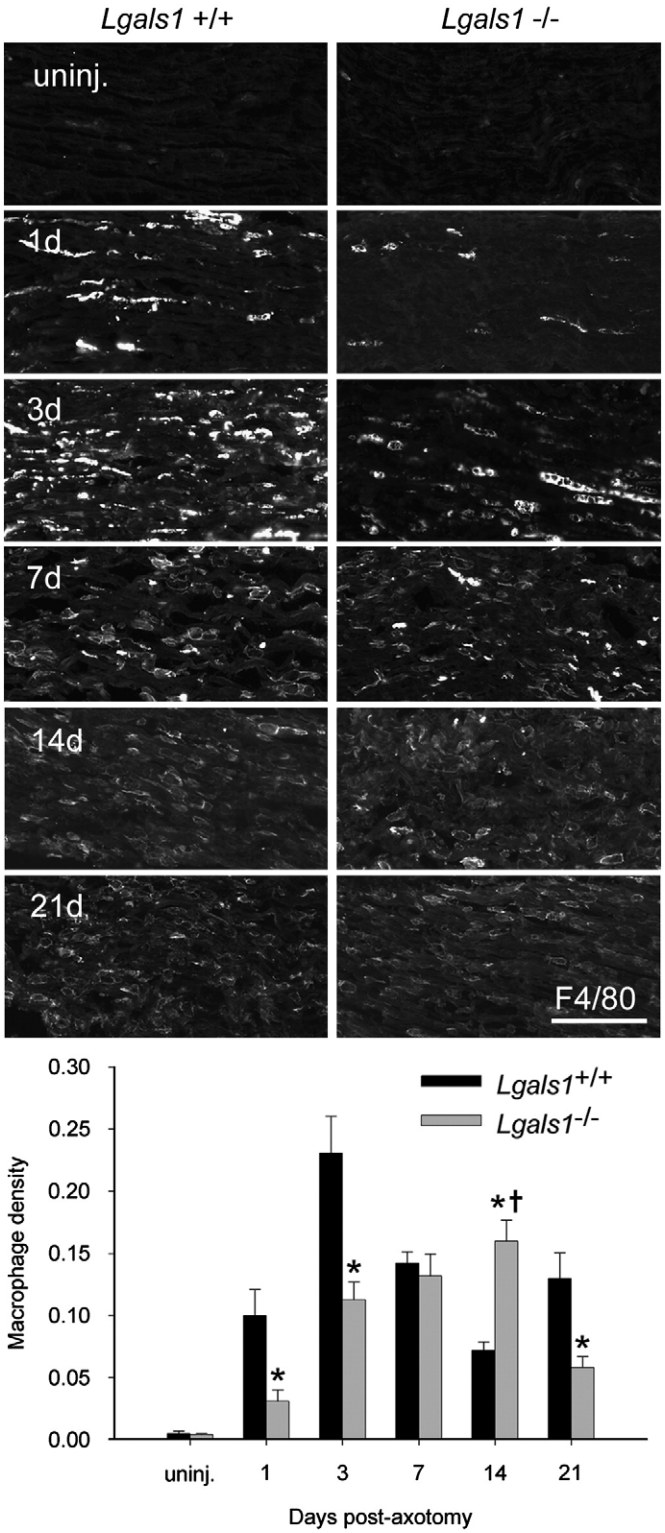

Fig. 3. Axotomy-induced accumulation of macrophages is delayed and diminished in sciatic nerves of *Lgals1*$^{-/-}$ mice, as visualized by F4/80 immunohistochemistry. The nerve distal to sciatic nerve ligation was examined. In the *Lgals1*$^{+/+}$ sciatic nerve ipsilateral to injury, peak macrophage density was observed at 3 days following injury. In *Lgals1*$^{-/-}$ mice, maximal macrophage accumulation was not achieved until 14 days after lesion, and the peak macrophage density was attenuated in these mice relative to *Lgals1*$^{+/+}$ mice. cont., contralateral. Unless indicated otherwise, photomicrographs represent tissue ipsilateral to injury. *, significant differences between *Lgals1*$^{-/-}$ nerves and *Lgals1*$^{+/+}$ nerves at same timepoint; ‡, significant difference between peak densities in *Lgals1*$^{-/-}$ (14 days) and *Lgals1*$^{+/+}$ (3 days) groups. Scale bar, 200 μm.

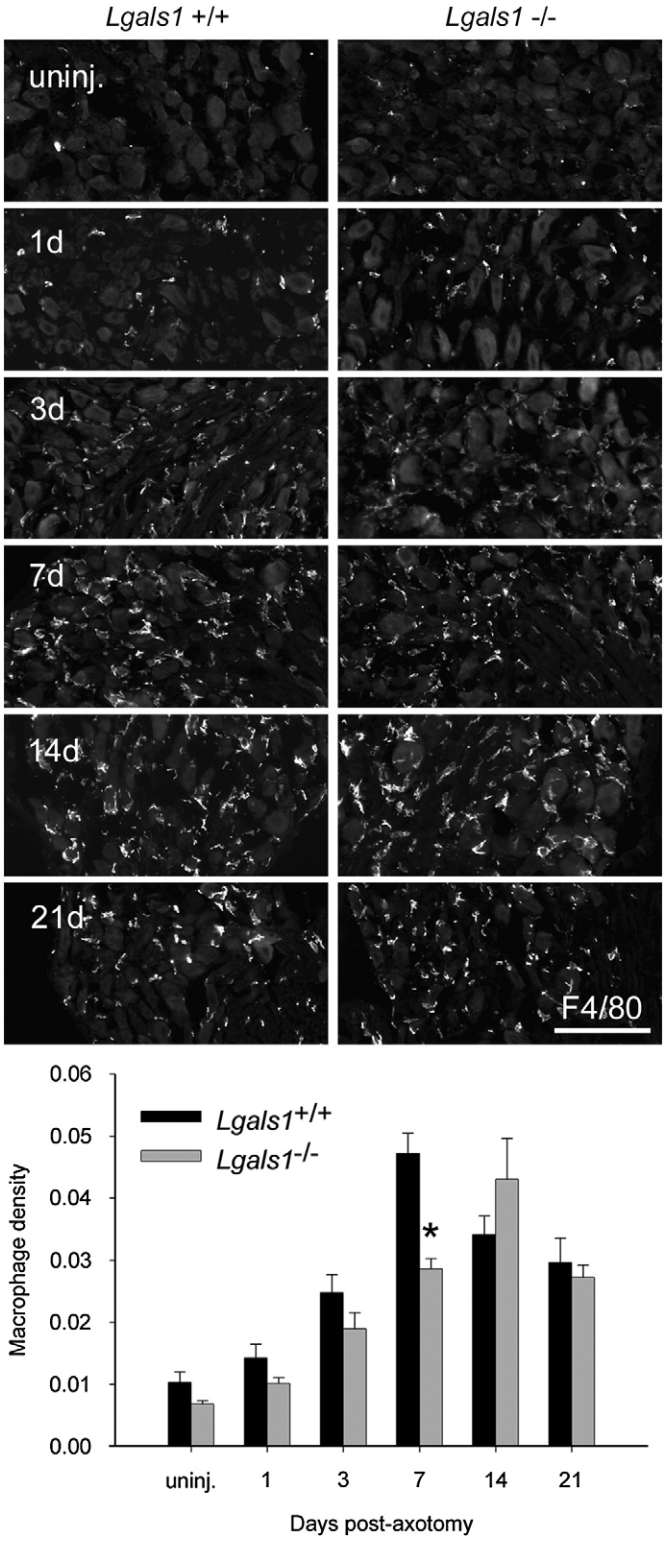

Fig. 4. Injury-induced macrophage accumulation is delayed in L5 dorsal root ganglia (DRGs) of *Lgals1*$^{-/-}$ mice, as visualized by F4/80 immunohistochemistry. In the *Lgals1*$^{+/+}$ DRG ipsilateral to injury, maximal macrophage accumulation was observed at 7 days following sciatic nerve ligation. In contrast, *Lgals1*$^{-/-}$ DRGs associated with injury exhibited maximal macrophage density at 14 days after injury. The peak macrophage densities are not significantly different between strains. cont., contralateral; ipsi., ipsilateral. Unless indicated otherwise, photomicrographs represent tissue ipsilateral to injury. *, significant difference between *Lgals1*$^{-/-}$ and *Lgals1*$^{+/+}$ DRGs at the same timepoint. Scale bar, 200 μm.

Lgals1$^{+/+}$ nerves, although F4/80 immunoreactivity was more widespread in zymosan-treated sciatic nerves. These results suggest that (1) Gal1/Ox is sufficient to direct the accumulation of *Lgals1*$^{+/+}$ macrophages in the absence of injury, and (2) macrophages from *Lgals1*$^{-/-}$ mice have a lower Gal1/Ox sensitivity than wild-type macrophages. It is possible that Gal1 is required for normal macrophage development: when differentiating monocytic cells do not express or are not exposed to Gal1 (in the *Lgals1*$^{-/-}$ mouse), they may not develop sensitivity to this protein or express its cognate receptor.

Nerve transplantation in Lgals1$^{-/-}$ *mice: compensation and (lack of) responsiveness to Gal1*

We performed a transplant experiment in order to establish whether macrophage accumulation in injured *Lgals1*$^{-/-}$ mice could be rescued by nerve grafts from wild-type animals. We transplanted

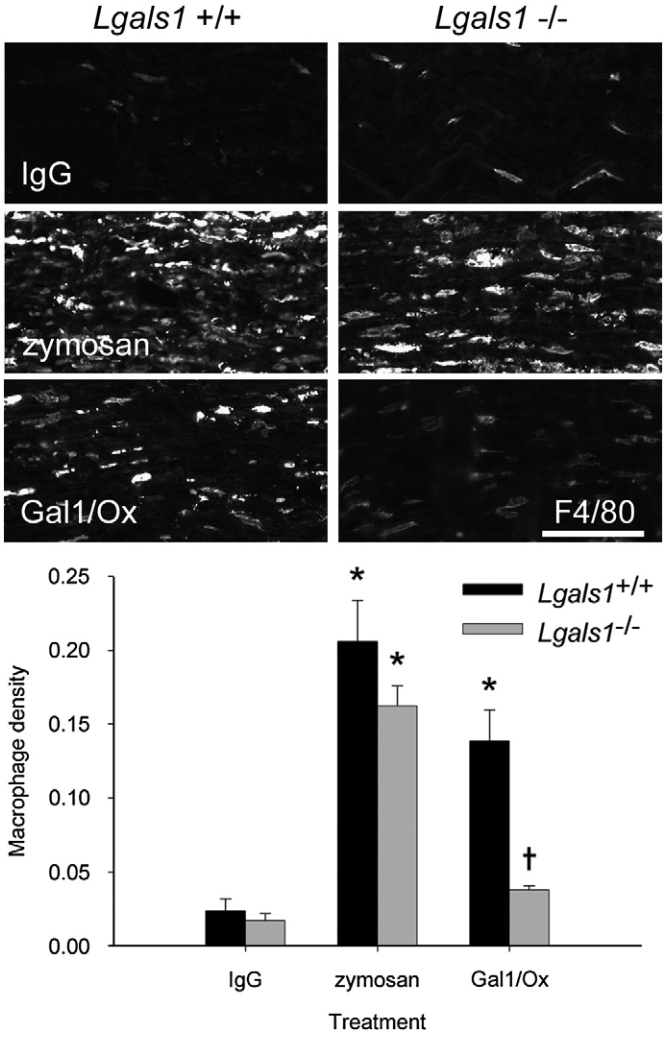

Fig. 5. Injection of oxidized galectin-1 (Gal1/Ox) into uninjured sciatic nerve is sufficient to facilitate the accumulation of macrophages in *Lgals1*$^{+/+}$ (but not *Lgals1*$^{-/-}$) mice. F4/80 immunohistochemistry was used to assess macrophage density in uninjured *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ mice injected with human IgG, zymosan, and Gal1/Ox 3 days after injection. As expected, nerves injected with zymosan had a significantly higher density of macrophages in both strains relative to the group that received IgG injection. In *Lgals1*$^{+/+}$ sciatic nerves, injection of Gal1/Ox induced greater accumulation of macrophages than IgG injection. In contrast, macrophage density in *Lgals1*$^{-/-}$ nerves injected with Gal1/Ox was not significantly different from those that were injected with IgG. *, significant differences between zymosan- or Gal1/Ox-treated groups and the IgG group; ‡, significant differences between *Lgals1*$^{-/-}$ Gal1/Ox-treated nerves and both *Lgals1*$^{+/+}$ Gal1/Ox-treated and *Lgals1*$^{-/-}$ zymosan-treated groups. Scale bar, 100 μm.

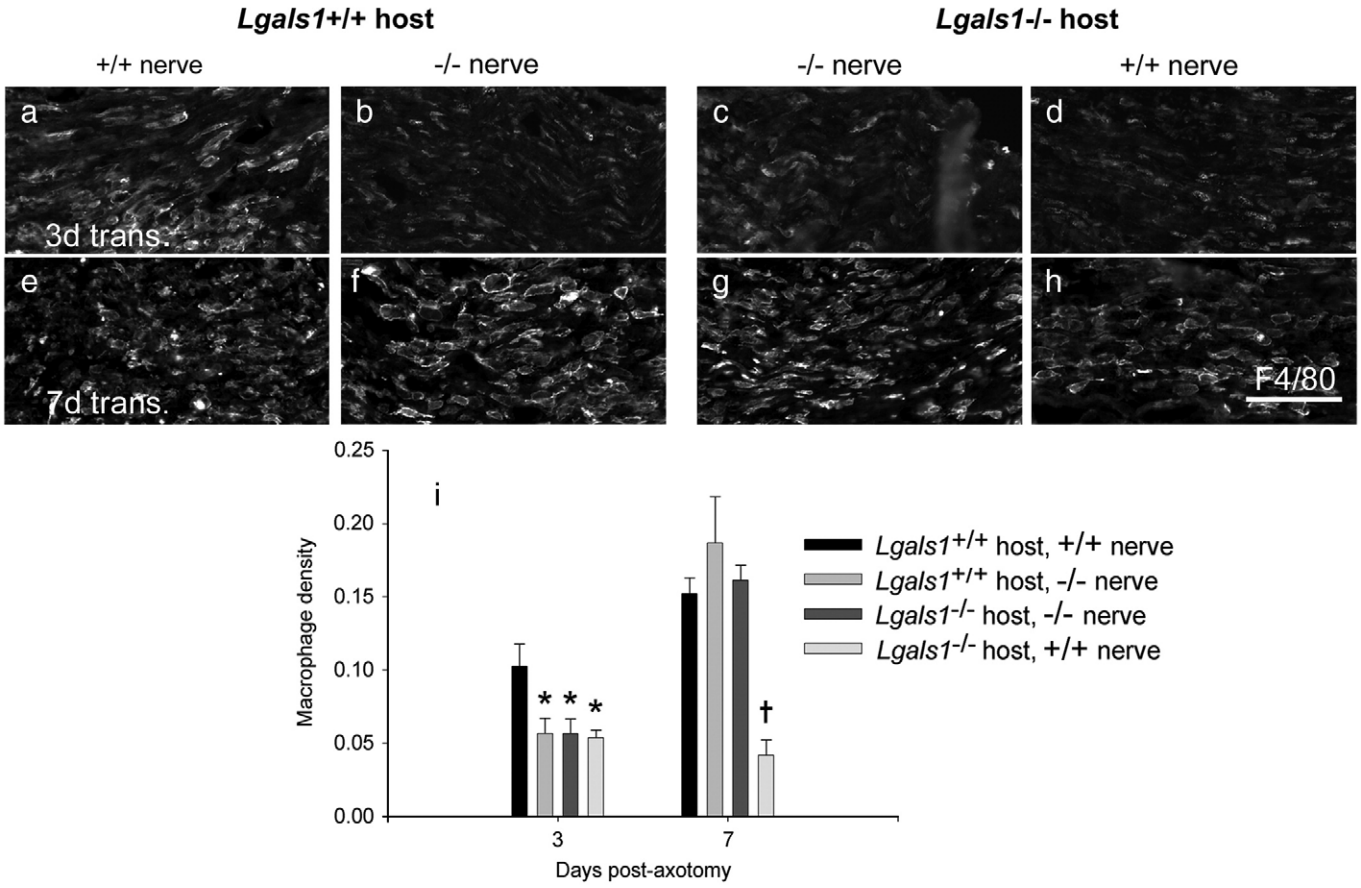

Fig. 6. Macrophage accumulation in segments of sciatic nerve transplanted to *Lgals1*$^{+/+}$ mice (left panels) or *Lgals1*$^{-/-}$ mice (right panels) 3 and 7 days post-transplantation. At 3 days post-transplant, macrophage accumulation was greatest in *Lgals1*$^{+/+}$ mice that received *Lgals1*$^{+/+}$ transplants (a, i). Interestingly, transplantation of a *Lgals1*$^{+/+}$ nerve segment into Lgals1$^{-/-}$ mice did not normalize macrophage accumulation at 3 days post-transplantation (d, i). At 7 days post-transplantation, macrophage density in the *Lgals1*$^{+/+}$ nerve segment transplanted into *Lgals1*$^{-/-}$ mice was significantly lower than all other transplant groups (h, i). trans, nerve transplanted during surgery. *, significant differences between *Lgals1*$^{+/+}$ → *Lgals1*$^{+/+}$ transplant and all other groups at 3 days; ‡, significant difference between *Lgals1*$^{+/+}$ → *Lgals1*$^{-/-}$ group and all other groups at 7 days. Scale bar, 200 μm.

Lgals1$^{+/+}$ and *Lgals1*$^{-/-}$ donor nerves into *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ host mice, and studied the density of F4/80 immunoreactivity at 3 and 7 dpi in the transplanted nerve (Fig. 6). At 3 dpi, *Lgals1*$^{+/+}$ transplant nerves in *Lgals1*$^{+/+}$ host mice had a higher density of macrophages than any other group ($p < 0.05$; Figs. 6a–d, i). This suggests that Gal1 is required for the typical response of macrophages to injury at this timepoint, and that Gal1 in the Gal1-positive nerve is not sufficient to rescue the phenotype of the *Lgals1*$^{-/-}$ nerve. By 7 dpi, the density of F4/80 immunoreactivity was significantly higher in both groups that received a *Lgals1*$^{-/-}$ nerve than at 3 dpi, and macrophage density in these nerves was not significantly different from the *Lgals1*$^{+/+}$ nerve in the *Lgals1*$^{+/+}$ host. Interestingly, macrophage density in *Lgals1*$^{+/+}$ nerves in *Lgals1*$^{-/-}$ hosts was significantly different from all other groups at 7 dpi ($p < 0.001$; Figs. 6e–i), and the density in the *Lgals1*$^{+/+}$ nerve into *Lgals1*$^{-/-}$ host group at 7 dpi was not different from that at 3 dpi. Taken together, these results suggest that Gal1 is required for the typical response of macrophages to injury, that Gal1 in wild-type nerve is not sufficient to rescue macrophage accumulation in *Lgals1*$^{-/-}$ nerve, and that there are compensatory mechanisms in place within the *Lgals1*$^{-/-}$ nerve that allow for the delayed accumulation of macrophages up to *Lgals1*$^{+/+}$ levels.

Discussion

In the present study, we have established that Gal1 activity in peripheral nerve positively regulates accumulation of immune cells in the nervous system following peripheral axotomy. Using a model of peripheral nerve injury in transgenic mice, we showed that (1) Gal1 is required for the normal injury-induced accumulation of macrophages in the nerve distal to injury, as well as to the ipsilateral L5 DRG; (2) exogenous Gal1/Ox facilitates the accumulation of macrophages in the uninjured *Lgals1*$^{+/+}$ sciatic nerve; (3) Gal1/Ox is not sufficient to direct accumulation of macrophages in *Lgals1*$^{-/-}$ sciatic nerve; and (4) compensatory mechanisms in Lgals1$^{-/-}$ mice allow for injury-induced macrophage accumulation to occur at later timepoints. These results show that Gal1, which is upregulated transiently in the distal nerve around 3 days post-axotomy, accelerates the early response of macrophages to nerve injury.

Gal1 and the immune response

Gal1 is expressed by many leukocytes (dendritic cells and activated macrophages, B cells, and T cells) (Blaser et al., 1998; Rabinovich et al., 1998; Zuniga et al., 2001), and has critical immunoregulatory functions (Rabinovich et al., 2007). Although Gal1 is generally thought to act as an immunosuppressive agent, previous studies have described conflicting roles for Gal1 in the modulation of the immune response. For instance, Gal1 regulates the survival of activated T cells, but not naïve T cells or monocytes (Perillo et al., 1995; Rabinovich et al., 1998; Barrionuevo et al., 2007). Moreover, Gal1 treatment increases phagocytosis by untreated monocytes, but decreases phagocytosis by monocytes activated by interferon-γ (Barrionuevo et al., 2007). Thus, the response of a particular immune cell to Gal1 *in vivo* is the net result of the complex

interplay between Gal1 (and its oxidation state), signaling by other cytokines, and receptors on the competent cell.

Role of Gal1 following peripheral nerve injury

Previous studies have shown that Gal1 promotes regeneration of axons following injury in a variety of models: mixed (sensory and motor) peripheral nerve transection and tubulization (Horie et al., 1999), facial (motor) nerve injury (McGraw et al., 2004a,b), and optic nerve injury (Okada et al., 2005). There are four mechanisms that may underlie the actions of Gal1 on repair of the injured nerve. First, Gal1 may be acting directly on the regenerating axon itself (Horie et al., 1999; Inagaki et al., 2000; Horie et al., 2004; Miura et al., 2004). Second, Gal1 promotes migration of Schwann cells following injury (Horie et al., 1999; Fukaya et al., 2003), presumably allowing these cells to expediate Wallerian degeneration and to form bands of Büngner for regrowing axons. Third, Gal1/Ox activates macrophages by binding to a cell surface receptor, and may recruit them to the site of injury through this ligand–receptor interaction (Horie et al., 2004). Finally, Gal1 may indirectly enhance regeneration by causing degeneration of detached axons following axotomy (Plachta et al., 2007). We will consider the latter two processes in more detail, since these involve or cause the accumulation of macrophages.

Gal1 may act directly on macrophages to elicit their accumulation following peripheral axotomy. This injury-induced accumulation is the result of both the invasion of hematogenous monocytes and the proliferation of macrophages resident to the nerve. Since blood-derived macrophages comprise the majority of the macrophage population in the injured nerve soon after injury (Mueller et al., 2003), it is possible that Gal1 is affecting the homing-in or extravasation of these cells. Gal1/Ox activates macrophages by binding an unidentified receptor that initiates an intracellular tyrosine phosphatase phosphorylation cascade (Horie et al., 2004), and a similar action could underlie axotomy-induced recruitment of these cells. However, Gal1 has been shown to inhibit trans-migration of bone marrow progenitor cells, granulocytes, and monocytes across an endothelial cell layer *in vitro* and prevents their mobilization from bone marrow *in vivo* (Kiss et al., 2007), and Gal1 treatment inhibits neutrophil extravasation, chemotaxis, and transendothelial migration (Rabinovich et al., 2000; La et al., 2003). These results suggest that Gal1 actually inhibits the mobilization, chemotaxis, and migration of these immune cells, and would attenuate their accumulation. Thus, Gal1 may not recruit hematogenous macrophages directly, and another mechanism might be responsible for the accumulation of phagocytes following nerve injury.

Gal1-induced degeneration of axons could also have a key role in the accumulation of immune cells following injury. Gal1 has recently been implicated in the degeneration of axons: this process involves signaling intrinsic to the neuron, as Gal1 treatment of isolated embryonic stem cell-derived neurons leads to the degeneration of their neurites (Plachta et al., 2007). These authors also showed that the elimination of peripheral nerve endings is delayed in *Lgals1*$^{-/-}$ mice following sciatic nerve injury. Interestingly, we found that injection of Gal1/Ox induced degeneration of axons in the sciatic nerve in our model, as axons (identified by PGP-9.5 immunoreactivity) were discontinuous and beaded up more in areas surrounding Gal1/Ox injection than in areas adjacent to IgG injection (data not shown). Thus, the oxidized form of Gal1 may be able to cause the local degeneration of axons, which would lead to the release of cytokines and other factors that promote the activation and recruitment of immune cells. The pro-inflammatory cytokine cascades initiated by Gal1-induced degeneration may overcome Gal1's signal to prevent immune cell extravasation, leading to the net influx of neutrophils and macrophages.

Degeneration of detached axon and myelin debris is crucial for efficient axonal regeneration following peripheral axotomy. The inability to remove this debris is the major factor that impedes axon regeneration in Wallerian degeneration slow (*Wld*^{S}) mice: fewer macrophages accumulate in the nerve distal to injury, and this results in delayed clearance of myelin and axonal debris (Brown et al., 1994). Interestingly, assisting degeneration by crushing the distal nerve at the time of injury increases the rate of regeneration in these mice, suggesting that the residual intact debris acts as a more robust barrier for regrowing axons. Thus, the delayed and diminished accumulation of macrophages in the distal nerves of injured *Lgals1*$^{-/-}$ mice may underlie delayed peripheral axon regeneration in nerves of these mice.

The timecourse of peripheral axotomy-induced macrophage accumulation has been characterized extensively. For instance, Bendszus and Stoll (2003) examined axotomy-induced macrophage infiltration in rat using ED1 immunoreactivity and found that macrophage density increased in the injured nerve up to 14 days after sciatic nerve crush. Likewise, Avellino et al. (2004) found the highest density of macrophages present between 14 and 21 days after sciatic nerve transection in rat. In contrast, Perry et al. (1987) and de la Hoz et al. (2003) showed that macrophage density peaked around 5 days after mouse sciatic nerve crush and transection, respectively. We found that macrophage accumulation peaked around 3 dpi in our wild-type mice, although we did not have a 5-day timepoint. As mentioned above, some previous studies showed a more protracted wave of macrophage accumulation. We attribute the disparities between reported timelines of macrophage infiltration to differences in techniques used: the animal model, injury model, antibody, and analysis method used all have important roles in defining how we observe macrophages' response to nerve injury (time and intensity of response).

The results of our nerve transplant experiment (Fig. 6) proved to be intriguing. Not surprisingly, macrophage density was highest in *Lgals1*$^{+/+}$ hosts with *Lgals1*$^{+/+}$ donor nerves at 3 dpi. *Lgals1*$^{+/+}$ transplant nerves into *Lgals1*$^{-/-}$ recipients did not rescue the knockout phenotype at this timepoint. This finding is congruent with the results of our gain-of-function experiment, which showed that *Lgals1*$^{-/-}$ macrophages are not responsive to Gal1/Ox. The fact that macrophage density in *Lgals1*$^{+/+}$ and *Lgals1*$^{-/-}$ animals that received *Lgals1*$^{-/-}$ transplants was at the same level as *Lgals1*$^{+/+}$ hosts with *Lgals1*$^{+/+}$ transplant nerves at 7 dpi suggests that there is a compensatory mechanism that is initiated in the absence of Gal1, which promotes the accumulation of macrophages at this later timepoint. Interestingly, this compensatory mechanism does not seem to be present in Gal1-positive nerves: macrophage density in *Lgals1*$^{+/+}$ nerves transplanted into *Lgals1*$^{-/-}$ hosts remains lower than in other groups at 7 dpi. Macrophage density in this group is lower than in others at both timepoints, which suggests that *Lgals1*$^{-/-}$ macrophages might not be responsive to Gal1. Taken together, these results suggest that Gal1 may be involved in a cytokine cascade evoked by nerve injury.

Although peripheral axons have the ability to regenerate following injury, repair and functional recovery in humans is usually incomplete and inadequate. Gal1 is one factor that may improve nerve repair following injury: Gal1 may act both within the injured neuron, and within its axon's environment, to promote regeneration. In the current study, we have defined a role for Gal1 in the axonal environment. We have shown that Gal1 is necessary and sufficient to direct typical macrophage accumulation following peripheral nerve injury, and our data suggest that Gal1 may enhance the efficiency of Wallerian degeneration in the PNS by promoting accumulation of phagocytes following nerve injury.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.expneurol.2009.09.007.

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