THE IMMUNOGENICITY OF MESENCHYMAL STEM CELLS

A Dissertation

by

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ABSTRACT

Mesenchymal stem cells (MSCs) have been used for decades to treat a variety of conditions in humans and animals, but consistent and reproducible efficacy has yet to be demonstrated. Two possible reasons for inconsistent efficacy are 1) xenogen contamination from the methods used for MSC preparation, or 2) the use of non-cross matched allogeneic (non-self) MSCs.

Fetal bovine serum (FBS) is the most used culture media supplement for the preparation of MSCs. However, proteins present in media become internalized during the culture period and foreign proteins may be recognized by the recipient immune system after MSC administration. To investigate immune recognition of FBS, we first developed an alternative method of MSC preparation that did not require FBS. We tested bone marrow supernatant (BMS) as an alternative to FBS and found no differences in MSC preparation other than greater MSC isolation with BMS compared to FBS. In vivo, we noted an adverse clinical response after FBS-MSC administration, which did not occur after BMS-MSC administration. Importantly, we documented antibody mediated destruction of FBS-MSCs, but not BMS-MSCs.

Our laboratory transitioned to exclusively using BMS in the preparation of clinical MSCs in 2018. Retrospective analysis of BMS-MSCs for the treatment of horses with naturally occurring joint disease showed greatly improved return to function with BMS-MSCs compared to our previous clinical experience as well as to previous reports with FBS-MSCs.

To investigate the use of allogeneic MSCs, we identified four MSC donors that were homozygous for well-characterized major histocompatibility complex (MHC) haplotypes. Matched (heterozygous, carrying donor haplotype) and mismatched (dissimilar to donor haplotype) recipients received two intra-articular injections of donor MSCs isolated and expanded in BMS. In all mismatched recipients, there was marked antibody development that resulted in in vitro MSC cytotoxicity, but no sign of immune recognition in MHC-matched recipients.

Our findings definitively demonstrate that MSCs are recognized by the recipient immune system due to xenogen contamination during MSC preparation as well as donor haplotype mismatch. This understanding will help to advance the use of MSCs clinically and explains why there has been inconsistent efficacy demonstrated in pre-clinical and clinical trials.

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Contributors

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NOMENCLATURE

BMS	Bone marrow supernatant
FBS	Fetal bovine serum
FES	Fetal equine serum
FGF	Fibroblast like growth factor
FITC	Fluorescein isothiocyanate
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IL-17a	Interlekin-17a
MHC	Major histocompatibility complex
MSC	Mesenchymal stem cell
NK	Natural killer

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1. INTRODUCTION

Mesenchymal stem cells (MSCs) have been the subject of extensive research for decades, but there are currently no FDA approved MSC-based therapeutics. Inconsistent efficacy has led to a failure of trials of MSC-based therapies to meet stringent clinical endpoints and has impeded regulatory approval of MSC use in the United States, despite numerous therapies being approved in other countries (1). Recently, it has been theorized that the reason for varying efficacy may be recipient immune recognition of MSC culture supplements or non-self (allogeneic) MSCs (2, 3).

It was previously understood that MSCs would not be recognized by the recipient immune system, meaning that allogeneic use of MSCs and foreign proteins in MSC culture media were not of consequence (4, 5). This was because MSCs have both relatively low expression of major histocompatibility complex I (MCH I) and potent immunomodulatory properties, leading researchers to believe they are immune privileged (4). However, that belief has been questioned in recent years. In humans, antibody production has been documented after administration of both allogeneic MSCs and those prepared using fetal bovine serum (FBS), but the consequence of these antibodies was not explored (6-8). Recognizing and then deciphering the recipient immune response in clinical trials has proven difficult because MSC production methods vary greatly and allogeneic MSC use is prevalent. Thus, controlled investigation of these potential issues is necessary.

1.1. Fetal bovine serum in MSC preparation

In the 1970s, the pioneer of modern stem cell study, Alexander Friedenstein, first described the use of fetal bovine serum (FBS) as a culture media additive for the isolation and expansion of MSCs (9). Fetal bovine serum provides growth factors, hormones, and other essential yet undefined nutrients, making FBS supplementation of MSC culture media essential to the isolation, expansion, and maintenance of MSC characteristics (9). However, inclusion of FBS in culture media is known to result in intracellular accumulation of bovine proteins (10, 11). In 2004, Spees et al. clearly demonstrated using FITC-labeled FBS in human MSCs that intracellular proteins are not washed away or removed during MSC processing, and that introduction of xenogen proteins to the recipient is a risk with FBS prepared MSCs (11). Spees et al. also showed a 100,000-fold reduction in intracellular FBS proteins when FITC-labeled FBS was replaced with adult human serum for 48 hours before assessment, but importantly there was not a complete elimination of FITC labeled FBS proteins (11). Likewise, Joswig et al. in 2017 reported a reduction, but not elimination, of intracellular FBS contamination in equine MSCs after 48 hours of FBS replacement with adult equine serum (10).

1.1.1. Recognition of FBS

Fetal bovine serum contains a variety of proteins, hormones, lipids, and unknown components (12, 13). The most abundant protein in serum is albumin, the structure of which varies greatly between species, making it a potential source of immune recognition (11, 14). Other proteins in serum that are bovine-specific may also trigger an

immune response, likely making the recognition of FBS multifactorial and thus difficult to prevent (11).

As a normal mechanism of cell surveillance, intracellular proteins are processed into peptides and presented on the surface of all nucleated cells via the major histocompatibility type I complex (MHCI) (15). Foreign peptides presented by MHCI are recognized by the host immune system and the presenting cell is targeted by cytotoxic T cells for destruction (15). Additionally, expulsion of bovine proteins, peptides, or other components as cellular metabolic waste can result in presence of these factors in the extracellular space and direct antibody binding or activation of the complement system (16, 17).

In humans, development of anti-bovine antibodies after FBS-prepared MSC administration was documented by Horwitz et al. in 2002 (18). This seroconversion to bovine proteins was implicated as a potential reason for poor clinical response in children with osteogenesis imperfecta treated with FBS-prepared MSCs (18). Importantly, the vast majority of humans and horses have pre-existing anti-bovine antibodies, meaning FBS prepared cells may be targeted on the first exposure (7, 19, 20). Anti-bovine antibodies often develop in humans and horses as a result of inadvertent sensitization from routine vaccination, as many anti-viral vaccines are produced using FBS; in humans, consumption of dairy products and beef can also lead to sensitization to bovine proteins (19, 20).

1.1.2. Fetal bovine serum in human clinical trials

As recently as 2014, more than 80% of registered clinical trials of MSC-based therapies reported using FBS to prepare the cells (21). While there has been a shift away from FBS use during MSC preparation, FBS usage is still prevalent. In 2019, it was reported that 5/16 (31%) of academic institutions are still using FBS during the preparation of human clinical MSCs (22). The shift away from FBS has been mostly motivated by ethical concerns, the risk of disease transmission, variability between lots of FBS, and the increase in demand that is likely to outpace supply as MSC use increases (23). However, xenogen contamination poses an overarching concern because of recipient immune recognition potentially interfering with MSC efficacy. Immune monitoring after administration of FBS prepared MSCs is almost never performed, and it is likely that recipient immune responses are is underrecognized and therefore underreported.

1.1.3. Fetal bovine serum in veterinary medicine and pre-clinical animal models

Fetal bovine serum continues to be industry standard for the supplementation of MSC culture media in veterinary medicine and pre-clinical animal models (24-26). Continued use of FBS is likely due to the high cost of chemically defined media as well as repeated failure of FBS alternatives to adequately support MSC isolation and expansion in some species (27-29). Alternatives to FBS have proven to be particularly difficult to develop for equine MSCs.

To date, little work has been done evaluating the recipient immune response to FBS, but a lack of change in anti-bovine antibodies after administration of FBS prepared MSCs has led some researchers to the conclusion that anti-bovine titers are not of consequence (26, 33). In 2016, Owens et al. reported anti-bovine antibodies in 89% of horses that received allogeneic FBS-prepared MSCs (34). While they did see any systemic adverse reactions or a change in anti-bovine titers after FBS-prepared MSC administration, they did not investigate if these antibodies were of consequence to the administered MSCs (34).

In humans, platelet products are well documented to support MSC isolation and expansion, but with equine MSCs, cellular senescence and alteration of MSC characteristics is a problem (23). Despite preliminary positive reports by Naskou et al. in 2018 showing "normal" expansion of equine MSCs using platelet lysate, a 2019 report from the same group noted lower proliferation rates and altered immunomodulatory capacity of platelet lysate cultured equine MSCs (30, 31). Other groups have reported similar results, with senescence and altered MSC characteristics after 3 passages in platelet lysate supplemented culture (29, 32).

1.2. Use of allogeneic MSCs

Allogeneic (non-self, intraspecies) MSCs have been used for decades with little concern regarding immune recognition (5). Relatively low MHCI expression combined with powerful immunomodulatory capabilities led researchers to trust that MSCs would not be recognized by the recipient immune system (4). This has led clinicians and

researchers to use allogeneic MSCs without immune monitoring (2, 5, 8, 24). We suspect that undetected recipient immune recognition may be a contributing to variable efficacy in late phase clinical trials.

1.2.1. Recognition of allogeneic MSCs

Despite relatively lower expression of MHCI on MSCs compared to other nucleated cell types, all MSCs still express MHCI (35, 36). The MHC structure is hereditary with one copy (haplotype) being inherited from each parent (37). Having genetically diverse MHC molecules is evolutionarily advantageous as the peptide binding cleft is highly polymorphic and therefore different haplotypes are able to present different peptides to varying degrees (37). Thus, individuals are able to present different peptides, increasing the likelihood of a population being able to mount an immune response to a large number of pathogens (37).

In addition to presentation of intra-cellular proteins, MHCI is also responsible for the recognition of self vs non-self (37). Because the MHC structure varies between individuals, the molecule itself serves as a direct mechanism of recognition, allowing immune cells (including helper T cells and dendritic cells) to be able to recognize differences in MHC structure and thus target foreign cells (37).

A confounding issue in the recognition of foreign cells due to the differences in MHC structure is that the elimination of MHCI expression does not eliminate recipient immune recognition. Natural killer (NK) cells, a component of the innate immune system and a necessary checkpoint for detecting pathogens, are signaled by a lack of

MHCI on a cell surface (38). Because non-mammalian cells do not express MHCI, a lack of MHCI is recognized as threat and the cell is destroyed (38).

1.2.2. Current allogeneic MSC therapeutics

Allogeneic MSCs have been used for decades and are still being used clinically with little or no monitoring of the recipient immune response. In a recent review of clinical trials in the United States from 2004-2018 by Kabat et al., a similar number of registered clinical trials involved autologous and allogeneic MSCs from 2004-2014 (39). After 2015, allogeneic MSC clinical trials increased compared to autologous MSC clinical trials (39). This trend away from autologous use is likely due in part to the fact that allogeneic MSCs offer the convenience of an off-the-shelf alternative and a possibility to produce a standardized product that can be FDA regulated (22, 39).

In 2017, the POSEIDON trial (FDA IND #14419), compared the use of autologous vs allogeneic bone marrow-derived human MSCs for the treatment of non-ischemic dilated cardiomyopathy (40). Based on their results, the group called for a clinical trial and further investigation of this therapeutic (40). However, two things are important to note when reading this report, and other similar reports, 1: MSCs were only administered at a single time point, and immunological monitoring was performed sporadically, and 2: MSC preparation technique was not reported (40). With limited immunological surveillance and single dose administration, Hare et al. still reported a seroconversion rate of 30% in the allogeneic group, but the functionality of the anti-HLA antibodies was not investigated (40). Similarly, MSC preparation technique was

not reported for this trial, but a 2012 POSEIDON trial by the same group evaluating autologous vs allogeneic MSCs for the treatment of ischemic cardiomyopathy reported using FBS-prepared MSCs (8). This study highlights the importance of complete reporting of methods and proper immunological monitoring when interpreting results.

1.2.3. Future clinical use of MSCs

It is likely that incomplete immunological monitoring and inconsistent MSC preparation combined with varying MSC administration in regard to dosing and timing, has led researchers to overlook potential recipient immune recognition. Investigation of both FBS alternatives and non-cross matched allogeneic MSCs needs to be performed with complete immunological monitoring. In vitro modeling has limitations in detection of recipient immune response, so in vivo studies should be performed to fully evaluate the immune compatibility prior to continuation of clinical MSC use.

2. PREPARATION TECHNIQUE AFFECTS RECIPIENT IMMUNE TARGETING OF AUTOLOGOUS MESENCHYMAL STEM CELLS

2.1. Introduction

Despite decades of work, consistent and reproducible clinical efficacy of mesenchymal stem cells (MSCs) has not been demonstrated (2, 3, 41). Failure to meet clinical endpoints in both late phase clinical trials and post-approval monitoring has precluded market authorization in the United States (1-3, 41). Likewise, lack of predictable efficacy of animal MSCs plagues the veterinary community and has casted doubt on the usefulness of MSCs, both translationally and clinically. One reason for the lack of consistent efficacy may be MSC preparation technique (2). Pittenger et al., recently emphasized the importance of MSC preparation technique, stating the preparation method of MSCs is the product (42). Preparation methods include culture media composition and serum supplementation sources.

Supplementation of MSC culture media with fetal bovine serum (FBS) has been a standard MSC preparation technique since MSCs were first described in the1970s, providing growth factors, hormones, and other undefined, yet essential, components to cell culture media (9). However, the use of FBS is decreasing because of ethical concerns, availability, and the risk of disease transmission from bovine products (43). Despite this shift in FBS acceptance, FBS supplemented MSCs have market approval in Canada and New Zealand, and FBS supplementation remains the industry standard in pre-clinical and veterinary MSC use (1, 24, 33, 42, 44, 45).

An important, but infrequently discussed, consequence of FBS supplementation during MSC preparation is the accumulation of intracellular bovine contamination that is presented on MHCI, leading to seroconversion of the recipient (7, 11, 46, 47). In horses, we confirmed that accumulation of intracellular bovine protein by MSCs leads to local inflammation after therapeutic administration, but did not assess anti-bovine titers (10). In that report, removal of FBS during the final 48 hours of culture markedly reduced intracellular bovine contamination, but all MSCs remained positive for bovine proteins (10).

A lack of change in anti-bovine titers in horses and cats after MSC therapy has led others to conclude that FBS contamination is not clinically relevant yet, in humans there is evidence that seroconversion against bovine proteins in MSC recipients correlates to poor clinical response (18, 26, 33). The question remains, what do pre- and post-MSC treatment anti-bovine titers mean in patients receiving FBS supplemented MSCs? Immune recognition of intracellular bovine proteins and resultant cytotoxicity could explain why pre-clinical study often fails to predict therapeutic response and why clinical trials have failed to meet rigorous clinical endpoints in the United States (3, 42, 45, 48).

Our objective was to determine if there is an immune response against autologous MSCs because of laboratory preparation with FBS. First, we confirmed that replacement of FBS supplementation with bone marrow supernatant (BMS) supplementation did not alter MSC growth or characterization. In the equine model, we then performed repeated intra-articular injections of autologous FBS supplemented

MSCs (FBS-MSCs) or autologous BMS supplemented MSCs (BMS-MSCs). We demonstrate immune recognition with antibody mediated death of MSCs, local inflammation, and reduced efficacy after FBS-MSC administration, which did not occur with BMS-MSCs. Given the historical and ongoing use of FBS in pre-clinical and clinical trials, identifying FBS use and potential recipient immune recognition with subsequent antibody mediated MSC death is imperative in interpreting results. In future study, especially pre-clinical study and veterinary applications where FBS supplementation remains the standard practice, FBS should not be utilized.

2.2. Materials and methods

2.2.1. Animals and experimental overview

All animals were cared for according to university standards and all procedures were approved by the animal care and use committee (AUP 2018-0003 and 2018-0118). Six horses were utilized for BMS characterization (5 females, 1 castrated male), and 18 horses were utilized for the equine model (13 females, 5 castrated males). All horses were Quarter Horse type and ranged from 4 - 22 years of age.

Bone marrow derived MSCs were isolated and expanded in culture media supplemented with either BMS or FBS. In vitro growth rate, characterization, and immunomodulation were compared. Intra-articular injection of autologous FBS-MSCs or BMS-MSCs was performed twice, one month apart. To mimic naturally occurring inflammation, lipopolysaccharide (LPS) was injected immediately prior to MSCs at the second injection. Clinical reaction, synovial cytology, synovial cytokines, synovial MSC

concentration and cytotoxicity against MSCs was evaluated for the week after each intra-articular injection.

2.2.2. MSC isolation and expansion

Bone marrow was collected from the sternum as previously described (49). Heparinized bone marrow was centrifuged at 300g for 5 minutes and the BMS was collected and filtered. The cellular fraction underwent red blood cell lysis as previously described (50). Briefly, red blood cell lysing solution (7.7 mg/ml NH4CL; 2.06 mg/ml hydroxymethane–aminomethane, pH 7.2) was added to the cellular fraction and centrifuged twice (300g for 5 minutes). The cells were washed in DPBS (Dulbecco's phosphate buffered saline, Corning) and resuspended in serum free media (Dulbecco's modified Eagle's medium (DMEM, Corning), 1 g/l glucose supplemented with 10,000 U/ml Penicillin; 10 mg streptomycin sulfate, 25 μg/ml amphotericin B (Gibco); 2.5% HEPES buffer (Life Technologies); 10 μg/ml human recombinant basic fibroblast growth factor (b-FGF, Corning)). Media contained either 10% BMS (autologous or pooled) or 10% FBS (HyClone) and MSCs were maintained at 37°C in 5% CO_2, humidified air with media exchanged 3 times per week. Pooled BMS was created with equal parts from each of the six autologous bone marrow collections.

After 7 days in culture, MSCs were passaged and replated at 5,000-7,000 MSCs/cm^2, which was repeated each time confluence reached 70-80% until the third passage. Once MSCs reach the third passage (P3), they were cryopreserved is cryopreservation media (95% autologous serum and 5% DMSO) as previously described (50).

2.2.3. Characterization of BMS-MSCs and FBS-MSCs

2.2.3.1. *In vitro* colony forming unit-fibroblast assay

The equivalent of 1ml of raw bone marrow was plated to 10 cm tissue culture dishes and maintained in media supplemented with 10% BMS or 10% FBS. After 10 days, colonies were stained with 3% crystal violet (Sigma Aldrich) and counted.

2.2.3.2. Population doubling time

Population doubling time was calculated using the following equation: PDT = days in culture * log 2 / (log$f$ – log$i$) where f is final cell count and i is the initial number of cells.

2.2.3.3. Trilineage differentiation and cell surface marker expression

MSCs at passage 3 underwent trilineage differentiation into adipocytes, chondrocytes, and osteocytes, and cell surface marker expression of MHCI, MHCII, CD29, CD45, and CD90 was evaluated as previously described (36, 50).

2.2.3.4. Mixed lymphocyte reactions

Previously cryopreserved MSCs were thawed and plated at 50,000 cells per well for 24 hours prior to inactivation with mitomycin C (Sigma Aldrich) as previously described (51). Responder and stimulator lymphocytes were isolated from two unrelated donors using a Ficoll (GE Healthcare) gradient with the addition of carbonyl iron (Sigma Aldrich) (52). Stimulator lymphocytes were inactivated by incubation with 50μg/ml mitomycin C for 30 minutes and then added at a density of 1×10^6 stimulator lymphocytes per well. Responder lymphocytes were stained with a commercially available nuclear stain (CellTrace® Violet, Thermo Fisher) and 2×10^6 responder

lymphocytes were added to each well. Cultures were maintained for 5 days, after which lymphocytes were collected and stained with anti-equine CD3+ antibody (UC Davis) at a 1:200 dilution. Flow cytometry was then performed on CD3+ T lymphocytes to assess proliferation with the use of commercially available software (FlowJo™ Software). Stained, unstimulated responder lymphocytes were used as a negative proliferation control, Concanavalin A (Sigma Aldrich) stimulated responder lymphocytes were used as a positive proliferation control, and changes in mean fluoresce intensity were evaluated as a percent change from the negative control as previously described (53).

2.2.4. Equine model

On day 0, 10 x 10^6 MSCs respective of group assignment were thawed at 37˚C and injected in cryopreservation media (95% autologous serum and 5% DMSO). On day 29, 25ng lipopolysaccharide (LPS) was injected immediately prior to MSCs.

2.2.4.1. Clinical evaluation

Physical examinations including heart rate, respiratory rate, and temperature were performed prior to and every 12 hours for 3 days after each injection. As a measurement of pain, gait asymmetry was quantified using an inertial-based sensor system (Lameness Locator, Equinosis®) prior to and after each injection (days 0, 1, 2, 3, 7, 29, 30, 31, 32, and 36). Peri-articular edema and synovial effusion were independently scored at the same time points; 0 = no edema/effusion, 1 = mild edema/effusion, 2 = moderate edema/effusion, 3 = severe edema/effusion. Limb circumference was measured at the level of distal metacarpophalangeal IV on days 29, 30, 31, 32 and 36.

2.2.4.2. Synovial cytology, cytokine, and chemokine analysis

Synovial fluid was collected prior to and after each injection on days 0, 1, 2, 3, 7, 29, 30, 31, 32, 36 and examined by a board-certified veterinary pathologist. Synovial fluid analysis including total nucleated cell count, cellular differential, and total protein measurement was performed on all samples.

Synovial fluid collected on days 1 and 30 was also analyzed using a 23 analyte, equine specific, multiplex kit (Millipore Sigma) as previously described (54). Analytes measured included: FGF-2, eotaxin, G-CSF, IL-1α, GM-CSF, fractalkine, IL-13, IL-5, IL-18, IL-1β, IL-6, IL-17a, IL-2, IL-4, IL-12, IFNγ, IL-8, IP-10, GRO, MCP-1, IL-10, TNFα, and RANTES.

2.2.4.3. Synovial colony forming unit-fibroblast assay

On days 1, 7, 30, and 36, 8 drops of synovial fluid were plated to a 10cm tissue culture dish along with MSC culture media. Media was changed after 24 hours and again 72 hours later. After 7 days in culture, dishes were stained with 3% crystal violet, allowed to dry overnight, and colonies counted without magnification.

2.2.4.4. Anti-FBS antibody ELISA

Blood was collected on all horses weekly prior to injection and for 8 weeks after the first injection (days 0, 7, 14, 21, 28, 35, 42, 49, and 56). An anti-FBS antibody ELISA was performed as previously described (55, 56). Briefly, plates were coated with FBS from the same lot as MSC preparation, and incubated overnight. Plates were washed, serum was added at a 1:3200 dilution, and for 30 minutes. Secondary antibody (Abcam) was added at 1:20,000 dilution for 30 minutes. After a final wash, 100 µl of TMB (Genway Biotech Inc.) was added followed by 100 µl of stop solution (Genway

Biotech Inc.) 15 minutes later. Plates were read at 450nm, and optical density (OD) reported. Fetal equine serum (FES) was used as a negative assay control, which had the same optical density as the blank control. Titers were then measured by repeating the above ELISA procedure with serial dilutions (from 1:1600 to 1:819,200) of serum collected from the FBS group on days 0 and 56.

2.2.4.5. Microcytotoxicity assay

Microcytotoxicity assays were performed using serum collected weekly (days 0, 7, 14, 21, 28, 35, 42, 49, and 56) and synovial fluid collected prior to and after the first and second injections (days 0, 1, 7, 30, and 36) with autologous or donor MSCs cultured in either BMS or FBS. Briefly, 2 µl of serum or synovial fluid was added to a Terasaki plate and 5 µl of paraffin oil (Sigma Aldrich) layered on top. Autologous MSCs were suspended in DPBS at a concentration of 1000 cells/µl and 2 µl of the suspension added to each well, ensuring that the cell solution was in contact with the serum or synovial fluid. After 30 minutes at room temperature, 5 µl of rabbit complement was added (One Lamda) and plates were incubated for another 60 minutes at room temperature. Two µl of 5% eosin (Sigma Aldrich) was then added to each well, after 5 minutes 5 µl of 10% formalin (Thermo Scientific) was added. Cells were allowed to settle overnight and cell death was assessed within 24hrs. Percentage of cell death was assessed in a blinded manner. Fetal equine serum was used as a negative control and MHCI specific monoclonal antibody (CZ3.2, provided by Donald Miller), was used as a positive control.

2.2.4.6. Immunoglobulin depletion

To confirm that cell death in the microcytotoxicity assays was anti-FBS antibody mediated, immunoglobulins were depleted from serum collected from FBS-MSC recipients on day 35 as previously described (57). Briefly, a commercially available kit with a Protein A column (ProteoExtract®, Merck KGaA) was used followed by manual depletion with Sepharose G beads (Millipore Sigma). One hundred µl of serum was diluted in 900 µl of 1x binding buffer. Samples were passed through the Protein A column to remove IgG, in a dropwise manner resulting in partial IgG removal. Two hundred µl of preconditioned Sepharose G beads was added to 300 µl of undiluted eluate and incubated for 1 hour with gentle mixing for complete IgG removal. Microcytotoxicity assays were repeated with undiluted serum, serum diluted in 1x binding buffer, partial immunoglobulin depleted serum, or complete immunoglobulin depleted serum. Assays were completed in duplicate, with donor MSCs cultured in either BMS or FBS.

2.2.5. Statistical analysis

Differences in in vitro data between groups were evaluated by paired Wilcoxon signed rank or Wilcoxon rank sum based on the distribution of data. In vivo, edema, effusion, limb circumference, and lameness were normalized to baseline (day 0 and day 29) prior to MSC injection. Differences between groups and over time in edema, effusion, limb circumference, lameness, and number of colonies present were tested using Kruskal Wallis or Wilcoxon rank sum tests. As a follow-up, a mixed model was used for all in vivo data, no differences were found between either analysis. Differences

in proportion of joints positive for MSC colonies were tested using Fisher's exact test at each time point. Groups were considered different when the p value was < 0.05.

2.3. Results

2.3.1. Greater MSC isolation with BMS, but no difference in expansion, characterization, or immunomodulatory capacity in BMS-MSCs compared to FBS-MSCs

First, we investigated whether BMS supports MSC isolation and expansion without differences to MSC characterization or immunomodulatory capacity compared to FBS supplementation. There was an increased rate of MSC colony isolation after BMS supplementation compared to FBS, but no differences in expansion rate between BMS-MSCs and FBS-MSCs (Fig. 1).

No differences in immunomodulatory function were seen using modified one-way mixed lyphocyte reactions (Fig. 1). After 3 passages, BMS-MSCs and FBS-MSCs were phenotypically similar without appreciable differences in morphology, and there were no differences in cell surface marker expression or trilineage differentiation into bone, cartilage, and fat (Fig. 1).

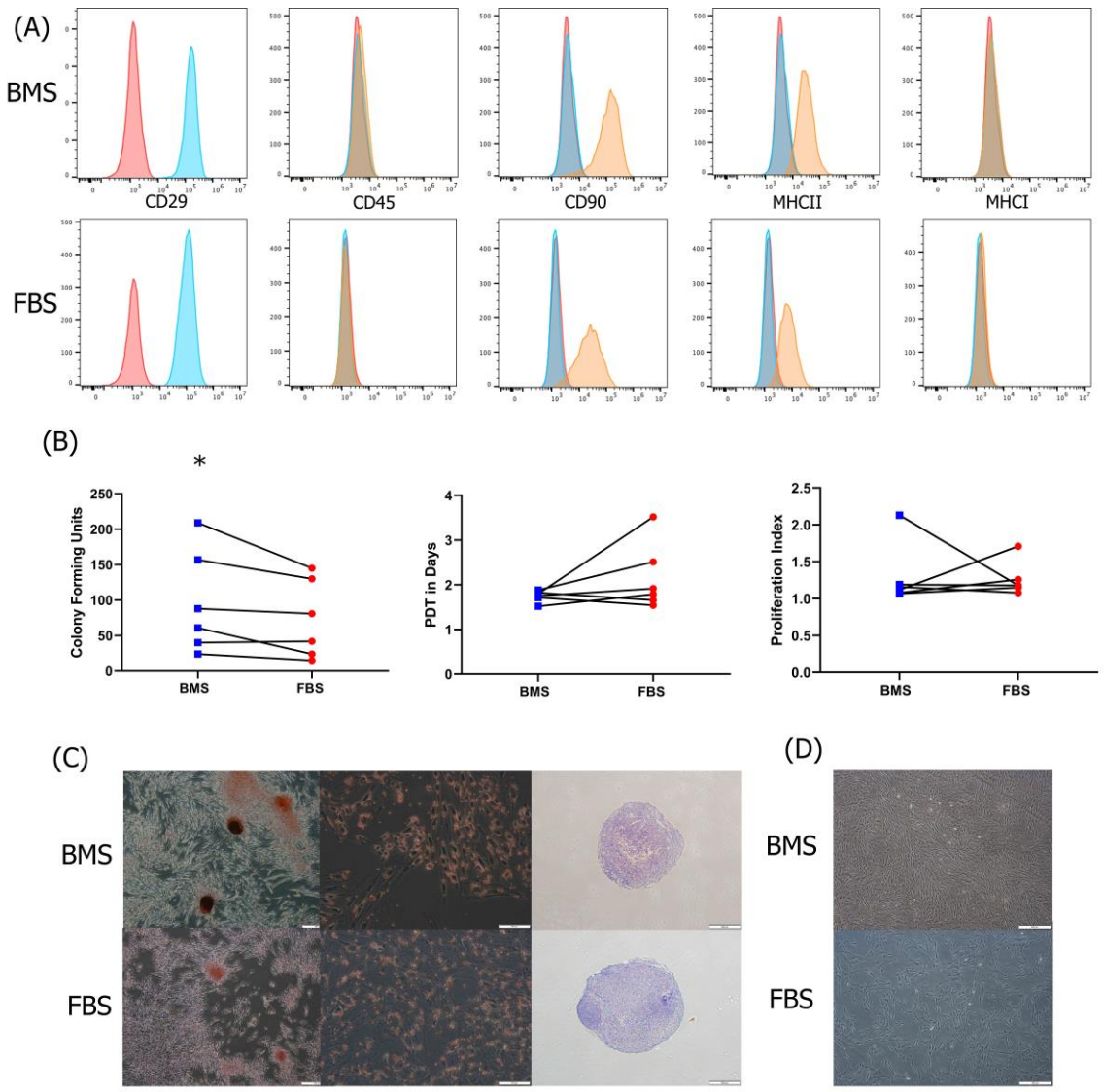

Figure 2.1 MSC isolation is increased in BMS-MSCs compared to FBS-MSCs; expansion and characterization are not different
(A) There was no difference in cell surface marker expression, (B) there was greater isolation of CFU-f colonies with BMS compared to FBS, but no difference in population doubling time (PDT) from isolation to passage 3, or immunomodulation, measured by proliferation index. (C) Trilineage differentiation into bone, fat, and cartilage was not different between BMS-MSCs and FBS-MSCs. (D) Phenotypically, BMS-MSCs were similar in appearance to FBS-MSCs.

2.3.2. FBS-MSCs, but not BMS-MSCs, cause local inflammation and are targeted by the recipient immune system

Eighteen horses received intra-articular injection of autologous MSCs prepared with media supplemented with autologous BMS (n = 6), pooled BMS (n = 6), or FBS (n = 6). Intra-articular injections occurred on experimental day 0 and 29. There were no differences in any data set between the autologous and pooled BMS groups; therefore, pooled and autologous BMS data were combined to a single group (BMS-MSC, n=12).

2.3.2.1. FBS contamination causes local inflammation and adverse clinical response

After each intra-articular injection, there was increased peri-articular edema and synovial effusion in FBS-MSC recipients compared to BMS-MSC recipients (Fig. 2 and 3) (58, 59). There were no differences in pain between groups after the first injection. One FBS-MSC recipient was removed from gait analysis for assessment of pain after the second injection because of a right forelimb lameness, not related to the study. After the second MSC injection, when LPS was also administered, there was reduced MSC efficacy with a trend of more pain in FBS-MSC recipients on day 30 and 31, and significantly worse pain in FBS-MSC recipients on day 32 (Fig. 2) (18, 45).

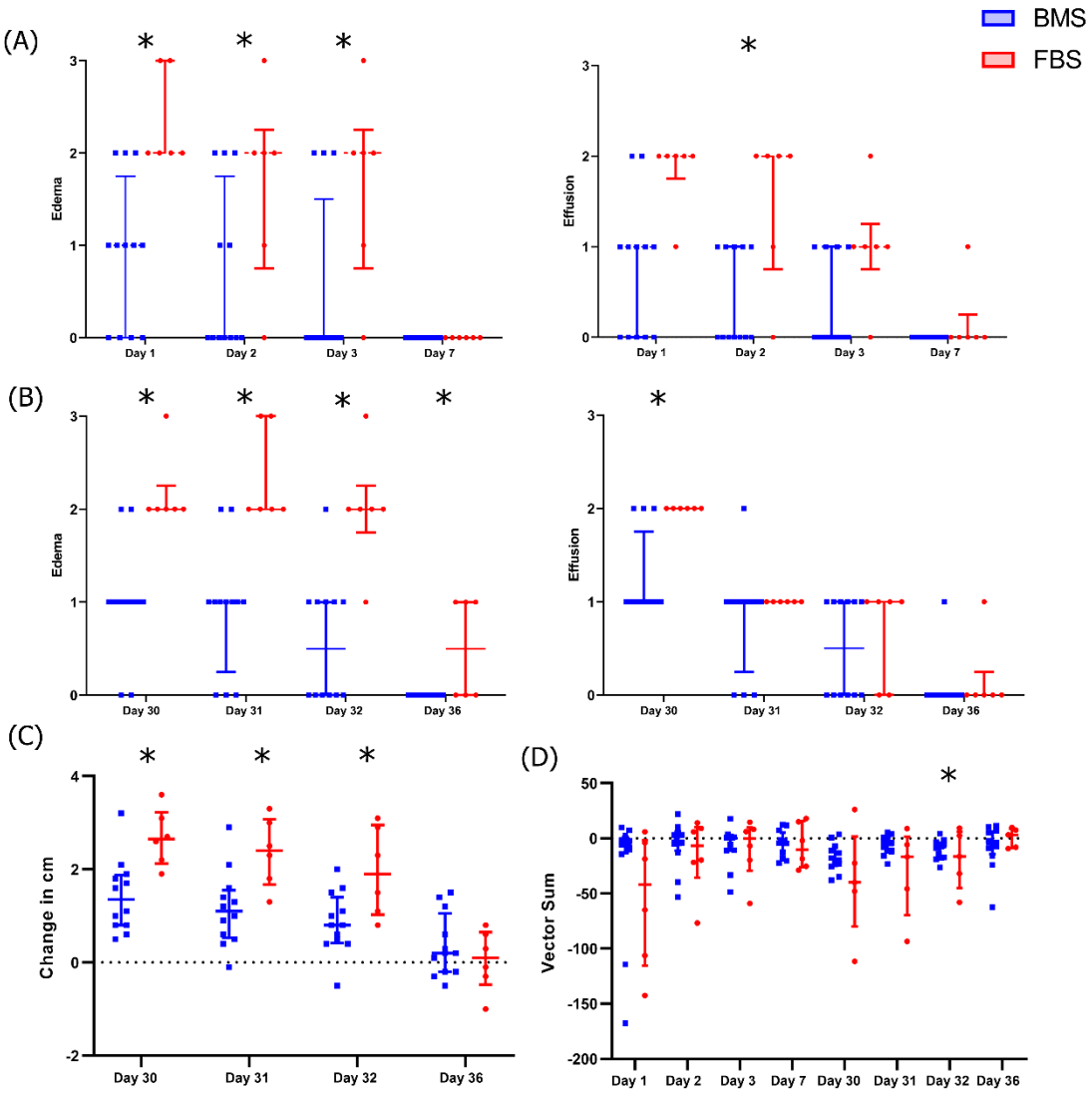

Figure 2.2 Local adverse response in FBS-MSC recipients compared to BMS-MSC recipients (A) Peri-articular edema and synovial effusion was worsened in FBS-MSC recipients (significance denoted by asterisks). (B) After the second injection, with concurrent administration of LPS, peri-articular edema and synovial effusion was again worsened in FBS-MSC recipients. (C) Likewise, limb circumference was increased after the second injection in FBS-MSC recipients. (D) There was no difference in gait asymmetry (pain) after the first injection. After the second injection, with concurrent LPS administration, there was a trend of worsened gait asymmetry in FBS-MSC recipients on day 30 and 31, and worsened gait asymmetry on day 32 in FBS-MSC recipients.

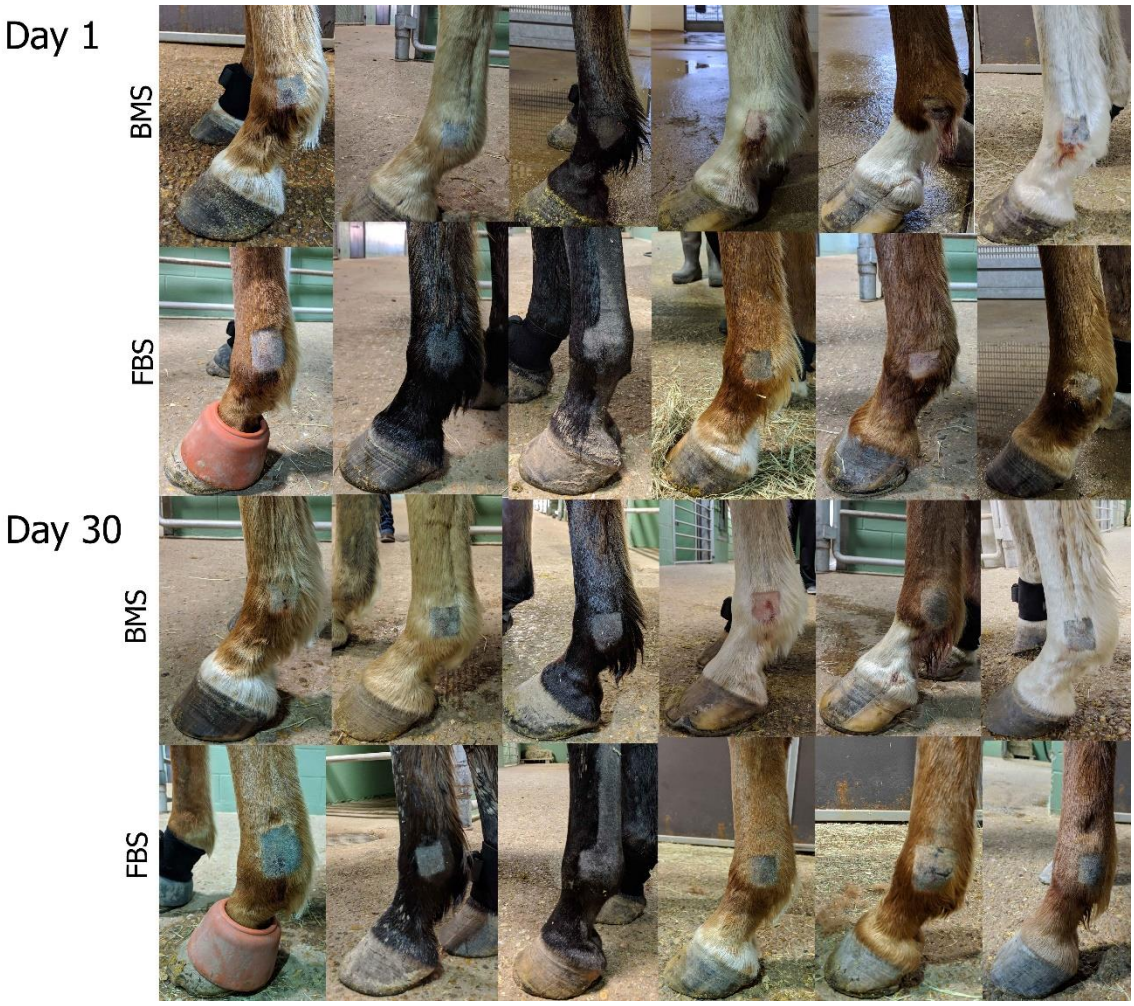

Figure 2.3 Post-injection edema was marked in FBS-MSC recipients compared to BMS-MSC recipients
Photographs taken on days 1 and 30, one day after FBS-MSC or BMS-MSC injection. There was worse swelling that retained impression (pitting edema) in FBS-MSC recipients.

2.3.2.2. No difference in synovial fluid cytology, cytokine, or chemokine concentrations

There were no differences in synovial fluid cytology (total nucleated cell count or proportion of cell type) after either each injection (Figure 2.4). Synovial cytokines or chemokines the day after each injection revealed measurable concentrations of IFNγ, MCP, IP-10, IL-10, IL-6, IL-4, and IL-1β, but no differences between groups (Figure 2.4).

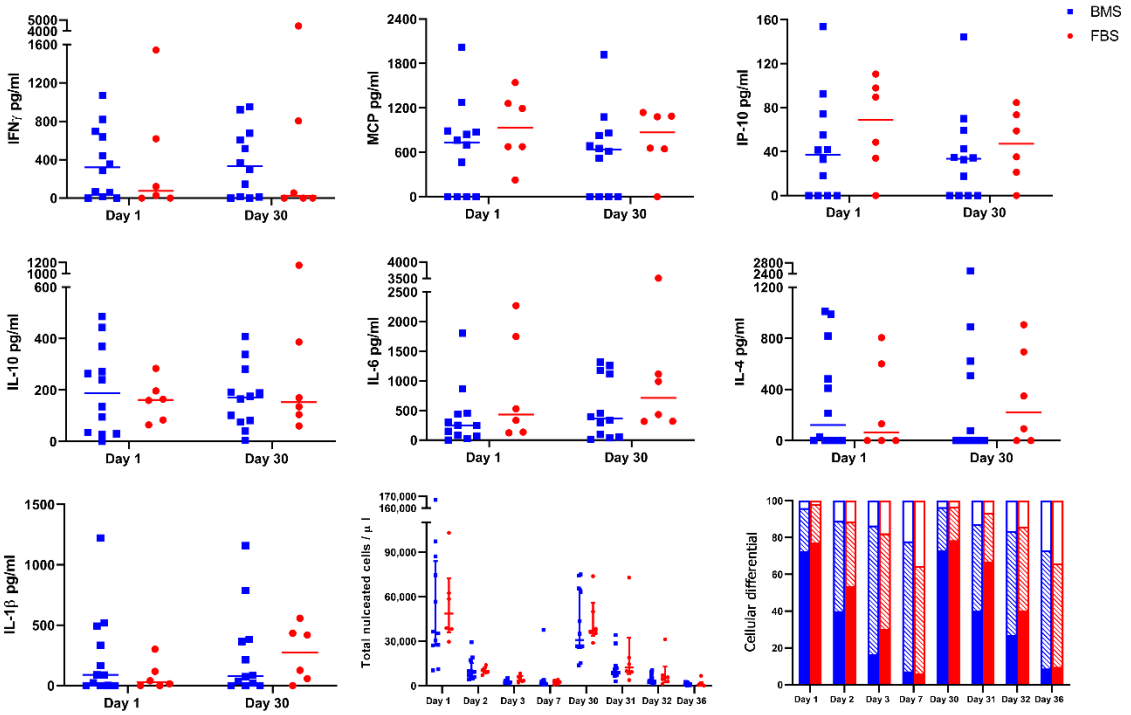

Figure 2.4 No differences in cytokine and chemokine concentration, total nucleated cell count, or cellular differential after either injection
There were no differences in concentration of IFNγ, MCP, IP-10, IL-10, IL-6, IL-4, IL-1β in synovial fluid on days 1 or 30 between groups. Likewise, there were no differences in synovial total nucleated cell count or cellular differential after either injection (solid portion = neutrophils; diagonal lines = small lymphocytes; empty portion = large mononuclear cells).

2.3.2.3. Anti-bovine antibodies were present and unchanged in all horses

Anti-bovine antibody concentrations were not different between groups or over time (Fig. 2.5). Titers varied by individual, with a median maximum titer of 1:204,800 (range, 1:12,800 – 1:409,600) without differences in anti-bovine antibody titers between day 0 and 56 (Figure 2.5).

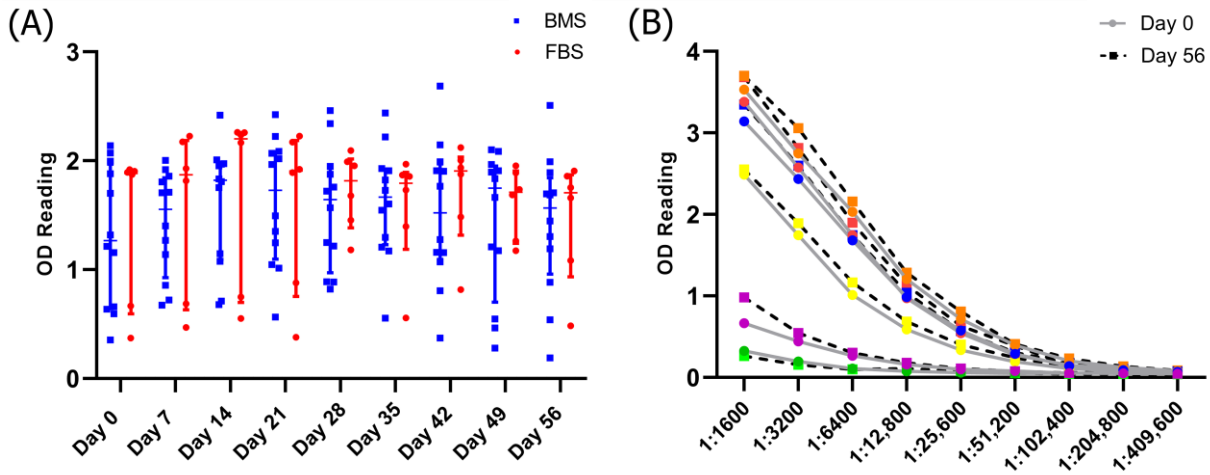

Figure 2.5 Anti-bovine titers were present in all horses prior to MSC administration that did not change after MSC administration
(A) There were no differences in antibody concentrations, measured by optical density (OD), between groups, and antibody concentrations did not change over time. (B) There was no difference in anti-bovine titers in FBS-MSC recipients prior to and after FBS-MSC exposure. Each individual is represented by a different color.

2.3.2.4. Antibodies induce cytotoxicity of FBS-MSCs, but not BMS-MSCs

While it is well documented that anti-bovine antibodies are present in human and equine serum, little is known about the consequence of these antibodies relative to FBS-MSCs (19, 20). Microcytotoxicity assays with recipient serum and autologous MSCs resulted in widespread death of FBS contaminated MSCs. In contrast there was virtually no cytotoxicity of BMS-MSCs (Figure 2.6).

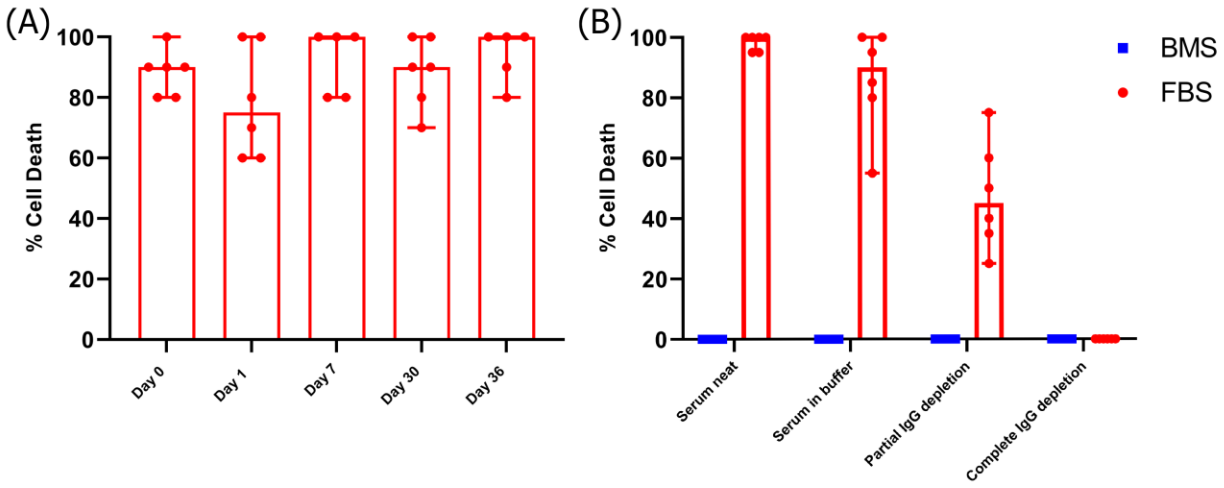

Figure 2.6 Antibodies against bovine proteins are present in serum and synovial fluid, and cause death of FBS prepared MSCs
(A) Synovial fluid collected on days 0, 1, 7, 30, and 36 produced significant cell death when combined with autologous FBS-MSCs. (B) To demonstrate that MSC cell death was antibody mediated, serum collected on day 35 was depleted of immunoglobulins partially and completely. All serum samples were combined with either BMS-MSCs or FBS-MSCs. There was no death of BMS-MSCs, there was significant cell death of FBS-MSCs both neat and in buffer, a reduction of cell death with partial IgG depletion, and elimination of cell death with complete IgG depletion.

To confirm that death of FBS-MSCs in the microcytotoxicity assay was due to antibody, we repeated mircrocytoxocity assays with partially and fully immunoglobulin depleted serum. There was virtually no cell death of BMS-MSCs when combined with serum in buffer, partial, or complete immunoglobulin depleted serum. In contrast, there was marked death of FBS-MSCs when combined with serum in buffer, a reduction in cell death with partial immunoglobulin depletion, and absence of cell death after complete immunoglobulin depletion (Figure 2.6).

2.3.2.5. Pre-existing anti-bovine antibodies in synovial fluid cause FBS-MSC death

After demonstrating the presence of consistent and unchanged anti-bovine antibodies in serum capable of causing FBS-MSC cytotoxicity, we wanted to evaluate if antibodies are of consequence in the articular environment. This is of particular importance because the articular environment is often considered to be immune privileged as it is nearly acellular with a distinct blood-joint barrier that minimizes diffusion of small molecules (60, 61). We repeated microcytotoxicity assays combining FBS-MSCs or BMS-MSCs with synovial fluid collected on days 0, 1, 7, 30, and 36 after intra-articular MSC administration. At all time-points, there was cytotoxic FBS-MSC death but not BMS-MSC, confirming that anti-FBS antibodies are present in synovial fluid in sufficient quantities to cause cytotoxic cell death of FBS contaminated MSCs before and after intra-articular injections (Figure 2.6).

2.3.2.6. Joints injected with FBS-MSCs have lower synovial MSC concentrations compared to those injected with BMS-MSCs

To assess for differences in MSC survival within the joint, we measured synovial MSC concentrations using CFU-f assays on days 1, 7, 30, and 36. The proportion of CFU-f plates with at least one colony was higher on days 1,7, and 36 in BMS-MSC recipients compared to FBS-MSC recipients (Figure 2.7). The total number of colonies from each joint was higher one week after each injection on days 7 and 36 in BMS-MSC recipients compared to FBS-MSC recipients (Figure 2.7).

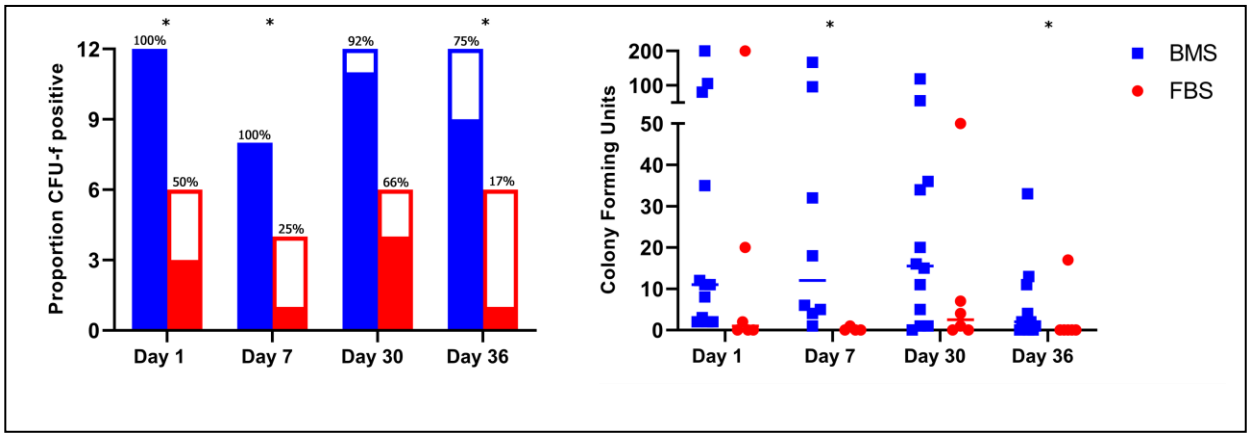

Figure 2.7 Increased synovial MSC isolation after BMS-MSC administration compared to FBS-MSC administration
The proportion of synovial fluid CFU-f plates with at least one colony was higher in BMS-MSC recipients (significance denoted by asterisks). Bars represent total number of cultures performed per day with solid portions denoting colonies present and clear portion denoting no colonies seen. (B) The synovial MSC concentration was higher in BMS-MSC recipients one week after each injection.

2.4. Discussion

We demonstrate that the recipient immune response to FBS prepared autologous MSCs results in MSC death, local inflammation, and reduced synovial MSC concentrations. Recent failures of therapeutic MSCs to achieve market approval in the United States, despite foreign regulatory approval, might be due to FBS use during MSC preparation and resultant altered clinical effect and failure to meet stringent end points (1, 19, 44, 45, 62). While clinical MSC preparation is transitioning away from FBS use, continued FBS supplementation of MSCs in animal models will obfuscate clinical translation (25, 45, 59).

Our findings refute the conclusion that anti-bovine titers are not of consequence because they do not change with repeated exposure to FBS-MSCs (7, 26, 33). We suggest the lack of change in anti-bovine titers, described by others and here, is because peak titers that are incapable of an anamnestic response exist prior to MSC therapy (26, 33). In horses, these peak titers are due to routine bi-annual vaccination against viral pathogens, which are also prepared with FBS (63). This frequent vaccination results in inadvertent, but thorough, vaccine induced immunity and peak titers against bovine proteins (63).

Veterinary reports have attributed inflammatory events after MSC therapy to a normal physiologic response to MSCs, and in people immunomodulatory drugs are commonly administered to mitigate inflammation during MSC administration (26, 45, 59, 64-66). We show that adverse reactions and local inflammation are not the normal physiologic response to MSCs, rather a consequence of FBS contamination of MSCs (59, 67). Moreover, the articular environment that we used is particularly well suited to

investigate these adverse events because the blood-joint barrier and large volume-to-surface area ratio sequesters the local response, augmenting detection of inflammation (60, 61). Given this exquisite sensitivity of the articular joint to inflammation, the absence of edema or effusion in BMS-MSC recipients without concurrent anti-inflammatory administration is remarkable.

In a recent report from our group using the same experimental model, we showed differences in synovial cytokine concentrations, including IFNγ, one day after mismatched allogeneic MSCs were administered compared to matched allogeneic MSCs (54). Intriguingly, in the current report, we did not see differences in synovial cytokines, despite marked synovial effusion and peri-articular edema that lasted several days in FBS-MSC recipients. This difference in degree and duration of inflammation, with greater degree of inflammation in mismatched recipients reported previously and longer duration of inflammation in FBS-MSC recipients reported here, is likely because of differences in the mechanism of antigen recognition. With allogeneic mismatch, MHCI incompatibility would result in every MSC being immediately identified as foreign. Whereas, FBS contaminated MSCs may not initially be presenting bovine antigen, may be presenting very little bovine antigen, or may not present bovine antigen until days after administration.

We also recently reported increased endogenous progenitors in joints injected with matched allogeneic MSCs compared to mismatched allogeneic MSCs (54). In the report here, all injected MSCs were autologous and whether the increased synovial MSC concentration after BMS-MSC administration was due to improved MSC persistence or

endogenous progenitor upregulation cannot be distinguished. Nonetheless, recipient immune targeting of FBS-MSCs resulted in lower synovial MSC concentrations.

2.5. Summary

We show that recipient anti-bovine titers cause antibody mediated death of MSCs with resultant local inflammation and reduced synovial MSCs after FBS-MSC administration. The historic and current use of FBS for MSC preparation is likely to misrepresent MSC effect because of cytotoxicity and adverse responses to FBS contamination (24, 64, 68). When evaluating reported pre-clinical, veterinary, and human clinical trials, the use of FBS should be recognized when interpreting results (1, 25, 45, 62). Bone marrow supernatant is a simple, inexpensive, and autologous replacement for FBS that eliminates immune targeting and resultant adverse clinical effects. Fetal bovine serum should not be used for MSC supplementation in veterinary, pre-clinical or clinical MSC preparation, and the use of BMS should be further investigated.

3. MESENCHYMAL STEM CELL THERAPY FOR JOINT DISEASE IN HORSES: PREPARATION MATTERS

3.1. Introduction

Mesenchymal stem cells (MSCs) are one of the most heavily investigated and utilized regenerative medicine therapies in the horse, yet there are no approved therapies in the United States (69, 70). Similarly in man, despite decades of research and massive clinical effort, failure to meet stringent clinical endpoints in late phase trials and post-approval monitoring has precluded market authorization of MSCs in the United States.(1) Variable laboratory preparation techniques have been identified as a possible reason for mixed treatment effects and approval failures (2, 3, 18, 42, 71).

To our knowledge, fetal bovine serum (FBS) is utilized as a media supplement in all reported equine MSC preparations or the preparation methods are not reported (24, 33, 68, 72, 73). Supplementation of cell culture media with FBS has been an industry standard since the 1970s because it results in highly predictable and reproducible MSC propagation (9). However, supplementation with FBS results in intracellular bovine contamination that is presented by MSCs on MHCI as part of normal cellular processing (11, 74). This is of particular importance in horses because of univerally high anti-bovine titers due to routine annual vaccination (20, 74). We recently demonstrated that these anti-bovine titers in horses result in recipient immune targeting and death of fetal bovine serum (FBS) prepared autologous MSCs resulting in reduced MSC efficacy (74). Given the immune targeting of bovine contaminated MSCs and adverse reactions in

horses, we have suggested that MSC efficacy would be improved with a xenogen-free preparation technique (10, 63, 74).

To eliminate immune recognition of MSCs as a result of xenogen contaminants, we developed a technique for autologous MSC preparation using autologous bone marrow supernatatnt (BMS). We have been using autologous BMS instead of FBS for the preparation of autolgous MSCs in equine clinical patients for the treatment of joint disease since 2018. Our objective is to report the outcome in horses with lameness due to osteoarthritis that were treated with autologous BMS-MSCs by intra-articular injection.

3.2. Materials and Methods

University large animal hospital medical records were searched from June 2018 to October 2020 for horses treated with intra-articular BMS-MSCs. The following was recorded from the medical record: age, breed, gender, discipline, treated joint, method of lameness localization (diagnostic anesthesia or clinical examination combined with diagnostic imaging), pre-treatment failure to respond to intra-articular corticosteroid, degree of injury (mild, moderate, marked), if and what surgery was performed on the treated joint, number of MSC injections, adverse reactions after treatment, and level of work up to 12 months after the first treatment. Exercise programs were categorized as stall rest, controlled exercise program (partial work), or full work. Based on diagnostic imaging, lesions were categorized as: OA (osteoarthritis with radiographically apparent enthesophytes and osteophytes), AC (osteoarthritis with articular cartilage damage), or SB (osteoarthritis with articular cartilage damage and subchondral bone loss).

Whether our standard protocol for MSC use was followed for each case was confirmed in the medical record. In brief, our standard protocol is as follows. Each treatment dose is approximately 10×10^6 MSCs per joint that are thawed and immediately injected, suspended in 1 ml of cryopreservation media (95% autologous serum and 5% DMSO). Non-steroidal anti-inflammatory medications are not administered, the treated limb is not wrapped, and the horse is rested for 3 days. The dosing protocol is 3 intra-articular injections at monthly intervals. After the initial 3 injections, monthly injections are continued until there is resolution of lameness and effusion.

3.3. Results

Eighteen horses received autologous BMS-MSCs by intra-articular injection from June 2018 – October 2020. There were 11 geldings, 5 mares, and 2 stallions. Ages ranged from 2 – 20 years, with the median of 10 years. Breeds included: Quarter Horses (n = 8), Warmblood (n = 8), Andalusian (n = 1), and Saddlebred (n =1). Disciplines included: dressage (n = 8), western show (n = 7), hunter (n = 1), barrel racing (n = 1), and reining (n = 1).

Primary joint injury was classified as osteoarthritis with articular cartilage injury (AC) in 11 cases, and osteoarthritis with articular cartilage injury and subchondral bone (SB) loss in 7 cases (Table 3.1 and Fig. 3.1 and 3,2). Three horses had joints treated bilaterally, and four horses also had secondary joints treated that were unrelated to the primary lesion (Table 3.1). Lameness was localized to the affected joint with regional anesthesia (4/18), intra-articular anesthesia (11/18), or clinical examination and imaging

(3/18). Diagnostic imaging for primary lesions included radiographs (18/18), magnetic resonance imaging (12/18), nuclear scintigraphy (1/18), ultrasound (1/18), or arthroscopy (7/18). Fourteen/18 (78%) of horses had lameness that was refractory to intra-articular corticosteroid injection of the affected joint. Surgery was performed in 7/18 (39%) horses prior to MSC injection. Joint disease severity was classified as mild in 3/18 (17%) horses, moderate in 6/18 horses (33%), and marked in 9/18 horses (50%).

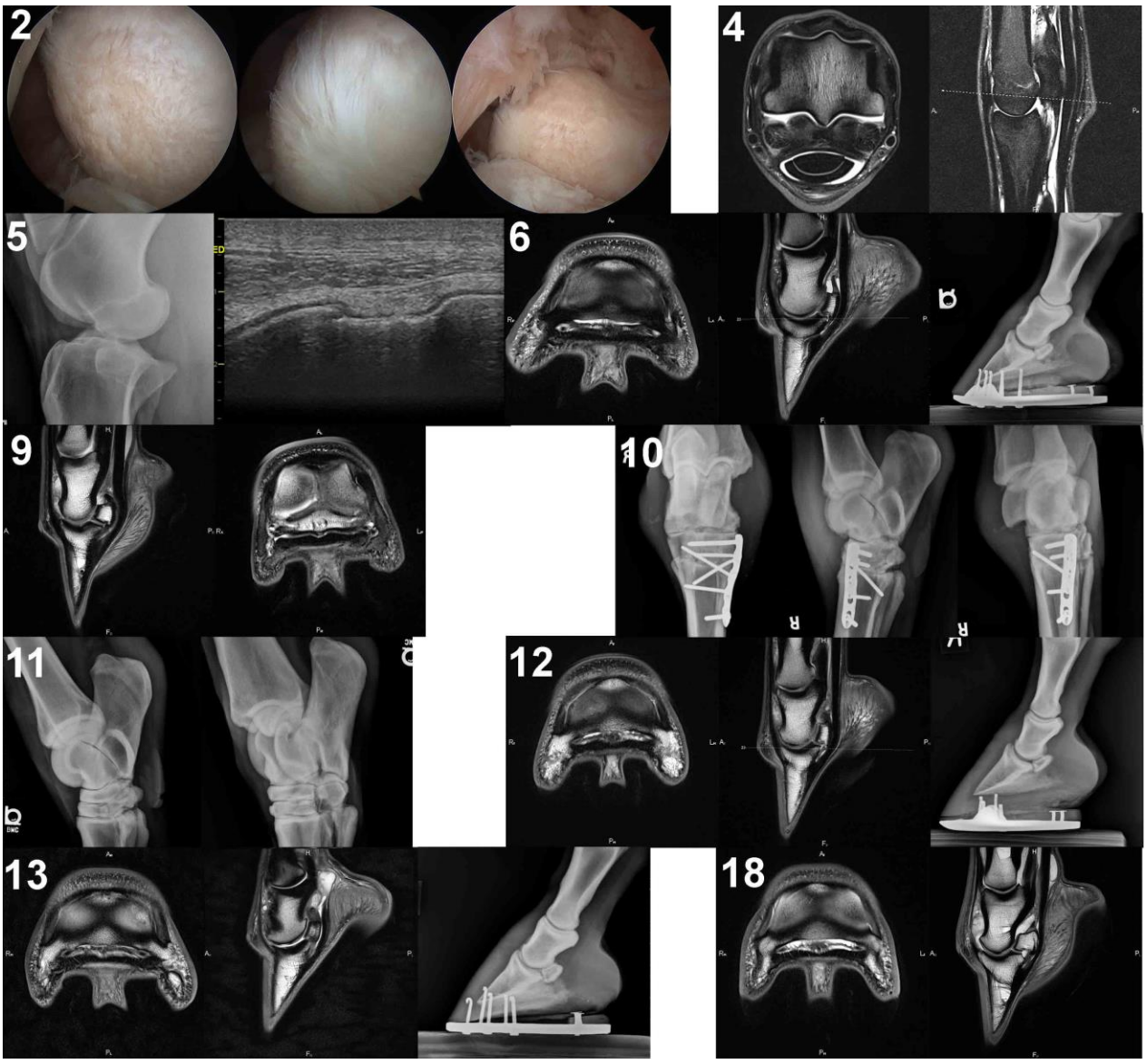

Figure 3.1 Composite of diagnostic images of primary lesions categorized as AC (articular cartilage damage) by horse. Of these lesions, 3 joint injuries were classified as mild, 3 as moderate, and 5 as severe.

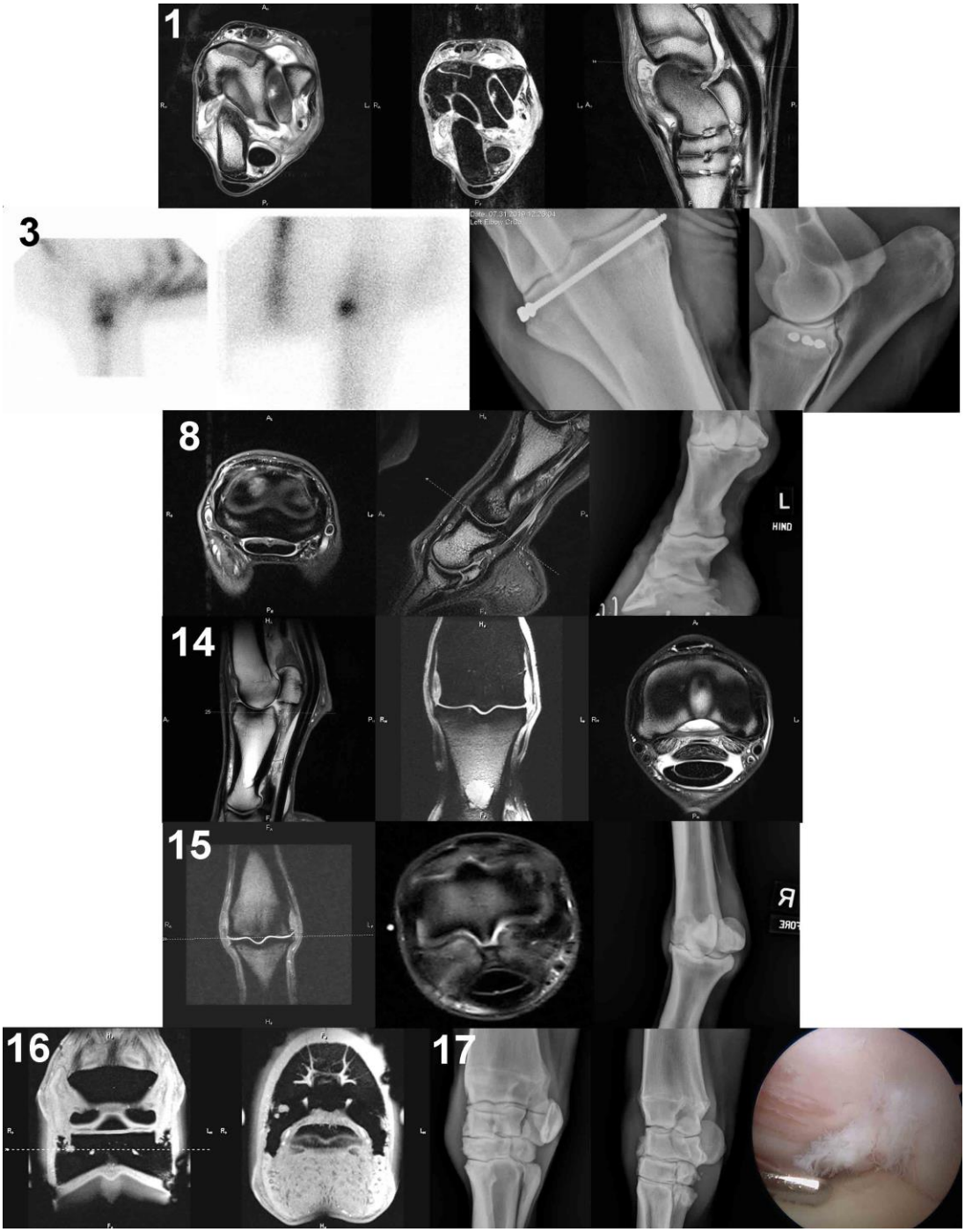

Figure 3.2 Composite of diagnostic images of primary lesions categorized as SB (subchondral bone damage) by horse. Of these lesions, 3 joint injuries were classified as moderate and 4 as severe.

	Age	Breed	Discipline	Joint	Lesion Type	Lesion Severity	Failed prior steroid	Surgery Performed	Additional Imaging	Total number of treatments
Horse 1	2	Saddlebred	Show	TC	SB	Mod	N	Arthroscopy	MRI	3
Horse 2	19	Holsteiner	Dressage	TC	AC	Mod	Y	Arthroscopy	NA	3
Horse 3	11	Quarter Horse	Western Show	HR	SB	Marked	Y	Cortical screw	Nuc med	4
				RC	OA		Y	N		3
Horse 4	4	Quarter Horse	Reining	MCP	AC	Mod	Y	Arthroscopy	NA	1
Horse 5	7	Andalusion	Dressage	MFT (bilateral)	AC	Mild	N	N	US	3 (each side)
Horse 6	10	Warmblood	Dressage	DIP	AC	Marked	Y	N	MRI	7
				MCP	AC		Y	N	MRI	7
Horse 7	6	Warmblood	Dressage	MCP	AC	Mild	Y	Arthroscopy	NA	3
Horse 8	16	Warmblood	Hunter	PIP	SB	Mod	Y	N	MRI	8
				MCP	SB		Y	N	MRI	8
				DIT	OA	Mild	N	N	NA	3
Horse 9	4	Quarter Horse	Western Show	DIP	AC	Marked	Y	N	MRI	6
Horse 10	10	Quarter Horse	Western Show	PIT (bilateral)	AC	Marked	N	N	MRI	5 (each side)
Horse 11	19	Holsteiner	Dressage	TMT/DIT (bilateral)	AC	Mild	N	N	MRI	2 (each side)
Horse 12	7	Quarter Horse	Western Show	DIP	AC	Marked	Y	N	MRI	8
Horse 13	13	Quarter Horse	Western Show	DIP	AC	Marked	Y	N	MRI	6
				RC	OA	Mod	N	N	NA	4
Horse 14	11	Warmblood	Dressage	MCP	SB	Mod	Y	N	MRI	4
Horse 15	17	Quarter Horse	Western Show	MCP	SB	Marked	Y	Cortical screw and arthroscopy	MRI	4
Horse 16	20	Warmblood	Dressage	DIP	SB	Marked	Y	N	MRI	6
Horse 17	5	Quarter Horse	Barrel Racing	RC	SB	Marked	Y	Arthroscopy	NA	3
Horse 18	11	Warmblood	Dressage	DIP	AC	Mod	Y	N	MRI	6

Table 3.1 Lesions for all horses treated with BMS-MSCs. Joint locations included: tarsocrural (TC), humeroradial (HR), metacarpophalangeal (MCP), medial femorotibial (MFT), proximal interphalangeal (PIP), proximal intertarsal (PIT), tarsometatarsal/distal intertarsal (TMT/DIT), and distal interphalangeal (DIP). Lesions were scored as: OA, osteoarthritis including enthesophytes and osteophytes; AC, osteoarthritis with articular cartilage damage; or SB, osteoarthritis with articular cartilage damage and subchondral bone loss.

Other than injection frequency, our standard MSC protocol was followed in all cases. The median number of treatments was 3 (range, 1 – 8) (Table 3.2). Post-injection, two horses (horse 6 and 13) had an acute exacerbation of self-limiting lameness with lameness seen at a walk within 24 hours and resolved by 48 hours of MSC injection. In horse 6, there was an acute and short-lived exacerbation of lameness after each injection, with decreasing severity at each subsequent injection. By the 7th intra-articular injection there was no post-injection lameness exacerbation. In horse 13 there was an acute exacerbation of lameness after the third injection, but not after the first and second or fourth-sixth injections. In total, 117 joint injections were performed, and incidence of joint flare was 7/117 (6%).

The owner could not be contacted for one horse after the first intra-articular injection thus this horse was considered lost to follow up. Eleven/17 (65%) horses with follow-up returned to and stayed in full work, 2/17 (11%) returned to full work, but had lameness develop in another limb, 2/17 (11%) improved but were retired, 1/17 (6%) elected other therapy, and 1/17 (6%) was euthanized due to severity of disease. There were two horses that did not have a full 12 months of follow-up (horses 17 and 18) (Table 3.2).

Horse	Age	Breed	Joint	Lesion Severity	Failed prior steroid	Surgery Performed
1	2	SB	TC	AC	N	Y
2	19	WB	TC	AC	Y	Y
3	11	QH	Elbow	SB	Y	Y
4	4	QH	MCP	AC	Y	Y
5	7	And	MFT	AC	N	N
6	10	WB	DIP	AC	Y	N
7	6	WB	MCP	AC	Y	Y
8	16	WB	PIP	SB	Y	N
9	4	QH	DIP	AC	N	N
10	10	QH	TC	AC	N	N
11	19	WB	TMT/DIT	AC	N	N
12	7	QH	DIP	AC	Y	N
13	13	QH	DIP	AC	Y	N
14	11	WB	MCP	SB	Y	N
15	17	QH	MCP	SB	Y	Y
16	20	WB	DIP	SB	Y	N
17	5	QH	RCJ	SB	Y	Y
18	11	WB	DIP	AC	Y	N

Table 3.2: Timing of MSC therapy, level of exercise, and outcome for all horses. Red indicates stall rest, yellow indicates partial work, and green indicates full work.

Horse	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9	Month 10	Month 11	Month 12
1	1st	2nd	3rd									In full work
2	1st	2nd	3rd									In full work
3	1st	2nd								3rd	4th	In full work
4	1st	Lost to follow-up										
5	1st	2nd	3rd									In full work
6	1st	2nd	3rd	4th	5th	6th	7th					In full work
7	1st	2nd	3rd									In full work
8	1st	2nd	3rd	4th	5th	6th	7th	8th				In full work
9	1st	2nd					3rd	4th	5th	6th		Primary lesion resolved, lame on another limb
10	1st	2nd	3rd	4th	5th				Euthanized due to severity of disease			
11	1st	2nd	Elected other therapy									
12	1st	2nd			3rd	4th	5th	6th	7th	8th		Primary lesion resolved, lame on another limb
13	1st	2nd	3rd		4th	5th	6th					In full work
14	1st	2nd	3rd	4th								In full work
15	1st	2nd	3rd	4th				Improved but not resolved, retired				
16	1st	2nd	3rd	4th	5th	6th						In full work
17	1st	2nd	3rd							In full work		
18	1st	2nd	3rd	4th	5th	6th	Improved but not resolved, retired					

Table 3.2 continued: Timing of MSC therapy, level of exercise, and outcome for all horses. Red indicates stall rest, yellow indicates partial work, and green indicates full work.

3.4. Discussion

Current literature would suggest that MSCs improve the outcome in musculoskeletal conditions in horses, but clinical and experimental reports that show efficacy are extremely limited (24, 68, 72, 75). We and others have suggested that immune targeting of MSCs, because of FBS contamination, prevents optimal clinical effect and may be the reason for the scarcity of reports documenting MSC efficacy (10, 11, 74). In a recent experimental report, we were the first to evaluate the use of autologous MSCs prepared without FBS at any time point in horses, and we showed enhanced efficacy of autologous BMS-MSCs, compared to FBS-MSCs (74). In the retrospective report here, the number of horses that were able to return to full work (76%) and stay in full work (65%) is remarkable given the severity of joint disease treated. This data supports the importance of MSC preparation technique, and the avoidance of bovine contamination in optimizing MSC efficacy.

As a retrospective report, there are no controls to show that the positive response is due to MSC therapy. However, most cases selected to receive MSC therapy were severe. All cases had documented full thickness articular cartilage injury, and many cases also had associated loss of subchondral bone, which would not be expected to have good outcomes with conventional therapy alone. In fact, lameness in 14/18 horses was recalcitrant to intra-articular corticosteroid injection prior to MSC therapy, indicating an end-stage joint. Of those 14 horses, 9/14 (64%) returned to full work and only 4/9 that returned to full work also received other interventions, such as surgery.

In the past, we have considered the flare reaction after MSC therapy to be due to FBS contamination, as we have shown in experimental reports (10, 74). Yet, the 6% incidence of joint flare in this group of horses without FBS, is similar to the 9% reported by Ferris et al, where FBS was used for MSC preparation.(68) Two key differences to the Ferris et al. report should be noted. One, unlike the Ferris et al report, horses in this report did not receive NSAIDs. Two, the horses in our report received multiple intra-articular MSC injections, totaling 117 joint injections in 18 horses, where the Ferris report was only one injection per horse. Additionally, there was no peri-articular edema in either horse that flared, which is a striking difference to the flare we have experienced and previously reported to be secondary to FBS contamination (10, 74).

The long-term lameness resolution in cases with severe joint injury and previous failure to respond to conventional therapy is remarkable and begs the question, what exactly are the MSCs doing? It has been suggested that age related osteoarthritis is because of an age related reduction in synovial MSC concentration.(76) Our recent report of increased endogenous MSCs after intra-articular MSC injection may indicate that MSC therapy increases synovial MSC concentration, which may explain the long term effect (54, 76). Further work on the possible effects of MSC on osteoarthritic joints is indicated.

To date, reliable and predictable efficacy of MSC therapy has been elusive (1, 69). In man, laboratory MSC preparation techniques have been identified as a reason for failure to meet clinical end points (2, 3). This may also be true in the horse, as all previous reports have utilized FBS prepared MSCs or the preparation methods are not

reported (59, 68, 77). We have previously shown that FBS use during MSC preparation results in adverse effects as well as MSC death and reduced efficacy.(10, 74) In this retrospective report with severe joint injury in 50% of the cases, we report a 65% rate of return to full work, which is higher than would be expected with conventional therapy and our previous experience with FBS prepared MSCs. This is strongly suggestive of enhanced efficacy of MSCs prepared without FBS in equine clinical patients. A prospective clinical trial with BMS-MSCs should be performed. Preparation techniques for MSC therapies should be transparently reported. Optimization of MSC therapy is key in advancing the use of MSCs and further work investigation of MSC dosing, dosing intervals, and mechanism of lameness reduction are indicated.

4. CROSS-MATCHING OF ALLOGENEIC MESENCHYMAL STROMAL CELLS ELIMINATES RECIPIENT IMMUNE TARGETING*

Introduction

Mesenchymal stromal cell (MSC) therapy is one of the most heavily studied therapeutic modalities for which there are no market authorizations in the United States. The reason for the lack of regulatory approval could be that despite decades of MSC research and repeated pre-clinical success, late phase clinical trials and post approval monitoring have failed to demonstrate consistent therapeutic effects (5, 78, 79). Lack of efficacy has been proposed to be due to non-uniformity of MSC preparation and application techniques (2, 79, 80). Certainly, the immune-privileged status of MSCs has been questioned and the possible negative effect of immunological incompatibility on primary efficacy endpoints of MSC therapy has been considered (3, 5).

Clinical investigations into the effect of MHC mismatched allogeneic MSCs suggest that mismatch does not alter efficacy, but reports have been limited and immunological monitoring has not been stringent (6, 8). While donor specific antibody production against allogeneic MSCs has been confirmed in people (81-87), non-human primates (88, 89), and horses (64, 90) the effects of mismatched allogeneic MSCs on MSC persistence remains unknown (5). Certainly, reduced persistence with concomitant loss of function of allogeneic MSC-gene therapy constructs compared to autologous or syngeneic MSCs has been confirmed in laboratory animals (89, 91-95). Still, the survival

* Rowland, Aileen L et al. “Cross-matching of allogeneic mesenchymal stromal cells eliminates recipient immune targeting.” *Stem cells translational medicine*, 10.1002/sctm.20-0435. 25 Dec. 2020, doi:10.1002/sctm.20-0435

time of allogeneic MSCs is significantly longer than allogeneic fibroblasts, likely due to MSC immune-evasiveness (92). This immune evasion may explain why there is clinical safety with allogeneic MSC injection, and acute rejection responses do not occur, even if there is allo-recognition with resultant cytotoxicity.

Despite the value of laboratory animal models in science, pre-clinical success often fails to result in clinical application (96). Poor translation of pre-clinical findings is in part due to the use of inbred laboratory animals that lack the diversity of man (79, 96). Specific to the study of MSC immune-compatibility, results from syngeneic or inbred animals are not translatable to human patients because of the lack of MHC diversity (3, 79).

Conversely, the horse has wide genetic diversity with frequent MHC recombination events and is an ideal model to study immune compatibility of allogeneic cell therapy (97-99). Additionally, the horse is well-recognized for its value as a pre-clinical model for joint injury as the equine articular joint closely mimics that of man in the cartilage thickness and collagen distribution as well as the architecture of subchondral bone (100, 101). Given that nearly 20% of clinical trials for MSC therapy in man are for bone and cartilage disease, the equine articular model is ideal for pre-clinical study of allogeneic MSC therapy (102).

We compare repeated intra-articular injection of clinically prepared MHC matched, mismatched, and autologous MSCs to confirm allo-recognition of MSCs by the innate and adaptive immune system because of MHC mismatch. For the first time, using genetically distinguishable but MHC matched MSCs, we demonstrate that immune

recognition has a negative effect on endogenous progenitor recruitment. Moreover, we show that the immunomodulatory effects of MHC mismatched allogeneic MSCs are insufficient to prevent or overcome recipient innate and adaptive immune responses, resulting in cytotoxicity and local inflammation.

4.1. Materials and Methods

4.1.1. Experimental design

Four horses with homozygous MHC haplotypes (2 ELA-A5a, 2 ELA-A3b) were used as donors and each paired with 3 MHC matched and 3 MHC mismatched recipients. Bone marrow derived xenogen-free donor MSCs were injected into the left metacarpophalangeal (MCP) joint and recipient serum-DMSO into the contralateral MCP on days 0 and 29. All 4 donors and 2 additional horses received autologous MSCs at the same time points. In 6 additional horses, the left MCP joint was injected with lipopolysaccharide (LPS) alone on day 0, and a repeat injection was not performed. Synovial fluid was collected on days 0, 1, 2, 3, 7, 29, 30, 31, 32, and 36 from the MSC-treated joint and on days 0, 1, 29 and 30 from the contralateral joint that received serum-DMSO (Figure 4.1).

4.1.2. Animals

All experimental procedures were performed according to the *United States Government and Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training* and were approved by the Institutional Animal Care and Use Committee at Texas A&M University (AUP 2018-0118). No animals were euthanized for the purpose of this study. In total, 35 Quarter Horse type horses were

included in the study. Horses ranged in age from 2 – 22 years, and there were 2 intact males, 10 altered males, 21 intact females, and 2 altered females.

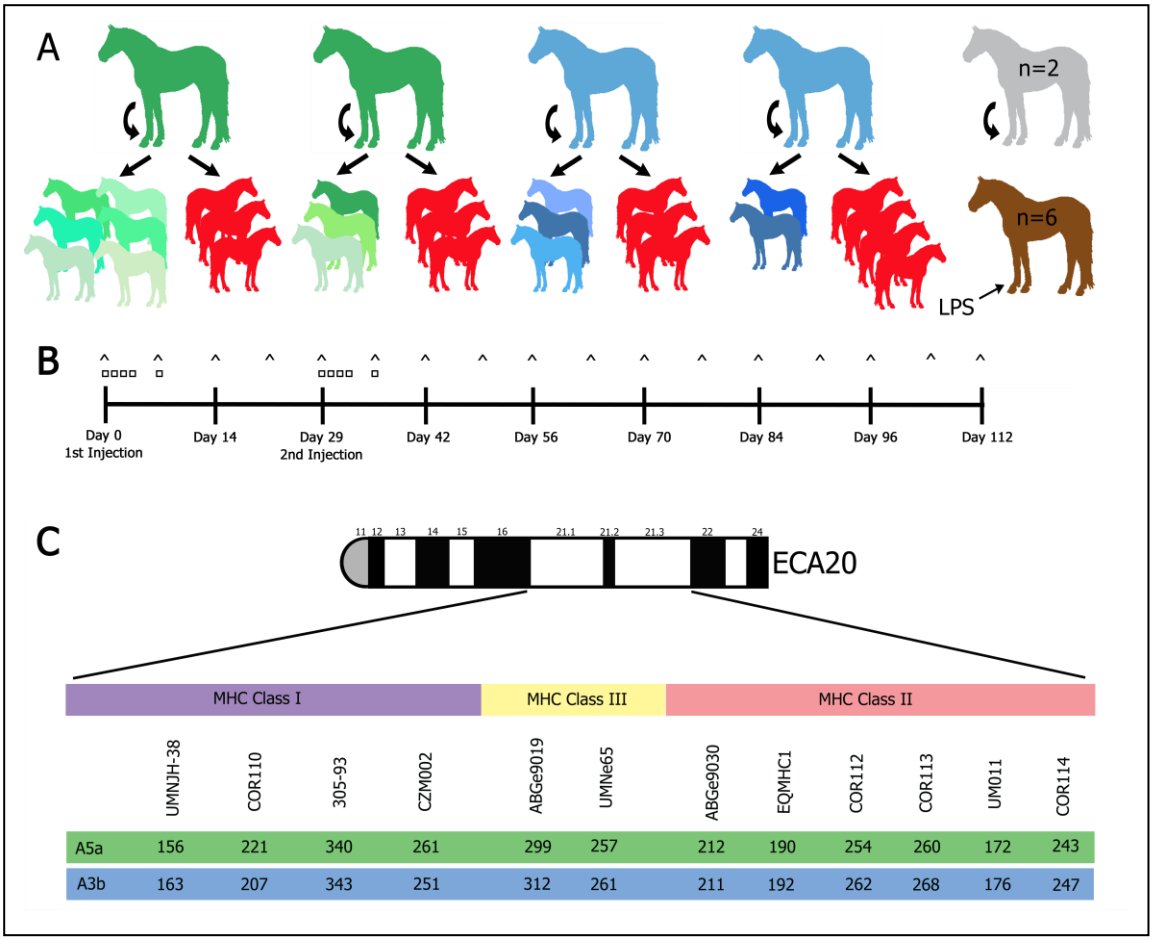

Figure 4.1 Study overview and schematic of intra-MHC microsatellite loci analyzed to determine donor and recipient MHC haplotypes

A) Donor and recipient pairings based on MHC haplotype (green, ELA-A5a; blue, ELA-A3b; red, mismatched). All donors received autologous MSCs, as did 2 additional unrelated horses (grey). Six additional horses were injected with 25 ng of lipopolysaccharide alone (LPS, brown).

B) Study timeline of MSC injection and sample collection (□, synovial fluid collection; ^, serum collection).

C) Base pair length at each microsatellite loci for ELA-A5a (green) and ELA-A3b (blue) haplotypes are noted. Horses homozygous for these haplotypes were used as MSC donors.

4.1.3. MHC haplotype identification

MHC haplotype analysis was performed on all horses. DNA was extracted from lymphocytes using a commercially available kit (Qiagen). Genomic DNA was amplified using multiplex fluorescent PCR with known primers for twelve microsatellite loci within the MHC region (103). PCR fragments were submitted to the Cornell University BioResource Center (BRC) and electrophoresed on an ABI 3700 instrument. Fragment analysis files were analyzed using GeneMarker software (SoftGenetics, State College, PA). Known haplotypes were reported when matched to a previously characterized haplotype, novel haplotypes were reported when two or more individuals with the same haplotype were identified in the cohort, and unknown haplotypes were reported when no individuals with the same haplotype had been previously identified (Table 4.1).

	I UMNJH-38	I COR110	I 305-93	I CZM002	III ABGe9019	III UMNe65	II ABGe9030	II EQMHC1	II COR112	II COR113	II UM011	II COR1144
ELA-A5a												
1	156	209	344	253	297	269	205	194	258	260	169	243
	156	211	345	261	305	253	207	190	254	260	172	243
2	156	221	342	259	299	257	207	190	237	266	179	241
	165	219	345	230	316	263	205	194	256	270	172	249
3	156	211	343	249	301	259	209	192	262	268	174	234
	156	215	345	247	307	257	207	190	254	260	172	243
4	156	194	336	230	307	257	207	190	254	260	172	243
	161	219	345	251	314	261	209	192	254	270	172	249
5	156	211	336	230	312	261	206	192	244	270	169	249
	163	217	345	255	305	259	209	192	262	272	172	255
6	156	211	343	249	301	259	209	192	262	268	174	234
	163	211	345	251	297	267	215	194	256	274	165	236
ELA-A3b												
7	156	221	342	259	312	261	207	190	237	264	180	243
	156	215	345	253	312	261	221	180	250	274	171	243
8	156	211	**	**	312	261	209	192	244	270	169	249
	156	221	**	**	312	249	206	190	237	266	172	249
9	156	211	343	249	301	259	209	192	262	268	174	234
	156	209	343	261	314	259	206	192	268	274	180	245
10	156	209	343	**	297	269	207	190	237	266	169	234
	156	211	345	**	310	259	215	190	260	272	170	255
11	156	215	345	253	312	261	221	180	252	274	171	243
	156	219	345	261	299	257	206	192	244	270	172	249
12	156	209	344	261	299	257	212	190	254	260	172	243
	156	211	345	240	318	257	212	190	262	270	184	245
13	156	221	342	259	312	261	207	190	237	264	180	243
	156	207	349	265	299	257	211	194	262	270	170	247

Table 4.1: Microsatellite haplotypes of all 13 mismatched recipients. Horses 1-6 received MSCs from an ELA-A5a donor, and horses 7-13 received MSCs from an ELA-A3b donor.

4.1.4. MSC preparation

Bone marrow was collected from the sternum of donor horses and autologous recipients as previously described (50). Heparinized raw bone marrow was centrifuged at 300g for 5 minutes and the bone marrow supernatant collected and filtered through a 100 µm filter to remove lipid aggregates. Red blood cell lysis was performed using

ammonium chloride (7.7 mg/ml NH_4Cl; 2.06 mg/ml hydroxymethane-aminomethane; pH 7.2). The remaining cellular portion was plated at 175 µl original bone marrow volume/cm^2 and maintained in Dulbecco's modified Eagle's culture medium 1 g/L glucose (Corning) that was supplemented with 2.5% HEPES buffer (Corning), 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B (Life Technologies), 1 ng/ml of basic fibroblast growth factor (b-FGF, Corning), and 10% bone marrow supernatant, and cultures were maintained at 37 ˚C, 5% CO^2 in humidified air and media exchanged three times per week (104). When colonies or monolayers reached 70% confluence, cultures were passaged and cells replated at 5000 cells/$cm^{2,}$ as previously described (50). After 3 passages, MSCs were cryopreserved in 95% recipient serum and 5% DMSO (Sigma Aldrich) with 10 x 10^6 MSCs per ml. Cryopreservation media alone (95% recipient serum and 5% DMSO without MSCs) was cryopreserved at the same time for injection into the contralateral joint. All MSCs used for injection were expanded from the same bone marrow aspirate and cryopreserved after 3 passages.

4.1.5. MSC characterization

Donor MSCs underwent trilineage differentiation and immunophenotyping as previously described (50). Briefly, expression of MHCII (Bio-Rad), CD45RB (VMRD Inc), CD90 (VMRD Inc), and CD29 (Beckman Coulter) were evaluated using commercially available antibodies, and MHCI was evaluated using our own anti-equine monoclonal antibody CZ3.2.

Primary antibodies (MHCII and CD29) were added to 1 million cells per antibody at a dilution of 1:100 and incubated for 45 minutes at 4 ˚C. When MSCs were

stained with secondary antibodies (MHCI, CD90, and CD45RB), MSCs were added to 1 million cells per antibody undiluted, 1:400, and 1:10 dilutions, respectively, and incubated for 15 minutes on ice prior to the addition of secondary antibody (Jackson Immunoresearch, 1:100) and then incubated again for 15 minutes on ice. All aliquots of cells had 5 µl of 7-AAD (Biolegend) added immediately prior to analysis and only live cells were included in analysis.

To assess multipotency of MSCs, trilineage differentiation into cartilage, bone, and fat was performed, all differentiations were performed in triplicate. For chondrogenic differentiation, 500,000 cells were pelleted via centrifugation and maintained in media containing Dulbecco's modified Eagle's medium with 4.5 g/L glucose (Corning), supplemented with 1% fetal bovine serum (FBS, GE Life Sciences), 2.5% HEPES buffer (Corning), 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B (Corning), 0.01 µg/ml transforming growth factor beta (Life Technologies), 0.1 nM dexamethasone (Sigma Aldrich), 0.05 mg/ml L-ascorbic acid (Sigma Aldrich), 0.04 mg/ml proline (Sigma Aldrich), and 1% ITS premix (VWR). Media was exchanged three times per week and after 21 days pellets were fixed in 4% paraformaldehyde (PFA, Sigma Aldrich) then embedded, sectioned, and stained with toluidine blue (Sigma Aldrich).

For adipogenesis, MSCs were plated to 6 well plates at 1000 cells/cm^2. Adipogenesis was induced using media containing Dulbecco's modified Eagle's medium F12 (Corning) supplemented with 3% FBS, 2.5% HEPES buffer (Corning), 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B (Life

Technologies), 1 ng/ml b-FGF, 5% rabbit serum (Thermo Fisher), 33 µM biotin (Sigma Aldrich), 17 µM calcium pantothenate (Sigma Aldrich), 1 µM insulin (Sigma Aldrich), 1 nM dexamethasone (Sigma Aldrich), 0.1 mg/ml isobutylmethylxanthine, and 1.78 ng/ml rosiglitazone (Sigma Aldrich). After 3 days, media was exchanged for the same media as above, without the addition of isobutylmethylxanthine and rosiglitazone. After a total of 6 days, plates were fixed and stained with Oil Red O (Sigma Aldrich).

For osteogenesis, MSCs were also plated to 6 well plates at 1000 cells/cm^2. Osteogenic induction media containing Dulbecco's modified Eagle's medium F12 supplemented with 10% FBS, 2.5% HEPES buffer (Corning), 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B (Life Technologies), 10 µM β-glycerophosphate (Sigma Aldrich), 1 ng/ml b-FGF, 20 nM dexamethasone (Sigma Aldrich), 0.05 mg/ml L-ascorbic acid (Sigma Aldrich). Media was exchanged three times per week. After 21 days, plates were fixed and stained with 2% Alizarin Red (Sigma Aldrich).

4.1.6. Intra-articular injections of MSCs or LPS

Horses were mildly sedated with 0.4 mg/kg xylazine hydrochloride (XylaMed, VetOne) intravenously and the left and right metacarpophalangeal (MCP) joints were aseptically prepared prior to intra-articular injection. Cryopreserved donor MSCs in recipient serum and recipient serum alone were thawed in a 37 ˚C water bath. The left MCP received 10 x 10^6 MSCs in freezing medium (recipient serum with 5% DMSO) and the right MCP was injected with freezing medium (serum-DMSO) alone. In 6 additional horses, the left MCP joint was injected with 25ng of lipopolysaccharide (LPS)

in DPBS without MSCs, and a repeat injection was not performed. Synovial fluid was serially collected on days 0,1, 2, 3, 7, 29, 30, 31, 32, and 36 from the treated joint, and on days 0, 1, 29, and 30 from the contralateral joint that received freezing medium alone (Figure 4.1).

4.1.7. Clinical assessment

Physical examinations including assessment of heart rate, respiratory rate, and temperature, were performed prior to each injection and daily for 3 days after injection. Gait asymmetry assessments were performed as an objective measure of pain using a commercially available system (Lameness Locator, Equinosis®). Baseline gait assessments were performed on days 0 and 29 prior to injection, and were repeated on days 1, 2, 3, 30, 31, and 32, or until the horse returned to baseline. Differences in gait were reported as a change from baseline (days 0 and 29), with a negative vector sum indicating a left forelimb lameness and a positive vector sum indicating a right forelimb lameness. Subjective evaluations of edema and effusion were performed at the same time points. Both scores were recorded independently: 0 = no edema or effusion; 1 = mild edema or effusion; 2 = moderate edema or effusion; and 3 = severe edema or effusion. All recipients were assessed prior to intra-articular injection.

4.1.8. Synovial fluid analysis

Synovial fluid collected from MSC (days 0, 1, 2, 3, 7, 29, 30, 31, 32, and 36), LPS (days 0, 1, 2, 3, and 7) and serum-DMSO (days 0, 1, 29, and 30) injected joints was evaluated for total nucleated cell count (TNCC) and nucleated cell differential. Synovial fluid samples collected on days 0 and 29 were all within normal limits (Appendix C,

Figure S4). Additional synovial fluid collected was centrifuged at 1600 RPM for 10 minutes to remove nucleated cells, and cryopreserved at -80 ˚C until assays were performed.

4.1.9. Microcytotoxicity assays

Microcytoxocity assays were performed as previously described (90). Briefly, serum was collected weekly from all recipients and 2 µl of recipient serum was combined with peripheral blood lymphocytes (PBLs, 3000 cells/well) or donor MSCs (1000 cells/well) under 5 µl of paraffin oil (Sigma Aldrich). A negative assay control was performed with donor PBLs or MSCs combined with autologous serum, and a positive control with donor PBLs or MSCs combined with anti-MHCI antibody (CZ3.2). After 30 minutes at room temperature, 5 µl of rabbit complement (Abcam) was added and the plates were incubated for 60 minutes at room temperature. Two µl of 5% eosin (Sigma Aldrich) was added, followed by 5 µl of 10% formalin (Sigma Aldrich). The experiment was repeated using synovial fluid collected on days 1 and 30, in the place of recipient serum. A masked evaluator estimated percentage of live and dead cells in each well.

4.1.10. Immunoglobulin depletion

To remove immunoglobulins, a combination of a commercially available IgG removal column utilizing Protein A (ProteoExtract®, Merck KGaA, Darmstadt, Germany) and manual depletion with Sepharose G beads (Millipore Sigma) was performed as previously described (57). Serum samples collected on day 35 from 6 MHC mismatched recipients (3, A5a recipients; 3, A3b recipients) were

immunoglobulin depleted. Briefly, 100µl of serum was added to 900µl of 1x Binding Buffer. The sample was passed through the IgG removal column in a dropwise manner. Three hundred µl of undiluted eluate was then combined with 200µl of preconditioned Protein G Sepharose beads and incubated at 20˚C for 1 hour with gentle mixing. After 1 hour, the samples were centrifuged at 4000g for 5 minutes and the immunoglobulin depleted supernatant collected. After sample processing, 2ml of Protein A Elution Buffer was passed through the IgG removal column and collected. Microcytotoxicity assays were repeated as above with respective donor PBLs being combined with serum diluted to a 1:10 dilution with 1x Binding Buffer, immunoglobulin depleted serum, or IgG removal column eluate.

4.1.11. Cytokine and chemokine analysis

Synovial fluid cytokine and chemokine concentrations were evaluated on days 1 and 30 using a commercially available kit (Luminex Multiplex, Millipore Sigma) according to manufacturer instructions. In brief, synovial fluid was thawed and centrifuged at 10,000g for 10 minutes before adding 25 µl of sample to each well along with 30 µl of premixed beads and 100 µl of assay buffer. Plates were incubated overnight at 4 ˚C with agitation. Plates were placed on a magnetic base and washed 5 times before 25 µl of detection antibodies were added and the plate incubated for 1 hour at room temperature with agitation. Twenty-five µl of streptavidin-phycoerythrin was added to each well and the plate incubated for an additional 30 minutes with agitation. Plates were washed and 200 µl of sheath fluid added and plates were read with 100 µl volume to be read. Analytes measured included: FGF-2, eotaxin, G-CSF, IL-1α, GM-

CSF, fractalkine, IL-13, IL-5, IL-18, IL-1β, IL-6, IL-17a, IL-2, IL-4, IL-12, IFNγ, IL-8, IP-10, GRO, MCP-1, IL-10, TNFα, and RANTES.

4.1.12. Synovial fluid CFU-f

MSC concentrations in synovial fluid were quantified by their ability to form colony forming units-fibroblasts (CFU-f). When assessed, 1ml of synovial fluid from day 30 was plated directly to 10 cm dishes supplemented with standard culture media (Dulbecco's modified Eagle's culture medium 1 g/L glucose with 2.5% HEPES buffer, 10,000 units/ml penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B, 1 ng/ml of b-FGF, and 10% FBS). Media was exchanged 24 hours after the synovial fluid was plated and again 3 days later. On the seventh day, plates were washed and stained with 3% crystal violet (Sigma Aldrich) and visible colonies counted and plates photographed.

4.1.13. Synovial fluid derived cell characterization and genotyping

Synovial fluid was plated to T75 tissue culture flasks and cells expanded until passage 3 at which point they were cryopreserved. Genotyping was performed by the Veterinary Genetics Laboratory at the University of California, Davis. Cryopreserved synovial MSCs, and hairs with roots attached from donors and recipients, were submitted for DNA analysis. Briefly, genomic DNA was extracted using a standard Proteinase-K digestion protocol and PCR for genotyping was performed. A panel of 17 microsatellite markers (AHT4, AHT5, ASB17, ASB2, ASB23, HMS2, HMS3, HMS6, HMS7, HTG10, HTG4, LEX3, LEX33, TKY333, TKY374, TKY394, and VHL20) and one gender marker (AME) were analyzed. Genomic DNA from synovial fluid MSCs

was compared to DNA from donor and recipient hair bulb to determine the origin of MSCs.

4.1.14. Statistical analysis

Statistical analysis was performed using commercially available software (JMP, Statistical Discovery from SAS, Cary, NC). Normality was not assumed, differences between groups was assessed using two-tailed Kruskall-Wallis, significance was set at $p < 0.05$. Unless otherwise indicated, error bars represent median values with inter-quartile range.

4.2. Results

4.2.1. Use of MHC homozygotic MSCs for matched and mismatched pairings

To evaluate the extent of immune recognition of allogeneic MSCs, we first identified donors and recipients to form MHC matched and mismatched pairings. Four homozygote donors of well-characterized equine leukocyte antigen (ELA) haplotypes (2 ELA-A5a, 2 ELA-A3b) were identified (Figure 4.1, Table 4.1)(105-107). Recipients with 1 copy of the donor haplotype were identified as matches. Three matched and 3 mismatched recipients were selected for each donor. Furthermore, one ELA-A5a homozygous donor was paired with an additional 3 recipients, for a total of 6 matched recipients. For one ELA-A3b donor 2 matched and 4 mismatched recipients were used. The 14 matched recipients were haploidentical to the donor, and the 13 mismatched recipients were haplo-dissimilar to the donor (Figure 4.1). All 4 donor horses, and 2 additional unrelated horses, received their own (autologous) MSCs.

Bone marrow derived MSCs for all donors were prepared entirely in xenogen-free culture media.(104) We confirmed MSC characteristics of donor cells through trilineage differentiation into fat, bone, and cartilage (Appendix C, Figure S1), and assessment of a panel of cell surface markers (Appendix C, Table S1). Passage 3 MSCs were cryopreserved in freezing medium (recipient serum with 5% DMSO) prior to intra-articular injection (50, 104). In all recipients, the contralateral joint was injected with recipient serum-DMSO alone.

4.2.2. No adverse clinical response after first injection, mild local adverse clinical response after second injections of MHC mismatched MSCs

The clinical safety of non-cross matched allogeneic MSC administration has been shown repeatedly, and safety of intra-articular injection of MSCs has been suggested (5, 108). In line with this, we saw no adverse clinical response after the first injection of any MSC type. After the second injection, there were no differences in signs of pain or in synovial cytology, but there were signs of local inflammation on physical examination in the mismatched group. On days 30 and 31, there was increased peri-articular edema and synovial effusion in the mismatched group as compared to the matched or autologous groups, or serum-DMSO (Appendix C, Figure S2). The increased edema and effusion in the mismatched recipients after the second injection indicate an increase in local inflammation, likely due to immune activation by mismatched MSCs.

4.2.3. MHC mismatched MSCs activate the innate and adaptive immune system

Next, we surveyed synovial cytokines to understand the etiology of the peri-articular edema and synovial effusion in mismatched injected joints. Analysis of

synovial fluid with an equine-specific 23-analyte cytokine and chemokine panel revealed factors associated with innate immune recognition and adaptive immune activation in the mismatched recipients. There were differences in IFNγ, TNFα, MCP-1, GRO, eotaxin, IL-10, IL-1β, IL-6, IL-4, IL-2, fractalkine, IL-5, IL-18, and IP-10 (Figure 4.2 and Appendix C, Figure S3).

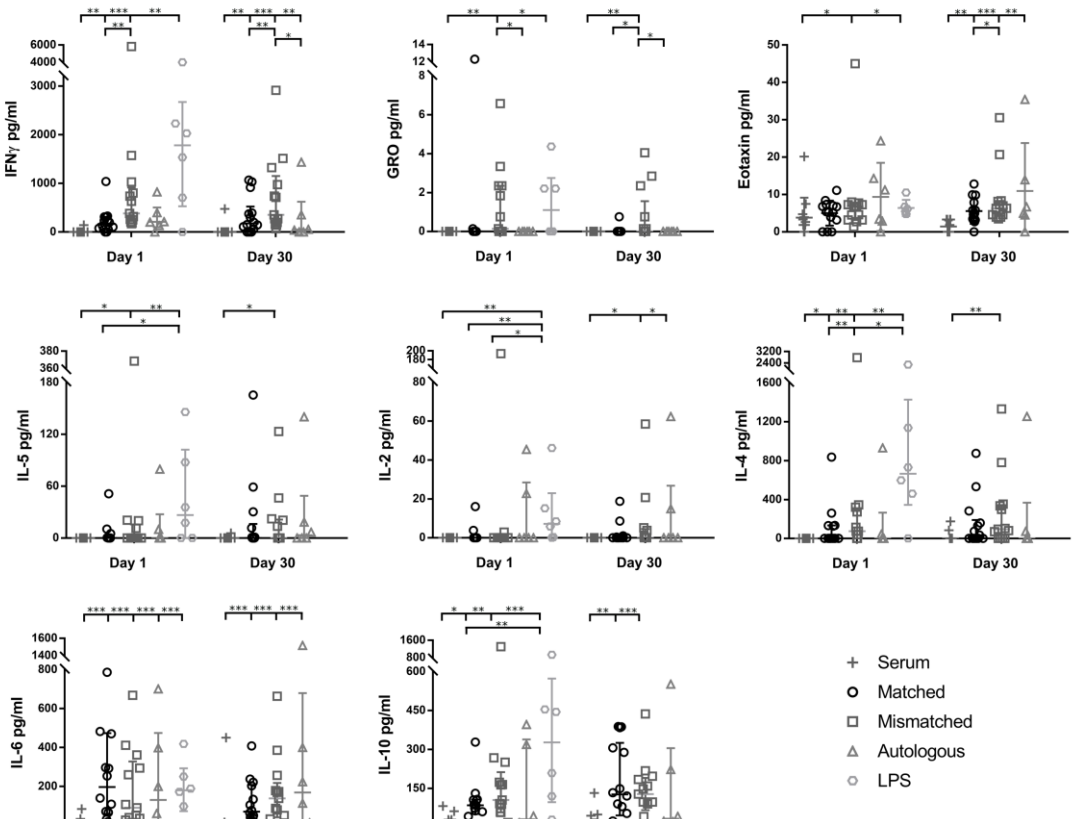

Figure 4.2: Innate and adaptive immune response occurs after MHC mismatched injection
Cytokines and chemokines measured in synovial fluid collected after the first and second intra-articular injection. Increased concentrations of IFNγ, GRO, eotaxin, and IL-5 demonstrates immune recognition in the mismatched group.
Lines and error bars represent median values and interquartile range, *p <0.05, **p<0.01, ***p<0.001

Innate and adaptive immune activation in the mismatched group was apparent after both injections, even though there were no clinical signs after the first injection. The master regulator of the innate immune system, and key factor in initiation of the adaptive immune response, interferon-γ (IFNγ) (109, 110), was increased in mismatched injected joints compared to matched or autologous injected joints, and was not different from LPS injected joints. Chemoattractants growth related oncogene (GRO) and eotaxin, were also increased in mismatched and LPS injected joints, but not in matched injected joints, compared to serum-DMSO. Similarly, IL-5 was elevated in mismatched and LPS injected joints, but not in matched or autologous injected joints compared to serum-DMSO. The increases in IFNγ, GRO, eotaxin, and IL-5 in mismatched joints are due to immune activation and all likely contributed to the increased peri-articular edema and synovial effusion noted after mismatched injection.

Importantly, some MSC immunomodulatory function was still present in the mismatched group, despite immune activation. Synovial concentrations of IL-2 were increased in LPS injected joints compared to MSC injected joints or joints injected with serum-DMSO alone. Similarly, after the first injection, IL-4 was increased in LPS injected joints compared to mismatched and matched injected joints. This preserved immunomodulatory function of surviving mismatched MSCs is likely why clinical safety has long been reported in the face of alloimmunization.

Furthermore, we noted no difference in IL-6 or IL-10 concentrations between MSC and LPS injected joints. Both IL-6 and IL-10 have been reported to be anti-inflammatory, but increased concentrations have also been reported in acute graft

rejection (111, 112). The lack of differences between MSC and LPS injected joints highlight the pleiotropic nature of these cytokines, and highlights significant crossover of pro and anti-inflammatory effects.

4.2.4. Systemic humoral immune response to mismatched allogeneic MSCs

The cytokine profile of MHC mismatched injected joints revealed innate and adaptive immune stimulation, but definitive proof that the humoral immune system was activated by mismatched MSCs is the development of donor-specific antibodies in the mismatched injected group, but not the matched injected group (Figures 4.3 and 4.4).

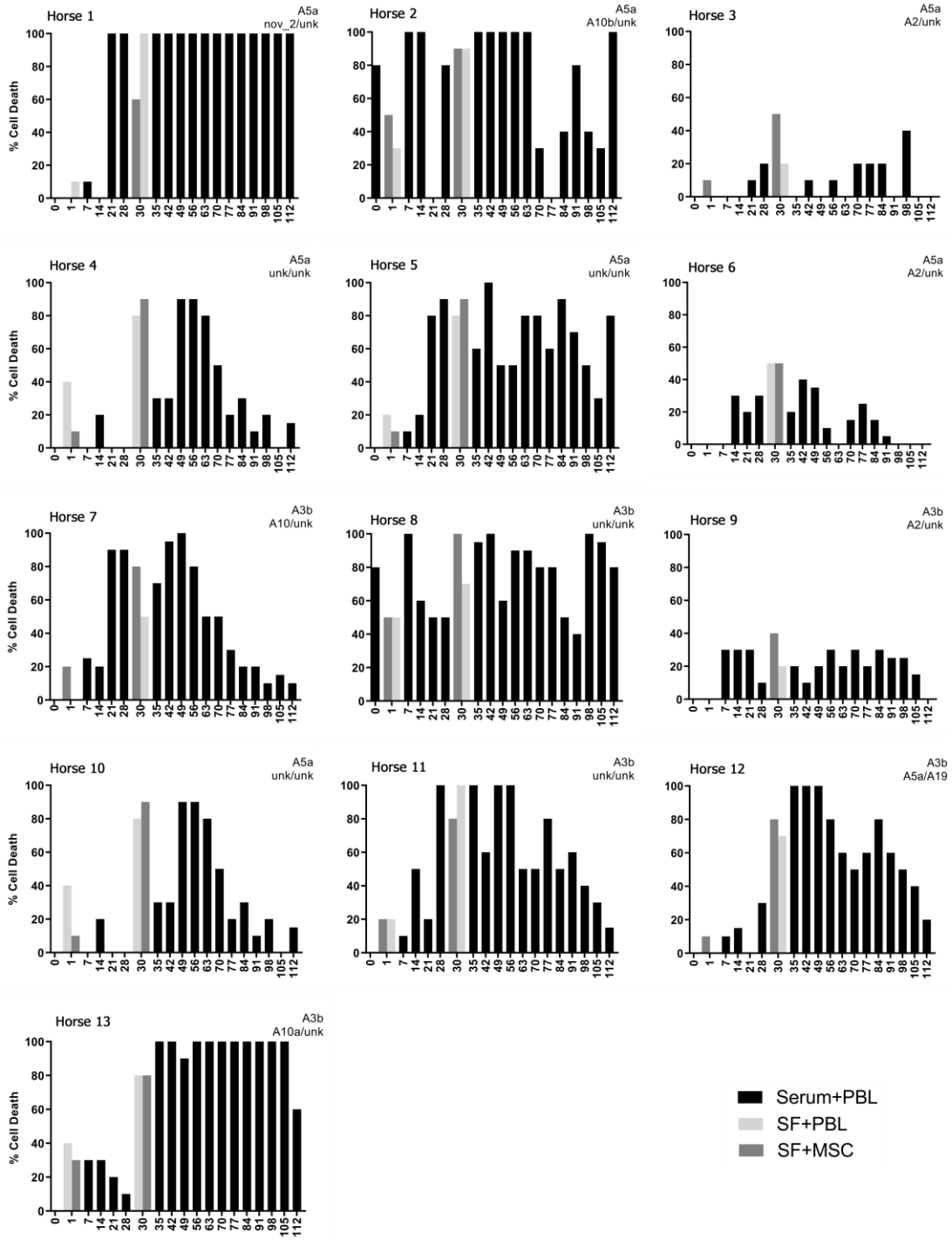

Figure 4.3: Antibody-mediated cytotoxicity in MHC mismatched MSC recipients
Donor peripheral blood lymphocytes (PBL) or MSCs were combined with recipient serum or synovial fluid (SF). Donor and recipient haplotypes listed in the top right corner.

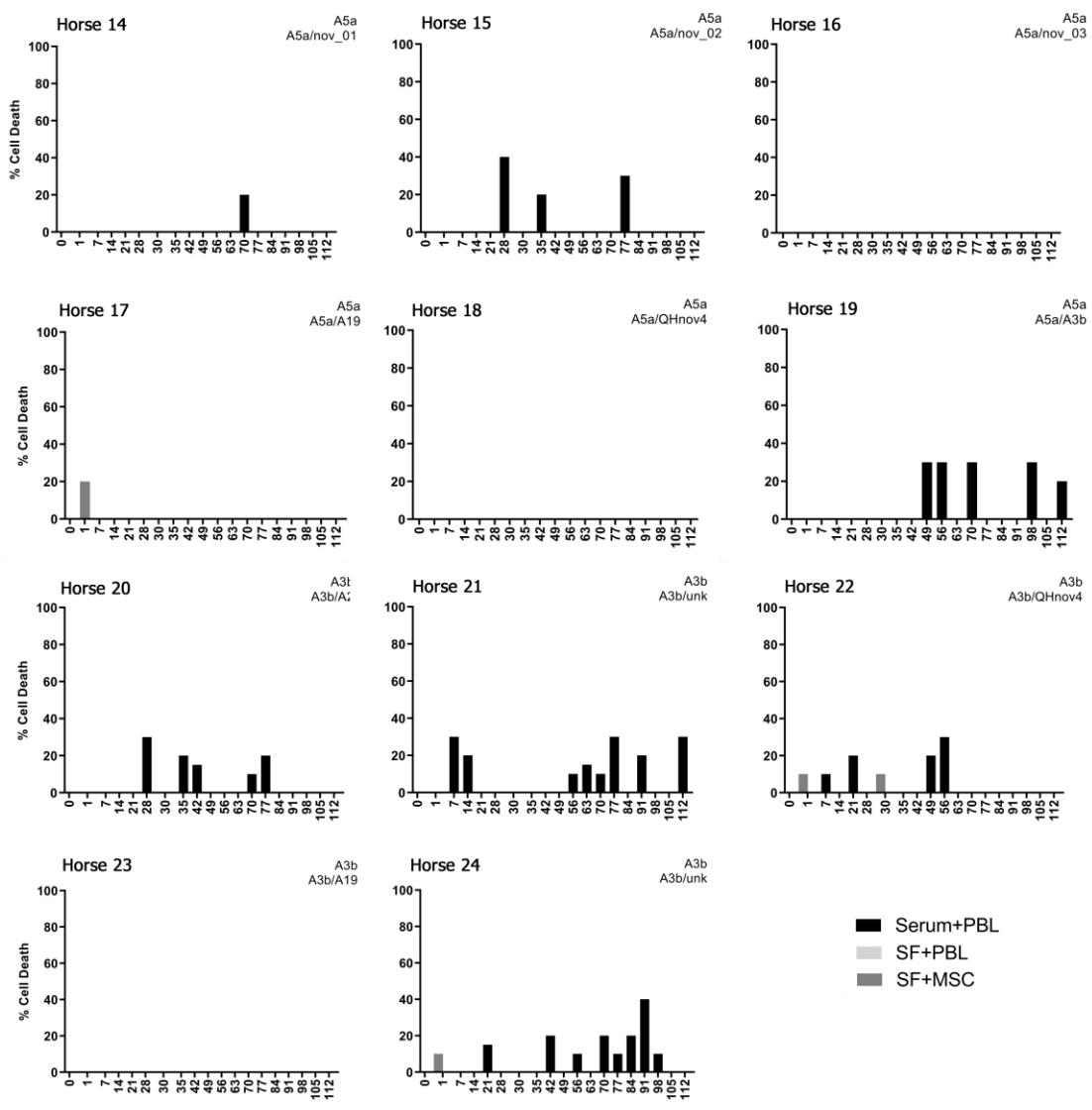

Figure 4.4: Little to no antibody-mediated cytotoxicity in MHC matched MSC recipients Donor peripheral blood lymphocytes (PBL) or MSCs were combined with recipient serum or synovial fluid (SF). Donor haplotype is listed in the top right corner, with the recipient haplotype listed below.

Microcytotoxicity assays, in which we combined donor lymphocytes with recipient serum, collected weekly throughout the experiment, showed virtually no detectable cell death when serum from the matched group was tested. In stark contrast, antibody-mediated cell death increased rapidly for serum collected after the first injection in all mismatched recipients, with 100% lymphocyte toxicity two weeks after the second injection in 10 of the 13 mismatched recipients (Figure 4.3). Immunological memory is a tenet of the adaptive immune system (113) and was clearly demonstrated in our mismatched recipients.

Of the three mismatched recipients that did not reach 100% lymphocyte toxicity, all were heterozygous for ELA-A2. ELA-A2 is a well characterized equine haplotype that is known to have differences in ability to present and recognize antigens (114). In support of the hypothesis of reduced antigen recognition by recipients that had an ELA-A2 haplotype, after the first and second injections each of these ELA-A2 recipients had synovial IFNγ levels that were below the median value of the mismatched injected group. Despite their reduced immune responsiveness, as reflected in lower antibody levels and lower synovial IFNγ, these ELA-A2 mismatched recipients mounted an antibody response that was greater than that seen in the matched group.

An unexpected finding in our study was that two mismatched recipients had pre-existing antibodies at the time of the first injection. This surprised us as we had documented lack of pre-sensitization during MHC haplotype screening for inclusion in the study, which occurred 9 months prior to the intra-articular injection arm of the experiment. We suspect that these 2 horses were sensitized to the donor MHC haplotype

during the interim. Classical sensitization events are blood transfusion and pregnancy; however, anti-MHC antibodies can develop due to cross-reactivity with epitopes on other antigens (8, 83, 115-118). Both sensitized horses were female, and one was bred in the interim and carried a conceptus to 40 days of gestation, at which point sensitization to the fetal haplotype can occur (119). Regardless of the mechanism of sensitization, these two horses highlight the possibility that sensitization against MHC occurs frequently, and thus a humoral immune response can occur even after a single therapeutic injection of mismatched MSCs when prior sensitization has occurred.

Finally, we used immunoglobulin depletion to confirm that the microcytotoxicity results were due to circulating antibody. Serum from the 6 mismatched recipients with the highest level of cytotoxicity on day 35 were antibody depleted by trapping with Protein A and G. Immunoglobulin depleted serum resulted in negligible lymphocyte toxicity. In contrast, cell death persisted in serum that was diluted in binding buffer, and in the eluate from the IgG binding column (Figure 4.5).

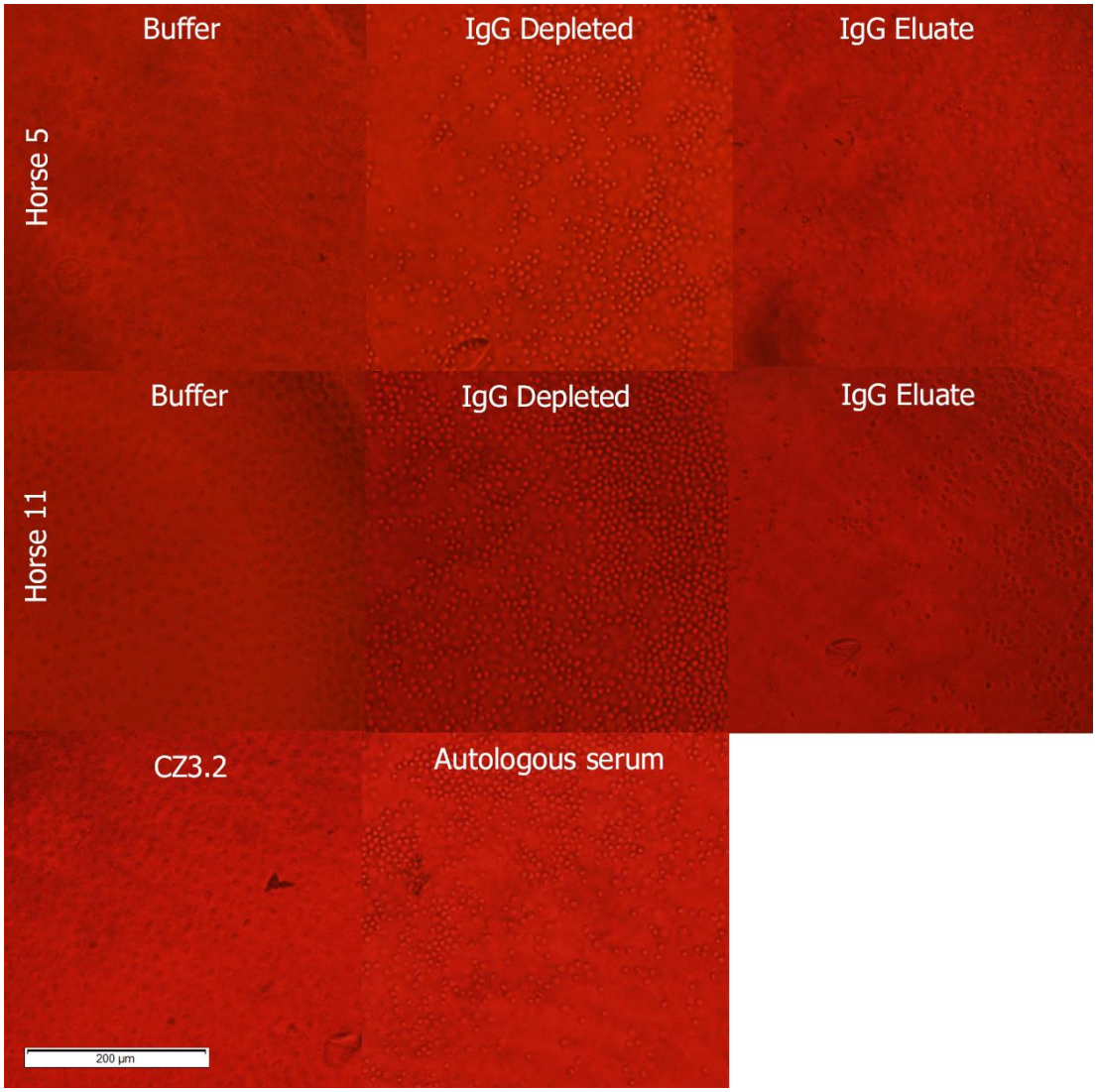

Figure 4.5: Immunoglobulin depletion eliminates cytotoxicity in MHC mismatched recipient serum
Serum collected on day 35 from 6 mismatched recipients (3, A5a recipients; 3, A3b recipients) was depleted of immunoglobulins and microcytotoxicity assays were performed again with donor lymphocytes. Microcytotoxicity images from two mismatched recipients (Horse 5 and Horse 11) with serum diluted in binding buffer, IgG depleted serum, and IgG column eluate (left to right). On the bottom are positive (CZ3.2, anti-MHCI antibody) and negative (right, autologous serum) controls for reference.

4.2.5. Pre-formed anti-MHC antibodies exist in synovial fluid

As shown by the microcytotoxicity assay, antibodies specific to donor haplotype developed in all mismatched recipients at levels sufficient to cause antibody mediated cytotoxicity. Given the unique environment of the synovial joint, considered to some extent immune-privileged (81, 90), we sought to determine if antibodies were present in synovial fluid at sufficient levels to result in cell death. We again performed microcytotoxicity assays, this time with donor lymphocytes and recipient synovial fluid collected the day after each intra-articular injection. The day after the first intra-articular injection there was little cell death in any group. The day after the second injection, synovial fluid from all non-ELA-A2 mismatched recipients caused greater than 60% cell death, and synovial fluid from mismatched recipients with the ELA-A2 haplotype caused 20-50% cell death. As expected, there was essentially no lymphocyte toxicity in matched injected joints (Figures 4.3 and 4.4).

4.2.6. Pre-formed anti-MHC antibodies induce cytotoxicity of MSCs

We then wanted to test if the anti-MHC antibodies would induce antibody mediated cytotoxicity of donor MSCs, as they had for donor lymphocytes. This is important because the immunomodulatory properties of MSCs include down-regulation of complement, which could protect them from antibody mediated cytotoxicity (120). We repeated the microcytotoxicity assay, combining donor MSCs, instead of lymphocytes, with recipient synovial fluid. The results paralleled those for lymphocytes. After the first injection, there was negligible MSC death either group (Figures 4.3 and 4.4). After the second injection, there was minimal cytotoxicity in the matched group,

but a median of 80% cell death in synovial fluid from mismatched recipients, confirming that MSCs induce and are susceptible to donor specific anti-MHC antibodies and complement-mediated cytotoxicity.

4.2.7. Matched allogeneic MSC injection increases endogenous progenitors but mismatched MSCs do not

The differences in local inflammation, innate and adaptive immune responses, and humoral cytotoxicity between recipients injected with matched and mismatched MSCs led us to evaluate the survival of MSCs within the joint the day following the second injection. To quantify the number of synovial MSCs, we used the colony forming units-fibroblasts (CFU-f) assay. We found that MSCs were present and abundant in synovial fluid from all matched and autologous MSC injected joints in which synovial fluid was assessed. In contrast, only 6 colonies were isolated from one of four mismatched MSC injected joints after the second injection. Concentrations of MSCs in synovial fluid after LPS injection were similarly low (Figure 4.6).

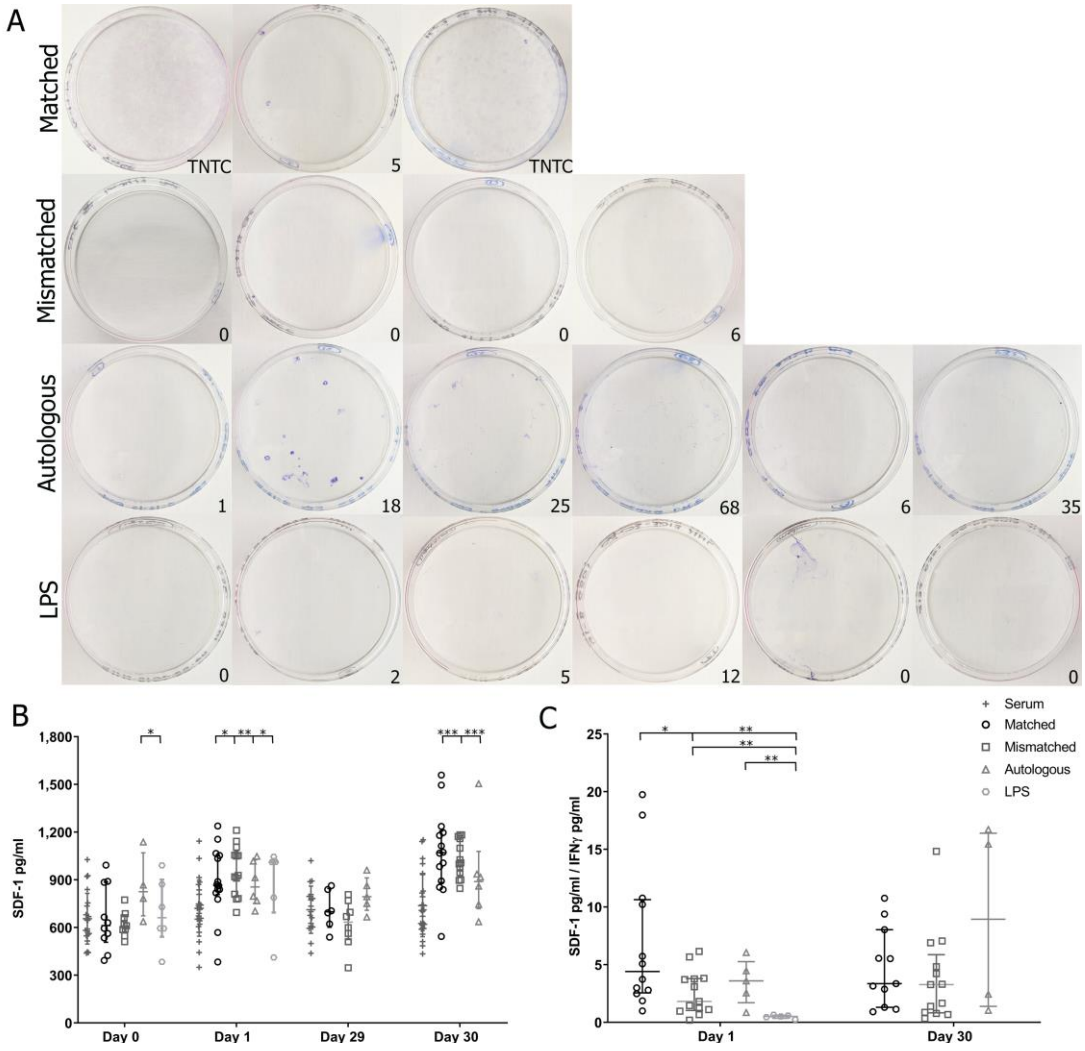

Figure 4.6: Upregulation of endogenous progenitors in MHC matched and autologous MSC recipients, but not after MHC mismatched or LPS injection and SDF-1 increases in matched and autologous injected joints

A) Composite of MSC CFU-f isolated from synovial fluid one day after the second injection. More colonies were isolated after matched and autologous compared to mismatched or LPS injection. All retrieved MSCs were recipient, indicating an upregulation of endogenous progenitors in matched and autologous groups.

B) Synovial fluid SDF-1 concentrations prior to (days 0 and 29), and after the first (day 1) and second injection (day 30). SDF-1 concentrations were increased in MSC treated joints compared to serum alone after both injections.

C) SDF-1 normalized to IFNγ to control for changes in SDF-1 due to inflammation. After normalization, SDF-1 was higher in the matched group compared to mismatched or LPS.

Lines and error bars represent median values and interquartile range, *p <0.05, **p<0.01, ***p<0.001

To our surprise, genotype analysis of MSCs retrieved from synovial fluid demonstrated that the MSCs were recipient in origin, and the apparent increase in synovial fluid MSC concentrations was in fact due to recruitment of endogenous progenitors, and not to persistence of injected MSCs (Table 2). To the best of our knowledge, this is the first direct evidence for local upregulation of endogenous progenitors after exogenous MSC treatment in a large animal model. This exciting finding sheds light on a widely held, but difficult to prove, therapeutic mechanism for local application of MSCs. Upregulation of endogenous progenitors may explain the lasting regenerative effects of MSCs, given the relatively short survival time of administered MSCs (121, 122).

4.2.8. SDF-1 increases after matched allogeneic and autologous MSC injection

Prior to this report, recruitment of endogenous progenitors has been difficult to prove in models other than genetically-engineered mice. For this reason, upregulation of chemokines known to recruit endogenous progenitors is a commonly used measure to estimate the degree of endogenous recruitment by exogenous MSCs. In mice and rats, increased stromal derived factor-1 (SDF-1) is used to confirm endogenous MSC recruitment after MSCs administration (123, 124). In our study, SDF-1 was increased in all groups compared to serum-DMSO alone, without differences to joints injected with LPS only. As SDF-1 increases during inflammation (125), as well as during non-inflammatory MSC recruitment, this finding was not surprising. To control for SDF-1 increases due to inflammation from immune activation and resulting synovitis, we normalized SDF-1 to IFNγ levels (Figure 4.6). When normalized, SDF-1 was

significantly increased, compared to injection of serum-DMSO, in matched and autologous injected joints, but not mismatched or LPS injected joints. Increased relative synovial SDF-1 concentrations in matched joints is mechanistic support for increased endogenous progenitor recruitment by matched, but not mismatched, MSCs.

Source	M/F	AME	AHT4	AHT5	ASB17	ASB2	ASB23	HMS2	HMS3	HMS56	HMS7	HTG10	HTG4	LEX3	LEX33	TKY333	TKY374	TKY394	VHL20
Synovial MSCs Recipient	F	X	KO	JK	GL	MQ	L	KR	IM	M	MO	IN	LM	OP	OR	KS	LM	LP	MO
	F	X	KO	JK	GL	MQ	L	KR	IM	M	MO	IN	LM	OP	OR	KS	LM	LP	MO
Donor	F	X	H	KM	MN	NQ	KL	KR	IM	MP	LM	MR	KM	HI	LR	K	MO	DL	MR
Synovial MSCs Recipient	F	X	KO	KN	LO	MQ	K	KL	IM	MP	JO	IR	MP	MO	QR	KS	JM	JL	LO
	F	X	KO	KN	LO	MQ	K	KL	IM	MP	JO	IR	MP	MO	QR	KS	JM	JL	LO
Donor	F	X	H	KM	MN	NQ	KL	KR	IM	MP	LM	MR	KM	HI	LR	K	MO	DL	MR
Synovial MSCs Recipient	F	X	JO	KN	OR	MQ	JK	KR	MP	LO	O	IO	MO	MN	M	KS	MN	JM	IL
	F	X	JO	KN	OR	MQ	JK	KR	MP	LO	O	IO	MO	MN	M	KS	MN	JM	IL
Donor	F	X	HK	JN	LR	Q	KL	KL	MP	MP	L	LO	M	MO	LQ	S	JM	JL	I
Synovial MSCs Recipient	M	YX	HO	JK	OR	KQ	IK	L	IP	MP	L	M	KN	L	L	RT	JM	O	M
	M	YX	HO	JK	OR	KQ	IK	L	IP	MP	L	M	KN	L	L	RT	JM	O	M
Donor	F	X	HK	JN	LR	Q	KL	KL	MP	MP	L	LO	M	MO	LQ	S	JM	JL	I

Table 4.2: Microsatellite data from genotype analysis of MSCs retrieved from synovial fluid compared to donor and recipient. Matching genotypes are highlighted.

4.3. Discussion

We show, for the first time, that innate and adaptive immune recognition of MHC mismatched MSCs negatively affects the local environment and reduces the critical therapeutic MSC action of endogenous progenitor recruitment. Our study highlights the complexity of immune recognition of mismatched MSCs by individual recipients of different MHC haplotype. We offer insight as to why numerous allogeneic MSC studies have shown clinical safety and lack of acute transplant rejection, but fewer have shown efficacy in advanced clinical trials (2, 3, 5, 78, 79).

Much effort has been made to identify donor factors that predict patient responsiveness to MSC therapy (6, 126-128). However, prior sensitization to donor MHC haplotype (8, 83, 118) and development of anti-MHC antibodies (6, 8) after multiple treatments may explain why donor MSC factors do not determine whether a patient will be a responder versus a non-responder (6, 126-128). Rather, our findings indicate that recipient factors, such as MHC compatibility with the donor, dictate response versus non-response in patients. Beyond this, the differences we noted in antibody development in mismatched recipients with the ELA-A2 haplotype suggests that additional recipient factors further influence the effect of allogeneic incompatibility.

The possibility for immune compatibility, coupled with small group size, is likely why early clinical trials report a significant treatment effect. In Phase I and II trials, happenstance immune compatibility between donors and recipients will greatly influence results, but as trials advance to Phase III and IV, increased group size and diversity of recipient MHC haplotypes inevitably leads to immune incompatibility and a variable

treatment effect overall (2). At a minimum, future allogeneic MSC studies, especially those with repeated treatments for chronic conditions, should document MHC haplotype of donors and recipients and perform evaluation for pre-sensitization as well as stringent assessment of anti-MHC antibody development after treatment.

Despite numerous previous reports on the lack of adverse effects of non-crossmatched allogeneic MSC therapy, we documented localized tissue inflammation secondary to mismatched MSC injection. The synovial joint has a large volume-to-surface area ratio and a blood-joint barrier, both of which limit diffusion of small molecules and transport of proteins (60, 61). This unique environment augments detection of inflammation, and allowed us to identify inflammation due to immune incompatibility of MHC mismatched MSCs.

We provide direct evidence of endogenous progenitor recruitment by MSCs. Increased SDF-1 concentrations relative to IFNγ in the matched and autologous groups provide mechanistic support for this finding (123, 129-131). This effect of MSC therapy is of particular importance in the synovial joint, where it is known that synovial fluid MSCs are likely responsible for articular cartilage repair, and their reduced concentration over time is in part responsible for age related osteoarthritis progression (76, 132).

In a similar experimental protocol, we previously reported an adverse clinical response with increased gait asymmetry and differences in synovial cytology with elevated synovial total nucleated cell count (TNCC), after a second exposure to intra-articular injection of mismatched allogeneic MSCs, but not autologous MSCs (10). In

the current report, we expected to find similar increases in pain and abnormal synovial cytology in mismatched injected joints. However, we did not find these adverse reactions, and there were no differences in gait asymmetry. The discrepancy between our two reports is likely due to the fact that the MSCs used in the current report were isolated and expanded entirely in xenogen-free media. Although the MSCs used by Joswig et al. (2017) were fetal bovine serum (FBS) reduced, all MSCs in that study were still positive for FBS contamination. The notion that FBS contamination of MSCs should be avoided is now well accepted, and this is particularly true in the synovial joint, where immune reaction to FBS can cause marked and severe adverse responses (108). In the Joswig et al. report, contamination of all MSCs by intracellular FBS, in the face of reduced MSC persistence of the allogeneic group, resulted in worsened inflammation in the allogeneic group compared to the autologous group (10, 74).

4.4. Conclusion

In summary, we report that repeated injection with MHC mismatched allogeneic MSCs results in an innate and adaptive immune response, local inflammation, and reduced MSC therapeutic action. Our data provides strong evidence that the use of non-crossmatched allogeneic MSCs may be the Achilles heel for reliable and predictable MSC efficacy and be the reason for lack of market authorization. Until immune recognition of MSCs can be avoided, repeated clinical use of MSCs, where alloimmunization is deleterious, should be limited to autologous or cross-matched allogeneic MSCs. When non-cross-matched allogeneic MSCs are used in single MSC dose applications, pre-sensitization should be assessed. This paradigm shift may offer

the opportunity for repeatable therapeutic results and lead to regulatory approval of MSC therapy.

5. SUMMARY AND CONCLUSIONS

In order to optimize MSC use in humans and veterinary species, understanding and avoiding the recipient immune response is imperative. Our work clearly demonstrates that MSCs are not immune privileged and that recipient recognition of either xenogen proteins or non-self MHC results in an adverse clinical response, antibody mediated cytotoxicity, and decreased efficacy. Importantly, the relatively low expression of MHCI and potent immunosuppressive properties of MSCs are not sufficient to overcome the recipient immune response (35).

Foreign intracellular proteins, as a result of xenogen serum supplementation during MSC preparation has been present for decades (9, 78). The use of FBS has persisted in MSC preparation despite previous reports of xenogen contamination causing allergic responses after the administration of FBS prepared dendritic cells as well as poor response to FBS-MSC therapy that correlated to anti-bovine antibodies (8, 18). In the veterinary community, a lack of change in anti-bovine titers has led researchers to conclude that FBS contamination was not of consequence (26, 33). However, we demonstrate that intracellular bovine proteins are presented on the MHCI molecule resulting in antibody mediated cytotoxicity and an adverse clinical response and that replacement of FBS with BMS eliminates immune recognition, and greatly improves efficacy.

In clinical patients, we saw a good rate of return to work (65%) in horses with osteoarthritis treated with BMS-MSCs. This rate of return to full work is higher than in our previous experience with FBS-MSCs, and is likely due to improved persistence of

the MSCs in the treated joint because they are no longer being targeted by the recipient immune response. Because of the retrospective nature of this study, a prospective clinical trial is warranted to contemporaneously investigate conventionally cultured FBS-MSCs compared to BMS-MSCs. It is also possible that the improved outcomes are due to a direct effect of BMS, and not just because the MSCs are xenogen-free. Bone marrow supernatant should also be further investigated.

Finally, we demonstrate seroconversion and antibody-mediated cytotoxicity directly in MHC mismatched, but not MHC matched, recipients after repeated intra-articular injection. This demonstrates the importance of immune compatibility, even when using an immunosuppressive cell type like MSCs. It is likely that the lack of stringent immune monitoring and single-dose administration protocol of many clinical trials has allowed the immune recognition of allogeneic MSCs to go unobserved. However, with over 50% of current MSC clinical trials utilizing allogeneic MSCs, our finding of recipient immune recognition is of paramount importance (39).

5.1. Future studies

Fetal bovine serum should no longer be utilized for MSC preparation in pre-clinical animal models, veterinary applications, or clinically. The use of FBS in previously reported studies should be considered when interpreting results. Bone marrow supernatant as replacement for FBS should be further investigated. Likewise, until the immune recognition of non-crossmatched allogeneic MSCs can be avoided, repeated clinical use of MSCs should be limited to autologous or cross-matched allogeneic MSCs.

When non-cross-matched allogeneic MSCs are used in single MSC dose applications, pre-sensitization against donor MHC should be assessed.

5.2. Final Remarks

Mesenchymal stem cells have a vast potential for therapeutic application, but much is still to be learned. Optimization of MSC preparation technique and elimination of immune recognition is crucial in developing a consistently efficacious product. Fetal bovine serum should not be used in MSC preparation, and until the MSCs can be altered to avoid recognition by the recipient immune system, only cross-matched allogeneic or autologous MSCs should be used.

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APPENDIX A

SUPPLEMENTARY FIGURES FOR CHAPTER 4

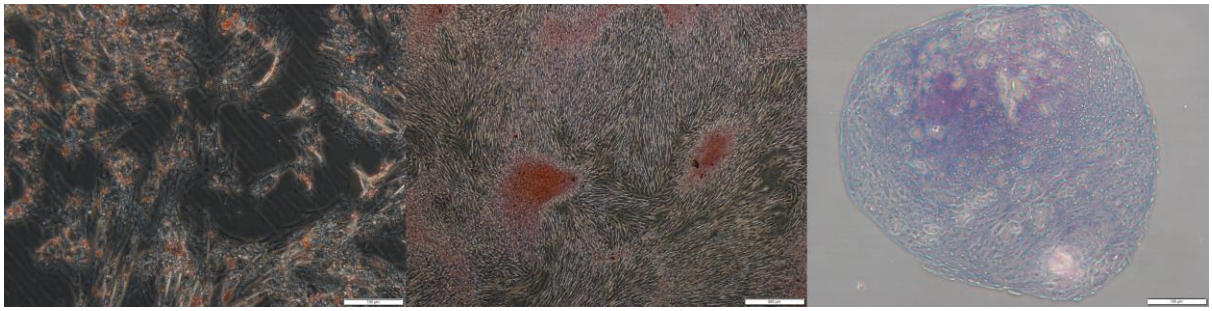

Figure S1: Trilineage differentiation into fat, bone, and cartilage from a single donor

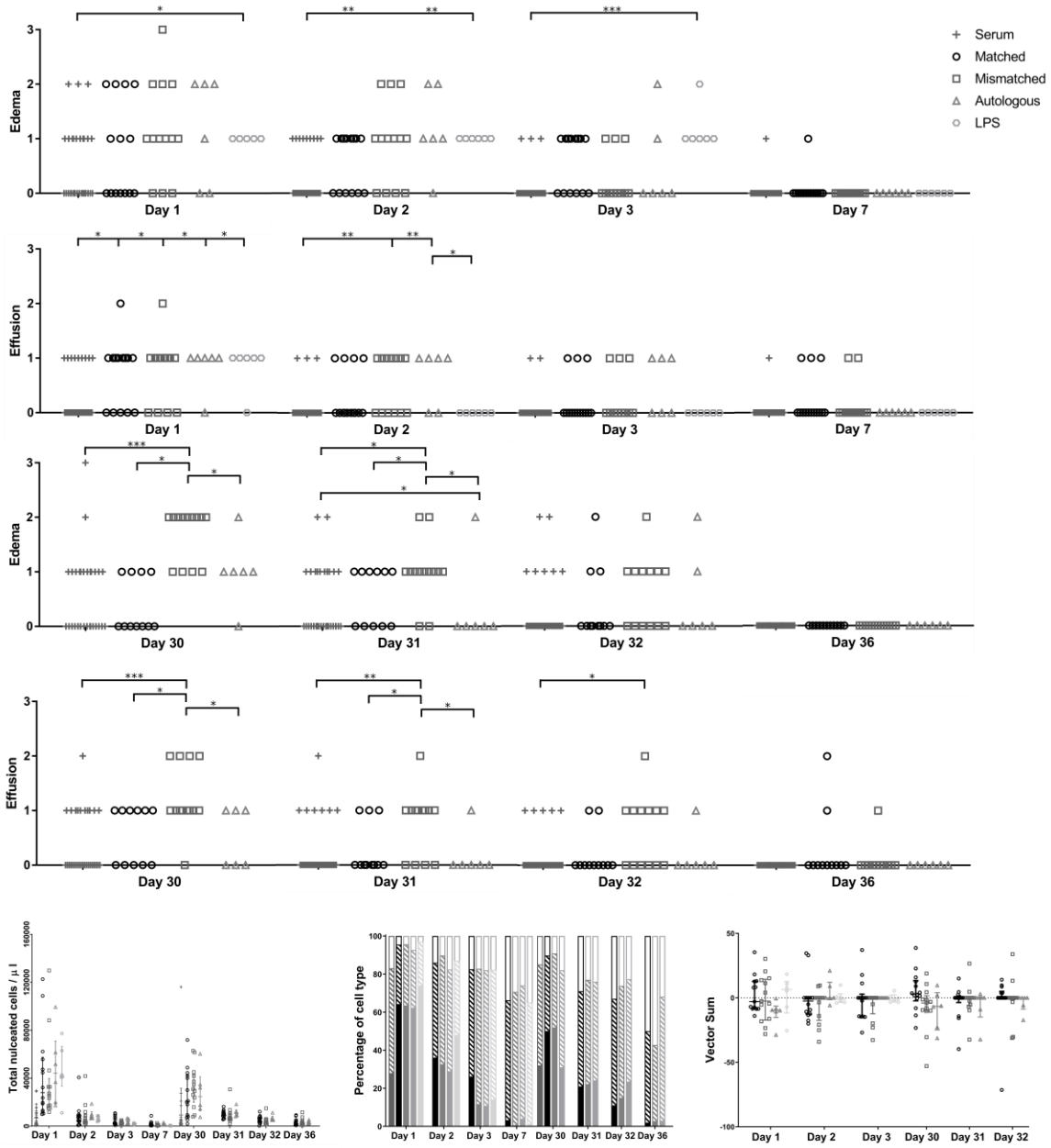

Figure S2: No adverse clinical response after first injection, mild local adverse clinical response after second injection of mismatched MSCs

There was increased edema (A) and effusion (B) in MHC mismatched injected joints compared to those injected with serum-DMSO alone after the first injection, but no important differences between MSC injected groups. After the second injection, there was increased edema and effusion in the mismatched group compared to the matched and autologous group as well as serum alone.

There were no differences in total nucleated cell count (TNCC, C), and cellular differential (D) of synovial fluid collected after the first (days 1, 2, 3, and 7) and second (days 30, 31, 32, and 36) injection. Bars represent median values of neutrophils (solid), large mononuclear cells (striped), and lymphocytes (empty). There was also no difference between groups in pain, as measured by gait asymmetry (E).
Bars represent median values, and lines represent interquartile range
*p <0.05
**p<0.01
***p<0.001

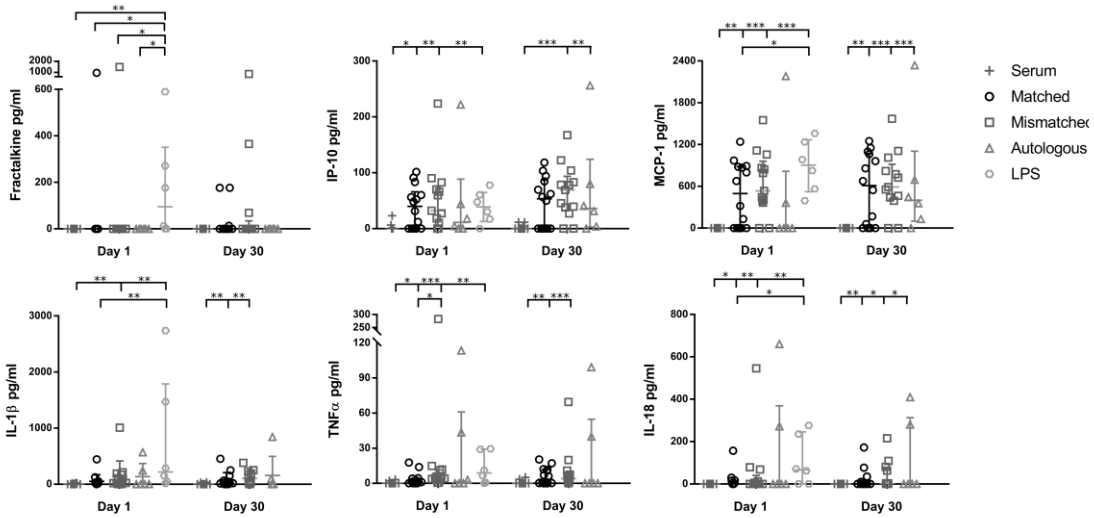

Figure S3: Additional cytokines and chemokines measured in synovial fluid
Cytokines and chemokines measured in the synovial fluid collected after the first and second intra-articular injection.
Lines and error bars represent median values and interquartile range
*p <0.05
**p<0.01
***p<0.001

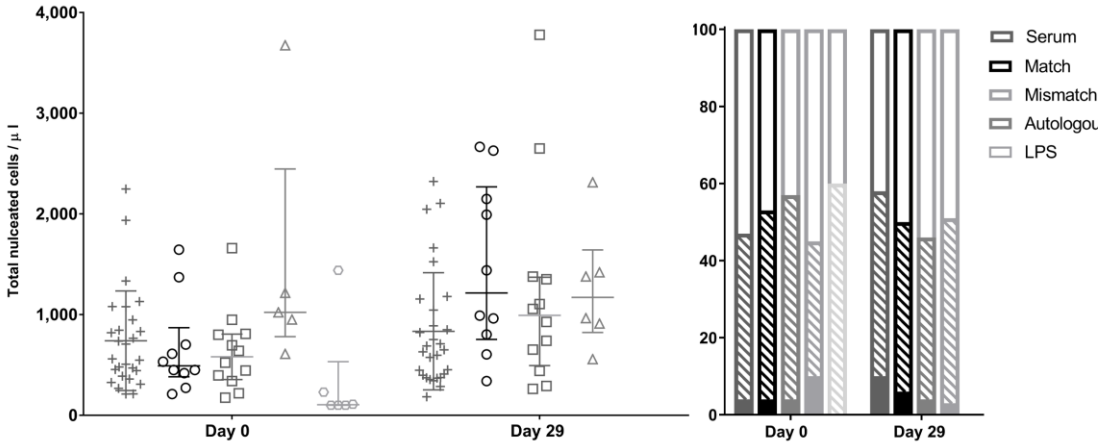

Figure S4: Total nucleated cell count (TNCC) and cellular differential prior to the first (Day 0) and second (Day 29) injection.
All parameters were within normal limits.
Bars represent median values of neutrophils (solid), large mononuclear cells (striped), and lymphocytes (empty).

	CD29	CD45	CD90	MHCI	MHCII
Donor 1					
ELA-A5a	99.9%	0.86%	99.5%	99.4%	1.18%
Donor 2					
ELA-A5a	100%	2.45%	100%	99.7%	0.54%
Donor 3					
ELA-A3b	97.8%	0.93%	95%	99.7%	0.15%
Donor 4					
ELA-A3b	100%	2.07%	73%	98.5%	0.70%

Table S1: Percent positive cells for MSC surface markers at the time of injection from each donor