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ENABLING TECHNOLOGIES FOR CELL-BASED CLINICAL TRANSLATION

STEM CELLS TRANSLATIONAL MEDICINE

Cross-matching of allogeneic mesenchymal stromal cells eliminates recipient immune targeting

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Abstract

Allogeneic mesenchymal stromal cells (MSCs) have been used clinically for decades, without cross-matching, on the assumption that they are immune-privileged. In the equine model, we demonstrate innate and adaptive immune responses after repeated intra-articular injection with major histocompatibility complex (MHC) mismatched allogeneic MSCs, but not MHC matched allogeneic or autologous MSCs. We document increased peri-articular edema and synovial effusion, increased synovial cytokine and chemokine concentrations, and development of donor-specific antibodies in mismatched recipients compared with recipients receiving matched allogeneic or autologous MSCs. Importantly, in matched allogeneic and autologous recipients, but not mismatched allogeneic recipients, there was increased stromal derived factor-1 along with increased MSC concentrations in synovial fluid. Until immune recognition of 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, presensitization against donor MHC should be assessed.

KEYWORDS

allogeneic, alloimmunization, antibody, autologous, cross-match, humoral, innate, intra-articular, major histocompatibility complex (MHC), MSC

1 | 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 preclinical success, late phase clinical trials and post approval monitoring have failed to demonstrate consistent therapeutic effects.[1-3] Lack of efficacy has been proposed to be due to nonuniformity of MSC preparation and application techniques.[3-5] 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.[1,6]

Clinical investigations into the effect of major histocompatibility complex (MHC) mismatched allogeneic MSCs suggest that mismatch

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does not alter efficacy, but reports have been limited and immunological monitoring has not been stringent.[7,8] Although donor-specific antibody production against allogeneic MSCs has been confirmed in people,[9-15] nonhuman primates,[16,17] and horses,[18,19] the effects of mismatched allogeneic MSCs on MSC persistence remains unknown.[1] Certainly, reduced persistence with concomitant loss of function of allogeneic MSC-gene therapy constructs compared with autologous or syngeneic MSCs has been confirmed in laboratory animals.[17,20-24] Still, the survival time of allogeneic MSCs is significantly longer than allogeneic fibroblasts, likely due to MSC immune-evasiveness.[21] 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 allorecognition with resultant cytotoxicity.

Despite the value of laboratory animal models in science, preclinical success often fails to result in clinical application.[25] Poor translation of preclinical findings is in part due to the use of inbred laboratory animals that lack the diversity of man.[3,25] 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.[3,6]

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.[26-28] Additionally, the horse is well-recognized for its value as a preclinical 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.[29,30] 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 preclinical study of allogeneic MSC therapy.[31]

We compare repeated intra-articular injection of clinically prepared MHC matched, mismatched, and autologous MSCs to confirm allorecognition 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.

Significance statement

The mesenchymal stromal cell (MSC) regenerative medicine dogma has been one of absolute immune privilege. This notion, taken together with the commercial advantages of allogeneic cell lines, has led to a majority of preclinical and clinical studies utilizing non–cross-matched MSCs despite lack of scientific evidence for allogeneic superiority. This study provides strong evidence that use of non–cross-matched allogeneic MSCs may be the Achilles' heel for reliable and predictable MSC efficacy. This article will accelerate a paradigm shift away from non–cross-matched allogeneic MSC clinical trials and pave the way to regulatory approval for disorders that currently do not have adequate medical therapies.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Four horses with homozygous MHC haplotypes (two ELA-A5a, two ELA-A3b) were used as donors and each paired with three MHC matched and three MHC mismatched recipients. Bone marrow-derived xenogen-free donor MSCs in cryopreservation media were injected into the left metacarpophalangeal (MCP) joint and cryopreservation media (recipient serum-DMSO) alone into the contralateral MCP on days 0 and 29. All four donors and two additional horses received autologous MSCs at the same time points. In six 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 cryopreservation media (Figure 1).

2.2 | 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 to 22 years, and there were 2 intact males, 10 altered males, 21 intact females, and 2 altered females.

2.3 | 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 polymerase chain reaction (PCR) with known primers for 12 microsatellite loci within the MHC region.[32] 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, Pennsylvania). 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

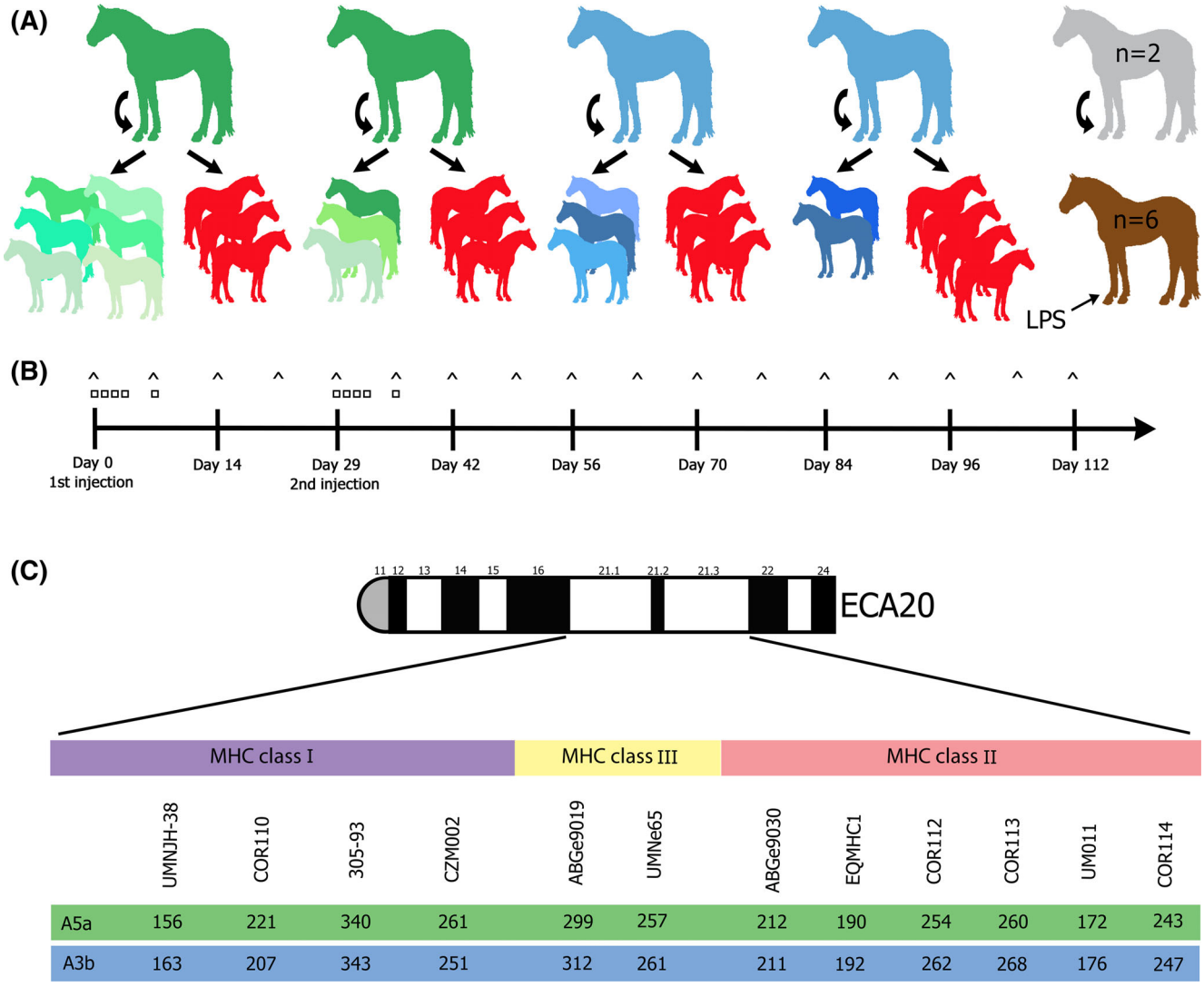

FIGURE 1 Study overview and schematic of intra-major histocompatibility complex (MHC) microsatellite loci analyzed to determine donor and recipient MHC haplotypes. A, Donor and recipient pairings based on MHC haplotype (green, ELA-A5a; blue, ELA-A3b; red, mismatched). All donors received autologous mesenchymal stromal cells (MSCs), as did two 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

cohort, and unknown haplotypes were reported when no individuals with the same haplotype had been previously identified (Table 1).

2.4 | MSC preparation

Bone marrow was collected from the sternum of donor horses and autologous recipients as previously described.[33] Heparinized raw bone marrow was centrifuged at 300*g* 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.[34] When colonies or monolayers reached 70% confluence, cultures were passaged and cells replated at 5000 cells/cm^2, as previously described.[33] After three passages, MSCs were cryopreserved in 95% recipient serum and 5% DMSO (Sigma Aldrich) with 10×10^6 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

TABLE 1 Microsatellite haplotypes of all 13 mismatched recipients. Horses 1 to 6 received mesenchymal stromal cells (MSCs) from an ELA-A5a donor, and horses 7 to 13 received MSCs from an ELA-A3b donor

	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 COR114
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

** Signifies microsatellite bands not present.

were expanded from the same bone marrow aspirate and cryopreserved after three passages.

2.5 | MSC characterization

Donor MSCs underwent trilineage differentiation and immunophenotyping as previously described.[33] 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 before 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 before analysis and only live cells were included in analysis.

To assess multipotency of MSCs, trilineage differentiation into cartilage, bone, and fat was performed, and 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 six 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 Scientific), 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 six 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).

2.6 | Intra-articular injection 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 before 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 10×10^6 MSCs in cryopreservation medium (recipient serum with 5% DMSO) and the right MCP was injected with cryopreservation medium alone. In six additional horses, the left MCP joint was injected with 25 ng of 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 cryopreservation medium alone (Figure 1).

2.7 | Clinical assessment

Physical examinations including assessment of heart rate, respiratory rate, and temperature were performed before each injection and twice 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 before injection, and were repeated on days 1, 2, 3, 30, 31, and 32, or until the gait returned to its baseline value. 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.

2.8 | 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 (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.

2.9 | Microcytotoxicity assays

Microcytotoxicity assays were performed as previously described.[18] Briefly, serum was collected weekly from all recipients and 2 μL of recipient serum was combined with donor 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 donor 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 incubated for 60 minutes at room temperature. Two microliter of 5% eosin (Sigma Aldrich) was added, followed by 5 μL of 10% formalin (Sigma Aldrich). A masked evaluator estimated percentage of live and dead cells in each well. The experiment was repeated using synovial fluid collected on days 1 and 30, in the place of recipient serum.

2.10 | 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.[35] Serum samples collected on day 35 from 6 MHC mismatched recipients (3, A5a recipients; 3, A3b recipients) were immunoglobulin depleted. Briefly, 100 μL of serum was added to 900 μL of 1× Binding Buffer. The sample was passed through the IgG removal column in a dropwise manner. Three hundred microliter of undiluted eluate was then combined with 200 μL 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 4000*g* for 5 minutes and the immunoglobulin depleted supernatant collected. After sample processing, 2 mL 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 recipient serum diluted to a 1:10 dilution with 1× Binding Buffer, recipient immunoglobulin depleted serum, or recipient serum IgG removal column eluate.

2.11 | 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's instructions. In brief, synovial fluid was thawed and centrifuged at 10 000*g* 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 microliters of streptavidin-phycoerythrin were 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.

2.12 | Synovial fluid CFU-f

MSC concentrations in synovial fluid were quantified by colony forming unit-fibroblast (CFU-f) assay. When assessed, 1 mL 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.

2.13 | 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 with DNA from donor and recipient hair bulb to determine the origin of MSCs.

2.14 | Statistical analysis

Statistical analysis was performed using commercially available software (JMP, Statistical Discovery from SAS, Cary, North Carolina). Normality was not assumed, differences between groups were assessed using two-tailed Kruskall-Wallis, and significance was set at $P < .05$. Unless otherwise indicated, error bars represent median values with interquartile range.

3 | RESULTS

3.1 | 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 and 2 ELA-A3b) were identified (Figure 1; Table 1).[36-38] Recipients with one copy of the donor haplotype were identified as matches. Three matched and three mismatched recipients were selected for each donor. Furthermore, one ELA-A5a homozygous donor was paired with an additional three recipients, for a total of six matched recipients. For one ELA-A3b donor two matched and four 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 1). All four donor horses, and two additional unrelated horses, received their own (autologous) MSCs.

Bone marrow-derived MSCs for all donors were prepared entirely in xenogen-free culture media.[34] We confirmed MSC characteristics of donor cells through trilineage differentiation into fat, bone, and cartilage (Figure S1), and assessment of a panel of cell surface markers (Table S1). Passage 3 MSCs were cryopreserved in freezing medium (recipient serum with 5% DMSO) before intra-articular injection.[33,34] In all MSC recipients, the contralateral joint was injected with recipient serum-DMSO alone.

3.2 | No adverse clinical response after first injection, mild local adverse clinical response after second injection 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.[1,39] 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 with the matched or autologous groups, or serum-DMSO (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.

3.3 | 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 2; Figure S3).

Innate and adaptive immune activation in the mismatched group was apparent after both injections, even though there were no clinical signs of local inflammation 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),[40,41] was increased in mismatched injected joints compared with 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 with serum-DMSO. Similarly, IL-5 was elevated in mismatched and LPS injected joints, but not in matched or autologous injected joints compared with 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 with MSC injected joints or joints injected with serum-DMSO alone. Similarly, after the first injection, IL-4 was increased in LPS injected joints compared with mismatched and matched injected joints. This preserved immunomodulatory function of surviving mismatched MSCs is likely why clinical safety of non-crossmatched allogeneic MSCs 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.[42,43] The lack of differences between MSC and LPS injected joints highlight the pleiotropic nature of these cytokines, and the significant crossover of pro- and anti-inflammatory effects.

3.4 | Systemic humoral immune response to mismatched allogeneic MSCs

The synovial 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 3 and 4).

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 2 weeks after the second injection in 10 of the 13 mismatched recipients (Figure 3). Immunological memory is a tenet of the adaptive immune system[44] 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

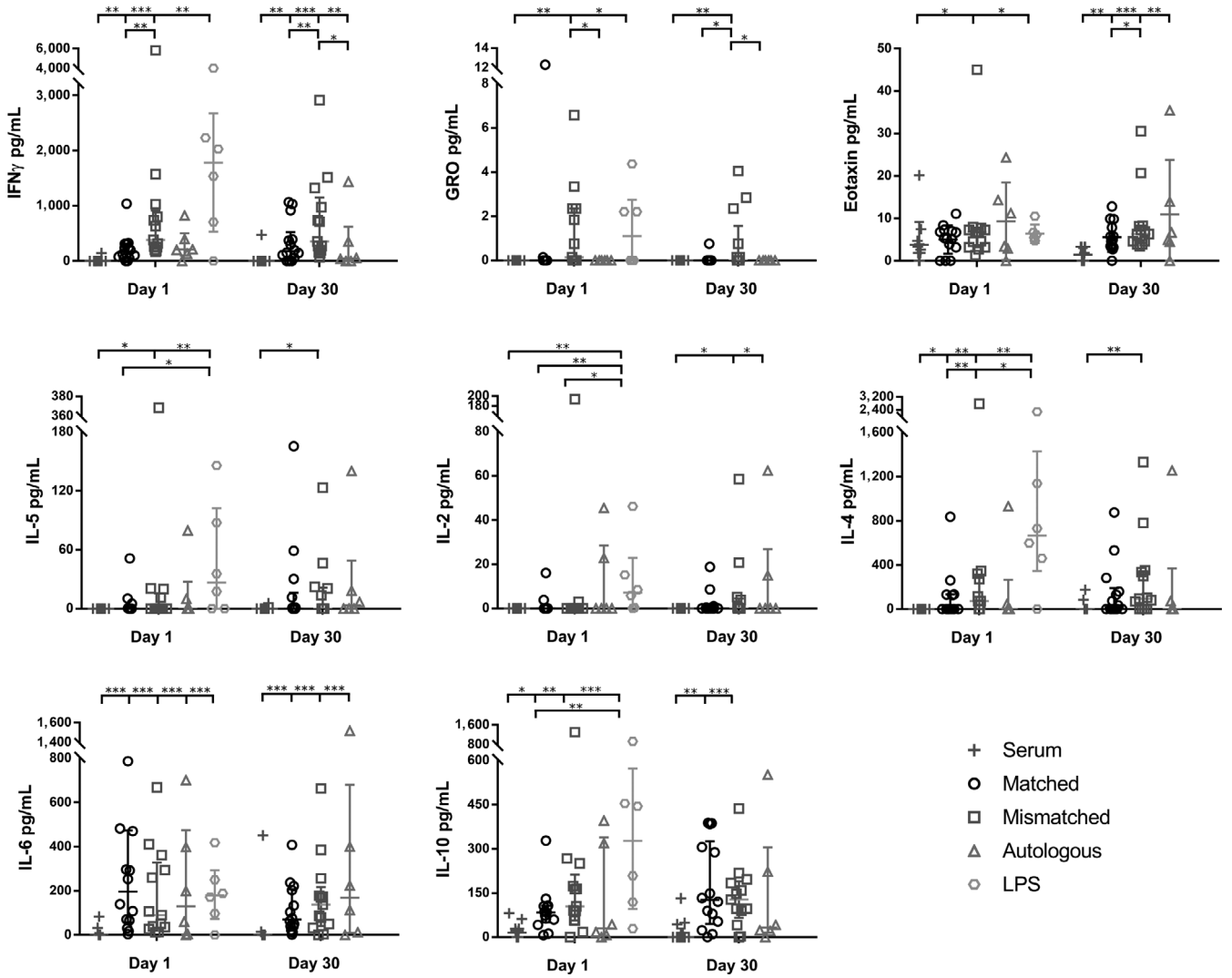

FIGURE 2 Innate and adaptive immune response occurs after major histocompatibility complex (MHC) mismatched MSC injection. Cytokines and chemokines measured in synovial fluid collected after the first and second intra-articular injection. Increased concentrations of IFNγ, GRO, eotaxin, and IL-5 demonstrate immune recognition in the mismatched group. Lines and error bars represent median values and interquartile range, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

well-characterized equine haplotype that is known to have differences in ability to present and recognize antigens.[45] 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 two mismatched recipients had pre-existing antibodies at the time of the first injection. This surprised us as we had documented the lack of presensitization during MHC haplotype screening for inclusion in the study, which occurred 9 months before the intra-articular injection arm of the experiment. We suspect that these two 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.[8,11,46-49] 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.[50] 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 six mismatched recipients with the highest level of cytotoxicity on day 35 was 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

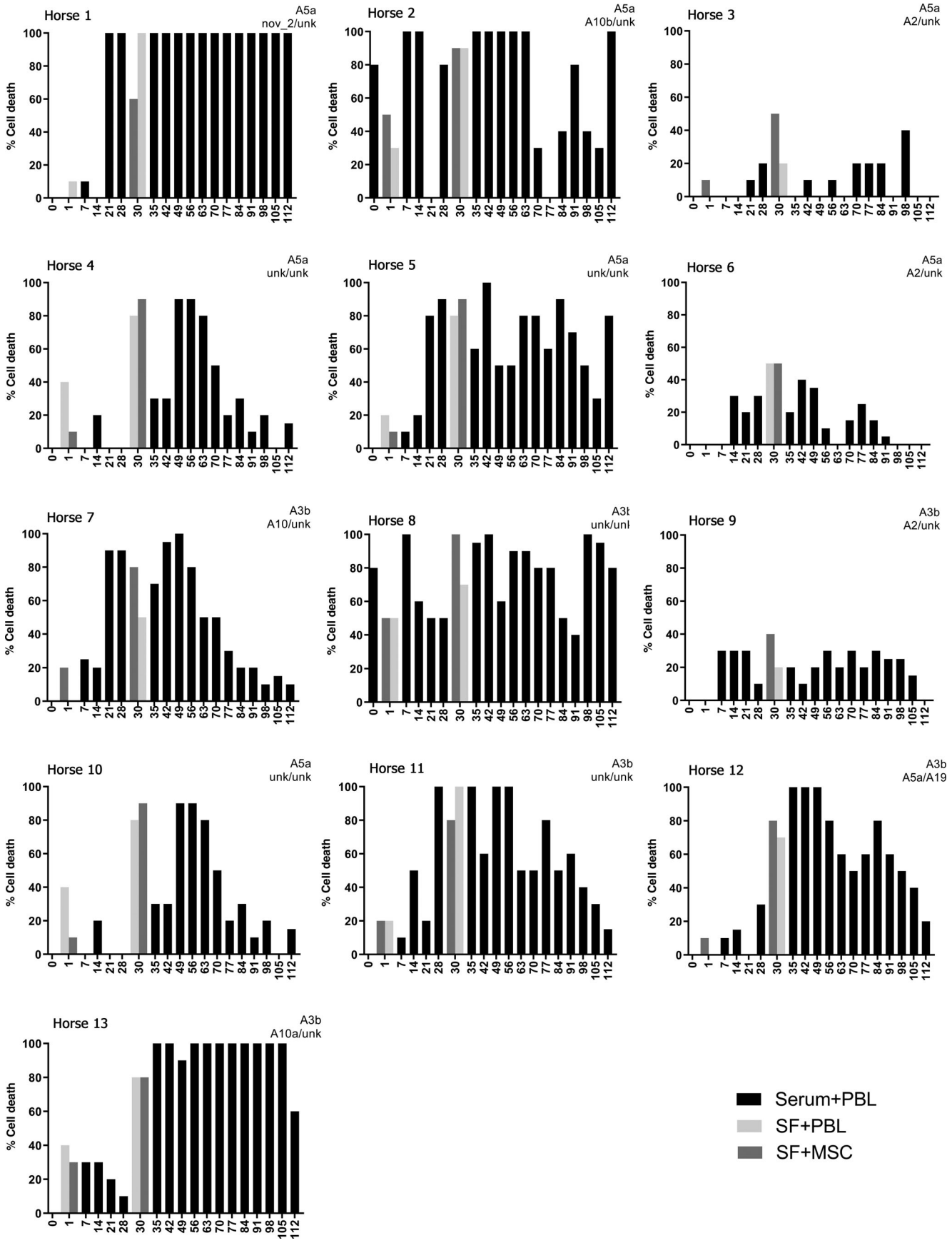

FIGURE 3 Antibody-mediated cytotoxicity in major histocompatibility complex (MHC) mismatched mesenchymal stromal cell (MSC) recipients. Donor peripheral blood lymphocytes (PBL) or MSCs were combined with recipient serum or synovial fluid (SF). Donor and recipient haplotypes listed in the top right corner

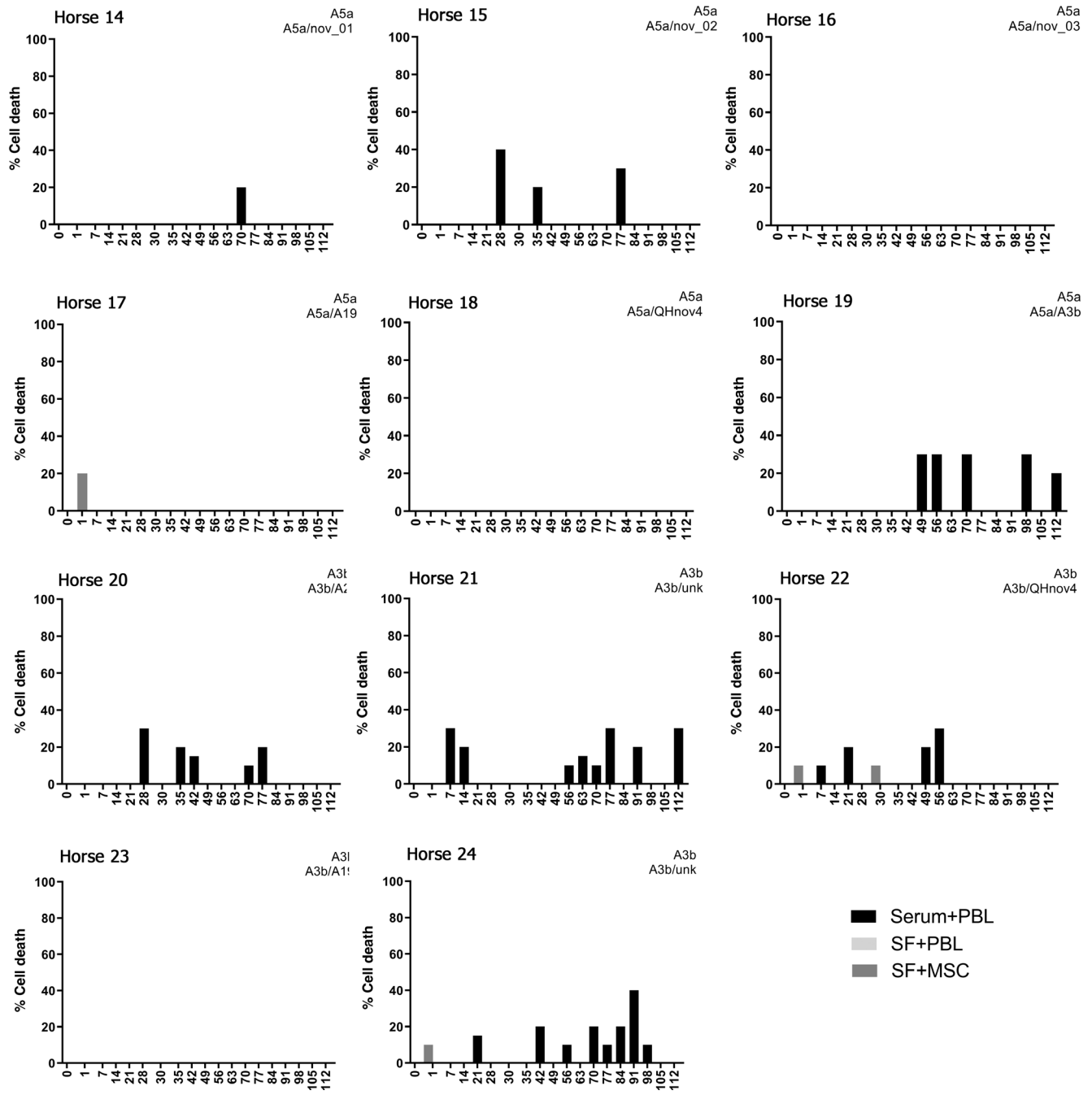

FIGURE 4 Little to no antibody-mediated cytotoxicity in major histocompatibility complex (MHC) matched mesenchymal stromal cell (MSC) recipients. Donor peripheral blood lymphocytes (PBL) or MSCs were combined with recipient serum or synovial fluid (SF). Donor haplotype is listed in the top right corner, with the recipient haplotype listed below

binding buffer, and in the eluate from the IgG binding column (Figure 5).

3.5 | Preformed 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,[9,18] 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% to 50% cell death. As expected, there was essentially no lymphocyte toxicity in matched injected joints (Figures 3 and 4).

3.6 | Preformed 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.[51] 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 3 and 4). After the second injection, there was minimal cytotoxicity in the matched group, but a median of 80% MSC death in synovial fluid from mismatched recipients, confirming that MSCs are susceptible to donor-specific anti-MHC antibodies and complement-mediated cytotoxicity.

3.7 | 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

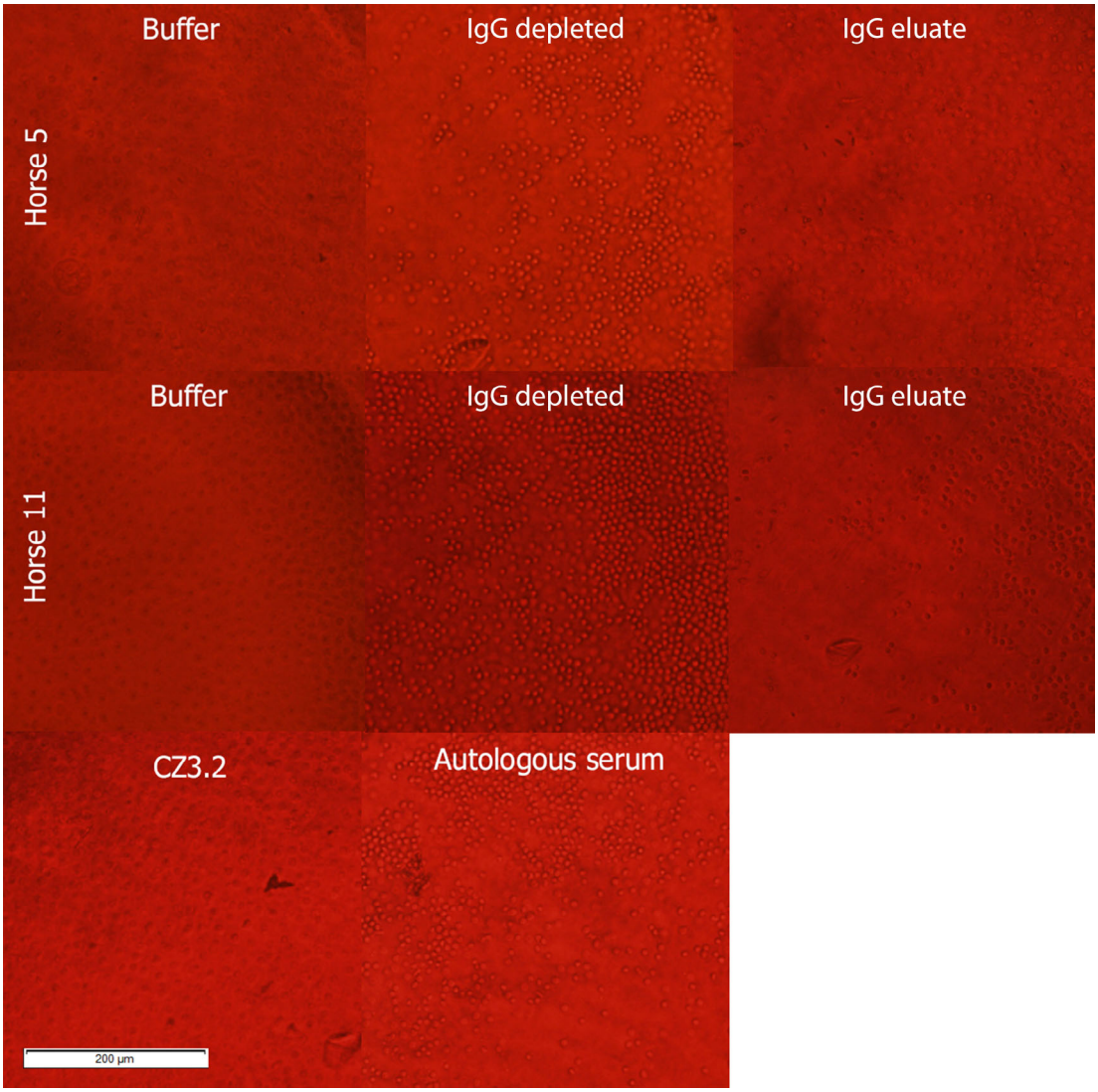

FIGURE 5 Immunoglobulin depletion eliminates cytotoxicity in major histocompatibility complex (MHC) mismatched recipient serum. Serum collected on day 35 from six mismatched recipients (3, A5a recipients; 3, A3b recipients) was depleted of immunoglobulins and microcytotoxicity assays were performed again 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

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 six 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 6).

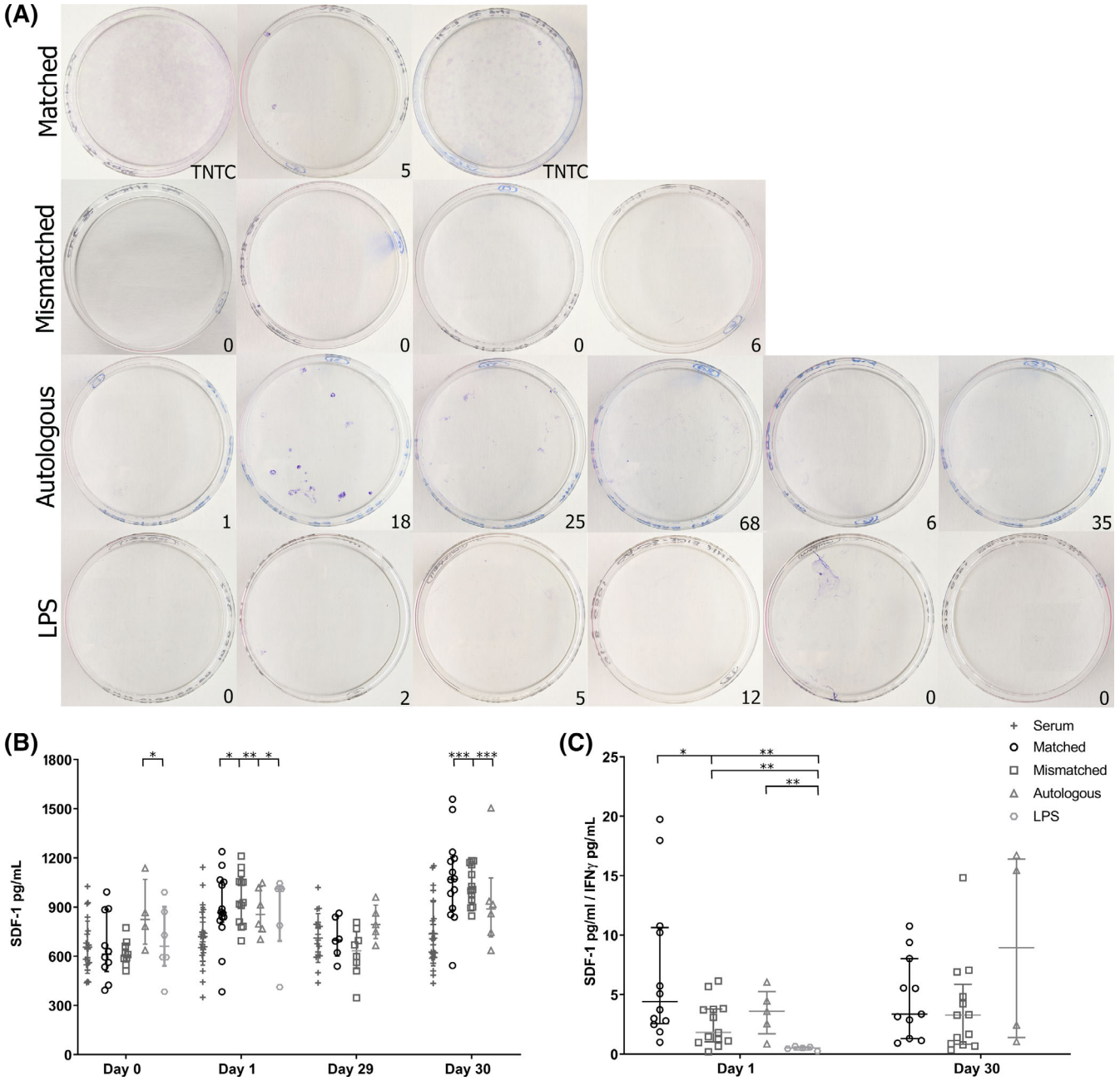

FIGURE 6 Upregulation of endogenous progenitors in major histocompatibility complex (MHC) matched and autologous mesenchymal stromal cell (MSC) recipients, but not after MHC mismatched or lipopolysaccharide (LPS) injection and stromal derived factor-1 (SDF-1) increases in matched and autologous injected joints. A, Composite of MSC CFU-f isolated from synovial fluid 1 day after the second injection. More colonies were isolated after matched and autologous compared with 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 before (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 with 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 with mismatched or LPS. Lines and error bars represent median values and interquartile range, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$

TABLE 2 Microsatellite data from genotype analysis of mesenchymal stromal cells (MSCs) retrieved from synovial fluid compared with donor and recipient. Matching genotypes are highlighted

Source	Gender	AME (sexing)	AHT4	AHT5	ASB17	ASB2	ASB23	HMS2	HMS3	HMS56	HMS7	HTG10	HTG4	LEX3	LEX33	TKY333	TKY374	TKY394	VHL20
Synovial MSCs	F	X	KO	JK	GL	MQ	L	KR	IM	M	MO	IN	LM	OP	OR	KS	LM	LP	MO
Recipient	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	F	X	KO	KN	LO	MQ	K	KL	IM	MP	JO	IR	MP	MO	QR	KS	JM	JL	LO
Recipient	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	F	X	JO	KN	OR	MQ	JK	KR	MP	LO	O	IO	MO	MN	M	KS	MN	JM	IL
Recipient	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	M	YX	HO	JK	OR	KQ	IK	L	IP	MP	L	M	KN	L	L	RT	JM	O	M
Recipient	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

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.[52,53]

3.8 | SDF-1 increases after matched allogeneic and autologous MSC injection

Before 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.[54,55] In our study, SDF-1 was increased in all groups compared with serum-DMSO alone, without differences to joints injected with LPS only. As SDF-1 increases during inflammation,[56] as well as during noninflammatory 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 6). When normalized, SDF-1 was significantly increased, compared with injection of serum-DMSO, in matched and autologous injected joints, but not in mismatched or LPS-injected joints. Increased relative synovial SDF-1 concentrations in matched joints are mechanistic support for increased endogenous progenitor recruitment by matched, but not mismatched, MSCs.

4 | DISCUSSION

For the first time, we show 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.[1-4,6]

Much effort has been made to identify donor factors that predict patient responsiveness to MSC therapy.[7,57-59] However, prior

sensitization to donor MHC haplotype[8,11,49] and development of anti-MHC antibodies[7,8] after multiple treatments may explain why donor MSC factors do not determine whether a patient will be a responder vs a nonresponder.[7,57-59] Rather, our findings indicate that recipient factors, such as MHC compatibility with the donor, dictate response vs nonresponse 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.[4] 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 presensitization as well as stringent assessment of anti-MHC antibody development after treatment.

Despite numerous previous reports on the lack of adverse effects of non–cross-matched 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.[54,62-64] 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.[65,66]

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.[67] 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 were 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.[39] 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 with the autologous group.[67,68]

5 | CONCLUSIONS

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 provide strong evidence that the use of non–cross-matched 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, presensitization should be assessed. This paradigm shift may offer the opportunity for repeatable therapeutic results and lead to regulatory approval of MSC therapy.

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CONFLICT OF INTEREST

L.V.S. declared honoraria from Arthrex Inc and cofounder of Vetletics Inc. The other authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

A.L.R., A.E.W.: conception and design, data collection, data analysis, manuscript writing, final approval of manuscript; D.M.: conception and design, provision of study material, data collection, data analysis, final approval of manuscript; A.B., L.V.S.: conception and design, final approval of manuscript; G.J.L.: data collection, data analysis, final approval of manuscript; D.F.A.: conception and design, data analysis, final approval of manuscript.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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