Title

Mature neutrophils promote long-term functional recovery after spinal cord injury in a sex-dependent manner

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Abstract

Following spinal cord injury (SCI), neutrophils are the first peripheral immune cells to infiltrate the injured spinal cord in large numbers. There is a growing body of evidence demonstrating sex differences in neutrophil function, however, the role of sex as a biological variable in neutrophil response following SCI remain unclear. Additionally, while divergent roles for mature and immature neutrophil subsets have been observed, subset-specific contributions of neutrophils to functional recovery following SCI have not been fully characterized. Here, we provide novel evidence that systemic and localized neutrophil responses differ by sex following SCI. Antibody-mediated depletion of neutrophils following SCI revealed a previously unidentified role for mature neutrophils in promoting long-term functional recovery in a sex-dependent manner. We performed single-cell RNA sequencing analysis using publicly available datasets and discovered dramatic shifts in the phenotype of intraspinal neutrophils across time following SCI. We identified that mature neutrophils in the acutely injured spinal cord upregulate genes associated with resolution of inflammation. Furthermore, we found that depletion of mature neutrophils exacerbates long-term macrophage accumulation following SCI in a sex-dependent manner. Finally, we show that the beneficial properties of neutrophils in the injured spinal cord are temporally specific. Collectively, these data provide a first account of sex differences in the response of neutrophils to SCI. Our findings elucidate a novel and sex-dependent role for mature neutrophils in promoting resolution of inflammation and long-term recovery following SCI.

Keywords

Spinal Cord Injury, Neurotrauma, Neuroinflammation, Innate Immunity, Neutrophils, Sex As A Biological Variable

Introduction

Spinal cord injury (SCI) is a devastating and life-altering event marked by irreversible tissue damage and loss of motor, sensory, and autonomic function. The acute phase of SCI is characterized by an extensive inflammatory cascade that substantially influences secondary tissue injury and long-term functional outcomes[1–3]. Neutrophils are the most abundant immune cell (leukocyte) type in human blood and are the first to infiltrate the injured spinal cord in large numbers, peaking within the first day following SCI in rodents[2,4–6]. While neutrophils have canonically been implicated in secondary pathogenesis in the acutely injured spinal cord, recent studies have demonstrated beneficial roles for neutrophils in central nervous system (CNS) injury, including SCI and optic nerve injury[7,8]. Outside of the CNS, there is now a large body of evidence demonstrating that neutrophils play an important role in promoting resolution of inflammation and tissue repair across multiple models of injury, disease, and infection[5,9–20]. The dichotomous role of neutrophils in tissue injury and wound healing may explain the inconsistent effects on long-term outcomes that have been reported with neutrophil depletion in murine models of SCI[21–25]. While some of the variability can be attributed to methodological differences, the dynamic and heterogenous nature of neutrophils, as well as prominent sex differences in neutrophil function[9,26–30], may also contribute to the variable effects observed between neutrophil depletion studies. Thus, it is imperative to re-examine the time- and sex-dependent roles of neutrophils in recovery processes following SCI.

The multi-faceted nature of neutrophils may be due in part to maturation-dependent changes in neutrophil function. During steady-state conditions, neutrophils predominantly mature within the bone marrow (BM) and are released into circulation in a circadian pattern[26,31]. During injury or infection, immature neutrophils are released from the BM *en masse* resulting in a rapid increase in circulating immature and mature neutrophils (neutrophilia). Recent studies support neutrophil maturation as a predisposition for phenotypic diversity in neutrophils, and disparate roles for mature and immature neutrophils have been reported for tumor progression[26,32,33], lymphocyte regulation[34], axon regeneration[29], and pathogen clearance[35]. However, neutrophil heterogeneity in the context of SCI, as well as time-dependent changes in granulopoiesis, neutrophil maturation, and other neutrophil responses, have yet to be characterized.

Prominent sex differences in neutrophil phenotype and functional responses have been recently reported for mice and humans. In humans, sex differences have been observed in circulating neutrophil maturation, neutrophil extracellular trap (NET) formation, reactive oxygen species production, apoptosis, metabolism, transcriptome, and chemorepulsion[36–41]. In mice, multi-omic profiling of primary BM neutrophils has demonstrated sex-dependent transcriptional signatures and chromatin accessibility profiles[42,43]. Despite the emphasis of sex as a biological variable in SCI research, sex differences in neutrophil responses to SCI, such as granulopoiesis, neutrophilia, and intraspinal accumulation, as well as the sex-dependent contribution of neutrophils to post-SCI recovery, remain unclear.

In this study, we provide the first report of sex differences in granulopoiesis and neutrophil mobilization following injury in a clinically relevant murine model of contusive SCI. Our findings demonstrate, for the first time, that the phenotype of circulating and intraspinal neutrophils rapidly and robustly changes after SCI, which is supported by our functional studies demonstrating the

effect of neutrophil depletion on functional recovery is dependent on the timing of depletion relative to SCI. In addition, we show that neutrophils mediate long-term functional recovery following SCI in a sex-dependent manner. These data provide critical insight into how neutrophil heterogeneity and sex as a biological variable contribute to the inconsistencies observed in previous neutrophil depletion studies in SCI. Remarkably, we show a novel reparative role for mature neutrophils in the injured spinal cord, and provide evidence that neutrophils regulate resolution of inflammation in a sex-dependent manner following contusive SCI.

Methods

Mice

All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at Texas A&M University. All experiments used adult male and female C57Bl/6J (WT) mice (age 12-20 weeks; age-matched within experiments) ordered from The Jackson Laboratory and maintained in our colony. Mice were housed in groups of two to five prior to antibody administration and surgery. Following antibody administration or injury, female mice were housed in groups of two to three, and male mice were single-housed. Mice were housed in a climate-controlled room on a 12 h light/dark cycle with food and water provided ad libitum.

Antibody-mediated neutrophil depletion

Animals were randomly assigned to groups and experimenters were blinded to treatment throughout the duration of each experiment. Animals received either anti-Ly6G (2.5mg/kg, clone 1A8, InVivoPlus, BioXCell) or the control non-specific IgG antibody (2.5mg/kg, clone 2A3, InVivoPlus, BioXCell) via intraperitoneal (i.p.) injection. Antibodies were diluted in sterile InVivoPure dilution buffer (pH=7.0, BioXCell). For studies assessing the effect of neutrophil depletion on the recovery process following SCI, mice were injected either 1-day prior to injury or immediately post-injury following closing of the skin incision. All intraperitoneally injected solutions were prepared in a sterile bio-safety cabinet.

Spinal cord injury

A moderate spinal cord contusion injury was performed for all studies. Mice were anesthetized using 2% isoflurane, a laminectomy was performed at thoracic vertebral level nine (T9), and a 60-kilodyne (kDyne) contusive SCI with a one-second dwell time was administered using the Infinite Horizon Impactor. Following the SCI, 50µl of 0.25% bupivacaine was administered directly onto the sutured musculature post-injury prior to closing the skin incision with wound clips, then animals received 1 mL of saline via subcutaneous (s.q.) injection. Animals were kept on a circulating water pad set at 37°C to maintain body temperature both before and after surgery.

Post-operative care

To ensure hydration and to prevent urinary tract infections, mice were given saline (1 mL, s.q.) and Baytril (2.5mg/kg) for the first 10 days post-injury. Food pellets were placed on the floor of the cages for easy access as animals recovered. Manual bladder expression was performed twice daily until euthanasia. Body weight was measured at 1 and 3 dpi and then weekly until euthanasia. Animals with significant reductions in body weight (>15%) were given Nutrical on food pellets to encourage eating.

Open field locomotor testing

The Basso Mouse Scale (BMS) was performed at 1, 3, 7, 14, 21, 28, and 35 days post-SCI to assess hindlimb locomotor recovery. The 9-point BMS test assesses multiple aspects of hindlimb function such as the degree of hindlimb movement, paw placement, stepping, and coordination where higher scores indicate greater levels of hindlimb functional recovery. For this testing, animals were placed into a rectangular open field and were rated by two independent scorers for 4-minutes. Scorers were blinded to treatment and group throughout the duration of the study.

Flow cytometry

Mice were anesthetized by i.p. injection of 2.5% avertin (0.02 mL/g body weight) and bilateral thoracotomy was performed. Peripheral blood was collected via cardiac puncture of the right ventricle using an insulin syringe containing 200 µL of 3.6mg/mL EDTA (Invitrogen) to prevent clotting. Blood was transferred into a microcentrifuge tube, placed on ice, and additional EDTA (3.6mg/mL) was added till a 1:1 ratio of blood to EDTA was reached. Blood/EDTA solution was then transferred into a 15 mL conical containing 10 mL of ice cold HBSS (without calcium, magnesium, or phenol red).

Following collection of peripheral blood, approximately 5 mm of spinal cord tissue (including meninges) centered on the injury site was rapidly dissected and placed into a microcentrifuge tube containing RPMI media on ice. While kept on ice, the spinal cord (SC) tissue was mechanically dissociated using a plastic tissue pestle. The solution was triturated before being filtered through a 70-µm nylon mesh cell strainer. The filtered solution was added to 10 mL of cold HBSS on ice.

Following collection of the spinal cord, the femur and tibia were dissected from one of the hindlimbs. Both bones were then transferred to 5mL of HBSS on ice. The lateral and medial condyles of the femur were removed as well as the femoral head using small dissecting scissors, and 10mL of ice cold HBSS was flushed through the BM lumen to flush the BM cells into an empty 50mL conical. The BM sample was then triturated on ice with a 19G needle to evenly suspend the BM cells in HBSS.

Blood, BM, and SC samples were centrifuged at 500 × *g,* 4°C for 5 min. Pellets were then lysed with 10 mL (blood) or 1mL (BM & spinal cord) of 1X red blood cell (RBC) lysis buffer (BD Pharm Lyse) at 4°C. Blood was lysed for 6 min while BM and SC were lysed for 5 min. Blood samples were quenched with 40mL ice cold FACS buffer (.02% .5 µM EDTA, 2% fetal bovine serum, and HBSS). BM and SC samples were quenched with 10mL ice cold FACS buffer. Samples were centrifuged at 400 × *g,* 4°C for 5 min, pellets were resuspended in 10mL cold HBSS, and 1x10^6 BM cells were transferred to a new 15 ml conical. Samples were centrifuged again, supernatant was tipped off, and the pellets were resuspended in Zombie Red Fixable Viability Kit (Biolegend; 1:500) for 30 min at 4°C for live/dead cell staining. Samples were quenched with 10mL cold FACS buffer and centrifuged. Supernatant was tipped off, and samples were resuspended in remaining supernatant and transferred to a 96-well round bottom plate. Samples were centrifuged at 800 × *g,* 4°C for 2 min, the supernatant was discarded, and the pellets were incubated with anti-mouse CD16/32 Fc blocking antibody (1:100; Biolegend, cat: 101302) at 4°C for 20 min. Samples were centrifuged, supernatant discarded, and pellets were resuspended in 200

μL cold FACS buffer. For isotyping staining, 100 μL of cell solution was split into a separate well and samples were centrifuged again.

Pellets were then incubated for 30 min at 4°C with a cocktail of the following antibodies: Pacific Blue-conjugated rat anti-mouse Ly6G (1:200; 1A8, Biolegend, cat: 127612), FITC-conjugated rat anti-mouse/human CD11b (1:200; M1/70, Biolegend, cat: 101206), APC-Cyanine7-conjugated rat anti-mouse CD45 (1:200; 30-F11, Biolegend, cat: 103115), and PE-Cyanine7-conjugated rat anti-mouse CD101 (1:100; Moushi101, Invitrogen, ref: 25-1011-82). PE-Cyanine7-conjugated rat IgG2α,κ (1:100; RTK2758, Biolegend, cat: 400521) served as an isotype control for CD101. Samples were quenched with 200 μL of cold FACS and centrifuged, supernatant discarded, and then resuspended in FACS and centrifuged again. For fixation and permeabilization of the cell membranes for intracellular Ly6G staining, pellets were then incubated with 100 μL of BD Fixation/Permeabilization solution (Biolegend) for 20 min at 4°C. Samples were washed with 200 μL of 1X BD Perm/Wash buffer (Biolegend), centrifuged, resuspended in cold FACS buffer, and stored at 4°C overnight. The following day, cells were centrifuged at 800 × *g* at 4°C for 2 min and pellets were resuspended in 1X BD Perm/Wash buffer containing PerCP-Cyanine5.5-conjugated rat anti-mouse Ly6G (1A8, Biolegend, cat: 127616) for intracellular Ly6G staining. Compensation beads were generated using ArC Amine Reactive Bead Compensation Kit (Invitrogen) for Zombie Red and AbC Anti-Rat/Hamster Bead Kit (Invitrogen) for all other staining antibodies. Cells and beads were washed and centrifuged twice, then resuspended in FACS buffer.

Flow cytometry was performed on the cell suspensions using a BD Fortessa X-20. For spinal cord samples, 10 μL of Accucheck Counting Beads (Invitrogen, ref: PCB100) were added to the cell suspension before the sample was run through the cytometer. At least 50,000 events for blood and BM and all events for spinal cord samples were collected. Data analysis was performed using FlowJo software. $CD101^+$ populations were gated using the CD101 isotype signal out of $Ly6G^+$ cells.

White matter sparing

Animals were euthanized at 35-days post-SCI and transcardially perfused with 25 mL of ice cold 1X PBS followed by 35 mL of cold 4% paraformaldehyde in PBS. Spinal columns were dissected and post-fixed in 4% paraformaldehyde overnight at 4°C. Spinal cords were then dissected and cryoprotected in 30% sucrose with 0.2% sodium azide. After cryoprotection, 4 mm of spinal cord tissue centered over the injury site was dissected and embedded in Tissue-Tek optimal cutting temperature medium (Sakura) and rapidly frozen on dry ice. Transverse tissue sections were cut in series along the rostral-caudal axis (25-μm thick, 20 slides per series) and every tenth section was selected for eriochrome cyanine staining. Images were captured using a Leica DM 6B microscope. Total area of residual myelin was quantified using ImageJ and the section with the least white matter area was selected as the lesion epicenter.

Lipid-laden macrophage staining and analysis

Spinal cords and transverse sections were collected as described for white matter sparing. Slides with transverse tissue sections were dried on a plate warmer set at 37°C for 10 min then washed with 1X PBS at room temperature (RT) for 5 min. Sections were incubated in blocking solution (5% normal donkey serum (NDS) and .1% BSA diluted in 1X PBS) for 1 h at RT in humidified chambers to prevent drying. Next, sections were incubated in blocking solution

containing rat anti-mouse F4/80 primary antibody (1:100; BM8, Biolegend) overnight at 4°C in humidified chambers. The following morning, slides were brought to RT for 30 min then washed 3X in 1X PBS. Sections were incubated for 2h at RT in blocking solution containing AlexaFluor (AF) 647-conjugated donkey anti-hamster secondary (1:1000; Jackson Labs, cat: 405510), washed 3X in 1X PBS, then incubated in 2 μM BODIPY diluted in 1X PBS for 1 h at RT. Slides were washed 3X in 1X PBS and coverslipped with Prolong Gold Antifade with DAPI. Images were captured using a Nikon Eclipse upright microscope equipped with a 10x objective (0.45 NA).

Custom MATLAB code was used to quantify F4/80$^+$ pixels for macrophages, BODIPY$^+$ pixels for lipid droplets, and F4/80$^+$BODIPY$^+$ pixels for lipid-laden macrophages. Sections were manually traced by 3 independent and blinded researchers. F4/80$^+$BODIPY$^+$ pixels were normalized to total number of F4/80$^+$ pixels in the selected region of interest (ROI). An average of the two closest values for each measurement was used for the final analysis. A total of 1500 μm spanning the lesion was analyzed and sum total of averaged values was calculated.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (GraphPad Software). Flow cytometry, white matter sparing, and immunoblotting data were analyzed using two-way ANOVA with Sidak's multiple comparison post hoc test with a 95% confidence interval. Prior to disaggregation by sex, flow cytometry and white matter sparing data were analyzed using an unpaired two-tailed Student's *t* test. BMS scores were first analyzed using a three-way, repeated measures ANOVA. Where appropriate, BMS scores were disaggregated by sex and analyzed using a two-way, repeated measures ANOVA followed by Bonferroni's multiple comparison test with a 95% confidence interval. Significance was designated as $p < .05$. Values are reported as the mean plus or minus standard error of the mean (SEM).

Results

Systemic neutrophil responses and phenotypes differ by sex after SCI

To characterize sex-dependent changes in neutrophil phenotype, as well as injury-induced neutrophilia following SCI, we performed a moderate thoracic contusion SCI in adult male and female mice followed by flow cytometry analysis of blood and BM samples collected up to 35-days post-injury (Fig 1a-b). We observed a rapid increase in circulating neutrophils 4-hours post-injury with sustained neutrophilia for up to 1 week following SCI in both sexes (Fig 1c). Interestingly, male mice had a substantially greater neutrophil mobilization response following SCI compared to females. While we observed injury-induced neutrophilia in female mice, the abundance of circulating neutrophils returned to pre-injury baseline by 3-days post-SCI. We next characterized the maturation phenotype of circulating neutrophils in male and female mice. In both sexes, there was marked reduction in the relative proportion of mature neutrophils early after SCI with neutrophils displaying a predominantly immature phenotype (CD101$^-$) by 1 dpi (Fig 1d-e). In addition to having a larger neutrophil mobilization response post-SCI, male mice had a greater proportion of mature (CD101$^+$) circulating neutrophils than female mice after SCI. Neutrophil maturation profiles recovered to pre-injury levels by 3 dpi in male mice, whereas neutrophils in female mice did not return to pre-injury levels until after 3dpi.

We also characterized the effect of SCI on bone marrow (BM) neutrophil populations. As expected, there was a reduction in the proportion of mature neutrophils in the BM in both male and female mice at 1 dpi, likely due to the generation of new immature neutrophils as well as mobilization of reserve mature BM neutrophils into circulation immediately after injury (Fig S1a). At 3-days post-SCI, we observed a sex-dependent recovery of the BM neutrophil populations, which returned to pre-injury mature neutrophil levels (~20% $CD101^+$/total $Ly6G^+$ cells) in male, but not female mice. To characterize if any sex differences in granulopoiesis were occurring following SCI, we injected EdU 1-day prior to injury, then performed flow cytometry analysis on BM and blood samples collected up to 35 dpi (Fig S1b-c). EdU should only label proliferative neutrophil precursors that then differentiate into non-proliferative immature neutrophils[26]. At 1-day post-injury, females had a higher proportion of $CD101^-/EdU^+$ immature neutrophils in the bone marrow (Fig S1d, whereas males had a higher proportion of $CD101^+/EdU^+$ mature neutrophils in the bone marrow at 3dpi (Fig S1e). Consistent with the increase in the percentage of mature neutrophils in the blood of male mice at 3dpi, there was a greater percentage of $CD101^+/EdU^+$ mature neutrophils in the blood of male mice relative to females at 3dpi (Fig S1f). Collectively, our data provide novel evidence of sex differences in SCI-induced neutrophilia, including sex-dependent changes in neutrophil maturation and granulopoiesis in the bone marrow and blood after SCI.

Intraspinal neutrophil accumulation and phenotype differ by sex after SCI

Since we observed prominent sex differences in neutrophilia and the maturation profile of circulating neutrophils following SCI, we also assessed if there were sex differences in neutrophil accumulation and phenotype in the injured spinal cord. Mice were perfused with 1X PBS to remove circulating neutrophils prior to fixation. Spinal cord tissue was then collected and sectioned for histological analysis of total intraspinal neutrophil accumulation (Fig 1f-g). In accordance with our findings that male mice have a greater neutrophilia response to SCI, we observed significantly higher neutrophil counts at the lesion epicenter across the first week post-injury in male mice relative to females (Fig 1h). Flow cytometry analysis of neutrophil maturation in the injured spinal cord revealed a marked increase in mature neutrophils in the injured spinal cords of female mice at 1 and 3 dpi relative to 0 hpi (Fig. 1i-k). There was a trend ($p=0.11$) towards an increase in the mature neutrophil proportion in male mice at 3 dpi compared to 0 hpi. The percentage of mature neutrophils at 3 dpi was greater in female mice relative to males (Fig. S1g). Altogether, we observed that the accumulation and maturation phenotype of intraspinal neutrophils rapidly changed in a sex-dependent manner after SCI.

We next examined whether there is a relationship between chronic neutrophil persistence in the spinal cord lesion and long-term functional recovery. We performed a moderate thoracic contusion SCI in male and female mice and long-term functional recovery of the hindlimbs was assessed using the Basso Mouse Scale (BMS)[44]. Spinal cords were collected at 35 dpi and intraspinal neutrophil numbers were quantified at the lesion epicenter. Interestingly, linear regression analysis showed that total neutrophil numbers within the lesion epicenter of male mice at 35 dpi positively correlated with functional outcomes ($R^2 = 0.38$, $p = .03$, Fig 1i), suggesting that neutrophils may be associated with greater recovery following SCI in male mice. However, no correlation was observed for female counterparts. These data support a novel and sex-dependent role for neutrophils in mediating recovery following SCI and provide additional evidence for sex differences in neutrophil function after injury.

Depletion of neutrophils impairs long-term functional recovery in a sex-dependent manner

Given the marked sex differences observed in neutrophil responses to SCI, we next wanted to assess the sex-dependent contribution of neutrophils towards long-term functional recovery. We administered anti-Ly6G depletion (clone 1A8, 2.5 mg/kg, i.p.) or control IgG antibody (clone 2A3) immediately following thoracic contusion SCI and long-term functional recovery was assessed using BMS (Fig 2a). Contrary to the prevailing dogma that neutrophils are detrimental after SCI, antibody-mediated neutrophil depletion significantly impaired long-term hindlimb functional recovery following SCI (Fig 2b). Remarkably, only male mice showed long-term functional impairment with neutrophil depletion (Fig 2c). Long-term functional recovery in female mice was not affected by depletion of neutrophils. Eriochrome cyanine staining revealed that depletion of neutrophils after SCI did not significantly alter long-term tissue sparing in male or female mice (Fig 4d), suggesting that tissue sparing alone may not be responsible for the observed impairment of functional recovery in males.

To ensure that anti-Ly6G antibody administration after SCI abrogates intraspinal neutrophil accumulation, we first validated our method for detecting potential remaining neutrophil populations. Staining for neutrophils that remain following administration of anti-Ly6G antibody can be difficult due to the recently described masking of the Ly6G surface antigen[45], which can be circumvented by intracellular Ly6G staining (Fig 2e). In our hands, staining for intracellular Ly6G was effective at labeling remaining neutrophil populations that could not be labeled with conventional surface Ly6G staining techniques following anti-Ly6G administration (Fig 2f). We performed flow cytometry analysis on spinal cord samples collected at 1 day after SCI and neutrophil depletion (Fig 2g). Anti-Ly6G depletion antibody administered immediately after SCI reduced total intraspinal neutrophil numbers at 1 dpi independent of sex (Fig 2h). Interestingly, neutrophil depletion abrogated the accumulation of mature ($CD101^{+}$) neutrophil at 1-day post-SCI in both sexes but did not alter immature ($CD101^{-}$) neutrophil accumulation (Fig 2i-j). No differences in the mean fluorescent intensity (MFI) of surface Ly6G (Fig S2a) were observed between immature and mature circulating neutrophils in uninjured mice, suggesting that surface Ly6G expression alone does not account for differences in depletion efficacy of mature and immature neutrophils in the acutely injured spinal cord. Our data suggest the sex-dependent effects of neutrophil depletion on long-term functional recovery may be due to inherent sex differences in the role of neutrophils in the injured spinal cord rather than sex differences in the efficacy of neutrophil depletion.

Intraspinal neutrophil phenotype and putative function are highly dynamic after SCI

To better understand how intraspinal neutrophil phenotype and function may contribute to the sex-dependent functional impairments observed following neutrophil depletion, we analyzed the transcriptional profile of neutrophils from three published scRNAseq datasets[46–48]. Clustering of neutrophils by time revealed a population of intraspinal neutrophils at 3-days post-injury that appeared distinct from other timepoints (Fig. 3a). For simplicity, neutrophils from sham and uninjured mice were combined into a single “sham” group. A correlation matrix of the transcriptional profiles of neutrophils at different timepoints after SCI, as well as sham animals, confirmed that intraspinal neutrophils at 3 dpi are transcriptionally distinct (Fig. 3b). Analysis of gene expression for neutrophil granule production and metabolism showed drastic differences in

transcript levels azurophilic granules, specific granules, and glycolysis across time, further demonstrating that neutrophil phenotype function is highly dynamic after SCI (Figs. 3c). In addition, analysis of gene expression associated with phagocytosis, chemotaxis, and NADPH oxidase (critical for ROS production) shows evidence that neutrophil function is also likely highly dynamic following injury.

To determine how neutrophil maturation changes after SCI in the scRNAseq data, we assessed the transcriptional similarity of intraspinal neutrophils to BM neutrophils using the previously described "bone marrow proximity score"[49]. Intraspinal neutrophils during the acute phase of SCI (1 and 3-days post-injury) had significantly lower bone marrow proximity scores relative to neutrophils from sham animals and all other timepoints post-SCI, suggesting that neutrophils at these timepoints are transcriptionally distinct from the BM neutrophil population (3d-d'). By assessing the early and late Neutrotime[50] scores of intraspinal neutrophils, which quantifies the transcriptional levels of early and late genes expressed during neutrophil development and maturation (Figs. 3e-e' and 3f-f'), we confirmed that intraspinal neutrophils at 1 and 3-days post injury had uniquely greater maturation profiles when compared to intraspinal neutrophils at other timepoints, as well as neutrophils from sham and uninjured mice. While intraspinal neutrophils at 1 and 3 dpi both had relatively high mature neutrophil scores, neutrophils at 3 dpi had remarkably unique transcriptional signatures, suggesting functionally distinct properties for these cells.

Mature neutrophils adopt a pro-resolving phenotype and limit macrophage accumulation in a sex-dependent manner

Since anti-Ly6G antibody administration mitigated the accumulation of mature neutrophils in the acutely injured spinal cord, we performed a gene ontology analysis of differentially expressed genes from neutrophils at 3 dpi relative to neutrophils at all other timepoints. Efferocytosis (mmu04148) was identified as a prominent pathway for neutrophils at 3 dpi, potentially indicating the production of "eat me" signals from neutrophils undergoing phagocytoses (Fig 4a). It has been

previously shown that macrophage phagocytosis of apoptotic neutrophils can shift macrophages towards an anti-inflammatory phenotype and induce a pro-resolution cascade that can promote tissue repair[10,11,51]. To further analyze the potential role of neutrophils in resolution of inflammation, we focused on the data from Wang et al. 2022[48], where scRNAseq was performed on leukocytes ($CD45^+$ cells) sorted and enriched from the injured spinal cord. Analysis of the ligand-receptor interaction strength of neutrophils at 3dpi revealed high incoming and outgoing interactions between neutrophils and macrophages, as well as monocytes, within the injured spinal cord (Fig S3a-b). Interaction of neutrophils with macrophages waned by 14 dpi, suggesting that the 3 dpi neutrophil population might play a unique role in regulating macrophage responses to SCI.

Neutrophils have also been shown to play a role in regulating the formation and function of myelin lipid-laden ("foamy") macrophages[52,53], therefore, we next determined if depletion of neutrophils increased lipid-laden macrophage formation and accumulation in the injured spinal cord. Histological analysis for lipid-laden macrophage accumulation was performed on spinal cord tissue sections at 35 days following SCI and neutrophil depletion. While we did not observe any effect of neutrophil depletion on total lipid droplet abundance or lipid-laden macrophage

accumulation, we did observe a striking sex-dependent increase in total macrophage accumulation in the injured spinal cord (Fig 4b-d). Depletion of neutrophils did not alter macrophage accumulation in the injured spinal cords of female mice, however, it significantly increased macrophage accumulation in male mice at 35 dpi. These findings provide evidence for a novel and sex-dependent role of mature neutrophils in promoting immune resolution in the injured spinal cord.

Pre-injury depletion of neutrophils does not alter functional recovery in either sex

In past studies, neutrophil depletion has been commonly initiated up to 24 hours prior to SCI. To determine if the sex-dependent effects of neutrophil depletion were also time-dependent, we administered anti-Ly6G depletion antibody or IgG control antibody in adult male and female mice at 1-day prior to SCI (Fig 5a). Surprisingly, no significant differences in long-term functional recovery were observed in either sex with pre-injury neutrophil depletion (Fig 5b). Consistent with this finding, pre-injury neutrophil depletion also did not alter long-term white matter sparing (Fig. 5c-d).

We next wanted to ensure that earlier administration of the depletion antibody was still effective at reducing intraspinal neutrophil accumulation following SCI. Antibody-mediated depletion of neutrophils starting 1-day prior to injury successfully reduced the accumulation of mature neutrophils ($CD101^{+}/Ly6G^{+}$ cells) in the acutely injured spinal cord (Fig. 5e-f). Consistent with post-SCI depletion, pre-injury administration of the anti-Ly6G depletion antibody did not alter the accumulation of immature neutrophils ($CD101^{-}/Ly6G^{+}$ cells). Collectively, our findings demonstrate that the sex-dependent role of mature neutrophils in mediating long-term recovery is also time-dependent.

Discussion

Neutrophils have historically been thought to exacerbate secondary tissue damage and worsen long-term outcomes following SCI. Neutrophils respond to injury and infection via cytotoxic effector functions essential for mitigating microbial spread, but often with bystander damage to nearby healthy tissue[5,6,9,54,55]. Limiting neutrophil accumulation in the acutely injured spinal cord, by blocking adhesion receptors or chemoattractants, has commonly led to tissue sparing and greater functional recovery[22,56,57]. However, neutrophil depletion has produced conflicting results and recent studies have implicated beneficial roles for neutrophils in CNS and peripheral nerve injury.

The contribution of neutrophils in SCI is likely multifaceted, with neutrophils having both harmful and reparative functions. Neutrophil heterogeneity may mediate this functional divergence, which could be further complicated by sex differences in neutrophil function. Here we show that neutrophils in the periphery and lesion site rapidly change in number and phenotype after SCI in a time- and sex-dependent manner. We demonstrate that neutrophil depletion worsens long-term functional recovery in male mice, but not females. We also show that mature neutrophils acquire an inflammation-resolving phenotype in the acutely injured spinal cord and that depletion of mature neutrophils exacerbates inflammation in a sex-dependent manner. Our findings demonstrate that neutrophils respond to SCI in a time- and sex-dependent manner and engage in

sex-dependent beneficial activities that are critical for mediating long-term functional recovery.

During maturation, neutrophils will increase CXCR2 expression and downregulate CXCR4 to facilitate release from the bone marrow into circulation[31]. Neutrophil mobilization after injury is coordinated in parting by an increase in granulocyte colony-stimulating factor (G-CSF), which stimulates the downregulation of CXCR4 to enhance neutrophil release into circulation[58]. While sex differences have been previously shown for circulating and BM neutrophils[38,41–43], our study is among the first to demonstrate sex differences in neutrophil mobilization as well as granulopoiesis after injury. Neutrophil abundance after SCI has been shown to be predictive of long-term functional recovery, which may be reflective of injury-induced neutrophilia. To the best of our knowledge, our study is the first to demonstrate that the maturation prolife of intraspinal neutrophils changes over time in a sex-dependent manner. Interestingly, the time course of maturation for intraspinal was distinct from the blood and bone marrow compartments, which may reflect differences in recruitment and migration of mature and immature neutrophils into the injured spinal cord. In our data and the published scRNAseq data, we found immature neutrophils associated with the spinal cord in sham mice or early after SCI, as well as at later time points after 7 dpi. A recent study found that skull and vertebral BM neutrophil populations were capable of bypassing circulation and rapidly respond to inflamed CNS tissue by migrating through microscopic channels connecting the BM lumen with the meninges[59]. By migrating directly from the BM, immature neutrophils may be able to enter the spinal cord meninges and possibly the tissue parenchyma, thus allowing for the disconnect between circulating and intraspinal neutrophil maturation states.

Neutrophil depletion in thoracic contusion models of SCI has produced varied effects on long-term outcomes ranging from impaired recovery[21], to no effects[23,60], to marked neuroprotection[22]. Early studies utilized the GR-1 depletion antibody which can target cells expressing Ly6G and Ly6C, including monocytes[61]. However, it is important to note that only a single sex was considered in these studies. In fact, the only study to use male mice (sex was not published, but confirmed by the Dr. Stirling) saw worse long-term functional recovery with neutrophil depletion, albeit with the GR-1 antibody[21]. The follow-up studies utilized female mice and predominantly found minimal effects on long-term recovery, particularly when using the Ly6G depletion antibody (clone 1A8). While many methodological differences exist between these studies, our study suggests that the inconsistent effects can be partially explained by sex- and time-dependent effects of neutrophil depletion after SCI.

Reparative roles for neutrophil subsets in models of sterile injury have largely been attributed to immature neutrophils. Within the CNS, immature neutrophils have been shown to promote neuron survival, promote axon regeneration in the injured spinal cord, and contribute to regenerative and neuroprotective processes[62]. In our study, we provide novel evidence of a sex-dependent reparative role for mature neutrophils in CNS injury. Neutrophils are capable of synthesizing many specialized pro-resolving mediators that work to regulate leukocyte accumulation and phenotype. Neutrophil extracellular traps can sequester cytokines and chemokines and matrix metalloproteinase-9, released from neutrophil granules, can degrade damage associated molecular patterns to mitigate further leukocyte recruitment[10,63,64]. In addition, apoptotic neutrophils can induce resolution of inflammation by promoting an anti-inflammatory phenotype in phagocytosing macrophages during efferocytosis[10,11,51,65]. Our data indicate a direct interaction of neutrophils

with macrophages, including the expression of genes involved in efferocytosis, which likely collectively work with indirect mechanisms (sequestration and degradation of immune stimulating cues) to mitigate subsequent inflammation. We focused on macrophages due to their prevalence in the SCI lesion site, however, future work should examine the effect of neutrophils on other leukocyte subsets, including lymphocytes.

While other studies on neutrophil heterogeneity have found mature and immature neutrophil subsets to have differing levels of Ly6G expression[26,29,66], we found that in circulation, mature and immature neutrophils equally express the Ly6G surface antigen. Additionally, we demonstrate anti-Ly6G administration in the context of SCI specifically abrogates mature neutrophil accumulation in the injured spinal cord in both male and female mice. A similar effect has been previously reported on splenic neutrophils with anti-Ly6G administration, where again only the mature neutrophil population was depleted by anti-Ly6G[67]. Additionally, because we only administer one acute dose of anti-Ly6G in both of our depletion studies, the depletion capacity of anti-Ly6G in our experiments is inherently transient. The pre-injury anti-Ly6G antibody administration might not deplete neutrophils long enough to significantly impact the later intraspinal population of mature neutrophils (e.g. 3 dpi) to the same extent as post-injury anti-Ly6G administration. Interestingly, one study that employed the anti-Ly6G antibody for neutrophil depletion in the context of SCI previously showed that one singular dose of anti-Ly6G was effective at depleting neutrophils for 3-days in uninjured female mice[68]. The duration of neutrophil depletion after SCI in both sexes remains to be determined.

Collectively, our results identify sex differences in neutrophil responses to SCI and demonstrate that neutrophils in the acutely injured spinal cord are predominantly beneficial for long-term functional recovery in male mice. To the best of our knowledge, our study is the first to demonstrate sex-dependent roles for mature neutrophils in resolution of inflammation after injury. Our work also addresses many of the inconsistent effects previously observed in past neutrophil depletion studies in SCI by demonstrating the sex- and time-dependent effects of neutrophils in long-term functional recovery. Future studies are needed to a determine why resolution of inflammation by neutrophils only occurs in male mice after SCI and if these activities can be augmented for future sex-specific strategies to promote long-term recovery after SCI.

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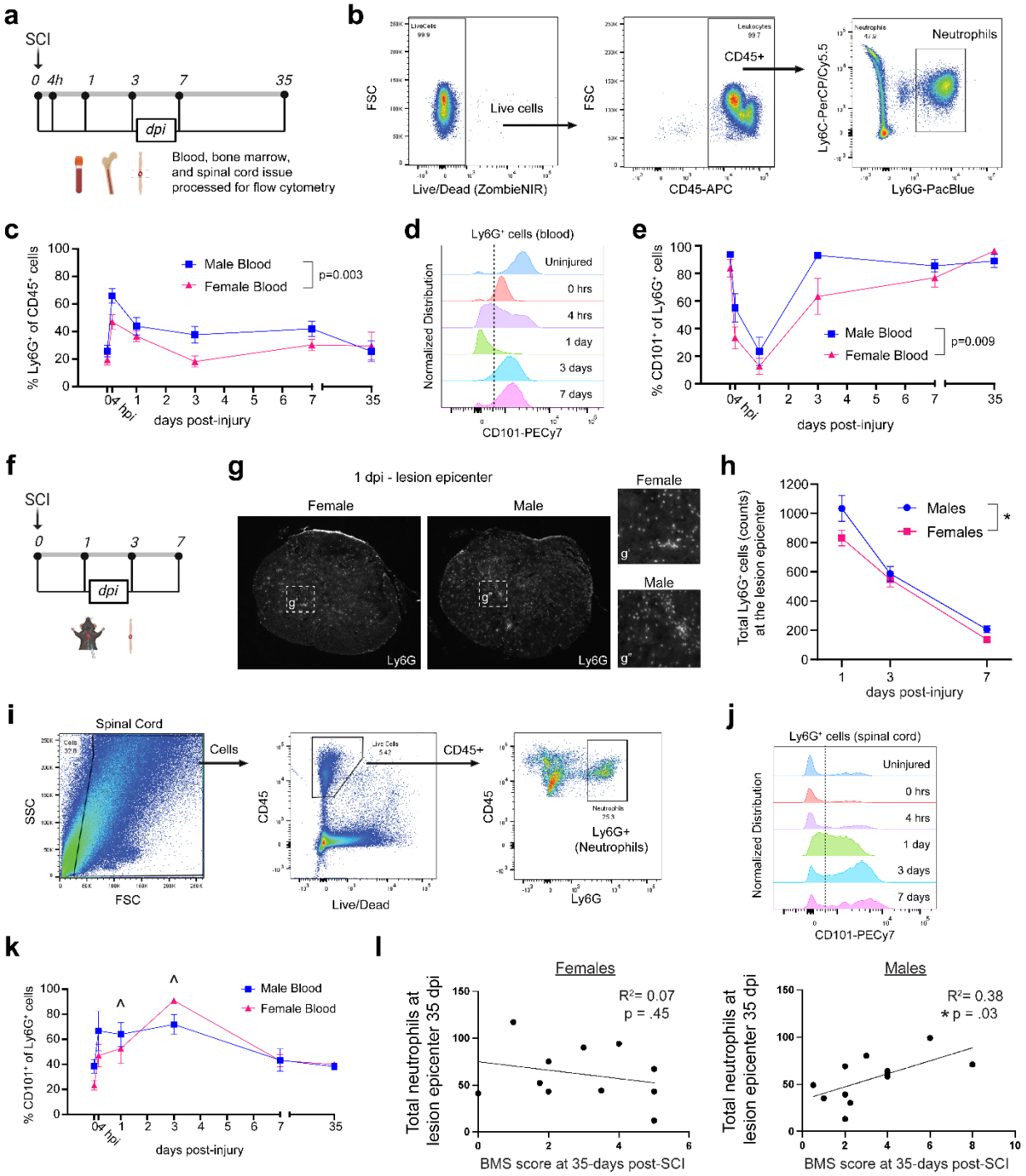

Figure 1: Sex-dependent neutrophil responses to spinal cord injury. (a) Schematic of SCI and tissue collection timepoints for flow cytometry analysis. (b) Representative flow cytometry gating of neutrophils in blood samples. (c) Quantification of neutrophil (Ly6G$^+$) percentage of total circulating leukocytes (CD45$^+$) after SCI. Mixed effects analysis with Sidak's multiple comparisons test. n=3-10/sex/timepoint. (d) Representative histograms for CD101 levels on circulating neutrophils (Ly6G$^+$ cells). (e) Quantification of the percentage mature neutrophils (CD101$^+$/Ly6G$^+$ cells) out of total neutrophils (Ly6G$^+$ cells) via flow cytometry in the blood after SCI. Mixed effects analysis with Sidak's multiple comparisons test. n=3-5/sex/timepoint. (f) Schematic of SCI and spinal cord tissue collection for histology. (g) Representative

images of Ly6G immunostaining (neutrophils) in the lesion epicenter at 1 dpi in female (left) and male (right) mice. g' and g'' are magnified insets. (h) Quantification of $Ly6G^+$ cells in the lesion epicenter at 1, 3, and 7 dpi. Mixed effects analysis with Sidak's multiple comparisons test. n=8-11/sex/timepoint. *$p<0.05$. (i) Representative flow cytometry gating of neutrophils in spinal cord samples. (j) Representative histograms for CD101 levels on intraspinal neutrophils ($Ly6G^+$ cells). (k) Quantification of the percentage mature neutrophils ($CD101^+/Ly6G^+$ cells) out of total neutrophils ($Ly6G^+$ cells) via flow cytometry in the spinal cord after SCI. Mixed effects analysis with Sidak's multiple comparisons test. n=3-6/sex/timepoint. (l) Correlation of BMS scores and intraspinal neutrophils at the lesion epicenter in female (left) and male (right) mice at 35 dpi. Linear regression analysis. n=11/sex. Abbreviations: SCI = spinal cord injury, BMS = Basso Mouse Scale, dpi = days post-injury. Mean +/- SEM.

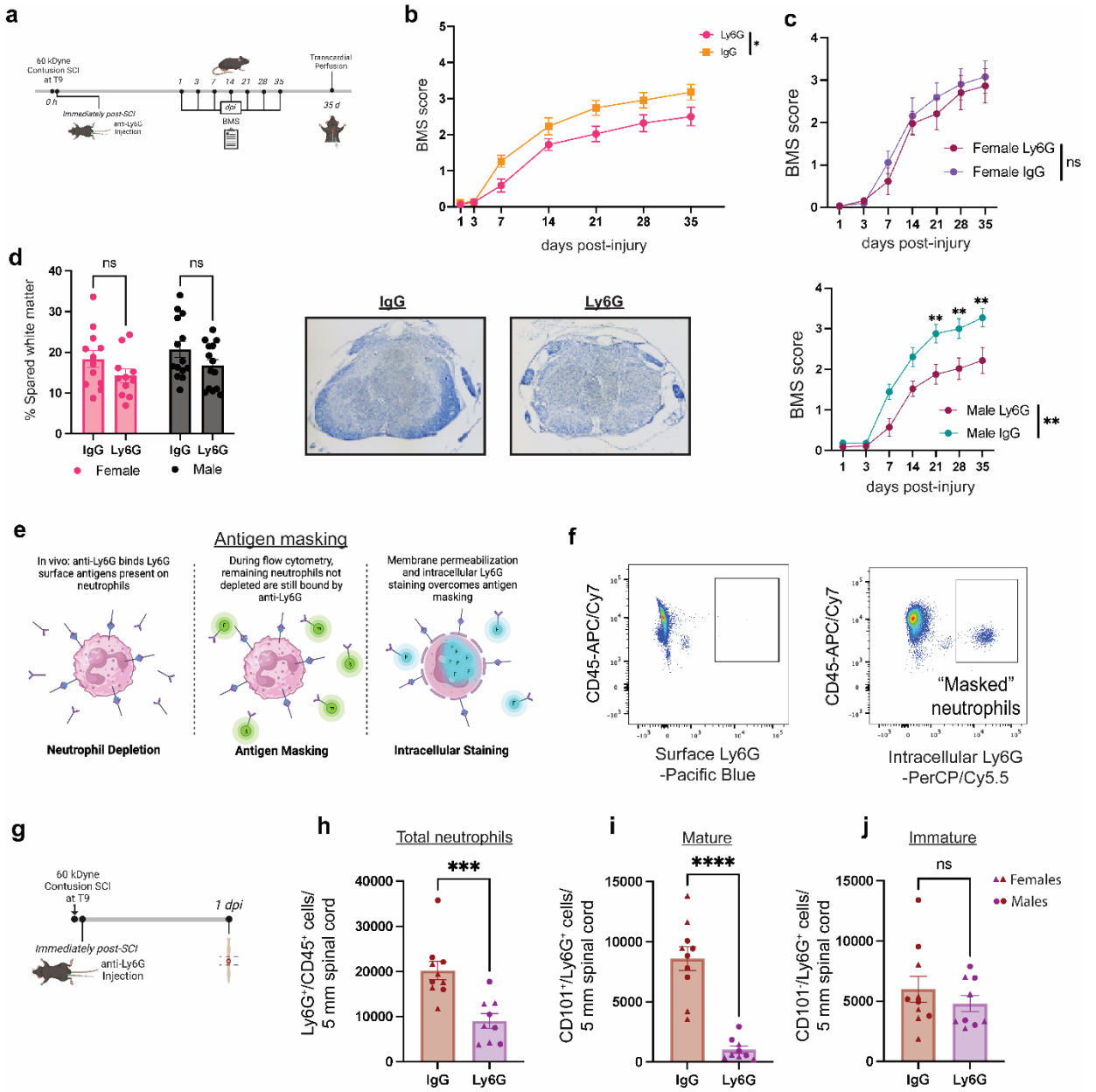

Figure 2. Depletion of mature neutrophils impairs long-term recovery in a sex-dependent manner. (a) Schematic of experimental paradigm for post-SCI depletion utilizing anti-Ly6G antibody (clone 1A8, 2.5 mg/kg) in male and female mice. (b) Depletion of neutrophils immediately after reduced BMS scores. n=13-14/sex/treatment. Repeated measures three-way ANOVA. (c) Disaggregation of BMS scores in (b) by sex. Depletion of neutrophils did not alter BMS scores for female mice. Neutrophil depletion reduced BMS scores after SCI in male mice. N=13-14/treatment. Repeated measures two-way ANOVA with Bonferroni's multiple comparison test, *p<0.05, **p<0.01, ***p <0.001, ****p<0.0001. (d) Quantification and representative images of white matter sparing determined by eriochrome cyanine staining. No differences in white matter sparing were observed with neutrophil depletion. Two-way ANOVA with Sidak's multiple comparisons test. n=11-14/sex/treatment. (e) Schematic of antigen masking circumvented by intracellular Ly6G staining. (f) Flow cytometry analysis of neutrophil populations with surface (left) and intracellular (right) Ly6G staining after neutrophil depletion. Intracellular Ly6G labels remaining masked

neutrophils not labeled with conventional surface staining of Ly6G. (g) Schematic of SCI, depletion of neutrophils, and tissue collection for flow cytometry analysis. (h) Anti-Ly6G antibody administration reduces total intraspinal neutrophils ($Ly6G^+$ cells percentage out of total $CD45^+$ population) at 1 dpi. (i) Anti-Ly6G antibody administration substantially reduces intraspinal mature ($CD101^+$) neutrophil counts. (j) Intraspinal immature ($CD101^-$) neutrophils were not altered by anti-Ly6G antibody administration. N = 4-5/sex/treatment. Unpaired two-tailed Student's t test. Abbreviations: SCI = spinal cord injury, BMS = Basso Mouse Scale, dpi = days post-injury. Mean +/- SEM.

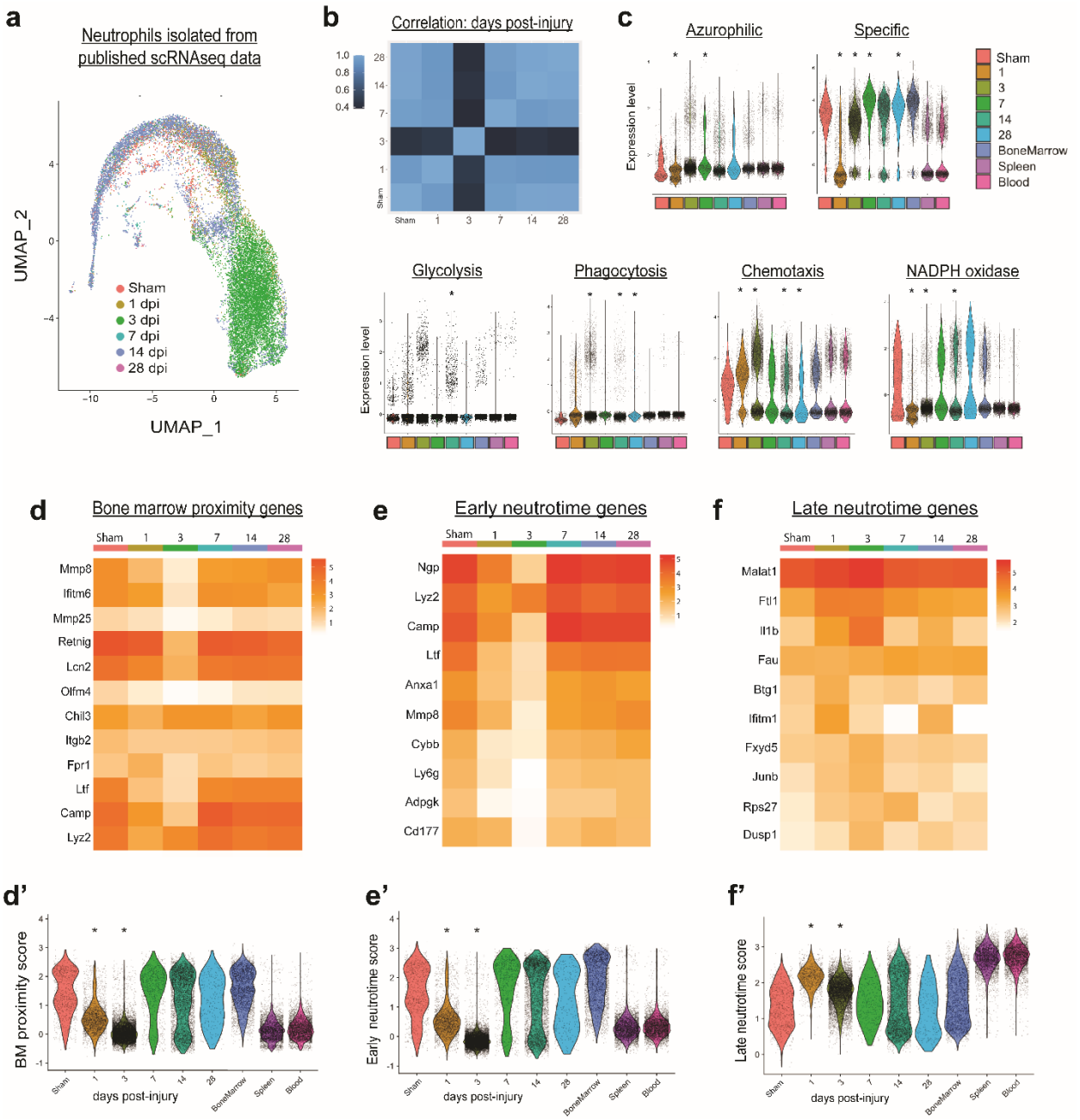

Figure 3. scRNAseq analysis reveals unique time-dependent neutrophil transcriptional signatures in the injured spinal cord. (a) UMAP of intraspinal neutrophils isolated from three published scRNAseq datasets for thoracic contusion SCI in mice. Neutrophils from sham and uninjured mice combined together in "sham" group. Neutrophils are transcriptionally distinct across time after SCI. (b) correlation matrix of the transcriptional profiles of neutrophils at different timepoints after SCI. Neutrophils at 3 dpi are transcriptionally distinct versus all other timepoints. (c) Expression levels of genes associated with azurophilic granules, specific granules, glycolysis, phagocytosis, chemotaxis, and NADPH oxidase (ROS production. (d) Heatmap for genes associated with the bone marrow proximity score. (d') Quantification of the bone marrow proximity score for neutrophils after SCI. (e) Heatmap for genes associated with the early Neutrotime score. (e') Quantification of the early Neutrotime score for neutrophils after SCI. (f) Heatmap for genes associated with the late Neutrotime score. (f') Quantification of the late Neutrotime score for

neutrophils after SCI. Neutrophils at 1 and 3dpi express genes indicative of mature neutrophils. Kruskal-Wallis test with Dunn's Test for post-hoc analysis. *$p<0.05$ vs. all other timepoints. Bone marrow, blood, and spleen neutrophils are not included in analysis. Abbreviations: SCI = spinal cord injury, dpi = days post-injury.

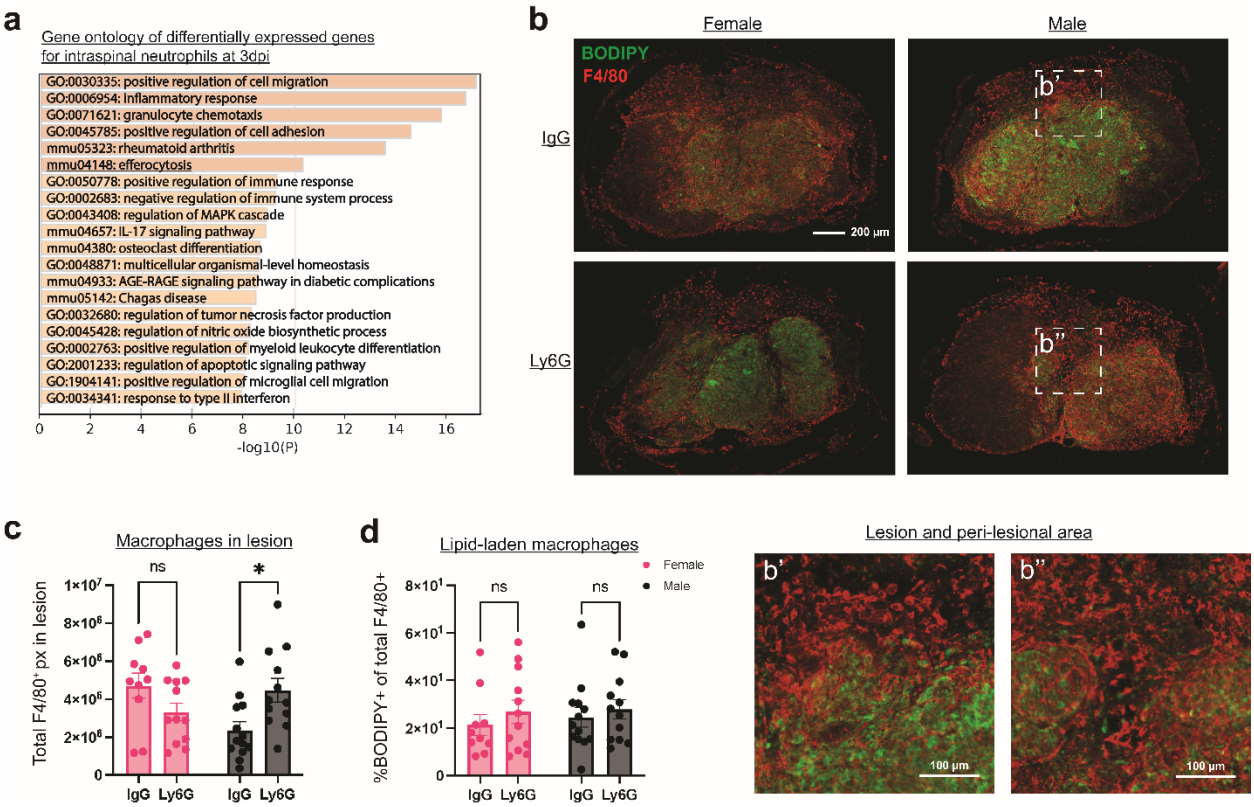

Figure 4. Neutrophils mediate resolution of inflammation after SCI in a sex-dependent manner. (a) Gene ontology analysis of differentially expressed genes in intraspinal neutrophils at 3 dpi vs. all other timepoints after SCI. (b) Representative images BODIPY labeling of neutral lipids (green) and immunostaining of F4/80 (red) in the spinal cord lesion epicenter at 35 dpi in female and male mice with neutrophil depletion (Ly6G) or control (IgG) antibody administered immediately after SCI. b' and b" are magnified insets. (c) Quantification of the total F4/80$^+$ pixels accumulated from seven serial sections spanning 1.5mm centered on the lesion. Neutrophil depletion exacerbates long-term macrophage accumulation in male, but not female neutrophils. n=10-13/sex/treatment. (d) Quantification of the total BODIPY$^+$ pixels out of total F40/80$^+$ pixels from seven serial sections spanning 1.5mm centered on the lesion. No differences in lipid-laden macrophages were observed. Two-way ANOVA with Sidak's multiple comparison test. *$p<0.05$. Abbreviations: SCI = spinal cord injury, dpi = days post-injury. Mean +/- SEM

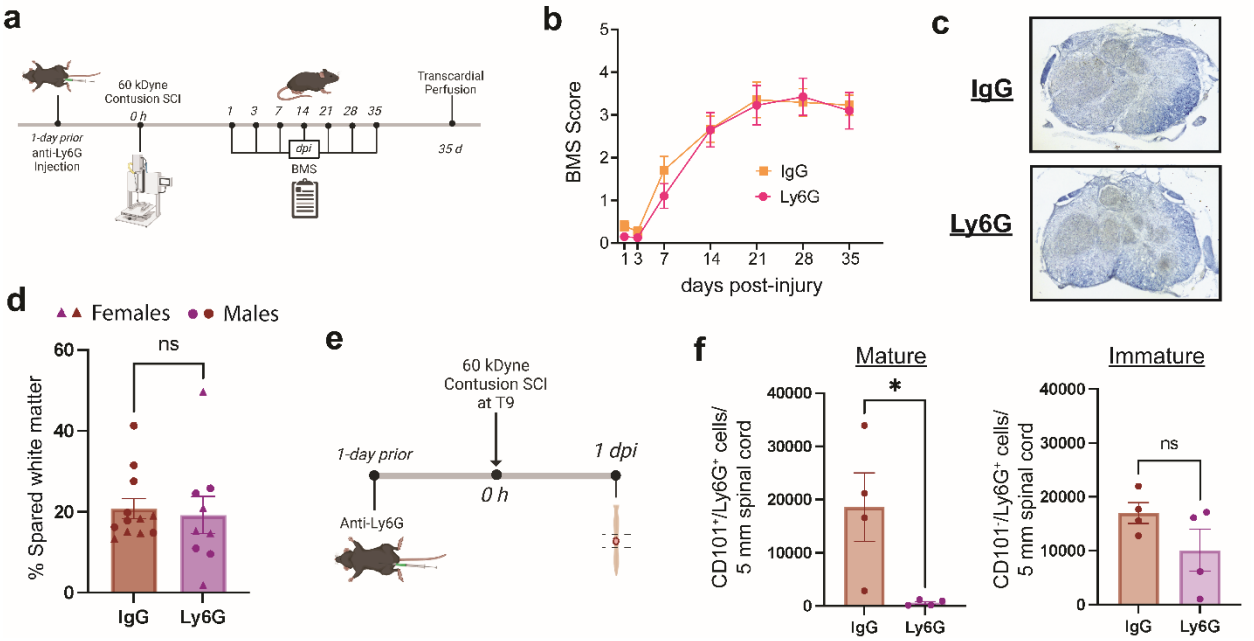

Figure 5. Depletion of mature neutrophils prior to SCI does not impair long-term recovery. (a) Schematic of experimental paradigm for pre-SCI neutrophil depletion. (b) Depletion of neutrophils at 1 day prior to SCI does not alter long-term BMS scores. n=5-7/sex/treatment. Repeated measures three-way ANOVA. (c) Representative images of the lesion epicenter stained with eriochrome cyanine for pre-injury neutrophil depletion (Ly6G) and control (IgG) animals. (d) Quantification of spared white matter at the lesion epicenter for male and female mice at 35 dpi. No sex-differences or effect of pre-injury neutrophil depletion were observed on long-term white matter sparing. n=4-7/sex/treatment. Unpaired two-tailed Student's t test. (e) Schematic of pre-injury depletion of neutrophils, SCI, and tissue collection for flow cytometry analysis. (f) Anti-Ly6G antibody administration substantially reduces intraspinal mature ($CD101^+$) neutrophil counts, but not intraspinal immature ($CD101^-$) neutrophils counts. n=4/sex/treatment. Unpaired two-tailed Student's t test. Abbreviations: SCI = spinal cord injury, BMS = Basso Mouse Scale, dpi = days post-injury. Mean +/- SEM.