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Glia Are Bussin': How Single-Cell and Spatial Transcriptomics Enlighten the Role of Neuroglia in Spinal Cord Injury and Regeneration

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ABSTRACT

The impact that single-cell and spatial genomic technologies have had on the scientific community since their advent has been widespread and profound. The field of spinal cord injury (SCI) is one such area that has experienced an increased implementation of genomic technologies within recent years. Innovative genomic tools have proven to be particularly useful for delving into the progression of SCI pathology and plasticity that is highly dynamic, temporal, and involves the close coordination and signaling of many different central nervous system resident and non-resident cells. Neuroglia, in particular, have been extensively profiled, resulting in a deeper understanding of how neuroglia phenotypes and molecular signaling interact and progress after a spinal cord injury. In this review, we summarize some of the major discoveries that have enlightened and/or shifted our current understanding of the role of neuroglia in injury potentiation and repair. Additionally, through a meta-analysis of five SCI single-cell time course atlases, we uncover insights and cautions regarding how injury model, time course, sample preparation, cellular categorization, and analytical approach impact the interpretation of any new genomic research in this field.

1 | Introduction

Traumatic spinal cord injury (SCI) is a severe affliction that currently impacts ~300,000 Americans and ~15.4 million people worldwide (Jain et al. 2015; WHO, n.d.). These injuries often lead to permanently impaired motor function, sensory deficits, costly rehabilitation, and shorter life expectancies, among many other pathologies (Spinal Cord Injury Facts and Figures at a Glance 2024). Resultantly, there has been a long-standing interest in assessing the cellular and temporal dynamics of SCI pathology and recovery in order to catalyze the development of new SCI-targeted therapies. Researchers have unraveled many of the intra- and intercellular events related to SCI through immunohistochemistry, genetic manipulation, lineage tracing, and bulk RNA-sequencing techniques. However, with the advent and popularization of single-cell technologies, we have seen a deepening in our understanding of cell identities and functions well beyond anything previously achieved. Single-cell RNA sequencing (scRNAseq) allows for the isolation and capture of an individual cell (or nucleus) transcriptomic profile at a given time. Since scRNAseq is not limited to any number of genes, probes, or the genetic averaging of pooled cells, it produces a higher-resolution transcriptomic cellular and phenotypic profile than probe-based or bulk RNA approaches. Indeed, scRNAseq has validated some predictions on cellular dynamics after injury while also illuminating novel cellular phenotypes and molecular interactions within SCI. These advances have challenged and updated our understanding of cellular plasticity, cellular coordination, cellular co-dependence, regional heterogeneity, temporal heterogeneity, and overall phenotype nomenclature as they relate to SCI. While scRNAseq has led to novel insights into neuronal lineages, the majority of published single-cell datasets are primarily comprised of and focused on

characterizing neuroglia and neuroglial interactions. Thus, in this review, we aim to convey key findings, as a direct result of single-cell and spatial genomics, in the field of SCI and regeneration with a large focus on neuroglia.

The goal of this review is to expand and, in some instances, challenge the conventional understanding of neuroglial dynamics, molecular interactions within neuroglia, and neuroglial phenotypic heterogeneity through a comparative review of scRNAseq data. To this end, we curated and compiled available single-cell genomic atlases, and we review the strengths and weaknesses of different protocols, injury models, time points, and genetic markers to reveal exciting details of cell genesis and cell functions after SCI (Table 1). In addition, we discuss the major pitfalls and technical limitations of the techniques presented and some suggestions for best practices and data interpretation when it comes to performing and analyzing scRNAseq data in SCI. The results presented herein are an exciting landscape of insight into the emergence of novel glial phenotypes, both temporally and spatially, that are likely to catalyze new ideas for targeting repair of the injured nervous system.

2 | The Centurion of the Spinal Cord: Microglia

In the initial acute/sub-acute phase after SCI (< 14 days in rodents), there is an immunological "call to arms" that creates a massive, complex, and highly dynamic response within resident and infiltrating immune cells. This acute immune cascade has been well described and is comprised of a rapid accumulation and proliferation of microglia, neutrophils, monocytes, macrophages, and lymphocytes within hours to days after injury (Donnelly and Popovich 2008). Some have reported that immunologic cascades, in part, may be dependent on species, strain, and/or injury model with respect to lymphocytes, neutrophils, and monocytes (Donnelly and Popovich 2008; Sroga et al. 2003). Alternatively, microglia/macrophages have demonstrated more consistent temporal dynamics between rats and mice with only minor variations in microglial morphology at more chronic time points (≥ 6 weeks in rodents) (Sroga et al. 2003). Moreover, select depletion and gain and loss of function studies within microglia/macrophages have illuminated the diversity of their roles from lesion resolution or expansion, production of neurotoxic molecules and cytokines, and pro-inflammatory cellular coordination to myelin remodeling and remyelination, synapse pruning, and providing pro-regenerative neurotropic support (Donnelly and Popovich 2008; Green et al. 2020; Olah et al. 2012; Paolicelli and Ferretti 2017; Paolicelli et al. 2022; Parkhurst et al. 2013; Schafer et al. 2012). This seeming duality of microglial function had been previously described as categorically good or bad, pro-regenerative or pro-inflammatory, M1 or M2 (Paolicelli et al. 2022). However, microglial nomenclature and phenotypic definitions are evolving with research. It has become clear that microglia should not be categorized in strict, functional bins as previously mentioned. Rather, they are more likely comprised of a spectrum of subphenotypes that are capable of transitioning between these states given the right environment and immunological cues (Paolicelli et al. 2022). This update in our understanding of microglial phenotypes is owed largely in part to single-cell transcriptomics, as its high genetic resolution allowed for a more holistic interpretation of microglial function that is not limited to a handful of genes or markers (Paolicelli et al. 2022). Even though many gaps in our knowledge of microglia still exist, scRNAseq has already revealed many novel insights within microglial molecular subtypes and their temporal dynamics after SCI. Indeed, it is an exciting time for immunotherapeutics as these and subsequent studies enlighten our overall understanding of the temporal and anatomical landscape of microglial phenotypes, and that knowledge can be used to better tailor drug or genetic interventions in future preclinical experiments.

Before we move any further, we would like to acknowledge that in an effort to simplify and aggregate many different naming conventions from the heretofore mentioned journal articles, we will occasionally refer to different microglial states or phenotypes as "activated," "pro-inflammatory," or "ameboid" versus "resting," "anti-inflammatory," or "ramified" even though these definitions are slowly being updated to more closely address the complexity of microglial phenotypes as a whole (Paolicelli et al. 2022). We only intend for these interpretations to be used within the context of central nervous system (CNS) injury as these categorizations, although broad, are distinct enough in this highly inflammatory environment. This can be seen on a single-cell level through the phenotypic shifts that microglia undergo after SCI, such as the acute presence of proliferative, phagocytizing, and antigen-processing microglia that are not seen within the intact spinal cord (Brennan et al. 2022; Hakim et al. 2021). However, we move forward with the understanding that these phenotypic labels have room for specification and improvement in the near future.

2.1 | Temporal Dynamics of Microglia and Chronic Microglia

First, to demonstrate how scRNAseq holds up to the known temporal dynamics of microglia in terms of general proportions and proliferation after SCI, we will compare time-course single-cell experiments to those published in seminal histological studies (Donnelly and Popovich 2008; Fleming et al. 2006; Popovich and Hickey 2001; Popovich et al. 1997; Schnell et al. 1999; Sroga et al. 2003). In general, scRNAseq recapitulates many of the known microglial relationships that have been previously reported within the acute phase after injury while also enriching our understanding of specific microglia roles. These single-cell time course experiments show that microglia begin expressing pro-inflammatory markers and cytokines, such as Il1a and Cd83, as soon as 0.5 h post injury (hpi) (Hakim et al. 2021). This insight expands on the previously reported immediate immune response to the SCI environment and cell death that demonstrates significant cytokine production at 6 hpi that peaks 12 hpi at the epicenter of injury (Donnelly and Popovich 2008; Hellenbrand et al. 2021; Pineau and Lacroix 2007; Streit et al. 1998). These data support a very rapid, molecular activation of microglia not previously observed. Further, snRNAseq data show microglia rapidly enter a proliferative subphenotype within 1–3 days post injury (dpi) that is not observed in the intact spinal cord (Gong et al. 2023; Hou et al. 2022; Milich et al. 2021). Eventually, both microglia abundance and the development of a pro-inflammatory phenotype peak at 7 dpi, which is also confirmed in spatial

transcriptomics (Hou et al. 2022; Matson et al. 2022; Milich et al. 2021). This supports previous lineage tracing and histological observations that quantified a peak in microglial proliferation at 7 dpi in mice (Bellver-Landete et al. 2019). Notably, the peak abundance for microglia was histologically determined to be ~14 dpi (Bellver-Landete et al. 2019). However, this finding, at 14 dpi, was either not captured in any current scRNAseq time course experiments or at least not quantified or reported.

As previously mentioned, the power of single-cell genomics emerges when analyzing genetic subphenotypes of cells to illuminate the complexity of cellular dynamics and function. This is clearly seen within microglia when analyzing sub-acute and chronic time points after SCI. It was first reported by Brennan and colleagues that at around 28 dpi, when the peak of inflammation has subsided, microglial genetic expression shifts into a lipid-phagocytizing and patrolling phenotype; this could be related to an increase in microglial-mediated synapse or myelin remodeling that we will consider later. Notably, at this stage, microglia still persist in a proliferating phenotype, though lower in abundance compared to the first 7 days after injury (Brennan et al. 2022). The genomic evidence of a novel late-proliferative phenotype has been confirmed histologically and detected well into the chronic injury stage at 84 dpi (Perez et al. 2023). A particularly interesting discovery from these pioneering single-cell studies is that microglia undergo a dramatic change in their gene expression profile and phenotype in the chronic phase after SCI. Microglia specifically transition from a resting (ramified) state after SCI toward a distinct molecular profile that remains stable until the latest timepoint published of 90 dpi (Hakim et al. 2021; Hou et al. 2022). Some refer to this chronic microglia molecular phenotype as damage-associated microglia (DAMs) because of their transcriptional similarity to other DAMs within mouse models of neurodegeneration, demyelination, and development (Hakim et al. 2021). More specifically, these SCI DAMs are transcriptionally colocalized with a subclass of white-matter ameboid microglia that are described as metabolically active and preferentially interacting with oligodendrocytes that express apoptotic factors during development, suggesting increased phagocytosis of these cells (Hakim et al. 2021; Li et al. 2019). Genetically, these SCI DAMs are distinguished by an upregulation of Apoe, Spp1, Lyz2, Cst7, Clec7a, Gpnmb, Igf1, Glmp, and Nfe2l2/Nrf2 when compared to healthy, uninjured microglia (Hakim et al. 2021). Notably, Nrf2 has been widely studied in a variety of neurodegenerative, cognitive, and CNS injury models but by and large, data indicates that Nrf2 upregulation within microglia has a protective and anti-oxidant impact on the system as a whole and that loss of expression is detrimental for disease pathology and recovery (Guo et al. 2022; He et al. 2022; Rojo et al. 2010; Saha et al. 2021; G. Wu and Liu 2016). Further, Glmp has also been implicated recently in the regulation of iron accumulation after SCI, knockdown of which leads to increased iron deposition, mitochondrial shrinkage and damage within microglia, increased lesional area, decreased neuronal survival, and worse functional recovery (Ouyang et al. 2025). Moreover, the overexpression of most of the remaining markers mentioned (i.e., Apoe, Cst7, Gpnmb, etc.) has been associated with pro-inflammatory microglia within models of neurodegeneration (Huttenrauch et al. 2018; Rachmian et al. 2024; Saade et al. 2021; Yao et al. 2022), but the implications for SCI have not yet been well described.

To date, the presence of DAM's within SCI has not been confirmed histologically, as in the seminal neurodegeneration studies, and research is needed to determine if this molecular phenotype plays an active role in pathologic or protective demyelination and debris removal after SCI. However, a recent study using a lateral hemisection mouse model of SCI demonstrated that a transient, one-week depletion of proliferating microglia with a Csf1r inhibitor (GW2580) decreased the abundance of myelin-phagocytizing microglia, resulting in increased myelin fiber density and myelin thickness at 42 dpi (Perez et al. 2023). These data suggest that microglia in the sub-chronic period may be preferentially focused on myelin remodeling, which correlates with the lipid-phagocytizing and processing phenotype within the single-cell data we previously mentioned. Moreover, Perez et al. (2023) show that the transient ablation of proliferating microglia beyond the acute phase may be sufficient to partially rescue myelin thickness after injury. However, researchers did not assess the impact on function with this form of microglial depletion, so the functional impact of myelin sparing is currently unknown. Nevertheless, these data are an example of how single-cell transcriptomic data can be used to guide the targeting of discrete microglial phenotypes (e.g., lipid-phagocytizing and processing microglial subtypes).

Thus, single-cell transcriptional evidence indicates that microglia gene expression is undergoing an immediate and drastic alteration after SCI associated with proliferation and a pro-inflammatory phenotype that is chronically sustained with the potential for them to be more focused on myelin removal than in a healthy system. If these findings hold true, it would be interesting to determine if this SCI DAM profile can be further manipulated to reduce their inflammatory nature and potentially restore a resting or pro-regenerative state. Moreover, if the connection between increased myelin remodeling and phagocytosis can be bolstered further within the microglia after SCI, it has the potential to lead to functional improvements and a novel avenue for immunomodulatory efforts in the future.

2.2 | Microglial Depletion and What Have We Learned?

Armed with a richer, single-cell data set, efforts to modulate the microglial response after SCI have resulted in a better and more complete understanding of what phase and subtype of cell they are targeting. Diving deeper into this field of study offers a potentially very fruitful area of research as genetic manipulation of microglia becomes a reality with the advent of new viral tools capable of infecting and manipulating microglia, a cell type previously intractable to gene therapy (Lin et al. 2022). In fact, the drive to better understand the microglial response is particularly evident in the case of drug-targeted depletion of microglia.

One of the more recent methods of microglial manipulation is the use of commercially available Csf1r inhibitors, most commonly PLX5622 or PLX3397, but other variations, such as GW2580, do exist, although they have different functionality. Usually placed into the chow for mice, after several days of treatment, these drugs can deplete > 90%–95% of all microglia within the spinal cord while leaving peripheral monocytes unchanged (Brennan et al. 2022; Li et al. 2020). While the use of these PLX

chows has become a very popular method in analyzing microglial functionality, we have only started to see the impact of microglial depletion on a single-cell level.

Recently, the Popovich group performed single-cell analysis on microglia-depleted spinal cords that were subjected to a contusive injury, and their findings are crucial to deciphering the role microglia have in the injury microenvironment (Brennan et al. 2022). As others have also reported, the near-complete depletion of microglia before injury dysregulated and increased the glial scar, increased lesion size, decreased axonal sparing, and ultimately worsened functional outcomes (Bellver-Landete et al. 2019; Fu et al. 2020; Zhou et al. 2023). However, the biggest enlightenment from this single-cell experiment was the significant reduction in macrophage and astrocytic abundance and the proportional shifts in phenotypic subtypes within these cell types when depleting microglia before SCI (Brennan et al. 2022). Perhaps unsurprisingly, without microglia to initiate the inflammatory recruitment process, infiltrating macrophages were largely absent from the injured spinal cord, with 20–30-fold reduced abundance compared to non-depleted control (Brennan et al. 2022). These recruited macrophages mainly consisted of lipid and antigen-binding phenotypes, overexpressing genes such as Gpnmb, Fabp4, Fcrls, and Stab1 at 7 dpi. By 28 dpi, they had shifted to a more matrix-remodeling and cholesterol-processing phenotype, overexpressing genes such as Mmp12, Ctnnb1, Lpl, and Ch25h. However, the overall distribution of macrophage subphenotypes appeared to be similar to that of the injured spinal cord containing an intact microglia population. These data indicate infiltrating macrophages are dependent on early microglia activation for injury site recruitment but to a lesser degree for guiding function of infiltrating macrophages. Microglia and macrophage crosstalk and interdependence is a challenge to study due, in part, to the remarkable genetic similarity of microglia and macrophages that share many of the same markers even in the context of injury (London et al. 2013; Paolicelli et al. 2022). These initial single-cell experiments should encourage further exploration in this area as it is increasingly important to distinguish macrophage and microglial function, and this technology allows for the level of resolution needed for this kind of experimentation.

In contrast, astrocytes showed almost the opposite effect compared to macrophages. Although not as drastic as the effect on macrophages, overall astrocyte abundance was roughly halved when microglia were depleted at all-time points (Brennan et al. 2022). Importantly, astrocyte cell number was low in all conditions, so this might be partially due to biological or technical variability. However, it was reported that the Gfap area and mitotically active Gfap cell abundance were decreased; thus, this could be a *bona fide* phenomenon, but further validation is required. Additionally, it becomes evident when analyzing astrocytic phenotypes that reactive astrocytes are suppressed or absent within the microglia-depleted spinal cord and the remaining astroglial cells have a measured decrease in notable inflammatory genes like Apoe, Tmsb4x, Ctsd, Hexb, and Fau while exhibiting increased expression of Ntm, Neat1, and Atp1b2 when compared to astroglial cells from a non-depleted, injured spinal cord. This suggests that astrocytes, at least in part, respond to signals from microglia that amplify the astrocyte inflammatory/reactive phenotype. These findings imply a critical role for microglia in regulating both astrocyte and macrophage abundance and phenotype after SCI. It is particularly interesting that when researchers exogenously provided recombinant-Ccl2 and Tlr2 agonists to mice that were both depleted of microglia and injured, they were able to partially rescue some of the negative side effects of microglial depletion previously mentioned (e.g., increased lesion volume and lesion length, decreased spared axons, decreased myelin spread, and overall worse hindlimb function) (Brennan et al. 2022). Thus, the negative impact microglial depletion has on SCI may not be entirely a function of cell loss, but rather it may also depend on microglia-delivered molecular cues and their impact on other cell types. This would be in line with other experiments that showed astrocytes were only sent into a reactive state when in the presence of microglia or Lippopolysaccharide (LPS)-activated microglial secretions, both in vivo and in vitro, but not by LPS alone (Liddelow et al. 2017). Notably, with microglial depletion, there was a strong reduction of cells in general within single-cell samples, with only about 1900 cells compared to 19,000 cells in the non-depleted samples (Brennan et al. 2022). Therefore, it is possible that microglial depletion leads to a much more pronounced loss in overall cell abundance. Thus, it is strongly implied that the absence of microglia leads to a depletion of reactive astrocytes after injury, which is created by the loss of microglial molecular cues. However, further validation of this theory would be required as reactive astrocyte abundance was not quantified in situ within any of the tested conditions.

In the end, single-cell genomics has started to shed light on microglial dynamics and function after SCI, from the emergence of late-proliferating microglia to the new understanding that microglia may sustain an altered genetic phenotype well into the chronic stage of injury, potentially making them more pro-inflammatory with a focus on myelin or synapse remodeling. Alongside this, microglia-depletion experiments revealed that astrocytes may require the presence of microglial contact or cytokines to develop a reactive phenotype and that infiltrating macrophages, though dependent on microglia to recruit them to the site of injury, may be less dependent on microglia to dictate their genetic phenotype/function. Although preliminary, these findings point to a close interdependence between microglia, macrophage, and astrocyte reactivity and may lead investigators into an exciting era of discovery of immunomodulatory therapeutics; however, the path to get there might be riddled with pitfalls along the way, particularly in the case of reactive astrocytes.

3 | Astrocyte Reactivity: A Red Herring or the Key to Gliogenesis?

Astrocytes have been well studied as one of the key cell types coordinating repair and regeneration at the site of injury after SCI. However, the field still lacks a deep understanding on a genetic level of the role astrocytes play, particularly how astrocytic sub-phenotypes participate in SCI and recovery. One of the most recognizable but controversial astrocytic phenotypes is their potential to become “activated” or “reactive.” This alludes to the fact that they can both react to immunological stimuli and drastically alter their gene and protein expression, morphology, and basic functions to accommodate a

more sensitive environment and potentially coordinate other cells to react accordingly (Escartin et al. 2021). Initial analysis of single-cell data focuses on understanding their "reactive" phenotype, but here, we will explore why conventional means of studying this phenotype might not apply and why a closer analysis might be required.

3.1 | Reactive Astrocyte Proliferation After SCI

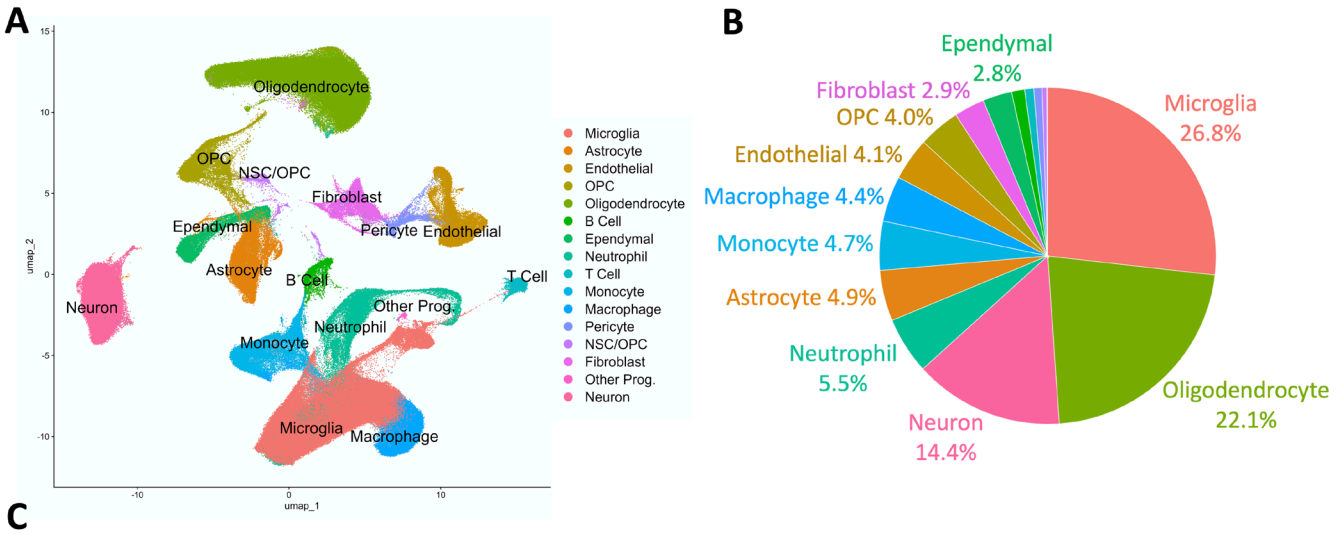
Now, here comes a potentially controversial question: are reactive astrocytes the source of astrocyte proliferation after SCI? It is well known that after SCI, astrocyte abundance and density increase around the injury and what becomes the glial scar, the majority of which are considered to be reactive astrocytes (Anderson et al. 2016; Faulkner et al. 2004; Silver and Miller 2004; Wanner et al. 2013). However, although the source of astrocyte gliogenesis after injury has been predicted with a variety of techniques, as of yet, the field has not reached a consensus (Barnabe-Heider et al. 2010; Ikeda-Yorifuji et al. 2022; Johansson et al. 1999; Mothe and Tator 2005; O'Shea et al. 2024; Wanner et al. 2013; Wei et al. 2023). Spoiler alert: in this section, single-cell data appears to generate more questions than answers, but we want to emphasize the fact that single-cell and spatial genomics have only begun to be widely implemented. Thus, we hope that future experiments can settle some of the inconsistencies we heretofore discuss. In an effort to keep naming conventions consistent, from herein, we will occasionally be referring to general astrocytes as Gfap^{+}, reactive astrocytes as Gfap^{Hi}, and non-reactive astrocytes as Gfap^{Lo}. Still, we will go into further detail in the next section about why these naming conventions leave room for refinement.

Astrocyte proliferation after SCI has been historically assessed in many studies, but sometimes with mixed or inconsistent results. Several histological studies quantified astrocyte proliferation with systemic application of the thymidine analog bromo-deoxyuridine (BrdU) and found that in mouse crush models of SCI, proliferated, BrdU+ astrocytes make up around 60%–80% of all Gfap^{+} and/or Aldh1l1-lineage astrocytes around the glial scar (Anderson et al. 2016; Liddelow and Barres 2017; O'Shea et al. 2024; Wanner et al. 2013). Moreover, histological and bulkRNAseq quantification of replicative genes within astrocytes, such as Mki67/Ki67 and Top2a, revealed a significant amount of active astrocyte proliferation takes place within the first 14 days after injury (O'Shea et al. 2024; Wanner et al. 2013). These findings, in conjunction with the previously stated fact that the majority of astrocytes are reactive after injury, are interpreted as evidence that reactive astrocytes actively divide and proliferate after any SCI. However, when assessing BrdU incorporation within intact and contusive injuries of the rat spinal cord, BrdU^{+} astrocytes appear to be much rarer at around 0.75% and 14% of total Gfap^{+} cells, respectively (Horner et al. 2000; McTigue et al. 2001; Zai and Wrathall 2005). This is more in line with other CNS injury models, such as cortical stab injuries in mice, demonstrating a more limited population of BrdU^{+} incorporating astrocytes at about 10% (Liddelow and Barres 2017; Simon et al. 2011). Therefore, it appears that significant astrocyte proliferation after SCI may not be a universal observation, as once thought, and might be heavily dependent on the injury model and species being studied. Perhaps more strikingly, when assessing our meta-analysis of single-cell data, we were struck by little to no expression of replicative genes (i.e., Mki67 and Top2a) within any of the astrocyte populations (Figure 2C,D), and this held true across all time points studied (Figure 1C). Even though some fluctuation in gene expression for these replicative genes is predicted with cell cycle progression and cell maturation, it is unexpected that these genes are expressed at such low levels across all time points studied. This is in direct contrast with the histological results observed with BrdU or lineage tracing experiments. Moreover, these replicative genes are not even observed in what would be considered the Gfap^{Hi} reactive astrocyte populations (Figure 2C,D). The only cell populations that were highly expressing replication genes were oligodendrocyte progenitor cells (OPCs), ependymal cells, a small group of dividing B cells, endothelial cells, and fibroblasts (Figure 2C,D). Thus, this begs the question: "Where are these astrocytes coming from?" and "Are reactive astrocytes actively dividing after injury?" Unfortunately, we do not have a definitive answer yet; but we have some hints as to what might be happening. First, we will explore the added complication of inconsistent genetic markers and overall nomenclature for reactive astrocytes, as this may give us some insight into how this field is changing and how defining the molecular diversity of astrocytes can improve the interpretation and design of experiments.

3.2 | Reactive Astrocyte Categorization and Nomenclature

As previously mentioned, reactive astrocytes in the context of SCI have been extensively studied. However, the current standard for determining astrocytic reactivity is a qualitative observation of morphological hypertrophy and overexpression of Gfap and Vim (Barrett et al. 1981; Bignami and Dahl 1974; Eng 1985; Hara et al. 2017; Silver and Miller 2004; Yang et al. 1994). These metrics might be sufficient for identifying reactive astrocytes histologically; but in the scenario where cell morphology cannot be assessed, having limited genetic markers for a reactive phenotype leads to challenges when analyzing single-cell RNA data.

Several scRNAseq time course experiments in mice demonstrate that after SCI, Gfap does show an initial and strong upregulation in certain astrocyte clusters. However, said upregulation quickly decreases as soon as 7 dpi and stays constant more chronically (Hou et al. 2022; Matson et al. 2022). This can also be seen in our compiled meta-analysis dataset, where overall Gfap expression within astrocytic clusters is not consistent among the time points post-injury (Figure 2B). Thus, if we were only to consider Gfap overexpression (compared to uninjured samples) when looking at reactive astrocyte abundance, we might interpret these data as reactive astrocytes are diminishing or absent beyond 14 dpi; a finding that does not correlate to histological analysis of the lesion (Silver and Miller 2004; Wanner et al. 2013). What we theorize could be happening in these subchronic and chronic time points is that astrocytes begin to shift to a different "activated" phenotype as characterized by high levels of Lcn2, C3, and CD44 expression (Hou et al. 2022). These genes generally have been associated with a reactive astrocytic phenotype in a variety of models (Liddelow and Barres 2017; Liddelow et al. 2017; O'Shea et al. 2024); thus, these markers could be indicative of a more chronic molecular phenotype of astrocytes that is not



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Publication	Species	Injury Type	Injury Level	SC Analysis Type	Timepoints	Notes
Brennan et al. 2022	Mouse (C57Bl6)	Contusion	T9	Whole-cell	Uninjured, 7, 28 dpi	Only Microglial Non-Depleted Data Used
Matson et al. 2022	Mouse (C57Bl6)	Contusion	T9	Nuclei	Uninjured, 1, 7, 21, & 42 dpi	
Milich et al. 2021	Mouse (C57Bl6)	Contusion	T8	Whole-cell	Uninjured, 1, 3, 7 dpi	
Skinnider et al. 2024	Mouse (C57Bl6)	Crush	T10	Nuclei	Uninjured, 1, 4, 7, 14, 30 (1 mpi), & 60 dpi (2 mpi)	Only Crush Timecourse Data Used
Hou et al. 2022	Mouse (C57Bl6)	Hemisection	T10	Whole-cell	Uninjured, 0.5, 1, 3, 7, 14, 60, & 90 dpi	

FIGURE 1 | Overview of compiled datasets for meta-analysis. (A) Uniform Manifold Approximation and Projection (UMAP) plot of all datasets compiled together and grouped by cell type. Unsupervised integration and clustering were performed using Seurat v4 algorithm, and cell categorization/labeling was determined by the expression of key genetic markers, as demonstrated in Figures 4, 5, and Table S1 (B) Pie chart showing the proportion of different cell types within the compiled single-cell datasets. (C) Tabular summary of experimental details for SCI datasets used in the meta-analysis compilation. Of note, all datasets are within C57Bl6 mice, all use 10X genomics single-cell platform (either whole-cell or nuclei), and all focus on thoracic injuries.

characterized by the typical Gfap^{Hi}/Vim^{Hi} patterns. However, this may not be a universal outcome, as some single-cell studies show a more consistent overexpression of Gfap throughout their studies (Skinnider et al. 2024) (Figure 2B). To add to the complexity, when enriching for Sox9+ cells (e.g., astrocytes and ependymal cells), one dataset shows Gfap overexpression in their uninjured astrocytic populations as well, making it even harder to distinguish this reactive phenotype (O'Shea et al. 2024). In addition, some researchers have further subcategorized astrocyte reactivity as either pro-inflammatory and neurotoxic or pro-regenerative and neurotrophic, referred to as A1 or A2 reactive astrocytes, respectively (Liddelow et al. 2017). This is based on reported differences in functionality in a variety of disease models and genetic distinctions, such as expression of S100a10, Psd95, or Stat3. However, these robust categorizations are no longer favored in the astrocyte research community. Instead, a consensus has been reached that there exists a spectrum of phenotypes and functions (Escartin et al. 2021; Escartin et al. 2019; Lawrence et al. 2023; Liddelow and Barres 2017; Liddelow et al. 2017). To solidify this point, reported A1 and A2 reactive astrocyte genes (Lawrence et al. 2023) do not translate well to identifying these reactive astrocyte subphenotypes in any of our compiled single-cell datasets. However, some genes associated with the regulation of proteolysis and inflammation, such as Serpina3n, Serping1, and Lcn2, do significantly segregate reactive (Gfap^{Hi}) vs. non-reactive (Gfap^{Lo}) astrocytes (Figure 2E). Notably, all three of these genes have been shown to mediate neuroinflammation, astrocyte reactivity, and/or neurodegeneration when overexpressed within astrocytes (Han et al. 2024; Ishiyama et al. 2025; Kim et al. 2022; Liu et al. 2023). Thus, genes identified in transcriptomic screens may enrich our molecular characterization of reactive astrocytes beyond Gfap and Vim overexpression, but more research needs to be done.

3.3 | No Answers but Some Theories

Here, we have touched on a few topics that are currently under heavy debate in the astrocyte community (e.g., astrocyte nomenclature, reactive astrocyte proliferation, and genetic markers for reactive astrocytes) and that lack a clear consensus (Escartin et al. 2021; Escartin et al. 2019; Liddelow and Barres 2017). Perhaps ironically, scRNAseq partially obfuscates our understanding because of its high genetic resolution and relative lack of spatial confirmation. It is still unclear what might be the cause of the discrepancies discussed above and within the overarching question of how to enrich the molecular depth of reactive astrocyte phenotypes and mechanisms of proliferation after SCI we posed at the beginning. Nevertheless, one can speculate on the insights derived from single-cell genomic data.

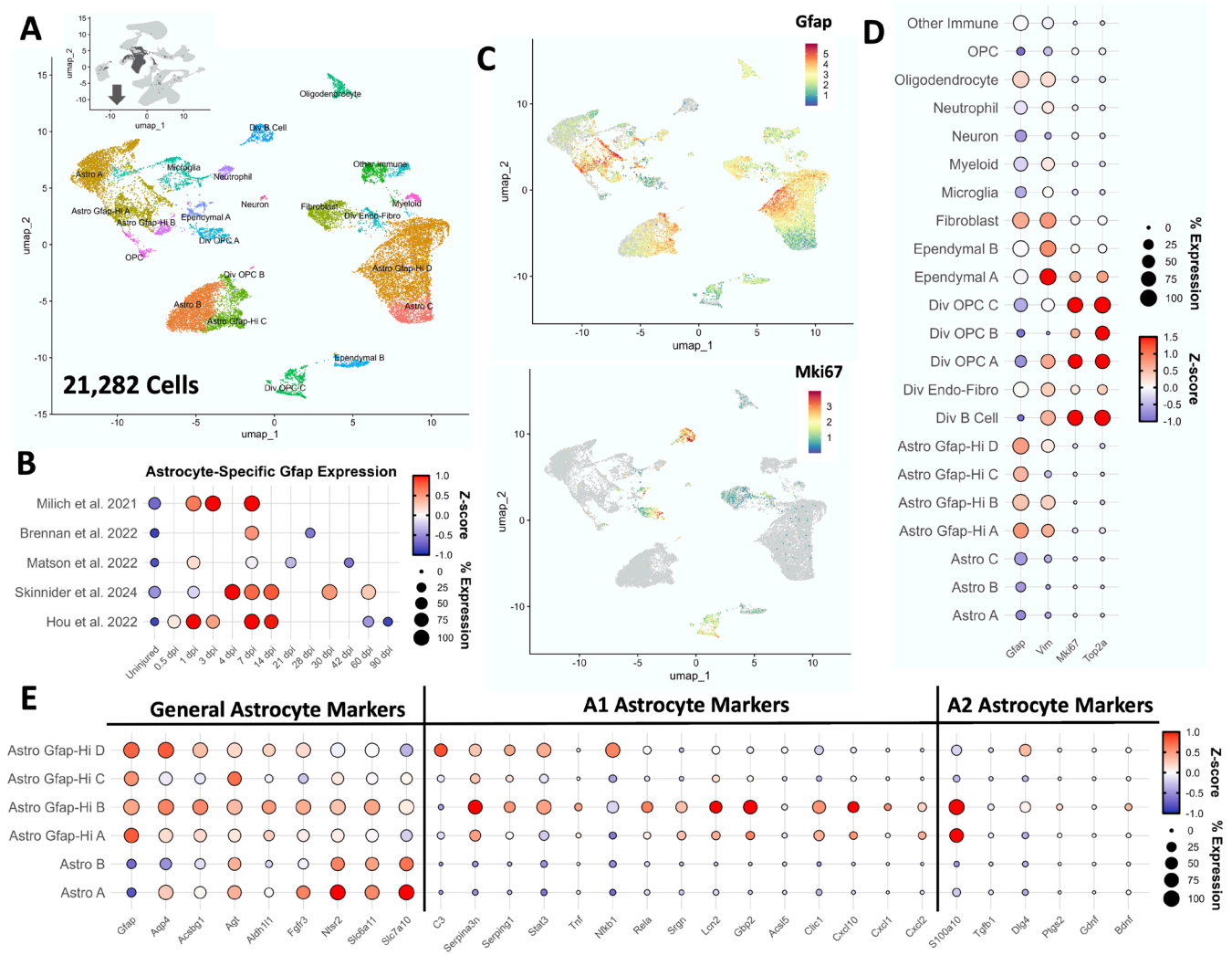

FIGURE 2 | Astrocyte reactivity and proliferation after SCI on a single-cell level. (A) subset UMAP plot of "Astrocyte" and "NSC/OPC" clusters from the entire compiled datasets in Figure 1A. Clustering and labeling were determined by the expression of key genetic markers as demonstrated in Figures 4, 5, and Table S1. Additionally, clusters with high Gfap (Gfap-Hi) expression were separated into individual clusters to segregate what would most likely be considered reactive astrocytes. (B) Dotplot showing the scaled expression of Gfap throughout time, within each dataset. Notably, Gfap overexpression seems to peak by 14 dpi but markedly decreases afterward. (C) Gene feature plots showing the transcriptomic distributions of Gfap and Mki67. It can be seen that replication genes are not abundant in any of the astrocytic clusters, indicating a potential loss of the phenotype in the datasets. (D, E) Dotplots summarizing differential gene expression of selected reactive astrocyte and proliferation genes based on clustering. It is evident that Gfap expression alone does not capture all astrocytic clusters, but it is possibly an adequate method for categorizing reactive astrocytes. However, A1 and A2 reactive astrocyte markers are not consistent and do not show clear distinctions between clusters.

First, discrepancies in histologic and genomic data could boil down to artifacts associated with cell isolation methodologies. It is possible that proliferative astrocytes or canonical reactive astrocytes are more sensitive to isolation and single-cell processing may result in the loss of cells before analysis. Although we do not believe this is the sole confounding variable, this may partially be the case given most scRNAseq datasets contain only ~3% astrocytes in whole-cell sequencing and ~6% in nuclei sequencing (Figure 3D). Without any additional enrichment, these datasets may fail to capture low-abundance or vulnerable astrocytic subtypes in the proportions being reported. Unfortunately, the only two scRNAseq SCI datasets published that contain a significant enrichment of astrocytes isolated the cells from spinal cords between 28 dpi and 1 month post injury (mpi) (O'Shea et al. 2024; Wei et al. 2023), thus missing the proliferative window of < 14 dpi. The Courtine group recently reported a proliferative astrocyte population within their moderate crush single-cell time course experiment, without significant enrichment of astrocytes (Skinnider et al. 2024). Within their dataset, the astrocytic proliferative population appears early after injury, peaks in abundance at 4 dpi, and disappears by 7 dpi. This aligns with the aforementioned proliferation window that has been well described histologically (Barrett et al. 1981; Wanner et al. 2013). However, these findings did not seem to translate to our meta-analysis, which includes said dataset (Figure 1A–C). So, it is possible that this specific cell type may have been categorized into one of the other proliferative cells within the astrocyte subset (Figure 2D), or the genetic markers for this cell do not lend themselves to being categorized as canonically astrocytic. A general weakness of the single-cell data that has been published so far is variability in cell type isolation and the generally limited correlation

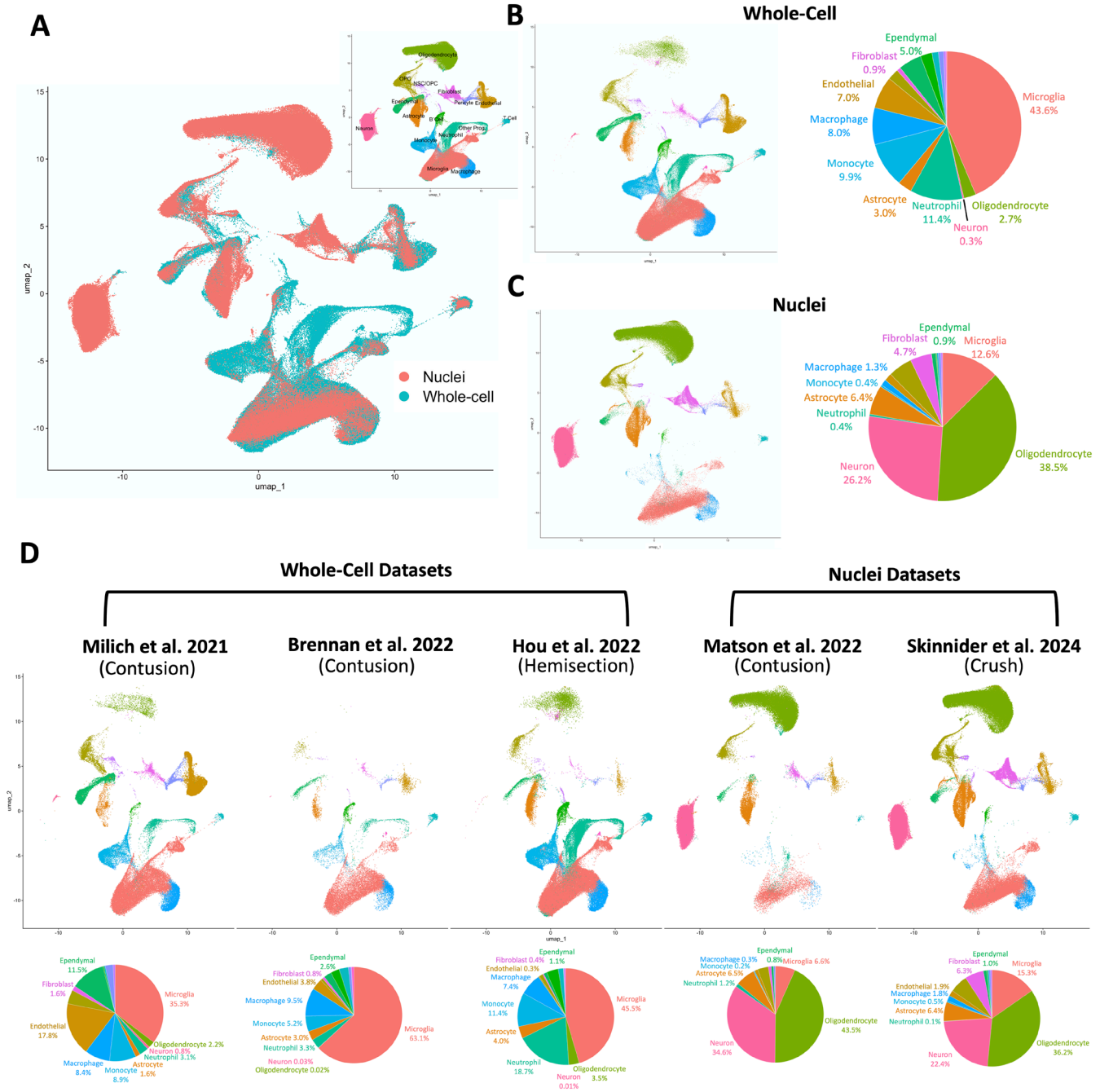

FIGURE 3 | Whole-cell and nuclei single-cell sequencing have significantly different outputs. (A) UMAP plot of all datasets compiled together, grouped by single-cell assay type (i.e., single-whole-cell or single-nuclei sequencing). (B, C) UMAP plots and pie charts showing the distribution and proportion of cells within the compiled single-whole-cell & single-nuclei sequencing datasets, respectively. These plots demonstrate a basic difference in the cell types that are most represented based on whether whole-cell or nuclei sequencing is chosen; neurons and oligodendrocytes are mostly only present in nuclei datasets, and microglia are the most abundant cell type within whole-cell datasets. (D) UMAP plot and pie charts showing cell type proportion split by publication. This more clearly shows the changes within the datasets, the variability between experiments, and the major differences between conditions (i.e., whole-cell vs. nuclei, time points, and injury model).

of genomic findings with histologic or spatial confirmation of RNA or protein. In other words, the absence of a genomic signature should not be interpreted as the absence of the cell type. Rather, it should be viewed as an important opportunity for further research to define astrocyte phenotypic and spatial domains by histology as well as gain and loss-of-function approaches.

A second hypothesis for the expansion of activated astroglia is the process of rapid dedifferentiation of mature astrocytes and downregulation of astrocyte gene expression. Dedifferentiation, or the loss of an astrocyte identity, could explain the lack of histologically observed proliferative phenotypes in single-cell data. Recent work from O'Shea and colleagues applied bulk RNA-sequencing to actively translated

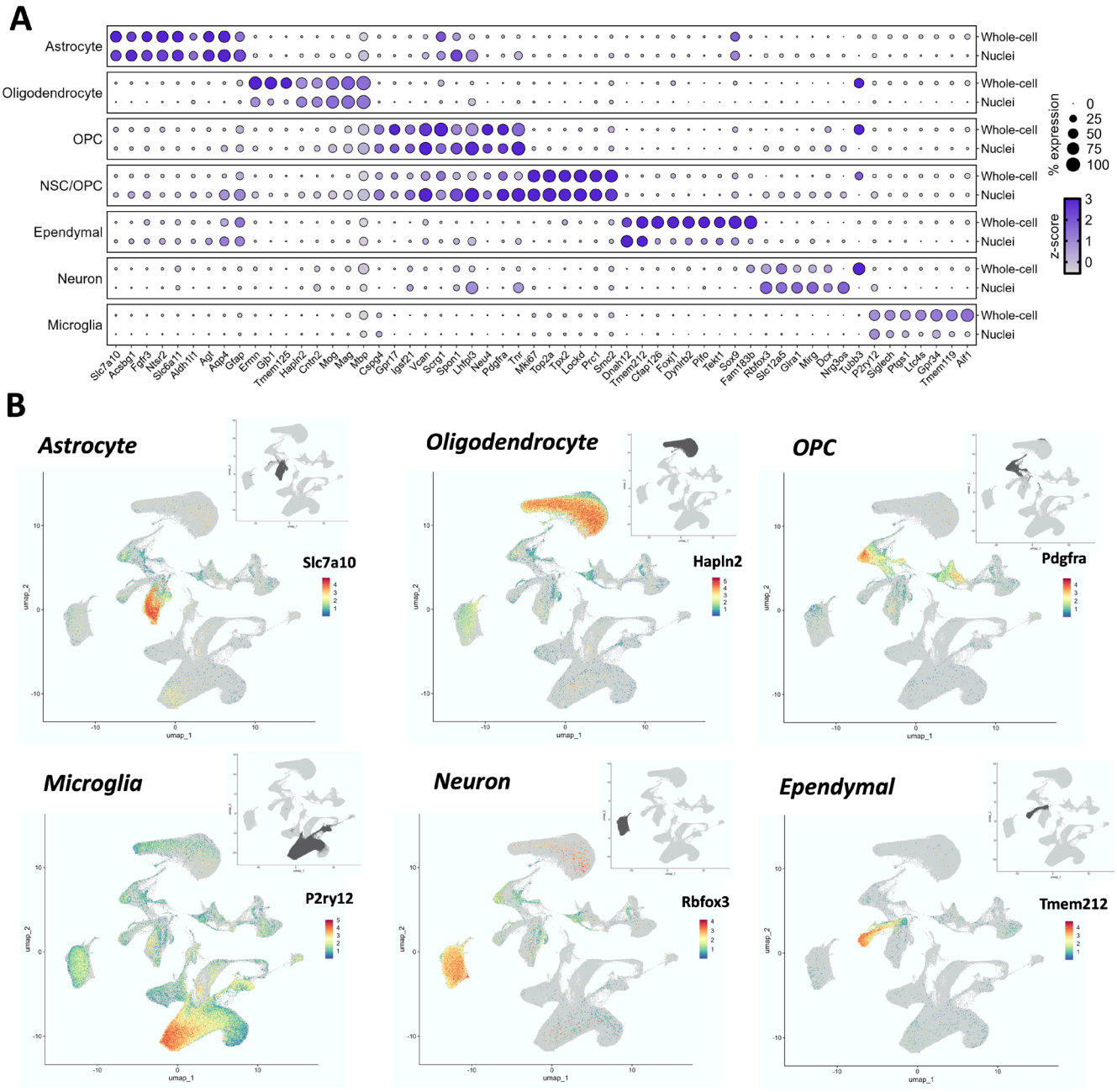

FIGURE 4 | Cellular and cluster differentiating markers and their specificity for neurons and neuroglia. (A) Dotplot summarizing selected common and novel genetic markers used to determine the cluster and cellular identity of neurons and neuroglia. Gene expression is depicted through scaled z-score and percentage expression within the specified cluster, split between whole-cell and nuclei assay types. (B) Gene feature UMAP plots showing gene expression distribution of selected genes demonstrating the abundance and specificity of each gene to their respective cluster and determined cell type. Additional UMAP highlighting the specified cluster in the original projection, seen in Figure 1A, is provided in the top right of each feature plot.

(ribosomal-tagged) mRNA from mouse astrocytes after a severe crush SCI (O'Shea et al. 2024). Their work demonstrates that astrocytes undergo a rapid (< 5 dpi) and significant downregulation of key astrocytic hallmark genes, such as Aqp4, S100b, and Slc7a10, within the first days after injury compared to uninjured controls (O'Shea et al. 2024). Moreover, the translatome profile of injured astrocytes was similar to that of immature postnatal astrocytes within the first 28 days after SCI, expressing fewer genes related to astrocyte maturity and more genes related to proliferation (O'Shea et al. 2024). Thus, these researchers hypothesize that astroglia acutely downregulate mature astrocyte genes and upregulate genes related to astrocyte immaturity and proliferation. If this is indicative of a potential transient dedifferentiation of astrocytes into a more progenitor-like state after SCI, then it could potentially explain why proliferation markers do not co-stratify with astrocytic lineage pathways in single-cell datasets. One caveat to this interpretation of the O'Shea data set is the finding that canonical activated-astroglial genes including Gfap, Lgals3, and C3 were significantly upregulated in astrocyte populations compared with uninjured controls at most time points after injury (O'Shea et al. 2024). Hence, a partial or

TABLE 1 | Tabular summary of experimental details for selected SCI datasets.

Publication	Species	Injury type	Injury level	Analysis type	Timepoints
Brennan et al. 2022	Mouse (C57Bl6)	Contusion and Crush	T9 and T9/L1 respectively	Whole-cell (Includes Microglia-depletion data)	Uninjured, 7, 28 dpi
Matson et al. 2022	Mouse (C57Bl6)	Contusion	T9	Nuclei	Uninjured, 1, 7, 21, & 42 dpi
Milich et al. 2021	Mouse (C57Bl6)	Contusion	T8	Whole-cell	Uninjured, 1, 3, 7 dpi
Skinnider et al. 2024	Mouse (C57Bl6 + Transgenic)	Mainly crush, contusion, hemisection	T10	Nuclei and Spatial (Visium)	Uninjured, 1, 4, 7, 14, 1, and 2 mpi
Hou et al. 2022	Mouse (C57Bl6)	Hemisection	T10	Whole-cell	Uninjured, 0.5, 1, 3, 7, 14, 60, & 90 dpi
Marques et al. 2016	Mouse (C57Bl6) (Transgenic: C57Bl6 Background)	Uninjured	Uninjured	Whole-cell (Fluidigm) and RNAscope	Uninjured: P21, P60
Marques et al. 2018	Mouse (Transgenic: C57Bl6 Background)	Uninjured	Uninjured	Whole-cell (Fluidigm), and RNAscope	Uninjured: E15.5, E12.5
Floriddia et al. 2020	Mouse (Transgenic: C57Bl6 Background)	Contusion & Dorsal Funiculi Transection	T9 & T10 respectively	Whole-cell and RNAscope	Uninjured, 3mpi
Gong et al. 2023	Mouse (C57Bl6)	Lateral Hemisection	T10	Spatial (Visium)	Uninjured, 3, 7, 14, 28 dpi
Hakim et al. 2021	Mouse (C57Bl6)	Contusion	T10	Whole-cell (plate/non-10X)	Uninjured, 0.5, 2, 6, 24, and 36 hpi, 3, 7, 21, 90 dpi
Wei et al. 2023	Mouse (Transgenic: C57Bl6 Background)	Compression	T10	Whole-cell (Gfap-lineage cells only)	Uninjured, Naïve, 1 mpi
O'Shea et al. 2024	Mouse (Transgenic: C57Bl6 Background)	Crush	T10	Nuclei (Sox9+ nuclei only)	Uninjured, 28 dpi

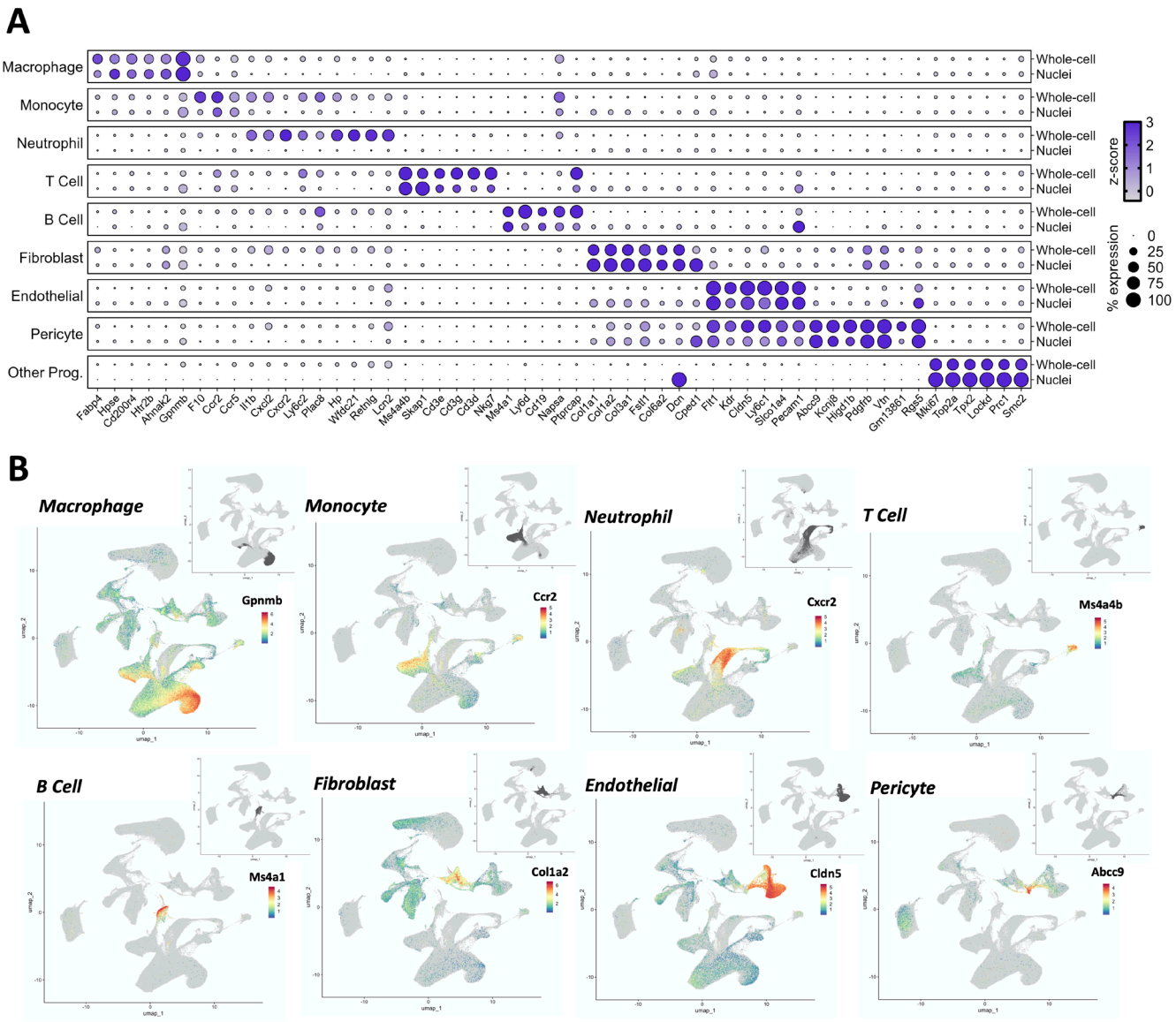

FIGURE 5 | Cellular and cluster differentiating markers and their specificity for immune and vascular cells. (A) Dotplot summarizing selected common and novel genetic markers used to determine the cluster and cellular identity of immune and vascular cells. Gene expression is depicted through scaled z-score and percentage expression within the specified cluster, split between whole-cell and nuclei assay types. (B) Gene feature UMAP plots showing gene expression distribution of selected genes demonstrating the abundance and specificity of each gene to their respective cluster and determined cell type. Additional UMAP highlighting the specified cluster in the original projection, seen in Figure 1A, is provided in the top right of each feature plot.

mosaic loss of astrocyte phenotypic genes as a form of dedifferentiation may be an explanation for a subset of astrocytes that is not distinguished from bulk RNA data, but further testing of this theory is required.

Lastly, there could be a unique subclass of cells that has been previously reported but not yet fully characterized and that does not fall into accepted astrocyte genetic categorization. One scRNAseq experiment in a contusive mouse model of SCI by the Lee group found a transient subclass of cells that exhibit unique molecular signatures of ependymal cells and astrocytes simultaneously. Gfap, Aldh1l1, Foxj1, Cfap126, Vim, and Crym expression was detected in this stratified population and labeled astroependymal cells (Milich et al. 2021). Transcriptionally, astroependymal cells only appeared acutely, 1–3 dpi, and were not present in the uninjured spinal cord. Moreover, these cells did not exhibit a proliferative genetic phenotype and were histologically confirmed, primarily at 1 dpi, through the presence of Crym around the central canal. Crym's specific role in astrocyte and ependymal function remains unclear. Others have reported similar RNA enrichment of Crym after SCI within ependymal populations (Chevreau et al. 2021), and repression of Crym expression within striatal astrocytes has led to perseveration of psychiatric behaviors and perturbed synaptic balance within the striatum of mice (Ollivier et al. 2024). In addition, a single-cell experiment by the Qian Wu group, this one enriching for Gfap-lineage cells, found another astrocyte-ependymal mixed phenotype cell characterized by its expression of Sox9, Foxj1, Nes, Gfap-lineage, acute and transient presence, along with

its potential for producing astrocytes, oligodendrocytes, and neurons in vitro (Wei et al. 2023). Together, these cells' transient nature, limited proliferation, presence around the central canal, and pluripotent potential allude to the likelihood that they are transitioning from a proliferative ependymal population and giving rise to some subsequent astrocytic cluster. However, lineage-tracing of Foxj1-ependymal cells in mice after dorsal funiculus transection only detected a modest, ~20% of scar-forming astrocytes derived from ependymal populations after SCI (Barnabe-Heider et al. 2010; Meletis et al. 2008). This is in line with other research that observed the pluripotency of ependymal cells during SCI with a limited proliferative phenotype outside their zone of origin (Horner et al. 2000; Ikeda-Yorifuji et al. 2022). Thus, niche astroependymal cells may only partially account for scar-forming astrocytes, resulting in the following critical questions: by what mechanism and from what progenitor do the majority of astrocytes emerge? How much of this process is lesion and model-dependent?

In the end, it looks like the question we posed earlier, "Are reactive astrocytes the source of astrocyte proliferation after SCI?" is potentially model-dependent, genetically inconsistent, and currently too complex to answer with the markers/tools available. However, it is exciting to see that single-cell transcriptomics has shifted the discourse on astrocytes in SCI by bringing to light limitations in our current understanding and illuminating unique astroglial phenotypes and molecular pathways that may transform our approaches to the study of astrogliogenesis and astroglial function after injury.

4 | Oligodendrocyte Heterogeneity Acutely Triggered After SCI

Oligodendrocytes often get overlooked when it comes to a deep characterization after SCI in favor of other neuroglia and neurons. However, several single-cell and spatial datasets reveal an interesting dynamic when it comes to oligodendrocytes. Here, we consider some of the sequencing data that might bring a new understanding not only to how oligodendrocytes repair myelin but also to novel, less studied regenerative functions after SCI. Oligodendrocytes have been shown to play a critical role in axonal maintenance, metabolic support, and conduction. More recently, they have proven to be remarkably plastic (Simons et al. 2024). In addition, several new functions have been attributed to oligodendrocyte cells and their precursors that are as far-ranging as participation in synaptic plasticity to antigen presentation (Auguste et al. 2022; Buchanan et al. 2023; Kirby et al. 2019; Zeis et al. 2016). Oligodendrocyte precursor cells tile the CNS and make up to 5% of all white matter glia (Dawson et al. 2003; Horner et al. 2000). After injury, oligodendrocyte precursor cells are a first responder, especially proximal to the lesion epicenter where they rapidly divide, producing bipolar migratory morphotypes, yielding complex pre-oligodendrocyte or polydendrocyte morphologies, and eventually ensheathing or myelinating oligodendrocytes (Hesp et al. 2015; Horky et al. 2006; Powers et al. 2013; Tripathi and McTigue 2007). Newly generated oligodendrocytes produce remarkable amounts of myelin after SCI, but the physiological relevance of the new myelin needs to be established (Duncan et al. 2018; Manesh et al. 2025; Plemel et al. 2014; Powers et al. 2012; Powers et al. 2013). Ultimately, there is much to be studied and myelin is not an all-or-none phenomenon (Arancibia-Carcamo et al. 2017; Dutta et al. 2018; Etxeberria et al. 2016; Freeman 1978; Gutierrez et al. 1995; Lasiene et al. 2008; Patzig et al. 2016; Powers et al. 2012; Scurfield and Latimer 2018; Waxman 1980; Wu et al. 2012). It is clear that subtle changes in myelin structure, both pathologic or regenerative, can have significant impact on motor timing, network fatigue, and axonal sprouting.

The first key finding from single-cell genomic data is the discovery of heterogeneous molecular phenotypes with developmental, anatomical, activity, or injury state dependence. Recent single-cell and spatial works revealed significant genetic differences within oligodendrocyte and progenitor populations when comparing regional subpopulations from the spinal cord, corpus callosum, and cortex (Floriddia et al. 2020; Marques et al. 2018; Marques et al. 2016). Researchers also demonstrated that these niches are dependent on the animal's age, white or gray matter sublocalization, and exposure to injury or stimuli (Floriddia et al. 2020; Marques et al. 2019; Marques et al. 2018; Marques et al. 2016). These data have led to the discovery of unique molecular phenotypes of oligodendrocytes that are spatially distributed proximal or distal to an injury or in specific regions of degeneration (Floriddia et al. 2020; Marques et al. 2019; Marques et al. 2018; Marques et al. 2016). The transcriptional profiles of myelin-producing cell subtypes and their reaction/localization to the injury environment suggest that certain oligodendrocyte subtypes, more closely associated with adaptive myelination, are more present in the spinal cord after injury around the site of injury and in areas of degeneration. Additionally, uninjured spinal cords contain subtypes that are more closely associated with stable neural circuits or ones favoring myelin production for fast conduction. For example, two distinct mature oligodendrocyte populations have been identified, Mol2 and Mol5, with unique molecular characteristics. Mol2 are Kallikrein-related peptidase 6 positive ($Klk6^+$), and they are concentrated in spinal white matter but more sparse in gray matter. Mol5 are marked by prostaglandin D2 synthase ($Ptgds^+$) protein expression and appear early in post-natal myelinating regions, suggesting a role in the myelination of early developing tracts. In contrast, Mol2 oligodendrocytes appear later in development and are very sensitive to spinal cord trauma. Could the unique transcriptional attributes of Mol2 yield insight into their vulnerability? In terms of repair, both mature oligodendrocyte populations, Mol2 and Mol5, appear to participate in unique aspects of tissue repair. For example, Mol2 oligodendrocytes are concentrated at sites of Wallerian Degeneration, while Mol6 populations are repopulated in proximity to axons at the lesion epicenter. Spared axons of passage at the injury penumbra exhibit extensive demyelination and do become remyelinated by dividing precursors (Powers et al. 2013). Remyelination even in the chronic stage continues, suggesting a cycle of demyelination and repair (Pukos et al. 2023). However, non-myelinating oligodendrocytes do repopulate the lesion region as well (Tripathi and McTigue 2007). Molecular markers such as Ptgds combined with lineage tracking studies or gain and loss of function studies will be important to better define the physiologic role of this population. Together, these

single-cell genomic studies generate new hypotheses and lead the field in new and exciting directions. For example, how long do each of the multiple oligodendrocyte subtypes persist after injury, and do they transition between phenotypes, or are their linear relationships from precursor to committed, mature phenotypes in response to pathologic or physiologic cues? Will we be able to capitalize on insights from oligodendrocyte molecular phenotypes to better understand and prove the mechanisms of oligodendrocyte-mediated repair?

Mature oligodendrocytes are known to undergo widespread, progressive apoptosis in response to SCI (Crowe et al. 1997). Histologically, oligodendrocytes are seen to decrease in abundance acutely and stabilize at 3–7 dpi in mice coincidentally with a decreased gene expression of myelin stacking proteins (Wrathall et al. 1998). These historical data are largely interpreted in the context that mature, myelinating oligodendrocytes are sensitive to excitotoxic stress and limited in their ability to launch a remyelination program (Pukos et al. 2019). These observations are all recapitulated in the published single-cell datasets; oligodendrocyte abundance drops significantly after injury, which is likely attributed to immediate cell death until around 7 dpi (Gong et al. 2023; Hou et al. 2022; Matson et al. 2022). From here, the oligodendrocyte and OPC populations become highly proliferative, confirming published OPC lineage tracking studies (Huang et al. 2018; Powers et al. 2013). Interestingly, even though oligodendrocyte abundance decreases in the acute phase, spatial transcriptomics has illuminated three distinct molecular phenotypes of oligodendrocytes that correlate with macrophage locations at 3 dpi within the peripheral edge of what will become a scar. These cells subsequently migrate into the white matter tracts in the spared penumbra by 7 dpi (Gong et al. 2023). These data indicate that some oligodendrocytes or their precursors are either migrating or proliferating into the injured areas parallel with the emergence of monocytes and macrophages. The three identified oligodendrocyte clusters include Cluster 2, a Ptgds expressing population which may share characteristics with that of the Mol5 population identified by Marques et al. (Marques et al. 2016). But perhaps more striking are the molecular signatures, identified by Gong and colleagues, of Cluster 0, which includes the expression of Klk8, serine (or cysteine) peptidase inhibitor, clade A, member 3 N (Serpin3na), and transferrin (Trf) (Gong et al. 2023). The latter two genes have been associated with tissue repair, endocytosis, and hemostasis (Lee et al. 2019; Zhu et al. 2024). It is interesting to speculate whether cluster 0 is derived from the same population of lineage-tracked oligodendrocyte precursors shown to have astrocytic functions, including endocytosis early after SCI (Huang et al. 2018; Sellers et al. 2009). These observations suggest potentially pivotal new roles for subpopulations of oligodendrocytes and their precursors in the resolution of the lesion environment. It is worth noting that in some cases when studying rats and non-human primates, oligodendrocyte depletion is observed well beyond the first week in certain areas of degeneration (Crowe et al. 1997; Pukos et al. 2023; Totoiu and Keirstead 2005). Thus, it is important to keep in mind that specific cell dynamics might be subject to change depending on the model, species, and timeline that is being studied. Together, single-cell and spatial genomic studies are adding tremendous insight into the molecular diversity of myelin-forming cells after injury, confirming some anatomical and lineage tracing studies and creating entirely new exciting hypotheses for their role in neural injury and repair.

One thing to keep in mind is that most of the data regarding oligodendrocytes comes from single-nuclei sequencing specifically and not whole-cell sequencing. We will touch on this phenomenon and other interesting technical cell dynamics in the following section.

5 | Garbage in, Roses Out: A Perspective on Technical Limitations, Replicability, and Insights for Single-Cell Genomics in SCI

One of the challenges when comparing and interpreting the data reviewed here is that they contain a surprising variety of cell/subtype categorization, a mix of analytic approaches (i.e., whole-cell vs. nuclei), and possible misinterpretations of data. Here, we summarize some potential guidelines and caveats for the design and analysis of SCI/spinal cord single-cell data. Specifically, we compiled five published SCI datasets: three whole-cell datasets and two nuclei datasets (Figure 1A) and performed a limited meta-analysis to illustrate technical insights and important assumptions.

5.1 | Whole-Cell Versus Nuclei Sequencing

A key decision when planning any single-cell experiment is the choice between whole-cell or nuclei sequencing. This might initially seem like a trivial distinction, but there is demonstrable evidence that each technique has its strengths and limitations. For example, it has been shown that whole-cell sequencing detects a higher number of genes and unique molecular identifiers than nuclei sequencing (Bakken et al. 2018). However, a distinct advantage of nuclei sequencing is that it can circumvent some bias from the tissue dissociation and sample preparations and possibly pick up novel, discrete, or rare cell types (van den Brink et al. 2017; Wu et al. 2019). Here, we present a limited comparison of whole-cell and nuclei sequencing within mouse models of SCI in an attempt to bring to light some key differences between the two assays. Please note, in this section, to help accentuate differences in technique, we will be referring to the traditional single-cell sequencing as "whole-cell sequencing," to the single-nuclei sequencing as "nuclei sequencing," and "single-cell sequencing" will refer to either technique.

The first major difference we observed is variability in cell type proportions between whole-cell and nuclei methodologies. A major consideration for any single-cell analysis is that of cell viability, as this directly correlates to the quality of the RNA and subsequent sequencing. It is likely that we are just beginning to understand how cell morphology, metabolic status, and other risk factors impact cell survival during isolation. Neurons are especially fragile and have limited capability to withstand the tissue dissociation process for whole-cell sequencing (Kulkarni et al. 2019). This is clearly illustrated in our compiled meta-analysis dataset. The

proportion of neuronal cells, out of all cells, averages ~0.4% within the whole-cell datasets and ~26.2% for the nuclei datasets (Figure 3B,C). It is worth noting that none of these compiled datasets intentionally selected against neurons in their sample preparation. This is a consequence of the cell stress and injury caused by whole-cell compared to single-nuclei isolation and sequencing. Thus, if an experimental goal is to measure gene expression in neurons, then nuclei sequencing is necessary. Going back to neuroglia, from this same comparison, whole-cell sequencing appears to underrepresent oligodendrocyte proportions when compared to nuclei sequencing (e.g., 2.7% vs. 38.5% of all cells), but overrepresent microglial proportions (e.g., 43.6% vs. 12.6%) (Figure 3B,C). Even though some of these differences can be attributed to changes in experimental methodology, such as injury model and time points chosen, the proportional representation of oligodendrocytes and microglia are more or less consistently different between individual whole-cell and nuclei datasets (Figure 3D). Interestingly, astrocytes were not significantly different between assay types, averaging 3.0% for whole-cell sequencing and 6.4% for nuclei sequencing (Figure 3B,C). However, astrocyte proportions in both assays without additional enrichment are considerably lower than the measured abundance in vivo and may limit our ability to capture the molecular diversity of astrocytes after SCI. This leads us to the question of whether either assay preserves the cell proportions during isolation that accurately represent cell populations in vivo.

Although not a definitive answer, the Levine group was the first to publish a direct comparison of neurons and neuroglia proportions before and after injury within immunohistological and nuclei sequencing samples (Matson et al. 2022). Their findings show that within the uninjured spinal cord, immunohistochemical and nuclei proportions of microglia, oligodendrocytes, and astrocytes did not differ significantly. However, by 7 and 21 dpi, snRNAseq underrepresented astrocytic populations by about half, overrepresented microglial populations by about a third, and did not significantly alter oligodendrocyte proportions when compared to quantitative histology. Interestingly, the neuronal populations were consistently overrepresented by about one-third with nuclei sequencing in both the uninjured and injured samples. Nevertheless, it becomes clear that nuclei sequencing, by far, maintains a better proportional representation of all neural and glial cells than whole-cell sequencing. For example, the proportions of cells captured by whole-cell sequencing were two times lower for astrocytes, 10 times lower for oligodendrocytes, three-and-one-half times higher for microglia, and essentially nonexistent for neurons (Figure 3B,C). This all highlights the fact that deciding between nuclei sequencing and whole-cell sequencing is an important choice and depends on experimental goals and the cell type of interest. Altogether, further validation is needed to empirically determine *how whole-cell or nuclei techniques generate biases in cell lineages, cell sub-types, or cell type morphologies* and how they can be interpreted in the context of disease state in vivo.

An additional point to be considered when analyzing these datasets is that genetic expression can vary depending on whether the source or RNA is from nuclei or whole cells. There have been a few studies that characterized differences between the observed transcriptomes from nucleus and whole-cell sequencing. They found high concordance between the two methods in isolated neurons but acknowledged that there were large differences when it came to certain gene subsets, particularly those related to certain parts of mitochondrial respiration (Lake et al. 2017). Moreover, when directly comparing mouse cortical neurons with either wcRNAseq or snRNAseq, the average number of genes detected was about 1.5× higher within the whole-cell samples, ~11,000 genes for whole-cell compared with ~7000 genes for nuclei (Bakken et al. 2018). However, no validation has been done between the neuroglial populations or in the context of CNS trauma. Thus, the level of agreement between these two methods is still unknown outside of neuronal populations in an uninjured setting. To this point, some canonically distinguishing genes in the curated datasets were differently expressed between the two modalities, particularly those of Iba1(Aif1), Tmem119, Foxj1, Sox9, and Tubb3 (Figure 4A). Thus, it is important for future analysis to take all of this into account when designing and analyzing nuclei or whole-cell data, as the two modalities are not exactly comparable in the output of cell type proportions and even gene expression per se.

It is worth mentioning that there are new probe-based single-cell options (10X Genomics' Flex Gene Expression Platform) that can provide researchers with the option to use flash-frozen or fixed tissues and still perform whole-cell or nuclei sequencing on said tissue samples. This circumvents some of the weaknesses discussed regarding cell viability and lets the user mix and match with preferred cell and tissue isolation methodologies. However, these new methods have yet to be widely implemented in part due to inherent weaknesses and cost. Probe-based single-cell methodologies require significant piloting and validation that is specific to a laboratory's tissue and experimental conditions. Probes are currently limited to mouse and human tissues and there has not been any detailed comparison of gene expression by tissue-based probes compared with non-fixed platforms.

5.2 | Gene Markers, Clustering, and Quality Control

One of the most important yet overlooked approaches when it comes to single-cell data interpretation is that of correctly categorizing cell phenotypes based on unique genetic signatures. This could be a complicated process, especially since some of these genetic signatures vary with anatomy (e.g., brain vs. spine, injured vs. non-injured segment), time after injury, type of injury, and various other factors. To help normalize these categories, we compiled a list of genes that can be used as a starting point for many single-cell analyses in SCI (Figures 4, 5, and Table S1). This is not intended to be a comprehensive gene list but rather a catalyst to both validate individual findings and help standardize datasets in the field as a whole.

Another important aspect of single-cell analysis that can vary between datasets and labs is how to properly check for quality, clean data, and cluster appropriately for the analysis type in question. Notably, quality control begins before any data is generated but is somewhat dependent on the isolation or analysis method being performed. For instance, for whole-cell sequencing, the viability of cells being processed is critical for a high-quality analysis downstream. However, for nuclei-sequencing, it is more

important that the quality of the isolated nuclei is preserved, which is more a factor of the tissue preservation and nuclei isolation protocol. Once data is generated, the main considerations in quality control should be the removal of any doublets and the removal of low-quality cells. This can be done with the use of more user-friendly packages such as Scrublet (Python) and DoubletFinder (R) or directly within the all-in-one analysis R pipeline package Seurat (Hao et al. 2024). Although there is no set researcher guidance here, each package can break down the individual gene/feature counts, UMI count, percentage of mitochondrial genes, and percentage of ribosomal genes, all of which can inform the researchers on the overall quality of the dataset and be used to filter out doublets and unwanted cells. For more detailed information on how these metrics are used to indicate quality of sample, the Theis group has made several great reviews on this subject that we recommend (Heumos et al. 2023; Luecken and Theis 2019). Moreover, to perform this kind of analysis within Seurat, we recommend you follow one of the Satija lab's readily available vignettes: https://satijalab.org/seurat/. Lastly, when it comes to clustering of single-cell data, it is important for researchers to appropriately assess the quantity of cells within a given cluster if they are doing any sort of major determination of their system as a whole. In general, simple categorizations of cell type can be done relatively easily with robust genetic markers such as those detailed in Figures 4 and 5. However, care should be taken when analyzing any sub-phenotypes within cell clusters, as the clustering can be impacted heavily by technical factors such as read depth, cell quantity/cluster abundance, clustering resolution, and cell quality. Unfortunately, this again does not have any set guidance and is entirely dependent on the kind of analysis that is necessary for the biological question. However, when it comes to clustering-based optimization, open source tools exist that can help researchers make the best determination of what clustering resolution to use in their datasets: Clustree for R and Pyclustree for Python (Zappia and Oshlack 2018). The clustering workflow needs to be verified to make sense biologically and ensure adequate cell numbers in any particular cluster before making conclusions on the system as a whole. As always, in situ RNA verification is ideal when translating any single-cell findings directly but researchers should always be aware of non-genetic regulators of pathways or specific protein modifiers. This is particularly evident with immune processes as it is well known that these pathways are heavily regulated through transcription factor binding, cytokine cascades, microRNA binding, and long non-coding RNA binding (Heward and Lindsay 2014; Liu et al. 2017; Megha et al. 2021; Mehta and Baltimore 2016); all of which are not captured in standard single-cell protocols that only interrogate mRNA abundance. This is not to say that nothing can be discerned from immune pathway data in single-cell analysis, but rather that researchers should take these additional regulatory modalities in mind when making determinations on how pathways are behaving in the system as a whole and validate such findings accordingly.

5.3 | Myelin and Mature Oligodendrocytes

Briefly, we wanted to address an interesting technical finding in regard to the oligodendrocyte lineage that we observed while analyzing our compiled datasets. As discussed above, the method in which a single-cell dataset is generated (i.e., whole-cell vs. nuclei) will significantly impact the overall proportions of the cells captured in the analysis. Further, the method by which the sample data is curated can also impact this outcome. It can be seen that the (Brennan et al. 2022) dataset is largely lacking oligodendrocytes in their final output at 0.02% of the overall proportion of cells (Figure 3D). This is not solely an artifact of the whole-cell analysis method as the other two whole-cell datasets have 2.2% and 3.5% oligodendrocyte density (Figure 3D); nor is this an issue of timing, as there is overlap with the time points chosen among all three whole-cell datasets and this includes uninjured controls (Figure 1C). The difference may be a result of the preparation process of the single-cell suspension for these samples. For example, the main difference in sample preparation is that the Brennan samples used anti-myelin magnetic bead sorting (Miltenyi, #130-096-733) followed by magnetic bead dead cell removal (BioPal, #CP-50VQ02). In comparison, the "Milich et al. 2021" samples were prepared using the same myelin removal beads but without the dead cell removal, and the "Hou et al. 2022" samples were prepared by centrifugation alone. It is possible that the mature myelinating oligodendrocytes are being removed and/or damaged by the anti-myelin beads, leading to the subsequent removal by live-dead exclusion. This would explain the sudden loss of mature oligodendrocytes from the Brennan dataset. Thus, if researchers are interested in oligodendrocytes, it might be important to use a more cell sparing clean-up method such as density gradients (Sucrose, Percoll, or Opti-Prep) instead of anti-myelin magnetic bead sorting. Or better yet, performing single-nuclei sequencing instead to preserve this population.

Nonetheless, we would like to emphasize that the choice in clean-up method for these single-cell datasets is important and that certain cell types might be more sensitive to one method of preparation over others. More generally, technical considerations are important when reviewing and comparatively interpreting glial RNAseq studies.

6 | Spatial Transcriptomics: The Final Frontier?

Although single-cell technologies have started to unravel deeper phenomena in almost all biological sciences, it is increasingly evident that the field is realizing the need to understand these transcriptional changes on a spatial level. Newly commercialized technologies such as 10X Genomics' Visium, Visium HD, and Xenium platforms, along with nanoString's GeoMx and CosMx spatial profilers, have made it much easier for researchers to include spatial transcriptomics/proteomics into their workflows. Of these, the Visium platform is the most widely used to date. The major drawback of 10X Genomics' Traditional Visium technology is that it lacks single-cell resolution as the 55-μm capture spots pool the RNA from 1 to 10 cells per spot, depending on cell size. This is a problem when interrogating highly heterogeneous tissues such as spinal cord or brain and relies on computational deconvolution of cell types post hoc. However, this technology can still be useful for researchers in need of some spatial transcriptomics to define gradients or compartmental changes in healthy or disease conditions.

The most comprehensive of these spatial SCI datasets is the Courtine group's recent publication in *Nature* (Skinnider

et al. 2024). They performed spatial sequencing using the Visium platform on uninjured, 7 dpi, and 2-month post-injury tissue slices using a mouse crush SCI model. With evenly spaced transverse sections, they were then able to approximately reconstruct the 3D environment of the mouse spinal cord, before and after injury. The spatial atlas validated some of the well-known temporal mechanisms of SCI in terms of inflammation, cell invasion, and astrocytic scar formation. Moreover, the data discerned the laminar organization of spinal neuron subtypes and their genetic signatures. While this atlas can prove to be a valuable tool for researchers interested in the spatial progression of severe crush SCI, future in-depth analysis and additional SCI models are needed to catalyze new hypotheses for how degeneration and regeneration can be targeted.

The future in this field is even more promising as the resolution of all commercial spatial platforms has increased drastically in recent years. In fact, the traditional Visium platform is now being replaced with the new Visium HD platform, which brings down the capture spot size to 2 μm. Similarly, the same company's Xenium platform uses a panel of multiplexing probes that allows for much higher subcellular resolution but is limited to fewer than 5000 genes with limited customizability. These technologies allow for cellular and sub-cellular resolution within a similar workflow. Moreover, non-commercial techniques such as those relying on in situ hybridization of probes (MERFISH, seqFISH+) and those that generate DNA amplicons (STARmap) have proven themselves incredibly useful at preserving three-dimensional transcriptomic data, although harder to implement (Chen et al. 2015; Eng et al. 2019; Wang et al. 2018).

Even though insights have been limited from the analysis of SCI spatial transcriptomics, progress in the resolution and decline in cost give confidence that we are on the precipice of a productive time ahead. In the future, more insightful discoveries from spatial biology will be made in the field of SCI that build and clarify the powerful single-cell work that is already being done.

7 | Final Thoughts

In the end, we reviewed many exciting discoveries researchers have brought to light using single-cell genomics applied to SCI and, more specifically, how these new findings have deepened or shifted our understanding of the neuroglial populations and their responses to injury. The rapid boom in the applications of single-cell techniques also highlights the differences and limitations of scRNAseq methodology, sample preparation, genetic markers for cellular classification, and injury and species model variability. Indeed, like a double-edged sword, single-cell genomics has rapidly left its mark on the SCI community and beyond, catalyzing new ideas and experiments to better interpret the very complex neuroglial processes after injury while simultaneously emphasizing the gaps in our knowledge of SCI as a whole. Gaps in knowledge or historical oversimplifications are being challenged by the publication of detailed genomic, single-cell data. In turn, genomic data are catalyzing a new field of research focused on genetic or pharmacological manipulation of discrete cellular lineages and subphenotypes to better understand mechanisms of injury, plasticity, and regeneration. Single-cell and spatial genomic technologies are driving new ideas to move the needle forward on new and improved therapeutics for spinal cord injury and regeneration.

Author Contributions

Data processing and meta-analysis was performed by Julio Mejia. This article was written by Philip J. Horner and Julio Mejia.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings and meta-analysis performed in this review are openly available in Brennan et al. at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE196928; Skinnider et al.: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE234774; Matson et al.: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE172167; Milich et al.: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE162610; Hou et al.: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE189070.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Single-cell markers for cell general categorization of cell types within SCI. All genes were selected based on uniqueness and expression profile within the compiled datasets described in Figure 1. The distribution and cluster expression profiles are also available in Figures 4 and 5. This is intended to be used as a starting point for most SCI single-cell dataset analysis in a broad setting, but more specific gene and subcategorization markers should be evaluated on a case-by-case basis. **Table S2:** Gene abbreviation appendix. All genes mentioned in the body of the paper, with their abbreviations and full gene names.