ORIGINAL ARTICLE

Horses with equine recurrent uveitis have an activated CD4+ T-cell phenotype that can be modulated by mesenchymal stem cells in vitro

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Abstract

Equine recurrent uveitis (ERU) is an immune-mediated disease causing repeated or persistent inflammatory episodes which can lead to blindness. Currently, there is no cure for horses with this disease. Mesenchymal stem cells (MSCs) are effective at reducing immune cell activation in vitro in many species, making them a potential therapeutic option for ERU. The objectives of this study were to define the lymphocyte phenotype of horses with ERU and to determine how MSCs alter T-cell phenotype in vitro. Whole blood was taken from 7 horses with ERU and 10 healthy horses and peripheral blood mononuclear cells were isolated. The markers CD21, CD3, CD4, and CD8 were used to identify lymphocyte subsets while CD25, CD62L, Foxp3, IFNγ, and IL10 were used to identify T-cell phenotype. Adipose-derived MSCs were expanded, irradiated (to control proliferation), and incubated with CD4$^+$ T-cells from healthy horses, after which lymphocytes were collected and analyzed via flow cytometry. The percentages of T-cells and B-cells in horses with ERU were similar to normal horses. However, CD4$^+$ T-cells from horses with ERU expressed higher amounts of IFNγ indicating a pro-inflammatory Th1 phenotype. When co-incubated with MSCs, activated CD4$^+$ T-cells reduced expression of CD25, CD62L, Foxp3, and IFNγ. MSCs had a lesser ability to decrease activation when cell-cell contact or prostaglandin signaling was blocked. MSCs continue to show promise as a treatment for ERU as they decreased the CD4$^+$ T-cell activation phenotype through a combination of cell-cell contact and prostaglandin signaling.

KEYWORDS
activated CD4$^+$ T-cells, equine recurrent uveitis, immunomodulation, mesenchymal stem cells

1 | INTRODUCTION

Equine recurrent uveitis (ERU), commonly known as moon blindness, is a devastating immune-mediated disease that affects between 2% and 25% of horses, with 1%-2% of American horses showing enough clinical signs to threaten vision.[1-4] ERU is characterized by recurring flare-ups of intraocular inflammation or low levels of persistent inflammation, predominantly of the iris, ciliary body, and choroid.[5] Acute symptoms of ERU include miosis, lowered intraocular pressure, aqueous flare, and iris adhesions, while chronic ERU can lead to cataracts, glaucoma, and blindness.[5] Three clinical forms of ERU have been described, with classic ERU being the most common, insidious ERU being characterized by constant and subtle intraocular inflammation, and posterior ERU being seen with inflammation mostly in the posterior segment of the eye.[6] Current treatments for ERU, including anti-inflammatories and immunosuppressive drugs, are not curative and only slow the progression of the ocular inflammation,[7] with approximately 56% of horses affected with ERU eventually becoming blind.[4,8] Euthanasia or change of ownership are commonly seen as sequelae to horses with chronic ERU, and over 60% of ERU horses are not able to return to their previous role as a working horse.[9] As a recurring uveitis targeting retinal proteins, ERU serves as the only naturally occurring model for human uveitis.[10-13]

Equine recurrent uveitis symptoms are primarily the result of T-cell activation (specifically Th1 and Th17 cells) causing destruction of the uveal tract of the eye.[14-16] In horses, the pro-inflammatory Th1 subset can be identified by cells expressing both interferon gamma (IFNγ) and FoxP3,[17] while the Th17 subset is associated with markers interleukin (IL)-6, IL-17, and IL-23.[18] Naturally occurring T regulatory cells (Tregs) express CD25 as well as CD4 or CD8, though activated, natural Tregs may also express FoxP3 and IL-10.[17,19] Histologically, ERU lesions are CD4+ predominant with lower percentages of CD8+ T-cells and increased transcription of interleukin IL-2 and IFNγ, supportive of Th1 inflammation.[16,18,20] Additionally, inflammation in the eye has strong immunoreactivity for Th17 cells (IL-6, IL-17, and IL-23), suggesting these cells play a role in ERU pathogenesis.[14,15,18] Eyes affected with ERU have significantly higher levels of the anti-inflammatory cytokine IL-10, indicating that IL-10 may play an important role in ERU.[21]

While the ERU inflammatory lesion is well characterized in the uveal tract and in the aqueous humor, there is a need to identify more accessible blood biomarkers to help identify horses and monitor response to therapeutic interventions. Models of ocular autoimmune diseases are characterized by activated CD4+ T-cells in both peripheral blood and in infiltrating leukocytes.[11,22] In the induced equine model of ERU specifically, autoreactive peripheral blood lymphocytes traffic in blood and migrate to the eye.[11,23] Lymphocytes dominate the leukocyte infiltrate in ERU, and the majority of these cells are CD4+ T-cells.[12] In proteomic studies, these autoreactive lymphocytes express proteins associated with cell migration and immunity,[11,15] including formin-like 1, which is highly expressed in peripheral blood lymphocytes.[23] This CD4+ T-cell membrane protein, involved in phagocytosis, cell adhesion, and cell migration, was found to be of significantly higher abundance in the proteome of horses with ERU.[23] Although activated T-cells in peripheral blood may not perfectly reflect the ocular infiltrate, changes in peripheral blood leukocytes may provide readily accessible data on immune cell activation, disease dynamics and remission, and therapeutic efficacy of novel immunotherapies. The percentages of blood CD4+ T-cells, CD8+ T-cells, and B-cells (CD21+) and the distribution of Th1 and Tregs in horses with ERU is currently unknown.

Most of our current knowledge of ocular inflammation, especially in the serum, focuses on pro-inflammatory cytokines such as IFNγ, IL-1a, IL-6, and IL-17.[21] However, autoimmune diseases, such as ERU, can also result from a lack of immunosuppression (ie, an absence of Tregs].[24,25] Tregs, also known as suppressor T-cells, are a subpopulation of T-cells which modulate the immune system and abrogate autoimmune disease in part via suppressing proliferation of effector Th1 and Th17 cells. There is a developing body of in vivo and in vitro data in veterinary medicine that shows that mesenchymal stem cells (MSCs) used in immune-mediated and inflammatory diseases can induce a switch from pro-inflammatory T-cell subsets (Th1/Th17) to regulatory suppressive T-cell subsets (Tregs).[26-29]

Mesenchymal stem cells are multipotent stromal cells with powerful pro-regenerative and anti-inflammatory properties.[30] MSCs have been isolated from a number of tissues and expanded ex vivo can be used as a therapeutic agent.[31] Equine MSCs derived from bone marrow, adipose, umbilical cord blood and umbilical cord tissue have all been shown to reduce activated T-cell proliferation in vitro though adipose-derived MSCs may be easier to access and grow.[32] MSC's regenerative ability is attributed in part to their ability to modulate both innate and adaptive immune responses,[33,34] as they produce a variety of immunomodulating factors including IL-6, prostaglandin E_2 (PGE$_2$) and nitric oxide.[32,35] These secreted factors inhibit activated T-cell proliferation, decrease pro-inflammatory cytokine secretion and increase Treg numbers.[27,34,35] Human MSCs decrease activated T-cell proliferation as well as decrease expression of IFNγ and CD25.[36] In rodent models of induced autoimmune uveitis, MSCs are immunosuppressive. They downregulate activated T-cells, increase number and function of Tregs and reduce inflammation for an extended amount of time.[37,38]

The goals of this study were to determine blood immune cell phenotype in horses with ERU and to then determine whether MSCs could alter this phenotype in vitro. We

hypothesized that horses with ERU would have a pro-inflammatory immune cell phenotype, showing Th1 activation, and that MSCs would decrease these T-cell subsets in vitro, shifting toward Treg subsets. We found that horses with ERU show an activated CD4$^+$ T-cell phenotype and that MSCs were able to reduce CD4$^+$ T-cell activation in vitro.

2 | MATERIALS AND METHODS

2.1 | Horses

Blood from 10 clinically healthy horses was collected for immune cell phenotyping. These horses were determined to be healthy based on a complete blood count (CBC), biochemical profile, physical examination and ocular examination. All horses had a clear medical history for any inflammatory disease or medical intervention besides vaccines for at least 6 months. Horses over the age of 20 were excluded due to age-associated alterations in CD4$^+$ FoxP3$^+$ cells.[17]

Blood from seven ERU horses was collected for immune cell phenotyping. These horses had a history of ERU (including anterior or posterior synechia, cataract, glaucoma, degenerative/discolored vitreous, and/or peripapillary chorioretinal scarring) and an active uveitis flare. Horses were not on systemic or topical steroidal or nonsteroidal therapies at the time of blood collection.

Healthy horses included the following: Hanoverians (2), American Quarter Horses (2), Czech warmblood (1), Belgian warmblood (1), Thoroughbred (1), Irish sport horse (1), Standardbred (1), and one of unknown breed. Both geldings (6) and mares (4) were represented, with ages ranging from 9 to 20. ERU horses included the following: Appaloosas (5), Dutch warmbloods (1), and one of unknown breed. Mares (2), geldings (4), and a stallion were represented, and ages ranged from 4 to 20.

Blood was also collected from healthy horses under the age of 15 housed at the Center for Equine Health (CEH), UC Davis, CA for use in in vitro studies. All protocols were approved by the Institutional Animal Care and Use Committee and the Clinical Trials Review Board at the University of California, Davis.

2.2 | Immune cell characterization

Equine whole blood was collected via jugular venipuncture into sodium heparin vacutainers (BD Biosciences). Peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll gradient and resuspended in lymphocyte stimulation media, consisting of RPMI 1640 media (Gibco, Invitrogen) with 10% fetal bovine serum (FBS; Atlanta Biologicals), 1% GlutaMax (Gibco), 1 mmol/L Na Pyruvate (Gibco), 75 μg/mL gentamicin (Gibco),

2 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Gibco), 1 μL/mL minimum essential medium nonessential amino acids (MEM NEAA; Gibco), and 55 μmol/L b-mercaptoethanol (Bio-Rad Laboratories).[32] PBMCs were variably labeled with antibodies that cross-react with equine leukocytes. Specifically, B-cells were labeled with CD21 (BD Pharmingen, Clone B-ly4),[39] T-cells with CD3 (Dr Jeffrey Stott, UCD, School of Veterinary Medicine, Clone UC F6G),[40] CD4 (Monoclonal Antibody Center, Washington State University, Pullman, WA, Clone HB61A),[41] CD8 (Dr Jeffrey Stott, Clone CVS8),[39] CD25 (R&D Systems, polyclonal anti-human CD25/IL-2R alpha Clone AF-223-NA),[17] and CD62L (MyBioSource, Clone LAM1-116). After surface markers were labeled, cells were labeled with Foxp3 antibody (eBioscience, clone FJK-16s,[17] using the manufacturer's fixation/ permeabilization buffer. Cells to be labeled for intracellular cytokines were stimulated with 25 ng/mL phorbol 12-myristate 13-acetate (PMA), 1 μmol/L ionomycin, and 10 μg/mL Brefeldin A for 3 hours to activate equine T-cells.[17,42] These cells were fixed with 2% paraformaldehyde and washed/permeabilized with a buffer containing 0.5% BSA and 0.1% saponin (Wagner), and then labeled for IFNγ (Bio-Rad, Clone CC302)[42] and IL-10 (Wagner Laboratories, Cornell University, Ithaca, NY, Clone 165-2).[42] Cells were resuspended in flow buffer (Dulbecco's phosphate-buffered saline (DPBS), Gibco) with 2% FBS and 2 mmol/L EDTA (Sigma-Aldrich) and read on a Cytomics FC500 flow cytometer (Beckman Coulter). Flow cytometry data were analyzed using FlowJo software (Tree Star Inc). The percentage of CD25$^+$, CD62L$^+$, FoxP3$^+$, IFNγ$^+$, and IL-10$^+$ cells were gated from CD4$^+$ or CD8$^+$ cells.

2.3 | Mesenchymal stem cells and CD4$^+$ T-cell co-incubations

2.3.1 | Mesenchymal stem cells collection and culture

Seven cryopreserved primary adipose-derived MSC lines were used in this study. MSCs were obtained from either healthy horses housed at the CEH or from the Regenerative Medicine Laboratory (RML) at UC Davis. All samples were obtained from adult horses following approved animal care and use protocols. All MSC lines were phenotyped by our laboratory prior to this study.[32,43] MSCs are CD90, CD44, MHC I, and CD29 positive and MHC II and F6B (pan leukocyte) negative. For experimental use, cells were thawed in a 37°C water bath, centrifuged (100x g, 10 minutes), and plated into a tissue culture flask in media (Dulbecco's Modified Eagle's Medium (DMEM), Gibco] with 10% FBS and 1% penicillin-streptomycin (Gibco)). Cells were cultured at 37°C in 5% CO_2. When cells were ~70% confluent,

they were trypsinized using 0.05% Trypsin/EDTA (Gibco) and resuspended in media to prepare for co-incubation studies. All MSCs were between passages 2 and 4 for experimentation.

2.3.2 | Irradiation

To prevent MSC proliferation, cells were irradiated at 10 Gy (Varian 2100C linear accelerator, Varian Medical Systems). After irradiation, MSCs were washed (100x g, 10 minutes), resuspended in media, and kept on ice until co-incubation plating.

2.3.3 | CD4 + T-cell isolation

Peripheral blood mononuclear cells were isolated from equine whole blood as described above and resuspended in DPBS. Cells were filtered through a 35 μm cell strainer cap (BD Biosciences), washed (400x g, 5 minutes), and resuspended in MACs buffer (DPBS, 0.5% bovine serum albumin (Gibco), 2 mmol/L EDTA). Cells were labeled for CD4 followed by anti-mouse IgG Microbeads (Miltenyi Biotec). Cells were run through a MACS LS separation column (Miltenyi Biotec) per manufacturer's recommendation, and purity of CD4+ T-cells after this column was confirmed by flow cytometry to be ≥95%. Isolated CD4$^+$ T-cells were resuspended in media.

2.3.4 | Co-incubation setup

CD4$^+$ T-cells were plated alone or at a ratio of 5:1 with MSCs in 12-well plates (Falcon, Corning, Inc, Corning, NY) with or without activation (0.5% phytohemagglutinin [PHA]). Plates were incubated at 37°C, 5% CO_2 for 4 days. For some experiments, MSCs were separated from CD4$^+$ T-cells using transwell inserts (Corning). For other experiments, 1% indomethacin (5 mmol/L in DMSO) was added to the wells to block prostaglandin signaling. After 4 days, cells were collected, labeled for CD4, CD25, CD62L, FoxP3, IFNγ, and IL-10, and read on a flow cytometer as previously described. Data were analyzed using FlowJo flow cytometry software. The percentage of

CD25$^+$, CD62L$^+$, FoxP3$^+$, IFNγ$^+$, and IL-10$^+$ cells were gated from CD4$^+$ cells.

2.4 | Data analysis

The Grubbs' test was used to detect outliers, although all outliers were included in additional tests and depicted in figures. Normality was determined using the Shapiro-Wilk test. For nonparametric results, statistical significance was determined using the Mann-Whitney test. For parametric results comparing two outcomes, data were analyzed by paired t tests. For parametric results comparing more than two conditions, the ANOVA was used (GraphPad InStat version 3.06). For all results, $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Healthy horses and horses with ERU have similar percentages of blood CD3+ T lymphocytes (CD4$^+$ and CD8$^+$subsets) and B lymphocytes

Normal horses and ERU horses had similar percentages of circulating T-cells (CD3$^+$), CD3$^+$CD4$^+$ cells (T helper cells), CD3$^+$CD8$^+$ cells (cytotoxic T-cells), and CD21$^+$ cells (B-cells) (Table 1). There was no significant difference between the percentages of these cell types for the horse groups.

3.2 | Equine recurrent uveitis horses have an activated CD4$^+$ blood T-cell phenotype

CD4$^+$ T-cells from ERU horses expressed significantly higher levels of IFNγ ($P = .01$, Figure 1A) than control horses, and showed a trend toward expressing lower levels of IL-10 ($P = .07$, Figure 1B), indicative of a shift toward a Th1 activation phenotype. There was no difference in the percentage of circulating in CD4$^+$ T-cells that were positive for FoxP3 or CD25, normally associated with CD4 Tregs, between ERU horses and control horses ($P = .32$, Figure 1C, $P = .2$, Figure 1D, respectively). The mean fluorescence of CD25 on CD4$^+$

TABLE 1 Normal horse and ERU horse lymphocyte ranges

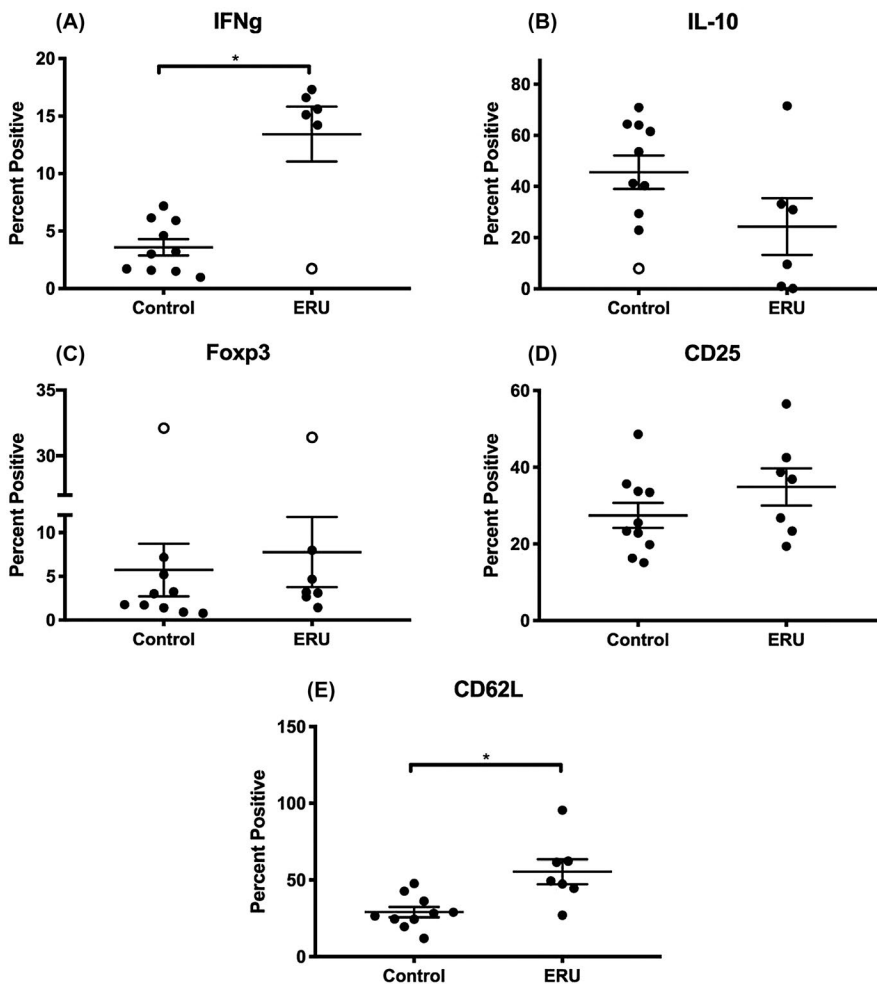| Marker	Horse group	Cell type	Ranges (%)	Average(%)	*P* Value
CD3	Normal	T-cell	39.2-73.8	59.8	.95
	ERU		15.2-79.7	55.7	
CD4	Normal	T helper cell	69.0-85.3	76.8	.18
	ERU		63.8-74.0	72.4	
CD8	Normal	Cytotoxic T-cells	6.4-27.0	15.3	.30
	ERU		13.1-26.3	20.0	
CD21	Normal	B-cells	2.8-19.9	11.8	.27
	ERU		3.6-12.9	8.7	

CD4+ T Cell Phenotype

FIGURE 1 CD4+ T-cells show increased levels of IFNγ expressing CD4+ T-cells. (A-C) ERU horses and control horses express similar levels of CD25+, IL10+ and FoxP3+ CD4+ T-cells. (D-E) ERU have significantly higher levels of IFNγ+ CD4+ T-cells and CD62L+ CD4+ T-cells. Data are shown as box and whisker plots with a mean value shown as the middle bar and the range being from minimum to maximum value. Open dots represent outliers. *P < .05

T-cells was also evaluated (CD25^{hi}) and not noted to be different between control and ERU horses. Lymphocytes from horses with ERU had significantly increased expression of CD62L ($P < .01$, Figure 1E), associated with a naïve or central memory phenotype, compared to healthy horses.

3.3 | CD8+ T-cells from ERU horses have increased expression of CD62L but otherwise do not reflect alterations noted in CD4+ cells

CD8+ T-cells from ERU horses did not have increased IFNγ compared to healthy horses ($P = .41$, Figure 2A) and had slightly lower levels of IL-10 ($P = .09$, Figure 2B). ERU horses did have slightly higher levels of FoxP3 ($P = .06$, Figure 2C) than healthy horses; however, this was not significant. The percentage of CD25+ CD8+ T-cells was not altered in ERU horses ($P = .89$, Figure 2D). Taken together, there was no distinct pattern indicating CD8+ T-cell activation or

Tregs in ERU horses. Similar to CD4+ T-cells, CD8+ T-cells had significantly increased CD62L expression ($P = .02$, Figure 2E).

3.4 | Mesenchymal stem cells decrease CD4+ T-cell activation phenotype

Phytohemagglutinin activation of equine CD4+ T-cells resulted in increased intracellular accumulation of IFNγ, IL-10, and FoxP3 ($P < .01$, Figure 3A, $P < .01$, Figure 3B, $P < .01$, Figure 3C) and increased surface expression of CD25 and CD62L ($P < .01$, Figure 3D, $P = .05$, Figure 3E). MSCs significantly decreased measured markers of T-cell activation including decreased intracellular IFNγ ($P < .01$, Figure 3A), intracellular FoxP3 ($P < .01$, Figure 3C), and surface CD25 ($P = .01$, Figure 3D). MSCs were able to downregulate CD25 even in the absence of activation ($P = .01$, Figure 3D). MSCs did not change CD4+ T-cell expression of IL-10, regardless

CD8+ T Cell Phenotype
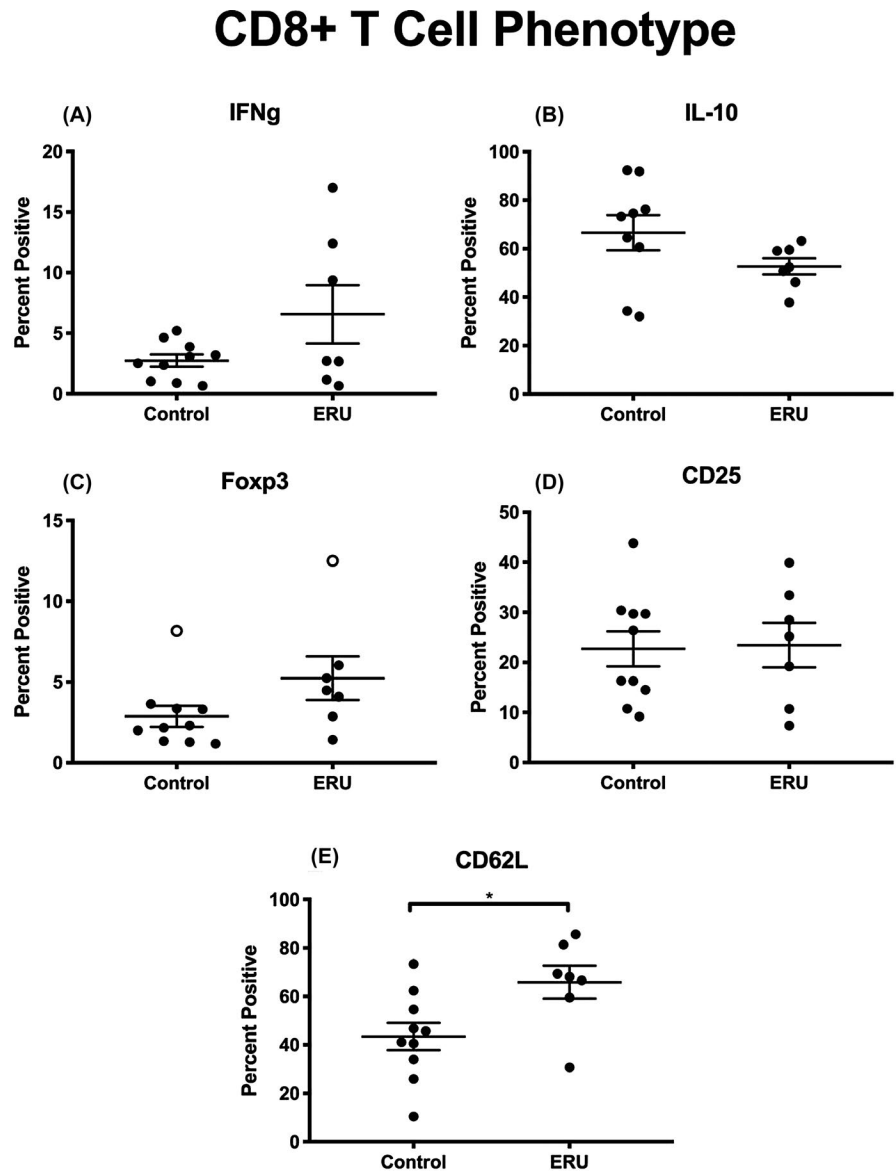
of activation ($P = .14$, Figure 3C). MSCs were also able to decrease surface CD62L ($P = .02$, Figure 3D) in activated CD4$^+$ T-cells.

3.5 | Soluble mediators produced by MSCs reduce CD4$^+$ T-cell activation whereas cell-cell contact is needed for MSCs to induce CD4$^+$ effector/effector memory cells

Mesenchymal stem cells reduced activated CD4$^+$ T-cell activation indicated by decreased expression of IFNγ ($P = .03$ Figure 4A) and FoxP3 ($P = .01$, Figure 4B) with or without cell-cell contact indicating a soluble mediator was responsible for these changes. However for another indicator of T-cell activation CD25 expression MSCs relied on direct CD4$^+$ T-cell-MSC interaction to reduce CD25 expression (as CD25 expression was largely restored when MSCs were

separated from T-cells by a transwell) ($P = .09$, Figure 4C). Similarly the induction of CD4$^+$ effector/effector memory cells (CD62L-) by MSCs requires cell-cell contact as there was no difference in CD62L levels between activated CD4$^+$ T-cells and CD4$^+$ T-cells separated from MSCs by a transwell ($P = .57$, Figure 4D).

3.6 | Prostaglandin is required for MSC reduction of CD25 and IFNγ expression in activated CD4 + T-cells

When prostaglandin signaling was blocked in the co-incubations, lymphocyte secretion of IFNγ was restored ($P = .67$, Figure 5A). However, MSCs were still able to reduce intracellular FoxP3 expression ($P = .01$, Figure 5B) suggesting that PGE$_2$ is not the soluble mediator responsible for reduction of FoxP3. In the absence of PGE$_2$, MSCs were unable to
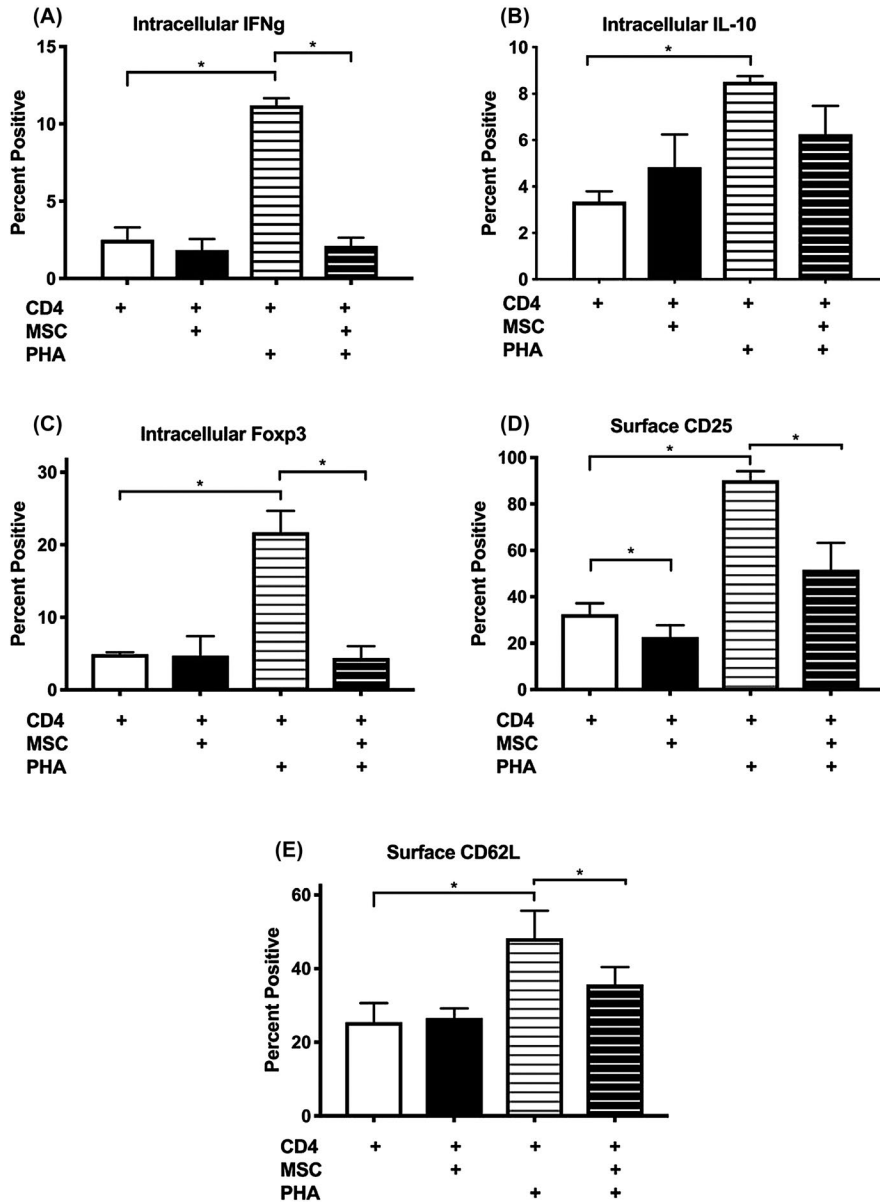
FIGURE 3 CD4^{+} T-cells have a lowered activation phenotype after four day co-incubation with MSCs. (A) CD4^{+} T-cells had lowered expression of CD25 when co-incubated with MSCs, both with and without activation by PHA. (B) Intracellular IL-10 showed no change based on co-incubated with MSCs. Intracellular FoxP3 (C), intracellular IFNγ (D), and surface CD62L (E) expression was lowered in activated CD4^{+} T-cells that were co-incubated with MSCs. Data are presented as mean ± standard error of the mean. *$P < .05$; CD4, CD4^{+} T-cells; MSC, mesenchymal stem cells; PHA, phytohemagglutinin

decrease CD25 expression suggesting that PGE$_2$ is partially responsible for this MSC-mediated decrease in lymphocyte activation. ($P = .14$, Figure 5C). MSCs were able to reduce CD62L ($P = .05$, Figure 5D) expression on activated CD4^{+} T-cells both with and without prostaglandin signaling.

4 | DISCUSSION

Equine recurrent uveitis is an inflammatory, autoimmune disease with characteristics similar to human uveitis.[13,21] Previous studies have shown that T lymphocytes, especially CD4^{+} T-cells, are the predominant inflammatory cell present in ERU tissues.[18,23] Our data, however, suggest that the percentage of CD3^{+}, CD8^{+}, and CD4^{+} lymphocytes in blood are similar between normal horses and horses

with ERU. Our work also showed an increase in activated CD4^{+} T-cells with increased IFNγ expression, which is similar to another study that found that horses with marked ocular inflammation have higher serum levels of IFNγ.[21] Overall, we saw a shift toward Th1 inflammation in horses with ERU, as CD4^{+} T-cells expressed increased levels of IFNγ, and some horses showed a concurrent expression of IFNγ from CD8^{+} T-cells. ERU horses showed no changes in CD4^{+} T-cell expression of FoxP3, IL-10, or CD25 or CD8^{+} T-cell expression of IL-10 or CD25. These data suggest that ERU horses do not have specific patterns of alterations in circulating markers of Tregs, though examination of additional ERU horses would help to confirm this lack of alteration. Differences in the subclinical type of ERU and genetic background may also contribute to patterns in T-cells that should be examined.

FIGURE 4 Differences between contact and no contact co-incubations of activated CD4^{+} T-cells with MSCs. Co-incubation with MSCs induced a reduction in expression of intracellular IFNγ (A) and FoxP3 (B) both with contact and without contact. (C) While co-incubation with MSCs reduced activated CD4 + T-cells expression of CD25 with contact, without contact, no reduction of CD25 was seen. (D) Co-incubations with MSCs without contact did not reduce CD4+ T-cell expression of CD62L. *P < .05
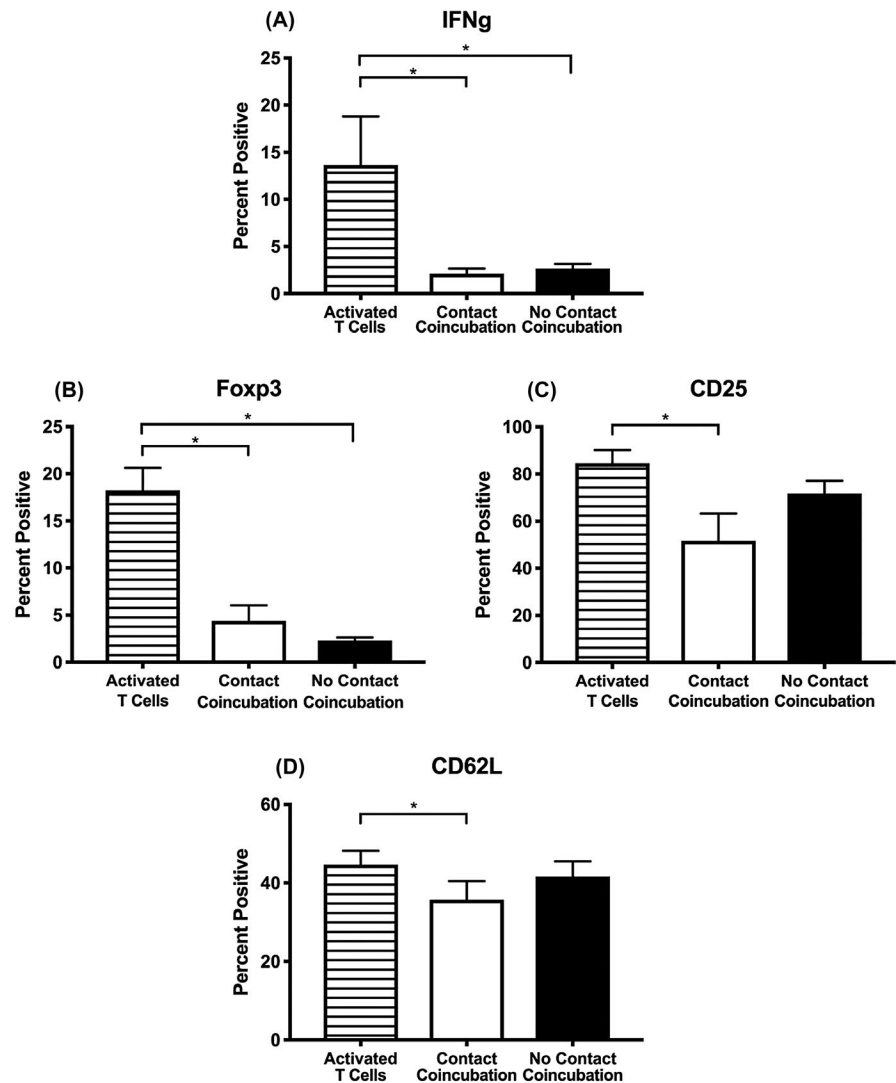
Equine recurrent uveitis horses may have a shift toward central memory cells, indicated by both circulating CD4^{+} and CD8^{+} T-cells having increased CD62L (aka L selectin) expression. CD62L is a lymphocyte adhesion molecule that plays an important role in lymphocyte-endothelial cell adhesion and lymphocyte homing. Central memory T lymphocytes have encountered antigen and express CD62L to localize in secondary lymphoid organs.

A number of studies have looked at the immunomodulatory effects of MSCs in horses, showing that equine MSCs, much like other species, can inhibit activated T-cell proliferation and decrease TNFα and IFNγ production[27,32] in part through the secretion of PGE$_2$. Our study has expanded on these findings and demonstrated that equine MSCs also decrease CD4^{+} T-cells expression of CD25 (IL-2 receptor), through cell-cell contact. Human MSCs also decrease CD25 through MSC-lymphocyte contact via PD-L1/ PD-1.[36] Equine adipose-derived MSCs, like we used in this study, induce lymphocyte apoptosis, which may also be done through

PD-L1/PD-1 pathway.[27] Equine MSC downregulation of CD25 on CD4^{+} T-cells is also facilitated by MSC secretion of PGE$_2$. These data confirmed our previous findings that equine MSCs decrease protein secretion of IFNγ (as measured by supernatant in co-culture assays),[27] by demonstrating the specific reduction of intracellular IFNγ accumulation in CD4^{+} T-cells mediated via PGE$_2$ signaling.

FoxP3 is a cytokine known to play many roles and is generally associated with Treg function,[17] but has also recently been associated with T-cell activation in humans and horses.[44,45] Furthermore, in horses, the induced expression of FoxP3 by activated T-cells was associated with a regulatory cytokine profile. Similar to Cavatorta et al, our data confirm that FoxP3 is regulated similarly in horses and humans and is an inherent component of T-cell activation and proliferation. Our data demonstrate that equine MSCs decrease FoxP3 expression by activated, proliferating CD4^{+} T-cells through a soluble mediator.[45] This mediator was not PGE$_2$ in our assays, and equine adipose-derived MSCs do not produce NO

(A) IFNg
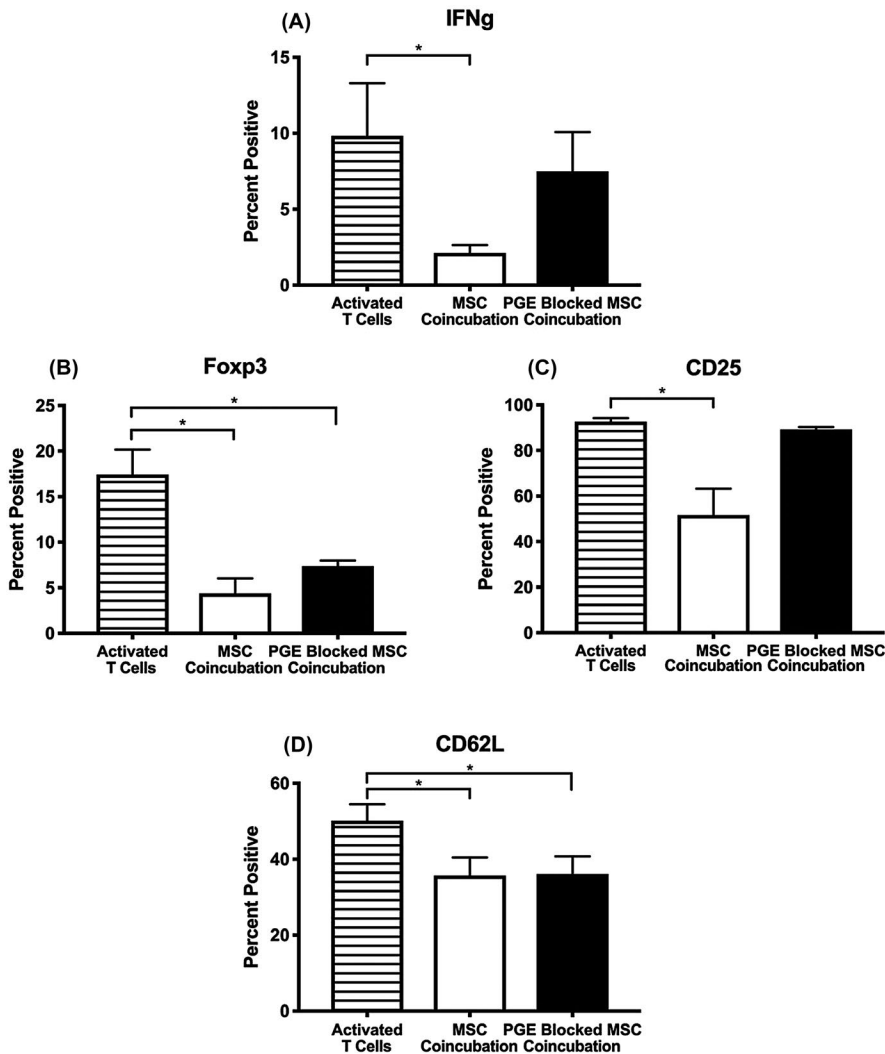

(B) Foxp3

(C) CD25

(D) CD62L

FIGURE 5 Differences between contact and prostaglandin blocked co-incubations of activated CD4$^+$ T-cells with MSCs. A, With contact, co-incubations with MSCs reduced CD4+ T-cell expression of IFNγ. However, without prostaglandin signaling, IFNγ expression was not reduced. B, Expression of intracellular FoxP3 was reduced in the presence of MSCs both with and without prostaglandin signaling. C, While co-incubations with MSCs reduced CD25 expression in contact co-incubations, without prostaglandin signaling, no reduction of CD25 was seen. D, CD62L expression by activated CD4+ T-cells was reduced in co-incubations both with and without prostaglandin signaling. *P < .05; PGE, prostaglandin

or IDO,[32] as such FoxP3 reduction may be mediated through TGFβ or IL-6. Cavatorta et al also determined that although FoxP3$^+$ T-cells were capable of producing IFNγ, they were more likely to produce IL-10 and less likely to produce IFNγ than equivalent FoxP3$^-$ cells. This may explain why T-cell activation in our hands increased IFNγ, FoxP3, and IL-10. The concurrent reduction in IFNγ and FoxP3 by equine MSCs provide some evidence that MSCs could be useful to decrease CD4$^+$ T-cell activation, and induce tolerance, in horses with diseases such as ERU that are characterized by T-cell activation.

CD62L was used in this study to look at T-cell activation, effector memory, and central memory/naive lymphocyte subsets. Interestingly, horses with ERU have higher percentages CD62L$^+$ T-cells compared to healthy horses suggesting increased numbers of central memory/naive cells. In vitro, equine MSCs induced a switch to effector memory cells as seen by decreased CD62L expression on activated CD4$^+$ T-cells after direct cell-cell contact with MSCs. Ligand pairs known to be important in MSC-lymphocyte adhesion and signaling include PD-L1/PD-1, ICAM-1/LFA-1, and VCAM-1/

VLA-4.[36,46,47] Our data do not agree with data from a murine study that demonstrated that CD62L was maintained/upregulated after MSC/lymphocyte co-incubation[48]; however, the induction of effector memory cells has been reported in a human study after MSC infusion.[49] ERU horses have high numbers of CD62L$^+$ CD4$^+$ and CD8$^+$ T-cells and equine MSCs in vitro can cleave CD62L resulting in a shift to effector memory cells. To confirm this finding, evaluating CD62L levels in additional ERU affected horses and controls are warranted.

Horses with ERU have a shift toward a Th1 inflammatory phenotype (CD4$^+$ IFNγ$^+$) with increased numbers of central memory/naïve CD4 and CD8 T-cells. In vitro, equine MSCs reduce IFNγ expression, decrease T-cell activation (and proliferation) in general (including decreased FoxP3 and CD25), and increase effector and effector memory cells via cleaving CD62L. Like other MSCs, equine MSCs appear to rely on both soluble mediators and cell-cell contact for their immunomodulatory properties. These data suggest that ERU may be an appropriate target for MSC therapy (along with other equine diseases characterized by this immune phenotype). In

addition, the data highlight some objective blood biomarkers that could be monitored in ERU horses prior to and post-MSC transfusion.

Future clinical applications for MSCs in treating ERU appear to be promising. Our laboratory has consistently found the intravenous (IV) administration of MSCs to be safe.[29] We have treated 3 ERU horses with MSCs IV, and the horses did very well after these infusions. However, it is unknown if these MSCs will disproportionately reach the eye or affect ocular inflammation. Intravitreal injections in horses have been attempted, but have been associated with significant adverse events and are not recommended (unpublished data). Recent studies have had success with subconjunctival MSC injections, which may be a promising method for treatment of ERU.[50] Additional prospective studies will be needed to assess the effect of MSC administration on ocular inflammation in vivo.

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SUPPORTING INFORMATION

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