USING CXCR4 ANTAGONISM AND G-CSF TO INDUCE SUSTAINED NEUTROPHILIA

IN MICE FOR APPLICATIONS IN SPINAL CORD INJURY RESEARCH

A THESIS

by

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ABSTRACT

In spinal cord injury, the immune response can affect the damage scope and functional recovery. However, without a comparable pre-clinical model of spinal cord injury, it is extremely difficult to research potential therapeutic treatments for inflammation-associated damage after spinal cord injury in humans. The goal of this project was to increase the abundance of circulating neutrophils in mice to a comparable human rate of 50-70% of total white blood cells to improve the murine pre-clinical model of spinal cord injury. To induce sustained neutrophilia, we assessed two independent methods. The first strategy utilized C-X-C chemokine receptor 4 (CXCR4) antagonism and we injected AMD3100 (Plerixafor), which is a drug known to target CXCR4. The second strategy we employed was to deliver G-CSF via a plasmid vector, injected directly into the bloodstream, to induce the proliferation of pre-neutrophil progenitor cells. To assess neutrophilia, we utilized flow cytometry and characterized neutrophil abundance out of total leukocytes in the peripheral blood and bone marrow, as well as neutrophil maturation. Using a 5 mg/kg dose of AMD3100, we did not observe an increase in neutrophils in the peripheral blood at 3 hours or 24 hours post-injection. Using a 10 mg/kg dose of AMD3100, we observed a significant increase in neutrophils in the peripheral blood at 3 hours that diminished by 24 hours post-injection. We also observed a significant decrease in the percentage of mature neutrophils in both the bone marrow and the peripheral blood 3 hours after the 10 mg/kg injection of AMD3100. Using the CSF3 (G-CSF) gene packaged in a non-viral vector and injected into the mouse retro-orbitally, we did not observe an increase in neutrophil abundance in the bone marrow or peripheral blood after a 1 or 2-week timepoint. We did, however, observe a marked increase in the number of cells in the bone marrow compared to a vehicle injection at both timepoints.

DEDICATION

I would like to dedicate this thesis to my mother, Kim Maale, who has always pushed me

to be the best and brightest version of myself, even if I did not always listen.

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Contributors

This work was supervised by a thesis committee consisting of Dr. McCreedy, Dr. Dulin, and Dr. Keene of the Department of Biology and Dr Hook of the School of Medicine.

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NOMENCLATURE

G-CSF   Growth Colony Stimulating Factor

IP   Intrperitoneal

SQ   Subcutaneous

PBS   Phosphate-Buffered Saline

APC   Allophycocyanin

SCI   Spinal Cord Injury

IV   Intravenous

RO   Retro-Orbital

TABLE OF CONTENTS

LIST OF FIGURES

1. INTRODUCTION

1.1 Background

Acute spinal cord injury (SCI) is a disastrous and costly neurological condition with over 17,800 new U.S. cases each year according to the 2020 SCI data sheet from the National Spinal Cord Injury Statistical Center. Primary injuries can range from motor vehicle accidents to falls and although there are treatments being tested, there is no cure for spinal cord injury. Secondary tissue damage after SCI occurs in the days to months following the initial or primary injury and results from a cascade of molecular and cellular processes including edema, apoptosis, inflammation, and various other biological events[1]. Although the mechanisms surrounding secondary injury and inflammation are being actively investigated, therapies targeting inflammation have yet to be successfully translated to a clinical setting. Mediated by chemokine (C-C motif) ligands and receptors, inflammation results from rapid recruitment of circulating innate immune cells, such as neutrophils, T-cells, and monocytes to the site of the primary SCI [2]. After infiltration, these immune cells influence recovery through various effector functions, such as phagocytosis, which can target invading pathogens.

Neutrophils are short-lived phagocytic leukocytes and are some of the first immune cells to arrive at the site of injury. Originating in the bone marrow, neutrophils are typically released into the blood in response to steady-state circadian rhythms, however immature neutrophils can still be released into the blood when CXCR4 is inhibited, affecting disease progression in tissues [3,4]. Once in the bloodstream, neutrophils can be recruited to a target tissue by trans-endothelial migration induced by various chemokine signaling [5]. When recruited to a target tissue, neutrophils can employ a variety of effector functions including reactive oxygen species (ROS) production, neutrophil extracellular traps (NET) formation, and degranulation [6]. While neutrophils have been

commonly shown to contribute to secondary tissue injury, recent studies have also indicated that specific neutrophil subpopulations can enhance regeneration after SCI [7,8].

In order to study the effect of neutrophils on tissue damage after SCI in mice and relate these findings to human SCI, the neutrophil populations in the murine blood should be at comparable levels to their human counterparts. Since human blood contains ~50-70% neutrophils out of total lymphocytes and mice blood only contains ~10-30% neutrophils, it is essential to increase and maintain the neutrophil abundance to more accurately relate the murine neutrophil responses to human neutrophil responses after SCI [9]. One way to accomplish greater neutrophil abundance (neutrophilia) is by inhibiting CXCR4, a chemokine that helps retain neutrophils in the bone marrow. AMD3100, also known as Plerixafor, is a CXCR4 antagonist and has been shown to increase short-term neutrophil abundance in the blood in mice [10]. Originally, AMD3100 was thought to recruit neutrophils from lung tissue, but recently it has been hypothesized that it directly effects the bone marrow neutrophil population[11,12]. Although further studies are needed to confirm this, it is a promising approach to inducing short-term neutrophilia. Another possible approach is using granulocyte colony-stimulating factor (G-CSF), a hematopoietic cytokine that increases proliferation of granulocyte precursor cells, to increase neutrophil counts in the blood as has been shown in multiple studies [13,14]. Unlike AMD3100, G-CSF acts indirectly on bone marrow neutrophil populations by increasing neutrophil progenitor cells and therefore acts as an alternative to induce neutrophilia in the blood[15]. Both approaches will help illuminate neutrophil recruitment routes in mice.

2. EXPERIMENTAL DESIGN

2.1 AMD3100

AMD3100 is a potent CXCR4 inhibitor, used as a treatment for some forms of cancer and HIV[16]. It has been shown to significantly increase neutrophils in the blood and spleen at 2.5 hours post injection[10]. However, the effective dosage and timeline of the drug as it relates to neutrophilia, is not well characterized. Furthermore, the specific neutrophil population (mature or immature) released into circulation has yet to be determined. To address these unknowns, we injected mice either IP or SQ with a 5 or 10 mg/kg dose of AMD3100 in sterile PBS. Control mice received an equal volume injection of sterile PBS (vehicle). We then collected the bone marrow and blood at either 3- or 24-hour post-injection using the tissue isolation and cell preparation protocol (see MATERIALS AND METHODS). Samples were then processed for flow cytometry to assess neutrophil abundance and maturation.

2.2 G-CSF

G-CSF is a common growth factor used to stimulate granulocyte production in the bone marrow. It targets multiple immune cell types, including the progenitor cells of neutrophils, thereby increasing the neutrophil count in the bone marrow. This in turn, should increase the neutrophil count in the blood in a steady-state system. Using a non-viral, long-term expression vector, pLIVE (Mirus Bio, Madison, WI), with a G-CSF insert, we injected purified plasmid in sterile PBS intravenously (IV) into mice. Control mice received an equal titer injection of the unmodified pLIVE vector in sterile PBS. We then collected the bone marrow and blood at either 1 or 2-weeks post-injection and processed the samples for flow cytometry to assess neutrophil abundance and maturation.

2.3 Flow Cytometry and Analysis

First, we separated the cells and debris, using previously established forward and side scatter gating strategies. Next, we gated for live leukocytes using CD45 and NIR Zombie Dye. After that, we separated cell lineages using CD45 and CD11b to gate for a myeloid lineage ($CD11b^+$) and lymphocytes ($CD45^+/CD11b^-$). Then, we further separate myeloid cells into inflammatory monocytes ($Ly6C^+/Ly6G^-$) and neutrophils ($Ly6C^{mid}/Ly6G^+$). Neutrophils were then separated into $CD101^{high}$ (mature) and $CD101^{-/low}$ (immature) populations. Neutrophil populations were quantified as percent of live $CD45^+$ leukocytes and maturation was quantified as the percent mature ($CD101^+$) out of total $Ly6G^+$ neutrophils. Bone marrow cell counts were quantified using the EVE automatic cell counter (NanoEntek America Inc, Waltham, MA). Statistical analysis was performed using the PRISM software (see Statistics under MATERIALS AND METHODS section).

3. RESULTS

3.1 AMD3100

With the 5mg/kg dose of AMD3100 we saw no significant change in neutrophil abundance in the peripheral blood in the mice, both at the 24-hour (Figure 1-2.) and 3-hour (Figure 3.) timepoints with either IP or SQ injection. We did not see significant change in neutrophil populations with the 10 mg/kg at 24-hours (Figure 4.). We did observe a significant decrease in bone marrow neutrophils, and an increase in blood neutrophils with 10 mg/kg ADM3100 at the 3-hour timepoint (Figure 5.). We also saw in Figure 5 that both bone marrow and blood neutrophils were significantly less mature in the treated compared to the control mice. No changes in maturation were observed at any of the other timepoints or dosages examined in this study.

Figure 1. AMD3100 5 mg/kg IP injection at 24-hr timepoint has no significant effect on neutrophilia.
WT mice (n=3-4/group) were injected IP with 5mg/kg AMD3100 or PBS and sacrificed at 24-hours post-injection. Peripheral blood and bone marrow were collected and analyzed by flow cytometry. A) Neutrophil abundance out of total leukocytes in the bone marrow. B) Neutrophil abundance out of total leukocytes in the peripheral blood. C) Neutrophil maturation rate (CD101$^+$ out of total neutrophils) in the bone marrow. D) Neutrophil maturation in the peripheral blood. Data is the mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001, two-way ANOVA.
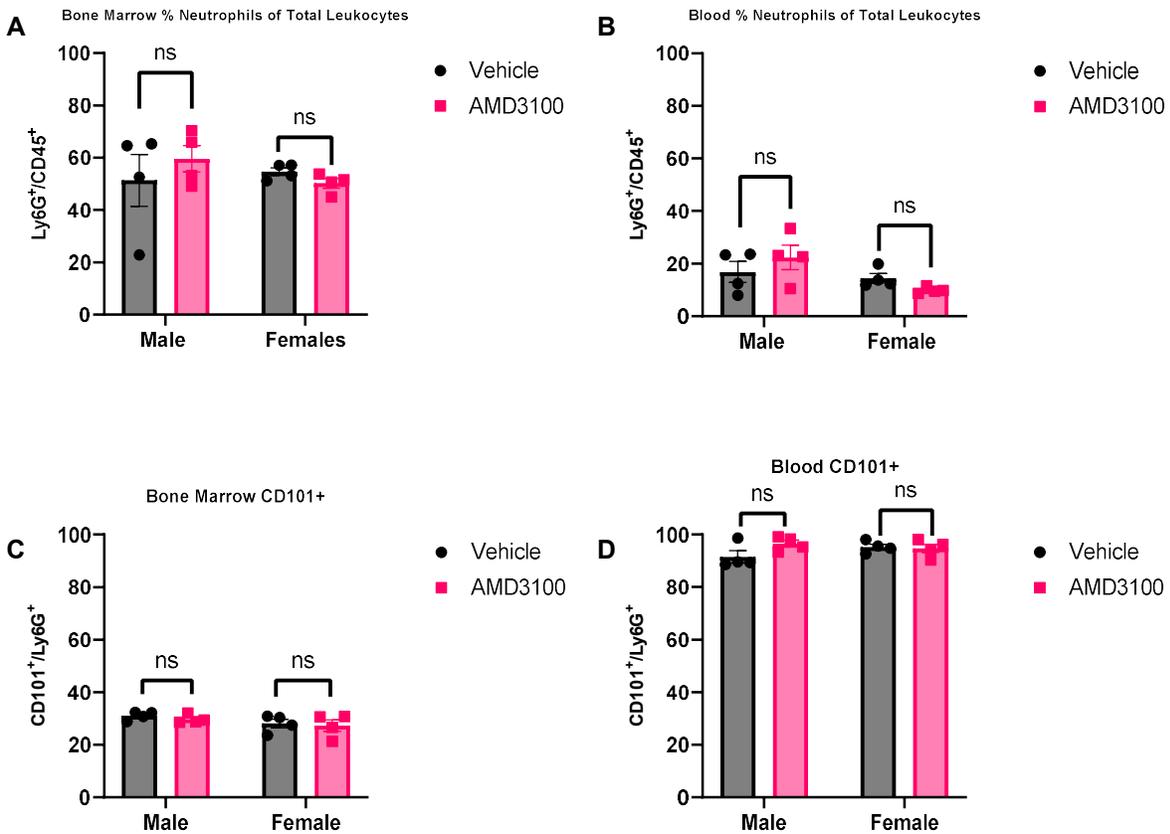
Figure 2. AMD3100 5mg/kg SQ injection at 24-hr timepoint has no apparent effect on neutrophilia.

WT mice (n=4/group) were injected SQ with 5mg/kg AMD3100 or PBS and sacrificed at 24-hours post-injection. Peripheral blood and bone marrow were collected and analyzed by flow cytometry. A) Neutrophil abundance out of total leukocytes in the bone marrow. B) Neutrophil abundance out of total leukocytes in the peripheral blood. C) Neutrophil maturation rate (CD101$^+$ out of total neutrophils) in the bone marrow. D) Neutrophil maturation in the peripheral blood. Data is the mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001, Unpaired t-test.

A Bone Marrow % Neutrophils of Total Leukocytes

B Blood % Neutrophils of Total Leukocytes

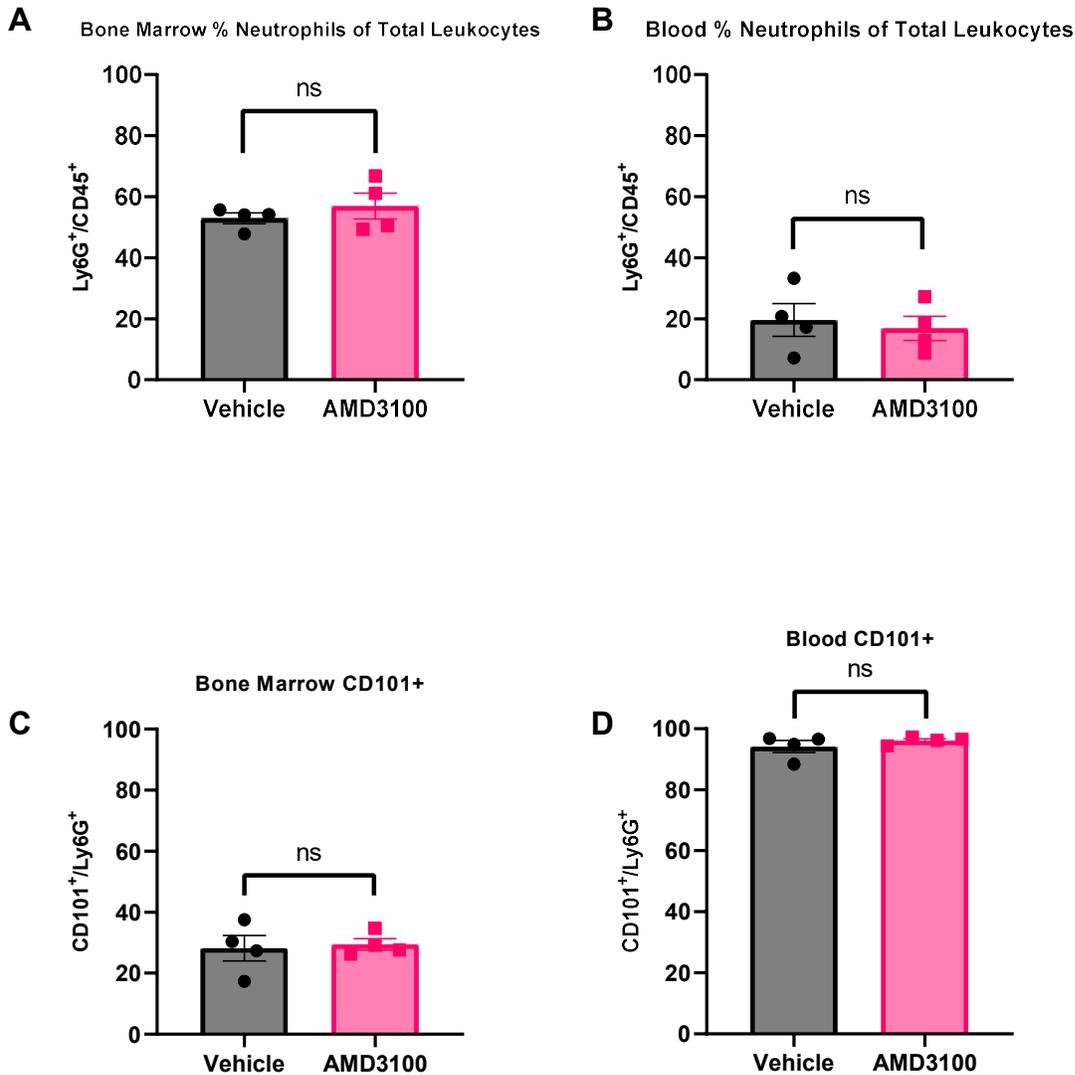**C** Bone Marrow CD101+

D Blood CD101+

Figure 3. AMD3100 5mg/kg SQ injection at 3-hr timepoint has no apparent effect on neutrophilia.
WT mice (n=2/group) were injected SQ with 5mg/kg AMD3100 or PBS and sacrificed at 3-hours post-injection. Peripheral blood and bone marrow were collected and analyzed by flow cytometry. A) Neutrophil abundance out of total leukocytes in the bone marrow. B) Neutrophil abundance out of total leukocytes in the peripheral blood. C) Neutrophil maturation rate (CD101$^+$ out of total neutrophils) in the bone marrow. D) Neutrophil maturation in the peripheral blood. Data is the mean ± SEM.
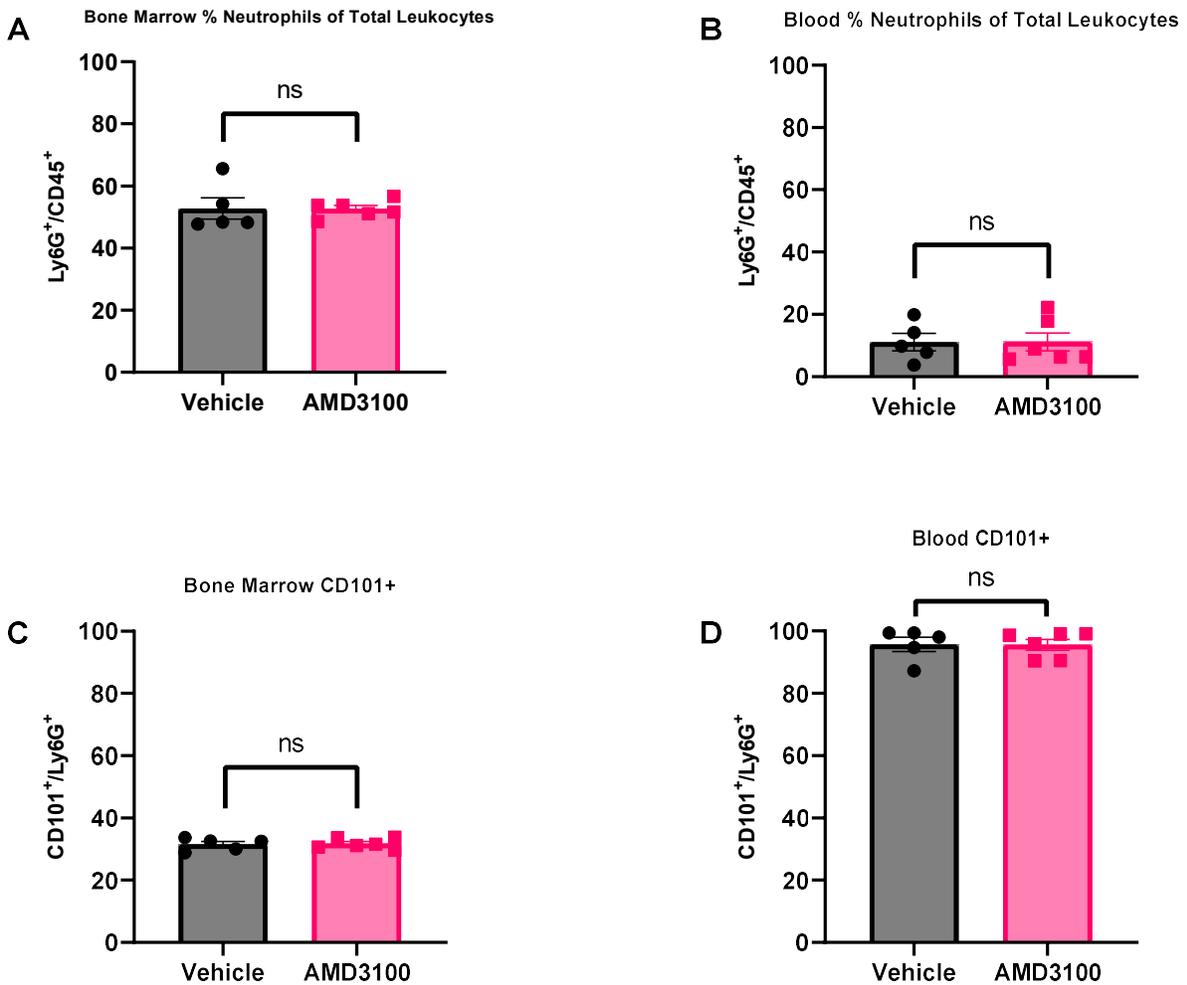
Figure 4. AMD3100 10mg/kg SQ injection at 24-hr timepoint has no apparent effect on neutrophilia.
WT mice (n=5-6/group) were injected SQ with 10 mg/kg AMD3100 or PBS and sacrificed at 24-hours post-injection. Peripheral blood and bone marrow were collected and analyzed by flow cytometry. A) Neutrophil abundance out of total leukocytes in the bone marrow. B) Neutrophil abundance out of total leukocytes in the peripheral blood. C) Neutrophil maturation rate (CD101$^+$ out of total neutrophils) in the bone marrow. D) Neutrophil maturation in the peripheral blood. Data is the mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001, Unpaired t-test.
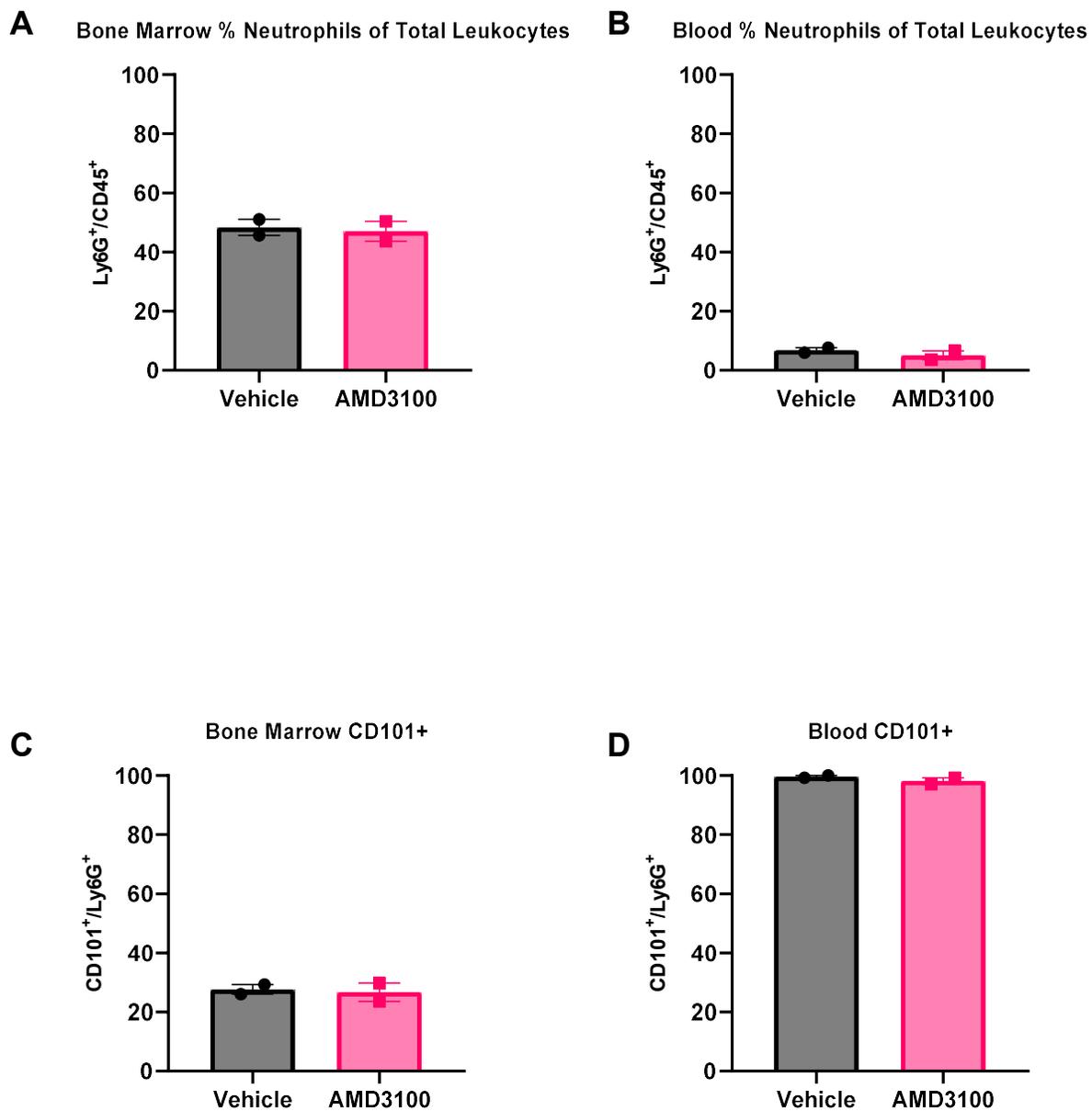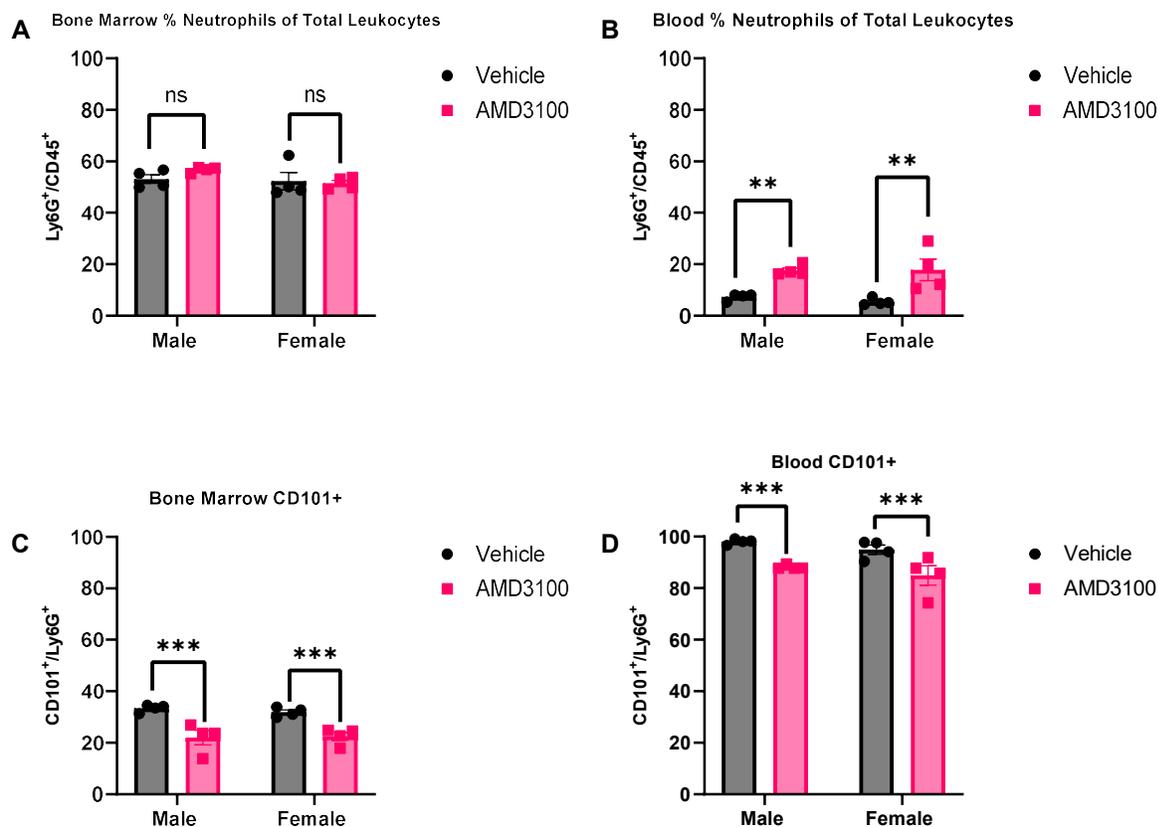
Figure 5. AMD3100 10mg/kg SQ injection at 3-hr timepoint has significant effect on neutrophilia.
WT mice (n=4/group) were injected SQ with 10mg/kg AMD3100 or PBS and sacrificed at 3-hours post-injection. Peripheral blood and bone marrow were collected and analyzed by flow cytometry. A) Neutrophil abundance out of total leukocytes in the bone marrow. B) Neutrophil abundance out of total leukocytes in the peripheral blood. C) Neutrophil maturation rate (CD101$^+$ out of total neutrophils) in the bone marrow. D) Neutrophil maturation in the peripheral blood. Data is the mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001, two-way ANOVA.

3.2 G-CSF

To determine the effects of G-CSF on neutrophilia, we injected the pLIVE plasmid with a CSF3 gene insert, encoding G-CSF, into WT mice and assessed neutrophil abundance and populations at 1 and 2-week timepoints. At the 1-week (Figure 6.) and 2-week (Figure 7.) timepoint, we saw no significant change in bone marrow or blood neutrophil abundances. We did not see a significant change in the neutrophil maturation populations either. However, at both timepoints we did see a drastic increase in the number of cells from the bone marrow (Figures 6E and 7E).

Figure 6. G-CSF has no effect on neutrophil abundance in the blood at the 1-week timepoint.

WT mice (n=3-4 per group) were injected RO with ~25µg of pLIVE-G-CSF plasmid or pLIVE control plasmid, in sterile PBS, and sacrificed at 1-week post injection. A) Neutrophil abundance out of total leukocytes in the bone marrow. B) Neutrophil abundance out of total leukocytes in the peripheral blood. C) Neutrophil maturation rate (CD101$^+$out of total neutrophils) in the bone marrow. D) Neutrophil maturation in the peripheral blood. E) Bone marrow cell counts pooled from one femur and tibia per mouse. Data is the mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001, unpaired t-test.
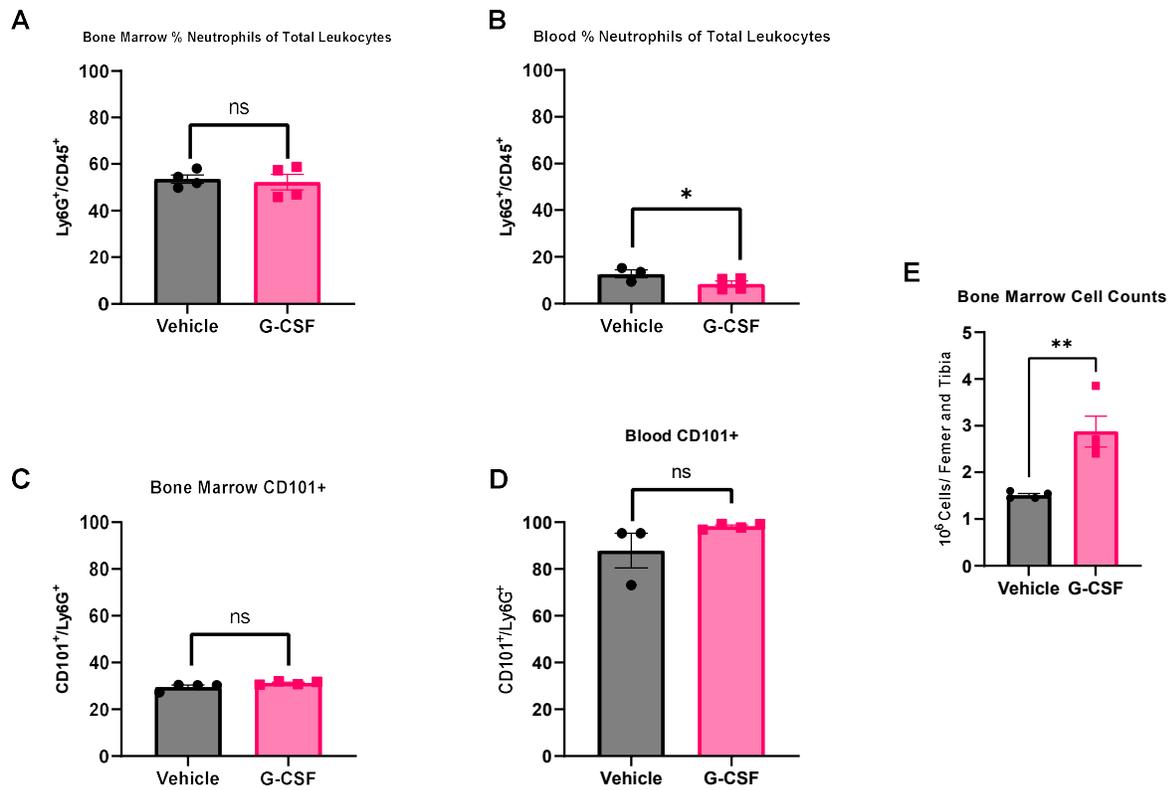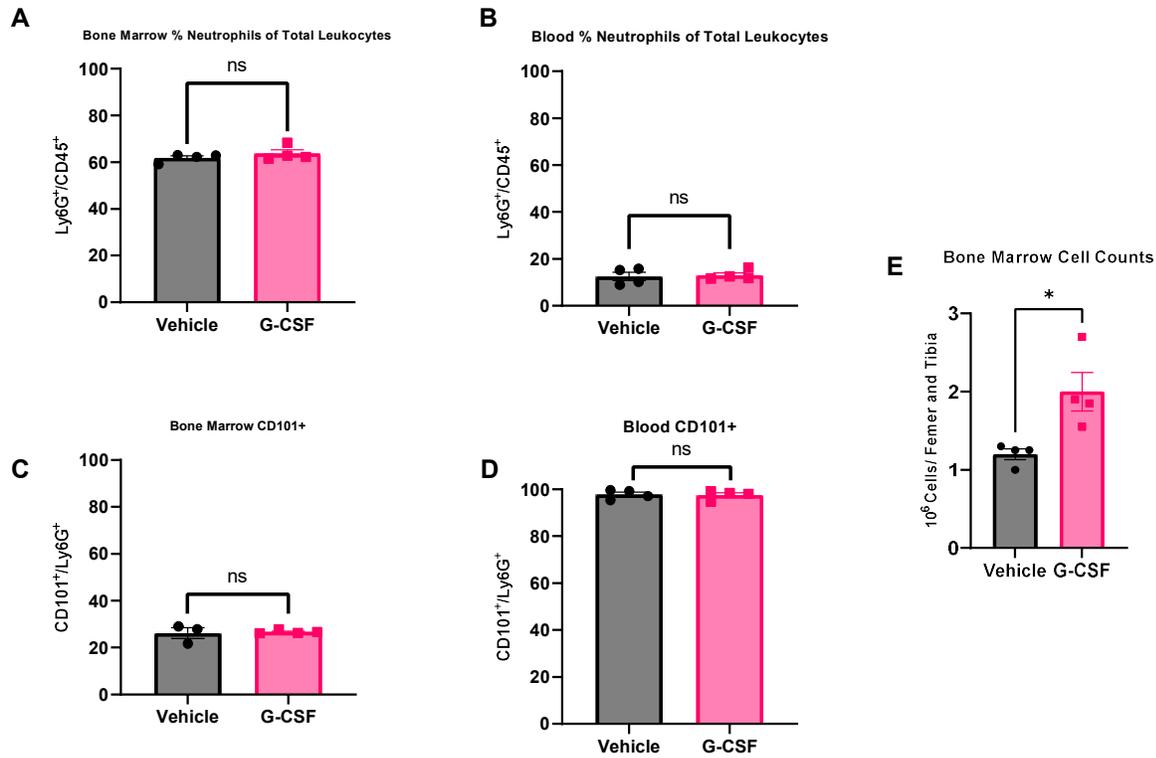
Figure 7. G-CSF has no effect on neutrophil abundance in the blood at the 2-week timepoint.

WT mice (n=3-4 per group) were injected RO with ~25µg of pLIVE-G-CSF plasmid or pLIVE control plasmid, in sterile PBS, and sacrificed at 1-week post-injection. A) Neutrophil abundance out of total leukocytes in the bone marrow. B) Neutrophil abundance out of total leukocytes in the peripheral blood. C) Neutrophil maturation rate (CD101$^+$ out of total neutrophils) in the bone marrow. D) Neutrophil maturation in the peripheral blood. E) Bone marrow cell counts pooled from one femur and tibia per mouse. Data is the mean ± SEM. *p<0.05, **p<0.01, ****p<0.0001, unpaired t-test.

4. MATERIALS AND METHODS

4.1 G-CSF Plasmid

The CSF3 gene was cloned into the multiple cloning site of the pLIVE vector (Mirus Bio LLC., Wilmington, MA) and then purified and mini-prepped along with the control pLIVE plasmid with the NucleoSpin Plasmid mini kit (Macherey-Nagel Inc., Allentown, PA). The pLIVE plasmid was verified to contain the CSF3 insert (Eton Bioscience, San Diego, CA) by Sanger sequencing and was purified in large quantities alongside the control plasmid with NucleoBond Xtra Maxi EF kit (Macherey-Nagel Inc., Allentown, PA) to be injected into the mice (see Drug Treatments below).

4.2 Animals

2–6-month-old male and female C57B1/6 mice from Jackson Laboratory (Bar Harbor, ME) and Charles Rivers (Wilmington, MA) were used for this study. All studies were approved by the Institutional Animal Care and Use Committees at Texas A&M University. All studies are in accordance with the United States Department of Agriculture guidelines.

4.3 Drug Treatments

Mice received 5 or 10 mg/kg AMD3100 IP or SQ (Sigma-Aldrich, St. Louis, MO) or PBS and were sacrificed at 3 or 24-hours post injection. G-CSF pLIVE vector or the unmodified pLIVE vector alone were injected retro-orbitally into an anesthetized mouse. Around 25 μg of plasmid was added to sterile PBS to make a total injection volume of 150 μl. Mice were later injected with 2.5% avertin overdose and bi-lateral thoracotomy was used as secondary euthanasia.

4.4 Tissue Isolation and Cell Preparation

Blood was collected through the heart after secondary euthanasia and added to equal volume EDTA (3.6 mg/ml in HBSS). The left femur and left tibia were collected and flushed with Hank's Balanced Salt Solution (HBSS) to harvest bone marrow cells. The cells were then washed and centrifuged to obtain single cell suspensions in HBSS.

4.5 Flow Cytometry

Single cell suspensions were labeled with Zombie NIR™ Fixable Viability Kit 4°C for 30 minutes and Fc-blocked with purified anti-mouse CD16/32 antibody at 4°C for 20 minutes (BioLegend San Diego, CA). The cells were then stained at 4°C for 30 minutes with the following mouse-specific antibodies (BioLegend San Diego, CA): CD45- APC, CD11b- FITC, Ly6C- PerCP-Cy5.5, Ly6G- Pacific Blue, CD101- PECy7. Gates were set as mentioned in the flow cytometry section under EXPERIMENTAL DESIGN. Data was acquired using the BD Fortessa X-20 flow cytometer (SMACC, College Station, TX) and analyzed with FlowJo software (version 10.8.1; Treestar, Ashland, OR)

4.6 Statistics

Statistical analysis was preformed using 2-way ANOVA or unpaired parametric t-tests (two-tailed) with Prism 9 (GraphPad Software, La Jolla, CA) and are presented as the mean ± SEM. The cut-off for statistical significance was defined as $P < 0.05$ (****$P < 0.0001$; ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns, $P \geq 0.05$).

5. CONCLUSIONS

5.1 AMD3100

As seen in Figures 1-3, a 5mg/kg dose of AMD3100 is not sufficient to induce neutrophilia as suggested in previous field-relevant literature[10]. However, the 10 mg/kg dose of AMD3100 did induce neutrophilia at 3 hours post-injection. Neutrophilia, however, was not sustained and no difference was observed with AMD3100 treatment at 24-hours post-injection. Bone marrow populations did not significantly change, likely due to the relatively small release of neutrophils into circulation.

We also saw a population shift from mature neutrophils to immature neutrophils in the blood and bone marrow of treated mice. We expected this result because AMD3100 antagonizes the CXCR4 retention mechanism in the bone marrow allowing mature and immature neutrophils to be released into the blood. The bone marrow may replace the released neutrophils through proliferation of neutrophil progenitors, which may have tilted the neutrophil populations to more immature neutrophils. We did not see significant sex differences in any of the AMD3100 studies.

Ultimately, we were able to induce short-term neutrophilia after three hours using a 10mg/kg injection of AMD3100. This led to an increase in the percentage of immature neutrophils in the peripheral blood, when compared to control.

5.2 G-CSF

Using ~25 µg of the pLIVE vector with a G-CSF insert injected retro-orbitally, we were unable to induce neutrophilia in the blood at a 1-week or 2-week timepoint. We had expected G-CSF to rapidly increase neutrophil populations in the bone marrow as it directly affected the neutrophil progenitors in the bone marrow. However, we did not see any effect on neutrophil

abundance or maturation at the tested timepoints. We did observe an increase in the number of cells in the bone marrow of G-CSF treated mice. This means that G-CSF may be having an effect on multiple cell populations, which could prevent the specific enrichment of neutrophils. It is possible that more time is needed for the neutrophil progenitors to differentiate into mature neutrophils and be released into the blood.

5.3 Future Directions

AMD3100 is a promising agent for inducing short-term neutrophilia. It would be beneficial to have a shorter timepoint (1 hour) with the 10mg/kg dosage to determine the minimum time necessary to induce neutrophilia. Also, increasing the dosage to 20 mg/kg could increase the length of sustainment of neutrophilia to 24-hours, which would be beneficial for spinal cord injury studies, where surgery is necessary. Although we did not see sex differences with an N=3/4, increasing the sample size could illuminate differences not seen in this study.

G-CSF has been shown to have an effect on neutrophil populations[13–15,17], however we only saw a significant change in the bone marrow cell counts and not specific neutrophil or leukocyte populations at the 1-week and 2-week timepoints. It would be beneficial to use neutrophil progenitor markers such as CD71 and CD117 to see if G-CSF is directly affecting pre-neutrophil populations in the future[18]. We also would like to test a 1-month long timepoint to see if longer timepoints are necessary for inducing sustained neutrophilia. Another approach using G-CSF, could be to alter the vehicle used to express G-CSF. The pLIVE vector is made for long-term, non-viral, expression in the liver and could be less useful for bone marrow and blood neutrophilia. It was also suggested that hydrodynamic tail injection could be a useful alternative to retro-orbital injection, but we found that it was not consistently reproducible in mice due to

the small tail vein and dark tail skin. Unfortunately, time was a large limitation in this study and therefore sex differences in the G-CSF experiments were not quantified. It would be beneficial to see the differences between male in female in a longer study.

Other methods for inducing neutrophilia could include utilizing lipopolysaccharide (LPS) injections to induce short-term neutrophilia, or act as a priming agent for the G-CSF pLIVE plasmid [19]. Another alternative pathway for priming is to utilize fecal microbiota transfer in mice to increase immune response, which in turn, would induce neutrophil proliferation and recruitment into peripheral blood [20,21].

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