Chondroitin sulfate proteoglycans prevent immune cell phenotypic conversion and inflammation resolution via TLR4 in rodent models of spinal cord injury

Isaac Francos-Quijorna1,6, Marina Sánchez-Petidier2,8, Emily R. Burnside1,7,8, Smaranda R. Badea1,8, Abel Torres-Espin3, Lucy Marshall1, Fred de Winter4, Joost Verhaagen4,5, Victoria Moreno-Manzano2 & Elizabeth J. Bradbury1

Chondroitin sulfate proteoglycans (CSPGs) act as potent inhibitors of axonal growth and neuroplasticity after spinal cord injury (SCI). Here we reveal that CSPGs also play a critical role in preventing inflammation resolution by blocking the conversion of pro-inflammatory immune cells to a pro-repair phenotype in rodent models of SCI. We demonstrate that enzymatic digestion of CSPG glycosaminoglycans enhances immune cell clearance and reduces pro-inflammatory protein and gene expression profiles at key resolution time points. Analysis of phenotypically distinct immune cell clusters revealed CSPG-mediated modulation of macrophage and microglial subtypes which, together with T lymphocyte infiltration and composition changes, suggests a role for CSPGs in modulating both innate and adaptive immune responses after SCI. Mechanistically, CSPG activation of a pro-inflammatory phenotype in pro-repair immune cells was found to be TLR4-dependent, identifying TLR4 signalling as a key driver of CSPG-mediated immune modulation. These findings establish CSPGs as critical mediators of inflammation resolution failure after SCI in rodents, which leads to prolonged inflammatory pathology and irreversible tissue destruction.

1 King’s College London, Regeneration Group, The Wolfson Centre for Age-Related Diseases, Institute of Psychiatry, Psychology & Neuroscience, Guy’s Campus, London Bridge, London SE1 1UL, UK. 2 Neuronal and Tissue Regeneration Laboratory, Prince Felipe Research Center, Carrer d’Eduardo Primo Yúfera 3, 46012 Valencia, Spain. 3 Brain and Spinal Injury Center, Neurological Surgery, University of California San Francisco, San Francisco, USA. 4 Laboratory for Neuroregeneration, Netherlands Institute for Neuroscience, Royal Netherlands Academy of Sciences, Meibergdreef 47, 1105 BA Amsterdam, The Netherlands. 5 Center for Neurogenomics and Cognition Research, Neuroscience Campus Amsterdam, Vrije Universiteit Amsterdam, 1081HV Amsterdam, The Netherlands. 6 Present address: Immunometabolism and Inflammation Laboratory, Centro de Biología Molecular Severo Ochoa (CBMSo), Consejo Superior de Investigaciones Científicas (CSIC)-Universidad Autónoma de Madrid (UAM), 28049 Madrid, Spain. 7 Present address: Laboratory of Axonal Growth and Regeneration, German Center for Neurodegenerative Diseases (DZNE), Venusberg Campus 1/99, 53127 Bonn, Germany. 8 These authors contributed equally: Marina Sánchez-Petidier, Emily R. Burnside, Smaranda R. Badea. ✉ Email: elizabeth.bradbury@kcl.ac.uk
Spinal cord injury (SCI) typically leads to severe and permanent motor, sensory, and autonomic dysfunction due to the inability of the adult mammalian CNS to regenerate lost neurons and re-establish functional connections. Several factors have been identified that influence this regeneration failure and one of the major contributors is the presence of inhibitory chondroitin sulfate proteoglycans (CSPGs), which accumulate in and around spinal injury scar tissue. Much attention has been focused on therapeutic strategies to reduce the growth inhibitory properties of CSPGs, such as Chondroitinase ABC (ChABC), a bacterial enzyme which, through catabolism of CSPG glycosaminoglycan (GAG) side chains has been shown to increase spinal and brain plasticity and facilitate functional recovery following injury. Thus, the role of CSPGs in restricting growth and neuroplasticity has been well-studied. However, new roles for CSPGs in modulating CNS pathology beyond neuronal growth inhibition are emerging. Recent evidence suggests that CSPGs play a key role in modulating immune cell responses in chronic inflammatory and demyelinating disorders of the CNS. Given their abundant expression, proximity, and interactions with multiple reactive cell types after SCI, CSPGs may also be important contributors to the pathological inflammatory response after SCI.

SCI triggers an aggressive immune response which is characterised by activation of resident microglia and recruitment of peripheral leukocytes to the site of injury. While the inflammatory response plays a critical role in tissue protection and wound healing after injury, an effective resolution is a prerequisite for return to homeostasis. Inadequate resolution can lead to chronic pathological inflammation that causes greater damage and impaired tissue healing. Inflammation resolution failure is particularly problematic after SCI and the mechanisms which propagate inflammatory pathology and prevent immune cell clearance after SCI are poorly understood. We and others have recently identified a pro-inflammatory role for CSPGs after SCI, where they amplify the immune response and contribute to poor functional outcomes. However, the mechanisms regulating CSPG-immune interactions remain poorly understood. How they affect dynamic responses of innate and adaptive immune cells, and which bioactive mediators and signalling pathways are involved is still unknown. Here we demonstrate that CSPGs have prominent immunomodulatory effects which impede multiple aspects of the resolution of inflammation after SCI. Digestion of CSPGs by lentiviral ChABC attenuated the pro-inflammatory environment, led to enhanced clearance/reduced recruitment of microglia and peripheral myeloid cells from the lesion site and activated resolution of the inflammatory response, modulating the phenotype of microglial cells and monocyte/macrophages and T lymphocyte infiltration and composition, with effects predominant at day 7 after SCI (a critical time point for inflammation resolution). Spatially, CSPGs prevent immune cells at the injury core from converting to a pro-repair phenotype, which can be reversed by their digestion. Furthermore, we reveal TLR4 signalling as a key mechanism underlying CSPG-mediated modulation of the inflammatory signature after SCI. Using TLR4 inhibitors and TLR4 knockout mice we demonstrate that activation of TLR4 signalling by CSPGs prevents macrophages and microglia from converting to a pro-resolution phenotype. We propose this as a key mediator of failed inflammation resolution after SCI. These data reveal a new role for CSPGs in preventing inflammation resolution and suggests they are a critical mediator of non-resolving inflammatory pathology after SCI.

**Results**

CSPG digestion promotes enhanced clearance/reduced recruitment of immune cells after spinal cord injury. To determine whether the failed resolution of inflammation after SCI is regulated by CSPGs, we used a lentiviral vector expressing chondroitinase ABC (LV-ChABC) to digest CSPGs in the injured rat spinal cord and used flow cytometry to evaluate the dynamics of the major inflammatory cell types over 28 days post injury (dpi; study design, Fig. 1a, c). To confirm efficient ChABC expression in the injured spinal cord during the neuroinflammatory period, qPCR was used to measure ChABC mRNA in spinal cord tissue, confirming high ChABC transgene expression in LV-ChABC-treated animals at all post-injury time points examined (from 6 h to 14 days, Extended Data Fig. 1a, b). Following confirmation of ChABC gene expression in the spinal cord and that contusion injuries were of equal severity across treatment groups (Fig. 1b), we first assessed the effects of CSPG digestion on immune cell dynamics using manual gating flow cytometry (Extended Data Fig. 1c, d and e–e). CSPG digestion did not affect recruitment of neutrophils (CD45+, CD11b+, CD43+, RP1+; Extended Data Fig. 1e) into the contused spinal cord as neutrophil numbers were unaltered by LV-ChABC treatment at 1 dpi, the peak time point for neutrophil accumulation (Extended Data Fig. 1d) or 3 dpi (3 dpi vs. 7 dpi—Extended Data Fig. 1e). However, LV-ChABC treatment accelerated the clearance of neutrophils from the contused spinal cord, evident by the reduced resolution index parameter compared with the LV-GFP control group (RI = 52.8 and 40.3 h for LV-GFP and LV-ChABC treatment, respectively; Extended Data Fig. 1d), leading to a significant reduction in neutrophil accumulation at 7 dpi in animals treated with LV-ChABC (Extended Data Fig. 1d and Extended Data Fig. 5e). We next studied whether CSPGs interfere with the recruitment of macrophages (CD45+, high, CD11b++; Extended Data Fig. 1c) after SCI. Peripheral macrophage infiltration into the contused spinal cord was not significantly different at 1 dpi or 3 dpi, the peak of macrophage infiltration, after CSPG digestion (Fig. 1d and 3 dpi vs. 7 dpi—Extended Data Fig. 5c). However, macrophage clearance during the later resolution phase was enhanced by LV-ChABC treatment, with macrophage numbers significantly reduced at 7 dpi compared to LV-GFP treatment (Fig. 1d). LV-ChABC treatment also attenuated the number of microglial cells (CD45+medium/low, CD11b++; Extended Data Fig. 1c) during the resolution phase of the inflammatory response, compared with LV-GFP control treatment (Fig. 1e and 3 dpi vs. 7 dpi—Extended Data Fig. 5a). These data indicate that CSPGs play a key role in hindering the clearance of immune cells after SCI.

Having used manual-gating flow cytometry to study dynamics, we next sought to evaluate the effects of CSPG digestion on immune cell recruitment at a more granular level, using unbiased t-distributed stochastic neighbour embedding (t-SNE) analysis, at a key resolution time point (7 dpi). We designed a 16-colour antibody panel for fluorescence cytometry (Supplementary Table 1—Panel 2) that identified all the major leukocytes in the CNS, including lineage markers (CD45, CD11b, CD43, HIS48, CD45RA, CD3, CD4, CD8) and markers linked to activation and phenotype differentiation (CD68, CD86, HMC-II, iNOS, CD163, CD206, and Arg1). Single, live, CD45+ cells from spinal cords at 7 dpi were analysed and samples were two-dimensionally mapped using t-SNE. Based on t-SNE data, we identified 9 phenotypically distinct clusters (Fig. 1f) and generated a heat map showing the distinct lineage marker expression profiles for each cluster (Fig. 1g). Lineage marker expression characterised cluster-1 as microglial cells. We observed clear visual changes in the size of cluster 1 between treatment groups (Fig. 1h) and cell number analysis showed a significant reduction of microglial cells after ChABC treatment (Fig. 1i), providing strong corroboration of the results obtained by manual-gating flow cytometry (Fig. 1e). Thus, CSPG digestion significantly reduces microglial cell accumulation. We next characterised the impact of CSPG digestion on the recruited monocyte/macrophage population at 7 dpi, identified as cluster 2 and 3 by the high expression of CD45 and CD11b...
In agreement with our observations with manual-gating flow cytometry analysis (Fig. 1d), CSPG digestion significantly reduced the monocyte/macrophage population within the injury epicentre at 7 dpi (Fig. 1i, k). Furthermore, t-SNE analysis identified two different monocyte/macrophage subsets based on differential expression of CD43, cluster 2 (CD43low) and cluster 3 (CD43high) (Fig. 1f, g, l). The effect of CSPG digestion was analysed on these two subsets separately. As observed in the total monocyte/macrophage population, CSPG digestion produced a significant reduction in both monocyte/macrophage subsets, being more prominent in the CD43low population (Fig. 1m).

These data provide evidence that CPSG digestion enhances the clearance of peripheral myeloid cells and reduces microgliosis in...
the injured spinal cord, suggesting an important role for CSPGs in the pathological chronication of the inflammatory response after SCI.

CSPG digestion alters inflammatory gene co-expression dynamics during the resolution phase of the inflammatory response. To generate an in-depth understanding of CSPG-mediated immunomodulatory effects, we performed a dynamic analysis of 29 target inflammatory-related genes (measured by qPCR on RNA extracted from the injury epicentre) at different post injury time points with or without CSPG digestion (study design, Fig. 2a). Multivariate pattern detection using dual multiplet factor analysis (dMFA) revealed 2 main dynamic patterns (dimensions) over time, explaining ~56% of the total variation in gene co-expression (35.1% and 20.6% for dimension 1 and 2, respectively; Fig. 2b). Analysis of the correlated loadings (the importance of each cytokine within a dimension), reveals that dimension 1 is characterised by an overall positive correlation in cytokine expression, likely indicative of injury-induced activation of glial and immune cells, and dimension 2 by a positive correlation in Matrix Metalloproteinases (MMPs) and microglia/macrophage activation markers (CD68, CD206, CD163), likely indicative of tissue remodelling and immune cell activation (Fig. 2c and Extended Data Fig. 2). Comparing the scores of each group, we observed that both SCI groups significantly differed from naive animals at 6 h, 12 h, 1 dpi and 3 dpi. However, at 7 dpi whilst LV-GFP-treated animals were still significantly different from naive animals, the profile of LV-ChABC-treated animals was restored to that of naive animals (Fig. 2d, Extended Data Fig. 3a and 3 dpi vs. 7 dpi—Extended Data Fig. 5g). Thus, CSPG digestion by LV-ChABC reduces pro-inflammatory cytokine gene expression to basal levels more quickly following injury. Dimensional plots highlight the divergence between groups in the dimension 1 vs. dimension 2 space, where LV-ChABC animals cluster closely with naive animals at 7 dpi (Fig. 2e and Extended Data Fig. 3b–d). We next sought to evaluate this at the cytokine profile level. Luminex multiplex immunoassay analysis revealed dynamic changes in cytokine expression levels from 6 h to 7 dpi, with several cytokines remaining elevated at 7 dpi (Extended Data Fig. 4). CSPG digestion reduced CXCL1 and IL6 at 1 dpi, which correlates with more rapid neutrophil clearance (resolution index: 52.8 vs. 40.3) (Extended Data Fig. 1d). However, little to no changes were observed at 6, 12 h or 3 dpi (Extended Data Fig. 4d–f, h and 3 dpi vs. 7 dpi—Extended Data Fig. 5f). A side by side comparison of data at 3 dpi vs. 7 dpi revealed no significant differences in immune cell recruitment and phenotype, inflammatory cytokine protein synthesis or inflammatory gene expression extracted from dMFA after LV-ChABC treatment at 3 dpi (Extended Data Fig. 5a–g). In contrast, we observed significant changes in immune cell number and phenotype (Extended Data Fig. 5a–e), significantly reduced levels of pro-inflammatory cytokines CCL3, IL1α, CCL5, IL18, and IL1β (Extended Data Fig. 5f) and a significant reduction in numerous prototypical pro-inflammatory genes (iNOS, CD68, MHCII, IL1β, TNFa, IL18, CCL2, CCL5, and CXCL10; Extended Data Fig. 5g) after lentiviral-ChABC treatment at 7 dpi. Thus, 7 dpi (which is a key time point in the resolution phase of inflammation) was the earliest time-point when CSPG digestion elicited significant changes in multiple aspects of the inflammatory response after SCI, providing evidence that CSPG digestion attenuates pro-inflammatory cytokine release during the resolution phase of the inflammatory response.

Having found that CSPGs exhibit the most pronounced immunomodulatory effects at 7 dpi, we set out to further characterise this. To this end we investigated a larger number of cytokine genes at this time point. Principal component analysis (PCA) of the expression of 42 genes revealed 3 dimensions which account for ~73% of gene expression variance (40%, 20.4%, and 12.9% for dimension 1, 2, and 3, respectively; Fig. 2f). Dimension 1 was characterised by most cytokines moving in the same direction (positive correlated loadings). However, dimensions 2 and 3 were characterised by a patchwork of different cytokines moving in positive and negative directions (Fig. 2f). Comparing the scores for each dimension we observed that the LV-GFP-treated group significantly differed from the LV-ChABC-treated group in dimension 1, but not dimension 2 or 3 (Fig. 2g). This difference was characterised by LV-GFP moving in a positive direction and LV-ChABC moving in a negative direction on dimension 1 (Fig. 2g, h), confirming that at 7 dpi the expression profile of studied cytokines was strongly modulated by CSPG digestion. mRNA expression heatmaps (Fig. 2i) and univariate analysis of mRNA expression (Fig. 2j) show significantly reduced expression of cytokines and inflammatory molecules, assessed by qPCR, in animals treated with LV-ChABC compared to LV-GFP at 7 dpi. Together, these data show that modulation of CSPGs with ChABC has a significant impact on inflammatory gene dynamics and cytokine protein profile 7 days after SCI. In addition, for some of the inflammation-associated genes analysed, the observed reduction in inflammatory gene
expression following CSPG digestion was maintained one week later (although to a lesser degree than at 7 dpi), alongside an observed increase in the pro-resolution marker CD163 (7 dpi vs. 14 dpi—Extended Data Fig. 6a). Thus, we demonstrate that the immunomodulatory effects of CSPGs after SCI are predominantly focused in the resolution phase of neuroinflammation where CSPGs modulate the inflammatory environment and obstruct the clearance of pro-inflammatory immune cells from the injury site.

CSPGs modulate macrophage and microglial cell phenotype during the resolution phase of the inflammatory response. Having probed gene expression at a whole tissue level, we next...
Fig. 2 CSGP digestion shifts inflammatory gene co-expression dynamics after spinal cord injury during the resolution phase of inflammation.

a Experimental design of inflammatory gene co-expression analysis. b–e Dynamic analysis of the expression of 29 target genes over time with or without CSGP digestion. Gene expression was measured by qPCR on RNA extracted from the injury epicentre at different time-points after SCI. b Bar graph showing that multivariate pattern detection using dual multiple factor analysis (dMFA) detected 2 dynamic patterns (dimensions) over time explaining <5% of the total variation in gene co-expression. c Loadings correlation bar graph to show which cytokines contribute to each of the patterns (interpreted as the Pearson r correlation coefficient ranging from −1 to 1). d Bar graph representing the Euclidean distance to the naïve centroid for each group at different time-points, showing the relative movement of treated animals with respect to naïve in the global inflammatory gene profile. Two-way ANOVA with time and group as factors using Tukey for multiple testing correction. *p < 0.05, **p < 0.01 versus uninjured (naïve) group; ***p < 0.001 versus LV-GFP group. Data are shown as mean ± SEM (n = number of animals/samples, with n = 6 for each group–time combination except LV-GFP at 12 h, LV-ChABC at 6 and 12 h post injury (n = 4); LV-GFP at 3 dpi (n = 5) and LV-ChABC at 7 dpi (n = 7). e Bi-dimensional plots of the component scores in dimensions 1 and 2. Ellipsoids represent the bivariate standard deviation and the coloured circles the centroid. There is little divergence of LV-ChABC and LV-GFP at any timepoint except 7 dpi, where LV-ChABC becomes highly diverged from LV-GFP and is proximal to naïve, reflecting a gene expression pattern comparable to uninjured animals at 7 dpi after CSGP digestion. f Further pattern analysis at 7 dpi was performed using principal component analysis (PCA) for 42 inflammatory-related genes, confirming a reduction of pro-inflammatory genes after CSGP digestion, assessed by qPCR. f Loadings correlation heat map show dimension 1 loadings are positive for almost all cytokines, indicative of a global higher cytokine co-expression in LV-GFP vs. LV-ChABC-treated animals. g Component score bar graphs for each group and dimension at 7 dpi show significant differences between LV-GFP and LV-ChABC in dimension 1. **p < 0.01 versus control (LV-GFP) group. Data were assessed for normality using the Shapiro–Wilk test and analysed using a two-tailed unpaired t test. Data are shown as mean ± SEM. (h) Bi-dimensional plot of the component scores for each group in dimension 1 and 2 at 7 dpi showing significant differences between LV-GFP and LV-ChABC in dimension 1. *p < 0.01 versus control group (LV-GFP) (n = 5 per treatment). i Heatmap showing gene expression data for 42 key genes in the inflammatory response at 7 dpi. LV-ChABC treatment elicits gene expression patterns closer to naïve than LV-GFP treated animals. j Bar graph showing all significant pro-inflammatory gene expression differences between LV-GFP and LV-ChABC treatments at 7 dpi. *p < 0.05, **p < 0.01 versus control (LV-GFP) group. Results were assessed for normality using the Shapiro–Wilks test and analysed using a two-tailed unpaired t test. Data are shown as mean ± SEM (n = 6 naïve group; n = 5 per treatment). Detailed statistics and exact p values are provided in Supplementary Table 8. Source data are provided as a Source Data file.

wanted to assess the phenotype of individual cell populations at 7 dpi. Using our t-SNE unbiased clustering based on lineage markers, we were able to identify microglia and monocyte/macrophages (Fig. 1f, g). We then sought to conduct an in-depth phenotype analysis of these populations. Firstly, in microglial cells (cluster 1, Fig. 1f–h) we assessed expression levels of particular markers linked to phenotype activation (M1-like: iNOS, CD68, CD86, MHC-II; M2-like: Arg I, CD206, and CD163; Fig. 3a–h). CSGP digestion significantly reduced the expression of MHC-II, a prototypical pro-inflammatory marker, in the microglial cell population (Fig. 3a, b). This MHC-II reduction at 7 dpi was maintained at least until 14 dpi (Extended Data Fig. 6b). These data indicate that CSGPs are a key mediator of inflammatory microglial activation after SCI. Next, we characterised the impact of CSGP digestion on the recruited monocyte/macrophage population, identified as cluster 2 and 3 by the high expression of CD45 and CD11b in our t-SNE unbiased clusterisation (Fig. 1f, g). Similar to microglial cells, infiltrated monocyte/macrophages from animals treated with LV-ChABC showed a significant reduction of MHC-II expression at 7 dpi (Fig. 3c, d), which was maintained at 14 dpi (Extended Data Fig. 6c). CSGP digestion also elicited additional immunomodulatory effects in monocytes/macrophages. Unlike in microglial cells, LV-ChABC treatment significantly reduced the expression of the pro-cytotoxic enzyme iNOS and the inflammatory activation marker CD68 in the monocyte/macrophage population (Fig. 3c, d). Although there appeared to be a trend for increased expression of anti-inflammatory M2-like markers with LV-ChABC treatment, this was not statistically significant (Fig. 3c).

The immunomodulatory effects of CSGP digestion on microglial and monocyte/macrophage immune cell populations at 7 dpi was further confirmed by phenotype gene expression analysis. Expression of pro-inflammatory and pro-repair-associated genes, assessed by qPCR on manual-gating microglia (GPR34high, FcRshigh, and CCR2low) and monocyte/macrophage (GPR34low, FcRslow, and CCR2high) (Fig. 3i, j) sorted cells, revealed that CSGP digestion with LV-ChABC treatment redirects monocytes/macrophages and microglial cells toward a pro-repair (M2-like) phenotype after SCI (Fig. 3k). Changes in M1-like and M2-like gene expression were no longer apparent by 14 dpi, other than reduced CD68 expression in microglia (Extended Data Fig. 6d, e). Finally, phenotype analysis was performed on the two monocyte/macrophage subsets identified in our t-SNE unbiased clusterisation (based on differential expression of CD43; Fig. 1i). This revealed that the immunomodulatory role of CSGPs in the total monocyte/macrophage population is predominantly mediated by their impact on the CD43low population, where CSGP digestion significantly reduced the expression of iNOS, CD68 and MHC-II (Fig. 3e–h).

Together these data demonstrate that CSGP digestion after SCI converts the phenotype of microglial cells and macrophages towards an anti-inflammatory, pro-repair state. This is particularly evident in the CD43low monocyte/macrophage population. Thus, CSGP digestion can activate inflammation resolution after SCI, thus overcoming the prolonged pro-inflammatory response that leads to detrimental/pathology and failure of tissue repair.

Differences in immune cell spatial distribution after CSGP digestion. We next sought to understand the immunomodulatory effects of CSGP digestion in-situ. To this end we used immunohistochemistry in spinal cord tissue sections to assess the expression and distribution of two prototypical markers for M1-like and M2-like cells following contusion SCI and treatment with either LV-GFP or LV-ChABC at 7 dpi. Expectedly, LV-ChABC treatment led to significantly less staining for intact CSGPs (CS-56 expression), in both perilesional and entire spinal cord areas (Fig. 4a, b, g, h). The spatial distribution of cells positive for the M2-like marker CD206 was dramatically altered by CSGP digestion. In LV-GFP treated control animals, we observed CD206+ cells to be localised almost exclusively in a ring-like pattern within the inner astroglial border in close proximity to GFAP+ projections (Fig. 4cii), while CD206+ cells were largely absent from the injury core (Fig. 4ci). In contrast, while a similar pattern of CD206+ cells was observed along the astroglial border in close proximity to GFAP+ projections (Fig. 4dii), we also observed an abundance of CD206+ cells within the injury core following CSGP digestion (Fig. 4di). Interestingly, the densely
packed core of CD206+ immune cells was mirrored (almost exactly) by an absence of CSPGs, as can be seen by comparing Fig. 4a, c (dense CSPG in core, CD206+ cells excluded from the core) with Fig. 4b, d (absence of CSPG in core, abundance of CD206+ cells in the core). Increased CD206 expression in LV-ChABC treated animals was also mirrored by a reduction in the M1-like marker iNOS (Fig. 4e, f, j) within the lesion core (Fig. 4h) and border (Fig. 4i), where iNOS expression appeared to be associated with areas of injury-induced loss of neurofilament (NFH). Therefore, these data confirm our molecular biology findings in-situ and provide spatial context to where CSPG-immune interactions occur. Notably, we provide evidence that CSPG deposition at the injury core restricts immune cells in this region from adopting a pro-repair phenotype.
CSPG digestion reduces adaptive immune cell infiltration and modulates \( CD^4 \) cell composition after SCI. To gain a more comprehensive understanding of CSPG-immune modulatory effects, we next evaluated the role of CSPGs in adaptive immune cell recruitment, namely T-helper lymphocytes (TCD4), cytotoxic T lymphocytes (TCD8) and B cells. Using flow cytometry, we assessed the recruitment of TCD4 (\( CD4^+ \), CD45\(^{high} \), CD11b\^−, CD3\^+, CD4\^+ and CD8\^−), TCD8 (CD45\(^{high} \), CD11b\^−, CD3\^−, CD4\^+ and CD8\^+), and B cells (CD45\(^{high} \), CD11b\^−, CD3\^−, and CD45RA\^+) into the injury epicentre at 7, 14, and 28 dpi (Fig. 5a-d). Animals treated with LV-ChABC showed a significant reduction of CD4\^+ and CD8\^+ T cell recruitment at 7 dpi (Fig. 5b). A trend for reduced TCD4 numbers at later time points (14 and 28 dpi) was observed, although this did not reach statistical significance (Fig. 5c, d). Thus, CSPG digestion with LV-ChABC modulates the infiltration of T lymphocytes into the injury epicentre, indicating a role for CSPGs in modulating the adaptive immune response after SCI. TCD4 lymphocytes, also called T helper (Th), are a heterogeneous population which once activated can adopt a myriad of phenotypes depending on environmental signals. We therefore investigated whether LV-ChABC treatment modulates the infiltration of specific Th subtypes. Th signature gene expression, assessed by qPCR in TCD4 sorted cells, showed that following LV-ChABC treatment there was a significant reduction of Th1-specific inflammatory gene expression at 7 dpi (Fig. 5e, f). We found that T-bet (a classic transcription factor for Th1 polarisation) and TNF-\( a \) (a prototypical Th1 cytokine) were significantly downregulated in sorted TCD4 lymphocytes after CSPG digestion (Fig. 5e). Furthermore, the expression of interferon gamma (IFN\( y \)) in TCD4 sorted cells from LV-ChABC-treated animals, in comparison to high expression in LV-GFP-treated animals (Fig. 5f). These data indicate that CSPGs are involved in the chronication of the inflammatory response after SCI by propagating the infiltration of TCD8 and TCD4 pro-inflammatory lymphocytes (Th1) at later stages of the inflammatory response.

Together, our in vivo data provides robust evidence that CSPG digestion can positively modulate both the innate and adaptive immune cell response after SCI, thereby enhancing multiple aspects of the resolution of inflammation.

CSPGs promote phenotypic conversion of bone marrow-derived macrophages and isolated microglia from pro-resolving to pro-inflammatory. In order to gain greater insight into the mechanisms by which CSPGs prolong inflammation, we next conducted cell culture experiments to assess the effect of CSPGs on specific immune cell-types. We first cultured bone marrow-derived macrophages (BMDMs) and polarised them towards M1-like (pro-inflammatory) and M2-like (anti-inflammatory) states to mimic their activation at early and resolution stages of inflammation, respectively (Fig. 6a). After evaluating purity (\( 98\% \)), confirming polarisation (by flow cytometry and qPCR) and optimising stimulation parameters (Extended data Fig. 7), we investigated the effects of CSPG treatment (for either 4 or 16 h) on inflammatory gene expression in both macrophage phenotypes. We observed a slight reduction in inflammatory-related gene expression in M1-like polarised macrophages following 4 h CSPG stimulation, however these were small in magnitude (fold change <1; Fig. 6c, d). In contrast, M2-like polarised macrophages underwent dramatic phenotypic conversion following 4 h CSPG stimulation. CSPG treatment caused an almost complete reversal of M2-like phenotype towards a more pro-inflammatory phenotype, with significantly enhanced expression of multiple pro-inflammatory genes related with M1-like activation (Fig. 6c, e). Immunomodulatory effects of CSPGs were maintained at 16 h, although to a lesser extent than at 4 h, evident by PCA analysis of inflammatory gene expression profiles, where gene expression changes induced by CSPGs are most pronounced in M2-like compared to M1-like polarised macrophages, and at 4 h compared to 16 h (Fig. 6b). As with 4 h treatment, the predominant effects with 16 h CSPG treatment were observed in M2-like polarised macrophages, where CSPG treatment stimulated gene expression changes indicating conversion of M2-like macrophages to a more M1-like phenotype (Fig. 6f-h). Having observed differential effects of CSPG stimulation on macrophage gene expression, we next asked how CSPG stimulation affects macrophage function/behaviour. Phagocytosis is an important mechanism to recover tissue homeostasis by which macrophages remove injury-induced cellular and environmental debris. In an assay which reflects the ability of macrophages to perform phagocytosis we observed that M2-like macrophages, but not M1-like macrophages, phagocytose significantly less when...
stimulated with CSPGs (Extended Data Fig. 8). Thus, at both a gene expression level and a functional level, CSPG stimulation makes M2-like macrophages more similar to M1-like macrophages. These data indicate that, in vitro, CSPGs have differential effects on macrophage phenotypes and a striking ability to convert pro-resolving macrophages to pro-inflammatory. Taken together with our in vivo findings, where positive immunomodulatory effects of CSPG digestion after SCI are prominent at the resolution phase, when it is important that macrophages undergo a phenotypic switch from pro-inflammatory to pro-repair, this provides evidence that CSPGs act directly on macrophages to prevent a switch to a reparative phenotype.

After evaluating the immunomodulatory effects of CSPGs in BMDMs, we next investigated CSPG-mediated immunomodulation in M2-like polarised isolated microglial cells. Primary microglial cell cultures were derived from P2-3 rat pups (Extended Data Fig. 9a) or adult rat brain and spinal cord (Extended Data Fig. 9b). After astrocyte and myelin removal and...

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evaluation of purity, microglial cells were polarised towards an M2 anti-inflammatory state and gene expression analysed by qPCR (Extended Data Fig. 9a–c). We showed that CSPG treatment (4 h) significantly enhances the expression of pro-inflammatory cytokine genes (IL1β, iNOS, TNFa, and CCL3) in both adult and neonatal M2 polarised microglial cells (Extended Data Fig. 9d). In line with our in vivo findings (Fig. 3), we found that both adult and neonatal microglial cells were less sensitive to CSPG treatment than BMDMs, exhibiting lower up-regulation of inflammation-related genes (BMDM data from Fig. 6 combined for comparison in Extended Data Fig. 9). These data demonstrate that CSPGs exert a direct effect on inflammatory gene activation in both macrophages and microglial cells.

**Immunomodulatory effects of CSPGs in M2 macrophages are attenuated by ChABC in vitro and in vivo.** Having shown that CSPGs directly modulate macrophage and microglia phenotypes, we next set out to address whether CS-GAG removal using ChABC would affect this. BMDMs were transduced with LV-ChABC and the conditioned media was harvested and used as a source of ChABC enzyme, with LV-GFP conditioned media as control (Fig. 7a). After evaluating the optimal vector titration (Fig. 7b–d), we assessed the effects of ChABC conditioned media on inflammatory cytokine expression in both M1-like and M2-like BMDMs (Fig. 7e, f). In M2-like BMDMs, incubation with ChABC conditioned media significantly attenuated the CSPG-mediated induction of IL1β, IL6, CCL5 and CXCL10 inflammatory gene expression (Fig. 7e). Thus, CSPG digestion with ChABC prevents M2-like BMDMs from phenotypic conversion upon exposure to CSPGs. Consistent with our previous observations (Fig. 6), M1-like polarised BMDMs did not upregulate IL1β, IL6, CCL5 and CXCL10 expression appreciably following CSPG stimulation (Fig. 7f). Interestingly, we observed that application of ChABC conditioned media reduced the basal levels of IL1β, IL6, CCL5, and CXCL10 in M1-like polarised BMDMs. This suggests that digestion products may positively modulate the inflammatory properties of M1-like macrophages. To this end, we asked whether CSPG digestion products themselves exert immunomodulatory effects. We evaluated the effect of the glycosaminoglycan digestion products (Chondroitin disaccharide Δdi-0S and Δdi-4S)
and compared with CSPGs on M1-like and M2-like polarised macrophages. Consistent with our previous experiments (Fig. 6) we observed that intact CSPGs cause a profound increase in pro-inflammatory gene expression in M2-like, but not M1-like macrophages (Extended data Fig. 10a, b). In contrast, the disaccharides did not elicit pro-inflammatory effects in either M1-like (Extended data Fig. 10a) or M2-like (Extended data Fig. 10b) polarised macrophages. One of the CSPG digestion products, the Chondroitin disaccharide Δdi-4S, was actually associated with higher expression of the M2-like signature marker CD206 in M2-like macrophages, which could indicate an additional beneficial role for the digestion product itself in promoting an M2-like phenotype. Both macrophage and microglial responsiveness to CSPG digestion was next corroborated in vivo in FACS sorted microglial and macrophage cells from contusion injured spinal cords at 7 dpi (Fig. 7g; phenotype characteristics of sorted cells confirmed in Fig. 3j). Both macrophages and microglial cells exhibited reduced expression of inflammatory cytokine genes (Il1β, CCL5 and CXCL0) after CSPG digestion at 7 dpi, with the effect more pronounced in macrophages (Fig. 7h).
in vitro stimulation of polarised M2-like immune cells with CSPGs can elicit conversion from pro-repair to a pro-inflammatory phenotype. Conversely, degradation of CSPGs (either in CSPG-stimulated cells in vitro or in a pro-inflammatory SCI environment in vivo) can elicit a pro-repair immune cell phenotype. Taken together, these data indicate that CSPGs play a central role in phenotypic conversion of immune cells, such that in the presence of CSPGs macrophages are not able to adopt a pro-reparatory phenotype, but this can be enabled when CSPGs are degraded.

The pro-inflammatory effect of CSPGs on M2 polarised macrophages is TLR4 dependent. Having found that CSPGs directly cause M2-like polarised macrophages to adopt a proinflammatory M1-like phenotype, we asked how this occurs mechanistically. The TLR4 pathway has been recently implicated in proteoglycan signalling and several of the proinflammatory genes that are degraded. CSPG treatment (5 μg/ml) in M1 (left) and M2 (right) polarised BMDMs. mRNA levels of inflammatory response genes were determined by qPCR. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus control (no CSPG). Results were assessed for normality using the Shapiro-Wilk test and analysed using a two-tailed unpaired t test. Data are shown as mean ± SEM (n = 3 per group). Bar graphs showing genes that were significantly altered by 4 h CSPG treatment in M1 and M2 polarised BMDMs. mRNA levels of inflammatory response genes were determined by qPCR. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 versus control (no CSPG). Results were assessed for normality using the Shapiro-Wilk test and analysed using a two-tailed unpaired t test. Data are shown as mean ± SEM (n = 3 per group). Detailed statistics and exact p values are provided in Supplementary Table 8. Source data are provided as a Source Data file.
phosphorylation of p38 in M2-like polarised macrophages at both 4 and 16 h but did not affect other MAPK or NF-κB signalling pathways (Extended Data Fig. 13b, d). The effect of CSPGs on p38 pathway activation was corroborated by fluorescence intensity in mouse M1/M2-like polarised macrophages (Extended Data Fig. 13e, f) where 4 h CSPG incubation induced higher p-p38/p38 ratio fluorescence intensity in M2-polarised macrophages compared with controls. The effect of CSPG on p38 pathway activation in M2-polarised macrophages was further corroborated in rat BMDMs, where p38 pathway inhibition using SB202190 (25 μM) significantly reduced the expression of several inflammatory cytokines (IL1β, iNOS, IL6, CXCL10, and CCL5) upregulated by CSPGs in M2-like macrophages. These data suggests that activation of the p38
pathway plays a role in mediating CSPG-induced proinflammatory cytokine gene expression.

We next examined the involvement of TLR4 in phenotypic conversion of macrophages in greater detail, using BMDMs derived from a TLR4 knockout mouse (BMDM<sub>TLR4<sup>−/−</sup></sub>) treated with the polarising factors used to establish M1-like and M2-like phenotypes in rat BMDMs (Fig. 8). As expected (due to the lack of TLR4 and in line with results obtained in Fig. 8b) with TLR4 pathway inhibition, the pro-inflammatory M1-activation was reduced, with M1-stimulated BMDM<sub>TLR4<sup>−/−</sup></sub> exhibiting an intermediate inflammatory phenotype with more amoeboid shape, reduced iNOS expression and lower basal inflammatory cytokine levels than their WT counterparts (Supplementary Table 6, Extended Data Fig. 14a–c). Furthermore, M1-like BMDM<sub>TLR4<sup>−/−</sup></sub> exhibited significantly higher CD206 expression, a well-known anti-inflammatory signature marker, than WT M1-like polarised macrophages, showing an intermediate inflammatory phenotype (Supplementary Table 6, Extended Data Fig. 14d). However, M2-like BMDM<sub>TLR4<sup>−/−</sup></sub> activation was not compromised by the lack of TLR4, with both BMDM<sub>TLR4<sup>−/−</sup></sub> and BMDM<sub>WT</sub> exhibiting typical M2 morphology and basal inflammatory gene expression levels (Supplementary Table 6, Extended Data Fig. 14a–c). E. We then explored the response of polarised BMDM<sub>TLR4<sup>−/−</sup></sub> to CSPG stimulation. Unlike BMDM<sub>WT</sub> M0, which become activated by CSPG stimulation and upregulate proinflammatory cytokines CXCL10, IL1ß and TNFα, BMDM<sub>TLR4<sup>−/−</sup></sub> M0 were largely unresponsive to CSPG stimulation (Extended data Fig. 14f). This suggests that TLR4 is critical for CSPG activation of M0 macrophages towards a proinflammatory status. In M1-stimulated BMDM<sub>TLR4<sup>−/−</sup></sub> due to their ineffective inflammatory activation, there was a significant upregulation of IL1ß, CXCL10, and CCL2 (Fig. 8f and Extended data Fig. 14d). This contrasts with fully M1-activated BMDM<sub>WT</sub> which did not undergo appreciable changes in inflammatory gene expression (Fig. 8e and Extended data Fig. 14d; and in line with rat BMDM data, Fig. 6d, g). This suggests that in M1 macrophages that are only partially activated, CSPGs elicit proinflammatory effects via a TLR4-independent mechanism. In contrast, TLR4 appears essential for inflammatory activation of M2 macrophages, demonstrated by the significant upregulation of a number of prototypical proinflammatory cytokines (IL1ß, iNOS, IL6, CXCL10, CCL2, CCL5, and TNFα) upon CSPG stimulation in M2-stimulated BMDM<sub>WT</sub>, whereas M2-stimulated BMDM<sub>TLR4<sup>−/−</sup></sub> only upregulated CXCL10 (Fig. 8h and Extended data Fig. 14e). Finally, the intermediate polarisation exhibited by M1-like BMDM<sub>TLR4<sup>−/−</sup></sub> (Extended Data Fig. 14d, Supplementary Table 6), namely the upregulation of IL1ß, CXCL10 in M1 BMDM<sub>TLR4<sup>−/−</sup></sub> and the upregulation of CXCL10 in M2-like BMDM<sub>TLR4<sup>−/−</sup></sub> may be partly explained by compensatory effects of other TLRs. Accordingly, we observed an increase in TLR2 in M1-like BMDM<sub>TLR4<sup>−/−</sup></sub> and an increase in TLR2 and TLR6 in M2-like BMDM<sub>TLR4<sup>−/−</sup></sub> (Extended data Fig. 14g, h). Thus, using two approaches to inhibit TLR4 signalling in rat and mouse models we demonstrate that TLR4 is essential for the phenotypic switch that converts polarised pro-repair macrophages to a pro-inflammatory phenotype in the presence of CSPGs.

We have demonstrated, using multiple methods and using both genetic and pharmacological approaches, that CSPGs affect immunomodulation via TLR4 signalling. As CSPGs have well described effects on neuronal growth inhibition,<sup>9,37–39</sup> we finally assessed whether CSPG interactions with TLR4 have any effect on neurite outgrowth. CSPG treatment caused a significant reduction in neurite length in both WT and TLR4 KO neurons, and this effect was rescued by application of ChABC equally in both groups (Extended Data Fig. 15). These results suggest that neuronal growth inhibition by CSPGs is not influenced by TLR4 signalling and that the main role of CSPG–TLR4 interactions is modulation of the immune cell response to injury.

Together these data reveal that CSPGs act via TLR4 to provoke a switch to a pro-inflammatory phenotype in pro-repair immune cells. This provides a new mechanism underlying the delayed resolution phase of inflammation following SCI, which leads to chronic pathology.

Discussion
CSPGs are well established to be potent inhibitors of axonal growth and neuroplasticity after SCI. Here we report a role for CSPGs that goes far beyond growth inhibition and demonstrate their critical function as pro-inflammatory mediators that prevent pro-repair phenotypic conversion of immune cells during the resolution stage of inflammation. Finally, we reveal that these immunomodulatory effects are driven by TLR4.

Neuroinflammation in response to injury or disease is critical for enabling wound healing and tissue repair. However, active termination of neuroinflammation is required to successfully restore tissue homeostasis. Failure of inflammation resolution can lead to impaired wound healing, chronic pathology and neurodegeneration, which are typical pathological hallmarks of SCI in humans<sup>40</sup> and in rodent models.<sup>41</sup> CSPGs have previously been implicated in neuroinflammation, although evidence as to their role is conflicting.<sup>26,42</sup> By conducting a dynamic and in-depth characterisation of the immunomodulatory role of CSPGs...
following SCI we delineate their effects on multiple cell types and cell phenotypes. We show that over-expression of the CSPG-digesting enzyme ChABC promotes enhanced clearance/reduced recruitment of immune cells, causes pro-resolution phenotypic changes in both innate and adaptive immune cells, and elicits dynamic immune signature changes in the tissue microenvironment at a key resolution time point.

Our data revealed that CSPGs are regulators of multiple cell types involved in innate and adaptive immune responses to SCI. The spinal injury epicentre is a complex milieu of cell types, activation states and phenotypes. Whilst CSPGs have previously been implicated in signalling with resident glia and innate immune cells, until now we had little understanding of this effect on precise populations, or their phenotypes. Furthermore,
whether the adaptive immune response is modified by CSPG digestion has never been explored. To capture the complexity of cell phenotypes, we used 15 colour flow cytometry and multiplex-omics approaches47 and these techniques are beginning to present as mean ± SEM (n = 3 per group). Detailed statistics and exact p values are provided in Supplementary Table 8. Source data are provided as a Source Data file.

Until now the effects of CSPG digestion on the adaptive immune system have not been studied. We found that CSPG removal leads to an overall decrease in lymphocyte recruitment to the lesion. T-lymphocyte recruitment progressively increases within the first week post injury53 and contributes to SCI pathology, by direct effects on neurons or glia or indirect effects on other CNS cells following the production of proinflammatory cytokines or chemokines55. There is evidence to suggest both positive and negative effects of T-lymphocyte recruitment following SCI56,57. We therefore sought to distinguish between T-lymphocyte subtypes in our analysis. Here we found that CSPG digestion results in significant reduction of CD4+ and CD8+ T cell recruitment at day 7 after contusion SCI. Furthermore, we found that CSPG digestion reduced type 1 T-helper (Th1) cells specifically. Within this population, CSPG digestion rendered IFNγ expression undetectable. IFNγ is a prototypical Th1 cytokine and thought to be an important mediator of cross-talk between T-lymphocytes and macrophages58-60. Our findings are consistent with previous work showing a modulation of Th1/Treg balance towards less inflammatory after inhibition of CSPG signalling via LAR and PTP receptors in a compressive SCI model66 and reduced CD4+ T cell infiltration after surfen treatment in a murine EAE model13. Thus, CSPG targeting dampens the T-lymphocyte response. Whether CSPGs activate T-lymphocytes directly, or whether this is an indirect effect of the pro-inflammatory microenvironment stimulated by CSPGs is the subject of further study. Collectively, these data demonstrate that CSPGs elongate the period of inflammation by modulating both the innate and adaptive immune response following SCI.

We further revealed that CSPG deposition at the injury epicentre restricts pro-repair immune cells from the lesion core. There is increasing appreciation as to the roles of specific cell types in the injured spinal cord and how their spatial distribution influences interactions51-53,61. Significant recent findings place activated microglial cells at a critical position at the interface between infiltrating leukocytes and border astrocytes, where they are associated with protection of neural tissue53.

Similar to these findings, here we observed a ring-like pattern of CD206+ cells along the inner astroglial border after SCI. Additionally, we observed that CSPG digestion changes this restricted distribution and results in a dramatic increase in CD206+ cells. Thus, the spatial distribution of CSPG-immune interactions could play an important role in recently identified wound healing processes, such as the microglial scar53 and/or the process of “corralling”, where phagocytic immune cells are confined to the injury core which is surrounded by an astrocytic border51. In support of this, we found that CSPG digestion dramatically altered the

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balance of pro- vs. anti-inflammatory immune markers within the lesion core. Notably, when CSPGs were degraded the core was no longer filled with toxic immune mediators such as iNOS but was abundant in pro-repair CD206+ immune cells (the densely packed core of CD206+ immune cells mirrored almost exactly by an absence of CSPGs). We therefore hypothesise that CSPGs prevent immune cells at the very injury core from converting to a pro-repair phenotype, and this leads to tissue necrosis at the injury epicentre and subsequent tissue cavitation. Thus, confinement of phagocytic immune cells may be beneficial early on, but then phenotype conversion is needed for inflammation resolution. If this does not occur, toxic macrophages at the core lead to tissue necrosis and central cavitation, which are classic pathological hallmarks of non-resolving SCI pathology. We demonstrate that CSPGs play a critical role in this process.

Our next key finding demonstrated that CSPGs prevent resolution of inflammation following spinal cord injury. Although there is increasing evidence for a role of CSPGs in modulating inflammation following SCI, until now it has remained unclear how and at which stage of neuroinflammation this occurs. Our phenotype analysis of specific immune cell subpopulations revealed CSPG immunomodulatory effects to be most prominent at day 7 following spinal contusion injury, which is considered a key resolution time point when inflammatory mediators and immune cells need to switch towards a repairing phenotype. Dynamic analysis of high-dimensional inflammatory gene expression and luminescent measurements of cytokine levels revealed that at this critical time point, the presence of CSPGs results in a complex proinflammatory profile at a whole tissue level, captured by multiparametric analysis. Digestion of CSPGs using lentiviral overexpression of ChABC was found to accelerate resolution of this profile towards homoeostasis. This suggests that CSPG immunomodulatory effects predominate in the resolution phase of the inflammatory response.

Inflammation resolution, once believed to be a passive process involving downregulation of pro-inflammatory chemokine gradients, is an active anti-inflammatory programme involving specific pro-resolving mediators aimed at restoring tissue homoeostasis. As outlined above, successful inflammation resolution requires activated pro-inflammatory immune cells to convert towards a more reparative phenotype. After SCI, microglia and macrophages display a predominant and sustained pro-inflammatory/M1-like phenotype, which is thought to mediate cytotoxic actions and lead to excessive tissue damage. There is currently limited information about the in vivo factors that prevent these immune cells from converting to an M2-like phenotype, which is associated with tissue healing and repair. Recent work has suggested that CSPGs may influence macrophage polarisation, and this may underlie the neuroprotective effects observed after large scale CSPG digestion. Here we have provided direct in vivo evidence that CSPGs are involved in pro-inflammatory polarisation of macrophages and microglial cells during the resolution phase of the inflammatory response and, together with our cytokine profiling, this identifies CSPGs as important contributors to the pathological chronicisation of the inflammatory response after SCI that leads to irreversible tissue damage and resulting poor functional outcome.

Notably, we show that CSPGs directly convert distinct macrophage subsets to a non-resolution phenotype. To further understand the mechanisms behind CSPG immunomodulation we used BMDM and microglial cell cultures to evaluate the direct effects of CSPGs on innate immune cells. Recent studies have indicated that CSPGs can cause microglia to display proinflammatory properties and to adopt an M1-like phenotype. Following stimulation of M2-polarised isolated microglia with CSPGs, we found a significant increase in classical M1-associated proinflammatory mediators (IL1b, iNOS, TNFa, CCL3) in both adult and neonatal cultures, indicating a direct influence of CSPGs in blocking M2-like microglial conversion. Furthermore, in isolated macrophages (rat BMDM cultures), we discovered an even more potent and specific phenotype-dependent effect of CSPG stimulation which we hypothesise underscores our in vivo observations of inflammation resolution. We found that CSPG stimulation has minimal effect on already proinflammatory M1-like polarised macrophages. Interestingly, application of ChABC conditioned media reduced their basal expression levels of several inflammatory genes. This suggests that breakdown products as a result of CS-GAG digestion may positively modulate the inflammatory properties of M1-like macrophages. Indeed, CSPG disaccharides have previously been shown to be neuroprotective against excitotoxic damage and digested oligosaccharide products have been shown to influence TLR4 pathway activation in M1-like macrophages. In contrast to the limited effects of CSPG stimulation on proinflammatory M1 macrophages, and more meaningful in an inflammatory resolution context, we found that CSPG stimulation elicited potent effects on pro-resolving M2-like polarised macrophages, where they acted to phenotypically reverse the M2-like phenotype to M1-like, upregulating pro-inflammatory signature genes and reducing phagocytosis, essential for recovery of tissue homoeostasis. These effects were attenuated by ChABC treatment (using ChABC enriched conditioned medium). Application of CSPG digestion product disaccharides did not induce inflammation. However, consistent with prior findings, chondroitin disaccharide Δ4S induced anti-inflammatory (CD206) marker expression. We cannot rule out that additional (indirect) effects of CSPGs may also modulate the inflammatory environment after SCI, for example by cytokine and chemokine retention. However, these in vitro data provide evidence that CSPGs directly convert recruited monocyte/macrophages towards a M1-like phenotype and hinder the transition to M2-like at a tissue resolution level, resulting in chronicisation of the inflammatory response. CSPG digestion therefore represents a potent regulator of timely resolution of inflammation. Given the reciprocal activation of multiple cell types, such as astrocytes, by macrophages this further underscores a role of CSPGs in being potential central regulators of the neuroinflammatory response to injury.

We finally demonstrate that CSPGs convert the phenotype of macrophages by a TLR4-dependent mechanism. Having discovered that CSPGs block macrophage phenotypic conversion to pro-resolution, we examined whether TLR4 is involved in this CSPG immunomodulatory role. TLRs recognise a wide variety of pathogen-associated molecular patterns, initiate acute inflammation through the production of inflammatory cytokines and play a pivotal role as an amplifier of the inflammatory response in “sterile” conditions. TLR4 activation by endogenous extracellular matrix (ECM) ligands has been explored in many inflammation and tissue injury paradigms. For example, biglycan is known to activate TLR4 in kidney injury and sepsis, activation of TLR4 by fibronectin fragments is proinflammatory in myocardial infarction and stroke, and tenascin is a major TLR4 ligand in rheumatoid arthritis. TLR4 activation by endogenous molecules has been less studied in the context of CNS pathology, but its enhanced expression in microglial cells and peripheral macrophages in neurodegeneration models make it a candidate to contribute to disease progression in the absence of pathogens. Indeed, persistent exposure to danger signals can cause aberrant microglial activation and produce proinflammatory mediators, reactive oxygen and nitrogen species that propagate pathology in nervous system disorders. After SCI, the role of TLR4 and its endogenous ligands is complex, with most reports showing
a detrimental inflammatory role of TLR4 activation and improved recovery after TLR4 inhibition, although TLR4 inactivation has also been shown to increase astrogliosis and lesion pathology. The role of CSPGs in TLR4 activation and its effect on the inflammatory response has not previously been established. Here, we provide data which links SCI upregulated CSPGs with TLR4 activation and detrimental consequences in inflammatory chronication. Using two approaches to inhibit TLR4 signalling (pharmacological inhibitors and a knockout model of TLR4), we show that TLR4 signalling is necessary for the CSPG-mediated phenotypic conversion of M0 and M2 macrophages towards a proinflammatory state. The significant upregulation of pro-inflammatory cytokines upon CSPG stimulation in M2 polarised BMDMs was consistently suppressed by TLR4 inhibition or deletion with the exception of CXCL10, which despite being linked with TLR4 activation by LPS, is mainly induced in response to IFN type-1 and type-2 receptor activation and could be related with compensatory upregulation of other receptors (e.g. other TLRs) and activation of other pathways. Our results suggest that SCI upregulated CSPGs can act through TLR4 signalling in inactivated (M0) macrophages or when macrophages try to adopt a more repair phenotype (M2), causing the switch to an inflammatory phenotype and delaying the resolution phase of inflammation with devastating consequences.

Thus, we have identified a TLR4-dependent mechanism by which CSPGs exert effects on macrophages. CSPGs are known to signal through PTPTs, and previous work has shown this pathway can mediate inflammatory processes. In agreement, we found that CSPGs do act on M1-like macrophages partially through PTPTs, since inhibiting PTPs increased CD206 in M1-like macrophages. However, we demonstrate that TLR4 inhibition exerted far greater effects than PTPs inhibition in the modulation of M2-like macrophages. As previously discussed, we found the effect of CSPGs on M1-like macrophages to be orders of magnitude lower than their role in converting M2-like macrophages to a more pro-inflammatory state. Here we find that TLR4 is critical to these effects, since TLR4 inhibition elicited a robust reduction in CSPG-induced pro-inflammatory gene expression in M2-like macrophages. In contrast, PTPs signalling plays no clear role in M2 phenotypic conversion, since PTPs inhibition did not reverse CSPG-induced pro-inflammatory gene expression in M2-like macrophages. Thus, CSPGs predominantly act via the TLR4 pathway to cause M2-like macrophages to switch to a proinflammatory phenotype. Furthermore, we show that CSPGs–TLR4 pathway activation does not mediate other well-known functions of CSPGs, such as their growth inhibitory effects, since growth of cultured neurons in response to CSPG activation or degradation was uninfluenced by TLR4 signalling. This confirms immunomodulation as the critical role of CSPG–TLR4 interactions.

This work has implications for immunomodulatory therapies for SCI. Together our data indicate that CSPGs in the injured environment play a critical role at multiple stages of the immune response. First, they activate innate immune cells to a proinflammatory state—stimulating macrophages and microglia to express prototypical M1 pro-inflammatory markers. Second, they contribute to the infiltration of adaptive immune cells—propagating the expression of Th1 pro-inflammatory lymphocytes. Third, their continued presence delays inflammation resolution—while M1-activated immune cells should naturally convert to a pro-resolving M2 phenotype, CSPGs block this transition, keeping them in an activated pro-inflammatory state and perpetuating inflammation. These effects are mediated via TLR4 signalling. Further understanding of the mechanisms that prolong inflammation and hinder resolution will aid the development of pro-resolution strategies. CSPG-targeting strategies, such as ChABC and ISP3 represent a particularly potent therapeutic approach for SCI, since they represent both a neuroplasticity and an immunomodulatory strategy, addressing two of the main goals for spinal cord repair. Developing further anti-CSPG strategies could be an important avenue for treatment of other complex chronic inflammatory diseases.

In summary, we have identified a new role for CSPGs in resolution failure after SCI, where they prevent pro-resolution phenotypic conversion of immune cells via a TLR4-dependent mechanism. Insights into the dynamic interactions of CSPGs and immune cells may aid immunomodulatory therapies for SCI and other neurological disorders with a marked inflammatory component.

Methods

Animals. One hundred and eighty-two adult female Lister Hooded (LH) rats (200–220 g; Charles River) were used for in vivo and in vitro studies. Rats were housed under a 12 h light/dark cycle with ad libitum access to food and water. All procedures were performed in accordance with the United Kingdom Animals (Surgical Procedures) Act 1986, approved by the Animal Welfare and Ethical Review Body (AWEB) of King’s College London and conducted under Home Office project license 70/8032 and HFEFSC02. Methods and results are written in accordance with the ARRIVE guidelines for publishing in vivo research.

Twenty-four 10-week-old female mice C57/B16 wild-type (WT; n = 12) and TLR4-Knock-out (TLR4−/−; n = 12) (23–25 g; C57/B16 background kindly provided by Dr. S. Akira, Osaka, Japan) were used for in vitro experiments. Mice were anaesthetised with isoflurane (1–2.5% in oxygen) under a 12 h light/dark cycle with ad libitum access to food and water, under controlled conditions of temperature (23 °C) and humidity (60%). All procedures were carried out in accordance with the guidelines approved by the European Communities Directive 2010/63/ECC and by Spanish Royal Decree 1201/2005 with the approval of the Ethical Committee of Animal Experimentation of the Príncipe Felipe Research Centre (Valencia, Spain).

Lentiviral delivery of ChABC. In order to express ChABC in the spinal cord, we used a lentiviral vector containing the cDNA coding for a mammalian-compatible engineered ChABC gene (termed LV-ChABC), with ChABC expression driven by the mouse phosphoglycerate kinase (PGK) promoter. The production of these vectors was described in detail elsewhere and second generation lentiviral vectors were generated as described previously. The resulting vectors were integrating, self-inactivating and pseudotyped with VSV-G (vesicular stomatitis virus G). Viral particles were harvested by ultracentrifugation and tittered by serial dilution of HEK293T cells followed by qPCR for the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) as described previously.

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SCI model and treatment. Adult female Lister Hooded rats (n = 158) were anaesthetised with isoflurane (O2, 2.1 L/min) and skin was shaved then cleansed with sequential chlorohexidine and iodine swabs. Skin and underlying muscle were retracted and a thoracic laminectomy of T10 vertebra was performed. Periosteum was removed. Rats then received a midline 150 kdyn spinal cord contusion injury at level T10 using an Infinite Horizon impactor and 2.5 mm diameter impact tip (Precision Systems Instrumentation). Immediately following injury, rats underwent midline intraspinal injection of viral vectors at two sites (0.5 µl, per site injected at 1 mm rostral and 1 mm caudal to the injury site), using a fine glass-pulled-pipette and Microdrive pump (NanoLitre 2010 Injector/Micro 4 Controller, World Precision Instruments). The pipette was lowered 1.5 mm in the dorsoventral axis then retracted 0.5 mm and vectors were injected at a rate of 200 nl/min. The pipette was left in place for a further 2 min to ensure vector diffusion. Following intraspinal injection, overlying musculature and skin were reapproximated. Body temperature was maintained at 37 °C using a self-regulating heating mat throughout and animals recovered for at least 1 h in an incubator (water base thermostat 32 °C, Thermore, and were then returned to home cages. Analgesia (Capriven, 5 mg/kg; was administered perioperatively, and animals received daily subcutaneous injection of 5 µg/kg of Bupivacaine and 5 µg/kg of Capriven for 3 days following surgery. Bladder expression was performed twice a day until reflexive bladder emptying was restored (typically by 7 dpi). In the acute postoperative period following surgery, extensive welfare checks were carried out, including provision of accessible chew, hydration gel and soft bedding in all cages, before returning to standard husbandry conditions.

Bone marrow-derived macrophage culture (BMDM). BMDMs were obtained from the tibia and femurs of LH rats and naïve C57/B16 mice. Animals were euthanized by CO2 and bone marrow was flushed from the bones with ice-cold sterile DMEM medium (Gibco) using a 23 G needle. The suspension was...
Pellets were resuspended in 7 ml of 30% Percoll (DPBS, Gibco) on ice and stripped of olfactory bulbs, cerebellum and midbrain cells/cm² concentration. Microglia culture purity was evaluated by qPCR, shaken for 5 h at 300 rpm at 37 °C to harvest microglial cells and seeded at 100,000 cells/cm² in 24-well plates coated with poly-D-lysine (PDL) (0.1 mg/ml. Sigma-Aldrich). Cells were incubated at 37 °C in a water saturated atmosphere with 5% CO₂, with 50% of the total media refreshed at day 3 and 7. Upon cell confluence (10–12 days in culture), flasks were shaken for 5 h at 300 rpm at 37 °C to harvest microglial cells and seeded at 100,000 cells/cm² concentration. Microglia culture purity was evaluated by qPCR, described below.

**Neonatal microglial cell culture.** Primary microglia were obtained from mixed glia culture from the forebrain of 2-day-old LH rat pups. Postnatal pups were anesthetised on ice and rapidly decapitated. Brains were isolated in Dulbecco’s PBS (DPBS, Gibco) on ice and stripped of olfactory bulbs, cerebellum and midbrain cells. Cells were minced using a sterile razor blade and tissue were centrifuged at 300 g for 5 min, the supernatant removed and 1 ml of 0.25% Trypsin EDTA (Gibco) with DNAseI (200 µg/ml) for 15 min at 37 °C. Cells were gently centrifuged and resuspended in complete medium (DMEM with high glucose and 10% foetal bovine serum and Pen-Strep) and were seeded alone or in poly-I-Lysine-coated plates at a density of 1–1.2 x 10⁶ cells/cm². Cells were polarised using an inflammatory stimulus (LPS, 1 µg/ml) and/or an immunological activator (0.2 µg/ml Phorbol 12-myristate 13-acetate, PMA) in the presence of either TNF-α (20 ng/ml) or M-CSF (50 ng/ml) for 16 h. After 16 h, microglia were evaluated for qPCR analysis, respectively, as described below. Polarisation of rat BMDM and mouse BMDM were evaluated using qPCR and flow cytometry or qPCR analysis, respectively, as described below.

**Adult microglial cell culture.** Adult microglia were isolated based on a modified version of an established protocol. LH rats were deeply anaesthetised with sodium pentobarbital (Euthatal, 80 mg/kg, administered intraperitoneally) and transcardially perfused with ice-cold DPBS containing 2 ml EDTA. Brains were dissected, homogenised with Dounce homogeniser in 5 ml ice-cold DPBS, filtered through a 70 µm cell strainer and centrifuged at 10,000 g for 5 min, 4 °C. Pellets were resuspended in 7 ml of 30% Percoll centrifuged at 400 x g, 5 min at 4 °C, resuspended in complete medium, filtered through a 70 µm cell strainer and seeded at 300,000 cells/ml to obtain a single-cell suspension. 10 ml of pre-warmed DMEM/F12 complete medium (DMEM/F12, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco) and 70 µl of Pen-Strep was added to the pellet. The cell suspension was centrifuged (400 x g, 5 min at 4 °C), resuspended in complete medium, filtered through a 70 µm cell strainer and seeded at 300,000 cells/ml to obtain a single-cell suspension. 10 ml of pre-warmed DMEM/F12 complete medium (DMEM/F12, Gibco) supplemented with 10% foetal bovine serum (FBS, Gibco) and 70 µl of Pen-Strep was added to the pellet. The cell suspension was centrifuged (400 x g, 5 min at 4 °C), resuspended in complete medium, filtered through a 70 µm cell strainer and seeded at 300,000 cells/ml to obtain a single-cell suspension. Cells were incubated at 37 °C in a water saturated atmosphere with 5% CO₂, with 50% of the total media refreshed at day 3 and 7. Upon cell confluence (10–12 days in culture), flasks were shaken for 5 h at 300 rpm at 37 °C to harvest microglial cells and seeded at 100,000 cells/cm² concentration. Microglia culture purity was evaluated by qPCR, described below.

**In vitro stimulation of microglia and macrophages.** M0 macrophages were incubated with 1, 2.5 and 5 µg/ml soluble CSPGs (Merck-Millipore, cc117) for 4 h to determine the optimal CSPG dose, evaluated by qPCR. M0, M1, and M2 macrophages were incubated with CSPG on a PDL + CSPG matrix or in a solubilized form from a 14-day-old C57Bl/6 mouse Chondroitin sulfate (PDL). 10 ml of pre-warmed DMEM/F12 complete medium (DMEM/F12, Gibco) supplemented with 10% foetal bovine serum (FBS) and 70 µl of Pen-Strep was added to the pellet. Cells were seeded at 100,000 cells/cm² concentration in 24-well plates coated with poly-D-lysine (0.1 mg/ml. Sigma-Aldrich).

**Endocytic TLR4 quantification.** M1 or M2 mouse derived macrophages were fixed with PFA 4%, permeabilized or not with 0.1% Triton X-100 and subsequently blocked with 5% normal goat serum in PBS. The primary antibody against TLR4 (Supplementary Table 4) incubated overnight at 4 °C. After washing, AlexaFluor-488 conjugated antibody (1:400, Invitrogen) against mouse IgG was incubated for 1 h at room temperature. Images were acquired using Nikon A1R Si Confocal Imaging system on an Eclipse Ti-E inverted microscope. Optimised conditioned media was used for further in vitro CSPG digestion experiments. For determining ChABC effects on polarised BMDMs, M0, M1 and M2 polarised macrophages were cultured for 4 h with complete medium derived from cells expressing GFP or ChABC enricned medium, both alone or supplemented with CSPGs (5 µg/ml). After incubation, medium was removed and after three washes cells were collected. The effect of CSPG digestion was assessed by qPCR.

**CSPG digestion products assay.** Generation of ChABC-conditioned media and CSPG digestion. Differentiated rat BMDM cells were plated on PDL precoated glass coverslips at 100,000–120,000 cells/cm² density in complete media. After 16 h, media was replaced with viral infection media (complete media supplemented with either LV-GFP or LV-ChABC, used in the in vivo experiments, at different concentrations (0, 2x10⁶, 4x10⁶ and 1x10⁹ CFUs/ml) for 4 or 16 h at 37 °C in a water saturated atmosphere with 5% CO₂ and 50% of the total media refreshed at day 3 and 7. Upon cell confluence (10–12 days in culture), flasks were shaken for 5 h at 300 rpm at 37 °C to harvest microglial cells and seeded at 100,000 cells/cm² concentration. Microglia culture purity was evaluated by qPCR, described below.

**Flow cytometry sample preparation.** To study the dynamics of immune cells after SCI, spinal cords from sham (laminectomy only) and injured LV-GFP or LV-ChABC treated rats were harvested at day 1, 3, 7, 14 and 28 days after lesion. Animals were deeply anaesthetised with sodium pentobarbital (Euthatal, 80 mg/kg, administered intraperitoneally) and transcardially perfused with ice-cold 1X phosphate buffered saline (PBS) containing 0.1% Sodium citrate and 0.1% Calcium chloride and 0.002% EDTA. Intraperitoneal infusion of 50% of the injured spinal cord centred around the lesion epicentre was disected and put into ice-cold PBS. Tissue was mechanically dissociated and then passed
through a 70 μm cell strainer (BD Falcon, Germany), and centrifuged at 300 g at 4 °C. The pellet was incubated with Myelin Removal Beads II (Millenyi Biotec, Germany) and passed through 15 cm column (Miltenyi Biotec) to date. For primary cell cultures, cells were detached by incubating with Enzyme Free cell dissociation buffer (Milipore). Remaining adherent cells were gently scraped and passed through a 70 μm cell strainer (BD Falcon, Germany). After centrifugation, cells were resuspended in DPBS (Gibco).

**FACS staining/gating and analysis.** Both isolated CNS cells and primary cell cultures were washed with cold PBS then incubated with a live/dead stain (eBioscience). After cell counts, samples were incubated with anti-CD16 and CD32 (1:50, BD Bioscience) for 15 min on ice to block the Fc receptors and stained with specific extracellular antibodies for 30 min (antibodies are detailed in Supplementary Table 1—Panel 1). For TLRA expression studies, cells were stained with CD45, CD11b, and TLRA (detailed in Supplementary Table 1—Panel 1) for FACS detection. For intracellular staining used in phenotype analysis experiments, cells were then washed, fixed using 2% PFA and permeabilized with cell permeabilization buffer (Invitrogen) containing intracellular antibodies (antibodies are detailed in Supplementary Table 1—Panel 2). Single-stained Compensation Beads (BD) were used according to manufacturer’s instructions to prepare compensation controls by incubating with fluorescently conjugated antibodies used in the experiments. Fluorescence minus one (FMO) experiment and isotype-matched control samples were run prior to this study to establish the positiveness of the samples and to aid the optimisation of the compensation matrix. Based on this, the compensation matrix was adjusted where necessary due to over- or under-compensation by the automated algorithm.

Cells were acquired on LSRFortessa III flow cytometry (BD) and the data was analysed with FlowJo (Treestar) software. Single live cells were gated on the basis of dead cell exclusion (L/D), side (SSC-A) and forward scatter (FSC-A) gating, and were then reselected using side scatter width (SSC-W) against SSC-A. To perform the analysis, cells were first gated for CD45 to ensure that only infiltrating leukocytes and resident microglia were selected. Then, for the single-stained, a combination of markers were added based on dead cell exclusion (L/D), side (SSC-A) and forward scatter (FSC-A) compensation by the automated algorithm. A compensation matrix was adjusted where necessary due to over- or under-compensation by the automated algorithm.

**Cytokine and chemokine expression analysis.** The complex maps of immune cells were plotted by t-distributed stochastic neighbour embedding (t-SNE) which, reduced dimensionality of multi-colour flow cytometry data into a 2-dimensional data space (SNE-1 vs. SNE-2). Concatenating graphs are generated from all samples in each group. Multiple criteria were used to identify the phenotype of microglia, in addition to prior described antibodies, these cells were further differentiated based on CD86, CD68, MHC II, iNOS, CD45, HSF4, CD163, CD206, and Arg1 expression. The FACSsymphony A5 flow cytometer, equipped with UV (355 nm), violet (405 nm), blue (488 nm), yellow/green (561 nm), and red laser (637 nm) was utilised for these experiments. Data was analysed and dot plots were generated using FlowJo (Treestar) software and population clustering was performed by t-SNE FlowJo Plugin.

**RNA extraction and reverse transcription.** Total RNA from frozen spinal cord and sorted immune cells were extracted using the NResearch Mini Kit including DNAse treatment (Qiagen) according to the manufacturer’s recommendations. For primary cultured cells, cells were homogenised in TRIzol® reagent (Thermo Fisher Scientific). An aqueous (RNA-containing) phase was generated using 1:5 bromo-chloro-propane, mixed 1:2 with 70% isopropanol and centrifuged at 12,000 g for 20 min. The aqueous phase was incubated with TRIzol® and pelleted at 12,000 g for 10 min. Total RNA was converted to cDNA using the reverse transcription kit (Applied Biosystems).

**Immunohistochemistry.** Rats were anaesthetised with an overdose of pentobarbital and perfused with PBS and 4% paraformaldehyde (PFA). Spinal cords were dissected and post-fixed at 4 °C. Spinal cords were dehydrated in 20% sucrose. 20-μm-thick sections were mounted on Super Frost slides after cryosectioning. For iNOS staining, heat mediated antigen retrieval was performed prior to blocking by incubating the slides in citrate buffer pH 6.0 at 90 °C for 30 min. After blocking, sections were incubated with primary antibodies overnight at 4 °C followed by appropriate secondary antibodies. Primary antibodies used are detailed in Supplementary Table 4. The sections were incubated with appropriate fluorescently conjugated secondary antibodies: goat anti-mouse IgM AF-568 (#A21043, Thermofisher, San Diego, USA), donkey anti-rabbit IgG Alexa Fluor® 488 (#A10044, Thermofisher, San Diego, USA) or goat anti-rat IgG Alexa Fluor® 568 (#A10042, Thermofisher, San Diego, USA) for 1 h at room temperature. Sections were washed in PBS and mounted with Fluoromount-G with DAPI mounting medium. Images were captured with a Zeiss LSM Z1 fluorescence microscope equipped with a Zeiss Axiocam MRm camera. Tissue sections were stained in post-acquisition processing using AxioVision software. Staining was quantified using ZEISS ZEN software by measuring the mean fluorescence over sections excluding roots and meninges.

**Immunoblotting.** Spinal cord samples used for the Luminex assay were also used for Western blotting. Protein extracts were denatured and reduced in 2× sample buffer (500 μL Tris, pH 6.8, 40% glycerol, 0.2% SDS, 2% β-mercaptoethanol, and 0.02% bromophenol blue), and boiled (98 °C, 10 min). For BMDM immunoblotting, proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) on ice for 30 min. Samples were centrifuged (20,000 g, 4 °C, 20 min) and protein quantified with DC protein assay (Bio-Rad) as described above. Twenty micrograms of protein per sample were loaded and separated on 15-well Bis-Tris 4–12% polyacrylamide gels (NuPAGE, Invitrogen). Proteins were then transferred on nitrocellulose membranes (ThermoFisher Scientific) for immunoblotting. Membranes were stained with Ponceau red stain to visualise protein transfer and total protein loading. Following blocking in 5% fat-free milk powder in PBS, membranes were incubated overnight at 4 °C with anti-rat or anti-mouse antibodies against phosphorylated protein form described in Supplementary Table 3. Following three washes in PBS TWEEN 20, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (Dako Cytomation) in 5% fat-free milk powder at 4°C for 1 h (room temperature). Enhanced chemiluminescence (ECL) reagents (GE Healthcare) and a Kodak processor and Alliance Q9 Advanced (Uvitec Cambridge) were used for detection and blot development. Membranes were restored using western blot stripping buffer (Thermo Scientific) and re-blocked in 5% BSA in PBS. The membranes were then incubated with the appropriate primary antibodies with anti-rabbit and anti-mouse antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with HRP conjugated secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight. The membranes were washed with 1× PBST and incubated with secondary antibodies at 4 °C overnight.
reaction was performed in triplicates. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Actb (β-actin) were used as housekeeping gene. The log fold change in mRNA expression was calculated from ΔΔCt values relative to control samples.

**Multidimensional analysis for gene expression.** Dual multiple factor analysis (dMFA) was conducted to study the longitudinal evolution of the multi-dimensional gene expression profile. Different groups of subjects (animals at different timepoints) were analysed on the same variables (mRNA levels of selected genes). Global patterns (consensus components or dimensions across time) were extracted and the effects of time in the gene expression patterns (time partial components or dimensions) were studied. Loadings (relationship of genes with the components) are interpreted as the correlation of a variable with a component (ranging from −1 to 1) where the value of each animal for each component (component scores) was extracted and dMFA performed using the R package FactoMineR, with scaling gene values to unit variance on samples at 6, 12, 1, 3, 4 and 7 days post-injury. Three Cattel’s criteria global components were determined as representative by Scree test, |loading| ≥ 0.3 are marked as the cutoff for the relative importance of each component on each gene. To study the impact of time and experimental group on the three extracted components, a two-full factorial ANOVA was performed taking time, group and their interaction as “factors” for each component and “group by time pairwise comparison” was performed using Tukey correction for multiple comparisons. The Euclidean distance to naïve centroid (average) for the three dimensions was calculated for each animal. A two- full factorial ANOVA was also conducted in these distances as described above. At 7 dpi (a key resolution time point), a second multidimensional analysis was conducted using PCA. Cattel’s criteria selected three components as before. A two- full factorial ANOVA was conducted (taking group, component and the interaction between them as factors), with alpha significance level of 0.05. Loading and score plots were generated using the ggplot2 R package.

**Statistical analysis.** Numerical values are reported as mean ± standard error of the mean (SEM). Datasets were tested for normality using Kolmogorov–Smirnov tests. Student’s t-test was used for single comparisons between two groups and one- or two-way ANOVA followed by Bonferroni’s multiple-comparison tests for more than two groups when the data were normally distributed. Mann–Whitney tests were performed for two-sample comparisons or Kruskal–Wallis tests with Dunn’s post hoc comparisons for more than two samples if the data were not normally distributed. Normal distribution was assumed if sample size was sub-threshold for normality testing. Statistical analyses were performed with GraphPad Prism v8 and v9 software and differences were considered significant *p < 0.05, **p < 0.01 and ***p < 0.001. All statistics and post hoc tests are stated in the figure legends and correction for multiple comparisons performed where appropriate. Multivariate analyses (dMFA and PCA) were performed in R package FactoMineR.

**Blinding and randomisation.** During surgical procedures blinding was ensured by the experimenter injecting the vectors being unaware of animal identification number. Animals across all groups were randomised into cages. Experimenter were also blinded to treatment groups for histological analyses. For in vitro experiments, activation, data collection and all statistical analysis was completed with the investigator blind to the experimental coding. In some cases, such as with TLR4 WT vs. KO experiments, blinding was not possible during cell isolation. However, as in the rest of in vitro experiments, activation, data collection and all statistical analysis was completed with the investigator blind to the experimental coding.

**Figure design.** Figures were designed using GIMP 2.10.22 and Biorender (with full licence to publish).

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

**Data availability** Complete source data are provided with this paper. Data that support the findings will be made available upon reasonable request to the corresponding author.

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**References**

1. Hutson, T. H. & Di Giovanni, S. The translational landscape in spinal cord injury: focus on neuroplasticity and regeneration. Nat. Rev. Neurol. 15, 732–745 (2019).

2. Bradbury, E. J. & Burnside, E. R. Moving beyond the glial scar for spinal cord repair. Nat. Commun. 10, 3879 (2019).

3. Tran, A. P., Warren, P. M. & Silver, J. The biology of regeneration failure and success after spinal cord injury. Physiol. Rev. 98, 881–917 (2018).

4. Moon, L. D. F., Asher, R. A., Rhodes, K. E. & Fawcett, J. W. Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. Nat. Neurosci. 4, 465–466 (2001).

5. Bradbury, E. J. et al. Chondroitin ABC esterases functional recovery after spinal cord injury. Nature 416, 636–640 (2002).

6. García-Alias, G., Barkhuyzen, S., Buckle, M. & Fawcett, J. W. Chondroitinase ABC treatment opens a window of opportunity for task-specific rehabilitation. Nat. Neurosci. 12, 1145–1151 (2009).

7. Allain, W. J., Horn, K. P., Hu, H., Dick, T. E. & Silver, J. Functional regeneration of sensory pathways after spinal cord injury. Nature 475, 196–200 (2011).

8. Warren, P. M. et al. Rapid and robust restoration of breathing long after spinal cord injury. Nat. Commun. 9, 4843 (2018).

9. Wu, D. et al. Chronic neuronal activation increases dynamic microtubules to enhance functional axon regeneration after dorsal root crush injury. Nat. Commun. 11, 6131 (2020).

10. Sorokin, L. The impact of the extracellular matrix on inflammation. Nat. Rev. Immunol. 10, 712–723 (2010).

11. Heindryckx, F. & Li, J.-P. Role of proteoglycans in neuro-inflammation and central nervous system fibrosis. Matrix Biol. 68–69, 589–601 (2018).

12. Pu, A. et al. The glycosyltransferase EXT12 promotes proteoglycan deposition and injurious neuroinflammation following demyelination. J. Neuroinflamm. 17, 220 (2020).

13. Warford, J. et al. Surfen, a proteoglycan binding agent, reduces inflammation but inhibits remyelination in murine models of Multiple Sclerosis. Acta Neuropathol. Commun. 6, 4 (2018).

14. Stephenson, E. L. et al. Chondroitin sulfate proteoglycans as novel drivers of neurodegenerative disease — Multiple Sclerosis. Brain 141, 1094–1110 (2018).

15. David, S. & Kroner, A. Repertoire of microglial and macrophage responses after spinal cord injury. Nat. Rev. Neurosci. 12, 388–399 (2011).

16. Prüss, H. et al. Non-resolving aspects of acute inflammation after spinal cord injury (SCI): indices and resolution plateau. Brain Pathol. 21, 652–660 (2011).

17. Brennan, F. H. & Popovich, P. G. Emerging targets for reprogramming the immune response to promote repair and recovery of function after spinal cord injury. Curr. Opin. Neurol. 31, 334–344 (2018).

18. Popovich, P. G. & Longbrake, E. E. Can the immune system be harnessed to repair the CNS? Nat. Rev. Neurosci. 9, 481–493 (2008).

19. Serhan, C. N. Pro-resolving lipid mediators are leads for resolution physiology. Nature 510, 92–101 (2014).

20. O’Reilly, M. L. & Tom, V. J. Neuroimmune system as a driving force for plasticity following CNS injury. Front. Cell. Neurosci. 14, https://doi.org/10.3389/fncel.2020.00187 (2020).

21. Buckley, C. D., Gilroy, D. W. & Serhan, C. N. Resolving lipid mediators and mechanisms in the resolution of acute inflammation. Immunity 40, 315–327 (2014).

22. David, S., López-Vales, R. & Wee Yong, V. in Handbook of Clinical Neurology Vol. 109 (eds Verhaagen, J. & McDonald, J. W.) 485–502 (Elsevier, 2012).

23. Hawthorne, A. L. & Popovich, P. G. Emerging concepts in myeloid cell biology after spinal cord injury. Neurotherapeutics 8, 252–261 (2011).

24. Bartus, K. et al. Large-scale chondroitin sulfate proteoglycan digestion with chondroitinase gene therapy leads to reduced pathology and modulates macrophage phenotype following spinal cord contusion injury. J. Neuroscience 34, 4822–4836 (2014).

25. Didangelo, A., Iberl, M., Vinsland, E., Bartus, K. & Bradbury, E. J. Regulation of IL-10 by chondroitinase ABC promotes a distinct immune response following spinal cord injury. J. Neuroscience 34, 16424–16432 (2014).

26. Dyck, S. et al. Perturbing chondroitin sulfate proteoglycan signaling through LAR and PTP receptors promotes a beneficial inflammatory response following spinal cord injury. J. Neuroinflamm. 15, 90–98 (2018).

27. Gaudet, A. D. & Popovich, P. G. Extracellular matrix regulation of inflammation in the healthy and injured spinal cord. Exp. Neurol. 258, 24–34 (2014).

28. Muir, E. M. et al. Modification of N-glycosylation sites allows secretion of Matrix Biol. 69, 636–640 (2014).

29. Serhan, C. N. et al. Resolution of inflammation: state of the art, definitions and terms. FASEB J. 21, 325–332 (2007).

30. Filiano, A. J., Gadani, S. P. & Kipnis, J. How and why do T cells and their derived cytokines affect the injured and healthy brain? Nat. Rev. Neurosci. 18, 375–384 (2017).

31. Schafer, L. et al. The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages. J. Clin. Investig. 115, 2223–2233 (2005).

32. Brancucci, A. et al. The proteoglycan biglycan mediates inflammatory response by activating TLR-4 in human chondrocytes: Inhibition by specific siRNA and high polymerized Hyaluronan. Arch. Biochem. Biophys. 640, 75–82 (2018).
Bundesen, L. Q., Scheel, T. A., Bregman, B. S. & Kromer, L. F. Ephrin-B2 and CD41 trafficking and its influence on LPS-induced pro-inflammatory signaling. Cell. Mol. Life Sci. 78, 1233–1261 (2021).

Tran, A. P., Sundar, S. Y., Lu, B. T. & Silver, J. Modulation of receptor protein tyrosine phosphatase sigma increases chondroitin sulfate proteoglycan degradation through Cathepsin b secretion to enhance axon outgrowth. J. Neurosci. 38, 5399–5414 (2018).

Rajaiah, R., Perkins, D. J., Ireland, D. D. & Vogel, S. N. CD14 dependence of TRL4 endocytosis and TRIF signaling displays ligand specificity and is dissociable in endothotoxin tolerance. Proc. Natl Acad. Sci. USA 112, 8391–8396 (2015).

Steri, S. et al. RhoA drives actin compaction to restrict axon regeneration and astrocyte reactivity after CNS injury. Neuron 109, 3436–3455.e3439 (2021).

Lang, B. T. et al. Modulation of the proteoglycan receptor PTPσ promotes recovery after spinal cord injury. Nature 518, 404–408 (2015).

Fidler, P. S. et al. Comparing astrocytic cell lines that are inhibitory or permissive for axon growth: the major axon-inhibitory proteoglycan is NG2. J. Neurosci. 19, 8778–8785 (1999).

Zravry, T. et al. Acute and non-resolving inflammation associate with oxidative injury after human spinal cord injury. Brain 144, 144–161 (2020).

Greenhalgh, A. D., David, S. & Bennett, F. C. Immune cell regulation of glia during CNS injury and disease. Nat. Rev. Neurosci. 21, 139–152 (2020).

Tran, A. P., Shechter, R. & Schwartz, M. The bright side of the glial scar in CNS repair. Nat. Rev. Neurosci. 10, 235–241 (2009).

Dyck, S. M. & Karimi-Abdolrezaee, S. Chondroitin sulfate proteoglycans: Key modulators in the developing and pathologic central nervous system. Exp. Neurol. 269, 169–187 (2015).

Ziegler-Hirthbrock, L. Monocyte subsets in man and other species. Cell. Immunol. 289, 338–359 (2014).

Mantovani, A. et al. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25, 677–686 (2004).

Kigerl, K. A. et al. Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord. J. Neurosci. 29, 13435–13444 (2009).

Dumas, A. A., Borst, K. & Prinz, M. Current tools to interrogate microglial biology. Neuron 109, 2805–2819 (2021).

Wahane, S. et al. Diversified transcriptional responses of myeloid and glial cells in spinal cord injury shaped by HDAC3 activity. Sci. Adv. 7, https://doi.org/10.1126/sciadv.abd881 (2021).

Shang, M. et al. Macrophage-derived glutamine boosts satellite cells and muscle regeneration. Nature 587, 626–631 (2020).

Kroner, A. et al. TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord. Neuron 83, 1098–1116 (2014).

Zhou, X. et al. Microglia and macrophages promote corollaring, wound contraction and recovery after spinal cord injury via Plexin-B2. Nat. Neurosci. 23, 337–350 (2020).

Li, Y. et al. Microglia-organized scar-free spinal cord repair in neonatal mice. Nature 587, 613–618 (2020).

Bellver-Landete, V. et al. Microglia are an essential component of the neuromorphic scar that forms after spinal cord injury. Nat. Commun. 10, 518 (2019).

Trivedi, A., Olivas, A. D. & Noble-Haeusslein, L. J. Inflammation and spinal cord injury: infiltrating leukocytes as determinants of injury and repair processes. Clin. Neurosci. Res. 6, 283–292 (2006).

Giuliani, F., Goodyer, C. G., Antel, J. P. & Yong, V. W. Vulnerability of human neurons to T cell-mediated cytotoxicity. J. Immunol. 171, 368–379 (2003).

Walsh, J. T. et al. MHCII-independent CD4+ T cells protect injured CNS neurons via IL-1β. J. Clin. Invest. 125, 699–714 (2015).

Fee, D. et al. Activated/effector CD4+ T cells exacerbate acute damage in the central nervous system following traumatic injury. J. Neuroinflamm. 136, 54–66 (2003).

Groth, P. et al. Kherzog, K., Hertzog, P. J., Ravasi, T. & Hume, D. A. Interferon-γ: an overview of signals, mechanisms and functions. J. Leukoc. Biol. 75, 163–189 (2004).

Hu, X. & Ivashev, L. B. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. Immunity 31, 539–550 (2009).

Ivashevich, L. B. & IFNγ: signaling, epigenetics and roles in immunity, metabolism, disease and cancer immunotherapy. Nat. Rev. Immunol. 18, 545–558 (2018).

Bundesen, L. Q., Scheel, T. A., Bregman, B. S. & Kromer, L. F. Ephrin-B2 and EphB2 regulation of astrocyte- meningial fibroblast interactions in response to spinal cord lesions in adult rats. J. Neurosci. 23, 7757–7780 (2003).

Ahuja, C. S. et al. Traumatic spinal cord injury. Nat. Rev. Dis. Prim. 3, 17018 (2017).

Norenberg, M. D., Smith, J. & Marcillo, A. The pathology of human spinal cord injury: defining the problems. J. Neurotrauma 21, 429–440 (2004).

Francois, M. & Jaijona, I. et al. Macrophage 1 promotes inflammatory resolution, neuroprotection, and functional neurological recovery after spinal cord injury. J. Neurosci. 37, 11731–11743 (2017).

Ortega-Gómez, Á., Perretti, M. & Soehnlein, O. Resolution of inflammation: an integrated view. EMBO Mol. Med. 5, 661–674 (2013).

Headland, S. E. & Northington, L. V. The resolution of inflammation: principles and challenges. Semin. Immunol. 27, 149–160 (2015).

Rolls, A. et al. A disaccharide derived from chondroitin sulphate proteoglycan promotes central nervous system repair in rats and mice. Eur. J. Neurosci. 20, 1973–1983 (2004).

Castro-Alves, V. C. & Nascimento, J. R. O. D. Site matters: TLR4-mediated effects of (α,1.5)-linear arabin-oligosaccharides in macrophage-like cells depend on their degree of polymerization. Food Res. Int. 141, 11093 (2021).

Arandjelovic, S. & Ravichandran, K. S. Phagocytosis of apoptotic cells in homeostasis. Nat. Immunol. 16, 907–917 (2015).

Rolls, A. et al. A sulfated disaccharide derived from chondroitin sulphate proteoglycan protects against inflammation-associated neurodegeneration. J. Neurosci. 20, 547–549 (2012).
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Author contributions

E.J.B. and I.F.Q. conceived the study. I.F.Q. carried out the majority of the experiments. I.F.Q., L.M., and E.R.B. performed the rodent surgeries. J.V. and F.D.W. generated viral vectors for the study. I.F.Q. performed the FACS/Flow experiments, T-SNE analysis, qPCR, Western blot and Luminex studies. I.F.Q. and A.T.E. performed the TLR4 KO studies. S.B. performed the histological procedures. I.F.Q., M.S.P., V.M.M., A.T.E. and S.B. analysed the data. I.F.Q., M.S.P. and S.B. generated the figures. E.J.B., I.F.Q. and E.R.B. wrote the paper. E.J.B., V.M.M. and J.V. secured funding.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Elizabeth J. Bradbury.

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