SEX DIFFERENCES AND CONSEQUENCES OF PERIPHERAL BLOOD LEUKOCYTOSIS AFTER SPINAL CORD INJURY IN FISCHER 344 RATS

By

CARLOS ARMANDO AYALA

A dissertation submitted to the School of Graduate Studies

Rutgers, The State University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Neuroscience

Written under the direction of Wise Young

And approved by

Wise Young

_____

_____

_____

_____

New Brunswick, New Jersey

January, 2021

ABSTRACT OF THE DISSERTATION

Sex Differences and Consequences of Peripheral Blood Leukocytosis after Spinal Cord Injury in Fischer 344 Rats

By CARLOS ARMANDO AYALA

Dissertation Director:

Wise Young

Four times as many men as women have chronic spinal cord injury (SCI), suggesting that women have less severe SCI than men. The mechanism of this large sex gap is not known. SCI changes peripheral blood leukocyte counts, causing acute neutrophilia chronic lymphopenia after injury. Neutrophils infiltrate the injured spinal cord. We compared peripheral blood leukocyte responses in male and female Fischer F344 rats. Males had greater blood neutrophilia than females during the first days after SCI. We also measured myeloperoxidase (MPO) by Western Blot injured spinal cords. Surprisingly, MPO did not differ between male and female rats, despite greater peripheral neutrophilia in males. We assessed effects of blood leukocyte responses on locomotor recovery. We treated male and female rats with the chemotherapeutic agent cyclophosphamide (CYP) 2 days before SCI to reduce acute blood neutrophilia and evaluated locomotor recovery and neuronal and myelin sparing in the spinal cords at 6 weeks after injury. CYP prevented increases in blood neutrophil counts after SCI.

However, blood neutrophil counts did not correlate with tissue damage or behavioral recovery. Male rats recovered walking earlier than female rats regardless of blood neutrophilia. Differences in recovery were transient and not significant 3 to 6 weeks after SCI. Quantitative analyses of the rat spinal cords showed that standardized contusion injury caused similar tissue loss, myelin sparing and neuron survival in both sexes. However, male spinal cords were bigger in age matched 100-day-old F344 rats. Preventing acute blood neutrophil responses did not change walking recovery or tissue damage in either sex. These findings argue strongly against the hypothesis that acute neutrophilia is responsible for sex differences in recovery after SCI. We conclude that acute blood leukocytosis do not affect recovery or tissue damage after spinal cord injury in F344 rats.

ACKNOWLEDGEMENT

I would like to express my deep gratitude to Dr. Wise Young, the kindest human being I have ever met and whom as my advisor, fully supported my Ph.D. studies. His patience, prodigious knowledge and fervor for robust planning of experiments helped guide my research, understanding of science and led to a productive doctoral thesis. Dr. Young's guidance has unequivocally evolved my critical thinking, shown to me our research's impact and prepared me for an effective career in Neuroscience. I would like to thank my thesis committee: Dr. Patricia Buckendahl, who inducted me into research and taught to me many things about life; Dr. Martin Grumet, whose ability to think critically helped focus our research questions; and Dr. Long-Jun Wu, whose phenomenal attention to detail taught me valuable lessons for my career. I would also like to extend my sincere thanks to Dr. Patricia Morton, always there for me through tough times inspiring me to never lose hope. I also very much appreciate all the help from Dr. Dongming Sun, whose many critical thinking sessions advanced my ability to understand data. I am very grateful to Dr. Shao-Yun (Charlotte) Hsu for teaching me surgery. Mr. Sean O'Leary played a critical role in my success and I will forever be thankful for his patience and love for our animals. I would like to thank the entire staff of the W.M. Keck Center with special thanks to Dr. Noriko Kane-Goldsmith and Iman Tadmori, who taught me critical technical skills that made my research possible. I would also like to thank Dr. Gregory Voronin and, postdoctoral fellows Thomas Theis and Suneel Patel, true friends in this journey. Many, many thanks to the undergraduate students that enthusiastically worked with me: Ms. Shambavi Metgud, Ms. Noelle Messina, Mr. Adin Aoki, Ms. Meher

Pandher, Ms. Avina Rami, Ms. Ashwini Chidirala, Ms. Morgan Fishman, Ms. Audrey Zhou, Ms. Samantha Young and Mr. Krishna Raja. I would also like to thank my dear friends Mrs. Laurie Harden, Dr. Peter Andrusciewicz Jr., Ms. Sujitha Jayaraj, Ms. Marisa Gorab, Mr. Tomas Kasza, Mr. Ryan Brown, Dr. Emily Chang and Dr. Eduardo Estades for their unwavering encouragement. Finally, I would like to thank my parents and my sister for their unconditional support, love, and opportunity to make my dreams come true.

Table of Contents

List of Abbreviations

AIS	American Spine Injury Association Impairment Scale
APC	Allophycocyanin
APCs	Antigen-Presenting Cells
ASIA	American Spine Injury Association
ATP	Adenosine Triphosphate
BBB	Basso, Beattie And Breshnahan Scale
BCA	Bicinchoninic Assay
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CCR2	C-C Motif Chemokine Receptor 2
CD	Cluster Differentiation
CNS	Central Nervous System
CVC	Cranial Vena Cava
CXCR1	C-X-C Motif Chemokine Receptor 1

CYP	Cyclophosphamide
DAMPs	Damage Associated Molecular Patterns
df	Degrees of Freedom
EDTA	Ethylenediaminetetraacetic Acid
F344	Fischer 344 Rat
FBS-PBS	Fetal Bovine Serum - Phosphate Buffer Saline
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
G-CSF	Granulocyte Colony Stimulating Factor
H&L	Heavy and Light Chain
HRP	Horse Radish Peroxidase
IL	Interleukin
M1	Pro-Inflammatory Phagocytes
M2	Tissue Repair-Promoting Macrophages
Mac-1	Macrophage-1 integrin

MASCIS	Multicenter Animal Spinal Cord Injury Study
MBP	Myelin Basic Protein
MD	Mean Difference
MHC	Histocompatibility Complexes
mL	Milliliter
mm	Millimeter
MPO	Myeloperoxidase
MVA	Motor Vehicle Accidents
NA	Neuroscience Associates
NIH	National Institute of Health
η^2	Eta Squared
η_p^2	Partial Eta Squared
p	Significance
PE-50	Polyethylene-50
RIPA	Radioimmunoprecipitation Assay

RJV	Right Jugular Vein
RT	Room Temperature
RWJ-UH	Robert Wood Johnson University Hospital
SCBB	Spinal-Cord-Blood-Barrier
SCI	Spinal Cord Injury
SCI-IDS	Spinal Cord Injury Immune Deficiency Syndrome
SD	Standard Deviation
SM-TBS	5% skim milk dissolved in TBS
TBS	Tris Buffered Saline
Th1	T Helper 1
Th2	T Helper 2
TNF-a	Tumor Necrosis Factor-Alpha
T-Reg	T-Regulatory
UV	Ultra Violet
WR	Working Reagent

ε	Epsilon Correction for Sphericity Violation
η_p^2	Partial Eta Squared

Chapter 1. Introduction and Literature Review

1.1 Sex Bias in Animal Research

Four times as many men than women live with chronic spinal cord injury (SCI) [1-5]. In contrast, animal studies that only include one sex dominate the SCI literature with a significant preference for female rodents. Beery and Zucker [6] found the neuroscience field in 2009 had the most studies with one sex in all biological science fields. The National Institutes of Health (NIH) recommends that animal research include both sexes [7] because males and females respond differently to many pathologies including brain and spinal cord trauma [8, 9], stroke [10], shock [11-17], sepsis [13] and autoimmune disease [18-20]. Sex differences [10] in recovery occur in these pathologies and some evidence suggests the mechanism may be immune-related [21-23].

1.2 Sex Differences after SCI in Humans

Historically, men were more prone to SCI than women. However, contemporary trends in spinal cord trauma indicate that SCI is no longer primarily a young man's condition resulting from war and high-risk behaviors [24, 25]. According to Chiu, et al. [26], the man to woman ratio of incidence for SCI ranges from 1.73:1 to 4.3:1 in developed countries, with China reporting the lowest male-female ratio of 1.73:1, the United States 3.8:1, and developing countries Pakistan and Bangladesh reported the highest ratio of about 7.5:1.

Motor vehicle accidents and falls are major causes of spinal cord trauma [1, 27-30]. Cervical level injuries are the most common [27, 30], classified as complete or incomplete and rarely transecting injuries [3, 28, 31]. In some countries, women are more likely to suffer SCI from motor vehicle accidents than men [32, 33]. The number of injuries from falls in elderly women is also rising [28, 31]. In underdeveloped countries, Pott's disease (tuberculosis of the spine) causes more cases of SCI in women than men [34]. Increasing numbers of spinal-injured women has shifted clinical focus away from psychosocial perspectives [35, 36] to analyses of clinical outcomes in both sexes [37-40].

Comparison of outcomes after SCI in both sexes led to findings that spinal-injured men fare worse than spinal-injured women in several respects. First, morbidity and mortality are greater in men than in women after traumatic than non-traumatic SCI [41-44]. Second, more men die acutely [25, 45] and women have a longer life-expectancy than men after SCI [25]. Third, more men had complications by the start of rehabilitation, even though spinal-injured women tend to have more comorbidities [32, 46, 47]. Lastly, chronic rehabilitation outcomes favor women compared to men [31, 42, 44, 48-50]

The American Spine Injury Association (ASIA) Impairment Scale (AIS) measures injury severity and rehabilitation [51]. Sex differences in ASIA and AIS outcome measures have been known for decades [52]. For example, Scivolette, et al. [47, 53] reported that spinal-injured men achieve better AIS scores than women early during rehabilitation. Likewise, in a cohort of 14 thousand patients, Sipski, et al. [50] found that men with complete SCI recovered better than women, but that long-term recovery favors women

with incomplete SCI. Women reach higher motor index scores, improve their AIS classification more often and achieve higher functional independence scores than men at 1 year after SCI [50]. Fisher, et al., [54] reported that women have better motor recovery at 2 years after complete SCI. Nevertheless, some studies reported no differences in recovery between spinal cord injured men and women [31, 55, 56]. Findings of sex differences in recovery and rising numbers of spinal cord injured women should motivate researchers to do experiments in both sexes.

1.3 Sex Differences in Animal SCI Research

Animal models [57, 58] provide an opportunity to study the effects of therapies after spinal cord trauma in males and females. Standardized contusion injuries cause clinically relevant examples of motion recovery and tissue sparing [59]. Paradoxically, even though sex differences in incidence and rehabilitation after SCI have been known for decades, experiments with rodents that only include one sex dominate the SCI literature with a significant preference for females [26]. Investigators typically exclude male rodents because bladder care of males after SCI is considered more difficult [60].

Several researchers [61-65] reported that female rodents spare more spinal cord tissue and have better long-term recovery than males after contusion injuries, others found that males recover earlier [66] and have better long-term hind-limb function [67] while others [68-71] found no sex differences. The mechanism for sex differences in recovery after SCI in animal models was first thought to relate to spinal cord size and sex hormones [65]. Hsu [65] and Young et al., [57] reported that spinal cord length or weight did not differ significantly in age-matched F344 and Long-Evans Hooded rats,

respectively. Injury severity parameters, like spinal cord contusion velocity [72] and 24-hour lesion volume [73], are also similar in both sexes. Since spinal cord sizes and lesion volumes are similar, like in brain injury [74], there may be sexually dimorphic mechanisms that affect injury spinal cord tissue repair [75].

Reports of sex differences in recovery after SCI in humans and rodents led to experiments with sex hormones [76]. Sex hormones have a significant effect on immune responses after infection and trauma [14]. Elkabes and Nicot [76, 77] reviewed preclinical literature and reported a strong indication that estrogen or progesterone agonists and testosterone antagonists improve recovery after SCI. Sex hormone therapy reduced apoptosis [78], autophagy and inflammation [79]. However, it is not well understood why female rats recovered better than males regardless of ovariectomies [64] and orchiectomies [63]. Despite testing the effect of sex hormones, most of these studies still focused on only one sex, typically female. SCI sex hormone therapy in humans is not widely used due to limited clinical success, because feminizing male patients is controversial and experimental animal evidence has been primarily in females, which does not represent the human patient population.

1.4 Sex Differences in Immune Responses after CNS Trauma

The effects of biological sex in immune responses have gained notoriety because inflammatory responses are sexually dimorphic and influence tissue repair, response to infections and autoimmunity [14]. Gene expression differs between males and females in the immune system [80-82]. Y-chromosome [83, 84] and over-expression of X-

chromosome [85, 86] genes cause critical sex differences in immune cell function thought to manifest in injury models.

Sex differences after CNS injury are not unique to spinal cord trauma. Several rodent [87-92] and human [23] studies reported that brain infarcts are larger in males than females. Autoimmune disease in the brain is also more prominent in women, with 2-4 times higher incidence than in men around the world [93, 94]. Sex differences in infarct size [88] and autoimmune responses correlate with more intense cellular immune activity [95].

Immune cells, like myeloid-derived neutrophils, monocytes and microglia, infiltrate within hours after spinal cord or brain injuries. More myeloid-cells infiltrate injured brain tissue in males than females, correlating with worse functional recovery and less tissue sparing [89]. Sex differences after brain injury are thought to occur from increased myeloid cell activity that phagocytose myelin debris and injured cells [92]. As neutrophils, monocytes and microglia respond to tissue during the first days after injury, these cells secrete a storm of enzymes, pro and anti-inflammatory cytokines thought to propagate tissue damage and glial cell apoptosis [89].

Little is known about the effect of sex on immune responses after SCI. Far more researchers have investigated the cause for sex differences after brain injury than spinal cord trauma. Standardized brain injury rodent models and attention to include both sexes in research led to important discoveries under investigation to improve recovery after

brain trauma and stroke in both sexes [74]. The same must happen for the spinal cord research.

1.5 History of SCI animal research

Most human spinal cord injuries are contusions and very rarely transections [26]. In the early nineteen hundreds, Reginald Allen [96] standardized a weight drop contusion model in dogs and Armando Ferraro [97] did so in rabbits. Other injury models include spinal cord compression [98] and transection [99]. Rodents, particularly rats, are most widely used for standardized contusion SCI studies. Wrathall, et al., [100], Kwo, et al. [101], Gruner, et al. [72] and Cao, et al. [102] developed calibrated mechanical weight drop devices to contuse rodent spinal cords. Our laboratory developed the Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor to contuse T9-T10 laminectomy exposed rat spinal cords with a 10g rod [72]. MASCIS graded contusions produce consistent spinal cord lesions [73, 101].

1.6 The Immune Response after SCI

1.6.1 Immediate tissue response to injury

Pioneer SCI researchers Bailey Pearce [103], Alfred Allen [96] and Armando Ferraro [97] described that contusion injury causes acute hemorrhage in gray matter and nerve cell damage. Contusion injury rapidly indents the spinal cord [57] and preferentially damages large myelinated axons [104]. The indentation translates to longitudinal movements of the spinal cord. At contusion velocities exceeding 0.5 meters / second, blood vessels and axons break, red blood cells and platelets accumulate on endothelium of capillaries and form thrombi that worsens ischemia. Post-contusion hypoxic

conditions and necrosis increase extracellular calcium and ionic shifts [105, 106], adenosine triphosphate (ATP) depletion and lactate acidosis [107, 108], that propagate hypoxic cell damage.

Dying neurons, glia and oligodendrocytes [205][217, 218] in the injured spinal cord release pro-inflammatory interleukin (IL)1b [109], IL-8 [110], TNF-alpha [111-113], prostaglandins [114, 115], leukotrienes [116] and many other inflammatory proteins. Cell death propagates of lipid peroxidation in the hypoxic tissue environment [117]. Physical trauma and inflammation make the spinal-cord-blood-barrier leaky [118-122], and stimulate recruitment of immune cells [123-125] like resident microglia-derived-macrophages (MDMs) [126-128], blood monocyte-derived macrophages (BMDM) [127], blood neutrophils [125] and lymphocytes [129]. Immune cells first enter injured spinal cord during the initial central hemorrhagic necrosis [130] and then during the delayed apoptosis phases in white matter undergoing Wallerian degeneration weeks and months after injury [130-132]. Progressive tissue damage after the initial spinal cord injury, sometimes called “primary injury”, is collectively called secondary injury [133, 134]. Sex differences in inflammation and immune cell activity at the injured spinal cord may contribute to better tissue recovery in males compared to females.

1.6.2 Myeloid Cell Responses after SCI

1.6.2.1 Microglia Activation

Microglia are the first neural tissue responders to spinal cord trauma [135]. Microglia responses are necessary for brain and spinal cord repair [136]. Receptors on microglial processes detect damage associated molecular patterns (DAMPs) [137], ionic imbalance

[138], extracellular adenosine triphosphate [139] and inflammatory cytokines [140]. Detection of these acute injury signals activate microglia [141] to proliferate [142], secrete proinflammatory cytokines and to become ameboid macrophages [143]. Microglia functions after SCI have been extensively investigated [144].

1.6.2.2 Blood and Tissue Neutrophilia after SCI

Although most studies of inflammation at the injured spinal cord were done with female animals, it is clear that injured spinal cord cells and activated resident glia secrete large numbers of inflammatory signals [112, 113]. Immune responses after SCI are both local and systemic, leading to acute blood neutrophilia, an abnormally high neutrophil count, in mice [145], rats [146] and humans [147]. Injured spinal cord cells secrete potent neutrophil-chemotactic cytokines IL-8 [110, 148, 149], prostaglandins and leukotriene B4 [150, 151] that demarginate neutrophils from the bone marrow and peripheral reservoirs [152] into the blood stream. Neutrophil chemotactic signals seem to be stronger in males than females [153, 154].

Neutrophils are the first leukocytes to invade the injured spinal cord in response to inflammatory signals [110, 150], a leaky spinal cord-blood barrier and increased cell adhesion molecules on endothelial cells [155]. Depending on the type and severity of injury, neutrophils may appear adjacent to venules and capillaries as early as 4 hours and phagocytize necrotic neurons in hemorrhagic areas [110, 150, 156-158]. Neutrophils have a short half-life of several hours [159] and have been reported to persist for weeks after SCI [125, 126, 160]. Peak neutrophil infiltration in injured spinal cord lasts for a few days and coincides with a proinflammatory environment [161, 162]. Nieto-

Sampedro et al., [160], reported that males and females had similar numbers of neutrophils in the injured spinal cord at 15 days after injury. It is not known if blood neutrophil responses are sexually dimorphic during peak neutrophil activity within hours and days after SCI.

Once in the injury site, neutrophils phagocytize dying neurons and release granule contents like myeloperoxidase (MPO) [156, 163, 164], metalloproteinases [165-167] and other proteases considered both neurotoxic and helpful for tissue remodeling [110, 120]. Short-lived migrated neutrophils undergo apoptosis and recruit phagocytic monocyte-derived macrophages [168] that respond to "eat me" [168-170] signals in a process called efferocytosis [171, 172]. It was classically believed that blood neutrophils were bad for CNS injury [110, 113, 173]. However, recent evidence suggests that neutrophils may be more friends than foes for nervous system tissue repair [174]. Depleting only blood neutrophils with Ly6G antibody prevents normal recovery after SCI [175]. Although neutrophils are phagocytic in nature, neutrophils are key determinants of angiogenesis, secreting significant VEGF and other growth factors to help revascularize the injured spinal cord [176].

1.6.2.3 Recruitment of Monocyte-Derived Blood Macrophages after SCI

Spinal cord trauma triggers the spleen, the biggest monocyte reservoir, to release large numbers of monocytes into circulation [177]. Along with responding to neutrophil-derived chemotactic signals [168-170], blood monocytes use C-C Motif Chemokine Receptor 2 (CCR2) [178] and C-X-C Motif Chemokine Receptor (CXCR1) cell-adhesion dependent mechanisms [179] to enter the injured spinal cord [180]. Peak infiltration by

blood-monocyte-derived-macrophages (BMDM) after SCI differs among species [69, 126, 127]. BMDM mostly gather in the spinal cord mainly within one week after injury and continue to infiltrate into the spinal cord for months thereafter [69, 126, 127].

BMDMs spread centrifugally and longitudinally through the injury site [181] and initially far outnumber resident microglial-derived-macrophages (MDM) at the injury epicenter [182, 183]. Myelin phagocytosis converts and maintains BMDMs as proinflammatory M1 "foamy macrophages" [182-184]. Myelin ingestion reduces the ability of M1 BMDM to digest and process apoptotic neutrophils [183, 185]. In contrast, myelin phagocytosis turns MDM into trophic M2 phenotype. Over several days, MDMs surround and have been described to "herd" blood macrophages to the injury epicenter, restricting them to the injured tissue while proliferating astrocytes repair the surrounding cord [183]. A second late wave of bone-marrow derived M2 macrophages that enters the central nervous system through the choroid plexus has been hypothesized to reduce Wallerian degeneration [186].

1.6.2.4 Are neutrophils and monocytes good or bad for SCI?

Several studies showed that depletion of both blood neutrophils and monocytes with clodronates [187, 188] improved spinal cord tissue repair. Reduced cell adhesion molecule expression prevented leukocyte migration [163, 175, 189-194] after SCI, lowered proinflammatory cytokines and improved spinal cord tissue repair. Depletion of blood leukocytes with nitrogen mustards in rodents [164, 195, 196] and cats benefited spinal cord neuroregeneration [197, 198]. However, depleting only blood neutrophils with antibodies has recently been found to worsen recovery after SCI [175, 199].

Katoh et al., [200] showed that spinal cord injured patients with less leukocytosis had better neurological outcomes. From a clinical point of view, testing the effect of acute blood leukocyte responses provides a potential therapeutic target. However, depletion of blood immune cells is dangerous, even for healthy adults. A more acceptable clinical approach would be to dampen peripheral immune responses instead of depleting blood leukocytes. Doing so would answer two important questions. First, is acute blood leukocytosis harmful for SCI? Second, is dampening immune responses after SCI harmful or beneficial?

1.6.2.5 Sex Differences in Myeloid Cell Responses Affect CNS injury

General trauma causes more intense blood myeloid cell responses in males than females [145, 200-202]. More intense myeloid cell activity after brain injury correlates with worse recovery [87-92]. Doran, et al. [90] and Banerjee, et al. [88] reported that females get smaller brain infarcts and less myeloid blood cell infiltrate. Villapol, et al. [89, 92] and others reported more activated microglia in male than female mice after brain ischemia. Although immune responses are more intense after contusion injury in brain than spinal cord, similar immune pathways get activated in both injuries [162].

Sex differences in cells involved in tissue repair, like neutrophils and monocytes, could account for previously reported sex differences in spinal cord gray and white matter sparing [52]. Since sex differences in myeloid cell activity affect brain tissue repair and females tend to spare more injured spinal cord tissue [61], it is reasonable to hypothesize that dampening blood myeloid cell responses would assimilate recovery in both sexes after SCI.

1.7 Effects of SCI on Adaptive Immunity

B and T lymphocytes play key roles in long-lasting immunity. Lymphocytes are part of the "adaptive" immune system and can have long half-lives [203, 204]. Adaptive immunity creates long-lasting protection against pathogens and tolerance of self-proteins. B and T cells detect self or foreign antigens and work in conjunction with antigen-presenting cells to produce antibodies that bind and label pathogens or infected cells for phagocytosis [205]. SCI leads to decreased B and T cells in the blood and at lymph nodes [206]. Targeted killing of circulating lymphocytes with anti-CD20 antibody [207] and gene knock-out animal models with dysfunctional adaptive immune responses [208, 209] have positive effects on recovery after SCI, even though lymphocyte function in spinal cord repair is not understood.

B and T lymphocyte function has gained attention in SCI research for several reasons. First, spinal cord injured patients have more respiratory [210] and cutaneous infections [211, 212] and have a higher death-risk from recurrent pneumonia [213-217]. Second, B and T cells infiltrate the injured spinal cord in rodents [129] and humans [126] but their function in the injured tissue is not clear. Third, people and animals produce significant higher quantities of autoantibodies after SCI [218]. B and T cells may detect neural injured tissue fragments as foreign antigens and cause autoimmunity.

Because SCI patients have higher rates of infection, several investigators [219-221] believe that SCI causes an immune deficiency syndrome (SCI-IDs). However, the reason why SCI increases auto-antibody production but reduces ability to fight infections

remains puzzling. Sex differences in adaptive immunity after spinal cord trauma may affect long-term host-defense mechanisms and autoimmunity [222, 223].

T cells express cluster differentiation (CD) 3 protein on their surface that serves as co-receptor for CD4 and CD8 to recognize new foreign antigens, those that previously attacked the host, self-peptides for self-recognition, or self-peptides that cause autoimmune attacks. T cell precursors originate in the bone marrow, differentiate in the thymus [224] into antigen-specific naïve cytotoxic T cells, naïve Helper T cells or regulatory T cells [225] and circulate to lymphatic organs where they await interaction with major glycoprotein histocompatibility complexes (MHC) on antigen-presenting cells (APCs).

Bone marrow lymphopoietic cells produce B cell precursors. B cell precursors rearrange their genes in a process called somatic recombination to express unique cell membrane bound immunoglobulins that recognize a specific antigen [226]. B cells recognize antigens by themselves [227], endocytose it, process it and present it on an MHC-II surface protein [228], or recognize antigens displayed on APCs and form an immunological "synapse" [229] with T cells that respond to the same antigen. B cells that have recognized antigen or formed an immunological synapse will expand clonally into plasmablasts [230]. Some plasmablasts remain in lymphatic organs and secrete short-term IgM antibodies and others will become memory B cells [231]. Eventually, plasmablasts will mature into plasma cells as they undergo class switching through somatic hypermutation [230] to produce high affinity IgG antibodies that protect against

repeated infections. Plasma cells migrate to the bone marrow, spleen, and lymphatic tissues for long-term surveillance against their respective antigen [232].

1.8 Immunosuppressive Treatment for SCI

Therapies that modify peripheral immune responses have been of interest for several decades because SCI causes acute blood and tissue neutrophilia, then migration and differentiation of blood macrophages followed by lymphocyte infiltration into the injury site [125, 127]. These blood leukocytes participate in injury repair and subsequent responses to infections. Depleting the number of blood leukocytes, a form of immunosuppression, has yielded mixed results to understand the role of peripheral blood leukocytes in spinal cord tissue repair.

Cancer drugs, like cyclophosphamide (CYP), cause dose-dependent apoptosis of circulating blood leukocytes [233] and a transient rebound of bone-marrow hematopoiesis afterward [234]. Researchers have used other non-specific immunosuppressants methotrexate [235, 236] and cyclosporine-A [237, 238] to improve functional recovery. Methylprednisolone, widely used to treat SCI, is frequently given to dampen systemic inflammation. Bracken and colleagues [239] found that one methylprednisolone intravenous bolus dose, given within 8 hours after SCI, can improve recovery. However, no convincing evidence indicates that pretreatment with methylprednisolone before spinal cord injury is similarly beneficial as chemotherapeutic drugs at depleting circulating leukocytes [134].

A single dose of CYP given two days before injury prevents acute blood neutrophilia, reduces MPO activity at the injury site and improves locomotor recovery [240, 241]. Feringa, et al. [197] reported that CYP improved spinal cord regeneration after spinal cord transection. Since a single dose of CYP has a transient effect, this drug can target acute blood leukocytosis (neutrophilia) after SCI [200]. Understanding the role of acute peripheral blood neutrophilia after SCI is important because it is treatable with several inexpensive FDA approved drugs. Unfortunately, previous studies that suppressed immune responses were mainly in females and we do not know if immunosuppressive therapy after SCI affects males and females differently.

1.9 Thesis Objectives

In this thesis, I studied the effect of acute peripheral blood leukocytosis in male and female F344 rats after moderate spinal cord contusion at the T9-T10 thoracic level. Undergraduate students helped collect the data. First, we developed and validated an inexpensive manual method to count total leukocytes per µl of blood. Second, we compared peripheral blood neutrophil, monocyte and lymphocyte responses, as well as spinal cord neutrophilia, in male and female spinal cord injured F344 rats. Experiments showed that males had more pronounced blood neutrophilia and monocyte responses than females after SCI but sex differences in neutrophilia did not occur at the injury site. Third, we found that more intense blood neutrophilia in males did not worsen locomotor or tissue sparing compared to females by six weeks after injury. Lastly, immunosuppressive therapy with one CYP dose, given 2 days before injury, prevented acute blood neutrophilia in both sexes but more effectively in females than males.

Unexpectedly, preventing acute blood neutrophilia did not worsen nor improve recovery after SCI in either sex. Overall, results in this thesis demonstrate that an inexpensive method to count blood leukocytes, combined with flow cytometry, can be used to study blood leukocyte responses after SCI in rodents. Importantly, more intense blood neutrophil responses in males did not worsen recovery compared to females. In fact, blood neutrophilia may be inconsequential, because preventing blood neutrophil responses after SCI did not worsen tissue sparing nor locomotor recovery in neither male nor female F344 rats.

Chapter 2. Methodology

2.1 Blood Collection

2.1.1 Jugular Vein Catheterization

2.1.1.1 Catheter Preparation

Catheters were prepared by linking 2.5 cm long silicon tubing [0.63 outer diameter (OD) x 0.76 internal diameter (ID) mm, (SIL065, Braintree Scientific)] with 15 cm of polyethylene (PE) tube [0.97 OD x 0.53 ID, (BD Biosciences, 427516)]. The PE tube was wedged 5 mm into the silicon tip with micro forceps to form a tight seal between both segments. The silicon tip was beveled, catheters were checked for patency with distilled water then sterilized in 70% ethanol overnight.

2.1.1.2 Catheterization

The right jugular vein (RJV) was catheterized before laminectomy [242]. F344 rats were anaesthetized with 5% isoflurane then maintained at 2.5% flow rate. The rats' neck and intrascapular area were shaved, scrubbed twice with betadine and 3 times with 70% ethanol. Catheter placement required two skin incisions: one 5 mm at the intrascapular area and a 1.5 cm incision mid-neck above the right sternocleidomastoid muscle to expose the carotid triangle. A stainless-steel trocar was used to guide the catheter under the neck skin towards the intrascapular incision. A clove-hitch knot with a silk suture occluded RJV blood flow below the external jugular vein. A v-shaped incision with microscissors at the RJV opened a flap to insert the catheter 2 cm towards the right atrium, then the catheter was secured to the RJV with two silk half-hitch knots at each side of the cannula. After testing for patency, catheters were filled with 10 IU

heparinized saline solution to prevent intra-tube thrombosis and endothelization then sealed with bone wax, and the neck incision was closed with micro-wound clips. The first 50 µl of blood collected from catheters was discarded to prevent dilution with heparin saline. Blood collected from RJV catheters was stored in ethylenediaminetetraacetic acid (EDTA) coated tubes (Thermofisher, 22-689-03) then rats received 200 µl subcutaneous saline to replace lost fluid volume.

2.1.2 Cranial Vena Cava Venipuncture

Cranial vena cava (CVC) venipuncture technique [243], with some modifications, was used to collect blood for experiments in Chapter 5. Rats were anaesthetized with 5% isoflurane then maintained at 2.5% flow rate. Rats' necks were shaved, then scrubbed twice with betadine and 3 times with 70% ethanol. Using one hand, the rat's right and left arms were pulled towards the back with the thumb and middle finger, respectively then the index finger knuckle extended the rat's neck (Figure 1A). This position was maintained to insert a 30g needle (305128, BD Biosciences) bevel down towards the bulge that connects the jugular vein and CVC (Figure 1B), then the needle was held in place as drops of blood entered the syringe reservoir (Figure 1C). The syringe plunger was slowly pulled with the hand holding the syringe to collect 100 µl of blood. Blood collected with CVC venipunctures was stored in EDTA coated tubes. After blood collection, light pressure with a sterile gauze contained hemorrhage at the venipuncture site then rats received 100 µl subcutaneous saline to replace lost fluid volume.

2.2 Spinal cord injury

2.2.1 Laminectomy

Rats were anaesthetized with 5% isoflurane and maintained at 2.5% flow rate, their backs were shaved, then scrubbed twice with betadine and 3 times with 70% ethanol. The T10 spinous process was localized by counting from the 13th to the 10th rib, then a 5-cm mid-dorsal cutaneous incision was made to expose the T7 to T11 vertebral levels. A bilateral 0.5 cm incision adjacent to T9-T10 and blunt dissection of paravertebral muscles exposed spinous processes and vertebral laminae. Without tearing the dura or ligamentum flavum, the T10 and T9 spinous processes and vertebral laminae were removed with micro rongeurs to make a laminectomy 6x3 mm in length and width, respectively. A sterile gauze pad was used to stop hemorrhage throughout the surgery.

2.2.2 Spinal Cord Contusion

Male and female 100 ± 3-day-old F344 rats were spinal cord injured at T11 with a MASCIS impactor Model 1, as previously described [114]. Rat spinal cords were contused by dropping a 10 g, 2.1-mm diameter rod from a 12.5 mm height onto the T11 spinal cord level, centered at the laminectomy site at T9 and T10 vertebral level. After placing a sponge under the rat's belly, the T8 and T11 spinous processes were secured with clamps and the impactor rod was centered to the laminectomy. The contusion height was calibrated by lowering the impactor rod to the spinal cord until the impactor's LED light and alarm indicated that the tip touched the spinal cord. The impactor rod was set to drop 12.5 mm then released to contuse the spinal cord. Rats that had impacts with >5% error in contusion velocity and height were excluded and replaced. Immediately

after contusion, the impactor rod was secured to prevent re-injury, rats were unclamped then returned to the surgical table. A small piece of subcutaneous fat was placed over the spinal cord contusion site, the paravertebral muscles and subcutaneous fascia adjacent to the laminectomy were sutured, and the wound was closed with stainless steel clips.

Shortly after surgery and on the first day after, the rats received one 5 ml or 10 ml of subcutaneous saline injection for females and males respectively, followed by 25 mg / kg Cefazolin (a broad-spectrum antibiotic). Injured rats were housed in sterile cages with fresh bedding and maintained on a heating pad overnight after surgery. Our animal care supervisor, Sean O'Leary, monitored the injured rats for autophagy, infections and expressed urine in the rats twice daily for 48 hours and then once daily until rats had empty bladders in the morning, indicating that they were able to urinate.

2.3 Quantification and Differentiation of Blood Leukocytes

2.3.1 Linear Smears

2.3.1.1 Linear Smear Blood Sample Preparation

390 µl of 10 µM Hoechst 33342 (Thermofisher, 62249) in 0.9% saline (Braun, S8004-5264) were added to an eppendorf tube and weighed on an analytical scale. 10 µl of blood were slowly pipetted with a 10-20 µl micropipette tip and added to the eppendorf tube. After rinsing the pipet tip 3 times with the diluent to wash all blood from the pipette tip, each sample was re-weighed to ensure blood samples were 10µg ± 0.1µg. Sample preparation was repeated until the blood weight criteria was met. The diluted blood was vortexed for 2 seconds and incubated at 37° in the dark for 30 minutes, then mixed gently half-way through the incubation period.

2.3.1.2 Linear Smear Slide Preparation

A linear smear template (Figure 2A) was placed under a positively charged microscope slide (Thermofisher, 22-037-246). A 10-20 µl micropipette tip was used to uptake 5 µl of well-mixed blood dilution, then the 5 µl were slowly released at a 45°- 60° angle onto the microscope slide to trace the entire linear smear template (Figure 2A). After air-drying for 10 minutes, linear smears were cover-slipped with organic non-aqueous mounting medium (Baxter, Protexx) and dried overnight at room-temperature protected from light.

2.3.1.3 Quantification of total leukocytes under the microscope

Linear smear preparations clearly distinguish brightly Hoechst-stained nucleated leukocytes from a sea of enucleate erythrocytes (Figure 2D). To count blood leukocytes, the proximal end of the linear smear was located with visible light, leukocytes were seen with an ultra violet (UV) epifluorescence lamp (Zeiss, Axiophot) and counted down the linear smear path. Blood leukocyte counts in linear smears represent a 1:8 dilution of leukocytes per µl of blood. Linear smear blood leukocyte counts multiplied by 8 equal the total number of leukocytes per µl blood.

2.3.2 Blood Smears

2.3.2.1 Blood Smear Preparation

5 µl of blood were placed on a microscope slide. Another clean slide was used to spread the blood drop at a ~30° angle (Figure 3A) to form a feather-like blood smear, (Figure 3B). After air-drying, blood smears were dipped into Wright stain (Sigma, SW-16) in a coplin jar for 20 seconds, then transferred to deionized water for 2.5 minutes, then quickly rinsed with 1 ml of deionized water to remove excess dye debris. Air-dried blood

smears were cover-slipped with non-aqueous histology mounting medium (Baxter, Protexx).

2.3.2.2 Differentiation of Leukocytes in Blood Smears

Blood leukocytes were identified at 63x magnification with a bright-field light microscope (Zeiss, Axiophot) as nucleated cells compared to enucleate erythrocytes. Wright stain colors leukocyte nuclei shades of blueish purple and enucleated erythrocytes pink. 300 blood leukocytes were defined as neutrophils, monocytes, lymphocytes and other granulocytes according to hematological criteria. Monocytes and macrophages were the largest leukocytes, with irregular rod-shaped nuclei, white vacuoles and lilac blue cytoplasm (Figure 3C). Lymphocytes were the smallest leukocytes with scant cytoplasm and large dark purple round or oval nuclei (Figure 3D). Neutrophils were larger than lymphocytes, had polysegmented nuclei and poly-chromatic granules (Figure 3E). Blood eosinophils, mast cells and basophils make up about 2% of the circulating blood leukocyte pool, have irregular shaped nuclei and prominent monochrome granules. Undergraduate Student Noelle Messina helped to count blood neutrophils, monocytes and lymphocytes from blood smears.

2.3.3 Flow Cytometry

2.3.3.1 Sample Preparation for Flow Cytometry

Erythrocytes in 50 µl of whole blood were lysed with 500 µl of ammonium chloride (Thermofisher, 00-4333-57) for 2.5 minutes at room temperature, then washed twice with 1mL of 2% Fetal Bovine Serum in Phosphate Buffer Saline (FBS-PBS) at 300g, 4°C and for 5 minutes (wash buffer). Blood leukocytes were incubated with mouse monoclonal

pre-conjugated antibodies and respective isotype controls. We used 1:200 anti-CD45-Vioblue (BD Biosciences, 561587), a pan-leukocyte antibody, to tell apart leukocytes from erythrocytes and platelets. $CD45^+$ leukocyte subpopulations were identified with 1:100 anti-CD11b-Flourescein isothiocyanate (FITC) (BD Biosciences, 554982) that labels myeloid-derived cells, 1:50 anti-RP-1-Phycoerythrin (PE) (BD Biosciences, 550002) for rat neutrophils [244], and 1:200 anti-CD3-Allophycocyanin (APC) (BD Biosciences, 557030) and 1:100 anti-CD45R-FITC (BD Biosciences, 554880), that distinguish T and B cells, respectively. All samples incubated in the dark for 30 minutes at 4°C, were washed twice with buffer then reconstituted to 100 µl. Dead cells were excluded with 1:100 propidium iodide, incubated for 1 minute, immediately before signal capture.

2.3.3.2 Flow Cytometric Differentiation of Blood Leukocytes

Leukocytes were differentiated with a MACSQuant flow cytometer (Miltenyi). An UV detection trigger excluded all non-CD45-$VioBlue^+$ erythrocyte debris and platelets. We determined optimal gating with isotype controls: IgG1-VioBlue-FITC (BD Biosciences, 561504), IgG2a-FITC (BD Biosciences, 556652), IgM-APC (BD Biosciences, 550883) and IgG2a-PE (BD Biosciences 553497). For experiments in Chapter 4, CD45-$VioBlue^+$ leukocytes were gated according to isotype controls to quantify the percent of $CD11b^+$ myeloid cells, $RP1^+$ neutrophils, $CD3^+$ T cells and $CD45R^+$ B cells.

For Chapters 5 and 6, $CD11b^+$ blood myeloid cells were further differentiated into neutrophils and monocytes based on scatter signals as previously shown by Stirling et al., [128]. Cells scatter light forward and sideways as they pass through interrogating lasers in flow cytometers. Optical detectors convert scattered light into electrical signals. Forward scatter is light diffracted around the cell and relates to cell size. Intracellular components reflect and refract light to cause side scatter. Neutrophils have large cytoplasmic granules and polymorphic nuclei while monocytes have fine cytoplasmic granules and one bilobed nuclei [245, 246]. Large granules and polymorphic nuclei cause high side scatter signals in neutrophils compared to monocytes (Figure 4). We defined high side scatter $CD45^+CD11b^+$ $SSC^{(hi)}$ myeloid cells as neutrophils and low side scatter $CD45^+CD11b^+$ $SSC^{(lo)}$ myeloid cells as monocytes.

2.4 Quantification of SCI Recovery

2.4.1 Assessment of locomotor function after SCI

Sean O'Leary and I graded bilateral hindlimb motor function after SCI with the open field Basso-Beattie-Bresnahan (BBB) locomotor scale [247]. Rats were placed one at the time to acclimate in a circular open field for 5 minutes, then hindlimb function was scored for 4 minutes. Animals with no detectable hindlimb movement received a zero score. Hind-limb function was assessed at 2 days after injury and weekly thereafter for 6 weeks. 1 male and 1 female rat with BBB scores above 2 at 2 days after SCI were excluded from the analysis.

2.4.2 Neuron and Myelin Immunohistochemistry

2.4.2.1 Spinal Cord Microtome Sectioning

Sean O'Leary euthanized 36 F344 rats by anesthetizing (45-65 mg/kg pentobarbital) and perfusing the rats with 0.9% saline and then 4% paraformaldehyde at 6 weeks after SCI. I dissected the spinal cords in 30 mm segments centered at the injury site, post-fixed in 4% paraformaldehyde overnight and then stored the spinal cords in 30% sucrose at 4°C. Spinal cords were sent to Neuroscience Associates (NA) for sectioning and staining (Knoxville, Tennessee, USA). Spinal cords were incubated overnight in 20% glycerol and 2% dimethyl sulfoxide to prevent freeze-artifacts and embedded in a gelatin matrix block. This block was flash frozen with chilled 2-methylbutane, mounted to an AO 860 sliding microtome freezing stage, and sectioned to 40 μm in thickness on the transverse plane. All sequential sections were collected in separate cup containers. All containers contained antigen preserve solution (50% PBS pH7.0, 50% ethylene glycol, 1% polyvinyl pyrrolidine). Tissue section sets contained one spinal cord slice spaced 1-mm apart.

2.4.2.2 Immunohistochemistry

NA did immunohistochemistry on free floating sections. Tissue sections were treated with hydrogen peroxide and blocked for non-specific binding with serum from the antibody host. Separate tissue section sets were immunostained simultaneously with either chicken anti-myelin Basic Protein (MBP) antibody at 1:250,000 (Encor, CPCA-MBP) or rabbit anti-NeuN at 1:50,000 (Abcam, Ab104225) overnight at room temperature. Primary antibody was removed, tissue sections were washed with Tris

buffered saline (TBS) with Triton X-100, rinsed with TBS then incubated with biotinylated secondary antibodies for one hour at room temperature. Antigen detection was visualized by incubating tissue sections with avidin-biotin-HRP complex (Vector, PK-6100), rinses, then treatment with diaminobenzidine tetrahydrochloride and hydrogen peroxide to create a visible reaction. The sections were then mounted on gelatin-coated glass slides to air-dry, cleared with xylene and cover-slipped with anti-fade mounting medium.

2.4.2.3 Immunohistochemistry and Quantification

All slides were scanned in monochrome at 100x magnification with each slide containing one transverse section from all samples. We used ImageJ to measure the spinal cord total cross-sectional and myelinated area in intact and injured spinal cord segments. To measure cross-sectional area (gross tissue sparing), ImageJ's edge detection tool outlined the spinal cord border and measured the area inside the spinal cord in mm^2. To measure myelin sparing, we applied a threshold to detected MBP signal without background noise, then ImageJ measured the area of MBP signal per tissue section in mm^2. To count neurons, I wrote a macros for ImageJ that used edge detection to outline the spinal cord, remove background noise, then signal from each $NeuN^+$ nuclei was condensed to one signal maxima. This strategy permitted the quantification of each individual $NeuN^+$ neuron nuclei present in a 40 μm section. For each spinal cord, the tissue section with the smallest spared area was designated as the injury epicenter (Figure 5).

2.5 MPO Protein Quantification by Western Blot

2.5.1 Tissue Lysis and Protein Quantification

At three days after contusion, rats were anaesthetized with 5% isoflurane and decapitated to harvest the injured spinal cords (M: n=8, F: n=8). The spinal cord was frozen with crushed dry-ice, then the injury epicenter (5 mm) was frozen at -80°C. Samples were thawed and shredded with a Dounce homogenizer (10 passes) in 300 µl of chilled 1x radioimmunoprecipitation assay (RIPA) buffer (Sigma, 20-188) with protease inhibitor (Sigma, 11836153001), then agitated for 2 hours at 4°C. Supernatants were collected after samples were centrifuged at 10,000g for 10 minutes at 4° C. We used a Pierce bicinchoninic assay (BCA) protein assay kit (Thermo Scientific, 23227) and a microplate reader (Thermo Scientific) to determine calorimetrically the protein concentration in each sample. BCA working reagent (WR) was prepared from 1-part Reagent B and 50 parts reagent A and bovine serum albumin (BSA)was serially diluted from 2 mg / ml as protein standard. 20 µl of sample or standard and 200µl WR were added to each well in a 96 well-plate, the plate was sealed with an adhesive cover and placed on shaker for 30 seconds. Samples then incubated for 30 minutes at 37 °C, cooled for 5 minutes, and calorimetric signal was detected with a microplate reader at 570 nm. Protein concentration in each injured spinal cord sample was determined in comparison to the standard curve. Undergraduate students Shambhavi Metgud and Morgan Fishman helped with Western Blot experiments.

2.5.2 Gel Electrophoresis

To prepare the sample for gel electrophoresis, we pipetted 5µl of NuPage antioxidant (Thermo Scientific, NP0005), 25µl of NuPage lithium dodecyl sulfate sample buffer (Thermo Scientific, NP0007), 100 µg of protein solute into an eppendorf tube and

standardized to 100 µl of solution with protease free deionized water. Samples were heated at 95 °C for 10 minutes and then chilled on ice. We placed a 10-well NuPage acrylamide 4-12% Bis-Tris gels (Thermo Scientific, NP04120BOX) in an electrophoresis tank with 4°C 1x sodium dodecyl sulfate running buffer (Thermo Scientific, NP0002). 1-well received 5 µl of protein ladder (Bio-Rad, 1610373) and 40 µg of protein per sample were loaded on the other wells. Gel electrophoresis separated proteins by size at 200V in 20 minutes. Gels were placed on an iBlot polyvinylidene difluoride transfer stack (Thermo Scientific, IB401031) to blot proteins with an iBlot2 system (Invitrogen) for 7 minutes at 20V and 1.3A current.

2.5.3 Myeloperoxidase Immunochemistry

All incubations and wash steps for Western Blot experiments took place on a shaker at 10 RPM. Blots incubated at room temperature (RT) for 2 hours with 5% skim milk dissolved in TBS (SM-TBS) to block non-specific protein binding sites. Blots then incubated overnight at 4°C with 1 µg / mL polyclonal rabbit anti-myeloperoxidase (Abcam, ab45977) in SM-TBS. We washed the blots 3 times for 5 minutes at RT with 0.1% Tween 20 (Sigma, P9416) in TBS (TBS-T). Blots then incubated with 1:5000 Horse Radish Peroxidase (HRP) donkey anti-rabbit polyclonal secondary antibody (Abcam, 16284) in SM-TBS for 1 hour at RT, then were washed 3 times for 5 minutes at RT with TBS-T.

2.5.4 Signal Acquisition

We mixed 1 ml of Pierce ECL Western Blotting Substrate Kit Reagent 1 and Reagent 2 (substrate) (Thermo Scientific, 32106), added the mix to the blots and incubated for 2

minutes. We used an Odyssey Fc imaging system (LI-COR) to capture an MPO protein band (84kDa) after a 2-minute exposure.

2.5.4 Blot Stripping

Blots rested in a container with Restore Western Blot Stripping Buffer (Thermo Scientific, 21059) for 10 minutes at RT then were washed 3 times for 5 minutes with TBS-T at RT.

2.5.5 Loading control

After stripping, non-specific protein binding sites were blocked again for 2 hours with SM-TBS at RT. Blots incubated for 2 hours at RT with 1:5000 mouse monoclonal anti-Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Abcam, 8245). Blots were washed 3 times with TBS-T for 5 minutes and incubated at RT with 1:5000 HRP goat anti-mouse IgG H&L (Abcam, Ab6789) in 5% skim milk in TBS for 1 hour. Blots were then washed 3 times with TBS-T for 5 minutes and signal for GAPDH was acquired with the Odyssey Fc imaging system (LI-COR) for 20 seconds.

2.5.6 MPO Quantification and Statistical Analysis

We exported images from the LI-COR Odyssey Fc imaging system, opened them with ImageJ, quantified the signal for each MPO and GAPDH band with the “Gels” ImageJ macros, calculated the ratio of MPO / GAPDH for each sample, and compared male and female samples with an ANOVA in SPSS.

2.6 Cyclophosphamide Treatment

I tested the safety of 80, 40, 20 mg / kg of cyclophosphamide (CYP) (Sigma, C7397-1G) dissolved in 1 ml of diH2O (distilled water) after SCI. Male and female rats received one subcutaneous CYP dose at 48 hours before SCI (3 groups, Female: n=1, Male: n=1, per group). Sean O'Leary and I monitored skin turgor, eye rheum build-up and cage-rummaging by each rat after dosing to assess dehydration, suffering and physical activity, respectively. Further experiments utilized one 25 mg / kg CYP dose or distilled water vehicle (VEH) treatment.

2.7 Statistical Analysis

Statistical analyses were carried out with SPSS version 25.0 and with Statview version 2.0. Graphs were made with OmniGraffle. We used *one-way repeated measures ANOVA* to determine if blood leukocyte counts changed significantly over 14 days after SCI in males and females. *Scheffé's' post hoc* tests identified from when to when blood leukocyte counts differed after SCI. *2-factor repeated-measures ANOVA* (Time*Sex) tested if biological sex had a significant effect on blood leukocyte responses after SCI. *2-factor repeated-measures ANOVA* (Time*Treatment) tested if CYP or VEH treated groups had a significantly different blood leukocyte responses after SCI in either males or females. Pairwise comparisons with unpaired T-tests revealed at which time-points sex differences in blood leukocyte counts were most pronounced.

We used a *2-factor repeated-measures ANOVA* that tested for biological sex differences in spinal cord area (Tissue-Sparing*Sex), myelinated tissue area (Myelin-sparing*Sex) and neuron counts (Neuron-survival*Sex). Since we found sex differences in blood neutrophilia and sex differences in response to therapies are well known, we restricted

our analysis of the effect of CYP treatment to each gender. A *2-factor repeated-measures ANOVA* (BBB*Treatment) assessed if CYP-treatment had a significant effect in locomotor recovery over 6 weeks after injury in males or females. *Scheffé's post hoc* tests identified when BBB scores differed most significantly between CYP and VEH treated groups. Similarly, *2-factor repeated-measures ANOVA* (Tissue*Treatment) assessed if CYP-treatment had a significant effect on tissue sparing and neuronal survival.

We analyzed both, the extent of tissue damage and the size of spinal cords. We used pairwise comparisons with unpaired T-tests to compare the volume of spared spinal cord tissue and myelin content between males and females at the injury site. Unpaired T-tests were also used to test the hypothesis that the volume in intact spinal cord segments did not differ between males and females. The analyses yielded *F* values for the ratio of variance between sample means to variance within samples. p values are the likelihood that the null hypothesis is correct (E.g. no effect of SCI, sex or treatment on leukocyte counts over time). Effect sizes (η^2) or (η_p^2) represent the proportion of variance contributed by the dependent variable. The null hypothesis was rejected at $p \leq 0.05$ and Scheffé's post hoc method identified when blood leukocyte counts differed after SCI and by how much. Blood leukocyte counts are shown as mean ± standard error of the mean per µl of blood.

Chapter 3: Blood Leukocyte Counts with a Linear Smear

3.1 Introduction

Clinical grade hematology analyzers are the gold standard for counting human blood leukocytes [248]. Veterinary hematology analyzers [249] and flow cytometers are used to count leukocytes in other species. However, access to hematological analyzers may be cost prohibitive or not feasible. A reliable and inexpensive manual method to count total white blood cells (WBCs, leukocytes) per μL of blood would be helpful.

The standard manual method of counting blood leukocytes uses a hemocytometer. A known blood volume is diluted with a stromatolyser agent to lyse cell membranes and a dye to label nuclei, like acetic acid and methylene blue, respectively [250]. Lysed blood cells are placed on a hemocytometer with a coverslip, labeled nuclei are counted and expressed in cells per box in the grid. Unfortunately, hemocytometer leukocyte counts are unreliable [251, 252] for several reasons. First, cell membrane lysis leads to fragments and cell settling may vary among samples [253, 254] and lead to miscounts of leukocyte nuclei. Second, blood must be diluted at least 1:20 for hemocytometer counts and low numbers of countable leukocytes causes high inter-sample variability [250]. Neutrophils may also be miscounted after membrane lysis because thin chromatin strings hold together their lobed polymorphic nuclei [255, 256].

We invented and tested the “linear smear” to serve as a manual method to count blood leukocytes. The linear smear uses inexpensive materials and rivals hematological

analyzers to count the number of white blood cells per microliter of blood. 10µl of blood sample are mixed in normal saline and Hoechst stain, then this blood dilution is traced on a microscope slide in a thin linear smear pattern. After airdrying, leukocytes on the linear smear path are easily seen under 100x magnification with a UV fluorescent filter. Blood leukocytes in linear smears are not lysed and stand out in contrast to a sea of enucleate red blood cells. Total blood leukocyte counts are a helpful addition to multicolor flow cytometry or blood smears that give results as percentage ratios. Absolute counts of blood neutrophil, monocyte and lymphocyte subtypes would be more helpful while studying pathologies that trigger abrupt changes in blood leukocytes, like spinal cord injury.

3.2 Linear smear protocol

The linear smear method requires the following steps. First, pipette 390 µl of 0.9% saline, containing 10 µM Hoechst 33342 and 5 µM EDTA into an eppendorf tube. Second, slowly pipette 10 µl of anticoagulated blood into the bottom of the tube, rinse the micropipette tip in untouched diluent to remove any remaining viscous blood, and weigh the tube before and after adding the blood to confirm that the blood weight is 10 ± 0.1 milligrams. Third, draw or print a S-shaped template with three connected 35-mm lines spaced 5 mm apart on paper and place the template under a positively charged glass microscope slide (Figure 2A). Fourth, vortex the sample for 2 seconds, incubate at 37°C for 30 minutes and aspirate 5 µl of the sample with a 2-20µl micropipette tip. Importantly, dispense the sample slowly at a ~45° angle following linear smear template (Figure 2A) to completion without touching the pipette tip touching the microscope slide

surface. Lastly, air-dry the linear smears, protected from light for 10 minutes, and coverslip with non-aqueous mounting medium (Protexx, Baxter). Properly dispensed linear smears should be ~1 mm in diameter (Figure 2C) and fit within a 100x magnification diameter field of view (Figure 2D).

Use a microscope with UV detection and100x magnification to count the total number of blood leukocytes in a linear smear. First, identify the proximal linear smear end with bright field illumination, then switch to the UV filter. Next, count fluorescent cells down the linear smear track (Fig. 3D). Hoechst 33342 permeates cell membranes, attaches to adenine rich nucleic acids in leukocyte nuclei to distinguish them from anucleate erythrocytes. Fluorescent leukocytes in linear smears stand out from a semi-confluent erythrocyte track (Fig. 3D). To calculate the total number of leukocytes per µl of blood from linear smears, multiply leukocyte counts by 8 to account for a 1:8 dilution factor.

3.3 Linear Smear validation

Leukocyte counts in linear smears were compared with veterinary hematological analyzers and a Cellometer (Auto-2000, Nexcelom). Rat blood leukocyte counts in linear smears correlated closely with a Sysmex XT 2000iV hematological analyzer (N=9, R= 0.94, Figure 2E). Mouse blood leukocytes correlated well with a Heska Element 5HT hematological analyzer (N=12, R= 0.97, Figure 2F). Rat leukocyte counts also matched those from a Cellometer (N=19, R= 0.95, Figure 2G). Leukocyte counts in different linear smears from one sample had a low coefficient of variability (N=8, CV= 1.43%). Likewise, repeated leukocyte counts from the same linear smear were consistent

(N=4, CV= 0.575%, Fig. 1H). These results indicate that leukocyte counts from linear smears are reliable and comparable to automated instruments.

3.4 Discussion

Linear smears have several advantages over automated hematological analyzers and hemocytometers. First, linear smears need inexpensive reagents and no automated instruments. Second, this method can be used to count blood leukocytes in 10 µl of blood from different mammalian species without species-specific instrument calibration [257]. Third, leukocyte counts in linear smears rely on a quality control step that ensures accurate blood volume for each sample. Accurate pipetting skills and calibrated equipment are key to the inexpensive quantification of blood leukocytes with this protocol. Fourth, sample slides may be cover-slipped, counted manually or digitally scanned before long-term storage. A disadvantage of the linear smear is that, although it provides absolutely blood leukocyte counts per µl of blood, it cannot be used differentiate leukocyte sub-populations.

Total blood leukocyte counts from linear smears are a helpful addition to pre-established flow cytometry work-flows [258]. Blood leukocyte subpopulations are detected with antigen-specific antibodies. Since blood samples for flow cytometry are typically reconstituted during erythrocyte lysis steps, the percentage of each leukocyte subpopulation is commonly reported. Absolute numbers of neutrophils, monocytes or lymphocyte subpopulations per µl or mL of blood can be calculated after multiplying to the total number of blood leukocytes counted in linear smears. Absolute leukocyte subpopulation counts are more useful to track cellular changes in leukocytosis,

leukopenia and immunosuppression [259]. We hope the linear smear will be used as an inexpensive, reliable and accessible method for laboratories around the world to count the total number of blood leukocytes from 10 μl of blood.

Chapter 4. Sex Differences in Blood Neutrophilia after SCI

4.1 Introduction

SCI was classically considered a young man's condition resulting from war and high-risk behavior. Today, more women than ever live with paraplegia and tetraplegia world-wide [32]. For unknown reasons, females tend to recover better than males after SCI in humans and rodent animal models [52]. Paradoxically, although more spinal cord injured patients are men and the female sex may be advantageous for recovery, animal studies that only include one sex dominate the SCI literature with a significant preference for female rodents [75]. Including animals of both sexes in SCI research will improve the quality of experimental findings and their clinical translation.

The National Institutes of Health (NIH) recommends that animal studies include males and females [7] because sex differences are prominent in immune responses [80, 82, 260] and recovery after brain injury [8-10, 23], shock [11-17], infection [261] and autoimmune disease [18-20]. Standardized spinal cord injury rodent models [57-59] provide an opportunity to study the causes for biological sex differences in functional recovery and tissue sparing.

Spinal cord contusion, the most clinically relevant spinal cord injury model, produces strong inflammatory responses both at the lesion site and in peripheral blood. Shearing forces from contusion injuries kill cells, break capillary endothelial and glial cell networks [262, 263], leading to significant lipid peroxidation products from broken membranes [117]. After SCI, dying neurons, glia and oligodendrocytes release a storm of proinflammatory signals like interleukin (IL)1b [109], IL-8 [110], TNF alpha [111-

113], prostaglandins [114, 115] and leukotrienes[116]. Both, immediate injury and subsequent proinflammatory signals make the spinal-cord-blood-barrier leaky [118-122], and stimulate recruitment of immune cells [123-125].

Immune blood cells: myeloid-derived blood neutrophils and monocytes and, lymphocyte-derived T-cells and B-cells infiltrate injured spinal cord tissue [125, 127]. Myeloid-derived blood neutrophils, monocytes and microglia are the first responder immune cells to arrive at injured spinal cord tissue. Blood neutrophilia, an abnormally high blood neutrophil count, occurs within hours after injury, in mice [145], rats [146] and humans [147]. SCI-induced blood neutrophilia correlates with neutrophil invasion and a proinflammatory environment at the injured spinal cord. Blood neutrophilia is also thought to damage peripheral organs and upregulate inflammatory cytokines in the liver [264, 265].

During peak neutrophil activity after SCI, the spleen [177] releases significant numbers of bone-marrow-derived-monocytes that have high expression of cell adhesion proteins [155]. These peripheral blood monocytes differentiate into pro and anti-inflammatory macrophages that infiltrate the injured spinal cord during first during the first days after injury and then for weeks thereafter. Peripheral blood myeloid cells, the neutrophils and blood-macrophages, help repair injured nervous tissue but their proinflammatory and phagocytic nature may lead bystander tissue damage during the first days after SCI [266].

Although the spleen releases many blood monocyte-derived macrophages destined for the injury site, other lymphatic cells do not have the same fate. Several groups have shown

that SCI in rodents leads to severe reduction in the number of splenic and circulating lymphocytes. Dysregulated adrenergic activity after SCI floods lymphatic organs with stress hormones that induce lymphocyte apoptosis [128, 145]. Paradoxically, although SCI leads to significant increases in circulating auto-antibodies [222], SCI patients are more prone to lethal infections from what some consider an "SCI-induced immune deficiency syndrome" [267].

Although blood leukocyte responses after SCI are well known, these experiments included only one sex and were mainly done with females. Studying immune responses after SCI in both sexes is relevant because more intense blood myeloid cell responses in males correlate with worsened recovery and more tissue damage than in females after brain trauma [90, 268]. Sex differences in immunity also affect responses to infections [82] and predisposition to auto-immunity [69, 71], both clinically relevant for SCI.

In this study, we counted the absolute number of blood leukocytes for two weeks with linear smears and flow cytometry for two weeks after SCI in male and female age-matched F344 rats. We found sex differences in acute blood neutrophilia and monocytosis within the first week after injury. SCI also caused long-term blood B-cell lymphopenia, but no significant changes in T cell numbers, in both sexes. Since blood neutrophils and monocytes actively infiltrate the spinal cord within hours and days after injury, sex differences in myeloid cell responses may affect acute inflammation, injury resolution and outcomes after SCI.

4.2 Results

4.2.1 SCI Leads to Chronic Leukopenia

Blood leukocyte (White Blood Cell, WBC) counts [128, 145] decline after SCI in female mice. We compared the number of WBCs per µl of blood counted with linear smears before and at 1, 3, 7 and 14 days after T9 spinal cord contusion in male (n = 6) and female (n = 7) F344 rats. Leukopenia, abnormally low WBC counts, occurred in males and females after SCI (Figure 6, Table 1). We used *Repeated Measures ANOVA* to test if the number of WBCs changed over 14 days after SCI (Time). WBC counts changed significantly after SCI in males ($F_{(4,24)} = 10.64$, $p < 0.001$, $\eta^2 = 0.64$, Table 2:A1) and females ($F_{(4,24)} = 10.43$, $p < 0.001$, $\eta^2 = 0.63$, Table 2:A2). *Post hoc* tests with *Scheffé's method* showed that compared to before injury, WBC counts fluctuated insignificantly over 7 days in both sexes, then blood leukopenia occurred by day 14 after injury in males (M, 0d vs 14d, $p = 0.001$) and females (F, 0d vs 14d, $p = 0.001$). *2-factor-repeated measures ANOVA* assessed the significance of being male or female (Sex: between-subjects factor) on the sequence of changes in WBCs over 14 days (Time: within-subjects factor) after SCI. The pattern of blood leukopenia was similar in males and females over 14 days after SCI ($F_{(4,48)} = 0.22$, $p = 0.93$, $p = 0.93$, $\eta_p^2 = 0.02$, Table 2:A3). The same statistical analysis was performed for all other blood leukocytes in this chapter.

4.2.2 Sex Differences in Blood Myeloid Cell Responses after SCI

Biological sex has a significant effect on the number of $CD11b^+$ blood myeloid cell counts over time after SCI (Figure 7, Table 3). The number of blood $CD11b^+$ cells changed significantly over 14 days after SCI in males ($F_{(4,24)} = 6.05$, $p = 0.002$, $\eta^2 = 0.5$, Table 4:A5) but less so in females ($F_{(4,24)} = 3.81$, $p = 0.02$, $\eta^2 = 0.39$, Table 4:A6).

Compared to pre-injury, *post hoc* tests with *Scheffé's' method* indicated that blood $CD11b^+$ counts in males increased at 1 day [Males (M), 0d vs 1d, $p = 0.09$] and peaked at 3 days after injury (M, 0d vs 3d, $p = 0.004$). In contrast, blood myeloid cell counts in females peaked at 1 day after SCI [Female (F), 0d vs 1d, $p = 0.03$]. 2-factor repeated measures ANOVA indicated that biological sex affects blood myeloid cell responses after SCI ($F_{(4,48)} = 11.89$, $p = 0.005$, $\eta_p^2 = 0.5$, Table 4:A7). *Pairwise comparisons* revealed that males had significantly more blood myeloid cells than females at day 1 (3247 ± 581 vs 2299 ± 588, $t_{(12)} = 3.04$, $p = 0.01$, $d = 1.62$) and day 3 after SCI (4150 ± 2118 vs 2032 ± 583, $t_{(12)} = 2.55$, $p = 0.03$, $d = 1.36$).

4.2.3 Sex Differences in $RP1^+$ Blood Neutrophilia

Previous studies with either male [145, 200, 201] or female [269] mice showed that SCI causes transient acute blood neutrophilia (Figure 8, Table 5). We report that blood neutrophil responses differed between males and females after SCI in F344 rats. Blood neutrophils changed significantly over 14 days after SCI in males ($F_{(4,24)} = 7.0$, $p = 0.001$, $\eta^2 = 0.54$, Table 6:A9) but less so in females ($F_{(4,24)} = 3.81$, $p = 0.02$, $\eta^2 = 0.39$, Table 6:A10). Males had higher blood neutrophil counts within 24 hours after injury and peak blood neutrophilia occurred at 3 days post-injury (M, 0d vs 1d, $p = 0.09$; 0d vs 3d, $p = 0.004$). In contrast, blood neutrophilia in females was transient and peaked at 1 day after SCI (F, 0d vs 1d, $p = 0.04$). Blood neutrophil counts returned near pre-injury levels at 7 and 3 days after injury in males and females, respectively. Sex had an effect on blood neutrophil responses over Time after SCI ($F_{(4,48)} = 2.34$, $p = 0.07$, $\eta_p^2 = 0.16$, Table 6:A11). *Pairwise comparisons* revealed that males had more blood neutrophils

than females at 1 day after injury (1808 ± 640 vs 1341 ± 509 neutrophils/µl, $t_{(12)} = 1.51$, $p = 0.16$, $d = 0.81$). Blood neutrophilia peaked at 3 days after SCI in males but not in females and blood neutrophil counts were two-times higher in males (2185 ± 1234 vs 1051 ± 366 neutrophils/µl, $t_{(12)} = 2.33$, $p = 0.04$, $d = 1.25$).

4.2.4 Sex Differences in Acute Blood Monocyte Responses after SCI

Previous studies with either male [145, 200, 201] or female [269] mice showed that blood monocyte counts increase after SCI (Figure 9, Table 7). We report slight sex differences in blood monocyte responses after SCI. The number of blood monocytes changed significantly over 14 days after injury in females ($F_{(4,24)} = 3.0$, $p = 0.04$, $\eta^2 = 0.33$, Table 8:A14) but less so in males ($F_{(4,24)} = 2.6$, $p = 0.062$, $\eta^2 = 0.3$, Table 8:A13). Blood monocyte counts in females increased gradually from before to 14 days after SCI (F, 0d vs 14d, $p = 0.04$). In contrast, blood monocyte counts in males fluctuated during the first and second weeks after injury but these changes did not reach statistical significance. Blood monocyte responses in both sexes were somewhat different over 14 days after SCI ($F_{(4,48)} = 1.64$, $p = 0.18$, $\eta_p^2 = 0.56$, Table 8:A15). Males had twice as many blood monocytes than females at 3 days after injury (1965 ± 1102 vs 947 ± 287 monocytes/µl, $t_{(12)} = 2.37$, $p = 0.04$, $d = 1.26$) but similar counts at all other time-points.

4.2.5 SCI Causes B cell Lymphopenia

SCI causes abnormally low B-cell counts in the blood (B-cell lymphopenia) in female mice [270, 271]. Our experiments indicate that SCI in F344 rats leads to B-cell lymphopenia in males and females (Figure 10A, Table 9). SCI caused significant changes over 14 days on the number of blood B-cell in males ($F_{(4,24)} = 10.87$, $p < 0.001$,

$\eta^2 = 0.64$, Table 10:A17) and females ($F_{(4,24)} = 3.19$, $p = 0.03$, $\eta^2 = 0.35$, Table 10:A18). Blood B-cell counts decreased significantly within 1 day after injury (M, 0d vs 1d, $p < 0.001$), B-cell lymphopenia persisted thereafter and was most severe at 14 days post-injury (M, 0d vs 14d, $p < 0.001$). Similarly, SCI in females reduced the number of blood B-cells within 1 day after SCI and lymphopenia persisted at 14 days after injury (F, 0d vs 14d, $p = 0.05$). Acute and prolonged B-cell lymphopenia occurred in both sexes over time after SCI ($F_{(4,48)} = 0.92$, $p = 0.46$, $\eta_p^2 = 0.07$, Table 10:A19).

4.2.6 SCI had no significant effect on T cell counts.

SCI causes T-cell lymphopenia in female mice [270, 271]. However, our data suggests this phenomenon does not occur in F344 rats (Figure 11, Table 12). SCI had a mild effect on the number of blood T-cell over 14 days in males ($F_{(4,20)} = 2.86$, $p = 0.05$, $\eta^2 = 0.32$, Table 12:A21) but not in females ($F(4,24) = 1.31$, $p = 0.30$, $\eta^2 = 0.18$, Table 12:A22). Blood T-cell counts in spinal-cord-injured males increased insignificantly over 7 days then returned near pre-injury levels by 14 days. In contrast, blood T-cell counts in females fluctuated for the first 7 days after injury then decreased insignificantly below pre-injury levels by 14 days. SCI caused similar changes in blood T-cell counts over 14 days in both sexes ($F_{(4,48)} = 0.74$, $p = 0.57$, $\eta^2 = 0.058$, Table 12:A24). Our data suggests that significant SCI-induced T cell lymphopenia does not occur in F344 rats.

4.3 Discussion:

4.3.1 Sex Differences in Blood Neutrophilia after SCI

Experiments in this study compared peripheral blood leukocyte responses after a moderate spinal cord contusion with a MASCIS impactor [114] in male and female F344 rats. As previously shown, independently in males [145, 200, 201] and females [269], blood neutrophilia and increased monocyte counts occur during the first week after spinal cord trauma. Our experiments showed significant sex differences in acute blood myeloid cell responses after spinal cord contusion. The number of blood $CD11b^+$ myeloid cells, $RP1^+$ neutrophils and blood smear monocytes peaked 1 day after injury in females but continued to increase for 3 days in males. The magnitude of sex differences in blood leukocyte responses after SCI was similar for $CD11b^+$ myeloid cells and $RP1^+$ neutrophils, indicating that neutrophils contribute most to sex differences in blood myelocytosis (abnormally higher counts of blood myeloid cells). The most significant sex difference in blood neutrophilia occurred at 3 days after injury, when blood neutrophil counts were two times higher in males than females.

Blood neutrophils [157] are the first blood leukocytes to reach the injured spinal cord. Although the role of blood neutrophils in spinal cord repair remains controversial, blood neutrophilia after SCI is classically thought to worsen recovery [164, 200]. Blood neutrophils secrete significant amounts of proinflammatory cytokines at the injured spinal cord and in peripheral organs [264, 272]. Sex differences in blood neutrophilia after SCI may lead more neutrophils at the injury site and cause sex differences in inflammation.

Sex differences in inflammation after SCI may contribute to differences in recovery between males and females.

4.3.2 Sex Differences in Blood Neutrophilia after other Injury Models

Sex differences in blood neutrophil responses are not exclusive to SCI. As we showed after SCI in rats, blood neutrophilia is also more pronounced in males than females after diffuse brain injury in mice [273]. Likewise, several studies [145, 200-202] reported that blood neutrophilia is more prominent in male than female rodents after infection and peripheral organ ischemia.

Injured tissue releases neutrophil-chemotactic factors. Higher blood concentrations of neutrophil-chemotactic factors IL-8, CXCL1 and CXCL5/6 in males cause sex differences in blood neutrophilia. Men produce more IL-8 than women after endotoxin challenges [154, 274, 275]. Crockett, et al. [276] reported that males produce more CXCL1 after hepatic ischemia. Likewise, male mice and rats have higher CXCL5/6 expression after renal ischemic-reperfusion [153].

Enhanced granulopoiesis and neutrophil survival in males may contribute to prolonged blood neutrophilia after SCI. SCI increases bone-marrow granulopoiesis and prolongs neutrophil half-lives in male mice [145]. Tanaka, et al. [277] found that Granulocyte Colony Stimulating Factor (G-CSF) stimulates bone marrow granulopoiesis more potently in male than female mice. Aoyama, et al. [12] reported more bone-marrow granulopoiesis and prolonged activated neutrophil half-lives in males compared to females after systemic endotoxic inflammation. Overall, male-specific increased

neutrophil-chemotactic signals that demarginate neutrophils from bone marrow and peripheral reservoirs, enhanced granulopoiesis and prolonged neutrophil half-life across different inflammatory models suggest that blood neutrophilia is a sex-specific trait that also occurs after SCI.

4.3.3 Sex Differences in Blood Monocytosis after SCI

SCI leads to higher blood monocyte counts in mice [145], rats [269] and humans [201, 278]. Blood-monocyte derived macrophages (BMDM) infiltrate the injured spinal cord in two waves, first M1 proinflammatory BMDM enter within days after injury [183] then M2 pro-repair BMDM surround areas of Wallerian degeneration [279]. We report that spinal cord injured male rats had more blood monocytes than females with the most significant differences occurring at 3 days after injury. Neutrophils produce monocyte chemotactic factors like cathepsin G, azurocidin and pro-inflammatory cytokines that recruit monocytes to the bloodstream and injured tissues [280]. Since neutrophils are the first leukocytes to respond to injury, higher blood monocyte counts may be a direct result of more intense blood neutrophilia in males compared to females.

Sex differences in blood monocyte counts before and after injury may relate to physical differences in monocyte reservoirs. The spleen is the largest monocyte reservoir in the body [281]. Male spleens are larger than female spleen in humans [282], macaques [206], rats [283], mice [284], guinea pigs [285] and birds [286]. Spleens in male F344 rats, used in our experiments, are twice as large as in females [283]. Compared to mechanisms that maintain blood neutrophilia [152, 287], blood monocytosis after SCI occurs when lymphoid monocyte reservoirs contract to release monocytes into circulation

[281]. Findings by Blomster, et al. [177] indicate that the spleen contributes the largest proportion of blood monocytes that infiltrate injured spinal cords. Sex differences in spleen monocyte numbers may be the reason why splenectomy reduces brain infarct volume in male but not female mice [288]. It is not known if splenectomy affects recovery after SCI differently in males and females.

Sex differences in blood monocyte responses after SCI may affect outcomes. Compared to women, men have more pronounced blood monocyte proinflammatory responses [289]. In brain injured mice, males have more activated tissue macrophages, higher concentrations of proinflammatory proteins and worse recovery than females [92]. Although, Walker et al., [71] and Luchetti et al., [69] did not find significant sex differences in macrophage activation after SCI, future studies should distinguish BMDM and MDM infiltration after SCI in both sexes.

Injured spinal cord tissue macrophages come from resident microglia and BMDM. However, distinction between BMDM and MDM, without chimeric animal models [183], is an unresolved challenge [290]. Further advances in macrophage phenotype differentiation will improve the ability to compare macrophage differentiation in both sexes after SCI.

4.3.4 No Sex Differences in Lymphopenia after SCI

The number of B-cells in circulation is tightly regulated. Nascent naïve B cells leave the bone marrow and circulate the blood until they identify target antigens and migrate to lymphatic organs [291]. Previous studies with either males or females demonstrated that

blood lymphopenia occurs within 24 hours after spinal cord injury [145, 270, 271, 292, 293]. We report that acute blood B-cell lymphopenia after SCI occurs in both male and female F344 rats. Our data is consistent with other studies that have reported reduced B-cell percentages after SCI in Sprague Dawley rats [146] and humans [220]. Male F344 rats in our study started with more blood B cells than females before injury. However, sex differences disappeared 24 hours after SCI when blood B cell counts plummeted and stayed low thereafter.

The causes for SCI-induced acute B cell lymphopenia are not clear. Some evidence [145, 294, 295] suggests that SCI reduces B cell hematopoiesis in the bone marrow. However, reduced B cell hematopoiesis [291] is unlikely to account for the rapid decline in blood B cells that we and others [270, 271, 292, 293] observed after SCI. Pruss et al., [270] showed that, regardless of injury level, SCI reduces the number of immature pre-B-cells and causes a 5-fold increase in naive B cells in the bone marrow. Pruss et al., [270] concluded that acute B cell blood lymphopenia occurs when large numbers of naive B cells migrate from the blood to the bone marrow in an LFA-dependent mechanism after SCI.

Dysregulated autonomic function is thought to cause chronic B cell lymphopenia after SCI. Pruss et al., [270] and several others [270, 271, 292] reported that spinal cord transection in female mice disinhibits adrenal gland function and leads to excess catecholamine secretion in lymphatic organs. Excess catecholamines correlate with shrinking of lymphatic organs [270] and increased B-cell apoptosis [271, 292] during chronic recovery from spinal cord trauma in rodents.

Findings of lymphopenia after SCI in mice have fueled speculation that dysfunctional adaptive immunity causes a higher incidence of infections in spinal cord injured patients [267]. However, B cell blood lymphopenia does not occur after SCI in humans. In fact, SCI patients tend to get high auto-antibody titers [223, 296, 297] over time, indicating that B cell differentiation into plasma cells and antibody synthesis remains functional.

Little progress has been made to understand how SCI affects B-cell differentiation into antibody-secreting plasma cells. Mature B cells that differentiate to plasma cells lose common identification antigens CD19 and CD45R [298]. It is possible that SCI causes a significant increase in differentiation of mature B-cells to plasma cells. B cell apoptosis in lymphatic organs, an LFA-dependent B-cell transmigration and retention in the bone-marrow and increased B-cell differentiation into plasma cells may account for the acute and chronic decline in blood B-cells after spinal cord trauma.

In contrast to acute B-cell lymphopenia, the number of T cells in the blood did not change significantly over 2 weeks after SCI in males or females. Other researchers [128, 146, 299] reported a rapid decline of T cells in the blood after spinal cord transection in mice. In contrast, spinal-cord-injured F344 rats of both sexes had an acute but non-significant increase of blood T cell counts at 3 days after injury. Conflicting findings between our study and others may be due to several reasons. First, T cell responses to SCI may differ between animal species and injury models [300]. Second, we quantified the absolute number of T-cells from whole blood rather than percent ratios. Third, we did not differentiate the number of $CD4^+$, $CD8^+$ or Treg T cell subpopulations. Some researchers have shown that SCI significantly changes some subpopulations of T-cells

and not others [225, 301]. Species specific T-cell responses and skewed changes within T cell subpopulations may account for the lack of chronological differences in blood T cell counts in a well-defined spinal cord contusion model [114].

Chapter 5. Sex Differences in Recovery and Blood Neutrophilia after Spinal Cord Injury

5.1 Introduction

Over the past century, many groups have reported a remarkable effect of biological sex on spinal cord injury outcomes [52]. Women, female mice and rats recover better long-term function than males after SCI, although some studies did not find significant differences. Some studies showed that women [49] and female rodents of different species [62], including in F344 rats [61], spare more spinal cord tissue and have higher BBB locomotor scores than males. Others found that males recover earlier [31, 71] and have better long-term hind-limb function, while some found no differences.

The causes for sex differences in recovery after spinal cord trauma are not yet fully understood. Young et al., [57] and Hsu et al., [65] showed that spinal cord weights and lengths did not differ significantly in age-matched Long-hooded Evans and F344 rats, respectively. Several authors [57, 65, 71] also did not find sex differences in spinal cord contusion parameters like impact velocity or compression rate. Since spinal cord sizes and injury severities are thought to be similar for age-matched males and females, longer acting mechanisms in tissue repair may cause sex differences in recovery after SCI.

More injured spinal cord tissue tends to survive in spinal cord injured females than male rodents [61-63]. This phenomenon suggests that tissue repair may differ according to biological sex. We previously reported that blood myeloid cell responses differed between males and females after SCI in F344 rats (Chapter 4). Dramatic sex differences in acute blood neutrophilia are particularly important after SCI because blood neutrophils

infiltrate spinal cord tissue within hours after injury [127], phagocytize dying neurons [156] and secrete proteases and reactive oxygen species thought to harm neuron survival [302]. Intense tissue inflammation and neutrophils undergoing apoptosis recruit blood macrophages that phagocytize dying cells and myelin debris, and secrete inflammatory cytokines to help injury repair [280]. More pronounced acute immune responses in males worsen tissue repair after traumatic brain injury compared to females [88]. Whether sex differences in acute immune responses affect recovery after SCI is not well understood.

Findings reported in this and the next chapter were part of the same large experiment evaluating the role of acute blood leukocytosis after SCI. This chapter focuses on the effect of sex differences in blood neutrophilia after contusion injury in age-matched F344 rats. As showed previously (Chapter 4), spinal cord injured males had more intense and prolonged blood neutrophilia than females. However, experiments evaluating injury site neutrophilia, locomotor recovery and tissue sparing in both sexes revealed unexpected findings. First, neutrophil infiltration at the injury site was similar for both sexes despite significant differences in blood neutrophilia. Second, spinal cord injured males recovered hind-limb function earlier than females but long-term recovery was similar in both sexes. Third, sex differences in blood myeloid cell responses also did not harm nor improved tissue loss, myelin sparing and neuronal survival at 6 weeks after injury. Lastly, spinal cord sizes were bigger in males than females, both at the injury site and intact segments. Overall, experiments in this chapter revealed that more intense blood myeloid cell responses did not worsen nor improve recovery in males compared to females after SCI.

5.2 Results

5.2.1 Blood Neutrophil Responses after SCI

Table 13 describes experimental design and antibodies used for this study. We counted CD11b neutrophils per µl of blood before and at 1, 3, 7 and 14 days after SCI (Figure 12, Table 14). *Repeated-measures ANOVA* indicated that blood neutrophil counts changed significantly over 14 days after SCI in males ($F_{(4,28)}$= 7.34, $p < 0.001$, $\eta^2 = 0.51$, Table 15:B1) but less so in females ($F_{(4,36)}$= 4.68, $p = 0.004$, $\eta^2 = 0.28$, Table 15:B2). Spinal cord injured males had a significant increase in blood neutrophils within 24 hours and peak blood neutrophilia occurred at 3 days post-injury (M, 0d vs 1d, $p = 0.040$ and vs 3d, $p = 0.0019$, *Scheffé's test*). In contrast, blood neutrophil counts in females fluctuated, increasing at 1 day after SCI (F, 0d vs 1d, $p = 0.027$, *Scheffé's test*), then decreased by 3 days but neutrophilia returned at 7 days after injury (F, 0d vs 7d, $p = 0.015$, *Scheffé's test*). The number of blood neutrophils returned gradually near pre-injury levels from 7 to 14 days after SCI in both sexes.

Blood neutrophil responses differed between males and females after SCI ($F_{(4,64)} = 2.64$, $p = 0.04$, $\eta_p^2 = 0.14$, Table 15:B3). Males tended to have more blood neutrophils than females at 1 day after injury and significant sex differences in blood neutrophilia occurred at 3 days after SCI, when blood neutrophil counts were two-times higher in males than females (2185 ± 1234 vs 1051 ± 366 neutrophils/µl, $t_{(16)} = 2.33$, $p = 0.04$, $d = 1.25$, *post hoc unpaired T-test*).

5.2.2 Blood Monocyte Responses after SCI

We counted CD11b monocytes per µl of blood before and at 1, 3, 7 and 14 days after SCI (Figure 13, Table 16). Compared to before injury, blood monocyte counts fluctuated for the first week after injury then decreased significantly by 14 days in males ($F_{(4,28)}$= 5.17, p = 0.002, η_p^2 = 0.36, Table 17:B4) and females ($F_{(1,36)}$= 4.83, p = 0.004, η_p^2 = 0.41, Table 17:B5). There were no significant sex differences in blood monocyte responses over 14 days after SCI ($F_{(4,64)}$= 0.64, p = 0.64, η_p^2 = 0.04, Table 17:B7). However, males had on average, more blood monocytes than females before and after SCI ($F_{(1,16)}$= 22.71, p < 0.001, η_p^2 = 0.59, Table 17:B8). *Post-hoc pairwise comparisons* with *unpaired T-tests* showed that males had more blood monocytes before and 1, 3, and 7 days after SCI (p < 0.05).

5.2.3 No sex differences in neutrophil infiltration at the injured spinal cord

Large numbers of blood neutrophils infiltrate the spinal cord during the first days after injury [164]. Since male rats had twice as many blood neutrophils than females during the first 3 days after SCI (Section 4.2.3), we hypothesized that more neutrophils infiltrate the injured spinal cords in males than females at this time. Myeloperoxidase (MPO) is an enzyme highly expressed in neutrophils [303] and commonly used to quantify neutrophil infiltration after spinal cord trauma [157, 193, 304]. We estimated neutrophil infiltration by Western Blot immunochemical detection of MPO compared to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) at 3 days after injury in spinal cord lysates from male (N = 8) and female (N = 8) F344 rats. The MPO / GAPDH signal ratio represented neutrophil infiltration and was 0.11 ± 0.05 and 0.10 ± 0.07 for males and females,

respectively (Figure 14A & B). ANOVA indicated that sex differences did not occur for neutrophil spinal cord infiltration at 3 days after injury ($p = 0.72$, $\eta^2 = 0.01$)

5.2.4 Sex differences in locomotor recovery after SCI

We assessed walking recovery with the BBB locomotor scale at day 2, then weekly for 6 weeks after SCI (Figure 15, Table 18). 2-factor-*repeated-measures ANOVA* indicated that the improvement of BBB scores was significantly different between males and females over 6 weeks after SCI ($F_{(6,96)}= 13.07$, $p < 0.001$, $\eta_p^2 = 0.45$, Table 19:B10). *Pairwise comparisons* with *unpaired T-tests* showed that BBB scores improved better in males than females at 1 week after injury (8.38 ± 2.87 vs 1.80 ± 2.62, $t_{(16)} = 5.07$, $p < 0.001$, $d = 2.39$), and this trend continued at week 2 (11.75 ± 1.83 vs 10.40 ± 0.70, $t_{(16)} = 2.15$, $p = 0.05$, $d = 0.97$), week 3 (12.13 ± 1.64 vs 11.0 ± 0.67, $t_{(16)} = 1.98$, $p = 0.06$, $d = 0.90$) and week 4 (13.88 ± 1.88 vs 12.1 ± 0.99, $t_{(16)} = 2.14$, $p = 0.02$, $d = 1.78$). Although BBB scores remained higher in males than females at weeks 5 and 6, differences were not statistically significant.

5.2.5 Sex differences in volume and myelin sparing at the injury site

After the last BBB score at 6 weeks post-injury, we euthanized and perfused the rats, sectioned the injured spinal cords at 1 mm intervals, then quantified the area of tissue damage, myelin sparing and number of surviving neurons in sequential tissue sections. 12.5 mm contusions led to a distinct injury epicenter with least area of spared tissue for each spinal cord and bilateral ~5mm tissue damage in both male and female F344 rats. We used a *2-factor-repeated measures-ANOVA* to assess for significant sex differences over the length of the injury for tissue and myelin sparing, and neuron survival.

Contusion injury caused similar gross tissue damage in both males and females ($F_{(9,134)}$ = 0.18, p = 0.99, η_p^2 = 0.01, Figure 16, Table 20:B12). There were also no sex differences in myelin loss at 6 weeks after SCI ($F_{(9,134)}$ = 0.25, p = 0.99, η_p^2 = 0.02, Figure 17, Table 21:B14). Lastly, males and females had similar numbers of surviving neurons at the injury site ($F_{(9,135)}$ = 0.71, p = 0.7, η_p^2 = 0.05, Figure 18, Table 22:B16).

Next, we transformed the area of spinal cord tissue and myelin throughout the injury site into an injury volume. The volume of spinal cord at the injury site was larger in males than females at 6 weeks after SCI (28.01 ± 2.46 mm^3 vs 25.57 ± 0.98 mm^3, $t_{(16)}$ = 2.86, p = 0.012, d = 1.3, Table 23). The volume of myelin in the injured spinal cord site was also larger in males than females (12.03 ± 1.42 mm^3 vs 10.11 ± 0.58 mm^3, $t_{(16)}$ = 3.88, p = 0.002, d = 1.77, Table 24). We then added the number of surviving neurons in 10 sequential sections epicentered at the injury site. In contrast to tissue sparing, there we no significant sex differences in the number of surviving neurons throughout the injury site (Table 25).

5.2.5 Sex differences in volume and myelin sparing in intact spinal cords

We quantified the volume of 4 intact spinal cord segments 1 mm cranial to the injury site border. *Unpaired samples T-Tests* indicated that intact spinal cord segments were larger in males than females at the cranial (14.37 ± 0.46 vs 13.76 ± 0.53 mm^3, $t_{(16)}$ = 2.46, p = 0.027, d = 1.23, Table 23) and caudal directions (17.21 ± 0.64 vs 16.55 ± 0.64, $t_{(16)}$ = 2.09, p = 0.05, d = 1.03, Table 23) from the injury site.

Lastly, we quantified the myelin volume of 4 intact spinal cord segments 1 mm cranial to the injury edge. Compared to females, males had more myelin volume in intact spinal cord segments at the cranial (7.69 ± 0.38 vs 6.95 ± 0.28, $t_{(16)}$= 4.63 $p < 0.001$, $d = 2.07$, *unpaired T-test,* Table 24) and caudal (8.08 ± 0.39 vs 7.45 ± 0.22, $t_{(16)} = 4.26$, $p < 0.001$, $d = 2.0$, *unpaired T-test,* Table 24) directions distal from the injury site.

5.3 Discussion

5.3.1 Sex differences in blood myelocytosis do not affect chronic SCI recovery

5.3.1.1 Blood neutrophilia does not reduce recovery after SCI in males

Causes for sex differences in recovery after SCI are not well understood. As first described in Chapter 4, these subsequent experiments indicated that sex differences in blood neutrophilia and monocyte responses are repeatable phenomena. Blood myeloid cells help repair injured nervous tissue but it is not fully understood if their inflammatory products and phagocytic nature cause bystander tissue damage [266, 305]. Our findings of sex differences in blood myeloid responses are of particular interest for this spinal cord contusion model.

Datto et al., [61] and Hsu [65] previously reported that female, compared to male F344 rats, recover better motor function and spare more spinal cord tissue after contusion injury. Further studies by Hsu [65], however, showed no sex differences in spinal cord sizes and injury severity, suggesting that a process beyond the initial injury may cause sex differences in tissue sparing after SCI. We hypothesized that sex differences in blood myeloid cell responses would contribute to the sexual dimorphism in locomotor recovery and tissue sparing after SCI.

To our surprise, our experiments revealed that more pronounced blood neutrophilia in males did not negatively affect recovery after SCI. In fact, males recovered earlier hind-limb function than females after thoracic contusion injury but long-term recovery (6 weeks) was similar in both sexes. Males and females also had similar loss of gross spinal cord tissue, myelin sparing and neuronal survival. These findings suggest that significant sex differences in blood neutrophilia do not negatively affect tissue repair even though neutrophils degranulate proteolytic and peroxidase enzymes thought to be harmful for spinal cord repair [156, 302].

Sex differences in blood neutrophilia did not affect SCI recovery because males and females had similar neutrophil content at the injury site. Most SCI studies have estimated tissue neutrophil infiltration by measuring myeloperoxidase (MPO) activity [157, 191]. MPO is an enzyme abundant in preformed neutrophil granules that catalyze formation of reactive oxygen species [306, 307]. Studies with mainly female rodents have shown that MPO activity declines steeply beyond 24 hours after SCI [157, 192, 308, 309] even though neutrophils are seen in tissue sections for weeks after injury [126, 310]. Since males had double the number of blood neutrophils than females at 3 days after injury, we expected to find sex differences in blood neutrophilia quantified as MPO present at the injury site. To our surprise, injured spinal cords in both sexes had similar amounts of MPO, suggesting similar neutrophil migration to the injury site at three days after injury despite significant sex differences in blood neutrophilia.

5.3.1.2 Higher monocyte counts in blood are not associated with more myelin loss after SCI

Monocyte-derived blood macrophages have both beneficial and harmful effects on spinal cord tissue repair [266]. Neutrophils [152] undergo apoptosis from 24-48 hours after tissue infiltration and, along with pro-inflammatory cytokines, secrete strong blood monocyte chemotactic factors [311] azurocidin [312], MCP-1, MIP-a and MIP-3a [313] from preformed granules. Early infiltrated blood monocytes at the injured spinal cord differentiate into M1 macrophages that phagocytose most myelin debris, secrete proinflammatory cytokines [142] and clear necrotic tissue [178, 183]. A second delayed wave of blood M2 macrophages at 2 weeks after SCI is thought to contribute neurotrophic factors that reduce Wallerian degeneration [186, 314]. Glial cells surround early infiltrated M1 blood macrophages and "herd" them to the injury epicenter to prevent further spread of secondary injury [181, 183].

Since males had more blood monocytes, especially during the first days after injury, we expected that males would lose more myelin than females. However, myelin sparing was similar in both sexes, suggesting that higher numbers of blood monocytes do not correlate with more myelin loss. In fact, males had more myelin volume than females at the injury site and in adjacent intact tissue segments. Myelin content at the injury site directly correlates with locomotor improvement [59]. Earlier stepping in males in our study may have occurred because male injured spinal cords had more surviving myelinated tracts.

Males tend to have more blood monocytes than females in rodents and these monocytes come mainly from the spleen [288]. A weakness of our study is that we did not compare

blood monocyte infiltration at the injury site in males and females. However, Walker et al., [71] and Luchetti et al., [69] both reported that macrophage infiltration at injured spinal cord is similar in both sexes during the first days after injury. Since we also did not see sex differences in myelin sparing, we hypothesize that higher numbers of blood monocytes do not correlate with more blood macrophage infiltration at the injury site.

Our results and those of others [69, 71], differ from findings in brain trauma models, in which sex differences in blood myeloid cell responses affect tissue repair [288]. After brain injury, more myeloid cells infiltrate injured brain tissue in male than female mice and, correlates with more pro-inflammatory proteins and tissue damage [92, 315]. Although brain and spinal cord injury share tissue repair mechanisms, blood-brain-barrier damage and inflammatory responses are more severe after brain than spinal cord contusions [162, 316]. Sex differences in peripheral myeloid cell responses may be less consequential after spinal cord injury.

5.3.2 Importance of methodology used to quantify tissue sparing after SCI

Sex differences in tissue sparing after spinal cord trauma may be subtle and more discernable with more severe injuries [64]. The methods used to compare tissue sparing in males and females after moderate SCI must be sensitive and accurate to identify small differences. Since damage from spinal cord contusion spreads over several millimeters, transverse sequential sections that span the width and length of the injury site are better-suited to quantify spinal cord tissue damage and myelin sparing than sections only at the injury epicenter.

Histological stains, like hematoxylin & eosin (H&E), Erichrome C and Cresyl Violet are widely used in SCI research to quantify tissue sparing. Swartz et al., [64] reported that sex differences in overall tissue sparing after SCI in rats depended on the loss of gray matter after Erichrome C staining. Datto et al., [61] and Hauben et a., [63] used H&E to show that females preserved more white and gray matter at the injury epicenter. Areas of stained surviving gray and white matter are typically outlined by the researcher on each tissue section. Although histological stains are less expensive, the quantification process is less accurate because areas of surviving gray and white matter are selected by the researcher on each tissue section and subjectivity in this process may introduce variability [317, 318].

Our finding that F344 males recover earlier hind-limb function without significant long-term sex differences in recovery differ from previous similar experiments by others [61, 65]. Datto et al. [61] reported that female F344 rats had better functional recovery and had more spared white and gray matter at the injured site. Hsu [65] observed that some cohorts of female F344 rats recover better than males but not others. Differences in results between our F344 study and Datto et al. [61] may be due to differences in the application of the injury model [114] and differences in methodology to compare spinal cord tissue sparing [319]. We contused rat spinal cords at the junction of T9-T10, as opposed to T8-T9 by Datto et al., [61]. As recommended by the MASCIS contusion model [57], we used only rats that had BBB scores of 2 or less at 2 days after injury and less than 5% error of expected contusion height, impact velocity and compression rate. These inclusion criteria ensured that all animals had similar severity injuries [57].

We also quantified tissue sparing from transverse serial sections 1 mm apart spanning 10 mm that covered the entire injury site, as opposed to sagittal sections at the injury epicenter [61]. Our strategy permitted us to compare sequential changes in tissue sparing throughout the lesion cavity and showed to clear sex differences in tissue volume at the injury site and adjacent intact segments. Injured spinal cord volumes in these experiments were approximately ~ 5-10 % bigger in male than female 100-day-old F344 rats in our study while Datto et al., reported that males had ~ 25% less spinal cord tissue volume than females at the injury epicenter.

Despite significant sex differences in blood neutrophilia and monocytes, we found no sex differences in neither neuron loss, the amount of gross tissue damage nor myelin sparing. In fact, the amount of injured tissue was almost identical in both sexes, supporting a good application of the contusion model for this study. To our knowledge, we are the first to use antigen specific immunohistochemistry combined with automated morphometry to assess the effect of biological sex on myelin sparing (myelin basic protein) and neuron survival (NeuN+) in transverse spinal cord tissue sections after a standardized contusion injury. Our study exemplifies that using antigen-specific immunohistochemistry, sequential transverse spinal cord sections and automated volumetric analysis will lead to less variability and improve the ability to compare tissue sparing after SCI in both sexes [318].

5.3.3 Sex differences in spinal cord sizes

Standardized contusion models recommend to match young male and female rodents by age to prevent differences in spinal cord sizes from affecting outcomes [57]. Young et

al., [57] studied hundreds of rats and found that 77-day-old Long-Hooded Evans rats had similar spinal cord weights at the T9-T10 segments. Hsu [65] reported that spinal cords tended to weigh more in male than female 100-day-old F344 rats but these differences did not reach statistical significance. The MASCIS model recommends the use of 77-day-old Long-Hooded Evans rats because sex differences in spinal cord size do not occur at this age [57]. Sex differences in spinal cord size are determined by age and not the weight of the rat [57].

Our experiments with age matched 100-day-old F344 rats showed that neuron survival, gross tissue damage and myelin sparing after contusion injury was similar between both sexes. However, on average, gross tissue and myelinated areas were bigger in males than females for all injured spinal cord tissue segments. Combined, these two results indicate that all animals received similar severity contusion injuries (comparable tissue loss) but that spinal cords segments at the injury site were bigger in male than female F344 rats. Because males had more injured spinal cord tissue and myelin volume, we made the *post-hoc* hypothesis that spinal cords from 100-day-old F344 rats were bigger in males than females. We tested this hypothesis by measuring the spinal cord and myelin volume in intact spinal cord segments adjacent to the injury site. Myelin makes up a large portion of the spinal cord and if intact segments are bigger in males than females, this difference should also be represented in myelin volume [319]. Accordingly, our analysis revealed that tissue sections from intact spinal cord segments had larger cross-sectional area in males than females. Consequently, there were sex differences in gross tissue and myelin volume in injured and non-injured spinal cord segments.

Sex differences in spinal cord sizes may occur depending on species [320], between rodent strains [321, 322] and in older rodents [57]. Our experiments suggest spinal cords in 100-day-old F344 rats may be bigger in males than females. Researchers should measure the spinal cord sizes from age-matched males and females in the chosen rodent species or strain by comparing uninjured spinal cord weight and volume. Furthermore, injured spinal cord tissue areas should be reported instead percent changes in tissue loss. This will help SCI researchers understand if spinal cords of male and female rodents differ in size and prevent erroneous conclusions regarding effects of therapies in both sexes.

5.3.5 Sex Differences in Neutrophilia May Affect Inflammation after SCI

As of today, it is not well understood if sex differences in immune responses affect inflammation at the injured spinal cord. Our findings of sex differences in acute blood myeloid cell responses are an important indicator that inflammation related experiments should include both, males and females [323]. Although more intense blood myeloid cell responses did not worsen recovery in males compared to females, different magnitudes of acute blood neutrophilia may be an important factor to consider while evaluating therapies that modulate inflammation [323]. Importantly, since sex differences in tissue sparing may be subtle, especially with mild to moderate injuries, the most specific, accurate and sensitive methods are recommended to compare the effect of therapies in both sexes after SCI [63, 64, 324, 325].

Chapter 6. Blood Neutrophilia Does Not Affect Recovery After Spinal Cord Injury

6.1 Introduction

Our previous experiments showed that blood neutrophilia after SCI occurs during the first days after injury and is more intense in males than females. A time-specific therapy that prevents blood neutrophilia, without severe immunosuppression, would elucidate the role of acute blood leukocytosis on injured spinal cord tissue repair. Nitrogen mustards, like cyclophosphamide (CYP), cause transient dose-dependent apoptosis of circulating blood leukocytes and reduced granulopoiesis then a rebound of bone-marrow hematopoiesis afterward [233, 234]. A single dose of CYP, given two days before injury, prevents blood neutrophil responses, lessens blood neutrophilia at the injury site and improves locomotor recovery [164, 193, 195, 196, 240, 241, 326]. Feringa et al., reported that CYP therapy improved spinal cord regeneration after spinal cord transection [197]. Although most studies have focused on depleting blood neutrophil and monocyte responses, less is known about the effect of higher blood myeloid counts and whether immunosuppressive therapy after SCI affects males and females differently.

In this study, we prevented acute blood myelocytosis with one dose of CYP, given two days before SCI, in age-matched 100-day-old F344 rats. Compared to vehicle (VEH, distilled water), CYP treatment reduced the duration and intensity of blood neutrophil responses and significantly decreased blood monocytes after SCI. However, our experiments indicated that blood neutrophilia and higher blood monocyte counts after SCI did not have a negative effect on locomotor recovery, tissue sparing or neuronal

survival in either male or female rats. Instead, males recovered hind-limb function earlier than females but sex differences disappeared in long term recovery, regardless of treatment. Sex differences occurred in response to CYP treatment for SCI. CYP therapy prevented acute blood neutrophilia more effectively in spinal cord injured females compared to males. Our study demonstrates that higher numbers of blood neutrophils and monocytes during the first week after SCI neither harmed nor improved functional recovery. Since we assimilated blood leukocyte responses in males and females, our study shows that sex differences in blood myeloid cell responses are not the cause for sex differences in functional recovery after SCI.

6.2 Results

6.2.1 Cyclophosphamide Reduces Blood Leukocyte Counts after SCI

We did a pilot study to assess the safety of cyclophosphamide (CYP) treatment in spinal cord injured F344 rats. 3 groups (Male: n=1, Female: n=1, per group) received one subcutaneous dose of 80 mg/kg, 40 mg/kg or 20 mg/kg aqueous CYP 2 days before injury. At 2 days before a 12.5 mm T12 contusion, rats received a subcutaneous dose of aqueous CYP. We collected blood with cranial vena cava (CVC) venipunctures before dose, and at 1, 2, and 3 days after injury and counted the number of leukocytes per µl of blood with linear smears. Compared to pre-injury, blood leukocyte counts (per µl) decreased to 23% (4944 ± 1165 vs 1140 ± 73 leukocytes/µl), 49% (4232 ± 961 vs 2080 ± 531 leukocytes/µl) and 56% (4999 ± 298 vs 2803 ± 426 leukocytes/µl) at 3 days post injury in the 80 mg/kg, 40 mg/kg and 20 mg/kg groups, respectively (Figure 19). Rats in the 80 and 40 mg/kg groups lost skin turgor through dehydration, had large rheum

buildup that indicated suffering, were shivering, did not rummage through their cage and were euthanized to prevent further suffering. In contrast, rats in the 20 mg/kg group had normal skin turgor, visibly less rheum build up than other groups and moved around their cage. CYP doses above 40 mg/kg were deemed unsafe for spinal cord injured F344 rats.

Experiments in Chapter 4 showed that males had more prominent blood neutrophilia and more blood monocytes than females during the first week after SCI. Blood neutrophils and monocytes are $CD45^{+}CD11b^{+}$ bone-marrow derived myeloid leukocytes that fight infections and repair injured tissue. We tested if one dose of cyclophosphamide (CYP) prevented acute blood neutrophil cell responses after SCI. At 2 days before a 12.5 mm T12 contusion, male F344 rats received a subcutaneous dose of 25 mg/kg aqueous CYP (CYP treated, n = 2) or distilled water (VEH treated, n = 2) at 2 days before injury. We collected blood with CVC venipunctures before dose, at 2, 4 and 6 days after SCI, counted the total number of leukocytes per µl of blood with linear smears, used a MACSQuant flow cytometer (Miltenyi) to quantify the percent of $CD45^{+}CD11b^{+}$ blood myeloid cells then multiplied the percent of $CD45^{+}CD11b^{+}$ and total leukocyte numbers to obtain the absolute myeloid cell count per µl of blood. Blood myeloid cell counts doubled by 4 days after injury in VEH treated rats but were lower than before injury in CYP treated rats (Figure 20). A 25 mg/kg subcutaneous dose of CYP prevented acute myeloid cell responses after SCI but did not deplete these cells from circulation.

6.2.2 CYP Reduced Blood Neutrophil and Monocyte Counts in Males after SCI

6.2.2.1 CYP prevented blood neutrophilia after SCI in male rats

SCI causes acute blood neutrophilia within hours after injury in rodents and humans. [145-147]. We counted the number of $SSC^{hi}CD45^{+}CD11b^{+}$ blood neutrophils in VEH or CYP treated spinal cord injured F344 male rats (VEH: n = 8, CYP: n = 8) (Figure 21A). As we have shown previously, the number of blood neutrophils changed significantly over 14 days after SCI in VEH treated males ($F_{(4,28)}$= 7.34, $p < 0.001$, $\eta_p^2 = 0.51$, Table 15:C1). In contrast, CYP treatment in males prevented significant changes in blood neutrophils after SCI ($F_{(4,28)}$= 1.38, $p = 0.27$, $\eta_p^2 = 0.17$, Table 26:C1). *2-factor-repeated measures ANOVA* indicated that blood neutrophil responses differed between spinal cord injured VEH and CYP-treated males ($F_{(4,64)}$= 2.45, $p = 0.056$, $\eta_p^2 = 0.15$, Table 26:C4). Blood neutrophil counts increased in both treatment groups at 1 day after injury but more so in VEH treated males. CYP treatment prevented prolonged blood neutrophilia after SCI. Peak blood neutrophilia occurred in VEH males at 3 days after injury but neutrophil counts decreased and were significantly lower in CYP treated males (1637 ± 477 vs 830 ± 597 neutrophils/µl, $t_{(14)} = 2.99$, $p = 0.01$, $d = 1.49$). Blood neutrophil counts remained higher in VEH males at 7 days after SCI compared to CYP treatment (1267 ± 683 vs 776 ± 332 neutrophils/µl, $t_{(14)} = 1.83$, $p = 0.09$, $d = 0.91$).

6.2.2.2 CYP reduced blood monocytes after SCI in male rats

We previously showed that blood monocyte counts increased slightly in males during the first week after injury (Chapter 4). We counted the number of $SSC^{lo}CD45^{+}CD11b^{+}$ blood monocytes in VEH or CYP treated spinal cord injured F344 male rats (Figure

22A). *Repeated-measures ANOVA* detected significant changes in blood monocyte counts over 14 days after SCI in VEH ($F_{(4,28)}$= 4.83, p = 0.004, η_p^2 = 0.41, Table 17:B4) and CYP treated males ($F_{(4,28)}$= 12.38, p < 0.001, η_p^2 = 0.64, Table 27:C5). Treatment had a significant effect on the number of blood monocytes in spinal cord injured males over two weeks after SCI ($F_{(4,56)}$ = 7.34, p < 0.001, η_p^2 = 0.40, *2-factor-repeated measures ANOVA,* Table 27:C7). Blood monocyte counts were significantly lower in CYP than VEH-treated males within 1 day after injury (633 ± 216 vs 395 ± 104 monocytes/µl, $t_{(14)}$ = 2.8, p = 0.001, d = 1.4, *unpaired T-test*). The number of blood monocytes then increased and peaked in VEH-treated males but decreased in CYP treated males at 3 days after SCI (742 ± 277 vs 306 ± 73 monocytes/µl, $t_{(14)}$ = 4.29, p = 0.001, d = 2.15, *unpaired T-test*). Blood monocyte counts decreased at 7 days after injury in both treatment groups but remained significantly higher in VEH than CYP treated males (547 ± 150 vs 321 ± 49 monocytes/µl, $t_{(14)}$ = 4.04, p = 0.001, d = 2.03, *unpaired T-test*), then treatment differences disappeared by 14 days.

6.2.2.3 CYP prevented blood neutrophilia after SCI in female rats

We counted the number of $SSC^{hi}CD45^{+}CD11b^{+}$ blood neutrophils in VEH or CYP treated spinal cord injured F344 female rats (VEH: n = 10, CYP: n = 10) (Figure 21B, Table 11). *Repeated-measures ANOVA* identified significant changes in blood neutrophil counts over 14 days after SCI in VEH ($F_{(4,36)}$= 4.68, p < 0.01, η_p^2 = 0.34, Table 15:B2) and CYP treated females ($F_{(4,36)}$= 11.32, p < 0.001, η_p^2 = 0.56, Figure 26:C2). *2-factor-repeated measures ANOVA* indicated that CYP treatment had a significant effect on blood neutrophil responses after SCI in female rats ($F_{(4,72)}$ = 5.57, p = 0.001, η_p^2 = 0.24,

Table 26:C4). Blood neutrophil counts increased in VEH females but remained stable with CYP treatment at 1 day after injury (980 ± 541 vs 352 ± 129 neutrophils/µl, $t_{(18)}$ = 3.58, p = 0.002, d = 1.6, *unpaired T-test*). This trend continued for 3 days (730 ± 281 vs 383 ± 198 neutrophils/µl, $t_{(18)}$ = 3.19, p = 0.005, d = 1.43, *unpaired T-test*). By 7 days, blood neutrophil counts were similar in both female treatment groups, as the number of blood neutrophils returned to pre-injury levels. However, by 14 days after injury, blood neutrophil counts were higher in CYP than VEH-treated females (985 ± 355 vs 668 ± 321 neutrophils/µl, $t_{(18)}$ = 2.1, p = 0.05, d = 0.94).

6.2.2.4 CYP reduced blood monocytes after SCI in female rats

We counted the number of blood monocytes in VEH or CYP treated spinal cord injured females over 14 days after SCI (Figure 22B). *Repeated-measures ANOVA* demonstrated that blood monocyte counts changed significantly over 14 days after SCI in VEH ($F_{(4,36)}$= 5.17, p = 0.02, η_p^2 = 0.36, Table 17:B5) and CYP treated females ($F_{(4,36)}$= 20.31, p < 0.001, η_p^2 = 0.69, Table 27:C6). Treatment had a significant effect on the number of blood monocyte counts over 14 days after injury ($F_{(4,72)}$ = 8.57, p < 0.001, η_p^2 = 0.32, *2-factor-repeated measures ANOVA,* Table 27:C8). *Post hoc pairwise comparisons* with *unpaired T-tests* showed that blood monocyte counts were significantly higher in VEH than CYP treated females at 1 day (451 ± 80 vs 263 ± 68 monocytes/µl, $t_{(18)}$ = 5.61, p < 0.001, d = 2.53), 3 days (530 ± 190 vs 193 ± 50 monocytes/µl, $t_{(18)}$ = 5.43, p < 0.001, d = 2.43) and 7 days after injury (413 ± 118 vs 214 ± 51 monocytes/µl, $t_{(18)}$ = 4.89, p < 0.001, d = 2.19).

6.2.3 Blood neutrophilia does not affect outcomes after SCI

6.2.3.1 Blood neutrophilia has no effect on locomotor recovery in males after SCI.

We used the BBB locomotor scale to evaluate locomotor recovery weekly for 6 weeks after SCI in VEH or CYP treated males and females (VEH: n= 8, CYP: n= 8). After the last BBB score at 6 weeks post-injury, we euthanized and perfused the rats, sectioned the injured spinal cords at 1 mm intervals, then measured the area of spinal cord preserved after injury, myelin sparing and neuronal survival in sequential tissue sections. 12.5 mm contusions led to a distinct injury epicenter with the least area of spared tissue and damage that extended for ~9 mm in both VEH and CYP treated male and female rats. We used a 2-factor repeated measures ANOVA to assess for significant Treatment (between-subjects factor) differences in locomotor recovery for 6 weeks, gross tissue loss, myelin sparing and neuron survival throughout the length of the injury site (Distance, within-subjects factor).

CYP and VEH treated males recovered some hind-limb function within 1 week after SCI and BBB scores increased thereafter for 6 weeks (Figure 23A). However, compared to VEH, preventing blood neutrophilia and decreasing blood monocyte counts with CYP had a minimal effect on walking recovery over 6 weeks in spinal cord injured male rats ($F_{(6,84)} = 0.01$, $p = 1.0$, $\eta_p^2 = 0.01$, Table 28:C9). Unexpectedly, even though large numbers of blood neutrophils and monocytes infiltrate spinal cord during the first days after injury, preventing acute blood myeloid cell responses in spinal cord injured males did not have a significant effect on spinal cord tissue damage ($F_{(1,13)} = 0.5$, $p = 0.49$, $\eta_p^2 = 0.04$, Figure 24A, Table 29:C11), myelin sparing ($F_{(9,134)} = 0.27$, $p = 0.98$, $\eta_p^2 = 0.02$,

Figure 25A, Table 30:C13) or neuron survival ($F_{(9,135)} = 0.5$, $p = 0.87$, $\eta_p^2 = 0.037$, Figure 26A, Table 31:C15).

6.2.3.2 Blood neutrophilia transiently effects walking recovery in females after SCI

Similar to males, hind-limb function improved in both female treatment groups within 1 week after injury and BBB scores continued to improve for 6 weeks (Figure 23B). Although long-term walking recovery was very similar in both female treatment groups, females treated with CYP recovered hind-limb movement earlier than VEH ($F_{(6,108)} = 3.24$, $p = 0.006$, $\eta_p^2 = 0.15$, *2-factor-repeated- measures ANOVA,* Table 28:C10). *Post hoc pairwise comparisons* with *unpaired T-tests* indicated that, compared to VEH treatment, BBB scores in CYP-treated females were significantly higher at 1 week after injury (1.8 ± 2.62 vs 4.70 ± 3.34, $t_{(18)} = 2.16$, $p = 0.04$, $d = 0.97$). However, both female treatment groups had very similar BBB scores from 2 to 6 weeks after SCI.

Since acute blood neutrophilia did not occur in CYP-treated females, we hypothesized that tissue sparing would be better in CYP than VEH treated groups. Unexpectedly, *2-factor-repeated measures ANOVAs (Effect: Tissue-sparing*Treatment)* indicated that preventing blood myeloid cell responses in spinal cord injured females had an insignificant impact on the amount of gross tissue damage ($F_{(9,162)} = 0.73$, $p = 0.68$, $\eta_p^2 = 0.039$, Figure 24B, Table 29:C12), myelin-sparing ($F_{(9,162)} = 0.71$, $p = 0.7$, $\eta_p^2 = 0.038$, Figure 25B, Table 30:C14) and neuron survival ($F_{(9,162)} = 1.42$, $p = 0.18$, $\eta_p^2 = 0.07$, Figure 26B, Table 31:C16).

6.2.4 Males Had Bigger Spinal Cords than Females

We compared the size of the injury site and adjacent spinal cord segments in CYP treated males and females (Table 23). Similar to results from sex-matched groups in the VEH-treated groups ("normal SCI") (Section 5.2.5), spinal cords were bigger in CYP-treated males than females on several measures. First, 10 mm of injured spinal cord that span the injury site had bigger volume in CYP treated males than females at 6 weeks after SCI (27.18 ± 2.12 mm^3 vs 24.73 ± 2.51 mm^3, $t_{(16)} = 2.2$, $p = 0.043$, $d = 1.05$). Second, injured spinal cords had more myelin volume in CYP males than females (11.56 ± 0.96 mm^3 vs 9.90 ± 1.35 mm^3, $t_{(16)} = 2.92$, $p < 0.01$, $d = 1.42$). Third, intact spinal cord segments (4 mm) were larger in CYP-treated males than CYP-treated females at the cranial (12.47 ± 0.47 vs 13.79 ± 0.62 mm^3, $t_{(16)} = 2.77$, $p = 0.014$, $d = 2.4$) and caudal segments (17.19 ± 0.69 vs 16.57 ± 0.79 mm^3, $t_{(16)} = 3.17$, $p = 0.006$, $d = .84$) from the injury site. CYP-treated males also had more myelin content (volume) than females in the cranial (7.69 ± 0.38 vs 6.93 ± 0.38 mm^3, $t_{(16)} = 4.22$, $p < 0.001$, $d = 2.0$) and caudal (8.09 ± 0.49 vs 7.31 ± 0.40 mm^3, $t_{(16)} = 3.72$, $p = 0.002$, $d = 1.74$) intact segments. However, in contrast to tissue sparing, there we negligible sex differences in neuron survival at the injury site from CYP-treated groups.

6.2.5 Reducing blood myelocytosis did not prevent sex differences in recovery

We previously reported that more intense blood neutrophil and monocyte responses in males after SCI did not worsen locomotor recovery or tissue sparing compared to females (Chapter 5). The consequences of sex differences in myeloid cell responses on spinal cord tissue repair are less understood. We had hypothesized that making blood

neutrophil and monocyte responses similar both sexes would assimilate their locomotor recovery and tissue sparing. Overall, treatment had a significant effect on locomotor recovery ($F_{(6,192)} = 2.94$, $p = 0.009$, Effect: *Time*Treatment,* Table 32:C17). However, treatment differences were only evident during the first week after injury in female but not in male rats (Section 4.2.3.1&2). Unexpectedly, males in both treatment groups had higher BBB scores than females during the first weeks after injury but long-term walking recovery was similar in both sexes, regardless of treatment ($F_{(6,192)} = 14.94$, $p < 0.001$, Effect: *Time*Sex,* Table 32:C18). Preventing blood neutrophilia and reducing blood monocyte counts in both sexes did not have a significant effect on tissue loss, myelin sparing or neuron survival at 6 weeks after SCI [*p = N.S., Effect:* Tissue-Sparing*Treatment (Table 33:C19), Myelin-sparing*Treatment (Table 34:C21), Neuron-survival*Treatment (Table 35:C23).

6.3 Discussion

6.3.1. Acute blood neutrophilia does not worsen nor benefit recovery after SCI

Understanding if blood neutrophilia affects tissue repair and functional recovery after SCI is important because intact spinal cord tissue has few neutrophils but tissue neutrophilia is a prominent feature during the first days after injury [126]. We used a time-restricted approach to prevent acute blood neutrophilia after SCI. Compared to vehicle treatment (VEH), one 25 mg/kg dose of CYP, given two days before injury, prevented significant changes in blood neutrophilia and reduced blood monocyte counts below baseline during the first 7 days after injury in male and female F344 rats. Blood neutrophil and monocyte counts returned near pre-treatment levels by 14 days after

injury. This strategy helped us investigate the effect of blood neutrophilia and monocyte responses, because the most extensive migration of these blood myeloid cells to injured tissue occurs during the first days after injury [129].

SCI leads to acute blood neutrophilia [145]. Neutrophils, the first blood leukocytes to infiltrate injured spinal cord, secrete proinflammatory cytokines and release granules rich in enzymes that catalyze neurotoxic reactive oxygen species, tissue remodeling and angiogenesis [128, 164]. Whether blood neutrophils are helpful or harmful for nervous tissue repair is still debated. Katoh et al., showed that spinal cord injured patients with more severe neutrophilia were more likely to suffer neurological deterioration during the first week after injury [200]. Several studies showed that depleting blood neutrophil and monocytes after CNS injury tends to improve neural tissue sparing [164, 191, 327]. However, others showed that depleting only blood neutrophils reduces recovery after SCI [175].

Neutrophils secrete significant quantities of cytokines and their granules release enzymes with high peroxidase and metalloproteinase activity that digest tissue matrix. Neutrophil responses after brain and spinal cord trauma have been classically thought as harmful for nervous tissue repair. We hypothesized that higher numbers of blood neutrophils, in the form of blood neutrophilia, would affect neuron survival and tissue sparing after SCI. However, our experiments did not refute the null. Neuron survival was similar whether blood neutrophilia occurred or not. Likewise, preventing blood neutrophilia had minimal impact on tissue sparing.

6.3.2 The effect of higher numbers of blood monocytes after SCI

Blood monocyte derived macrophages phagocytize myelin debris, mainly differentiate into M1 phenotype and release significant amounts of proinflammatory cytokines [185, 305]. It is thought that reactive resident glia "herd" early infiltrated blood macrophages into the injury epicenter in an attempt to reduce excess myelin phagocytosis and a proinflammatory environment [181, 183]. Hence, we hypothesized that more monocytes circulating in blood would correlate with less myelin sparing. However, our experiments showed that higher numbers of blood monocytes had an insignificant effect on myelin sparing throughout the injury site.

Although we did not compare blood derived-macrophage infiltration at the injury site, similar myelin sparing in both treatment groups suggests that having more macrophages in circulation may not lead to more myelin phagocytosis. This conclusion is strengthened by our previous analysis where spinal-cord-injured male rats had significantly more blood monocytes than females but myelin and tissue sparing was similar in both sexes. Walker et al., [71] and Luchetti et al., [69] also showed that injured spinal cords in male and female rats had similar numbers of macrophages at the lesion epicenter. Further experiments should investigate the mechanisms that prevent excess blood leukocytes from infiltration injured spinal cord tissue.

6.3.3 Control of Neutrophil Migration to the Injured Spinal Cord

Our findings that significant differences in blood neutrophil and monocytes did not affect tissue damage, myelin sparing and neuronal survival suggest that mechanisms exists to prevent excess leukocyte migration into the injured spinal cord. We provide evidence for

this hypothesis in two forms. First, our previous study (Chapter 5) showed that sex differences in blood neutrophilia did not correspond to sex differences in neutrophil content at the injury site, even though males had twice the number of blood neutrophils than females at 3 days after SCI. Second, males and females had similar tissue sparing and neuronal survival regardless of significant differences in the number of blood myeloid cells. Third, even though CYP treatment prevented blood neutrophilia and reduced blood monocyte counts over the first week after injury, CYP and vehicle-treated rats had almost identical myelin sparing and long-term walking recovery. Although we did not compare neutrophil or macrophage infiltration in both treatment groups, our results, and those of others, indicate that blood myeloid cell migration into injured spinal cord tissue may be tightly regulated. Several neutrophil-chemotactic proteins are released after SCI and in higher quantities in neutropenic spinal cord injured animals [175]. However, less is known about signals that limit neutrophil migration into the injured spinal cord.

Blood neutrophils and macrophages do not remain static in the injured spinal cord. Live-imaging experiments have shown showed that blood neutrophils and monocytes migrate to injuries, act on injured tissue, then most reverse-migrate to peripheral storage pools [328]. Blood neutrophils are found in peripheral areas surrounding the injury epicenter, adjacent to areas of neovascularization and veins and venules [156]. As more neutrophils infiltrate the injured spinal cord, neutrophil derived granule proteins, like MPO, catalyze the production of free oxygen radicals, further depleting oxygen concentration in the injured tissue. Oxygen responsive tissue elements, like HIF-1, down regulate the

expression of cell adhesion proteins on adjacent endothelium to prevent further migration of neutrophils into injured tissue [329, 330]. Neutrophils are also short lived and undergo apoptosis within 48 hours and secrete macrophage chemotactic signals for their removal [331-333]. Macrophages phagocytize apoptotic neutrophils at the injury site and through contact mediated guidance [334], signal surviving neutrophils to reverse migrate to peripheral reservoirs [183]. Only a limited number of neutrophils may remain in injured spinal cord, regardless of the magnitude of blood neutrophilia.

6.3.4 The effect of Immunosuppression after SCI

Understanding whether acute blood leukocyte responses are harmful or beneficial for SCI recovery have important clinical implications because several inexpensive treatments are immediately available in clinics to dampen inflammatory immune responses. However, acute immunosuppressive approaches to treat SCI patients are not viewed favorably because this patient population is more prone to pneumonia and urinary tract infections during the first weeks after injury. The recommended treatment for SCI, one 30 mg/kg bolus dose of methylprednisolone (MP) within 8 hours after injury [335], is no longer used widely due to fears that MP-induced immunosuppression may put patients at more risk for internal bleeding and infections [336]. Findings from several studies [110, 155, 163, 193, 195, 304, 337, 338] indicate that profound myeloid-suppression improves recovery after SCI but our results suggest that preventing acute blood leukocyte responses has no effect on tissue repair or motor function. Hence, we conclude that maintaining blood leukocyte counts at homeostasis for the first week after SCI is sufficient for proper recovery after SCI.

6.3.5 Sex Differences in Response to Immunosuppressive Therapy after SCI

Assuming that treatments have similar effects in males and females may be harmful [323]. Therapeutic efficiency, especially to treat immune responses, differs between males and females [81]. Our experiments showed that CYP therapy prevented blood neutrophilia and reduced blood monocytes more efficiently in females than in males. Since the number of blood neutrophils still somewhat increased acutely after SCI in CYP treated males compared to females, there may be sex differences in the concentration of neutrophils in peripheral pools and demargination mechanisms.

Circulating neutrophils are a minor fraction of neutrophil populations. The bone marrow, lymphatic organs, lungs and superficial capillaries serve as the largest neutrophil reservoirs [152]. Compared to females, bone marrow granulopoiesis and neutrophil demargination to the blood are more pronounced in males than females after injury [274, 339]. Males also produce more chemotactic signals that promote blood neutrophilia [153, 154, 202]. Our results showed that CYP had stronger effect on blood neutrophilia in females, keeping blood neutrophil counts unchanged, but that a mild increase in blood neutrophils still occurred in male rats. These findings suggests that sex differences may occur in the mechanism that maintains blood neutrophil homeostasis after SCI and that the same dose of CYP therapy was more effective in females than males.

7. Conclusions

7.1 Sex differences in blood neutrophilia after SCI

This thesis tackled a longstanding question in spinal cord injury research. Men outnumber women by a ratio of 4:1 and women tend to recover better than men after spinal cord trauma but the mechanisms are not well understood [52]. Spinal cord injury elicits intense peripheral inflammatory and immune responses, manifested by an initial increase in neutrophils (neutrophilia) followed by a delayed and prolonged decline of lymphocytes (lymphopenia) [128]. We hypothesized that differences of peripheral blood leukocytosis contribute to differences in spinal cord injury and recovery in males and females.

To test this hypothesis, we measured peripheral blood leukocyte responses in male and female Fischer 344 (F344) rats after standardized spinal cord contusion, correlated these responses with preservation of myelinated tracts, survival of neurons in the spinal cord, and locomotor recovery. To measure the leukocyte counts, I developed and worked with undergraduate students to validate a "linear smear" method to count total leukocytes per μl of blood. The linear smear is an inexpensive and accurate method to count blood leukocytes on a microscope slide, allowing quantitative differential counts of neutrophils, monocytes, B- and T-cells by flow cytometry and blood smear.

As others have reported, SCI causes acute blood leukocytosis and then leukopenia from one to two weeks after injury [128, 145]. Our results showed that blood neutrophilia occurs earlier, is more intense, and lasts longer in spinal cord injured male than female

F344 rats. Blood neutrophils infiltrate the injured spinal cord during the first days after spinal cord contusion [241].

We hypothesized that male F344 rats would have greater neutrophil infiltration, less neuron survival and myelin sparing, and worse locomotor recovery than females. Our experiments refuted the hypothesis. First, male F344 rats did not have more myeloperoxidase in the injured spinal cord even though peak blood neutrophilia was 3-fold higher in males compared to females. Thus, more blood neutrophils in circulation did not correlate with more neutrophils at the injury site. Second, BBB locomotor scores initially improved more in F344 males compared to females but did not differ significantly beyond 3 weeks after injury. Third, long-term neuronal survival at the injury site was nearly identical in both sexes. Lastly, gross spinal cord and spared myelin volumes were bigger in males than females. These findings suggest that higher blood neutrophilia is associated with less spinal cord damage and better early locomotor recovery.

Our hypothesis predicted that less blood neutrophils would lead to better recovery in both males and females after SCI. We reduced blood neutrophil counts with a dose of cyclophosphamide (CYP) two days before injury. CYP treatment prevented the injury-induced blood neutrophilia in male and female rats. However, male F344 rats continued to walk better than females even though CYP prevented sex differences in blood neutrophilia. Treated males also had more myelin and spared white matter volume at the injury site than treated females. To our surprise, neuron survival was nearly identical after treatment in both sexes. Thus, both “normal” and CYP treated sex-matched pairs

had similar recovery patterns despite significant differences in the magnitude of blood neutrophilia. Our experiments showed that sex differences in blood neutrophil responses do not cause sex differences in locomotor recovery or tissue sparing after SCI.

Gross tissue and myelin volume at the injury site were significantly bigger in males than females. Spinal cord segments distal to the injury site were also bigger in males than females in both treatment groups. Because BBB scores correlate directly with the amount of spared myelin, these findings are consistent with the observation that males recovered locomotor function earlier and better than females [59]. However, BBB scores from both groups converged after 3 weeks [340, 341]. Recent clinical trials have shown that more physical activity leads to better functional recovery after SCI [342]. One possibility is that female F344 rats are more active but this will need to be confirm in future experiments.

The finding that 100-day-old male F344 rats had bigger spinal cords than females would explain why male F344 rats recovered better and earlier than female F344 rats. The original impactor model in Long-Evans hooded rats and Sprague-Dawley rats standardized the age of injury to 77±1 day old because this was the age at which spinal cord weights of males and females of these two strains were most similar [57]. F344 rats are significantly smaller than Long-Evans and Sprague-Dawley rats, particularly the females [343]. So that the females would fit in the impactor devices, the F344 model was standardized at 100±1 day of age. Our data suggests that more studies should be done to find the age, probably 77 days, that male and female F344 rats would be more similar in terms of spinal cord size.

Several studies in animal models have shown no sex differences . Our current study may be the most detailed and careful study of sex differences in a well-standardized rodent spinal cord injury. While one should be cautious generalizing from rodents to human, our results rule out neutrophils as one mechanism of sex difference in spinal cord injury.

7.2 Effects of blood neutrophilia on SCI recovery

Our results indicate that preventing blood neutrophil responses, by giving cyclophosphamide 2 days before injury, did not change locomotor recovery in male or female F344 rats. The experiments, however, yielded several unexpected findings. First, CYP reduced blood neutrophil counts more in females than in males. Second, greater blood neutrophilia was not associated with neurotoxicity. Third, preventing blood neutrophil responses after SCI did not spare myelin or locomotor recovery in males or females. Our results suggest that acute blood neutrophilia is neither damaging nor helpful for spinal cord repair because all outcome measures were nearly identical for both treatment groups.

From an evolutionary point of view, in retrospect, it is not surprising that blood neutrophilia does not have detrimental effects after SCI. Neutrophils are the first peripheral blood leukocytes to respond to injury. Our experiments suggest that baseline blood neutrophil counts after SCI are sufficient for normal tissue repair and that excess blood neutrophilia is neither harmful nor beneficial for recovery. Animals must have evolved mechanisms to limit excessive neutrophil infiltration into the injured spinal cord.

8. Future Directions

Our experiments provided several experimental opportunities. We found that F344 male rats have 3 times more blood neutrophils than females during the first 3 days after SCI. Higher blood leukocyte counts are associated with worse clinical outcomes after SCI [200]. In humans, blood neutrophil counts 3 times higher than normal would be alarming to doctors. The few clinical studies published on differences of leukocytosis after SCI have been retrospective. A prospective controlled study of blood leukocytes responses to SCI should be done to correlate recovery with leukocytosis in men and women.

The causes of the differences in SCI-induced blood neutrophilia in male and female F344 rats are not known. Several possible explanations should be considered and experimentally ruled out or confirmed. First, the number of blood neutrophils in peripheral reservoirs may be higher in male than female F344 rats. Second, SCI-related complications may contribute to greater blood neutrophilia in male rats. Third, sex differences in blood neutrophilia may be a specific for Fischer 344 rats. Each of these will be discussed below.

The spleen, the largest leukocyte reservoir, is twice as big in male than female F344 rats [283]. SCI increases splenic catecholamines thought to kill lymphocytes and contract the spleen to expel monocytes into blood [271]. The effects of SCI on splenic neutrophil reservoirs need to be confirmed. Neutrophil chemotactic factors like IL-8, that recruit these cells into circulation, are more pronounced in males than females after ischemic injuries [275]. More neutrophils in reservoirs and increased neutrophil-chemotactic

signals would explain the higher neutrophil response to SCI in males. Studying lymphatic reservoirs after SCI may show the reasons for sex differences neutrophils and monocytes but not lymphocytes.

SCI researchers have long preferred to use female rodents because males are considered harder to care for. Male rodents need manual bladder expression after SCI compared to females. Spinal cord injured male F344 rats develop hematuria several days after injury. Hematuria rarely occurred in our spinal cord injured F344 female cohort. The causes for hematuria and urinary retention may contribute to the greater neutrophilia in male rats at 3-4 days after injury.

Our experiments refuted the hypothesis that sex differences in blood neutrophilia worsen recovery after SCI in males compared to females. Having more or less blood neutrophils did not alter recovery after SCI. These findings raise questions about decades of published studies reporting that neutrophils contribute to tissue damage. Depleting blood neutrophils after SCI may be either beneficial or detrimental [158].

Few neutrophils are present in intact spinal cord vasculature [126]. The extent of neutrophil infiltration in injured spinal cord is debated. While implantation of umbilical blood mononuclear cells and mesenchymal stem cells helps recovery after SCI [342], to our knowledge, no one has transplanted neutrophils into the injured spinal cord. Such transplants would answer several questions. First, we could label these cells to determine if implanted neutrophils remain or exit the spinal cord and which cells react to the implanted neutrophils. Second, does activation play a significant role on neutrophil

function in the spinal cord? Third, the experiment would tell us whether are neutrophils neurotoxic and interact with glial cells. Fourth, is neutrophil implantation a therapeutic beneficial option? Lastly, these experiments may define if microglia or monocyte derived macrophages can contain neutrophil activity and protect the injured spinal cord [183, 344].

Figures and Illustrations

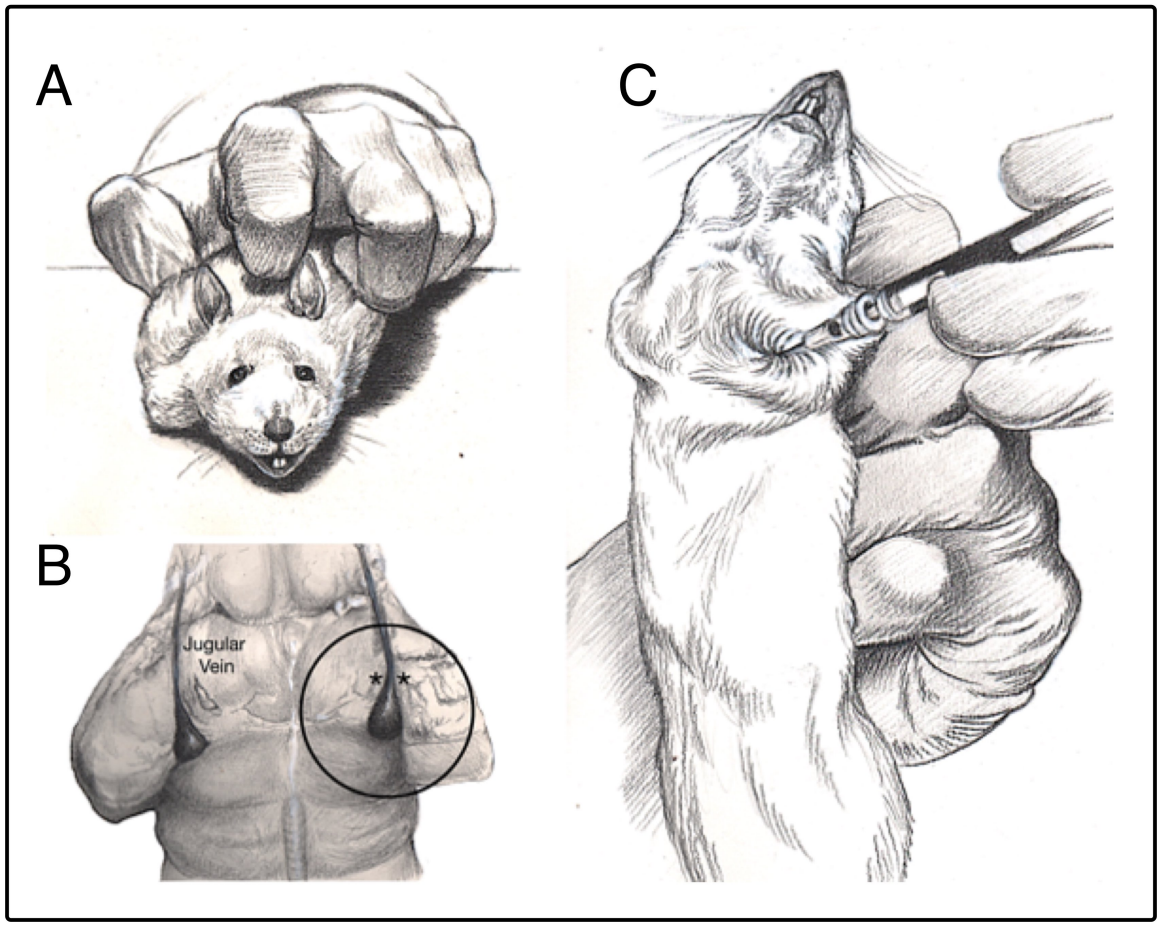

Figure 1. Cranial vena cava venipuncture

A shows how to position a rat for a cranial vena cava (CVC) venipuncture. The thumb and middle finger secure the rats' right arms behind its back and the index finger presses on the scruff of the neck. **B** ** shows the gross anatomy for the target area to insert the needle at the cranial vena cava. **C** shows that proper hand placement and needle insertion at the thoracic cowlick will permit blood flow to the needle.

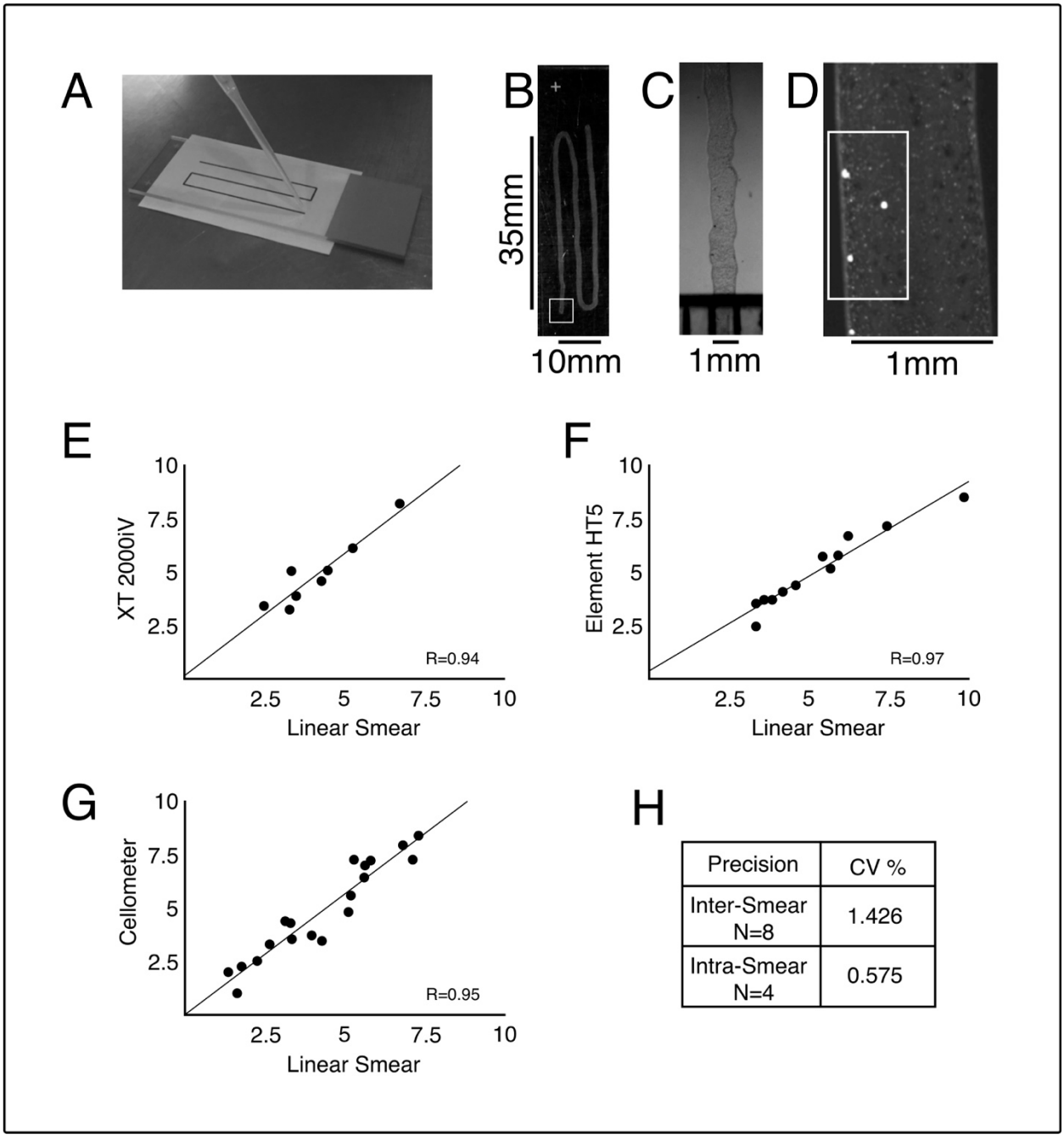

Figure 2. Linear smear technique and validation

A shows a linear smear template and pipette position to make a linear smear. **B** shows each linear smear is 35mm in length, has 3 continuous arms 5 mm apart and has a well-defined proximal end. **C** shows linear smears must be ~1 mm wide. **D** is a representative image of a linear smear under UV fluorescence at 100x magnification, showing bright Hoechst 33342^{+} leukocytes. **E-G** Graphs for logistic regression comparing leukocyte counts in linear smears to counts in XT 2000iv (N = 9, R = 0.94) and Element 5HT (N = 12, R = 0.97) automated hematological analyzers and a Cellometer nucleated cell counter (n = 19, R = 0.95) **H** shows that leukocyte counts in eight different linear smears had low inter-smear variability, CV(8) = 1.426 %. Leukocytes in one linear smear were counted 4 times with low intra-smear variability, CV(4) = 0.575%.

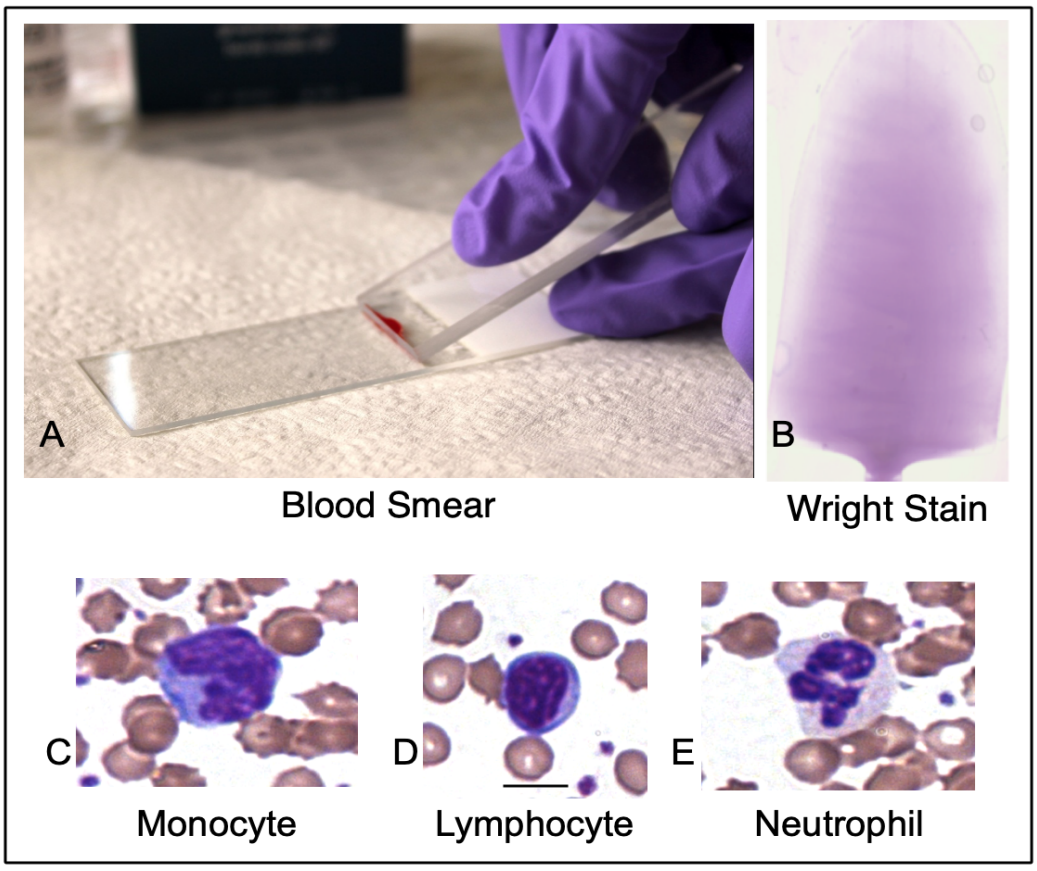

Figure 3. Identification of blood leukocytes in a blood smear

A shows one drop of blood immediately before smearing along the length of microscope slide **B** shows a feather shaped blood smear after Wright Stain **C** shows monocytes and macrophages have ellipsoid bean-shaped nuclei, white circular vacuoles and lilac blue cytoplasm, **D** lymphocytes have lilac blue cytoplasm, dark purple circular nuclei and low cytoplasm to nucleus ratio, E neutrophils have poly-segmented nuclei and light blue granules. Scale bar represents 10 μm.

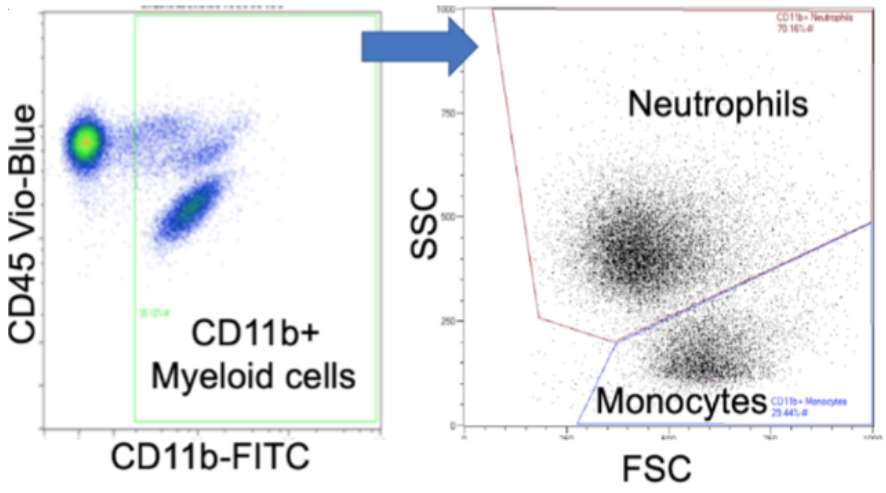

Figure 4. CD11b^{+} myeloid cells separated into neutrophils and monocytes

Flow cytometry plot showing a primary gate for CD45^{+} and CD11b^{+} double positive myeloid cells then secondary gates that isolate CD11b^{+} neutrophils and monocytes, based on high or low side scatter (SSC), respectively.

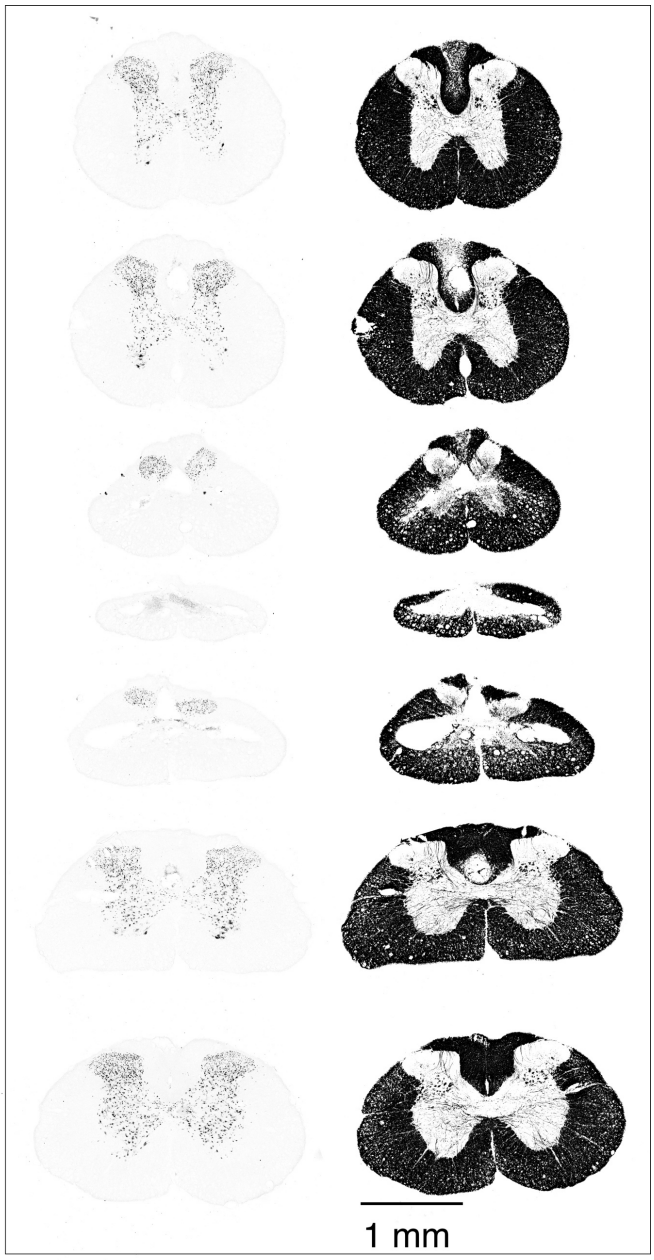

Figure 5. NeuN and Myelin Basic Protein in injured spinal cords

Figure shows immunohistochemistry of neuron nuclear protein NeuN (left) and myelin basic protein (right). Neuron and myelin loss was most pronounced at the injury epicenter. Scale bar is 1 mm.

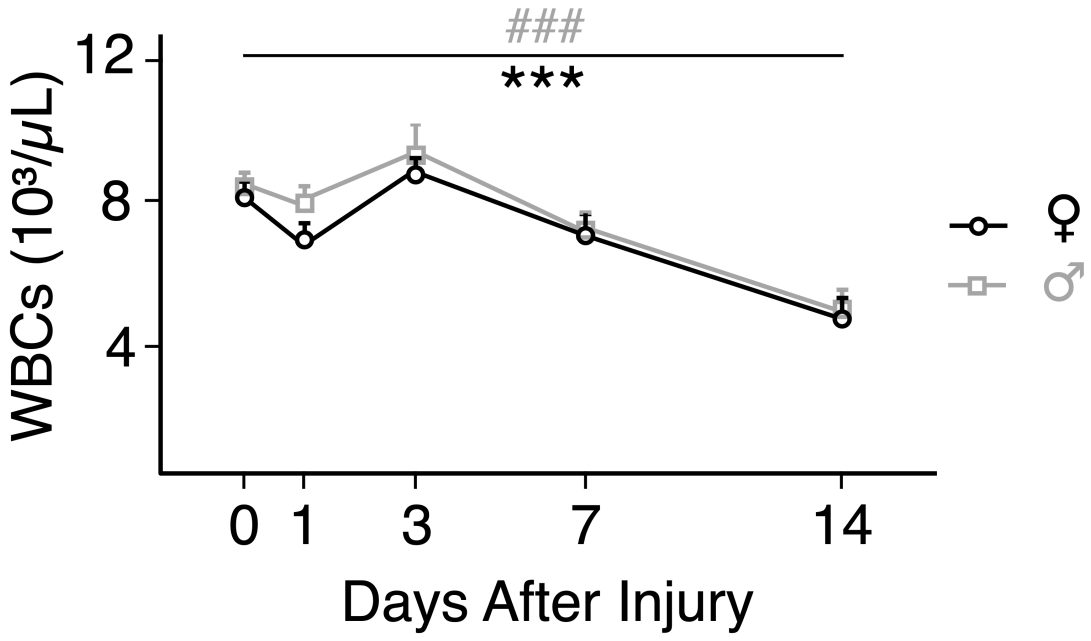

Figure 6. SCI caused blood leukopenia in males and females

Blood leukocytes were collected through a jugular vein catheter and counted with a linear smear. Graph shows mean (+ SEM, 10^3 cells/μl) blood leukocyte counts before and at 1, 3, 7 and 14 days (d) after a 12.5mm spinal cord contusion in male and female F344 rats. Blood leukocyte counts decreased significantly over 14 days after SCI in both sexes (Male, $p < 0.001$; Female, $p < 0.001$, repeated-measures ANOVA, Effect: Time. However, blood leukopenia progressed similarly in both sexes after SCI (p = N.S., 2-factor-repeated-measures-ANOVA, Effect: Time*Sex). (***) indicates ($p < 0.001$) for the number of blood leukocytes from 0d to 14 after SCI using post-hoc paired T-tests. Female: n = 10; Male: n = 10.

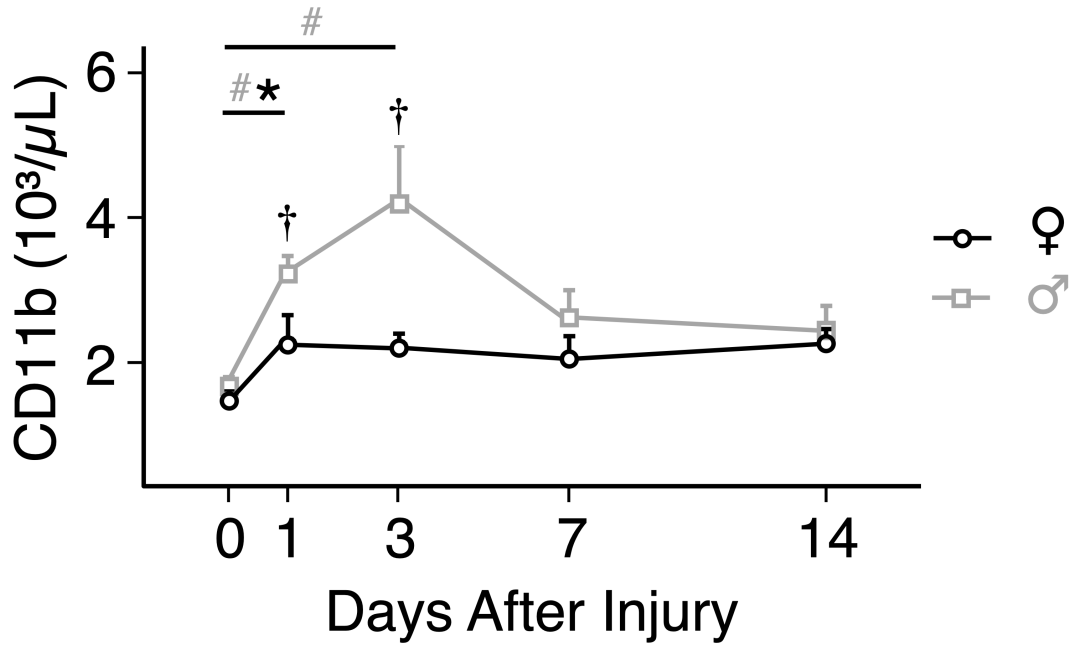

Figure 7. Sex differences in acute blood myeloid cell responses after SCI

Graph shows mean (+ SEM, 10^3 cells/µl) blood $CD11b^+$ myeloid cell counts before and through 14 days (d) after spinal cord contusion in male and female rats. Blood myeloid cell counts increased significantly after SCI in both sexes (Male, $p = 0.002$, Female, $p = 0.02$, repeated-measures ANOVA, Effect: Time). Biological sex had a significant effect on blood myeloid cell responses over 14 days after SCI ($p = 0.005$, 2-factor-repeated-measures-ANOVA, Effect: Time*Sex). (#) and (*) indicate ($p < 0.05$) for changes in the number of blood myeloid cells between those time-points in males and females, respectively (*Post hoc Scheffé's test*). (†) indicates significant sex differences ($p < 0.05$) at each time-point (*Post hoc unpaired-T-tests*). Female: n = 7; Male: n = 6.

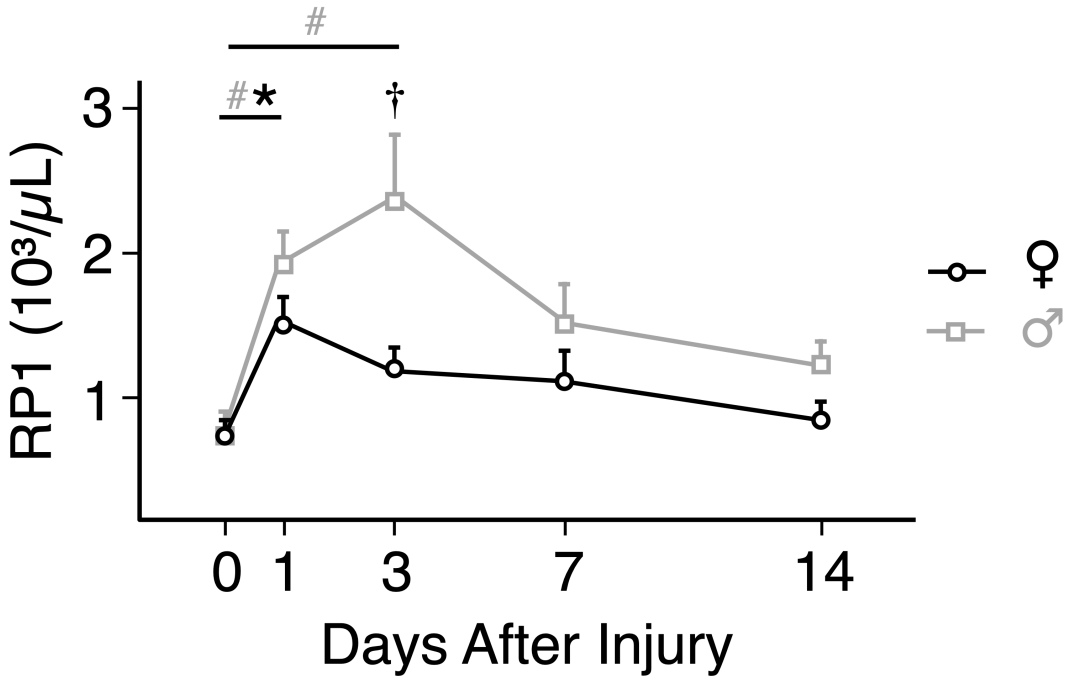

Figure 8. Sex differences in blood neutrophilia after SCI

Blood RP1$^+$ neutrophil counts increased significantly after SCI in both sexes (Male, p = 0.001; Females, p = 0.02; repeated-measures ANOVA, Effect: Time). Biological sex affected the magnitude of blood neutrophilia after SCI (p = 0.07, 2-factor-repeated-measures-ANOVA, Effect: Time*Sex). (#) and (*) indicate ($p < 0.05$) for changes in the number of blood neutrophils between those time-points in males and females, respectively (*Post hoc Scheffé's test*). (†) indicates significant sex differences ($p < 0.05$) at each time-point (*Post hoc unpaired-T-tests*). Female: n = 7; Male: n = 6.

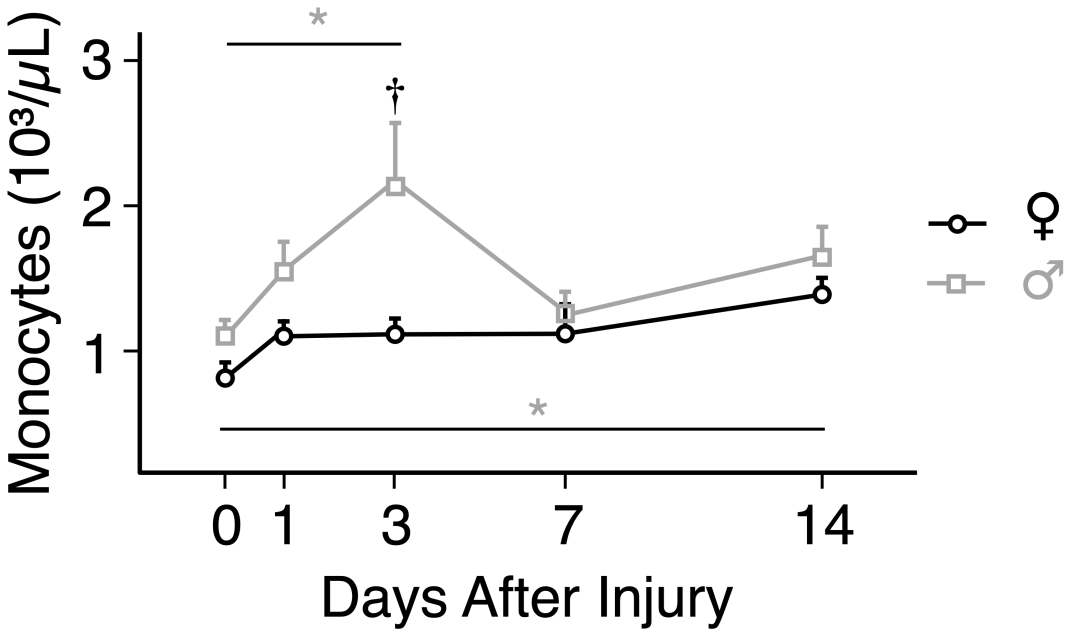

Figure 9. Sex differences in blood monocyte responses after SCI

Graph shows changes in blood monocyte counts (average + SEM, 10^3 cells/μl) from before to 14 days after spinal cord contusion in male and female rats. Blood monocyte counts changed over time after injury in both sexes (Males, $p = 0.062$; Females, $p = 0.04$; repeated-measures ANOVA, Effect: Time). Although blood monocyte counts tended to be higher in males than females, there were no significant sex differences in blood monocyte responses after spinal cord contusion (p = N.S., 2-factor repeated-measures ANOVA, Effect: Time*Sex). (*) indicate ($p < 0.05$) for changes in the number of blood neutrophils between those time-points in male rats (*Post hoc Scheffé's test*). Female: n = 7; Male: n = 6.

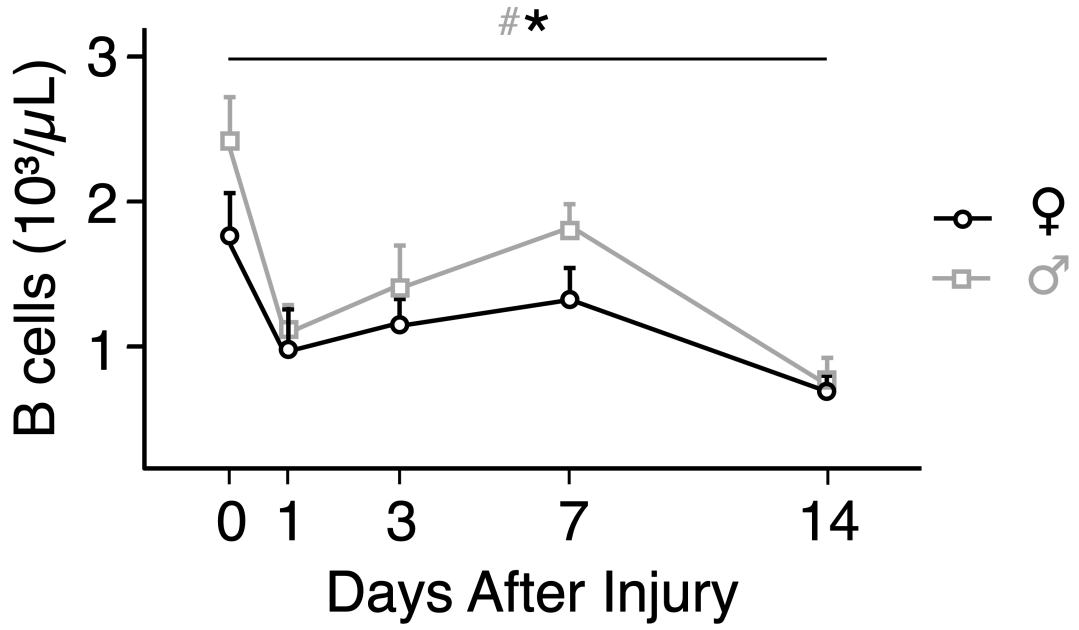

Figure 10. SCI causes lymphopenia for B-cells not but T-cells

Graph shows changes in blood B cell counts (average + SEM, 103 cells/µl) from before to 14 days after spinal cord contusion in male and female rats. Blood B cell counts decreased significantly over 14 days after injury in both sexes (Males, $p < 0.001$; Females, $p = 0.03$; *repeated-measures ANOVA*, Effect: *Time*). Blood B cell lymphopenia progressed similarly in both sexes after SCI (p = N.S., 2-factor-repeated-measures-ANOVA, Effect: Time*Sex). (#) and (*) indicate ($p < 0.05$) for changes in the number of B-cells between those time-points in males and females, respectively (*Post hoc Scheffé's test*). Female: n = 7; Male: n = 6.

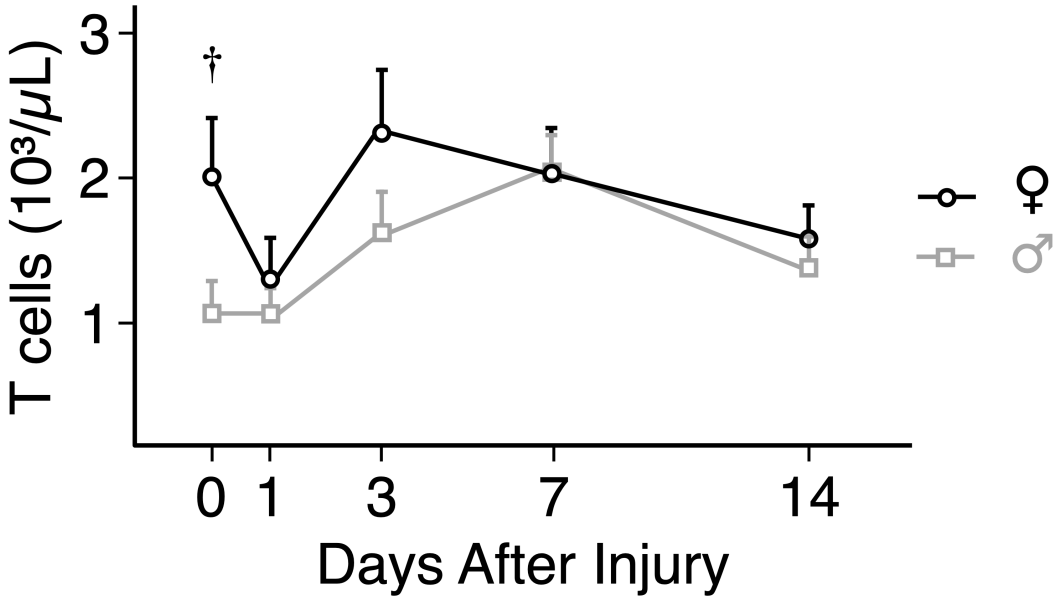

Figure 11. Insignificant changes in blood T cell counts after SCI

Graph shows changes in blood T cell counts (average + SEM, 103 cells/µl) before and over 14 days after spinal cord contusion in male and female rats. Although the number of T cells fluctuated after SCI in males or females, these changes were not statistically significant for neither sex (Male, p = N.S.; Female p = N.S.; *repeated-measures ANOVA,* Effect: *Time*). Changes in blood T cell counts after SCI did not differ significantly between male and female rats (p = N.S., *2-factor repeated-measures ANOVA*, Effect: *Time*Sex*). (†) indicates significant sex differences ($p < 0.05$) in T cell counts before spinal cord contusion (Post hoc unpaired T-tests). Female: n = 7; Male: n = 6.

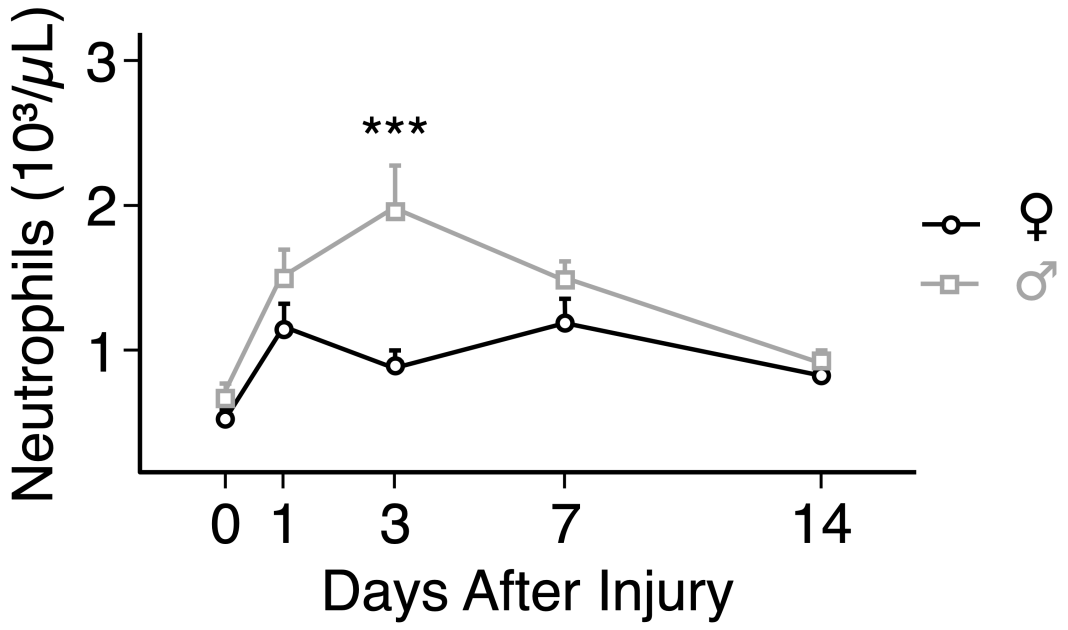

Figure 12. Sex differences in blood neutrophilia after SCI

Graph shows mean (+ SEM, 10^3 cells/µl) blood neutrophil counts before and through 14 days (d) after spinal cord contusion in male and female rats. Blood was collected with jugular venipunctures before and at 1, 3, 7 and 14 days (d) after SCI in 100 ± 3-day-old male (n=8) and female (n=10) F344 rats. Blood neutrophils were identified with flow cytometry as $CD45^+CD11b^+SSC^{high}$. The percent of neutrophils was multiplied by the total number of leukocytes counted in linear smears to obtain the absolute counts per µl of blood. SCI caused blood neutrophilia in both sexes (Male, $p < 0.001$; Female, $p = 0.004$, repeated-measures ANOVA, Effect: Time). The magnitude and duration of peak blood neutrophilia differed between males and females after SCI but this effect did not reach statistical significance ($p = 0.07$, $\eta p2 = 0.16$, Effect: Time*Sex). (*) indicates sex differences ($p < 0.05$) in blood neutrophilia at 3 days after injury (Post hoc unpaired T-tests).

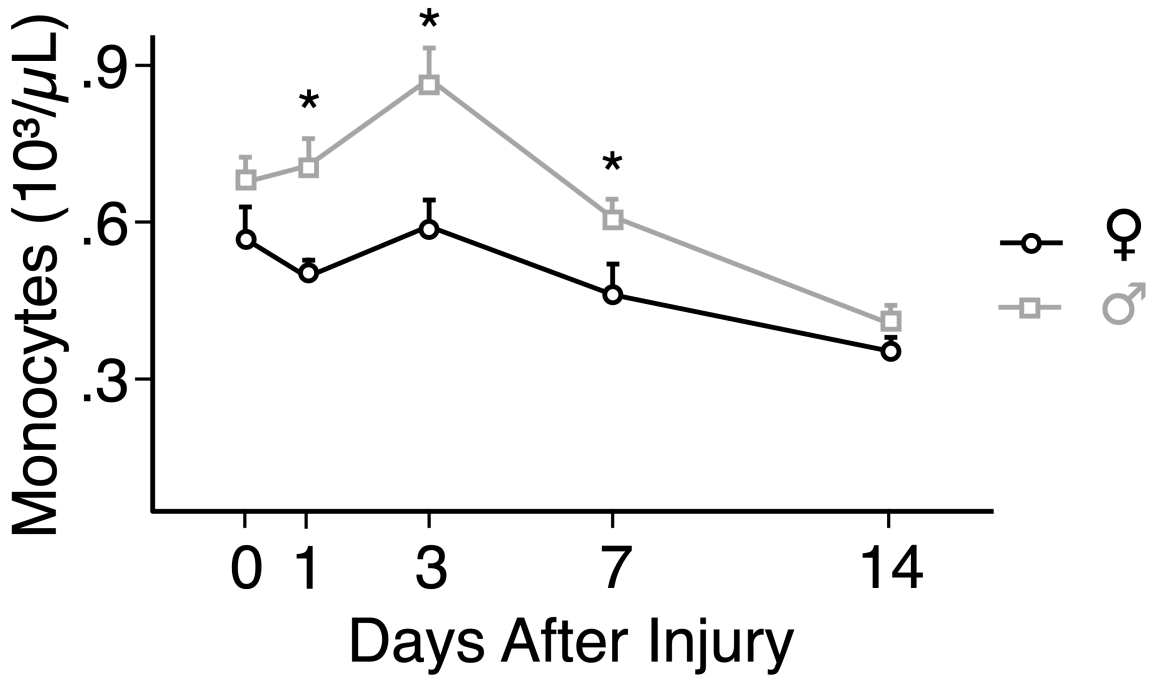

Figure 13. Sex differences in blood monocyte responses after SCI

Graph shows mean (+ SEM, 10^3 cells/µl) blood monocytes identified with flow cytometry as $CD45^+CD11b^+SSC^{low}$. The percent of monocytes was multiplied by the total number of leukocytes counted in linear smears to obtain the absolute counts per µl of blood. Blood monocyte counts increased then decreased over 14 days after SCI in both sexes (Male, $p < 0.001$; Female, $p < 0.001$; repeated-measures ANOVA, Effect: Time). Males had on average, more blood monocytes than females before and after SCI ($p < 0.001$, 2-factor repeated-measures ANOVA, Effect: Sex). Males had more blood monocytes before and 1, 3, 7 and 14 days after SCI ($p < 0.05$, *post-hoc unpaired T-tests*). (*) indicates $p < 0.05$.

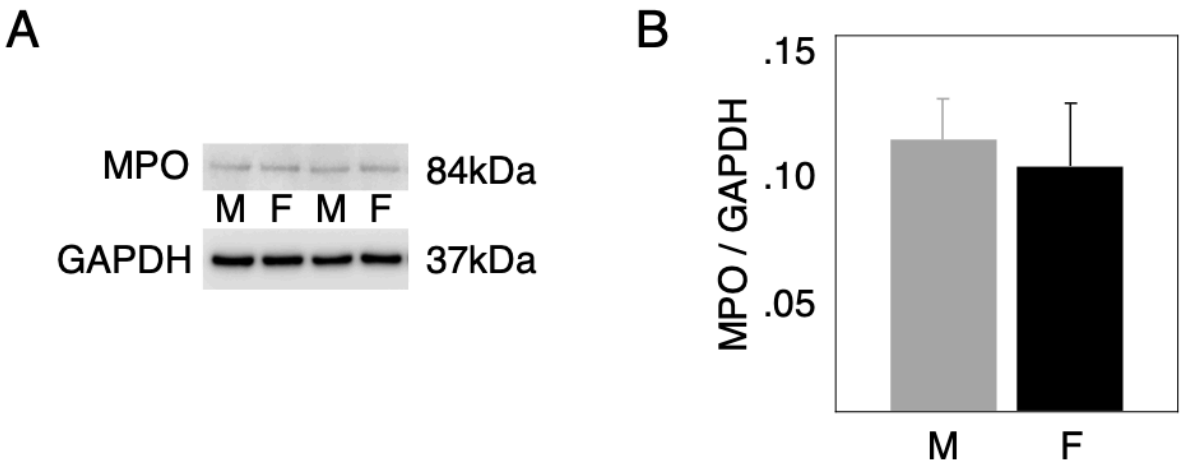

Figure 14. No sex differences in neutrophil infiltration at the injured spinal cord **A** Representative Western blots of myeloperoxidase (MPO) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) content in injured spinal cords from males (M) and females (F) at 3 at days after 12.5mm spinal cord contusion. **B** Bar graph shows the ratio of MPO / GAPDH in injured spinal cords at 3 days after injury in males and females. (n = 8 per sex).

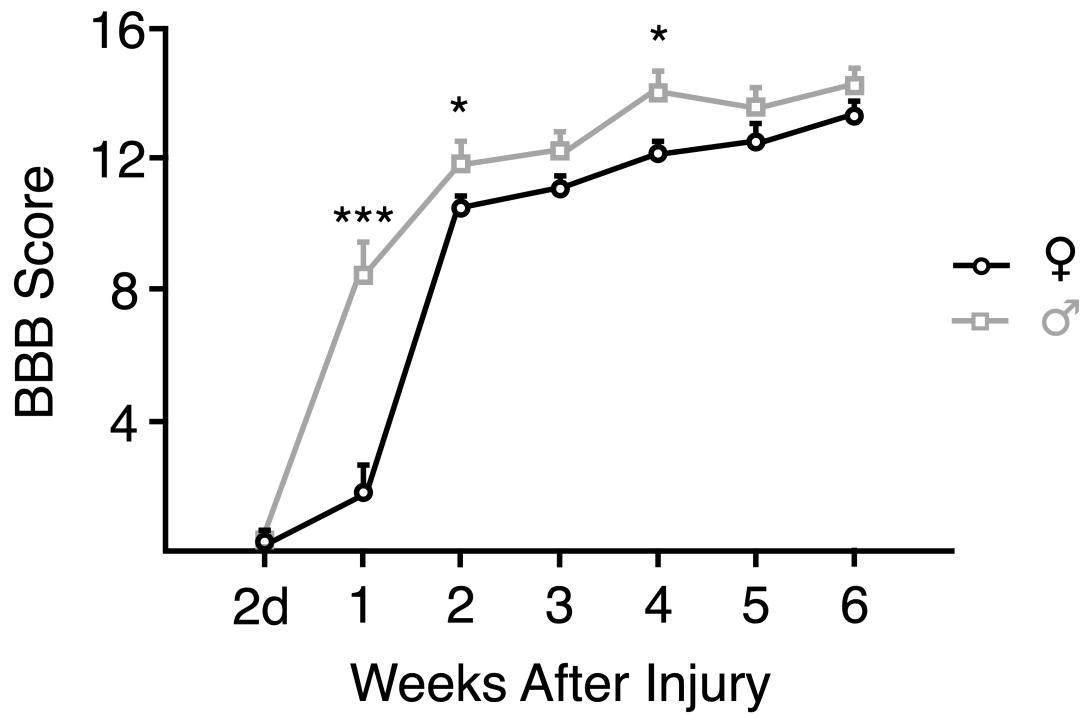

Figure 15. Early sex differences in locomotor recovery at 6 weeks after SCI

Graph shows the time-course of BBB scores (average + SEM) in 100-day-old spinal cord injured male and female F344 rats at 2 days (d) after injury then weekly for 6 weeks. BBB scores improved better in males than females, but long-term locomotor recovery was similar in both sexes ($p < 0.001$, repeated measures ANOVA, Effect: Time*Sex). (* and ***) indicate significant differences of ($p < 0.01$ and $p < 0.001$) in BBB scores between male and female spinal cord injured rats at the respective time-point (*Scheffé's post hoc test*).

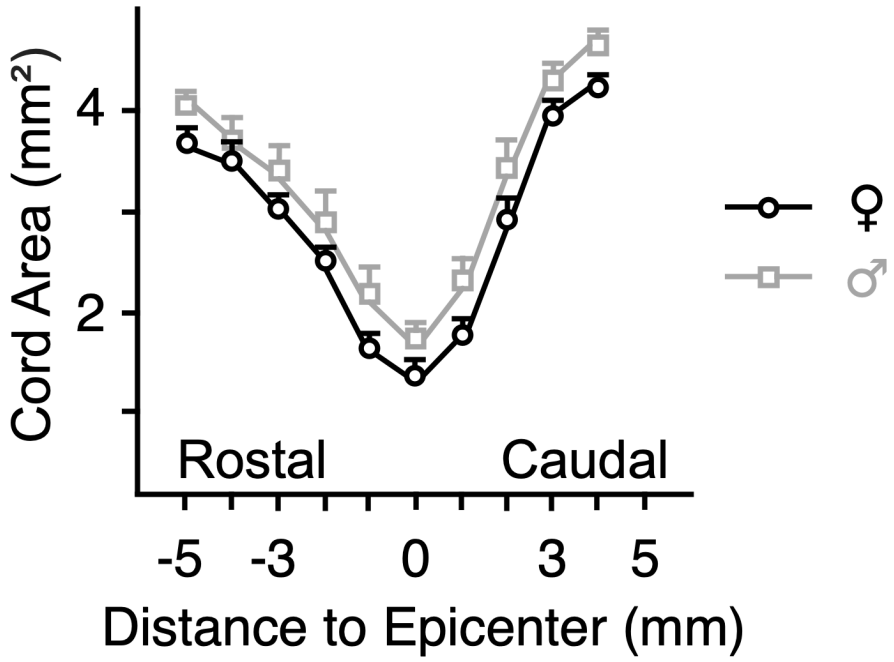

Figure 16. Sex differences in cord area but not tissue sparing after SCI

Graph shows mean cross-sectional area (+ SEM, mm2) from transverse spinal cord tissue sections at 6 week after contusion injury in male and female rats. There were no sex differences in spinal cord tissue sparing at 6 weeks after injury (p = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Spared-Tissue*Sex*). However, cross-sectional spinal cord areas were on, average, bigger in males than females ($p < 0.01$, *2-factor-repeated-measures-ANOVA*, Effect: *Sex*). (0) represents the injury epicenter. Cross-sectional spinal cord areas were recorded 1-millimeter (mm) apart at the rostral and caudal directions from the injury epicenter. Male: n = 7; Female: n = 10.

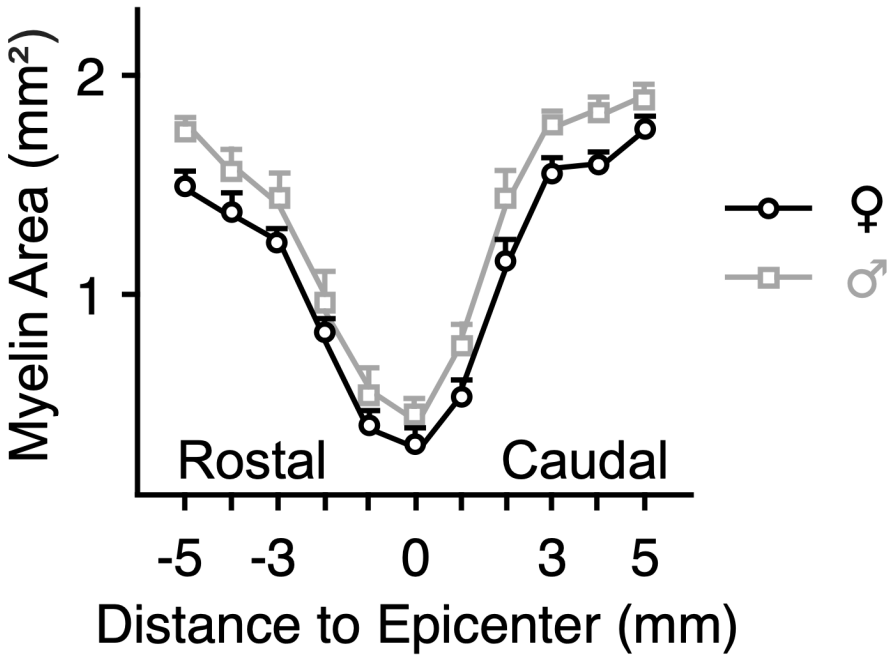

Figure 17. Sex differences in area but not myelin sparing after SCI

Graphs shows the cross-sectional area (+ SEM, mm2) of myelin basic protein (MBP) signal from transverse spinal cord tissue sections at 6 week after spinal cord contusion in male and female rats. There were no sex differences in myelin sparing at 6 weeks after injury (p = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Spared-myelin*Sex*). However, cross-sectional myelinated areas were on, average, bigger in males than females ($p < 0.01$, *2-factor-repeated-measures-ANOVA*, Effect: *Sex*). (0) represents the injury epicenter. Cross-sectional spinal cord areas were recorded 1-millimeter (mm) apart at the rostral and caudal directions from the injury epicenter. Male: n = 7; Female: n = 10.

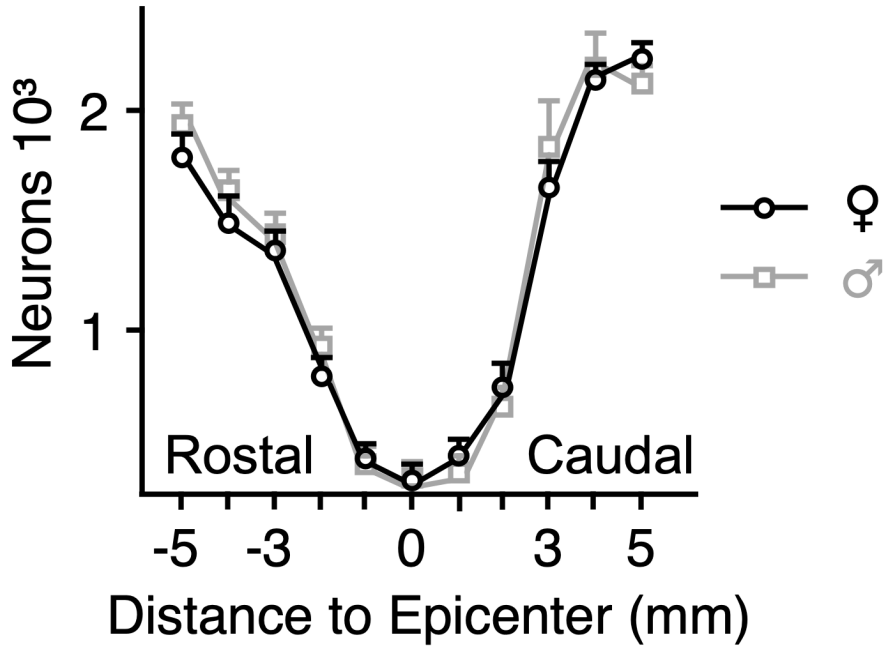

Figure 18. No sex differences in neuron survival after SCI

Graph shows mean (+ SEM) counts of NeuN+ from transverse spinal cord tissue sections at 6 week after contusion injury in male and female rats. There were no sex differences in neuron survival at 6 weeks after injury (p = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Neuron-Survival*Sex*). Males and females also had similar numbers of surviving neurons per tissue section across the injured spinal cord (p = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Sex*). (0) represents the injury epicenter. Cross-sectional spinal cord areas were recorded from tissue sections 1-millimeter (mm) apart at the rostral and caudal directions from the injury epicenter. Male: n = 7; Female: n = 10.

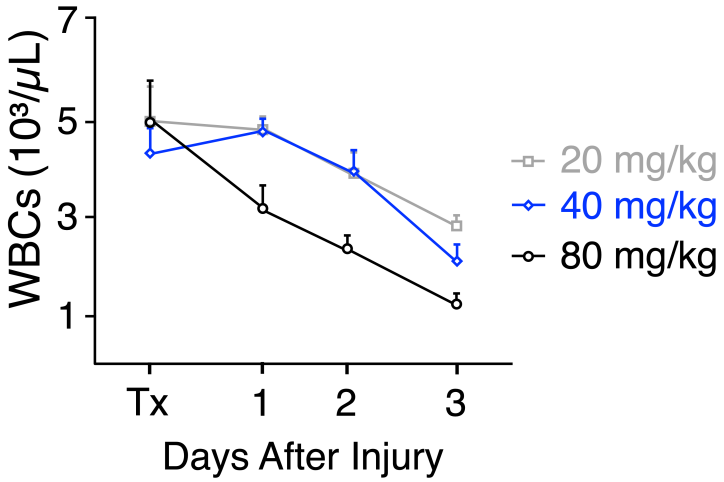

Figure 19. CYP dose-dependent reduction in blood leukocytes after SCI

Graph shows mean (+ SEM, 103 cells/µl) white blood cells (WBCs, leukocytes) in male F344 rats before 20 mg/kg, 40 mg/kg or 80 mg/kg subcutaneous cyclophosphamide (CYP) treatment (given two days before injury), then at 1, 2 and 3 days after injury. 20 mg/kg: n = 2; 40 mg/kg: n = 2, 20 mg/kg: n = 2.

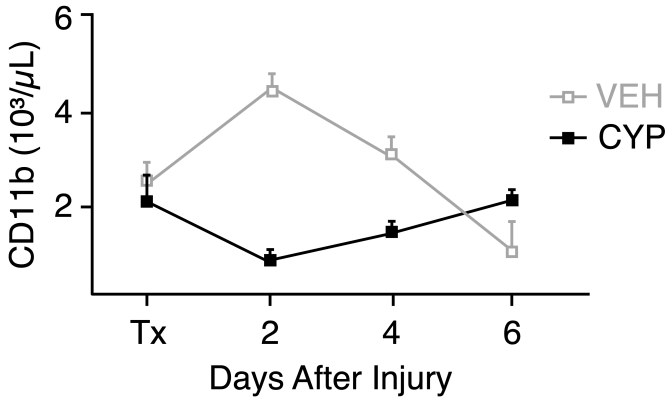

Figure 20. CYP prevented blood myeloid cell responses after SCI

Graph shows the number of CD45+CD11b+ blood myeloid cells (average + SEM, 103 cells/µl) in male F344 rats before 25 mg/kg subcutaneous cyclophosphamide (CYP) treatment (given two days before injury), then at 2, 4 and 6 days after injury. VEH: n = 2, CYP: n = 2.

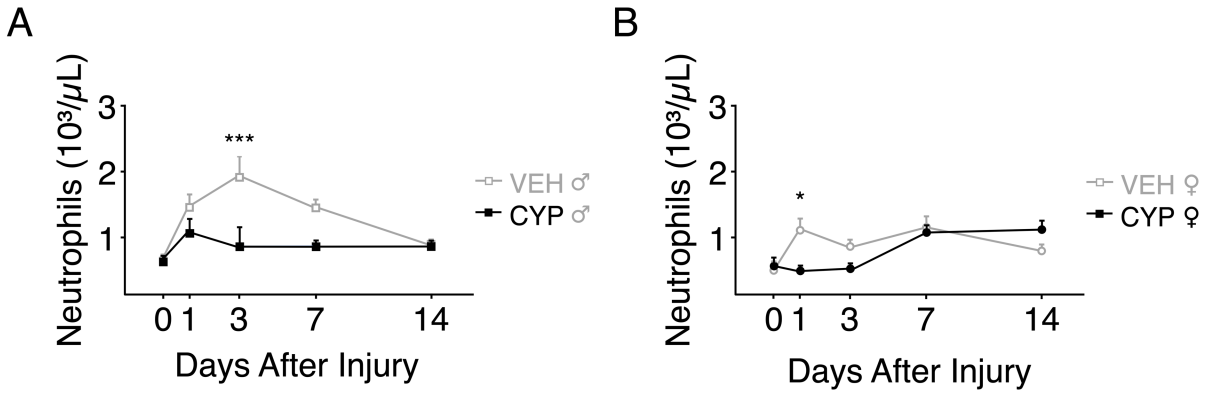

Figure 21. CYP prevented blood neutrophilia in males and females after SCI

Graphs show mean (+ SEM, 103 cells/μl) blood neutrophil counts before and over 14 days after SCI. Blood neutrophils were identified as $CD45^{+}CD11b^{+}SSChigh$ with flow cytometry and the percent of blood neutrophils was multiplied by the total number of blood leukocytes obtained from linear smears. **A** Blood neutrophil responses differed significantly after SCI in VEH and CYP treated male rats ($p = 0.002$, *2-factor-repeated-measures-ANOVA*, Effect: *Male*Time*Treatment*). (***) indicates $p < 0.001$ using post-hoc unpaired T-tests. Male-VEH: n = 8; Male-CYP: n = 8. **B** Blood neutrophil responses differed significantly in VEH and CYP treated spinal cord injured female rats ($p = 0.001$, *2-factor-repeated-measures-ANOVA*, Effect: *Female*Time*Treatment*). (*) indicates $p < 0.05$ using post-hoc unpaired T-tests. Female-VEH: n = 10; Female-CYP: n = 10.

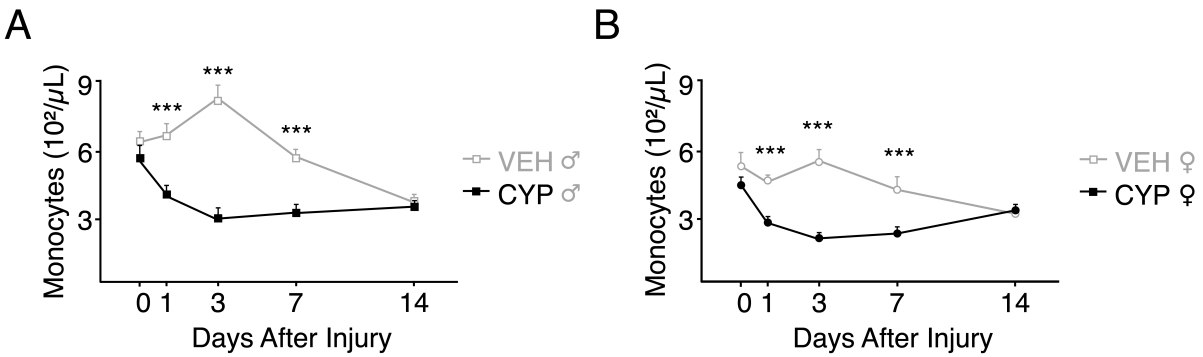

Figure 22. CYP reduced blood monocyte counts after SCI

Blood monocytes were identified as $CD45^{+}CD11b^{+}SSClow$ with flow cytometry and the percent of blood monocytes was multiplied by the total number of blood leukocytes obtained from linear smears. Graphs show mean (+ SEM, 103 cells/μl) blood monocyte counts before and over 14 days after SCI. **A** Compared to VEH, CYP treatment significantly reduced the number of blood monocytes in spinal cord injured in male rats ($p = 0.002$, *2-factor-repeated-measures-ANOVA*, Effect: *Male*Time*Treatment*). Male-VEH: n = 8; Male-CYP: n = 8. **B** Compared to VEH, CYP treatment significantly reduced the number of blood monocytes after SCI in female rats ($p < 0.001$, *2-factor-repeated-measures-ANOVA*, Effect: *Female*Time*Treatment*). (***) indicates $p < 0.001$ using *post-hoc unpaired T-tests*. Female-VEH: n = 10; Female-CYP: n = 10.

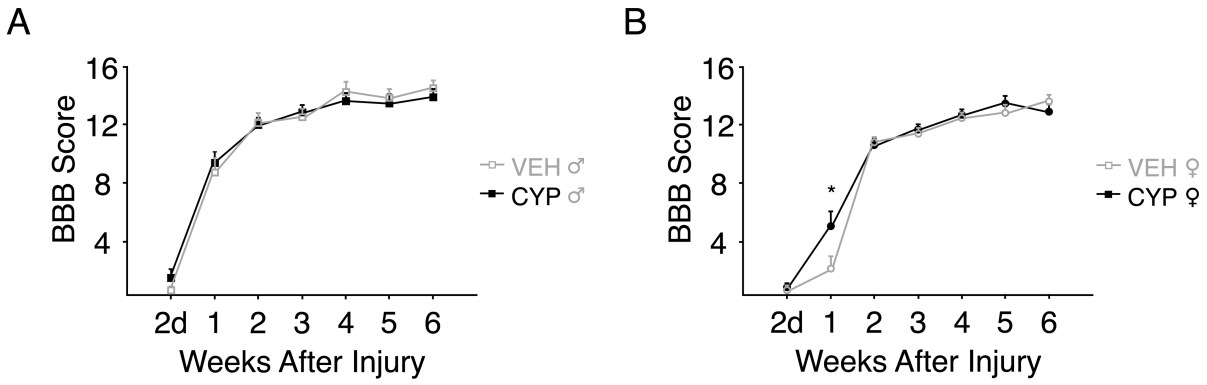

Figure 23. Preventing blood myelocytosis did not improve walking recovery

A Graph shows mean BBB locomotor scores (+ SEM) from 2 days (d) to 6 weeks after SCI in VEH and CYP treated male rats. Compared to VEH, CYP treatment that prevented blood neutrophilia and reduced blood monocyte counts had a minimal effect on locomotor recovery in spinal-cord-injured males (p = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Male*Time*Treatment*). Male-VEH: n = 8; Male-CYP: n = 8. **B** Graph shows mean BBB locomotor scores (+ SEM) from 2d to 6 weeks after SCI in VEH and CYP treated female rats. Compared to VEH, treatment that prevented blood myeloid cell responses had an acute and transient benefit on recovery of hind-limb function in female rats but long-term recovery was nearly identical in both female treatment groups ($p = 0.006$, *2-factor-repeated-measures-ANOVA*, Effect: *Female*Time*Treatment*). Female-VEH: n = 10; Female-CYP: n = 10. (*) indicates $p < 0.05$ using *post-hoc unpaired T-tests*.

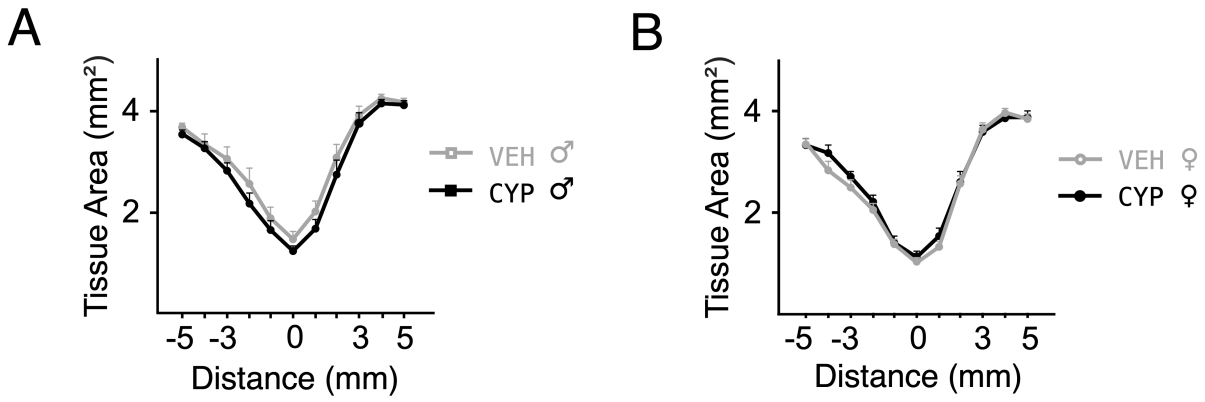

Figure 24. Preventing blood myelocytosis did not improve tissue sparing

Graphs show mean spinal cord cross-sectional area (+ SEM) in transverse tissue sections at 6 week after spinal cord contusion in **A** VEH and CYP treated male rats and **B** VEH and CYP treated female rats. Compared to VEH, CYP treatment that prevented acute blood neutrophilia and reduced blood monocyte counts after SCI did not have significant effect on tissue sparing in neither males nor females (p = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Cord-Area*Treatment*). (0) represents the injury epicenter and cross-sectional spinal cord areas were recorded 1-millimeter (mm) apart at the rostral and caudal injured spinal cord segments. Male-VEH: n = 7; Male-CYP: n = 8; Female-VEH: n = 10; Female-CYP: n = 10.

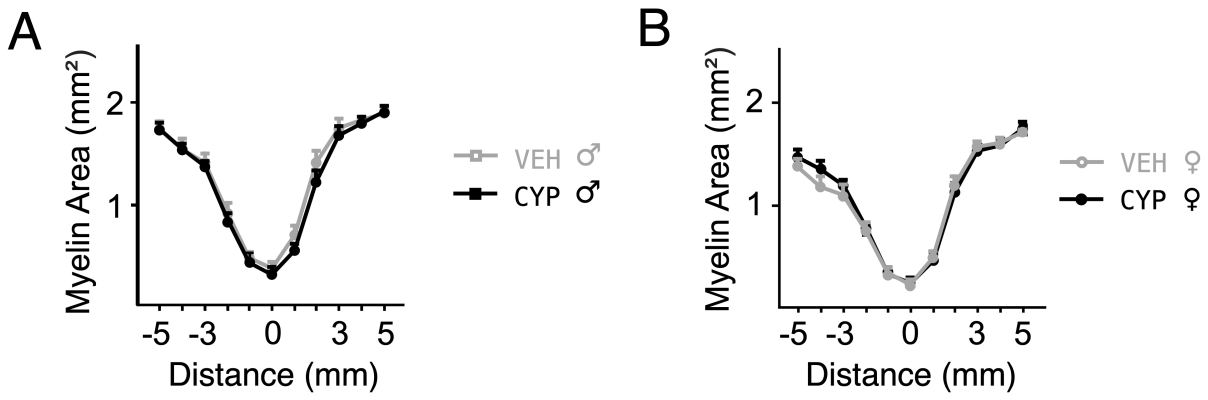

Figure 25. Preventing blood myelocytosis did not improve myelin sparing

Graphs shows the cross-sectional area of myelin basic protein (MBP) signal in transverse spinal cord tissue sections at 6 week after spinal cord contusion in **A** VEH and CYP treated male rats **B** VEH and CYP treated female rats. Compared to VEH, CYP treatment that prevented acute blood myeloid cell responses after SCI did not have significant effect on myelin sparing in neither males nor females (*p* = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Myelin-Area*Treatment*). (0) represents the injury epicenter and cross-sectional spinal cord myelinated areas were recorded 1-millimeter (mm) apart at the rostral and caudal injured spinal cord segments. Male-VEH: n = 7; Male-CYP: n = 8; Female-VEH: n = 10; Female-CYP: n = 10.

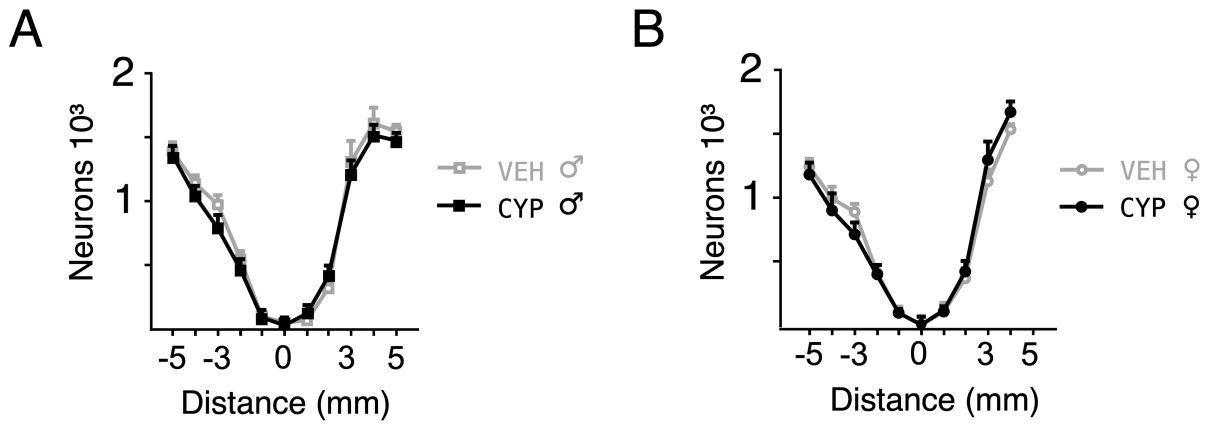

Figure 26. Preventing blood myelocytosis did not improve neuron survival

Graphs show the number of NeuN+ neurons in transverse spinal cord tissue sections at 6 weeks after spinal cord contusion in **A** VEH and CYP treated male rats and **B** VEH and CYP treated female rats. Compared to VEH, CYP treatment that prevented acute blood neutrophilia and reduced blood monocyte counts did not have a significant effect on neuron survival in neither male nor female groups (p = N.S., *2-factor-repeated-measures-ANOVA*, Effect: *Neuron-Counts*Treatment*). (0) represents the injury epicenter and neuron counts were recorded 1-millimeter (mm) apart at the rostral and caudal injured spinal cord segments. Male-VEH: n = 7; Male-CYP: n = 8; Female-VEH: n = 10; Female-CYP: n = 10.

Tables

Table 1

Leukocytes per µl of blood before and after SCI

Days After	Male		Female	
Injury	Mean	SD	Mean	SD
0	8137	703	7558	1178
1	7211	1162	6361	1156
3	8083	1679	8210	761
7	6768	1278	6547	1693
14	4168	441	4229	1113

Table 2

Effect of Time and biological Sex on blood leukocyte counts after SCI assessed with Repeated-measures ANOVA (RMANOVA)

ID	Groups	Effect	*SS*	*df*	*MS*	*F*	*p*	η_p^2
A1	Males	Time	71693906	4,24	17923477	10.66	< 0.001	0.64
		Error	40369991		1682083			
A2	Females	Time	64343490	4,24	16085872	10.43	< 0.001	0.63
		Error	37019761		1542490			
A3	All	Time*Sex	1424975	4,48	4880832	2.25	0.16	0.16
		Error	77389753		2172847			
A4	All	Sex	4880832	1,12	356244	20.87	0.93	0.02
		Error	26074160		1612287			

Table 3

$CD11^+$ per µl of blood before and after SCI

Days After	Male		Female	
Injury	Mean	SD	Mean	SD
0	1513	237	1245	312
1	3258	635	2299	588
3	3609	1710	2032	583
7	2456	873	1885	853
14	2197	852	2093	485

Table 4

Results from RMANOVA for the effects of Time after SCI and Biological Sex on the number of $CD11^+$ blood myeloid cells

ID	Groups	Effect	*SS*	*df*	*MS*	*F*	*p*	η_p^2
A5	Males	Time	29001089 28768888	4,24	7250272 1198704	6.05	0.002	0.50
A6	Females	Time	4500512 7090738	4,24	1125128 295447	3.81	0.02	0.39
A7	All	Time*Sex	11453377 11557914	4,48	1145337 963159	11.89	0.005	0.50
A8	All	Sex	8832536 35859625	1,12	2208134 747076	2.96	0.03	0.20

Table 5

$RP1^+$ cells per µl of blood before and after SCI

Days After	Male		Female	
Injury	Mean	SD	Mean	SD
0	443	162	585	312
1	1940	588	1341	509
3	2023	268	1051	366
7	1342	641	947	606
14	804	344	689	332

Table 6

Effects of Time after SCI and Sex on blood neutrophil counts assessed with RMANOVA

ID	Groups	Effect	*SS*	*df*	*MS*	*F*	*p*	η_p^2
A9	Males	Time	13630695 11687247	4,24	3407674 486969	7.0	0.001	0.54
A10	Females	Time	2522130 3981169	4,24	630532 165882	3.80	0.02	0.39
A11	All	Time*Sex	3051907 15668416	4,48	762977 326425	2.34	0.07	0.16
A12	All	Sex	2818127 4640586	1,12	2818127 386716	7.29	0.02	0.38

Table 7

Monocytes per µl of blood before and after SCI

Days After	Male		Female	
Injury	Mean	SD	Mean	SD
0	1070	236	660	267
1	1318	579	958	235
3	1585	494	945	287
7	1115	333	937	469
14	1393	618	1232	283

Table 8

Effects of Time after SCI and Sex on blood monocyte counts assessed with RMANOVA

ID	Groups	Effect	*SS*	*df*	*MS*	*F*	*p*	η_p^2
A13	Males	Time	4083358	4,24	1020840	2.60	0.06	0.30
		Error	9433871		393078			
A14	Females	Time	1146143	4,24	286536	3.00	0.04	0.33
		Error	2291781		95491			
A15	All	Time*Sex	1598996	4,48	399749	1.64	0.18	0.12
		Error	11725652		244284			
A16	All	Sex	3530018	1,12	3530018	9.87	0.009	0.45
		Error	4291140		357595			

Table 9

B cells per µl of blood before and after SCI

Days After	Male		Female	
Injury	Mean	SD	Mean	SD
0	2244	1077	1576	784
1	889	348	802	766
3	1132	750	964	429
7	1555	442	1135	593
14	429	284	515	181

Table 10

Effects of Time after SCI and Sex on blood B cell numbers assessed with RMANOVA

Test	Measure	Effect	*SS*	*df*	*MS*	*F*	*p*	η_p^2
A17	Males	Time	126704546992017	4,24	3167613291334	10.87	0.001	0.64
A18	Females	Time	43763778238707	4,24	1094094343279	3.19	0.03	0.35
A19	All	Time*Sex	117036415230724	4,48	292591317307	0.92	0.46	0.07
A20	All	Sex	16663885862050	1,12	1666388488504	3.41	0.09	0.22

Table 11

T cells per µl of blood before and after SCI

Days after	Male		Female	
Injury	Mean	SD	Mean	SD
0	1020	575	1840	1043
1	854	630	1139	759
3	1281	653	2147	1191
7	1883	673	1861	805
14	1031	446	1429	560

Table 12

Effects of Time after SCI and Sex on blood T cell counts assessed with RMANOVA

ID	Measure	Effect	*SS*	*df*	*MS*	*F*	*p*	η_p^2
A21	Males	Time	4447821 9324063	4,24	1111955 388503	2.86	0.05	0.32
A22	Females	Time	4427456 20347219	4,24	1106864 847801	1.31	0.30	0.18
A23	All	Time*Sex	2749907 6447753	1,12	2749907 537313	5.12	0.04	0.30
A24	All	Sex	1818601 29671281	4,48	454650 618152	0.74	0.57	0.06

Table 13

Experimental design to treat acute blood leukocyte responses after SCI

Injury Model	N
F344 rats 100 ± 3 days old	
12.5mm 10g contusion at T11 with a MASCIS Impactor	36
Males	16
Females	20
Treatment	N
Subdermal 1mL of distilled water vehicle 2 days before injury	18
Subdermal 25 mg / kg CYP in distilled water 2 days before injury	18
Blood collection and leukocyte quantification	
Cranial Vena Cava Venipuncture, 100µl of blood per collection	
Endpoints: before treatment, 1 day (d), 3d, 7d, 14d after injury	
Linear smear to count total leukocytes per µl of blood	
Flow cytometry to differentiate $CD45^{+}CD11^{+}$ blood myeloid cells	
Blood CD11b+ monocyte and neutrophil differentiation with side scatter	
Outcome Measures	
CD11b high side scatter neutrophil counts	
CD11b low side scatter monocytes counts	
BBB scores at 2d, 1 week (w), 2w, 3w, 4w, 5w, 6w	
Spinal cord sparing area 6 weeks after injury	
Myelin sparing area 6 weeks after injury	
Neuron survival at the injury site	
Injury site gross spinal cord and myelin volume at week 6	
Intact spinal cord volume 4mm caudally and rostrally from injury site	
Intact myelin volume 4mm caudally and rostrally from injury site	

Table 14

$CD11b^+$ neutrophils per µl of blood before and after SCI in male and female rats

	Male		Female	
Days	Mean	SD	Mean	SD
0	511	161	317	142
1	1319	693	980	541
3	1637	477	730	281
7	1267	683	1027	522
14	756	123	668	321

Table 15

Effects of Time after SCI and Sex on $CD11b^+$ neutrophil counts assessed with RMANOVA

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
B1	Male	Time	6637483	4,28	1659371	7.34	<0.001	0.51
		Error	6330189		226078			
B2	Female	Time	2801859	4,36	700465	4.68	.004	0.34
		Error	5385459		149596			
B3	All	Time*Sex	1934660	4,64	483665	2.64	.04	0.14
		Error	11715648		183057			

Table 16

$CD11b^+$ monocytes per µl of blood before and after SCI

	Male		Female	
Days	Mean	SD	Mean	SD
0	654	110	514	135
1	633	216	451	80
3	742	277	530	190
7	547	150	413	118
14	360	67	304	79

Table 17

RMANOVA results for the effects of Time after SCI and biological Sex on $CD11b^+$ blood monocyte counts

Test	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
B4	Male	Time	669258	4,28	167315	5.17	0.002	0.36
		Error	969024		34608			
B5	Female	Time	329995	4,36	82499	4.83	0.004	0.41
		Error	574292		15953			
B6	All	Time*Sex	61431	1,4	15358	0.64	0.64	0.04
B7	All	Sex	465697	1,16	465697	22.71	<0.001	0.59
		Error	328125					

Table 18

BBB locomotor scores after SCI

	Male		Female	
Time	Mean	SD	Mean	SD
2d	0.25	0.46	0.20	0.42
1w	8.38	2.88	1.80	2.62
2w	11.75	1.83	10.40	0.70
3w	12.13	1.64	11.00	0.67
4w	13.88	1.89	12.10	0.99
5w	13.38	1.92	12.40	1.51
6w	14.13	1.36	13.20	1.23

Table 19

The effect of biological sex on locomotor recovery after SCI assessed with RMANOVA

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
B9	All	Time	2720.98	6	453.50	286.06	<0.001	0.95
B10		Time*Sex	124.28	6,96	20.71	13.07	<0.001	0.45
		Error	152.19		1.59			

Table 20

RMANOVA results for the effect of biological Sex on tissue sparing 6 weeks after SCI

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
B11	All	Area	137.92	9	15.32	96.88	< 0.001	0.87
B12		Area*Sex	0.26	9,135	0.03	0.18	0.99	0.01
		Error	21.35		0.16			

Table 21

The effect of biological sex on myelin sparing 6 weeks after SCI assessed with RMANOVA

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
B13	All	Myelin	41.42	9	4.60	129.16	< 0.001	0.90
B14		Myelin*Sex	0.08	9,135	0.01	0.25	0.98	0.02
		Error	4.81		0.04			

Table 22

RMANOVA results for the effect of biological sex on neuron survival 6 weeks after SCI

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
B15	All	Neurons	46931092	9	5214566	126.62	< 0.001	0.89
B16		Neurons*Sex	263353	9,135	29261	0.71	0.698	0.05
		Error	5559471		41181			

Table 23

Gross spinal cord volume (mm^3) in the injury site and at intact cranial and caudal segments

Location	Sex	Treatment	Volume
Injury Site	Male	VEH	28.01 ± 2.46
Injury Site	Male	CYP	27.18 ± 2.12
Injury Site	Female	VEH	25.57 ± 0.98
Injury Site	Female	CYP	24.73 ± 2.51
Cranial	Male	VEH	14.47 ± 0.47
Cranial	Male	CYP	14.27 ± 0.44
Cranial	Female	VEH	13.73 ± 0.45
Cranial	Female	CYP	13.79 ± 0.62
Caudal	Male	VEH	17.24 ± 0.63
Caudal	Male	CYP	17.19 ± 0.69
Caudal	Female	VEH	16.54 ± 0.50
Caudal	Female	CYP	16.57 ± 0.79

Table 24

Spinal cord myelin volume (mm^3) in the injury site and at intact cranial and caudal segments

Location	Sex	Treatment	Volume
Injury Site	Male	VEH	12.03 ± 1.42
Injury Site	Male	CYP	11.56 ± 0.96
Injury Site	Female	VEH	10.11 ± 0.57
Injury Site	Female	CYP	9.90 ± 1.35
Cranial	Male	VEH	7.68 ± 0.45
Cranial	Male	CYP	7.69 ± 0.38
Cranial	Female	VEH	6.95 ± 0.28
Cranial	Female	CYP	6.93 ± 0.38
Caudal	Male	VEH	8.08 ± 0.39
Caudal	Male	CYP	8.09 ± 0.49
Caudal	Female	VEH	7.45 ± 0.22
Caudal	Female	CYP	7.31 ± 0.40

Table 25

Neuron counts at the injured spinal cord

Location	Sex	Treatment	Neuron Count
Injury Site	Male	VEH	74339 ± 7713
Injury Site	Male	CYP	70734 ± 9831
Injury Site	Female	VEH	70566 ± 9467
Injury Site	Female	CYP	70943 ± 8416

Table 26

Effect of CYP treatment on blood neutrophil responses after SCI assessed with RMANOVA

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
C1	CYP Male	Time	956009	4,28	239002	1.38	0.27	0.17
		Error	4837347		172762			
C2	CYP Female	Time	4029991	4,36	1007498	11.32	< 0.001	0.56
		Error	3204229		89006			
C3	VEH and CYP	Time	5637526	4	1659371	7.34	< 0.001	0.40
	Male	Time*Tx	1955966	4,56	488992	2.45	0.056	0.15
		Error	11167535		199420			
C4	VEH and CYP	Time	4172214	4	1043053	8.74	< 0.001	0.33
	Female	Time*Tx	2659636	4,72	664909	5.57	0.001	0.24
		Error	8589688		119301			

Table 27

Effect of CYP treatment on blood monocyte responses after SCI assessed with RMANOVA

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
C5	CYP Male	Time	370220	4,28	92555	12.38	< 0.001	0.64
		Error	209266		7474			
C6	CYP Female	Time	355328	4,36	88832	20.31	< 0.001	0.69
		Error	157440		4373			
	VEH and CYP	Time	5637526	4	1659371	7.34	< 0.001	0.40
C7	Male	Time*TX	1955966	4,56	488992	2.45	0.056	0.15
		Error	11167535		199420			
	VEH and CYP	Time	4172214	4	1043053	8.74	< 0.001	0.33
C8	Female	Time*TX	2659636	4,72	664909	5.57	0.001	0.24
		Error	8589688		119301			

Table 28

RMANOVA results for the effect of CYP treatment on gross tissue sparing in both sexes at 6 weeks after SCI

Test	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
C9	VEH and CYP	Time	128.39	9	14.27	82.97	< 0.001	0.86
	Male	Time*Tx	0.10	9,117	0.01	0.07	1.000	0.01
		Error	20.12		0.17			
C10	VEH and CYP	Time	177.58	9	19.73	147.45	< 0.001	0.89
	Female	Time*Tx	0.88	9,162	0.10	0.73	0.681	0.04
		Error	21.68		0.13			

Table 29

The effect of CYP treatment on BBB Scores after SCI assessed with RMANOVA

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
C11	VEH and CYP	Time	2105.55	6	350.93	224.59	< 0.001	0.94
	Male	Time*Tx	8.62	6,84	1.44	0.92	0.485	0.6
		Error	131.25		1.56			
C12	VEH and CYP	Time	3066.39	6	511.06	251.41	< 0.001	0.93
	Female	Time*Tx	39.50	6,108	6.58	3.24	0.006	0.15
		Error	219.54		2.03			

Table 30.

RMANOVA results for the effect of CYP treatment on myelin sparing at 6 weeks after SCI

Test	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
C13	VEH and CYP	Time	41.32	9	4.59	129.62	< 0.001	
	Male	Time*Tx	0.09	9,117	0.01	0.27	0.981	
		Error	4.14		0.04			
C14	VEH and CYP	Time	46.29	9	5.14	145.04	< 0.001	
	Female	Time*Tx	0.23	9,162	0.03	0.71	0.696	
		Error	5.74		0.04			

Table 31.

Effect of CYP treatment on neuron survival at 6 weeks after SCI in both sexes

ID	Group	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
C15	VEH and CYP	Time	43375159	9	4819462	106.02	<0.001	0.89
	Male	Time*Tx	204609	9,117	22734	0.50	0.872	0.04
		Error	5318619		45458			
C16	VEH and CYP	Time	55833829	9	6203759	163.78	<0.001	0.90
	Female	Time*Tx	484086	9,162	53787	1.42	0.183	0.07
		Error	6136493		37880			

Table 32.

Effect of CYP treatment and biological Sex on BBB scores for 6 weeks after SCI

ID	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
	Time	4910	6	816.9	447.1	< 0.001	0.89
C17	Time*Tx	32.2	6,192	5.4	2.9	0.009	0.04
C18	Time*Sex	163.8	6,192	27.3	14.9	< 0.001	
	Time*Tx*Sex	12.46	6,192	2.1	1.1	0.34	
	Error	350.79		1.8			

Table 33

Effect of CYP treatment and biological Sex on tissue sparing at 6 weeks after SCI

ID	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
	Area	298.6	9	33.2	221.4	< 0.001	0.87
C19	Area*Tx	0.27	9,279	0.03	0.2	0.99	0.01
C20	Area*Sex	0.43	9,279	0.05	0.32	0.97	0.01
	Area*Tx*Sex	0.44	9,279	0.05	0.33	0.96	0.01
	Error	41.8		1.8			

Table 34.

Effect of CYP treatment and biological Sex on myelin sparing at 6 weeks after SCI

ID	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
	Myelin	86.5	9	9.6	271	< 0.001	0.9
C21	Myelin*Tx	0.06	9,279	0.01	0.19	0.99	0.01
C22	Myelin*Sex	0.43	9,279	0.05	1.35	0.21	0.04
	Myelin*Tx*Sex	0.23	9,279	0.03	0.73	0.68	0.02
	Error	9.9		0.04			

Table 35.

Effect of CYP treatment and biological Sex on neuron survival at 6 weeks after SCI

ID	Effect	*SS*	*df*	*MS*	*F*	*Sig.*	η_p^2
	Neurons	96986440	9	10776271	262	< 0.001	0.9
C23	Neurons*Tx	415565	9,279	46174	1.13	0.35	0.04
C24	Neurons*Sex	399958	9,279	44440	1.08	0.38	0.03
	Neurons*Tx*Sex	248203	9,279	27578	0.67	0.73	0.02
	Error	11455113		41058			

Literature Cited

1. Furlan, J.C., et al., *Global incidence and prevalence of traumatic spinal cord injury.* Can. J. Neurol. Sci., 2013. 40(4): p. 456-464.

2. Jazayeri, S.B., et al., *Incidence of traumatic spinal cord injury worldwide: a systematic review.* Eur. Spine J., 2015. 24(5): p. 905-918.

3. Sekhon, L.H. and M.G. Fehlings, *Epidemiology, demographics, and pathophysiology of acute spinal cord injury.* Spine, 2001. 26(24 Suppl): p. S2-12.

4. Singh, A., et al., *Global prevalence and incidence of traumatic spinal cord injury.* Clin. Epidemiol., 2014. 6: p. 309-331.

5. Wyndaele, M. and J.J. Wyndaele, *Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey?* Spinal Cord, 2006. 44(9): p. 523-529.

6. Beery, A.K. and I. Zucker, *Sex bias in neuroscience and biomedical research.* Neurosci. Biobehav. Rev., 2011. 35(3): p. 565-572.

7. Clayton, J.A. and F.S. Collins, *Policy: NIH to balance sex in cell and animal studies.* Nature, 2014. 509(7500): p. 282-283.

8. Herrera-Melero, M.C., et al., *Acute predictors for mortality after severe TBI in Spain: Gender differences and clinical data.* Brain Inj., 2015. 29(12): p. 1439-1444.

9. Kim, T., et al., *Age and sex differences in the pathophysiology of acute CNS injury.* Neurochem. Int., 2019. 127: p. 22-28.

10. Kim, J.-S., et al., *Gender differences in the functional recovery after acute stroke.* J. Clin. Neurol., 2010. 6(4): p. 183-188.

11. Angele, M.K., et al., *Effect of gender and sex hormones on immune responses following shock.* Shock, 2000. 14(2): p. 81-90.

12. Aoyama, M., J. Kotani, and M. Usami, *Gender difference in granulocyte dynamics and apoptosis and the role of IL-18 during endotoxin-induced systemic inflammation.* Shock, 2009. 32(4): p. 401-409.

13. Bauer, I., et al., *[Influence of gender on stimulated cytokine response in patients with severe sepsis].* Anaesthesist, 2006. 55(5): p. 515-527.

14. Bösch, F., M.K. Angele, and I.H. Chaudry, *Gender differences in trauma, shock and sepsis.* Mil Med Res, 2018. 5(1): p. 35.

15. Choudhry, M.A., K.I. Bland, and I.H. Chaudry, *Trauma and immune response--effect of gender differences.* Injury, 2007. 38(12): p. 1382-1391.

16. Marriott, I., K.L. Bost, and Y.M. Huet-Hudson, *Sexual dimorphism in expression of receptors for bacterial lipopolysaccharides in murine macrophages: a possible mechanism for gender-based differences in endotoxic shock susceptibility.* J. Reprod. Immunol., 2006. 71(1): p. 12-27.

17. Pietropaoli, A.P., *Gender differences in the sepsis syndrome: Clinical evidence and potential mechanisms.* principles of gender-specific medicine, 2004: p. 360-373.

18. Du, S., et al., *XY sex chromosome complement, compared with XX, in the CNS confers greater neurodegeneration during experimental autoimmune encephalomyelitis.* Proc. Natl. Acad. Sci. U. S. A., 2014. 111(7): p. 2806-2811.

19. Smith-Bouvier, D.L., et al., *A role for sex chromosome complement in the female bias in autoimmune disease.* J. Exp. Med., 2008. 205(5): p. 1099-1108.

20. Voskuhl, R.R. and K. Palaszynski, *Sex hormones in experimental autoimmune encephalomyelitis: implications for multiple sclerosis.* Neuroscientist, 2001. 7(3): p. 258-270.

21. Mollayeva, T., S. Mollayeva, and A. Colantonio, *Traumatic brain injury: sex, gender and intersecting vulnerabilities.* Nat. Rev. Neurol., 2018. 14(12): p. 711-722.

22. Rahimian, R., P. Cordeau, Jr., and J. Kriz, *Brain Response to Injuries: When Microglia Go Sexist.* Neuroscience, 2019. 405: p. 14-23.

23. Zuo, W., W. Zhang, and N.-H. Chen, *Sexual dimorphism in cerebral ischemia injury.* Eur. J. Pharmacol., 2013. 711(1-3): p. 73-79.

24. Donovan, W.H., *Donald Munro Lecture. Spinal cord injury--past, present, and future.* J. Spinal Cord Med., 2007. 30(2): p. 85-100.

25. Frankel, H.L., et al., *Long-term survival in spinal cord injury: a fifty year investigation.* Spinal Cord, 1998. 36(4): p. 266-274.

26. Chiu, W.-T., et al., *Review paper: epidemiology of traumatic spinal cord injury: comparisons between developed and developing countries.* Asia. Pac. J. Public Health, 2010. 22(1): p. 9-18.

27. Bellucci, C.H.S., et al., *Contemporary trends in the epidemiology of traumatic spinal cord injury: changes in age and etiology.* Neuroepidemiology, 2015. 44(2): p. 85-90.

28. Kannus, P., et al., *Continuously increasing number and incidence of fall-induced, fracture-associated, spinal cord injuries in elderly persons.* Arch. Intern. Med., 2000. 160(14): p. 2145-2149.

29. Sivak, M., *Female drivers in the United States, 1963-2010: from a minority to a majority?* Traffic Inj. Prev., 2013. 14(3): p. 259-260.

30. Tafida, M.A., et al., *Descriptive epidemiology of traumatic spinal injury in Japan.* J. Orthop. Sci., 2018. 23(2): p. 273-276.

31. Furlan, J.C., A.V. Krassioukov, and M.G. Fehlings, *The effects of gender on clinical and neurological outcomes after acute cervical spinal cord injury.* J. Neurotrauma, 2005. 22(3): p. 368-381.

32. Furlan, J.C., B.C. Craven, and M.G. Fehlings, *Sex-related discrepancies in the epidemiology, injury characteristics and outcomes after acute spine trauma: A retrospective cohort study.* J. Spinal Cord Med., 2019. 42(sup1): p. 10-20.

33. Shackelford, M., T. Farley, and C.L. Vines, *A comparison of women and men with spinal cord injury.* Spinal Cord, 1998. 36(5): p. 337-339.

34. Equebal, A., S. Anwer, and R. Kumar, *The prevalence and impact of age and gender on rehabilitation outcomes in spinal cord injury in India: a retrospective pilot study.* Spinal Cord, 2013. 51(5): p. 409-412.

35. Calancie, B., M.R. Molano, and J.G. Broton, *Epidemiology and demography of acute spinal cord injury in a large urban setting.* J. Spinal Cord Med., 2005. 28(2): p. 92-96.

36. Scivoletto, G., et al., *Traumatic and non-traumatic spinal cord lesions: an Italian comparison of neurological and functional outcomes.* Spinal Cord, 2011. 49(3): p. 391-396.

37. Davidoff, G., et al., *Depression among acute spinal cord injury patients: A study utilizing the Zung Self-Rating Depression Scale.* Rehabil. Psychol., 1990. 35(3): p. 171-180.

38. Estores, I. and M. Sipski, *Women's issues after SCI.* Top. Spinal Cord Inj. Rehabil., 2004. 10(2): p. 107-125.

39. McColl, M.A., *A house of cards: women, aging and spinal cord injury.* Spinal Cord, 2002. 40(8): p. 371-373.

40. Samuel, V.M., et al., *Spinal cord injury rehabilitation: the experience of women.* Spinal Cord, 2007. 45(12): p. 758-764.

41. Catz, A., et al., *Recovery of neurologic function following nontraumatic spinal cord lesions in Israel.* Spine, 2004. 29(20): p. 2278-82; discussion 2283.

42. Kay, E., et al., *Effects of gender on inpatient rehabilitation outcomes in the elderly with incomplete paraplegia from nontraumatic spinal cord injury.* J. Spinal Cord Med., 2010. 33(4): p. 379-386.

43. McKinley, W.O., et al., *Nontraumatic vs. traumatic spinal cord injury: a rehabilitation outcome comparison.* Am. J. Phys. Med. Rehabil., 2001. 80(9): p. 693-9; quiz 700, 716.

44. New, P.W. and M.C. Epi, *Influence of age and gender on rehabilitation outcomes in nontraumatic spinal cord injury.* J. Spinal Cord Med., 2007. 30(3): p. 225-237.

45. Chamberlain, J.D., et al., *Mortality and longevity after a spinal cord injury: systematic review and meta-analysis.* Neuroepidemiology, 2015. 44(3): p. 182-198.

46. van den Berg, M.E.L., et al., *Survival after spinal cord injury: a systematic review.* J. Neurotrauma, 2010. 27(8): p. 1517-1528.

47. Scivoletto, G., et al., *Prediction Model for the Presence of Complications at Admission to Rehabilitation After Traumatic Spinal Cord Injury.* Top. Spinal Cord Inj. Rehabil., 2018. 24(2): p. 151-156.

48. Hatchett, P.E., et al., *Impact of Gender on Shoulder Torque and Manual Wheelchair Usage for Individuals with Paraplegia: A Preliminary Report.* Top. Spinal Cord Inj. Rehabil., 2009. 15(2): p. 79-89.

49. Scivoletto, G., B. Morganti, and M. Molinari, *Sex-related differences of rehabilitation outcomes of spinal cord lesion patients.* Clin. Rehabil., 2004. 18(6): p. 709-713.

50. Sipski, M.L., et al., *Effects of gender on neurologic and functional recovery after spinal cord injury.* Arch. Phys. Med. Rehabil., 2004. 85(11): p. 1826-1836.

51. Maynard, F.M., et al., *International standards for neurological and functional classification of spinal cord injury.* Spinal Cord, 1997. 35(5): p. 266-274.

52. Chan, W.M., et al., *Effect of gender on recovery after spinal cord injury.* Transl Stroke Res, 2013. 4(4): p. 447-61.

53. Scivoletto, G., et al., *An international age- and gender-controlled model for the Spinal Cord Injury Ability Realization Measurement Index (SCI-ARMI).* Neurorehabil. Neural Repair, 2015. 29(1): p. 25-32.

54. Fisher, C.G., et al., *Motor recovery, functional status, and health-related quality of life in patients with complete spinal cord injuries.* Spine, 2005. 30(19): p. 2200-2207.

55. Harrop, J.S., et al., *Neurologic Improvement After Thoracic, Thoracolumbar, and Lumbar Spinal Cord (Conus Medullaris) Injuries.* Spine, 2011. 36(1): p. 21-25.

56. Greenwald, B.D., et al., *Gender-related differences in acute rehabilitation lengths of stay, charges, and functional outcomes for a matched sample with spinal cord injury: a multicenter investigation.* Arch. Phys. Med. Rehabil., 2001. 82(9): p. 1181-1187.

57. Young, W., *Spinal cord contusion models.* Prog. Brain Res., 2002. 137: p. 231-255.

58. Tarlov, I.M. and H. Klinger, *Spinal cord compression studies. II. Time limits for recovery after acute compression in dogs.* AMA Arch. Neurol. Psychiatry, 1954. 71(3): p. 271-290.

59. Basso, D.M., M.S. Beattie, and J.C. Bresnahan, *Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection.* Exp. Neurol., 1996. 139(2): p. 244-256.

60. Ferrero, S.L., et al., *Effects of lateral funiculus sparing, spinal lesion level, and gender on recovery of bladder voiding reflexes and hematuria in rats.* J. Neurotrauma, 2015. 32(3): p. 200-208.

61. Datto, J.P., et al., *Female Rats Demonstrate Improved Locomotor Recovery and Greater Preservation of White and Gray Matter after Traumatic Spinal Cord Injury Compared to Males.* J. Neurotrauma, 2015. 32(15): p. 1146-1157.

62. Farooque, M., et al., *Gender-related differences in recovery of locomotor function after spinal cord injury in mice.* Spinal Cord, 2006. 44(3): p. 182-187.

63. Hauben, E., et al., *Sexual dimorphism in the spontaneous recovery from spinal cord injury: a gender gap in beneficial autoimmunity?* Eur. J. Neurosci., 2002. 16(9): p. 1731-1740.

64. Swartz, K.R., et al., *Gender differences in spinal cord injury are not estrogen-dependent.* J. Neurotrauma, 2007. 24(3): p. 473-480.

65. Hsu, S.-Y., *The Fischer 344 spinal cord contusion model and effects of cyclosporin-A on the model*. 2016, Rutgers University - Graduate School - New Brunswick.

66. Fukutoku, T., et al., *Sex-Related Differences in Anxiety and Functional Recovery after Spinal Cord Injury in Mice.* J Neurotrauma, 2020.

67. McFarlane, K., et al., *Effect of Sex on Motor Function, Lesion Size, and Neuropathic Pain after Contusion Spinal Cord Injury in Mice.* J Neurotrauma, 2020. 37(18): p. 1983-1990.

68. Emamhadi, M., et al., *Influence of Sexuality in Functional Recovery after Spinal Cord Injury in Rats.* Arch Bone Jt Surg, 2016. 4(1): p. 56-59.

69. Luchetti, S., et al., *Comparison of immunopathology and locomotor recovery in C57BL/6, BUB/BnJ, and NOD-SCID mice after contusion spinal cord injury.* J. Neurotrauma, 2010. 27(2): p. 411-421.

70. Ung, R.V., et al., *Spontaneous recovery of hindlimb movement in completely spinal cord transected mice: a comparison of assessment methods and conditions.* Spinal Cord, 2007. 45(5): p. 367-379.

71. Walker, C.L., et al., *Functional and Histological Gender Comparison of Age-Matched Rats after Moderate Thoracic Contusive Spinal Cord Injury.* J. Neurotrauma, 2019.

72. Gruner, J.A., *A monitored contusion model of spinal cord injury in the rat.* J. Neurotrauma, 1992. 9(2): p. 123-6; discussion 126-8.

73. Constantini, S. and W. Young, *The effects of methylprednisolone and the ganglioside GM1 on acute spinal cord injury in rats.* J. Neurosurg., 1994. 80(1): p. 97-111.

74. Gupte, R., et al., *Sex Differences in Traumatic Brain Injury: What We Know and What We Should Know.* J. Neurotrauma, 2019. 36(22): p. 3063-3091.

75. Stewart, A.N., et al., *Considerations for Studying Sex as a Biological Variable in Spinal Cord Injury.* Front Neurol, 2020. 11: p. 802.

76. Elkabes, S. and A.B. Nicot, *Sex steroids and neuroprotection in spinal cord injury: a review of preclinical investigations.* Exp. Neurol., 2014. 259: p. 28-37.

77. Aminmansour, B., et al., *Effects of progesterone and vitamin D on outcome of patients with acute traumatic spinal cord injury; a randomized, double-blind, placebo controlled study.* J. Spinal Cord Med., 2016. 39(3): p. 272-280.

78. Yune, T.Y., et al., *Systemic Administration of 17β-Estradiol Reduces Apoptotic Cell Death and Improves Functional Recovery following Traumatic Spinal Cord Injury in Rats.* J. Neurotrauma, 2004. 21(3): p. 293-306.

79. Zendedel, A., et al., *Estrogen Attenuates Local Inflammasome Expression and Activation after Spinal Cord Injury.* Mol. Neurobiol., 2018. 55(2): p. 1364-1375.

80. Giefing-Kröll, C., et al., *How sex and age affect immune responses, susceptibility to infections, and response to vaccination.* Aging Cell, 2015. 14(3): p. 309-321.

81. Klein, S.L. and K.L. Flanagan, *Sex differences in immune responses.* Nat. Rev. Immunol., 2016. 16(10): p. 626-638.

82. Pennell, L.M., C.L. Galligan, and E.N. Fish, *Sex affects immunity.* J. Autoimmun., 2012. 38(2-3): p. J282-91.

83. Case, L.K., et al., *The Y chromosome as a regulatory element shaping immune cell transcriptomes and susceptibility to autoimmune disease.* Genome Res., 2013. 23(9): p. 1474-1485.

84. Teuscher, C., et al., *Evidence that the Y chromosome influences autoimmune disease in male and female mice.* Proc. Natl. Acad. Sci. U. S. A., 2006. 103(21): p. 8024-8029.

85. Libert, C., L. Dejager, and I. Pinheiro, *The X chromosome in immune functions: when a chromosome makes the difference.* Nat. Rev. Immunol., 2010. 10(8): p. 594-604.

86. Wang, J., et al., *Unusual maintenance of X chromosome inactivation predisposes female lymphocytes for increased expression from the inactive X.* Proc. Natl. Acad. Sci. U. S. A., 2016. 113(14): p. E2029-38.

87. Alkayed, N.J., et al., *Gender-linked brain injury in experimental stroke.* Stroke, 1998. 29(1): p. 159-65; discussion 166.

88. Banerjee, A., et al., *Phenotypic changes in immune cell subsets reflect increased infarct volume in male vs. female mice.* Transl. Stroke Res., 2013. 4(5): p. 554-563.

89. Villapol, S., D.J. Loane, and M.P. Burns, *Sexual dimorphism in the inflammatory response to traumatic brain injury.* Glia, 2017. 65(9): p. 1423-1438.

90. Doran, S.J., et al., *Sex Differences in Acute Neuroinflammation after Experimental Traumatic Brain Injury Are Mediated by Infiltrating Myeloid Cells.* J. Neurotrauma, 2018.

91. Yanguas-Casás, N., et al., *Sex differences in the phagocytic and migratory activity of microglia and their impairment by palmitic acid.* Glia, 2018. 66(3): p. 522-537.

92. Villapol, S., et al., *Early Sex Differences in the Immune-Inflammatory Responses to Neonatal Ischemic Stroke.* Int. J. Mol. Sci., 2019. 20(15).

93. Whitacre, C.C., et al., *A Gender Gap in Autoimmunity.* Science, 1999. 283(5406): p. 1277-1278.

94. Trojano, M., et al., *Geographical variations in sex ratio trends over time in multiple sclerosis.* PLoS One, 2012. 7(10): p. e48078.

95. Gandhi, R., A. Laroni, and H.L. Weiner, *Role of the innate immune system in the pathogenesis of multiple sclerosis.* Journal of Neuroimmunology, 2010. 221(1-2): p. 7-14.

96. Allen, A.R., *REMARKS ON THE HISTOPATHOLOGICAL CHANGES IN THE SPINAL CORD DUE TO IMPACT. AN EXPERIMENTAL STUDY.* J. Nerv. Ment. Dis., 1914. 41(3): p. 141.

97. Ferraro, A., *EXPERIMENTAL MEDULLARY CONCUSSION OF THE SPINAL CORD IN RABBITS: HISTOLOGIC STUDY OF THE EARLY STAGES.* Arch NeurPsych, 1927. 18(3): p. 357-373.

98. Eidelberg, E., et al., *A model of spinal cord injury.* Surg Neurol, 1976. 6(1): p. 35-8.

99. Matthews, M.A., et al., *Spinal cord transection: a quantitative analysis of elements of the connective tissue matrix formed within the site of lesion following administration of piromen, cytoxan or trypsin.* Neuropathol Appl Neurobiol, 1979. 5(3): p. 161-80.

100. Wrathall, J.R., R.K. Pettegrew, and F. Harvey, *Spinal cord contusion in the rat: production of graded, reproducible, injury groups.* Exp. Neurol., 1985. 88(1): p. 108-122.

101. Kwo, S., W. Young, and V. Decrescito, *Spinal cord sodium, potassium, calcium, and water concentration changes in rats after graded contusion injury.* J. Neurotrauma, 1989. 6(1): p. 13-24.

102. Cao, Q., et al., *Functional and electrophysiological changes after graded traumatic spinal cord injury in adult rat.* Exp. Neurol., 2005. 191 Suppl 1: p. S3-S16.

103. Pearce, B., *PRIMARY FOCAL HÆMATOMYELIA FROM TRAUMATISM--A FREQUENT BUT OFTEN UNRECOGNIZED FORM OF SPINAL-CORD INJURY.* Med. Rec., 1898. 54(21): p. 731.

104. Blight, A.R. and V. Decrescito, *Morphometric analysis of experimental spinal cord injury in the cat: the relation of injury intensity to survival of myelinated axons.* Neuroscience, 1986. 19(1): p. 321-341.

105. Goodman, J.H., W.G. Bingham, Jr., and W.E. Hunt, *Edema formation and central hemorrhagic necrosis following impact injury to primate spinal cord.* Surg. Forum, 1974. 25(0): p. 440-442.

106. Nelson, E., et al., *Spinal cord injury. The role of vascular damage in the pathogenesis of central hemorrhagic necrosis.* Arch. Neurol., 1977. 34(6): p. 332-333.

107. Carafoli, E. and M. Crompton, *The regulation of intracellular calcium by mitochondria.* Ann. N. Y. Acad. Sci., 1978. 307: p. 269-284.

108. Roos, A. and W.F. Boron, *Intracellular pH.* Physiol. Rev., 1981. 61(2): p. 296-434.

109. Wang, C.X., J.A. Olschowka, and J.R. Wrathall, *Increase of interleukin-1beta mRNA and protein in the spinal cord following experimental traumatic injury in the rat.* Brain Res., 1997. 759(2): p. 190-196.

110. Tonai, T., et al., *A neutrophil elastase inhibitor (ONO-5046) reduces neurologic damage after spinal cord injury in rats.* J. Neurochem., 2001. 78(5): p. 1064-1072.

111. Lee, Y.B., et al., *Role of tumor necrosis factor-alpha in neuronal and glial apoptosis after spinal cord injury.* Exp. Neurol., 2000. 166(1): p. 190-195.

112. Yune, T.Y., et al., *Increased production of tumor necrosis factor-alpha induces apoptosis after traumatic spinal cord injury in rats.* J. Neurotrauma, 2003. 20(2): p. 207-219.

113. Pan, J.Z., et al., *Cytokine activity contributes to induction of inflammatory cytokine mRNAs in spinal cord following contusion.* J. Neurosci. Res., 2002. 68(3): p. 315-322.

114. Young, W., *MASCIS Spinal Cord Contusion Model*, in *Animal Models of Acute Neurological Injuries*. 2009. p. 411-421.

115. Horrocks, L.A., et al., *The degradation of phospholipids, formation of metabolites of arachidonic acid, and demyelination following experimental spinal cord injury.* Cent. Nerv. Syst. Trauma, 1985. 2(2): p. 115-120.

116. Lu, J., K.W. Ashwell, and P. Waite, *Advances in secondary spinal cord injury: role of apoptosis.* Spine (Phila Pa 1976), 2000. 25(14): p. 1859-66.

117. Hall, E.D. and J.M. Braughler, *Role of lipid peroxidation in post-traumatic spinal cord degeneration: a review.* Cent Nerv Syst Trauma, 1986. 3(4): p. 281-94.

118. Goodman, J.H., W.G. Bingham, Jr., and W.E. Hunt, *Ultrastructural blood-brain barrier alterations and edema formation in acute spinal cord trauma.* J. Neurosurg., 1976. 44(4): p. 418-424.

119. Griffiths, I.R., M. McCulloch, and R.A. Crawford, *Ultrastructural appearances of the spinal microvasculature between 12 hours and 5 days after impact injury.* Acta Neuropathol., 1978. 43(3): p. 205-211.

120. Noble, L.J. and J.R. Wrathall, *Distribution and time course of protein extravasation in the rat spinal cord after contusive injury.* Brain Res., 1989. 482(1): p. 57-66.

121. Engelhardt, B., *The blood-central nervous system barriers actively control immune cell entry into the central nervous system.* Curr. Pharm. Des., 2008. 14(16): p. 1555-1565.

122. Sharma, H.S., *Pathophysiology of blood-spinal cord barrier in traumatic injury and repair.* Curr. Pharm. Des., 2005. 11(11): p. 1353-1389.

123. Adrian, E.K., *Cell division in injured spinal cord.* Am. J. Anat., 1968. 123(3): p. 501-519.

124. Adrian, E.K., Jr. and M.G. Williams, *Cell proliferation in injured spinal cord. An electron microscopic study.* J. Comp. Neurol., 1973. 151(1): p. 1-24.

125. Beck, K.D., et al., *Quantitative analysis of cellular inflammation after traumatic spinal cord injury: evidence for a multiphasic inflammatory response in the acute to chronic environment.* Brain, 2010. 133(Pt 2): p. 433-447.

126. Fleming, J.C., et al., *The cellular inflammatory response in human spinal cords after injury.* Brain, 2006. 129(Pt 12): p. 3249-3269.

127. Popovich, P.G., P. Wei, and B.T. Stokes, *Cellular inflammatory response after spinal cord injury in Sprague-Dawley and Lewis rats.* J. Comp. Neurol., 1997. 377(3): p. 443-464.

128. Stirling, D.P. and V.W. Yong, *Dynamics of the inflammatory response after murine spinal cord injury revealed by flow cytometry.* J. Neurosci. Res., 2008. 86(9): p. 1944-1958.

129. Sroga, J.M., et al., *Rats and mice exhibit distinct inflammatory reactions after spinal cord injury.* J. Comp. Neurol., 2003. 462(2): p. 223-240.

130. Liu, X.Z., et al., *Neuronal and glial apoptosis after traumatic spinal cord injury.* J. Neurosci., 1997. 17(14): p. 5395-5406.

131. Crowe, M.J., et al., *Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys.* Nat. Med., 1997. 3(1): p. 73-76.

132. Shuman, S.L., J.C. Bresnahan, and M.S. Beattie, *Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats.* J. Neurosci. Res., 1997. 50(5): p. 798-808.

133. Tator, C.H. and M.G. Fehlings, *Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms.* J. Neurosurg., 1991. 75(1): p. 15-26.

134. Young, W., *Secondary injury mechanisms in acute spinal cord injury.* J. Emerg. Med., 1993. 11 Suppl 1: p. 13-22.

135. Davalos, D., et al., *ATP mediates rapid microglial response to local brain injury in vivo.* Nat. Neurosci., 2005. 8(6): p. 752-758.

136. Bellver-Landete, V., et al., *Microglia are an essential component of the neuroprotective scar that forms after spinal cord injury.* Nat Commun, 2019. 10(1): p. 518.

137. Savage, C.D., et al., *NLRP3-Inflammasome Activating DAMPs Stimulate an Inflammatory Response in Glia in the Absence of Priming Which Contributes to Brain Inflammation after Injury.* Front Immunol, 2012. 3: p. 288.

138. Stebbing, M.J., J.M. Cottee, and I. Rana, *The Role of Ion Channels in Microglial Activation and Proliferation - A Complex Interplay between Ligand-Gated Ion Channels, K(+) Channels, and Intracellular Ca(2.).* Front Immunol, 2015. 6: p. 497.

139. Wu, L.-J., K.I. Vadakkan, and M. Zhuo, *ATP-induced chemotaxis of microglial processes requires P2Y receptor-activated initiation of outward potassium currents.* Glia, 2007. 55(8): p. 810-821.

140. Haynes, S.E., et al., *The P2Y12 receptor regulates microglial activation by extracellular nucleotides.* Nat. Neurosci., 2006. 9(12): p. 1512-1519.

141. Impellizzeri, D., et al., *Role of Toll like receptor 4 signaling pathway in the secondary damage induced by experimental spinal cord injury.* Immunobiology, 2015. 220(9): p. 1039-1049.

142. Greenhalgh, A.D. and S. David, *Differences in the phagocytic response of microglia and peripheral macrophages after spinal cord injury and its effects on cell death.* J. Neurosci., 2014. 34(18): p. 6316-6322.

143. Koshinaga, M. and S.R. Whittemore, *The temporal and spatial activation of microglia in fiber tracts undergoing anterograde and retrograde degeneration following spinal cord lesion.* J Neurotrauma, 1995. 12(2): p. 209-22.

144. Rezvan, M., et al., *Time-dependent microglia and macrophages response after traumatic spinal cord injury in rat: a systematic review.* Injury, 2020.

145. Rouleau, P., et al., *Hormonal and immunological changes in mice after spinal cord injury.* J. Neurotrauma, 2007. 24(2): p. 367-378.

146. Wu, F., et al., *Cellular Inflammatory Response of the Spleen After Acute Spinal Cord Injury in Rat.* Inflammation, 2019. 42(5): p. 1630-1640.

147. Furlan, J.C., A.V. Krassioukov, and M.G. Fehlings, *Hematologic abnormalities within the first week after acute isolated traumatic cervical spinal cord injury: a case-control cohort study.* Spine, 2006. 31(23): p. 2674-2683.

148. Mukhamedshina, Y.O., et al., *Systemic and Local Cytokine Profile following Spinal Cord Injury in Rats: A Multiplex Analysis.* Front. Neurol., 2017. 8: p. 581.

149. Stammers, A.T., J. Liu, and B.K. Kwon, *Expression of inflammatory cytokines following acute spinal cord injury in a rodent model.* J. Neurosci. Res., 2012. 90(4): p. 782-790.

150. Saiwai, H., et al., *The LTB4-BLT1 axis mediates neutrophil infiltration and secondary injury in experimental spinal cord injury.* Am. J. Pathol., 2010. 176(5): p. 2352-2366.

151. Xu, J.A., et al., *Leukotriene B4 release and polymorphonuclear cell infiltration in spinal cord injury.* J. Neurochem., 1990. 55(3): p. 907-912.

152. Summers, C., et al., *Neutrophil kinetics in health and disease.* Trends Immunol., 2010. 31(8): p. 318-324.

153. Madalli, S., et al., *Sex-specific regulation of chemokine Cxcl5/6 controls neutrophil recruitment and tissue injury in acute inflammatory states.* Biol. Sex Differ., 2015. 6: p. 27.

154. Spitzer, J.A. and P. Zhang, *Gender differences in neutrophil function and cytokine-induced neutrophil chemoattractant generation in endotoxic rats.* Inflammation, 1996. 20(5): p. 485-498.

155. Fleming, J.C., et al., *α4β1 integrin blockade after spinal cord injury decreases damage and improves neurological function.* Exp. Neurol., 2008. 214(2): p. 147-159.

156. Means, E.D. and D.K. Anderson, *Neuronophagia by leukocytes in experimental spinal cord injury.* J. Neuropathol. Exp. Neurol., 1983. 42(6): p. 707-719.

157. Carlson, S.L., et al., *Acute inflammatory response in spinal cord following impact injury.* Exp. Neurol., 1998. 151(1): p. 77-88.

158. Neirinckx, V., et al., *Neutrophil contribution to spinal cord injury and repair.* J. Neuroinflammation, 2014. 11: p. 150.

159. Tak, T., et al., *What's your age again? Determination of human neutrophil half-lives revisited.* J Leukoc Biol, 2013. 94(4): p. 595-601.

160. Nieto-Sampedro, M., et al., *Sex differences in the effect of MK-801 on normal and spinal cord injured rats.* Restor. Neurol. Neurosci., 1991. 2(4): p. 175-179.

161. Bethea, J.R., et al., *Traumatic Spinal Cord Injury Induces Nuclear Factor-κB Activation.* J. Neurosci., 1998. 18(9): p. 3251-3260.

162. Schnell, L., et al., *Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord.* Eur. J. Neurosci., 1999. 11(10): p. 3648-3658.

163. Kubota, K., et al., *Myeloperoxidase exacerbates secondary injury by generating highly reactive oxygen species and mediating neutrophil recruitment in experimental spinal cord injury.* Spine, 2012. 37(16): p. 1363-1369.

164. Taoka, Y., et al., *Role of neutrophils in spinal cord injury in the rat.* Neuroscience, 1997. 79(4): p. 1177-1182.

165. Cochrane, C.G., *Immunologic tissue injury mediated by neutrophilic leukocytes.* Adv. Immunol., 1968. 9: p. 97-162.

166. Goussev, S., et al., *Differential temporal expression of matrix metalloproteinases after spinal cord injury: relationship to revascularization and wound healing.* J. Neurosurg., 2003. 99(2 Suppl): p. 188-197.

167. Zhang, H., et al., *Role of matrix metalloproteinases and therapeutic benefits of their inhibition in spinal cord injury.* Neurotherapeutics, 2011. 8(2): p. 206-220.

168. Savill, J.S., et al., *Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages.* J. Clin. Invest., 1989. 83(3): p. 865-875.

169. Bratton, D.L. and P.M. Henson, *Neutrophil clearance: when the party is over, clean-up begins.* Trends Immunol., 2011. 32(8): p. 350-357.

170. Ravichandran, K.S., *Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways.* Immunity, 2011. 35(4): p. 445-455.

171. Elliott, M.R., K.M. Koster, and P.S. Murphy, *Efferocytosis Signaling in the Regulation of Macrophage Inflammatory Responses.* J. Immunol., 2017. 198(4): p. 1387-1394.

172. Meszaros, A.J., J.S. Reichner, and J.E. Albina, *Macrophage-induced neutrophil apoptosis.* J. Immunol., 2000. 165(1): p. 435-441.

173. Pineau, I. and S. Lacroix, *Proinflammatory cytokine synthesis in the injured mouse spinal cord: multiphasic expression pattern and identification of the cell types involved.* J. Comp. Neurol., 2007. 500(2): p. 267-285.

174. Peiseler, M. and P. Kubes, *More friend than foe: the emerging role of neutrophils in tissue repair.* J. Clin. Invest., 2019. 129(7): p. 2629-2639.

175. Stirling, D.P., et al., *Depletion of Ly6G/Gr-1 leukocytes after spinal cord injury in mice alters wound healing and worsens neurological outcome.* J. Neurosci., 2009. 29(3): p. 753-764.

176. McCourt, M., et al., *Proinflammatory mediators stimulate neutrophil-directed angiogenesis.* Arch Surg, 1999. 134(12): p. 1325-31; discussion 1331-2.

177. Blomster, L.V., et al., *Mobilisation of the splenic monocyte reservoir and peripheral CX_3CR1 deficiency adversely affects recovery from spinal cord injury.* Exp. Neurol., 2013. 247: p. 226-240.

178. Ma, M., et al., *Monocyte recruitment and myelin removal are delayed following spinal cord injury in mice with CCR2 chemokine receptor deletion.* J. Neurosci. Res., 2002. 68(6): p. 691-702.

179. Prinz, M. and J. Priller, *Tickets to the brain: role of CCR2 and CX3CR1 in myeloid cell entry in the CNS.* J. Neuroimmunol., 2010. 224(1-2): p. 80-84.

180. Donnelly, D.J., et al., *Deficient CX3CR1 signaling promotes recovery after mouse spinal cord injury by limiting the recruitment and activation of Ly6Clo/iNOS+ macrophages.* J. Neurosci., 2011. 31(27): p. 9910-9922.

181. Kobayakawa, K., et al., *Macrophage centripetal migration drives spontaneous healing process after spinal cord injury.* Sci Adv, 2019. 5(5): p. eaav5086.

182. Kigerl, K.A., et al., *Identification of two distinct macrophage subsets with divergent effects causing either neurotoxicity or regeneration in the injured mouse spinal cord.* J. Neurosci., 2009. 29(43): p. 13435-13444.

183. Wang, X., et al., *Macrophages in spinal cord injury: phenotypic and functional change from exposure to myelin debris.* Glia, 2015. 63(4): p. 635-651.

184. Kopper, T.J. and J.C. Gensel, *Myelin as an inflammatory mediator: Myelin interactions with complement, macrophages, and microglia in spinal cord injury.* J. Neurosci. Res., 2018. 96(6): p. 969-977.

185. Kroner, A., et al., *TNF and increased intracellular iron alter macrophage polarization to a detrimental M1 phenotype in the injured spinal cord.* Neuron, 2014. 83(5): p. 1098-1116.

186. Norden, D.M., et al., *Bone Marrow-Derived Monocytes Drive the Inflammatory Microenvironment in Local and Remote Regions after Thoracic Spinal Cord Injury.* J. Neurotrauma, 2019. 36(6): p. 937-949.

187. Lee, S.M., et al., *Prevention of both neutrophil and monocyte recruitment promotes recovery after spinal cord injury.* J. Neurotrauma, 2011. 28(9): p. 1893-1907.

188. Popovich, P.G., et al., *Depletion of hematogenous macrophages promotes partial hindlimb recovery and neuroanatomical repair after experimental spinal cord injury.* Exp. Neurol., 1999. 158(2): p. 351-365.

189. Bao, F., et al., *Early anti-inflammatory treatment reduces lipid peroxidation and protein nitration after spinal cord injury in rats.* J. Neurochem., 2004. 88(6): p. 1335-1344.

190. Bao, F., et al., *A selective phosphodiesterase-4 inhibitor reduces leukocyte infiltration, oxidative processes, and tissue damage after spinal cord injury.* J. Neurotrauma, 2011. 28(6): p. 1035-1049.

191. Chatzipanteli, K., et al., *Posttraumatic hypothermia reduces polymorphonuclear leukocyte accumulation following spinal cord injury in rats.* J. Neurotrauma, 2000. 17(4): p. 321-332.

192. Taoka, Y., et al., *Role of neutrophil elastase in compression-induced spinal cord injury in rats.* Brain Res., 1998. 799(2): p. 264-269.

193. Taoka, Y., et al., *Reduction of spinal cord injury by administration of iloprost, a stable prostacyclin analog.* J. Neurosurg., 1997. 86(6): p. 1007-1011.

194. Xu, J., et al., *Xanthine oxidase in experimental spinal cord injury.* J. Neurotrauma, 1991. 8(1): p. 11-18.

195. Taoka, Y., et al., *Gabexate mesilate, a synthetic protease inhibitor, prevents compression-induced spinal cord injury by inhibiting activation of leukocytes in rats.* Crit. Care Med., 1997. 25(5): p. 874-879.

196. Hirose, K., et al., *Activated protein C reduces the ischemia/reperfusion-induced spinal cord injury in rats by inhibiting neutrophil activation.* Ann. Surg., 2000. 232(2): p. 272-280.

197. Feringa, E.R., J.S. Wendt, and R.D. Johnson, *Immunosuppressive treatment to enhance spinal cord regeneration in rats.* Neurology, 1974. 24(3): p. 287-293.

198. Feringa, E.R., et al., *Regeneration of corticospinal axons in the rat.* Ann. Neurol., 1977. 2(4): p. 315-321.

199. Saiwai, H., et al., *Ly6C+ Ly6G- Myeloid-derived suppressor cells play a critical role in the resolution of acute inflammation and the subsequent tissue repair process after spinal cord injury.* J. Neurochem., 2013. 125(1): p. 74-88.

200. Katoh, S., et al., *Possible implication of leukocytes in secondary pathological changes after spinal cord injury.* Injury, 1997. 28(3): p. 215-217.

201. Bao, F., et al., *Increased oxidative activity in human blood neutrophils and monocytes after spinal cord injury.* Exp. Neurol., 2009. 215(2): p. 308-316.

202. Kay, E., et al., *Sexual dimorphisms in leukocyte trafficking in a mouse peritonitis model.* J Leukoc Biol, 2015. 98(5): p. 805-17.

203. Feichtmeir, T.V., et al., *Electronic counting of erythrocytes and leukocytes.* Tech. Bull. Regist. Med. Technol., 1961. 31: p. 39-43.

204. Tough, D.F. and J. Sprent, *Life span of naive and memory T cells.* Stem Cells, 1995. 13(3): p. 242-249.

205. den Haan, J.M., R. Arens, and M.C. van Zelm, *The activation of the adaptive immune system: cross-talk between antigen-presenting cells, T cells and B cells.* Immunol Lett, 2014. 162(2 Pt B): p. 103-12.

206. Spoor, M.S., Z.A. Radi, and R.W. Dunstan, *Characterization of age- and gender-related changes in the spleen and thymus from control cynomolgus macaques used in toxicity studies.* Toxicol. Pathol., 2008. 36(5): p. 695-704.

207. Casili, G., et al., *B-Cell Depletion with CD20 Antibodies as New Approach in the Treatment of Inflammatory and Immunological Events Associated with Spinal Cord Injury.* Neurotherapeutics, 2016. 13(4): p. 880-894.

208. Potas, J.R., et al., *Augmented locomotor recovery after spinal cord injury in the athymic nude rat.* J. Neurotrauma, 2006. 23(5): p. 660-673.

209. Wu, B., et al., *Improved regeneration after spinal cord injury in mice lacking functional T- and B-lymphocytes.* Exp. Neurol., 2012. 237(2): p. 274-285.

210. Garcia-Arguello, L.Y., et al., *Infections in the spinal cord-injured population: a systematic review.* Spinal Cord, 2017. 55(6): p. 526-534.

211. Fuhrer, M.J., et al., *Pressure ulcers in community-resident persons with spinal cord injury: prevalence and risk factors.* Arch Phys Med Rehabil, 1993. 74(11): p. 1172-7.

212. Stover, S.L., A.M. Hale, and A.B. Buell, *Skin complications other than pressure ulcers following spinal cord injury.* Arch. Phys. Med. Rehabil., 1994. 75(9): p. 987-993.

213. Brommer, B., et al., *Spinal cord injury-induced immune deficiency syndrome enhances infection susceptibility dependent on lesion level.* Brain, 2016. 139(Pt 3): p. 692-707.

214. Held, K.S., et al., *Impaired immune responses following spinal cord injury lead to reduced ability to control viral infection.* Exp. Neurol., 2010. 226(1): p. 242-253.

215. Winslow, C., et al., *Impact of respiratory complications on length of stay and hospital costs in acute cervical spine injury.* Chest, 2002. 121(5): p. 1548-54.

216. Soden, R.J., et al., *Causes of death after spinal cord injury.* Spinal Cord, 2000. 38(10): p. 604-10.

217. Failli, V., et al., *Functional neurological recovery after spinal cord injury is impaired in patients with infections.* Brain, 2012. 135(Pt 11): p. 3238-3250.

218. Arevalo-Martin, A., et al., *Elevated Autoantibodies in Subacute Human Spinal Cord Injury Are Naturally Occurring Antibodies.* Front Immunol, 2018. 9: p. 2365.

219. Cruse, J.M., et al., *Immune system-neuroendocrine dysregulation in spinal cord injury.* Immunol. Res., 1996. 15(4): p. 306-314.

220. Riegger, T., et al., *Spinal cord injury-induced immune depression syndrome (SCI-IDS).* Eur. J. Neurosci., 2007. 25(6): p. 1743-1747.

221. Riegger, T., et al., *Immune depression syndrome following human spinal cord injury (SCI): a pilot study.* Neuroscience, 2009. 158(3): p. 1194-1199.

222. Davies, A.L., K.C. Hayes, and G.A. Dekaban, *Clinical correlates of elevated serum concentrations of cytokines and autoantibodies in patients with spinal cord injury.* Arch. Phys. Med. Rehabil., 2007. 88(11): p. 1384-1393.

223. Hayes, K.C., et al., *Elevated serum titers of proinflammatory cytokines and CNS autoantibodies in patients with chronic spinal cord injury.* J. Neurotrauma, 2002. 19(6): p. 753-761.

224. Schuurman, H.J. and V.F.J. Quesniaux, *Development and maturation of T and B cells.* Principles of, 1999.

225. Kipnis, J., et al., *Neuroprotective autoimmunity: naturally occurring CD4+CD25+ regulatory T cells suppress the ability to withstand injury to the central nervous system.* Proc. Natl. Acad. Sci. U. S. A., 2002. 99(24): p. 15620-15625.

226. Coffman, R.L., *Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development.* Immunol Rev, 1982. 69: p. 5-23.

227. LeBien, T.W. and T.F. Tedder, *B lymphocytes: how they develop and function.* Blood, 2008. 112(5): p. 1570-80.

228. Rodriguez-Pinto, D., *B cells as antigen presenting cells.* Cell Immunol, 2005. 238(2): p. 67-75.

229. Dustin, M.L., *The immunological synapse.* Cancer Immunol Res, 2014. 2(11): p. 1023-33.

230. Sablitzky, F., G. Wildner, and K. Rajewsky, *Somatic mutation and clonal expansion of B cells in an antigen-driven immune response.* EMBO J, 1985. 4(2): p. 345-50.

231. Schittek, B. and K. Rajewsky, *Maintenance of B-cell memory by long-lived cells generated from proliferating precursors.* Nature, 1990. 346(6286): p. 749-751.

232. Winkelmann, R., et al., *B cell homeostasis and plasma cell homing controlled by Kruppel-like factor 2.* Proc Natl Acad Sci U S A, 2011. 108(2): p. 710-5.

233. Cawley, S., G. Findon, and T.E. Miller, *Peripheral blood leukocyte count as an index of defense status in the leukopenic host.* J. Lab. Clin. Med., 1988. 112(1): p. 52-57.

234. DeWys, W.D., et al., *Hematopoietic recovery after large doses of cyclophosphamide: correlation of proliferative state with sensitivity.* Cancer Res., 1970. 30(6): p. 1692-1697.

235. Liu, J.-T., et al., *Methotrexate combined with methylprednisolone for the recovery of motor function and differential gene expression in rats with spinal cord injury.* Neural Regeneration Res., 2017. 12(9): p. 1507-1518.

236. Rong, F., et al., *Methotrexate remediates spinal cord injury in vivo and in vitro via suppression of endoplasmic reticulum stress-induced apoptosis.* Exp. Ther. Med., 2018. 15(5): p. 4191-4198.

237. Hayashi, Y., et al., *Immunosuppression with either cyclosporine a or FK506 supports survival of transplanted fibroblasts and promotes growth of host axons into the transplant after spinal cord injury.* J. Neurotrauma, 2005. 22(11): p. 1267-1281.

238. Ibarra, A., et al., *Effects of cyclosporin-A on immune response, tissue protection and motor function of rats subjected to spinal cord injury.* Brain Res., 2003. 979(1-2): p. 165-178.

239. Bracken, M.B., et al., *Methylprednisolone or naloxone treatment after acute spinal cord injury: 1-year follow-up data. Results of the second National Acute Spinal Cord Injury Study.* J. Neurosurg., 1992. 76(1): p. 23-31.

240. Fujimoto, T., et al., *Effects of EPC-K1 on lipid peroxidation in experimental spinal cord injury.* Spine, 2000. 25(1): p. 24-29.

241. Taoka, Y. and K. Okajima, *Role of leukocytes in spinal cord injury in rats.* J. Neurotrauma, 2000. 17(3): p. 219-229.

242. Yoburn, B.C., R. Morales, and C.E. Inturrisi, *Chronic vascular catheterization in the rat: comparison of three techniques.* Physiol Behav, 1984. 33(1): p. 89-94.

243. Jekl, V., et al., *Blood sampling from the cranial vena cava in the Norway rat (Rattus norvegicus).* Lab Anim, 2005. 39(2): p. 236-9.

244. Gotoh, S., et al., *Enhancement of the expression of a rat neutrophil-specific cell surface antigen by activation with phorbol myristate acetate and concanavalin A.* J Immunol, 1986. 137(2): p. 643-50.

245. van der Rhee, H.J., C.P. de Winter, and W.T. Daems, *Fine structure and peroxidatic activity of rat blood monocytes.* Cell Tissue Res, 1977. 185(1): p. 1-16.

246. Nichols, B.A., D.F. Bainton, and M.G. Farquhar, *Differentiation of monocytes. Origin, nature, and fate of their azurophil granules.* J Cell Biol, 1971. 50(2): p. 498-515.

247. Basso, D.M., M.S. Beattie, and J.C. Bresnahan, *A sensitive and reliable locomotor rating scale for open field testing in rats.* J. Neurotrauma, 1995. 12(1): p. 1-21.

248. Verso, M.L., *The Evolution of Blood Counting Techniques.* Med. Hist., 1964. 8: p. 149-158.

249. Stender, R.N., et al., *Establishment of blood analyte intervals for laboratory mice and rats by use of a portable clinical analyzer.* J. Am. Assoc. Lab. Anim. Sci., 2007. 46(3): p. 47-52.

250. Gagon, T.E., et al., *An Evaluation of the Variance of Leukocyte Counts as Performed with the Hemocytometer, Coulter, and Fisher Instruments.* Am. J. Clin. Pathol., 1966. 46(6_ts): p. 684-691.

251. Faulkner, M., J. Dixon, and J. Green, *Discrepancy between haemocytometer and electronic leucocyte counts in neutrophilic dogs.* Veterinary Record, 1982. 110(9): p. 202-202.

252. Whittington, R.J. and D.A. Comer, *Discrepancy between hemocytometer and electronic counts of blood cells.* J. Wildl. Dis., 1984. 20(3): p. 258-260.

253. Berkson, J., T.B. Magath, and M. Hurn, *The error of estimate of the blood cell count as made with the hemocytometer.* American Journal of Physiology-Legacy Content, 1939. 128(2): p. 309-323.

254. Turner, M.E., *The Distribution of Red Blood Cells in the Hemacytometer.* Biometrics, 1957. 13(4): p. 485-495.

255. Aquiles Sanchez, J., R.J. Karni, and L.J. Wangh, *Fluorescent in situ hybridization (FISH) analysis of the relationship between chromosome location and nuclear morphology in human neutrophils.* Chromosoma, 1997. 106(3): p. 168-177.

256. Carvalho, L.O., et al., *The Neutrophil Nucleus and Its Role in Neutrophilic Function.* J. Cell. Biochem., 2015. 116(9): p. 1831-1836.

257. Ji, P., M. Murata-Hori, and H.F. Lodish, *Formation of mammalian erythrocytes: chromatin condensation and enucleation.* Trends Cell Biol., 2011. 21(7): p. 409-415.

258. Craig, F.E., *The utility of peripheral blood smear review for identifying specimens for flow cytometric immunophenotyping.* Int. J. Lab. Hematol., 2017. 39 Suppl 1: p. 41-46.

259. Weitzman, M., *Diagnostic utility of white blood cell and differential cell counts.* Am. J. Dis. Child., 1975. 129(10): p. 1183-1189.

260. Oertelt-Prigione, S., *The influence of sex and gender on the immune response.* Autoimmun. Rev., 2012. 11(6-7): p. A479-85.

261. Cannon, J.G. and B.A. St Pierre, *Gender differences in host defense mechanisms.* J. Psychiatr. Res., 1997. 31(1): p. 99-113.

262. Mautes, A.E., et al., *Vascular events after spinal cord injury: contribution to secondary pathogenesis.* Phys. Ther., 2000. 80(7): p. 673-687.

263. Popovich, P.G., et al., *A quantitative spatial analysis of the blood-spinal cord barrier. I. Permeability changes after experimental spinal contusion injury.* Exp. Neurol., 1996. 142(2): p. 258-275.

264. Gris, D., E.F. Hamilton, and L.C. Weaver, *The systemic inflammatory response after spinal cord injury damages lungs and kidneys.* Exp. Neurol., 2008. 211(1): p. 259-270.

265. Hundt, H., et al., *Assessment of hepatic inflammation after spinal cord injury using intravital microscopy.* Injury, 2011. 42(7): p. 691-696.

266. Hawthorne, A.L. and P.G. Popovich, *Emerging concepts in myeloid cell biology after spinal cord injury.* Neurotherapeutics, 2011. 8(2): p. 252-61.

267. Brommer, B., et al., *Secondary immunodeficiency (immune paralysis) following spinal cord injury.* e-Neuroforum, 2010. 16(3): p. 361.

268. Ross, A.M., C.S. Lee, and H. Lutsep, *Influence of Gender and Age on the Peripheral Immune Response in Stroke.* J. Cardiovasc. Nurs., 2016. 31(4): p. 331-335.

269. Javdani, M., et al., *Effect of Selenium Nanoparticle Supplementation on Tissue Inflammation, Blood Cell Count, and IGF-1 Levels in Spinal Cord Injury-Induced Rats.* Biol. Trace Elem. Res., 2018.

270. Prüss, H., et al., *Spinal cord injury-induced immunodeficiency is mediated by a sympathetic-neuroendocrine adrenal reflex.* Nat. Neurosci., 2017. 20(11): p. 1549-1559.

271. Lucin, K.M., V.M. Sanders, and P.G. Popovich, *Stress hormones collaborate to induce lymphocyte apoptosis after high level spinal cord injury.* J. Neurochem., 2009. 110(5): p. 1409-1421.

272. Sun, X., et al., *Multiple organ dysfunction and systemic inflammation after spinal cord injury: a complex relationship.* J. Neuroinflammation, 2016. 13(1): p. 260.

273. Saber, M., et al., *Acute peripheral inflammation and post-traumatic sleep differ between sexes after experimental diffuse brain injury.* Eur J Neurosci, 2020. 52(1): p. 2791-2814.

274. Abbasi, A., et al., *Changes in spontaneous and LPS-induced ex vivo cytokine production and mRNA expression in male and female athletes following prolonged exhaustive exercise.* Exerc. Immunol. Rev., 2013. 19: p. 8-28.

275. Clark, J.A. and T.C. Peterson, *Cytokine production and aging: overproduction of IL-8 in elderly males in response to lipopolysaccharide.* Mech. Ageing Dev., 1994. 77(2): p. 127-139.

276. Crockett, E.T., et al., *Sex differences in inflammatory cytokine production in hepatic ischemia-reperfusion.* J. Inflamm., 2006. 3: p. 16.

277. Tanaka, H. and T. Kaneko, *Sex differences in the pharmacokinetics of recombinant human granulocyte colony-stimulating factor in the rat.* Drug Metab. Dispos., 1991. 19(6): p. 1034-1039.

278. Huang, W., et al., *Identification of distinct monocyte phenotypes and correlation with circulating cytokine profiles in acute response to spinal cord injury: a pilot study.* PM R, 2014. 6(4): p. 332-341.

279. Shechter, R., et al., *Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus.* Immunity, 2013. 38(3): p. 555-569.

280. Soehnlein, O., L. Lindbom, and C. Weber, *Mechanisms underlying neutrophil-mediated monocyte recruitment.* Blood, 2009. 114(21): p. 4613-4623.

281. Swirski, F.K., et al., *Identification of splenic reservoir monocytes and their deployment to inflammatory sites.* Science, 2009. 325(5940): p. 612-616.

282. Chow, K.U., et al., *Spleen Size Is Significantly Influenced by Body Height and Sex: Establishment of Normal Values for Spleen Size at US with a Cohort of 1200 Healthy Individuals.* Radiology, 2016. 279(1): p. 306-313.

283. Toyoda, T., et al., *A 13-week subchronic toxicity study of sodium iron chlorophyllin in F344 rats.* J. Toxicol. Sci., 2014. 39(1): p. 109-119.

284. Naimi, W.A., et al., *Differential Susceptibility of Male Versus Female Laboratory Mice to Anaplasma phagocytophilum Infection.* Trop Med Infect Dis, 2018. 3(3).

285. Andreasen, E., *Sex difference in the size of the lymph nodes and spleen in guinea-pigs.* Acta Anat., 1946. 1(4): p. 359-364.

286. Moller, A.P., J. Erritzoe, and L.Z. Garamszegi, *Covariation between brain size and immunity in birds: implications for brain size evolution.* J Evol Biol, 2005. 18(1): p. 223-37.

287. Manz, M.G. and S. Boettcher, *Emergency granulopoiesis.* Nat Rev Immunol, 2014. 14(5): p. 302-14.

288. Dotson, A.L., et al., *Splenectomy reduces infarct volume and neuroinflammation in male but not female mice in experimental stroke.* J. Neuroimmunol., 2015. 278: p. 289-298.

289. Bouman, A., et al., *Gender difference in the non-specific and specific immune response in humans.* Am J Reprod Immunol, 2004. 52(1): p. 19-26.

290. Haage, V., et al., *Comprehensive gene expression meta-analysis identifies signature genes that distinguish microglia from peripheral monocytes/macrophages in health and glioma.* Acta Neuropathol Commun, 2019. 7(1): p. 20.

291. Cabatingan, M.S., et al., *Naive B lymphocytes undergo homeostatic proliferation in response to B cell deficit.* J. Immunol., 2002. 169(12): p. 6795-6805.

292. Lucin, K.M., *Mechanisms of impaired humoral immunity after high thoracic spinal cord injury*. 2007, The Ohio State University.

293. Popovich, P.G., et al., *Alterations in immune cell phenotype and function after experimental spinal cord injury.* J. Neurotrauma, 2001. 18(9): p. 957-966.

294. Chernykh, E.R., et al., *Characteristics of bone marrow cells under conditions of impaired innervation in patients with spinal trauma.* Bull. Exp. Biol. Med., 2006. 141(1): p. 117-120.

295. Iversen, P.O., et al., *Depressed immunity and impaired proliferation of hematopoietic progenitor cells in patients with complete spinal cord injury.* Blood, 2000. 96(6): p. 2081-2083.

296. Ankeny, D.P., et al., *Spinal cord injury triggers systemic autoimmunity: evidence for chronic B lymphocyte activation and lupus-like autoantibody synthesis.* J. Neurochem., 2006. 99(4): p. 1073-1087.

297. Ulndreaj, A., et al., *Characterization of the Antibody Response after Cervical Spinal Cord Injury.* J. Neurotrauma, 2017. 34(6): p. 1209-1226.

298. Tellier, J. and S.L. Nutt, *Standing out from the crowd: How to identify plasma cells.* Eur. J. Immunol., 2017. 47(8): p. 1276-1279.

299. Monahan, R., et al., *Circulating T cell subsets are altered in individuals with chronic spinal cord injury.* Immunol. Res., 2015. 63(1-3): p. 3-10.

300. Haley, P.J., *Species differences in the structure and function of the immune system.* Toxicology, 2003. 188(1): p. 49-71.

301. Monahan, R., et al., *Circulating T cell subsets are altered in individuals with chronic spinal cord injury.* Immunol Res, 2015. 63(1-3): p. 3-10.

302. Liu, C., et al., *Resveratrol improves neuron protection and functional recovery in rat model of spinal cord injury.* Brain Res, 2011. 1374: p. 100-9.

303. Schultz, J. and K. Kaminker, *Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization.* Arch Biochem Biophys, 1962. 96: p. 465-7.

304. Harada, N., Y. Taoka, and K. Okajima, *Role of prostacyclin in the development of compression trauma-induced spinal cord injury in rats.* J. Neurotrauma, 2006. 23(12): p. 1739-1749.

305. David, S., et al., *Myeloid cell responses after spinal cord injury.* J. Neuroimmunol., 2018. 321: p. 97-108.

306. Schultz, J. and K. Kaminker, *Myeloperoxidase of the leucocyte of normal human blood. I. Content and localization.* Arch. Biochem. Biophys., 1962. 96: p. 465-467.

307. Lacy, P., *Mechanisms of degranulation in neutrophils.* Allergy Asthma Clin. Immunol., 2006. 2(3): p. 98-108.

308. Li, X., et al., *Peroxisome proliferator-activated receptor-γ agonist rosiglitazone reduces secondary damage in experimental spinal cord injury.* J. Int. Med. Res., 2013. 41(1): p. 153-161.

309. Naruo, S., et al., *Prostaglandin E1 reduces compression trauma-induced spinal cord injury in rats mainly by inhibiting neutrophil activation.* J. Neurotrauma, 2003. 20(2): p. 221-228.

310. Albadawi, H., et al., *Spinal Cord Inflammation: Molecular Imaging after Thoracic Aortic Ischemia Reperfusion Injury.* Radiology, 2017. 282(1): p. 202-211.

311. Prame Kumar, K., A.J. Nicholls, and C.H.Y. Wong, *Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease.* Cell Tissue Res., 2018. 371(3): p. 551-565.

312. Chertov, O., et al., *Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils.* J Exp Med, 1997. 186(5): p. 739-47.

313. Ousman, S.S. and S. David, *MIP-1α, MCP-1, GM-CSF, and TNF-α Control the Immune Cell Response That Mediates Rapid Phagocytosis of Myelin from the Adult Mouse Spinal Cord.* J. Neurosci., 2001. 21(13): p. 4649-4656.

314. Shechter, R., et al., *Infiltrating blood-derived macrophages are vital cells playing an anti-inflammatory role in recovery from spinal cord injury in mice.* PLoS Med., 2009. 6(7): p. e1000113.

315. Dotson, A.L. and H. Offner, *Sex differences in the immune response to experimental stroke: Implications for translational research.* J. Neurosci. Res., 2017. 95(1-2): p. 437-446.

316. Zhang, B. and J.C. Gensel, *Is neuroinflammation in the injured spinal cord different than in the brain? Examining intrinsic differences between the brain and spinal cord.* Exp Neurol, 2014. 258: p. 112-20.

317. Gittins, R. and P.J. Harrison, *Neuronal density, size and shape in the human anterior cingulate cortex: a comparison of Nissl and NeuN staining.* Brain Res Bull, 2004. 63(2): p. 155-60.

318. Zhu, Y., et al., *Comparison of unbiased estimation of neuronal number in the rat hippocampus with different staining methods.* Journal of Neuroscience Methods, 2015. 254: p. 73-79.

319. Jakeman, L.B., *Assessment of Lesion and Tissue Sparing Volumes Following Spinal Cord Injury*, in *Animal Models of Acute Neurological Injuries II*. 2012. p. 417-442.

320. Forger, N.G. and S.M. Breedlove, *Sexual dimorphism in human and canine spinal cord: role of early androgen.* Proceedings of the National Academy of Sciences, 1986. 83(19): p. 7527-7531.

321. Roderick, T.H., et al., *Genetic and phenotypic variation in weight of brain and spinal cord between inbred strains of mice.* Brain Research, 1973. 64: p. 345-353.

322. Kigerl, K.A., V.M. McGaughy, and P.G. Popovich, *Comparative analysis of lesion development and intraspinal inflammation in four strains of mice following*

spinal contusion injury. The Journal of Comparative Neurology, 2006. 494(4): p. 578-594.

323. Klein, S.L. and R. Morgan, *The impact of sex and gender on immunotherapy outcomes.* Biol Sex Differ, 2020. 11(1): p. 24.

324. Fee, D.B., et al., *Effects of progesterone on experimental spinal cord injury.* Brain Res., 2007. 1137(1): p. 146-152.

325. Colon, J.M., et al., *Continuous tamoxifen delivery improves locomotor recovery 6h after spinal cord injury by neuronal and glial mechanisms in male rats.* Exp Neurol, 2018. 299(Pt A): p. 109-121.

326. Fujimoto, T., et al., *Potent protective effects of melatonin on experimental spinal cord injury.* Spine, 2000. 25(7): p. 769-775.

327. Holtz, A., B. Nyström, and B. Gerdin, *Spinal cord injury in rats: inability of nimodipine or anti-neutrophil serum to improve spinal cord blood flow or neurologic status.* Acta Neurol. Scand., 1989. 79(6): p. 460-467.

328. Gray, C., et al., *Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish.* Thromb Haemost, 2011. 105(5): p. 811-9.

329. Kong, T., et al., *Leukocyte adhesion during hypoxia is mediated by HIF-1-dependent induction of beta2 integrin gene expression.* Proc Natl Acad Sci U S A, 2004. 101(28): p. 10440-5.

330. Wang, J., et al., *Visualizing the function and fate of neutrophils in sterile injury and repair.* Science, 2017. 358(6359): p. 111-116.

331. Godson, C., et al., *Cutting edge: lipoxins rapidly stimulate nonphlogistic phagocytosis of apoptotic neutrophils by monocyte-derived macrophages.* J. Immunol., 2000. 164(4): p. 1663-1667.

332. Soehnlein, O., et al., *Neutrophil secretion products pave the way for inflammatory monocytes.* Blood, 2008. 112(4): p. 1461-71.

333. Greenlee-Wacker, M.C., *Clearance of apoptotic neutrophils and resolution of inflammation.* Immunol. Rev., 2016. 273(1): p. 357-370.

334. Tauzin, S., et al., *Redox and Src family kinase signaling control leukocyte wound attraction and neutrophil reverse migration.* J Cell Biol, 2014. 207(5): p. 589-98.

335. Bracken, M.B., et al., *A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury: results of the Second*

National Acute Spinal Cord Injury Study. N. Engl. J. Med., 1990. 322(20): p. 1405-1411.

336. Bartholdi, D. and M.E. Schwab, *Methylprednisolone inhibits early inflammatory processes but not ischemic cell death after experimental spinal cord lesion in the rat.* Brain Res, 1995. 672(1-2): p. 177-86.

337. Campbell, S.J., et al., *Central nervous system injury triggers hepatic CC and CXC chemokine expression that is associated with leukocyte mobilization and recruitment to both the central nervous system and the liver.* Am. J. Pathol., 2005. 166(5): p. 1487-1497.

338. Nguyen, H.X., et al., *Systemic Neutrophil Depletion Modulates the Migration and Fate of Transplanted Human Neural Stem Cells to Rescue Functional Repair.* J. Neurosci., 2017. 37(38): p. 9269-9287.

339. Clark, J.A. and T.C. Peterson, *Cytokine production and aging: overproduction of IL-8 in elderly males in response to lipopolysaccharide.* Mech Ageing Dev, 1994. 77(2): p. 127-39.

340. Rosenfeld, C.S., *Sex-dependent differences in voluntary physical activity.* J Neurosci Res, 2017. 95(1-2): p. 279-290.

341. Stohr, T., et al., *Rat strain differences in open-field behavior and the locomotor stimulating and rewarding effects of amphetamine.* Pharmacol Biochem Behav, 1998. 59(4): p. 813-8.

342. Zhu, H., et al., *Phase I-II Clinical Trial Assessing Safety and Efficacy of Umbilical Cord Blood Mononuclear Cell Transplant Therapy of Chronic Complete Spinal Cord Injury.* Cell Transplant, 2016. 25(11): p. 1925-1943.

343. Tordoff, M.G., L.K. Alarcon, and M.P. Lawler, *Preferences of 14 rat strains for 17 taste compounds.* Physiol Behav, 2008. 95(3): p. 308-32.

344. Neumann, J., et al., *Very-late-antigen-4 (VLA-4)-mediated brain invasion by neutrophils leads to interactions with microglia, increased ischemic injury and impaired behavior in experimental stroke.* Acta Neuropathol, 2015. 129(2): p. 259-77.