UNIVERSITY OF CALIFORNIA

IRVINE

Navigating the Aftermath: CD44 and The Cellular Response to Spinal Cord Injury

DISSERTATION

submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in Biomedical Sciences with a concentration in Anatomy & Neurobiology

by

Dana Alison Creasman

DEDICATION

To

My family and friends, for all their support and patience

My partners, for the many ways they fill my life with all the hues of love

David, for surviving a world that was not kind

Two reminders:

The philosophers have only interpreted the world, in various ways. The point, however, is to change it.

(Karl Marx, epitaph)

im so ruthlessly commited to Dialectics that i am constantly at war with the person i was two days ago, who is a clown and a coward

(@unhaunting, twitter)

An aspiration

I will be good, though my body be broken

(Typhoon, Common Sentiments)

TABLE OF CONTENTS

LIST OF FIGURES/TABLES

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I would like to thank my committee members, Professor Brian Cummings, Professor Robert Hunt, and Professor Matthew Blurton-Jones, who were additional and also essential sources of wisdom throughout the development of completion of these studies, and whose broad collective knowledge base on stem cell biology and neuroimmune interactions was an invaluable boon to me as I advanced through my PhD.

I would also like to thank Professor Andrea Tenner, who served on an earlier stage of my committee and provided incredibly important insight into complement biology, especially interactions between complement components and the nervous system.

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Finally, I want to thank the following authors of the R packages I used for statistics, data visualization, and figure assembly throughout this document:

tidyverse

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LIST OF ABBREVIATIONS

SCI: Spinal cord injury

BBB: Blood brain barrier

BSCB: Blood spinal cord barrier

HA: Hyaluronic acid

NSC: Neural stem cells

hNSC: Human neural stem cells

KD: Kilodyne

DPI: Days post injury

WPT: Weeks post transplant

VITA

Dana Creasman

EDUCATION

2016	**Campbell University, BS, Biochemistry - Summa cum laude (GPA 4.0)**
2022 (expected)	**University of California-Irvine, PhD, Neuroscience (GPA 3.99)**

RESEARCH INTERESTS

Using statistical, computational, and bioinformatics-based approaches to define molecular mechanisms regulating cellular behavior, including in the context of development, neurotrauma, and stem cell-transplantation.

APPOINTMENTS AND RESEARCH EXPERIENCE

September 2016-Present	**Graduate Researcher, University of California-Irvine** School of Medicine, Department of Anatomy and Neurobiology Laboratory of Dr. Aileen Anderson
August 2013-May 2016	**Undergraduate Researcher,** Campbell University Biology Department Laboratory of Dr. Karen Guzman
May 2014-July 2014	**Undergraduate Researcher, UNC Chapel Hill** School of Medicine Laboratory of Dr. Stephanie Gupton

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Teaching Assistant, University of California, Irvine

BIO SCI 99 – Molecular Biology - Discussion section leader:

- Course includes three one-hour lectures per week

BIO SCI M114L – Biochemistry Lab – Research group leader

- Course includes one three-hour lecture per week
- Met with sub group of students weekly to over see their research assignments

BIO SCI 100 - Scientific Writing - Discussion section leader:

- Course includes one three-hour lecture per week
- Selected articles for course readings, gave lectures, devised exam questions

BIO SCI D170 - Anatomy - Laboratory instructor

- Course includes three one-hour lectures and 1 3-hour lab section
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Bio 199 – Undergraduate Research – Undergraduate Overseer for Anderson lab

- Oversee welfare of all undergraduate researchers in lab
- Mediate conflicts between students and their mentors
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RESEARCH MENTORING

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2019-2021 Ying Zhong '21

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2020-Present **Club Advisor, UCI REAL Self Care Club**

- Working with undergraduate members of club board to plan events and activities designed to help undergraduate students at UCI practice mental and physical selfcare
- Give talks to students on strategies for maintaining mental health

2019-Present **Queer Graduate Caucus of UCI, Council Member**

- Scheduling and coordinating volunteer and social events for the queer graduate student community at UCI
- Planning initiatives and policies to ensure UCI's campus is an inclusive space for students of diverse identities and backgrounds

2013-2016 **Collegiate Academy of the North Carolina Academy of Sciences**
Historian 2013-2014
President 2014- 2015
Campus Ambassador 2015- 2016

• Worked with team of officers to plan events designed to equip undergraduate students for careers in research

• Recruited speakers to give presentations at CANCAS events.

PUBLICATIONS

Rebecca Nishi, Anna Badner, Mitra Hooshmand, **Dana Creasman**, Hongli Liu, Aileen Anderson. The effects of mouse strain and age on a model of unilateral cervical contusion spinal cord injury. PLOS One. 2020.

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Dana Creasman, Francisca Benavente ,Pooja Sakthivel, Aileen Anderson. CD44 Regulates Interactions Between Human Neural Stem Cells and the Immune Microenvironment in a Rodent Model of Chronic Spinal Cord Injury Transplantation. *In preparation.*

ABSTRACTS

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Francisca Benavente, **Dana Creasman**, Mitra Hooshmand, Katja Piltti, Aileen Anderson. Novel C1q-Receptor Interaction Mediates Chemotaxis in Neural Stem Cells. International Complement Workshop. Sante Fe, New Mexico. September 2018.

Francisca Benavente, **Dana Creasman**, Mitra Hooshmand, Katja Piltti, Aileen Anderson. Novel C1q-Receptor Interaction Mediates Chemotaxis in Neural Stem Cells. American Society for Neurochemistry. Riverside, California. March 2018.

Dana Creasman, Francisca Benavente, Katja M. Piltti, Sarah Royer, Diane Su, Aileen Anderson. Examining the role of the C1q/C-Met Signaling Axis in Mediating Neuroimmune Interactions in Stem Cells and Glioblastoma. University of California Irvine. November 2017.

Dana Creasman, Karen Guzman. Investigating the Interactions of SR-A, TLR3, and TLR4 Receptors and the Impact on Cytokine Production. North Carolina Academy of Science Annual Meeting. Methodist University. April 2016.

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Dana Creasman, Krysti Byrd, Devin Kallam, Karen Guzman. Investigating the Role of SR-A in the Modulation of TLR3-and TLR4- Dependent Cytokine Production. Campbell University Wiggins Research Symposium. Campbell University. March 2015.

Dana Creasman, Cortney Winkle, Stephanie Gupton. Loss of DCC Function and Protein Degradation Reduces Netrin- Stimulated Axon Branching. UNC-Summer Research Symposium. University of North Carolina Chapel Hill. July 2014.

INVITED TALKS

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2016	B.S. Awarded Summa Cum Laude, Campbell University
2016	Who's Who Among Students in American Universities & Colleges
2015	NCICU Research Award
2012-2016	Campbell University President's List
2012-2016	Campbell University Presidential Scholarship

TRAINING AND PROFESSIONAL COURSES

2021	"Visualizing and Communicating Data to the Public and Policymakers" Course
2021	UCI 390X "Teaching Excellence" Course
2018,2019,2020	Neurotranslational T32 Training Grant Retreat
2019	UCI DTEI "Instructional Technology" Workshop
2019	UCI DTEI "Accessibility" Workshop
2019	"Activate to Captivate Improv for Teaching" Certification

ABSTRACT OF THE DISSERTATION

Navigating the Aftermath: CD44 and The Cellular Response to Spinal Cord Injury

by

Dana Allison Creasman

Doctor of Philosophy in Anatomy and Neurobiology

University of California, Irvine, 2023

Professor Aileen Anderson, Chair

Spinal Cord Injury (SCI) is a devastating condition that results in a serious burden on the health care system. Currently, no therapeutics for the disease exist, and thus research into generating effective therapies for SCI is a paramount concern. One exciting avenue for treatment is neural stem cell (NSC) transplantation. This method has shown efficacy in rodent models, but the therapeutic outcome is highly dependent upon the timing of the transplantation relative to injury. Acutely transplanted cells will migrate primarily towards the injury epicenter, exhibiting pronounced astrogliosis, and not improving animal recovery vs. vehicle control. NSC transplanted with a delay of 9 or more days relative to injury will instead migrate more distally from the epicenter, exhibit a more neuronal and oligodendrocytic phenotype, and will improve animal locomotor recovery vs. vehicle control. This dramatic shift in outcome is due to parallel shifts in the post-SCI microenvironment, including an increase in immune components such as C1q, the recognition molecule of the complement cascade.

Here, I first highlight recently published data from my lab showing that C1q signals to NSC through several receptors, including CD44, a receptor known to regulate a wide variety of cellular behaviors. We have shown that C1q signals to NSC through CD44, serving

as a chemotactic cue. CD44 knockout (KO) NSC, when transplanted into an acute SCI niche, exhibited significantly reduced astrogliosis and migration to the epicenter compared to wild-type (WT) NSC. Additionally, CD44 KO NSC were able to restore locomotor function beyond vehicle control, whereas the WT NSC remained unable to.

Next, I then show data from three distinct projects. First, I examine the effect of CD44 KO on human NSC transplanted into the chronic phase of SCI, where I observe that CD44 is necessary for transplant engraftment. Then, I test the effect of CD44 KO on the endogenous response to SCI, discovering that CD44 KO animals have dramatically altered cellular responses to SCI, including acutely activated immune cell recruitment to the injury site, scar formation, and, strikingly, improved locomotor outcomes vs. WT animals. Finally, I show the development of a new strategy to facilitate the analysis of animal behavioral data obtained from SCI studies, utilizing methods from computational biology. I demonstrate the power of this approach via a pair of use cases, and detail plans to make these methods widely and easily implemented by the field at large by releasing it as a publicly available R package.

INTRODUCTION

SCI Progression and Overview

SCI is a devastating condition, causing degeneration of nervous tissue and a loss of both motor and sensory function [1]. The current global prevalence of SCI is over 27 million [2]. This volume combined with the complete lack of effective treatments for SCI represents a massive loss in length and quality of human life, necessitating basic research into the basic biology behind SCI, and translational research into possible therapies for restoring function to SCI patients. Broadly, SCI consists of an initial insult to the spinal cord (primary injury), followed by waves of inflammation over the following days/weeks (secondary injury) [1, 3, 4]. It is this multi-phasic response, involving elements from the immune, nervous, and other systems, that makes SCI such a complicated challenge

Injury Types

Another factor adding to the complexity of SCI is that it is not a monolithic "injury", but an umbrella term for a variety of different insults to the spinal cord. This includes punctures/lacerations of the spinal cord, compressions, and impact-induced contusions [1]. The rest of this document will primarily discuss SCI from the perspective of contusion injuries, both because it is the most common type, and because it is the injury model utilized in the animal study data presented. However, it is worth acknowledging that each of these injury types has a distinct pathophysiological profile, and findings in one injury model are not guaranteed to hold in another model.

Post- SCI, there is a Breakdown of the Blood Spinal Cord Barrier and a Shift in the Spinal Cord Microenvironment

Whatever its origin, the initial physical insult to the spinal cord sets off the chain of events. Much of post-SCI dynamics revolve around changes in the interaction between the "immune privileged" spinal cord and peripheral factors in the circulatory system. In a homeostatic environment, the vasculature of the spinal cord is ensheathed in a blood-spinal cord barrier (BSCB), akin to the blood-brain barrier (BBB). Though the BSCB is believed to be slightly more permeable than the BBB, its function is still to keep the spinal cord parenchyma a relatively "immune privileged" environment, tightly regulating the entry of peripheral immune cells and large molecules [5]. In addition to the damage to the spinal cord and neural tissue, the BSCB is compromised after SCI, increasing its permeability [6-8]. This breakdown reaches its peak at 24 hours post-injury, and while the barrier will begin to restore itself, evidence suggests it may remain excessively permeable indefinitely [5, 6, 9-11].

This allows circulating cells and molecules from the blood to access the spinal cord parenchyma. Neutrophils make up a large proportion of the cells infiltrating early (1-2 dpi), with macrophages and microglial being present at high numbers first at 7 dpi, then again 4-12 weeks after injury [12, 13]. Humoral components such as complement proteins are present as well, both as a result of serum influx, and local synthesis/secretion by spinal cord-endogenous cells and resident microglia/infiltrating immune cells [14]. Between the activation of spinal cord-native cells and the rapid infiltration of non-native cells and proteins, the spinal cord microenvironment is dramatically and acutely shifted after SCI, setting the stage for a myriad of different cellular responses, some regenerative/protective, others damaging.

SCI Induces the Formation of a Lesion/Cyst

Between primary damage from mechanical forces and secondary damage from inflammation, SCI induces high amounts of necrosis and apoptosis in neural cells, along with degeneration of damaged axons [1, 15, 16]. The lesioned area can develop into a collagenous scar or a cystic, fluid-filled cavity [1, 17, 18]. Which of these outcomes occurs depends both on the species being studied (humans and rats form cystic cavities, mice form collagenous scars), and the type of injury (contusion via impact, crush/compression, etc) [19]. Consistently, however, the resulting region is unfavorable to axonal regeneration, posing a serious obstacle to functional restoration [1].

An Astroglial Scar Forms to Contain the Inflamed Lesion

To prevent the necrotic, inflamed region from spreading and causing further damage to the spinal cord, an astroglial scar is formed, outlining the lesioned tissue. This scar consists of elongated astrocytes that link their processes to form a barrier between spared tissue and the necrotic, inflamed tissue at the injury epicenter [18]. Other cells, including microglia, are present within this scar region as well, and help regulate the behavior of astrocytes to properly form this barrier between dead tissue and tissue that is reactive, but spared [18] [20].

The source of these scar-forming astrocytic cells remains a point of dispute. There is a body of literature that suggests that they arise from an endogenous NSC population located in the central canal of the spinal cord [21-26]. This model poses that these ependymal cells proliferate acutely after SCI, migrating out of the canal and towards the injury epicenter where they differentiate into an astroglial lineage and form the scar. However, there is an opposing argument that scar-forming astrocytes are strictly the progeny of mature astrocytes in the spinal cord that proliferate in response to injury, and

that the ependymal cells do not migrate extensively out of the canal, if at all [27, 28]. The differences in these sets of literature appear to stem from a complex mixture of injury models (stab wound vs hemisection vs compression vs contusion) and the precise transgenic mouse model used to trace ependymal cells after injury [26, 29]. More work remains to properly understand how each cell population contributes to scarring post-injury and to generalize these findings across injury models.

In both cases, the initial wave of proliferation from scar-forming cells appears to occur in the first few days after injury, with the scar itself reaching maturity around 3-4 weeks after injury [21, 27, 30]. While it is often suggested that the scar is a barrier to axonal regeneration and functional restoration, it is critical to note that several studies have shown the necessity of scar formation to ensure the protection of spared neural tissue. Removal of or prevention of the formation of the astroglial scar has consistently devastating outcomes in terms of lost neural tissue and worsened locomotor performance [24, 31-33]. Instead, the introduction of a specific cocktail of signaling factors is a way to induce axonal regrowth through injured regions [34]. Critically though, this regrowth did not manifest as restored locomotor function, which highlights that the mere generation of new fibers is not enough for meaningful therapeutic benefit, as those fibers must appropriately integrate and connect to their appropriate target circuits.

Stem Cell Transplantation as an SCI Therapeutic

The debilitating nature of SCI and the absence of therapies for it means there is a great need for research into new methods of treatment. Transplantation of neural stem cells (NSC) is one promising avenue. NSC are resident stem cells of the CNS, capable of generating neurons, astrocytes, and oligodendrocytes; the bulk of the CNS is built from

these NSC during development [35, 36]. Transplanted NSC can aid in post-SCI recovery and repair in a myriad of ways, including engraftment into the host, neuroprotective and neurotrophic mechanisms, and immunomodulation that limits the damage done by the post-SCI immune response [37]. However, our lab has consistently seen in our studies that the behavior of transplanted NSC depends not only on the nature of the cell line transplanted but also on the timing and location of the transplantation relative to the injury. For example, transplantation into the injury epicenter results in decreased cell survival/proliferation compared to transplantation into the intact parenchyma [38]. In terms of timing, sub-acute (9dpi) or chronic (30dpi, 60dpi) NSC transplantation results in transplanted human NSC (hNSC) migrating away from the injury epicenter, differentiating primarily into neurons and oligodendrocytes, and aiding in locomotor recovery [38-41]. However, acute transplantation leads to hNSC clumping around the lesion, forming primarily astrocytes, and failing to contribute to the animal's locomotor recovery [42]. In sum, these data indicate that hNSC are highly sensitive to the microenvironment into which they are transplanted, highlighting that careful study of NSC-niche interactions is still needed.

The post-SCI Niche is highly dynamic

As highlighted in the above sections, the post-SCI response consists of numerous coordinated and interconnected processes involving a wide range of systems and cell types. As expected, this results in a microenvironment that is highly dynamic both spatially and temporally. Transplanted NSC will be exposed to many different molecular signals that can greatly impact both behavior and ultimately potential efficacy as an SCI therapeutic. For instance, cytokines are a major molecular subtype present in the injured spinal cord and

other neurotraumatic environments. These molecules can exert multiple different effects that dramatically alter the behavior of NSC. IL-6 regulates the proliferation and differentiation of NSC [43-45]. Chemokines and their receptors (such as SDF-1/CXCR4 and CCR2/CCL2), guide NSC migration towards injured sites [46, 47] [48] [49] [46, 50, 51] [52] [53]. Still, other inflammatory factors, including LIF and CNTF, can activate JAK-STAT pathways in NSC and drive astroglial differentiation [54]. These examples represent only a small slice of the myriad of immune factor-NSC interactions present in neurotraumatic niches. A more thorough review of NSC-cytokine interactions has been provided by Covacu et. al [55] [56]. As I will cover in more detail later on, the extracellular matrix is also highly dynamic post-SCI, with components such as hyaluronic acid or osteopontin also having been established as exerting effects on NSC.

C1q as a Novel Regulator of NSC Behavior

The above section has established the broad background spinal cord injury, the dynamic microenvironment that follows it, and how the outcomes of hNSC transplants into the injured spine are seemingly highly dictated by this microenvironment. Now, I will transition to discussing a recently published set of data from my lab that seeks to explain in part why hNSC respond to the post-SCI niche in the way that they do [42, 57]. The crux of this data revolves around a novel role for C1q, the recognition molecule of the classical cascade in function as a signaling ligand, binding to cell surface receptors expressed on NSC and exerting a wide variety of effects on NSC behavior, both in *in vitro* cultures and in an *in vivo* rodent model of post-SCI transplantation. This data is of particular intrigue because C1q has, with a few exceptions, not previously been shown to function as a signaling ligand outside its traditional role in initiating the complement cascade [14]. The first clue to

relevance of C1q to hNSC transplantation is that post-SCI, there is an acute increase in the level of C1q present near the injury epicenter (FIG 1B), along with phasic infiltration of immune cells that secrete C1q, such as macrophages and neutrophils (FIG 1C). We found discrete concentrations of C1q in serum (C1q[serum]), and in conditioned media (CM) from macrophages (C1q [MΦ]) and neutrophils (C1q [PMN]) (Table 1). Using *in vitro* NSC cultures, it was discovered that NSC migration, proliferation, and fate were all affected by C1q in a dose-dependent manner. Critically, C1q also activated intracellular signaling pathways, indicating function as a true signaling ligand on NSC. These data are summarized in FIG 0.2.

These effects were maintained in *in vivo* transplantation experiments. Administration of a C1q-neutralizing antibody was able to transiently prevent NSCs from migrating towards the injury epicenter after acute transplantation [58]. Similarly, depletion of neutrophils (which infiltrate into the spinal cord post-SCI and secrete C1q) from the perimeter overcomes the recruitment of transplanted NSCs to the SCI epicenter and improves motor outcomes [59].

Novel Interactions between C1q and Cell-Surface Proteins Expressed by NSC

Having established that C1q not only alters the behavior of NSC but activates intracellular signaling pathways in NSC, it was hypothesized that there would exist C1q receptors expressed on the surface of NSC that were mediating these effects. A forward, unbiased screen was conducted by incubating cultured hNSC with biotinylated C1q, followed by immunoprecipitation to isolate C1q and any cell-surface proteins bound to it. These proteins were then analyzed via mass spectrometry, resulting in a list of 5 candidate C1q-binding cell-surface proteins expressed on NSC. Interactions between C1q and the

candidate proteins were then verified via proximity ligation assay (PLA, FIG 3). Among these proteins was CD44, a protein with an established role in mediating cellular migration, proliferation, and fate [60-62]. The functions of CD44, both broadly and specifically on NSC, are discussed in the following sections.

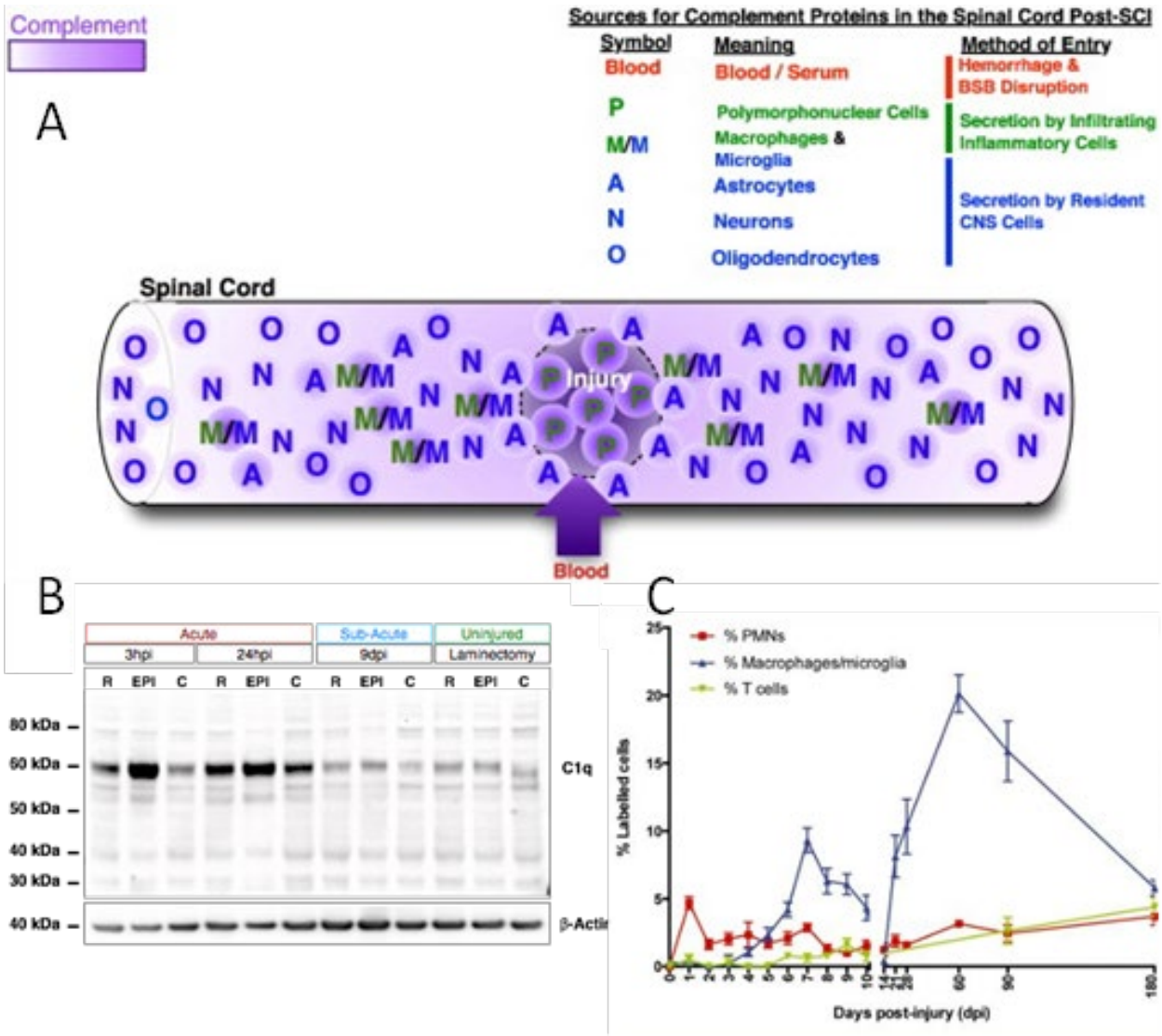

Fig 0.1. Inflammation in the spinal cord post-SCI. A) Complement comes from multiple sources, including serum, infiltrating immune cells, and resident CNS B) Complement C1q is elevated in the post-SCI cord 24 hrs following SCI or laminectomy (control). Spinal cords from NOD-scid mice were freshly dissected and cut into 3 mm sections: 1 at the injury epicenter, 1 rostral, and 1 caudal to the injury. C1q levels were analyzed via PAGE-SDS/Western blotting, with actin serving as a loading control. C) Flow cytometry on cell extracts from the cords of SCI nod-SCID mice show a phasic immune cell response post-SCI. Neutrophils (PMN) peak in number acutely after injury, whereas macrophages/microglia

have an initial peak at 7 dpi followed by a larger one at 60 dpi. In contrast, T cells have a steady increase over the 0-180 dpi window.

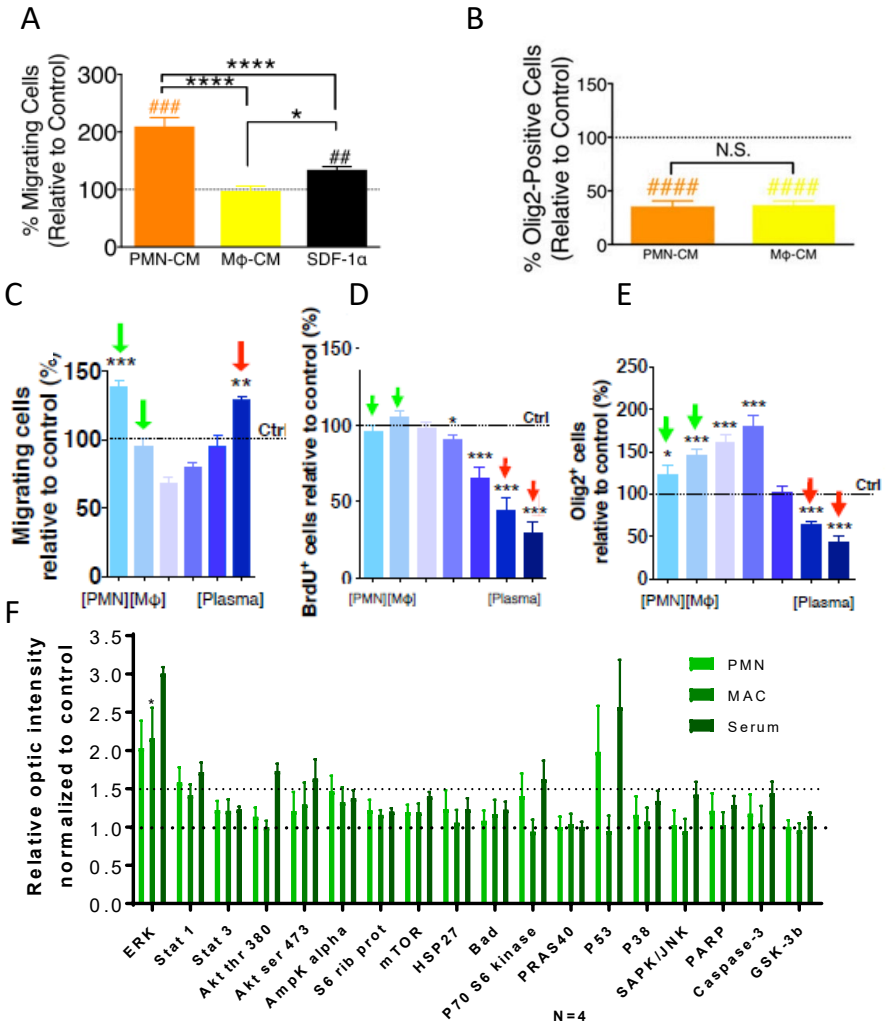

Fig 0.2. Inflammatory conditions induce changes in vitro hNSC behavior. All data normalized to control A) PMN CM, but not MΦ CM is capable of inducing migration in hNSC. Known hNSC chemoattractant SDF-1α was used as a positive control. One sample test vs control: ## $p<0.01$, ### $p<0.001$. ANOVA with posthoc Student's t-test vs. group *** $p<0.001$, **** $p<0.0001$ B) PMN CM and MΦ CM reduce oligogenesis hNSC, as indicated by immunocytochemistry staining for Oligo2+ hNSC in vitro after 14 days of exposure. One sample test vs control #### $p<0.0001$. C) C1q treatment induces migration in hNSC at [PMN] and [Serum] doses as indicated by transwell migration assay. ANOVA with posthoc Student's t-test vs. group *** $p<0.001$, ** $p<0.01$. D) high-dose C1q treatment reduces hNSC proliferation, as indicated by BrdU assay. ANOVA with posthoc Student's t-test vs. group *** $p<0.001$, * $p<0.1$ E) C1q treatment modulates oligogenesis in hNSC, as indicated by immunocytochemistry staining for Oligo2+ hNSC after 14 days of exposure. ANOVA with posthoc Student's t-test vs. group *** $p<0.001$, * $p<0.1$ F) C1q treatment induces activation of intracellular signaling pathways, including ERK, AKT, and P53 in hNSC, as indicated by phosphoarray analysis.

Source	C1q Concentration
Mouse Serum	80 µg/mL
Mouse Neutrophil CM	28 ng/ml
Mouse Macrophage CM	400 ng/ml

Table 0.1. Summary of C1q concentrations found in different

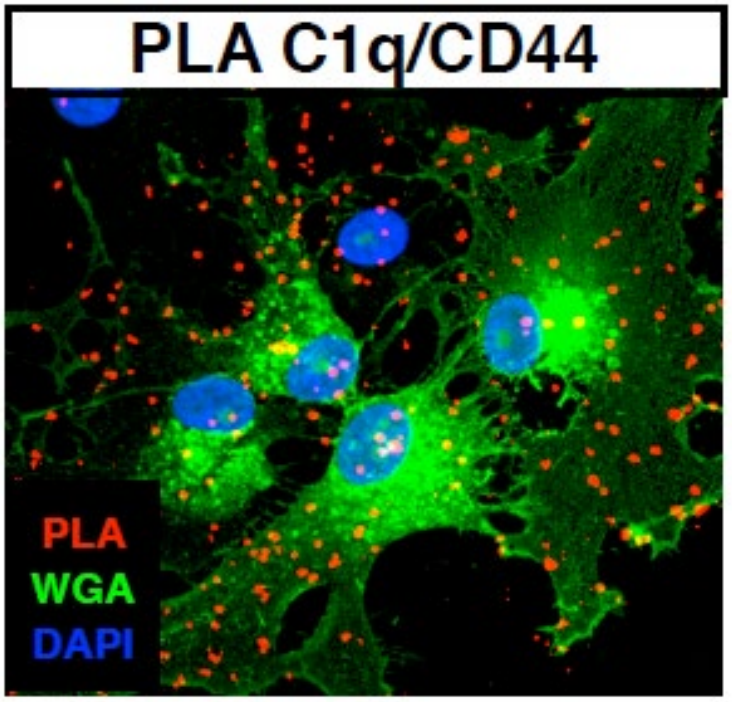

Fig 0.3. CD44-C1q binding on hNSC was confirmed via in vitro proximity-ligation assay. Red puncta indicate binding.

CD44: A Regulator of Cellular Behavior

The CD44 protein family consists of the various products of the CD44 gene. Alternative splicing allows for the generation of multiple variants of CD44, with the standard form of CD44 (CD44s) containing exons 1-5 and 16-20 (other variants will include one or more of exons 6-15)[60]. CD44 has a diverse set of ligands, including hyaluronan (HA), osteopontin, and matrix metalloproteases. As one might expect given its wide variety of forms and ligands, CD44 has a multitude of functions. It is involved in both cell-cell and cell-matrix interactions, and cell migration, proliferation, and fate [60, 63, 64]. CD44 has been previously shown to modulate the behavior of multiple cell types in the CNS, especially astrocytes [64]. CD44 expression is linked to astroglial differentiation and reactive astrogliosis, with CD44 also having been shown to regulate cytoskeletal function in astrocytes [65-67]. CD44 also has established functions in NSC. CD44 has been used as a marker for stem cells, specifically cancer stem cells, for years [62, 68]. More recently, it has been shown that HA-CD44 signaling negatively regulates proliferation in brain NSC both *in vitro* and *in vivo [69].* CD44 has also been shown to be expressed by spinal cord-derived NSC [70]. The novelty of CD44-C1q interactions combined with this established literature makes CD44 an exciting target of study for the sake of both basic biology and therapeutic concerns.

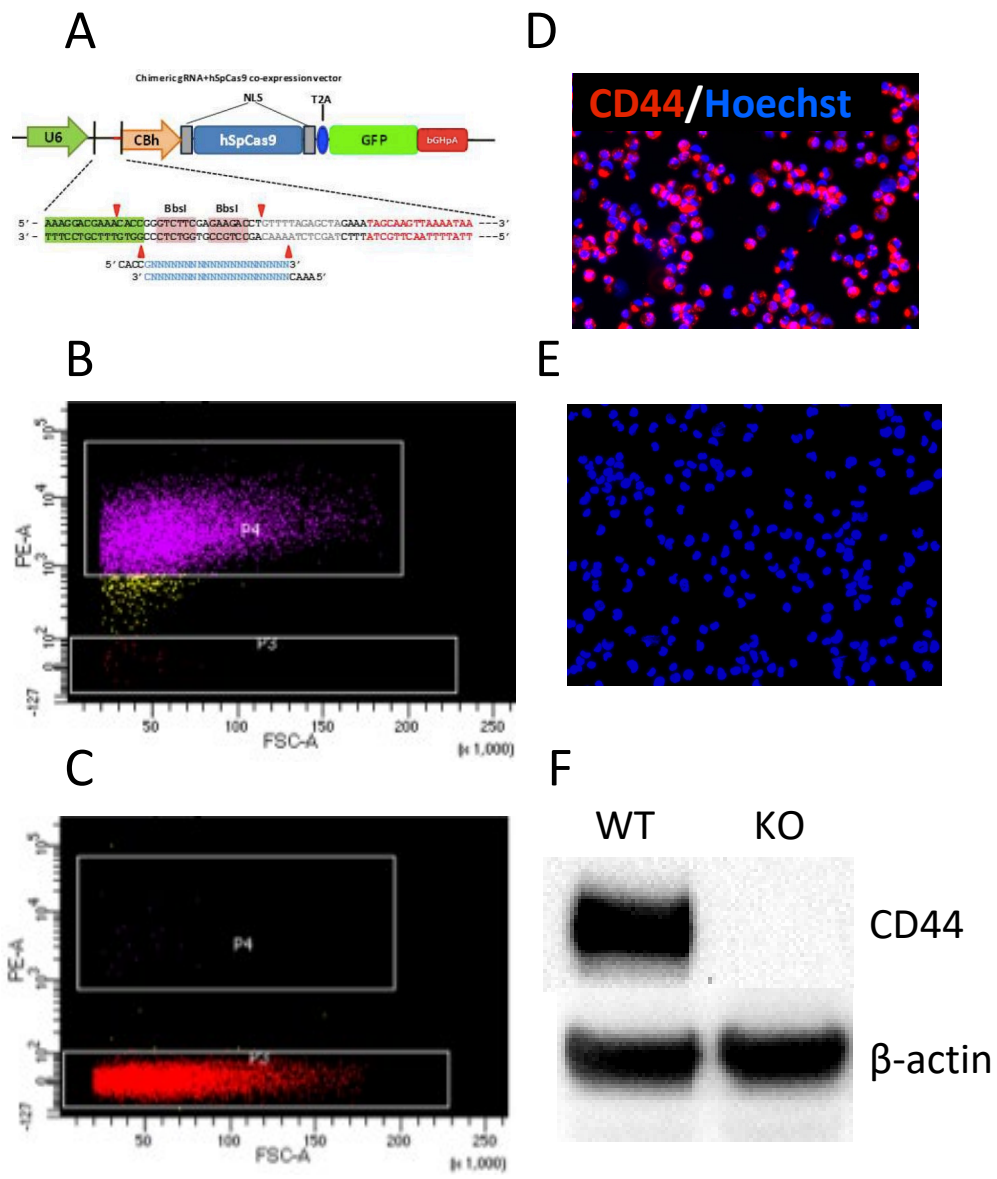

Fig 0.4. Generation of CD44 KO hNSCs. A) Schematic of the targeting vector and cloning strategy. C-D) Flow cytometry staining for CD44 of CD44 WT (B) and KO (C) cells. E-F) Hoechst/CD44 staining of CD44 WT and KO cells shows clear elimination of CD44 expression in KOs. G) Western Blot for CD44 showing complete absence of CD44 protein in whole-cell lysates of CD44 KO cells.

CD44 and CD44-C1q interactions regulate NSC behavior in vitro

To test the role of CD44 in hNSC, we generated CD44 KO hNSC via a CRISPR-Cas9 method. hNSC were transfected with guide RNAs for CD44 and a Cas9 protein. The resulting heterogeneous population of CD44-positive and negative cells was sorted by flow cytometry to obtain CD44 WT and KO cells. This method preserves the natural heterogeneity of NSC.[57] The efficacy of the KO was determined via sequencing and Western blotting. These data are summarized in FIG 4.

Next, we examined how the behavior of CD44 KO NSC was altered vs. WT, both at baseline and in response to inflammatory conditions. CD44 KO NSC exhibited a slight but significant elevation in baseline oligogenesis compared to WT. In parallel, CD44 KO significantly reduced NSC chemoattraction to C1q (FIG 5). These results are well aligned with the established role of CD44 in regulating cellular migration and fate [60, 64, 67, 71]. I was an author on the resulting publication from this work, and it led directly my primary dissertation studies, particular Chapter 1 of this document.

Current methods for measuring animal behavior and their limits/misapplication

Beyond exploring how CD44 effects the behavior of NSC and other cell types relevant to SCI, I am also highly concerned with a question that is parallel, but still central to improving the efficacy of SCI treatments: finding more robust methods of measuring animal locomotor recovery/behavior. Indeed, to make more efficacious treatments, we must first be able to define what “efficacy” looks like, which is more complex than is often assumed. Over the history of neurotrauma research, numerous scales and tasks have been developed, each with their respective strengths, weaknesses, and intended uses. Live-

graded, observation-based tasks such as BBB and BMS are sensitive, but their ordinal scaling results in a very uneven, non-linear progression of recovery through said scale. For example, several points on a scale may be dedicated to variations in an animal's gait that occur before it has even recovered the ability to step, but a difference of only one point separates an animal that can step vs. one that cannot. This creates regions of intense scale compression and scale expansion, which can greatly warp the analysis and interpretation of data [72]. Horizontal ladderbeam asks animals to walk across a horizontal ladder and scores them on successful steps made [73]. This task is highly sensitive, but only over a narrow region of recovery, as animals must be consistently stepping enough to perform the task, but not stepping so well as to create a ceiling effect.

Additionally, the clear "better/worse" dichotomy present in tasks such as BBB, BMS, or ladderbeam has the added benefit of making them easy to interpret in the context of a study. However, it is this simplicity that is also a downside, as it does not allow for robust analysis of exactly how animals are performing, only if they are doing "good" or "bad". This is where the idea of gait analysis becomes relevant. The goal here is to empirically measure different aspects of animal locomotion, such as stride length, speed, stepping pattern, etc. The early method of obtaining this info consistently of dipping animals' paws in ink and letting them run across a sheet of paper, then physically measuring the desired variables from the tracks. Currently, advanced machine-learning techniques are being developed that allow raw video of animal locomotion to be turned into kinematic information [74]. Sitting in between these two ends of the technological scale is the CatWalk kinematic testing apparatus. The CatWalk suite consists of running freely moving animals through a tunnel with a transparent walkway, specially lit and filmed from below to create a high-contrast

video of paw placement during locomotion. Videos are semi-automatically analyzed by a software package (with human quality control), providing detailed, robust insight into a myriad of aspects of how an animal is moving [75].

Like most gait analysis methods, the number of variables CatWalk outputs is both its biggest strength and its largest drawback: when dozens of variables are present, determining what the sum of the data means can be a difficult and confusing task. The potential for cherry-picking variables which show the "desired" outcome is high, as is the chance of missing potentially insightful results simply by never looking at the data in total, and instead focusing only on a few "gold standard" variables. Some approaches have gone further, attempting to use the massive data output from CatWalk to generate a linear model, correlated to another, established measure of locomotor function, such as BMS/BBB or ladder beam [76, 77]. While useful under certain circumstances, this approach has two primary issues. One is that animal locomotion, both at baseline and in response to injury, is highly dependent on factors such as sex, age, strain, genetics, injury/disease model, etc. This means that whatever model is generated may only work under a narrow set of conditions. Second, this approach ultimately seeks to reduce CatWalk data to a binary axis of "better" and "worse", usually with the hope of showing that a given intervention was efficacious in promoting recovery post-injury. This misses an opportunity to use CatWalk data to answer a more nuanced question: do different injuries and/or treatments alter locomotion in different ways? Post-injury recovery is not a binary axis; animals can exhibit functional recovery in ways that may not resemble uninjured performance. CatWalk data provides the chance to examine how different interventions result in different compensatory mechanisms, which could provide insight into the treatment's mechanism of

action. In the third chapter, I detail a methodological approach for how to extract useful information more fully from CatWalk data. Critically, this approach is not exclusive to just CatWalk data but is instead applicable to any gait analysis method with a high dimensional output.

Summary of background

The above literature cumulatively highlights the devastating nature of SCI, and that the cellular response to SCI is a complex phenomenon that involves numerous cell types. NSC transplantation offers a promising avenue for functional restoration after SCI. In the chapters below I investigate: 1) the effect of CD44 KO on hNSC transplanted into the chronic phase of SCI; 2) the effect of CD44 KO on the endogenous response to SCI; 3) the development of a new strategy to facilitate analysis of animal behavioral data obtained from SCI studies.

Chapter 1: Transplantation of CD44 KO hNSC into a chronic SCI niche

As explained in the introduction, NSC transplantation is a promising therapeutic avenue for SCI, but the potential efficacy of this approach is highly dependent on the timing of the transplant relative to the injury, and the corresponding host microenvironment encountered by donor NSC [39, 41, 42, 57, 78, 79]. C1q-CD44 interactions appear to be a critical regulator of transplanted NSC behavior, with CD44 KO hNSC exhibiting significantly less recruitment to the injury epicenter, reducing the commitment of these cells to the astroglial lineage and promoting therapeutic efficacy vs. WT hNSC [57]. Our lab has shown that C1q is secreted in large quantities by macrophages/microglial and that this same cellular population peaks dramatically in the chronic (60 dpi) phase of injury, a phenomenon that is paralleled in humans [12, 13].

Because of these data, I sought to compare CD44 KO vs WT hNSC transplantation during the chronic phase of SCI. Beyond the highly relevant basic biology questions, this transplantation time point is also critical from a purely translation point of view. Given the aforementioned large volume of patients living with SCI chronically, virtually any treatment for SCI should be tested for efficacy in this time window.

For this study, immunocompromised Rag1 mice received bilateral T9 injuries, and then 60 days later were injected with WT hNSC, CD44 KO hNSC, or vehicle control. Mice were then sacrificed 16 weeks later, and their spinal cords were dissected and stained for relevant hNSC markers. In parallel, animal locomotor behavior was recorded to measure each treatment's impact on recovery. Given the data from the acute transplantation experiments, my initial hypothesis was that CD44 KO hNSC would again exhibit less clumping around the injury epicenter and increased therapeutic efficacy compared to WT

hNSC. In contrast, I instead saw that neither hNSC treatment significantly increased recovery vs. vehicle, and the CD44 KO hNSC had significantly lower engraftment than WT hNSC.

Methods

NSC lines and culture

All procedures using human cells were conducted in accordance with the Human Stem Cell Research Oversight (hSCRO) committee at UCI, and mNSC generation was approved by IACUC at UCI. hNSCs used were UCI161 cells, a cell line isolated from 16-week gestation human fetal brain, and enriched for CD133, a stem cell marker shown to enhance the NSC population that exhibits neurosphere-initiating capacity. [80]. Cells derived in this manner retain the multipotent capacity for over 20 passages in vitro and differentiate in a site-specific manner upon transplantation, generating neurons, oligodendrocytes, and astrocytes[80].

Spinal cord contusion injuries for hNSC chronic transplantations

Immunodeficient mice were anesthetized using 2.5% isofluorane and received a laminectomy at the thoracic vertebrae 9 (T9) using a surgical microscope. All animals received 50 kilodyne (kD; one dyne = 10 μN) contusion injury using the Infinite Horizon Impactor (Precision Systems and Instrumentation), as previously described (Hooshmand et al., 2009). For hNSC transplantation immunodeficient mice were used to enable long-term hNSC engraftment. Rag1 mice exhibit deficits in adaptive immunity, however, they demonstrate innate immune responses and histopathological characteristics comparable to other mouse strains following SCI (Luchetti et al., 2010).. hNSC were dissociated into a single-cell suspension and concentrated to a final density of 75,000 cells/μL in X-vivo

media-(vehicle) on the day of SCI, and transplants were conducted, as described previously (Hooshmand et al., 2009; Hooshmand et al., 2017). Briefly, siliconized beveled glass pipettes (bevel: inner diameter = 70 μm, outer diameter = 100–110 μm; Sutter Instruments) were loaded with freshly triturated hNSC or vehicle, and injections were made into the intact parenchyma (two sites bilaterally above and below the injury epicenter) at T9 using a NanoInjector system and micropositioner (WPI Instruments) immediately after SCI contusion. Each site received 250 nL of cells or vehicle as described (Cummings et al., 2005). Previous studies in our laboratory have demonstrated that this volume does not exacerbate damage to the spinal cord.

Histology and Stereological Quantification

At 16WPT (24WPI) mice were terminally anesthetized, and transcardially perfused with PBS, followed by 4% PFA. Spinal cord regions corresponding to dorsal roots at T2–T6, T6–T12, and T12-L2 were post-fixed and cryoprotected overnight in 4% PFA plus 20% sucrose and, flash frozen at −55°C in isopentane, and stored at −80°C until tissue processing (Hooshmand et al., 2009; Hooshmand et al., 2017). T6–T12 spinal sections were embedded in Neg50 Frozen Section Medium (Thermo) and 30 μm thick transverse-coronal sections were sectioned in a Cryostat with a CryoJane tape transfer system (Leica), as previously described [81]. Frozen sections were collected on slides and stored at −20°C until processed for immunohistochemistry. All sectioning and immunohistochemistry procedures were conducted, as previously described [81], and dilutions used in immunohistochemistry are listed in Table 1.1. Unbiased histological quantification was conducted using stereology by investigators blinded to experimental groups. A random set of animals (n = 6-8/group) were selected for cell counts. The optical fractionator probe was

used for the estimation of the number of hNSC (STEM121+), human astrocytes (STEM123+), human oligodendrocytes (STEM121+ / Olig2+), and human neurons (STEM121+ / DCX+) in StereoInvestigator (Microbrightfield). Grid size measurements were determined based on preliminary experimentation to establish a low coefficient of error (CE ~0.08). Cell migration was reported as the total estimated number of cells per 1 mm section, in a distribution graph where the injury epicenter is defined as the area of the spinal cord with the largest Fibronectin-positive area.

Target	Host	Manufacturer	Catalog #	Lot #	Dilution
DCX	Goat	Santa Cruz	Sc-086	I1615	1:2000
SC121	Mouse	Takara Bio	YK40410	AK80005S	1:500
SC123	Mouse	Takara Bio	YK40420	AJZ0005s	1:3000
Olig2	Rabbit	Abcam	AB9610	3259538	1:500
Mouse IgG	Donkey	Jackson	715066151	138382/124850	1:500
Goat IgG	Donkey	Jackson	705066147	121985	1:500
Rabbit IgG	Donkey	Jackson	711066152	122168	1:500

Table 1.1: List of antibodies and dilutions used.

Behavioral tasks and assessments of locomotor recovery

All behavioral data were collected and analyzed by blinded observers. Gross locomotor recovery in mice (n = 11-12/group) was assessed using the Basso Mouse Scale [82] during the following time points: pre-injury and 2 d/7 d/2 weeks/4 weeks/6 weeks/8 weeks/12 weeks/16 weeks post-injury. Additionally, more sensitive behavioral parameters were utilized, including horizontal and declined ladder beam and CatWalk gait analysis to assess kinematic parameters [73, 75]. Horizontal ladder beam and CatWalk tasks were performed pre-injury and every 4 weeks after injury until animals were sacrificed 24 weeks post-injury.

Results

Neither WT nor CD44 KO hNSCs contribute to locomotor recovery in a chronic transplantation paradigm

Based on the combined data from rats, humans, and mice, I chose a transplantation timepoint of 60 dpi, near the zenith of macrophage presence. Previously, my lab has demonstrated efficacy for hNSC transplantation in treating SCI at sub-acute (9dpi) and early chronic (30dpi), in addition to late chronic (60dpi), albeit with a cervical model of injury that differs from the thoracic one used in this study [39, 41, 79, 83]. We have also have demonstrated that while WT hNSC are not efficacious in an acute transplantation paradigm, CD44 KO hNSC are [refs]. As a result, I hypothesized that at least CD44 KO hNSC, and possibly also CD44 WT hNSC, would be efficacious when transplanted at 60dpi. To the contrary, however, all behavioral data indicated that in fact, neither line was able to improve locomotor recovery beyond that of the vehicle-treated group (Fig 1.1). BMS and a declined ladder beam task failed to show evidence of any between-group effects. Given the

relatively high BMS scores and a low number of errors on the ladder beam task, I hypothesized that a ceiling effect might be obscuring the impact of cell treatment. However, even a more sensitive gait analysis conducted via CatWalk software failed to reveal any meaningful differences between groups.

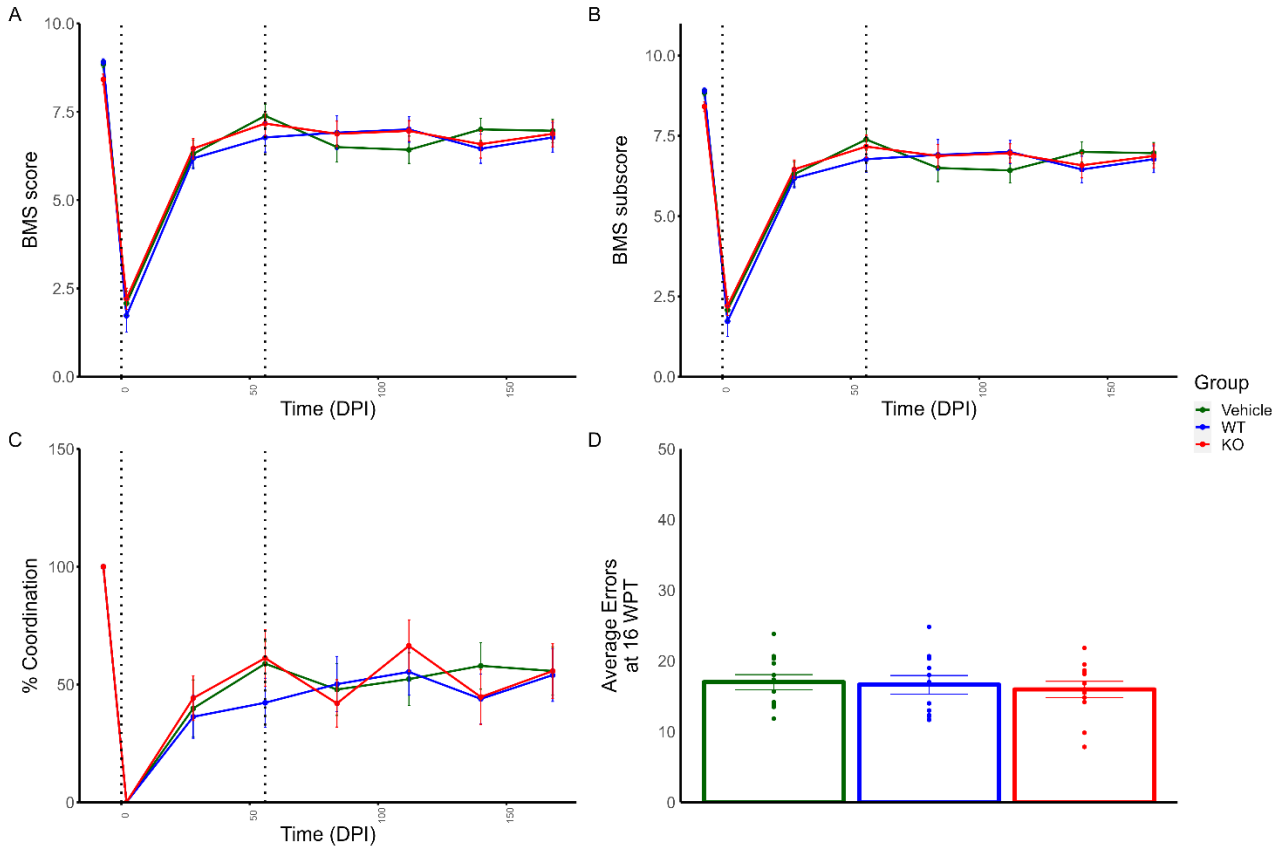

Figure 1.1 Analysis of locomotor behavior reveals no benefit of either WT or CD44 KO hNSC transplantation. A) BMS score. B) BMS subscore. C)BMS percent coordinated passes. D) Average errors per run out of three runs on a declined ladder beam task. All tasks performed at 16 wpt.

CD44 KO hNSC have significantly reduced engraftment vs. WT hNSC

I next examined the histological outcomes for insight into the lack of treatment efficacy. The most immediate and striking concern is the level of long-term (16WPT) hNSC engraftment. Whereas WT hNSC had proliferated well beyond their initial transplantation amount of 75,000, CD44 KO hNSCs decreased in number (Fig 1.2 A). This poor a level of engraftment is exceedingly rare in our experience. Pre-transplantation, both cell lines measured near or above 90% viability via a trypan blue assay, confirming the baseline health of both lines was good at the time of transplantation. In sum, this data suggests that CD44 may be required for long-term engraftment when transplanted 60dpi. Critically, such a requirement was not observed in our previously published acute transplantation study, where both lines exhibited similar and robust levels of engraftment. It is also critical to note that while the WT hNSC engrafted successfully in comparison to KO counterpart cells, cell numbers were still relatively low compared to other studies from our lab. Of particular note is a previous study using a different pair of cell lines and a chronic cervical model of SCI, where one cell line proliferated abundantly and prompted locomotor recovery, and the other showed much lower final cell numbers and did not improve locomotor outcomes [83]. Additionally, no differences in proportional fate were observed between the two lines, indicating that while the absence of CD44 greatly affected the number of engrafting cells, it did not alter the differentiation of cells that did engraft. Consistent with previous studies, transplanted hNSC primarily differentiated into Olig2+ oligodendrocytes, with a smaller number becoming GFAP+ astrocytes or DCX+ neurons (Fig 1.2 C-D).

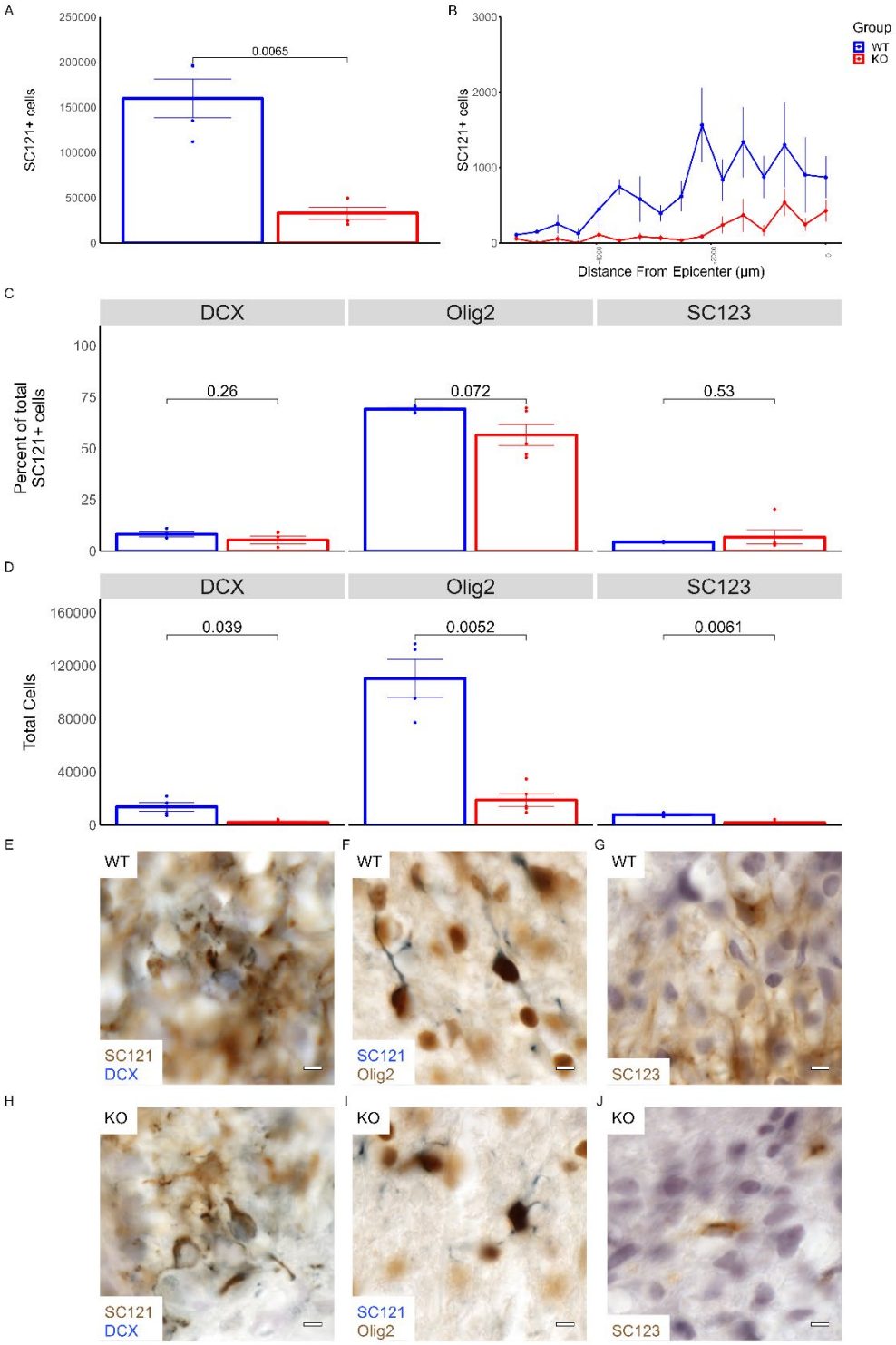

Figure 1.2 Transplanted CD44 KO hNSC engraft at a significantly lower level than WT hNSC. A) Number of SC121+ cells in each transplantation group at 16 wpt. B) Spatial distribution of transplanted hNSC. C) Fate distribution of transplanted NSC, delineated between neurons (DCX+), oligodendrocytes (Olig2+), and astrocytes (SC123+). D) Total numbers of each of these three lineages. E-J) Representative images of the stainings quantified.

Transcriptomic analysis of the post-SCI microenvironment reveals a temporal gradient of expression for several CD44-related proteins

Given the striking differences in both behavioral and histological outcomes in our previous acute transplantation compared to the current chronic one, I probed for differences between the acute and chronic microenvironments using a combination of publicly available datasets, originating from both rats and mice. Interestingly, this analysis revealed that osteopontin, a CD44 ligand known to increase NSC proliferation, is elevated in the chronic post-SCI period. I hypothesize that osteopontin-CD44 signaling aids in the proliferation and ultimately the engraftment of transplanted hNSC, which explains at least in part the struggle of CD44 KO hNSCs to engraft. Critically, osteopontin-CD44 signaling has been shown to promote growth in stem cell populations in previous literature [84], suggesting that osteopontin signaling could sustain WT hNSC transplanted in the chronic post-SCI period, but not CD44 KO hNSC. As to why this difference did not manifest in the acute transplantation paradigm, previous research using northern blotting indicates that osteopontin mRNA levels do not begin increasing until several days post-SCI [85]. This means the acutely transplanted cells will not be exposed to elevated osteopontin during the critical window immediately following transplantation. The fact that both WT and CD44 KO cells robustly engraft in an acute transplantation paradigm suggests the existence of a CD44-independent mechanism of inducing cell proliferation that is present acutely, but not chronically post-SCI. While more *in vitro* and *in vivo* experiments will need to be done to properly test this hypothesis, it does provide a useful framework for interpreting and following up on the results seen in the chronic transplantation paradigm.

Discussion

The post-SCI microenvironment is highly dynamic and has a massive impact on the efficacy of hNSC-based therapies for SCI. The lab has previously demonstrated this through a sequence of studies highlighting the importance of transplantation timing and neuroimmune interactions on both histological and behavioral outcomes [13, 38-42, 57, 78, 79, 81]. Critically, we had previously shown that while WT hNSC are unable to improve recovery post-SCI when transplanted acutely after injury, CD44 KO hNSC were [57]. In the same paper, we established a role for CD44 in mediating C1q-induced chemotaxis in hNSC, which we hypothesize is one of the key mechanisms causing acutely transplanted hNSC to clump up at the injury epicenter, where C1q is abundant. Given that macrophages, which secrete C1q at high levels, are present in abundance in the chronic phase post-SCI, I hypothesized that CD44 would again play a critical role in mediating the behavior of transplanted hNSC. While this hypothesis was broadly true, it was not primarily migration that was affected in CD44 KO hNSC, but engraftment, with CD44 KO cell engraftment far less than WT counterpart cells.

More surprising was that neither cell line improved locomotor recovery beyond that of vehicle treatment. This outcome could be the result of several factors. 60 dpi may be simply too late a treatment to see a meaningful recovery in this specific combination of thoracic SCI model, mouse strain, and cell line. Similarly, a potential treatment benefit might be obscured by a ceiling effect in our injury model. I saw relatively low locomotor deficits as measured via BMS and Ladderbeam, indicating animals were functioning fairly normally. I chose to match our injury model from the acute transplantation study for the sake of consistency, but future studies may benefit from switching to a more severe injury model. It is also quite likely that neither cell line is engrafted sufficiently to promote

recovery. This fact makes any interpretation of the behavioral data difficult, as it cannot be known if either cell type is fundamentally unable to enable recovery in this model, or if it is simply an issue of the number of engrafted cells not clearing a threshold. However, histological data such as the difference between the two genotypes in terms of engraftment or the proportional fate data should still be considered valid, and others useful insights into the role CD44 plays in regulating NSC behavior.

The clear next steps for this project involve further defining the precise mechanisms and microenvironmental components responsible for the unexpectedly low engraftment of the CD44 KO hNSC. A good preliminary experiment, given the prominent chronic presence of macrophages post-SCI, will be to expose CD44 WT and KO to macrophage-conditioned media and determining if survival/proliferation is impacted selectively across the two genotypes. I hypothesize that CD44 KO hNSC will show a greater decrease in proliferation when exposed to macrophage-conditioned media compared to WT hNSC, and from there we can begin determining exactly what factor/combination of factors is causing this effect. In parallel, similar experiments can be run using known CD44 ligands such as osteopontin and HA. If time and resources allow, another experiment that would provide critical insight would be to repeat the above study with a 30 dpi and/or 9 dpi injection, where we know the macrophage response is more mild and we have seen a more robust recovery in past studies. This will help elucidate how the CD44 KO hNSC function under conditions where the WT hNSC have been shown to provide therapeutic efficacy, as both the acute and late chronic time points present unique challenges to hNSC-induced recovery.

In summary, while these results ran strongly counter to my initial hypotheses, they did provide insights into both the basic biology of how CD44 interactions with the post-SCI

microenvironment, and shone a light on important obstacles to navigate to make hNSC transplantation a viable therapeutic for chronic SCI patients.

Chapter 2: The role of endogenous CD44 in regulating the cellular response to SCI

While the previous chapter focused primarily on the role of CD44 expressed on the surface of transplanted hNSC, CD44 is also highly involved in several cell types critical to the endogenous response to SCI. This includes various immune cells, and endogenous neural stem cells [60, 62, 64, 69, 86, 87]. This data, combined with how robust an effect CD44 had on transplanted hNSC behavior, prompted us to consider what role CD44 might play in the endogenous response to SCI. We performed cervical contusion injuries on CD44 KO mice and examined both their behavioral recovery post-SCI along with histological factors such as immune cell response, cell proliferation in response to SCI, and scar/lesion formation. The results indicate a wide-reaching role for CD44 in regulating the post-SCI response, and highlight CD44 not only as a target for further study in elucidating the basic biology of SCI but also as a potential therapeutic target for ensuring better outcomes in SCI patients.

Methods

Animal Models

All experiments were carried out in accordance with the Institutional Animal Care and Use Committee at the University of California, Irvine, and were consistent with Federal guidelines. All animals utilized are B6.129(Cg)-Cd44tm1Hbg/J) mice (Jackson Laboratories #005085) between 6-8 weeks of age at the time of injury [88]. A total of 46 animals were used between the two genotypes and three sacrifice time points.

Surgery

We used 2.5% isoflurane to anesthetize mice and gave them 30 kilodyne (KD) cervical vertebrae 5 (C5) contusions using the Infinite Horizon Impactor (Precision Systems and Instrumentation)

BrdU Injections

BrdU was injected into animals intraperitoneally at 12,24, and 36 hours post-injury, at a concentration of *(check for exact concentration in Francisca's notes).*

Histology and Stereological Analysis

At 2DPI, 7DPI or 28DPI mice were terminally anesthetized and transcardially perfused with PBS, followed by 4% PFA. Spinal cord regions corresponding to dorsal roots at C2-T1 were post-fixed and cryoprotected overnight in 4% PFA plus 20% sucrose and, flash frozen at −55°C in isopentane, and stored at −80°C until tissue processing spinal sections were embedded in Neg50 Frozen Section Medium (Thermo) and 30 µm thick transverse-coronal sections where sectioned in a Cryostat with a CryoJane tape transfer system (Leica), as previously described [81]. Frozen sections were collected on slides and stored at −20°C until processed for immunohistochemistry. All sectioning and immunohistochemistry procedures were conducted, as previously described [81]. Dilutions used in immunohistochemistry are listed in Table 1. Unbiased histological quantification was conducted using stereology by investigators blinded to experimental groups. A random set of animals (n = 5-6/group) were selected for cell counts. The optical fractionator probe was used for the estimation of the number of BrdU+, GFAP+, PU.1+, and Sox9 positive cells. in StereoInvestigator (Microbrightfield). Grid size measurements were determined based on preliminary experimentation to establish a low coefficient of error (CE ≤0.08). Cell migration was reported as the total estimated number of cells per section, in a distribution

graph where the injury epicenter is defined as the area of the spinal cord with the largest Fibronectin-positive area. A Cavalieri probe was used to estimate total volumes of fibronectin+ lesion volume, in 1 of 6 intervals from coronal spinal cord sections 360 µm apart, at 10× magnification.

Microscopy and Imaging

Stereology was performed on Zeiss Imager M.2 scopes using Axio MRc or MRm cameras for brightfield or fluorescent imaging, respectively. Information on how each image was acquired can be found in table 2. Image adjustments (color balance, brightness, contrast) and annotations were performed in Adobe Photoshop (V23).

Isolation, culture, and staining of spinal cord NSC

Multipotent mouse spinal cord neural stem cell line (mNSC) was isolated from 16-day-old C57BL/6 spinal cord tissue. Briefly, a 3-5 mm piece of the thoracic spinal cord was isolated and dissociated manually into single cells for a free-floating neurosphere culture in Temple DMEM-based growth medium supplemented with 10mg/ mL ciprofloxacin. After the first passage, mNSC were cultured as monolayers on poly-L-ornithine (PLO)/laminin (LAM) coated vessels in X-vivo 15 (Lonza) based growth medium (GM) supplemented with 20 ng/mL bFGF (Invitrogen PHG0026), 2 ng/mL EGF (Invitrogen PHG0311), 2 µg/mL Heparin (Sigma H-3149), 63 µg/mL write open <= (NAC) (Sigma A9165), 10 µg/mL N2 (Invitrogen A13707-01), and 10 ng/mL LIF (Invitrogen PHG0026) as previously described (PMID: 29050826, PMID: 32894219). mNSC exhibit a stable growth rate, sustained neurosphere-initiating capacity, multipotency, and responsiveness to migration cues in vitro similarly to human neural stem cell lines [80]. mNSC were fed twice a week. Once at ~80% confluency, mNSC were passaged to PLO and Laminin coated chamber well slides.

Once cells were grown to ~80% confluency, media was aspirated from slides, and cells were fixed in freshly thawed 4% PFA for 10 minutes. Cells were washed once with PBS, then blocked in PBS + 5% donkey serum (017-000-121, Jackson Labs), then stained for CD44 (ab189524, abcam) overnight at 4^{o} C. The next day, the primary antibody was removed, cells were washed 3 times with PBS for 5 minutes, and a secondary antibody was added for 1 hour (slides were subsequently kept in the dark). Cells were again washed 3 times with PBS for 5 minutes, then block in PBS + 5% donkey serum + 0.3% Triton X to permeabilize cell membranes. Cells were then stained for Nestin (NB100-1604, Novus) overnight at 4^{o} C. The next day, the primary antibody was removed, cells were washed 3 times with PBS for 5 minutes, and the secondary antibody was added for 1 hour. Finally, cells were washed 3 times with PBS for 5 minutes then slides were mounted with Flouromount-G (Southern Biotech).

Locomotor recovery

All behavioral analysis was performed by researchers blinded to experimental group designation. Ladderbeam performance tests were conducted by recording an animal's attempts to cross a 50-rung ladder at either a horizontal or 50-degree decline. Recordings were reviewed and the average number of errors over 3 runs per animal was quantified. Additional gait analysis was performed using the CatWalk gait analysis task/software. Both CatWalk and ladderbeam recordings were collected before the injury, and at 2 and 4 weeks post-injury.

Results

CD44 expression

I first performed staining for CD44 to both confirm the legitimacy of the CD44 KO mouse model, and to examine the pattern of CD44 staining in the spinal cord. As previous literature has shown, the pattern of CD44 expression in the spinal cord closely resembles that of GFAP, as astrocytes prominently express CD44 in the spinal cord (Fig 2.1A-B). Of note, I also show that CD44 is expressed by the ependymal cells of the central canal, which are the endogenous population of NSC in the spinal cord (Fig 2.1C). To further validate CD44 expression in spinal cord NSC, we isolated NSC from mouse spinal cord tissue, and stained the cultured cells for NSC marker nestin and CD44, and found robust colocalization (Fig 2.1D).

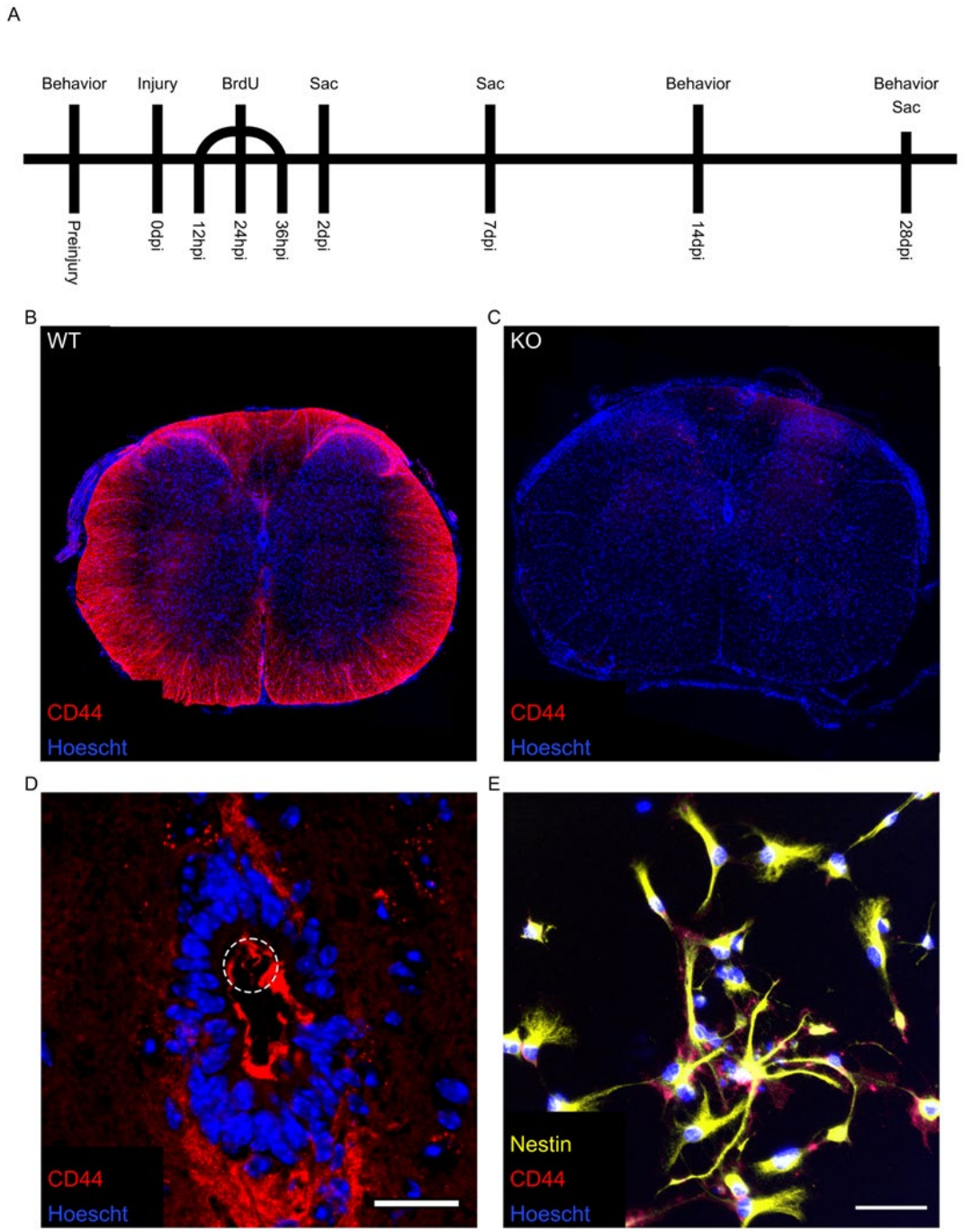

Fig 2.1: Experimental design and expression of CD44 in the spinal cord. A) Experimental timeline showing time points at which locomotor behavior was collected, BrdU was injected, and animals were sacrificed, perfused, and dissected (Sac). HPI = hours post-injury, DPI = days post-injury. B) CD44 expression in the spinal cord. C) CD44 staining in the spinal cord of CD44 KO mice, showing complete ablation of CD44 expression. D) High power (60x) image of CD44 expression in the central canal. The white circle indicates a CD44-positive process extended from the apical end of a canal-resident NSC. Scale bar = 10μm. E) CD44 and Nestin co-labeling in mouse spinal-cord derived NSC. Scale bar = 50μm.

Change in Brdu cell distribution

Given the rapid nature of the response to SCI, I wanted to determine how CD44 altered the response of acutely-dividing cells post-SCI. To examine this, we gave unilateral contusion injuries to CD44 WT and KO mice, followed by BrdU injections at 12,24, and 36 hours post-injury (Fig 2.2A). First, we examined the BrdU signal in the central canal ependymal cells. In this region, there was a rapidly arising and persistent level of BrdU labeling present in both CD44 WT and KO mice, detectable at 2, 7, and 28 dpi (Fig 2.2B). Likewise, there was also a high amount of BrdU-labeled cells in the spinal cord parenchyma, peaking at 7 dpi (Fig 2.2C).

Critically, while there was no difference between genotypes in terms of total BrdU labeling in either anatomical region, the spatial distribution of BrdU labeling was markedly different in the spinal cord parenchyma, specifically at 7 dpi (Fig 2.2C). While WT mice exhibited a sharp peak of BrdU labeling at the injury epicenter, labeling in KO mice was much more spread out rostral and caudal of the injury (Fig 2.2C-D). This result was in line with the established function of CD44 as a regulator of cellular migration and with our previously published data demonstrating the role of CD44 in C1q-induced hNSC migration, a molecule that is abundant at the injury epicenter [58, 89].

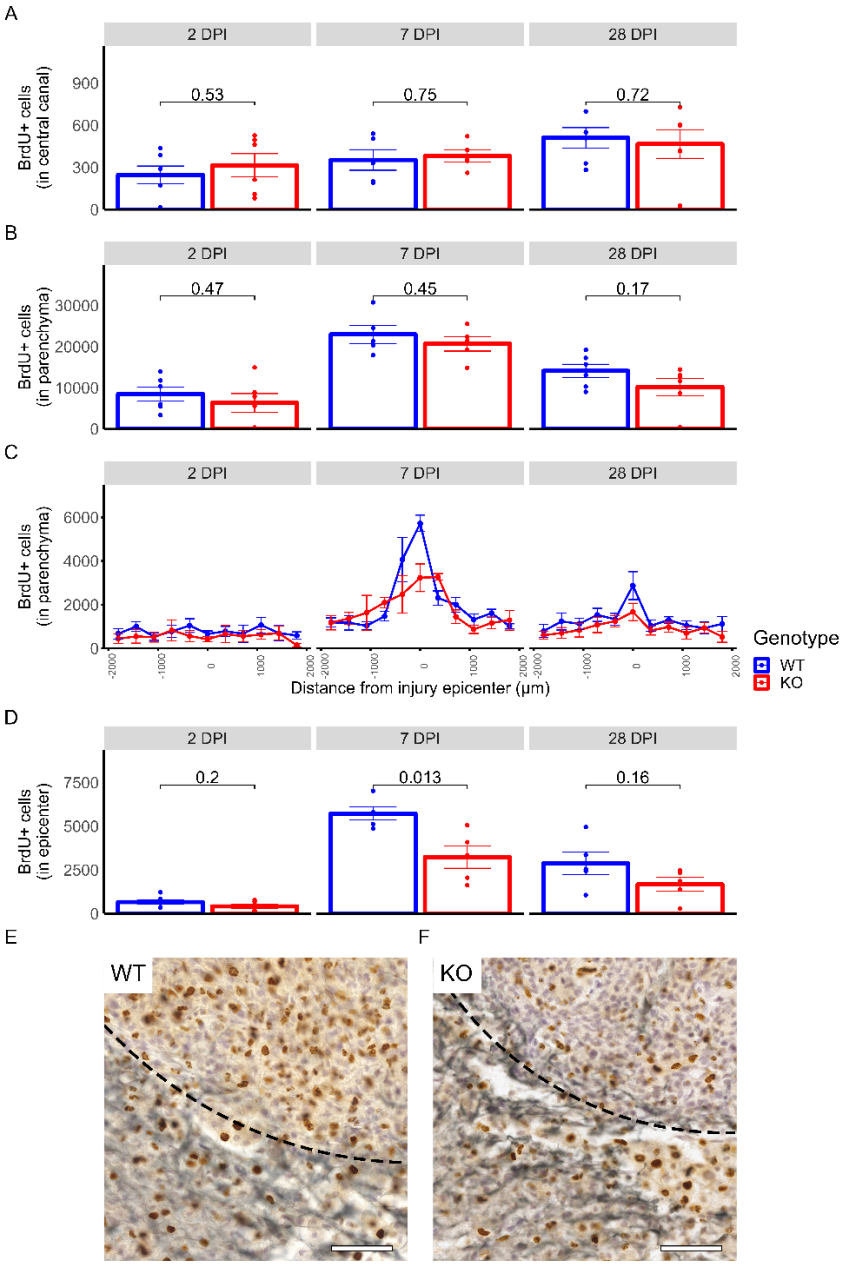

Fig 2.2: Temporal and spatial distribution of acutely activated, BrdU-labeled cells post-SCI shows distributional differences in WT vs CD44 KO mice at 7 DPI, as determined by unbiased stereology. Time points shown a re at 2, 7, and 28 DPI in both WT and CD44 KO mice. A) Total BrdU+ cells in the central canal. B) Total BrdU+ cells in the spinal cord parenchyma. C) Section-by-section analysis demonstrating the spatial distribution of BrdU+ cells in the spinal cord parenchyma. D) Total BrdU+ cells in epicenter sections. E) BrdU labeling in Two-tailed student's t-test as indicated by P values listed. F-G) Images at the injury epicenter of WT and CD44 KO mice, showing BrdU labeling in brown and GFAP labeling in blue. Scale bar = 50µm. Graphs show mean +/- SEM; N=5-6 for each genotype and time point.

Change in astrocyte recruitment, scarring

Given the evidence that post-SCI cellular recruitment had been altered in CD44 KO mice, I next examined how this affected scar formation. Scar formation is a critical part of the post-SCI response, requiring proper recruitment and alignment of astrocytes and progenitor/stem cells, with failures in this process resulting in significantly worse post-injury outcomes [18, 24, 25, 27, 32]. To determine if the recruitment of acutely-dividing astrocytes had been altered in CD44 KO mice, I performed GFAP/BrdU co-labeling followed by stereology. Counter to my expectations, however, there was a negligible amount of GFAP/BrdU++ cells in either genotype, suggesting that whatever astrocyte proliferation occurred happened outside the 12-36 hours post-injury time window. However, these findings are in line with literature that suggests that acutely dividing cells post-SCI consist primarily of NG2+ glial progenitors, rather than astrocytes [90].

I decided to instead examine the number and distribution of all astrocytes, with or without BrdU labeling and found that the spatial distribution was again significantly different between genotypes at 7DPI. In a reverse of our BrdU data, however, CD44 KO mice had a sharp peak of astrocytes at the injury epicenter, whereas this peak was broadened in WT mice (Fig 2.3A-B). I was curious about what effect this would have on scar morphology and lesion size, so I used unbiased stereological analysis via the Cavalieri probe to determine the volume of GFAP+ (scar) and fibronectin+ (lesion) areas. Interestingly, while the volume of the lesion remained unchanged between genotypes, the scar volume was actually less in CD44 KO mice, despite the increased number of astrocytes at the lesion epicenter (Fig 2.3C-D). However, this is actually in line with the idea that an increased number of astrocytes will enable improved compaction of the scar [27]. It is also

important to note that, given that the genetic model is a constitutive KO, these effects on astrocyte behavior and scar formation are likely a combination of astrocyte-intrinsic factors and interactions between astrocytes and other cell types regulated by CD44.

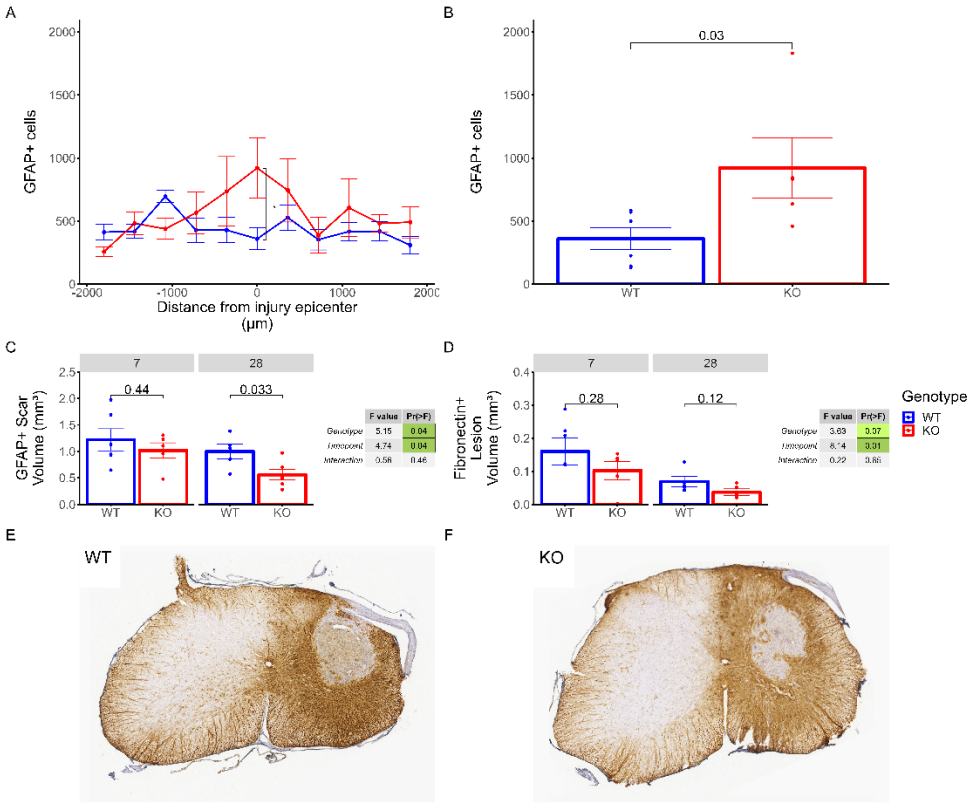

Fig 2.3: Scar formation post-SCI is altered in WT vs. CD44 KO mice, as determined by unbiased stereology. A) Section-by-section analysis demonstrating the spatial distribution of GFAP+ cells at 7DPI. B) Total GFAP+ cells in epicenter sections at 7 DPI. C) GFAP+ volume at 7 and 28 DPI. D) Fibronectin+ volume at 7 and 28 DPI. Two-way ANOVA tables for time and genotype. E-F) Images at the injury epicenter of WT and CD44 KO mice, showing GFAP labeling in brown. Two-tailed student's t-test as indicated by P values listed. Graphs show mean +/- SEM. N=5-6 for each genotype and time point.

Fate of BrdU labeled cells

Since I had observed a few GFAP+/BrdU+ cells, I next sought to determine the identity of the BrdU-labeled cells at the 7dpi injury epicenter. Two likely sources were local/infiltrating macrophages/microglia and endogenous neural stem/progenitor cells. I used Pu.1 as a marker for myeloid cells and SOX9 as a marker for progenitors. I found that these two markers accounted for most BrdU-labeled cells in the injury epicenter of both genotypes, with approximately 20% of BrdU-labeled cells in the injury epicenter section being SOX9 positive and 80% being Pu.1 positive (data not shown). In KO animals, there was a non-significant reduction in total BrdU+/SOX9+ cells vs. WT counterparts ($p = .21$) and a significant reduction in BrdU+/Pu.1+ Cells ($p = 0.012$). In sum, these data indicate that myeloid cells were both the primary population labeled by BrdU under this labeling/injury paradigm, and the primary cell group affected by CD44 ablation with respect to acute recruitment to the epicenter site. This aligns with our previous data on the time course of myeloid cell (specifically microglial/macrophage) infiltration post-SCI, and with the known role, CD44 has in immune cell migration/infiltration [13, 60, 91].

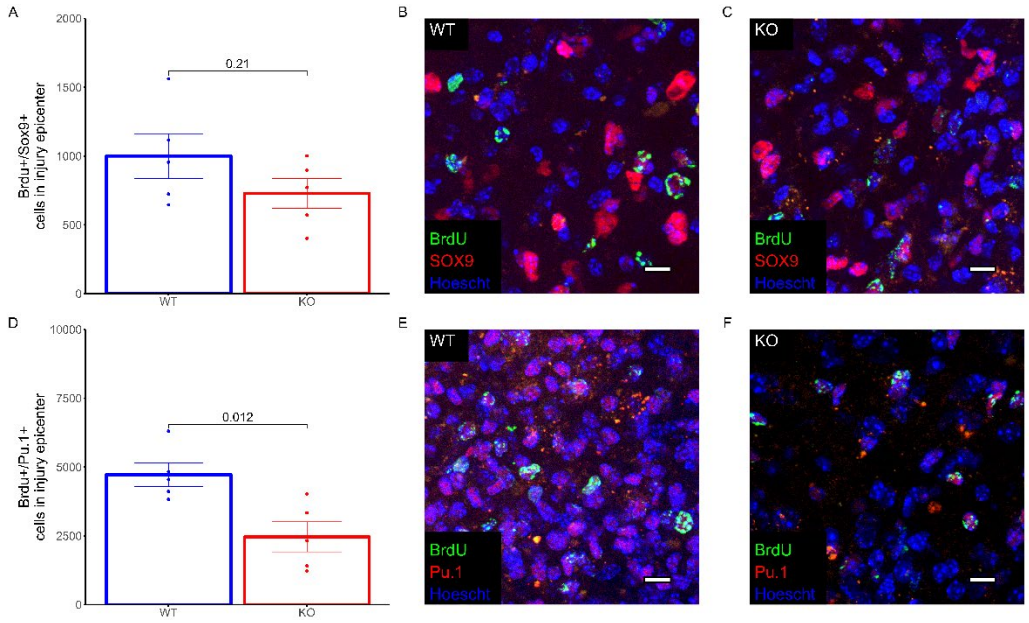

Fig 2.4: BrdU-labeled cells at the SCI epicenter at 7 DPI are primarily Pu.1+ and these Pu.1+/BrdU+ cells are reduced by CD44 KO. A) Total SOX9+/BrdU+ cells in the injury epicenter. B-C) Images at the injury epicenter of WT and CD44 KO mice, showing BrdU and SOX9 colabeling. D) Total Pu.1+/BrdU+ cells in the injury epicenter. E-F) Images at the injury epicenter of WT and CD44 KO mice, showing BrdU and Pu.1 colabeling. Two-tailed student's t-test as indicated by P values listed. Graphs show mean +/- SEM. N=5-6 for each genotype. All scale bars = 10μm.

Locomotor recovery

To determine the effect that this myriad of cellular-level changes had on locomotor outcomes in animals, I performed horizontal ladderbeam analysis at 14 and 28 dpi. There was an overall decrease in the number of errors in CD44 KO vs. WT mice, suggesting a net locomotor benefit of CD44 KO (Fig 2.5A). CatWalk gait analysis further revealed that CD44 KO mice had better locomotor recovery post-SCI. CD44 KO mice spent more of their time walking in a "diagonal" arrangement of their paws vs. having three paws on the ground at once, a behavioral phenotype typically seen pre-injury (Fig 2.5B-C). This behavioral data will be explored in even greater detail in Chapter 3, where I use this study as a case study for my more advanced catwalk analysis techniques.

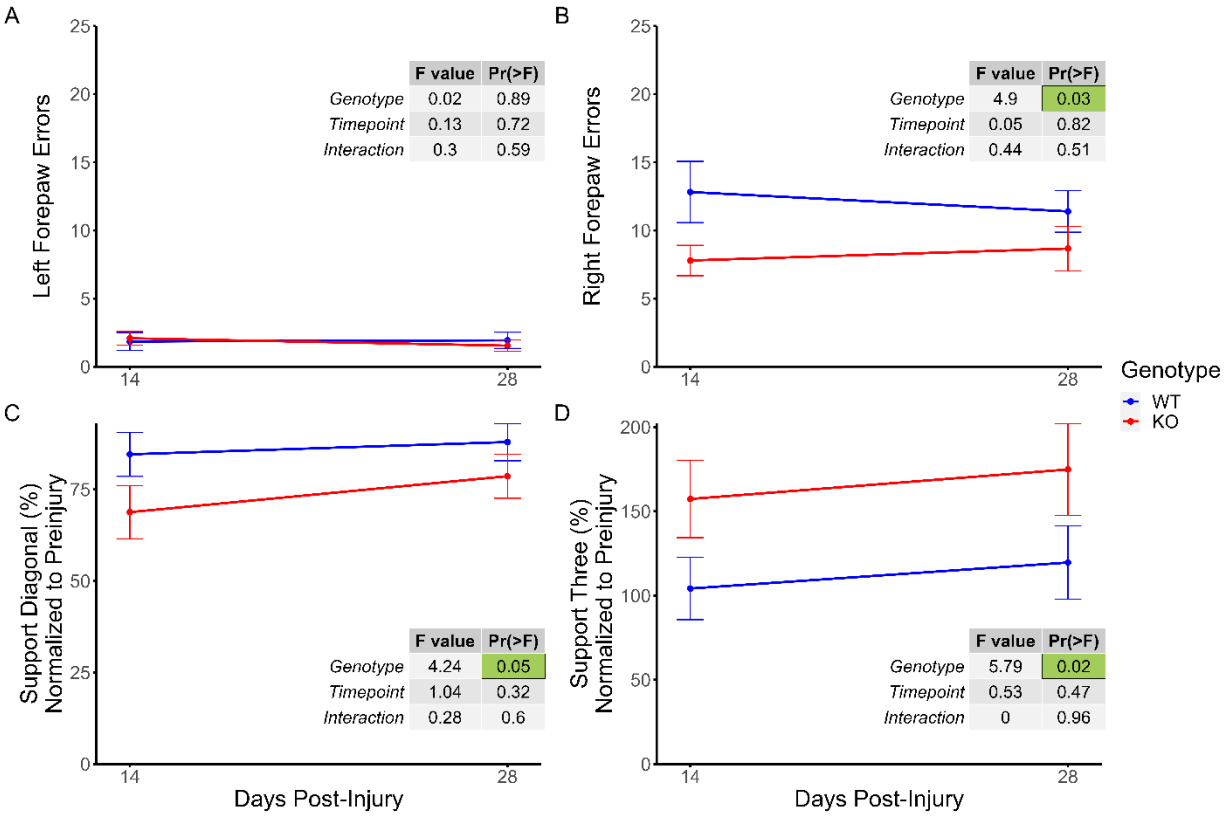

Fig 2.5: Functional locomotor recovery post-SCI demonstrates improvement in CD44 KO vs. WT mice. Horizontal ladder beam errors for the Left (A) and right (B) forepaw. CatWalk kinematic analysis of percent of time spent on diagonally positioned paws (C) or three paws (D). Two-way ANOVA tables for time (repeated measures at 14 and 28 DPI) and genotype are shown for each statistic (n = 8-10 per genotype).

Discussion

SCI is a debilitating injury associated with massive healthcare costs. A better understanding of the fundamental cellular mechanisms in play during the post-SCI response is critical to developing viable therapeutic candidates. CD44 is a critical regulator of cellular behavior and a potential therapeutic target for a post-SCI intervention. Here, I provide evidence that ablation of CD44 results in marked changes to the post-SCI response, altering the behavior of astrocytes, immune cells, and endogenous stem/progenitor cells, and that the net result of these changes is protective in terms of animal locomotor ability.

CD44 has long been established as a regulator of cellular migration and cell-cell matrix interactions in a variety of cell types and tissues (including in the CNS) and our results align with that established literature[62, 64, 68, 91-93]. Our lab has additionally shown that CD44 exerts a myriad of novel effects on NSC both *in vitro* and *in vivo* in a post-SCI transplantation paradigm, further validating its importance in a neurotrauma context [57]. Critically, here I investigate how endogenously expressed CD44 guides and shapes the response to neurotrauma, specifically SCI. The microenvironment and extracellular matrix are drastically different post-SCI vs in a homeostatic spinal cord, posing numerous questions about how CD44 will respond to these changes and how this will effect CD44-expressing cells [6, 14, 57, 64, 94-96]

Given that CD44 is both expressed in a high number of cell types, and regulates multiple aspects of cell behavior, it makes sense that the results obtained from this study are so multi-faceted. I observed a clear difference in the recruitment of acutely-activated cells to the injury epicenter. I determined that while ~20% of acutely-activated cells were Sox-9 positive stem/progenitor cells, typically restricted to the astrocyte lineage, these

cells were not the ones whose recruitment to the epicenter was being affected. Instead, it was Pu.1+ immune cells that were the much more significantly affected population, with significantly fewer reaching the epicenter in CD44 KO mice vs. WT. While both cell types have to migrate after injury, many immune cells present post-SCI are not native to the spinal cord, and thus have to infiltrate into the spinal cord via the periphery. This longer journey to the spinal cord could make these cells more reliant on CD44 to reach their target, and thus more likely to be affected by its absence [13, 86, 97].

Adding to this complexity is the fact that at none of the time points surveyed did I see significant numbers of GFAP+/BrdU+ cells, meaning that not only were astrocytes not proliferating at the 12-36 hour window in which BrdU was injected, but that the Sox9+/BrdU+ cells that were labeled did not differentiate into mature astrocytes. This occurred despite robust BrdU labeling of central canal cells, which are believed to contribute to the glial scar post-SCI [22-25, 98]. Given the distribution of Sox9+/BrdU+ cells around the perimeter of the lesion, these cells may contribute to scar formation in a way that did not involve differentiation into mature astrocytes. It is also critically important to acknowledge the role that the injury model and labeling paradigm play in studies like ours, as studies that variate these parameters have obtained deeply contrasting results regarding what cells contribute to glial scarring and what extent [21-24, 28, 29]. Prior studies have primarily utilized either a crush or hemisection injury, whereas here we performed a contusion. It has been argued that activation of endogenous NSC post-SCI relies on the occurrence of direct damage to the central canal itself [28]. However, within that study, the contrast was, again, between a crush injury and various types of hemisection injuries. A controlled, side-by-side comparison of those models to the contusion model

used in this study would be necessary to fully disentangle the effect of the injury model on endogenous NSC activation and recruitment, and even still such a study would also need to account for other variables such as animal strain.

While I did not observe BrdU labeling of mature astrocytes, I did still detect significant changes in the astrocytic response to SCI. This included a significant increase in astrocyte numbers at the injury epicenter at 7DPI in CD44 KO vs. WT mice and a significant decrease in the long-term GFAP+ volume in CD44 KO mice. These results might initially seem contradictory, as one would expect an increase in astrocyte number to correspond to an increase in GFAP+ volume. However, GFAP+ volume is highly sensitive to factors like astrocyte hypertrophy and other morphological changes astrocytes undergo post-SCI. Scar formation is a multi-cellular, multi-phase process [20, 27, 99]. Additionally, an increase in astrocyte recruitment could actually enable greater scar compaction [27].It is also critical to remember that not only were multiple aspects of astrocytes affected but also the cells they interact with, like stem/progenitor cells and immune cells. To better understand the role of CD44 in altering specifically astrocyte behavior post-SCI, we would need to utilize an astrocyte-restricted CD44 KO, along with more robust techniques for tracking scar formation [27].

Finally, the behavioral results indicate that CD44 KO improved functional locomotor recovery. Given the complexity of our histological results, this benefit is likely the accumulation of multiple changes to the SCI response, some of which are beneficial and perhaps some which are detrimental to recovery, with the net result being a positive one. One critical change to investigate more is the effect CD44 had on immune cell behavior. I observed a robust change in the recruitment of Pu.1+/BrdU+ cells at 7DPI, at a time point

that marks a critical influx/increase of macrophages and microglial in the injured spinal cord [13]. Given both CD44's established role in altering immune cell behavior and the critical importance of the immune response in altering SCI outcomes, I hypothesize that immunomodulatory changes due to CD44 KO are driving the protective effects observed. Like our questions about CD44's role in astrocytes, addressing this question requires utilizing a more cellular and temporally specific CD44 KO model.

The complex and broad nature of the impacts seen in this study is not surprising given the wide expression of CD44 across multiple and varied cell types in the body, and its extensive list of functions and interactions with other proteins and signaling pathways [60, 62, 64, 68, 91, 100]. The fact that CD44 plays such a pivotal role in cellular function makes it an ideal therapeutic target. Indeed, our parallel research to this study has highlighted an exciting immediate impact of CD44 manipulation on the efficacy of hNSC transplantation as a treatment for SCI [57]. This study highlights the potential for manipulation of endogenous CD44 as another SCI therapy, perhaps in the form of a CD44 blocking agent administered post-SCI to minimize damage to the patient's spinal cord, or a virally targeted approach to ablate CD44 expression in a specific cell population. While highly promising, these approaches will require further study.

Chapter 3: A novel approach for improving the standard of animal gait analysis

Here, I describe a novel approach for analyzing CatWalk data, utilizing an unbiased "forward screen" method to identify variables affected by the treatment/intervention in question. Next, I analyze the isolated variables through a variety of methods to further hone in on precisely how animal gait was affected. To demonstrate the flexibility of this approach, I illustrate two unique use cases from two distinct studies. The first uses data from an SCI study examining the efficacy of stem cells as a treatment for SCI. There are 3 groups (a vehicle group and two stem cell groups), and data were obtained pre-injury, pre-transplantation, and at 4, 8,12, and 16 weeks post-transplantation. The second uses a single-timepoint SCI dataset with 2 binary variables (hNSC transplantation vs. vehicle and bridge implantation vs. gel foam), and 4 unique groups. To make this analysis easier for both myself and others, I have created a soon to be publicly available R package called runway that allows for streamlined importing, processing, analysis, and visualization of CatWalk data.

Methods

Data processing and statistics

Analysis was performed using R version 4.0.3 within R Studio. Data processing was performed using the *dplyr* package. ANOVA modeling was performed using the *aov* function in base R. Complete list of packages used can be found in the "Acknowledgements" section.

Data compilation, cleaning, and processing

The first step in the analysis is the compilation of all relevant CatWalk data. Ideally, data will have been obtained at multiple time points, at least before and after injury. For

ease of data import and proper blinding, the best approach is to run each animal only entering its numerical identifier, without entering any group information into CatWalk. In parallel, a master lookup table is kept matching each ID to the animal group along with any other important metadata. Then, each exported set of CatWalk data is annotated with group info by aligning it to the lookup table. Finally, the individual time points are combined into one continuous data set.

The next step is the elimination of any unusable variables, such as variables with all missing or all 0 values. After this, data can be normalized. The standard way to do this is to normalize each subject to a baseline time point. Typically, this will be the pre-injury timepoint, but in some cases, it be will more appropriate to choose a timepoint such as post-injury but pre-intervention instead. In the case that an individual subject does not have data at the normalization time point, there are two options. If the study power is sufficiently high, the subject can be completely excluded from the analysis. Alternatively, the group means for each variable can be used in place of the individual value. Finally, it typically makes sense to exclude the normalization timepoint from future analysis, to prevent it from distorting results.

Isolation of significant variables using analysis of variance models

Once data is imported and pre-processed, the first step of the analysis is determining what gait variables are being affected by the independent variables in the study. In a typical study, this will mean looking at the interaction of a grouping variable (treatment, genotype, etc) and timepoint post-injury. I then apply this model to each normalized CatWalk variable in our complete dataset. I can use the p-values obtained from

this analysis to extract gait variables influenced by single independent variables, or the interaction between independent variables.

It is worth discussing how best to determine the correct alpha value to as a cutoff for measuring significance. In analyses such as these involving evaluating several variables, it is typically best practice to adjust the alpha value accordingly to compensate for the multiple comparisons and prevent false positives. However, in this case, there is frequently an issue of low power vs. high dimensionality. Indeed, the dimensionality is often inflated by the fact that CatWalk provides many variables which are highly related to one another or otherwise superfluous. For this reason, adjusting the cutoff for multiple comparisons is likely to lead to an excess of false negatives. The most straightforward way to avoid this is to not adjust for multiple comparisons, and simply use an alpha value appropriate for single variable comparisons. This is essentially the current state of practice in analyzing CatWalk in most published studies, where single variables are pulled out of a data set and analyzed with no adjustment for how many total variables were analyzed/available for analysis. Given the exploratory and descriptive goals of this analysis method, I consider this an acceptable concession. If desired, dimensionality can be reduced a priori via removing highly correlated variables/redundant variables, and/or performing a variable reduction method such as principal component analysis (PCA).

Interpretation of results

At this point, a set of affected gait variables will have been obtained for each independent variable (and variable interaction) in the study. There are several methods for interpretation of these results that can be used in parallel with each other.

One method is to simply look at the number of significant variables extracted for each independent variable. In the case of a combinatorial treatment study (such as the one covered in Use Case 2) if one treatment variable affects multiple gait variables and another does not, this could be considered as a proxy measure for the efficacy of one treatment method vs another. Similarly, if each treatment variable extracts multiple gaits variables, but the interaction of the two variables do not, this implies that the mechanisms alter locomotion via parallel, non-synergistic pathways. One issue to be careful of when using this approach is that, given that some variables are so intrinsically tightly correlated (i.e. print width, print length, and print area), the number of gait variables extracted by an independent variable can be artificially inflated.

For both this reason, and to gain a more thorough understanding of the effects of each independent variable, it is necessary to examine the nature of the singular gait variables extracted, beginning by graphing each variable to see how they are affected by time, treatment, genotype, etc. This will reveal how exactly animal gait is being affected. Perhaps animals in a given group are disproportionately shifting their weight onto their fore or hind paws, exhibiting a particular stepping pattern, or moving more steadily. If a given treatment affects an intuitively related set of variables, such as contralateral paw variables or variables relating to limb speed, this can help elucidate the mechanism via which an intervention is altering animal gait, informing what molecular targets or neurological pathways to target in subsequent studies. One way to test this statistically is to fragment each variable into “tags”, such as “right forepaw” or “couplings”, and then perform a hypergeometric test to determine if certain tags are being affected in a statistically significant manner.

While this type of descriptive, annotative analysis is arguably what CatWalk is best suited for, it is nonetheless usually desirable to try and obtain a measure of what group of animals is walking "best", especially in cases where more black and white locomotor tasks such as BMS or ladder beam fail to discriminate between groups. The main issue with using CatWalk data in this way is that it requires us to define what "good" walking looks like, which can be a deceptively difficult task because animals progress through compensatory locomotion adaptations, and cannot be assumed a priori to recover pre-injury (intact) locomotor gait patterns.

One method is to look at a restricted set of relatively well-understood, easy-to-interpret variables. For example, healthy animals tend to spend a lot of their gait time with their paws in a "diagonal" orientation, with 2 paws off the ground at any given time. In contrast, injured animals often struggle with balance and thus spend more time on 3 or 4 paws. This restricted approach can be used with the more open and exploratory one described above to obtain both a coarse and fine picture of animal recovery. However, one should not assume that a given variable is inherently indicative of recovery without first validating this fact for the animal/strain/injury being used, ideally by correlating it with one of the more straightforward locomotor tasks.

Another approach is to examine which group closest resembles their pre-injury baseline, as intuition suggests this should correlate with better outcomes. However, this is a dangerous assumption, as post-injury recovery involves a high degree of compensation. Thus, a treatment that helps animals to properly compensate may in fact cause them to look less and less like their pre-injury baseline.

A more nuanced version of comparing animals to baseline is to compare them against historic data from prior studies where recovery was observed. This is especially pertinent if a similar mechanism of recovery is expected from one intervention to the next, for example trying an identical treatment at different doses/time points. A powerful tool for this comparison, and indeed for any study where one wants to see how multiple groups compare to one another, is a dimensionality reduction tool such as UMAP. In this way, one can quickly see how different groups cluster relative to one another. A graphical generalization of this entire approach can be found in Fig 3.1

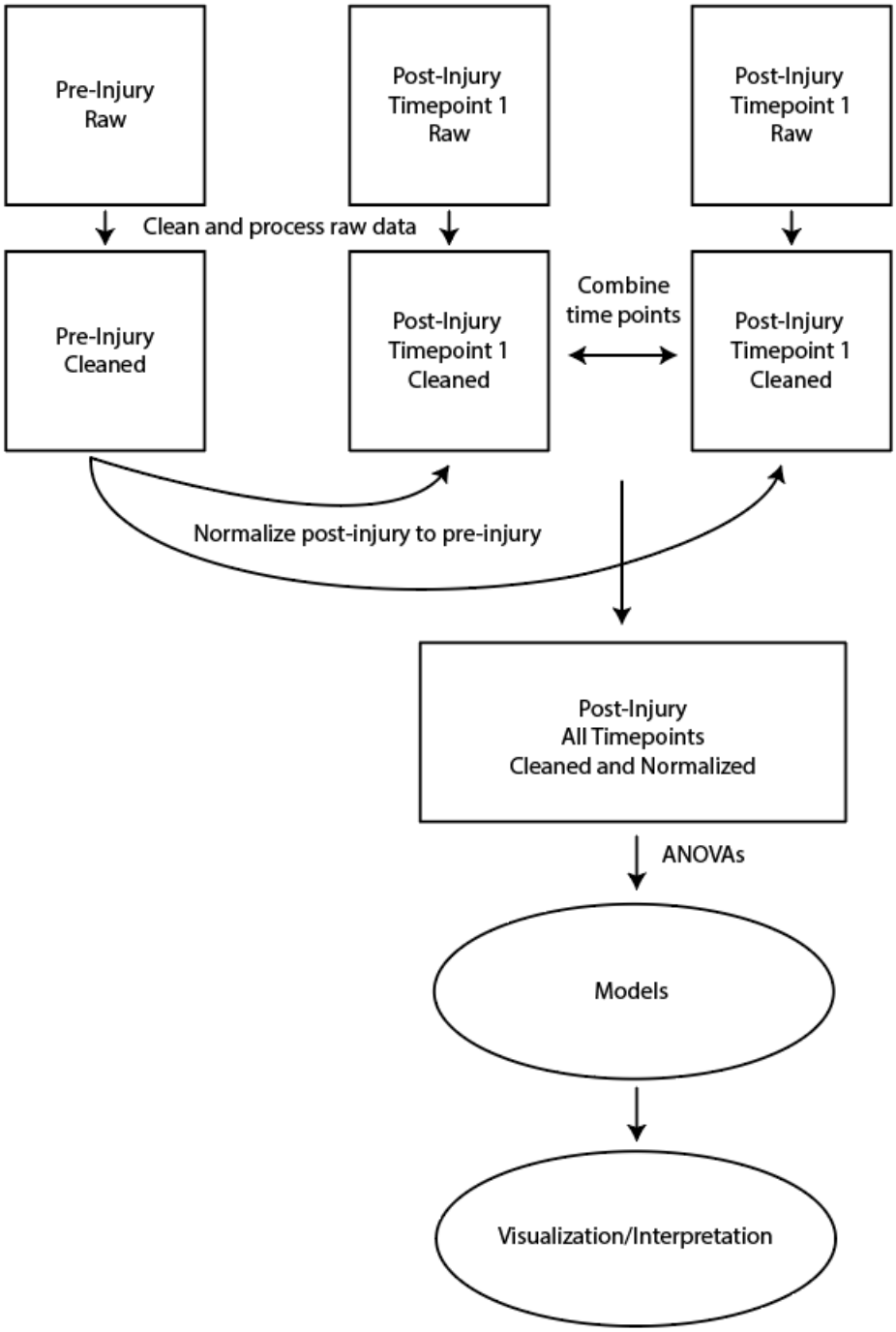

Figure 3.1: Graphical summary of analysis method utilized by runway package. Raw CatWalk data from separate timepoints is processed, then post-injury data is normalized to pre-injury data and combined. Normalized post-injury data is then analyzed using the appropriate ANOVA model, and then the results are visualized and interpreted.

Case One: A multiple timepoint data set with one independent variable.

In the first example, the experimental paradigm was as follows. Mice of two different genotypes (WT and CD44 KO) were given unilateral C5 contusion injuries. CatWalk data was obtained pre-injury, and at 14 and 28 days post-injury (dpi). I normalized data to the pre-injury timepoint and performed a repeated measures ANOVA to compare the effects of time vs. genotype on animal gait. A p-value of 0.05 was considered significant. This gave 31 variables significantly affected by time, another 31 affected by genotype, and only 1 affected by the interaction of time and genotype. A very useful way to quickly visualize this fact is using a heatmap and/or a Venn diagram (Fig 3.2 A-B). I can first conclude that while the progression of time after an injury and animal genotype both significantly impacted locomotor outcomes, they did so independently of one another. That is to say, the two genotypes had different locomotor outcomes, but the genotype did not affect how locomotion changed from 14 to 28 dpi. This implies that the differences between genotypes were already apparent by 14 days, indicating CD44 KO altered something about the early phase of the SCI response.

Next, I shifted our analysis to an examination of exactly what variables were affected and how focusing specifically on the effects of genotype. Three of the affected variables were “Diagonal Paw Support Percentage”, “Three Paw Support Percentage”, and “Lateral Paw Support Percentage”, all measures of what paw animals have in contact with the ground at any point. This implies a difference in genotypes in terms of how they are distributing their weight. Related to this, I saw several variables measuring the behavior of the right-fore and left-hind paws. This asymmetry aligns with the fact that our injury model

was a unilateral cervical injury primarily impeding right forepaw function. Given that the left hind paw and right forepaw contact the ground in a coupled manner during healthy animal movement, it makes sense that impacting the right forepaw would also affect the left hind paw. Similarly, several "couplings" variables were extracted, which measure how movement is coordinated between paws. Critically, however, the tag identified via a hypergeometric test as being most affected by genotype was actually "left forepaws", despite ladderbeam data indicating that left forepaw function was not impaired by injury. This suggests the effect of CD44 KO primarily altered how animals compensated post-injury with their unimpaired limbs.

Looking at precisely how these variables were affected by CD44 KO revealed that CD44 KO had an overall beneficial effect on animal locomotion. CD44 KO animals spent more time with a "diagonal" paw contact arrangement and less time with a "three" arrangement compared to WT mice, indicating a better ability to balance and support the weight. This aligned with our results from a ladder beam task, which showed fewer errors being committed by CD44 KO mice vs. WT mice.

Taken together, these data show that CD44 KO had a beneficial effect on animal locomotion post-SCI and that this benefit was likely due to neuroprotective modulation of the early response to SCI. Indeed, histology revealed the recruitment of immune cells was reduced in CD44 KO mice, along with reductions in the lesion and astroglial scar volume. This study provides an excellent example of the power of this method of gait analysis. Using CatWalk data, I was able to go beyond asking a simple yes or no question on animal recovery and instead provide insightful detail on exactly when and how our genetic manipulation was affecting the response to SCI.

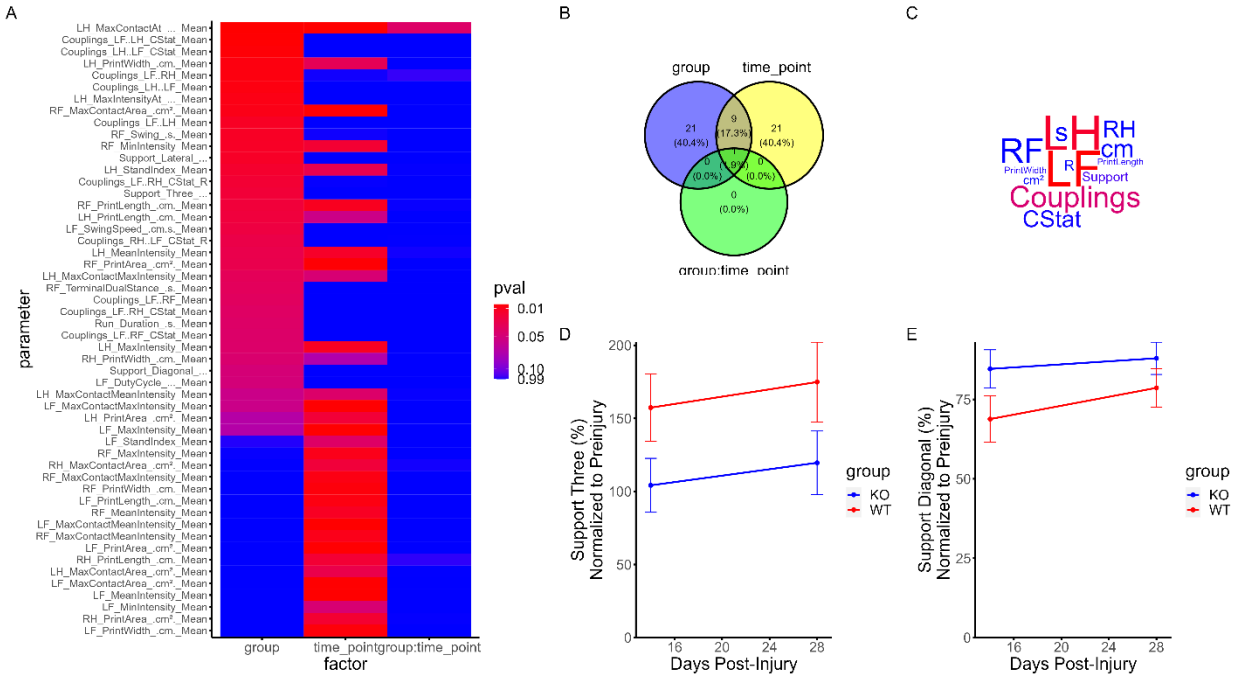

Fig 3.2 Summary of the findings from analysis of case one. A) Heatmap of isolated significant variables from repeated measures ANOVA of group vs. time, indicating that a high number of variables were affected by both time and group (columns 1 and 2, respectively), but few to no variables exhibit a combinational effect where time group alters how a variable changes over time. B) Venn diagram showing the number of "hits" (variables with a p-value less than 0.05) from each of the three columns in the heatmap in A. C) Word cloud visualization of the most common strings from the "hit" variables in the "group" category. Redder coloring indicates disproportionate hits vs. expecting as determined by a hypergeometric test, indicating that the group primarily affected left paw parameters. D-E) Line graphs showing the progression through time of two parameters identified by this analysis, "support percent three" and "support percent diagonal", showing that animals in the knockout group were spending more time in a diagonal arrangement than WT counterparts.

Case Two: A Single-Timepoint SCI Dataset With 3 Independent Variables

The second case uses data from another SCI study, where animals were given a unilateral hemisection injury, followed by treatment with four unique combinations of two treatments: bio-material bridge implementation and hNSC transplantation. In this case, CatWalk data was only obtained at a terminal time point, so pre-injury/pre-transplantation normalization was not possible. As in case one, I used ANOVAs to isolate relevant variables, and so much of the methodological approach used was identical to that described in case one. However, in this case, I used a single measure, two-way ANOVA for each of the independent variables. Then I isolated CatWalk variables affected by each individual variable (Fig 3.2 A-B). Critically, treating the data as having three independent variables rather than four unique groups allowed us to determine the unique ways in which each treatment affected gait. Interestingly, the results showed that cell treatment altered five variables, most dealing with right paws, whereas bridge affects ten completely different variables, mostly dealing with left paws. This suggests that the two treatments affected animal gait via different, parallel mechanisms, a fact that was independently verified via other histological and behavioral data from the study.

It's worth noting that one variable exhibited a significant combinatorial effect, where treatment with cells altered how the variable was affected by bridge treatment. This might seem in contradiction to the earlier statement about each treatment affecting a completely distinct subset of variables, though it is not. In case this is unclear, I will take a moment to explain the difference between interaction and main effects.

In this context, main effects are the effects that either bridge treatment or cell transplantation alone have on a given CatWalk parameter, ignoring the impact of the other

invention. An interaction effect in this case would examine how the presence of bridge treatment impacts the effect cell transplantation has on a given CatWalk parameter, and vice versa. Interaction effects do not require the presence of main effects. That is to say, an independent variable can significantly impact the effect another independent variable has on a dependent variable, even though the overall impact both independent variables have on the dependent variables is insignificant. In terms of how to interpret this outcome, for this case, given that only one variable showed interaction vs several variables showing main effects for only one independent variable, the simplest conclusion is that the two treatments were working via separate mechanisms.

Again, this method of analysis allowed us to gain critical insights into the mechanism of effect for each intervention. I could not only determine what treatments/combinations of treatments were efficacious, but I could also determine if the effects observed were due to overlapping/synergistic mechanisms or parallel/additive ones. Critically, a benefit of the runway R package is that it greatly facilitates this process, making designing and applying the right model to a given data set quite straightforward and flexible. Additionally, runway allows for the types of additional visualizations shown (such as the heatmaps, word clouds, etc.) that grant greater insight into a data set than a simple list of p-values would. When this work is published, the package will be made publicly available via GitHub in parallel.

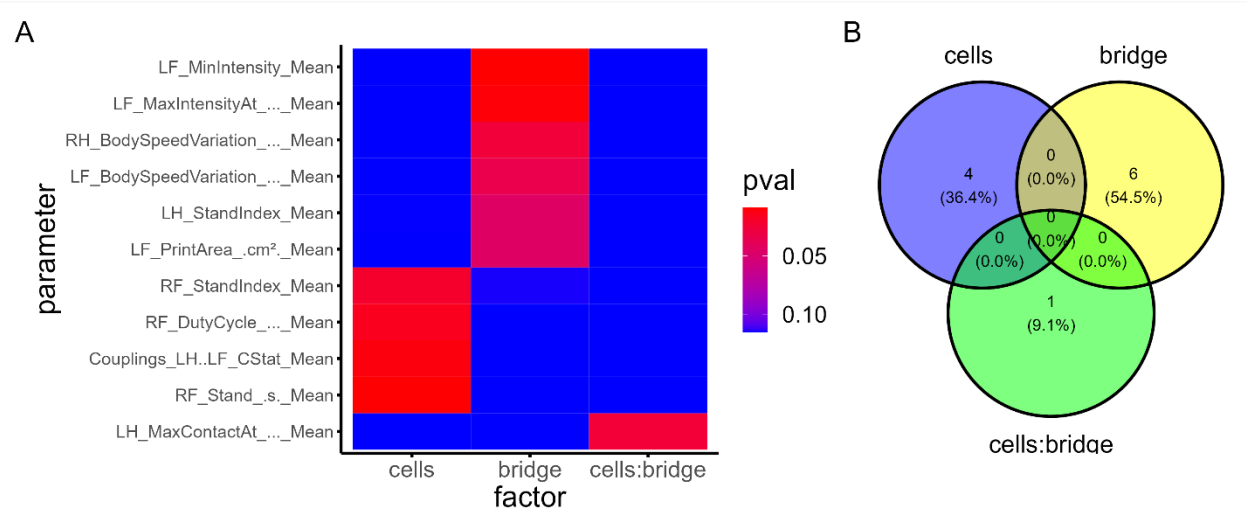

Figure 3.3 Summary of the findings from analysis of case two. A) Heatmap of isolated significant variables from repeated measures ANOVA of group vs. time, indicating that several of variables were affected by both cells and bridge (columns 1 and 2, respectively), relatively fewer variables exhibit a combinational effect. B) Venn diagram showing the number of "hits" (variables with a p-value less than 0.05) from each of the three columns in the heatmap in A. Further illustrates that cells and bridge treatment affected isolated sets of 4 and 6 variables, respectively, with no overlap.

Discussion

Gaining a clear picture of subject locomotion is critical to any research involving locomotor impairment. CatWalk represents a robust tool for obtaining a vast amount of behavioral data from animal studies. However, making sense of the vast amount of data it provides can be a difficult task, and it is very easy to draw incorrect conclusions or miss important findings in the process of analysis. Here, I provide a novel schema for analyzing CatWalk data that uses an unbiased, forward-screening approach to highlight the unique impact of a given injury/disease/intervention on locomotor behavior.

It is important to note that this method, while capable of providing valuable insights into animal locomotion, does contain drawbacks. Chief among these is that it is first and foremost descriptive of animal behavior and is less robust at providing a clear answer to the question "has a given intervention improved or worsened recovery?". Because of this, I recommended coupling CatWalk analysis with another behavior task such as ladderbeam or BMS that can provide clear conclusions to this question. In this way, one can examine both coarse and fine aspects of behavior.

Critically, beyond the technical statistical methods described above, the primary goal of this paper is to advocate for a more nuanced approach regarding interpreting data from CatWalk and other similar gait analysis programs/methods. The specific approach outlined may not be perfectly suited to a given experiment/tool. However, the overall philosophy of taking an exploratory approach to analysis and asking refined questions about behavior can be applied across any study to maximize the level of insight from the data obtained.

The longer-term goals of this project will be continuing to update and strengthen the runway package, aiming to making it both as powerful and accessible as possible in order to facilitate use by as many individuals working in the neurotrauma field and related disciplines as possible. Additionally, another goal is to make the public sharing of animal behavioral data more common and standardized, in line with the precedent set by the genomics/transcriptomics fields. Among many potential applications of such public, ideally centralized data sharing, would be the simple comparison of pre-injury data across a multitude of different studies. From that alone, a wealth of knowledge could be gained about how gait is altered by factors such as strain, age, sex, weight, time of year experiments are performed, or any other relevant metadata. Beyond that, such a public database could be used to compare gait response to different injury models, treatments, etc. While this is a longer term goal that will require spearheading on the author's part and broad cooperation from the field, it nonetheless others a wealth of highly exciting opportunities for collaboration's, both within the field of neurotrauma, and between this field and fields such as bioinformatics and data science. Indeed, projects such as the Open Data Commons for Spinal Cord Injury are already in development that share this ethos of data-sharing [101]. The hope is that by promoting such crosstalk and providing tools to make in-depth analysis more easily performed, we will see a surge in knowledge gained and ultimately treatments developed.

CONCLUDING REMARKS

The multi-faceted nature of SCI necessitates equally varied approaches in order to properly study and ultimately treat it. It is a condition that involves the complex interaction of numerous bodily systems, and care has to be taken to not become to fixated on one aspect of the disease, or one particular cell type. CD44 is thus an apt target to study in the context of SCI, as it is expressed by and regulates the behavior of cells from virtually every part of the body. Here, I follow up on my lab's previous work demonstrating a novel role for CD44 in regulating the behavior of neural stem cells, specifically their interaction with the immune microenvironment, namely C1q, the recognition molecule of the complement cascade. I expand on this work to show the CD44 KO hNSC transplanted into a chronic SCI niche exhibited significantly lower survival compared to WT counterpart cells, though neither treatment was able to restore locomotor recovery in animals beyond that of the vehicle control group. This has significant implications for the use of hNSC as an SCI therapeutic. In previous work from my lab, CD44 KO hNSC transplanted into an acute context performed better than WT cells, and unlike WT cells, CD44 KO hNSC were able to restore locomotor function beyond that of the vehicle group. These dramatically different results obtained from using the same cell line in the same mouse strain with the same injury model highlights just how much influence timing of transplantation matters. The next steps will be to more thoroughly examine what about the chronic timepoint leads to this outcome, then determine if the survival of CD44 KO hNSC can be rescued. If so, the question is then whether the CD44 KO hNSC are able to aid in recovery at the chronic timepoint provided they are able to survive and engraft into the cord.

However, it would be very shortsighted to only consider CD44's role in regulating the behavior of transplanted cells, as it also is known to be expressed by all manner of endogenous cells critical to the SCI response, such as astrocytes, immune cells, and endogenous NSC. Using a CD44 KO mouse model, I was able to probe what role CD44 plays in the post SCI response. The results of this experiment showed that, in line with its diverse expression, CD44 is very important for a myriad of aspects of the SCI response. CD44 animals exhibited less recruitment of acutely activated stem/progenitor and immune cells to the injury epicenter, dramatic alterations to scar formation, and overall improved locomotor outcomes. That last finding opens up the intriguing possibility of using a CD44 antagonist as a means of preventing damage in SCI patients, though more research remains to be done to ensure any therapeutic is as targeted and efficacious as possible.

Besides CD44, the connective tissue tying these two studies together is that they both rely on proper measurement of animal gait as one of their key readouts. As discussed in the third chapter, despite how key a goal this is to developing SCI treatments, our techniques for being abled to accurately analyze animal behavior remain startingly crude. Critically, the field's issue is less one of being able to obtain information on animal gait than it is extracting meaningful insights from that information. We have tools such as Catwalk which provide us with a wealth of measurements about every aspect of how an animal is moving. However, we are then often too reductive and simplistic in our approach to using this data, seeking to reduce the complexity of locomotion down to a binary "good/bad" spectrum. As I demonstrate, there can in fact by far more utility gained by taking a more open-ended philosophical and statistical approach, one that uses data from programs like Catwalk to tell us about how animals are walking, not just if they are.

SCI is a devastating condition that robs its victims of both length and quality of life. It is both an immediate and growing healthcare concern of enormous proportions. Combating it requires contributions from a multitude of disciplines working in concert to better understand the disease and develop treatments for it. By combining techniques and knowledge from the fields of neuroscience, immunology, cellular biology, kinematics, and data science, these studies aim to contribute to this process by not only providing key insights into a variety of facets of SCI, but critically by illustrating that those facets are all linked as but of a complex whole.

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