IL-4 drives microglia and macrophages towards a phenotype conducive for tissue repair and functional recovery after spinal cord injury

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Main points

- IL-4 protein levels are undetected in the spinal cord after contusion injury
- Administration of IL-4 induces the expression of M2 markers in microglia and macrophages and reduces tissue damage and functional deficits after spinal cord injury.

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ABSTRACT

Macrophages and microglia play a key role in the maintenance of nervous system homeostasis. However, upon different challenges, they can adopt several phenotypes, which may lead to divergent effects on tissue repair. After spinal cord injury (SCI), microglia and macrophages show predominantly pro-inflammatory activation and contribute to tissue damage. However, the factors that hamper their conversion to an anti-inflammatory state after SCI, or to other protective phenotypes, are poorly understood. Here, we show that IL-4 protein levels are undetectable in the spinal cord after contusion injury, which likely favors microglia and macrophages to remain in a pro-inflammatory state. We also demonstrate that a single delayed intraspinal injection of IL-4, 48 hours after SCI, induces increased expression of M2 marker in microglia and macrophages. We also show that delayed injection of IL-4 leads to the appearance of resolution-phase macrophages, and that IL-4 enhances resolution of inflammation after SCI. Interestingly, we provide clear evidence that delayed administration of IL-4 markedly improves functional outcomes and reduces tissue damage after contusion injury. It is possible that these improvements are mediated by the presence of macrophages with M2 markers and resolution-phase macrophages. These data suggest that therapies aimed at increasing IL-4 levels could be valuable for the treatment of acute SCI, for which there are currently no effective treatments.

INTRODUCTION

SCI elicits an inflammatory response that comprises mainly of microglia and peripheral blood-derived macrophages (Alexander and Popovich 2009; David et al. 2012). These cells, which remain chronically in the spinal cord in humans after injury (David et al. 2012; Fleming et al. 2006), contribute directly or indirectly to tissue damage and functional loss in SCI, as well as, in other central nervous system disorders. However, they can promote repair in other experimental paradigms (Alexander and Popovich 2009; David et al. 2012; Mills et al. 2000; Popovich and Longbrake 2008; Yong and Rivest 2009). These paradoxically conflicting actions of microglia and macrophages may depend on their activation state and the signals in the lesion milieu. In response to IFNγ and/or LPS, macrophages undergo "classical" M1 activation (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010). However, upon IL-4 and IL-13 stimulation, they acquire "alternative" M2 differentiation (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010; Mills et al. 2000; Murray et al. 2014). M1 macrophages release high levels of pro-inflammatory cytokines and free radicals that are crucial for killing microbes and tumor cells, but also induce damage in healthy neighboring tissue, contributing to cell loss and pathology (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010). M2 macrophages, display anti-inflammatory features, and are involved in parasite containment, tumor progression, as well as, in promoting tissue repair (David and Kroner 2011; Dey et al. 2014; Gordon and Martinez 2010). Recently, concerns have been raised about such classification since macrophages in tissues are influenced by multiple factors that can influence their polarization state (Murray et al. 2014). Indeed, different macrophage subsets have been identified in several pathologies, including $Ly6C^{high}$ and $Ly6C^{low}$ monocytes (Arnold et al. 2007), tumor-associated macrophages (Noy and Pollard 2014) and resolution-phase macrophages (Bystrom et al. 2008), among others. However,

the concept of a pro-inflammatory/M1-like phenotype and an anti-inflammatory/M2-like phenotype, is a useful tool to probe for responses to injury (Murray et al. 2014).

Microglia and macrophages display a predominantly pro-inflammatory/M1-like phenotype after SCI (Kigerl et al. 2009; Kroner et al. 2014). However, there is currently limited information about the factors that prevent these cells from acquiring a pro-repair M2-like phenotype after SCI. This is likely related to the post-injury milieu of the spinal cord, since M2 macrophages rapidly lose such markers when transplanted into the contused spinal cord (Kigerl et al. 2009). A recent report reveals that phagocytosis of erythrocytes, which occurs between day 7 and 14 after SCI, prevents macrophages to adopt a pro-repair/M2-like polarization via release of TNFα (Kroner et al. 2014). There is also evidence that TNFα can prevent the myelin phagocytosis-induced shift of M1 macrophages to M2 (Kroner et al. 2014). However, the existance of mechanisms occuring at early stages after SCI that may hamper the convertion of microglia and macrophages from pro-inflammatory towards a phenotype that is more conducive for tissue repair has yet to be elucidated. Moreover, it is also not known whether effective induction of such phenotype in microglia and macrophages in the injured spinal cord attenuates functional deficits and tissue damage. Answers to these questions are highly relevant to advancing therapeutics to treat SCI in humans.

In the present work, we demonstrate that IL-4 protein levels are undetected after SCI. We reveal that a single administration of IL-4 delayed 48h after injury is sufficient to induce the expression of M2 markers in microglia and macrophages, as well as, to promote the appearance of a macrophage subset that has a phenotype compatible with resolution-phase macrophages. Finally, we show that such changes in microglia and macrophage phenotype triggered by delayed IL-4 treatment is associated with improved tissue protection and functional recovery.

MATERIALS AND METHODS

All the experimental procedures were approved by the Universitat Autònoma de Barcelona Animal Experimentation Ethical Committee (CEEAH 1188R3-DMAH 6131) and followed the European Communities Council Directive 2010/63/EU, and the methods for each procedure were carried out in accordance with the approved guidelines.

Surgical procedure

Adult (8-10 weeks old) female C57Bl/6 mice (Charles River) were anesthetized with ketamine (90 mg/kg, i.m.) and xylazine (10 mg/kg, i.m.). After performing a laminectomy at the 11th thoracic vertebrae, the exposed spinal cord was contused with a force of 50 kdynes using the Infinite Horizon Impactor device (Precision Scientific Instrumentation) (Klopstein et al. 2012). 15 minutes or 48 hours after SCI, 1µl of saline or saline containing 100 ng of recombinant mouse IL-4 (eBioscience) was injected into the injured spinal cord at the lesion site by means of a glass micropipette (30 µm internal diameter, Eppendorf, Hamburg, Germany) coupled to a 10 ml Hamilton syringe (Hamilton #701, Hamilton Co, Reno, NV, USA). 100ng of IL-4 was chosen based on a recent report from our laboratory using another anti-inflammatory cytokine (Coll-Miro et al. 2016). Injections were made at a perfusion speed of 2 µl/min controlled by an automatic injector (KDS 310 Plus, Kd Scientific, Holliston, MA, USA), and the tip of the needle was maintained inside the cord tissue 3 min after each injection to avoid liquid reflux.

Cytokine Protein Expression

Adult female C57/Bl6 mice were perfused with sterile saline and a 5 mm length of intact or contused spinal cord was collected at 1, 6, 12 hours and at 1, 3, 7, 14 and 28 days after contusion (n=4 each group and time point) and snap-frozen. Spinal cords were homogenized and protein concentration was determined using the DC Protein Assay (Bio-Rad). Samples were concentrated

to 4µg/µl using MicroCon centrifugation filters (Millipore) to ensure equal amounts of protein. Cytokine protein levels were then analyzed using the Milliplex MAP Mouse Cytokine/Chemokine magnetic bead panel (Millipore) on a Luminex (Millipore) as per manufacturers' protocol.

Flow Cytometry

Immune cells from the injured spinal cord were analyzed by flow cytometry. Briefly, spinal cords were cut in little pieces and mechanically dissociated through a cell strainer of 70 µm (BD falcon) and the cell suspension was centrifuged twice at 300g for 10 minutes at 4ºC. Cell suspension from each sample was divided in 6 tubes, and each of them was incubated with combination of different antibodies. The following rat conjugated antibodies were used: CD45-PerCP (1:150 eBioscience), CD11b-PE-cy7 (1:150 eBioscience), Ly6G-PE (1:150 eBicoscience), F4/80-APC or PE, (1:150 eBioscience), CD16/32-PE (1:100 eBioscience) CD206-FITC (1:100 eBioscience), IL-4Rα-APC (1:100 Miltenyi Biotec) MHC-II (1:100 eBioscience), CD11c (1:100 eBioscience), TGFβ1-Alexa647 (1:100 Abcam), IL-10-Alexa488 (1:100 eBioscience) and unconjugated rabbit antibodies against 15-LOX (1:100 Bioss Biotechnology), COX-2 (1:100 Cayman Chemical), iNOS (1:100 Abcam) and goat antibodies against Arg1 (1:100 Santa Cruz). After 30 min of incubation with combinations of antibodies at 4°C cell were then fixed in 1% paraformaldehyde. For intracellular staining, cells were permeabilized with Permeabilization Wash Buffer (Invitrogen) and followed by staining with Alexa488 or Alexa647 conjugated donkey secondary antibodies against rabbit or goat (1:500 Molecular Probes) for 30 min when needed. When intracellular cytokine staining was performed, cells were incubated with brefeldin (1:1000, Biolegend) at 37º for 4 hours before the incubation with the antibodies against cytokines. Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences) and results analyzed using FlowJo® software version 10.0.7.

To perform the analysis, cells were first gated for CD45 to ensure that only infiltrating leukocytes and resident microglia are selected, and then, the following combination of markers were used to identify microglia ($CD45^{low}$, $CD11b^{+}$, $F4/80^{+}$), macrophages ($CD45^{high}$, $CD11b^{+}$, $F4/80^{+}$), and neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+}$). Since cell-associated fluorescence varies between the different immune cell subsets, the cut off expression was defined based on isotype controls for each of the immune cell subsets and sample. The number of neutrophils in the injured spinal cord was estimated by counting all the $CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+}$ cells present in one of the six tubes each spinal cord cell suspension was divided, and then, multiplied 6 times.

Functional assessment

Locomotor recovery was evaluated at 1, 3, 5, 7, 10, 14, 21 and 28 days post-injury (dpi) in an open-field test using the nine-point Basso Mouse Scale (BMS), which was specifically developed for locomotor testing after contusion injuries in mice (Basso et al. 2006). The BMS analysis of hindlimb movements and coordination was performed by two independent assessors and the consensus score taken. In addition, at the end of the follow up (day 28 post-injury) the highest locomotion speed of the mice was evaluated on a belt of a motorized treadmill. Briefly, each mouse was allowed to explore the treadmill compartment, with the motor speed set to zero, for 5min. Then speed was gradually increased from 0 up to 35 cm/s and the maximum speed at which each mouse performed for at least 5 seconds was recorded (Santos-Nogueira et al. 2015).

At day 28, electrophysiological tests were used to evaluate spared motor central pathways after SCI. Motor evoked potentials (MEPs) were recorded from the *gastrocnemius* muscle (GM) with microneedle electrodes, in response to transcranial electrical stimulation of the motor cortex by single rectangular pulses of 0.1ms duration. Pulses were delivered through needle electrodes inserted subcutaneously, the cathode over the skull overlying the sensoriomotor cortex and the

anode at the nose (Santos-Nogueira et al. 2015). All potentials were amplified and displayed on a digital oscilloscope (Tektronix 450S)

Histology

At 28 days post-injury mice were perfused with 4% paraformaldehyde in 0.1M-phosphate buffer (PB) at 12h, 3 and 28 dpi. A 5mm length of spinal cord containing the lesion site was removed, cryoprotected with 30% sucrose in 0.1M PB at 4ºC, and 6 series of 10µm thick section were picked up on glass slides. Adjacent sections on the same slide were therefore 100µm apart. For demyelination analyses, sections were stained with Luxol fast blue (LFB) (Sigma). After graded dehydration, sections were placed in a 1 mg/ml LFB solution in 95% ethanol and 0.05% acetic acid overnight at 37ºC. Sections were then washed in 95% ethanol and distilled water before place them into a solution of 0.5 mg/ml Li2CO3 in distilled water for 1 min at RT. After washes in distilled water, sections were dehydrated and mounted in DPX mounting media (Sigma-Aldrich). For neuronal assessment, sections were incubated overnight at 4ºC with biotinilated antibodies against NeuN (1:500; Millipore). After several washes in PBS, sections were incubated with Alexa 594-conjugated streptoavidin, and then coverslipped in Mowiol mounting media (Sigma-Aldrich).

The epicenter of the injection or contusion injury impact was determined for each mouse spinal cord by localizing the tissue section with the greatest damage using LFB stained section. Myelin sparing after SCI was calculated by delineating the spared LFB stained tissue, whereas neuronal survival was assessed by counting the number of $NeuN^+$ cells in the ventral horns at the injury epicenter and at rostral and caudal areas. The NIH ImageJ software was used to quantify all the histological parameters.

Statistical analysis

All analyses were conducted with SPSS version19. Changes in the expression of M1 and M2 markers after spinal cord were analyzed by using one-way ANOVA with Tukey post-hoc comparisons. Functional follow-up for BMS score and subscore, histological analysis of myelin and neuronal sparing were analyzed using two-way repeated measure ANOVA with Bonferroni's correction for multiple comparison. Student t-test was used for single comparisons between two groups. Results are expressed as mean ± SEM. Differences were considered significant at $p < 0.05$.

RESULTS

Microglia and macrophages do not acquire anti-inflammatory phenotype after SCI

Previous reports describe that microglia and macrophages express predominantly M1 markers for the first 2 weeks after SCI, whereas the expression of M2 signatures is scarce (Kigerl et al. 2009; Kroner et al. 2014). Here we first extended these observations using fluorescence-activated cell sorting analysis (FACS) to assess the temporal changes in expression of M1 (iNOS and CD16/32) and M2 (Arg1 and CD206) markers in microglia and macrophages for the first 4 weeks after spinal cord contusion injury in mice. We confirmed that these cells express mainly M1 markers after SCI, whereas the expression of M2 markers is restricted to a small population of microglia and macrophages (Fig. 1A-E). However, the expression of the M1 markers did not show a similar pattern. Most microglial cells and macrophages expressed CD16/32 for the entire 4 weeks period, however, this expression was more pronounced in microglia than macrophages (Fig. 1E). The expression of iNOS, which was scarcely detected in microglial cells from uninjured spinal cords (~15%), was induced in ~70% of microglia and found in ~55% of macrophages for the first 3 days

post-injury. However, we found that iNOS levels markedly dropped in microglia at day 28, when it reached basal levels, but also, although to lesser extent, in macrophages (Fig. 1E).

These results suggest that microglia and macrophage express predominantly M1 markers during the first days after SCI, and fail to adopt an M2-like phenotype.

The lack of IL-4 expression in the injured spinal cord favors pro-inflammatory activation of microglia and macrophages

Since cytokines are one of main factors that regulate the functional phenotype of microglia and macrophage *in vitro* (Boche et al. 2013; David and Kroner 2011; Mantovani et al. 2013), we monitored the expression of the main cytokines in the spinal cord tissue following contusion lesion. We found that protein levels for most pro-inflammatory cytokines peaked between 6-12 hours post-injury (Fig. 2). The reduction of cytokines was already evident at 24 hours post-injury, and most of them reached basal levels at day 3. A small number of cytokines, however, remained elevated up to day 28, although to much lower levels as compared to the first 12 hours post-injury (Fig. 2). Interestingly, the protein levels of IL-4, one of the main M2 inducers, was undetected at any time point (Fig. 2), which led us to hypothesize that the insufficient expression of IL-4 after SCI could account for the deficit of microglia and macrophages to adopt an M2-like phenotype.

To address this hypothesis, we first characterized the expression of IL-4Rα in the spinal cord by FACS analysis. We found that IL-4Rα was not expressed in the uninjured spinal cord spinal but was found after contusion injury (Fig. 3A). Interestingly, we observed that ~90% of the IL-4Rα+ cells are also CD45+, indicating the presence of this receptor is mainly restricted to microglia and infiltrating myeloid cells in the injured spinal cord. We then studied whether IL-4Rα was present in microglia and macrophages based on CD45, CD11b and F4/80 expression. Although microglia did not show constitutive expression of IL-4Rα, it was induced in ~55 % of microglia at 18 hours

after injury, remaining at steady levels at day 2 (Fig. 3B). In addition, peripheral macrophages, which migrate into the spinal cord after injury, showed similar proportion of IL-4Rα at the same time points (Fig. 3B).

Since IL-R4α was found in microglia and macrophages only after SCI, we then assessed the effects of increased levels of IL-4 in the contused spinal cord parenchyma, by injecting recombinant mouse IL-4 into the spinal cord, 15 minutes after contusion injury in adult mice. We observed that IL-4 induced the expression of Arg1 in ~30% of microglia and macrophages at day 3, but failed to modulate the levels of CD206 (Fig. 4, Fig. 5A). IL-4, however, did not reduce CD16/32 and iNOS expression in these cells (Fig. 4, Fig. 5A). This data shows that a single acute administration of IL-4 after SCI drives some aspects of M2 polarization in a small proportion of microglia and macrophages, suggesting that the insufficient levels of this cytokine in the contused spinal cord contributes to hamper the conversion of microglia and macrophages towards a more pro-repair phenotype after SCI.

Effects of delayed administration of IL-4 on microglia and macrophage phenotype

We hypothesized that the high levels of pro-inflammatory cytokines in the contused spinal cord within the first 6-12 hours, some of them being well known M1 inducers, together with the absence of IL-4R in the intact spinal cord, may have minimized the effects of *acute* administration of IL-4 (15 minutes after SCI) on the phenotype of microglia and macrophages. We therefore evaluated whether delaying the administration of IL-4 to 48 hours post-injury, when levels of most pro-inflammatory cytokines have markedly dropped and the expression of IL-4R is found in ~55% of microglia and macrophages (Fig. 3B), would be more effective in inducing an anti-inflammatory/pro-repair phenotype. In contrast to Arg-1, being expressed in ~30% of microglia and macrophages after acute injection (15 minutes post-SCI), delaying the injection of IL-4 to 48

hours post-SCI, increased Arg-1 expression in ~55% macrophages and in about 35% of microglia (Fig. 5B; Fig. S1). Moreover, in contrast to acute injection, delayed IL-4-administration also induced the expression of the M2 marker CD206 in microglia and macrophages (~25%) (Fig. 5B), indicating that delayed IL-4 treatment leads to rapid and more effective induction of M2 markers in a subset of microglia and macrophages in the injured spinal cord.

We also found that the expression of CD16/32 is increased slightly in microglia and macrophages after delayed injection of IL-4, whereas the expression of iNOS remained unaltered (Fig. 5B, Fig. S1).

Given previous reports showing that M2 macrophages lose Arg1 expression within 3 days after their transplantation into the injured spinal cord (Kigerl et al. 2009; Kroner et al. 2014), we assessed whether the responses of microglia and macrophages to delayed administration of IL-4 were acute or persisted for a few days. We found that 4 days post-injection (i.e., 6 days after SCI), the expression of Arg1 and CD206 in microglia and macrophages remained at similar levels as compared to day 1 post-injection (Fig. 5C, Fig. S2), indicating that the expression of M2 markers is maintained for up to 4 days. The expression of CD16/32 in microglia and macrophages, which was increased at day 1 post-delayed injection, was reduced back down to saline-injected mice, whereas the expression of iNOS was significantly reduced in both immune cell populations at 4 days after IL-4 injection (Fig. 5C, Fig S2). These results therefore suggest that microglia and macrophages are more skewed toward anti-inflammatory/M2-like activation over time after a single delayed injection of IL-4.

Delayed administration of IL-4 induces the appearance of resolution-phase macrophages

Spinal cords that received a delayed injection of IL-4 and taken 6 days post-injury for flow cytometry analysis of CD11b and CD45 showed the 2 expected populations of macrophages

(CD11b+, CD45^{high}) and microglia (CD11b+, CD45^{low}) (Fig 6A). Strikingly, the IL-4 treated spinal cord also showed another cell population that has higher levels of CD45 and lower levels of CD11b as compared to the infiltrating macrophage population. On further characterization of this immune cell subset we found that they expressed F4/80 and MHCII, but did not display CD11c, suggesting they were macrophages (Fig. 6B). Moreover, they expressed iNOS, CD16/32, CD206 and Arg1, indicative of a mixed M1/M2 phenotype (Fig. 6B). A variant activated form of macrophage was reported recently in the resolution phase of inflammation elicited by intraperitoneal administration of zymozan (Bystrom et al. 2008; Stables et al. 2011). These macrophages, referred to as "resolution-phase" macrophages express a mixed M1/M2 phenotype (expressing MHCII but lacking CD11c expression) (Bystrom et al. 2008; Li et al. 2013; Stables et al. 2011), like the new macrophage subset we see in the spinal cord after delayed IL-4 injection. In addition, these cells were reported to express anti-inflammatory cytokines as well as lipoxygenase (LOX) and cyclooxygenase (COX-2), key enzymes in the synthesis of the specialized pro-resolving lipid mediators, which turn on the resolution programs of inflammation (Bystrom et al. 2008; Li et al. 2013; Schwab et al. 2007; Serhan 2014). We therefore assessed whether the new macrophage subset found after delayed IL-4 injection also displayed these resolution-phase markers. Interestingly, these macrophages expressed 5-LOX, 15-LOX and COX-2 (Fig. 6B), as well as the anti-inflammatory cytokines IL10, and TGFβ1 (Fig. 6b; table 1). Note that IL-4 only increased the expression of these anti-inflammatory/resolving markers in this new macrophage subset, but not in microglia or macrophages, with the exception for 15-LOX, which was increased by ~25% in macrophages after IL-4 administration (Table 1). Therefore, these data indicate that delayed administration of IL-4, which modulates microglia and macrophage

phenotype after SCI, also leads to the appearance of a macrophage subset, which phenotypically resembles "resolution-phase" macrophages.

Since "resolution-phase" macrophages are important in resolving inflammation, we next investigated whether the delayed intraspinal injection of IL-4 enhanced the clearance of neutrophils from the contused spinal cord, a key step in the resolution of inflammation (Schwab et al. 2007; Serhan 2014). Neutrophil infiltration peaks in the contused spinal cord at day 1 after injury and decreases gradually in 7-10 days (David et al. 2012; Stirling and Yong 2008), which allowed us to assess whether their elimination was speeded by delayed administration of IL-4. Interestingly, we found that IL-4 injection reduced the number of neutrophils ($CD45^{high}$, $CD11b^{+}$, $F4/80^{-}$, $Ly6G^{+}$) by ~40% in the injured spinal cord at 6 days post-injury (i.e. 4 days post-injection) (Fig. 6c), supporting the idea that the resolution of inflammation is enhanced by IL-4.

Effects of delayed administration of IL-4 on functional and histopathological outcomes after SCI

We finally assessed whether delayed administration of IL-4 also led to functional recovery and reduced tissue damage after SCI. We found that delayed IL-4 treatment conferred significant protection against loss of locomotor function after SCI based the on the BMS scale (Fig. 7A,B). At day 28 post-injury (the latest time point examined), 90% of saline-injected (control) mice showed plantar placement of the hindlimbs and only 40% displayed occasional stepping. However, all the IL-4-injected mice showed plantar placement and 83% of them showed occasional or frequent plantar stepping. In addition, mice treated with IL-4 were able to perform faster locomotion on the treadmill (Fig. 7C). Additionally, electrophysiological evaluation of motor evoked potentials revealed that IL-4 treatment led to ~2.5-fold greater preservation of central

descending axonal pathways, and conduction was slightly but significantly faster (Fig. 7D-E), indicating that delayed IL-4 administration led to greater integrity of spinal motor pathways.

In line with the functional data, histopathological analysis revealed that IL-4 treatment led to significant enhancement in myelin sparing at the injury epicenter and in adjacent areas (Fig. 7F-G), as well as, greater preservation of neurons but only at caudal regions to the injury epicenter (Fig. 7H-I). Overall, this data provides clear evidence that delayed administration of IL-4 enhances functional and histological outcomes after SCI.

DISCUSSION

At present, there is no effective treatment for acute SCI, however, a large body of experimental reports reveal that inflammation exacerbates tissue damage and functional loss after SCI (David et al. 2012; Hawthorne and Popovich 2011; Ren and Young 2013). This is due, in part, to the release of numerous mediators from microglia and macrophages that exert cytotoxic effects on CNS cells that can lead to demyelination and neuronal loss (David et al. 2012; Hawthorne and Popovich 2011; Ren and Young 2013). Nevertheless, these cells also produce a variety of factors that promote cell survival and tissue healing, and under certain circumstances, may lead to protective effects in the injured CNS (David et al. 2012; Popovich and Longbrake 2008). These conflicting actions of microglia and macrophages are due to the plasticity of these cells that depends on factors in the immediate environment. Thus, understanding the mechanisms that drive microglia and macrophage activation toward a phenotype that is conducive for tissue repair can lead to therapies that are more effective to treat acute SCI.

In agreement with previous studies, we show here that microglia and macrophages express predominantly M1 markers after SCI, whereas the expression of M2 markers is restricted to a small

subpopulation, indicating that these cells adopt predominantly a pro-inflammatory phenotype after SCI (Kigerl et al. 2009; Kroner et al. 2014). Our data, in agreement with previous reports, also highlights that microglia and macrophages after SCI cannot be defined within the simple M1-M2 classification described for cells in culture, but fall into a spectrum of activation states depending the stimuli acting on these cells and the markers examined (David et al. 2015; Murray et al. 2014). This is reflected in our data, since the expression of the M1 markers (iNOS and CD16/32) was found in most microglial cells and macrophages for the first three days following injury, but the expression of iNOS, but not CD16/32, was progressively lost over time. Similarly, a recent study reports that the expression of CD86, another M1 marker, is also lost from microglia and macrophages over time (Kroner et al. 2014). We also observed, in agreement with a previous report (Kroner et al. 2014) that only a small subset of microglia and macrophages expressed M2 markers. This is not surprising as microglia and macrophages *in vivo* in the injured spinal cord are influenced by a variety of both pro and anti-inflammatory stimuli (David et al. 2015). In contrast, a previous study showed that M2 macrophages are abundant in the spinal cord of irradiated mice at 2 weeks post-injury, and reach the injury site through the choroid plexus migrating along the central canal (Shechter and Schwartz 2013). However, these M2 macrophages were classified based on the lack of Ly6C expression and not to the presence of classical M2 markers. This may account for the difference in interpretation of how many M2 cells are present after SCI between this study and our data. However, the majority of the evidence in the literature suggests that there is heterogeneity in microglia/macrophage phenotypes in the injured spinal cord but they are skewed mainly to a M1 polarization state.

Previous studies indicate that the environment of the injured spinal cord does not favor M2 polarization (Kigerl et al. 2009; Kroner et al. 2014) as M2 macrophages obtained from cell culture

rapidly lose Arg1 expression when transplanted into the injured spinal cord (Kigerl et al. 2009). Polarization of microglia/macrophages into M1/M2 *in vitro* is induced upon stimulation with different cytokines (Mills et al. 2000; Murray et al. 2014; Sica and Mantovani 2012). Therefore, it is likely that the functional phenotype of microglia and macrophages after SCI might depend on the balance between the levels of pro-inflammatory M1 and anti-inflammatory M2 cytokines found in the injured spinal cord environment (David et al. 2015). Our analysis of cytokine expression shows that most pro-inflammatory cytokines are increased at the protein levels in the spinal cord for the first 24 hours post-injury, and most of them dropped to basal levels by day 3. This pattern of changes is also seen at the mRNA level (Pineau and Lacroix 2007). Interestingly, the expression of cytokines that induce distinct alternative activation of macrophages was either undetectable (i.e. IL-4) or expressed at low levels and only for the first 24 hours post-injury (i.e. IL-10 and IL-13). Although IL-4 was not detected in the contused spinal cord, it is likely to be present at very low levels, as a previous study reported that IL-4Rα null mice show greater functional deficits after SCI (Fenn et al. 2014). In the current work, however, we found that increasing the levels of IL-4 in the injured spinal cord by direct intraspinal injection of IL-4 induces the expression of M2 markers in microglia and macrophages. These data suggest that the low levels of IL-4 in the contused spinal cord may underlie the low numbers of M2 polarized cells seen after SCI. Previous *ex-vivo* studies have shown that activated microglia from adult and aged brain can be redirected toward a M2-like phenotype by IL-4 (Fenn et al. 2012).Our work suggests that IL-4 may also induce such as shift *in vivo* after SCI. Additionally, our work also revealed this effect of IL-4 on M2 polarization is more effective when administered after a delay of 48 hours post-injury. This may be due to the high levels of pro-inflammatory cytokines in the spinal cord during the first 24 hours post-injury (Pineau and Lacroix 2007) that may hamper the effectiveness of IL-4 when

injected immediately after injury (15 minutes). Indeed, a recent work reveals that TNFα, which is increased in the injured spinal cord for the first 24 hours, prevents the induction of M2 markers in microglia and macrophages after SCI (Kroner et al. 2014), as well as in tumor macrophages (Kratochvill et al. 2015). It is likely that other pro-inflammatory cytokines and/or mediators synthesized during the first few hours post-injury could also exert a similar effect. Our data, along with another report (Fenn et al. 2014), indicates that microglia do not show constitutive expression of IL-4Rα. This may also limit the capacity of acute IL-4 administration to induce an anti-inflammatory phenotype. In addition, the very low levels of infiltration of blood borne monocytes into the spinal cord during the first 24 hours post-injury may also explain the low effectiveness of acute IL-4 to induce M2 markers in this immune cell population. However, at 48 hours post-injury, IL-4R is expressed on microglia, macrophage infiltration is abundant, and the levels of most of the pro-inflammatory cytokines are low at close to basal levels. At this point, the administration of IL-4 has greater effects in counteracting the pro-inflammatory polarizing environment of the injured spinal cord. This approach induced ~40% of microglia and ~55% of macrophages to acquire M2 markers, since only ~55% of these two immune cells expressed IL-4R at 48 hours post-injury. To our knowledge, this is currently the most effective strategy in inducing M2 markers in microglia and macrophages after SCI.

In many tissues, a switch in macrophage phenotype accompanies the resolution of inflammation. Transcriptomic profiling of isolated resolution-phase macrophages reveal that they show a mixed M1/M2 phenotype (Bystrom et al. 2008). Resolution-phase macrophages are enriched with MHCII expression and lack CD11c (Stables et al. 2011). They also express anti-inflammatory cytokines, such as TGFβ1 and IL-10 (Li et al. 2013; Stables et al. 2011). Importantly, they have elevated expression of COX-2 and LOX (Li et al. 2013; Stables et al. 2011), key enzymes in the synthesis

of specialized pro-resolving mediators that actively turn off inflammation (Schwab et al. 2007; Serhan 2014). Our data reveal that only a small proportion of macrophages display M2 markers, suggesting that resolution-phase macrophages are scarce in SCI, which may explain, in part, why inflammation remains active for several months or even years in this pathology. However, we found that delayed IL-4 treatment induced the appearance of a new immune cell subset in the spinal cord. Further characterization of this immune cell subset revealed that they were macrophages with a phenotype similar to resolution-phase macrophages (5-LOX, 15-LOX, COX-2, TGFβ1, IL-10). Whether this is due to either direct or indirect effects of IL-4 on macrophages is not known. In line with these results, we also found that the clearance of neutrophils from the injured spinal cord, which is a key step in the resolution of inflammation (Li et al. 2013; Schwab et al. 2007; Serhan 2014), was markedly accelerated after delayed IL-4 injection, linking the lack of IL-4 with a deficit in resolution of inflammation after SCI.

Delayed administration of IL-4 not only modulated microglia and macrophage phenotype after SCI, but more importantly, was accompanied by a substantial reduction in tissue damage and functional deficits, assessed by a variety of functional, electrophysiological and histopathological analysis. Since IL-4Rα was mainly found in microglia and macrophages, our results indicate that the effects of delayed administration of IL-4 on microglia and macrophage exerted restorative effects after SCI.

Taken together, our data reveals that the insufficient induction of IL-4 after SCI is responsible, in part, to drive microglia and macrophages towards a phenotype that is not conducive to tissue repair and functional recovery. We show that a single, delayed injection of IL-4 induces the appearance of a resolution-phase macrophage subpopulation and increased numbers of M2 macrophages in the injured spinal cord. Delayed IL-4 injection also reduced tissue damage and locomotor

impairment after SCI. As IL-4 is undetected in human CSF samples after acute SCI (Kwon et al. 2010), these findings may also be of relevance to SCI in humans. Therefore, therapies that increase IL-4 levels in the injured spinal cord could be a good approach with a wide therapeutic window for the treatment of acute SCI.

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FIGURE LEGENDS

Figure 1. Microglia and macrophages show predominant expression of M1 markers after SCI. (**A**) Representative FACS dot plots of injured spinal cord showing how microglia and macrophages were gated. Note the CD45+ cells (panel I) were further gated in CD11b+ expression to distinguish between microglia ($CD45^{low}$ $CD11b^{+}$) and myeloid cells ($CD45^{high}$ $CD11b^{+}$) (panel II). Myeloid cells were further differentiated based on F4/80 expression to identify macrophages ($F4/80^{+}$) (panel III). (**B-D**) Representative FACS histograms plots of M1 and M2 markers in uninjured spinal cord for microglia (**B**) and of microglia (**C**) and macrophages (**D**) at 3 days after SCI. These plots show the labelling for different M1 and M2 markers, as well as, for their respective isotype controls. (E) Graphs showing the quantification of microglia and macrophages expressing M1 and M2 markers after SCI. Note the percentage of microglia and macrophages expressing M1 markers (CD16/32 and iNOS) is markedly higher as compared to the scarce expression of M2 markers (Arg1 and CD206). Mean ± SEM. (n=4 per group). *$p<0.05$ compared to microglia from uninjured spinal cord; # $p<0.05$ compared to day 1 post-injury; + $p<0.05$ compared to microglia at the same day post-injury. One-way ANOVA with Bonferroni's post hoc correction was performed for the analysis.

Figure 2. Dynamic changes in cytokine levels in the spinal cord after contusion injury. Heat map showing the changes in protein levels of relevant pro-inflammatory cytokines and anti-inflammatory in the injured spinal cord obtained by doing Luminex analysis. Mean ± SEM. (n=4 per time point).

Figure 3. Expression of IL-4Rα after SCI. (**A**) Flow cytometry plots of showing the cut off of the isotype control (panel I), and the expression of IL-4Rα in the uninjured spinal cord (panel II), and at 18 post-injury (panel iii). Note that IL-4Rα is lacking in the uninjured spinal cord but it is rapidly detected after spinal cord injury, mostly in CD45+ cells (panel IV). (**B**) FACS plot histogram gated on the microglia and macrophage population (as shown in Fig. 1a) of intact and injured spinal cord at 18 hours and 48 hours post-injury. Note that IL-4Rα is not constitutively expressed in microglia, but it is found in >50% of microglia cells and infiltrated macrophages after SCI. Mean ± SEM (n=4 per time point).

Figure 4. Acute administration of IL-4 induces Arg1 expression in microglia and macrophages at day 3 after SCI. (**A**) Flow cytometry density plots showing the effects of acute intraspinal injection of IL-4 in the expression of M1 markers (iNOS and CD16/32) and M2 markers (Arg1 and CD206) in microglia and macrophages at day 3 after SCI. Note that IL-4 increased the proportion of microglia and macrophages expressing Arg1, but did not modulate the expression of iNOS, CD16/32 or CD206.

Figure 5. Delayed administration of IL-4 has greater effects in inducing M2 markers in microglia and macrophages than acute IL-4 injection. (**A-C**) Graphs showing the changes in the expression of M1 and M2 markers in microglia and macrophages at 3 days post-injury after acute IL-4 injection (**A**) or delayed IL-4 injection (**B**), and at 6 days post-injury after delayed IL-4 injection (**C**). Mean ±SEM. (n=4 per group) Note that microglia and macrophages adopt greater expression of M2 markers after delayed injection of IL-4, and the expression of M2 markers is maintained for at least 4 days. *$p<0.05$ vs saline. Student t test was used to analyze significant differences between IL-4 and saline injected mice.

Figure 6. Delayed administration of IL-4 triggers the appearance of resolution-phase macrophages. (**A**) Flow cytometry density plot showing CD45+ and CD11b+ cells from 6 days post-injury spinal cords of mice receiving delayed injection of saline or IL-4. Note the presence of resolution-phase macrophages in the contused spinal cord only after delayed IL-4 administration. (**B**) Representative flow cytometry histograms characterizing the expression of resolution-phase macrophages. (**C**) Representative flow cytometry dot plots showing granulocytes in the contused spinal cord at 6 days post-injury. Note that neutrophil clearance is enhanced after delayed IL-4 injection. Mean ± SEM. (n=4 per group). *$p<0.05$ vs saline. Student t test was used to analyze significant differences between IL-4 and saline injected mice.

Figure 7. Delayed intraspinal injection of IL-4 reduces functional deficits and tissue damage after SCI. (**A-B**). Mice treated with of IL-4 at 48 hours post-injury showed significant improvement in locomotor skills using the (**A**) BMS score and (**B**) BMS subscore, as well as (**C**) faster locomotion on a treadmill. Arrows indicate the day IL-4 was injected in the spinal cord. (D-E) Administration of IL-4 led to greater preservation of MEPS. (**D**) Quantification MEPs recordings in the gastrocnemius muscle at day 28 post-injury and (**E**) representative MEPs recordings from saline and IL-4 treated mice. (**F-I**) Delayed IL-4 treatment reduced secondary tissue damage after SCI. (**F**) Quantification of myelin sparing at various distances rostral and caudal to the injury epicenter reveals significant reduction in myelin loss at the epicenter of the injury and in adjacent sections in mice treated with IL-4. (**G**) Representative micrographs showing myelin sparing at the injury epicenter in section stained against LFB from mice treated with saline or IL-4. (**H**) Quantification of ventral horn neuron survival at various distances rostral and caudal to the injury epicenter reveals significantly greater neuronal survival in caudal regions to the injury

epicenter in mice treated with IL-4. (**I**) Representative micrographs showing sparing of ventral horn neurons in mice administered with saline or IL-4 in sections stained against NeuN at 1000 μm caudal to the injury epicenter. Mean ± SEM. (n=8 per group). *$p<0.05$ vs saline. Note the greater preservation of neurons after delayed IL-4, especially, in the most ventral region (white arrowheads). Two-way repeated-measures ANOVA with Bonferroni's post hoc correction was used to analyze significant differences between saline and delayed IL-4 injection in the BMS score and subscore, as well as, in histological parameters, and t-test for electrophysiological data.