Periocular and Intra-Articular Injection of Canine Adipose-Derived Mesenchymal Stem Cells: An *In Vivo* Imaging and Migration Study

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Abstract

Purpose: Immune-mediated diseases affect millions of people worldwide with an economic impact measured in the billions of dollars. Mesenchymal stem cells (MSCs) are being investigated in the treatment of certain immune mediated diseases, but their application in the treatment of the majority of these disorders remains largely unexplored. Keratoconjunctivitis sicca can occur as a result of progressive immune-mediated destruction of lacrimal tissue in dogs and humans, and immune-mediated joint disease is common to both species. In dogs, allogeneic MSC engraftment and migration have yet to be investigated *in vivo* in the context of repeated injections.

Methods: With these aims in mind, the engraftment of allogeneic canine MSCs after an injection into the periocular and intra-articular regions was followed *in vivo* using magnetic resonance and fluorescent imaging.

Results: The cells were shown to be resident near the site of the injection for a minimum of 2 weeks. Analysis of 61 tissues demonstrated preferential migration and subsequent engraftment of MSCs in the thymus as well as the gastrointestinal tract. These results also detail a novel *in vivo* imaging technique and demonstrate the differential spatial distribution of MSCs after migration away from the sites of local delivery.

Conclusion: The active engraftment of the MSCs in combination with their previously documented immunomodulatory capabilities suggests the potential for therapeutic benefit in using MSCs for the treatment of periocular and joint diseases with immune involvement.

Introduction

GLOBALLY, DRY EYE DISEASES (DED) are common reasons that patients seek eye care.^{1–4} Significant morbidity, in the form of superficial corneal erosions, corneal ulcers, and decreased visual acuity, dramatically reduce quality of life.^{1–3} The overall financial burden of DED is ~\$3.84 billion annually in the United States alone.⁴ Keratoconjunctivitis sicca (KCS) is one form of DED that results in reduced or complete absence of tear production by the lacrimal tissue.⁵ The cause of KCS is frequently Sjögren's syndrome, an autoimmune disease that results in destruction of the lacrimal gland.^{5,6} Treatment of DED that includes an autoimmune or inflam-

matory component typically consists of a topical immunosuppressant (cyclosporine) and/or an anti-inflammatory agent (corticosteroids).²

The coincidence of other systemic autoimmune or inflammatory diseases in patients with DED is as high as 71.8%.^{3,7} Rheumatoid arthritis (RA) is an autoimmune disease that is associated with DED. RA affects the joints and synovium of ~1.3 million adults in the United States with an annual economic cost of ~\$14.8 billion.^{8,9} The significant morbidities associated with RA dramatically reduces quality of life and can result in premature death.^{8,10} In RA-related DED cases, treatment using systemic and topical immunosuppressants is recommended by the American Academy of

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Ophthalmology.^{2,3} Additionally, immune-mediated mechanisms contribute to induction and progression of joint diseases of diverse origins in humans and dogs.¹¹

One possible therapeutic approach for treatment of immune mediated disorders is the use of mesenchymal stem cells (MSCs). MSCs are adult-derived stem cells that can be obtained from a variety of tissue sources including bone marrow^{12,13} and adipose tissue.^{14–16} Adipose-derived (Ad) MSCs have been isolated and characterized from several animal species including dogs.^{15,17,18} MSCs are defined by their ability to undergo tri-lineage differentiation (osteocytes, chondrocytes, and adipocytes) and their surface expression of a cluster of differentiation (CD) markers. Although there is no single definitive MSC marker, a consensus is that MSCs should express CD44 and CD90, and should not express CD34, CD45, CD80, CD86, or major histocompatibility class (MHC)-II.^{14,15,19,20}

Ad-MSCs are being investigated for the treatment of a variety of disorders and diseases including skin regeneration, spinal cord injury, diabetes, and immune disorders (including RA).^{19,21,22} MSCs are also being investigated for treatment of immune disorders due to their ability to modulate both innate and adaptive immunity.²² Although the mechanism(s) of immunomodulation remain incompletely understood, MSCs inhibit T-cell proliferation, and alter B-cell function.^{22,23} MSCs also downregulate MHC II, and inhibit dendritic cell maturation and differentiation.²² In addition to preclinical investigations, Ad-MSCs and MSCs from other sources are currently being used as therapeutics for a number of clinical applications ranging from bone grafting to treatment of immune disorders including Crohn's disease and graft versus host disease.^{21,22,24,25} Ad-MSCs can be obtained from both autologous and allogeneic sources. The use of allogeneic MSCs in patients is possible due their low immunogenicity.^{23,26,27} The use of cells from allogeneic sources allows for rapid initiation of therapy without the need for harvesting MSCs from each patient. Ad-MSCs can be administered both systemically and locally in the treatment of disorders. Several routes of administration have been investigated and shown to impact engraftment and migration.^{28–32} The fate of locally administered MSCs, however, remains under investigation.

The ultimate site of MSC engraftment is an important aspect of any cellular therapy. Although there is a rich literature investigating systemic and local delivery of MSCs, there is an appreciable knowledge gap regarding the migratory potential and ultimate site of MSC engraftment after repeated delivery of MSCs. In order to investigate the engraftment and migratory potential of allogeneic MSCs in the context of repeated doses, we labeled allogeneic Ad-MSCs with iron oxide nanoparticles or a fluorescent dye and tracked these cells *in vivo* and *ex vivo*. We monitored MSC engraftment and migration of periorbital injected MSCs in a large animal model. In order to investigate safety and engraftment of larger doses of MSCs, we also monitored the engraftment and migration of intra-articular injected MSCs.

Methods

Animals use and tissue culture

All animals and protocols in this study were approved by the Institutional Animal Care and Use Committee (IACUC)

at the University of California, Davis. MSCs were isolated from subcutaneous fat collected from the tail head of 6 clinically normal, 1 year-old purpose bred beagles and were cultured as previously described.^{17,33} Briefly, 10–13 g of fat was minced and rocked at 37°C for 2 h in 50 mL of Dulbecco's Phosphate Buggord Saline (DPBS) (Invitrogen, Carlsbad, CA) with 0.1% collagenase/1% bovine serum albumin (Worthington, Lakewood, NJ) followed by centrifugation to remove the lipid layer and repeated washes with DPBS. Cell pellets were re-suspended with culture media [low-glucose Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Manassas, VA), 10% fetal bovine serum (HyClone, Inc., Logan, UT), and 1% Pen Strep (Invitrogen)], plated, and incubated at 37°C, 5% CO₂. Cells were passaged at ~70% confluence.

Ad-MSC phenotype

Three Ad-MSC lines were characterized using a panel of 12 monoclonal antibodies (including a negative control) using flow cytometry. Briefly, 1×10^6 Ad-MSCs were incubated with 25 μ L of antibody for 30 min at room temperature (Table 1). The cells were then washed and pelleted twice followed by secondary labeling with 50 μ L FITC conjugated horse anti-mouse IgG (Vector Laboratories, Carpinteria, CA) for 20 min followed by washing. Flow cytometric measurement of 20,000 cells per antibody was performed using an FACScan flow cytometer (Becton Dickinson, San Jose, CA) with analysis using FlowJo software (version 8.6.3; Tree Star, Inc., Ashland, OR).

Iron oxide labeling of Ad-MSC

NIMT™ FeO label (275 μ g; Genovis, Lund, Sweden) was dissolved in 5 mL sterile distilled water. The iron oxide was added to MSCs in 55 mL of culture media, and MSCs were incubated at 37°C, 5% CO₂ overnight. Excess label was removed by washing with DPBS (Invitrogen), and cells were centrifuged for 5 min and then resuspended in DPBS 3 times, resulting in an FeO label of 25 pg/cell. Cells were then suspended in DPBS at a concentration of 10^6 cells/mL for injection. Viability was assessed by Trypan blue exclusion before and after labeling with iron oxide. After labeling, MSCs were plated at 5,000 cells/cm² in a T25 tissue culture flask to determine cell proliferative ability post iron oxide labeling. A cytospin of labeled cells was also prepared for Prussian blue staining (iron stain) to confirm strong MSC uptake of iron particles.

Magnetic resonance imaging

To evaluate proof of concept for monitoring persistence of MSCs after local delivery, in 1 dog, 2×10^6 MSCs in 0.2 mL DBPS were injected into the periorbital tissues of the right eye both superficially and adjacent to the orbital ligament under ultrasound guidance. DBPS injections of an equal volume were performed in the corresponding location of the left eye as a negative control. Magnetic resonance images of the head were acquired before FeO-labeled MSC injection, immediately after injection, and 2 days after injection. Images were acquired on a 1.5T scanner (Signa LX; General Electric Co., Milwaukee, WI) and included T1-, T2-, and proton density-weighted spin echo images at 3 mm image collimation, and 3D T1 spoiled gradient echo (SPGR) contrast-enhanced

TABLE 1. MONOCLONAL ANTIBODIES USED TO CHARACTERIZE ADIPOSE-DERIVED-MESENCHYMAL STEM CELLS

Antigen	Clone	Source	Label	Expression
CD44	S5	B. Sandmaier ^a	Adhesion	Positive
CD54 (ICAM-1)	CL18.1D8	C. Smith ^b	Adhesion	Positive
CD90 (Thy-1)	CA1.4G8	P.F. Moore ^c	Stem cell marker	Positive
anti-feline CD1a	FE1.5F4	P.F. Moore	Negative Control	Negative
CD3	CA17.2A12	P.F. Moore	T-cells	Negative
CD18	CA1.4E9	P.F. Moore	Leukocytes	Negative
CD34	1H6	P. McSweeney ^a & R. Nash ^a	Hematopoietic lineage cells	Negative
CD45	CA12.10C12	P.F. Moore	Pan-leukocytes	Negative
CD49d (VLA-4)	CA4.5B3	P.F. Moore	Adhesion	Negative
CD80	CA24.5D4	V.K. Affolter ^c	T-cell costimulator	Negative
CD86	CA24.3E4	V.K. Affolter	T-cell costimulator	Negative
MHC class II	CA2.1C12	P.F. Moore	MHC-II	Negative

Ad-MSCs were characterized by flow cytometry and positive for the adhesion markers CD44 and CD54 as well as the stem cell marker CD90 (Thy-1). In conjunction, Ad-MSC population did not express CD34 (hematopoietic lineage marker), CD45 (pan-leukocytes), or MHC class II. Isolated Ad-MSC did not express T-cell markers: CD3, CD80, or CD86 nor leukocyte marker CD18 or adhesion marker CD49d.

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^bHouston, TX.

^cUniversity of California, Davis.

Ad-MSCs, adipose-derived mesenchymal stem cells; CD, cluster of differentiation; MHC, major histocompatibility class.

images (0.1 mmol Gd/kg Gadopentetate dimeglumine, IV, Magnevist; Bayer HealthCare Pharmaceuticals, Inc., Wayne, NJ) with 1 mm image collimation. A T2* Gradient echo sequence was acquired on the day of injection, and an STIR sequence was acquired 2 days after injection, both with 3 mm collimation.³⁴

DiD labeling of Ad-MSC

In vivo fluorescent detection of the DiD signal was used to determine the residence time of periacral and stifle injected Ad-MSCs and their subsequent distribution. Ad-MSCs were labeled according to the manufacturer's protocol with Vybrant DiD (Invitrogen). Briefly, adherent Ad-MSC were washed twice with DPBS and removed from the flask with 5 mL of HyQase (HyClone, Inc.) for 10 min at 37°C, 5% CO₂. MSCs were pelleted, washed twice with DPBS, and re-suspended at 2 × 10⁶ cells/mL in warm, serum-free, DMEM (Invitrogen). Ad-MSCs were then incubated for 15 min at 37°C, 5% CO₂ with 5 μL/mL of DiD. After labeling, the cells were washed thrice, pelleted with DPBS, re-suspended in

DPBS, and transferred to a sterile glass vial with protection from the light.

Transplantation

Six dogs that were not included in the MRI injection course received repeated allogeneic, unlabeled injections of 2 × 10⁶ cells in 0.2 mL into the right periacral area and the region surrounding the gland of the third eyelid (Fig. 1). The left side was injected with DPBS. These injections were performed on a weekly basis for the first 6 weeks of the 10 week study course. Two injections of labeled cells were performed in an attempt to determine both residence time and whether the dynamics of the migration of the injected cells were altered by previous exposure. Three dogs were injected with allogeneic, DiD labeled Ad-MSCs (2 × 10⁶ cells in 0.2 mL) into the right periacral and the region surrounding the gland of the third eyelid on the 1st and 6th weeks of the study. If labeled cells were injected, unlabeled cells were not injected on those weeks. Additionally, intra-articular injections of 5 × 10⁶ unlabeled cells in 0.5 mL were performed on all 6 dogs

FIG. 1. Injection protocol of Ad-MSCs. Two groups of dogs containing 3 animals per group were injected with MSCs. In the first group, 2 × 10⁶ cells in 0.2 mL of DPBS were injected into the periacral region in conjunction with injection of 2 × 10⁶ cells in 0.2 mL of DPBS into the region of the third eye lid. These periocular injections were performed weekly for 6 consecutive weeks. The dogs in this group also received 5 × 10⁶ unlabeled cells in 0.5 mL of DPBS into the stifle joint on the 1st, 3rd, and 5th weeks of the study. In the 6th week, each dog in this group received 66 × 10⁶ DiD labeled cells in

3 mL of DPBS into the stifle joint. The dogs in the second group received the same injection course with the exceptions that the periocular cells that were injected in the 1st and 6th weeks of the study were DiD labeled and the dogs in this group did not receive an injection into the stifle joint in the 6th week. In all cases, cell injections were performed on the right side of the dogs, and injections of equivalent volumes of DPBS were performed on the left side. Ad-MSCs, adipose-derived mesenchymal stem cells; DPBS.

on the 1st, 3rd, and 5th weeks of the study. Corresponding DBPS (vehicle) injections were performed on the left side at all injection time points. The 3 dogs that did not receive labeled periocular cells were injected with 66×10^6 labeled cells in 3 mL of DPBS into the right stifle joint in the 6th week of the study (Fig. 1).

In vivo fluorescent imaging

Animals were initially anesthetized with 2.5–5 µg/kg of dexmedetomidine (WMI Veterinary Supply, Boise, ID) and maintained with no more than 4 mg/kg of propofol (WMI Veterinary Supply; 1–3 mL at 10 mg/mL) during imaging. The scanning stage of a Maestro 2 imaging system (CRi, Woburn, MA) was modified to accommodate the dogs. All *in vivo* imaging employed the “orange” filter set with excitation from 586 to 631 nm and 645 nm longpass emission filters. Emission images from 640 to 820 nm in 10 nm increments were taken at 1,827 ms of exposure for lacrimal region images and 7,500 ms for stifle region images with an F-stop setting of 8.4 in both cases and the focal plane set to zero. Image acquisition was set to 2×2 binning. The animals were held in the same positions for each image set throughout the course of the study. Maestro 2.10.0 software was used for all analysis. Identical background subtraction was performed on all lacrimal region images, and all stifle region images and quantitative measurements of 10^6 phot/cm²/s were used in all analyses to normalize for variations in animal size.

Postmortem imaging of tissues

Six animals that received fluorescent MSC injections were euthanized at 10 weeks using 165 mg/kg of Beuthanasia-D (Schering-Plough Animal Health, Boxmeer, The Netherlands) intravenously administered. Dissection of tissues was performed by a board certified veterinary pathologist (C.M.R.) on all study animals within 15 min after euthanasia to minimize tissue autofluorescence. Similarly, sized tissue samples were taken from 61 locations including all major organs and lymph nodes (Supplementary Table S1 Supplementary Data are available online at www.liebertonline.com/jop). All tissues were thoroughly rinsed in 10% formalin to remove exogenous particulate matter such as blood or food before fixation and storage in 10% formalin in phosphate-buffered saline (PBS).

Tissue procurement and ex vivo imaging

Tissues from all fluorescent injected dogs ($n=6$) were obtained immediately after euthanasia. A total of 61 tissues were harvested in the course of a complete necropsy. Tissue samples were rinsed thoroughly with 10% formalin and immersion fixed with 10% formalin in PBS before optical imaging. Identical tissues were obtained within 2–4 h after euthanasia from 3 control dogs euthanized for reasons unrelated to this study to provide comparison with tissues obtained from periocular and intra-articular injected dogs.

Each tissue sample was imaged in a single scan from all animals (e.g., all 6 study dog colon samples from MSC injected animals and all 3 control colon samples were imaged together) with 20,000 ms exposures per specimen. Larger tissues such as excised orbital tissue (removed en bloc) and stifle joints were imaged with appropriate vehicle injected controls, and samples from each animal were imaged con-

secutively with identical image settings. Background subtraction and quantitation were performed as previously described to normalize for variations in sample size.

Statistical analysis

Data were analyzed using Sigma Plot 11 (Systat Software, Chicago, IL) and StatXact 8 (Cytel Software, Cambridge, MA). Analysis of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks were used depending on the results of chi-square normality tests to determine significance between multiple treatment groups. Paired *t*-tests were used to compare right versus left sides of the same animal and 2-tailed Student's *t*-tests or exact Mann–Whitney rank sum tests were used to determine significance between 2 groups: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Results

Magnetic resonance imaging of Ad-MSC

In the single dog imaged, FeO-labeled Ad-MSCs were visible lateral to the orbit as a susceptibility induced signal void at both postinjection time points (Supplementary Fig. S1). The signal intensity of remained subjectively static over the study time course. The signal void was most pronounced on the T2* sequence (Supplementary Fig. S2), but was easily seen on the T1 postcontrast 3D SPGR with the added advantage of positive contrast enhancement of surrounding tissues and improved anatomic definition due to the 1 mm image collimation. This also allowed for more precise localization of labeled cells in relation to the orbit and lacrimal gland. No comparable susceptibility associated signal void was detected on the contralateral, DPBS injected side. We also found no evidence of labeled MSC migration into the targeted right lacrimal gland. Although gas trapped in the palpebral fissure caused small signal voids at the ocular surface of both the left and right eyes, this was easily distinguishable from susceptibility effects within tissues.

On the second day after the labeled MSC injection, there was mild peripheral positive contrast enhancement surrounding the labeled cell signal void on the gadolinium contrast enhanced 3D SPGR sequence (Supplementary Fig. S1), and a corresponding area of increased signal intensity on the STIR sequence (Supplementary Fig. S3) that were not seen on the day of injection, and were not present in the DBPS injected side. The central signal void was also visible on the STIR sequence but was smaller than the signal void seen on the 3D T1 SPGR and T2* sequences. The main aggregate of FeO-labeled MSCs was similar in size, shape, and signal characteristics compared with the initial postinjection images; however, a second, smaller aggregate was visible several millimeters rostral and distal to the first (Supplementary Fig. S4).

In vivo fluorescence of perilacrimal injected Ad-MSCs

Labeled Ad-MSCs were detected in the right perilacrimal region immediately after the injection ($n=3$) with only background labeling detected in the saline injected control left perilacrimal region (Fig. 2A). Fluorescence in the region of the third eyelid was not observed at any time point. The perilacrimal signal persisted for up to 3 weeks with increasing intensity being observed in the first 2 weeks

FIG. 2. *In vivo* fluorescence of Ad-MSC persists up to 3 weeks following transplantation into the lacrimal region. Representative images demonstrate the persistence of the fluorescent signal in the right perilacrimal region of injected animals compared to vehicle injected, left perilacrimal regions (**A**) for up to 3 weeks after injection. The average signal intensity ($n=3$) was significantly brighter than left perilacrimal controls immediately after injection and for 1 subsequent week ($*p<0.05$) after injection. Average signal intensity was also significantly brighter ($*p<0.05$) than left perilacrimal controls immediately after the 2nd injection of DiD labeled Ad-MSC (**B**). All data are presented as mean $\pm$ SEM. Color images available online at www.liebertonline.com/jop

FIG. 3. *In vivo* fluorescence of Ad-MSC persists up to 2 weeks following transplantation into the stifle region. Representative images demonstrate the persistence of the fluorescent signal in the right medial stifle region of injected animals compared to vehicle injected, left medial stifle regions (**A**) for up to 2 weeks after injection. The combined average signal intensity ($n=3$) from both the lateral and medial regions of the right stifle was significantly brighter than combined left stifle controls 1 week after injection ($*p<0.05$) (**B**). All data are presented as mean $\pm$ SEM. Color images available online at www.liebertonline.com/jop

FIG. 4. Ad-MSC engraft in the perilacrimal and third EL gland regions. *Ex vivo* imaging over 4 weeks after the transplantation of labeled MSC demonstrates persistence of the cells in representative images of the lacrimal region and region of the third EL gland (A). Heat maps demonstrate localization of the cells around the lacrimal and third EL glands compared with vehicle injected, left side controls (B). Analysis of variance results revealed statistically significant differences ($P < 0.05$) between the treatment groups for both lacrimal and third EL samples but there was insufficient statistical power elucidate the level significance between individual groups using paired sample analysis (C, D). Heat map signal intensity is presented as 10^6 phot/cm²/s, and all data are presented as mean $\pm$ SEM. EL, eyelid. Arrows indicator the presence of the fluorescent signal. Color images available online at www.liebertonline.com/jop

followed by decreased signal intensity in the third week. The average signal intensity after injection of labeled Ad-MSCs was significantly brighter than the background signal intensity of the control side (Fig. 2B). The right side perilacrimal signal levels were again significantly brighter than left side controls immediately after the second injection of DiD labeled cells in the 6th week; however, the signal was not significantly brighter 1 week later or throughout the remainder of the *in vivo* imaging time course (data not shown). Signal diminution was at least partially due to increased skin pigmentation as a result of repeated shaving.

In vivo fluorescence of intra-articular injected Ad-MSC

DiD labeled Ad-MSCs were detected in both the lateral (data not shown) and medial (Fig. 3A) sides of the right hind-limb after the injection ($n=3$). *In vivo* detection of the fluorescent signal was discernable from background for up to 2 weeks after injection. Peak fluorescent intensity was observed 1 week after injection with an average signal intensity of $23,650 \times 10^6$ (phot/cm²/s), which was significantly brighter than background on the left side (Fig. 3B).

Ex vivo imaging of periocular injected Ad-MSCs

The fluorescence from DiD labeled Ad-MSCs persisted for more than 4 weeks after the final injection in both the perilacrimal region and around the gland of the third eyelid (Fig. 4A). Heat map images demonstrate the concentration of the Ad-MSCs in the injection sites (Fig. 4B). The average signal intensity in the perilacrimal region of the right eye was more than twice the average signal intensity of the left eye. This background level was similar to right and left eyes of animals injected with labeled cells into the joint but with no labeled cells injected into the ocular region (Fig. 4C). Similarly, the average signal intensity in the region around the right gland of the third eyelid was nearly twice that found for the contralateral region and the corresponding region in animals that received intra-articular labeled cell injections (Fig. 4D).

Ex vivo imaging of intra-articular injected Ad-MSC

Ex vivo images of the left and right stifle cartilage and joint capsule from the hind-limbs of intra-articular injected animals demonstrate the persistence of fluorescence from labeled Ad-MSCs for greater than 4 weeks after injection (Fig.

FIG. 5. Ad-MSC engraft in the cartilage and joint capsule after transplantation. *Ex vivo* imaging over 4 weeks after the transplantation of labeled MSC demonstrates persistence of the cells in representative images of the cartilage region and joint capsule (**A**). Heat maps demonstrate localization of the signal to the compared to vehicle injected, left side controls (**B**). Engraftment of labeled MSC was found in the right cartilage and joint capsule regions of directly injected animals compared with the respective regions of the left legs and both right and left respective regions of animals receiving lacrimal injections. Average signal intensity in the cartilage surrounding the stifle joint is more than 7 times greater than the average signal intensity from the left stifle joint cartilage and nearly 70 times greater than the average signal intensities from cartilage from the left and right stifle joints of periocular injected animals (**C**). Average signal intensity in the joint capsule of the right stifle joint of intra-articular injected animals is more than 150 times greater than the joint capsule of either the left stifle joint of intra-articular injected or either stifle joint of periocular injected animals (**D**). Heat map signal intensity is presented as 10^6 phot/cm²/s and all data are presented as mean $\pm$ SEM. Color images available online at www.liebertonline.com/jop

5A). Heat map images demonstrate the localization of the cells around the synovium of the joint (Fig. 5B). The average signal intensity in the cartilage surrounding the stifle joint was more than 7 times greater than the average signal intensity from the left stifle joint cartilage and nearly 70 times greater than the average signal intensities from the left and right stifle joints of periocular dye injected animals (Fig. 5C). The average signal intensity in the joint capsule of the right stifle joint was more than 150 times greater than the joint capsule of the control stifle joint of intra-articular injected or stifle joints of periocular dye injected animals (Fig. 5D).

Migration of Ad-MSCs

To investigate Ad-MSC migration after transplantation, *ex vivo* imaging of 61 tissues was performed (Supplementary Table S1). Animals that received labeled intra-articular injections with a greater overall number of cells demonstrated a greater degree of fluorescence in imaged tissues (Fig. 6A). For example, the average signal intensity in the thymus of intra-articular injected animals was more than twice the

average signal intensity from the periocular injected animals (Fig. 6B).

Significant fluorescence was found in the thymus and the tissues of the gastrointestinal (GI) tract, including stomach, duodenum, jejunum, and colon. The majority of the tissues imaged, including the lung, did not contain a measurable fluorescent Ad-MSC signal (data not shown). *Ex vivo* heat map images of the GI tract demonstrate Ad-MSC engraftment (Fig. 7A). Significant levels of fluorescence were found in the stomach, duodenum, and jejunum with the levels higher in the animals injected in the synovium compared with periorbital injected animals. Similarly, significant levels of fluorescence compared to noninjected donor controls were found in the duodenum, jejunum, and colon (Fig. 7B).

Discussion

This is the first investigation that evaluates persistence and migration patterns away from sites of local delivery in dogs. In this study, we sought to investigate the impact of repeated MSC injections on residence time at the site of injection and

FIG. 6. Ad-MSc migration and engraftment to the thymus after injection. *Ex vivo* imaging over 4 weeks after the transplantation of labeled MSC demonstrates migration of the cells in heat map images of the thymus (**A**). Significant engraftment ($*p < 0.05$) was found in the stifle injected animals compared with periocular injected animals (**B**). Heat map signal intensity is presented as 10^6 phot/cm²/s, and all data are presented as mean $\pm$ SEM. Color images available online at www.liebertonline.com/jop

subsequent engraftment throughout the dogs. In the dog that received FeO-labeled MSCs, these cells can be visualized in the periorbital tissues using 3D T1 SPGR post-gadolinium, T2*, and STIR sequences. This technique may be useful in future studies aimed at developing MSC therapeutics for the eye and surrounding structures. The main cell population injected in this dog remained static with some migration of a subpopulation of cells rostrally and ventrally. However, in such a short time period, it is difficult to determine whether this was true trafficking or clearance via the lymphatic system without tissue sampling. Quantitative imaging studies have shown that viable cell populations migrate more readily than nonviable cells, which is consistent with the magnitude of cell movement in this dog.³⁵ Although it is not possible to document whether the iron oxide remained within the cells or was scavenged by macrophages, the label has been previously shown to remain within cells after injection into tissues.³⁶ In our study, STIR and post-gadolinium imaging indicated possible edema or inflammation surrounding the labeled cells. Although this was not documented histopathologically, it may indicate that the cells initiate a mild inflammatory response after deposition. This may be of importance when designing therapeutic protocols that allow for safe injection of iron oxide labeled cells in live animals.

The novel application of *in vivo* fluorescent imaging used for small animals (normally rodents) is less taxing on the animals, allows for greater control when tracking MSC

FIG. 7. Ad-MSc migration and engraftment to the gastrointestinal tract following injection. *Ex vivo* imaging over 4 weeks after the transplantation of labeled Ad-MSc demonstrates migration of the cells in heat map images of the tongue, stomach, duodenum, jejunum, ileum, and colon (**A**). Significant engraftment ($*p < 0.05$; $**p < 0.01$; $##p < 0.01$) was found in the stomach, duodenum, jejunum, and colon of stifle injected animals compared with either periocular injected (*) or control (#) animals (**B**). Heat map signal intensity is presented as 10^6 phot/cm²/s and all data are presented as mean $\pm$ SEM. Color images available online at www.liebertonline.com/jop

in vivo and *ex vivo*, and is less expensive than MRI. Therefore, it was preferentially employed over MRI imaging for use in 6 dogs in this study. Analysis after local delivery of labeled cells conclusively demonstrated local engraftment of Ad-MSCs following both periocular and joint injections. The ability to detect the fluorescent signal in subsequent weeks, however, was limited by the decrement in signal intensity.

Signal intensity is strongly affected by the ability of the fluorescent signal to penetrate the layers of tissue between the light source and cell source (during excitation) and the cell pool and the detector (during emission). This is clearly demonstrated by the diminished signal intensity observed in the *in vivo* images (Fig. 2B) compared with the brighter signals measured from *ex vivo* images more than 2 weeks later (Fig. 4B). Hyperpigmentation of the skin was noted during the later time points of our study that would also reduce fluorescent detection. Repeated shaving has been previously reported to cause hyperpigmentation of the skin in dogs.^{37–41} The development of increased pigmentation represents a confounding event in terms of fluorescent signal detection and likely contributed to the relatively low signal intensity after the second labeled cell injection (week 6) and the inability to detect the signal thereafter.

The initial increase in background signal intensity even of DPBS injected locations may be the result of fur removal by the initial shaving before the first injection. This average background signal intensity began to decline in the 4th week and steadily declined throughout the remainder of the study (Fig. 2B). The steady decline in average background signal intensity also correlated with the increased skin pigmentation.

Although successful survival of Ad-MSCs after the injection is well documented,^{42–44} possible Ad-MSC cell death after the injection could have been responsible for the diminution of the signal. In the event of cell death, lipophilic dyes, such as DiD, are released and spread to surrounding cells and distributed systemically. The migration of the labeled cells and subsequent localized fluorescence documented *ex vivo* demonstrate that this is unlikely in our study. In the event that released dye diffused systemically, the fluorescence would be diffusely distributed throughout the body and not consistently locally concentrated in identical tissues in multiple animals. We found labeling of specific segments in the GI tract and the thymus but no labeling of lymphatic vessels, local lymph nodes, or distal lymph nodes. We also did not find labeling of the blood vasculature. Absence of labeling in the lymph nodes also suggests that phagocytosis by macrophages and innate or adaptive immune responses to the cells are unlikely. This is consistent with previous reports that show MSCs to largely evade immune detection.^{32,45–47}

A likely contributor to the diminution of the signal at the injection site is cell migration away from the site of local delivery. MSCs from multiple sources have been shown to migrate after the injection in several animal models including dogs and in human patients.^{31,32,48} In the present study, we have shown the engraftment of allogeneic MSCs in the thymus. The contribution of autologous, labeled MSC to the thymic epithelium to support lymphopoiesis, has been previously demonstrated in mice⁴⁹ as has the contribution of human MSCs to the thymus of sheep after *in utero* transplantation.³² The primary mechanism for MSC inhibition of T-cell response is thought to be through down regulation of activated T-cell proliferation.⁴⁵ Combined with previous findings, the contribution of the MSCs to the thymus of intra-

articular injected dogs (Fig. 6) suggests that the transplanted MSCs may be influencing the immune system.

We also found preferential migration and engraftment of the Ad-MSCs throughout the GI tract (Fig. 7). Previous investigations with bone marrow-derived MSCs demonstrate their migration to the intestine.⁵⁰ Endothelial progenitor cells (EPCs), which have been shown to originate from a common progenitor source with MSCs,⁵¹ contribute to the stem cell pool of the small intestine. EPCs have also been shown to support the stem cell niche in the small intestine after the injection into fetal sheep.⁵² MSCs have also been used to regenerate small intestine tissue after transplantation on scaffolds.^{53,54} Our data quantitate the relative level of engraftment throughout the entire GI tract by Ad-MSC in a large animal model. Migration away from the injection site suggests that the matrix/soluble signaling environment in these healthy young dogs did not completely limit emigration and that the microenvironment of the GI tract encourages engraftment. Significant results were more apparent in the intra-articular injected animals most likely, because they received a much greater number of labeled Ad-MSCs (66 million) in 1 bolus dose as compared with the labeled Ad-MSCs injected into the periorbital region (8 million) at different time points. Engraftment of Ad-MSCs from intra-articular injected animals was statistically higher in the colon compared with non-injected control animals but a difference was not detected with periocular dye injected animals in this tissue.

In contrast to previous studies where migration to the lung was observed after intravenous injection of MSCs,^{31,32,55} local (periorbital and intra-articular) injection of MSCs did not lead to lung engraftment. The stifle skin immediately above the right stifle injection site was found to be fluorescent during *ex vivo* examination. A possible explanation is the spreading of cells along the injection path. Overall, only the GI tract and thymus evidenced significant MSC engraftment in our study and fluorescence was not found in any of the other tissue locations tissues sampled from each animal.

The active engraftment of the MSCs in combination with their previously documented immunomodulatory capabilities²² suggests the potential for therapeutic benefit in using MSCs for the treatment of periocular and joint diseases with immune involvement.

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Author Disclosure Statement

No competing financial interests exist.

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