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Research Paper

Overexpression of the monocyte chemokine CCL2 in dorsal root ganglion neurons causes a conditioning-like increase in neurite outgrowth and does so *via* a STAT3 dependent mechanism

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ABSTRACT

Neuroinflammation plays a critical role in the regeneration of peripheral nerves following axotomy. An injury to the sciatic nerve leads to significant macrophage accumulation in the L5 DRG, an effect not seen when the dorsal root is injured. We recently demonstrated that this accumulation around axotomized cell bodies is necessary for a peripheral conditioning lesion response to occur. Here we asked whether overexpression of the monocyte chemokine CCL2 specifically in DRG neurons of uninjured mice is sufficient to cause macrophage accumulation and to enhance regeneration or whether other injury-derived signals are required. AAV5-EF1α-CCL2 was injected intrathecally, and this injection led to a time-dependent increase in CCL2 mRNA expression and macrophage accumulation in L5 DRG, with a maximal response at 3 weeks post-injection. These changes led to a conditioning-like increase in neurite outgrowth in DRG explant and dissociated cell cultures. This increase in regeneration was dependent upon CCL2 acting through its primary receptor CCR2. When CCL2 was overexpressed in CCR2 −/− mice, macrophage accumulation and enhanced regeneration were not observed. To address the mechanism by which CCL2 overexpression enhances regeneration, we tested for elevated expression of regeneration-associated genes in these animals. Surprisingly, we found that CCL2 overexpression led to a selective increase in LIF mRNA and neuronal phosphorylated STAT3 (pSTAT3) in L5 DRGs, with no change in expression seen in other RAGs such as GAP-43. Blockade of STAT3 phosphorylation by each of two different inhibitors prevented the increase in neurite outgrowth. Thus, CCL2 overexpression is sufficient to induce macrophage accumulation in uninjured L5 DRGs and increase the regenerative capacity of DRG neurons *via* a STAT3-dependent mechanism.

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1. Introduction

Injury to peripheral nerves initiates an inflammatory response that plays a significant role in axonal regeneration and functional recovery (Bastien and Lacroix, 2014; DeFrancesco-Lisowitz et al., 2014; Popovich and Longbrake, 2008). In response to axotomy, both injured peripheral neuronal cell bodies and Schwann cells distal to the injury site upregulate and release the C–C class chemokine 2 (CCL2), also known as monocyte chemoattractant protein-1 (MCP-1; Schreiber et al., 2001; Subang and Richardson, 2001; Tanaka et al., 2004; Toews et al., 1998). As a result of CCL2 expression, monocytes extravasate into both peripheral ganglia and the nerve distal to the injury site, and macrophage accumulation in these areas remains elevated for several weeks (Lu and Richardson, 1993; Perry et al., 1987; Schreiber et al., 1995). Traditionally, investigation of this inflammatory process has focused on actions in the nerve at, or distal to, the site of injury. After axotomy, the distal nerve undergoes Wallerian degeneration, a process that includes fragmentation of the axon and myelin sheath and phagocytosis of debris by macrophages and Schwann cells (Rotshenker, 2011). Clearance of this debris is considered to be a prerequisite for regeneration, as blockade of macrophage accumulation yields incomplete degeneration, suboptimal regeneration, and limited functional recovery (Barrette et al., 2008; Bisby and Chen, 1990; Brown et al., 1994; Brown et al., 1991; Dahlin, 1995; Dailey et al., 1998; Reichert et al., 1994).

It should be noted, however, that in these experiments macrophage accumulation in peripheral ganglia was also almost certainly blocked and this might account for some of the decreased regeneration (Niemi et al., 2013). In fact, recent evidence has shown that the inflammatory response near axotomized neuronal cell bodies is necessary for axonal regeneration following a peripheral nerve injury. Utilizing a global knockout for the C–C class chemokine receptor 2 (CCR2), the primary receptor for CCL2, we showed that if macrophage accumulation in the dorsal root ganglion (DRG) does not occur then axonal regeneration is

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significantly diminished (Niemi et al., 2013). A related study showed that intrathecal administration of minocycline, which blocked injury-induced ganglionic macrophage accumulation, also reduced regenerative capabilities of DRG neurons (Kwon et al., 2013).

Exogenous activation of inflammation near neuronal cell bodies has also been shown to increase the regenerative capacity of neurons. Injection of *Cryptosporidium parvum* into the DRG, creating a local inflammatory response characterized by macrophage accumulation, significantly increased expression of growth-associated protein-43 (GAP-43) and calcitonin gene-related peptide (CGRP), two regeneration-associated genes (RAGs), and axonal regeneration after a dorsal root crush injury, though interestingly not after a sciatic nerve injury (Lu and Richardson, 1991, 1993, 1995).

The most widely studied example of inflammation-induced regeneration is in the optic nerve. Retinal ganglion cells (RGCs) are normally unable to regenerate their axons following optic nerve injury, but are able to do so after inducing an inflammatory reaction in the eye (Leon et al., 2000; Yin et al., 2003). Intraocular injection of zymosan leads to a dramatic increase in the expression of oncomodulin (OCM), a protein that has been proposed to play a key role in inflammation-induced regeneration in the optic nerve (Kurimoto et al., 2010; Yin et al., 2006). Other investigators have shown that lens injury-induced optic nerve regeneration is dependent upon the upregulation and actions of two gp130 cytokines, ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF; Leibinger et al., 2009).

While exogenous activation of local inflammation near neuronal cell bodies is able to greatly increase the regenerative capacity of those neurons, the mechanism (s) by which this occurs and the specific role macrophages play in this response are poorly understood. The inflammatory responses activated by zymosan, and other inflammatory agents, are complex and involve several immune cell types, including CD4-positive cells, neutrophils, and macrophages (Baldwin et al., 2015; Gantner et al., 2003; Kurimoto et al., 2013). We sought to determine if macrophage accumulation alone, without an axonal injury, could increase the regenerative capacity of DRG neurons. We addressed this question by overexpressing the monocyte chemokine CCL2 in uninjured lumbar DRGs and examined whether this singular change was sufficient to cause macrophage accumulation and increased axonal regeneration.

2. Materials and methods

2.1. Generation and production of an adeno-associated virus (AAV5) CCL2 overexpression vector

Mouse cDNA encoding the open reading frame of CCL2 (Origene; Rockville, MD) was cloned into a pAAV shuttle vector containing AAV inverted terminal repeats and an expression cassette composed of the human elongation factor-1 alpha (EF1α) promoter, a multiple cloning site, the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE), and a polyadenylation signal. The CCL2 insert was generated by PCR using the following primers: forward 5′ ATA GTC GAC ATG CAG GTC CCT GTC ATG CTT CTG G 3′ and reverse 5′ ATA AAG CTT CTA GTT CAC TGT CAC ACT GGT CAC TCC TA 3′. PCR products were digested by SalI and EcoRV and subcloned into the multiple cloning site of the pAAV shuttle vector to generate the pAAV-EF1α-CCL2 vector. Recombinant AAV5 vectors were generated by transient co-transfection of HEK-293T cells with 37.5 μg of total DNA containing 7.5 μg of pAAV-EF1α-CCL2, 7.5 μg of AAV5 rep–cap plasmid, and 22.5 μg of pAD helper plasmid using the calcium phosphate method. Seventy-two hours after transfection, cells were collected and lysed with 10% chloroform and AAV particles were purified using PEG8000 (Promega; Madison, WI) and benzonase nuclease (Life Technologies; Carlsbad, CA), and concentrated using an Ultra-4 centrifugal filter (EMD/Millipore; Billerica, MA). Titers were determined by quantitative PCR for viral genomic copies extracted from proteinase K-treated viral particles using the following primers: forward: 5′ ATA GTC GAC ATG CAG GTC CCT GTC ATG CTT CTG G 3′ and reverse 5′ TTG TAG CTC TCC AGC CTA CTC ATT GG 3′. For the present experiments, the following vectors were produced: AAV5-EF1α-CCL2: 2.2×10^{13} GC/ml and AAV5-EF1α-eYFP: 1.52×10^{13} GC/ml.

2.2. Animals and sciatic nerve transection injury (SNI)

Eight to twelve-week-old male wild type (WT) mice [C57BL/6J (Jackson Laboratories; Bar Harbor ME)] and mutant mice [C57B6.129S4-Ccr2^{tm1Ifc}/J (CCR2 −/−; Jackson Laboratories)] were utilized for this study. The animals were housed under a 12 h:12 h light:dark cycle with *ad libitum* access to food and water. SNI was performed on WT mice under isoflurane anesthesia. Briefly, the right sciatic nerve was exposed and transected proximal to the trifurcation and the wound closed with a wound clip. The left sciatic nerve was then exposed but not transected to serve as an internal control. One, two, three, or four weeks after intrathecal injection or 7 days after SNI, the animals were sacrificed by CO_2 inhalation, and the lumbar-level DRGs, sciatic nerves, and spinal cords were removed for immunohistochemical or molecular biological analysis. All animal procedures were approved by the Case Western Reserve University's Institutional Animal Care and Use Committee.

2.3. L5 laminectomy and intrathecal injection

The intrathecal injection method was modified from Parikh et al. (2011). Briefly, mice were anesthetized using isoflurane. A small incision was made in the back skin of the mouse and the spinal column was exposed. The site of viral injection was between lumbar levels L5 and L6, where a small laminectomy was performed to expose the dura. The injection was performed with a borosilicate glass capillary (WPI; Sarasota, FL) pulled to a fine point, attached by polyethylene tubing (Thermo Fisher Scientific; Waltham, MA) to a Hamilton syringe. The glass capillary remained at midline and was slowly inserted underneath the dura and further advanced in the subarachnoid space. Ten microliters of AAV5-EF1α-CCL2 or AAV5-EF1α- eYFP were slowly injected using 1% trypan blue added to the AAV particle solution to visualize the injection, and the capillary remained in place for 2 min after the injection. The paraspinal muscles and fascia were repositioned, and the incision closed with wound clips. One, two, three, or four weeks after injection mice were sacrificed and lumbar DRG were harvested for qPCR, immuno-labeling, or explant and dissociated culture.

2.4. Immunohistochemistry (IHC)

Generally, L5 DRGs from CCR2 −/− and WT mice were removed, and the ganglia were desheathed and fixed by immersion in 4% paraformaldehyde. The tissues were cryoprotected in 30% sucrose and embedded in Tissue-Tek O.C.T. compound (Electron Microscopy Sciences; Hatfield, PA). IHC was performed on 10 μm cryostat sections. For quantification of macrophages, a rat monoclonal antibody to CD11b (also known as Mac1, CR3, and integrin α_M; 1:100; EMD/Millipore) or CD68 (1:200; AbD Serotec; Oxford, UK) was incubated with tissue sections overnight at 4 °C. Given that the same effect was observed with CD11b and CD68, the cells labeled by either of these markers will be referred to as macrophages throughout the paper. For quantification of RAG proteins, a rabbit monoclonal antibody to pSTAT3 (Y705; 1:100; Cell Signaling; Danvers, MA), a rabbit polyclonal antibody to activating transcription factor 3 (ATF3; 1:200; Santa Cruz Biotechnology; Dallas, TX), or a rabbit polyclonal antibody to small proline-rich repeat protein 1a (SPRR1a), generously provided by Dr. Stephen Strittmatter and Dr. William Cafferty (Yale University), was incubated with tissue sections overnight at 4 °C. For YFP detection, a rabbit antibody against GFP (1:500; Life Technologies) was incubated with tissue sections for 3 days at 4 °C. After washing, the sections were incubated in Cy3 secondary antibody (1:400; Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA) or AlexaFluor 488 secondary antibody (1:500; Life

Technologies) for 1 h. In all experiments, sections not exposed to the primary antibody were included for each experimental group. Images were captured at 25× magnification using HCImage software (Hamamatsu Corporation; Bridgewater, NJ) and then quantified using MetaMorph software (Version 7.6.3.0, Molecular Devices; Downingtown, PA). Data are represented as the percentage of the imaged area that was positively immuno-labeled.

2.5. Real time PCR (RT-PCR)

The expression of CCL2 mRNA was analyzed by RT-PCR. One, two, three, or four weeks after intrathecal injection both L5 DRGs were removed and stored in RNAlater (Life Technologies) at 4 °C. RNA was extracted from pairs of ganglia using the Ambion RNAqueous micro kit. Five samples were included for each time point. Total RNA was quantified and 400 ng were reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; Carlsbad, CA). RT-PCR was performed in an ABI Step-One Plus, using prevalidated TaqMan expression assays [CCL2, Mm00441242; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Mm99999915; ATF3, Mm00476032; GAP-43, Mm00500404; Jun, Mm00495062; Smad1, Mm00484723; Sox11, Mm01281943; galanin, Mm00439056; interleukin (IL)-6, Mm00446190; LIF, Mm00434762; iNos, Mm00440502; CD86, Mm00444543; CD16, Mm00438875; arginase 1, Mm00475988; CD206, Mm00485148; Fizz1, Mm00445109; and Ym1, Mm00657889; Applied Biosystems], and samples were assayed in triplicate. Relative expression was determined using the Comparative Ct Model (ΔΔCt) with GAPDH as the housekeeping gene.

2.6. DRG explants

To assess the outgrowth of peripheral neurons in response to injury, we evaluated neurite outgrowth in explanted ganglia (Shoemaker et al., 2005). Three weeks after intrathecal injection of the virus, L5 DRGs from uninjured CCR2 −/− and WT mice were removed, desheathed, placed on coverslips, and overlaid with 7.5 μl Matrigel (Becton Dickinson; Franklin Lakes, NJ). Culture plates were placed in an incubator at 37 °C for 5 min to allow gelling of the Matrigel before adding 1 ml F12 medium with the additives described in Hyatt Sachs et al. (2010). Phase-contrast images of neurite outgrowth from each DRG were captured at 24 and 48 h after explantation using an Axiovert 405M microscope at 10× magnification. Neurite outgrowth was assessed using MetaMorph software by measuring the distance between the edge of the ganglion and the leading tip of the longest 20 processes in each explant. The average length of the 20 longest neurites was taken for each ganglion. At 48 h, explants were fixed and labeled with an antibody against βIII tubulin (1:500; Promega) to visualize the outgrowth. Representative images were taken at 10×. In some experiments, 200 ng/ml of recombinant mouse CCL2 (R&D Systems; Minneapolis, MN) was added to the culture medium at the time of plating.

2.7. DRG dissociated cell culture

To assess neurite outgrowth from isolated sensory neurons, we dissociated and cultured DRG neurons from uninjured WT mice or mice injected with AAV5-CCL2 or AAV5-YFP (Sachs et al., 2007). L5 DRGs were removed, cleaned and desheathed. Except where noted all reagents used in the DRG dissociations and culture were from Sigma-Aldrich (St. Louis, MO). Ganglia were incubated in 0.125% collagenase A at 37 °C for 1.5 h. The cells were then dissociated by gentle trituration using a P200 pipet in Neurobasal A medium containing 2% B-27 serum free supplement, 2 mM glutamine (both from Life Technologies), 10 U/ml penicillin, and 10 μg/ml streptomycin. The dissociated cells were purified following the procedure of Gavazzi et al. (1999), by centrifugation through 15% BSA at 600 rpm for 6 min. Neurons were resuspended in Neurobasal A containing 50 μg/ml DNase (Type I) and centrifuged at 1000 rpm for 2 min. Supernatants were removed, and cells were resuspended in Neurobasal A. Cells were gently plated (1/2 DRG/coverslip) onto 12 mm coverslips coated with 0.01% poly-L-lysine and 10 μg/ml laminin in a 12-well culture plate. Cells were allowed to adhere undisturbed for 20 min. Each coverslip was then overlaid with 1 ml of Neurobasal A and cultured for 24 h at 37 °C in 95% air/5% CO_2. In some experiments, 200 ng/ml of recombinant mouse CCL2 (R&D Systems) was added to the culture medium at the time of plating.

2.8. Inhibitors of signal transducer and activator of transcription 3 (STAT3) phosphorylation: AG490 and STATTIC

Two inhibitors of STAT3 phosphorylation were utilized in DRG dissociated cell culture to assess the role STAT3 plays in CCL2 overexpression-induced neurite outgrowth. AG490 (50 μM; EMD Millipore; Meydan et al., 1996), a Janus kinase (JAK) inhibitor, and STATTIC (10 μM; EMD Millipore; Schust et al., 2006), a STAT3 SH2 domain binding inhibitor, were used. L5 DRGs from mice 3 weeks after intrathecal injection with AAV5-YFP or AAV5-CCL2 were removed, dissociated and cultured as described above. At the time of plating, cells were cultured in Neurobasal A medium containing AG490 (50 μM), STATTIC (10 μM), or DMSO (0.17 μl/ml) for 24 h.

2.9. Analysis of neurite outgrowth in cultured neurons

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed in PBS, and labeled with a mouse monoclonal antibody to βIII tubulin (1:900 for DRG; Promega), followed by a 45 min incubation in an AlexaFluor 488 labeled secondary antibody (1:400, Life Technologies). Coverslips were placed onto slides with FluoroGel (Electron Microscopy Sciences). Fourteen-bit images were collected on a Leica DMI 6000 B inverted microscope (Leica Microsystems; Wetzlar, Germany) using a Retiga Aqua Blue camera (Q-imaging; Vancouver, British Columbia). Briefly, the entire coverslip was imaged at 10× and all resulting images were stitched together using the scan slide function in MetaMorph Imaging Software (Molecular Devices) to generate a full resolution composite image. The composite image was then subjected to neurite outgrowth analysis using the neurite outgrowth module in MetaMorph software. The longest neurite from each βIII tubulin-positive neuron with a process of at least 1.5 times the diameter of the cell body was measured.

2.10. Analysis of pixel intensity of pSTAT3 in cultured neurons

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature, washed in PBS, and labeled with a mouse monoclonal antibody to βIII tubulin (1:900; Promega) and a polyclonal antibody against pSTAT3 (Y705; 1:80; Cell Signaling), followed by a 1 h incubation in an AlexaFluor 488 labeled secondary antibody (1:400, Life Technologies) to visualize βIII tubulin and an AlexaFluor 647 labeled secondary antibody (1:200; Jackson Immuno) to visualize pSTAT3. Coverslips were placed onto slides with FluoroGel (Electron Microscopy Sciences) and imaged as described above. The longest neurite from each βIII tubulin-positive neuron with a process of at least 1.5 times the diameter of the cell body was measured using the MetaMorph software. Data for neurite outgrowth was then expressed as the average length of the longest neurite for each group. To assess the average pixel intensity of pSTAT3 in culture, the nucleus of each neuron was outlined and the average pixel intensity within the nucleus was measured using the MetaMorph software. The same neurons were measured for both neurite outgrowth and pSTAT3 nuclear pixel intensity.

2.11. Statistics

Data are expressed as the mean ± S.E.M. and were analyzed by one-way or two-way ANOVA followed by Tukey's post-hoc test. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Intrathecal injection of AAV5-CCL2 results in a time-dependent overexpression of CCL2 mRNA in uninjured L5 DRGs

To overexpress CCL2 in uninjured DRGs, we constructed an AAV5 vector encoding for CCL2 driven by the EF1α promoter (AAV5-CCL2; Fig. 1A). Intrathecal delivery of various AAV serotypes has been shown to allow for infection of DRG neurons (Iwamoto et al., 2009; Parikh et al., 2011; Wang et al., 2005), and a screen of AAV serotypes revealed AAV5 as the most efficient for infection of DRG neurons (Mason et al., 2010). To test the efficiency and localization of intrathecal delivery of AAV5, we first injected an AAV5 vector expressing eYFP driven by the EF1α promoter (AAV5-YFP; Figs. 1B–E'). Three weeks after injection of 10 μl of high-titer virus, lumbar DRGs, lumbar spinal cord, and sciatic nerves were removed and immuno-labeled with an antibody against YFP to assess infection efficiency and localization. Lumbar DRGs exhibited significant YFP labeling of neuronal cell bodies with minor localization to axons (Figs. 1B–C'), resulting in a DRG neuron labeling efficiency of 37.2% ± 5.7%. Only sparse axonal YFP labeling was found in the sciatic nerve (Fig. 1D). AAV5-YFP also resulted in significant labeling of neurons in the lumbar spinal cord (Fig. 1E), with some specificity to neurons and processes in the dorsal horn (Fig. 1E').

Intrathecal administration of AAV5-CCL2 resulted in a time-dependent overexpression of CCL2 mRNA in L5 DRGs (Fig. 1F). By 1 week after delivery, CCL2 mRNA showed a 2.9-fold increase and by 3 weeks there was a 9.1-fold overexpression compared to AAV5-YFP controls. This level of overexpression is comparable to the injury-induced increase in CCL2 mRNA in the L5 DRG seen at 1 day after sciatic nerve transection (Niemi et al., 2013). The intrathecal delivery method was chosen specifically because it allows for significant CCL2 overexpression in DRGs, while leaving the ganglia uninjured.

3.2. CCL2 overexpression leads to increased CD11b-positive macrophage accumulation

Immuno-labeling with CD11b, a common marker employed for detecting macrophages, was used to assess if CCL2 overexpression was sufficient to increase macrophage accumulation within L5 DRGs. The immune response to injury, and specifically macrophage accumulation near injured neuronal cell bodies, has been shown to be an important mediator of regenerative capacity (Kwon et al., 2013; Lu and

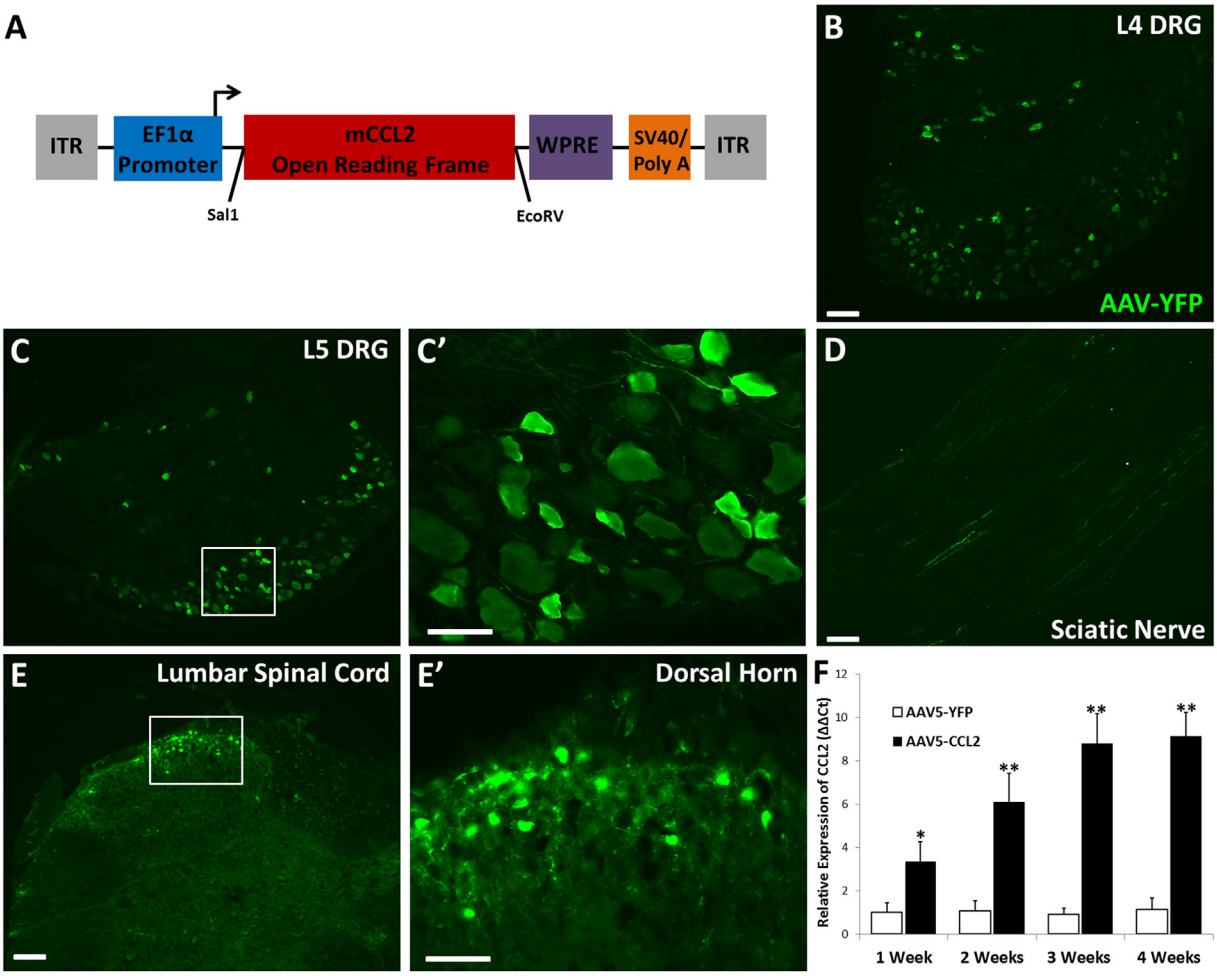


Fig. 1. Intrathecal injection of AAV5 infects lumbar DRG neurons and leads to a time-dependent overexpression of CCL2. Schematic of AAV5-EF1α-CCL2 vector. We used AAV5-EF1α-eYFP as a control (A). Representative images of AAV5-YFP localization in L4 (B) and L5 DRG (C) at 10×, visualized by anti-YFP labeling. High magnification image of YFP labeling in L5 DRG at 25× (C'). AAV5-YFP injection led to a 37.2% ± 5.7% DRG neuron infection efficiency. Representative image of sparse YFP axonal labeling in the sciatic nerve (D). A representative image of YFP localization in the lumbar spinal cord at low magnification (E) and high magnification (E') shows preferential expression in the dorsal horn. Injection of AAV5-CCL2 resulted in a time-dependent overexpression of CCL2 mRNA in L5 DRG quantified using the ΔΔCt method normalized to GAPDH (F). For images: scale bar = 100 μm. For RT-PCR: n = 5 per group. $^{*}p < 0.05$. $^{**}p < 0.001$.

Richardson, 1991; Niemi et al., 2013). A time-dependent increase in CD11b-positive macrophages was seen after injection of AAV5-CCL2 compared to AAV5-YFP controls (Figs. 2A–E). AAV5-YFP administration did not alter CD11b-positive macrophage accumulation compared to uninjured WT L5 DRGs (data not shown). Two weeks after viral delivery, there was a 3-fold increase in macrophage accumulation which increased to 4-fold by 3 weeks in AAV5-CCL2 mice compared to AAV5-YFP controls (Fig. 2A). This increase in CD11b-positive macrophages following CCL2 overexpression is similar to the injury-induced increase seen in WT mice at 7 days (Niemi et al., 2013). We also utilized a second macrophage marker, CD68, and saw a time-dependent increase in CD68 labeling compared to YFP controls (Figs. 2F–J). CD68 showed considerably more staining than CD11b, which could indicate that some of the CD68-positive macrophages are CD11b-negative.

Peripheral nerve injury has been shown to induce monocyte entry into ganglia through CCL2/CCR2 signaling (Niemi et al., 2013). The present data show that CCL2 overexpression is sufficient to cause macrophage accumulation within the L5 DRG even in the absence of nerve injury.

3.3. CCL2 overexpression and macrophage accumulation cause a conditioning-like increase in neurite outgrowth in vitro

To test if CCL2 overexpression and the subsequent increase in macrophage accumulation in the absence of injury enhance the regenerative capacity of DRG neurons, L5 DRGs from AAV5-CCL2 and AAV5-YFP mice were placed in explant culture 3 weeks after intrathecal injection. After 48 h in culture, DRG explants overexpressing CCL2 showed significantly longer neurite outgrowth than DRGs from mice receiving the control virus (Figs. 3A–C). We also measured regeneration in DRG dissociated cell cultures. We have found that dissociation of the DRG does not completely eliminate glial cells from the culture (data not shown); however, it does disrupt the proximity of non-neuronal cells and neurons. After 24 h in culture, DRG neurons overexpressing CCL2 grew significantly longer neurites than neurons from mice receiving the control virus (Figs. 3D–F). To examine whether the increased regenerative responses observed from both explant and dissociated cell cultures might be due to a direct effect of CCL2 acting on the DRG neurons at the time of regeneration, recombinant mouse CCL2 protein (200 ng/ml; Bianchi et al., 2005) was added to DRG explant cultures from WT mice. Exogenous CCL2 addition to culture medium did not increase the length of axon regeneration after 48 h in culture when compared to controls (Figs. 3G–I). The lack of an effect of exogenous CCL2 on neurite outgrowth could be due to inefficient penetration of the protein into the explants. To allow for better exposure of DRG neurons to CCL2, exogenous CCL2 was added to DRG dissociated cell cultures. However, exogenous CCL2 addition still had no effect on neurite outgrowth after 24 h in culture (Figs. 3J–L). This suggests that CCL2 does not act directly on neurons to induce increased regeneration. Thus, CCL2 exerts a conditioning-like effect to increase the regenerative capacity of neurons *in vivo*, prior to placing them in culture.

Macrophages have been shown to increase the regenerative response of DRG neurons through releasable factors (Kigerl et al., 2009); however, this ability was dependent upon the activation state of the macrophages. Macrophages stimulated to an anti-inflammatory state were growth-promoting, and pro-inflammatory macrophages were growth-inhibiting (Gordon and Taylor, 2005; Kigerl et al., 2009).

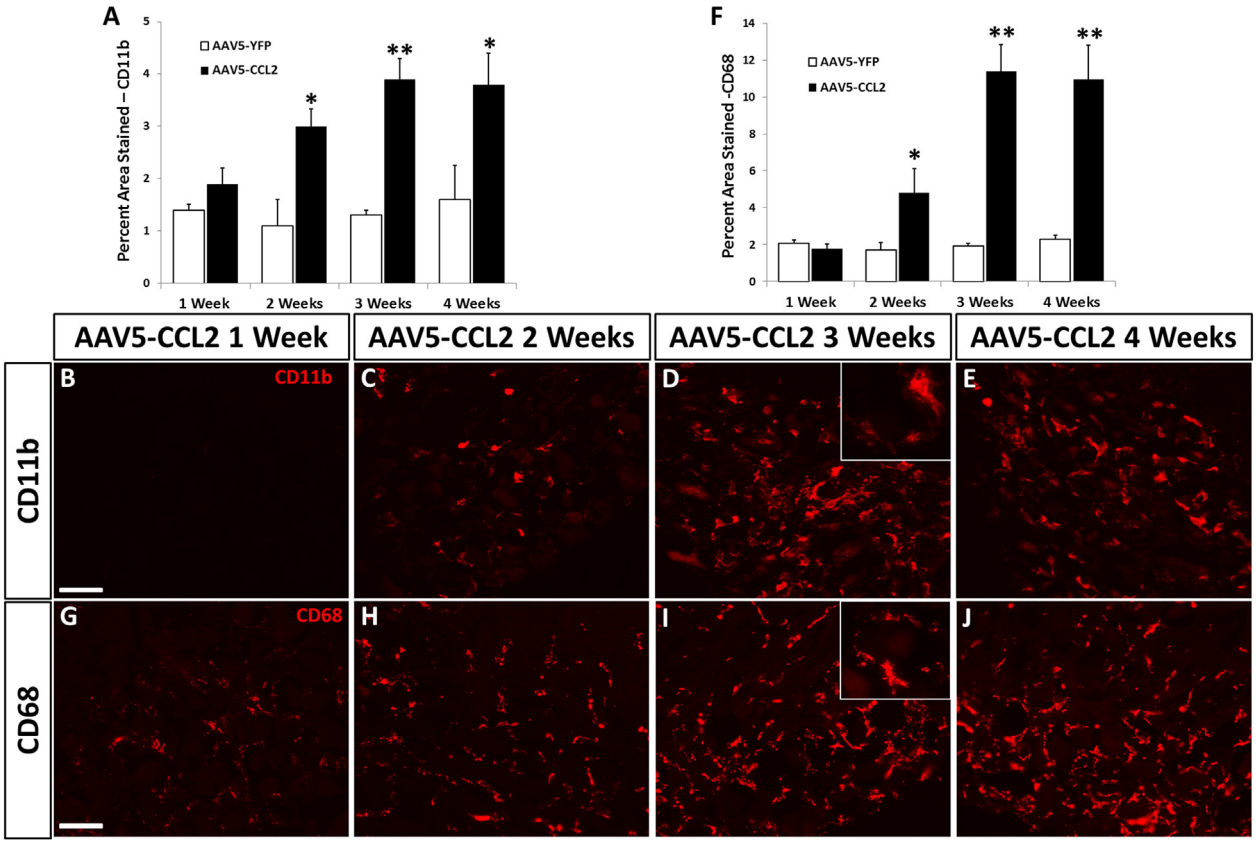


Fig. 2. CCL2 overexpression causes a time-dependent increase in CD11b- and CD68-positive macrophage accumulation in L5 DRGs. Immunohistochemical labeling of DRG sections with an antibody against CD11b (A) or CD68 (F) shows significant increases at 2, 3, and 4 weeks after injection of AAV5-CCL2 compared to AAV5-YFP (A, F). Data were quantified as a percentage of the tissue area positively immuno-labeled. Representative images of CD11b staining in L5 DRG 1, 2, 3, and 4 weeks after intrathecal injection of AAV5-CCL2 (B–E). Representative images of CD68 staining in L5 DRG 1, 2, 3, and 4 weeks after intrathecal injection of AAV5-CCL2 (G–J). Inset images taken at 63×. Scale bar = 100 μm. n = 5 per group. $^{*}p < 0.05$. $^{**}p < 0.001$.

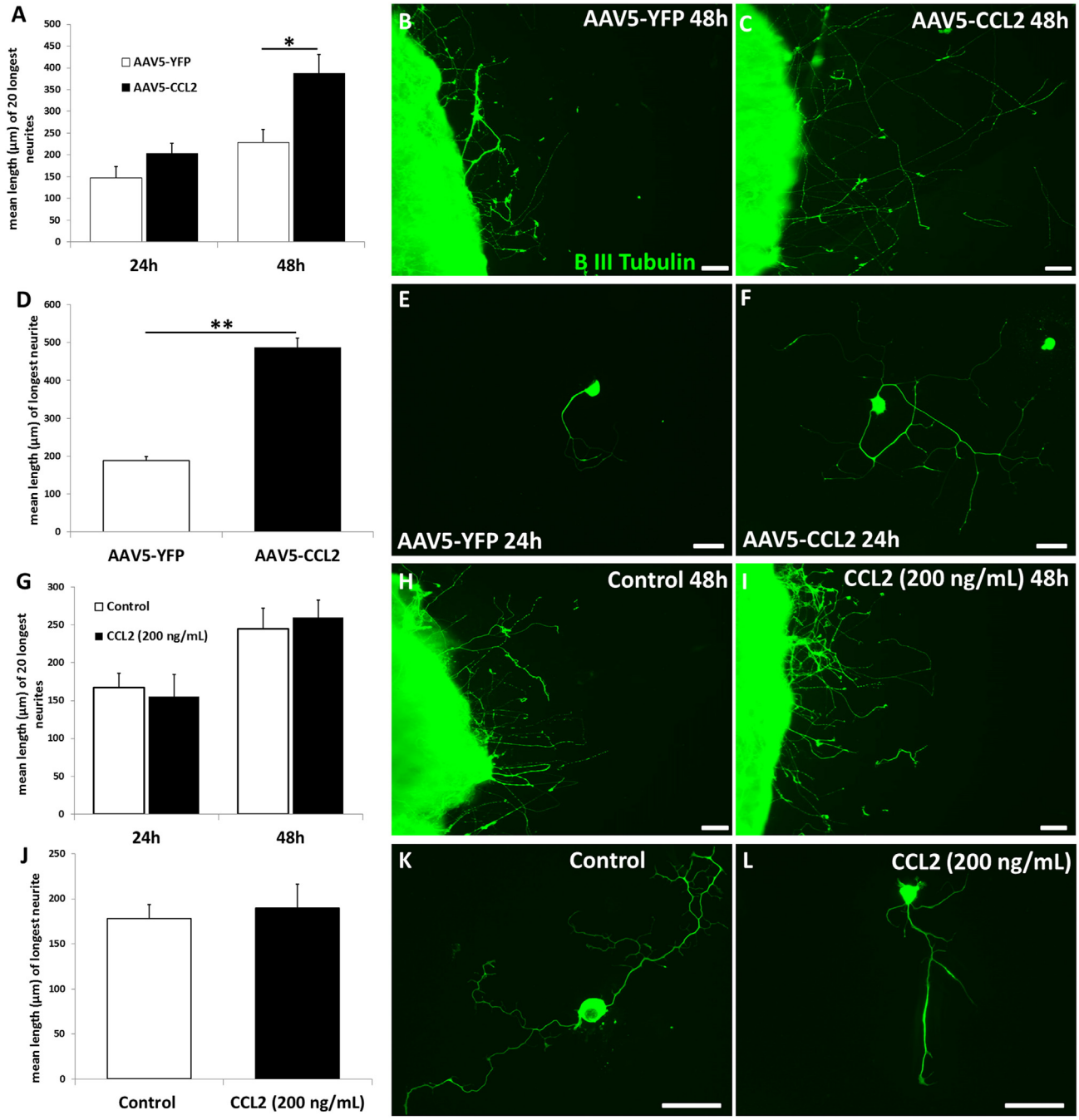


Fig. 3. A conditioning-like increase in neurite outgrowth from DRG neurons is observed with *in vivo* CCL2 overexpression but not after exogenous CCL2 addition at the time of culturing. Quantification of neurite outgrowth in explant culture measured after 24 and 48 h, 3 weeks after intrathecal injection of AAV5-CCL2 or AAV5-YFP (A). Data is represented as the mean length, in microns, of the 20 longest neurites. Representative images of neurite outgrowth after 48 h in explant culture, visualized with βIII tubulin, for AAV5-YFP (B) and AAV5-CCL2 (C). Quantification of neurite outgrowth in dissociated cell culture measured at 24 h, 3 weeks after intrathecal injection of AAV5-CCL2 or AAV5-YFP (D). Representative images of neurite outgrowth in dissociated cell culture at 24 h for AAV5-YFP (E) and AAV5-CCL2 (F). Quantification of neurite outgrowth in WT DRG explant culture measured after 24 and 48 h, with or without exogenous CCL2 (200 ng/ml) added to the culture medium at the time of plating (G). Representative images of neurite outgrowth after 48 h in culture for control (H) and exogenous CCL2 (I). Quantification of neurite outgrowth in WT DRG dissociated cell culture at 24 h, with or without exogenous CCL2 (200 ng/ml) added to the culture medium at the time of plating (J). Representative images of neurite outgrowth in dissociated cell culture for control (K) and exogenous CCL2 (L). Scale bar = 100 μm. For CCL2 overexpression explant cultures: n = 4 per group. $^{*}p < 0.05$. For CCL2 overexpression dissociated cell cultures: n = 180–251 neurons per group. $^{**}p < 0.001$. For exogenous CCL2 explant cultures: n = 6 per group. For exogenous CCL2 dissociated cell cultures: n = 253–397 neurons per group.

Given, the increased regeneration we see with CCL2 overexpression, we sought to determine if macrophages in our system expressed an activation state which would be expected to support regeneration. We assayed the mRNA expression of a subset of anti-inflammatory (arginase 1, CD206, Fizz 1 and Ym 1) and pro-inflammatory (iNos, CD86 and CD16) macrophage markers in L5 DRG (Fig. 4A; Gordon and Taylor, 2005; Martinez and Gordon, 2014). Expression of the anti-inflammatory markers CD206 and Fizz 1 significantly increased, 3.1 and 11.2-fold, respectively, 3 weeks after injection of AAV5-CCL2 compared to AAV5-YFP. mRNA levels for the pro-inflammatory macrophage markers did not change with CCL2 overexpression. This profile of anti-inflammatory macrophage marker expression is similar, though not

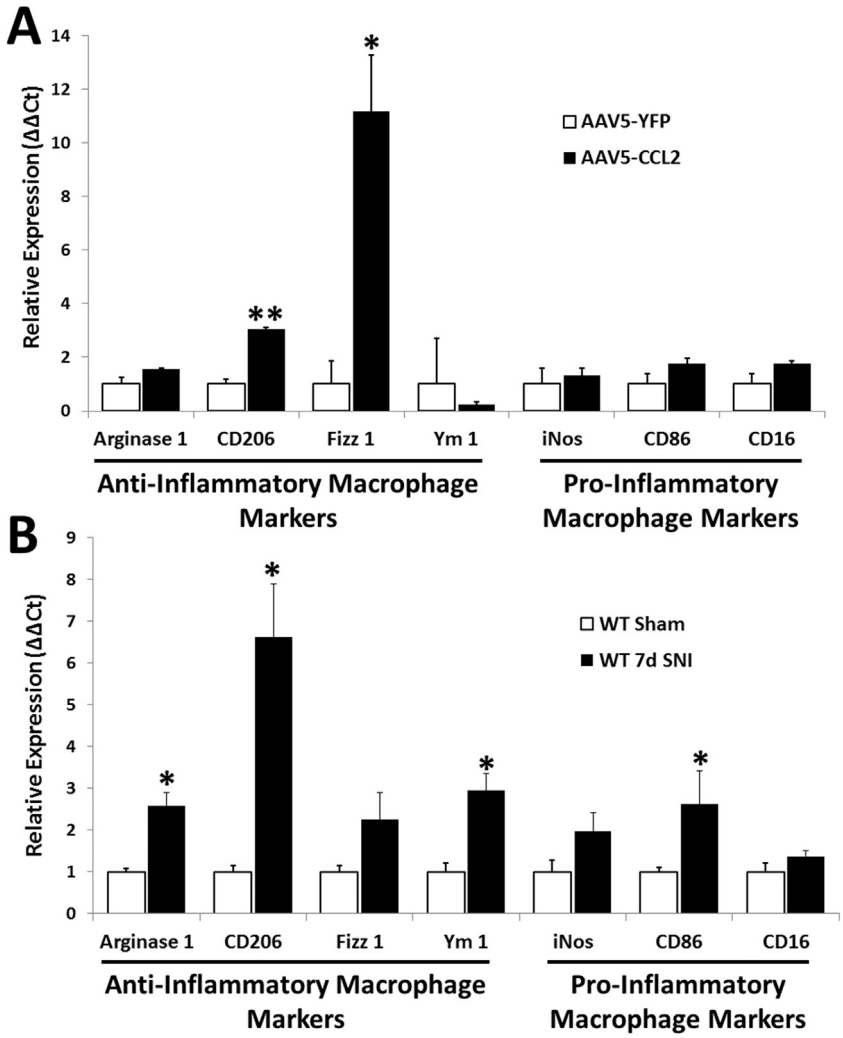


Fig. 4. Two anti-inflammatory macrophage markers and no pro-inflammatory markers are significantly increased in uninjured L5 DRGs 3 weeks after intrathecal injection of AAV5-CCL2. Relative expression of mRNA for 4 anti-inflammatory [arginase 1, CD206, Fizz1, and Ym1] and 3 pro-inflammatory [iNos, CD86, CD16] macrophage markers in L5 DRGs 3 weeks after injection of AAV5-CCL2 or AAV5-YFP (A) or 7 days after SNI in WT mice compared to sham controls (B). Data was quantified using the ΔΔCt method normalized to GAPDH. n = 5 per group. $^{*}p < 0.05$. $^{**}p < 0.001$.

identical, to the expression observed in WT L5 DRGs 7 days after SNI (Fig. 4B).

3.4. CCL2 acts through CCR2 to elicit changes in macrophage accumulation and regeneration

To determine if the changes mediated by CCL2 overexpression require the chemokine receptor CCR2, AAV5-CCL2 was delivered to CCR2 −/− mice (Boring et al., 1998). CCR2 is localized to a subpopulation of monocytes (Geissmann et al., 2003; Taylor and Gordon, 2003) and is necessary for their chemotactic responses to CCL2. CCR2 also plays an important role in monocyte exit from the bone marrow (Han et al., 1998; Serbina and Pamer, 2006). Three weeks after intrathecal injection of AAV5-CCL2, a significant increase in CCL2 mRNA (>7 fold) was found in CCR2 −/− L5 DRGs compared to that after injection with AAV5-YFP (Fig. 5A). However, CCL2 overexpression did not yield a significant increase in CD11b-positive macrophages in the L5 DRG 3 weeks after virus administration in CCR2 −/− mice (Fig. 5B). Furthermore, overexpression of CCL2 in CCR2 −/− mice did not result in increased axonal regeneration, measured at 24 and 48 h in DRG explant culture 3 weeks after intrathecal injection (Fig. 5C). These data show that CCL2 must act through its primary receptor CCR2 to elicit increases in macrophage accumulation and regeneration in the DRG.

3.5. CCL2 overexpression leads to a selective increase in LIF mRNA and activation of STAT3

To begin to ascertain the mechanism by which CCL2 overexpression in DRG neurons could lead to a conditioning-like increase in neurite outgrowth, the expression of various RAGs were screened in L5 DRGs 3 weeks after intrathecal injection of AAV5-CCL2 or AAV5-YFP. The mRNA expression of LIF, IL-6, GAP-43, JUN, ATF3, galanin, Smad1, and Sox11 were assayed using RT-PCR and quantified using the comparative Ct method with GAPDH as the housekeeping gene. CCL2 overexpression resulted in a significant increase in LIF expression (4-fold) compared to YFP controls (Fig. 6A). None of the other mRNAs showed a significant change in ganglia overexpressing CCL2. This is in contrast to the RAG expression profile observed 7 days after SNI where all of the RAGs measured, with the exception of LIF, show significant upregulation (Fig. 6B).

LIF signals through a heterodimeric receptor containing the signaling subunit gp130, and activates the transcription factor STAT3 by inducing its phosphorylation and nuclear translocation (Symes et al.,

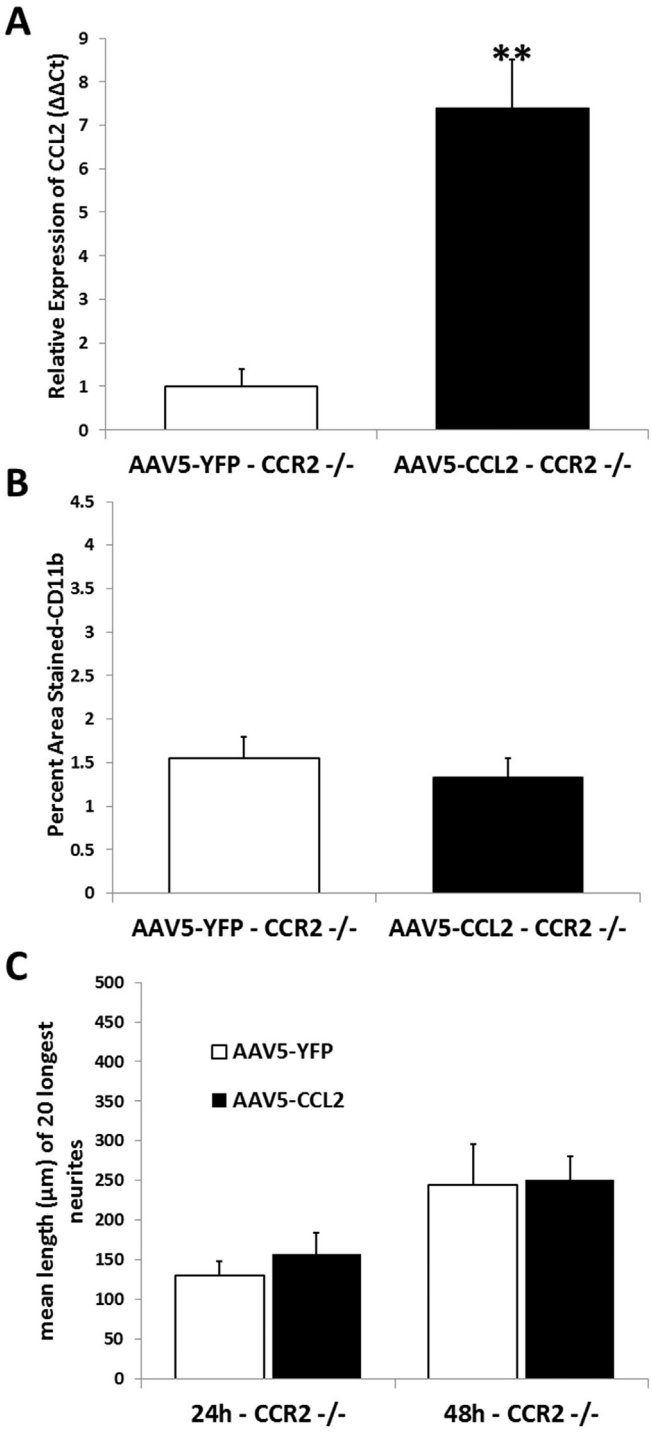


Fig. 5. Overexpression of CCL2 in CCR2 −/− mice does not cause macrophage accumulation or increased neurite outgrowth in L5 DRGs. Expression of CCL2 mRNA measured 3 weeks after intrathecal injection of AAV5-CCL2 or AAV5-YFP in CCR2 −/− mice, quantified using the ΔΔCt method normalizing to GAPDH (A). Immunohistochemical labeling of DRG sections with an antibody against CD11b shows no difference between AAV5-CCL2 and AAV5-YFP 3 weeks after injection (B). Data was quantified as a percentage of the imaged area positively immuno-labeled. Quantification of neurite outgrowth in explant culture measured at 24 and 48 h, 3 weeks after intrathecal injection of AAV5-CCL2 or AAV5-YFP in CCR2 −/− mice (C). Data are represented as the mean length, in microns of the 20 longest neurites. For RT-PCR: n = 5 per group. $^{**}p < 0.001$. For IHC: n = 5 per group. For explant cultures: n = 5 per group.

1994). Therefore, we next tested whether STAT3 phosphorylation was increased in DRG neurons following CCL2 overexpression. Three weeks after intrathecal injection of virus, a 3-fold increase in pSTAT3-positive labeling was seen in AAV5-CCL2 L5 DRGs compared to AAV5-YFP controls and CCR2 −/− mice overexpressing CCL2 (Figs. 6C–E,G). This increased activation of STAT3 in uninjured DRGs following CCL2 overexpression was nevertheless significantly lower than the activation of STAT3 7 days after sciatic nerve transection in WT mice (Figs. 6F,G). ATF3 and SPRR1a, two other well-known RAGs (Bonilla et al., 2002; Seijffers et al., 2007), were also assayed by IHC. Neither ATF3 (Figs. 6H–J,L) nor SPRR1a (Figs. 6M–O,Q) was upregulated following CCL2 overexpression, though both were upregulated 7 days after sciatic nerve transection (Figs. 6K,L,P,Q). Thus, the CCL2 overexpression-induced increase in axonal regeneration may result from activation of STAT3.

3.6. pSTAT3 is necessary for the conditioning-like increase in neurite outgrowth observed with CCL2 overexpression

To test whether the CCL2 overexpression-induced increase in neurite outgrowth is dependent upon STAT3 activation, we treated DRG cultured neurons with STAT3 phosphorylation inhibitors. We utilized the Janus kinase (JAK) inhibitor AG490 (Meydan et al., 1996) and the SH2-binding inhibitor STATTIC (Schust et al., 2006). Previous literature has shown that conditioning lesion-induced neurite outgrowth can be abolished through inhibition of STAT3 activation with AG490 in culture (Liu and Snider, 2001) or *in vivo* (Qiu et al., 2005).

After 24 h in culture, DRG neurons overexpressing CCL2 grew significantly longer neurites and showed significantly higher intensity nuclear pSTAT3 labeling than neurons from mice receiving the control virus (Figs. 7A–H). The addition of AG490 or STATTIC to the culture medium at the time of plating significantly reduced the CCL2 overexpression-induced neurite outgrowth compared to vehicle-treated CCL2 overexpressing neurons (Fig. 7A). This reduction in neurite outgrowth, similar to AAV5-YFP levels, coincided with a significant reduction in the pSTAT3 labeling intensity following treatment with AG490 or STATTIC (Fig. 7B). These data suggest that the CCL2 overexpression induced increase in neurite outgrowth is dependent upon the activation of pSTAT3.

4. Discussion

In the present study, we have demonstrated that overexpression of CCL2 in uninjured DRG neurons is sufficient to cause macrophage accumulation in the ganglion. We further showed that this overexpression alone results in a conditioning-like increase in neurite outgrowth, increased LIF mRNA, and activation of STAT3 in DRG neurons.

Sciatic nerve injury produces dramatic changes in DRG neurons and in non-neuronal cell populations both distal to the site of injury and in the ganglion (Bastien and Lacroix, 2014; Lieberman, 1971). These include activation and proliferation of satellite glial cells (Hanani et al., 2002), dedifferentiation and proliferation of Schwann cells distal to the injury site (Napoli et al., 2012), and macrophage accumulation distal to the site of injury and in the DRG (Lu and Richardson, 1993; Perry and Brown, 1992). In addition, the expression of hundreds of genes is altered (Costigan et al., 2002; Nagarajan et al., 2002). Therefore, the fact that overexpression of one gene, CCL2, is sufficient to increase the regenerative capacity of DRG neurons is remarkable.

4.1. Macrophage stimulation of axonal regeneration

Studies on the immune consequences of nerve injury have focused on actions at or distal to the injury site, in particular the role of macrophages in Wallerian degeneration (for reviews see Bruck, 1997; DeFrancesco-Lisowitz et al., 2014). The actions of multiple immune cells in the distal nerve may also have a bearing on the process of regeneration (*e.g.*, Brown et al., 1991; Schmid et al., 2013; Vargas et al., 2010). For example, inhibition of macrophage accumulation in the distal nerve following injury showed significant reductions in myelin clearance and significant delays in functional recovery of hind paw motor control

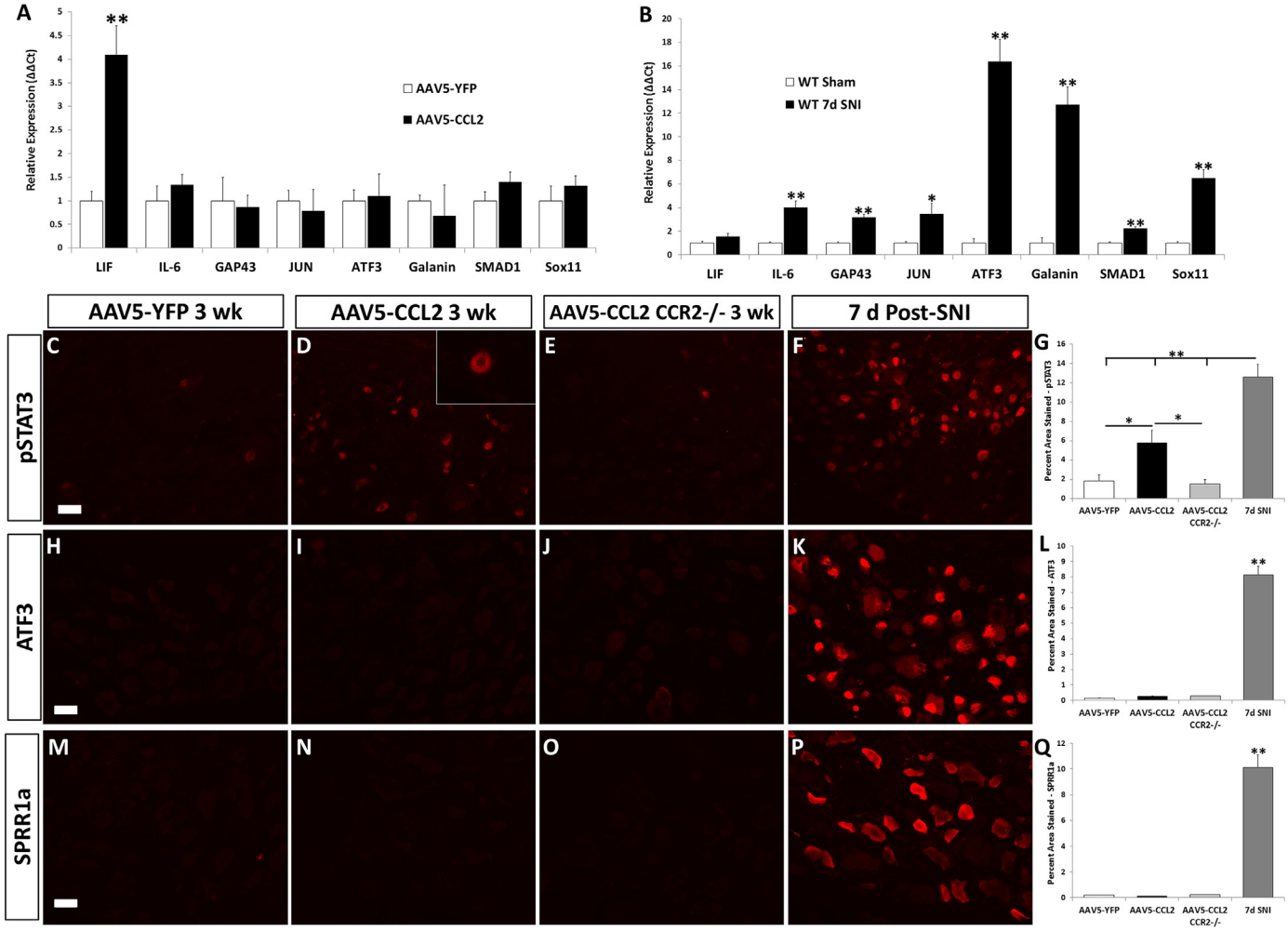
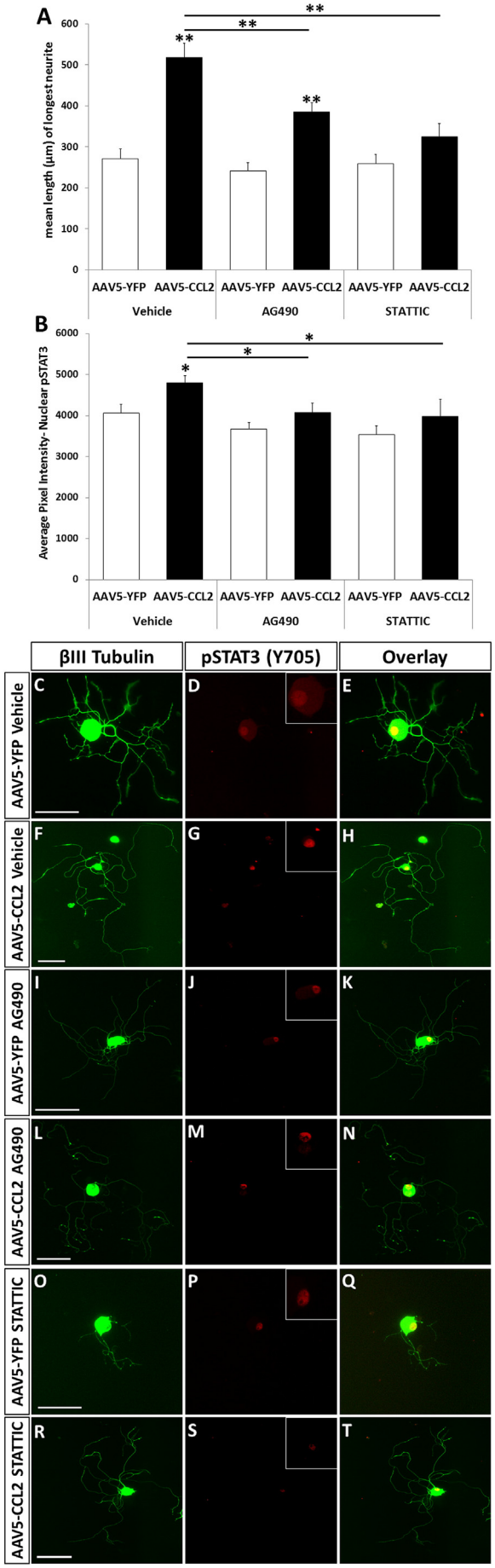


Fig. 6. LIF mRNA and pSTAT3 are significantly increased in uninjured L5 DRGs 3 weeks after intrathecal injection of AAV5-CCL2. Relative expression of various RAGs in L5 DRGs 3 weeks after intrathecal virus infection of uninjured DRGs quantified using the ΔΔCt method normalizing to GAPDH (A). Relative expression of various RAGs in L5 DRGs 7 days after SNI in WT mice compared to sham controls (B). Representative images of pSTAT3 staining in L5 DRGs 3 weeks after injection of AAV5-YFP (C), AAV5-CCL2 with inset image taken at 63× (D), AAV5-CCL2 in CCR2 −/− mice (E), or 7 days after SNI in WT mice (F). Quantification of pSTAT3 staining represented as the percentage of the imaged area positively immuno-labeled (G). Representative images of ATF3 staining in L5 DRGs 3 weeks after injection of AAV5-YFP (H), AAV5-CCL2 (I), AAV5-CCL2 in CCR2 −/− mice (J), or 7 days after SNI in WT mice (K). Quantification of ATF3 staining (L). Representative images of SPRR1a staining in L5 DRGs 3 weeks after injection of AAV5-YFP (M), AAV5-CCL2 (N), AAV5-CCL2 in CCR2 −/− mice (O), or 7 days after SNI in WT mice (P). Quantification of SPRR1a staining (Q). For RT-PCR: $^{*}p < 0.05$. $^{**}p < 0.001$. n = 5 per group. For IHC: scale bar = 100 μm. n = 8 per group. $^{*}p < 0.05$. $^{**}p < 0.001$.

(Barrette et al., 2008). Whether these two effects are causally related is not clear, however, because of the likelihood that in these experiments there were also decreases in macrophage accumulation in the DRG.

While macrophage accumulation in peripheral ganglia following axotomy was described in the 1990s (Lu and Richardson, 1993; Schreiber et al., 1995), their functions have not been clear. Injection of peritoneal macrophages or bacteria (*C. parvum*) into the DRG to induce a local inflammatory response resulted in increases in RAG expression and increased regeneration after a dorsal root injury though not after sciatic nerve transection (Lu and Richardson, 1993, 1995). Recent evidence has shown that inhibition of the injury-induced macrophage accumulation in lumbar DRGs results in the abolishment of the conditioning-lesion response both *in vitro* and *in vivo* (Kwon et al., 2013; Niemi et al., 2013).

What remains unclear is how macrophages influence the regenerative capacity of peripheral neurons. Neuroinflammation is often referred to as a "double-edged sword" as illustrated by the divergent role macrophages play in two CNS injury models. Depletion of hematogeneous macrophages leads to improved functional recovery after spinal cord injury (Popovich et al., 1999). However, stimulation of an immune response in the eye in conjunction with optic nerve injury significantly increases regeneration of retinal ganglion cell axons, though the involvement of both neutrophils and macrophages has been implicated (Kurimoto et al., 2013; Yin et al., 2003; Yin et al., 2006). These differing effects presumably result from the heterogeneity of macrophage activation following nervous system injury. Upon entry into tissue, monocyte-derived macrophages can take on a spectrum of activation states (for review see Gordon and Taylor, 2005; Martinez and Gordon, 2014). Representing the polar opposites on this spectrum, macrophage activation states have been described as pro-inflammatory/M1 or anti-inflammatory/M2 (Gordon and Taylor, 2005; Martinez and Gordon, 2014). Work by Kigerl et al. (2009) demonstrated that conditioned media from bone marrow-derived macrophages stimulated to an anti-inflammatory/M2 activation state with IL-4, induced significant increases in neurite outgrowth from DRG neurons on both growth-permissive and growth-inhibiting substrates. The identity of the factor(s) released from these M2 macrophages to stimulate axonal regeneration remains unknown. Given the increases in regeneration observed as a result of CCL2 overexpression, we hypothesized that the macrophages in our system are anti-inflammatory in nature. The upregulation of CD206 and Fizz 1 mRNA, two anti-inflammatory macrophage markers, and the lack of change in three pro-inflammatory markers are consistent with this hypothesis. CD206, a mannose receptor, is a useful marker of anti-inflammatory/M2 polarization as pro-inflammatory/M1



macrophages do not express this receptor (Alan et al., 1984). Fizz-1, a cysteine-rich secreted protein, has been implicated in the regulation of extracellular matrix and in wound healing (Holcomb et al., 2000), and is specifically expressed by macrophages in response to IL-4 both *in vivo* and *in vitro* (Raes et al., 2002).

4.2. Activation of a regeneration-associated signaling pathway

In a search for the mechanism by which CCL2 overexpression leads to a conditioning-like increase in neurite outgrowth, we assayed the expression of various RAGs, expecting to observe an increase in a number of mRNAs. Surprisingly of the eight RAGs we examined, we found that only LIF was significantly increased 3 weeks after intrathecal delivery of AAV5-CCL2. In addition, the main signaling mechanism by which LIF acts, namely the phosphorylation of STAT3, increased as a result of CCL2 overexpression. This specificity in CCL2 overexpression-mediated RAG expression suggests that LIF and STAT3 may be responsible for the increase in axonal regeneration. LIF has been implicated previously in conditioning lesion-induced sensory and sympathetic neuron regeneration (Cafferty et al., 2001; Hyatt Sachs et al., 2010).

Following sciatic nerve injury, LIF is expressed by Schwann cells in the nerve and is retrogradely transported to the DRG (Banner and Patterson, 1994; Curtis et al., 1994; Thompson et al., 1997). In addition, LIF expression increases in non-neuronal cells in the axotomized ganglia (*i.e.*, superior cervical ganglion), probably in satellite cells (Banner and Patterson, 1994; Sun et al., 1994). In our overexpression system, the cell expressing LIF has yet to be identified. While the 4-fold increase in LIF mRNA seen in DRGs following CCL2 overexpression is impressive, LIF expression in ganglia following axotomy reaches increases of >100-fold (Banner and Patterson, 1994; Habecker et al., 2009; Sun et al., 1996).

Axotomy induces the expression of both IL-6 and LIF in peripheral ganglia (Cafferty et al., 2004; Cafferty et al., 2001; Habecker et al., 2009; Sun et al., 1994). Thus, it was unexpected to see only LIF, and not also IL-6, mRNA increase with CCL2 overexpression. We also assayed the expression of two known gp130 cytokine/STAT3-dependent genes, galanin and GAP-43 (Ogai et al., 2014; Qiu et al., 2005; Rao et al., 1993; Zigmond, 2012) and found that their expression remained unchanged following CCL2 overexpression. Although initially surprising, this may indicate that LIF-induced gene expression requires more than a 4-fold increase in the cytokine. Another common RAG, ATF3, also remained unchanged following CCL2 overexpression. While ATF3 has been shown to play an important role in axon regeneration (Seijffers et al., 2006; Seijffers et al., 2007), its expression is not known to be gp130-dependent (Habecker et al., 2009).

Activation of the gp130 receptor subunit by LIF and other IL-6 family cytokines results in the activation of JAK2 and the phosphorylation of STAT3 (Heinrich et al., 1998). STAT3 activation has been shown to play a major role in axon regeneration following injury. STAT3 is activated in DRG neurons after a sciatic nerve injury, but not after a dorsal column lesion (Qiu et al., 2005). Inhibition of STAT3 phosphorylation, through administration of the JAK inhibitor AG490, results in significantly diminished DRG axonal regeneration *in vitro* after a conditioning lesion (Liu and Snider, 2001), as well as significantly reduced

Fig. 7. pSTAT3 is required for the CCL2 overexpression-induced increase in neurite outgrowth. Quantification of neurite outgrowth in DRG dissociated cell culture in the presence of inhibitors of STAT3 phosphorylation, AG490 (50 nM) or STATTIC (10 nM), or vehicle control, measured after 24 h, 3 weeks after intrathecal injection of AAV5-CCL2 or AAV5-YFP (A). Quantification of pSTAT3 nuclear pixel intensity in dissociated cell culture in the presence of the STAT3 phosphorylation inhibitors, AG490 or STATTIC, or vehicle control, measured after 24 h, 3 weeks after intrathecal injection of AAV5-CCL2 or AAV5-YFP (B). Representative images of neurite outgrowth and pSTAT3 are shown for AAV5-YFP vehicle (C–E), AAV5-CCL2 vehicle (F–H), AAV5-YFP AG490 (I–K), AAV5-CCL2 AG490 (L–N), AAV5-YFP STATTIC (O–Q), and AAV5-CCL2 STATTIC (R–T). Insets are digital magnifications of the neuronal cell body. Scale bar = 100 μm. For neurite outgrowth: n = 51–99 neurons per group. $^{**}p < 0.001$. For pixel intensity: n = 51–99 neurons per group. $^{*}p < 0.05$.

conditioning lesion-dependent outgrowth after a dorsal column lesion (Qiu et al., 2005). Utilizing *in vivo* imaging and selective deletion of STAT3 in DRG neurons, STAT3 was shown to play a role in the initiation of axonal regeneration following a sciatic nerve injury (Bareyre et al., 2011). Our finding that CCL2 overexpression-induced increases in neurite outgrowth are abolished through the application of STAT3 phosphorylation inhibitors aligns with this previous work. Thus, the conditioning-like increase in neurite outgrowth caused by CCL2 overexpression occurs *via* a STAT3-dependent mechanism.

4.3. CCL2 action in the ganglia

CCL2 is upregulated and expressed by neurons in peripheral ganglia following nerve injury (Niemi et al., 2013; Schreiber et al., 2001; Tanaka et al., 2004). This upregulation and its action through the chemokine receptor, CCR2, are necessary for the injury-induced increase in macrophage accumulation in the DRG (Niemi et al., 2013). CCL2 primarily acts as a monocyte chemokine by inducing CCR2-positive monocyte extravasation into tissue (Geissmann et al., 2003). Yet, CCL2 has also been shown to contribute to the development and maintenance of pain by acting directly on sensory neurons (Abbadie et al., 2009), and it has been demonstrated that CCL2 can depolarize DRG neurons, following pain-causing injuries, through direct action on the neurons (White et al., 2005). While CCL2 has also been shown to cause neurite outgrowth from embryonic avian statoacoustic ganglia, *in vitro* (Bianchi et al., 2005), in our preparation, CCL2 does not act directly on neurons to stimulate an increase in outgrowth.

Previous studies have also suggested that CCL2 can stimulate IL-4 production, a cytokine implicated in anti-inflammatory macrophage activation (Karpus et al., 1997). Evaluation of the general immune responses mounted in a CCL2 −/− mouse revealed decreased anti-inflammatory cytokine expression in response to bacterial immunization (Gu et al., 2000). Furthermore, macrophages stimulated with M-CSF *in vitro* in the presence of a CCL2 blocking antibody show significant increases in M1 or pro-inflammatory marker expression, while the presence of CCL2 induced expression of the anti-inflammatory cytokine IL-10 (Sierra-Filardi et al., 2014). It is interesting to note that we have observed increased protein expression of M-CSF in peripheral ganglia following nerve injury (Niemi and Zigmond, unpublished observations). These data suggest that CCL2 can lead to an anti-inflammatory stimulation of macrophages in addition to being chemotactic to CCR2-positive monocytes. This would favor a macrophage activation state which is capable of promoting axonal growth.

5. Conclusions

In conclusion, CCL2 overexpression induces macrophage accumulation in uninjured lumbar DRG and leads to a conditioning-like increase in axonal outgrowth. CCL2 overexpression leads to activation of STAT3, a regeneration-associated signaling molecule. CCL2 must act through its primary receptor, CCR2, to elicit subsequent changes in macrophage accumulation and neurite outgrowth. Finally, the conditioning-like increase in neurite outgrowth induced by CCL2 overexpression is abolished through inhibition of STAT3 phosphorylation. Taken together, CCL2 overexpression in DRG neurons is sufficient to increase the regenerative capacity of DRG neurons through a STAT3-dependent mechanism.

Author contributions

J.P.N. and R.E.Z. designed research; J.C. contributed materials and reagents; J.P.N., A.D.-L., and M.H. performed research; J.P.N. and R.E.Z. analyzed data; and J.P.N. and R.E.Z. wrote the paper.

Conflicts of interest

None.

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