EFFECTS OF HOST/GRAFT SEX MISMATCH ON NEURAL PROGENITOR CELL GRAFTS FOR SPINAL CORD INJURY

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Effects of Host/Graft Sex Mismatch on Neural Progenitor Cell Grafts for Spinal Cord Injury

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Spinal cord injury (SCI) is an extremely devastating injury that can result in complete loss of all motor and sensory functions. Numerous studies have shown transplantation of neural progenitor cells (NPCs) has great promise in restoring lost neural circuitry following SCI. However, little is understood about the biological factors that determine the success of the transplanted graft. One of these potential factors is the role of sex as a biological variable. We sought to determine whether sex mismatch between graft and host tissue influences the survival, differentiation, and integration of transplanted NPCs in a mouse model of SCI. Donor sex was determined for individual GFP+ mouse embryos through rapid genotyping of the X chromosome gene Rbm31x and its divergent Y chromosome gametolog Rbm31y. Either male or female NPCs were then isolated and acutely transplanted into lesion sites of either male or female adult mice. Four weeks following the transplantation, we analyzed neuron and astrocyte differentiation, glial scar formation, and extension of graft-derived axons. Although we did not detect any significant differences in these outcomes, we observed significant hypervascularization in grafts derived from male NPCs within female host animals. This observed vasculature was also associated with

abnormally high levels of perivascular density only in this treatment group. Immunohistochemical analysis revealed increased levels of infiltrating immune cells, including leukocytes and lymphocytes, in and around the male-to-female grafts. Altogether, these findings suggest that expression of sex-specific antigens on male donor cells may provoke an inflammatory response in female host animals.

DEDICATION

To my family, instructors, and friends who supported me throughout the research process. I would not be able to pursue my academic and career aspirations if it were not for their continued guidance and support.

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All other work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

SCI	Spinal Cord Injury
NPC	Neural Progenitor Cell
iPSC	Induced Pluripotent Stem Cell
ROI	Region of Interest
PBS	Phosphate Buffered Saline
TBS	Tris Buffered Saline
TBS+T	Tris Buffered Saline + Triton
LHRH	Luteinizing Hormone Releasing Hormone
GFP	Green Fluorescent Protein
HBSS	Hanks' Balanced Salt Solution
IP	Intraperitoneal
PB	Phosphate Buffer

1. INTRODUCTION

Spinal cord injury (SCI) is a devastating event that affects nearly 300,000 people in the United States [1]. Unfortunately, SCI includes many debilitating deficits for affected individuals, such as loss of motor and sensory function, along with hindered autonomic functions. This leads to a poor prognosis for these individuals, especially since no clinical treatments that restore these functions are currently available. Due to the lack of a restorative treatment, there currently are many preclinical and clinical studies evaluating different types of treatment, such as electrical neural stimulation, robotic therapy, and transplantation of neural progenitor cells (NPCs) [2-5].

Neural progenitor cells are one of the many types of multipotent stem cells in the body. They arise in the developing brain and spinal cord of an embryo and have a limited differentiation capacity as they are destined to give rise to the neuronal and glial cells of the nervous system. Recent biotechnology has also allowed us to derive NPCs from non-embryonic cell lines through the use of induced pluripotent stem cells (iPSCs), which could also be used in a similar capacity to restore neural circuitry following SCI [6, 7]. The capacity of NPCs to differentiate specifically into neuronal cell types have made them a prime candidate for a potential treatment of SCI in studies seeking to restore lost or damaged neural circuitry [8]. Many of these studies have now suggested that transplantation of NPCs following SCI has a very high therapeutic potential for successful treatment of SCI. Following transplantation into the injured spinal cord, these cells successfully proliferate into mature neurons and glial cells and functionally integrate into the host neural circuitry [9, 10]. These results suggest that transplantation of NPCs could restore circuitry that was lost following SCI, which would potentially restore many of the aforementioned deficits seen in individuals with this injury.

However, little is known about the biological factors involved in moderating the successful integration of NPC grafts into the host tissue [11]. An incomplete lack of understanding about how biological variables affect the therapeutic efficacy of NPC grafts currently limits progress in the development of a successful treatment using an NPC-based therapy.

A successful NPC graft can be defined in many ways, depending on the context of the study. In clinical trials, cell transplantation approaches must be determined to be both safe and effective before advancing to FDA approval. A treatment would be deemed *safe* if no harmful side effects are observed, such as immune rejection, the development of pain or spasticity, or uncontrolled cellular proliferation. The treatment would be deemed *effective* if significant improvements in motor, sensory, and/or autonomic functions are observed following treatment. In preclinical studies, the measure of a graft's effectiveness might include successful axonal extension and innervation from the grafted cells into the host nervous tissue or improved functional outcomes, depending on the aims of the study [9, 12]. Due to the high levels of complex cellular coordination needed to achieve these results, there are a myriad of biological variables that could affect each of these results *in vivo*.

Several preclinical and clinical studies have suggested that a mismatch in biological sex of host and graft tissue in human organ transplantation is associated with an immunological response in the recipient (host) [13, 14]. To date, no studies have investigated if similar effects may be seen following NPC transplantation into the injured spinal cord. We hypothesize that one of the biological variables that affects the success of NPC grafts in the injured spinal cord is the compatibility of the sex of the host tissue with the sex of the grafted cells.

We tested this hypothesis by transplanting either sex-matched or sex mismatched NPCs into sites of SCI in male and female mice. In this study, we show that female subjects treated

with dorsal column lesions and then grafted with male embryonic NPCs show marked hypervascularization, increased levels of perivascular cellular density, along with heightened concentrations of CD3+ cell types in comparison to subjects grafted with sex-matched NPCs. These results all suggest that the subjects in this group are displaying an amplified immune response that is commonly seen in rejection of organ tissue transplants. This reveals that biological sex match or mismatch may play a role in modulating the immune response of the host individual, which in turn affects the success of the graft. The heightened T-cell density also could be playing a larger role than just indicating the presence of an immune response. The results of one study have actually suggested that T-cells play a role in the normal functioning of the central nervous system, which could also translate into differences in the development of NPC grafts [15]. All of these results suggest that biological sex as a variable plays a significant role in NPC graft success in the injured spinal cord. This could greatly impact protocols for performing cellular transplantation studies as it could dictate a more effective way to perform these studies, furthering the field closer to elucidating a treatment for SCI.

2. MATERIALS AND METHODS

2.1 Preparation of NPCs

Adult C57BL/6J female mice (Jackson Laboratories, #000664) were intraperitoneally injected with luteinizing hormone releasing hormone (LHRH) five days prior to pairing with adult homozygous GFP+ male mice (Jackson Laboratories, #003291). The mice were then paired together at 1700 and then unpaired the following morning by 0900 to control timing of fertilization. The females were then separated and allowed to progress in their pregnancy for 12 days where they were then sacrificed so the embryos could be harvested. These embryos were classified as embryonic day (E) 12.5, which is most widely used embryonic stage for NPC transplant studies [16]. Following embryo isolation, the developing spinal cords enriched with NPCs were harvested from individual embryos and kept on ice until genotyping was complete. After pooling spinal cords according to biological sex, NPCs were isolated from spinal cord tissue according to previously published protocols [9, 17-19]. Briefly, tissue was incubated with 0.25% trypsin, triturated, washed with Hank's Buffered Salt Solution (HBSS), and filtered through a 40-μm filter. Isolated NPCs were kept on ice until the genotyping was complete.

2.2 Genotyping of embryos

Following extraction of the E12.5 embryos from the female mice, tail tissue was removed from each of the embryos to provide genetic material for genotyping. The method utilized for this study was originally published by the Tunster laboratory at the University of Cambridge [20]. The *Rbm31x* and/or *Rbm31y* genes were amplified by simplex PCR using primers that allow for detection of an 84-bp deletion of the X-linked *Rbm31x* gene that is not present in the *Rbm31y* gametolog (forward: 5' - CACCTTAAGAACAAGCCAATACA - 3'; reverse: 5' –

GGCTTGTCCTGAAAACATTTGG – 3'). The genotyping process lasted approximately 4 hours. Female samples were identified as exhibiting one PCR product of 269 bp (*Rbm31x*), and male samples were identified as exhibiting two PCR products of 269 bp and 353 bp (*Rbm31x* and *Rbm31y*, respectively).

2.3 Spinal cord injury and NPC transplantation

A total of twenty-seven adult male and female C57BL/6J mice (N=16 males and N=11 females) were subjected to SCI and transplantation surgeries. All animals were given free access to food and water throughout the study. National Institutes of Health guidelines for laboratory animal care and safety were strictly followed. All animal procedures were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

The SCI model used in this study was a dorsal column lesion at the spinal cord cervical level 4 (C4) [17]. Prior to surgery, mice were anesthetized using a combination of the inhalation general anesthetic isoflurane (0.5%) and an intraperitoneal injection of ketamine (50 mg/kg), xylazine (2.6 mg/kg), and acepromazine (0.50 mg/kg). Anesthetized animals were prepared for surgery by shaving the dorsal surface of the upper back and applying 70% ethanol and betadine scrub. A midline incision was then made on the upper back starting at the base of the skull to between the shoulder blades. The muscular tissue was then dissected away and retractors were used to allow for increased visualization of the vertebrae. A laminectomy was then performed at the C4 spinal level. Next, a retractable wire knife was retracted and inserted into the spinal cord to a depth of 0.8mm, extruded, and raised dorsally to transect the dorsal columns. Immediately following SCI, a total volume of 2.5 uL containing 10^6 NPCs in HBSS was transplanted into the injury site. Animals were randomly assigned to receive either sex-matched or -mismatched NPCs. Following transplantation, the incised muscle tissue was closed using simple interrupted

4-0 silk sutures. Neo-predef antibiotic powder with tetracaine was applied to the incision site. Finally, the incised skin was closed using wound clips. Following surgery, a prophylactic subcutaneous injection of lactated Ringer's solution (0.5 mL) containing ampicillin (0.15 mg/kg) and banamine (2.5 mg/kg) was provided to avoid bacterial infection of the incision site. These injections were administered postoperatively daily for 2 additional days.

2.4 Immunohistochemistry

At four weeks following transplantation, animals were deeply anesthetized and sacrificed by transcardial perfusion with 0.1M phosphate buffer (PB) followed by 4% paraformaldehyde in 0.1M PB, and spinal columns were harvested and post-fixed overnight. The tissue was then transferred to a solution of 30% sucrose in 0.1M PB for 3 days in order to cryopreserve it. Then, the spinal cord tissue was removed and a portion of the cervical spinal cord approximately 1cm long, centered around the NPC graft, was isolated to prepare for cryosectioning. The cervical portion of the spinal cord tissue was sectioned in the sagittal plane on a cryostat to 30-μm thickness. These sections were stored as a 1:6 series. For all of the immunostaining, the sections were washed in tris-buffered saline (TBS), blocked for 1 hour at room temperature in 5% normal donkey or goat serum in TBS containing 0.025% Triton-X-100 (TBS-T), and then incubated with primary antibodies diluted in TBS overnight at 4°C. The primary antibodies utilized for each of the stains can be found in the Table 1 below. The next day, the samples were washed in TBS again and then incubated in AlexaFluor-conjugated secondary antibodies for 2 hours. The sections were then washed one more time, with one of the washes containing the nuclear counterstain, 4′,6-diamidino-2-phenylindole (DAPI), at 0.1 μg/mL. Sections were then mounted onto gelatin-coated slides and coverslipped using the anti-fading mounting medium, Mowiol. These slides were allowed to set overnight and then were stored at -20°C until imaging.

Table 1: Summary of all primary antibodies used along with their respective dilutions in the immunohistochemical protocols.

Antibody	Manufacturer and Catalog Number	Dilution Used
Rabbit anti-GFP	Life Technologies; A6455	1:1500
Guinea Pig anti-NeuN	Millipore; abn90	1:3000
Chicken anti-GFAP	Encor Bio; CPCA-GFAP	1:2000
Hamster anti-CD31	Millipore; MAB1398Z	1:500
Rat anti-CD3	Biolegend; 100202	1:100

2.5 Image acquisition and analysis

Imaging of tissue sections was performed using a Nikon Eclipse fully motorized upright fluorescent microscope equipped with a monochrome camera. Entire sagittal sections, including the grafted NPC tissue as well as rostral and caudal host spinal cord, were imaged. Imaging settings were optimized to capture fluorescent signal while avoiding oversaturated pixels in all cases, except for the acquisition of GFP+ graft-derived axons which required overexposure in the GFP channel. Image files were exported as 8-bit monochromatic TIFF files for analysis.

All image analysis was performed in ImageJ. Regions of interest (ROIs) were drawn manually using the Polygon tool around GFP+ grafts. The ROIs were then overlaid onto the various image channels where then an automated image thresholding macro was utilized to count the number of cells positive for the respective antibody being investigated. The results from automated image analysis were also verified using hand counting methods in ImageJ. For axon outgrowth quantification, intervals of 500 μm rostral and caudal to the graft/host borders were generated using ImageJ, and total numbers of axons traversing each interval were counted. Graft

volume was calculated by quantifying the area of graft in each section and multiplying by the inter-section interval.

2.6 Statistical analysis

All statistical analysis was performed in GraphPad Prism 7.0 software. Multiple group comparisons were performed using one-way ANOVA with appropriate post tests. P values less than 0.05 were considered to be statistically significant.

3. RESULTS

3.1 Delayed transplantation does not affect graft survival

To distinguish male versus female donor embryos, we took advantage of a previously published protocol for determination of genetic sex in mice via simplex PCR [20]. Figure 1 shows a sample Southern blot performed on individual E12.5 mouse embryonic tissue samples. The distinct 269-bp and 353-bp bands correspond to *Rbm31x* and *Rbm31y* PCR products, respectively (Fig. 1). In this way, female and male embryos were easily identified through genotyping.

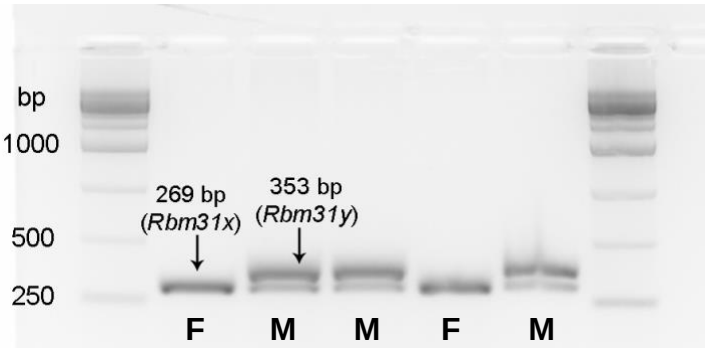

Figure 1: Representative Southern blot of the PCR product from amplification of Rbm31x and Rbm31y gametologs in male (M) and female (F) embryonic mice.

The genotyping protocol that was employed to determine the biological sex of each of the subjects took approximately 4 hours. Embryonic spinal cords isolated from E12.5 embryos were therefore stored on ice for at least four hours during the genotyping process. Since the isolated NPCs are typically transplanted immediately following isolation [9, 17-19], we wanted to determine if the delayed transplantation period affected survival of grafted cells. To investigate the effects of this process on the cells, we transplanted E12.5 embryonic NPCs into the site of SCI in host animals. These cells were recombinantly labeled with GFP so that they could be

easily identified with immunohistochemical staining. These grafts were allowed to grow and proliferate for 4 weeks in the host mouse prior to sacrifice. To visualize the survival of grafts, sagittal spinal cord sections were immunohistochemically labeled for GFP and NeuN, a marker for mature neurons. As seen in Figure 2, we found that 4-week-old GFP+ NPC grafts exhibited good survival, integration with host spinal cord, and were populated with NeuN+ neurons. We did not observe poor survival or integration in any of the grafts. This observation is consistent with previous reports [9, 17-19], indicating that the delayed transplantation protocol used for isolating and genotyping the NPCs did not adversely affect the survival and differentiation of the graft. Therefore, any differences seen between the each of the treatment groups should not result from the delayed transplantation process.

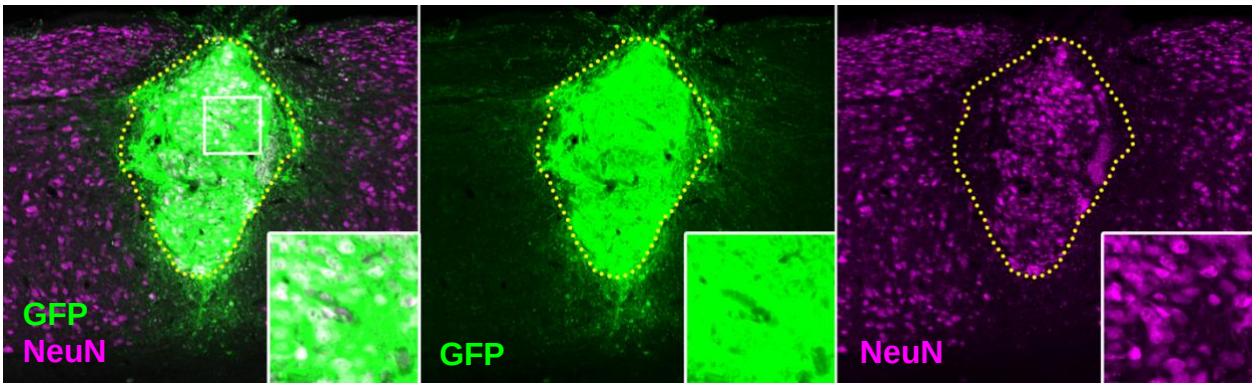

Figure 2: Embryonic spinal cords were kept on ice for approximately 4 hours while genotyping was performed. Once dissociated and transplanted, these cells produced viable grafts (GFP) populated with mature neurons (NeuN). Graft/host border is indicated with dotted lines.

3.2 Donor cell sex does not influence graft cell differentiation, glial scar formation, or graft axon outgrowth

As mentioned previously, a successful NPC graft in the laboratory would be defined as a graft that survives and matures into adult neuronal cell types and extends axons from the grafted tissue into the host tissue. To determine if there were any differences with respect to this

definition of graft success, we first began by quantifying the neuron density within each of groups (Fig. 3 A), since differences in the density of neurons within each graft could indicate different survival rates of the transplanted NPCs. Sagittal spinal cord sections were taken from each animal and immunohistochemically stained for the mature neuron marker, NeuN, as well as for GFP, representing the transplanted NPCs. The number of NeuN+ cells inside the GFP+ graft area was quantified and normalized to the graft volume using ImageJ. This data revealed that with respect to graft neuron density, there was no statistically significant difference between each of the groups (Fig. 3 B and C).

Next, we investigated whether there were differences in the glial scar formation between each of the groups. Several previous studies have shown that the presence of large glial scars around the borders of the site of SCI can prevent successful innervation of the graft into the host tissue, therefore hindering the success of the graft [21]. The extent of glial scar formation was determined by immunohistochemically staining sagittal spinal cord sections from each group with GFP and glial fibrillary acidic protein (GFAP), a marker of astrocytes, the cell type that typically represents the glial scar [22]. We located the GFP+ graft area and then quantified the total GFAP+ area in the region from the edge of the graft to 500 microns outside the edge using ImageJ (Fig. 3 D). Following normalization to the graft volume and statistical analysis, this data revealed that there was no significant difference between each of the groups with respect to glial scar formation (Fig. 3 E and F).

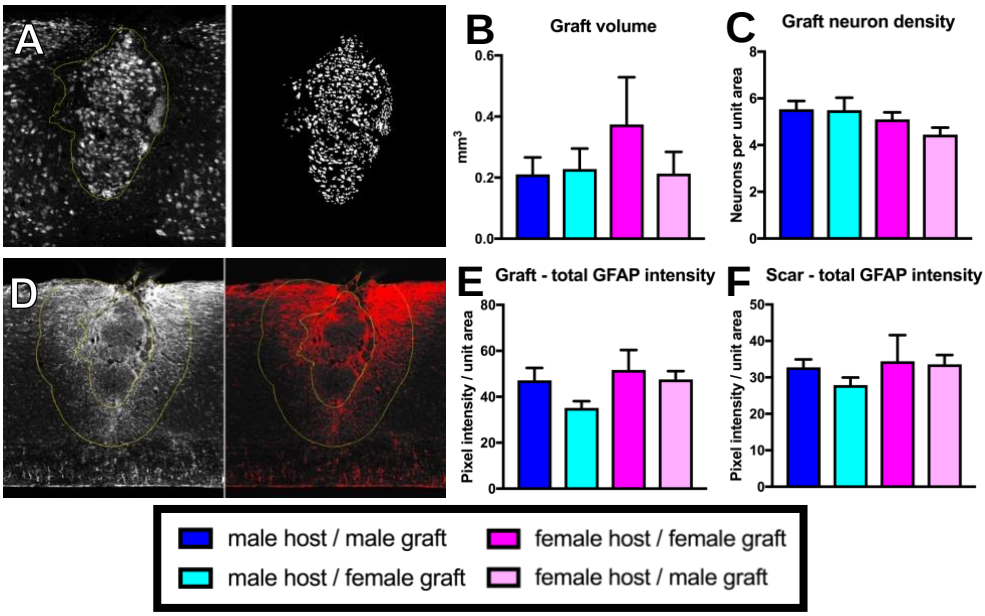

Figure 3: (A) Automated neuron quantification. (B) Total graft volume and (C) neuron density is not significantly different between treatment groups. (D) Automated quantification of astrocyte (GFAP+) immunoreactivity in grafts (inner) and surrounding region (outer). Total GFAP pixel intensity is not significantly different between treatment groups in (E) graft tissue or (F) surrounding scar region in host tissue. Comparison by one-way ANOVA followed by Tukey's multiple comparison test.

Finally, we investigated whether there were any significant differences between each of the groups with respect to the extent of axon outgrowth from the grafted NPCs into the host tissue. Once again, a graft with significantly more axonal extension from the graft into the host would have an increased potential in restoring lost neural circuitry in the site of a SCI, making a more successful graft in laboratory standards. The axonal outgrowth in each site was quantified utilizing the same sagittal sections from the previous data collection processes, only this time the GFP channel of the fluorescent imaging microscope was over-exposed (Fig. 4 A), allowing enhanced visualization of graft-derived axons both directly proximal (Fig. 4 A') and more distal (Fig. 4 A") to the graft. These images were then uploaded into ImageJ and ROIs were once again drawn around the perimeter of the implanted graft. The lateral portions of the ROIs were then extended both rostrally and caudally in 500-micron increments, and the number of axons

crossing the ROI line were counted. Once again, this data showed no statistically significant difference was found between each of the groups (Fig. 4 B), suggesting that a sex match or mismatch between the host and grafted cells does not affect the extent of axonal extension from the grafted NPCs into the host tissue.

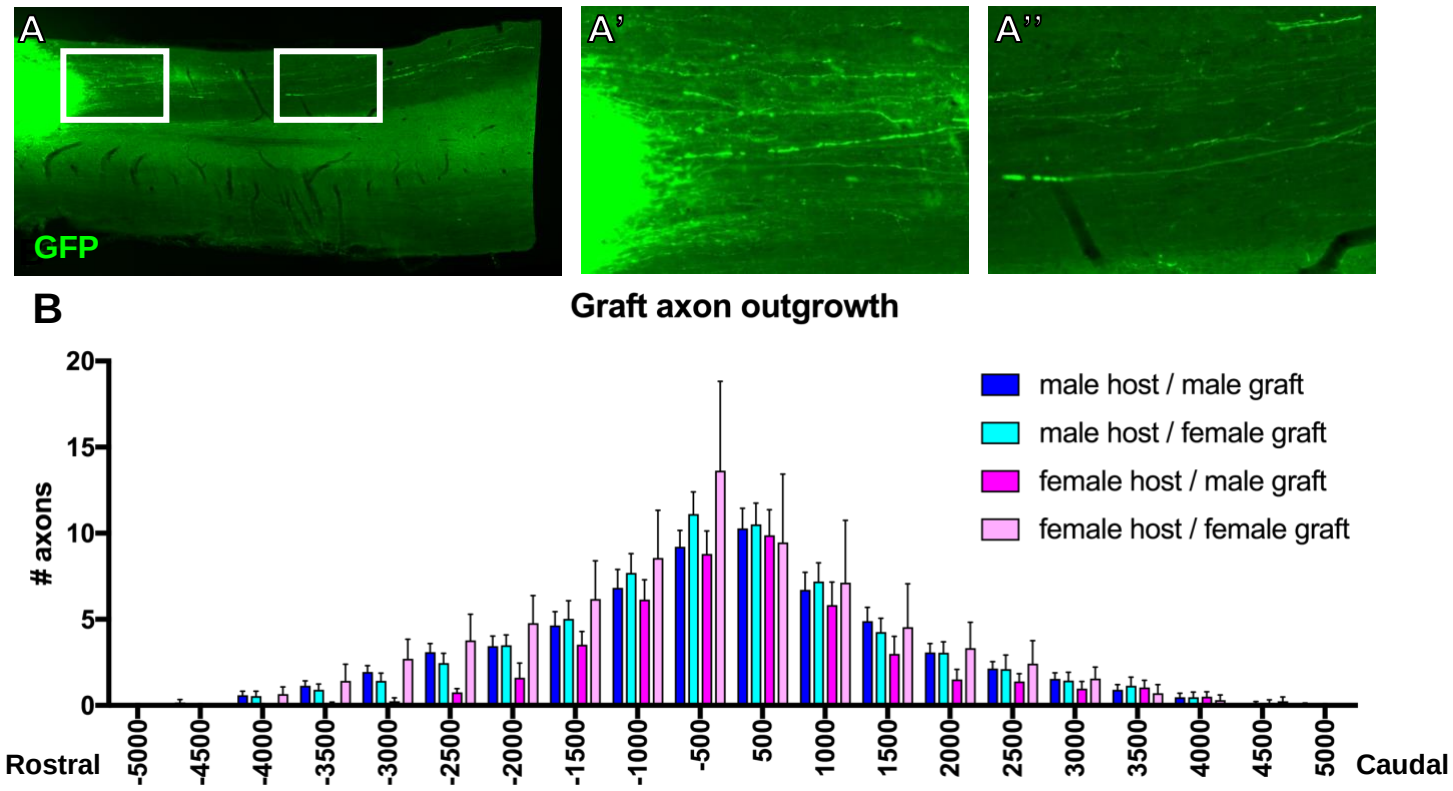

*Figure 4: (**A**) Representative image of GFP+ graft-derived axon outgrowth into the host spinal cord. **A'** and **A"** are high-magnification insets of graft-derived axons (**A'**) immediately proximal to the graft and (**A"**) within host spinal cord white matter 2.5 mm caudal to the graft. (**B**) Quantification of the number of axons at 500-μm intervals from the rostral or caudal graft/host boundary.*

3.3 Male-to-female grafts are hypervascularized

Despite the absence of significant differences between groups with regards to the above outcome measures, we made a surprising observation about the extent of vascularization in grafts.

Upon visually reviewing grafts that were immunohistochemically labeled for GFP and NeuN, we made an interesting and unexpected observation, specifically in the female host / male graft group. Figure 5 displays representative images of transplanted GFP+ grafts into the NeuN+ host tissue from each group. When compared to the other groups, the female host / male graft group displayed a graft morphology that is not seen in any of the other groups. We noted that the grafts in these groups had abnormal fissures that were morphologically similar to vasculature of the cardiovascular system. Vascularization of NPC grafts is a normal phenomenon, but the blood vessels seen in the female host / male graft group were much larger in diameter than what we have historically observed [9, 17-19]. This result suggests that in the female host / male graft, a hypervascularization event may be occurring, causing the abnormal enlargement of vascular structures in the grafts.

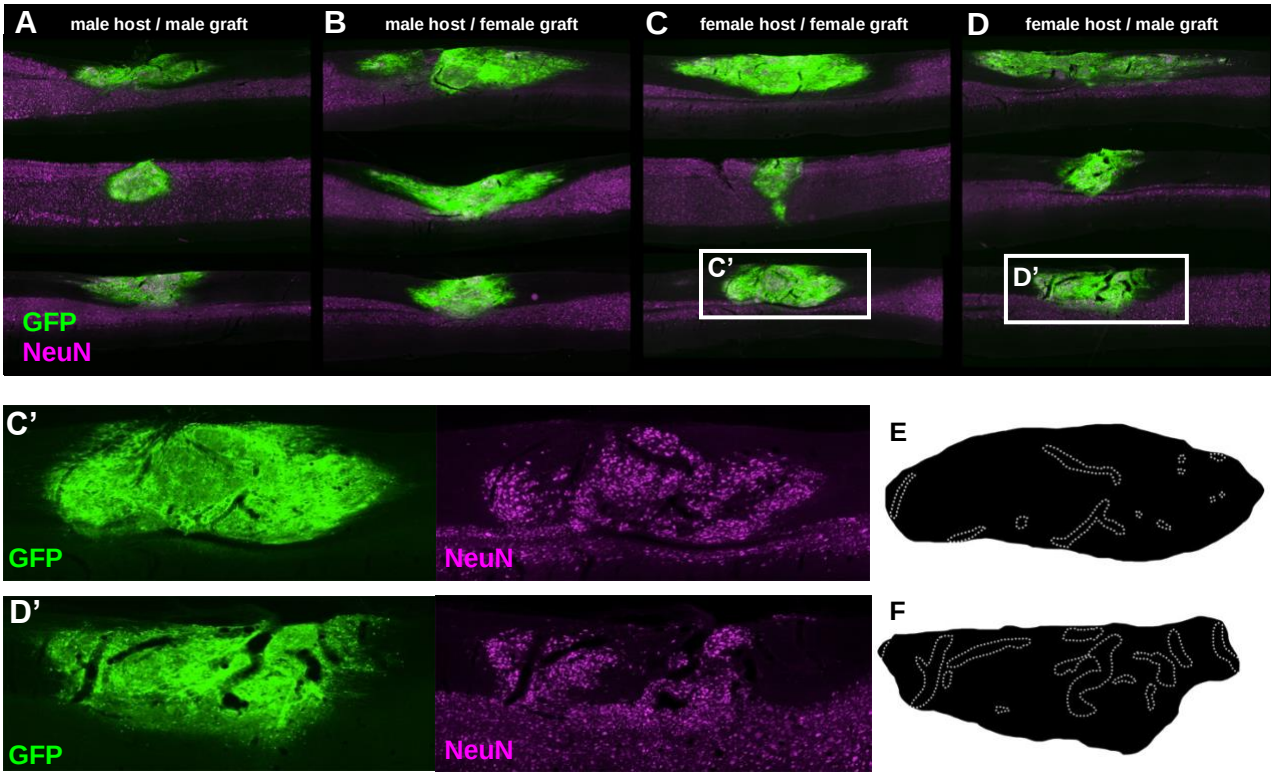

Figure 5: Grafts of neural progenitor cells derived from male embryonic donors and placed into female host animals exhibit abnormal vascularization. Representative images of GFP+ grafts of (A) male NPCs into male host mice, (B) female NPCs into male host mice, (C) female NPCs into female host mice, and (D) male NPCs into female host mice. Neurons in both host and graft tissue are labeled with NeuN. (C' and D') High magnification insets of individual (C') female NPC grafts in female host mice and (D') male NPC grafts in female host mice. (E, F) Outlines of blood vessels in the corresponding grafts are shown with dotted lines.

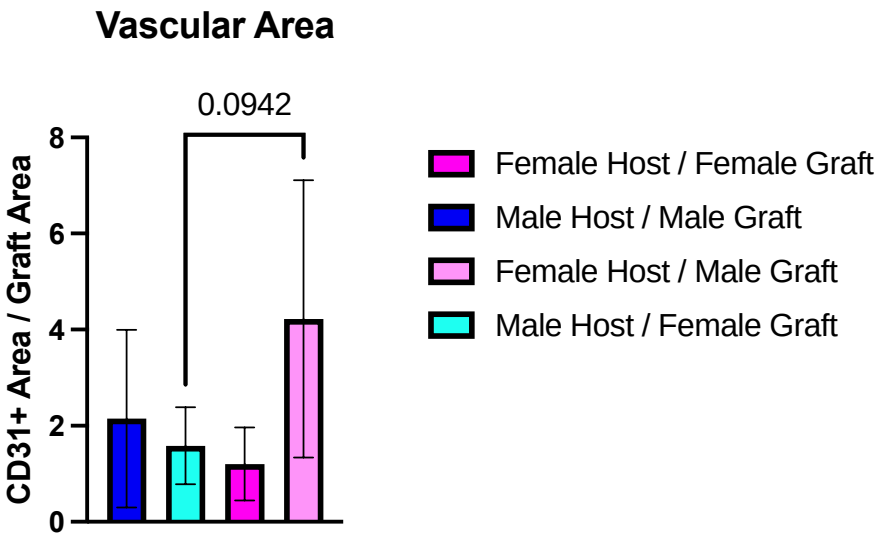

Figure 6: Quantification of the CD31+ vascular area in the NPC grafts of animals from each group. After comparison by one-way ANOVA followed by Tukey's multiple comparison test, no significant difference between the groups was found, but the female host / male graft group came close to significance when compared to the male host / female graft group with a p value of 0.0942.

With the findings discovered in the qualitative investigation of the grafted tissue, a more quantitative analysis was warranted. Therefore, an immunohistochemical stain was done of all of the tissue with CD31, an antibody that found on endothelial cells and is widely used as a vascular marker. This was in addition to staining with GFP in order to label the grafted cells. These images were then uploaded onto ImageJ where an ROI was drawn around each of the grafts. This was then overlaid onto the CD31 channel where ROIs were then drawn around the vascular area marked by CD31. The areas of both the graft ROIs and the vascular + graft ROIs were calculated via ImageJ. These values were then subtracted from each other to provide the vascular area in each of the grafts. In order to normalize the data, each of the vascular areas were divided by the graft areas. This data is summarized in figure 6. No statistically significant difference was found between each of the groups. However, a very low number of subjects were included in this quantification due to the low number of tissue available for staining. We also observed a p value

of 0.0942 between the female host / male graft group when compared to the male host / female graft group, which is close to being significant. Continued investigation of this phenomenon with more studies has the potential to reveal a statistically significant difference between these groups with an increased number of subjects.

3.4 Male-to-female grafts exhibit abnormal perivascular cellular density

Due to the observation of hypervascularization in the female host / male graft group, we began to further investigate this observed phenomenon, which revealed even more abnormal results. Once again, we used the same sagittally sectioned tissue that was immunohistochemically labeled with GFP for the grafted cells, NeuN for the mature neurons, and DAPI for the nuclei of all the cells in the tissue. Using this tissue, we found that the perivascular areas within the graft contained an increased concentration of DAPI+ cells (Fig. 7 D). The increased number of DAPI+ cells around these blood vessel structures suggests that in the female host / male graft group, there was an increased perivascular cell density in the NPC grafts.

Interestingly, we also found that these perivascular regions in the female host / male graft group contained atypical NeuN immunoreactivity, which did not appear to specifically label neurons (Fig. 7 D' and D"). This suggests either the presence of cellular debris that nonspecifically binds to the anti-NeuN antibody, or that that neuronal death may be occurring in these regions.

Hypervascularization along with increased perivascular cell density and cellular debris are archetypal events of an immune response of the transplanted tissue by the host [23, 24]. These results suggest that, in conjunction to a hypervascularization event occurring in the female host / male graft group, an inflammatory immune response by the host may also be occurring in

the grafts. This immune response would be indicative of the host rejecting the implanted cells, which at prolonged time points may cause death of the NPCs thus decreasing the successful proliferation and integration of these cells into the host tissue.

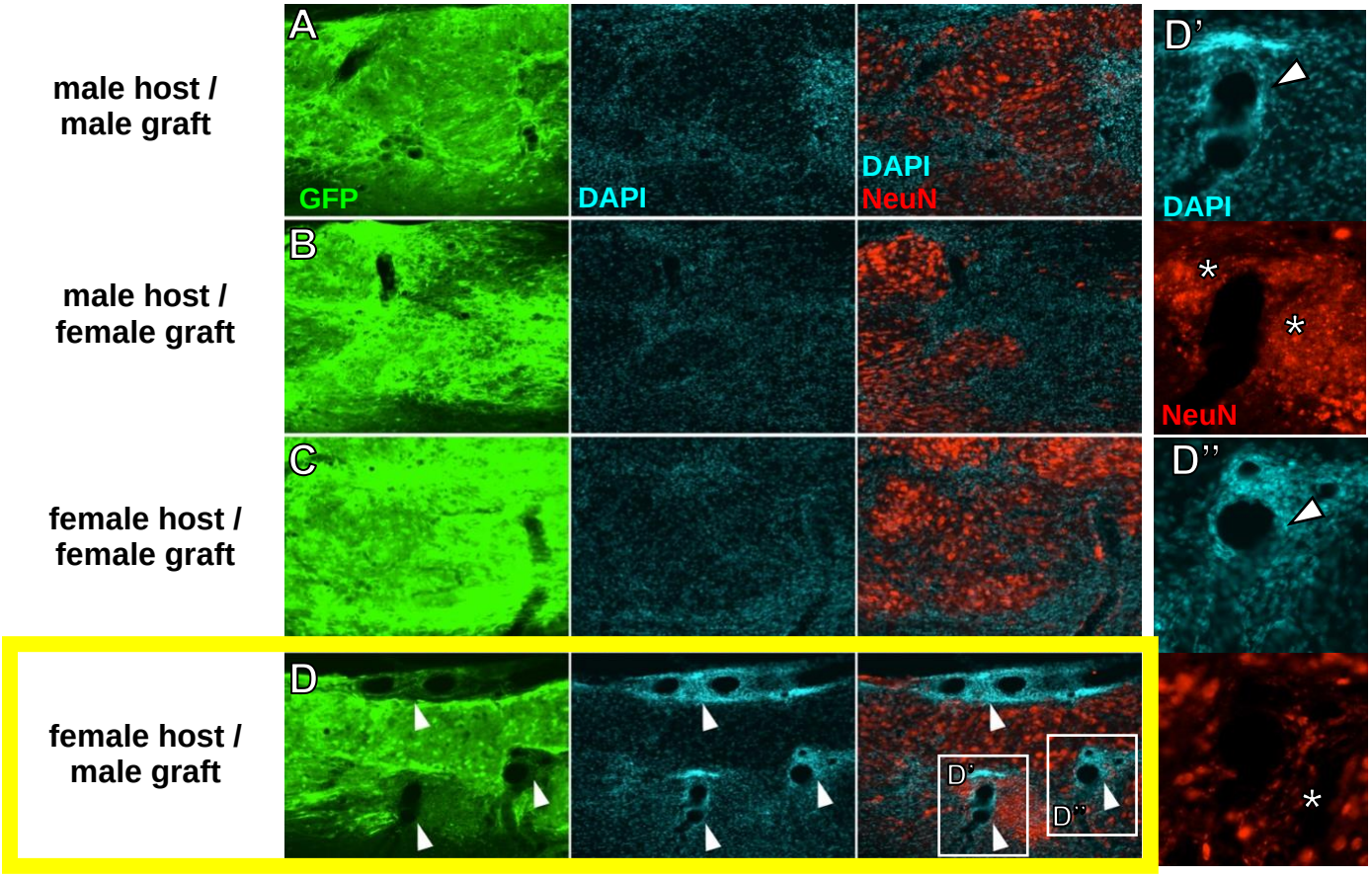

*Figure 7: High-magnification images of **GFP+** grafts of **(A)** male NPCs into male host mice, **(B)** female NPCs into male host mice, **(C)** female NPCs into female host mice, and **(D)** male NPCs into female host mice. Nuclei are labeled with **DAPI** and graft neurons are labeled with **NeuN**. Cross-sections of vasculature in grafts are indicated with arrowheads. **(D', D")** Insets of male NPC grafts into female host mice reveal high cell density (arrowheads) and cellular debris (asterisks) in perivascular regions.*

3.5 Male-to-female grafts exhibit abundant T cell infiltration

Since we hypothesized the occurrence of an immune reaction in the female host / male graft group, we wanted to further investigate the cell types that were involved in the perivascular cell density to confirm our hypothesis. This led us to once again immunohistochemically stain sagittal spinal cord sections of the grafted area with GFP for the grafted cells and the antigen

CD3, which labels any T lymphocytes present in the tissue. Interestingly, we found that even qualitatively, the female host / male graft group exhibited a significantly larger density of T lymphocytes in the grafted area (Fig. 8 A). Due to this, we quantified the average number of T lymphocytes per graft area by using ImageJ to automatically count the number of CD3+ cells in the graft and normalized this over the graft area (Fig 8 B) for samples from each group. The results revealed a statistically significant difference between the female host / male graft group and the male host / female graft group. We also found that a p-value of 0.058 was present between the data of the female host / male graft group and the male host / female graft group, coming very close to being statistically significant. This suggests that blood-born, CD3+ T lymphocytes are entering the grafted tissue from the host immune system in the female host / male graft group, which is not seen in any of the other groups. This enhanced infiltration of host lymphocytes is not typically found in the spinal cord, suggesting an abnormal immune response due to the grafted NPCs [25-27].

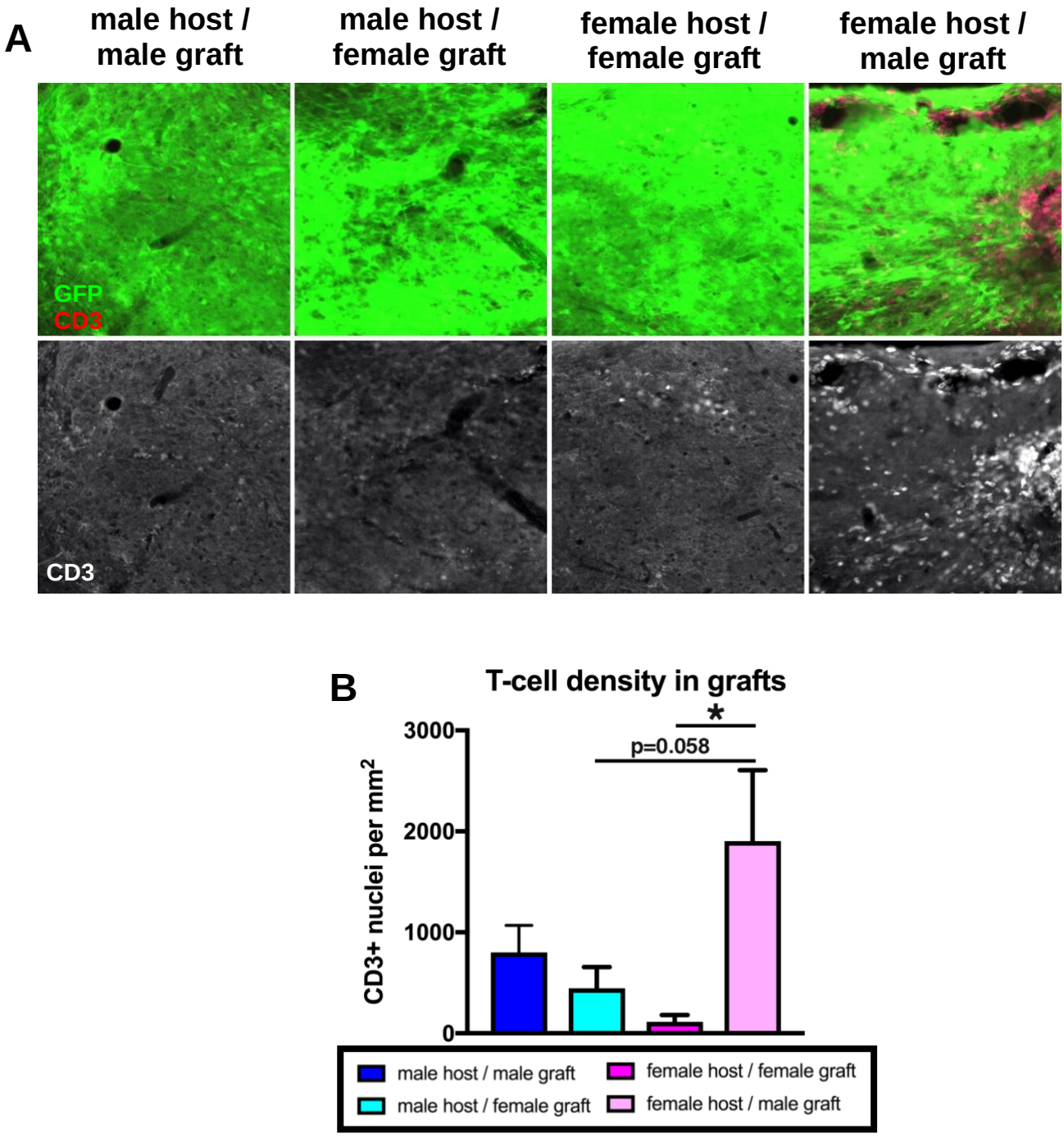

*Figure 8: Grafts of neural progenitor cells derived from male embryonic donors and placed into female host animals exhibit heightened T cell infiltration. Representative images of **GFP+** grafts immunolabeled against T-cell antigen **CD3**. (**B**) Quantification of the total number of CD3+ T cells per mm^2 of graft tissue. *p<0.05 by one-way ANOVA followed by Tukey's multiple comparisons test.*

4. DISCUSSION

4.1 Biological sex mismatch plays a role in NPC transplant success

Both the adult mice and the embryos used for NPC generation were from syngeneic (inbred) mouse lines, meaning they have the same genetic identity. This limits many of the immune effects sometimes seen in transplantation, allowing us to draw conclusions about the role of biological sex in the observed phenomena. Through our multiple analyses, we revealed that when male NPCs are transplanted into sites of SCI in female mice, these grafts display atypical perivascular cell density and cell debris (Fig. 7) that is associated with hypervascularization (Fig. 5 and 6). We also found that this group displayed heightened levels of CD3+ T lymphocytes infiltrating the graft tissue when compared to the other sex-matched and -mismatched groups (Fig. 8). All of these results are physiological events that are usually associated with an immunological rejection of the tissue [26, 28], meaning that the host immune system does not recognize the transplanted cells as "self." Since the genetic identity of the cells are the same between the mice and the embryos, this eliminates the possibility of these results being attributed to genetic incompatibility, except with regards to sex. Notably, the female host / male graft group was the only experimental group in this study that received cells containing a foreign (Y) chromosome. This suggests that the results we observed were due to the presence of antigens expressed by the Y chromosome of the male cells.

As is widely known in the field of biology, biologically male mammals contain both the X and Y sex chromosomes, whereas the females only contain two of the X chromosomes. This disparity between the biological sexes allows for differential development and provides many major phenotypic differences, both anatomically and physiologically. With this phenotypic

difference also comes a difference in the production of antigens on the extracellular face of each of male and female cells [29-31]. Interestingly, several human studies have shown that the HY antigens present on male cells have been attributed to rejection of organ transplants [27, 32-34]. The rejection of these organs, even on a cellular level, sparks the question of whether such immunological reactions also occur in cellular transplants, such as NPC transplantation for SCI. In our study, we observed characteristic immune reactions only within the female host / male graft group, whereas male host /female graft animals did not display these results. We attribute this to the lack of the Y chromosome in the female tissue, whereas the male tissue contains one X chromosome, allowing it to recognize the antigens from the X chromosome. This finding is incredibly important to the field of cellular transplantation as it reveals a potential way to increase the efficacy of cellular transplantation. Therefore, if we continue to see this enhanced immune reaction occurring in biologically female mice receiving grafts from biologically male mice, then we would be able to significantly improve our cellular transplant techniques. Within the field of NPC transplantation into sites of SCI, this finding would enhance successful survival and integration of NPCs into sites of SCI, allowing us to take a great step forward in SCI treatment. These results would provide an increased success rate of the grafts within the host tissue, allowing the field of neuroscience to move one step closer to elucidating the first cure for SCI.

4.2 Implications for clinical treatment

With many experiments involving model organisms to study disease or injury models found in human tissue, there is always the question of how the results will carry over into human physiology. Despite our studies being performed in genetically identical mice, several studies in the past have displayed how physiological results in mice can reflect results that would occur in

humans [13, 23, 33]. This further increases the impact of the results we found in this study since they can be applied to human treatment of SCI to a certain extent. We also have found that the results that we have seen in our cell transplantation study have been similar to what has been studied with human organ transplantation. Several studies have shown that a sex mismatch of male tissue being transplanted into a female host has been associated with an increased risk of immunological rejection in organ transplants performed in humans [13, 33, 34]. Many of these studies associated this rejection with the presence of HY antigens on the male tissue, which are not recognized by the female tissue. The kind of rejection of transplanted tissue by the host immune system significantly decreases the success rates of the tissue integrating into the host physiological tissue. This usually leads to transplant failure and need for another intervention in order to alleviate the diseased physiological state. In the context of cellular transplants, a rejection of this nature would almost certainly mean a failure in the treatment due to the sensitivity and fragility of the NPCs. The site of a spinal cord injury already is a very toxic environment [35, 36] for transplanted cell survival, so adding in an immunological attack from the host tissue would further decrease the chances of successful survival, integration, and proliferation of the NPCs within the host tissue.

With all of this information, it is now easy to see the importance of further investigation of the phenomena we describe in this study, as it may play a significant role in the success of the treatment. This is especially true as we begin to transition this kind of treatment into the clinical setting. If we continue to see these results as we make this transition, then we would be able to significantly increase the efficacy of NPC graft survival and integration within the hosts. Therefore, we would increase the chances of having a successful NPC graft within the patient,

providing the best opportunity for the NPCs to rebuild the patients' lost neural circuitry and potentially restore their lost motor and sensory functions.

This study provides evidence of an immunological reaction that has never been described before in NPC transplantation studies. Our results suggest that biological sex is a major biological variable that may influence the success of NPC transplantation in humans. Given the fact that several clinical trials to examine the safety and efficacy of human NPCs for SCI are currently underway, future studies of immunogenicity in sex-mismatched human cells are warranted. With these results, we could enhance our current protocols for NPC transplantation and then carry this over to our clinical trials in humans. This would be monumental in the field of NPC transplantation since it would provide us with one more answer that moves us another step closer to elucidating a cure for SCI.

4.3 Future directions

The results of this study are truly exciting and have the potential to have an incredible impact on the field of NPC transplantation for SCI. However, there are still many questions that need to be answered before we truly understand the impacts of sex on NPC transplant success, both in the laboratory and in the clinic. In future studies, we plan to investigate the physiological and immunological impact of transplanting NPC grafts comprised of a mixture of male and female cells. This would allow us to continue to optimize the transplantation protocols we have so that we can enhance the efficacy of our NPC transplants. We will begin by analyzing grafts made of 50% male cells and 50% female cells, but depending on the results we will experiment with other mixture ratios. Given that the vast majority of rodent NPC transplantation studies are conducted using a mix of male and female donor cells, this would provide useful insight that would assist with the interpretation of physiological results.

In the current study, we have limited our observations to a time point of four weeks post-transplantation, representing a subacute phase following transplantation. With regards to the immunological reaction we have described in this study, we next plan to determine the time course of lymphocyte infiltration within the graft. Specifically, we would like to establish at what points after transplantation CD3+, CD4+, and CD8+ cells arise within the grafts. Once this is known, we would also like to see if the immune reaction that is described in this study continues to develop over a longer period than the one month that we allowed for graft proliferation. All of these details would continue to strengthen our understanding of the immune reaction we described, allowing for immunosuppressive drugs to be applied at correct time to prevent this reaction. Once again, this would improve the graft's success within the host, which increases the potential of it restoring lost circuitry.

Even with answers to all of these questions, we still have many other questions that would need to be answered before a potential cure could be made. However, with the recent advancements in technology, the cure for SCI is becoming more of a reality than a dream. One avenue of research that displays this is the development of iPSCs from host cells that could be pushed to neuronal fate [6]. Once this technology is further studied and refined, it would eliminate the potential for rejection and provide the best opportunity for regeneration of lost neural circuitry following SCI. This along with all of the aforementioned questions will help us create the most successful outcomes for the patient and bring us closer to developing a safe and effective treatment for SCI.

5. CONCLUSION

5.1 It's not me, it's you.

This study has brought forth many results and conclusions that have the potential to impact the way NPC transplantation is performed in the field of spinal cord injury. With the findings that there is extensive hypervascularization, increased perivascular cell density, along with heightened T-cell density in the female host / male graft group, it is clear that biological sex mismatch provokes an immune response in our model. This result would provide researchers, not only in the field of neuroscience, but also even researchers in all other fields of cell transplantation to refine their methods in a way that would increase the overall success of the grafts. With this, we would be able to move our fields forward significantly and, in the case of this study, bring us closer to a treatment that could change the lives of hundreds of thousands of patients across the world. However, this does not mean that this research should stop here. There are still many questions that need to be answered before the most efficient and effective method can be elucidated for NPC transplantation into sites of SCI. As was previously mentioned, the time course of the phenomena described in this study needs to be characterized and more study of the potential reactions involved with grafts composed of mixed male and female NPCs. The answers to these and many other questions would provide information and evidence that would be able move the field of SCI research forward significantly.

Altogether, this study provides evidence for phenomena never previously seen that have the potential to impact many current and future studies, including current clinical trials. Additionally, since these results show a female host potentially rejecting a male graft, they can

also be closely analogized to every day interactions amongst our peers. This allowed us to bestow a fitting and memorable title for the study: "*It's not me, it's you*".

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