A new neutrophil subset promotes CNS neuron survival and axon regeneration

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Transected axons typically fail to regenerate in the central nervous system (CNS), resulting in chronic neurological disability in individuals with traumatic brain or spinal cord injury, glaucoma and ischemia–reperfusion injury of the eye. Although neuroinflammation is often depicted as detrimental, there is growing evidence that alternatively activated, reparative leukocyte subsets and their products can be deployed to improve neurological outcomes. In the current study, we identify a unique granulocyte subset, with characteristics of an immature neutrophil, that had neuroprotective properties and drove CNS axon regeneration in vivo, in part via secretion of a cocktail of growth factors. This pro-regenerative neutrophil promoted repair in the optic nerve and spinal cord, demonstrating its relevance across CNS compartments and neuronal populations. Our findings could ultimately lead to the development of new immunotherapies that reverse CNS damage and restore lost neurological function across a spectrum of diseases.

Axonal transection and neuronal death are pathological features of a wide range of CNS disorders, including traumatic brain or spinal cord injury (SCI), multiple sclerosis and motor neuron disease. They are also hallmarks of glaucoma and ischemia–reperfusion injury of the eye. Protracted disability in individuals with these conditions is due, in large part, to the poor regenerative capacity of the CNS, which includes the retina, optic nerves, brain, brainstem and spinal cord. There is a dire need for novel therapies that not only mitigate but also reverse chronic neurological deficits. One potential strategy that has been proposed to promote neuroprotection and regeneration involves modulation of the local immune response to CNS damage. Studies in cutaneous wound healing, atherosclerosis and myocardial infarction have elucidated alternative immune pathways that most likely evolved to repair bystander tissue damage associated with the clearance of microbial infections. Although CNS-infiltrating, proinflammatory leukocytes are detrimental in the context of multiple sclerosis and neuromyelitis optica, and possibly Alzheimer’s disease and stroke, there is growing evidence that alternatively activated, regenerative leukocyte subsets and their products can improve clinical outcomes in a range of neurological disorders. A deeper understanding of the phenotypes of beneficial immune cells, their developmental pathways and mechanisms of action, could lead to the development of novel neurorestorative immunotherapies.

A classic model of immune-driven CNS axon regeneration involves crush injury to the murine optic nerve, resulting in extensive neuronal loss and axonal transection, which is mitigated by intraocular (i.o.) injection of zymosan, a fungal cell wall extract. Similarly to white matter tracts in the brain and spinal cord, transected axons in the optic nerve fail to undergo long-distance regeneration. However, i.o. zymosan injection, at the time of optic nerve crush (ONC) injury or up to 3 d afterwards, induces vitreal inflammation associated with rescue of retinal ganglion cells (RGCs, the neurons that give rise to the axons in the optic nerve) from death, and RGC axon regeneration. We previously showed that β-1,3-glucan, a cognate ligand of the innate immune pattern recognition receptor dectin-1, is the active ingredient in zymosan responsible for its pro-regenerative properties in this model. We detected dectin-1 on eye-infiltrating myeloid cells, as well as on retina-resident microglia and dendritic cells (DCs), in i.o. zymosan-injected mice, but not on RGCs, Muller cells, oligodendrocytes or astrocytes. The cellular and molecular mediators that trigger neurorepair downstream of dectin-1 signaling are poorly understood. In the current study, we identify a unique granulocyte with characteristics of an immature neutrophil that accumulated in the vitreous fluid in response to i.o. zymosan and exerted neuroprotective and axonogenic effects that are, in part, secondary to the secretion of a cocktail of growth factors. We found that the same myeloid cell subset was capable of driving axon regeneration in the spinal cord, demonstrating its relevance across CNS compartments. A human cell line with characteristics of immature neutrophils also exhibited neuroregenerative capacity, suggesting that our observations might be translatable to the clinic.

Results

Intraocular Ly6Glo neutrophils drive RGC axon regrowth. We used flow cytometric analysis to determine the cellular composition of infiltrates that accumulated in the posterior chamber of the eye following i.o. zymosan injection and ONC injury (gating strategy is illustrated in Extended Data Fig. 1a). Vitreous fluid was serially collected until day 14 after ONC injury, a standard time point when optic nerves are harvested to assess axon regeneration. Myeloid cells (neutrophils and monocytes/macrophages) were predominant at every time point (Fig. 1a). Neutrophils outnumbered all other leukocyte subsets from days 1–5 following i.o. zymosan injection, previously established as the critical time window for instigating...
RGC rescue and axonal regeneration\(^1\). Lymphocytes and DCs were minor constituents of the vitreal infiltrates, irrespective of the day of harvest. Intracocular zymosan-driven RGC axon regeneration was unimpaired in \(Rag^1\^-\) mice, indicating that mature T and B cells are dispensable in this model (Extended Data Fig. 1b).

To assess the contribution of neutrophils, we attempted to impede their entry into the posterior chamber of the eye with a blocking antisera specific for CXCR2, the predominant chemokine receptor expressed by mature murine neutrophils. Unexpectedly, systemic administration of anti-CXCR2, from the day of ONC injury/i.o. zymosan injection onward, significantly enhanced RGC survival and axon regeneration (Fig. 1b–e). The augmentation of axonal regeneration by administration of anti-CXCR2 was sustained through day 28 following ONC injury/i.o. zymosan injection (Extended Data Fig. 1c), with some axons reaching the optic chiasm (data not shown). Flow cytometric analyses revealed that i.o. neutrophil accumulation was delayed, but not prevented, in the cohort treated with anti-CXCR2, such that the number of neutrophils peaked on day 5 rather than days 1–3 (Fig. 1a). The peak of i.o. monocyte and DC accumulation was delayed until day 7. Furthermore, the characteristics of the infiltrating leukocyte population were altered. Intracocular inflammatory cells, isolated between days 3 through 7 after ONC injury/i.o. zymosan and anti-CXCR2 treatment, directly stimulated the neurite outgrowth of primary isolated 5 d following ONC injury/i.o. zymosan and anti-CXCR2 treatment, contained an expanded subpopulation of CD11b+ myeloid cells that expressed relatively low amounts of Ly6G and Ly6B and had relatively high expression of CD14, when compared with i.o. infiltrates isolated from the mice treated with control sera (Fig. 2a). The majority of these Ly6G\(^+\)CD14\(^+\) cells had ring-shaped nuclei and were CD101\(^+\)hi, indicative of an immature neutrophil phenotype (Fig. 2a,b). In contrast, their Ly6G\(^-\)CD14\(^-\) counterparts (the most populous subset in the control group), universally had segmented nuclei and were CD101\(^-\), consistent with conventional mature neutrophils. Both Ly6G\(^+\)CD14\(^+\) and Ly6G\(^-\)CD14\(^-\) cells possessed cytoplasmic granules and expressed comparable amounts of elastase and myeloperoxidase (Fig. 2b). Intracocular Ly6G\(^+\) cells, isolated 5 d following ONC injury/i.o. zymosan and anti-CXCR2 treatment, directly stimulated the neurite outgrowth of primary RGCs in vitro (Fig. 2c). The frequency of Ly6G\(^+\)CD101\(^+\)myeloid cells was elevated in the blood and spleen of the cohort treated with anti-CXCR2, suggesting that the combination of i.o. zymosan administration and CXCR2 blockade accelerates mobilization of immature neutrophils from the bone marrow to the circulation, from where they are recruited to the eye (Extended Data Fig. 2; data not shown). These results implicate a subpopulation of immature neutrophils in i.o. zymosan-induced optic nerve axon regeneration.

**Transcriptome profiling of intracocular Ly6G\(^+\) cells.** For in-depth analysis, we purified Ly6G\(^+\) cells from the vitreous fluid of mice 5 d following i.o. zymosan injection and treatment with either anti-CXCR2 or control sera. Whole-transcriptome analysis with single-cell RNA sequencing (scRNA-seq) was then performed. Three distinct clusters were observed in the single-cell datasets that were representative of seven independent experiments. Each cluster contained cells expressing distinct sets of genes, with the central cluster containing a high proportion of genes that were uniquely expressed in Ly6G\(^+\) cells (Fig. 2d). These genes were enriched for those involved in immune cell activation and response to injury, indicating a role for Ly6G\(^+\) cells in the resolution of inflammation after ONC injury.
Fig. 2 | Treatment of mice with αCXCR2 antiserum, starting on the day of i.o. zymosan injection, skews eye-infiltrating neutrophils toward an immature phenotype. a–c, Mice were injected i.o. with zymosan on day 0 of ONC injury and i.p. with either αCXCR2 (red) or NRS (blue) on days 0, 2 and 4. Inflammatory cells were isolated from the vitreous fluid on day 5. a, b, Surface expression of myeloid cell markers. a, Upper, representative histograms. Lower, geometric mean fluorescence intensity (MFI) of Ly6G (n = 5 mice per group), CD14 (n = 5 mice per group), Ly6B (n = 5 mice per group), myeloperoxidase (MPO; n = 3 mice per group) and elastase (n = 3 mice per group) on Ly6G+ gated cells and the percentage of Ly6G+ gated cells that were CD101+ (n = 8 mice per group). Each symbol represents data obtained from a single mouse. Data are from one experiment representative of three independent experiments. Statistical significance was determined by a two-tailed unpaired Student’s t-test. b, Cytospins of purified vitreal Ly6G+ cells, stained with Wright-Giemsa solution. Top: scale bar, 10 μm. Bottom: scale bar, 6 μm. Arrows point to granules. c, Mean length of the longest neurite grown by primary RGCs, following 24-h co-culture with i.o. Ly6G+ neutrophils (Nϕ) that were purified from the NRS or αCXCR2 treatment groups. RGCs were cultured with recombinant CNTF as a positive control or with particulate zymosan (zym) or medium alone (no tx) as negative controls (n = 200 RGCs per condition). Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test. Right, representative images; scale bar, 20 μm.

a–c, Data are shown as the mean ± s.e.m. NS, not significant.
Fig. 3 | Neuroregenerative neutrophils have characteristics of alternatively activated cells. a–d. Mice with ONC injury were injected i.o. with zymosan on day 0 and i.p. with aCXCR2 or NRS on days 0, 2 and 4. Ly6G+ cells were purified from i.o. infiltrates on day 5. a, b, Single-cell analysis of pooled i.o. Ly6G+ cells from the NRS or aCXCR2 treatment groups using 10x Genomics (n = 5 mice per group). a, Uniform manifold approximation and projection (UMAP) visualizations showing 5,909 cells (left, NRS treatment group) and 4,844 cells (right, aCXCR2 treatment group) by cluster. Pie charts indicate the percentage of cells in each cluster. Lower, feature plots showing cluster-specific expression of arginase-1 (Arg1). b, Heat maps depicting the scaled expression of genes related to neutrophil maturation (left) and classical or alternative activation markers (right). c, RNA was extracted from Ly6G+ cells purified from the i.o. infiltrates of individual mice. Arg1, Il4Ra and CD206/mannose receptor (MRC) transcripts were quantified by qPCR and normalized to β-actin (n = 4 mice per group). Data are from one experiment representative of three independent experiments. d, Representative flow cytometric dot plots showing intracellular Arginase-1 protein expression in Ly6G+ gated cells. Right, percentage of Ly6G+ cells that are Arginase-1+ (n = 5 mice per group). Data are from one experiment representative of two independent experiments. FITC, fluorescein isothiocyanate. c, d, Each symbol represents the results obtained from an individual mouse. Data are the mean ± s.e.m. Statistical significance was determined by a two-tailed unpaired Student’s t-test.
derived from the groups treated with either the anti-CXCR2 or control sera, with profiles indicative of immature (cluster 3), intermediate (cluster 2) or mature (cluster 1) stages of neutrophil development (Fig. 3a,b). Consistent with our flow cytometric data, the percentage of i.o. Ly6G⁺ cells that fell within the immature neutrophil cluster was more than threefold higher in mice treated with anti-CXCR2 compared to mice treated with control sera (36.8% versus 10.6%, respectively). These results confirm that RGC axon regrowth is associated with the accumulation of immature neutrophils close to the site of nerve injury.

**Zymosan-induced Ly6G⁻ cells are alternatively activated.** Novel subpopulations of neutrophils were recently described in animal models of cancer, myocardial infarction and infection. ‘N2’ neutrophils, which express arginase-1, vascular endothelial growth factor (VEGF)α and bear a ring-shaped nucleus, were initially identified in intratumoral infiltrates. These cells are distinct from conventional ‘N1’ neutrophils, which express proinflammatory cytokines and chemokines and bear a multi-segmented nucleus. Enrichment of N2 neutrophils in the tumor bed is associated with impaired antitumor immunity and accelerated tumor growth. Unconventional neutrophils with features similar to N2 cells have subsequently been described in animal models of helmint and staphylococcus infection, stroke and myocardial infarction. In all of these models, unconventional neutrophils are characterized by ring-shaped nuclei, as well as expression of genes encoding type 2 cytokines (IL4, IL5 and/or IL13 mRNA) and genes traditionally associated with alternatively activated ‘M2’ macrophages (such as Arg1/arginase-1, Mrc1/ mannose receptor (CD206), Il4ra/ interleukin (IL)-4 receptor α-chain, Itga6/VL A-6, Tgfb1/transforming growth factor-β1, Iγf1/insulin-like growth factor 1 and Ccl5). However, the ability of alternatively activated neutrophil subsets to induce neuroprotection and neuroregeneration has yet to be demonstrated.

Reminiscent of the ‘N2’ and the other alternatively activated neutrophils described above, Ly6G⁻ cells, purified from the vitreous fluid of mice on day 5 after ONC injury/i.o. zymosan/αCXCR2 treatment, exhibited elevated abundance of transcripts for Arg1, Mrc1 and Il4ra relative to their Ly6G⁺ counterparts (Fig. 3c). Furthermore, a significant percentage of those cells expressed arginase-1 protein (Fig. 3d). Consistent with these results, scRNA-seq of i.o. Ly6G⁻ cells revealed that transcripts for Arg1 and Mrc1, as well as Il4ra, Tgfb1, Ilgf1 and Ccl5, were enriched in the immature neutrophil subset (cluster 3) that was expanded in the cohort treated with αCXCR2 (Fig. 3a,b and Extended Data Fig. 3). Thus, zymosan-induced Ly6G⁻ neutrophils have characteristics of an alternatively activated myeloid cell.

**Donor Ly6G⁻ cells promote RGC survival and axon regrowth.** We used an adoptive transfer approach to directly evaluate the neuroprotective and pro-regenerative potential of zymosan-elicited Ly6G⁻ neutrophils in vivo. The low yield of Ly6G⁻ cells sorted from vitreal infiltrates of mice injected with i.o. zymosan prohibits adoptive transfer experiments with those cells. As an alternative approach, we isolated Ly6G⁻ cells from the peritoneal cavities of naive mice at serial time points following intraperitoneal (i.p.) injection of zymosan, and characterized them by flow cytometric analysis and quantitative PCR with reverse transcription (RT–qPCR). We found that neutrophils isolated on day 3 after i.p. zymosan injection (3 d Nϕ) simulated a number of the properties of the pro-regenerative neutrophils harvested from the eyes of the i.o. zymosan/anti-CXCR2 treatment group, including relatively low expression of cell surface markers Ly6G and CD101, high expression of cell surface CD14 and high expression of Arg1, Il4ra and Mrc1 transcripts (Fig. 4a,b). In contrast, neutrophils isolated from the peritoneal cavity of mice 4 h following i.p. zymosan injection (4 h Nϕ) resembled conventional mature neutrophils in that they were Ly6G⁺CD14⁻/CD101⁺ and expressed undetectable to low amounts of Arg1, Il4ra and Mrc1 transcripts.
Ly6G<sup>lo</sup> neutrophils secrete an array of growth factors. Conditioned culture medium harvested from 3 d Nφ (3 d NCM) promoted neurite outgrowth of primary RGCs, indicating that soluble factors are, at least in part, responsible for the pro-regenerative properties of 3 d Nφ (Fig. 5a). The neurite growth-promoting effect of NCM was heat sensitive, suggesting that proteins were responsible for this activity. We performed a multiplexed antibody array assay to screen NCM for a panel of candidate growth factors. Nerve growth factor (NGF) and IGF-1 were highly elevated in NCM versus unconditioned medium (Extended Data Fig. 6a). High expression levels of NGF and IGF-1 by 3 d Nφ, in comparison to 4 h Nφ, were corroborated by RT–qPCR and ELISA (Fig. 5b). NGF and IGF-1 proteins were readily detectable in the vitreous fluid on days 3 and 5 following i.o. zymosan injection, and their abundance was enhanced by the administration of αCXCR2 antiserum (Fig. 5c).

Neutralization of either NGF or IGF-1 with antagonistic antibodies mitigated NCM-driven RGC neurite outgrowth in vitro (Fig. 5d). Neutralization of both growth factors together had a greater impact than blocking either one alone. Similarly, i.o. administration of either anti-NGF or anti-IGF-1 neutralizing antibody, at the time of 3 d Nφ adoptive transfer, impeded RGC protection and axon regeneration in vivo; administration of both antibodies had an additive effect (Fig. 5e,f). Conversely, recombinant NGF and IGF-1 acted in a collaborative fashion to promote RGC neurite outgrowth in vitro and RGC axon regeneration in vivo (Extended Data Fig. 6b,c). These findings indicate that zymosan-modulated Ly6G<sup>lo</sup> neutrophils promote neuronal survival and axon regeneration, in part, via secretion of NGF and IGF-1.

Ly6G<sup>lo</sup> cells drive repair in the spinal cord. To determine whether the neuroregenerative effects of Ly6G<sup>lo</sup> neutrophils are limited to RGCs and the microenvironment of the eye, or are more broadly applicable, we assessed their effects on dorsal root ganglia (DRG) cells. Importantly, 3 d Nφ stimulated axon outgrowth of primary DRG neurons when added at the initiation of culture or up to 8 h later, whereas naive bone marrow neutrophils and 4 h Nφ were ineffectual (Fig. 6a; Extended Data Fig. 5c). The CM of 3 d Nφ also induced neurite outgrowth of DRG (Fig. 6a). To test the regenerative potential of alternatively activated neutrophils in the setting of traumatic SCI, we injected 3 d Nφ, 4 h Nφ, naive bone marrow neutrophils or PBS alone into sciatic nerves 5 d before laceration of spinal cord dorsal columns at the T4 level. For a positive control, a separate group of mice was subjected to conditioning injury (that is, sciatic nerve crush) 5 d before SCI, which is a widely used

Fig. 5 | CD14<sup>−</sup>Ly6G<sup>lo</sup> cells induce RGC axon outgrowth, in part, via secretion of growth factors. a–d, Ly6G<sup>lo</sup> cells were purified from peritoneal lavage fluid that was collected 4 h (4 h Nφ) or 3 d (3 d Nφ) following i.p. zymosan injection. a, 3 d Nφ, 4 h Nφ, 3 d NCM or heat-shocked conditioned medium of 3 d Nφ (HS NCM) was added to primary RGC cultures, and neurite outgrowth was measured 24 h later. RGCs were cultured with recombinant CNTF or particulate zymosan as positive and negative controls, respectively. Each circle represents the mean neurite length of 200 RGCs counted in one experiment; data are representative of three independent experiments. Statistical significance determined by one-way ANOVA followed by Tukey’s post hoc test. Right, representative images. b, Upper, NFG and IGF-1 mRNA levels in 4 h or 3 d Nφ, quantified using qPCR and normalized to Actb. Lower, NFG and IGF-1 protein levels, measured in the CM of 4 h or 3 d Nφ by ELISA. c, NFG and IGF-1 protein levels, measured by ELISA, in vitreous fluid collected on day 5 following ONC injury and i.o. zymosan or PBS injection. Mice were injected i.p. with either NRS or αCXCR2 on days 0, 2 and 4 after ONC injury. b–c, Statistical significance was determined by a two-tailed unpaired Student’s t-test (n = 3 mice per group). One of two experiments is shown. d, Primary RGCs were cultured with the CM of 3 d Nφ in the absence or presence of neutralizing antibodies against NGF and/or IGF-1 or isotype-matched control antibodies. Neurite length was measured 24 h later. Each symbol represents the mean neurite length of 100 RGCs counted in one independent experiment; n = 10 experiments. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test. e, f, Purified 3 d Nφ were adoptively transferred, with or without neutralizing antibodies against NGF and/or IGF-1 or isotype-matched control antibodies, into the eyes of mice with ONC injury, as in Fig. 4c. A negative control group was injected with PBS alone. Optic nerves and retinas were harvested 14 d later. e, Density of regenerating axons in optic nerve sections at serial distances from the crush site (n = 10 nerves per group). Data are from one experiment representative of three independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001, compared with PBS; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001, compared with αNGF + αIGF-1). f, Frequency of viable BRN3a<sup>+</sup> RGCs in whole mounts, normalized to healthy retina (n = 10 retinas per group). Data are from one experiment representative of two independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test. a, d and f, *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001, compared with PBS; *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 and ****P < 0.0001, compared with NCM + αIGF-1. a–f, Error bars depict the mean ± s.e.m.
method for priming the regeneration of severed dorsal column axons\textsuperscript{19}. Spinal cords were harvested from all groups on day 52 after SCI and subjected to immunohistological analysis. We found severed dorsal column axons retracted proximally, away from the injury site, in the groups of mice that had been injected in the sciatic nerves with either vehicle or bone marrow neutrophils (Fig. 6b). Administration of 4 h N\(\phi\) prevented axonal retraction but did not trigger regeneration past the injury site. Conversely, severed axons regrew rostrally in the mice that had been injected with 3 d N\(\phi\), passing beyond the injury site by a distance commensurate to that reached by axons in mice subjected to a conditioning injury. Thus, 3 d N\(\phi\) displayed neuroprotective and pro-regenerative functions in several CNS compartments, namely the optic nerve and the spinal cord.
Human neutrophil-like cells are neuroregenerative. We next investigated the capacity of human myeloid cells, with characteristics of an immature neutrophil, to initiate neurorepair. HL-60 is a human promyelocytic leukemia cell line that, following short-term resting culture with dimethylsulfoxide (DMSO), exhibits morphological, enzymatic and chemotactic properties, and a cell surface antigen profile, indicative of immature neutrophils (that is, promyelocytes and blasts)\(^2\)\(^{}\)\(^{}\)\(^{}\)\(^{}\). At that point, they are deficient in secondary granules, suggestive of an early developmental stage in the granulocyte lineage\(^2\)\(^{}\)\(^{}\). Similarly to zymosan-modulated Ly6G\(^{\text{hi}}\) murine neutrophils, HL-60 cells expressed Arg1 and NGFB (Fig. 7a). Furthermore, they stimulated the regrowth of severed RGC axons upon adoptive transfer into the eyes of RAG1-deficient mice with ONC injury (Fig. 7b). In contrast, adoptive transfer of DG-75 cells, a human B cell lymphoma line, had no measurable effect. CM of HL-60 but not DG-75 cells induced neurite outgrowth by explanted RGC and DRG neurons (Fig. 7c and data not shown). Similarly to 3 d NΦ NCM, the axonogenic effect of HL-60 NCM was heat sensitive and partially suppressed by neutralization of NGF (Fig. 7c). HL-60 but not DG-75 cells directly stimulated neurite outgrowth by human cortical neurons in co-cultures (Fig. 7d). These results illustrate the neuroprotective and reparative potential of immature human granulocytes, mediated in part by a growth factor-dependent mechanism.

**Discussion**

In this study, we identify a novel myeloid cell phenotype with neuroprotective and axonogenic properties that arise in the setting of optic nerve and spinal cord injury. In contrast to the widely held notion that myeloid cells that promote tissue repair are generally of the monocyte/macrophage lineage\(^3\)\(^{}\)\(^{}\)–\(^{}\)\(^{}\)\(^{}\), the reparative cell that we characterize here is a CD14\(^{\text{hi}}\)Ly6G\(^{\text{hi}}\) granulocyte with features of an immature neutrophil. This finding is particularly surprising since numerous studies have highlighted the destructive impact of neutrophils infiltrating the CNS\(^7\)\(^{}\)–\(^{}\)\(^{}\)\(^{}\)\(^{}\). However, as opposed to pro-regenerative CD14\(^{\text{hi}}\)Ly6G\(^{\text{hi}}\) cells, the neutrophils that mediate CNS damage in these experimental systems are mature hypersegmented neutrophils, that access the CNS in a CXCR2-dependent manner. Although a few published studies have suggested that neutrophils may play a beneficial role in the context of CNS trauma\(^3\)\(^{}\)\(^{}\)\(^{}\)–\(^{}\)\(^{}\)\(^{}\)\(^{}\), here we characterize a CD14\(^{\text{hi}}\)Ly6G\(^{\text{hi}}\) neutrophil subset and its biological functions. The neuroprotective and axonogenic properties of the CD14\(^{\text{hi}}\)Ly6G\(^{\text{hi}}\) cells are, in part, secondary to secretion of the growth factors NGF and IGF-1. Although highly effective, treatment with a combination of antibodies against NGF and IGF-1 did not completely abrogate CD14\(^{\text{hi}}\)Ly6G\(^{\text{hi}}\) neutrophil-mediated axonal regeneration in vivo, implicating the presence of additional growth-promoting factors. Proteomics analyses have revealed numerous growth factors, besides NGF and IGF-1, in vitreous fluid isolated from i.o. zymosan-injected mice, as well as supernatants of pro-regenerative 3 d NΦ, that could collectively contribute to RGC rescue and axon regrowth (data not shown). Interestingly, we have found that adoptively transferred 3 d NΦ spatially align against the inner retina; the role of cell-to-cell interactions in the RGC regenerative process is a subject of ongoing investigation. We have considered the possibility that pro-regenerative neutrophils, and their products, modulate Muller glia and/or retinal astrocytes, and that these glial cells, in turn, promote or amplify RGC survival and axon regrowth, possibly via production of NGF and IGF-1 or other factors such as apolipoprotein E and ciliary neurotrophic factor (CNTF)\(^2\)\(^{}\). GFAP expression is elevated in the retina of mice following i.o. injection of 3 d NΦ, indicative of reactive astrogliosis. Future experiments with conditional knockout or transgenic mice might help clarify the roles and mechanisms of action of different glial populations in the regenerative process.

Our findings add to a growing body of literature that attests to the heterogeneity and functional subspecialization of circulating and tissue-infiltrating neutrophils. In particular, zymosan-elicted neuroregenerative neutrophils are reminiscent of recently described subpopulations of neutrophils that bear the signatures of an early developmental stage (based on cell surface phenotype and nuclear morphology), as well as alternative activation (based on expression of markers associated with M2-like macrophages), and play immunoregulatory and/or reparative roles in murine models of cancer, chronic infection and myocardial ischemia\(^3\)\(^{}\)–\(^{}\)\(^{}\)\(^{}\)\(^{}\)\(^{}\)\(^{}\)–\(^{}\)\(^{}\)\(^{}\)\(^{}\)\(^{}\)\(^{}\)\(^{}\). Alternatively activated neutrophils have been detected in ischemic brain tissue...
in rodent models of stroke, and their frequency correlates with increased neuronal survival, reduced infarct size and enhanced clinical recovery\(^\text{35,36}\). The extent to which the alternatively activated neutrophils characterized in these diverse models are biologically or developmentally related to one another, or share common mechanisms of action, remains to be determined. The existence of human neuroregenerative neutrophils is supported by our finding that HL-60 cells, widely used as a surrogate for immature human neutrophils, have axonogenic properties comparable to zymosan-elicited CD14\(^+\)Ly6G\(^–\) murine neutrophils. HL-60-mediated neurite outgrowth is also partially dependent on NGF but not IGF-1 (data not shown). However, HL-60 is a transformed myeloid leukemic cell line, as opposed to a subset of naturally occurring, primary neutrophils. An atypical subpopulation of Arg1\(^+\) primary human neutrophils, isolated in the low-density mononuclear layer of Ficoll gradients, has been described in the setting of advanced-stage cancer, HIV infection, pregnancy and systemic lupus erythematosus\(^\text{37-40}\). Although heterogeneous, the majority of these low-density neutrophils are immature on the basis of nuclear morphology (banded) and cell surface phenotype (CD33\(^+\)CD10\(^–\)CD16\(^–\)). In a number of studies, low-density human neutrophils were shown to possess immunosuppressive properties\(^\text{41-44}\). It is unknown whether they also possess reparative properties and/or they mobilize to sites of CNS injury.

ONC injury, in and of itself, induces the upregulation of dectin-1 on retinal microglia and DCs\(^\text{45}\). We hypothesize that engagement of dectin-1 on resident myeloid cells triggers the generation of a local inflammatory milieu conducive to the recruitment and polarization of neuroregenerative Ly6G\(^–\) neutrophils. Granulocyte colony stimulating factor levels rise in the vitreous fluid and the serum following i.o. zymosan injection, in conjunction with the mobilization of CD101\(^+\)CD14\(^+\)Ly6G\(^–\) neutrophils, which bear ring-shaped nuclei, into the bloodstream (data not shown). These circulating Ly6G\(^–\) cells do not express Arg1 or other markers of alternative activation, suggesting that they have not acquired neuroprotective or growth-promoting properties in the periphery (data not shown). Hence, it is likely that Ly6G\(^–\) neutrophils are polarized toward a neuroregenerative phenotype only after they cross the blood–eye barrier. Zymosan-elicited Ly6G\(^–\) neutrophils express low levels of CXCR2 and infiltrate the vitreous fluid by a CXCR2-independent pathway. We are currently interrogating candidate chemokine receptors, other than ELR\(^+\)CXCL\(^+\) chemokines, that might orchestrate their migration to the posterior chamber of the eye, as well as candidate

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**Fig. 7 | Human cell line–derived immature neutrophils are neuroregenerative.**

**a.** NGF protein levels were measured in CM of HL-60 and DG-75 cells by ELSA; Arg1 and NGF mRNA levels were measured in HL-60 and DG-75 cells by qPCR and normalized to Actb; data are representative of four independent experiments. Statistical significance was determined by a two-tailed unpaired Student’s t-test. **b.** Human HL-60 or DG-75 cells or murine 3 d Nφ were adoptively transferred into the vitreous fluid of C57BL/6 RAG1\(^–/–\) mice on days 0 and 3 after ONC injury. Optic nerves were harvested 14 d later. Bar graph shows the density of regenerating axons in optic nerve sections at serial distances from the crush site (n = 8 nerves per group). Data are from one experiment representative of two independent experiments. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (**P < 0.01, ***P < 0.001 and ****P < 0.0001, compared with DG-75). **c.** Primary RGCs were cultured with unconditioned medium (no tx) or HL-60 CM in the presence of either isotype control, anti-NGF antibodies or heat-shocked HL-60 CM. Neurite length was measured 24 h later (n = 200 RGCs per condition). One of two independent experiments is shown. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (**P < 0.01, ***P < 0.001 and ****P < 0.0001, compared with DG-75 CM). **d.** Primary human cortical neurons were cultured with unconditioned medium alone (no tx), NGF (positive control), HL-60 cells or DG-75 cells. Neurite length was measured 24 h later (n = 1,000 neurons per condition). One of two independent experiments shown. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (**P < 0.001, compared with no tx; ****P < 0.0001, compared with DG-75 cells). a-d. Data are shown as the mean ± s.e.m.
polarizing factors that might drive their differentiation locally. We are hopeful that this research will inform the development of protocols for the generation of neuroregenerative neutrophils from bone marrow precursors ex vivo, with the ultimate goal of reinfusing the polarized neutrophils into individuals with axonal injury, as an autologous cellular therapy. Alternatively, selected mobilizing and polarizing factors could be administered systemically, or at sites of CNS injury, to expand endogenous populations of alternatively activated neutrophils. Finally, identification of CD14+Ly6G– neutrophil chemoattractants may lead to the development of strategies that promote the migration of endogenous, as well as adoptively transferred, reparative neutrophils to sites of CNS injury while blocking the migration of potentially toxic mature neutrophils or other pathogenic leukocytes. In previous studies of the ONC model, multimodal therapeutic approaches were more effective than single agents, in some cases achieving RGC axon regeneration across the optic chiasm (such as i.o. zymosan injection combined with a CAMP analog and Pten deletion)49. The distinctive mechanism of action of the Ly6G– neuroregenerative neutrophil subset makes it an attractive candidate for multimodal therapy, in synergy with agents that block cell-intrinsic or cell-extrinsic suppressors of axon growth, to rescue dying neurons and enhance axonal regeneration after CNS injury.

Online content

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Methods

Mice. C57BL/6 WT, Ccr2−/− (B6.129S4-Ccr2tm1Brj/J), Clec7a−/− (B6.129S6-Clec7atm1Brj/J), Tbr2−/− (B6.129-Thr2tm1Brj/J) and RAG−/− (B6.129S7-Rag1tm1Sor/J) mice were purchased from the Jackson Laboratory. Dectin-1–TLR2 double-deficient (Clec7a−/−, Tbr2−/−) mice were bred and maintained in-house. Mice were group housed with a 12-h light/dark cycle and ad libitum access to food and water. All animal handling and surgical procedures were performed in compliance with national guidelines and approved by the University of Michigan and the Ohio State University Committees on Use and Care of Animals.

Optic nerve crush surgery and intraocular zymosan injection. Adult male or female mice (age 8–10 weeks) were anesthetized with 100 mg per kg body weight (mg kg−1) ketamine and 10 mg kg−1 xylazine i.p. The optic nerve was exposed through an incision in the conjunctiva while being visualized under a Nikon microscope. The nerve was then compressed, ∼1–2 mm behind the eye, for 5 s using a curved forceps (Dumont no. 5; Roboz). Immediately after ONC injury, the posterior chamber of the eye was injected with 3 μl of zymosan (12.5 μg μl−1 in PBS) or PBS alone, using a Hamilton syringe with a 30-gauge removable needle. The eyes were then rinsed with a few drops of sterile PBS. Ophthalmic ointment (Puralube; Fera Pharmaceuticals) was applied on the operated eye. For all surgical procedures, buprenorphine (0.1 mg kg−1, subcutaneous) was given before and after surgery (12, 24, 36, and 48 h). All operated mice were closely monitored until the endpoint. Two weeks after surgery, mice were given a lethal dose of ketamine/xylazine i.p. and then perfused transcardially with PBS for 5 min. Mice were inspected for lens injury after each i.o. injection and those with cataracts were eliminated.

Administration of antisera. Mice were injected, i.p., every 2 d with 500 μl of rabbit polyclonal antiCXR2 neutralizing sera (Cocalico) or control sera (NRS; Sigma), as previously described.

Intraocular levage and neutrophil isolation. Mice were injected i.p. with 500 μl of zymosan (2 μg μl−1 in PBS) and euthanized either 4 h or 3 d later via CO2 fixation. Next, 10 ml of sterile ice-cold PBS was injected into the peritoneal cavity and aspirated after 5 min. Neutrophils were isolated from lavaged cells with magnetic-activated cell sorting (MACS) Ly6G magnetic beads (Miltenyi Biotec) following the manufacturer’s instructions. Purity (95–99%) was confirmed using flow cytometry.

Intraocular adoptive transfer. Purified Ly6G+ cells were resuspended in sterile PBS at a concentration of 1×10^6 cells μl−1 and loaded into a Hamilton syringe with a 30-gauge needle. In some cases, anti-NGF (Alomone) and/or anti-IGF-1 (Sigma) antibodies (goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594) in blocking solution (PBS 5% goat serum) overnight at 4 °C were added to the adoptive transfer mixture. Recombinant mouse NFG (R&D) and/or IGF-1 (Peprotech) were reconstituted in sterile PBS at a concentration of 1 μg μl−1. Recombinant growth factors or PBS alone were injected into the posterior chamber of the eye (1–2 μl per eye) of anesthetized mice immediately following ONC injury and on post-op day 3.

Dorsal spinal cord injury. Male mice were anesthetized with 100 mg kg−1 ketamine and 10 mg kg−1 xylazine i.p. The T8 lamina were removed under a stereomicroscope using micro-rongeurs. The spinal column was exposed, and Roboz McPherson-Vannas Micro Dissecting Spring scissors were inserted 1 mm deep. A hemisection of the dorsal spinal cord was performed to transect axons in the dorsal columns. Muscle layers were closed with Perma-Hand Black sutures (5–0, Ethicon) and skin incisions were closed with coated Vycryl sutures (5–0, Ethicon). Six weeks after SCI, rhodamine-conjugated dextran MW 3,000 (microruby, Life Technology; 1.5 μl in 1 ml of 0.05% trypsin (Gibco). Retinas were triturated 20x with a fire polished glass pipette, incubated in a water bath at 37 °C for 5 min, after which trituration was repeated. Trypsin was quenched by adding 10 ml of 10% PBS in PBS. Tubes were centrifuged at 1,500 r.p.m. for 5 min. Supernatant was removed. Cells were suspended in MACS buffer (2% BSA and 0.004% EDTA in PBS). RGCs were isolated with MACS Thy1.2 magnetic beads (Miltenyi Biotec). Purified RGCs were suspended in Neurobasal medium (Gibco) supplemented with B-27 (Gibco), glutamine (2 mM; Gibco) and penicillin–streptomycin (100 units per ml; Gibco) and plated at a concentration of 5×10^4 per well on laminin/poly-1-lysine-coated 24-well plates. DRG neurons were isolated at 37 °C and 5% CO2. DRG neurons were counted, resuspended in DMEM/F12 (Gibco) with PBS (10%; Atlanta Biologicals), glutamine (2 mM; Gibco), and penicillin–streptomycin (100 units per ml; Gibco) and were plated at a concentration of 5×10^5 per well on laminin/poly-1-lysine-coated 24-well plates. DRG neurons were isolated at 37 °C and 5% CO2. When plated with the absence of L6G+ MACS bead-purified neutrophils (1×10^5 cells per well) or NCM. After 20 h, cultures were fixed with 4% formaldehyde (PFA) in PBS for 30 min before immunohistochemical staining and analysis.

Intraperitoneal levage and neutrophil isolation. Mice were injected i.p. with 500 μl of zymosan (2 μg μl−1 in PBS) and euthanized either 4 h or 3 d later via CO2 fixation. Next, 10 ml of sterile ice-cold PBS was injected into the peritoneal cavity and aspirated after 5 min. Neutrophils were isolated from lavaged cells with magnetic-activated cell sorting (MACS) Ly6G magnetic beads (Miltenyi Biotec) following the manufacturer’s instructions. Purity (95–99%) was confirmed using flow cytometry.

Primary human cortical neuron in vitro culture. Primary human cortical neurons (Sciencell) were thawed, counted, diluted in neutral medium with neurogrowth supplement (NGS) (Sciencell) and plated at 1.2×10^5 cells per well in a 96-well plate precoated with poly-1-lysine (Sigma) and laminin (Millipore). Neurons were either cultured alone or in the presence of HL-60 cells, DG-75 cells (1×10^5 cells per well) or recombinant human NGF (10 μg μl−1) at 37 °C and 5% CO2. After 20 h, neurons were fixed with ice-cold 4% PFA in PBS for 30 min before immunohistochemical staining and imaging.

Immunohistochemistry and quantification of regenerating axons, viable RGCs and neurite length. Optic nerves. Mice were perfused, optic nerves and eyes were dissected and post-fixed overnight in 4% PFA at 4 °C. Nerves were transferred to a 30% sucrose/PBS solution and maintained at 4 °C for at least 2 h and up to 2 weeks. Optic nerves were imbedded in Tissue-Tek OCT compound (Sakura Finteck) and stored at −80 °C. Longitudinal sections (10-μm thick) were cut on a cryostat, mounted on Superfrost Plus microscope slides (Fisher Scientific) and stained with polyclonal rabbit anti-GAP-43 (Abcam). Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (Invitrogen) was used for fluorescence labeling. Images were acquired using an Olympus IX71 inverted microscope with Olympus Cellsens Dimension software, attached to an Olympus IX71 digital camera. Regenerating GAP-43+ axons were counted at each 0.2-mm interval past the injury site, up to 1.6 mm, using a superimposed grid (3×5 sections per neuron). The number of labeled axons per section was normalized to the width of the section and converted to the total number of regenerating axons per optic nerve, as described previously.

Retinas. For cross-sectional images, eyes were dissected, post-fixed as described above and cryoprotected. Sections (25-μm) were cut with a cryostat and mounted on Superfrost Plus microscope slides, rinsed with PBS, blocked with 5% normal goat serum in PBS with 0.25% Triton X-100 (PBS-T) at 25 °C and incubated with primary antibodies (anti-GAP-43 at 1:1,500 dilution, Sigma; anti-Iba-1 at 1:300, Invivogen) diluted in blocking solution (PBS-T 4% goat serum) overnight at 4 °C. The next day, sections were washed with PBS-T and incubated with secondary antibodies (goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594) in blocking solution (PBS-T 3% goat serum) for 2 h at 25 °C. DAPI (300 nM) was used to stain nuclear counterstain.

For whole-mount analysis, PFA-fixed retinas were placed in a 35-mm dish (Corning) with sterile PBS. The retinal neural layer was dissected out of the globe under a dissecting microscope. Retinas were incubated with goat anti-mouse Bm3a (Santa Cruz) for 3 d, followed by Alexa Fluor 488-conjugated donkey anti-goat secondary antibody (Invitrogen). Retinas were washed, placed on Superfrost Plus microscope slides and imaged. Images were acquired using an Olympus IX71 inverted microscope attached to an Olympus DP72 digital camera. Bm3a+ RGCs were counted over eight fields distributed between four quadrants per retina at prespecified distances from the optic disc using Image J software.

Spinal cords. Dissected spinal cords were post-fixed overnight and transferred to 30% sucrose in solution for cryoprotection. Spinal cords were cut into 25-μm thick sagittal sections using a cryostat and processed for immunostaining. Briefly, spinal cord tissue sections were rinsed with PBS, blocked with 5% normal donkey serum in PBS-T at 25 °C and incubated with primary antibodies (anti-GFAP and anti-laminin)
Primary murine DRG, murine RGC and human cortical neurons. Plates were washed in 0.1% PBS-T, then blocked with 10% goat serum in PBS-T for 1 h at 25°C. After washing twice in PBS-T, samples were incubated with anti-JIL1 tubulin (TU1, Promega) in 3% BSA in PBS-T overnight at 4°C. Following two PBS-T washes, the samples were incubated with an Alexa Fluor 488-conjugated secondary antibody (Invitrogen) for 1–2 h at 25°C. Samples were washed twice with PBS and left in a solution of PBS and DAPI (NucBlue Fixed Cell ReadyProbes Reagent, two drops per ml PBS; Thermo Fisher Scientific). RGCs and human cortical neurons were imaged at 20X and DRG neurons at 10X, using an Olympus IX71 inverted microscope attached to an Olympus DP72 digital camera. Images were analyzed with Neumath (Weizmann Institute of Science). The longest neurite of each neuron was measured in GraphPad (Prism).

Neutrophil conditioned medium. MACs-purified neutrophils were placed into the RGC base medium (Neurobasal medium with B-27, penicillin–streptomycin and 2 mM l-glutamine) at a concentration of 5 × 10^5 cells/ml and incubated at 37°C and 5% CO_2 for 24 h. Supernatant was collected and spun at 8,000 rpm.

Cell morphology. Purified neutrophils were subjected to cytospin for 5 min at 5,500 × 10^3 r.p.m. Slides were air-dried, stained with Wright-Giemsa solution (Thermo Fisher Scientific) and imaged using an Olympus IX71 inverted microscope.

Flow cytometry. Flow cytometric analysis was performed as previously described.34 Mice were euthanized by isoflurane overdose. Blood was collected from the left ventricle in an EDTA tube. Peripheral blood mononuclear cells were isolated over a lymphocyte gradient (Cedarlane), and incidental red blood cells were lysed using an Ammonium-Chloride-Potassium Lysing Buffer (Quality Biological). Eyes were dissected, rinsed in PBS and placed in a 35-mm dish (Corning). Each eye was opened, vitreous fluid was collected and retinas were rinsed with 200 μl of PBS. Cells in the vitreous fluid and retinal rinse were combined. Cells were labeled with fixable viability dye (eFluor 506 or eFluor 780; eBioscience), blocked with anti-CD16/32 (clone 2.4G2) and stained with fluorochrome-conjugated antibodies specific for CD11b (clone M1/70), CD45 (30-F11), CD11c (N418), CD3 (145–2C11), CD4 (GK1.5), CD8 (53–6.7), CD19 (1D3), CD14 (61D3), Ly6B (7/4), CD101 (polyclonal) and F4/80 (BM8), all purchased from eBioscience; and Ly6C (AL-21) and Ly6G (1A8), purchased from Pharmingen. For intracellular staining, cells were fixed with 4% PFA, permeabilized with 0.5% saponin and stained with fluorescent antibodies specific for arginase-1 (polyclonal; Abcam), myeloperoxidase (polyclonal; Abcam) and 2 mM l-glutamine to process Chromium single-cell 3′ RNA-seq data. Once the gene-by-cell data matrix was generated, poor quality cells were excluded, such as cells with less than 500 unique genes or more than 7,000 unique genes expressed. Only genes expressed in three or more cells were used for further analysis. Cells were also discarded if their mitochondrial gene percentages were over 20%. The data were natural log transformed and normalized for scaling the sequencing depth to a total of 1 × 10^6 molecules per cell, followed by regressing out the number of UMIs using Seurat package (version 2.3.3).

Seurat downstream-analysis steps included SCTransform, dimensionality reduction (principal-component analysis) and UMAP, standard unsupervised clustering and the discovery of differentially expressed cell-type-specific markers. The SCTransform functionality embedded in Seurat R package include normalization and scaling of UMIs, batch information and mitochondrial content. We included the batch information as a variable in the SCTransform to scale out the differences between the batches. For the unsupervised clustering, we chose a low-resolution parameter (0.1). Differential gene expression analyses, to identify cell-type-specific genes, were performed using the non-parametric Wilcoxon rank-sum test. To compare the average gene expression of genes in the cell types identified in the two single-cell datasets, heat maps using Pearson's correlation values were generated.

Statistical analysis. RGC frequency and axon density data were analyzed by one-way ANOVA followed by Tukey's post hoc test using GraphPad 8.0 (Prism). The unpaired Student's t-test was used to analyze flow cytometry, ELISA and qPCR data with only two groups, and one-way ANOVA followed by Tukey's post hoc test was used to analyze flow cytometry, ELISA, RGC cultures and qPCR data with more than two groups.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Single-cell RNA-seq data are available in the Gene Expression Omnibus database (https://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE144637. Other data that support the findings of this study are available from the corresponding author on request.

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**Author contributions**

A.R.S., K.S.C., A.D.J., C.Y. and A.L.K. performed experiments and data analysis. R.M. oversaw RNA-seq analysis. B.M.S. wrote the manuscript and coedited it with the help of the other authors. B.M.S., R.J.G. and A.R.S. directed the studies.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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Extended Data Fig. 1 | Zymosan-induced RGC axon regeneration is independent of mature T and B cells. a, Gating scheme for analysis of intraocular infiltrates by flow cytometry. b, C57BL/6 WT or RAG1 deficient mice were injected i.o. with zymosan or PBS on the day of ONC injury. Optic nerves were harvested 14 days later. Longitudinal sections were stained with fluorochrome-conjugated anti-GAP-43 antibodies to enumerate the density of regenerating axons at serial distances from the crush site (n = 6 nerves/group). Data are shown as mean±SEM. One of two independent experiments with similar results is shown. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (P < 0.05, **P < 0.01, ***P < 0.001, compared with the PBS→WT group). c, Optic nerves were harvested on day 28 following i.o. injection of either PBS or zymosan. Mice received i.p. injections of either αCXCR2 antisera or control sera every other day from the day of ONC onward. The density of GAP-43+ regenerating axons was measured in optic nerve longitudinal sections at serial distances from the crush site (n = 10 nerves per group). Data are shown as mean±SEM; statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with the i.o. PBS/i.p. NRS group; *P < 0.05, **P < 0.01, ***P < 0.001, compared with the i.o. zymosan/i.p. NRS group).
Extended Data Fig. 2 | Immature neutrophils are mobilized into the circulation following treatment with i.o. zymosan and i.p. αCXCR2. Mice received an i.o. injection of zymosan on day 0, and i.p. injections of NRS (blue) or αCXCR2 (red) on days 0, 2 and 4, post ONC injury. Peripheral blood cells were obtained on day 5 and analyzed by flow cytometry. a, Cell surface expression of Ly6G, CD14 and CD101. Upper panels, representative histograms. Lower panels, geometric Mean Fluorescence Intensity on gated Ly6G+ cells and percentage of CD101+ neutrophils. Each symbol represents data from an individual mouse (n = 3 mice/group). Data are shown as mean ± sem. One experiment representative of 3 with similar results is shown. Statistical significance was determined by two tailed unpaired Student’s t-test. b, Representative dot plots.
Extended Data Fig. 3 | A population of alternatively activated, immature neutrophils is expanded in intraocular infiltrates following treatment with i.o. zymosan and i.p. αCXCR2. Single-cell analysis using 10X Genomics of intraocular Ly6G+ cells from the NRS (left panels) or αCXCR2 (right panels) treatment groups, as in Fig. 3. a, Violin plots showing the cells expressing Arg1, Mrc, HexB, Sgrn and Fpr1 in clusters 1 and 3 of the NRS and αCXCR2 treatment groups. b, Featureplots showing cluster-specific expression of Mrc (CD206, alternative activation marker), CXCR2 and S100a8 (maturation markers).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Adoptively transferred CD14$^+$Ly6G$^+$ cells induce RGC axon regeneration independent of TLR2 and dectin-1 or CCR2 signaling.

a, Mice were subjected to ONC injury on day 0 and received i.o. injections of either PBS, 4 h NΦ, or 3d NΦ, on days 0 and 3. Retina were harvested on day 14. The frequency of viable BRN3a$^+$ RGC neurons in whole mounts, normalized to healthy retina (n = 10 retina per group). One experiment representative of 2 is shown. Statistical significance determined by one-way ANOVA followed by Tukey’s post hoc test. b, Peritoneal Ly6G$^+$ cells were purified 3 days after i.p. zymosan injection (3d NΦ), and adoptively transferred into the eyes of naïve C57BL/6 WT or TLR2$^-$dectin-1$^-$ double knock-out (dko) mice on days 0 and 3 post ONC injury. For negative controls, additional groups were injected i.o. with PBS. Optic nerves were harvested 14 days later and analyzed by GAP-43 immunohistochemistry. The figure shows the density of regenerating axons, at serial distances from the crush site (n = 8 nerves per group). One of 2 independent experiments is shown. Statistical significance determined by one-way ANOVA followed by Tukey’s post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with PBS/WT; *P < 0.05, **P < 0.01, ***P < 0.001 compared with PBS/dKO). c, GFAP (green) and IBA1 (red) IHC of retinal cross-sections obtained 7 or 14 days following ONC and i.o injection of either 3d NΦ or PBS. Representative images shown (n = 3 mice, 1 of 3 independent experiments, scale bar 80 μm). d, eGFP labeled 3d NΦ were injected i.o. on the day of ONC injury. Representative microscopic image of retinal cross-section prepared 3 days later (n = 3 mice, 1 of 2 independent experiments scale bar 200 μm). e, Representative flow cytometric analysis of intraocular infiltrates harvested from WT or Ccr2$^{-/-}$ mice on day 3 post ONC injury and i.o. injection of 3d NΦ (n = 5 mice per group). f, 3d NΦ were adoptively transferred into the eyes of C57BL/6 WT or Ccr2$^{-/-}$ mice on days 0 and 3 post ONC injury. Axonal densities at serial distances from the crush site, on day 14 post ONC injury (n = 6 nerves, 1 of 2 independent experiments is shown). Statistical significance determined by one-way ANOVA followed by Tukey’s post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, compared with PBS/WT; *P < 0.05, **P < 0.01, ***P < 0.001 ****P < 0.0001, compared with PBS/ Ccr2$^{-/-}$). a, b, f Data are shown as mean± sem.
Extended Data Fig. 5 | Pro-regenerative neutrophils retain therapeutic efficacy when administered following CNS injury. a, 3d NΦ were adoptively transferred into the eyes of mice on the day of ONC injury, or after a delay of 6, 12, or 24 hrs. NΦ adoptive transfer was repeated 3 days later. A control group was injected i.o. with PBS alone on days 0 and 3. Optic nerves were harvested on day 14 for quantification of axonal densities by GAP-43 IHC (n = 8 nerves per group). *P < 0.05; **P < 0.01; ***P < 0.001 compared with PBS). b, 4 h or 3 d NΦ were added to primary RGC cultures 4 hrs after RGC plating. In other wells, RGC were cultured in media alone (No T x), or in the presence of recombinant CNTF, as negative and positive controls, respectively. Neurite outgrowth was measured 24 hours later (n = 2000 RGCs per condition, one of two independent experiments shown). Statistical significance determined by one-way ANOVA followed by Tukey’s post hoc test. c, 4 h or 3 d NΦ were added to primary DRG cultures 8hrs after DRG plating. In other wells, DRG were cultured in media alone (No T x), or in the presence of recombinant NGF, for negative and positive controls, respectively. Neurite outgrowth was measured 24 hours later (n = 300 DRGs per condition, one of two independent experiments shown). Statistical significance determined by one-way ANOVA followed by Tukey’s post hoc test. a–c, Data shown as mean ± sem.
Extended Data Fig. 6 | NGF and IGF-1 drive RGC axon regeneration in a collaborative manner. a, Quantification of a panel growth factors in unconditioned media (circles) and NCM (squares) by multiplexed antibody array. b, Primary RGC were cultured in the absence or presence of recombinant mouse CNTF, IGF-1, NGF, or a combination of IGF-1 and NGF. Neurite length was measured 24 hours later. Each symbol represents the mean of 200 RGCs in one independent experiment (n = 6 independent experiments shown). Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (**P < 0.01, ***P < 0.001 compared with No Tx; *P < 0.05 compared with NGF; ++P < 0.01, compared with IGF-1). c, Recombinant IGF-1 (blue bars), NGF (green), a combination of NGF and IGF1 (white), or PBS alone (black) was injected into the vitreous on days 0 and 3 post ONC injury. Optic nerves were harvested 14 days later. Density of regenerating axons in optic nerve sections, at serial distances from the crush site (n = 8 nerves per group). One experiment representative of 2 with similar results is shown. Statistical significance was determined by one-way ANOVA followed by Tukey’s post hoc test (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with PBS; *P < 0.05 compared with NGF; *P < 0.05, **P < 0.01, compared with IGF-1). b,c, Data shown as mean ± sem.
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- A description of all covariates tested
- A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  Give P values as exact values whenever possible.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Data collection
FACS DIVA v8.0.1 was used to collect flow cytometry data. Olympus CellSens Dimension v2.0 was used for imaging on the Olympus IX-71 microscope. Ly6G+ vitreal cells were purified with MACS beads and purity (>98%) and viability (99%) were confirmed by flow cytometric analysis. The samples were then submitted to the University of Michigan Advanced Genomics Core facility.

Data analysis
Flow cytometry was analyzed using Flowjo v10. RT-PCR was analyzed by Quant Studio Design and analysis software v1.3. Neurite length was quantified with Neuromath v3.4.8. Statistical analysis was completed using Prism GraphPad v8, and single cell RNAseq was analyzed with Cell Ranger v1.3 and the Seurat R Package v 2.3.3

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Mouse neutrophil single cell RNA seq raw data will be available in the Gene Expression Omnibus (GEO) with accession number GSE144637.
Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences  - [ ] Behavioural & social sciences  - [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nm-reports-summary-list.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size: Sample sizes were chosen based on our previous publication (Baldwin, K. T., Carbajal, K. S., Segal, B. M. & Giger, R. J. Neuroinflammation triggered by beta-glucan/dectin-1 signaling enables CNS axon regeneration. Proc Natl Acad Sci USA 112, 2581-2586, doi:10.1073/pnas.1423221112 (2015)) as well as published studies using the same experimental approaches (Neumann, S. & Woolf, C. J. Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. Neuron 23, 83-91 (1999); Lorber, B., Berry, M., Douglas, M. R., Nakazawa, T. & Logan, A. Activated retinal glia promote neurite outgrowth of retinal ganglion cells via apolipoprotein E. J Neurosci Res 87, 2645-2652, doi:10.1002/jnr.22095 (2009); Stoolman, J. S., Duncker, P. C., Huber, A. K. & Segal, B. M. Site-specific chemokine expression regulates central nervous system inflammation and determines clinical phenotype in autoimmune encephalomyelitis. J Immunol 193, 564-570, doi:10.4049/jimmunol.1400825 (2014); Ma, S. F. et al. Adaptive transfer of M2 macrophages promotes locomotor recovery in adult rats after spinal cord injury. Brain Behav Immun 45, 157-170, doi:10.1016/j.bbi.2014.11.007 (2015)).

Data exclusions: No data were excluded. No exclusion methods were used.

Replication: Experimental findings were consistently confirmed by at least 2 independent experiments.

Randomization: For each experiment, C57Bl/6 mice of the same sex and similar age, that were obtained from the same vendor, were randomly assigned to experimental groups prior to the initiation of each experiment. For cell line experiments, randomization was not relevant as all cells came from the same stock provided by the company, each experiment utilized cells from the same passage, and experimental conditions were applied uniformly to each cell line for comparison.

Blinding: Investigators who quantified axon regeneration and neurite length, and counted live retinal ganglion cells, were blinded to the experimental conditions and treatment groups. Blinding was not possible for flow cytometry analysis due to the necessity of compensating and gating against isotype matched controls. RNAseq analysis was conducted at a core facility with technicians blinded to experimental conditions and treatment groups, and the data were analyzed by a biostatistician blinded to the experimental groups.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

**Materials & experimental systems**

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | Antibodies |
| [x] | Eukaryotic cell lines |
| [ ] | Palaeontology |
| [ ] | Animals and other organisms |
| [x] | Human research participants |
| [ ] | Clinical data |

**Methods**

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | ChiP-seq |
| [ ] | Flow cytometry |
| [x] | MRI-based neuroimaging |

**Antibodies**

Antibodies used: All antibodies used were obtained from the commercial vendors listed. Antibody details, including conjugate, clone, and manufacturer are listed. All antibodies were validated by the manufacturer for the application and species used in the study. All antibodies were used at a dilution of 1:100 unless otherwise noted.

- Fixable Viability Dye - eFluor506 / eBioscience / 65-0866 (1:500)
- Fixable Viability Dye - eFluor780 / eBioscience / 65-0865 (1:500)
- CD16/32 / 2.4G2 / Mouse / eBioscience / 14-0161 (1:200)
- CD11b-PE-Cy7 / M1/70 / Mouse / eBioscience / 14-0112 (1:1000)
- CD11b-APC / M1/70 / Mouse / eBioscience / 14-0112 (1:1000)
- CD45-e450 / 30-F11 / Mouse / eBioscience / 12-0451
- CD11c-PE / N418 / Mouse / eBioscience / 12-0124
- CD3-HITC / 145-2C11 / Mouse / eBioscience / 16-0031
- CD4-PE / GK1.5 / Mouse / eBioscience / 11-0041
Validation

In preparation for experiments, each antibody was used to stain cells known to express the relevant marker, across a range of serial dilutions, starting at concentrations based on the recommendations of the manufacturer, and compared against isotype controls. We used the lowest dilution of antibody that provided reproducible staining (MFI) above background levels.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) Both the HL-60 and DG-75 human cell lines were purchased from ATCC. Human cortical neurons were purchased from Sciencell.

Authentication

The manufacturers provided detailed documentation regarding the authenticity of HL60 (ATCC) and DG75 (ATCC) human cell lines and human cortical neurons (Sciencell). For both HL60 and DG75 cells: [i] species was determined by COI assay (interspecies) and STR assay (intraspecies); [ii] sterility testing (BacT/ALERT 3D) via IAST bottle (aerobic) at 32.5°C and NIST bottle (anaerobic) showed no growth, and [iii] PCR-based assays for HIV, HepB, HPV, EBV, and CMV were negative (none detected). Furthermore, we performed qRT-PCR to measure abundance of mRNA encoding human beta-actin, G-CSF receptor, dectin-1, IL-4 receptor alpha chain, NGF and arginase-1 in the HL-60 and DG75 cell lines. For human cortical neurons: [i] morphology was neuronal; [ii] phenotype characterization by B- Tubulin III immunostaining was positive; [iii] HIV-1, HCB and HCV DNA were not detected. Bacteria and fungi were also not detected.

Mycoplasma contamination

For HL60 and DG75 cell lines: Mycoplasma detection was negative by Hoechst DNA stain (indirect), Agar culture (direct), and PCR assays. For human cortical neurons Mycobacteria DNA was not detected via PCR assay.

Commonly misidentified lines

No commonly misidentified cell lines were used in the study

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals All mice were on the C57Bl/6 background and housed under specific pathogen-free conditions. Mice were housed in ventilated cages in rooms maintained at 72 +/- 4 degrees Fahrenheit and at a humidity between 30-70%. Experiments were performed with 8-10 week old male or female mice. 7-8 day old pups from C57Bl/6 breeding pair were used to isolate retinal ganglion cells for primary neural cultures. All mouse strains used are listed in the Mice section of the methods.

Wild animals No wild animals were used in this study.

Field-collected samples No field collected samples were used in this study.

Ethics oversight All protocols were approved by the University of Michigan and Ohio State University Animal Care and Use Committees.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

- Confirm that:
  - The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
  - The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
  - All plots are contour plots with outliers or pseudocolor plots.
  - A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Mice were euthanized by isoflurane overdose or CO2 fixation. Blood was collected from the left ventricle in an EDTA tube. PBMC were isolated over a lympholyte gradient (Cedarlane labs), and incidental red blood cells were lysed using an Ammonium-Chloride-Potassium (ACK) Lysing Buffer (Quality Biological). Eyes were dissected, rinsed in PBS, and placed in a 35 mm dish (Corning). Each eye was opened, vitreous fluid was collected; retinas were rinsed with 200 ul of PBS. Cells in the vitreous fluid and retinal rinse were combined.
Intra-peritoneal cells were collected with a 10 ml of PBS lavage and aspirated after 5 minutes.

Instrument
- FACS Canto II flow cytometer (BD Biosciences)

Software
- FACS DIVA and FlowJo v10

Cell population abundance
Intra-ocular and intra-peritoneal neutrophils were isolated using MACS Ly6G beads (Miltenyi Biotec) per their protocol. Purity of the isolated cells and viability were confirmed via flow cytometry. Neutrophils were identified as CD45+ Ly6G+ CD11b+ and gated as described below and illustrated in extended figure 1. Purity of neutrophils used in experiments was 95-98% based on flow cytometry analysis, and viability was consistently >98%.

Gating strategy
Gating strategy is described as follows and outlined in extended figure 1A. FSC-A/SSC-A was used for gating mononuclear cells. SSC-W/SSC-H was used for gating on singlets. Viability dye negative cells were gated on to exclude dying cells. Hematopoietic cells were identified as CD45+. Neutrophils were identified as Ly6G+ CD11b+ CD45+. Monocytes CD11b+ CD45+ Ly6G+. Dendritic cells CD11c+ CD45+ CD11b+, T cells CD3+ CD45+ CD11b+, B cells CD19+, CD45+ CD11b-

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.