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Monocyte-derived SDF1 supports optic nerve regeneration and alters retinal ganglion cells' response to Pten deletion

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Although mammalian retinal ganglion cells (RGCs) normally cannot regenerate axons nor survive after optic nerve injury, this failure is partially reversed by inducing sterile inflammation in the eye. Infiltrative myeloid cells express the axogenic protein oncomodulin (Ocm) but additional, as-yet-unidentified, factors are also required. We show here that infiltrative macrophages express stromal cell–derived factor 1 (SDF1, CXCL12), which plays a central role in this regard. Among many growth factors tested in culture, only SDF1 enhances Ocm activity, an effect mediated through intracellular cyclic AMP (cAMP) elevation and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) activation. SDF1 deficiency in myeloid cells (*CXCL12*$^{flx/flx}$*LysM-Cre*$^{-/+}$ mice) or deletion of the SDF1 receptor CXCR4 in RGCs (intraocular AAV2-Cre in *CXCR4*$^{flx/flx}$ mice) or SDF1 antagonist AMD3100 greatly suppresses inflammation-induced regeneration and decreases RGC survival to baseline levels. Conversely, SDF1 induces optic nerve regeneration and RGC survival, and, when combined with Ocm/cAMP, SDF1 increases axon regeneration to levels similar to those induced by intraocular inflammation. In contrast to deletion of phosphatase and tensin homolog (Pten), which promotes regeneration selectively from αRGCs, SDF1 promotes regeneration from non-αRGCs and enables the latter cells to respond robustly to Pten deletion; however, SDF1 surprisingly diminishes the response of αRGCs to Pten deletion. When combined with inflammation and Pten deletion, SDF1 enables many RGCs to regenerate axons the entire length of the optic nerve. Thus, SDF1 complements the effects of Ocm in mediating inflammation-induced regeneration and enables different RGC subtypes to respond to Pten deletion.

optic nerve regeneration | inflammation | macrophages | RGC subtypes | SDF1

Significance

The optic nerve conveys information from retinal ganglion cells (RGCs) to visual processing areas of the brain. Although this pathway normally cannot regenerate when injured nor in degenerative diseases such as glaucoma, this failure can be partially reversed by eliciting a controlled immune reaction in the eye. We show here that the chemokine SDF1 (stromal cell–derived factor 1) is an important contributor to this phenomenon. SDF1 is produced by infiltrative monocytes and acts through its cognate receptor to enhance RGC survival, promote optic nerve regeneration, and sensitize subtypes of RGCs that normally fail to respond to a complementary treatment to exhibit robust, long-distance regeneration. These findings establish SDF1 as an important therapeutic candidate for repairing the injured optic nerve.

Inflammation plays a critical role in peripheral nerve regeneration (1) and, within the central nervous system (CNS), partially enables peripheral sensory neurons to regenerate axons in the spinal cord (2–4) and retinal ganglion cells (RGCs) to regenerate axons through the optic nerve (5–8). The 12-kDa neutrophil-derived protein oncomodulin (Ocm) is important in this regard, but the ability of Ocm to stimulate regeneration requires additional factors to elevate intracellular cyclic AMP (cAMP) and to enhance RGC survival (9, 10). Identification of these factors could enable us to mimic or exceed the beneficial effects of inflammation in a clinically relevant manner.

Several growth factors have been reported to promote regeneration and/or RGC survival after optic nerve injury (8, 11–16), as do manipulations of cell-intrinsic and cell-extrinsic suppressors of axon growth (17–25), suppression of A1 astrocyte polarization (26), physiological activity (27, 28), and chelating free zinc (29, 30). Nonetheless, the extent of central reinnervation achieved to date remains modest (27, 31–33), underlining the need to identify ways to enhance regeneration beyond current levels.

The chemokine stromal cell–derived factor 1 (SDF1, CXCL12) is involved in retinal development and can be expressed in multiple cell types. Based on our early screen and other studies showing that SDF1 stimulates axon outgrowth from RGCs (13, 14, 34), we investigated the possible involvement of SDF1 in inflammation-induced optic nerve regeneration, its downstream signaling pathways, the RGC subtypes that it targets, and synergy with other treatments. We report that SDF1 is highly expressed in activated macrophages and that, among many growth factors tested in cell culture, SDF1 alone augments the effects of Ocm. SDF1 deficiency in inflammatory cells or loss of its receptor CXCR4 in RGCs suppresses inflammation-induced RGC survival and axon regeneration after optic nerve injury, whereas exogenous SDF1 combined with Ocm/cAMP fully mimics the effects of intraocular inflammation in enhancing RGC survival and optic nerve regeneration. SDF1 induces regeneration from RGC subtypes other than αRGCs, the primary subtype that responds to deletion of phosphatase and

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tensin homolog (Pten), and enables non-αRGCs to respond robustly to Pten deletion. Finally, combining SDF1 with intraocular inflammation, cAMP elevation, and Pten deletion augments the number of RGCs that regenerate axons the full length of the optic nerve.

Results

SDF1 Is Expressed in Infiltrative Macrophages. Intraocular injection of zymosan stimulates neutrophil infiltration that peaks at 12 to 24 h and a slower infiltration of macrophages that peaks at 4 to 7 d (5, 6, 10). Infiltrative cells express a multiplicity of factors that can be detrimental or beneficial to RGCs, among which Ocm has been identified as an important contributor to axon regeneration (3, 6, 9, 10, 35). In light of our preliminary data showing that SDF1 induces outgrowth in cell culture, and because SDF1 was reported elsewhere to be expressed in inflammatory cells (34), we investigated whether SDF1 might become elevated during intraocular inflammation and contribute to optic nerve regeneration. SDF1 was not detected in the vitreous or inner retina of normal mice nor in mice examined 1 or 4 d (D1, D4) after optic nerve crush (NC) alone (Fig. 1*A* and *SI Appendix*, Fig. S1). Intraocular zymosan resulted in strong SDF1 immunoreactivity in F4/80-positive cells that lie outside the ganglion cell layer (GCL) at D1 (Fig. 1*A*, red and *SI Appendix*, Fig. S1*B*). F4/80 is a marker for macrophages. No staining was seen in cells that stain for Gr1 or TUJ1, markers for neutrophils and βIII-tubulin–positive RGCs, respectively (Fig. 1*A*). SDF1 expression in macrophages is also apparent when infiltrative cells extracted from the vitreous are examined ex vivo. At D1 and D4, nearly every F4/80$^+$ cell in the vitreous expresses SDF1, which is concentrated in peripherally clustered vesicles, whereas Gr1$^+$ neutrophils show little or no SDF1 staining (Fig. 1 *B* and *C* and *SI Appendix*, Fig. S1*C*).

Four days after intraocular inflammation, F4/80$^+$/SDF1$^+$ cells appeared in the inner plexiform layer (IPL), which contains synaptic contacts from retinal interneurons onto RGC dendrites and onto other interneurons, radial extensions of Müller cells, and microglia, as well as in the outer plexiform layer (OPL) (36) (*SI Appendix*, Fig. S1). SDF1$^+$ cells in the IPL and OPL take on the appearance of activated microglia with enlarged somata, few thin extensions (cf Iba1$^+$ cells in normal controls or after injury alone), and positive staining for the microglial marker Iba1 and for CD68, a marker for active phagocytosis (37) (*SI Appendix*, Fig. S2). At D4, SDF1 immunoreactivity in Iba1$^+$ and/or F4/80$^+$ cells was somewhat lower than at D1 (Fig. 1*A* and *SI Appendix*, Figs. S1 and S2).

At the messenger RNA (mRNA) level (Fig. 1*D*), the eye as a whole showed a small, nonsignificant elevation of SDF1 mRNA following NC alone at D1 and D4 (dark bars), with no detectable changes in the neural retina (light gray bars). Intraocular zymosan increased SDF1 mRNA levels in the eye 22-fold compared with normal at D1 ($P < 0.001$) and 7-fold compared with normal at D4 ($P < 0.05$), with little change in the neural retina. These results imply that the zymosan-induced elevation of SDF1 seen in the whole eye reflects a source from infiltrative cells. The elevation of SDF1 detected by immunostaining in retinal microglia at D4 is not paralleled by measurable changes in retinal SDF1 mRNA levels, suggesting that those microglial cells might be infiltrative macrophages that migrate into the retina.

Unexpectedly, intraocular zymosan also elevated SDF1 levels systemically. Whereas SDF1 was not detected in either F4/80$^+$ macrophages nor Gr1$^+$ neutrophils isolated from normal blood, levels rose dramatically in F4/80$^+$ macrophages 1 d after intraocular zymosan (though not in neutrophils; *SI Appendix*, Fig. S3). While the basis for the observed systemic up-regulation of SDF1 in macrophages after intraocular zymosan application remains unclear, a similar phenomenon was previously observed for Ocm expression in systemic neutrophils after intraocular inflammation (10).

SDF1 Stimulates Axon Outgrowth in RGCs. We next characterized the effects of SDF1 on adult RGCs in culture. RGCs were retrogradely labeled by injecting Fluorogold (FG) into the superior colliculus and dissociated retinal cultures were prepared 1 wk later and maintained for 3 d (6, 9, 38). SDF1 induced a dose-dependent increase in RGC outgrowth (assessed as the percentage of RGCs extending axons ≥30 μm), with a 2.5-fold increase above baseline at the higher concentration ($P < 0.05$ for 12 nM, $P < 0.01$ for 127 nM SDF1; Fig. 2 *A* and *B*). The effects of SDF1 declined at higher concentrations, a result that may be linked to the formation of inactive dimers at high concentrations (39, 40). SDF1 did not affect cell survival in our cultures (*SI Appendix*, Fig. S4*A*), possibly due to the presence of other prosurvival factors in the media that could mask such effects (6).

Using immunopurified postnatal day 5 mouse RGCs, we investigated whether SDF1 acts directly on RGCs. Following 3 d in culture, SDF1 increased neurite outgrowth in RGCs at concentrations between 2 nM ($P < 0.05$) and 32 nM ($P < 0.001$) (*SI Appendix*, Fig. S5 *A* and *B*). Higher concentrations (127 nM and above) were ineffective, reminiscent of the inverted U dose–response curve we observed for mature rat RGCs in mixed cultures. Under the different culture conditions used to study neonate RGCs, SDF1 increased cell survival ($P < 0.001$ at 2 to 127 nM, $P < 0.05$ at 506 nM; *SI Appendix*, Fig. S5 *A* and *C*). Although immunopanned RGC cultures are not 100% pure, immunopurification is a well-established method to produce cultures in which >95% of cells are RGCs (17, 41). These results indicate that SDF1 most likely acts directly on RGCs, consistent with previous reports on the role of SDF1 in RGC survival and outgrowth during development (42, 43).

SDF1 Enhances the Effects of Ocm on Axon Outgrowth. We next examined the effects of combining SDF1 with Ocm and other agents. As reported (6, 9), D-mannose and cAMP elevation (using forskolin to activate adenylate cyclase) induced moderate outgrowth in cultured RGCs ($P < 0.001$), and this response was doubled by the addition of Ocm (*SI Appendix*, Fig. S6*A*). SDF1 combined with mannose produced similar levels of outgrowth as mannose plus forskolin, and SDF1 combined with mannose and forskolin increased regeneration ∼4-fold above baseline ($P < 0.001$), as was seen with Ocm (*SI Appendix*, Fig. S6*A*). The addition of SDF1 to Ocm/mannose/forskolin increased outgrowth above the level seen with the latter combination alone ($P < 0.001$; Fig. 2*C*). This latter effect was not duplicated by any other trophic factor tested (brain derived neurotrophic factor: BDNF; cilliary neurotrophic factor: CNTF; glial cell-derived neurotrophic factor: GDNF; leukemia inhibitory factor: LIF; transforming growth factor beta: TGFβ; epidermal growth factor: EGF; basic fibroblast growth factor: bFGF or FGF2; insulin like growth factor 1: IGF1; Fig. 2*C*). None of the combinations tested altered RGC survival (*SI Appendix*, Fig. S4 *B* and *C*).

Mechanisms of Action. SDF1 can act through two different receptors, CXCR4 and CXCR7 (44, 45). In mature rat retinal cultures, the axon-promoting effects of SDF1 were fully

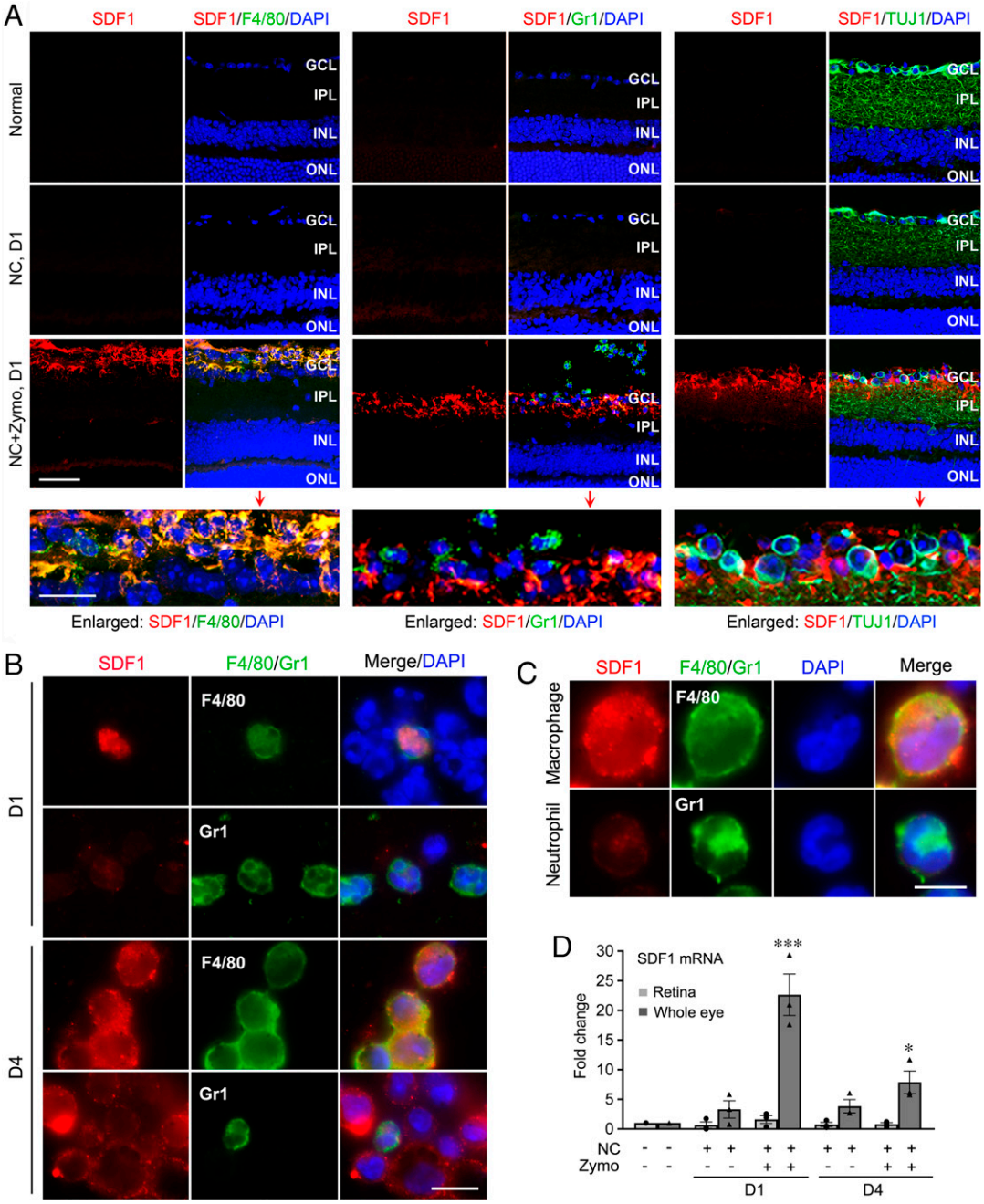

Fig. 1. Infiltrative macrophages express SDF1. (*A*) Retinal cross-sections from normal mouse and mice D1 after optic nerve crush alone (NC) or NC plus intraocular zymosan (NC + Zymo) costained for SDF1 (red) and either the macrophage marker F4/80, neutrophil marker Gr1, or RGC marker TUJ1 (for βIII-tubulin; green). DAPI is a nuclear marker (blue). Note the colocalization of SDF1 with F4/80$^+$ cells lying outside or invading the GCL but not with Gr1 or TUJ1. (*A, Bottom*) Enlarged images of SDF1 coimmunostained with the same three cell markers 1 d after NC + Zymo. INL, inner nuclear layer; ONL, outer nuclear layer. (*B*) Immunostained cells extracted from the vitreous D1 or D4 after intraocular zymosan show colocalization of SDF1 with F4/80 but not Gr1. (*C*) Enlarged images showing SDF1 immunostaining in vesicles within a mononuclear macrophage and weak staining in a polymorphonuclear neutrophil. (*D*) SDF1 mRNA distribution. Whole eyes (dark gray bars), which include infiltrative cells, show large increases in SDF1 mRNA D1 and D4 after zymosan injections whereas neural retina (light gray) shows little elevation. (Scale bars, 75 μm [*A*], 45 μm [*A, Bottom*], 20 μm [*B*], and 10 μm [*C*].) (*D*) One-way ANOVA plus Sidak correction; error bars in *D*: standard error of mean; $*P < 0.05$, $***P < 0.001$ (whole eye: NC + Zymo vs. NC alone).

suppressed by 100 μM AMD3100, a specific blocker of CXCR4 ($P < 0.001$; Fig. 2*D*), pointing to the latter being the relevant receptor in RGCs. AMD3100 also caused a nonsignificant trend toward diminished cell survival (*SI Appendix*, Fig. S4*D*). Based on studies showing that SDF1 increases cAMP levels in developing zebrafish RGCs by activating Ca^{2+}-calmodulin–dependent adenylate cyclase (42, 43, 46), we investigated whether cAMP elevation contributes to the effects of SDF1 in mature mammalian RGCs. In support of this hypothesis, outgrowth induced by SDF1 was partially suppressed by the cAMP antagonist Rp-cAMP ($P < 0.05$; Fig. 2*D*). SDF1 is also known to activate the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)–Akt and –Erk signaling pathways in neurons and in nonneuronal cells (47). The PI3K inhibitor LY294002 fully abrogated the neurite-promoting effects of SDF1 on RGCs, while the mitogen-activated protein kinase (MAPK) inhibitor PD98059 had no effect (Fig. 2*D*); neither inhibitor altered RGC survival (*SI Appendix*, Fig. S4*D*).

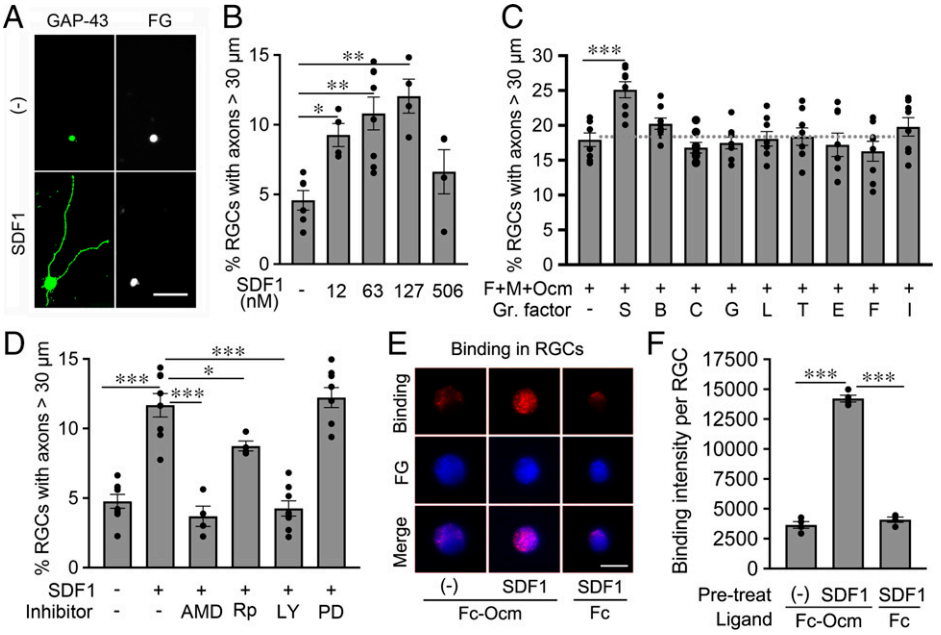

Fig. 2. SDF1 stimulates axon outgrowth in RGCs. Adult rat retinas were dissociated and maintained in defined, serum-free medium. RGCs are identified by virtue of being back-labeled with FG injected 2 wk earlier in the superior colliculus. Axon outgrowth was visualized 3 d later by GAP-43 immunostaining and quantified as the percentage of FG^+ cells with axons >30 µm in length. (*A* and *B*) SDF1 exerts maximal effects at 63 to 127 nM (*P < 0.05, **P < 0.01 compared with medium alone). (Scale bar, 30 µm.) (*C*) Effects of multiple growth factors (Gr. factor) on axon growth induced by F + M + Ocm (dotted line; B, BDNF; C, CNTF; E, EGF; F, FGF2; G, GDNF; I, IGF1; L, LIF; S, SDF1; T, TGFβ). Only SDF1 (S) shows positive effects (***P < 0.001). Concentrations are given in *SI Appendix, Methods*. (*D*) SDF1-induced outgrowth is fully blocked by CXCR4 antagonist AMD3100 (AMD; ***P < 0.001, SDF1 vs. SDF1 + AMD), partially blocked by protein kinase A (PKA) antagonist Rp-cAMPs (Rp; *P < 0.05), and completely blocked by PI3K inhibitor LY294002 (LY; ***P < 0.001), but is unaffected by MAPK inhibitor PD98059 (PD). (*E* and *F*) SDF1 enables Ocm to bind to its receptor. An Fc-Ocm fusion protein shows little binding to RGCs in culture unless cells are pretreated with SDF1. SDF1 pretreatment does not enable Fc alone to bind. ***P < 0.001, SDF1 + Fc-Ocm vs. Fc-Ocm, or vs. SDF1 + Fc. (Scale bar, 20 µm.) One-way ANOVA plus Dunnett correction (*B–D*) and Tukey correction (*F*). Error bars in *B–D*, and *F*: standard error of mean.

Elevation of intracellular cAMP ($[cAMP]_i$) enables Ocm to bind to its cognate high-affinity receptor on RGCs (9, 48). In conformity with our finding that SDF1 elevates $[cAMP]_i$, pretreatment of mature rat RGCs with SDF1 increased the binding of an Fc-Ocm fusion protein (Fc-Ocm) to RGCs about fourfold relative to controls without SDF1 pretreatment or relative to RGCs with SDF1 pretreatment but exposed to Fc alone (P < 0.001; Fig. 2 *E* and *F*).

To examine whether the ability of SDF1 to enhance the effects of Ocm depends on mechanisms beyond $[cAMP]_i$ elevation, we tested whether the benefits of SDF1 are still seen in the presence of saturating concentrations of CPT-cAMP (a nonhydrolyzable, cell-permeable cAMP analog; *SI Appendix*, Fig. S6*B*). SDF1 strongly increased the effects of Ocm/mannose/cAMP at the lowest concentration of CPT-cAMP tested (12.5 µM) and continued to show added benefits even when the effects of CPT-cAMP reached plateau levels (at ∼50 µM; *SI Appendix*, Fig. S6*B*). These results indicate that SDF1 exerts its effects on RGCs through mechanisms beyond cAMP elevation. As above, the effects of SDF1 on axon growth in culture were independent of RGC survival (*SI Appendix*, Fig. S4*E*).

SDF1 Contributes to Inflammation-Induced Regeneration In Vivo: Loss-of-Function Experiments. To investigate the role of SDF1 in vivo, we crossed mice with a conditional deletion of the gene encoding SDF1 ($CXCL12^{flx/flx}$) with mice expressing Cre recombinase under control of the myeloid cell–specific LysM promoter ($LysM\text{-}Cre^{-/+}$), yielding offspring that fail to express SDF1 in myeloid cells. One day after nerve injury and intraocular zymosan, levels of SDF1 mRNA in whole eyes of $CXCL12^{flx/flx}LysM\text{-}Cre^{-/+}$ (conditional knockout; cKO) mice were ∼96% lower than in similarly treated wild-type (WT) littermates (P < 0.001; *SI Appendix*, Fig. S7*A*). By immunostaining, experimental mice showed a complete loss of SDF1 in the inner retina (*SI Appendix*, Figs. S7*B* and S8*B*, D1) and in blood macrophages (*SI Appendix*, Fig. S8*A*) following intraocular inflammation. SDF1 cKO in myeloid cells also affected levels in the outer retina (*SI Appendix*, Fig. S8*B*, D4, red arrowheads), as examined using a horseradish peroxidase–conjugated secondary antibody and 3,3′-diaminobenzidine (DAB) histochemistry (to avoid the autofluorescence often seen in this area). SDF1 staining in the outer retina was dark 4 d after nerve injury and intraocular inflammation, and was largely, though not completely, suppressed in $CXCL12^{flx/flx}LysM\text{-}Cre^{-/+}$ cKO mice (*SI Appendix*, Fig. S8*B*, D4, red arrowheads). Note that DAB-stained SDF1 was also apparent in the inner retina following intraocular inflammation and disappeared in the cKO mice (*SI Appendix*, Fig. S8*B*, D1), as seen in our immunofluorescence results. In all, these results confirm the myeloid origin of SDF1 in the inner retina and suggest that SDF1 in the outer retina may be of mixed myeloid and nonmyeloid origin.

Following NC and intraocular zymosan, myeloid cell–specific SDF1 deletion reduced optic nerve regeneration by ∼40% (Fig. 3 *A* and *B*; P < 0.05) and decreased RGC survival to nearly the baseline level seen following NC alone in WT littermate controls (Fig. 3*C*; P < 0.001).

In complementary studies, we tested the effects of deleting the SDF1 receptor CXCR4 in RGCs. For this, we injected an adeno-associated virus expressing Cre recombinase (AAV2-Cre) into the eyes of mice bearing a conditional deletion of CXCR4 ($CXCR4^{flx/flx}$) 2 wk prior to NC. The effects of RGC-selective CXCR4 deletion were nearly identical to those of CXCL12 deletion in myeloid cells, diminishing zymosan-induced axon regeneration by ∼40% (P < 0.05) and RGC survival to the level seen after NC alone (P < 0.001) (Fig. 3). Despite the possibility that AAV-Cre might transfect other cells beyond RGCs (and therefore delete CXCR4 in both RGCs and other cells), these data support the idea that myeloid cell–derived SDF1 acts via CXCR4 to promote RGC survival and axon regeneration.

We also tested the role of SDF1 pharmacologically using the CXCR4 antagonist AMD3100. Intraocular AMD3100 decreased inflammation-induced regeneration by 50% (P < 0.01) and RGC survival by ∼25% (P < 0.001) (Fig. 3). Combining AMD3100 with P1, a peptide antagonist of Ocm, decreased regeneration by 70% (P < 0.001) with no further effects on RGC survival (Fig. 3 *B* and *C*). Although AMD3100 has been extensively used as a specific CXCR4 antagonist, it is

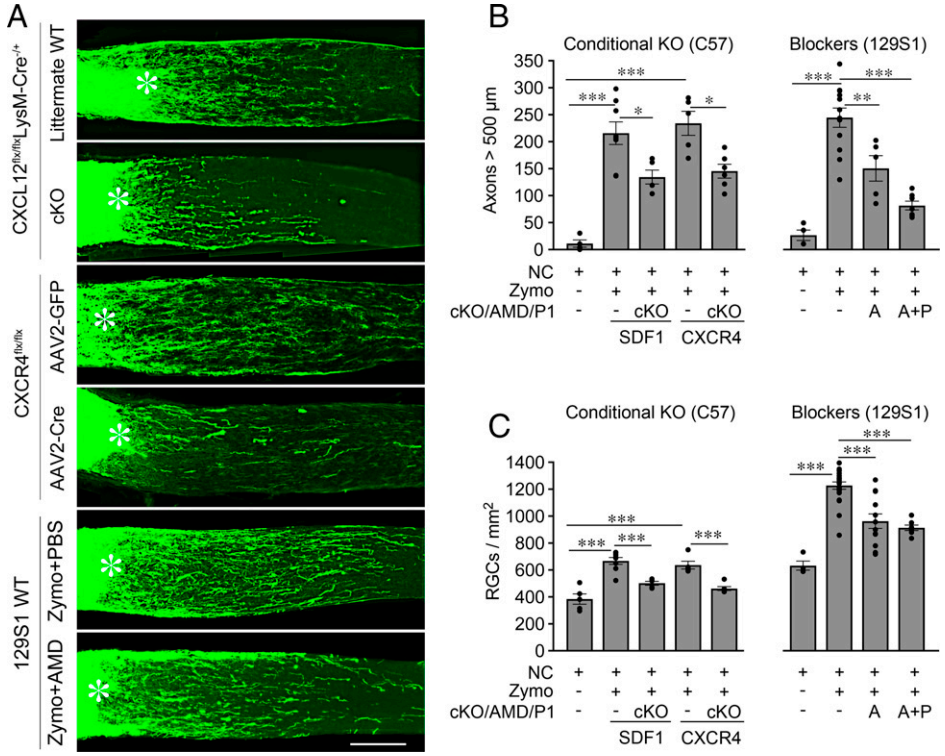

Fig. 3. SDF1 and CXCR4 contribute to inflammation-induced regeneration and RGC survival: loss of function. (*A* and *B*) Diminished regeneration following SDF1 deletion in myeloid cells, CXCR4 deletion in RGCs, or intraocular AMD3100. (*A*) Longitudinal sections through the mouse optic nerve immunostained for the anterograde tracer CTB to visualize regenerating axons 2 wk after NC + Zymo. Appreciable numbers of CTB^+ axons (green) appear distal to the injury site (asterisks) in WT littermates of mice bearing conditional gene deletions and in WT mice. (*B*) Zymosan-induced regeneration is reduced by SDF1 deletion in myeloid cells (*CXCL12$^{flx/flx}$LysM-Cre$^{-/+}$*; $*P < 0.05$), CXCR4 deletion in RGCs (*CXCR4$^{flx/flx}$*, AAV2-Cre; $*P < 0.05$), and AMD3100 in WT 129S1 mice (AMD or A; $**P < 0.01$). AMD3100 combined with Ocm inhibitor P1 (A + P; $***P < 0.001$) has an even stronger effect. (Scale bar, 200 µm.) (*C*) RGC survival 2 wk after NC + Zymo. Cell type–specific deletion of CXCL12 or CXCR4 or AMD3100 ± P1 treatment in 129S1 WT mice all reduce RGC survival close to the level seen without zymosan (RGCs were immunostained for βIII-tubulin and quantified as described in *SI Appendix, Methods*). One-way ANOVA plus Bonferroni correction (*B*) and Tukey correction (*C*); error bars in *B* and *C*: standard error of mean; $***P < 0.001$, $*P < 0.05$; ns, not significant, $P > 0.05$.

also reported to bind to CXCR7 as an allosteric agonist (49). Nonetheless, the present results, together with the CXCR4 cKO results shown above, point to the likelihood that the majority of the SDF1 effects described here are mediated through CXCR4. The overall differences in RGC survival between the pharmacological and genetic studies may reflect differences in the genetic backgrounds of the 129S1 WT mice used in the former study and C57-derived knockout mice used in the latter.

Using PCR, we examined whether SDF1 cKO affects the expression of other factors in myeloid cells that have been implicated in RGC survival or axon regeneration, including Ocm, CNTF, and LIF. In WT mice, Ocm and LIF mRNA levels increased dramatically 1 d after NC plus intraocular zymosan ($P < 0.001$, $P < 0.01$, respectively), whereas CNTF mRNA did not change significantly (*SI Appendix*, Fig. S8*C*). SDF1 cKO did not alter levels of Ocm or CNTF mRNA following NC and zymosan but decreased LIF mRNA ($P < 0.01$; *SI Appendix*, Fig. S8*C*).

Gain-of-Function Studies. We next investigated whether SDF1 complements the effects of Ocm in vivo. Exogenous SDF1 (0.3 µg per eye: estimated intraocular concentration ∼3 µM, ca 50× the effective concentration found in vitro; Fig. 2*B*) increased axon regeneration ∼7-fold compared with nerve injury only ($P < 0.05$; Fig. 4 *A* and *B*) and increased RGC survival ($P < 0.01$; Fig. 4*C*) to a similar extent as zymosan. A single injection of recombinant Ocm (rOcm; ∼1 µM) and CPT-cAMP (50 µM) was insufficient to increase axon regeneration (Fig. 4). However, the combination of SDF1 with Ocm/cAMP induced about 80% of the level of regeneration and a similar level of RGC survival as zymosan ($P < 0.01$ compared with Ocm/cAMP, $P < 0.05$ compared with SDF1 alone; Fig. 4). Note that in our earlier study, we found that slow-release polymer beads loaded with rOcm and CPT-cAMP induced considerably more regeneration than blank beads alone, an outcome that may have benefited from continuous release of Ocm/cAMP on top of the mild inflammatory reaction seen by injecting beads alone (9). A higher concentration of SDF1 (0.9 µg per eye) combined with rOcm/CPT-cAMP raised the level of axon regeneration even closer to that of zymosan (Fig. 4). Thus, a combination of identified factors can promote considerable repair without introducing potentially deleterious side effects of intraocular inflammation.

To investigate the generality of our results, we carried out experiments in rats using lens injury (LI) as an alternative way to induce intraocular inflammation (5, 6). As in mice, intraocular injection of AMD3100 immediately after NC decreased LI-induced regeneration by ∼50% compared with controls ($P < 0.01$; *SI Appendix*, Fig. S9 *A* and *B*) and diminished RGC survival ($P < 0.05$; *SI Appendix*, Fig. S9*C*), though to a lesser extent than seen in mice. The Ocm antagonist P1 had a greater negative effect on regeneration than AMD3100 ($P < 0.001$ compared with LI alone; *SI Appendix*, Fig. S9*B*) and, as expected, no effect on RGC survival (*SI Appendix*, Fig. S9*C*). Simultaneous inhibition of SDF1 and Ocm decreased LI-induced regeneration by ∼80% ($P < 0.05$ comparing LI/AMD/P1 vs. LI/AMD; *SI Appendix*, Fig. S9 *A* and *B*) and diminished LI-induced survival by ∼60% (*SI Appendix*, Fig. S9*C*; $P < 0.001$). In gain-of-function studies, a single injection of SDF1 immediately after NC increased axon regeneration in adult rats to about half the level induced by LI, with a nonsignificant effect on RGC survival (*SI Appendix*, Fig. S9 *A* and *B*). Combining SDF1 with LI resulted in stronger regeneration and RGC survival than LI alone ($P < 0.01$ for regeneration, $P < 0.001$ for survival; *SI Appendix*, Fig. S9).

SDF1 Induces Axon Regeneration from Non-αRGCs. Using a Kcng4-cas9-GFP (green fluorescent protein) mouse line in which αRGCs and their axons are genetically labeled (Kcng4-$Cre^{+/-}$ crossed with *STOP$^{flx/flx}$-cas9-GFP*), we investigated whether the regeneration induced by SDF1, like regeneration induced by Pten deletion and several other treatments (15, 50), arises from αRGCs. These studies used either recombinant SDF1 (rSDF1) or a previously validated adeno-associated virus

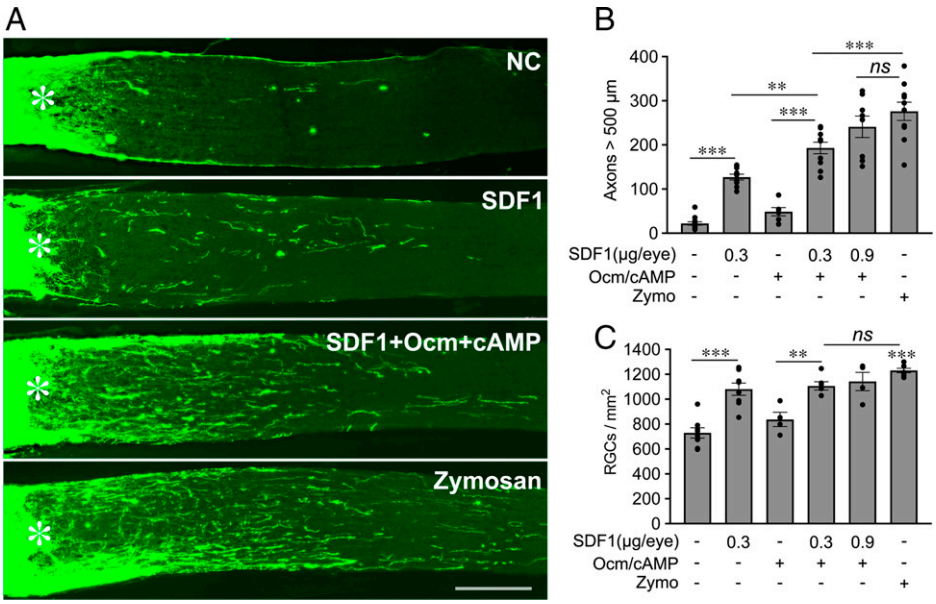

Fig. 4. SDF1 complements the effects of Ocm and other treatments on optic nerve regeneration. (*A* and *B*) Exogenous SDF1 (0.3 µg per eye) stimulates axon regeneration (***P < 0.001 compared with NC only) and augments the effects of Ocm combined with 50 µM CPT-cAMP (Ocm/cAMP: **P < 0.01, SDF1 + Ocm/cAMP vs. SDF1; ***P < 0.001, SDF1 + Ocm/cAMP vs. Ocm/cAMP). A higher concentration of SDF1 (0.9 µg per eye) plus Ocm/cAMP increases regeneration to a similar extent as zymosan. Asterisks in *A* indicate the injury site. (Scale bar, 200 µm.) (*C*) SDF1 increases RGC survival. Whereas Ocm/cAMP does not alter RGC survival, SDF1, either alone or combined with Ocm/cAMP, increases RGC survival to the same extent as zymosan (**P < 0.01, ***P < 0.001). Error bars in *B* and *C*: standard error of mean; one-way ANOVA plus Bonferroni correction (*B* and *C*).

expressing a monomeric form of SDF1 (AAV2-mSDF1) (39, 40). As a positive control, we deleted Pten via intraocular injection of an AAV2 expressing a verified short hairpin RNA (shRNA) (AAV2-shPten) (51). The latter virus increased phospho-S6 immunostaining in the GCL (*SI Appendix*, Fig. S10), providing confirmation of Pten knockdown. SDF1 (either rSDF1 or mSDF1), AAV2-shPten, or both were injected intraocularly immediately after optic nerve injury in Kcng4-cas9-GFP mice, which then survived for 3 wk. The total complement of regenerating axons was labeled with the anterograde tracer CTB (red fluorescence), enabling us to distinguish non-αRGC axons (red only) from axons arising from αRGCs (green + red = yellow; Fig. 5*A*). With SDF1 treatment, whereas some CTB-only axons regenerated beyond the injury site, no Kcng4$^+$-GFP–labeled axons (from αRGCs) extended past this point. It is unlikely that SDF1 altered the reporter expression in αRGCs because GFP$^+$ axons were still seen in the proximal optic nerve, although they appeared to be repulsed from the injury site (Fig. 5*A*, *Top*). Thus, axons that regenerate in response to SDF1 arise from non-αRGCs (Fig. 5 *A*, *A1*, *B*, and *C*). In marked contrast, about 70% of the axons that regenerated in response to Pten deletion were GFP$^+$ (i.e., arise from αRGCs), consistent with prior results (15) (Fig. 5 *A*, *A1*, *B*, and *C*). Combining SDF1 and Pten deletion resulted in far more regenerating axons than with SDF1 treatment alone, yet ca 90% of these axons arose from non-αRGCs (i.e., GFP-negative; Fig. 5 *A*, *A1*, *B*, and *C*). The underlying basis for the altered response to Pten deletion induced by SDF1 is as yet unknown. It is unlikely to be an artifact of dual-virus transfection, as combining the SDF1 virus and shPten virus did not alter the latter's transfection efficiency (*SI Appendix*, Fig. S11), increased outgrowth from non-αRGCs, and altered expression of the regeneration-associated gene Sprr1a in αRGCs (*SI Appendix*, Fig. S12). The shift in regenerative subtypes from αRGCs to non-αRGCs did not occur when combining zymosan with AAV2-shPten (*SI Appendix*, Fig. S13). Zymosan-induced regeneration arose mostly from non-αRGCs and, when combined with shPten, greatly increased outgrowth from non-αRGCs without diminishing the effect of Pten deletion on outgrowth from αRGCs (*SI Appendix*, Fig. S13).

To further investigate the effects of SDF1 in altering RGCs' response to Pten deletion, we carried out an in vitro study using adult rat RGC cultures as described above. In addition to retrogradely labeling RGCs with FG, we injected AAV2-mSDF1, AAV2-shPten, or the two viruses combined into the rat vitreous 2 wk before establishing cultures. After 3 d, cultured cells were fixed and double-immunostained with the antibodies SMI32 (against NFH, red, for αRGCs) and TUJ1 (against βIII-tubulin, green, for all RGCs and their processes) (Fig. 5*G*). The shift in responsiveness of different RGC subtypes to Pten deletion occurred again in culture when treatment changed from shPten to shPten + SDF1 (Fig. 5 *G* and *H*). While not increasing the level of growth from αRGCs above the untreated control group, SDF1 stimulated non-αRGCs to extend axons (P < 0.01). Pten deletion greatly increased growth from αRGCs and to a lesser degree from non-αRGCs (shPten-αRGC vs. control-αRGC: P < 0.001; shPten–non-αRGC vs. control–non-αRGC: P < 0.05). The addition of SDF1 dramatically reduced axon outgrowth from αRGCs while increasing outgrowth in non-αRGCs, yielding similar overall levels of axon growth (shPten-αRGC vs. [shPten + SDF1]–αRGC: P < 0.001; shPten–non-αRGC vs. [shPten + SDF1]–non-αRGC: P < 0.001). These findings confirm that SDF1 shifts the RGC pool that regenerates axons in response to Pten deletion to non-αRGCs.

To examine whether this subtype shift reflects a decrease in αRGC survival, we carried out double immunostaining (Fig. 5 *D*–*F* and *SI Appendix*, Fig. S14) with SMI32 (red) and TUJ1 (green). In the normal retina, αRGCs represent ∼9% of the total RGC population, as reported (52) (Fig. 5*F* and *SI Appendix*, Fig. S14). Three weeks after nerve injury, although the absolute number of surviving SMI32$^+$ cells decreased (Fig. 5*E*), the percentage of these cells in the total surviving population increased (Fig. 5*F*), as noted previously (15). SDF1 did not further alter the percentage of surviving αRGCs (Fig. 5*F*; P > 0.05), although the absolute number showed a near-significant increase (Fig. 5*E*; P = 0.07). Pten deletion greatly increased both the absolute and relative numbers of surviving αRGCs, consistent with an earlier report (15), and the addition of SDF1 did not change this outcome (Fig. 5 *E* and *F*). This finding indicates that the failure of αRGCs to regenerate axons in response to SDF1 and Pten deletion occurs despite an increase in αRGC survival, and dissociates the effects of Pten deletion on αRGC survival and axon regeneration. Note that these studies used 129S1 mice because, in the

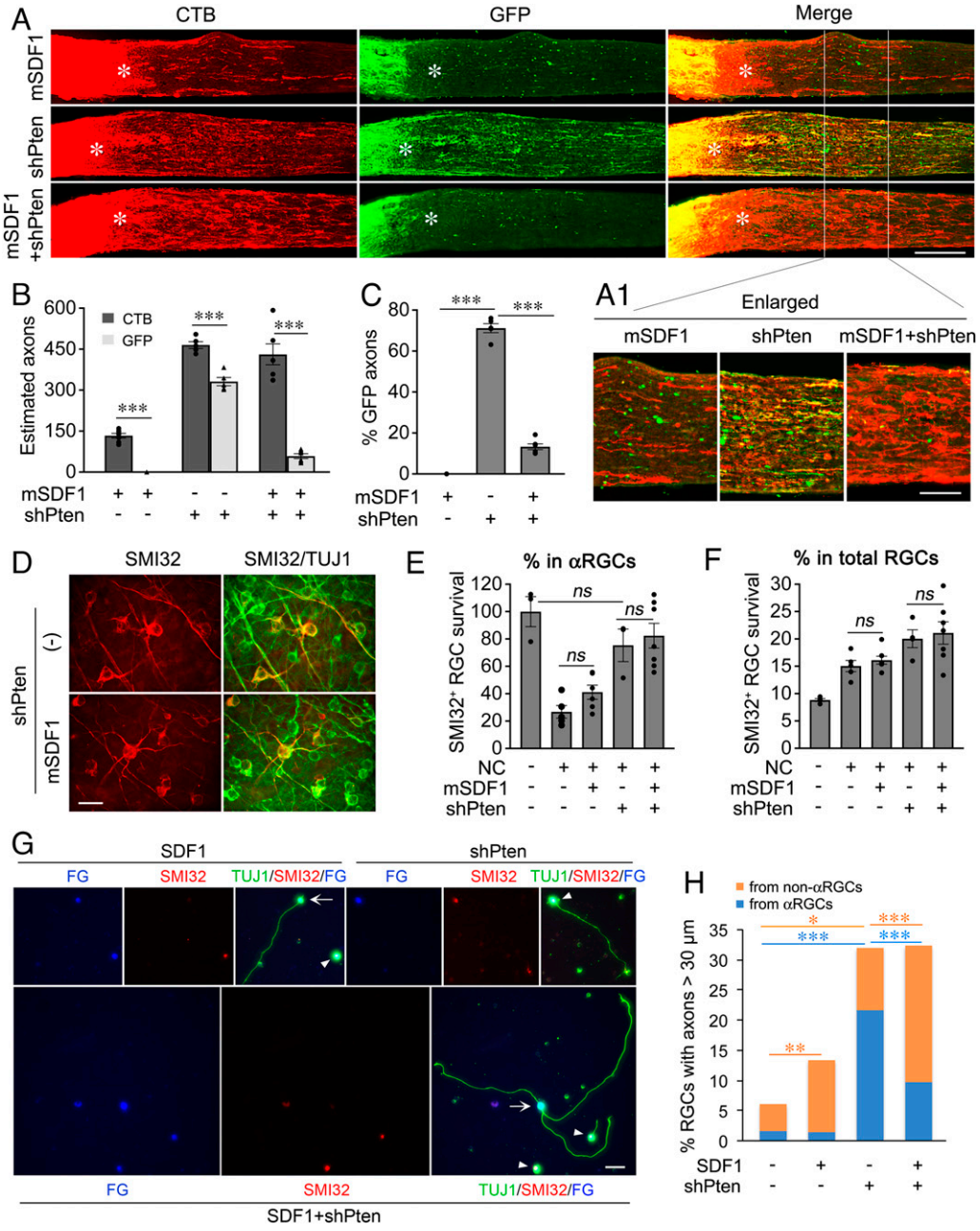

Fig. 5. SDF1-induced axon regeneration is not from αRGCs. (*A–C*) Mice expressing GFP in Kcng4-positive RGCs (i.e., αRGCs) were used to investigate the source of regenerating axons. Intraocular treatments: AAV2s expressing monomeric SDF1 (mSDF1), AAV2-shPten (shPten), or both (mSDF1 + shPten) administered immediately after optic nerve injury. Total regeneration, evaluated by anterograde CTB staining (red in *A* and *A1*), and regeneration arising from αRGCs (green in *A* and *A1*; light gray bars in *B*), are quantified 0.5 mm distal to the injury site (asterisks in *A*; dark gray bars in *B*). (*C*) Percentage of GFP$^+$ axons in total CTB$^+$ axons. ***$P < 0.001$. (*D–F*) αRGC survival in normal retina or in retinas of mice with optic NC without or with treatments that include mSDF1, shPten, or shPten + mSDF1. (*D*) Coimmunostaining with antibodies SMI32 (red; αRGCs) and TUJ1 (green; pan-RGC marker). (*E*) Survival of αRGCs as a percentage of the normal αRGC population. (*F*) Survival of αRGCs as a percentage of total TUJ1$^+$ RGCs. ns, $P > 0.05$. (*G* and *H*) Shifts in the response of different RGC subtypes visualized in culture. Rats received intraocular injection of AAV2-SDF1 or AAV2-shPten or both (2 wk), and FG (blue) retrograde labeling from the superior colliculus (1 wk) prior to establishing mixed retinal cultures. After 3 d, cells were immunostained with antibodies SMI32 (for neurofilament heavy-chain protein; red) and TUJ1 (for βIII-tubulin; green). (*G*) Examples of growing αRGCs (red/blue/green) or non-αRGCs (blue/green). (*H*) Quantitation of RGC-subtype outgrowth under different treatment. AAV2-monomeric SDF1 virus and recombinant SDF1 (SDF1); AAV2-shPten virus (shPten). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. One-way ANOVA plus Bonferroni correction (*B*, *F*, and *H*) and Tukey correction (*C* and *E*). (Scale bars, 200 µm [*A*], 80 µm [*A1*], 30 µm [*D*], and 40 µm [*G*].) Error bars in *B*, *C*, *E*, *F*, and *H*: standard error of mean.

retinas of Kcng4-cas9-GFP mice, GFP labels bipolar cells in addition to αRGCs (53, 54).

We also investigated the survival of two other RGC subtypes based on the availability of cell type–specific markers (*SI Appendix*, Fig. S15): CART-positive on–off direction-selective RGCs (ooDSGCs) and melanopsin-positive M1 to M5 intrinsically photosensitive RGCs (ipRGCs). Three weeks after optic nerve injury, more than 95% of CART$^+$ RGCs (*SI Appendix*, Fig. S15 *A* and *B*) and about 60% of melanopsin$^+$ ipRGCs (*SI Appendix*, Fig. S15 *C* and *D*) had died. SDF1 did not alter the survival of these populations (*SI Appendix*, Fig. S15). Further studies will be required to identify the cell types that show increased survival and regeneration in response to SDF1 treatment.

Intracellular Signaling. Based on our in vitro studies (Fig. 2*D*) and prior work showing that SDF1 activates PI3K, Akt, and mTOR (55, 56), we investigated whether we could detect a change in phosphorylation of ribosomal protein S6 (pS6) (57), a

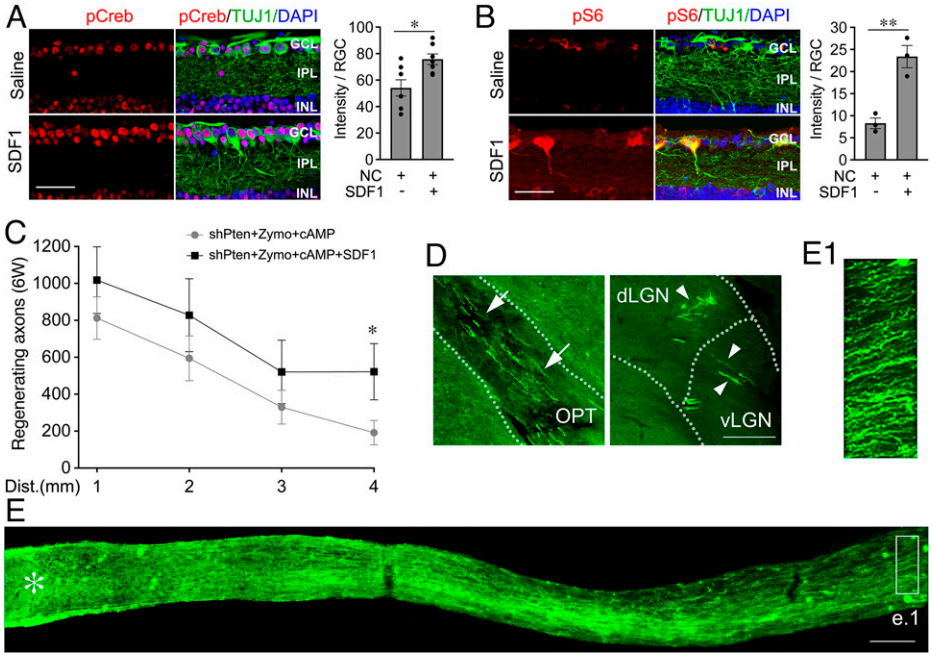

Fig. 6. SDF1 activates downstream effectors of axon growth and enhances long-distance regeneration. (*A* and *B*) SDF1 increases phosphorylation of Creb and ribosomal protein S6 (pCreb, pS6; red) in RGCs (green; stained with antibody TUJ1$^+$ for βIII-tubulin) 1 d after intraocular injection (*$P < 0.05$, **$P < 0.01$, SDF1 vs. saline). (Scale bars, 25 μm.) (*C*–*E*) Mice received intraocular AAV2-shPten 2 wk prior to NC and Zymo/cAMP, with or without SDF1, immediately after NC and again 3 wk later. Regenerating axons were visualized by CTB (green) injected intraocularly 2 d before euthanasia. (*C*) Quantitation of regenerating axons at the indicated distances from the injury site (*$P < 0.05$ comparing shPten/Zymo/cAMP/SDF1 vs. shPten + Zymo/cAMP + PBS). (*D*) Regenerating axons extending into the optic tract (OPT; *Left*, arrows) and entering the ventral and dorsal lateral geniculate nuclei (vLGN, dLGN; *Right*, arrowheads) in a mouse treated with AAV2-shPten, zymosan/cAMP, and SDF1. (Scale bar, 150 μm.) (*E*) Longitudinal section (14-μm thickness) through the optic nerve of a mouse treated with AAV2-shPten, zymosan/cAMP, and SDF1. Note the numerous CTB$^+$ axons regenerating the full length of the optic nerve after 6 wk. (*E1*) The enlarged image shows individual axons or axon bundles at the distal end of the optic nerve. The asterisk indicates the injury site. (Scale bar, 200 μm.) Error bars in *A*–*C*: standard error of mean; Student *t* test (*A* and *B*) and multiple *t* test (*C*).

downstream target of the latter pathway, or of CREB, a cAMP- and/or Ca^{2+}-dependent transcription factor, in vivo. One day after optic nerve injury, intraocular SDF1 increased phosphorylation of CREB ($P < 0.05$ compared with saline; Fig. 6*A*) and of S6 in RGCs ($P < 0.01$, ~3-fold increase; Fig. 6*B*), consistent with SDF1 activating multiple signaling pathways.

Taken together, our results show that SDF1 complements the effects of Ocm and that SDF1 combined with Ocm and elevated cAMP mimics the effects of intraocular inflammation on optic nerve regeneration and RGC survival. In addition, SDF1 changes the response of different RGC subtypes to Pten deletion, enabling non-αRGCs, which normally do not respond to Pten deletion, to respond robustly.

Potentiation of Pten Deletion Combined with Intraocular Inflammation. Combining zymosan, CPT-cAMP, and Pten deletion results in some of the strongest long-distance axon regeneration reported to date (31, 32, 48, 58). To investigate whether SDF1 can increase the effects of this treatment even further, we injected AAV2-shPten 2 wk prior to nerve injury and introduced the other treatments at the time of optic nerve injury and again 3 wk later. The anterograde tracer CTB was injected 19 d after the second treatment, and mice survived for a total of 6 wk post-injury before being prepared for histology. SDF1 strongly enhanced the level of regeneration induced by zymosan/CPT-cAMP and Pten deletion (Fig. 6 *C*–*E*). Whereas the latter combination enabled ca 200 axons to regenerate the full length of the optic nerve (Fig. 6 *C*, *E*, and *E1*), the addition of SDF1 increased long-distance regeneration 2.7-fold ($P < 0.05$; Fig. 6 *C*, *E*, and *E1*), allowing many axons to regrow up to the point at which they enter the optic chiasm (Fig. 6 *E* and *E1*; 14 μm in thickness). Among the mice with the three highest numbers of axons reaching the distal end of the optic nerve in each group, none of the mice without exogenous SDF1 showed central target reinnervation. In contrast, three mice with SDF1 included in the treatment showed axons growing further into the optic tract (white arrows, Fig. 6*D*), and one showed axons in the dorsal and ventral lateral geniculate nuclei (Fig. 6*D*).

Discussion

The innate immune system is essential for peripheral nerve regeneration and can also be harnessed to promote axon regeneration in the CNS (1–4, 59, 60). In the visual system, where this phenomenon has been widely studied, zymosan or other proinflammatory agents lead to an influx of neutrophils and macrophages expressing factors that enable injured RGCs to regenerate axons into the distal optic nerve (6–10, 16, 35, 58, 61–63). Ocm plays a major role in this phenomenon but its effects require additional factors (8–10, 35, 64). The present results show that SDF1 is important in this regard. SDF1 is highly expressed by activated macrophages and, in cell culture, SDF1 alone among many growth factors tested complements the effects of Ocm. In vivo, exogenous SDF1 elevates the effects of Ocm/cAMP on axon regeneration and RGC survival to a similar extent as zymosan, whereas deleting SDF1 in leukocytes, deleting the SDF1 receptor CXCR4 in RGCs, or the CXCR4 antagonist AMD3100 all strongly suppress inflammation-induced regeneration. Surprisingly, we also find that SDF1 alters the response of different RGC subtypes to Pten deletion. Pten deletion is perhaps the strongest single proregenerative treatment reported to date and normally stimulates axon regeneration preferentially from αRGCs (15). In contrast, we find that SDF1 promotes outgrowth from non-αRGCs and enables non-αRGCs to respond robustly to

Pten deletion. When combined with zymosan, cAMP, and Pten deletion, SDF1 increases the number of axons that regenerate the full length of the optic nerve.

Among more than 40 distinct subtypes of RGCs identified to date, αRGCs and M1 ipRGCs are the most resilient following optic nerve injury (15, 65–68), and αRGCs show a preferential increase in survival and capacity to regenerate axons after Pten deletion and other treatments (15, 69, 70). The RGC subtypes that regenerate axons in response to SDF1, Pten deletion, zymosan-induced intraocular inflammation, or combinations of these are shown schematically in Fig. 7. In sum, our results indicate that 1) αRGCs are not unique in their capacity to regenerate axons, 2) non-αRGCs can be induced to respond to Pten deletion, and 3) the pool of RGC subtypes that regenerates axons in response to a combinatorial treatment is not a simple addition of the subtypes that respond to each treatment alone. Further research will be required to identify the RGC subtypes that survive injury and respond to SDF1, alone or combined

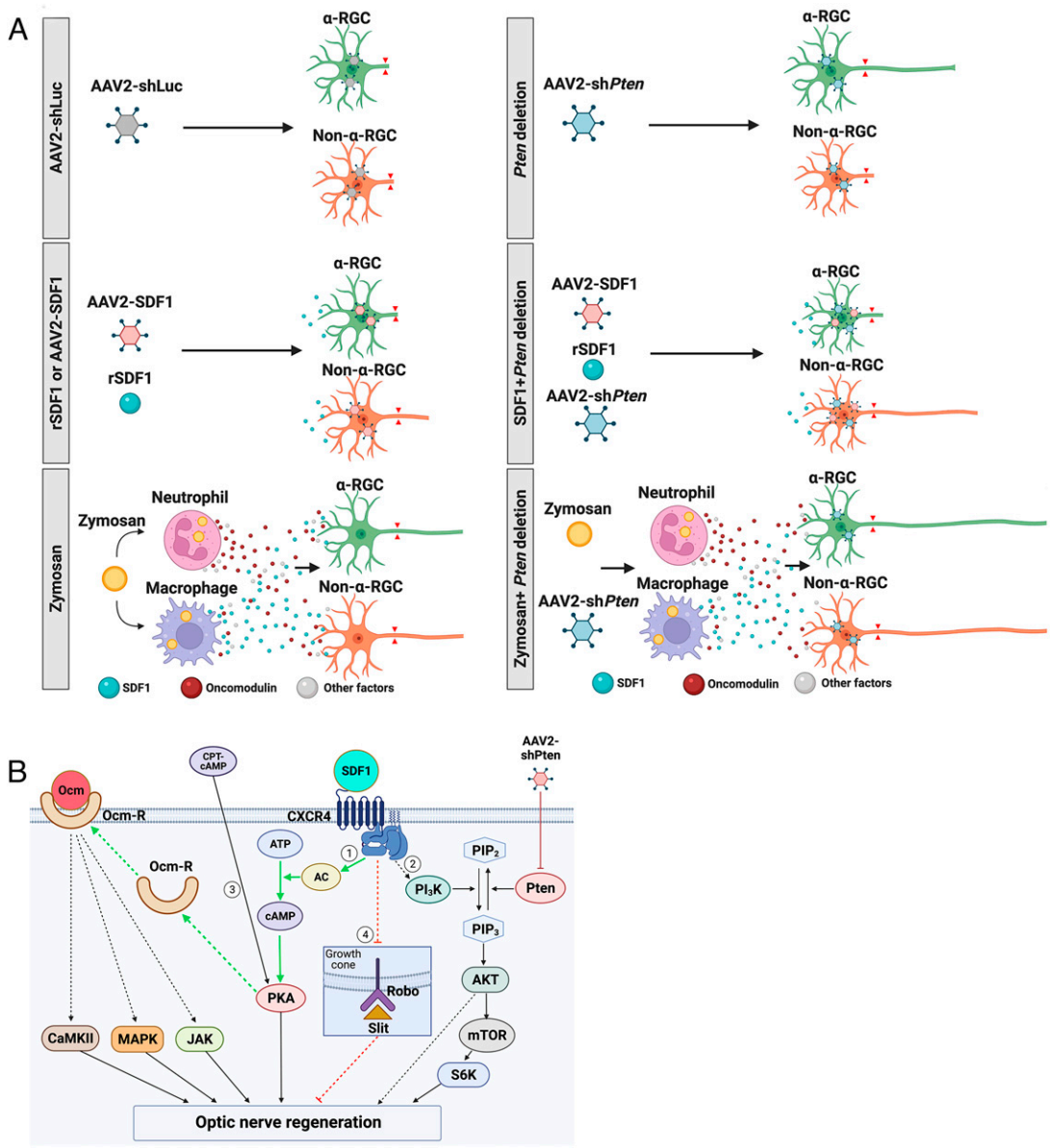

Fig. 7. Schematic summary of results and signaling pathways. (*A*) Effects of myeloid cell–derived growth factors SDF1 and Ocm on optic nerve regeneration: cellular sources and responses of RGC subtypes. (*A*, *Top*) AAV2 expressing an shRNA to suppress Pten expression (AAV2-sh*Pten*) induces axon growth primarily from αRGCs (15) (green cells); the control AAV2-shLuciferase virus (AAV2-shLuc) has no effects. (*A*, *Middle*) SDF1, either as a recombinant protein (rSDF1) or via AAV2-mediated expression of monomeric SDF1 (AAV2-SDF1), induces moderate axon growth primarily from non-αRGCs (*Middle Left*, orange cells). SDF1 combined with Pten deletion enables non-αRGCs to regenerate lengthy axons but simultaneously suppresses the ability of αRGCs to respond to Pten deletion (*Middle Right*). (*A*, *Bottom*) Zymosan, by elevating levels of neutrophil/macrophage-derived Ocm, macrophage-derived SDF1, and other factors, stimulates regeneration from both αRGCs and non-αRGCs (*Bottom Left*); growth from both subtypes is strongly augmented by combining zymosan with Pten deletion (*Bottom Right*). (*B*) Signaling pathways activated by SDF1, Ocm, CPT-cAMP, and Pten deletion. *SDF1* binds to its cognate G protein–coupled receptor CXCR4, thereby 1) activating adenylate cyclase (AC) to elevate cAMP and consequently PKA, which phosphorylates the Ocm receptor (Ocm-R, or a chaperone protein), causing it to translocate to the cell membrane (9, 10) (green arrows); 2) activating PI3K and its downstream effectors, including an AKT-dependent pathway (AKT, mTOR/S6K, to increase phosphorylation of ribosomal subunit pS6 and other substrates) and AKT-independent pathways (90) (dashed arrow); 3) besides cAMP generated downstream of SDF1, *CPT-cAMP*, a membrane-permeable, nonhydrolyzable cAMP analog, also activates PKA and potentiates the effects of Ocm; and 4) SDF1 antagonizes the repellent effects of slit/robo signaling at the optic chiasm and diencephalon (43). The binding of *Ocm* to Ocm-R promotes axon growth via CaMKII and other kinases. Inhibiting CaMKII alone, or any combination of MAPK, JAK, and PI3K, blocks the effects of Ocm (9, 10). *AAV2-shPten* decreases Pten expression, thereby augmenting PIP_3 phosphorylation and activating AKT, mTOR, and S6K. Pten deletion also promotes axon growth through AKT-independent pathways (e.g., focal adhesion kinase, RAS, and others; not shown) (90). Figure created with Biorender.com.

with Pten deletion. Like SDF1, overexpression of the transcription factor Sox11 enables non-αRGCs to regenerate axons (67). However, whereas Sox11 elevation is lethal to αRGCs (67), αRGCs remain resilient in the presence of SDF1. Understanding the mechanisms underlying the shift in responsiveness of different RGC populations to Pten deletion could lead to treatments that enable multiple RGC subtypes to regrow their axons.

SDF1 is highly conserved across vertebrates and is expressed in many tissues and cell types, contributing to organogenesis, including in the eye, and to stem cell migration, tumorigenesis, angiogenesis, cardiac development, axon guidance, spinal cord regeneration, and more (71, 72). Of the six known isoforms generated by alternative splicing, SDF1α is the most abundant and widely studied in the brain (73). Although SDF1 can bind to and initiate signaling through either of two G protein–coupled receptors, CXCR4 and CXCR7 (44, 45, 74, 75), our studies point to CXCR4 as most likely the relevant receptor in RGCs. In the normal retina, SDF1 is mainly expressed in the pigmented epithelium and is important in photoreceptor development and protection (76, 77). SDF1 contributes to the survival of embryonic RGCs (78) and to the guidance of developing RGC axons from the retina into and through the optic nerve and beyond (79), in part by antagonizing the repellent effects of slit/robo signaling at the optic chiasm and diencephalon (43). Earlier studies reported that SDF1 levels are slightly up-regulated in the retina and optic nerve glia after optic nerve injury (80) and that exogenous SDF1 stimulates a modest level of axon regeneration on its own. However, those studies did not explore the relationship of SDF1 to inflammation-induced regeneration (13). A recent paper (81) reported that RGCs are the primary source of SDF1, that SDF1 in RGCs suppresses inflammation-induced regeneration, and, conversely, that suppressing SDF1- (CXCL12-)CXCR4 signaling enhances regeneration. These statements are contradicted by our data showing monocytes to be by far the primary source of SDF1 (Fig. 1*A*, merge of SDF1 and TUJ1 immunostaining), an earlier report from the same group (13), the current data showing that SDF1 augments inflammation-induced regeneration (*SI Appendix*, Fig. S9), and our genetic and pharmacological data showing that blocking SDF1- (CXCL12-)CXCR4 signaling suppresses inflammation-induced regeneration (mice, zymosan; rats, LI; Fig. 3 and *SI Appendix*, Fig. S9). Also, whereas the other paper reports that 94% of CXCR4 is expressed in αRGCs, single-cell RNA sequencing shows CXCR4 to be expressed in multiple RGC subtypes (52) (*SI Appendix*, Table S1), consistent with our findings that SDF1 induces axon regeneration from non-αRGCs. The sources of these many differences will require further investigation.

The ability of SDF1 to complement the activity of Ocm is mediated in large part by elevation of $[cAMP]_i$ and by PI3K–Akt activation. In addition, enhancement of long-distance regeneration may reflect a role for SDF1 in counteracting signals that repel or suppress axon growth in the distal optic nerve and chiasm, including semaphorins, slits, and myelin (13, 43, 46). In several cases, SDF1 combined with zymosan/CPT-cAMP and Pten deletion enabled some axons to continue regenerating into the optic tract and, in at least one case, to enter the dorsal lateral geniculate nucleus. The signaling pathways associated with SDF1, Ocm, CPT-cAMP, and Pten deletion are shown schematically in Fig. 7*B*.

In addition to Ocm and SDF1, inflammatory cells express NGF, IGF1, and other growth factors (3, 8, 10, 82), and antibodies to NGF and IGF1 suppress neutrophil-induced regeneration in vivo (8). Gain-of-function data were not reported for the latter factors, however, nor is it known whether they act directly on RGCs or indirectly via other cell types. In our previous and current in vitro or in vivo studies, NGF, IGF1, and multiple other growth factors failed to either induce growth on their own or mimic the ability of SDF1 to complement the effects of Ocm (5, 6). In other studies, BDNF was shown to enhance RGC survival while counteracting the effects of intraocular inflammation on axon regeneration (83), and high concentrations of recombinant CNTF (rCNTF) were reported to promote axon regeneration and RGC survival, though others have found little effect of rCNTF unless *socs3*, a suppressor of the signaling pathway activated by CNTF, is deleted in RGCs (5, 84). CNTF gene therapy promotes considerable regeneration in vivo (33, 85, 86), but this effect is mediated largely via inflammation and the chemokine CCL5 (16). In addition to Ocm and SDF1, we found that zymosan increases intraocular levels of LIF (but not CNTF) mRNA in an SDF1-dependent manner. In keeping with studies showing that global deletion of LIF plus CNTF accelerates RGC death after NC (12), it is possible that LIF contributes to the residual regeneration and RGC survival seen after blocking SDF1 and Ocm signaling. CXCL5 was also recently found to contribute to LI-induced optic nerve regeneration and RGC survival (82), perhaps by modulating the inflammatory response. A role for additional factors seems likely in view of the fact that cAMP elevation is required for Ocm plus SDF1 to achieve the level of regeneration induced by zymosan. Nonetheless, it is important to note that, with cAMP elevation, recombinant Ocm and SDF1 mimic the effects of intraocular inflammation in a translationally relevant manner.

In conclusion, SDF1 is an important contributor to inflammation-induced regeneration of the optic nerve, and SDF1 combined with Ocm/cAMP mimics most of the beneficial effects of intraocular inflammation. Future translational studies might take advantage of these findings using viral vectors to express Ocm, SDF1, and constitutively active adenylate cyclase for continuous stimulation of the optic nerve and RGC survival while minimizing inflammation. In addition, the observation that SDF1 enables non-αRGCs to regenerate axons and respond to Pten deletion may afford insights into ways to promote axon regeneration from multiple RGC subtypes, bringing us closer to eventually achieving meaningful visual recovery.

Materials and Methods

In addition to the information provided in this section, further details regarding reagents, growth factors, antibodies, and animal lines used in this work are described in *SI Appendix*. Each statistical analysis is described in the appropriate figure legend and in Dataset S1. Details of previously reported methods, portions of lengthy methods, and methods related to the supporting figures are also included in *SI Appendix*.

Optic Nerve Injury, Intraocular Injections, and Lens Injury. Adult 129S1, $CXCL12^{flx/flx}$LysM-$Cre^{-/+}$, $CXCR4^{flx/flx}$, and Kcng4-Cre:$STOP^{flx/flx}$-cas9-GFP mice (8 to 10 wk old; original lines from The Jackson Laboratory), as well as adult Fischer rats (200 to 250 g; Charles River) were used in this study. Experiments performed at Boston Children's Hospital and the Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong were approved by the respective institutional animal care and use committees. Surgical procedures for optic nerve injury and intraocular injections in mice and rats were similar to those described previously (5, 6, 31, 35, 48) (*SI Appendix*).

Evaluating Optic Nerve Regeneration and RGC Survival. Methods are similar to those we reported (5, 6, 9, 10, 16, 30, 35). Briefly, CTB or GAP-43 (87) was used to visualize regenerating axons in the optic nerve, and TUJ1 was used for RGC survival (*SI Appendix*).

Long-Distance Regeneration and Brain Reinnervation. Mice received intraocular injections of AAV2-shPten or AAV2-shLuciferase 2 wk prior to nerve

injury, followed by intraocular zymosan plus CPT-cAMP, with or without SDF1 (100 μg/mL; other doses are described above), immediately after nerve injury (D0) and again 3 wk later. In the second injection, zymosan was used at half of the original concentration (6.3 μg/μL), whereas other factors were kept at the same concentrations as in the first injection. CTB was injected intravitreally 2 d before killing animals to trace axons extending through the nerve and into the brain. After a total postsurgical survival time of 6 wk, mice were perfused and nerves were processed as described above. Brains were postfixed for 48 h at 4 °C and then transferred to 30% sucrose until they sank, embedded in O.C.T., and cryostat-sectioned in the coronal plane at 40 μm. Sections were collected in three parallel series and stained free-floating to detect CTB-labeled growing axons and NeuN (to show the brain nucleus location), and then mounted onto slides.

Preparation, Staining, and Quantitation of Vitreal Cells and Retinal Cross-Sections. WT 129S1 mice were killed 1 or 4 d after intraocular injection of zymosan and a mixture of vitreous/aqueous fluid was collected and spread on glass coverslips. After fixation with 4% paraformaldehyde (PFA) for 10 min, vitreal cells were stained with an anti-SDF1 antibody (Santa Cruz, sc-74271; 1:500) together with either anti-F4/80 (for mouse macrophages; Bio-Rad, MCA497RT; 1:500) or anti-Gr1 (for mouse neutrophils; Bio-Rad, MCA2387; 1:500). In some material, zymosan particles stuck to the cell surface and absorbed antibodies nonspecifically, but these particles are easily recognized by their size and regular morphology. At each time point, ca 50 $Gr1^+$ or $F4/80^+$ cells from four to six different vitreal cell smears were quantified for SDF1 staining intensity.

Immunostaining in retinal cross-sections was used in multiple experiments. The methods are similar to those previously reported (16, 48) except using different antibodies according to individual experiments (*SI Appendix*).

qRT-PCR Analysis of Retinas and Whole Eyes. Retinas and whole eyes were collected from normal mice or mice 1 or 4 d after optic nerve injury with or without intraocular injection of zymosan. After tissue sonication, total RNA was extracted with the RNeasy Mini Kit (Qiagen), complementary DNA (cDNA) was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad), and real-time PCR was carried out with the following primers: SDF1-F (forward): 5′-ATGGACGCCAAGGTCGTCGCCGT-3′; SDF1-R (reverse): 5′-TCGGGTCAATGCACACTTGTC-3′; CD68-F: 5′-ATGAGGTTCCCTGTGTGTCTGACC-3′; CD68-R: 5′-TGTTCGGGTTCAATACAGAGAGGC-3′; Ocm-F: 5′-CCAAGACCCAGACACCTTTGA-3′; Ocm-R: 5′-GGCTGGCAGACATCTTGGAG-3′; CNTF-F: 5′-ATGGCTTTCGCAGAGCAATCACCT-3′; CNTF-R: 5′-CTACATTTGCTTGGCCCCATA-3′; LIF-F: 5′-ATGAAGGTCTTGGCCGCAGGGAT-3′; LIF-R: 5′-CTAGAAGGCCTGGACCACCACACT-3′; 18S-F: 5′-CGGCTACCACATCCAAGGAA-3′; 18S-R: 5′-GCTGGAATTACCGCGGCT-3′. Relative expression levels in experimental groups were first normalized to those of the reference gene 18S ribosomal RNA, and then normalized by the relevant control group depending on the experimental design.

Retrograde Labeling of RGCs and Preparation of Adult Dissociated Retinal Cultures. The procedure for retrograde labeling of RGCs and adult dissociated retinal cultures has been described previously (6, 9), except for the multiple growth factors, antagonists, and blockers used in this work (*SI Appendix*).

Ligand-Binding Assays. Plasmids encoding Fc or an Fc-Ocm fusion protein were expressed in 293T cells in Dulbecco's modified Eagle's medium (Cytiva, SH30021) plus 1% ultralow immunoglobulin G fetal bovine serum (Gibco, A3381901) and 1% penicillin/streptomycin (Invitrogen, 15140122). The supernatants were collected and the recombinant Fc or Fc-Ocm was verified and quantitated by comparing standard Ocm on Western blots. Binding assays were carried out using a modified protocol (88, 89) in rat adult retinal cells in which RGCs were retrogradely labeled with FG injected into the superior colliculus 1 wk before establishing cultures. Cultured cells were first treated with SDF1 (0.5 μg/mL) or medium only as a negative control. After one night in a CO_2 incubator, the cells were lightly fixed for 5 min with 4% PFA prior to incubation with recombinant proteins Fc or Fc-Ocm for 3 h at 37 °C. Cells were then washed with phosphate-buffered saline (PBS) three times and postfixed with 4% PFA for another 6 min. Positive signals were visualized by Cy3-conjugated Fc immunostaining. Binding intensity was quantified by selecting consecutively encountered FG-labeled RGCs in each well and was repeated in four different wells in each group.

Data Availability. All study data are included in the article, *SI Appendix*, and Dataset S1.

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