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Cross-species comparisons between pigs and mice reveal conserved sex-specific intraspinal inflammatory responses after spinal cord injury

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Abstract

Objective Therapeutic translation is challenging in spinal cord injury (SCI) and large animal models with high clinical relevance may accelerate therapeutic development. Pigs have important anatomical and physiological similarities to humans. Intraspinal inflammation mediates SCI pathophysiology. The purpose of this study was to evaluate the effect of sex on inflammation and outcomes in a pig thoracic contusion/compression SCI model.

Methods Adult (gonad-intact) male and female Yucatan miniature swine were subjected to either SCI or sham (laminectomy-only) injury.

Results SCI caused locomotor dysfunction (measured with the Porcine Thoracic Injury Behavior Score) with some recovery over 6 weeks and limited tissue sparing at 6 weeks with no difference between sexes. Immunohistological evaluations of spinal cord tissue at 2 days and 6 weeks post-injury revealed intraspinal microglia/macrophage (IBA-1, CD68) and lymphocyte responses (T-cells (CD3) and B-cells (CD79a)) consistent with observations in rodents and humans. Astrocyte (GFAP) immunoreactivity was observed within the lesion core at 6 weeks in contrast to observations in rodents. No differences were seen for astrocytes, microglia, macrophages, B-cells, and neutrophil infiltration between males and females. Intraspinal CD3 + T-cell counts and T-cell microclusters were significantly higher in females compared to males 6 weeks post-injury. Interestingly, we observed a similar significant increase in intraspinal CD3 + T-cell accumulation in female vs. male mice at 6 weeks post-thoracic contusion SCI.

Interpretation Our observations indicate that sex is a potential biological variable for T-cell infiltration and may contribute to sex-based differences in SCI pathophysiology and recovery outcomes.

Keywords Gender, Immune, Neurotrauma, Adaptive, Innate, Neuron, Porcine, Swine

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Introduction

Researchers in the spinal cord injury (SCI) field have predominately utilized rodents for modeling and experimentation. Despite significant advances in the development of therapeutic approaches in rodent SCI models, few effective therapies have translated to clinical application. The lack of translation raises concern that preclinical testing in rodents has limitations in predicting outcomes of clinical trials; thus, the evaluation of SCI neuropathology in large animal species is gaining attention as a necessary intermediate step between discovery research in rodent models and clinical application [1]. Researchers are increasingly using swine (e.g., pig or porcine) SCI models to mimic human disease conditions based on conserved neuroanatomical, physiological, and inflammatory similarities between the two species [2]. For example, the porcine corticospinal tract resides laterally in the white matter within the spinal cord with greater similarity to the human anatomical organization than rodents [3]. The pig immune system also shares remarkable similarities with humans specifically in microglia gene signatures and T-cell responses to trauma [4–6].

One important consideration for the development of translational therapies is understanding the potential for sex as a biological variable that affects neuropathy, recovery, and ultimately therapeutic efficacy [7]. We previously observed that contusion SCI yields subtle sex differences in mice. Although motor function was largely similar between sexes, all of the male mice in our previous study, but only half of the females, recovered plantar stepping after moderate-severe thoracic SCI [8]. In the same model, we observed that the response to analgesic therapy for SCI pain is sex-specific [9]. Coincident with these sex-specific motor and sensory outcomes, we observed that intraspinal inflammation after SCI significantly differs between male and female mice and that lesion sizes tended to be sex-specific [8, 10, 11]. Since intraspinal inflammatory responses to SCI are implicated in the progressive loss of spared and damaged tissue, sex-specific inflammatory responses to SCI may be of translational relevance.

Similar evaluations of sex as a biological variable in SCI recovery and inflammation have not been reported after pig SCI. Indeed, most porcine SCI studies utilize female pigs. This may be due, in part, to the challenges of neurogenic bladder management after SCI in male pigs. Retrograde urethral catheterization in male swine is complicated by the anatomy of the swine penile urethra which has a urethral sigmoid flexure and diverticulum that misdirects the catheter into the recess and not the bladder ([12] Chap. 9). To overcome this challenge, we have developed techniques to manage neurogenic bladder after SCI in male pigs using a cystostomy and temporary indwelling suprapubic urinary catheter that drains into an external collection bag based on techniques back-translated from human clinical practice.

Here we used immunohistochemical methods to evaluate and compare the cellular inflammatory response to SCI in male and female pigs. We explored the activation and infiltration of astrocytes, neutrophils, mononuclear phagocytes, T-lymphocytes, B-lymphocytes, and macrophages after porcine thoracic contusion/compression SCI at 2- and 42- days post-injury (DPI). We compared and validated our observations in pigs to a mouse thoracic contusion model. In the pig model, we also examine sex as a biological variable in locomotor recovery.

Materials and methods

Experimental design

Adult male and female gonad-intact Yucatan pigs were randomly assigned to receive a moderate contusion/compression spinal cord injury (SCI) or sham injury (laminectomy only) in the mid-thoracic region (thoracic level 10, T10). Estrous cycle of female pigs was determined by vaginal smears at the time of SCI (data not shown), but not used in subsequent analyses. The pigs were individually housed in our specialized acute care housing with 24-hour attendance for up to 10 days after injury and then returned to modified (food, water, enrichment lowered for access) group housing for the duration of the study. Study endpoints were set to assess acute injury responses (~48 h, 2 days) or sub-acute responses (6 weeks) after SCI or sham surgery. All in vivo animal procedures including handling/acclimation, behavioral training and assessment, SCI and cystotomy surgery, acute and sub-acute daily animal care, and humane euthanasia, tissue extraction, cryomolding and cyrosectioning were conducted at University of Utah (UA) in the Floyd-Schneider Lab. The spinal cord tissue was collected and shipped to the Gensel Lab at the University of Kentucky for subsequent histological analysis. Mouse histological outcomes utilized tissue generated in the Gensel Lab and reported previously in [8] and confirmed in an independent tissue cohort (data not shown).

Porcine model of spinal cord injury and associated cystotomy

All experimentation was approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah, the Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals [13], and U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training as well as the Animal Welfare Act. Prior to SCI, adult male and female gonad-intact Yucatan miniature swine were weight-matched (30–40 kg) and then randomly assigned to receive either an SCI or laminectomy only (LAM;

sham control injury) at the thoracic vertebral level (T10). Before surgery, sedation/anesthesia was induced by intramuscular injection of Telazol (4.4 mg/kg), ketamine (2.2 mg/kg) and xylazine (2.2 mg/kg). Pigs were then intubated with an endotracheal tube and maintained on 1–4% inhaled isoflurane anesthesia. Lidocaine (1–2%) was infused into the perioperative area prior to incision. All procedures were conducted in a designated large animal surgery suite under strict aseptic conditions with continuous monitoring of heart rate, respiratory rate, end tidal carbon dioxide and oxygen saturation. Normothermic (38–39 °C) temperatures were maintained during the surgical procedures. Hydration was maintained with IV saline. For induction of the SCI, overlaying muscle and skin layers were dissected from the spinal column and a laminectomy was performed at the 10th thoracic vertebra to expose the spinal cord. The weight drop apparatus that was used for SCI induction was affixed to the pig spinal column. To achieve this, 4 multi-axial pedicle screws (3.5 × 2.4 mm Medtronic screw) were implanted into the T8-9 and T11-12, pedicles. Next, 3.2 mm titanium rods were affixed to the screws to secure the weight drop guidance system to the spinal column. A short-acting paralytic (succinylcholine 1 mg/kg, intravenous) was administered prior to injury to prevent motor discharge upon impact of the spinal cord. The impactor (50 g) was then dropped onto the spinal cord from a height of 20 cm, which corresponds to a displacement of ~ 2 mm and a force of ~ 35 N. An additional 100 g was added to the dropped weight to induce sustained spinal cord compression (150 g) for 5 min. After the 5 min. compression, the weight, impactor, and guidance system were removed, and the bone sealed with sterile bone wax. Note that the dura remains intact but visible bruising of the spinal cord tissue was observed. The same procedures were followed for LAM controls except for weight drop and subsequent compression. After completion of the procedure, the incision was closed in layers.

In the same surgical session for pigs that received an SCI, a cystotomy and implantation of an indwelling urinary catheter was performed. The scientific justification and rationale for the cystostomy is that the SCI induces a transient state of impaired bladder function wherein the animals cannot voluntarily urinate. As male pigs have a sigmoid flexure with an acute bend in the distal loop in the body of the penis that renders clean intermittent catheterization untenable, an indwelling urinary catheter was required. To keep the surgical interventions consistent for males and female, pigs of both sexes received an indwelling urinary catheter. For the cystotomy, a midline posterior abdominal incision was conducted, and the bladder was localized. A purse-string suture was placed in the ventral surface of the bladder to surround a small stab incision through which a Foley catheter (14-18FR) was placed. The Foley balloon was inflated, and the purse-string suture secured. The urinary catheter was attached to a closed system such that sterile tubing ran from the catheter to the sterile collection bag. The urine collection bag was emptied and changed as needed, using aseptic techniques.

Upon completion of all surgical procedures and after closing of surgical incisions, anesthesia was ceased and animals extubated once spontaneous respiration occurred. Animals were allowed to recover in a warmed room and once awake and alert, were then moved to our specialized acute care housing. Pigs were housed for ~ 10 days after SCI in our customized acute care setting. In acute care, pigs were housed in individualized beds comprised of the lower half of a clamshell dog kennel (XL) equipped with a memory foam dog bed, blankets, pillows, and enrichment items. Pigs were hand-fed and watered with frequent health checks and repositioning to reduce the formation of pressure sores. Feces were manually removed, and skin kept clean and dry by trained attendants. While in the acute care setting, pigs have visual, auditory, and olfactory contact with other pigs. Once robust voluntary micturition via the urethra was observed (typically 7–10 days after SCI), the urinary catheter was removed and the pig returned to a modified (food, water, enrichment lowered) group home cage environment. At the pre-specified endpoint, 48 h or 6 weeks after induction of SCI, pigs were sedated, anesthetized, and then humanely euthanized by barbiturate overdose following the American Veterinary Medical Association guidelines for the euthanasia of animals. The spinal cord tissue was collected and shipped to the Spinal Cord and Brain Injury Research Center (SCoBIRC) at the University of Kentucky (UK) for subsequent histological analysis.

Locomotor outcomes

Motor function in pigs was assessed before injury as a baseline and then weekly after SCI or LAM, starting at week 1. Briefly, animals were trained to traverse a rubber mat placed on the floor (width 1.2 m. length 5 m), with food rewards at either end. Video recordings using high-definition cameras from 4 opposing angles at the investigated time points (pre-surgery, and weekly for 6 weeks post-surgery) were assessed by 2 independent raters. Raters used the Porcine Thoracic Injury Behavioral Scale as previously described [14]. Briefly, the scale ranges from 1, no active hindlimb movements to 10, grossly normal ambulation with normal balance. Scores from 1 to 3 are associated with hindlimb movements, scores 4–6 are associated with weight-bearing extensions or limited stepping, and scores of 7–9 are associated with weight-bearing stepping with full knee extension. Scores were assigned to videos using operational definitions and the

individual conducting the scoring was naive to the group membership and time point.

Tissue processing

After humane euthanasia, pigs were exsanguinated by transcardiac perfusion with 0.1 M cold phosphate buffered cold saline (PBS). A ~ 15 cm segment of thoracic spinal cord centered around the injury site was collected. Tissue was immersion fixed in 4% paraformaldehyde (PFA) for 72 h, and cryoprotected in an increasing sucrose gradient of 10–30% over a subsequent 7 days. Spinal cord segments were divided into ~ 1 cm long block corresponding to approximately one vertebral segment. Segments at the lesion epicenter and areas immediately rostral or caudal to the SCI site were collected. Blocks were frozen in O.C.T. Compound (Tissue-Tek, Sakura Finetek USA, Inc, Torrance CA) in a dry ice/ isopentane slurry and then stored at − 80 °C for later cryostat sectioning at 30 µm thickness in the axial (transverse) plane. For slide mounting, the intersection interval was 10 sections resulting in 10 sets of tissue with a 300 µm distance between sections on the same slide.

Mice

As described earlier, we utilized tissue from 4-month-old male and female C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) generated for [8]. Animals were individually housed in ventilated cages with ad libitum access to food and water. Housing was set to maintain a 14 h light/10 hrs. night cycle at 70 degrees F and 50% humidity. All experimental strategies were conducted during the light cycle and with the approval of the Institutional Animal Care and Use Committee at the University of Kentucky.

Mouse SCI was performed as described previously [8]. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injections and the thoracic region above the spinal cord was shaved to remove overlying hair. The vertebral column from T8–T10 was exposed by skin incision and subsequent dissection of the connective and muscle tissue. A dorsal laminectomy was performed at T9. SCI animals were subjected to a moderate-severe contusion SCI (75 kdyn force) using the Infinite Horizons (IH) injury device (Precision Systems and Instrumentation) [15]. Spinal cord displacement at the time of SCI was not statistically significant between sexes (female 594 ± 20 µm; male 580 ± 24 µm; $p = 0.65$ unpaired t-test). Muscle and skin incisions were then closed using monofilament sutures. All mice were given one subcutaneous injection of buprenorphine-SR (1 mg/kg) and one subcutaneous injection of an antibiotic agent (5 mg/kg, enrofloxacin 2.27%: Norbook Inc, Lenexa, KS, dissolved in 2mL saline) immediately after surgery. Animals recovered on paper towels in cages on a 37^0C heating pad overnight. Animals were then returned to home cages. Subcutaneous antibiotic injections (5 mg/kg, enrofloxacin) were given in 1mL saline for five days. Manual bladder expression was performed on injured mice twice daily throughout the study. The current study utilized tissue from mice reported in [8]. Locomotor recovery (Basso Mouse Scale, BMS [16]) for these mice was previously reported [8]. BMS recovery was the same for both male and female mice (main effect of sex $p = 0.5$) with both groups achieving plantar stepping by 42 days post-injury (dpi); however, a significantly greater proportion of males achieved plantar stepping abilities compared with females at 42 dpi (chi-square, $p = 0.04$). Additional details can be found in [8].

Mice were anesthetized and transcardially perfused with cold phosphate-buffered saline (PBS) (0.1 M, pH 7.4: Na2HPO4 (Fisher, Cat#7558-79-4), NaH2PO4 (Fisher, 10049-21-5), NaCl (Fisher, 7210-16)), followed by perfusion with cold 4% paraformaldehyde (PFA, Alfa Aesar, 30525-89-4) at 42 days post-injury (dpi). One cm of the spinal cord centered on the laminectomy site was dissected from each animal and then post-fixed in 4% PFA for 2 h and subsequently rinsed and stored overnight in phosphate buffer (PB, 0.2 M, pH 7.4) at 4 degrees C. The following day, tissues were cryoprotected in 30% sucrose (Sigma Aldrich CO., St.Louis, MO) for 3–5 days at 4^0C, followed by rapid freezing and blocking in optimal cutting temperature (OCT) compound (Sakura Finetek USA, Inc.) on dry ice. Spinal cords were distributed randomly (by an experimenter blinded to group assignment) to tissue blocks to ensure that both sexes were represented on each slide. The blocked tissue was stored at -80^0C before sectioning. Transverse serial Sect. (10 µm) were cut through each block and mounted in serial order, with 100 µm between serial sections on Color frost Plus Slides (Fisher, 12-550-17), and then stored at -80^0C before staining.

Eriochrome cyanine and neurofilament heavy chain (NF) staining

The Eriochrome cyanine (EC) stain, in combination with NF labeling, was used as an alternative to luxol fast blue [17] to identify areas of frank tissue pathology and myelin damage. Briefly, sections were incubated in acetone following standard immunohistochemistry (IHC) protocols (described below) for neurofilaments antibody NFH (neurofilaments heavy chain; Chicken anti-NF; Cat# NFH, Aves Inc., Davis, CA, 1:1500 dilution) and washed for 5 min in water and incubated with EC stain (Sigma, E2502) for myelin for 30 min and washed with water until clear. Stained slides were then dehydrated in ethanol series (70-100%) and cleared in histoclear and cover slipped with cytoseal (Cat#23-244257, Thermo Fisher Scientific).

Hematoxylin and eosin (H&E) staining

First, the tissue sections were passed through xylene and rehydrated in ethanol series (100%, 95%). Sections were stained with Harris' hematoxylin solution for 4 min and were then rinsed in tap water until the water was colorless. Next, 1% acid alcohol was used to differentiate the tissue (quick dip) and the slides were rinsed with tap water. In the bluing step, we soaked the slides in lithium carbonate solution for 5 s and then rinsed them with tap water, and then dipped them in 80% Ethanol. Finally, staining was performed with eosin Y ethanol solution for 3 min. Slides were then dehydrated through the ethanol in series (70–100%) and cleared in xylene and mounted with cytoseal mounting media.

Immunohistochemistry

For immunohistochemistry (IHC), embedded sections were cleared in xylene and rehydrated in ethanol series (100%, 95%) and subjected to antigen retrieval in a rice steam cooker using Target retrieval solution (0.1 M Sodium citrate buffer pH 6, Sigma Cat# 6132-04-3) at 80^0C for 5 min after rehydration in phosphate buffer saline (PBS). After retrieval, sections were blocked with quenching buffer (20% Methanol, and 0.3% H_2O_2 in PBS) for endogenous peroxidases. Samples were washed 3 times in PBS. The immunoglobulin blocking step was performed for 1 h in PBS containing 0.1% Triton-X100 (Cat# T9284 Sigma Aldrich) and 5% normal goat serum (Cat# G9023, Sigma Aldrich) [18]. Samples were washed 3 times in PBS. Pig samples were incubated with the following primary antibodies: mouse anti-pig GFAP; (astrocytes; ab4648, abcam,1:500 dilution), NFH (neurofilaments heavy chain; Chicken anti-pig; NFH, Aveslabs 1:500), CD3 (T cells; Rabbit anti-pig; MA1-90582, ThermoFisher Scientific; 1:500 dilution), CD68 (activated macrophages; Mouse anti-pig MCA2317GA, Bio-Rad;1:500 dilution), IBA-1 (microglia/macrophage; Rabbit anti-pig 019-19741, Wako Chemicals USA; 1:4000), CD79a/B220 (B cell; Mouse anti-pig; MA5-13212, ThermoFisher Scientific;1:300) for overnight. After incubation with primary antibody slides were washed 3 times with PBS and followed by incubation with respective biotinylated secondary antibodies anti-chicken (cat#BA-9010, Vector laboratories) or anti-rabbit (Cat#BA-1000 Vector laboratories) or anti-mouse (cat# BA-9200 Vector laboratories) at 1:500 dilution for 1 h. Mouse samples were incubated in primary antibodies for CD3 (T cells, rabbit anti-mouse MA1-90582, ThermoFisher Scientific, 1:500) or B220/CD45R (B cell, Rat anti-Mouse; Cat# 14045281, Invitrogen, 1:200 dilution). Overnight primary antibody incubation was followed by washing in PBS, 3 times and then incubation with respective secondary antibody for 1 h; anti-rabbit (Cat#BA-1000 Vector Laboratories, 1:500) or anti-rat (Cat#BA-9400, Vector laboratories,1:500) respective to the primary antibody.

Samples stained with secondary antibodies were washed 3 times with PBS. Samples were then incubated with VECTASTAIN Elite ABC reagent (Cat# PK-6100, Vector laboratories) prepared in PBS containing 0.1% Triton-X100. Samples were washed again 3 times with PBS. Immunolabeling was visualized using 3,3′-diaminobenzidine (DAB; Vector Laboratories, Inc. Burlington, CA) as a chromogen. Cresyl violet was used as a counter stain only while staining for T and B cells. Slides were then dehydrated through the ethanol in series (70–100%) and cleared in xylene and mounted with cytoseal mounting media.

Each set of slides for immunohistochemistry, imaging, and analysis was performed for all animals (either pig or mouse) in a single cohort, using identical staining, imaging acquisition, and analysis settings. Images were acquired using Zeiss Axioscan (model Z1, Carl Zeiss AG., Oberkochen, GE) and analyzed using Halo software (Indica Labs, Albuquerque, NM). Histology and immunohistochemistry were performed by individuals blind to group inclusion. Immune cell quantification was performed within areas of frank tissue pathology identified or surrounding "spared" tissue areas through H&E or EC/NF stains as outlined in Fig. 1 and Fig. 2. Manually traced areas of pathology were superimposed on adjacent slides for immune cell quantification.

Quantification of microglia/macrophages (IBA-1, CD68) and astrocytes (GFAP)

Previously, we determined that proportional area measurement of antigen labeling is a consistent and reliable method for quantifying intraspinal inflammation after injury [19]. Areas of positive staining were identified using the algorithm modified from Indica Labs area quantification v1.0. Figure 1 depicts the schematic representation of the histological analysis of targeted markers.

Quantification of neutrophil infiltration

As was performed previously with human tissue, regions of interest included the lesion core ('core') as well as the directly surrounding 'spared' area [20, 21]. For the quantitative evaluation of Neutrophils, H&E-stained sections were overlaid by a morphometric grid (200 μm^2) and 10 grids per region of interest spanning the entire lesion were selected. Polymorphonuclear (PMN) cells were characterized as neutrophils and were manually counted and the values were expressed as cell counts per 200 μm^2.

Quantification of T cells (CD3) and B cell infiltration

For quantification of lymphocytes (T and B cells), sections were analyzed using the modified Indica Labs multiplex IHC v1.2 algorithm to identify immunopositively

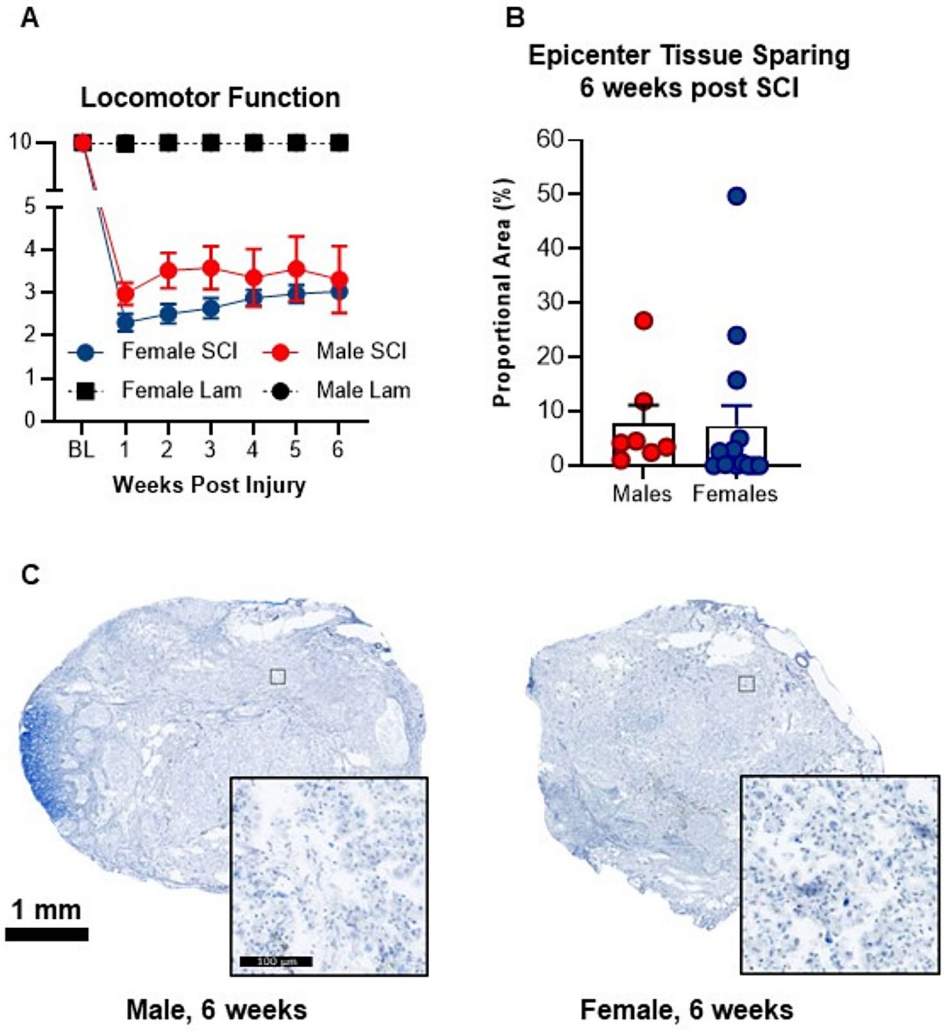

Fig. 1 Sex has no effect on locomotor recovery and tissue sparing after SCI in pigs. (**A**) Behavioral recovery on the Porcine Thoracic Injury Behavioral Scale (PTIBS) during the 6 weeks of survival post-SCI. Thoracic contusion SCI resulted in sustained hindlimb dysfunction relative to shams. Locomotor functional significantly improved over time ($p = 0.02$ main effect of time for SCI groups) with no significant differences between males and females. (**B**) There was little spared tissue at the lesion epicenter 6-weeks after injury. (**C**) EC stained (blue = myelin) sections representative of lesion pathology 6-weeks after SCI show very little spared tissue with no significant effects between males and females. ($n = 8–14$) Mean ± SEM

labeled cells. In addition, T cell clusters were counted in the lesion area of the spinal cord and defined as >5 cells within a diameter circle of 200 µm for pigs and 55 µm for mice samples respectively. All counts were done by one blinded researcher and confirmed by another.

Statistical analysis

Each animal was considered a separate subject (n) and the data are presented as means ± standard error of means (SEM). For statistical comparison, unpaired Student t-tests or Mann-Whitney tests were performed for outcomes with a single independent variable. One-way ANOVA or Mixed-effects analysis (REML) followed by post hoc multiple comparisons (Sidak's) were used when appropriate. P-values less than 0.05 were considered statistically significant during the analyses. Statistical analyses were performed using GraphPad Prism Version 9. Quantification was performed by investigators blinded to group inclusion.

Results

Sex does not affect locomotor recovery or tissue sparing in pigs

We observed a score of 10, corresponding to no locomotor deficits, with the Porcine Thoracic Injury Behavioral Scale (PTIBS) before injury and in animals receiving sham injury (laminectomy) without SCI (Fig. 1A). Consistent with previous reports examining contusion/compression SCI in female pigs [14, 22], we observed significant behavioral deficits in SCI injured animals. Specifically, by one-week post-injury, both male and female SCI animals showed a significant reduction in motor

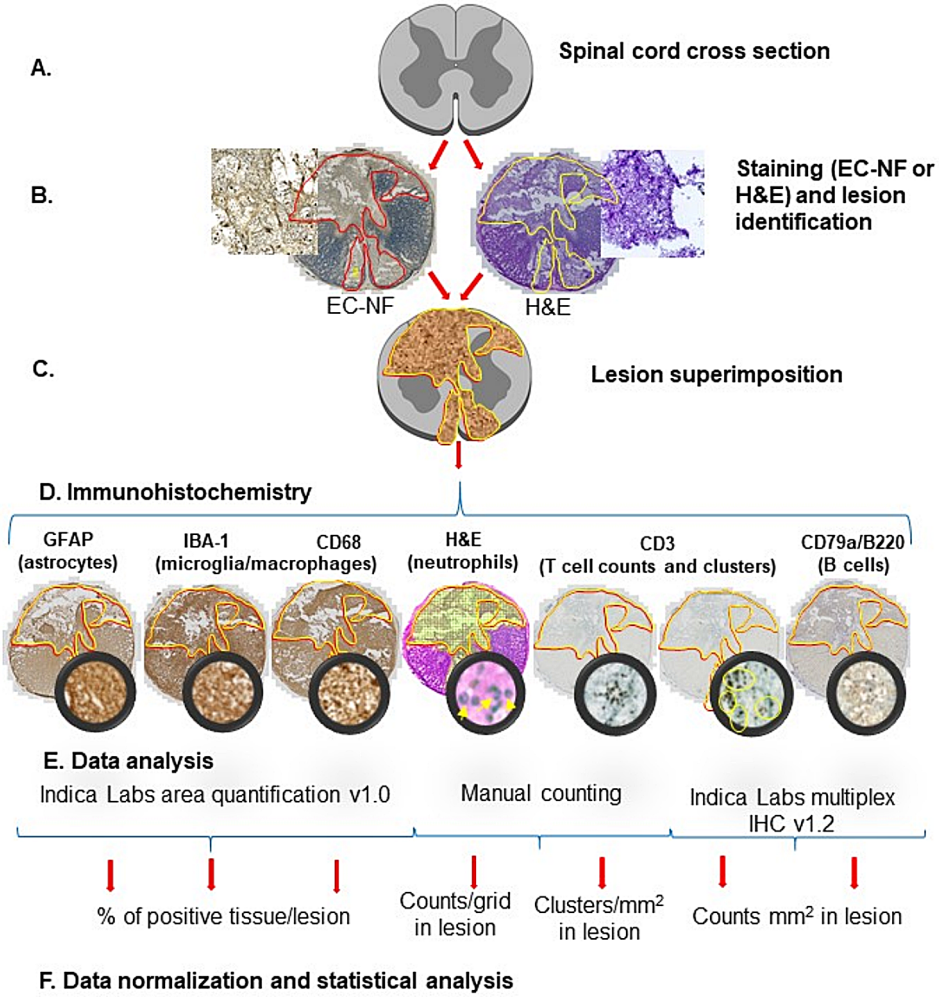

Fig. 2 Schematic representation of histological analysis of targeted markers. (**A**) Analyses were performed on transverse spinal cord cross-sections. (**B**) Spinal cord sections were stained with Eriochrome Cyanine-Neurofilament (EC-NF) or Hematoxylin-Eosin (H&E) to identify areas of frank tissue pathology. (**C**) Lesioned areas traced in EC-NF (red line) or H&E (yellow line) stained sections were superimposed on adjacent tissue sections for inflammatory cell quantification. (**D**) Immunohistochemistry for GFAP, IBA-1, CD68, neutrophils, T cells, and B cells. **(E)** Cellular quantification was performed by applying the respective method of analysis such as algorithm-based thresholding to generate proportional area or manual counting. (**F)** Data normalization and statistical analysis. All lesion tracing and data analysis was performed on Halo v2.2.1870

function with PTIBS scores of 2–3 indicative of some hindlimb movements sufficient to raise the rump and knees off the ground but no weight-supported hindlimb steps (Fig. 1A). SCI animals exhibited a statistically significant ($p = 0.02$, main effect of time for SCI groups, Mixed-effects model (REML)), but functionally modest improvements over time, gaining some rhythmic hindlimb movements and weight-supported hindlimb extensions (PTIBS ~ 3–4) (Fig. 1A). There was no significant effect of sex on locomotor recovery (main effect of sex $p = 0.13$ for SCI groups) (Fig. 1A).

At 6 weeks post-injury, we evaluated tissue sparing using standard stains for myelin (Eriochrome Cyanine) [17]. As described below, SCI resulted in a central lesion with a small rim of spared tissue. Most animals had < 5% sparing at the lesion epicenter indicative of a moderate-severe injury. There was no significant effect of sex on the percentage of spared tissue at the lesion epicenter ($P = 0.30$, Mann-Whitney Test) (Fig. 1B and C).

Distribution of injury and histopathological characterization

We next performed in-depth qualitative and quantitative analyses of cellular responses within the spinal cord in male and female pigs. In the sham injured control cases, (LAM; laminectomy only), no histopathological abnormalities were identified, apart from rare petechiae in the grey matter (data not shown). As illustrated in the experimental overview (Fig. 2), in SCI animals we used both EC/NF and H&E stains to assess tissue integrity and frank pathology. Spared white matter was defined as a region showing dense blue (Eriochrome Cyanine-myelin)

staining in EC/NF-stained tissue sections. Preserved gray matter was defined by the color of the stain as well as the appearance of normal gray matter cytoarchitecture and shape (Fig. 2B). H&E-stained sections of the injured spinal cord showed distortion and disruption of parenchyma and vessels with ensuing hemorrhage and leukodiapedesis (extravasation) in the lesion core; tissue necrosis with typical spongiotic tissue changes was also observed. Every effort was made to select representative images for illustrative purposes based on subjects that represented the statistical group mean for either time or sex for each stain. Insets for representative images were in areas of frank tissue pathology based on respective EC/NF staining.

Astrocytes form hypertrophic processes encapsulating numerous cavities post-SCI and infiltration into the lesion does not differ between male and female pigs

GFAP-positive astrocytic hypertrophic processes encapsulating numerous cavities were seen in the acute (2 days) and chronic time points (6 weeks post-injury). Some cavities were continuous with the surface of the spinal cord and may have been caused by arachnoiditis or the result of expanded central hemorrhagic necrosis [23]. Regardless, GFAP-positive cells were significantly lower within areas of damaged tissue at 6 weeks compared to 2 days ($P < 0.0001$, Unpaired T-test with Welch's correction; Fig. 3A, D and B, E and F). Ramified, GFAP-positive cells indicative of a resting/homeostatic astrocyte were abundant in tissue sections from LAM-only controls (Fig. 3C). At 6 weeks post-injury, GFAP staining was reflective of a matured glial scar organized around the perimeter of the lesion (Fig. 3D-E). Astrocyte infiltration was evident in the lesion core at 6 weeks with ~20–30% of the proportional area of the lesion positive for GFAP immunoreactivity. GFAP staining did not significantly differ between males and females at either time point (Fig. 3G and Supplementary Fig. 1A).

Intraspinal macrophage activation is rapid after SCI and does not differ between male and female pigs

We used immunohistochemical labeling with IBA-1 as a pan microglia and macrophage cell marker. CD68 was used to label activated/phagocytic myeloid cells. There was little evidence of activated macrophages within the spinal cord in the absence of SCI; in both male and female LAM controls, presumptive microglia (IBA-1$^+$) had a ramified phenotype indicative of a quiescent or resting state and there were few CD68$^+$ cells (Fig. 4C and J). In contrast to LAM controls, microglial and macrophage activation was observed at both 2 days and 6 weeks post-injury (Fig. 4A-E and H-L). Specifically, with both histologic markers, densely packed, ovoid cells were evident within lesion areas. The density of IBA1$^+$ or CD68$^+$ positive cells was significantly higher at 6 weeks compared to 2 days ($P < 0.0001$ for both stains, unpaired t-test with

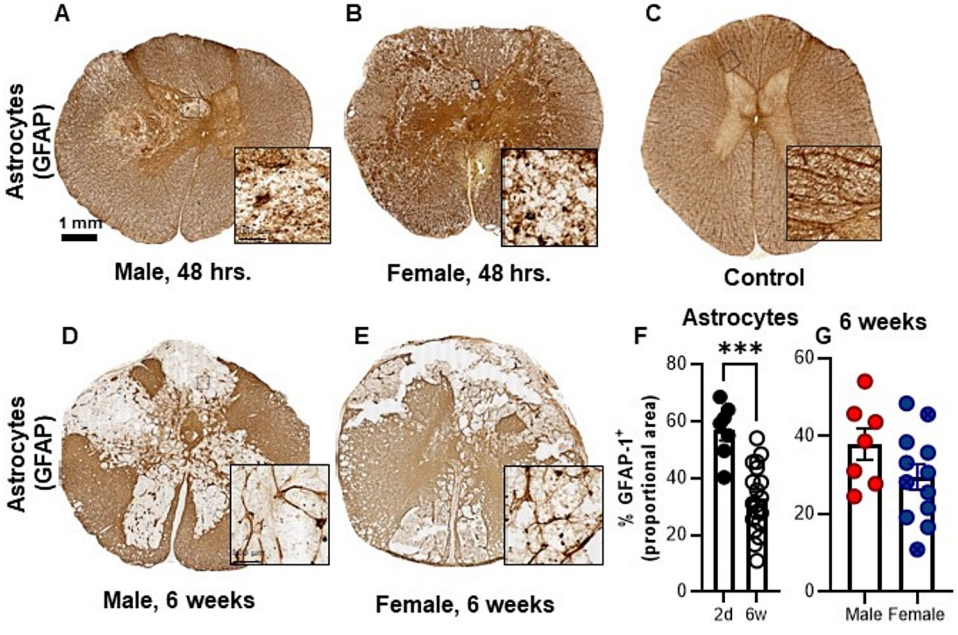

Fig. 3 Intralesional infiltration of astrocytes is evident by 6 weeks post-injury but does not differ by sex in pigs. Representative images of spinal cord sections stained with anti-GFAP antibody to label astrocytes at 2 days post-injury (**A**) in males, (**B**) in females, or (**C**) laminectomy-only, control animals. (**D-E**) Representative images of astrocytes in males and females at 6 weeks post-SCI. Insets are higher magnification of boxed, injured regions in lower power images. Notice the presence of GFAP-positive staining within the lesion epicenter. (**F**) Comparison of GFAP + proportional area in spinal cord lesion by immunohistochemistry (IHC) revealed a significant effect of time when collapsed across sex (***$p < 0.001$; 2 days $n = 7$; 6-weeks $n = 19$; mean ± SEM). (**G**) There was no significant effect of sex at 6 weeks post-SCI although ~ 20% of the area of frank tissue pathology was GFAP-positive ($n = 7$ m/12f, mean ± SEM)

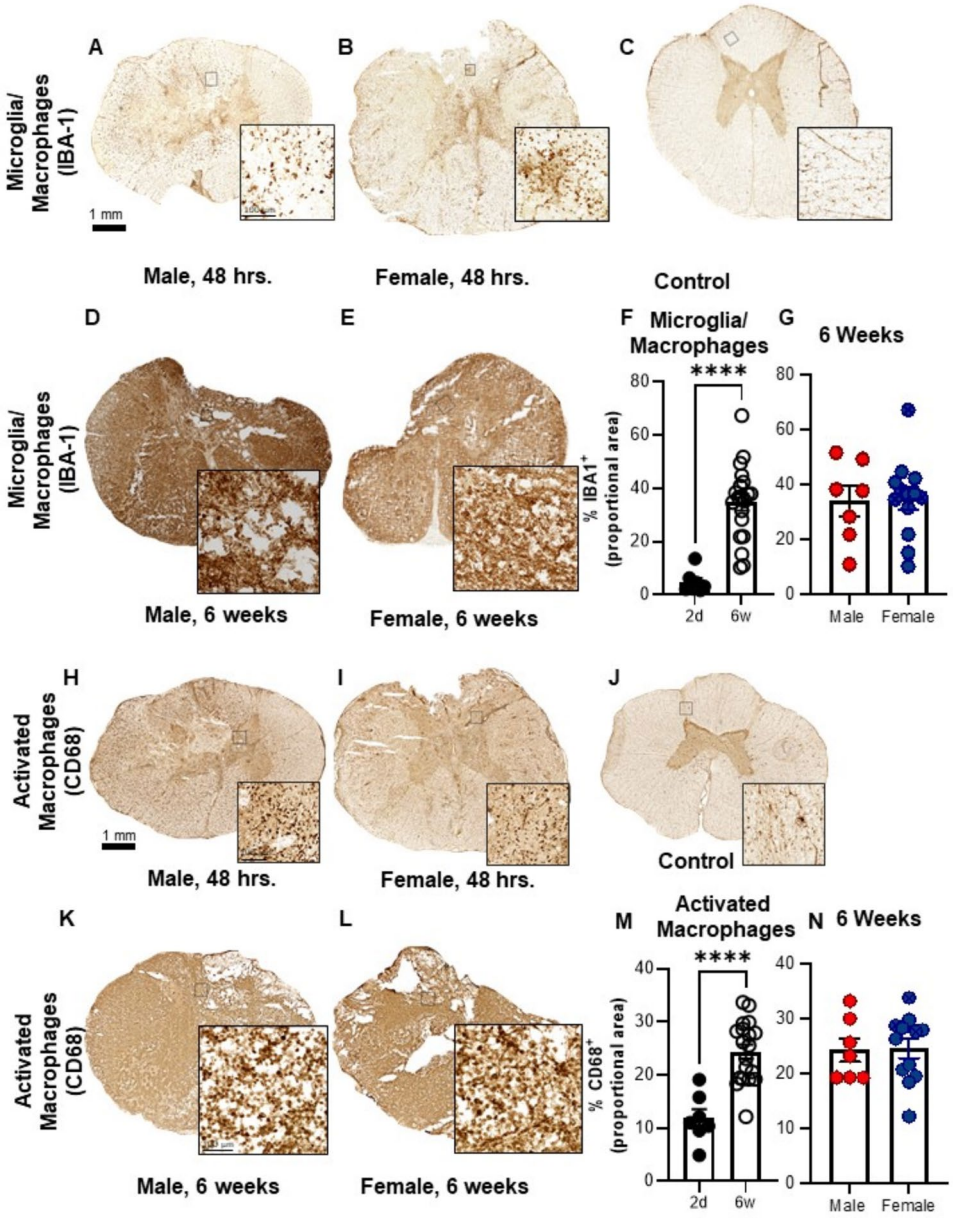

Fig. 4 Intraspinal microglia/macrophages activation increase over time after SCI but does not differ by sex in pigs. Representative pictures of spinal cord section stained for microglia/macrophages (IBA-1 marker) at 2 days after SCI for (**A**) male, (**B**) female, or (**C**) laminectomy only, control animals. (**D-E**) Representative images of microglia/macrophages in males and females at 6 weeks post-SCI. Insets are higher magnification of boxed, injured regions in lower power images. (**F**) Comparison of IBA-1 + proportional area in spinal cord lesion by immunohistochemistry (IHC) revealed a significant effect of time when collapsed across sex. (**G**) There was no significant effect of sex at 6 weeks post-SCI. Representative pictures of spinal cord section stained for activated macrophages (CD68) at 2 days after SCI for (**H**) male, (**I**) female, or (**J**) laminectomy only, control animals. Notice that very few spheroid, CD68 + macrophages appear in the absence of SCI in (**J**). (**K-L**) Representative images of microglia/macrophages in males and females at 6 weeks post-SCI. Insets are higher magnification of boxed, injured regions in lower power images. Spheroid, CD68 + macrophages are densely packed within areas of frank tissue pathology. (**M**) Comparison of CD68 + proportional area in spinal cord lesion by immunohistochemistry (IHC) revealed a significant effect of time when collapsed across sex. (**N**) There was no significant effect of sex at 6 weeks post-SCI. (****$p < 0.0001$; 2 days $n = 7$; 6 weeks, $n = 19$; mean ± SEM) ($n = 7$ m/12f, mean ± SEM)

Welch's correction; Fig. 4F and M). When macrophage/microglial densities were compared across sexes, we observed no significant differences between males and females at either time point post-injury (Fig. 4A, B and D, E, G and H, I and K, L, N and Supplementary Fig. 1B-C).

Neutrophils infiltrate the spinal cord early after SCI, are rarely seen at 6 weeks post-injury, and infiltration does not differ between male and female pigs

As in other species, immunohistochemical detection of neutrophils is challenging in the pig. Therefore, we

examined neutrophils using H&E stain based on their distinct polymorphonuclear morphology (Fig. 1D). Non-phagocytosed extravasated erythrocytes were also visible at 2 days in post-injury samples (Fig. 5G-H, green arrowhead); however, there were no overt differences between males and females. We did not observe neutrophil infiltration in LAM controls (Fig. 5C). We observed neutrophil infiltration mainly at 2 days in both male and female samples (Fig. 5A-B and D-H). We detected neutrophils within the lesion 6 weeks after SCI; however, levels were significantly lower (8.4-fold) than neutrophil counts at 2 days post-injury ($p < 0.0001$, Unpaired T-test with Welch's correction; Fig. 5F). No differences were observed for neutrophil infiltration within the lesion core between male and female pigs at either time point post-injury (Fig. 5I and Supplementary Fig. 1D).

Intraspinal T-cell infiltration differs significantly between male and female pigs after SCI

We used the pan T-cell marker, CD3 to analyze the T-cell infiltration in the spinal cord. $CD3^+$ cells were rarely observed in the uninjured spinal cord of LAM controls from either sex (Fig. 6C). Intraspinal $CD3^+$ cells were present in both sexes at 48 h post-injury, however, most were perivascular in nature (Fig. 6A-B) and there were no significant differences between sexes at this timepoint (Supplementary Fig. 1E). Intraspinal $CD3^+$ cells significantly increased by 6-weeks post-injury relative to 2 days after SCI ($P < 0.0001$, Unpaired T-test with Welch's Correction; Fig. 6D-H). At this later time point, scattered cells were present in areas of spared tissue while both scattered cells and clusters of CD3 + cells were evident within the lesion (Fig. 6I-K). Within the spared tissue,

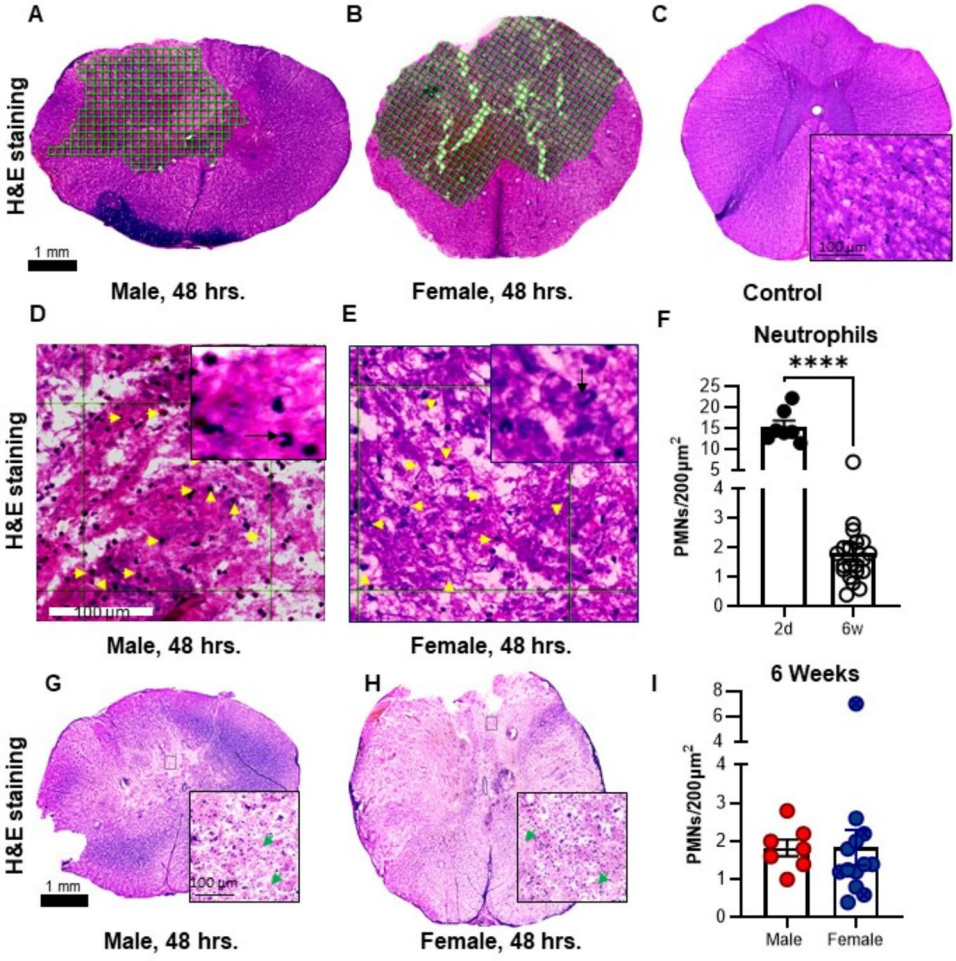

Fig. 5 Intraspinal infiltration of neutrophils occurs acutely after SCI but does not differ by sex in pigs. Representative pictures of neutrophil infiltration in spinal cord lesions after SCI for (**A**) male, (**B**) female, or (**C**) laminectomy only, control animals in H&E stained cross sections. Zoomed in representative pictures of neutrophils quantification in spinal cord lesions of (**D**) male and (**E**) female pigs at 2 days and (**G-H)** showing non-phagocytosed extravasated erythrocytes at 48 h., after SCI. Polymorphonuclear neutrophils (yellow arrowhead in **D**, **E**,) were not present in uninjured conditions and were scant at 6 weeks post-injury. (**F**) Collapsing by sex, H&E staining revealed a significant decrease (almost 10-fold) in intraspinal neutrophil activation between 2 days and 6 weeks post-injury (****$p < 0.0001$; 2 days $n = 7$; 6 weeks $n = 20$, mean ± SEM). (**I)** There was no significant effect of sex on neutrophil accumulation at 2 days (see supplemental material) or 6 weeks post-SCI ($n = 7$ m/13f, mean ± SEM)

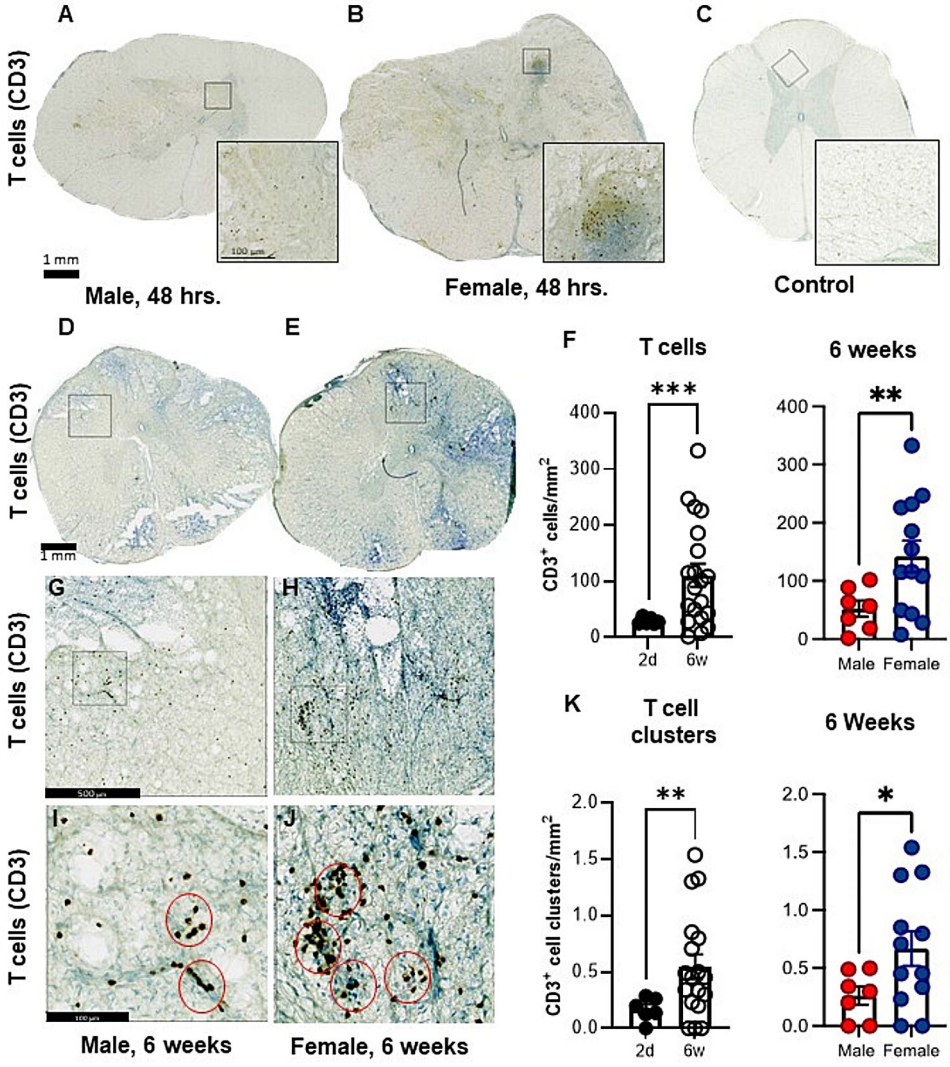

Fig. 6 Intraspinal T-cell infiltration increases after SCI and is significantly higher in female compared to male pigs at 6 weeks post-SCI. Representative pictures of $CD3^+$ T-cells in spinal cord lesion after (**A**) male, (**B**) female, or **c**) laminectomy only, control animals at 2 days after injury. Notice that CD3 + T cells were not present in the absence of SCI. (**D**-**E**) Representative images of T-cells in males and females at 6 weeks post-SCI. (**G**-**H**) Insets are higher magnification of boxed, injured regions in lower power images. Collapsing by sex, CD3 staining revealed a significant increase in intraspinal T cell counts and clusters between 2 days and 6 weeks post-injury (*$p < 0.05$, ***$p < 0.001$; 2 days $n = 6$; 6 weeks $n = 20$, mean ± SEM). (**G**-**J**) Higher powered magnification of CD3 staining at 6 weeks post-SCI. (**I**-**J**) T cell clusters (red circles) were defined as > 5 cells in 200 µm diameter. Notice the increase in the overall number and clusters in the female vs. male samples. **F** & **K**) There was a significant increase in CD3 + T cell numbers and clusters in female pigs at 6 weeks post-SCI (*$p < 0.05$, **$p < 0.01$; $n = 7$ m/13f, mean ± SEM)

CD3 + cells were sporadically distributed near neurons in some cases, diffusely distributed in spared white matter areas, and with some aggregates outside the spinal cord in the dura matter. Interestingly, dura matter aggregates were not observed in LAM conditions.

When compared across sexes, we observed a significant increase in females vs. males in the density of intraspinal CD3 + T cells within the lesion site at 6 weeks post-injury ($P = 0.01$, Unpaired T-test with Welch's Correction; Fig. 6F). When T-cell receptors (TCRs) engage with stimulatory ligands, one of the first microscopically visible events is the formation of microclusters at the site of T-cell activation [24]. Considering T-cell cluster formation as the first microscopic event indicating T-cell activation, next, we counted CD3 + T-cell clusters (> 4 cells within 200 µm distance/diameter) in the lesion core of the spinal cord 6 weeks post-injury. Similar to T-cell counts, CD3 + T-cell clusters were significantly higher at 6 weeks compared to 2 days post-injury ($P = 0.004$, Unpaired T-test with Welch's Correction, Fig. 6K). Intraspinal T-cell clusters were significantly higher in females vs. males at 6 weeks post-injury ($P = 0.038$, unpaired t-test with Welch's correction; Fig. 6K) but not at 2 days post-injury (Supplementary Fig. 1E, F).

B-Lymphocyte infiltration in the injured pig spinal cord is not sex-dependent

T- and B-cell collaboration is a hallmark of an active adaptive immune response. Therefore, based on our observation of increased T-cells in females, we examined intraspinal B-cell activation using CD79a/B220 post-SCI. As with T-cells and neutrophils, we did not observe $B220^+$ cells in the absence of SCI (LAM controls-Fig. 7C) and we observed scant $B220^+$ cells at 2 days post-SCI independent of sex (Fig. 7A-B). B220 + cell numbers significantly increased between 2 days and 6 weeks after injury (Unpaired t-test with Welch's correction; $p < 0.001$, Fig. 7A-F). We observed no significant differences in B-cell infiltration between males and females at either timepoint (Fig. 7A-G and Supplementary Fig. 1G). Qualitatively, we observed infrequent B- and T-cell clusters in both sexes.

Intraspinal T-cells numbers are significantly increased in female vs. male mice 6 weeks after SCI

To determine whether our observation of increased T-cell infiltration in females is specific to pigs or may represent an evolutionarily conserved sex-specific effect, we quantified the intraspinal T-cell response in male and female mice after a moderate-severe T9 contusion SCI. Using the same quantification method and T-cell marker, CD3, we observed significantly greater T-cell counts in the lesioned spinal cord of female mice compared to male mice 6 weeks post-SCI (Fig. 8A-B). These data were confirmed through independent analyses of mouse tissue generated 4 years apart (second cohort, data not shown). There were no significant differences in B-cell numbers between male and female mice (Fig. 8D-E). There was no significant difference in T-cell cluster formation between male and female mice (data not shown).

Discussion

Pig and other large animal models play an important role in the translational pipeline for advancement in SCI research [1], however, few studies have considered sex as a biological variable in these SCI models [25]. Further, comparative studies across rodent and pig SCI models are limited. The demographics of SCI are changing with increased incidence in females reducing the historic trends of SCI occurring primarily in males [26, 27] and the understanding of the effects of sex as a biological variable affecting immune responses is increasing (for review see [28]). Therefore, insight into the role of sex in SCI outcomes is critical to translate therapies across animal models and into clinical application. In the current study, we examined immune cell infiltration after SCI in male and female pigs and mice after contusion/compression SCI. Our data provide evidence that lymphocyte responses differ in males and females across multiple species after SCI. Specifically, we observed significantly higher numbers of intraspinal, CD3 + T-cells in female

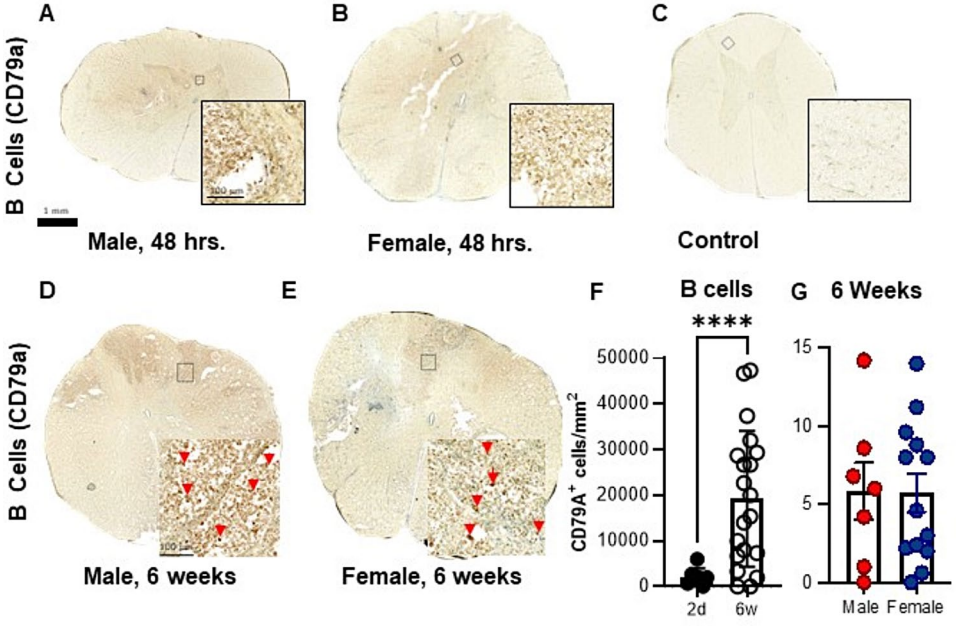

Fig. 7 Intraspinal B-cell activation increases over time but does not differ by sex in pigs after SCI. Representative pictures of spinal cord section stained for B-cells (CD79a) at 2 days after SCI for (**A**) male, **B**) female, or **C**) laminectomy only, control animals. CD79a + B cells are not evident in the absence of SCI. (**D-E**) Representative images of B-cells in males and females at 6 weeks post-SCI. Insets are higher magnification of boxed, injured regions in lower power images. **F)** Comparison of CD79a + cells (red arrowheads) in the spinal cord lesion by IHC revealed a significant effect of time when collapsed across sex (****$p < 0.0001$; 2 days $n = 7$; 6 weeks, $n = 20$; mean ± SEM). **G**) There was no significant effect of sex at 6 weeks post-SCI ($n = 7$ m/13f, mean ± SEM)

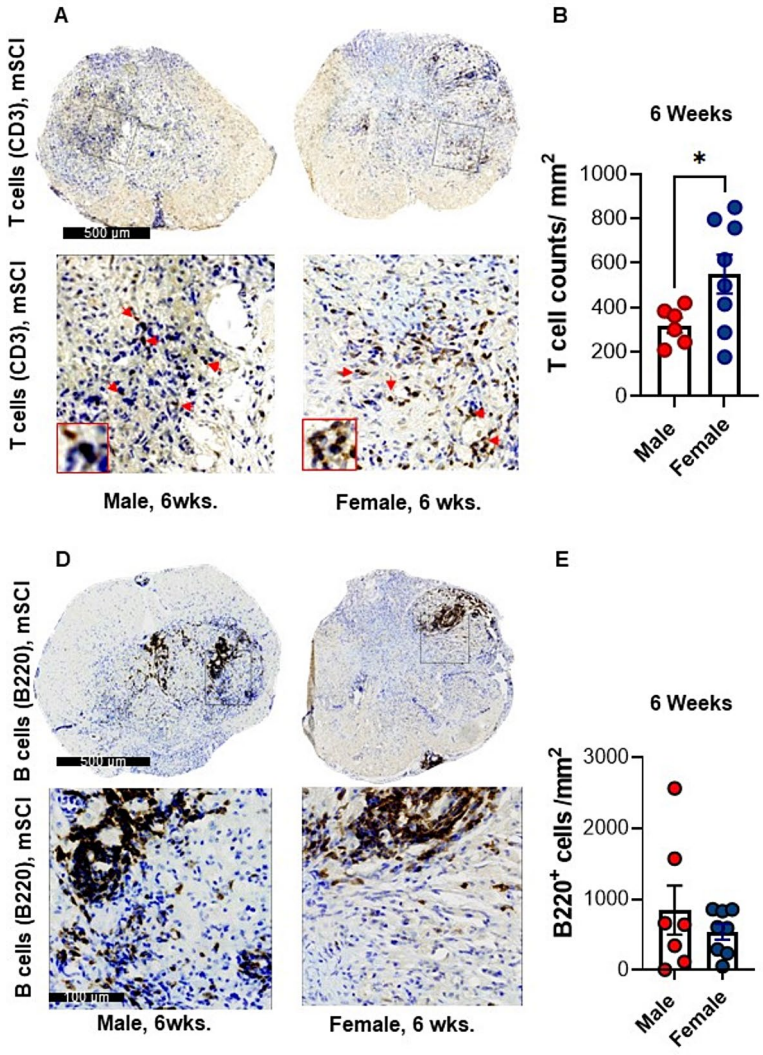

Fig. 8 Intraspinal T cell infiltration is higher in female compared to male mice at 6 weeks post-SCI. **A-B**) Representative images of male and female mouse spinal cords stained for T-cell (CD3) 6 weeks after SCI. Insets are higher magnification of boxed, injured regions in lower power images. Notice the increased CD3 cells (red arrowheads) in female vs. male samples. (**C**) T-cell counts were significantly higher in female vs. male mice 6 weeks after SCI (*$p < 0.05$; $n = 6$ m/8f, mean ± SEM). (**D-E)** Representative images of male and female mouse spinal cords stained for B cells (B220) 6 weeks after mouse SCI. There was no significant effect of sex on B-cell number ($n = 6$ m/8f, mean ± SEM)

pigs and mice, relative to males, at 6 weeks post-injury. Since inflammation is an important mediator of secondary injury, pain, and functional recovery after SCI, our findings have implications for translational, neurotrauma research.

We observed no significant differences between male and female pigs in locomotor recovery or tissue sparing 6 weeks after SCI. Our results are consistent with a growing body of preclinical work in rodents and clinical reports that gross motor recovery after SCI is subtly affected by sex, if at all [7, 29, 30]. Most sex-specific long-term, tissue-sparing outcomes are reported after moderate injuries and are of modest effect [7, 8, 30]. The relative severity of the current injury (< 10% sparing) likely limited the ability to detect sex-specific tissue-sparing outcomes.

We and others reported that the magnitude of the microglia and macrophage responses in rodents to SCI is sexually dimorphic 3 days after injury [11, 31]. One limitation of the current study is that our animal numbers by sex were insufficient for detailed comparisons between males and females at 2 days post-injury. Instead, sexes were collapsed to examine cellular trends over time. We were able to compare the effect of sex at 6 weeks post SCI and observed no significant differences in microglia/macrophage responses between male and female pigs. This observation is consistent with previous observations in rodents that sexually dimorphic responses in intraspinal macrophage accumulation normalize by chronic

timepoints [30, 32]. Our observation that astrocyte responses 6 weeks after injury do not differ between male and female pigs is also consistent with previous observations in rodents [30, 32].

Overall, the timing and morphological properties of the inflammatory responses to SCI that we observed in pigs are comparable to reports in humans and rodents. Specifically, with the exception of neutrophils, all other inflammatory cells (microglia and macrophages, astrocytes, T-cells, and B-cells) significantly increased from 2 days to 6 weeks post-SCI. Activated microglia and macrophages were prominent within the lesion core at 6 weeks post-injury as reported in rodents and humans [20, 33–35]. Neutrophils were prominent within the lesion at 2 days post-injury but were largely absent at later time points. Similar to humans and rodents, adaptive immune cells, B and T-cells, were almost undetectable in sham conditions and at 2 days after SCI but significantly increased within areas of frank tissue damage by 6-weeks [20, 33–35].

Astrocyte responses followed a similar time course in pigs as reported in other species with increased activation and cellular hypertrophy over time. GFAP + astrocytes clearly demarked areas of damage and spared tissue in the pig at 6 weeks post-SCI, indicative of a perimetric glial scar as reported in rodents [36]. Dense areas of astrocytic activation can encapsulate areas of hemorrhagic necrosis as well as areas of expanded arachnoiditis [23]. Interestingly, we detected GFAP + processes within areas of frank tissue pathology at 6 weeks post-injury which is uncommon after rodent SCI [36]. In contrast, GFAP + cells are reported in lesioned areas in humans and non-human primates [36]. Arachnoiditis has been posited to contribute to GFAP-positive fragments in damaged areas after rat SCI [23]. Although mechanistic interrogations of arachnoiditis after SCI have concluded: "(1) arachnoiditis is cleared in a timely fashion following injury, or (2) it is not severe enough to lead to alterations in parenchymal pathophysiology" [37] the presence of arachnoiditis increases with injury severity [38]. Given the severity of the injury modeled here, we cannot determine nor discriminate between astrocytic processes in lesioned areas caused by arachnoiditis vs. central hemorrhagic necrosis. Nonetheless, to the best of our knowledge, the infiltration of astrocytes into areas of frank tissue pathology at chronic timepoints has not been reported in pigs and supports the concept that pigs may have important predictive value for translational therapies targeting the glial scar.

One important aspect of this study is that female pigs and mice had increased $CD3^+$ T-cell accumulation in the spinal cord 6 weeks after thoracic contusion/compression injury. To the best of our knowledge, this is the first report of a sexually dimorphic adaptive immune response in either species. These data are somewhat inconsistent with a previous study that examined T-cells after rats. Specifically, Osimanjiang W. et al., [30] reported no significant effect of sex on intraspinal CD4 + and CD8 + T-cell accumulation 42 days after rat thoracic contusion SCI. In that study, $CD8^+$ cells were largely absent as reported previously in rats [35]. The different T-cell markers likely contribute to the conflicting results of the current study and Osimanjiang W. et al., We utilized CD3, a pan T-cell marker not expressed by pig or mouse microglia [5]. In contrast, CD4 and CD8 are specific for helper and cytotoxic T-cell subsets respectively. A limitation of the current study is that we did not look at specific T-cell subsets. The availability of tissue sections and the cost-benefit ratio of homogenizing expensive to generate pig tissue for flow cytometry vs. anatomical evaluation limited our ability to do deeper phenotyping in the current study. CD4 and CD8 can be expressed by microglia and macrophages in the injured spinal cord [34], further complicating phenotypic analyses on tissue sections. Future investigations into T-cell subsets after pig SCI are needed and may provide consistency when markers are directly compared to the observations made in rodents.

Despite only subtle sex differences in overt SCI outcomes, there is growing evidence that secondary injury processes and secondary physiological consequences of SCI differ between males and females. Multiple independent research groups and clinical studies report sexual dimorphisms in SCI-induced pain, cardiovascular disease risk, respiratory outcomes, depression, etc [7, 30, 31, 39–43]. Increasingly, therapeutic interventions to treat these conditions have sex-specific efficacy [9, 29] and SCI-induced inflammation contributes to secondary complications. Our current report identifies a cross-species increase in intraspinal T-cells in pigs and mice after SCI. This sexually dimorphic adaptive immune response likely contributes to several comorbidities of SCI and should be considered in translational research and application.

Conclusion

We report novel data regarding SCI recovery and neuropathology in male and female pigs. We also discovered and report for the first time that intraspinal t-cell responses are sex-specific and that this response is conserved across species. Our data fulfill an important translational research gap by bridging rodent and large animal models of SCI.

Supplementary Information

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Supplementary Material 1

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Author contributions
Conceptualization: CF & JG. Experimental design and analyses: RK, AS, TN, CF, JG. Experimental analyses and execution of experiments: RK, GH, HH, AS, SM, TN, LS, CF, JG. Manuscript preparation and writing: RK, CF, JG. Material support CF & JG.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethical approval
All experimentation was approved by and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah and University of Kentucky, the Office of Laboratory Animal Welfare Guide for the Care and Use of Laboratory Animals, and U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training as well as the Animal Welfare Act.

Consent to participate
not applicable.

Consent for publication
not applicable.

Competing interests
The authors declare no competing interests.

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