

Derivation and transcriptional reprogramming of border-forming wound repair astrocytes after spinal cord injury or stroke in mice

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Central nervous system (CNS) lesions become surrounded by neuroprotective borders of newly proliferated reactive astrocytes; however, fundamental features of these cells are poorly understood. Here we show that following spinal cord injury or stroke, 90% and 10% of border-forming astrocytes derive, respectively, from proliferating local astrocytes and oligodendrocyte progenitor cells in adult mice of both sexes. Temporal transcriptome analysis, single-nucleus RNA sequencing and immunohistochemistry show that after focal CNS injury, local mature astrocytes dedifferentiate, proliferate and become transcriptionally reprogrammed to permanently altered new states, with persisting downregulation of molecules associated with astrocyte–neuron interactions and upregulation of molecules associated with wound healing, microbial defense and interactions with stromal and immune cells. These wound repair astrocytes share morphologic and transcriptional features with perimeningeal limitans astrocytes and are the predominant source of neuroprotective borders that re-establish CNS integrity around lesions by separating neural parenchyma from stromal and immune cells as occurs throughout the healthy CNS.

All organs share the ability to rapidly repair tissue lesions by generating newly proliferated cells that derive from stromal-cell, immune-cell and parenchymal-cell lineages. This multicellular proliferative wound response is a protective adaptation that limits tissue damage, sustains organ integrity and function, and is essential for organism survival¹. In the CNS, astrocytes are key components of a multicellular proliferative wound response that is stimulated by tissue damage across a broad cross-section of CNS disorders including traumatic injury,

stroke, infection, autoimmune inflammation and certain neurodegenerative diseases^{2–6}. Understanding the derivation and temporally regulated proliferation, maturation, functions and potential failures of newly proliferated astrocytes during this wound response is fundamental to understanding and ameliorating the pathophysiology of many different CNS disorders.

Astrocytes are CNS parenchymal cells of neural progenitor cell origin⁷. They contiguously tile the entire CNS and provide multiple

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activities essential for CNS function in health and disease^{2,8–12}. Astrocytes rarely divide in healthy adult CNS and exist in a state of potentially reversible cell cycle arrest (G0)^{13,14}. Astrocytes respond to all forms of CNS injury and disease with molecular, structural and functional changes commonly referred to as astrocyte reactivity^{2,6,15}. Notably, astrocyte reactivity can be either nonproliferative or proliferative^{2,13,16} and is tailored to different disorder contexts by multifactorial signaling mechanisms¹⁷.

Proliferative astrocyte reactivity occurs in response to overt CNS tissue damage and results in the formation of borders that surround tissue damaged by trauma, ischemia, infection, autoimmune inflammation, fibrosis, neoplasm, foreign bodies or pronounced neurodegeneration^{4,13,16,18–25}. Multiple genetically targeted loss-of-function studies demonstrate that newly proliferated astrocyte borders serve essential functions that protect adjacent viable neural tissue, such that transgenic ablation or attenuation of border-forming astrocytes leads to impaired neural parenchymal wound repair with greater spread of destructive inflammation, larger fibrotic lesions, increased loss of neural tissue and impairment of neurological recovery^{4,13,17,18,23,24,26–29}. Aging-associated perturbation of astrocyte proliferation and border formation is associated with increased loss of neurons and decreased functional recovery after spinal cord injury (SCI) in mice³⁰. Despite the increasingly recognized importance of newly proliferated border-forming astrocytes, fundamental features of these cells are poorly understood. Here, we used a combination of transcriptionally targeted lineage tracing^{31,32}, astrocyte-specific transcriptome analysis^{17,33}, single-nucleus RNA sequencing (snRNA-seq) and immunohistochemical protein detection in mice to identify and selectively profile the transcriptional changes over time after traumatic injury of the predominant cellular source of newly proliferated astrocytes.

Results

Derivation of lesion border astrocytes

Studies from multiple laboratories implicate two main potential cellular sources for newly proliferated astrocytes around CNS injuries: local astrocytes^{18–20,26,34} and local oligodendrocyte progenitor cells (OPCs)^{34–37}. Here, we used lineage tracing based on tamoxifen-regulated Cre-reporter expression³¹ to determine the proportional contributions of these cell types to newly proliferated border-forming astrocytes around hemorrhagic lesions after crush SCI or around ischemic lesions after forebrain stroke caused by infusion of N5-(1-iminoethyl)-l-ornithine (L-NIO, Fig. 1 and Extended Data Fig. 1). We targeted the reporter, tdTomato (tdT), to mature astrocytes by using Aldh1l1-CreERT³⁸, and to OPCs by using either Pdgfra-CreERT-tdT³⁹ or NG2-CreERT-tdT^{40,41}, and induced temporary Cre expression with a 5-day regimen of tamoxifen dosing in healthy young adult (>8 week old) mice (Fig. 1a).

Newly proliferated astrocytes around CNS lesions organize into permanent, high cell density borders with overlapping cell processes that surround non-neural lesion cores of stromal and fibrotic tissue after SCI or stroke (Fig. 1b,c,i and Extended Data Fig. 1a)¹³. We quantified tdT-labeled, lineage-traced, border-forming astrocytes within representative 250 µm zones immediately adjacent to lesion core stromal tissue at 14 days after SCI or forebrain stroke (Fig. 1a–d,i,j), a timepoint by which border formation is largely complete¹³. As benchmarks against which to compare tdT labeling, we used Gfap and Sox9, which together label essentially all newly proliferated border-forming astrocytes around lesions^{13,26,32} (Fig. 1c,d,i,j).

In healthy adult spinal cord or striatum, essentially all astrocytes expressed Sox9 and Aldh1l1-CreERT-tdT, confirming previous reports³⁸, and no astrocytes were detectably derived from OPCs as indicated by Pdgfra lineage tracing (Extended Data Fig. 1b). In uninjured spinal cord, all Sox9-positive and Aldh1l1-tdT-positive astrocytes expressed detectable Gfap, whereas in uninjured striatum, only about 13% did so (Extended Data Fig. 1b).

Lineage tracing showed that after both SCI and stroke, over 90% of Gfap plus Sox9-positive lesion border astrocytes also expressed Aldh1l1-CreERT-tdT, indicating that these cells derived from local mature astrocytes (Fig. 1c,d,i,j). Approximately 10% of Gfap plus Sox9-positive lesion border astrocytes expressed Pdgfra-CreERT-tdT and transcription factors Sox10 and Id3, indicating that these cells derived from local OPCs and exhibited molecular features of reactive astrocytes^{17,42} (Fig. 1c,d,f,i,j and Extended Data Fig. 1c–e). The proportion of OPC-derived border-forming astrocytes was essentially equivalent using NG2-CreERT-tdT lineage tracing (Fig. 1f and Extended Data Fig. 1c,d). In both SCI and stroke, about 75% of the Pdgfra-CreERT-tdT-positive cells in the lesion border zone were Olig2-positive but Gfap-negative and Sox9-negative OPCs (Extended Data Fig. 1f).

To identify newly proliferated cells, bromodeoxyuridine (BrdU) was administered during a 6-day period from 2 to 7 days after injury (Fig. 1a). In healthy adult spinal cord or striatum, no astrocytes were detectably BrdU-labeled, whereas about 8–10% of OPCs were (Extended Data Fig. 1g). At 5 days after injuries, lineage-traced astrocytes expressed the active proliferation marker Ki67 (Fig. 1e). Quantification of BrdU showed that at 14 days after either SCI or stroke, at least 75–85% of lesion border astrocytes that were derived from either astrocytes or OPCs were newly proliferated (Fig. 1g,h,k,l and Extended Data Fig. 1h), and this is probably a conservative estimate because BrdU was administered only once daily.

Injury-induced transcriptional reprogramming

As mature Aldh1l1-expressing astrocytes are the predominant source of border-forming cells, we characterized their temporally dependent transcriptional changes after SCI. We used young adult (3–4 months old) male and female Aldh1l1-CreERT-RiboTag mice for hemagglutinin-positive ribosome immunoprecipitation and cell-specific transcriptome profiling of astrocytes^{33,38} in healthy, uninjured spinal cord and at 2, 5, 14, 28, 42 and 70 days after SCI (Fig. 2a, Extended Data Fig. 2a and Supplementary Data 1), spanning periods of proliferation, border formation and chronic border persistence^{13,17,25}. Specificity of Aldh1l1-CreERT-RiboTag for astrocyte transcriptome analyses has been demonstrated in brain³⁸ and was confirmed here for spinal cord (Extended Data Fig. 2a–k). Sequencing of mRNA from the flow-through solution after RiboTag immunoprecipitation was used to characterize gene expression by other local cells and enabled assessment of astrocyte enrichment (Extended Data Fig. 2a,d–k). Aldh1l1-CreERT-RiboTag gave equivalent astrocyte-enriched transcriptional profiles as mGfap-Cre-RiboTag^{17,27} but showed depleted genetic signatures originating from ependyma or OPC-derived cells that acquire Gfap expression after SCI; and astrocytes derived from OPCs after SCI had immunohistochemically detectable hemagglutinin expression in mGfap-Cre-RiboTag but not in Aldh1l1-CreERT-RiboTag spinal cords (Extended Data Fig. 2a,d–l). Consistent with previous reports after stroke⁴³, we detected negligible sex-dependent transcriptomic differences in astrocytes after SCI, with four Y-chromosome and two X-chromosome genes detected as the only differentially expressed genes (DEGs) (false discovery rate of <0.01) across two independent post-SCI timepoints comparing eight female and eight male mice (Extended Data Fig. 2m–o).

Using Aldh1l1-CreERT-RiboTag (Astro-RiboTag) across all six post-SCI timepoints, a total of 13,527 unique DEGs were identified compared to healthy baseline levels, with more genes upregulated than downregulated at all timepoints (Fig. 2b and Supplementary Data 2). The most DEGs, 9,924, were detected at 2 days, with over 6,000 DEGs persisting at 28, 42 and 70 days (Fig. 2b). The greatest changes occurred between 0–2 and 2–5 days, with far fewer changes after 14 days (Fig. 2c).

Over 15,722 genes were identified as expressed by healthy spinal cord astrocytes. Of these genes, 37% (5,755) were primarily downregulated after SCI, 36% (5,598) were primarily upregulated, 7% (1,153) were

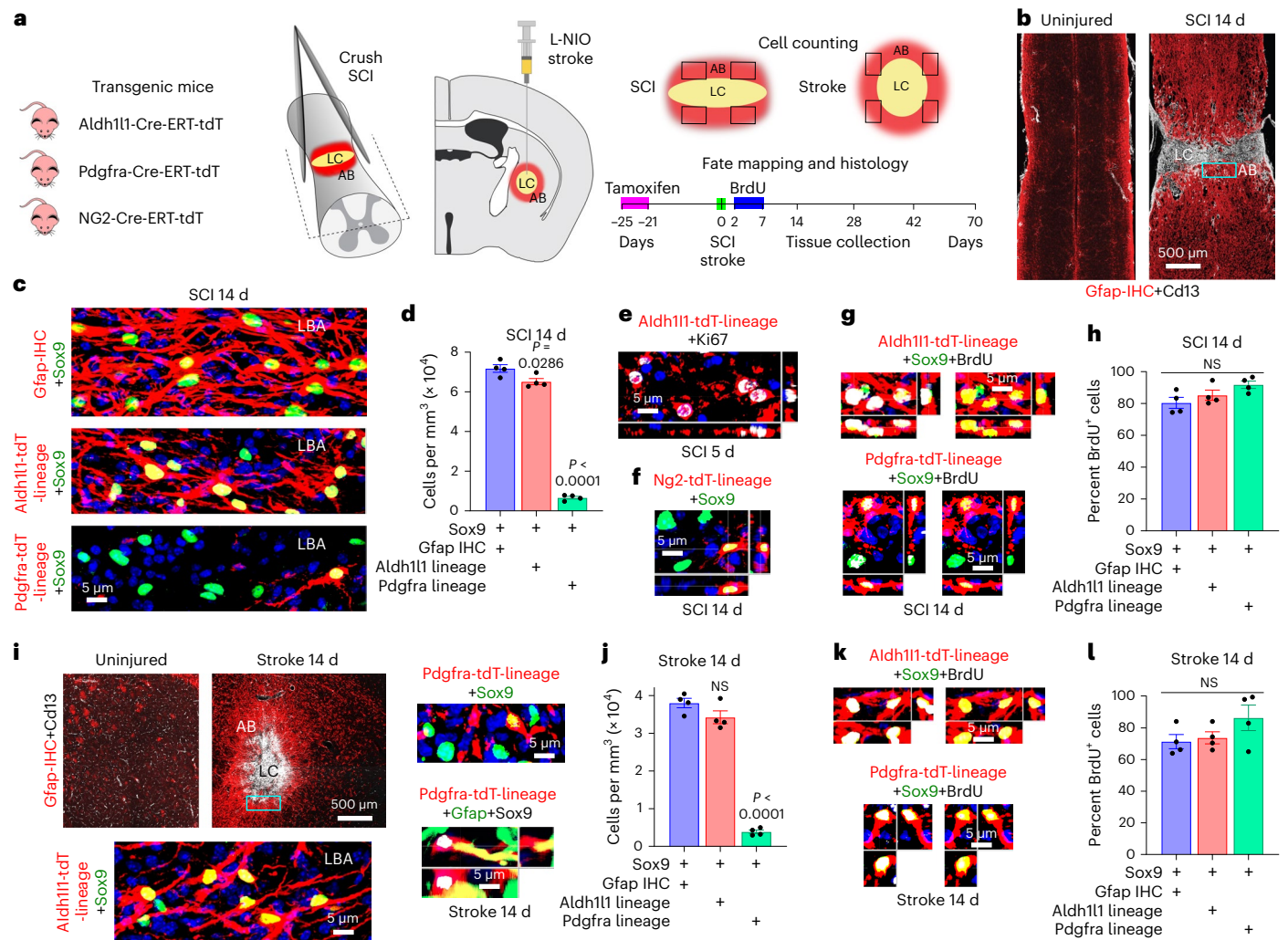


Fig. 1 | Lineage of border-forming astrocytes that surround CNS lesions. **a**, Lineage tracing procedures. **b**, Spinal cord, uninjured and after SCI, stained by immunohistochemistry for astrocytes (Gfap) or stromal cells (Cd13). **c, d**, Images (c) and cell counts (d) of Sox9-positive lesion border astrocytes (LBAs) plus Gfap-IHC or of lineage tracing with Aldh1l1-tdT or Pdgfra-tdT after SCI. **e**, Proliferating astrocytes labeled with Ki67. **f**, Staining for Sox9 plus Ng2-tdT. **g, h**, Newly proliferated BrdU-labeled astrocytes positive for Aldh1l1-tdT or Pdgfra-tdT after SCI (individual fluorescence channels are shown in Extended Data Fig. 1h).

i, j, Striatum uninjured and after L-NIO stroke, with images (i) and cell counts (j) of Sox9-positive LBAs plus Gfap-IHC or of lineage tracing with Aldh1l1-tdT or Pdgfra-tdT after stroke. **k, l**, Newly proliferated BrdU-labeled astrocytes positive for Aldh1l1-tdT or Pdgfra-tdT after stroke. *n* = 4 mice per group. All graphs were evaluated with one-way ANOVA with Tukey's post-hoc comparison. Bar graphs are mean values; error bars, s.e.m. *P* values are indicated on graphs. NS, nonsignificant; LC, lesion core; AB, astrocyte border.

dynamically regulated down or up at different timepoints and 20% (3,216) were not significantly different at any timepoint examined (Fig. 2d). Notably, 92% (12,506 out of 13,527) of the total DEGs identified across all times after SCI were expressed by astrocytes in healthy tissue (Fig. 2d). Induction of newly expressed genes that were not detectable in healthy astrocytes accounted for only 12% (889 out of 7640) of DEGs upregulated by astrocytes after SCI (Fig. 2d and Supplementary Data 2).

Principal component analysis (PCA) of the 13,527 DEGs identified across all timepoints revealed a clear temporal progression of astrocyte transcriptional responses, with two principal components that accounted for over two-thirds of the total system variation (Fig. 2e and Extended Data Fig. 2p). PC1, defining the most dominant effect, revealed acute and transient changes. DEGs with a PC1 factor loading of $>|0.8|$ in the positive or negative direction peaked at 2–5 days after SCI before returning essentially to baseline healthy astrocyte levels by 28 days, with roughly equal numbers transiently upregulated or downregulated (Fig. 2f, g). DEGs defining PC2 increased quickly and largely persisted with mean values of >2 or less than -1 across the entire time course, with more upregulated (854) than downregulated (547) (Fig. 2f, h).

Unsupervised analysis of Gene Ontology Biological Processes (GO-BPs) showed that transiently upregulated DEGs defining PC1 were associated with the regulation of gene expression and translational, cell proliferation, innate immune signaling and antigen presentation, whereas transiently downregulated DEGs defining PC1 were associated with mature CNS structure and glia cell differentiation (Fig. 2i). Persistently upregulated DEGs defining PC2 were associated with cytokine production, innate and adaptive immune regulation and extracellular matrix (ECM) organization, whereas persistently downregulated DEGs defining PC2 were associated with cholesterol and lipid metabolism, neurotransmitter transport and synapse organization (Fig. 2i).

These findings demonstrate that local healthy mature Aldh1l1-expressing astrocytes undergo pronounced, temporally dependent transcriptional changes during border formation after SCI, including both transient and persisting changes. Remarkably, 88% of DEGs upregulated by astrocytes after SCI were already expressed at detectable levels by healthy astrocytes. Astrocyte border formation involves permanent transcriptional reprogramming, given that almost 50% of total DEGs persist at 70 days post SCI, including many genes

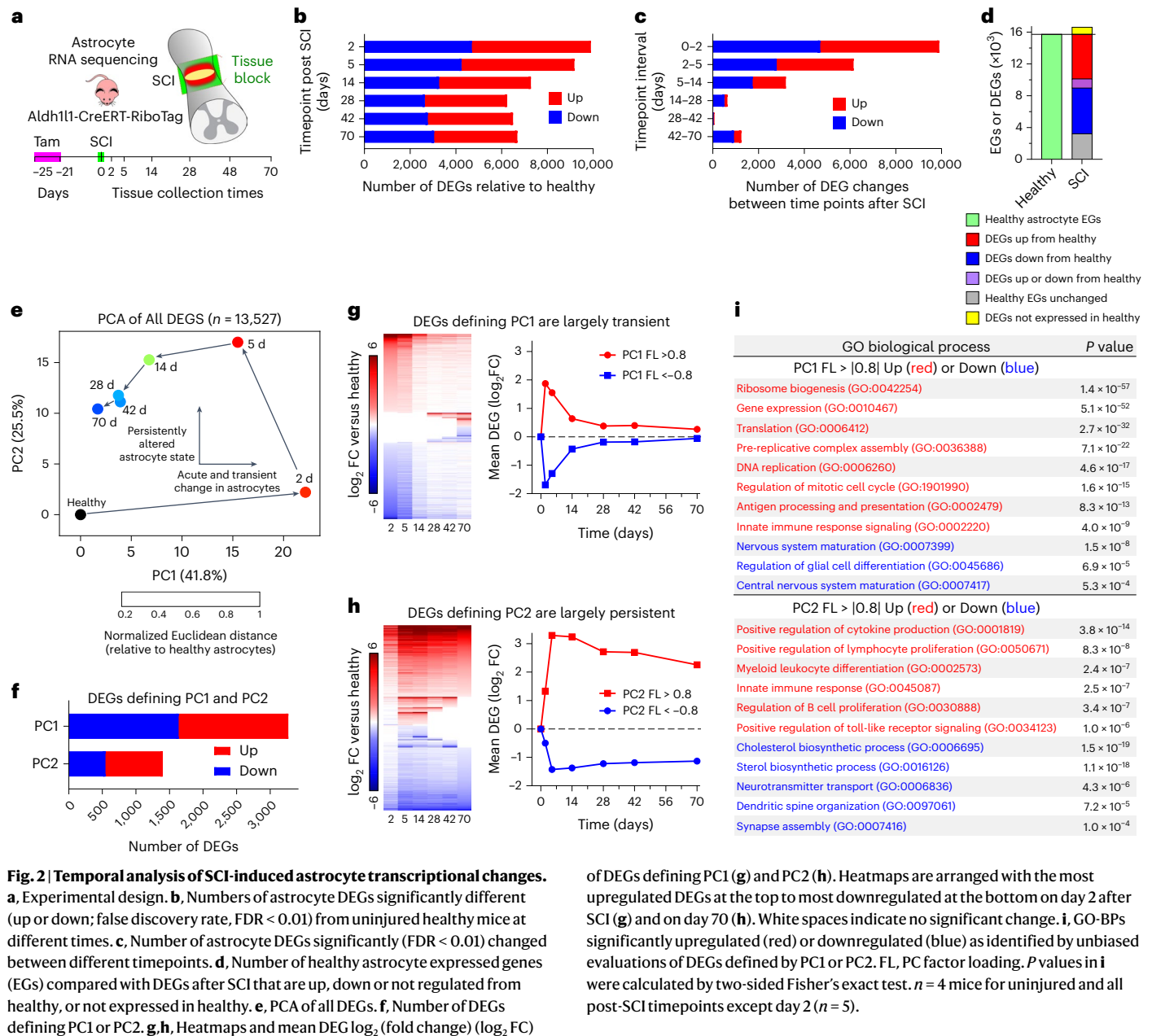


Fig. 2 | Temporal analysis of SCI-induced astrocyte transcriptional changes.

a, Experimental design. **b**, Numbers of astrocyte DEGs significantly different (up or down; false discovery rate, FDR < 0.01) from uninjured healthy mice at different times. **c**, Number of astrocyte DEGs significantly (FDR < 0.01) changed between different timepoints. **d**, Number of healthy astrocyte expressed genes (EGs) compared with DEGs after SCI that are up, down or not regulated from healthy, or not expressed in healthy. **e**, PCA of all DEGs. **f**, Number of DEGs defining PC1 or PC2. **g, h**, Heatmaps and mean DEG \log_2 (fold change) (\log_2 FC)

of DEGs defining PC1 (**g**) and PC2 (**h**). Heatmaps are arranged with the most upregulated DEGs at the top to most downregulated at the bottom on day 2 after SCI (**g**) and on day 70 (**h**). White spaces indicate no significant change. **i**, GO-BPs significantly upregulated (red) or downregulated (blue) as identified by unbiased evaluations of DEGs defined by PC1 or PC2. FL, PC factor loading. P values in **i** were calculated by two-sided Fisher's exact test. $n = 4$ mice for uninjured and all post-SCI timepoints except day 2 ($n = 5$).

not detectably expressed by healthy astrocytes. GO analyses parsed astrocyte transcriptional changes into profiles related to astrocyte dedifferentiation and proliferation, astrocyte reactivity, regulation of inflammation and immune signaling, wound healing and persisting border formation, examined in more detail below.

Dedifferentiation, proliferation and loss of functions

We next defined more precisely how local mature astrocytes change in response to SCI. Of the 15,722 total genes expressed by healthy astrocytes (Fig. 2d), about 60% were upregulated or downregulated at 2 and 5 days, and about 35–40% from 28 through 70 days, with the rest remaining unchanged (Fig. 3a). To characterize astrocyte-enriched genes, we first examined a panel of 429 consensus healthy astrocyte-enriched genes (cAEGs) identified in at least five of eight published archival datasets⁴², which we confirmed as enriched in our healthy astrocytes by at least twofold and up to over 50-fold (Extended Data Fig. 3a,b and Supplementary Data 2). Nearly all cAEGs (97%; 417 out of 429) were DEGs after SCI and only 3% (12 out of 429) were not significantly different at

any timepoint. Remarkably, 74% (317 out of 429) of cAEGs were primarily downregulated and only 11% (49 out of 429) were primarily upregulated, with 15% changing in either direction at different times (Fig. 3b). These changes were reflected in a pronounced decrease in mean expression of all 429 cAEGs, which had a negative peak at 2 days followed by a return towards baseline by 14 and 28 days, but with an overall downregulation persisting through 70 days (Fig. 3c). Notably, cAEGs with the highest enrichment in healthy astrocytes relative to other local cells were the most downregulated (Extended Data Fig. 3b).

Prominent cAEGs that were acutely downregulated and returned to baseline included the water channel (*Aqp4*), the calcium binding protein (*S100b*) and *Hepacam4*, which regulates astrocyte branching complexity⁴⁴ (Fig. 3d). Prominent cAEGs that were persistently downregulated included transporters for glutamate (*Slc1a2* and *Slc1a3*), GABA (*Slc6a1* and *Slc6a11*), glutamine (*Slc38ac*) and D-serine (*Slc7a10*), potassium channels (*Kcnj10* and *Kcnj16*), glutamate receptor subunits (*Grin2c* and *Grm*) and synapse modulating molecules (*Gpc5* and *Sparcl1*)^{10,45} (Fig. 3d). Immunohistochemistry (IHC) confirmed

certain changes at the protein level and showed, for example, that many, but not all, lesion border astrocytes had low or undetectable levels of *Gpc5* and *Kcnj10* (Fig. 3e,f and Extended Data Fig. 3c).

To explore more broadly how local healthy astrocytes acutely changed functional states after SCI, we examined additional specific DEG cohorts. We first examined 2,806 genes identified by our RiboTag immunoprecipitation as significantly enriched in healthy astrocytes by at least twofold and up to 50-fold versus other local cells (Extended Data Fig. 3a,b and Supplementary Data 2). Similar to cAEGs, these immunoprecipitation astrocyte-enriched genes (ipAEGs) exhibited a preponderance of downregulation after SCI, particularly among those ipAEGs most highly enriched in healthy astrocytes (Extended Data Fig. 3b). We conducted unsupervised analysis of GO-BPs associated with significant changes among these 2,806 ipAEGs after SCI and tracked over time after SCI the mean expression of ipAEGs associated with representative examples of the most significantly changed GO-BPs. The majority of significantly altered GO-BPs were associated with downregulated ipAEGs related to cell differentiation, fatty acid metabolism, general metabolic processes, vascular morphogenesis, transport across the blood–brain barrier, synapse assembly, glutathione production and cholesterol production (Fig. 3g). The few GO-BPs associated with upregulated ipAEGs were related to epithelial-to-mesenchymal transition (EMT), immune functions and ECM reorganization.

We next examined GO-BPs associated with all 5,755 DEGs downregulated by astrocytes at any time after SCI, and again tracked over time the mean expression of DEGs associated with representative examples of the most significantly changed. This analysis further confirmed a pronounced acute and persistent attenuation in the expression by astrocytes of genes associated with astrocyte–neuron interactions, neurotransmitter transport, synapse organization, synaptic transmission and potassium regulation (Fig. 3h).

Cell dedifferentiation can be associated with proliferation⁴⁶. Past¹³ and present Ki67 and BrdU evaluations (Fig. 1e,g,h,k,l) and GO-BP analysis (Fig. 2i) indicate pronounced astrocyte proliferation starting around 2 days after SCI. We tracked over time after SCI changes among genes associated with three of the most significantly upregulated cell proliferation-related GO-BPs identified by PCA (Figs. 2i and 3i,j). Astrocyte DEGs associated with each of these GO-BPs, and the mean expression of the unsupervised 91 proliferation-related genes associated with all three GO-BPs, were highly upregulated by local astrocytes at 2 and 5 days and returned to near baseline levels by 14 days (Fig. 3i,j and Supplementary Data 2).

Dedifferentiation and proliferation of local healthy Aldh1l1-expressing astrocytes after SCI suggested a potential return to an immature or progenitor-like state⁴⁷. We compared astrocyte DEGs after SCI with DEG panels positively or negatively associated with astrocyte maturation during postnatal development derived by PCA of transcriptomes from healthy astrocytes at postnatal days (P)

from P0 to P63 (Extended Data Fig. 3d–h). By 2 and 5 days after SCI, astrocytes had markedly downregulated mean expression of 2,417 genes associated with maturity and upregulated mean expression of 2,120 genes associated with immaturity, and these changes returned to essentially baseline levels by 28 days, with a modest upregulation of some immaturity genes persisting to 70 days (Fig. 3k and Extended Data Fig. 3d–h). Of the 429 cAEGs, 192 were positively associated with the progression toward maturity, and their mean expression declined and remained persistently low after SCI (Fig. 3l,m). Notably, healthy PO astrocytes expressed high levels of *Mki67* and *Top2a* associated with active proliferation (Fig. 3j), whereas mature astrocytes in healthy CNS are proliferation-dormant and exist in a potentially reversible G0 state (Extended Data Fig. 1g). They required two or more days after injury to express these genes and never reach fragments per kilobase of transcript per million mapped reads (FPKM) levels of healthy proliferating PO astrocytes (Fig. 3j).

These findings demonstrate that local healthy mature Aldh1l1-expressing astrocytes respond acutely to SCI with downregulation of most astrocyte-enriched genes and a transient phase of proliferation and immaturity. This is followed by a return of many transcriptional features of mature astrocytes but with persisting differences, including a persistent downregulation of molecules associated with astrocyte–neuron interactions such as maintenance of extracellular neurotransmitter and ion homeostasis, and synapse organization and function. These findings point towards persistent transcriptional reprogramming of newly proliferated lesion border astrocytes to new and different functional states.

Reactivity and gain of functions

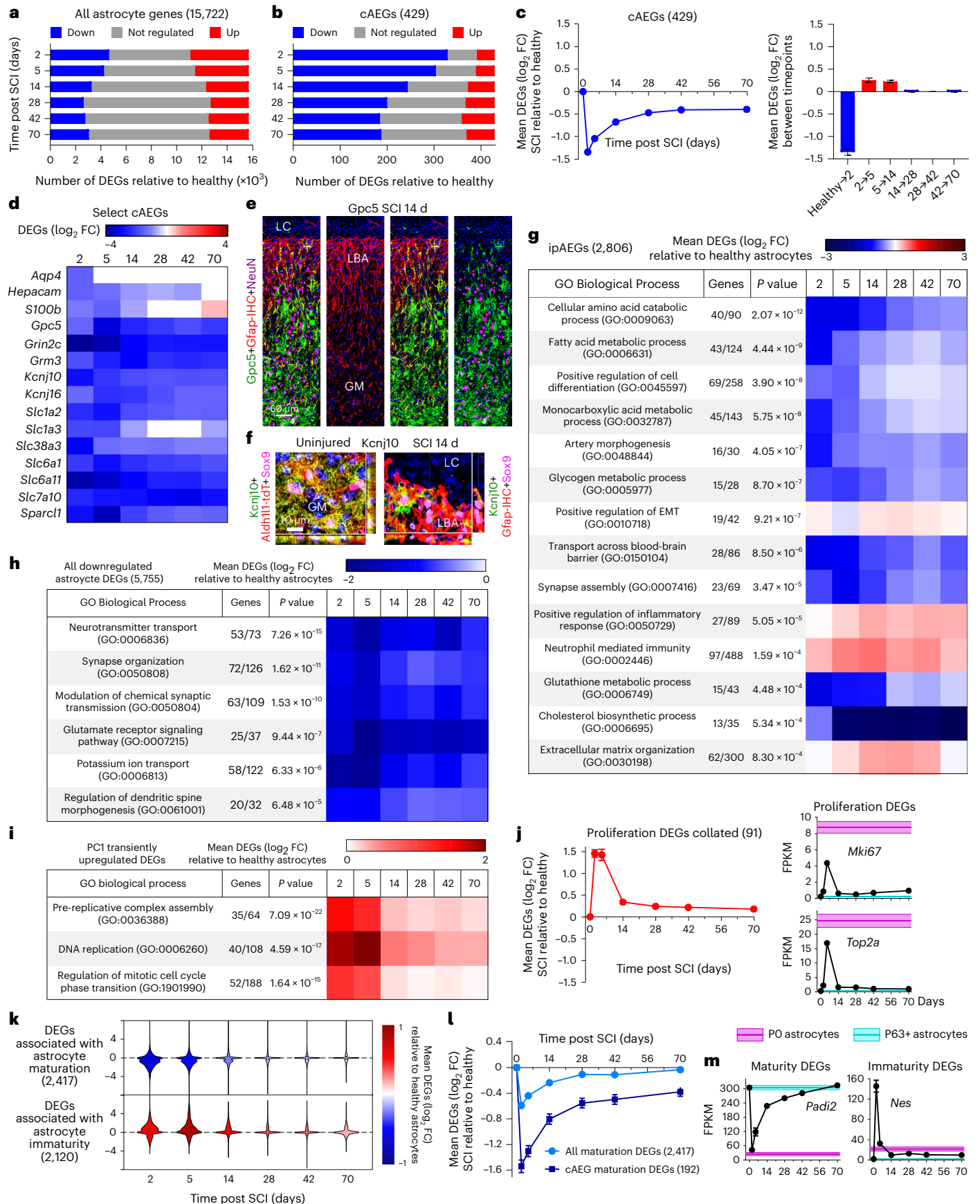
To look for potential new functions adopted by newly proliferated and reprogrammed astrocytes after SCI, we first examined changes in 170 consensus astrocyte reactivity genes (cARGs) derived from a meta-analysis of six archival datasets from multiple laboratories⁴² (Supplementary Data 2). All 170 cARGs were upregulated on at least one timepoint after SCI, 93% (158 out of 170) were upregulated at all timepoints and 92% (157 out of 170) were detectably expressed by healthy astrocytes (Fig. 4a). Mean cARG expression increased 16-fold by 2 days after SCI, increased to over 50-fold by 5 days and then declined moderately but remained persistently elevated by over eightfold at 70 days (Fig. 4b), consistent with reprogramming to an essentially permanent reactive state after SCI. Notably, 41 of the top 50 GO-BPs most significantly associated with cARGs upregulated at all timepoints involved regulation of inflammation, while other upregulated GO-BPs included phagocytosis, ECM organization, synapse pruning and homotypic cell–cell adhesion (Extended Data Fig. 4a). cARGs that were upregulated by 14 days after SCI and remained persistently and highly upregulated at 70 days included well-studied cARGs such as *Gfap*, *Vim* and *Lgals3*, as well as molecules that appear in multiple reactive astrocyte RNA evaluation studies such as *S100a6*, *Serpina3n*, *Lyz2*, *Lcn2*, *Hsbp1*, *C1qa*,

Fig. 3 | SCI-induced astrocyte dedifferentiation and proliferation. **a**, Numbers of up, down or nonsignificant changes (FDR < 0.01) in 15,722 genes expressed by uninjured astrocytes at different times after SCI. **b**, Numbers of up, down or nonsignificant changes (FDR < 0.01) in 429 cAEGs at different times after SCI. **c**, Mean log₂ FC of downregulated cAEGs at different times after SCI relative to uninjured, and changes between individual timepoints. **d**, Heatmap of mean log₂ FC of selected downregulated cAEGs at different times after SCI. **e**, Four different fluorescence channels illuminating different molecular markers within the same region to reveal the spatial distribution and colocalization of *Gpc5* staining at LBA and GM astrocytes. Markedly *Gpc5* immunoreactivities are reduced in LBAs compared with more distal gray matter (GM) astrocytes after SCI. **f**, *Kcnj10* immunoreactivities in LBAs compared with more distal GM astrocytes after SCI. For individual fluorescence channels, see Extended Data Fig. 3c. **g**, Time course of mean expression changes after SCI of ipAEGs (see main text) associated with representative examples of the most significantly changed

GO-BPs associated with ipAEGs. **h**, Highly downregulated GO-BPs associated with all downregulated astrocyte DEGs (whether enriched versus other cells or not). **i**, Time course after SCI of mean changes of DEGs associated with cell proliferation-related GO-BPs identified by PCA in Fig. 2i. **j**, Time course after SCI of mean changes in all unsupervised 91 proliferation-related astrocyte DEGs examined in **i**, plus two additional canonical proliferation DEGs, *Mki67* and *Top2a*. **k**, Time courses after SCI of mean changes in consensus genes associated with astrocyte maturity or immaturity. **l**, Time courses after SCI comparing downregulation of all maturation-associated genes versus consensus genes expressed by mature astrocytes (cAEGs). **m**, Time courses after SCI of mean changes in two specific examples of DEGs, *Pad12* and *Nes*, associated with astrocyte maturity or immaturity, respectively. $n = 4$ mice for uninjured and all post-SCI timepoints except at day 2 ($n = 5$). Bar and line plots are mean values; error bars, s.e.m. P values in **g–i** calculated by two-sided Fisher's exact test.

Tyrbp, *Trem2*, *Tgm1*, *Ccl3* and *Ccl4* (Fig. 4c). IHC confirmed protein expression by border-forming astrocytes for many of these molecules after SCI and stroke, and revealed that whereas certain proteins such as

Gfap and S100a6 were readily detectable in essentially all Sox9-positive lesion border astrocytes, many proteins, such as *Lgals3* and others discussed below, were highly expressed in some lesion border



astrocytes that were intermingled with other astrocytes with low or no detectable expression (Fig. 4d and Extended Data Fig. 4b).

Given that the injury response involves many cell types³, we compared the expression of the same genes by astrocytes and other cells (Extended Data Fig. 3a). Many highly upregulated cARGs (Fig. 4c) were also highly enriched in other cells, such as *Tgm1*, *Steap4*, *Serpina3n* and others (Extended Data Fig. 4c). Nevertheless, many highly upregulated cARGs were de-enriched in astrocytes relative to other cells, such as *Tyrobp*, *Trem2*, *C1qc*, *Ccl4* and others, indicating that although these genes were used by reactive astrocytes, they were also more prominently used by other cells (Extended Data Fig. 4d). Notably, astrocytes and other cells often exhibited different temporal patterns of upregulation or downregulation of these DEGs after SCI, including changes in opposite directions at different times, suggesting potentially different roles exerted by different cell types at different times and supporting the specificity of the expression of these transcripts by astrocytes (Extended Data Fig. 4e).

We next examined 1,129 DEGs upregulated by at least twofold at all timepoints after SCI (Fig. 4e,f and Supplementary Data 2). Most of these DEGs had peak expressions at 5 days and remained markedly elevated for at least 70 days (Fig. 4e). We then tracked over time the mean expression changes of astrocyte DEGs associated with representative examples of the top GO-BPs associated with these 1,129 DEGs (Fig. 4g). Remarkably, all top 15 and 40 of the top 50 GO-BPs involved regulation of inflammation, including both innate and adaptive immune responses such as cytokine production, granulocyte chemotaxis, macrophage activation and lymphocyte regulation (Fig. 4g). Other persistently upregulated GO-BPs included regulation of angiogenesis, phagocytosis and cell adhesion (Fig. 4g).

To identify functions that might be preferentially associated with transcriptional changes in reactive astrocytes, we compared GO-BPs associated with upregulated DEGs enriched either in astrocytes or in other cells. As noted above, ipAEGs exhibited a mean downregulation of DEGs but included some upregulated DEGs (Extended Data Fig. 3b), of which only 40 were also enriched in astrocytes by over twofold (Fig. 4h). The top GO-BPs associated with these 40 upregulated and astrocyte-enriched ipAEGs included response to interferon-gamma and cytokines, cytosolic calcium regulation and negative regulation of programmed cell death (Fig. 4i). The top GO-BPs associated with DEGs enriched and upregulated in other cells (non-astrocytes) were reorganization of ECM and angiogenesis, and the top GO-BPs associated with DEGs similarly upregulated by both astrocytes and non-astrocytes were cytokine signaling and regulation of innate immunity and inflammation (Fig. 4i). These findings indicated that gene expression changes in astrocytes and non-astrocytes contribute to overlapping as well as to differing biological functions after SCI. Notably, ECM reorganization was more prominently associated with DEGs deriving from non-astrocytes (Fig. 4i). Chondroitin sulfate proteoglycans (CSPGs) are ECM components that have received prominent attention in SCI and have previously been attributed primarily to astrocytes. Nevertheless, three of six CSPG transcripts were more highly expressed by non-astrocytes at all timepoints after SCI, and not a single CSPG transcript exhibited prominent or persistent upregulation by astrocytes above baseline healthy levels (Extended Data Fig. 4f).

These findings show that as newly proliferated astrocytes reprogram after SCI, they exhibit both transient and persistent upregulation of many different DEGs. Analyses of GO-BPs associated with persistently upregulated DEGs point towards reprogrammed astrocytes adopting new functions after SCI that contribute to innate immune responses, regulation of inflammation, control of infection, debris phagocytosis and regulation of angiogenesis, which we examined further.

Immune regulation, antigen presentation, antimicrobial defense

The most prominent gain-of-function GO-BPs associated with astrocyte transcriptional reprogramming after SCI were related to innate and adaptive immune responses such as neutrophil recruitment, microglial and macrophage activation, antigen processing and presentation, antimicrobial activity, and B cell and T cell recruitment (Figs. 2i, 3g and 4g). To examine the time course of individual DEG changes related to these functions, we compiled a list of 2,766 unique genes associated with these GO-BPs. Of these 2,766 genes, 1,708 exhibited changes by astrocytes after SCI consisting primarily of acute upregulation at 2–14 days, with some returning towards baseline levels and many exhibiting long-term upregulation for up to 70 days (Fig. 5a,b and Supplementary Data 3).

In addition to multiple cytokines, chemokines and their receptors, notable immune regulatory DEGs acutely upregulated by astrocytes at 2 days after SCI included *Arg1*, *Ctss*, *Hmox1*, *Hspb1*, *Serpine1* and *Lgals3*; persistently upregulated immune regulatory DEGs at 70 days included *Cd74*, *H2-Ab1*, *H2-Eb1*, *H2-Aa*, *C1qa*, *C3*, *Trem2*, *Tyrobp*, *Serpina3n*, *Lcn2* and *Ctss* (Fig. 5b,c). Cytokines included many pro-inflammatory but also some anti-inflammatory (*Il11*, *Tgfb* and *Il6*) molecules (Fig. 5b,c). Most cytokines peaked rapidly followed by some decline and moderate long-term persistence (*Ccl2*, *Ccl3*, *Cxcl2*, *Tnf* and *Spp1*), some cytokines peaked acutely and were downregulated to baseline (*Cxcl3*, *Il6* and *Il11*) and a few gradually increased their expression over time (*Ccl8*) (Fig. 5b,c). Upregulated cytokine and chemokine receptors included *Ccr1*, *Ccr5*, *Il1rn* and *Il10ra* (Fig. 5c). We also found evidence consistent with astrocyte pyroptosis and inflammasome generation⁴⁸ with upregulation of *Dsdmd*, *Nlrp3*, *Pycard*, *Casp1*, *Casp4* and *Casp8* (Extended Data Fig. 5a). In addition, in agreement with findings from multiple laboratories⁴⁶, we found that multiple toll-like receptors were markedly upregulated by astrocytes from 2 to 14 days, including *Tlr1*, *Tlr4*, *Tlr6*, *Tlr7*, *Tlr8* and *Tlr9*, and some of these remained persistently elevated (Extended Data Fig. 5b), consistent with astrocyte involvement in diverse innate immune responses.

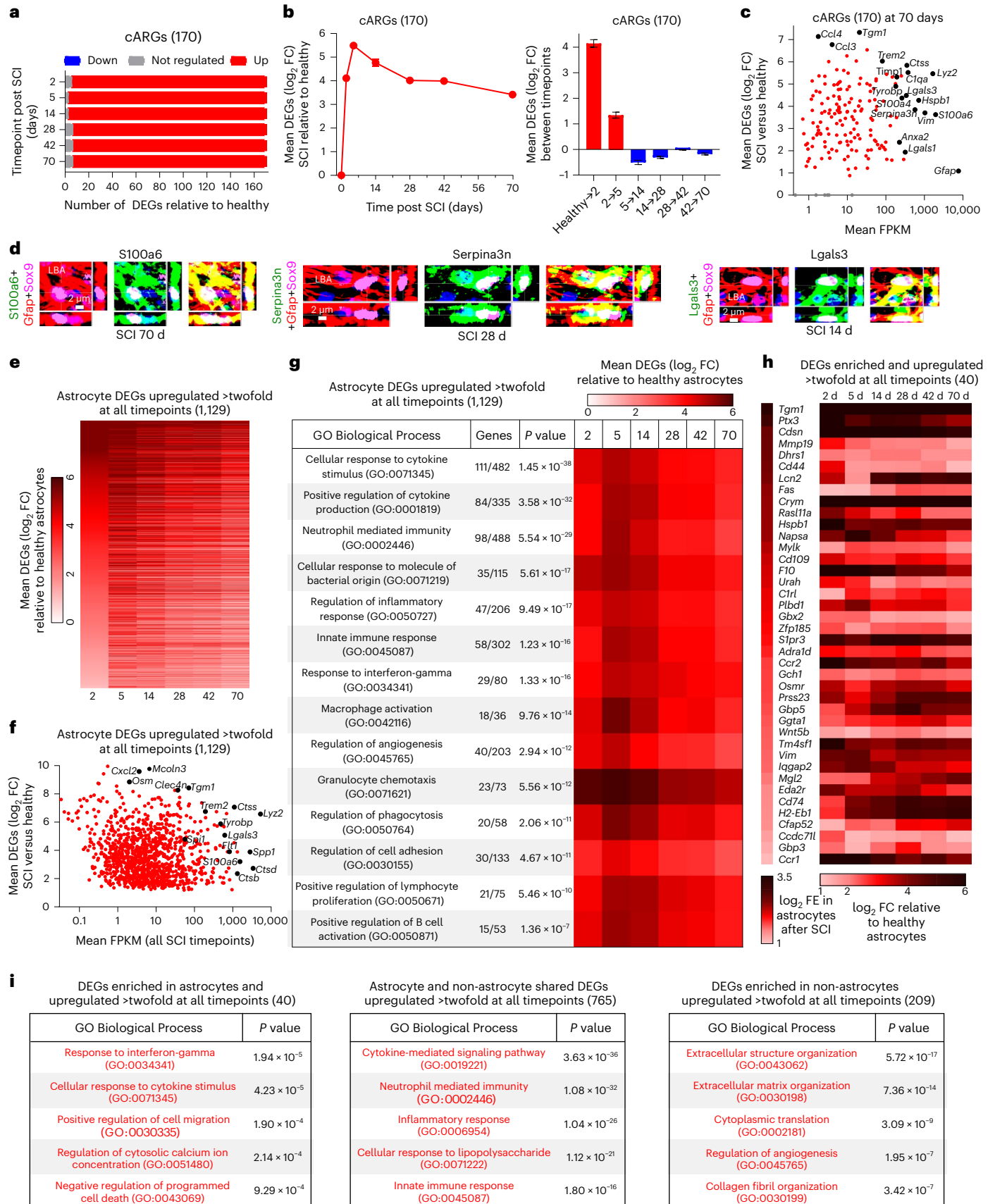
We compared expression of the same cytokines and receptors in astrocytes and other cells (Fig. 5d). At 2 days after SCI, *Cxcl3* and *Cxcl2* were the most highly upregulated and highly enriched in astrocytes, suggesting potentially unique roles for astrocytes compared with other cells (Fig. 5d). Additional cytokines that were both highly upregulated and enriched in astrocytes at 2 days included *Ccl2*, *Ccl6*, *Ccl9*, *Ccl24*, *Cxcl10*, *Il1b*, *Il11* and *Tnfsf14* (Fig. 5d). Notably, many cytokines were upregulated by astrocytes but were nevertheless more highly expressed by other cells at 2 days, such as *Ccl3*, *Ccl8* and *Cxcl16* (Fig. 5d). By 70 days, *Cxcl3* had returned to baseline in astrocytes, whereas *Cxcl2*,

Fig. 4 | SCI-induced astrocyte transcriptional changes associated with reactivity and gains of functions. **a**, Numbers of up, down or nonsignificant (FDR < 0.01) changes in 170 cARGs at different times after SCI. **b**, Mean log₂ FC of upregulated cARGs at different times after SCI and changes between individual timepoints. **c**, Scatterplot showing log₂ FC and FPKM of cARGs at 70 days after SCI. Selected examples of highly expressed and highly upregulated DEGs are labeled. **d**, Examples of cARGs with high levels of protein immunoreactivity in LBAs. **e**, Heatmap of 1,129 astrocyte DEGs upregulated at least twofold at all times after SCI. **f**, Scatterplot showing log₂ FC and FPKM of 1,129 astrocyte DEGs upregulated at least twofold at all times after SCI. **g**, Top GO-BTs associated

with 1,129 astrocyte DEGs upregulated at least twofold at all times after SCI. **h**, Heatmaps of log₂ (fold enrichment) (log₂ FE) and log₂ FC of 40 DEGs enriched in astrocytes by a mean of at least twofold compared with other cells and upregulated by at least twofold at all timepoints after SCI. **i**, Top GO-BTs associated either with 40 DEGs expressed more highly by astrocytes than other cells, or with 765 DEGs expressed at similar levels by astrocytes and other cells, or with 209 DEGs expressed more highly by other cells. *n* = 4 mice for uninjured and all post-SCI timepoints except at day 2 (*n* = 5). Bar and line plots are mean values; error bars, s.e.m. *P* values in **g** and **i** were calculated by two-sided Fisher's exact test.

Ccl8, and *Ccl2* remained upregulated at levels comparable to other cells and only *Cxcl10* was persistently upregulated and enriched in astrocytes, as confirmed also by IHC (Fig. 5d and Extended Data Fig. 5c). At 2

days after SCI, upregulated cytokine receptors enriched in astrocytes included *Ccr1*, *Ccr2*, *Ccr5*, *Cxcr2* and *Il6ra*, whereas at 70 days, various receptors were upregulated but were present at levels comparable to



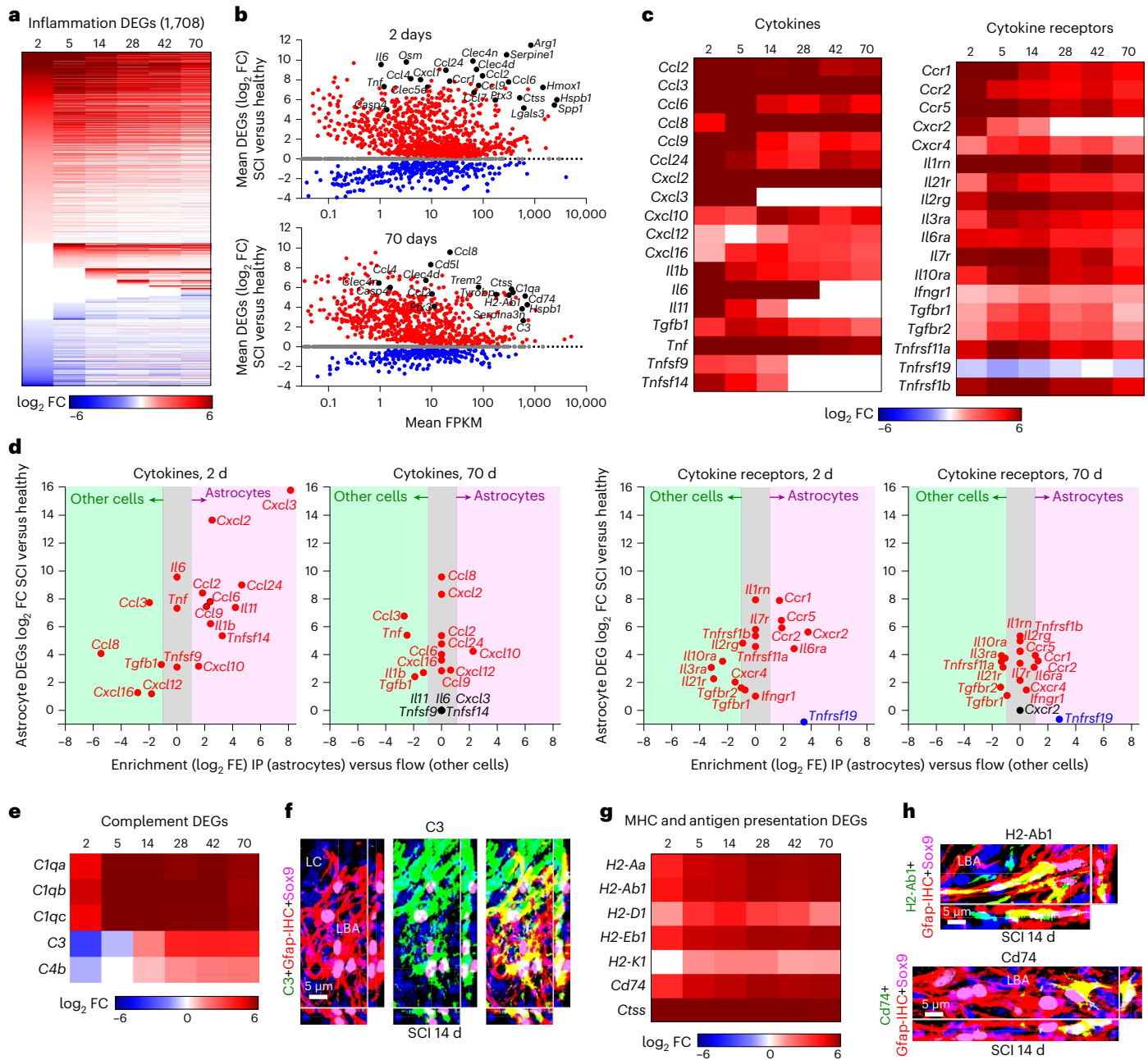


Fig. 5 | Innate and adaptive immune signaling, complement signaling and antigen presentation. **a**, Heatmap of 1,708 consensus inflammation-associated DEGs upregulated or downregulated by astrocytes at different days after SCI. **b**, Scatterplots showing \log_2 FC and FPKM of 1,708 astrocyte consensus inflammation-associated DEGs at 2 or 70 days after SCI. **c**, Heatmaps of mean \log_2 FC of selected cytokines and cytokine receptors expressed by astrocytes after SCI. **d**, Comparison of relative enrichment and expression levels of various cytokines and cytokine receptors by astrocytes and by other cells at 2 and

70 days after SCI. **e**, Heatmap of mean \log_2 FC of selected complement-related DEGs expressed by astrocytes after SCI. **f**, High levels of C3 protein immunoreactivity in LBAs after SCI. **g**, Heatmap of mean \log_2 FC of selected DEGs associated with antigen presentation and expressed by astrocytes after SCI. **h**, High levels of H2-Ab1 or Cd74 protein immunoreactivity in scattered individual LBAs intermingled among many negative LBAs after SCI. $n = 4$ mice for uninjured and all post-SCI timepoints except at day 2 ($n = 5$).

other cells (Fig. 5d). These findings suggest the potential for certain unique, and many shared, cytokine-related functions among astrocytes and other cell types after SCI.

Astrocytes also upregulated genes associated with antimicrobial defense in complement pathways and antigen presentation. *C1qa*, *C1qb* and *C1qc* were upregulated by over 50-fold from 5 days through at least 70 days after SCI (Fig. 5e). Microbiocidal^{49,50} C3 and *C4b* were initially downregulated at 2 and 5 days but were then persistently upregulated through 70 days (Fig. 5e), and immunoreactive C3 protein

was prominently detected in lesion border astrocytes that interfaced with non-neural lesion core cells in SCI and stroke (Fig. 5f and Extended Data Fig. 5d). Notably, although C1q was upregulated by astrocytes, it was more highly expressed by other cells, whereas C3 was both upregulated and enriched in astrocytes (Extended Data Fig. 5e). Complement receptors *C3ar1* and *C5ar1* were also upregulated by over 50-fold and were initially enriched in astrocytes but became equally or more highly expressed by other cells by 28 days and longer after SCI (Extended Data Fig. 5e).

Additional antimicrobial defense DEGs included *Clec4d*, *Clec4n*, *Clec5a* and *Clec7a*, which encode pathogen-associated molecular pattern receptors for viruses, bacteria and fungi, as well as *Ptx3*, *Lyz2* and *Lgals3* (Figs. 4c,d and 5b and Extended Data Fig. 5f,g). Multiple DEGs associated with antigen presentation and major histocompatibility complex (MHC) class II were not only prominently upregulated from 5 through 70 days after SCI but in many cases were enriched in astrocytes compared with other cells, including *H2-Aa*, *H2-Ab1*, *H2-Eb1* and *Cd74* (Fig. 5g,h and Extended Data Fig. 5h,i). Notably, immunoreactive protein for these major histocompatibility complex class II and related molecules was high in scattered lesion border astrocytes but not detectable in others (Fig. 5h and Extended Data Fig. 5h), consistent with specialized expression among some but not other astrocytes.

These findings show that the transcriptional reprogramming of newly proliferated astrocytes after SCI includes both transient and persistent changes in many DEGs associated with multiple innate and adaptive immune functions, including diverse pro-inflammatory and some anti-inflammatory cytokine signaling, antigen presentation and antimicrobial defense. The persistent upregulation of these DEGs points towards a long-term contribution to immune preparedness by border-forming astrocytes around persisting CNS lesions.

EMT, wound repair, cell adhesion and border maturation

Another prominently upregulated GO-BP associated with ipAEGs was ‘regulation of EMT’ (Fig. 3g). We examined a panel of 197 consensus EMT-associated DEGs adapted from Msigdb gene sets⁴² and found a rapid acute increase in mean expression that peaked at 2 to 5 days and declined thereafter but remained persistently elevated above baseline, including prototypical EMT genes such as *Vim*, *Fn1* and *Acta2* (Fig. 6a–c and Supplementary Data 3). Previous studies have identified EMT genes in astrocyte responses to SCI^{42,51,52}, and EMT-associated DEGs in adult cells have been implicated in wound healing^{52–54}. Therefore, we conducted a hypothesis-driven analysis of 278 DEGs associated with the GO-BP ‘wound healing’ (GO:0042060) and also found a rapid acute increase in mean expression in astrocytes at 2 to 5 days after SCI that declined gradually but remained persistently elevated (Fig. 6d and Supplementary Data 3). Notable and unexpected wound-healing-associated DEGs that were highly expressed by astrocytes sub-acutely from 2–14 days after SCI included coagulation factors *F7*, *F10* and *F13a1*, which were upregulated over 50-fold to 100-fold and enriched in astrocytes compared with other cells (Fig. 6e and Extended Data Fig. 6a); heparin-degrading and hemostasis molecules *Pf4*, *Hpse* and *Fermt*; heme-degrading enzyme *Hmox1*; phagocytosis promoting *Cd44*; membrane repair *Dysf*; tissue remodeling *Mmp12* and *Timp1*; free-radical scavenger *Gpx1*; and others including *Hbegf*, *Cd109*, *Cd151*, *Mylk* and *Pdpr* (Fig. 6e,f and Extended Data Fig. 6b). Remarkably, many of these DEGs were both upregulated and enriched in astrocytes compared with other cells, suggesting unique and important wound repair functions for reactive astrocytes (Extended Data Fig. 6c), and immunoreactive protein for certain molecules such as *Mmp12* was high in some lesion border astrocytes and low or not detectable in others (Extended Data Fig. 6b).

As noted above, newly proliferated lesion border astrocytes downregulate the domain-associated cell adhesion molecule (CAM) *Hepacam44* (Fig. 3d), and as they mature do not adopt individual domains but instead reorganize with highly overlapping and intermingled cell processes (Fig. 1c). Therefore, we looked for changes in DEGs associated with cell–cell interactions by examining a panel of 425 CAMs and cell–cell interaction-associated genes compiled from gene set enrichment analysis, GO and the literature. After SCI, astrocytes exhibited an overall early and persisting mean upregulation in expression of CAM-associated DEGs, but with about 40% of individual DEGs exhibiting upregulation, 40% downregulation and 20% no change (Fig. 6g–i, Extended Data Fig. 7a and Supplementary Data 3). Notable

upregulated CAM-associated DEGs included *Ccn5*, *Gpnmb* and *Spp1* involved in integrin binding; *Lgals1* and *Fxyd5* involved in matrix adhesion; *Cdsn*, *Cd9* and *Mfge8* involved in cell–cell adhesion; and *Itgb2* and *Icam1* involved in leukocyte adhesion (Fig. 6h,i and Extended Data Fig. 7b,c). Notable downregulated CAMs included *Hepacam*, *Cldn10*, *Cntn1*, *Kirrel3*, *Ncam1*, *Nrxn1* and *Nlgn3* involved in homophilic astrocyte–astrocyte interactions and interactions with neurons (Fig. 6h and Extended Data Fig. 7b). Many of these CAMs were enriched in astrocytes compared with other cells (Extended Data Fig. 7d).

The DEGs and associated GO-BPs examined thus far peaked acutely after SCI. To identify potential features of border-forming astrocytes that might emerge after the acute period, we next examined 848 DEGs that were upregulated from 14 to 70 days. PCA revealed a prominent difference defined by PC1 between 5 days and the more chronic timepoints, and a smaller difference defined by PC2 between 14 and 28–70 days (Fig. 6j). Factor analysis of the 757 DEGs defining PC1 revealed a cohort of delayed astrocyte reactivity genes (dARGs) whose mean expression declined acutely and then increased and remained high from 14 to 70 days (Fig. 6k and Supplementary Data 3). These dARGs exhibited GO Cellular Components associated with ciliated cells, ECM interactions and intercalated disk cell–cell contact interactions (Fig. 6l). Notable dARGs at 70 days after SCI included the transcription factor *Id3*; proteinase inhibitors *A2m*, *Lxn* and *Serpina3i*; the neuroprotective endozepine *Dbi*⁵⁵; antioxidants *Mt1*, *Mt2* and *Prdx6*; ion transport regulator *Fxyd1*; chaperone *Clu*; multifunctional *Igfbpl1*, antimicrobial *Lcn2*, *Nrdg1* and *Igha*; heat shock protein *Cryab*; and cilia-associated proteins including *Dnal1* and *Cfap126* (Fig. 6m,n and Extended Data Fig. 7e,f). Many of these dARGs were enriched in astrocytes (Extended Data Fig. 7d). Protein expression for various dARGs was confirmed by IHC and *Id3*, *Fxyd1* and *Prdx6*, in particular, were robustly detected in most if not all lesion border astrocytes (Fig. 6n and Extended Data Fig. 7e). Delayed expression of *Id3* and various cilia-related genes including *Dnal1* and *Cfap126* (Fig. 6m and Extended Data Fig. 7f) is consistent with their established roles in proliferation arrest and promotion of astrocyte differentiation of immature progenitor-like cells and points towards their possible involvement in ending the transient phase of proliferation and immaturity observed in the initial post-injury response^{14,56–58}.

Together, these findings show that the transcriptional reprogramming of newly proliferated astrocytes after SCI equips astrocytes with the potential to contribute to important wound healing processes, including hemostasis, coagulation and breakdown of heme in hemorrhagic CNS wounds, free-radical scavenging, and tissue and matrix remodeling. Remarkably, many of the associated DEGs were not only upregulated but were also enriched in astrocytes compared with other cells, suggesting unique and important wound repair functions for newly proliferated reactive astrocytes. The findings also reveal major changes in CAM expressions (both up and down) by lesion border astrocytes around CNS lesions, indicating major readjustments in their homophilic and heterophilic cell–cell interactions consistent with their major change in morphology from cells with unique non-overlapping cellular domains⁴⁴ to cells with highly overlapping and intertwined cellular processes (Fig. 1c).

Phenotypic features of mature lesion border astrocytes

We conducted snRNA-seq and immunohistochemical protein analyses to look for phenotypic features of mature lesion border astrocytes compared with astrocytes in healthy spinal cord. We isolated and sequenced 139,981 nuclei from the same region of thoracic spinal cord in uninjured mice ($n = 4$) and at 28 days after SCI ($n = 4$) when borders are mature (Fig. 7a). Nuclei were filtered and annotated using panels of multiple cell-type-specific marker genes, and clusters identified as astrocytes were extracted for further processing (Extended Data Fig. 8a–c). Re-clustering and further extraction based on multiple astrocyte

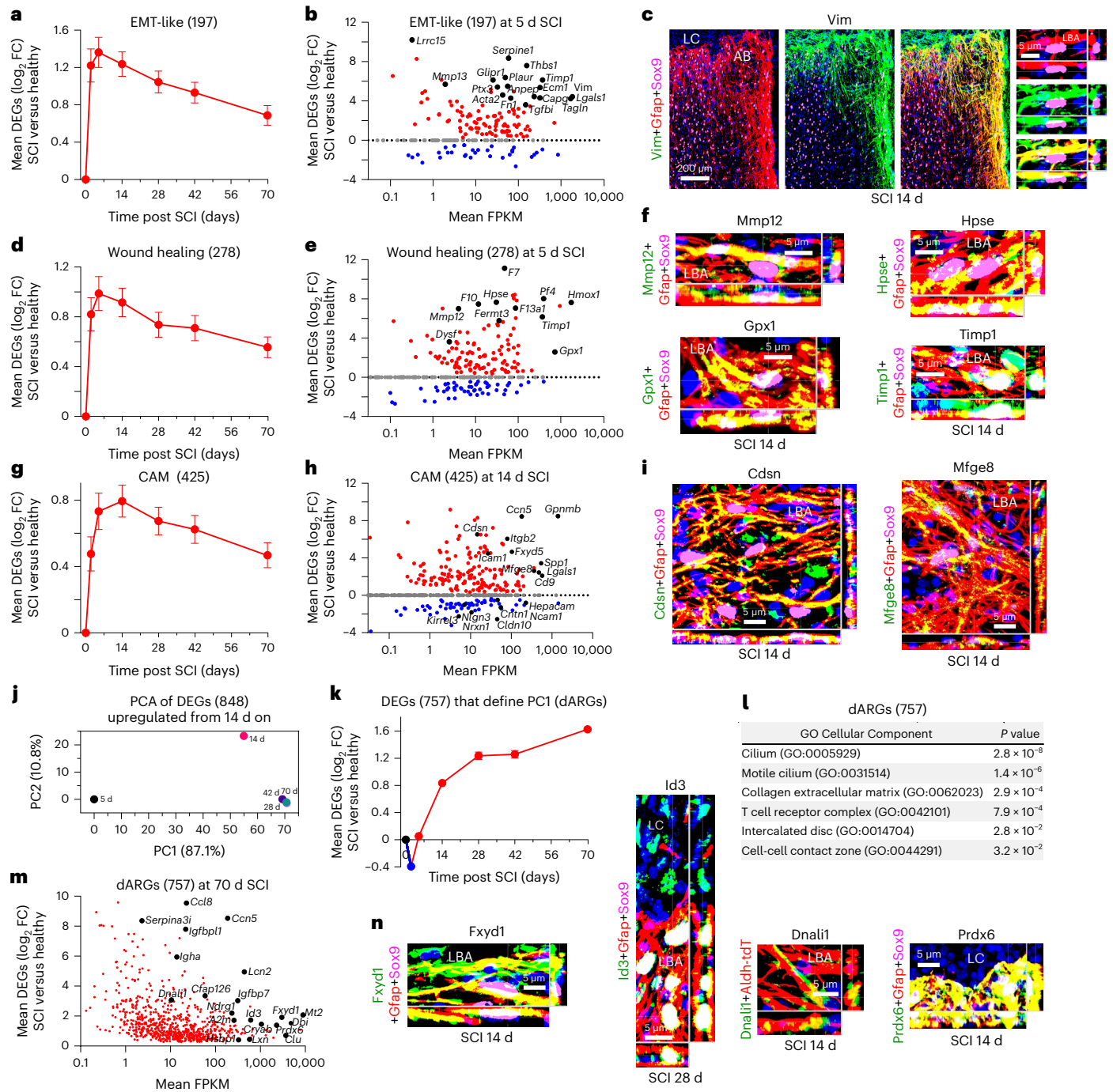


Fig. 6 | Wound healing, cell adhesion changes and border maturation. **a**, Mean log₂ FC of a panel of 197 EMT-like DEGs at different times after SCI relative to uninjured. **b**, Scatterplot showing log₂ FC and FPKM of EMT-like DEGs at 5 days after SCI. **c**, High protein immunoreactivity of the canonical EMT marker, Vim, in LBAs. **d**, Mean log₂ FC of a panel of 278 wound-healing-associated DEGs at different times after SCI. **e**, Scatterplot showing log₂ FC and FPKM of wound healing DEGs at 5 days after SCI. **f**, High protein immunoreactivity of selected wound-healing-associated molecules in LBAs. **g**, Mean log₂ FC of a panel of 425 cell adhesion molecule (CAM) DEGs at different times after SCI. **h**, Scatterplot

showing log₂ FC and FPKM of CAM DEGs at 14 days after SCI. **i**, High protein immunoreactivity of selected CAMs in LBAs. **j**, PCA of 848 DEGs upregulated from 14 days onwards after SCI relative to 5 days after SCI. **k**, Mean log₂ FC of the 757 dARGs that define PC1. **l**, Top GO Cellular Components associated with the 757 dARGs. **m**, Scatterplot showing log₂ FC and FPKM of dARGs at 70 days after SCI. **n**, Examples of high protein immunoreactivity in LBAs of selected dARGs. *n* = 4 mice for uninjured and all post-SCI timepoints except at day 2 (*n* = 5). Bar and line plots are mean values; error bars, s.e.m. *P* values in **l** were calculated by two-sided Fisher's exact test.

marker genes confidently identified 15,637 astrocyte nuclei that were used for final analyses (Fig. 7a–e and Extended Data Fig. 8d–h). Expression changes among astrocyte genes detected by both snRNA-seq and RiboTag at 28 days after SCI were significantly correlated, including DEGs highlighted in our analyses (Extended Data Fig. 8i).

Uniform manifold approximation and projection (UMAP) analysis showed clear separation of uninjured and SCI-derived nuclei (Fig. 7b–e). A similar clear separation was also found in another study³⁰ in an independent UMAP evaluation of astrocyte nuclei derived from a multicellular evaluation of lower thoracic spinal cord nuclei of uninjured mice

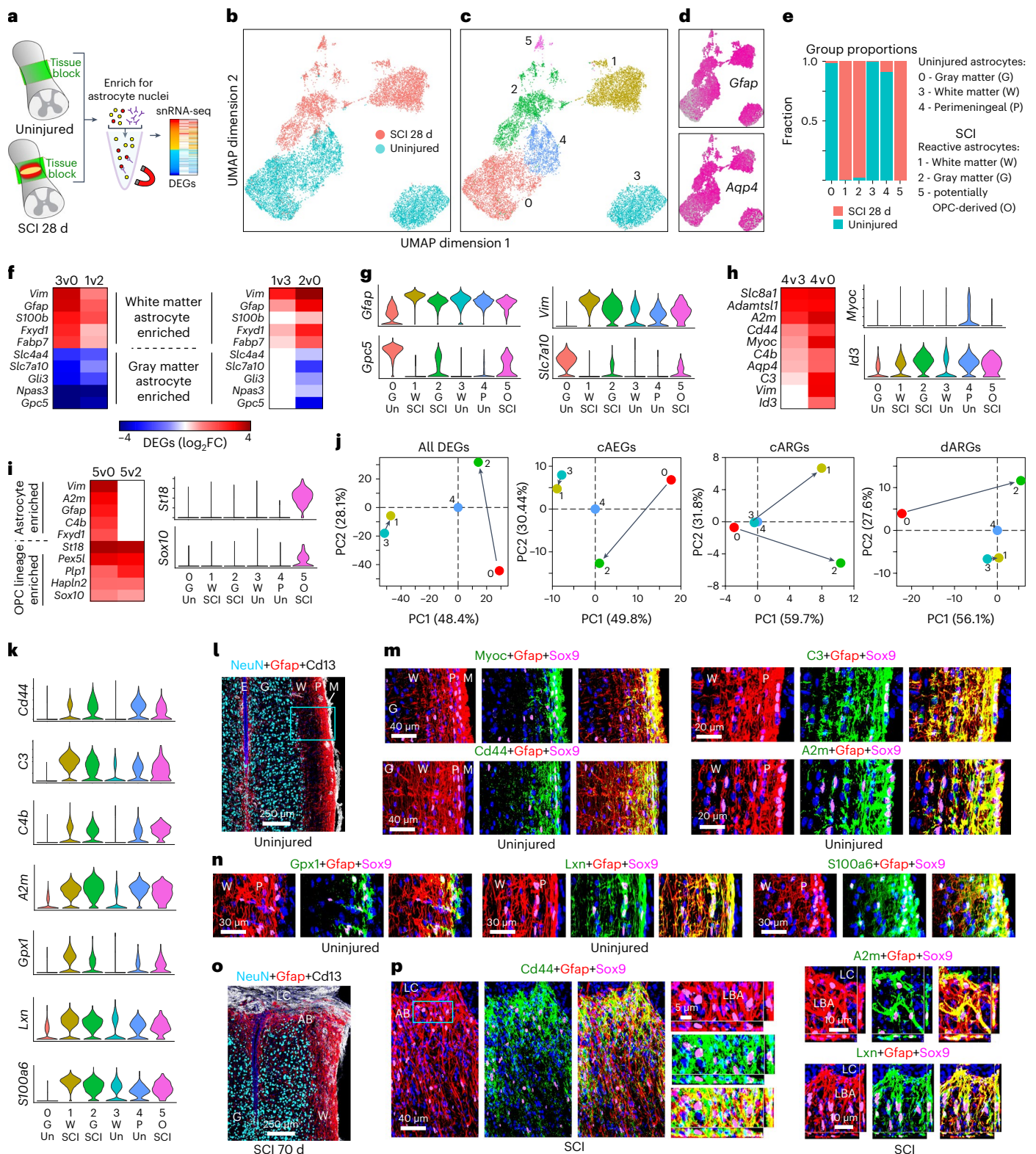


Fig. 7 | Shared and distinct molecular features of uninjured astrocytes and mature LBAs. **a**, snRNA-seq procedures. **b,c**, UMAP clusters of astrocyte nuclei. **d**, *Gfap* and *Aqp4* expression across clusters. **e**, Proportion of nuclei from uninjured or SCI mice per cluster. **f,g**, Heatmaps (**f**) and violin plots (**g**) comparing marker genes enriched in gray (G) or white matter (W) astrocytes across different clusters. P, perimeningeal; O, OPC-derived; Un, uninjured. **h,i**, Heatmaps and violin plots of genes enriched in perimeningeal astrocytes (**h**) or in OPC-derived

astrocytes (**i**). **j**, PCA comparing changes in different gene cohorts across clusters after SCI. **k**, Violin plots of genes enriched in both perimeningeal and reactive astrocytes compared with uninjured. **l-n**, IHC of proteins enriched in uninjured perimeningeal astrocytes and LBAs after SCI. **l,o**, Survey images of uninjured (**l**) and SCI (**o**). **m,n**, Details from boxed region in **l** showing proteins enriched in perimeningeal astrocytes. **p**, Proteins enriched in LBAs. E, ependyma; M, meninges.

and mice at 2 months after SCI (Extended Data Fig. 8k,l). Moreover, expression changes among consensus astrocyte genes detected by the present study at 28 days after SCI and by the previous study³⁰ at 2 months after SCI were significantly correlated (Extended Data Fig. 8k,l).

In our present data, we categorized six major clusters of astrocytes: three from uninjured and three from SCI (Fig. 7b–e). Canonical markers of healthy and reactive astrocytes such as *Gfap* and *Aqp4* were expressed throughout all clusters (Fig. 7d). Consensus markers discriminated healthy gray matter (cluster 0) and white matter (cluster 3) astrocytes and identified separate major clusters of reactive astrocytes with transcriptional features associated with gray matter (cluster 2) and white matter (cluster 1) astrocytes (Fig. 7c–g and Extended Data Fig. 8e,f). Healthy spinal cord astrocytes also separated into a third cluster (cluster 4) with enriched expression of molecules such as *Myoc*, *Cidea* and *Id3* (Fig. 7c,h and Extended Data Fig. 9a) that have previously been associated with astrocytes that form so-called limitans borders adjacent to meninges^{42,59,60}. IHC confirmed robust protein expression of *Myoc* and *Id3* proteins in healthy perimeningeal astrocytes (Fig. 7m and Extended Data Fig. 9b). An additional small cluster (cluster 5) expressed reactive astrocyte markers and was enriched for OPC lineage markers *St18*, *Sox10*, *Plp1*, *Hapln2*, *Ninj2* and *Enpp6* (Fig. 7c,g,i and Extended Data Fig. 9c), suggesting that these cells may have derived from OPCs, although further experiments will be needed to directly test this possibility. Nevertheless, our findings provide both snRNA-seq and lineage tracing (Fig. 1 and Extended Data Fig. 1) evidence consistent with previous observations^{34–37} that local OPCs can give rise to a small contingent of lesion border astrocytes, as also noted in another recent single-cell RNA-seq study⁶¹.

We next evaluated how different gene cohorts consisting of all DEGs, cAEGs, cARGs or dARGs changed in the different major astrocyte clusters after SCI. PCA analyses showed that in all cases, gray matter astrocytes exhibited the greatest changes at 28 days after SCI relative to healthy, including markedly reduced expression of cAEGs, and markedly upregulated cARGs and dARGs (Fig. 7j). By contrast, white matter astrocytes exhibited minimal changes in gene expressions except for markedly upregulating cARGs (Fig. 7j).

Historical evidence has suggested similarities between astrocyte borders around CNS lesions and limitans astrocytes adjacent to meninges^{4,62–64}. We therefore looked for potential similarities and differences. Notable DEGs enriched in both healthy perimeningeal astrocytes (cluster 4) and lesion border reactive astrocytes (clusters 1, 2 and 5) compared with healthy gray or white matter astrocytes (clusters 0 and 3) included *Id3*, *Cd44*, *C3*, *C4b*, *A2m*, *Gpx1*, *Lxn*, *Vim*, *S100a6*, *Lgals3*, *Spp1*, *Fxyd1*, *Padi2* and *Prdx6* (Fig. 7g,h,k–p and Extended Data Fig. 9b,d–g). Both *Myoc* and *Cidea* were selective for healthy perimeningeal astrocytes and were essentially not detected in reactive astrocytes or in healthy gray matter or white matter astrocytes (Fig. 7h,m and Extended Data Fig. 9a). Conversely, healthy perimeningeal astrocytes did not express appreciable levels of multiple markers associated with overt astrocyte reactivity, including *Lyz2*, *Serpina3n*, *Fcerg1*, *Ctss*, *Trem2*, *Tyrobp*, *C1qa*, *Hspp1* and others (Fig. 8a, Extended Data Figs. 8f,g and 9h). Reactive perimeningeal astrocytes were not discriminated as a separate cluster, most likely because they become indistinguishable from other reactive astrocytes owing to the pronounced and persistent downregulation of *Myoc* and other genes that distinguish them (Extended Data Fig. 9i). As indicated by our Astro-RiboTag data, certain reactivity DEGs peaked acutely from 2 to 14 days and then declined by 28 days but continued to be expressed thereafter at levels higher than in healthy astrocytes, such as *Tyrobp*, *Trem2*, *Spi1*, *Lyz2*, *Lgals3*, *Ccl4* and others (Fig. 8b,c and Extended Data Fig. 9j), and DEGs with this pattern were generally not shared with perimeningeal astrocytes (Fig. 8a and Extended Data Fig. 9h). By contrast, certain other reactivity DEGs only reached their peak expression at later times, generally after 14 days, and then persisted at those levels, and this pattern associated with mature lesion border astrocytes was particularly exhibited by DEGs

such as *Gfap*, *C3*, *Id3*, *S100a6*, *Prdx6*, *Fxyd1*, *A2m* and others, which were also enriched in perimeningeal astrocytes that also form borders to stromal cells of the meninges (Figs. 7k–p and 8d–g and Extended Data Fig. 9f,g,k). These findings indicate that healthy perimeningeal astrocytes exhibit unique features that distinguish them from healthy gray matter and white matter astrocytes and share some features with reactive astrocytes but do not exhibit overt reactivity profiles.

Trem2 and *Tyrobp* were not detectably expressed in healthy astrocytes but were robustly upregulated by reactive astrocytes after SCI as detected by both snRNA-seq and Astro-RiboTag RNA-seq, and we confirmed protein expression by IHC (Fig. 4c,f and 8a–c,h and Extended Data Figs. 4e, 8f and 9i). We have previously shown that reactive astrocytes can adopt gene expressions that are unexpected based on their absence in healthy CNS, such as the transcription factor *Spi1* (ref. 17) (Fig. 8c). *Trem2*, *Tyrobp* and *Spi1* are widely regarded as uniquely expressed by microglia in the CNS^{65,66} but can be expressed by reactive astrocytes, albeit at levels lower than other cells (Fig. 8i).

The antimicrobial defense factor *C3* (refs. 49,50) became persistently upregulated by over sixfold from 28 to 70 days in astrocytes after SCI, and immunoreactive protein was highly concentrated in lesion border astrocytes (Figs. 5e,f and 8d,e). *C3* has been proposed as a marker of a subtype of reactive astrocytes that are neurotoxic to neurons across multiple CNS disorders, and generation of saturated lipids by astrocyte *Elovl1* has been proposed as the mediator of their neurotoxicity⁶⁷. We noted here that *C3* is expressed by certain astrocytes in healthy tissue, particularly perimeningeal and scattered white matter astrocytes, and that astrocyte *C3* expression declines acutely after SCI during periods when neurons are lost, after which *C3* becomes highly and persistently upregulated in many border-forming astrocytes that separate inflamed tissue lesions from immediately adjacent surviving neurons (Figs. 7o and 8d,e and Extended Data Fig. 8e). In addition, *C3* and *Elovl1* expression levels changed in opposite directions over time after SCI (Extended Data Fig. 9m). These findings suggest that *C3* may be expressed by astrocytes to exert natural antimicrobial functions^{49,50} around sites of tissue damage and that *C3* expression is not necessarily an absolute indicator of a neurotoxic reactive astrocyte phenotype.

Qualitative and quantitative IHC at single-cell resolution revealed considerable heterogeneity among lesion border astrocytes. Some immunoreactive proteins, such as *Gfap*, *Prdx6*, *Fxyd1*, *Timp1*, *S100a6*, *Id3*, *A2m*, *Gpx1* and *Cd44*, were detectable at persistently high levels in all or most mature lesion border astrocytes from 28 to 70 days after SCI (Figs. 4d, 6n, 7p and 8f,g and Extended Data Figs. 4b, 7e and 9n). By contrast, other proteins, such as *Mmp12*, *Hpse*, *Cdsn*, *Lgals3*, *H2-ab1*, *Cd74*, *C3* and *Tyrobp*, were detectable only in variable subsets of lesion border astrocytes that were intermingled with others with undetectable levels (Fig. 5h and 8e and Extended Data Figs. 4b, 5h, 6b and 9l,o,p). Moreover, detectable levels of immunoreactive protein for certain molecules showed temporal variation after SCI. For example, the number of border-forming astrocytes exhibiting detectable immunoreactive *Tyrobp* declined from 41% at 14 days after SCI to 20% or fewer at 28 days after SCI (Extended Data Fig. 9l,p), a trend that mimicked the decline in *Tyrobp* gene expression (Fig. 8c). Notably, those astrocytes expressing immunoreactive *Tyrobp* did so at a high level (Extended Data Fig. 9i). By contrast, the number of border-forming astrocytes exhibiting detectable immunoreactive *C3* increased from 14 to 28–70 days (Extended Data Fig. 9p), similar to the increase in *C3* gene expression over time (Fig. 8c,d).

Certain DEGs, such as the calcium binding protein *S100a6*, that are not expressed by healthy gray matter astrocytes became highly upregulated by lesion border astrocytes that exhibit gray matter features (cluster 2), and immunoreactive *S100a6* protein was prominent in astrocyte borders along lesions adjacent to spared gray matter (Figs. 7k and 8f and Extended Data Fig. 9k). Other DEGs, such as the antioxidant *Prdx6* and the intercalated disc protein *Fxyd1*, that were moderately expressed by healthy gray and white matter astrocytes

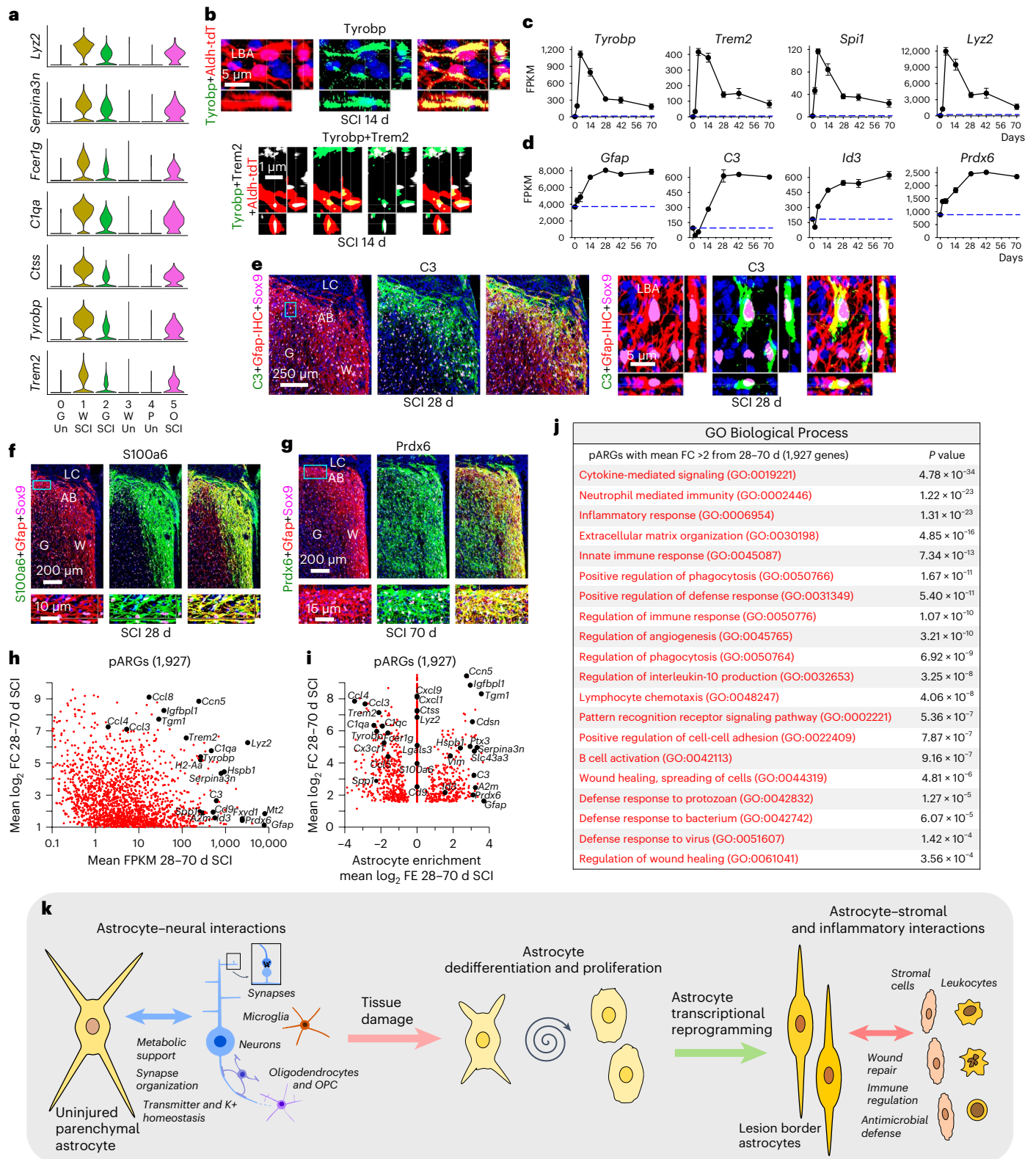


Fig. 8 | Features of mature LBAs. a, Violin plots of snRNA-seq detected genes enriched in reactive astrocyte clusters compared with uninjured. **b**, IHC of proteins *Tyrobp* and *Trem2* in LBAs after SCI. **c, d**, Selected astrocyte DEGs exhibiting different patterns of expression changes in the form of acute rise followed by decline (**c**) or delayed but persistent increase (**d**) after SCI as detected by Astro-RiboTag RNA-seq. **e, f, g**, IHC of proteins *C3* (**e**), *S100a6* (**f**) and *Prdx6* (**g**) in LBAs after SCI. Boxes show locations of expanded regions. **h**, Scatterplot comparing mean \log_2 FC and mean FPKM of 1,927 pARGs upregulated at least twofold from 28 to 70 days after SCI as detected by Astro-RiboTag RNA-seq.

i, Scatterplot comparing mean \log_2 FC and mean \log_2 FE of 1,927 pARGs upregulated from 28 to 70 days after SCI. **j**, GO-BPs associated with 1,927 pARGs upregulated at least twofold from 28 to 70 days after SCI. **k**, Summary schematic showing local astrocyte responses to CNS tissue damage by dedifferentiation, proliferation and transcriptional reprogramming into border-forming wound repair astrocytes. Line plots are mean values; error bars, s.e.m.; $n = 4$ mice for uninjured and all post-SCI timepoints except day 2 ($n = 5$). P values in (**j**) calculated by two-sided Fisher's exact test.

as well as perimeningeal astrocytes, became prominently and persistently upregulated by lesion border astrocytes (Fig. 8d,g and Extended Data Fig. 9f,g,k). Notably, lesion border astrocytes and perimeningeal astrocytes shared prominent expression of various immunoreactive proteins, such as Cd44, A2m, Id3, C3, S100a6 and Gpx (Fig. 7k–p), that were expressed at low or undetectable levels in healthy gray matter or white matter astrocytes and therefore have the potential to serve as combinatorial markers of border astrocytes (Extended Data Fig. 10a,b).

To identify phenotypic features preferentially associated with mature reactive lesion border astrocytes compared with other cells in the lesion area, we examined a cohort of 1,927 DEGs persistently upregulated by more than twofold from 28 to 70 days after SCI in our Astro-RiboTag data, which we refer to as persisting astrocyte reactivity genes (pARGs) (Fig. 8h and Supplementary Data 3). We compared the enrichment of pARG transcripts in astrocytes versus other cells (Fig. 8i). The majority of the 1,927 pARGs that were highly and persistently upregulated by border astrocytes were either expressed at equal (1,330 pARGs) or greater (335 pARGs) levels by other cells, including *Tyrobp*, *Trem2*, *Lyz2*, *Lgals3*, *Cxcl1*, *Cxcl9*, *Ccl3*, *Ccl4*, *C1qa*, *C1qc*, *Fcer1g* and *Spp1* (Fig. 8c,i and Extended Data Fig. 9j). Nevertheless, 262 pARGs were both highly upregulated and highly enriched in astrocytes, including *Gfap*, *Tgm1*, *Ptx3*, *A2m*, *C3*, *Cdsn*, *Serpina3n*, *Hspb1*, *Id3*, *Timp1*, *Vim* and others (Fig. 8d,i and Extended Data Fig. 9k), suggesting that gene expression changes in astrocytes and other cells contribute to overlapping as well as differing functions. The top GO-BPs associated with all 1,927 pARG changes in astrocytes prominently included multiple functions associated with innate immune responses, regulation of inflammation, wound healing and antimicrobial defense (Fig. 8j).

Discussion

In this Article, we show that after focal CNS tissue damage caused by SCI or stroke, local mature astrocytes dedifferentiate, proliferate and become transcriptionally reprogrammed into permanently altered new functional states. They downregulate molecules associated with homeostatic astrocyte–neuron interactions and upregulate molecules associated with wound healing, immune regulation and microbial defense (Fig. 8k). These wound repair astrocytes share morphologic and transcriptional features with perimeningeal limitans astrocytes and are the predominant source of neuroprotective borders that re-establish tissue integrity around CNS lesions by separating neural parenchyma from stromal and immune cells as occurs throughout the healthy CNS.

These findings have implications for understanding the diversity and complexity of astrocyte responses to injury and disease in different contexts. For example, increasing evidence indicates that astrocytes, for unknown reasons, attenuate homeostatic functions in aging and neurodegenerative disorders. Our findings indicate that the downregulation of astrocyte homeostatic functions is part of a natural wound repair response controlled by specific signaling mechanisms. Our findings suggest potential links of such responses to neurodegenerative conditions such as Alzheimer's disease, in which astrocyte loss-of-functions and gain-of-functions may contribute to disease progression⁶⁸ and in which the top 30 most consistently upregulated proteins across multiple studies included at least 11 molecules identified here not only as upregulated by, but also enriched in, wound repair astrocytes: Cd44, S100a6, Padi2, Prdx6, C3, Gpx1, Hspb1, Gpnmb, Clu, Vim and Gfap⁶⁹. Understanding the regulation of astrocyte reactivity responses in different contexts will help identify ways to selectively attenuate detrimental responses or augment beneficial ones. In this regard, we posit that the potential dysfunction of astrocyte wound repair responses is likely underestimated in CNS disorders. The identification here of additional protein markers associated with these cells may facilitate future neuropathological studies.

We demonstrate, by lineage tracing and transcriptional profiling, that over 90% of newly proliferated border-forming wound repair astrocytes derive from local Aldh1l1-expressing astrocytes,

and we confirm, by lineage tracing and snRNA-seq, that about 10% derive from local OPCs^{34–36}. Future studies using techniques such as RABID-seq⁷⁰ or STICR⁷¹ will be useful to examine whether OPC-derived or astrocyte-derived border-forming cells contribute overlapping or differing functions. Ependymal cells have been proposed as the major source of lesion border astrocytes⁷², but this could not be confirmed by previous lineage-tracing studies, which showed that no meaningful number of border-forming astrocytes derived from ependymal cells after SCI³² or forebrain stroke⁷³. Consistent with these observations, our lineage tracing here accounts for essentially all Gfap-positive and Sox9-positive lesion border astrocytes as derived from local astrocytes or OPCs.

The question arises as to why newly proliferated astrocytes form borders around CNS lesions as part of wound repair. Such astrocyte borders have long been proposed to comprise a 'glial scar' that contributes to inferior wound repair and regeneration failure. Preventing or removing these astrocytes was long regarded as a major goal to improve outcome. Challenging this notion, a large and accumulating body of evidence indicates that preventing or attenuating astrocyte border formation disrupts wound repair and causes increased spread of inflammation, failure of blood–brain barrier repair, increased loss of neural tissue, decreased functional recovery and in some cases increased mortality^{4,17,18,23,24,26–29}, whereas augmenting astrocyte border formation reduces lesion size^{23,30}.

In healthy CNS, all neural parenchyma is segregated from non-neural stromal cells either by astrocyte endfeet along blood vessels or by so-called limitans astrocytes that abut meningeal cells around the entire CNS. Structural similarities between perimeningeal astrocytes and lesion border astrocytes have been noted previously^{4,62–64}. Here, we extend these observations by showing that lesion border astrocytes share molecular similarities with healthy perimeningeal astrocytes but also exhibit additional transcriptional profiles associated with wound repair and heightened levels of microbial defense and immune regulation. Previous histological observations have suggested that astrocytes surrounding CNS lesions form new limitans borders rather than scar tissue, particularly in human pathology^{62–64}. Notably, true scar tissue derives from proliferating stromal cells that are unable to reconstitute lost parenchymal cell functions. In pro-regenerative mammalian organs, such as liver, intestinal epithelia and skin, injury induces parenchymal cell proliferation that enables tissue regeneration and recovery of organ function^{74–76}. Proliferating parenchymal cells in these other organs are not considered to form scars; instead, scars are tissue formed by the proliferation of stromal cells when parenchymal regeneration is inadequate^{74–76}. Astrocytes are key neural parenchymal cells that derive from the same neural stem cells as neurons⁷. Unlike mature neurons that are post-mitotic, mature astrocytes can re-enter the cell cycle and proliferate after injury. Newly generated and reprogrammed astrocytes around lesions exert a natural CNS parenchymal cell function when they surround and isolate stromal cell scar tissue and thereby re-establish CNS tissue integrity. In this manner, they preserve CNS function by separating functioning neural parenchyma from stromal and immune cells as occurs throughout the healthy CNS. These findings advocate that astrocyte borders around lesions should no longer be referred to as scars.

The appealing notion of potentially achieving scar-free wound healing in the adult CNS is supported by observations in mammalian neonates in which microglia and astrocytes sustain high levels of proliferation that enable a rapid glia-based repair rather than fibrotic repair, which is sufficient to promote neural circuit regeneration and recovery of function^{77,78}. This may occur in part because neonatal astrocytes are already proliferative at the time of injury and may rapidly increase proliferation to replace lost neural parenchyma without stromal scarring in a manner similar to pro-regenerative organs. By contrast, astrocytes in mature CNS are dormant with respect to proliferation, and their proliferative response to injury is delayed, spatially restricted and

transient. This response is adequate to generate new astrocyte borders around the rapidly formed stromal cell scars but is insufficient to rapidly achieve scar-free parenchymal repair. These observations support the pursuit of strategies that accelerate and extend astrocyte post-injury proliferative and immaturity states to augment neural parenchymal repair^{23,30} or rapidly replace lost glia⁴² in a variety of contexts associated with neural parenchymal loss.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41593-024-01684-6>.

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Methods

Animals

Young adult male and female C57BL/6 mice between 2 and 4 months of age at the time of experimental procedure were used for all studies. For lineage tracing, Ai14 mice expressing the reporter tdT (JAX, 007914) were crossed with different Cre-driver lines: Aldh1l1-CreERT2 (ref. 38) (JAX, 031008), Pdgfra-CreERT³⁹ (JAX, 018280) or NG2-CreERT^{40,41} (JAX, 008538). For sequencing of astrocyte ribosome-associated RNA, mice expressing RiboTag³³ (JAX, 029977) were crossed either with Aldh1l1-CreERT2 (ref. 38) (JAX, 031008) or with mGfap-Cre-73.12 (ref. 79) (JAX, 012886). For postnatal astrocyte evaluations, mGfap-RiboTag mice were used at postnatal days P0, P3, P7, P14, P21, P35 and P63. Transgene expression for each sample was confirmed by genotyping of collected tail samples before processing for astrocyte-specific RNA. Mice were housed in a specific pathogen-free facility with a 12 h light–dark cycle and controlled temperature (20–25 °C) and humidity (50–70%) and were allowed free access to food and water. All *in vivo* experiments involving the use of mice were conducted according to protocols approved by the Animal Research Committee (ARC) of the Office for Protection of Research Subjects at the University of California Los Angeles (UCLA), under ARC numbers ARC-2017-044, ARC-2008-051, ARC-2015-073 and ARC-2000-001. Mice were assigned to experimental groups randomly although ensuring a balanced number of males and females. Animals were coded numerically, and all surgical procedures and subsequent analyses were conducted blind to experimental condition.

Surgical procedures

All surgeries were performed under general anesthesia with isoflurane in oxygen-enriched air using an operating microscope (Zeiss) and rodent stereotaxic apparatus (David Kopf). All animals received the opiate analgesic buprenorphine (0.1 mg kg⁻¹) subcutaneously before surgery and every 12 h for at least 48 h post surgery.

SCI. Laminectomy of a single vertebra was performed at cord level T10. A timed (5 s) lateral compression, complete crush SCI was made using No. 5 Dumont forceps (Fine Science Tools) with a tip width of 0.5 mm. Daily bladder expression was performed for the duration of experiments or until voluntary voiding returned.

Stroke. After a small craniotomy over the left coronal suture, 1.5 µl of L-NIO (N5-(1-iminoethyl)-L-ornithine) (cat. no. 0546, Tocris solution) (27.4 mg ml⁻¹ in sterile PBS) was injected into the caudate putamen nucleus at 0.15 µl min⁻¹ using target coordinates relative to bregma of +0.5 mm A/P, +2.5 mm L/M and -3.0 mm D/V by using a glass micropipette.

Lineage tracing

Cell lineage tracing was conducted by using tdT reporter protein targeted to specific cells and temporally regulated via CreERT-loxP in transgenic mice³¹. tdT expression was activated in young adult mice by administering tamoxifen (Sigma, T5648-1G, 20 mg ml⁻¹ in corn oil) by intraperitoneal injection (100 mg kg⁻¹, once a day) for 5 days followed by clearance for 3 weeks before SCI or stroke, so that no residual tamoxifen remained. Using this approach, tdT becomes constitutively expressed by cells in which it has been activated by Cre during the period of tamoxifen delivery, and this expression is passed on to all progeny cells. Once tamoxifen is no longer administered and has cleared, only the originally targeted cells and their progeny express tdT.

BrdU

BrdU (Sigma-Aldrich), 100 mg kg⁻¹ day⁻¹ dissolved in saline plus 0.007 N NaOH, was administered as single daily intraperitoneal injections on days 2 through 7 after SCI.

Histology and immunohistochemistry

After terminal anesthesia by barbiturate overdose, mice were perfused transcardially with 4% paraformaldehyde (Electron Microscopy Sciences). Spinal cords were removed, post-fixed overnight and cryoprotected in buffered 30% sucrose for 48 h. Frozen sections of the spinal cord were prepared in the horizontal plane at 30 µm thickness using a cryostat microtome (Leica) and processed for immunofluorescence as previously described²⁷.

Primary antibodies. Primary antibodies used included goat anti-A2m (1:300, AF1938; R&D Systems), rabbit anti-Aldh1l1 (1:1,000, Ab87117; Abcam), sheep anti-BrdU (1:800, NB-500-235; Novus), rat anti-C3 (1:400, NB200-540; Novus), goat anti-CD13 (1:600, AF2335; R&D Systems), rat anti-Cd44 (1:400, 14-0441-82; Invitrogen), rat anti-CD68 (1:1,000, MCA1957; Biorad), rabbit anti-Cd74 (1:200, A13958; Abclonal), rabbit anti-Cd3n (1:800, 13184-1-AP; Proteintech), goat anti-Cxcl10 (1:200, AF-466; Novus), rabbit anti-Dnal1 (1:500, 17601-1-AP; Proteintech), rabbit anti-Fxyd1 (1:800, A15082; Abclonal), rabbit anti-GFAP (1:2,000, GA524, Z033401-2; Dako/Agilent), rat anti-GFAP (1:1,000, 13-0300; ThermoFisher), rabbit anti-hemagglutinin (1:1,000, H6908; Sigma-Aldrich), goat anti-Gpc5 (1:200, AF2607; R&D Systems), rabbit anti-Gpx1 (1:200, 29329-1-AP; Proteintech), goat anti-hemagglutinin (1:800, NB600-362; Novus Biologicals), rabbit anti-H2-Ab1 (1:200, A18658; Abclonal), rabbit anti-Hpse (1:200, 24529-1-AP; Proteintech), guinea pig anti-Iba1 (1:1,000, 234004; Synaptic Systems), rabbit anti-Iba-1 (1:800, 019-19741; Wako), rabbit anti-Id3 (1:500, 9837; Cell Signaling), rabbit anti-Kcnj10 (Kir4.1) (1:400, APC-035; Alomone Labs), rat anti-Lgals3 (1:200, 14-5301-82; ThermoFisher), rabbit anti-Lxn (1:500, 13056-1-AP; Proteintech), rabbit anti-Mfge8 (1:200, A12322; Abclonal), rabbit anti-Mmp12 (1:200, 22989-1-AP; Proteintech), goat anti-Myoc (1:400, AF2537; Novus), guinea pig anti-NeuN (1:1,000, 266004; Synaptic Systems), rabbit anti-NeuN (1:1,000, ab177487; Abcam), guinea pig anti-Olig2 (1:800, ABE1024; Millipore), rabbit anti-Olig2 (1:200, AB9610; Millipore), rabbit anti-Padi2 (1:300, 12110-1-AP; Proteintech), rabbit anti-Prdx6 (1:500, 13585-1-AP; Proteintech), sheep anti-S100a6 (1:300, AF4584; R&D Systems), rabbit anti-S100a6 (1:200, A3461; Abclonal), goat anti-Serpina3n (1:200, AF4709; R&D Systems), goat anti-Sox9 (1:800, AF3075; R&D Systems), rabbit anti-Sox9 (1:800, 702016; ThermoFisher), goat anti-Sox10 (1:500, AF2864; R&D Systems), guinea pig anti-tdT (RFP) (1:1,500, 390-004; Synaptic Systems), rabbit anti-RFP (1:1,500, 600-401-379; Rockland), rabbit anti-Timp1 (1:800, 16644-1-AP; Proteintech), sheep anti-Trem2 (1:400, AF1729; Novus), rabbit anti-Tyrobp (1:400, 12492S; Cell Signaling) and rat anti-Vim (1:200, MAB2105; Novus). All antibodies were sourced from commercial vendors and selected because they had previously been validated for fluorescent immunohistochemistry in mouse tissue and had a manufacturer-provided demonstration of specificity based on western blots and, in most cases, validation in peer-reviewed publications.

Fluorescence secondary antibodies. Alexa 488 (green), Cy3 (550, red) or Alexa 647 (far red) were used, all from Jackson ImmunoResearch Laboratories. Mouse primary antibodies were visualized using the Mouse-on-Mouse detection kit (M.O.M., Vector). The nuclear stain was 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, blue; 2 ng ml⁻¹; Molecular Probes). Sections were cover-slipped using ProLong Gold anti-fade reagent (Invitrogen). Sections were examined and photographed with an epifluorescence microscope using structured illumination hardware and deconvolution software (Zeiss).

Quantification of positively stained cells. The number of lesion border astrocytes expressing specific individual immunoreactive proteins or incorporating BrdU was evaluated by using double and triple labeling immunohistochemistry combined with analysis of single cells in three dimensions. Stacks of optical slices were collected through the z axis

(15–25 μm) in the astrocyte borders immediately adjacent to and within 250 μm of fibrotic lesion cores and were evaluated using the software Imaris 9.2 (Bitplane), Zen 3.1 (Zeiss) and ImageJ 1.53 (NIH). Values were expressed as the proportion of Gfap+Sox9-positive cells that were also positive for a given protein or BrdU. At least 30 cells were evaluated per animal examined.

Fresh tissue collection and freezing for RiboTag or snRNA-seq

Uninjured mice and mice at various timepoints after complete crush SCI were perfused with ice-cold heparinized saline prepared using RNase-free and DNase-free water and $10\times$ PBS for 2 min at 7 ml min^{-1} for blood clearance. Spinal cords were rapidly dissected on ice-chilled blocks. For SCI samples, the lesion core center was identified and a tissue block of 1 mm rostral and caudal was then rapidly removed. We have previously shown that astrocyte proliferation after SCI in mice is proportionally highest in the 250 μm zone immediately adjacent to the stromal cell lesion core and is almost as high in the next adjacent 250 μm zone and then tapers gradually over about 1 mm¹³. Our observations were equivalent here. Histological evaluations indicated that the lesion core extends rostro-caudally for about 500 μm and contains few astrocytes. Thus, the block of tissue collected here included the lesion core and about 750 μm of border tissue on either side of the lesion core, and the overwhelming majority of astrocytes in this tissue block will have been newly proliferated. We purposely made our dissections of tissue samples as small as possible to include primarily newly proliferated border-forming astrocytes while excluding nonproliferative astrocytes from adjacent spared neural tissue. Anatomically equivalent regions of spinal cord were taken from uninjured mice, including from postnatal samples. Tissue samples were rapidly snap-frozen in microcentrifuge tubes maintained in a dry ice bath and stored at $-80\text{ }^{\circ}\text{C}$ until further processing.

RiboTag immunoprecipitation and RNA-seq

Frozen spinal cord tissue was processed by RiboTag immunoprecipitation³³ using established methods^{27,42}. In brief, tissue was homogenized in RiboTag lysis buffer and centrifuged to remove tissue debris. Immunoprecipitation of hemagglutinin-positive ribosomes was performed by incubating with anti-HA.11 Epitope Tag Antibody (Biolegend, 901515) for 4 h in a microcentrifuge tube on a microtube rotator kept at $4\text{ }^{\circ}\text{C}$. Immunoprecipitation solutions were combined with Pierce A/G Magnetic Beads (ThermoFisher, PI88803) and incubated overnight on a microtube rotator at $4\text{ }^{\circ}\text{C}$. On the second day, the solution was separated from the magnetic beads and processed as the ‘flow through’ sample representing mRNA of other cells not from RiboTag-positive cells. Magnetic beads were washed three times with high salt solution (50 mM Tris pH 7.4, 300 mM KCl, 12 mM MgCl_2 , 1% NP-40, 1 mM dithiothreitol (DTT), 100 mg ml^{-1} cyclohexamide). Unpurified RNA was collected from the magnetic beads by addition of RLT Plus buffer with BME and vigorous vortexing. RNA was then purified using RNeasy Plus Mini (for in vitro cell pellets) or Micro Kits (for spinal cord tissue) (QIAGEN, 74134 and 74034). Total mRNA derived from the RiboTag immunoprecipitation was quantified using a 2100 Bioanalyzer (Agilent), and RNA samples having RNA integrity numbers greater than seven were processed for RNA-seq. Sequencing was performed on poly-A selected libraries using Illumina NovaSeq S2 (UCLA Technology Center for Genomics & Bioinformatics) using paired-end reads ($2\times 50 - 50\text{ bp}$ length) with an average of 50–100M reads per sample split over two lanes of the S2 flow cell.

Transcriptomics analysis of RiboTag RNA-seq data

Analysis of RNA-seq raw data was performed in Galaxy using standardized workflows as previously described⁴². The R1 and R2 FASTQ files from lanes 1 and 2 that were obtained directly from the Illumina NovaSeq S2 run were concatenated and cleaned up using the Trimmomatic tool. Data were then aligned to the *M. musculus* (mm10) reference genome

using the HISAT2 tool applying default parameters. Gene counts from the aligned datasets were performed using the featureCounts tool, applying default parameters. FPKM values were calculated for each gene directly in Excel (Microsoft) using standardized lists of gene lengths and normalization of the count data. DEG analysis on raw gene count data was conducted using Edge-R in Galaxy, applying Benjamini and Hochberg *P* value adjustment and TMM normalization. Across all studies, we used a conservative false discovery rate cutoff of <0.01 to define the significance of DEGs and evaluated at least four unique samples per experimental group. GO analyses were performed using the Enrichr tool (<https://maayanlab.cloud/Enrichr>). Differences in transcript expression across samples were evaluated using data-dimensionality reduction techniques, including principal component analysis and Euclidian distance as described below. Heatmaps of DEG data were generated using NG-CHM BUILDER (<https://build.ngchm.net/NGCHM-web-builder>). Violin plots of DEGs were generated using Prism 10.

Threshold criteria for gene expression in healthy astrocytes

Healthy astrocyte expressed genes (AEGs) were defined as having an FPKM value greater than 0.1, which was a conservative cutoff that accounted for genes that were no more than one standard deviation below the mean of the log-transformed dataset and represented the upper 77% of all genes that had detectable counts in the dataset. Genes with an FPKM value at or above this threshold in healthy astrocytes were considered healthy expressed genes.

Isolation and precipitation of astrocyte nuclei for snRNA-seq

Tissue samples were collected from $n = 4$ uninjured mice and $n = 4$ mice at 28 days after SCI and were rapidly frozen as described above. To avoid potential batch effects, frozen tissue samples were then all processed at the same time. To enrich for astrocyte nuclei, we used Sox9 antibody-binding and magnet-assisted nuclear immunoprecipitation using well-characterized procedures^{80–82} as follows. Tissue samples were first gently dissociated by trituration and pelleted by centrifugation. Nuclei were extracted from cell pellets by gentle resuspension in ice-cold lysis buffer (10 mM Tris buffer, 10 mM NaCl, 3 mM MgCl_2 , 0.1% Nonidet P40 Substitute). Nuclei were pelleted by centrifugation (model 5415R, Eppendorf; 500 r.p.m. for 5 min at $4\text{ }^{\circ}\text{C}$) and then resuspended in nuclei wash and resuspension buffer ($1\times$ PBS, 1% BSA, 0.2 U ul^{-1} RNase inhibitor) before being washed once more in nuclei wash and resuspension buffer and then filtered using a 5 ml polystyrene round-bottom tube with 35 μm cell-strainer cap and concentrated to a nuclei concentration of 1,000 nuclei per μl (1×10^6 nuclei per ml), resuspended and incubated with Sox9 rabbit monoclonal antibody (ThermoFisher, 72016) for 30 min and then centrifuged at $700\times g$ for 10 min. Pellets were resuspended in 80 μl of MACS buffer composed of $1\times$ PBS (tissue culture grade; Ca^{2+} , Mg^{2+} free), 0.5% nuclease-free BSA, and 2 mM EDTA, and then incubated with anti-Rabbit IgG Microbeads (Miltenyi, 130-048-602) for 20 min at $4\text{ }^{\circ}\text{C}$. After washing with 1 ml of MACS buffer at $300\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$, immunolabeled nuclei were enriched by magnetic separation using MACS MS columns (Miltenyi, 30-042-201, 130-042-102 and 130-042-303). Pellets were resuspended in an appropriate volume of resuspension buffer to achieve a final concentration of 1,000 nuclei per μl (1×10^6 nuclei per ml) and immediately processed for library preparation using Chromium Next GEM Single Cell 3' v3.1 kits following the manufacturer's instructions. Eight library samples were processed simultaneously for RNA-seq on two lanes (lane 1: injured-1, injured-2, uninjured-1, uninjured-2; lane 2: injured-3, injured-4, uninjured-3, uninjured-4) of Illumina NovaSeq S4 with at least 300 M reads per library sample, conducted in-house by the UCLA Technology Center for Genomics & Bioinformatics using paired-end reads (2×100).

Analysis of snRNA-seq data

Raw single-nuclei sequencing data was processed using RNA StarSolo on the Galaxy Single Cell Omics platform using *M. musculus* (mm10)

reference genome, 3M-February-2018 barcode whitelist, the gencode vM25 annotation list and Cell Ranger v3 configure chemistry options. Scanpy tools were used through the Galaxy platform to convert genes, barcodes and matrix files derived from RNA StarSolo into an Ann-Data matrix h5ad format. Downstream analysis was performed in R (v4.1) using the Seurat package (v4). The dataset was pre-processed to remove cells with high mitochondrial reads, filter out genes that were detected in less than 200 cells and filter out low-quality cells that had less than 500 attributed genes. We then imposed stringent quality control metrics on unique molecular identifiers, the number of genes detected per cell and the proportion of mitochondrial reads (Extended Data Fig. 8b), thereby effectively mitigating the likelihood of doublet incorporation. Second, we used the CellBender algorithm⁸³ to correct for ambient RNA contamination, a factor that can artificially inflate doublet rates. CellBender's robust ambient RNA removal further contributed to the enhancement of data quality. Our prior investigations⁸⁴ have revealed a lack of consensus among various doublet detection algorithms when identifying doublet cells in identical datasets. We therefore focused on rigorous upfront quality control measures and ambient RNA correction as more reliable avenues for enhancing data fidelity.

Next, we \log_2 normalized the dataset, identified the 4,000 most variable genes across the total population, computed principal components and summarized the top 50 principal components using the UMAP projection. Clustering of nuclei was performed on the UMAP data through Seurat using Louvain clustering algorithms with a resolution of 0.3, which resulted in 20 discrete clusters for nuclei from both uninjured and SCI tissue. Based on feature plot analysis of the distribution of marker genes in individual nuclei, clusters of nuclei were annotated as enriched for astrocytes, neurons, oligodendrocytes, OPCs or microglia on the basis of multiple cell-type-specific marker genes. Clusters broadly identified as enriched for astrocytes based on high expression of *Gfap*, *Aqp4*, *Slc1a2*, *Aldoc* and *Clu* with low or absent expression of markers for other cell types (Extended Data Fig. 8c) were extracted and re-clustered with a resolution of 0.3. We then selected clusters of astrocytes and eliminated clusters of other cell types based on panels of multiple consensus marker genes highly enriched in or selective for uninjured or reactive astrocytes⁴² or other specific cell types⁸⁵. Examples of marker genes used to identify clusters included (but were not limited to) *Gfap*, *Aqp4*, *Gpc5*, *Slc7a10*, *Slc1a2*, *Slc4a4*, *Slc39a12*, *Fgf3*, *Aldoc*, *Clu*, *Pad12*, *Fxyd1*, *Prdx6*, *Lcn2*, *Serpina3n*, *A2m*, *Spp1* and *C3* for uninjured or reactive astrocytes; *Dnah12*, *Pifo*, *Odf3b*, *Dynlrb2*, *Cfap126* and *Fam183b* for ependyma; *Pecam1*, *Ptprb*, *Adgrf5*, *Cyt11* and *Lmcd1* for endothelia; *Csf1r*, *P2ry12* and *Ptgs1* for microglia; *Ptprc* (*Cd45*) for leukocytes; *Mog* and *Cldn14* for oligodendroglia; *Pdgfra* and *Cspg4* (*Ng2*) for OPCs; *Pdgfra* for pericytes; *Fbn1* and *Lum* for stroma and fibroblasts; and *Syt1* and *Stx1a* for neurons. Final selection of astrocyte clusters was based on high expression of multiple astrocyte markers combined with low or absent expression of markers of other cell types as shown for selected examples in Extended Data Fig. 8h. This process confidently identified 15,637 astrocyte nuclei from $n = 4$ uninjured mice (1,585, 1,576, 2,961 and 2,648 nuclei per mouse) and $n = 4$ SCI mice (1,967, 1,859, 1,709 and 1,332 nuclei per mouse) that were used for final analyses after re-clustering with a resolution of 0.15 (Extended Data Fig. 8d–g).

PCA

PCA and Euclidean distance analysis were performed using XLStat (Addinsoft)⁴². For PCA presented throughout, the first two principal components were used to display two-dimensional scatterplots. PCA factor analysis to identify genes correlated with a particular PC used a factor loading threshold of $>|0.8|$. PCA data were represented as Euclidean distance plots throughout. Euclidean distance magnitude calculations were derived by assessing the vector magnitude in PCA space of a specific sample referenced to another sample as an initial point.

Statistics, power calculations, group sizes and reproducibility

Graph generation and statistical evaluations of repeated measures were conducted by one-way or two-way ANOVA with Tukey's post hoc independent pair-wise analysis or by one-sample t -tests in which the null hypothesis was equal to 100%, as appropriate, using Prism 10 (GraphPad Software). P values for GO evaluations were calculated by two-sided Fisher's exact test using the Enrichr tool. Data distribution was assumed to be normal but this was not formally tested. Statistical details of experiments can be found in the figure legends including the statistical tests used and the number of replicative samples. Across all statistical tests, significance was defined as $P < 0.05$. Power calculations to determine group sizes were performed using G*Power Software v3.1.9.2. For immunohistochemical quantification analysis and RNA-seq, group sizes were calculated to provide at least 80% power when using the following parameters: probability of type I error (α) = 0.05, a conservative effect size of 0.25 and 2–5 treatment groups with multiple measurements obtained per replicate. All graphs show mean values \pm s.e.m. as well as individual values as dot plots. All bar graphs are overlaid with dot plots in which each dot represents the value for one animal to show the distribution of data and the number (n) of animals per group. Lineage-tracing studies were repeated independently at least three times in different cohorts of mice across a 3-year period with similar results. All immunohistochemistry analyses were reproduced in at least $n = 4$ biological replicates.

Reporting summary

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

Data availability

Raw FASTQ sequencing files and processed count data have been deposited at Gene Expression Omnibus and are publicly available with accession number [GSE241628](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE241628) for RiboTag data, accession number [GSE247844](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE247844) for our snRNA-seq data from this study and [GSE234774](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE234774) for snRNA-seq data from a previous publication³⁰. All data generated for this study are included in the main and extended data figures and supplementary data files with lists of different gene cohorts examined. For all quantitative figures, source data are provided in Supplementary Information. Other data that support the findings of this study are available on reasonable request from the corresponding authors. Source data are provided with this paper.

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Author contributions

T.M.O'S. and M.V.S. conceptualized and led the overall project. T.M.O'S. and M.V.S. designed, guided and supervised all experiments. T.M.O'S., Y.R. and S.W. performed surgeries. T.M.O'S., Y.A., Y.R. and A.L.C. conducted histological processing. T.M.O'S. and S.W. conducted

biochemical and transcriptome processing. T.M.O'S, Y.A., S.W., R.K., Z.S., V.S. and M.V.S. analyzed data. T.M.O'S. and M.V.S. wrote the paper with input from all co-authors.

Competing interests

The authors declare no competing interests.

Additional information

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