The search for an ideal mesenchymal stromal cell donor in the horse

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Abstract

The search for an immune privileged allogeneic mesenchymal stromal cell (MSC) line has been an interest for many biomedical researchers. This holds true for the field of equine medicine where MSCs are frequently used in research and clinical cases for the treatment of musculoskeletal disease. An ideal allogeneic MSC suppresses the immune system of the recipient leading to decreased inflammation in the face of disease. The ideal MSC also expresses the markers of a multipotent cell, retains a high level of viability and is able to perform anabolic activities to enhance repair.

Our research sought to more clearly define the expression of MSC markers harvested from different equine MSC donors. Bone marrow-derived MSCs from Thoroughbreds, Standardbreds, and a subset of universal blood donor-type Standardbreds were compared. Standardbred MSCs showed significantly less MHC class II expression at early passages as compared to Thoroughbreds. When universal blood donor Standardbreds were compared to non-blood donor Standardbreds, the only significant variation was that CD90 was expressed more highly on universal blood donor MSCs as compared to non-blood donor Standardbred MSCs. The conclusion from stage one of our research was that universal blood donor-type Standardbred horses appeared less likely to cause an MHC II driven immune reaction and had the highest levels of bone marrow-derived MSC markers expressed at passage 2-4.

We then compared the MSC donor cells in an *in vitro* trial exploring several arms of the immune system to understand the effects of the MSCs without prior

activation of the immune cells, as has been done previously. Overwhelmingly, we found that MSCs of allogeneic origin cause very little to no activation of the immune system as compared to autologous MSCs. B cell and activated T lymphocyte populations were similar between the autologous and allogeneic MSCs. Those allogeneic MSCs that expressed little MHC II prior to interaction with the immune cells (MHC II-low MSCs) had reduced activation of recipient lymphocytes and neutrophils as compared to those MSCs expressing high levels of MHC II prior to interaction with immune cells (MHC II-high MSCs).

MHC II-low MSCs, both of universal blood donor and non-blood donor origin, had higher expression of the genes we studied when placed in an allogeneic environment. These include both anabolic molecules known to assist in healing and some catabolic molecules. This knowledge combined with published information that ‘activated’ MSCs can be more beneficial to healing than unactivated MSCs, support the use of the more metabolically active MHC II-low MSCs as compared to MHC II-high MSCs.

Based upon a wide array of testing, allogeneic MHC II-low MSCs created a low level of immune activation and an increased level of gene anabolic gene expression as compared to autologous MSCs. In conclusion passage 2-4 MHC II-low MSCs are preferred for use in allogeneic therapy.

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This work would not have been possible without the constant investment of my supervisors. I cannot thank them enough. Dr. Chris Riley is a strong advocate for the student, and though he is both a researcher and a surgeon, likely his greatest gift to the world is his ability to inspire his students. He put my benefit before his agenda, he gently steered me back on course when I went astray, and his intentions always had my best interest at heart. His kindness pushed me through many disappointing days. The amount of appreciation I have for him cannot be expressed.

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Dr. Erica Gee guided the way when completion of this project seemed the least likely possibility. She taught me how to break down an immense amount of work into a multitude of steps which would lead me from start to end. It was only with this understanding that the path was feasible.

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I must thank Dr. Joanna Roberts for her expertise and precision in cytometry. This field can appear like one of modern art, but she firmly plants it into the realm of science.

My partner Benjamin Cunningham deserves most of the recognition for the completion of this PhD. During the completion of this work, we had 2 children and both of us carried on with full-time careers. Ben has given up an equivalent amount time to be a father and caretaker as I have to be a student. I am sure that there have been many times that he has regretted the timing of my pursuit of a PhD, but he has only ever been supportive. His love and guidance mean everything to me. He has made all of my dreams come true.

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It is my intention to return my debts to all these amazing people by giving as much as I have taken as the chain of knowledge and support passes from one person to the next.

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Kamm JL, Riley CB, Parlane NA, Gee EK, McIlwraith CW. Immune response to allogeneic equine mesenchymal stromal cells. Stem Cell Res Ther. 2021;12(1):570.

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Riley CB, Kamm JL, Parlane N, Gee E, Dittmer KE, McIlwraith CW. Differences in cell marker expression by equine bone marrow-derived mesenchymal stem cells associated with blood antigen type and breed. ANZCVS Equine Chapter Proc 2018, Science Week, Gold Coast, Australia 2018:67

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Chapter 1. General Introduction

1.1 Introduction

Selecting the optimal mesenchymal stomal cell source is critical for obtaining favorable results from their use in regenerative medicine (Richardson *et al.* 2016). This has led to an ongoing search for mesenchymal stromal cells (MSCs) with the best capacity to replace or restore function to damaged tissues and a low occurrence of side effects (Joswig *et al.* 2017). In equine medicine, autologous MSCs derived from bone marrow are frequently used in clinical cases as their ability to enhance repair of tissues damaged by musculoskeletal disease is supported by a growing body of evidence from experimental and clinical studies (McIlwraith *et al.* 2011, Godwin *et al.* 2012, Ferris *et al.* 2014).

There is a move in equine medicine to use allogeneic MSCs instead of autologous MSCs due, in part, to the immediate availability of allogeneic MSCs and the inconsistent quality of autologous cells (Garvican *et al.* 2014, Schnabel *et al.* 2014, Pezzanite *et al.* 2015, Colbath *et al.* 2017). Perhaps the most important advantage of an allogeneic source of MSCs is the benefit afforded by a uniform MSC treatment for efficacy research into the therapeutic use of MSCs for equine diseases. An allogeneic cell line with a consistent phenotype would allow patients in clinical trials to be treated with characterised MSCs from the same donor, and therefore all cases would receive a repeatable treatment. The current use of autologous MSCs in clinical studies adds an element of variability in the therapeutic efficacy of MSCs and standardized comparisons in clinical trials

(Portalska *et al*. 2013). MSC function has been shown to vary in older humans, and the cell phenotype can vary from one bone marrow draw to the next (Schnabel *et al.* 2014, Pezzanite *et al.* 2015, Colbath *et al.* 2017).

When considering treatment with allogeneic MSCs, the potential for immunologic reactions by the host is a likely cause of treatment failure (Joswig *et al.* 2017, Colbath *et al.* 2017, Griffin *et al*. 2013). MSCs are acutely or progressively rejected by the cell-mediated and humoral arms of the immune system leading to MSC death and local inflammation (Zangi *et al.* 2009, Consentius *et al.* 2015, Berglund *et al.* 2017). The major histocompatibility complex (MHC) class I and II molecules present on the cell surface facilitate allorecognition when foreign cells are transplanted into a recipient (Benichou *et al.* 2011, Griffin *et al.* 2013, Schnabel *et al.* 2014). MHC class I and II molecules on the surface of the donor MSCs are identified by the recipient's immune system leading to T and B lymphocyte activation (Griffin *et al.* 2013, Schnabel *et al*. 2014).

We hypothesized that one group of equids of a particular phenotype may have a preferential MSC phenotype as compared to another group of equids. Ideally, this would allow the MSC to retain viability in the recipient, provide immunosuppression and provide beneficial anabolic effects as MSC are known for. We used three groups of horses for comparison: Standardbreds, Thoroughbred and blood donors. Research has demonstrated that erythrocyte and leukocyte antigen expression vary between horse breeds (Becht and Semrad 1985, Angelos *et al.* 1988). Our aim was to determine if there is some correlation between the expression of immunogenic antigens on erythrocytes and those

immunogenic antigens that are expressed on MSCs as it is well known that a series of equine erythrocyte antigens causes immune reaction leading to hemolysis after blood transfusion (Tomlinson *et al.* 2015). After determining the phenotype of the MSCs from our three groups, we then tested these MSCs in immunological assays to determine which was most capable of immunosuppression. Assays tested all branches of the immune system to find an MSC with optimal qualities to be used as a donor MSC for the treatment of equine disease.

1.2 Conflict of interest statement

Lacy Kamm is the director and shareholder of Advanced Regenerative Therapies NZ. This company offers autologous mesenchymal stromal cells. The author has no conflict of interest with the results in this body of work.

1.3 Dissertation Structure

This dissertation is submitted as a “Doctoral thesis with publications.” The dissertation begins with an overview of the research topic including a literature review on the use of mesenchymal stromal cells (MSCs), methodology used in the assays, and finally the immunologic implications of allogeneic MSC use. Gaps in the literature led to our goal of determining whether one type of equine donor would more immunopriviledged than another donor. Following the introductory chapter, there are three chapters aimed at answering this question. These chapters are comprised of published manuscripts that encompass the body of research performed in pursuit of this PhD. We start our research by identifying three distinct sets of MSC donor types (Chapter 2). We then perfect novel methods for comparison of these MSCs for immunological testing (Chapter 3). We then compare the performance of our group of MSCs when co-cultured with lymphocytes (Chapters 4 and 5), neutrophils and complement (Chapter 5). Our final chapter clarifies our conclusions by describing why our ideal MSC donor is preferable to the other groups.

Each chapter containing a published scientific study is comprised with a prelude, introduction, methods, results, discussion, references, supplemental information and an epilogue. The prelude serves to tie each of the chapters together. The introduction, methods, results, discussion, and references are the published work. The supplemental information contains those figures and tables published as supplemental information for the manuscript and additional figures

and tables to better illustrate the data. The epilogue serves to further discuss the published findings.

1.4 Literature review

1.4.1 Mesenchymal stromal cell treatment for equine musculoskeletal disease

Published in the Equine Veterinary Practitioner (2016; 40(3):18-21).

Prelude

This section of the literature review reflects upon the current use of MSCs in common practice in equine medicine. It is important to understand how this therapy is used in order to determine the level of need and functional application for an allogeneic donor cell in clinical practice. This needs to be done prior to undertaking further research on the topic. If there were little need for an allogeneic MSC in clinical practice or in clinical research, then continuing on with this body of work would be unnecessary. By completing the following review, the level of need was determined to be substantial and the research worthy of pursuit (Kamm and McIwraith 2016). This information was also necessary for obtaining financial support for the project.

Introduction

Mesenchymal stromal cells (MSCs) have a distinct set of cell surface markers and multipotent potential to differentiate down adipocyte, chondrocyte and osteoblast lineages (Viswanathan *et al.* 2019). The use of MSCs for disease treatment has been advocated for over 10 years, and the scientific evidence in

favor of their use in musculoskeletal disease is mounting (Clegg *et al.* 2011, Broeckx *et al.* 2019). Reports have shown a definitive benefit from MSC treatment for equine joint disease (Ferris *et al.* 2014, Broeckx *et al.* 2019) and tendonitis (Godwin *et al.* 2011, Smith *et al*. 2013). However, it is to be noted that MSC treatments have not consistently shown benefit in experimental studies (Arhberg *et al.* 2018) and their effectiveness in treating other diseases still needs to be defined (Schnabel *et al.* 2013).

This report gathers data from both clinical and experimental studies on humans and animals with a focus on horses. The intention of this report is to provide a balanced and objective analysis of these studies in order to determine if MSCs should be established as an effective treatment for specific equine musculoskeletal diseases.

Mechanisms of action

The actions of mesenchymal stromal cells in a disease process can be broken down into 2 main categories:

1. Direct contribution of healing involving differentiation into tissue-specific cell phenotypes and the production of appropriate extracellular matrix products (Alves *et al*. 2011).
2. Indirect mechanisms include trophic effects through the production of active proteins (such as growth factors), induction of nearby cells to become tissue-

specific cells, anti-apoptotic factors, chemotactic agents, and anti-inflammatory mechanisms (Caplan and Dennis 2006).

MSCs can assist in tendon repair by differentiation into tenocytes (Schneider *et al.* 2011, Tong *et al.* 2012). MSCs undergo this differentiation by co-culture with tendon explants or under monolayer conditions with tenocytes and added growth factors (Schneider *et al*. 2011, Tong et at. 2012). After differentiation, the MSCs become elongated in a tenocyte phenotype and express tenocyte molecules such as collagen type I and III, tenomodulin, and scleraxis (Schneider *et al*. 2011).

MSCs can also differentiate into chondrocytes (Coates *et al.* 2014). MSC can be manipulated down the chondrocyte lineage by co-culture with cartilage explants (Coates *et al.* 2014). After differentiation, the MSCs increased their collagen type II expression 11-fold as compared to prior to differentiation (Coates *et al*. 2014). As collagen type II is an important and unique component of articular cartilage, its expression is a fundamental activity of chondrocytes. Another fundamental molecule to the extracellular structure of articular cartilage is aggrecan. Kisiday *et al*. (2008) found significant aggrecan deposition by MSCs when the cells were cultured in a chondrogenic environment.

The second mechanism of action of MSCs, as listed above, is their ability to decrease inflammation, increase growth factors in diseased tissue (Caplan and Dennis 2006), and promote healing by induction of other cells (Chen *et al.* 2018). MSCs can act with potent anti-inflammatory effects that result in their somewhat immune-tolerant cell phenotype (Ripoll *et al.* 2011). The cells decrease

inflammation by upregulation of chemokines, suppression of cytokine secretion from dendritic cells and reduction in populations of T cells and natural killer cells (Nixon *et al.* 2012). These induced anti-inflammatory effects of MSCs in tendon disease results in reduced fibre degeneration (Nixon *et al.* 2012).

Finding the right mesenchymal stromal cell

Several stromal cell lines are available for use in horses. Mesenchymal stromal cells derived from bone marrow or adipose tissue are the most common cell types used (Schnabel *et al*. 2013). As MSCs comprise only a small fraction of the total population of nucleated cells from adult tissue sources, MSCs must be harvested and cultured in order to expand their number to an amount that will be sufficient for treatment (Alves *et al*. 2011). Clinical and experimental reports have generally utilized 10-20 x 10^6 cells per treatment (Schnabel *et al*. 2009, Ferris *et al*. 2014).

Stromal cells have also been derived from umbilical cord blood, embryos, synovial tissue and joint fluid (Prado *et al*. 2015, Burk *et al*. 2014). All of these sources have been less frequently studied in the horse as they are less cell-dense and/or less readily-available for autologous use. If, in the future, cells from these sources are found to be immune privileged for allogeneic use, then these sources may become more interesting.

When attempting to determine which MSC line is the best line to use for orthopaedic disease, one must compare the MSC's ability to treat disease with both direct and indirect mechanisms (as previously discussed). Bone marrow and

adipose-derived MSCs have been compared in their ability to decrease inflammation and immunomodulate activated leukocytes. *In vitro* work showed no significant difference in immunosuppressive effects of adipose-derived MSCs as compared to bone marrow-derived MSCs (Remacha *et al*. 2015). Frisbie *et al*. (2009) found a significantly decreased amount of prostaglandin E2 in bone marrow-derived MSC-treated arthritic joints as compared to adipose-derived MSC-treated arthritic joints (Frisbie *et al.* 2009). As prostaglandin is a molecule upregulated by inflammatory cytokines and considered to correlate with joint pain, the fact the bone marrow-derived MSCs cause a significant reduction in prostaglandin would make them the more effective anti-inflammatory cell. In controversy, one human study found that an equal number of adipose-derived MSCs provided greater immunomodulation of activated peripheral blood mononuclear cells (PBMCs) as compared to bone marrow-derived MSCs (Melief *et al.* 2013).

Studies have compared bone marrow-derived MSCs and adipose-derived MSCs in their capability to form articular cartilage (Kisiday *et al*. 2008, Teunissen *et al.* 2021). Kisidat *et al.* (2008) found that bone marrow-derived MSCs were more capable of forming the extracellular matrix of articular cartilage created by chondrocytes than adipose-derived cells. Both type II collagen production and aggrecan gene expression were greater in bone marrow-derived MSCs as compared to adipose-derived progenitor cells (Kisiday *et al*. 2008). Teunissen *et al*. found that canine bone marrow-derived MSCs had improved ability to form cartilage *in vitro* as compared to adipose derived MSCs (Teunissen *et al.* 2021).

This data would lead us to consider bone marrow- derived MSCs as the better MSC to contribute to direct mechanisms of joint repair.

A comparison of the capability of adipose and bone marrow-derived MSCs for in treating tendon injuries has been performed. Burk *et al*. (2015) found MSCs derived from adipose displayed the highest expression of collagen type I and III as compared to MSCs from embryos, cord blood and bone marrow (Burk *et al.* 2015). Burk *et al.* found that scleraxis expression was highest in MSCs derived from cord blood as compared to MSCs derived from other sources. Scleraxis has been proposed previously to be the primary controller of directing MSCs along the tenocyte lineage (Nixon *et al.* 2012, Li *et al*. 2015). Another study showed an equal capability of adipose and bone marrow-derived MSCs to assume a morphology similar to tenocytes, express tendon marker genes, and improve tissue mechanical properties when grown on decellularized tendon scaffolds (Youngstrom et al. 2016).

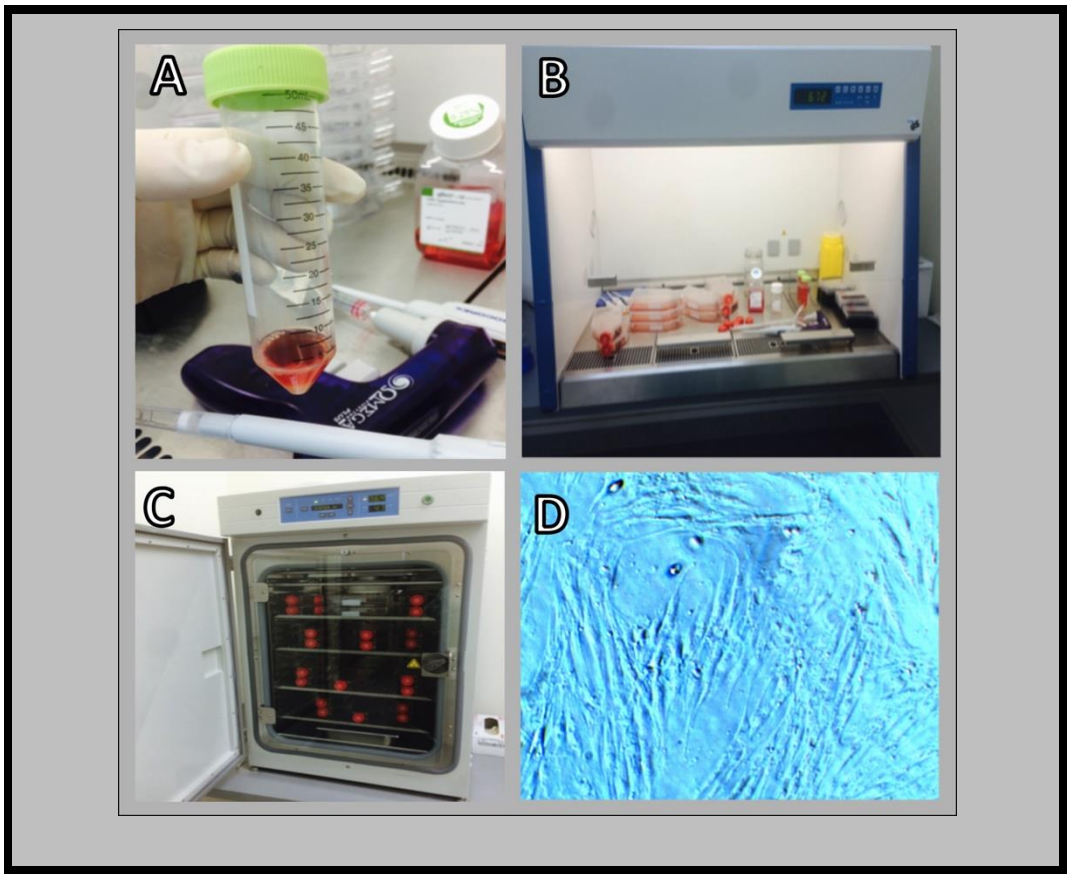

Figure 1. Culture of MSCs.

Meticulous preparation of MSCs, both in experimental and in clinical settings is paramount to successful outcomes. Aseptically harvested progenitor cells are prepared as a suspension in nutrient medium (A), placed in culture flasks in a ventilated hood (B), incubated (C) and microscopically evaluated as a cell culture (D).

MSCs in orthopaedic disease

MSCs have been used to treat many equine musculoskeletal disorders including tendonitis, desmitis, joint injury and joint disease as well as laminitis (Schnabel *et al*. 2013). Clinical results in humans and horses are described here for soft tissue and joint diseases. Laminitis treatment with MSCs will not be further discussed as no scientific studies have been performed to date that assess their effectiveness in treatment of this disease.

Tendonitis and desmitis

Treatments for tendon and ligament injuries include rest, rehabilitation, surgical reconstruction, injectable biologics (MSCs, platelet rich plasma [PRP]), extracorporeal shockwave, magnetic therapy, and treatment with deep tissue laser energy (Nixon *et al*. 2012). When considering human tendon and ligament diseases that have been treated with intra-lesional MSCs, there are few studies published to date. Experimental studies have found improved histological and biomechanical properties, but mineralization at the location of bone marrow-derived MSC therapy has also occurred (Long and Sun 2016). A common location of soft tissue injury in people are rotator cuff injuries (Teunis *et al.* 2014). Two published reports on human rotator cuff repair using cultured MSCs showed improved recovery with MSCs and surgery as compared to surgery alone (Ellera Gomez *et al*. 2012, Kim *et al.* 2017). Kim *et al.* (2017) showed that one treatment with MSCs at the time of surgery decreased the MRI indicated re-tear rate from 28.5% in the conventional group to 14.3% in the MSC injection group ($p < .001$). Another study showed that all patients treated with MSCs and surgery had no loss of integrity of repair as seen on MRI at one year post-treatment while the current research on rotator cuff repair has concluded that integrity is complete in less than 75% of cases treated with surgery alone (Ellera Gomez *et al*. 2012).

Experimental *in vivo* research has been performed using MSCs in equine superficial digital flexor tendonitis (Crovace *et al.* 2007, Schnabel *et al.* 2009, Smith *et al*. 2013, Ahrberg *et al*. 2018). Smith *et al*. (2013) used clinical cases of career-ending superficial digital flexor tendonitis. Cases were either treated with bone

marrow-derived MSCs or were treated with saline. After 6 months of rehabilitation, the horses were euthanized (Smith *et al*. 2013). Tendons treated with MSCs showed significant improvement in mechanical and histological tests over the control group (Smith *et al*. 2013). In two *in vivo* experimental studies using a collagenase model of tendonitis, bone marrow-derived MSC treatment resulted in improved histological score when compared with scores for untreated tendons (Crovace *et al.* 2007, Schnabel *et al.* 2009). Tensile strength using mechanical testing was improved in MSC treated tendons, but this was not significant (Schnabel *et al*. 2009). Cross sectional area of the lesion was smaller on ultrasound of MSC treated tendons as compared to untreated tendons by eight weeks post-treatment and continued to be significantly smaller until the end of the study (Crovace *et al*. 2007). Collagen type I, the most common type of collagen seen in normal tendons, was more highly expressed in MSC treated tendons lesions as compared to untreated lesions (Crovace *et al*. 2010). Two studies showed no major improvement in histologic score nor musculoskeletal marker expression 24 weeks after treatment with adipose derived MSCs (Geburek *et al*. 2017, Ahrberg *et al*. 2018). These results are mixed as to whether there is a biologic basis for the use of MSCs in soft tissue disease. These results may be extrapolated to suggest the bone marrow-derived MSCs were superior to adipose derived MSCs, though side-by-side comparison was not performed.

There are several reports of clinical cases of MSC use in tendon lesions in racehorses (Pacini *et al*. 2007, Smith 2008, Godwin *et al.* 2012, Renzi *et al.* 2013). One study on lesions in the superficial digital flexor tendon (SDFT) showed an

improved rate of return to full athleticism for steeplechase/hurdle racehorses (82% of 71 horses) as compared to another study which used rehabilitation alone (56%) (Dyson *et al*. 2004, Smith 2008). It should be noted that clinical comparisons with historical controls such as this are difficult to compare due to inherent variation. There was no improvement for flat racehorses with a return to full athleticism rate of 55% (of 23 horses) as compared to rehabilitation alone (66%) (Dyson *et al*. 2004, Smith 2008). A similar study described improved rates of return to athleticism for steeplechase/hurdle racehorses (n=105) with 74.3% returning to full work for at least 2 years following treatment (Godwin *et al.* 2012). This study showed no improvement over rehabilitation alone (using historical controls) for flat racehorses (n=8) with only 50% returning to full athleticism and not re-injuring for 2 years following MSC treatment.

Another study found that 13/19 steeplechase/hurdle racehorses returned to full athleticism after a superficial or deep digital flexor tendon or suspensory ligament tear was treated with MSCs (Renzi *et al.* 2013). This report did carry its own control population and found that only 3/12 of the horses that were not treated with MSCs returned to full athleticism (Renzi *et al.* 2013). This data trended towards statistical significance (p=0.06). It should be noted though that in this report, many of these cases received less than 10×10^6 cells/ treatment. Godwin *et al.* (2012) and Pacini *et al*. (2007) showed a decreased prognosis when fewer cells were used.

Pacini *et al*. (2007) showed very good return to athleticism for a small group of racehorses. Nine of 11 horses returned to full work after treatment with MSCs into a SDFT tear.

These clinical case reports are important as we can then translate the effectiveness of MSC therapy to clinical cases. These reports show improvement in return to athleticism for jumping race horses with soft tissue injuries as compared to horses that were treated with rehabilitation alone (Smith 2008, Godwin *et al.* 2012, Renzi *et al.* 2013).

Joint injury and joint disease

Osteoarthritis is a frequent cause of pain, loss of function and disability in people and affects 5-30% of the adult population (Arshi *et al.* 2020). Clinical studies of intra-articular injection of MSCs in people are becoming more common. Nineteen studies on MSC use for osteoarthritis were being completed or ongoing as of 2015 (Pers *et al.* 2015). In 2019, this number increased to 84 studies for knee arthritis alone (Arshi *et al.* 2020). A meta-analysis of published human studies on clinical knee arthritis found that 15 of 17 studies (6 randomized controlled trials, 8 prospective observational studies, and 3 retrospective case-control studies) showed positive clinical effects in the intra-articular MSC-treated groups (Ha *et al.* 2019).

In the United States' horse industry, joint disease is the leading cause of economic loss (Frisbie 2012). Many equine studies on the use of MSCs in joint disease have been performed to date. The number of studies is steadily increasing

with more of these studies focusing on allogeneic MSCs as compared to autologous MSCs as time goes by (PubMed 2021). One of the earliest studies tested horses with surgically induced carpal osteoarthritis (Frisbie *et al*. 2009). Horses were injected with 5-15 x 10^6 MSCs at two weeks post joint injury induction, and horses were regularly scored for lameness, effusion and the presence of inflammatory mediators in the synovial fluid. Pain and effusion were not significantly improved in the MSC treated joints (Frisbie *et al*. 2009) although prostaglandin content was significantly reduced in MSC treated joints as compared to untreated joints (Frisbie *et al*. 2009).

A clinical study in horses with naturally-occurring femorotibial joint injury showed an improved rate of return to athleticism with MSC treatment compared to previous authors' work (Ferris *et al*. 2014). All horses had arthroscopic surgery and bone marrow derived MSC treatment post-operatively. With MSC treatment, 75% (25/33) of the patients returned to some level of work compared to those in previous reports that indicated a 60–64% (42/70 and 14/22, respectively) return to work for horses that were treated with arthroscopy alone (Walmsley *et al.* 2003, Cohen *et al*. 2009, Ferris *et al*. 2014). It should be noted that clinical comparisons with historical controls such as this are difficult to compare due to inherent variation.

More recent equine studies have used allogeneic MSCs with various degrees of manipulation of the cell selection and culture processes. Several studies using selected allogeneic MSCs or manipulated allogeneic MSCs have shown benefit in *in vivo* studies (Barrachina et al. 2018, Delco et al. 2020, Broeckx

et al. 2019). Intra-articular therapy with tumor necrosis factor (TNF)-α- and interferon-γ- activated MSCs caused an increased amount of cartilage extracellular matrix components and decreased the expression of inflammatory mediators (TNFα in IL-1β) (Barrachina *et al.* 2018). Delco *et al.* 2020 found that treatment of traumatically-induced OA with integrin α10high MSCs decreased bone sclerosis and cartilage fibrillation. Chondrogenically-induced MSCs improved athleticism in a placebo-controlled study treating horses with naturally occurring OA (Broeckx *et al.* 2019).

Conclusion

MSCs have the ability to decrease inflammation and improve the quality of healing in tendons, ligaments and joints (Crovace *et al*. 2007, Frisbie *et al*. 2009, Orozco *et al*. 2013). Their use in equine clinical cases of tendon tears and osteoarthritis is widespread (Schnabel *et al.* 2013, Frisbie 2016). The number of clinical reports showing beneficial results in humans and horses is steadily increasing (Godwin *et al.* 2012, Orozco *et al*. 2013, Ferris *et al*. 2014). This being said, further case controlled studies are in needed in order to better understand MSC's place in the treatment of orthopaedic diseases.

1.4.2. Mesenchymal stromal cell culture methodologies

Prelude

After determining there was a need for research in the field of allogeneic MSCs, we then needed to gather the technical knowledge of MSC culture methodologies. We decided to focus our work on *in vitro* models due to the capacity to control for variables and determine, on the cellular basis, the level of immune response to allogeneic MSCs. MSC isolation from equine bone marrow has been performed in a variety of ways and laboratories are not unified in the preferred method (Bourzac *et al*. 2010, Kisiday *et al*. 2013). Cell culture methods are somewhat more standardized though the cell media additives vary from laboratory to laboratory. MSC cryopreservation methods are largely standardized (Freshney 2010), though the effect of freezing on the MSC can vary (Chatzistamatiou *et al.* 2014, del Pino *et al*. 2015). The effect of freezing on the MSCs was important for us to determine, as the cell surface markers can be altered during the freezing process. These markers were critical for determining immunogenicity of allogeneic MSCs in our assays. After reviewing the literature, methods for equine bone marrow-derived MSCs in our laboratory conditions were compared and validated in order to assure consistent and appropriate results (Appendices A and B).

Introduction

The successful isolation and culture of mammalian cells has been performed since the early 1900s (Freshney 2010). Cell isolation and culture is performed so that sought-after cells may be isolated and preferentially expanded

in order to create a uniform sample, as tissue samples are invariably heterogeneous (Freshney 2010). In the case of mesenchymal stromal cells (MSCs), they must be selectively cultured in order to increase their population as the number of MSCs in tissue is very low, and the number of MSCs harvested from a bone marrow aspirate is typically considered insufficient for clinical treatment (Jung *et al.* 2012).

There is a large number of techniques described for MSC separation, culture, and storage process which determines the end product. Ultimately, the cells must maintain their ability to become many different cell phenotypes, also known as pluripotency, and the MSCs should divide rapidly in order to efficiently create a relatively homogenous population (Bourzac *et al*. 2012). The purity and properties of the resulting populations can be affected greatly by the conditions under which they are collected, transported and cultured (Jung *et al*. 2012). Major variables that may influence the number and quality of MSCs recovered include the conditions of transport of the raw sample, method of cell separation, type of media and growth factors used, incubation parameters, and method of cell freezing (Horn *et al.* 2008, Turnovcova *et al.* 2009, Garvican *et al.* 2014). This section will present what is known of these different variables.

Bone marrow harvest

The sternum and the wing of the ilium are the two primary locations for bone marrow derived MSC harvest in the horse (Goodrich *et al.* 2008). A trephine bone marrow aspiration needle is used to penetrate the cortex of the bone allowing the cannulated needle to enter the bone marrow cavity (Kisiday *et al.* 2013). At this

point, a syringe loaded with anticoagulant is attached to the needle to aspirate the bone marrow.

When comparing the MSCs retrieved from the sternum to those retrieved from the ilium, Adams *et al*. (2013) showed that the number of MSCs collected after up to three passages in culture was not significantly different between the sternum and ilium (Adams *et al*. 2013). Kisiday *et al.* (2013) had conflicting data that found there was an increase in mesenchymal stromal cell number derived from the ilium as compared to the sternum at the end of passage 2. The ilium yielded 2.1 times as many MSCs as the sternum ($p<0.05$) (Kisiday *et al.* 2013).

The volume of equine MSCs collected does not correlate with the amount of bone marrow harvested (Kisiday *et al*. 2013). Five ml samples of marrow from the sternum and ilium were compared to 50ml samples collected from an adjacent sternebrae or the opposite iliac crest. There was no significant difference in the number of MSCs cultured at 8 days nor after passage 2. With increasing aspiration volumes, connective tissue progenitor cells concentration decreases due to dilution of the aspirate with peripheral blood (Smith *et al*. 2003).

Transportation of bone marrow sample

Transportation of the sample from the animal to the laboratory in critical as MSCs can die if transported at the wrong temperature or if kept out of culture for an extended period. Four degrees Celsius is an often-referenced temperature when searching literature pertaining to the topic (Martin and Rowley 1986, Hahn *et al.* 2015). Higher temperatures tend to increase the acidity of the media due to

lactic acid build up (Kao *et al.* 2011). Storage of bone marrow up to 72 hours at 4^0C reportedly does not significantly decrease the amount of mitoses when these cells are then cultured as compared to bone marrow cultured directly after harvest (Martin and Rowley 1986). Uchida *et al.* 2011 showed that preservation of cardiomyocytes in media at temperatures between 3 and 20^0C for 3 days decreased the number of viable cells to no less than 80% of those cells cultured immediately. The majority of MSCs from bone marrow aspirates left in the refrigerator (4^0C) or transported at temperatures below 20^0C and greater than 3^0C will retain their viability for up to 3 days (Martin and Rowley 1986, Hahn *et al.* 2015).

Transport media is usually not used for specimens intended for culture, and its use is not included in the methods in the recent equine bone marrow derived MSC publications (Bourzac *et al.* 2010, Kisiday *et al.* 2013, Garvican *et al.* 2014). It appears unnecessary to add transport media as cell survival was good without its addition (Kisiday *et al.* 2013, Garvican *et al.* 2014). However, an anticoagulant, is added to prevent blood clot formation and cell-cell adhesion. Two types of anticoagulant have been compared; heparin and acid citrate dextrose (ACD) (Kao *et al.* 2011). The addition of ACD causes less change in the pH of the media, but cell viability is unchanged as compared to heparinized bone marrow over 72 hours of storage (Kao *et al.* 2011). It has been concluded that either anticoagulant can be used (Kao *et al.* 2011).

Bone marrow separation

Once the bone marrow sample has arrived at the laboratory, MSC isolation is performed by separating the components of bone marrow. Separation can be

performed using one of three general methods: centrifugation, erythrocyte lysis, or the use of a density gradient (Bourzac *et al.* 2010, Zhang *et al*. 2014). More advanced cell separation methods such as magnetic bead and fluorescence-activated cell separation can be performed but have a low yield. These methods are less commonly used for MSC isolation from equine bone marrow as a large number of cells are needed (Freshney 2010). The centrifugation, erythrocyte lysis, and density gradient separation processes will be described in the following sections.

In brief, bone marrow is comprised of MSCs, mature and immature erythroid and myeloid cells, immature lymphoid cells, fat, and serum (Jafari *et al.* 2017). The myeloid cells include agranulocytes (monocytes) and granulocytes (neutrophils, basophils, eosinophils, and mast cells). Electrolytes and proteins (globulins, albumin) are found in the serum. The intention of the bone marrow separation is to separate the mononuclear cells including the MSCs from the other components of the bone marrow in order to get a high yield of MSCs at the end of the culturing procedure.

Centrifugation

Centrifugation separates a solution by the density of its components with the most dense elements falling to the base of the sampled tube. In the case of bone marrow separation, the most dense cells and biomolecules fall to the bottom of the tube (Freshney 2010). Centrifugation for cell separation can be used in one of two basic ways: slow or fast. The speed of the centrifugation determines what cells and molecules will fall to the base of the tube and form a pellet.

Slow centrifugation can be performed to separate the erythrocytes from the mononuclear cells and plasma. Slow centrifugation (around 100 *x* g) will pellet only the most dense cells and particles to the base of the tube as described in the protocol by Kisiday *et al*. 2013. The erythrocytes are more dense than mononuclear cells and fall to the bottom of the centrifuge tube when separated by slow centrifugation. Kisiday *et al.* 2013 uses slow centrifugation at 100 *x* g for five minutes to form a plasma and mononuclear cell layer above the erythrocyte pellet. These researchers found that there was a 25-50% loss of mononuclear cells (including MSCs) to the erythrocyte pellet layer (Kisiday *et al.* 2013). This minimal centrifugation protocol appears to be the least complex of the cell separation methods, creating the least number of variables. On the other hand, this protocol loses a significant number of sought-after MSCs with the 25-50% mononuclear cell loss (Kisiday *et al.* 2013).

Higher speed centrifugation (1000 *x* g) will pellet the erythrocytes, platelets and mononuclear cells, leaving the serum at the top of the tube (Bourzac *et al.* 2010 and Fortier 2005). After the erythrocytes and mononuclear cells are separated from the serum, the erythrocytes and mononuclear cells are then plated together. The erythrocytes and leukocytes are later removed when the media is changed as they do not adhere to the plate. Recovery of MSCs has been reported to be poor for equine MSCs using this method, as it produces <25% of the number of MSCs as compared to other methods (Bourzac *et al.* 2010). In contrast, a report describing separation of rabbits marrow described no significant decrease in

mesenchymal stromal cell number was seen with this method as compared to other methods (Zhang *et al*. 2014).

Erythrocyte lysis

The second main type of bone marrow cell separation is erythrocyte lysis. Erythrocyte lysis removes the erythrocyte population by colloid osmotic hemolysis using a variety of solutions (Cherneyshev *et al*. 2008). Motais *et al.* 1995 used a hypotonic solution to cause cell swelling and lysis. Many companies offer a proprietary solution for erythrocyte lysis.

In human and animal MSC isolation by erythrocyte lysis methods, the most commonly used lysis solution is a 15mM buffered ammonium chloride solution (Cherneyshev *et al.* 2008). The erythrocyte swells and ruptures preferentially to mononuclear cells due to the presence of Cl-/HCO_3- ion exchangers in the cell membrane of erythrocytes but these components are not present in mononuclear cells (Cherneyshev *et al*. 2008). Centrifugation (1000 x g) can be performed after erythrocyte lysis in order to create a mononuclear cell pellet. The mesenchymal stromal cells will be among those in the mononuclear pellet. This salvages a greater number of sought-after MSCs but introduced the possibility of mononuclear cell lysis when human MSCs were isolated (Horn *et al.* 2008).

Density gradient

A variety of density gradient media are available to separate erythrocytes from mononuclear cells. These density gradient media contain molecules that do not dissolve in liquid. When centrifuged, these media separate with the more dense

molecules moving towards the bottom of the tube while the less dense molecules stay near the top of the tube. The suspension medium is mixed with the bone marrow, and when they are both centrifuged together, the cells separate based on their density along with the medium. In the density gradient, the cells settle to a position that is in equilibrium with their own density (Freshney 2010). The areas of gradient 'bands' can be visualized. The bands are preferentially aspirated to collect a group of cells. Common agents used as separation media include Percoll® (GE Healthcare), a silica based solution; Ficoll®-Paque (GE Healthcare), a hydrophilic polysaccharide; and Histopaque® (Sigma-Aldrich) or Lymphoprep™ (Alere Technologies) solutions containing a polysaccharide and sodium diatrizoate.

Comparison of separation procedures

Studies comparing the high-speed centrifugation, erythrocyte lysis and density gradient methods of separation have been performed (Horn *et al*. 2011, Bourzac *et al*. 2010). When comparing these methods for the use of human MSC isolation, Percoll® produced the most pure population of MSCs as this method resulted in the highest number of colony forming units as compared to the number of cells originally plated (Horn *et al.* 2008). When examining the methods by comparing colony forming units created per bone marrow sample, erythrocyte lysis had the greatest potential for maximum cell harvest as there were a greater number of colony forming units in total and larger colonies in the erythrocyte lysis group (Horn *et al.* 2008). This study found that if efficiency was the primary goal, then Percoll® density gradient was the best method. If total MSCs attained after a period of culture was the primary goal, then erythrocyte lysis method would be

preferred (Horn *et al.* 2008). This study did not include a comparison with the slow centrifugation method.

One equine bone marrow specific study compared high-speed centrifugation to the density gradients (Bourzac *et al*. 2010). This study found that Percoll® gradient dramatically increased the number of mesenchymal cells recovered after 14 days of culture as compared to high-speed centrifugation alone (Bourzac *et al*. 2010).

When comparing high speed centrifugation, erythrocyte lysis and density gradients, it appears that erythrocyte lysis and density gradient are the most productive means of cell separation (Horn *et al*. 2011, Bourzac *et al*. 2010). It must be remembered that slow speed centrifugation was not included in these assay comparisons, and it is unclear as to how this method compares to the others.

Plating density

Cell separation, once complete, produces a subset of cells containing the MSCs which is then transferred to a culture plate, and media is added. The MSCs adhere to the plastic substrate of the plate by transmembrane proteins (Horn *et al.* 2011). Other cells do not adhere to the plastic and are removed during the media changing process. Cell separation and quantification allows for placement of the cells an optimal distance from one another (also known as cell density) when plated and prevents the consumption of nutrients in the media by non-MSCs. As the media and additives are often the most expensive part of the culturing process, providing nutrients for only the sought-after cells is ideal. Additionally, cells must

be plated at a specific density in order to allow the cells to proliferate. Cells plated too densely can slow or stop the process of cell division by contact inhibition or alter the phenotype of the cell (Horn *et al.* 2011). Cells plated at an insufficient density do not have the cell-cell signaling required for cell proliferation and differentiation (Horn *et al.* 2011).

Cell media

Once the cells are transported and separated, they must be cultured in an appropriate environment. This includes an appropriate incubation temperature, atmospheric gas concentration, humidity and nutritive media (Freshney 2010). Media provides calories for energy consumption, a buffered environment for optimal metabolic activity, growth factors to enhance mitosis, and amino acids for protein synthesis (Verma and Singh 2013). Most recent common equine MSC media protocols use Dulbecco's Modified Eagle Medium (DMEM) or alpha minimum essential medium (aMEM) (Bourzac *et al.* 2010, Kisiday *et al.* 2013). It has been shown that aMEM improves the population doubling rate as compared to DMEM in human MSCs (Chen *et al*. 2015). These media contain 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), or HEPES must be added separately in order to buffer the solution. Glutamine is added to provide a nitrogen source and an energy source. Glutamine decomposes more quickly when mixed with fetal bovine serum (FBS) or at an elevated pH, so a higher concentration may be needed when under these conditions (Lin and Agrawal 1988). Penicillin and streptomycin (1000U/ml with or without an antimycotic) may also be added depending on the likelihood of contamination. This use of antibiotics

is beneficial if minor infection exists in a culture, but its use may promote drug resistant bacteria and can affect the MSC's ability to differentiate (Llobet *et al*. 2015).

FBS is often added to provide a milieu of growth factors. Equine adipose derived stromal cells have a significantly shorter population doubling time with serum-added media as compared to serum-free media (Schwarz *et al*. 2012). A recent study using human MSCs showed that human adult serum provided improved colony formation and sped mitosis in the human cells as compared to those cultured with FBS (Turnovcova *et al.* 2009). Joswig *et al.* (2017) showed that the use of FBS in MSC media caused a greater immune reaction when MSCs were injected intra-articularly as compared to cells grown for 48 hours in autologous serum. The regime in this study seems the ideal compromise for a busy laboratory setting where storing and using large batches of autologous serum is less than ideal. It combines the ease of using FBS for a majority of the culturing process and then finishing the cells in autologous serum.

Additional growth factors can be added in order to maintain undifferentiated MSCs or induce MSCs to differentiate towards an intended cell type. Basic fibroblast growth factor (bFGF) (10ng/ml) is commonly used as an additive to MSC media and causes a dramatic increase in cell mitosis as compared to cells cultured without it (Auletta *et al.* 2011). This growth factor also causes MSCs to maintain their pluripotency by stimulating mitogen-activated protein kinase via FGF receptors (Onuma *et al.* 2015). There is an autocrine FGF signaling pathway in undifferentiated MSCs, inhibition of which quickly leads to differentiation (Dvorak

et al. 2005). Basic FGF in culture medium is rapidly lost due to its vulnerability to heat and proteases (Onuma *et al.* 2015). Therefore, bFGF should be added to the media just prior to its use, and media changes should be performed every 3-4 days in order to maintain FGF levels (Auletta *et al.* 2011). Although comparable studies have not been published on equine cells, it is likely that FGF in equine MSC cultures acts similarly.

Fibroblast growth factor supplementation can cause variability in cell markers on the surface of MSCs (Hagmann *et al.* 2013). It has been shown that the presence of CD90, a MSC marker, is significantly decreased when using DMEM and FGF as compared with aMEM with or without FGF. The presence of CD44, a cell surface glycoprotein associate with cell-cell interaction, and adhesion and migration is unchanged with the use of FGF in both DMEM and aMEM (Hagmann *et al.* 2013).

Incubation temperature

An additional environmental factor that causes great variability in cell proliferation and protein production is the temperature at which the cells are incubated (Ito *et al*. 2014, Natale and McCullough 1998). Based upon the available evidence, the current recommendation is to maintain cells at the body temperature of the donor species during incubation (Natale and McCullough 1998). High temperatures (41^0C) significantly decrease cell expression and synthesis of the extracellular matrix components including proteoglycan, collagen type 2, and glycosaminoglycan in human dedifferentiated chondrocytes as compared to human body temperature of 37^0C (Ito *et al*. 2014). Lower temperatures (32^0C)

caused a significant decrease in extracellular matrix expression compared to body temperature, but not to the degree of compromise caused by high temperatures (Ito *et al*. 2014).

Cryopreservation

Freezing allows for the banking of cells and coordination of culturing and testing of multiple samples (Chatzistamatiou *et al.* 2014). Freezing cells simplifies studies by allowing all the cells to be cultured and tested by batches rather than individually. MSCs can be stored at 4°C for up to three days in a buffered trehalose solution or commercially available solutions (Petrenko *et al.* 2019). Beyond this three day period, cells lose viability quickly when stored under nonproliferative conditions. For this reason, cells are cryopreserved for storage. A specifically selected medium is used for cell freezing. Cryopreservation media is used to prevent cell death until the time comes for their use (Garvican *et al.* 2014). Dimethyl sulfoxide (DMSO) or glycerol are the cryoprotectants that are most commonly used as freeze media with DMSO at 5-10% being the most frequently used due to its ability to penetrate the cell wall (Freshney 2010). These agents prevent crystallization within the cell which would lead to cell rupture (Carpenter and Hansen 1992).

The freezing of MSCs does not appear to greatly alter their immunophenotype after they have been thawed, or thawed and re-cultured (Chatzistamatiou *et al.* 2014, del Pino *et al*. 2015). Human MSCs harvested from umbilical cord blood have little change in marker expression at passage 1 or 2 as compared to passage after freezing at -196^{0}C for 1 week to 6 months and re-

culturing (Chatzistamatiou *et al.* 2014). There are no consistent significant differences in cell marker expression prior to freezing as compared to after being frozen when measuring the expression of CD90, CD44, and nine other cell markers (Chatzistamatiou *et al.* 2014). This has been verified by a study that showed a similar lack of significant marker expression change when human MSCs are frozen at -196^{0}C and re-cultured for 7 days (del Pino *et al*. 2015).

Questions remain as to whether all of the cell surface markers remain stable after freezing. Although previous studies have shown no consistent change in expression, the introduction of variation caused by freezing may need to be evaluated on an individual cell marker basis (Chatzistamatiou *et al.* 2014, del Pino *et al*. 2015). A marker-specific evaluation of fresh versus frozen MSCs can be performed to increase confidence in the results of MSC analysis. The expression of markers not previously assessed on frozen cells should be compared to their expression without freezing. This can be done with flow cytometry by measuring the marker expression for those cells that have been frozen as compared to cells that are fresh. When doing such a comparison, fresh and frozen cells should be at the same passage number and should be from the same sample as there can be sample to sample variation (Schnabel *et al.* 2014). This will determine if there is an effect on the markers due to the freezing process.

Conclusion

A large number of the variables in mesenchymal stromal cell culture have been studied. Optimal techniques have been identified in order to improve stromal cell colony formation and increase cell mitosis rates while maintaining MSC purity

and pluripotency. A review of the recent literature shows that some fundamental guidelines should be followed:

1. Storage of the cells at 4^0C after harvest and prior to culture appears to be the optimal method of transport and storage. Cell viability decreases with storage time greater than 72 hours (Martin and Rowley 1986).
2. Separation with erythrocyte lysis seems to allow for the largest amount of MSCs recovered with culturing, but Percoll density gradient removes more of the unwanted cells prior to plating (Horn *et al.* 2008, Bourzac *et al.* 2010).
3. MSCs should be cultured at 37^0C in order to promote optimal cell growth (Ito *et al.* 2015).
4. Media should contain FGF in order to maintain self-renewal (Onuma *et al.* 2015). FGF causes inconsistent changes in some markers when used with DMEM or aMEM as compared to media without FGF (Hagmann *et al.* 2013). Therefore, FGF should be supplemented as consistently as possible through the culturing process if variation in cell marker expression is to be assessed.
5. Freezing cells after a period of culture causes no consistent variation in MSC marker expression, though occasional variation exists and may need to be examined on a case-by-case basis (Chatzistamatiou *et al.* 2014).

1.4.3 Flow cytometry instrumentation and methods

Prelude

After culture methodologies were validated, flow cytometry protocols for equine MSCs needed to be developed. Flow cytometry in horses was much in its development stage when we began to undertake our assays. Advances in fluorochrome technology and machinery occurred while we were performing our preliminary testing, and we were able to encorporate some of the most advanced methods in flow cytometry to our assays.

One of the most significant advances in flow cytometry was the use of a large number of fluorochromes in a single assay. This would potentially allow us to identify leukocyte population flux by characterizing many different types of cells in the same sample. This type of an assay would lead to a better understanding of the reciprocal effect of our MSCs on leukocytes. The knowledge gained from the flow cytometry assay is central to our hypothesis that the presence of MHC II on the surface of allogeneic MSCs is influential on the immune response.

Introduction

Flow cytometry combines the study of the cell with fluid dynamics and photoexcitation to provide a “picture” of the cell populations in a fluid sample. This modality uses optical analysis for the identification of cells by its internal characteristics and the molecules contained on its surface (Shapiro 2003).

The first flow cytometer dates back to the 1950s and was composed of a microscope and a separate light source (Macey 2007). Cytometers became more

complicated and automated during the following decades to allow for a suspended cell sample to be passed in front of a light source (Macey 2007). This caused the differential illumination of specific cell types. Later, automation of the process allowed the illumination data to be quantified by a computer (Givan 2001).

Flow cytometry holds great promise for quickly identifying cell types, and its use in medicine is growing (Aebisher *et al.* 2017). Haematology relies on cytometers to quantify and categorize cells. This allows for the diagnosis of infection, inflammation, anemia and plethora of other systemic diseases. The field of oncology is increasingly using cytometers to isolate and quantify neoplastic cells to determine if there is improvement in the patient from one time point to another (Givan 2001).

Current use of flow cytometry includes not only the identification of cells, but also the identification of different types of bacteria, viruses and intracellular events (Lloyd 1993, Carson *et al.* 1999, Marie *et al.* 1999). Minor alterations to the cell assay can allow flow cytometry to be useful in a variety of fields, although these assays are less frequently performed than cell identification.

The mechanics of flow cytometry

Flow cytometers are used in diagnostic and research laboratories to identify the components of a liquefied sample. The primary components include a cell suspension which is streamed through a series of lasers. Each cytometer can carry up to ten different lasers (Telford 2011). Lasers come in a variety of wavelengths from ultraviolet to the near infrared (Telford *et al*. 2017). Lasers are able to create

fluorescence when exciting fluorochromes that absorb the light and then release it as light (also called photons) of a longer wavelength (McCarthy 2007). The corresponding emitted light is received by a lens and photodiode filter. The lens and photodiode filter increase the intensity of the light so that the data can be interpreted by a photodetector. The photodetector then sends the signal to a computer which compiles the events read by the photodetector.

Forward and side scatter light

Flow cytometers are used primarily to identify cells by their size, granularity, and cell membrane proteins (Givan 2001). The cytometer measures the granularity and size of a cell by measuring the amount of scattered light produced by the laser as it passes through the stream of cells (Givan 2001). Cells with greater granularity scatter light at greater angles (15-150°) and compared with cells with round, consolidated nuclei (Shapiro 2003). Granularity is determined by a lens set at 90 degrees to the laser. This is called “side scatter light” or SSC.

Cells with a greater size scatter the light more than cells that are smaller. The light scattered by larger cells is only scattered 0.5-5° (Shapiro 2003), The size of the cell is determined by a lens set in the forward direction of the laser. This is called “forward scatter light” or FSC. By understanding the granularity and size of the cell, the researcher can identify the cell as a specific cell type.

Antigens, antibodies, and fluorochromes

Cell membrane proteins are called markers or antigens and are involved in cell signaling or attachment (Givan 2001). These surface proteins are specific to

each type of cell as they determine the function, lineage and developmental stage of the cell (Overton 2005). Identification of cells is particularly useful in the field of haematology as millions of cells at a time must be rapidly differentiated from one another.

In flow cytometry, antibodies that recognize specific cell surface markers are used to identify the cell type (Boenisch and Hudson 2005). Antibodies are mixed with the cells causing binding between the antibody and the cell antigen by ionic interactions, hydrogen bonding and van der Waals forces (Wulff 2005). This is a similar mechanism to immune system antibodies that bind to bacteria and foreign material.

Once the appropriate antibody clone that will bind to a specific protein is determined, the method of fluorescence must then be considered. Antibodies are added to the cell sample using one of two general immunofluorescence techniques in flow cytometry, called direct and indirect immunofluorescence (Serke *et al.* 1998). Direct immunofluorescence is a flow cytometry technique that uses an antibody that is linked to a fluorescing molecule called a fluorochrome. Indirect immunofluorescence requires a secondary anti-immunoglobulin antibody which can identify the primary antibody. The secondary antibody is linked to a fluorochrome.

After the appropriate protocol has been completed that would allow for fluorochrome-linked antibodies to bind to cells, the liquefied cell sample is placed in a flow cytometer. The cytometer discharges the sample allowing it to pass through a series of lasers (Shapiro 2003). The interaction of the laser with the

fluorochrome causes the fluorochrome to emit photons (fluorescence) (McCarthy 2007). This fluorescence is then received by the photodetector. With current technology, up to 12 fluorochrome-linked antibodies can be used in a single experiment order to identify multiple surface markers on a population of cells (Autissier *et al*.2010).

Antibody titration

To obtain accurate data from the flow cytometer, the amount of antibody added to a quantity of cells must be appropriate. Too high of a concentration of antibodies will encourage nonspecific binding which will give a falsely elevated reading (McCarthy 2007, McClellan 2014). Too low of a concentration will not allow all of the antigens to be identified, thus falsely decreasing your reading (McCarthy 2007, McClellan 2014). As antibodies are an expensive component of flow cytometry, it is also important to find the lowest optimal concentration to make the assays economically feasible.

The optimal antibody concentration is 2-5 times the number of antibody molecules necessary to achieve saturation of the antigen sites (McCarthy 2007). Often the quantity of antigen sites is unknown, so determining the optimal concentration is best done using a titration curve. To do this, a flow cytometry assay is prepared using 1:2 dilutions of the antibody (Collino *et al.* 2007). The mean fluorescence intensity is compared between the positive staining cells and the unstained cells at each antibody dilution. The antibody concentration for which there is the greatest difference between the mean fluorescence intensities of the unstained cells and the stained cells is the optimal antibody concentration

(McCarthy 2007). There are other mathematical methods of determining the optimal antibody concentration, but most adhere to this basic principle (Collino *et al.* 2007).

Fluorochrome selection

The set of fluorochromes used in an assay depends on a few important principles. First, fluorochromes only fluoresce in the presence of lasers of a specific color or wavelength (Overton 2005). Only lasers of the color absorbed by the fluorochrome will excite the fluorochrome. This excitation causes an electron in the fluorochrome to jump to a higher energy state, absorbing the laser's energy and releasing heat. Then the electron falls back down to its original energy state and releases the rest of the energy absorbed from the laser as light energy, called photons.

Fluorochromes have different emission intensities, or ability to fluoresce when energized by a laser. The intensity of fluorescence depends on the magnitude of light absorbed by the fluorochrome and the ratio of light absorbed to light emitted, called quantum yield (McCarthy 2007). Ideally, fluorochromes with high intensity should be used for antigens that are rare so that they may become 'visible' to the photodetector. Meanwhile common antigens should be labeled with a less bright fluorochrome so that their fluorescence will not be so intense that their fluorescence prevents the photodetector from detecting other fluorochromes.

When creating a flow cytometry assay, fluorochromes must be chosen which will be compatible with one another. Fluorochromes emit their photons at a

short spectrum of wavelengths with the most intense fluorescence existing at a specific wavelength, called the wavelength of peak emission (Figure 2). In order for multiple fluorochromes to be used to quantify multiple antigens in a single sample, fluorochromes must be used which have maximum emission wavelengths that are sufficiently different from one another (Baumgarth *et al*. 2000). The spectrums of fluorescence for different fluorochromes can overlap, and this is called spectral overlap or spillover. Spillover will be further discussed in future sections. The wavelength of peak emission must be sufficiently different from the other fluorochromes in order to determine the correct origin of the photons emitted.

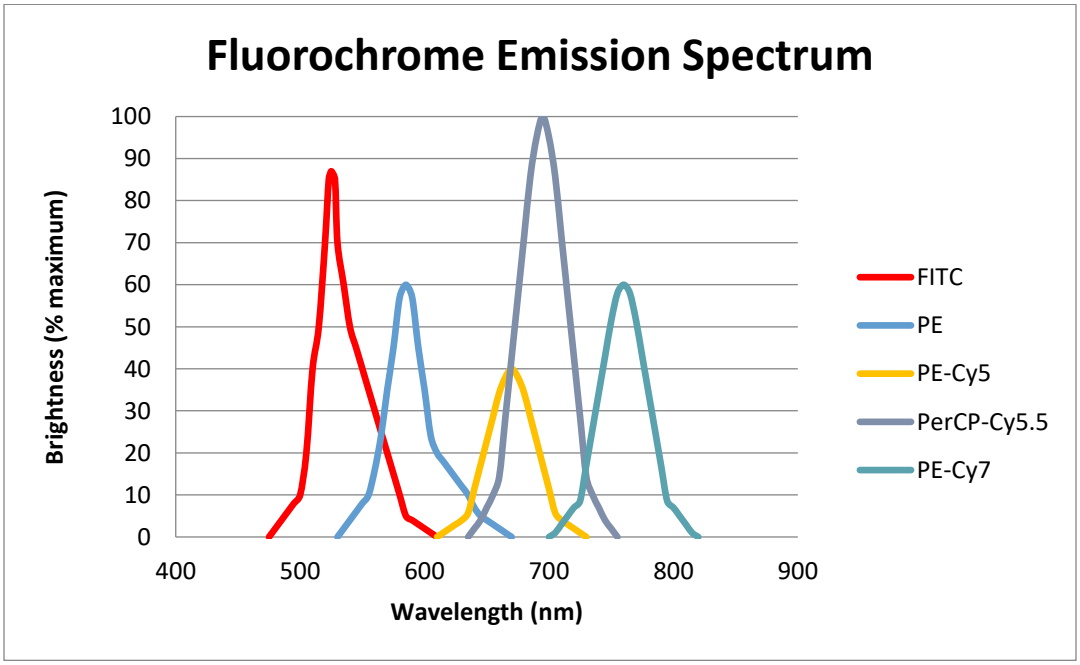

Figure 2. Spectrum of fluorochrome emission.
The spectrum of fluorescence for different fluorochromes can overlap with adjacent fluorochromes.

Flow cytometry data analysis

Once the data have been collected by the photodetector, it must be analysed to determine if there is fluorescence produced by each labelled cell. These data are then correlated to the cell size and granularity. The flow cytometry software is used to create plots of the data with the cells being plotted according to their fluorescence or scatter (SSC and FSC). Dot plots are created that show the amount of fluorescence for multiple fluorochromes, or histograms can be made to show the relative number of cells fluorescing at different wavelengths.

Gating

Once the data is plotted, regions of the graph containing cells of interest can be selected (Givan 2001). This is called ‘gating.’ Using software analysis of the data, a specific region of cells can be selected. This selection can be based on the antibody binding of the cell, the size of the cell, and the granularity of the cell. Gating is performed to examine a specific subset of cells. Nonviable cells can be removed from the examined population in this way (McClellan 2014). Gating allows the software to quantify the proportion of cells that is included in the gate so that the number of cells as a percent of the total cell population can be known. The gated cells can be examined independently of the cell population. Once the cells have been gated, they can be plotted to determine if they express other antigens.

An example of gating can be seen in Figure 3, indicated by the area called ‘MHC-II Positive.’ This group of circled cells express the cell surface marker MHC class II, as these cells bind the MHC class II antibody which is linked to the fluorochrome fluorescein isothiocyanate (FITC.)

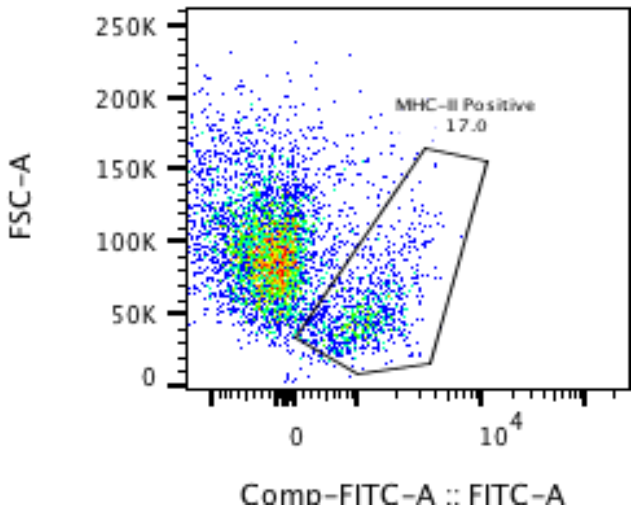

Figure 3. Cell population distributed by FSC (cell size) and FITC fluorescence.

Cells to the right are gated and labeled as positive for the antigen to which the FITC-linked antibody binds (MHC-II).

Nonspecific binding

One routine concern in flow cytometry is nonspecific binding of antibodies to Fc-receptors on leukocytes. Fc receptors (FcR) bind to IgG-covered targets including pathogens immune complexes (Junker *et al.* 2020). Fc receptors include CD16, CD32, and CD64, among others, which are expressed on all leukocytes except for T lymphocytes (Anderson *et al.* 2016). In order to prevent antibodies from binding nonspecifically, a few different reagents are routinely used. These include a commercial Fc-blocking reagent, serum, or a high concentration of purified IgG (Junker *et al.* 2020). It is still controversial as to whether the blocking

agent needs to be of the same species as the cells being assayed (Stewart et al. 2001, Junker *et al.* 2020). Without these blocking agents, false positive events commonly occur (Junker *et al.* 2020).

Autofluorescence

Autofluorescence in flow cytometry is the condition where the cells emit detectable fluorescence, independently from the antibody-linked the fluorochromes. Autofluorescence is due to the normal expression of highly fluorescent biomolecules such as NADH, FAD, riboflavin, flavin coenzymes and lipofuscins (Aubin 1979). Autofluorescence can also be attributed to metabolically active mitochondria (Levitt *et al.* 2006).

Autofluorescence causes an error in data analyses as it may create the appearance of the presence of a cell marker when there is no marker present on the cell. The spontaneous fluorescence emitted by the cells may emit at the same wavelengths as the fluorochromes being used in the assay. Highly autofluorescent events can be seen on a dot plot as diagonal streaks across graphs when plotting the fluorochrome channels in which the autofluorescence is emitted (Donnenberg and Donnenberg 2015). Compensation must be used to account for this autofluorescence and will be discussed in a future section.

Spillover

Complications arise when multiple cell markers are analyzed simultaneously. This allows for multiple fluorochromes to fluoresce and be detected on the same cell. The flow cytometer measures how much fluorescence

is created by each cell at each wavelength. The amount of fluorescence detected at a specific wavelength is equal to a specific fluorochrome's emission intensity plus the intensity of all other fluorochromes' spectra where they overlap at that wavelength (Roederer 2002). This overlap of the fluorochromes' emission is called spillover. Spillover occurs because the fluorochromes do not emit at exactly one wavelength and their fluorescence can be detected by the photodetector when measuring nearby wavelengths.

Consider for example Figure 2. To determine the amount of fluorescence of the fluorochrome PerCP-Cy5.5, one would examine the amount of signal recorded by the flow cytometer at its peak emission wavelength, 690 nm. As the PE-Cy5 fluorochrome also has some emission at this wavelength, some of the signal comes from this fluorochrome while the rest of the signal comes from the fluorochrome of interest, PerCP-Cy5.5. The signal that comes from the PE-Cy5 fluorochrome at the 690 nm wavelength is spillover, and must be accounted for by compensation of data.

Compensation of data

Compensation is the process by which the spillover between different fluorochromes is mathematically eliminated so that only the fluorescence from the fluorochrome of interest is quantified (Baumgarth and Roederer 2000). The most basic way to compute compensation is by running a series of samples through the flow cytometer, each containing cells stained with only one fluorochrome. This illustrates the amount of fluorescence derived from each fluorochrome identified in portions of the electromagnetic spectrum. The region of the spectrum where the

fluorochrome emits the greatest fluorescence intensity is called the ‘channel.’ By staining a sample with only one fluorochrome, the contribution of each fluorochrome to each channel can be identified (Roederer 2002). The total signal to remove from each channel can be computed by solving a set of equations based on this data to produce a spillover matrix (Bagwell 1993). When the spillover equations are used with the raw data from the cytometer, compensated data is produced (Bagwell 1993).

This method of compensation using single-stained samples is now considered to be less rigorous than what is needed to create an accurate compensation matrix (Sugar *et al.* 2011). A more sophisticated means of computing compensation is performed with samples containing all fluorochromes to be used in an assay except for one fluorochrome. This method shows the amount of spillover into the excluded fluorochrome’s wavelength. This amount can then be subtracted from the raw data gathered when all fluorochromes are included to compensate for the spillover effect (Sugar *et al.* 2011). This will also include the compensation for autofluorescence as long as the samples used for compensation contain the cells of interest.

Artifacts due to nonviable cells

A common cause of artifact creation in flow cytometry is nonspecific binding of antibodies. This can be caused by the presence of dead cells or debris in the sample as these will bind antibody without the presence of the antigenic marker to which the antibody binds (Johansson 2007, McClellan 2014). Debris is usually small in size, so it can be eliminated by gating in only larger structures using the

results from the forward scatter. To eliminate the dead cell population, a viability stain should be used. Viability stains will cause fluorescence of dead cells so that they can be excluded from further analysis (McClellan 2014).

Conclusion

Flow cytometry is an important tool to identify cells according to their phenotype. If the sample contains a variety of cells, the cells are differentiated by their size and granularity in addition to those markers the cells express. The antibodies and fluorochromes chosen to evaluate cells must bind efficiently and be detectable to the cytometer so that the individual fluorescence can be attributed to the correct antibody/ fluorochrome pair.

Flow cytometry is also being used to learn more about the phenotype of specific cells including better identification and understanding of the highly-studied mesenchymal stromal cell (Radcliffe *et al.* 2010, Maia *et al.* 2013, Boxall and Jones 2015). This final application is the one that we will in our studies of mesenchymal stromal cells and how they may vary from one breed to another and from one time point to the next.

1.4.4 Equine erythrocyte typing

Through our investigations, we found universal blood donor horses have MSCs that are MHC class II - negative. Hematopoietic stem cells and MSCs have common lineage at the embryonic level, though literature has shown that their relationship may continue to adulthood (Ogawa *et al.* 2010). Although this genetic link is weak, we sought to determine if the universal blood donor horses may have reduced immunogenicity as compared to horses of non-universal blood donor types.

Horses have a large number of different erythrocyte antigens with approximately 400,000 different blood types (Tomlinson *et al*. 2015). These are grouped into 7 antigen systems recognized by the International Society for Animal Genetics: A, C, D, K, P, Q, and U (Proverbio *et al*. 2020). Antibodies against Ca are most commonly found in the horse population, while antibodies against Aa and Qa cause the most transfusion-related complications (Tomlinson *et al*. 2015, Casenave *et al*. 2019). For these reasons, erythrocyte antigen testing for Aa, Ca, and Qa is performed to determine whether a horse may be a universal blood donor (Proverbio *et al*. 2020). Those horses whose erythrocytes express none of these antigens are considered a universal blood donor.

1.4.5 Bone marrow derived- mesenchymal stromal cell surface inclusion and exclusion markers

Prelude

Identification and phenotypic characterization of equine mesenchymal stromal cells is important to assure that the sample used for treatment is a well-defined population of MSCs. This is critical to our hypothesis as we require a pure population of MSCs to determine the degree of immune reaction in an allogeneic model. To include MSCs and exclude other cell types, a few important cell markers need to be present. Below is an outline of common equine bone marrow-derived MSC (BM-MSC) inclusion and exclusion markers for horses. As much of the information on these markers has been extrapolated from human or lab animal studies, studies from several species will be discussed. This review is not intended to be exhaustive, but will focus only on those markers most likely to be of value to the current research programme.

CD11a/18

CD11/18 is a cell adhesion molecule that is found on all erythroid and myeloid cells (Mazzone and Ricevuti 1995). The individual components, CD11a, CD11b or CD11c, and CD18, together form a glycoprotein complex (Thompson and Matsushima 1992). The CD11/18 complex provides the receptor for the lymphocytes, monocytes, neutrophils and other leukocytes to adhere to endothelial cells for extravascular migration or for adhesion of leukocytes to their target cell (Mazzone and Ricevuti 1995). More specifically, the CD11a/18 complex

appears to allow for adhesion under non-inflammatory conditions while the CD11b/18 complex allows adhesion under inflammatory conditions (Thompson and Matsushima 1992). The CD11b/18 complex, for example, allows neutrophils to migrate to a location of inflammation.

CD11a/18 has been studied in equine tissues and is found on equine leukocytes (Radcliffe *et al*. 2013). CD11a/18 is not found on BM-MSCs and therefore can be used to differentiate MSCs from erythroid and myeloid cells in bone marrow (Radcliffe *et al*. 2013).

CD44

CD44 is a glycoprotein which primarily binds hyaluronic acid in diseased tissues or the endosteum which lines the medullary cavity (Herrera *et al.* 2007, Zoller 2015, Thapa and Wilson 2016). The CD44/ hyaluronic acid complex triggers a milieu of cellular processes including cell proliferation. For this reason, CD44 is commonly seen on cancer cells of epithelial origin as neoplastic cells have this enhanced proliferative ability (Thapa and Wilson 2016).

There is some dispute as to whether human MSCs express CD44 *in vivo*. Several papers have reported that early passage human MSCs express CD44 (Herrera *et al.* 2007, Quian *et al*. 2012, Wystrychowski *et al*. 20 16). In contrast, one paper reported that the MSC population found in the bone marrow does not express CD44, and it is only when the cells are plated that the expression of this molecule is initiated (Quian *et al*. 2012). This study found that no fresh human bone marrow-derived MSCs expressed CD44 while 98% of first passage MSCs

expressed the marker (Quian *et al*. 2012). Another study showed that MSCs use CD44 to diseased areas of the kidney during acute renal failure (Herrera *at al.* 2007). It is possible that MSCs acquire CD44 expression as a part of their maturation process.

Several institutes have performed work to identify equine bone marrow-derived MSCs by their cell surface markers (Maia *et al.* 2011, de Schauwer *et al.* 2012, Radcliffe *et al.* 2013, Peabst *et al.* 2015). The literature appears consistent in that equine BM-MSCs are CD44 positive during all stages of growth *in vitro* (Ranera *et al.* 2011, Radcliffe *et al.* 2013, Peabst *et al.* 2015). Freshly isolated equine bone marrow-derived MSCs showed variable CD44 expression (Radcliffe *et al.* 2013).

CD59

CD59 (protectin) is a small protein that is anchored to the cell membrane. It has been well defined as the sole membrane complement regulatory protein that inhibits cell death by restricting complement formation in humans (Du *et al.* 2014). Complement formation is an important immune system mechanism of cell destruction (Soland *et al.* 2013). Human CD59 is widely expressed on all circulating cells and in almost all tissues, except for the central nervous system (Meri *et al.* 1991). Therefore, CD59 plays a crucial role in protecting autologous cells from destruction.

Investigations into CD59 expressed on equine cells not been previously performed. Human BM-MSCs are positive for CD59 (Moll *et al.* 2011). It has been

shown that the CD59 expression in human MSCs protects them to a degree from complement-mediated cell death (Li and Lin 2012). In the face of activation of the complement system, the level of CD59 expression is overwhelmed and the MSCs can be damaged or killed (Li and Lin 2012).

CD90

CD90 is a heavily glycosylated cell surface protein. It is usually expressed on human MSCs, natural killer cells, neurons, endothelial cells, and fibroblasts (Kumar *et al.* 2016). CD90 plays a role in cell adhesion, cell migration, apoptosis, cell to cell interactions and cell to matrix interactions (Kumar *et al.* 2016).

The reports of CD90 expression on equine BM-MSCs are conflicting (Radcliffe *et al.* 2013, Peabst *et al.* 2015, Ranera *et al.* 2011). Many reports have described BM-MSCs as CD 90 positive (Ranera *et al.* 2011, de Schauwer *et al.* 2012). Radcliffe *et al.* (2013) reported an increase in CD90 expression over time. Peabst *et al.* (2014) states that BM-MSCs are heterogenous in their CD90 expression with high individual horse variability. These researchers also found that the mechanism of lifting the cells from the plate caused variation in CD90 expression results (Peabst *et al.* 2015). When cells were mechanically lifted from the plate, the MSCs were found have higher CD90 expression as compared to cells that were enzymatically lifted using trypsin or Accutase (Peabst *et al*. 2015). It is likely that enzymatic cell lifting may cleave the CD90 marker from the cell surface and cause the variation observed in these studies.

Human BM-MSCs are reportedly CD90 positive (Weissman *et al.* 2006, Lee *et al.* 2014). Mechanical stimulation, though, can decrease the expression of CD90 on MSCs (Weissman *et al.* 2006). A study in rats showed that CD90 positive BM-MSCs had a decreased ability for multilineage differentiation as compared to heterogenous expression of CD90 (Davies *et al*. 2015). As the ability to differentiate into a variety of tissues is one of the key characteristics of being a MSC, it is possible that CD90 expression is not a true phenotype of the most proliferative MSCs.

MHC class I

The expression of MHC I motifs on MSCs is important in that it along with MHC class II are the key cell markers utilized for alloimmunity by the host's immune system, and expression of these markers identifies the MSCs as targets for destruction. MHC I is expressed on most cells of the body and on all equine bone marrow-derived MSCs though the degree of expression varies (Berglund *et al.* 2017). MHC I expression are increased in the face of culture with foreign lymphocytes, when MSCs are cultured with inflammatory cytokines, or as the MSCs differentiate (Cassano *et al.* 2018, Hill *et al.* 2017, Barrachina *et al.* 2018, Barrachina *et al.* 2020). Further discussion of MHC class I and II is discussed in section 1.3.5.

MHC class II

One of the key cell markers utilised for antigen recognition by the host's immune system is the major histocompatibility class II molecule (MHC class II). Cells showing this marker are known to increase T cell responses *in vitro*

(Schnabel *et al.* 2014). Schnabel *et al.* (2014) found that there is a direct correlation between the amount of MHC class II expression and the T lymphocyte immune response. Later studies have shown that even MSCs with low MHC class II expression could incite an adaptive immune response (Pezzanite *et al.* 2015, Joswig *et al*. 2017). When MSCs are used for treatment of disease in the horse, the cells must ideally not cause increased inflammation. For this reason, it is imperative to understand the expression of MHC class II in equine MSCs and then to determine if this expression is indeed significant.

The information is conflicting as to the expression of MHC class II in horses. Some published reports have found that equine BM-MSCs are MHC class II negative (Barberini *et al*. 2014, Paebst *et al.* 2014). The MSCs for these horses were only tested at one time point in these studies. Another study tested MHC class II expression as cells were passaged over time (Schnabel *et al.* 2014). These researchers found that horses were positive in their expression of MHC class II with 80% of the horses sampled having BM-MSCs with moderate to high expression (>30% of cells expressing the marker) at low passages and 60% of horses having moderate to high expression at later passages (Schnabel *et al.* 2014). Furthermore, two studies have found that some MHC class II negative cells stimulated with IFNγ upregulated the production of the MHC class II marker (Schnabel *et al.* 2014, Hill *et al.* 2017). The addition of TGF-β2 *in vitro* decreases the MHC II expression on IFNγ stimulated MSCs (Berglind *et al.* 2017). Equine MSCs appear to be very capable of expressing MHC class II and are not the universally immune privileged cells they perhaps were once considered to be.

Human BM-MSCs are negative for MHC class II (Lee *et al.* 2014, Huang *et al*. 2016). In saying this, human BM-MSCs can express MHC class II when they are stimulated to differentiate down a specific cell lineage (Huang *et al*. 2016). Is it possible that the equine BM-MSCs express MHC class II in a similar manner, and those cells seen to express the marker in horses aren't really mesenchymal stromal cells but instead are early differentiated cells? Or are equine MSCs indeed different from human MSCs in their MHC class II expression? We aim to answer these questions through our research into MSC surface markers.

Conclusion

Bone marrow-derived equine mesenchymal stromal cells are largely positive for CD44 and CD90. CD59 has not been studied in equine MSCs, but its expression on human MSCs is immunoprotective. Equine BM-MSCs are positive for MHC class I and vary in their MHC class II expression. The expression of MHC class II on equine BM-MSCs is still contentious and needs to be better evaluated. The expression of both MHC class I and II on the surface of BM-MSCs makes immunorecognition a potential complication of allogeneic administration.

1.4.6 Interactions between allogeneic mesenchymal stromal cells and the recipient immune system: A comparative review with relevance to equine outcomes.

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Prelude

Once methodologies were validated, published immunologic studies using allogeneic MSCs needed to be evaluated so that our base of knowledge in the topic was as current as possible. Admittedly, much new information and data was published over the course of our study. Our research was consistently updated to be as current as possible.

The groundwork laid by the following set of literature was crucial to the direction of our project. Early work on equine allogeneic MSCs and their potential for use as a therapy focused on the presence of a clinical immune reaction after injection of the cells. Mixed lymphocyte reactions or other types of assays causing activation of lymphocytes followed by the addition of MSCs to culture were then used to determine the immunosuppressive ability of allogeneic MSCs. More recently, serologic testing for the presence of antibodies after allogeneic MSC administration has been performed. Haplotyping the gene encoding the MHC molecules has become a popular method to determine the potential for an immune response *in vivo*. The performance of the equine allogeneic MSCs in the published literature guided both the MSC types we decided to utilize for our assays and the types of assays we performed.

Abstract

Despite significant immunosuppressive activity, allogeneic mesenchymal stromal cells (MSCs) carry an inherent risk of immune rejection when transferred into a recipient. In naïve recipients, this immune response is initially driven by the innate immune system, an immediate reaction to the foreign cells, and later, the adaptive immune system, a delayed response that causes cell death due to recognition of specific alloantigens by host cells and antibodies. This review describes the actions of MSCs to both suppress and activate the different arms of the immune system. We then review the survival and effectiveness of the currently used allogeneic MSC treatments.

Introduction

Bone marrow-derived mesenchymal stromal cells (MSCs) possess immense potential for the treatment of many diseases (Squaillaro *et al.* 2016, Chahal *et al. 2019*), and there has been rapid acceleration in the clinical use of MSCs (Chahal *et al.* 2019). Bone marrow-derived MSCs have become the “gold standard” MSC for use in musculoskeletal therapies (Frisbie *et al.* 2010, Wilson *et al.* 2019) though adipose-derived and umbilical cord-derived MSC products are also commonly available (Wilson *et al.* 2019). The use of allogeneic MSCs for treatment is less costly as it can be prepared for multiple animals and is immediately available for treatment (Peeters *et al.* 2013, Huang *et al.* 2016). An additional benefit is for older patients whose MSCs are known to have lower proliferation rates as compared to MSCs from younger donors (Brohlin *et al.* 2012).

Allogeneic MSCs as an off the shelf product will likely be the main mode of MSC treatment in the future.

When treating an individual, be it human or equine, with allogeneic stromal cell therapy, prevention of allorecognition of the recipient to the transplanted foreign antigens is an important component of achieving a persistent and potent effect. Medium- or long-term survival of the MSCs to exert their desired anabolic effects is likely to promote their effectiveness as a treatment as compared responses associated with short-term survival. Certainly, short-lived therapy with MSCs can be the catalyst for improvement of disease processes, as several studies have reported that the number of implanted MSCs detected in target tissue was too low to explain the improvement in disease state (Squillaro *et al.* 2016). However, without survival of the MSCs, there would be no source of ongoing therapeutic effect nor involvement of the MSC in the structural integrity of repair. Another concern regarding allorecognition is the described side effects of intra-articular allogeneic MSC injection in people and horses (Ardanaz *et al.* 2016). These include pain, swelling of the joint, and urticaria (Peeters *et al.* 2013, Ardanaz *et al.* 2016). For these reasons, a complete understanding of the interaction of the MSCs with the immune system is necessary to foresee the risks and predict the effectiveness of allogeneic MSCs as a treatment.

Many studies have found that bone-marrow-derived MSCs are capable of substantial anti-inflammatory effects (Semani *et al.* 2008, Ge *et al.* 2010, Remacha *et al.* 2015, Ranera *et al.* 2016, Colbath *et al.* 2017) The immunomodulation caused by MSCs is dependent on inhibitory molecule secretion, direct cell contact

and induction of regulatory leukocyte populations (Consentius *et al.* 2015, Moravej *et al.* 2016, Girdlestone *et al.* 2016, Khosaravi *et al.* 2017). Over 350 human studies are currently underway that investigate the ability of MSCs to limit immune reactions related to auto-immunity and tissue transplantation (clinicaltrials.gov). Previous studies have shown that allogeneic MSCs suppress immune reactions in graft-versus-host disease and organ transplantation even when steroids are unable to provide suppression (Dunavin *et al.* 2017*).*

From understanding the published literature, we know that allogeneic MSCs have both immunostimulatory and also immunosuppressive actions. What we must determine is the overall effect. Are allogeneic MSCs used in the equine patient able to provide anti-inflammatory and anabolic effects or does immune recognition negate these therapeutic benefits?

The interaction of the innate immune system with allogeneic MSCs

The cascade of events that occurs when MSCs encounter the immune system can be broken down into phases of the immune system response. These include the acute reaction by the innate immune system, and then the slightly delayed specific adaptive immunity of both cell-mediated and humoral (antibody) responses that result in long-term memory cells (Murphy 2012a). It is important to understand how the MSCs are affected through each of these steps in order to determine the potential for efficacy and the side effects of allogeneic treatment.

The innate immune system responds quickly and non-specifically to foreign antigens. This involves the release of anti-microbial enzymes and peptides, complement activation, recruitment of inflammatory cells, phagocytosis and destruction of foreign pathogens and cells (Murphy 2012a). Endothelial cells are one of the first cells to detect foreign pathogens, resulting in release of chemokines which allow the blood vessels to dilate leading to the extravasation and migration of phagocytes such as neutrophils and macrophages (Murphy 2012a).

Complement

The complement system is an important part of innate immunity. Components of the complement cascade are released from the liver in their inactive forms. In the blood they are cleaved to create their activated forms by proteases derived from inflammation. This initiates a cascade that culminates in the complement components binding directly to alloantigens or antibodies to mark an antigen for removal (Murphy 2012b). The foreign cell is then removed by forming a membrane attack complex or by facilitating leukocyte phagocytosis (Murphy 2012b, Gavin *et al. 2019).*

When the effects of complement are considered alone without accounting for the actions of other immune cells, this noncellular agent has been shown to cause a decrease in viability of human allogeneic MSCs (Li *et al.* 2012, Li *et al.* 2016a, Gavin *et al.* 2019). Two studies found >40% of human adipose-derived MSCs were damaged upon culture with naïve human serum containing activated complement (Li *et al.* 2012, Li *et al.* 2016a). Another study found minimal damage to MSCs when complement alone was added, but complement-mediated

phagocytosis caused MSC death when monocytes were added in vitro (Gavin *et al.* 2019). Means of resolving complement-mediated cytotoxicity have been created, but thus far each requires manipulation of the MSCs by means of application of complement-inhibiting materials (Factor H or N-glycolylneuraminic acid) to the cells' surface which is likely impractical from a licensing perspective at this point in time (Li *et al.* 2016a, Li *et al.* 2016b). CD59, a molecule found on some MSCs can prevent complement opsonization (Gavin *et al.* 2019). Sourcing MSCs with high surface expression of CD59 may also be a potential means to mitigate complement-mediated MSC death (Gavin *et al.* 2019).

The effects of the complement system on equine MSCs have not yet been reported in the horse.

Neutrophils

Neutrophils are the most numerous cell of the innate response and often the first leukocyte to infiltrate an allogeneic tissue. (Murphy 2012a). Neutrophils are recruited to areas of inflammation by vascular endothelium and likely recruited to MSCs by chemokine proteins such as CXCL8 (IL-8) (Scozzi *et al.* 2017, Mardpour *et al.* 2019). Once extravasated into allogeneic tissue, neutrophil infiltration leads to increased antigenicity and reduced allograft function (Mumaw *et al.* 2015). This may not occur when MSCs are administered as MSCs cause minimal activation of neutrophils *in vivo* by allogeneic MSCs (Mittal *et al.* 2018). Allogeneic MSCs appear to be immunomodulatory in that they can suppress neutrophil activation by causing a significant reduction in ROS when neutrophils

were activated prior to the addition of MSCs (Mumaw *et al.* 2015, Mittal *et al.* 2018, Salami *et al.* 2018, Joswig *et al.* 2017).

Although neutrophils in isolation are not activated by MSCs, one of the most concerning effects of the innate immune system in the horse is the rapid influx of neutrophils following intra-articular (both autologous and allogeneic) MSC injection (Ardanaz *et al.* 2016, Joswig *et al.* 2017). Numerous studies investigating the effect of MSC injection into equine joints show an increase in neutrophil count in synovial fluid lasting 48-72 hours after administration of autologous and allogeneic MSCs (Ardanaz *et al.* 2016, Joswig *et al.* 2017, Barrachina *et al. 2018,* Colbath *et al. 2020).* An increase in effusion (as measured by joint circumference) with or without a mild increase in lameness also occurs at similar time points (Ardanaz *et al.* 2016, Joswig *et al.* 2017, Barrachina *et al. 2018,* Colbath *et al. 2020).* There are several confounding factors for this neutrophil invasion. Joswig *et al.* (2017) showed this increase in cell infiltration and swelling occurs to the same degree when MSC freeze media (autologous serum and 5% DMSO) is injected alone without MSCs, as when freeze media is injected with autologous or allogeneic MSCs. The authors determined that in these cases, MSCs may not be the primary cause of neutrophil infiltration (Joswig *et al.* 2017). Another contributor to neutrophil activation found in earlier studies is the use of FBS in MSC media (Joswig *et al.* 2017). There is a significant increase in nucleated cell counts in the synovial fluid of joints injected with FBS-cultured autologous MSCs as compared to autologous or allogeneic MSCs cultured in equine serum during the final 48 hours of incubation (Joswig *et*

al. 2017). Because of this finding, where possible, studies are performed without this confounding factor.

Another possible cause of neutrophil influx may be due to a small proportion of MSCs in a cryopreserved or fresh MSC sample that become nonviable prior to administration (Chatzistamatiou *et al.* 2014). Activated neutrophils participate in the clearance of apoptotic cells; therefore, neutrophils enter the joint following an injection of dead cells. Interestingly, because apoptotic cells inhibit the proinflammatory functions of neutrophils, uptake of apoptotic cells by neutrophils can contribute to the resolution of inflammation in areas where dead cells are present (Esmann *et al.* 2010). The degree to which dead MSCs cause neutrophil influx as compared to live MSCs is unknown.

In a different type of study, MSCs had immunosuppressive effects on neutrophils in an inflamed equine joint (Williams *et al.* 2016). In this study lipopolysaccharide (LPS) was injected into one joint to stimulate an inflammatory response, and LPS and umbilical cord-derived MSCs were injected into the contralateral joint. This study saw a significant decrease in neutrophil influx into the joint after injection of both MSCs and LPS compared to the injection of LPS alone (Williams *et al.* 2017). The interpretation of these findings is that the presence of MSCs suppresses the activation of innate immune system.

Overall, there is concern when a horse is treated with either autologous or allogeneic MSCs and the joint then becomes acutely swollen and/or lame. In layman's terms this reaction is called a 'flare'; a short-lived inflammatory response that resolves without treatment or with anti-inflammatory medication. Flares in

clinical cases have been reported to occur in between 1.8-9% of equine cases receiving autologous or allogeneic MSCs (Broeckx *et al.* 2014, Ferris *et al.* 2014). No long-term negative effects were seen in either of these studies. Human studies using allogeneic MSCs and hyaluronic acid had a 25-53% rate of significant effusion after intra-articular treatment of the knee (Vega *et al.* 2015, Gupta *et al.* 2016), while administration of autologous MSCs and hyaluronic acid had a 45% rate of effusion (Lamo-Espinosa *et al. 2016)*. When, hyaluronic acid was used alone, 60% of human patients suffer from significant effusion (Vega *et al.* 2015).

Although these brief incidents of soreness and swelling can be worrying to the client, there is no evidence of long-term negative effects nor lack of response to treatment (Ferris *et al.* 2014*,* Vega *et al.* 2015*)*. Additionally, as laboratories replace FBS during the final 48 hours of culture, these 'flares' should be less common. Therefore, neutrophil influx after allogeneic MSC treatment in the horse does not appear to be an impediment to the use of allogeneic MSCs.

Macrophages

Macrophages are the most efficient type of phagocyte and are able to eliminate a large variety of pathogens, including foreign cells (Murray and Wynne 2011). When human allogeneic MSCs are cultured with macrophages, the macrophages become immunosuppressive, inhibiting natural killer (NK) cells and pushing T lymphocytes down a regulatory pathway (Chiossone *et al.* 2016*)*. At this time there are only two equine studies that have reported the reciprocal effects of MSCs and macrophages. Cassano *et al.* (2018) found minimal effect of MSCs on activated macrophages in vitro showing that MSCs may not have a strong

immunoregulatory ability to deactivate macrophages (Cassano *et al.* 2018a). Those MSC exposed to activated macrophages, though, then became immunosuppressive in an activated T lymphocyte proliferation assay (Cassano *et al.* 2018b). Although data in this area are extremely limited, allogeneic MSCs may be less capable of immunomodulation of activated macrophages (Cassano *et al.* 2018a).

Natural killer cells

Natural killer cells are a part of the innate immune system that can cause cell death through the targeted release of cytotoxins (Murphy 2012c). NK cells can attack cells lacking major histocompatibility complex (MHC) I on the surface of cells (Murphy 2012c). As bone marrow-derived equine MSCs express MHC I (Berglund *et al.* 2017, Kamm *et al.* 2019), NK cells may be less likely to pose a threat for these MSCs. Any hypothesizing on this issue is debatable at this point as appropriate antibodies for recognition of NK cells in the horse are lacking. MSCs have been found capable of suppressing NK cytotoxic activity in a murine hepatotoxicity model and using human cells in vitro (Li *et al.* 2015, Milosavljevic *et al.* 2017).

Dendritic cells

Dendritic cells capture and process alloantigens and serve to activate the adaptive immune system by presenting the alloantigens to B and T lymphocytes (Eisenbarth *et al.* 2019). Dendritic cells cultured with murine allogeneic MSCs cause the dendritic cells to decrease their surface expression of stimulatory molecules including CD80, CD83, CD86, and MHC II (Zhang *et al.* 2017). In

response to pathogens, these molecules are normally up-regulated to aid in activation of cell-mediated immunity. After interaction of the dendritic cells with murine allogeneic MSCs, the dendritic cells then cause a decrease in lymphocyte proliferation in mixed lymphocyte reactions (Zhang *et al.* 2019). Here we see evidence of the inhibition of adaptive immune system through MSC effects on the innate responses.

The interaction of the adaptive immune system with allogeneic MSCs

As previously mentioned, the adaptive immune response consists of two primary pathways; one is cell-mediated and the other is antibody-mediated (i.e. humoral immunity). T lymphocytes are needed for both pathways. In the humoral response of the adaptive immune system, B cells or antigen presenting cells bound with alloantigens in association with major histocompatibility type II (MHC II) receptor interact with helper T cells (i.e. CD4 T lymphocytes) (Haabeth *et al.* 2014, Hickey *et al.* 2016). Upon interaction with CD4 lymphocytes, B cells then are activated to differentiate into plasma cells which secrete antibodies to the alloantigen (Hickey *et al.* 2016). The earliest antibodies are seen in circulation after invasion of the organism is just less than 1 week (Pei *et al.* 2005, Berglund *et al.* 2017), and these antibodies can circulate for a long duration (Pei *et al.* 2005, Wood *et al.* 2013). This may be important in clinical scenarios where repeat treatments with allogeneic equine MSCs are warranted.

The cell-mediated component of the adaptive immune response requires cytotoxic T cells (i.e. CD8 T lymphocytes). Cytotoxic T cells take part in both direct

and indirect alloimmunity with cells bearing MHC I receptors that are bound with an alloantigen. In this way, cytotoxic T cells attack those cells that are foreign to the organism or cells that have taken up a foreign antigen. After a pathogen is recognized, a subset of CD8 cytotoxic T cells mature to form memory T cells (Akondy *et al.* 2017). Memory T cells rapidly respond upon subsequent antigen recognition, triggering the removal of the foreign antigens even many years later (Akondy *et al.* 2017). Both CD4+ and CD8+ lymphocytes are important when considering the use of allogeneic MSCs as these immune cells may recognize allogeneic MSCs due to their expression of MHC I and II.

MHC I and II expression on MSCs

After some debate about the presence of major histocompatibility markers on equine MSCs, it is now known that the cell surface expression of MHC I and II on MSCs is variable from one donor to another and even one MSC sample to another (Schnabel *et al.* 2014, Berglund *et al.* 2017, Kamm *et al.* 2019). MHC I is expressed on all equine bone marrow-derived MSCs though the degree of expression varies (Berglund *et al.* 2017). Conversely, some MSCs do not express MHC II antigens, while others have a strong positive expression (Schnabel *et al.* 2014, Kamm *et al.* 2019). Most problematically, MHC I and II expression are increased in the face of culture with foreign lymphocytes, when MSCs are cultured with inflammatory cytokines, or as the MSCs differentiate (Cassano *et al.* 2018b, Hill *et al.* 2017, Barrachina *et al.* 2018, Barrachina *et al.* 2020). The expression of MHC I and II motifs on MSCs are important in that they are the key cell markers utilized for alloimmunity by the host's immune system, and expression of these

markers identifies the MSCs as targets for destruction. Not only is the expression of these molecules important, but the degree to which these molecules are similar between the donor and recipient is also critical. The structure of each MHC molecule is defined by the human leukocyte antigen (HLA) or equine leukocyte antigen (ELA) haplotype (Kol *et al.* 2015). Horses are haplotyped using microsatellites to the ELA gene (Miller *et al.* 2017). The ELA haplotype and degree of mismatching determines the recognizability of donor cell to the recipient's immune system. Therefore, an MSC that expresses MHC I or II would be minimally immunogenic if the ELA haplotype is 'matched' to the recipient (Barrachina *et al.* 2018, Berglund *et al.* 2017).

T Lymphocyte responses to MSCs

What is the overlying result when allogeneic MSCs are exposed to lymphocytes? Are the lymphocytes activated or suppressed? When suppression of activated lymphocytes is considered, studies have overwhelmingly shown that allogeneic equine MSCs are capable of preventing lymphocyte proliferation in response to an activating agent (phytohaemaglutinin, foreign leukocytes, etc), thereby quelling an immune response (Remacha *et al.* 2015, Colbath *et al.* 2017, Ranera *et al.* 2016, Bloom *et al.* 2015). This immunosuppression occurs subsequent to the MSC-mediated increase in regulatory T lymphocytes (Tregs) which serve to dampen the adaptive immune response and can prevent rejection of foreign cells by the host (Owens *et al.* 2016). MSCs secrete immunomodulatory cytokines, including transforming growth factor beta (TGF-β), indoleamine 2,3-deoxygenase 1, IL-2, IL-10, IL-1beta receptor antagonist, hepatocyte growth factor

and PGE2 (Prasanna *et al.* 2010, Colbath *et al.* 2017, Klinker *et al.* 2017, Liu *et al.* 2019, Darlan *et al.* 2020). These cytokines serve to push the T lymphocytes down the path to create more T regulatory cells and to suppress leukocyte activation (Colbath *et al.* 2017, Darlan *et al.* 2020).

Many in vitro studies have been performed looking into lymphocyte behaviour after interaction with MSCs. Two studies using equine MSCs, showed that both autologous and allogeneic MSCs have an equal immunosuppressive capacity when MSCs are cultured with activated lymphocytes (Ranera *et al.* 2016, Colbath *et al.* 2017). This may indicate that immunosuppression is the predominant response when compared with immunoactivation by allogeneic MSCs. Another study examined activated lymphocytes and how they interacted with different types of allogeneic equine MSCs (Schnabel *et al.* 2014). Suppression of the lymphocytes occurred when MSCs expressing low levels of MHC II were co-cultured, but increased activation occurred when MSCs expressing high levels of MHC II were co-cultured (Schnabel *et al.* 2014). A study using eleven different human allogeneic MSC products found that every product tested was capable of immunosuppression when cultured with activated lymphocytes (Bloom *et al.* 2015). These studies indicate allogeneic MSCs are repeatedly shown to be capable of suppressing activated T lymphocytes. It must be acknowledged that each of these studies were performed in vitro, and previous studies in the horse have shown a lack of correlation in immunomodulatory properties between in vitro and in vivo results (Schnabel *et al.* 2014, Pezzanite *et al.* 2015).

Do allogeneic MSCs cause activation of unactivated lymphocytes? Colbath *et al.* (2017) has shown that allogeneic and autologous equine MSCs cause mild lymphocyte proliferation in vitro, the extent of which was similar for both groups (Colbath *et al.* 2017). Similarly, in humans, lymphocyte proliferation occurs when lymphocytes are co-cultured with allogeneic MSCs (Montespan *et al.* 2014). Interestingly, several human studies found an immunosuppressive form of the MHC I antigen, called HLA-G, which is expressed on some human MSCs (Nasef *et al.* 2007, Selmani *et al.* 2008, Montespan *et al.* 2014). Nasef *et al.* (2007) found that by adding an antibody against HLA-G, effectively inhibiting it from performing its function, activated lymphocytes proliferate when mixed with allogeneic MSCs (Nasef *et al.* 2007). Without the neutralizing antibody, human allogeneic MSCs prevent lymphocyte activation. Other work has shown HLA-G causes lymphocyte suppression and increases the number of immunosuppressive Tregs (Selmani *et al.* 2008). This HLA-G form of the MHC I molecule, which provides an innate ability to prevent the recognition of foreign cells, has likely evolved from the need to prevent fetal attack during gestation (Nasef *et al.* 2007, Selmani *et al.* 2008). This immunosuppressive isoform of MHC I is likely to exist in the ELA system, though no evidence has yet been published for the horse.

Does repeat exposure of the T lymphocytes to an allogeneic MSC cause lymphocyte activation? Piggot *et al.* (2014) co-cultured allogeneic MSCs with lymphocytes from horses that had previous exposure to the allogeneic MSCs and found no CD4+ lymphocyte proliferation signifying a lack of CD4+ memory cells (Pigott *et al.* 2014). Koi *et al.* (2015) found that the systemic CD8+ population of

lymphocytes, not the CD 4+ lymphocytes, increased when horses were treated for a second time with intravenous allogeneic MSCs (Koi *et al.* 2015). This suggests that CD8+ memory T cells are generated upon original exposure leading to cytotoxic lymphocyte proliferation upon re-injection with MSCs (Koi *et al.* 2015).

B cells and alloantibody responses to MSCs

Antibody production has been shown to be a limitation for allogeneic MSC survival. There is significant antibody production to allogeneic MSCs across species (Pezzanite *et al.* 2015, Gu *et al.* 2015, Owens *et al.* 2016). Barrachina *et al*. (2020) found that all equine patients receiving intra-articular allogeneic mis-matched MSCs formed antibodies after injection (Barrachina *et al.* 2020). Pezzanite *et al.* (2015) used MSCs of a mis-matched ELA haplotype and injected these cells intradermally in horses (Pezzanite *et al.* 2015). After 21 days, all horses had synthesized antibodies against the ELA type of the MSC that had been administered (Pezzanite *et al.* 2015). These antibodies are capable of targeting the MSCs for destruction (Berglund *et al.* 2017). Of the six horses tested, one also created an antibody response to another ELA type (Pezzanite *et al.* 2015). This cross reactivity has been reported previously in the human literature (Sernee *et al.* 1998, Owens *et al.* 2016).

The synthesis of antibodies capable of destruction of the MSCs after allogeneic treatment may limit the survival of the MSCs and therefore decrease the potency of therapeutic effect. Overcoming the undesirable consequences of the adaptive immune response is important when repeat MSC treatment is required as antibodies to the MSC may be present on administration (Schnabel *et*

al. 2014*)*. There are several methods to mitigate alloantibody production. One way forward is to ELA type donors and recipients to find a ‘matched’ pair. This is challenging as there hundreds of variations in ELA haplotypes (Holmes *et al.* 2019). Another strategy is to ELA type the donor horses of the MSCs and give subsequent treatments with MSCs of a different haplotype. Using this technique, only the horses that have cross-reactive antibodies would carry antibodies against the MSCs at the time of treatment. A third possible technique relies upon the manipulation the MSCs to prevent expression of MHC I and II. The reduction of MHC I and II expression has been successfully performed in human and murine MSCs using molecular biologic techniques (Huang et al. 2016, Broeckx *et al.* 2019). The addition of TGF β2 has also been shown to reduce MHC I and II expression (Berglund *et al.* 2017*)*.

Even without these techniques to decrease the effects of the major histocompatibility molecules, the MSCs that are currently being utilized provide beneficial treatment effects despite alloimmunity being present (Lange-Consiglio *et al.* 2013, Broeckx *et al.* 2014, Van Loon *et al.* 2014*,* Beerts *et al.* 2017, Magri *et al.* 2019, Delco *et al.* 2020, Vagnozzi *et al.* 2020).

Allogeneic MSC survival in vivo

There is some controversy as to whether there is a considerable beneficial effect of longer-term MSC survival in damaged tissue as compared to a short-lived effect. One study found that dead MSCs used to treat cardiac ischemia-reperfusion injury in mice had the same beneficial effect as viable MSCs (Xia and Cao 2013).

This study determined that the effect of MSCs on macrophages caused the improvement in cardiac output. Another study with the same method of cardiac insult found a significant effect between MSC survival and improved cardiac function (Guest *et al.* 2008). The MSCs in this second study were tracked over 30 days and were found to be present in the myocardium throughout the study period. These studies seem to conflict with one another, but perhaps this is due to the method of improvement in function seen in the different studies. An immune-mediated effect may not necessitate long term MSC survival as some reports suggest (Guest *et al.* 2010, Squillaro *et al.* 2016, Xia and Cao 2013), while a structural effect may require long-term MSC incorporation.

Few equine studies focusing on the duration of survival of allogeneic MSCs have yet been published. Furthermore, it is largely unknown what percent of the original dose of MSCs that is given to a patient survives long term, but generally this is believed to be a very small proportion for both autologous and allogeneic MSCs (Lacitignola *et al.* 2014, Braid *et al.* 2018). Guest *et al.* (2008, 2010) found that approximately 2% of the originally injected equine bone marrow-derived allogeneic MSCs survived to 30 days in the lesion and 1% survived to 60 days in the lesion (Table 1) (Guest *et al.* 2008, Guest *et al.* 2010). Ovine bone marrow-derived allogeneic MSCs survive at least 6 weeks after intra-tendinous injection though the percent survival was not measured (Table 1) (Lacitignola *et al.* 2014). Human MSCs injected into mice survive longer than 5 months when injected intramuscularly, 1-4 weeks when injected subcutaneously or intraperitoneally, but only a few days when injected intravenously (Table 1) (Braid *et al.* 2018). When

allogeneic adipose-derived MSCs were used intra-articularly after disease induction in the femorotibial joint, MSCs survived 10 weeks in the rat and 14 weeks in sheep (Table 1) (Li *et al.* 2016, Feng *et al.* 2019). By extrapolating the data in these studies, it appears that allogeneic MSCs survive for a longer period in areas of lower vascularity.

Table 1. Relevant allogeneic MSC survival studies.

Study	Recipient specie, Tissue treated, number of cases	MSC origin	Survival measurement method	Survival duration (days)
Guest *et al.* 2008, Guest *et al.* 2010	Equine, experimental tendon lesion, n=8	Equine bone marrow	Green fluorescent protein (GFP)	<5% of MSCs survive past 10 days, present in lesion >60 days, no difference between allogeneic and autologous
Lactignola *et al.* 2014	Ovine, Achilles tendon, n=9	Ovine bone marrow	Red florescent protein	>6 weeks (all allogeneic)
Braid *et al.* 2018	Murine, n=3-5/ location	Human bone marrow or umbilical cord	Luciferase lentivirus	>110 days intramuscular, 7 days subcutaneous, 21 days intraperitoneal, 3 days intravenous
Li *et al.* 2016	Murine, intra-articular, n=3 at each time point	Human adipose	DiD fluorescent dye	2/3 rats at 14 days and 1/3 rats at 70 days
Feng *et al.* 2018	Ovine, n=24	Human adipose	Iron visualization via MRI	All sheep at 98 days

Results of allogeneic MSC therapy for musculoskeletal disease

Above and beyond the possible mechanisms for deleterious effects on MSCs by the immune system, the results of in vivo clinical trials and experimental studies must be considered. The use of bone marrow-derived allogeneic MSCs for

joint disease has gained popularity, likely due to largely positive results (Chahal *et al.* 2019, Mahmoud *et al.* 2019). A large equine clinical trial of 165 horses treated with allogeneic MSCs and platelet rich plasma for clinical joint disease has been described (Broeckx *et al.* 2014). In this report 45% of cases at 6 weeks post-treatment, and 78% of cases by 18 weeks returned to athleticism, though this study lacked a control population (Table 2) (Broeckx *et al.* 2014). A study using a chemically induced- model of arthritis in the horse showed significant upregulation of type 2 collagen and significantly decreased expression of inflammatory mediators in cartilage at 6 months post-treatment when allogeneic MSC-treated joints were compared to untreated joints, though no significant gross nor histologic improvement was seen (Table 2) (Barrachina *et al.* 2018). In a similar study, allogeneic MSCs did not cause significant clinical improvement in IL-1beta-induced arthritis, however, this was a very acute and severe inflammatory model (Table 2) (Dyson *et al.* 2004). Additional allogeneic MSC studies focusing on joint disease in the horse have shown beneficial clinical and histologic results using blood-derived, neonatal-derived (Delco *et al.* 2020), or adipose-derived MSCs (Table 2) (Van Loon *et al.* 2014). In people with severe knee osteoarthritis, Vega et al. (2015) showed improved function and cartilage grade on MRI as compared to hyaluronic acid when MSCs were used intra-articularly (Vega *et al.* 2015). Experimentally created knee arthritis in rabbits was improved when treated intra-articularly, but only when the animals were treated on three occasions as one injection was insufficient to improve outcomes (Yuksel *et al.* 2016). The use of allogeneic MSCs in joint disease appears to be beneficial though in some studies, this benefit was not clinically relevant.

The use of bone marrow-derived allogeneic MSCs for soft tissue lesions show promise when the treatment is administered directly into the injured tissue. A clinical study of 40 horses treated with adipose-derived MSCs for tendon lesions concluded that 77% of those horses returned to full athletic function of equal or higher levels than prior to the injury (Table 2).84 Another study using 44 clinical cases of tendon or ligament lesions showed a similar proportion of horses returning to athleticism after bone marrow-derived allogeneic MSC therapy (Table 2) (Beerts et al. 2017). A recent large clinical equine study on soft tissue lesions found 18% of horses reinjuring within 2 years of follow up (Table 2) (Lange-Consiglio et al. 2013). These data appear favorable in comparison to the 44% re-injury rates among horses treated with rest and simple rehabilitation techniques alone (Khan et al. 2018).

When evaluating the therapeutic potential of allogeneic MSCs in experimental models of soft tissue lesions, laboratory animals were the only populations examined to date. Direct injection into a rat Achilles tendon rupture model results in improved elasticity and strength of treated tendons as compared to untreated tendons at 30 days post-treatment (Yuksel *et al.* 2016). Intrathecal injection of bone marrow-derived MSCs to treat a surgically created defect in the intra-synovial portion of the Achilles tendon in sheep does not improve healing of the treated tendons at 24 weeks post-injury (Giri *et al*. 2020). A study using adipose-derived allogeneic MSCs in a rat Achilles tendon tear model showed improved strength of the injured tendon when treated into the lesion with MSCs (Gao *et al.* 2016). Based on the evidence to date, tendons appear to have

improved healing when treated with allogeneic MSCs, and the use of these treatments in equine tendon and ligament lesions is warranted.

Table 2. Relevant equine studies evaluating the use of allogeneic MSCs in clinical and experimental musculoskeletal disease.

Study	Type of MSC used	Disease treated, number of cases	Negative effects?	Positive effects?
Broecx *et al.* 2014	MSC from peripheral blood or chondrogenic induced MSC	Clinical osteoarthritis, n=165	Flare in 1.8% of 165 horses	78% return to athleticism for native MSCs and 86% for chondrogenic induced MSCs
Broecx *et al.* 2019	Chondrogenic induced MSC	Clinical metacarpophalangeal osteoarthritis, n=75	No	Significant improvement in lameness, flexion, joint effusion score by 18 weeks post-injection
Barrachina *et al.* 2018	Bone marrow-derived MSCs	Chemically induced arthritis, n=14	No negative reactions in repeatedly treated cases	Decreased effusion, improved synovial score, improved histochemistry, no change in radiograph or MRI score as compared to control
Colbath *et al.* 2019	Bone marrow-derived MSCs	Chemically induced arthritis, n=8	No difference in nucleated cell count between autologous and allogeneic MSCs	No improvement in clinical nor cytologic parameters
Magri *et al.* 2019	Umbilical cord-derived MSCs	Metacarpo- or metatarsophalangeal joint arthritis, n=28	12% reported mild, transient heat or effusion	Significantly improved lameness and clinical score, 68% of horses back to athleticism
Delco *et al.* 2020	Adipose derived MSCs (integrin $\alpha10^{high}$)	Tarsocrural impact model, n=8	No	Significantly improved radiographic, gross and histological score

Van Loon *et al.* 2014	Umbilical cord-derived MSCs	Clinical tendon and ligament injuries, n=40	No	77% returned to equal or higher athleticism
Lange-Consiglio *et al.* 2013	Placenta-derived (n=51) and bone marrow-derived MSCs (n=44)_	Clinical tendon and ligament injuries	No	4.00% of placenta derived and 23.08% of bone marrow-derived re-injured post treatment
Beerts *et al.* 2017	Peripheral blood-derived MSCs (tenogenic induced)	Clinical tendon and ligament injuries, n=104	No	18% re-injury rate after 2 year follow-up

Repeated allogeneic MSC administration for treatment of disease

Few studies have been completed to determine if repeat administration of allogeneic MSCs is more beneficial than a single treatment. As we have detailed, there would likely be antibody presence in the animal upon repeat treatment along with memory Tcells (Pezzanite *et al.* 2015, Kol *et al.* 2015, Barrachina *et al.* 2020). Repeat treatment using allogeneic MSCs has shown to cause an increase in leukocyte recruitment when used intra-articularly (Joswig *et al.* 2017). One study using umbilical-derived MSCs showed no improvement in therapeutic efficacy when clinical cases of equine joint disease were treated twice in a one month interval as compared to only treated once (Delco *et al.* 2020). As previously discussed, one rabbit study saw no improvement in arthritis when only one treatment of bone marrow-derived MSCs was given, while repeat therapy proved beneficial (Yuksel *et al.* 2016). In contrast, a study using mouse model of colitis

showed that allogeneic MSCs improved the disease upon initial treatment, but when mice were again inflicted with colitis, only syngenic MSCs were beneficial, not the allogeneic MSCs that had provided therapy upon initial treatment (Tasso *et al.* 2012). It is a common concern that repeat allogeneic therapy may lead to reduced therapeutic benefit in the horse, and we have yet to fully answer this question. Judging from the great amount of research showing immune response to interaction of MSCs and leukocytes, adaptive immunity likely will limit the functional ability of allogeneic MSCs upon repeat administration unless a means to mitigate MHC expression has been reconciled.

Conclusion

Allogeneic MSCs have both immunostimulatory and immunosuppressive effects. Resounding immunosuppressive effects are seen when MSCs are mixed with activated neutrophils or activated lymphocytes (Remacha *et al.* 2015, Colbath *et al.* 2017, Ranera *et al.* 2016, Mumaw *et al.* 2015, Mittal *et al.* 2018, Salami *et al.* 2018, Jiang *et al.* 2016). Allogeneic MSCs are recognized by the innate and adaptive arms of the immune system and their viability may be decreased following immune recognition (Pezzanite *et al.* 2015, Berglund *et al.* 2017, Barrachina *et al.* 2020). An antibody response is generated post-injection in the horse which likely would inhibit their therapeutic efficacy upon repeat treatment (Tasso *et al.* 2012, Joswig *et al.* 2017, Berglund *et al.* 2017, Barrachina *et al.* 2020). Allogeneic bone marrow- derived MSCs can survive in the recipient long term when delivered into

low vascularity regions such as tendons and muscle (Guest *et al.* 2008, Lacitignola *et al.* 2014, Feng *et al.* 2018).

There is evidence that use of allogeneic MSC therapy is beneficial to the patient (Lange-Consiglio *et al.* 2013, Beerts *et al.* 2017, Broeckx *et al.* 2014, Van Loon *et al.* 2014, Magri *et al.* 2019, Vagnozzi *et al.* 2020, Delco *et al.* 2020). Results of several studies have shown allogeneic MSCs carry no greater rate of short-term complications when used as a one-off therapy as compared to other biologic therapies (Ardanaz *et al.* 2016, Joswig *et al.* 2017, Barrachina *et al.* 2018, Colbath *et al.* 2020), and improving laboratory techniques will continue to lower the occurrence of side effects (Joswig *et al.* 2017). These side effects seen thus far have no relation to the level of success of the treatment (Broeckx *et al.* 2014, Vega *et al.* 2015). The response generated from current allogeneic MSC therapies that may not survive long-term is substantial and should not be disregarded. Potentially, a more potent response will be generated from an MSC that is minimally recognized by the recipient immune system and allowed to have a longer time frame to exert a therapeutic effect (Cassano *et al.* 2018). Methods to mitigate alloantibody production are being researched. ELA matching can be performed between recipient and donor. Molecular manipulation the MSCs to prevent expression of MHC I and II would decrease immune recognition. If repeat MSC therapy is given, variation of the donor MSC haplotype could minimize the immediate adaptive immune response. These options deserve continued investigation to improve upon the therapeutic benefits of allogeneic MSC therapy.

1.4.7 A brief report on the state of human MSC research

Both autologous and allogeneic MSCs have been utilized to treat human diseases caused by damaged tissue or for their immunomodulatory effects (Attia and Mashal 2021). Diseases such as acute respiratory distress syndrome, pulmonary fibrosis, peripheral neuropathy, paraplegia, alveolar bone engineering, glaucoma, osteoarthritis, and cardiomyopathy have undergone clinical studies for treatment with MSCs (Attia and Mashal 2021). Many of these studies have shown strong benefit to MSC therapy (Caplan 2019). Even to get the clinical testing phase, numerous in vitro and pre-clinical studies must show positive results. MSCs are now being investigated as vehicles in cancer therapy as they are often naturally located in the tumor microenvironment (Li *et al.* 2019).

Human and equine research is similar in that many diseases have undergone testing, but no MSC therapies have yet received FDA approval for use. The FDA has provided guidance for the use of MSCs as therapeutics. They allow for use of minimally manipulated regenerative therapies when used in an autologous manner. Culturing of MSCs is not included under this umbrella. Therefore, all of the therapies discussed thus far would require FDA approval or would need to be used as a part of a research study. The European Union, South Korea, and Japan have MSC therapies that have been approved by their medical agencies (alliancerm.org/available-products, December 2021). Canada and New Zealand have conditional approval of MSC therapies (alliancerm.org/available-products, December 2021).

An area of MSC research that varies somewhat between human and equine studies are cell surface markers for equine and human MSCs. Human MSCs should express CDs 29, 44, 73, 90, and 105 and not express CDs 11b, 14, 19, 34, and 79a and MHC I and II (Attia and Mashal 2021). Equine research has not come to the same consensus as to equine MSC marker expression. Equine MSC marker expression was discussed in Section 1.4.5.

During the period of this research project, the nomenclature of MSCs has changed. The International Society of Cell and Gene Therapies (ISCT) has provided a position statement on the proper nomenclature for cells that are cultured from bone marrow and have shown the cell surface marker expression and trilineage differentiation ability (Viswanathan *et al.* 2019) such as those we refer to in our research. These cells have been defined as ‘mesenchymal stromal cells’ rather than their previous name of ‘mesenchymal stem cells.’ We have utilized the nomenclature ‘mesenchymal stromal cell’ in this body of work in all areas except for those titles of manuscripts that had already been published prior to the change in nomenclature.

1.5 Aims and hypothesis

Through our investigation into different types of equine MSCs, we sought to determine the most ideal type of MSC that could be used for donation. We hypothesized that the ideal MSC would be immunosuppressive in order to be minimally recognized by the immune system while maintain its ability to produce anabolic factors, contribute to the structural healing and, overall, improve healing in the disease state. We chose to focus on the major histocompatibility complexes as these structures are known to be the principal target of allorecognition (Ayala Garcia *et al.* 2012).

We further hypothesised that a comparative investigation of specific breed and red blood cell antigen phenotypes would identify corresponding variation in phenotypes of their MSCs. To evaluate our hypothesis, we utilised cells sourced from these phenotypes in a variety of *in vitro* assays in order to determine the interaction between our MSCs and the leukocyte subtypes. Assay validation was necessary as many of these tests had not previously been performed on equine cells.

We aimed to determine the immunogenicity of our MSCs with lymphocytes, neutrophils, and complement. We considered these three arms of the immune system to be crucial in the overall success of the donor MSC. When the MSCs interact with each of these arms, we aimed to find the donor MSC that caused the greatest amount of immunosuppression while producing high levels of anabolic factors and maintaining viability.

We hypothesised that the use of autologous MSCs was the most appropriate reference for comparison to the various allogeneic MSC groups. By comparing their effects, we hypothesized that we could determine how the allogeneic MSCs may potentially behave in the recipient as compared to what is already known of the behaviour of autologous MSCs in the treatment of disease.

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Chapter 2. Blood type and breed-associated differences in cell marker expression on equine bone marrow-derived mesenchymal stromal cells including major histocompatibility complex class II antigen expression

2.1 Prelude

After gaining our base of knowledge through the literature review, we then started the first phase of our project. The aim of the following manuscript was to differentiate phenotypic groups of equine MSCs and determine if there was a breed- or blood donor-based correlation with MSC phenotype. MHC class I was not evaluated in this study as all BM-MSCs were known to be positive for this marker (and this marker was evaluated in future studies). CD59 was not included in this manuscript as the MSCs did not bind a substaintial amount of antibody. It was not possible to determined whether this low binding was due to the MSCs being negative for CD59 antigen or due to inappropriate antibody binding as no equine-specific antibody was available.

Blood type and breed-associated differences in cell marker expression on equine bone marrow-derived mesenchymal stem cells including major histocompatibility complex class II antigen expression.

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2.2 Abstract

As the search for an immune privileged allogeneic donor mesenchymal stromal cell (MSC) line continues in equine medicine, the characterization of the cells between different sources becomes important. Our research seeks to more clearly define the MSC marker expression of different equine MSC donors. The bone marrow-derived MSCs from two equine breeds and different blood donor-types were compared over successive culture passages to determine the differential expression of important antigens.

Bone marrow-derived MSCs from 18 Thoroughbreds and 18 Standardbreds, including 8 blood donor (erythrocyte Aa, Ca, and Qa antigen negative) horses, were evaluated. Bone marrow was taken from each horse for isolation and culture of MSCs. Samples from passages 2, 4, 6, and 8 were labelled and evaluated by flow cytometry. The cell surface expression of CD11a/18, CD44, CD90 and MHC class II antigens were assessed. Trilineage assays for differentiation into adipogenic, chondrogenic and osteogenic lines were performed to verify characterization of the cells as MSCs.

There were significant differences in mesenchymal stromal cell marker expression between breeds and blood antigen-type groups over time.

Standardbred horses showed a significantly lower expression of MHC class II than did Thoroughbred horses at passages 2, 4 and 6. CD90 was significantly higher in universal blood donor Standardbreds as compared to non-blood donor Standardbreds over all time points. All MSC samples showed high expression of CD44 and low expression of CD11a/18.

Universal blood donor- type Standardbred MSCs from passages 2- 4 show the most ideal antigen expression pattern of the horses and passages that we characterized for use as a single treatment of donor bone marrow-derived MSCs. Further work is needed to determine the significance of this differential expression along with the effect of the expression of MHC I on equine bone marrow-derived MSCs.

2.3 Introduction

Selecting the optimal stromal cell source is critical for obtaining favorable results from their use in regenerative medicine (Richardson *et al.* 2016). This has led to an ongoing search for mesenchymal stromal cells (MSCs) with the best capacity to replace or restore function to damaged tissues and a low occurrence of side effects (Joswig *et al.* 2017). In equine medicine, autologous MSCs derived from bone marrow are frequently used in research and clinical cases as their ability to enhance repair of tissues damaged by musculoskeletal disease is supported by a growing body of evidence from experimental and clinical studies (McIlwraith *et al.* 2011, Godwin *et al.* 2012, Ferris *et al.* 2014).

There is a move in equine medicine to use allogeneic MSCs instead of autologous MSCs due in part to the immediate availability of allogeneic MSCs and the inconsistent quality of autologous cells (Garvican *et al.* 2014, Schnabel *et al.* 2014, Pezzanite *et al.* 2015, Colbath *et al.* 2017). Perhaps the most important advantage of an allogeneic source of MSCs is the benefit afforded by a uniform MSC treatment for efficacy research into the therapeutic use of MSCs for equine diseases. An allogeneic cell line with a consistent phenotype would allow patients in clinical trials to be treated with MSCs from the same donor, and therefore all cases would receive a repeatable treatment. The current use of autologous MSCs in clinical studies adds an element of variability in the therapeutic efficacy of MSCs and standardized comparisons in clinical trials (Portalska *et al.* 2013). MSC function has been shown to vary in older humans, and the cell phenotype can vary from one bone marrow draw to the next (Schnabel *et al.* 2014, Pezzanite *et al.* 2015, Colbath *et al.* 2017).

When considering treatment with allogeneic MSCs, the potential for immunologic reactions by the host is a likely cause of treatment failure (Joswig *et al.* 2017, Colbath *et al.* 2017, Griffin *et al.* 2013). MSCs are acutely or progressively rejected by the cell-mediated and humoral arms of the immune system leading to MSC death and local inflammation (Zangi *et al.* 2009, Consentius *et al.* 2015, Berglund *et al.* 2017). The major histocompatibility complex (MHC) class I and II molecules present on the cell surface facilitate allorecognition when foreign cells are transplanted into a recipient (Benichou *et al.* 2011, Griffin *et al.* 2013, Schnabel *et al.* 2014). MHC class I and II molecules on the surface of the donor MSCs are

identified by the recipient's immune system leading to T and B lymphocyte activation (Griffin *et al.* 2013, Schnabel *et al.* 2014).

In horses, MHC class I molecules are expressed by most cells of the body including equine bone marrow-derived MSCs (Schnabel *et al.* 2014,Barrachina *et al.* 2016). The appearance of MHC class I on the cell surface causes immunorecognition and antibody formation when administered in an allogeneic manner (Pezzanite *et al.* 2015, Berglund *et al.* 2017). This reaction becomes apparent on serologic testing no less than seven days after administration of the foreign MSCs (Pezzanite *et al.* 2015, Berglund *et al.* 2017). This allorecognition may be eliminated or reduced by matching of the donor and recipient, to administer cells with MHC antigens that are as similar as possible to that of the donor (Benichou *et al.* 2011, Alonso *et al.* 2012, Berglund *et al.* 2017). The need for donor-recipient genotype matching (haplotyping) is currently under investigation, as some studies have shown no significant immune response to one injection of MHC I-nonmatched allogeneic MSC administration *in vivo* (Huang *et al.* 2016, Joswig *et al.* 2017, Mei *et al.* 2017). Additionally, a beneficial therapeutic effect has been seen with the use of one injection of MHC I-nonmatched allogeneic MSCs *in vivo* (Huang *et al.* 2016, Mei *et al.* 2017).

Unlike MHC class I expression, MHC class II expression on equine bone marrow-derived MSCs varies from almost non-existent to high from one horse to another (Schnabel *et al.* 2014, Paebst *et al.* 2014, Barrachina *et al.* 2016). MHC class II expression by equine MSCs may predispose these cells to immune recognition when used in an allogeneic manner (Schnabel *et al.* 2014). MHC class

II is known to activate the innate immune system which causes a rapid immune response and T lymphocyte proliferation (Schnabel *et al.* 2014). In horses, those MSCs expressing MHC class I and not MHC class II have been shown to not cause T cell proliferation (Schnabel *et al.* 2014). This leads one to believe that MHC class II is possibly the primary antigen for acute cell mediated allorecognition in the horse, while both MHC class I and II cause an adaptive immune response driven by alloantibodies (Alonso *et al.* 2012, Schnabel *et al.* 2014, Pezzanite *et al.* 2015, Berglund *et al.* 2017).

Several cell surface markers are important for MSC identification and exclusion of non-MSCs. CD44 and CD90 are consistently considered as markers for MSC identification (De Schauwer *et al.* 2012, Radcliffe *et al.* 2012, Paebst 2014, Zahedi *et al.* 2017, Song *et al.* 2017). MSC markers CD44 and CD90 are used as inclusion markers to confirm the identity of the cells as MSCs. CD11a/18 is used in our study to show contaminating cells and is commonly an exclusion marker for MSCs in culture (Dvorak *et al.* 2008, Radcliffe *et al.* 2012, Zhao *et al.* 2015).

We hypothesize that one group of equids of a particular phenotype may have differing antigen expression on their MSCs as compared to another group of equids. Previous research has demonstrated that erythrocyte and leukocyte antigen expression varies between horse breeds (Becht and Semrad1985, Angelos *et al.* 1988). Furthermore, it is well known that a series of erythrocyte antigens causes immune reaction leading to hemolysis after blood transfusion (Tomlinson *et al.* 2015). Hematopoietic stem cells and MSCs have common

lineage at the embryonic level, though literature has shown that their relationship may continue to adulthood (Ogawa *et al.* 2010). We intend to determine if there is some correlation between the expression of immunogenic antigens on erythrocytes and those immunogenic antigens that are expressed on MSCs. For this reason MSC marker expression from cells sourced from universal blood donor type horses and non-blood donor type horses were compared. The effect of blood donor status on MSC phenotype has not previously been described in horses.

The aim of this study was to determine the frequency of expression of several cell markers in populations of MSCs derived from Thoroughbreds, Standardbreds and horses characterized as universal blood donor horses. Thoroughbreds and Standardbreds were chosen for comparison as they are two common breeds in New Zealand and many other countries, and these horses suffer from injuries that may benefit from treatment with MSCs (Waselou *et al.* 2008, Laacitignola *et al.* 2008, Godwin *et al.* 2012) . Additionally, these breeds are known to have differences in erythrocyte antigen expression as a Standardbred horse is more likely to be a universal blood donor as compared to a Thoroughbred (Becht *et al.* 1985). The study sought to determine if one a particular phenotype of equids studied has an MSC passage number that yielded bone marrow-derived MSCs with the most ideal cell surface antigen presentation that would decrease recipient immune system recognition (low MHC II expression) while showing optimal ability to proliferate and differentiate (high CD44 and CD90 expression).

2.4 Materials and Methods

Experimental design

In brief, 36 horses were classified into groups according to their breed and erythrocyte antigen status. These included registered Thoroughbreds (n = 18) and Standardbreds (n = 18) of ages ranging from 2-13 years (median 4 years, interquartile range 4-6 years). Of the Standardbreds, 8 were erythrocyte antigen negative (blood donor type) and 10 were positive for erythrocyte antigens (non-blood donors). None of the Thoroughbreds were erythrocyte antigen negative. All of the horses were either owned independently or by Massey University and consent for their use was granted by all parties. Bone marrow was harvested from horses for MSC culture. MSC samples taken from passage 2, 4, 6 and 8 were assessed for their surface marker phenotype using flow cytometry. Trilineage testing was performed on a sample from each group of horses.

Bone marrow harvest, isolation and culture

Following ethics approval by the Massey University Animal Ethics Committee (MUAEC Protocol 15/13), MSCs were harvested from the sternum of all 36 horses. In brief, 15 mL of bone marrow was aseptically harvested and added to 3 mL of 1000 IU/mL heparin (Pfizer®, New York, NY, USA), using previously described techniques (Kisiday *et al.* 2008). Blood (25 mL) was collected via the jugular vein and placed in blood tubes (Rapid Serum Tube, BD Vacutainer®, San Jose, CA, USA) for serum collection. The bone marrow aspirates and blood tubes were transported to the laboratory on cold saline bags (3-5°C).

MSCs were isolated within 12 hours of harvest. Bone marrow aspirates were centrifuged at 200 X *g* at room temperature for 2 minutes. The supernatant was centrifuged at 1,000 X *g* for 10 minutes to pellet the nucleated cells. The supernatant was discarded and the pellet suspended in low-glucose Dulbecco modified Eagle's medium (DMEM, Gibco™, Thermo Fisher®, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Gibco™, Thermo Fisher®), penicillin (100 IU/ml), streptomycin (100 ug/ml) and amphotericin B (0.25ug/ml) (Sigma-Aldrich®, St Louis, MO, USA) and 2.5% 1M HEPES buffer (Gibco™, Thermo Fisher®). The same FBS batch was used throughout the study. Polystyrene tissue culture flasks (CellStar®, Greiner Bio-one, Monroe, NC, USA) were plated at a concentration of 0.267×10^6 cells/cm^2 and incubated at 37°C in 5% CO_2. The culture media was completely replaced after 24 hours. Once MSC colonies had formed, the cells were lifted from the flasks using Accutase (StemPro®, Thermo Fisher®) and plated onto new flasks. Cells were then fed with MSC proliferation media comprised of Alpha modification of Eagle's medium (AMEM, Gibco™, Thermo Fisher®) with 10% FBS, 1% penicillin/streptomycin/ amphotericin B and 2.5% 1M HEPES buffer.

Following passaging, cells were grown in culture flasks to 80% confluence. Cells from passages 2, 4, 6, and 8 were frozen at a concentration of 10^7 cells/mL in freezing media (autologous equine serum and 10% dimethylsulfoxide (Molecular Probes™, Eugene, OR, USA). Cryovials (2mL, Greiner Bio-one, Monroe, NC, USA) were cooled to -80°C using a slow-cooling container (Mr Frosty™, Thermo Fisher®) followed by storage in liquid nitrogen.

Trilineage potential

MSCs from passage 4 of four horses from the Standardbred, Thoroughbred and blood donor groups were assessed for trilineage potential. Each horse's cells were sampled in triplicate. The potential for adipogenic, osteogenic and chondrogenic differentiation was determined for the MSCs samples through cell expansion according to the manufacturer's instructions. Briefly, MSCs were plated on chamber slides (Lab-Tek, Thermo Fisher®) at at 1 x 10^4 cells/cm^2 for the evaluation of adipogenesis, and at 5 x 10^3 cells/cm^2 for the determination of osteogenic potential. The chondrogenesis assay used 0.25 x 10^6 cells that were centrifuged at 1000 x g for 5 minutes to form a cell pellet. After 24 hours of growth in proliferation media, MSCs were grown using specialized media (StemPro® Adipogenesis, Osteogenesis, and Chondrogenesis Differentiation Kits, Thermo Fisher®). The cells were grown in the differentiation media in monolayer for 14 days for adipogenic and osteogenic lineage assays. Cells were grown in pellet culture for 21 days for the chondrogenic lineage assay.

An additional set of cells was made by combining the Thoroughbred, Standardbred and blood donor cells in equal proportions. These cells were used as a control. A control sample was made for each lineage (adipogenic, osteogenic, and chondrogenic). These cells were cultured and treated in a similar manner as the trilineage groups except that only proliferation media was used (no induction media).

All cells were fixed in 4% formaldehyde at the end of the culture periods and stained as described for the respective differentiation protocols (34). Adipogenic

cells were stained with Oil Red O. Osteogenic cells were stained with Alizarin Red S. Chondrogenic pellets were embedded in paraffin and stained with Alcian Blue and counterstained with hematoxylin and eosin. Five randomly selected regions of each of the samples were assessed, providing 45 images to be used for evaluation of each of the Standardbred, Thoroughbred, and blood donor groups. The presence or absence of differentiation was evaluated using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Adipogenesis was determined by percentage Oil Red O staining over total area of cell coverage. Osteogenesis was measured as percentage of alizarin red-positive area over total area. Chondrogenesis was measured as percentage of alcian blue-positive area over total area of cell coverage.

Blood typing

Five mL of blood was collected in heparinized tubes (Heparin Tube, BD Vacutainer®, San Jose, CA, USA) for blood typing at the Equine Parentage and Animal Services Centre at Massey University. Blood was screened for Aa Ca and Qa antigens as horses that are used for blood donation (universal donors) should be negative for Aa, Ca, and Qa antigens (Hardy *et al.* 2008, Snyder *et al.* 2012).

Flow cytometry

The methods and the efficacy of the selected cell markers were first validated in a pilot study prior to use on the study population. All antibodies used in the main assay were first validated in the pilot study. Flow cytometry assays for CD 11a/18, CD 44, CD 90, and MHC class II antigens were validated using bone marrow-derived MSCs or leukocytes (Radcliffe *et al.* 2010, De Schauwer *et al.*

2012, Barrachina *et al.* 2016). The specific antibodies used are included in the supporting information. Erythrocytes were added to exclude non-specific binding (Radcliffe *et al.* 2010). Erythrocytes autofluorescence did not cause these cells to appear positive for the fluorochromes as has been seen in other studies (Whittington and Wray 2017). Samples from three horses were used for each antigen for validation assays. MHC class I molecules were not tested as they are consistently expressed at high levels in equine bone-marrow derived MSCs (Schnabel *et al.* 2014, Barrachina *et al.* 2016). Antibodies used were those previously reported and listed in the supporting information (Radcliffe *et al.* 2010, De Schauwer *et al.* 2012). All of the antibodies used were fluorescence conjugated for direct immunofluorescence. Those antibodies that were distributed without a conjugated fluorescing label were conjugated using an antibody labelling system (Mix-n-Stain™ Dye Antibody Labelling Kit, Biotium, Fremont, CA, USA; LYNX Rapid Antibody Conjugation Kit, Bio-Rad Laboratories, Hercules, CA, USA) (see supporting information). Antibody titration was performed to assure the optimal dilution was used. Antibody concentrations of 1:10, 1:50, 1:100, and 1:200 were compared using the stain index equation (Maecker *et al.* 2004). The dilution with the highest stain index was used. The most appropriate dilutions identified are listed in the supporting information, and these dilutions were used in subsequent assays.

For the validation study, aliquots of MSCs or leukocytes were suspended in phosphate-buffered saline (PBS) to obtain a concentration of 25 x 10^3 cells/µL. A 40 µL aliquot (1 x 10^6 cells) was used for each flow cytometry assay. The cells

were incubated with a viability stain (1ul/500ul cells, Efluor 780™, eBioscience™, San Diego, CA, USA) for 30 minutes on ice and protected from visible light. The cells were then washed with PBS and the diluted antibodies for CD11a/18, CD44, CD90, and MHC class II molecules added were added at the same time. The mixture was incubated on ice and protected from visible light for 30 minutes. The samples were then washed with 2mL PBS to remove excess (non-bound) antibody and fixed in 3% paraformaldehyde for 20 minutes. After a final wash and dilution in 1mL PBS, the cells were evaluated on a flow cytometer (BD FACSVerse^{TM}, San Jose, CA, USA). Data were collected on 1 x 10^4 large cell events (small debris was ungated) for each sample.

All data were compensated and corrected for autofluorescence using cytometric capture beads (BD™ CompBeads, San Jose, CA, USA), single stains, and all-fluorochromes-minus-one compensation tubes. Compensation for any spectral overlap between fluorochromes and data evaluation was performed using specialized flow cytometry software (FlowJo®, Ashland, OR, USA).

Gating was performed on a hierarchy format with, first, cells being isolated over a time frame that provided consistent cell acquisition data. Then viable cells were selected according to their low viability stain uptake. A mononuclear cell subset was selected by graphing on forward cell scatter area and height. Finally, a large cell population was selected. This gated cell population was used to determine cell marker expression.

After initial gating to identify an appropriate cell population for further analysis, these cells were gated to identify populations of cells positive and

negative for each of the markers. The populations were gated using both unstained cells and stained cells known to be negative or positive for the marker respectively. Data were reported as the percent of cells in this population that showed fluorescence for a specific marker. Both LK and JR (acknowledgements) performed independent data analysis prior to finalizing the results.

After antibody validation, a sample of 1 x 10^6 equine MSCs in the fourth passage was used to compare expression levels from MSCs immediately removed from culture and those that had been cryopreserved 24 hours prior. Samples from three horses were used in this part of the study. Expression of the cell markers were compared using a Chi-Square test for proportional populations. This pilot study was performed to confirm that cryopreserved cells could be used to accurately depict the cell marker expression.

After these validation steps were performed, MSCs derived from bone marrow samples of the 36 test horses were examined. Cell surface expression of CD11a/18, CD44, CD90, and MCH class II molecules at culture passages 2, 4, 6 and 8 were analysed for each of these horses. These passages were selected to give an overview of marker expression during the early culture period, when MSCs are commonly utilized for therapy because they are more proliferative and therefore provide sufficient numbers for treatment, and have a greater potential for differentiation than later stage passages (Bonab *et al.* 2006).

Data analysis

Flow cytometry and trilineage data were not normally distributed, and followed a beta distribution. Data transformation did not produce normally distributed data. Summary statistics for cell marker expression are expressed as median (interquartile range [IQR]). Data points were classified as outliers if they were greater than 1.5 times the IQR below the 25^{th} quartile or greater than 1.5 times the IQR above the 75^{th} quartile. Data for each molecular marker were plotted and each variable had a beta distribution. Beta regression was performed to identify breed (Standardbred; Thoroughbred), blood donor status (universal donor; non-donor) and temporal effects (passage 2, 4, 6 and 8) on cell marker expression of the gated cell population using statistical software (Betareg package in R, Version 3.4.3, R Core Development Team). Goodness of fit of the model was determined with a likelihood ratio test, with significance at $p < 0.05$. Post-hoc analyses by Wilcoxon rank sum and Mann-Whitney U tests were performed to identify the source of significant differences (if identified) among passages within breed, and between breeds at each passage. Similarly, Wilcoxon rank sum tests were used to then identify significant differences among passages within the universal donor and non-donor horse groups, and between universal donor and non-donor horses at each passage. The latter comparisons were restricted to Standardbreds, as there were no Thoroughbred universal donors. All differences were considered significant at $p<0.05$. Chi-Square statistics were calculated for cryopreservation assays to determine if there was a difference in marker

expression between fresh and cryopreserved cells. Differences and correlations were considered significant at $p<0.05$.

2.5 Results

Standardbred, Thoroughbred and blood donor MSCs show appropriate trilineage differentiation

Four Standardbred, Thoroughbred, and blood donor MSC samples from passage 4 were tested in triplicate for differentiation towards adipogenic, osteogenic and chondrogenic lineages (Figure 4). These groups were compared to MSCs treated with MSC proliferation media only (no differentiation media). Lipid deposits could be seen in the adipogenic induction plates and lipid staining was significantly greater than control (non-induced) MSCs for the Standardbred ($p=0.010$), Thoroughbred ($p=0.00039$) and blood donor ($p=0.020$) groups. Calcium deposits were present in the osteogenic induction plates and staining was significantly greater than control (non-induced) MSCs for the Standardbred ($p=0.00016$), Thoroughbred ($p=0.000076$) and blood donor ($p=0.000057$) groups. Glycosaminoglycan staining was seen in the chondrogenic induction pellets and staining was significantly greater than control (non-induced) MSCs for the Standardbred ($p<0.00001$), Thoroughbred ($p<0.00001$) and blood donor ($p=0.00027$) groups.

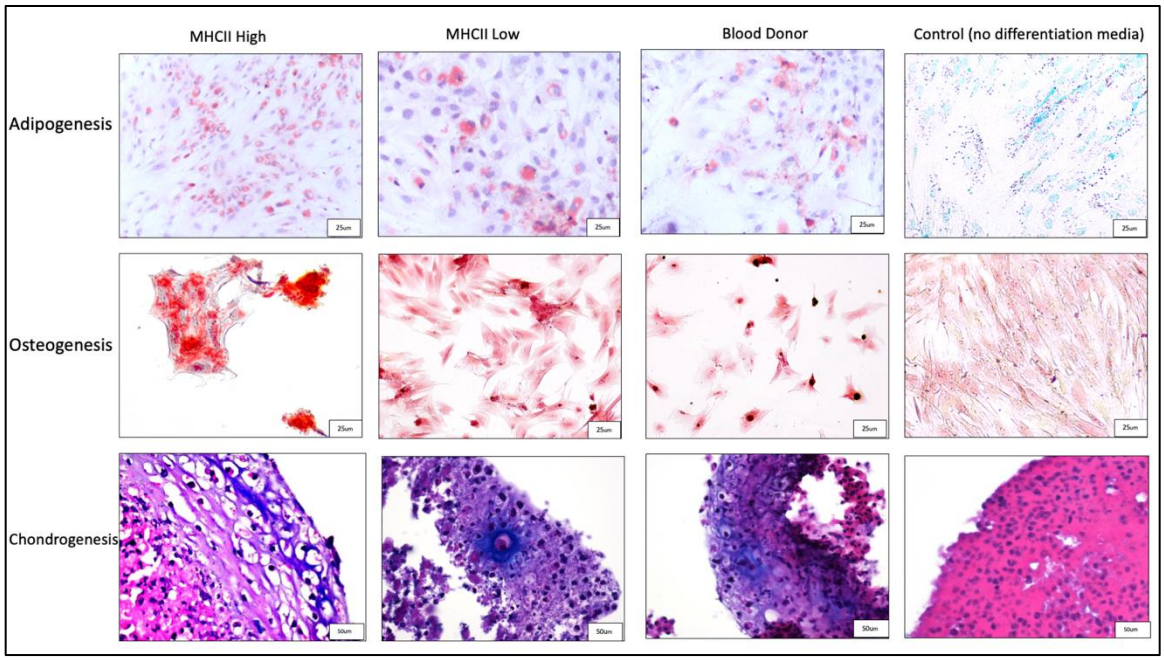

Figure 4. Trilineage differentiation is shown for the three types of horses.

All groups showed appropriate differentiation of MSC treated with induction media. The control group treated with no induction media showed no differentiation down adipogenic, osteogenic nor chondrogenic lines.

Trilineage differentiation assays were performed on Standardbred, Thoroughbred, and blood-donor MSCs. Cells placed in induction media showed differentiation down adipogenic, osteogenic, and chondrogenic lines. Control MSCs cultured in media without induction agents showed no differentiation.

MSC inclusion and exclusion antibodies were validated and cryopreservation did not alter marker expression

A full description of the antibody validation and dilutions are included in the supporting information.

Cryopreserved and fresh MSCs at the fourth passage were compared for their cell marker expression levels to assure that cryopreserved cells would appropriately represent fresh cell expression. There was no significant difference in surface marker expression on fresh samples as compared to cryopreserved samples (Chi-Square values 0.133-0.602) for CD11a/18 ($p=0.44$), CD44 ($p=0.64$), CD90 ($p=0.53$) and MHC class II ($p=0.71$). Cryopreserved cells were used for subsequent assays.

The gating scheme used for flow cytometric evaluation of a final large, viable cell population is shown in Figure 5. The antibodies used in this study showed appropriate binding to PBMCs or MSCs and did not bind to erythrocytes (Figure 6). Positively- and negatively-gated populations for each antibody are shown in Figure 6.

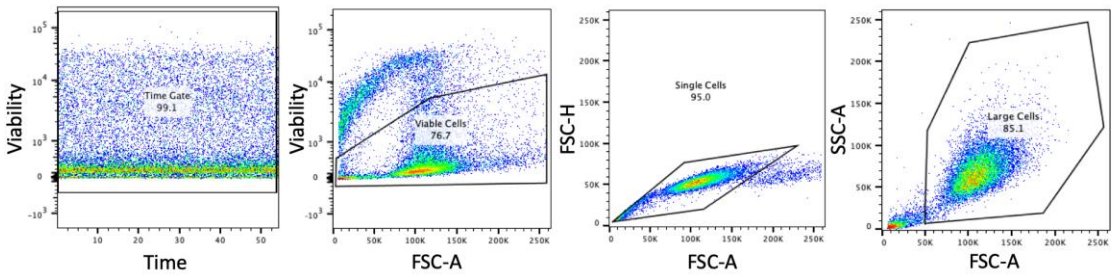

Figure 5. Gating scheme for MSC selection used in flow cytometry.
Representative dot plots show the gating scheme that was used prior to quantification of MSCs positive and negative for the desired marker.

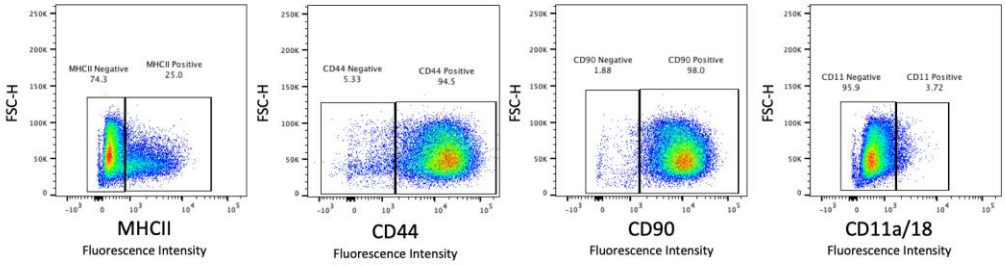

Figure 6. Positive and negative cell populations for each antibody illustrate marker expression in MSCs.

A representative MSC sample from passage 2 shows MHC class II, CD44, CD90 and CD11a/18 expression.

Blood typing reveals blood donor and non-blood donor type horses

All Standardbreds and Thoroughbred horses were blood-typed to identify the presence of Aa, Ca and Qa antigens on their erythrocytes. All Thoroughbred horses were positive for at least one of the erythrocyte antigens. Eight of the 18 Standardbreds were negative for all three antigens. These 8 horses were categorized as universal blood donor horses for comparison of universal blood donor and non-blood donor groups.

Beta regression models to understand multiple variables

In the multivariable model for MHC II expression, passage number ($p<0.001$) and breed ($p<0.001$) but not donor status ($p = 0.70$) were significant contributors to variance; the model was significant ($df=5$; $Chisq=30.67$; $p<0.001$). In the multivariable model for CD 11a/18 expression, breed ($p=0.003$) and blood donor status ($p=0.04$) but not passage number ($p=0.11$) were significant

contributors to variance; the model was significant (df=5; Chisq=13.32; p=0.004). In the multivariable model for CD 44 expression, breed (p=0.005) and blood donor status (p=0.04) but not passage number (p=0.19) were significant contributors to variance; the model was significant (df=5; Chisq=10.60; p=0.014). In the multivariable model for CD 90 expression, breed (p<0.001), blood donor status (p=0.001) and passage number (p=0.013) were significant contributors to variance; the model was significant (df=5; Chisq= 26.11; p<0.001).

Analysis of marker expression by breed shows significant differences between Standardbred and Thoroughbred MSCs

When marker expression was compared between the breeds, several markers showed significant differences (Figure 7). Standardbreds were significantly lower in their expression of MHC class II overall (p<0.001) and in particular during the early and middle passages as compared to Thoroughbreds (p<0.001 at passage 2; p=0.02 at passage 4, p=0.008 at passage 6) (Figure 7). Expression levels were similar at passage 8 only. Overall, MHC class II expression was low for both phenotypes, though Thoroughbreds showed higher variation and were more likely to be high at early passages (Figure 7).

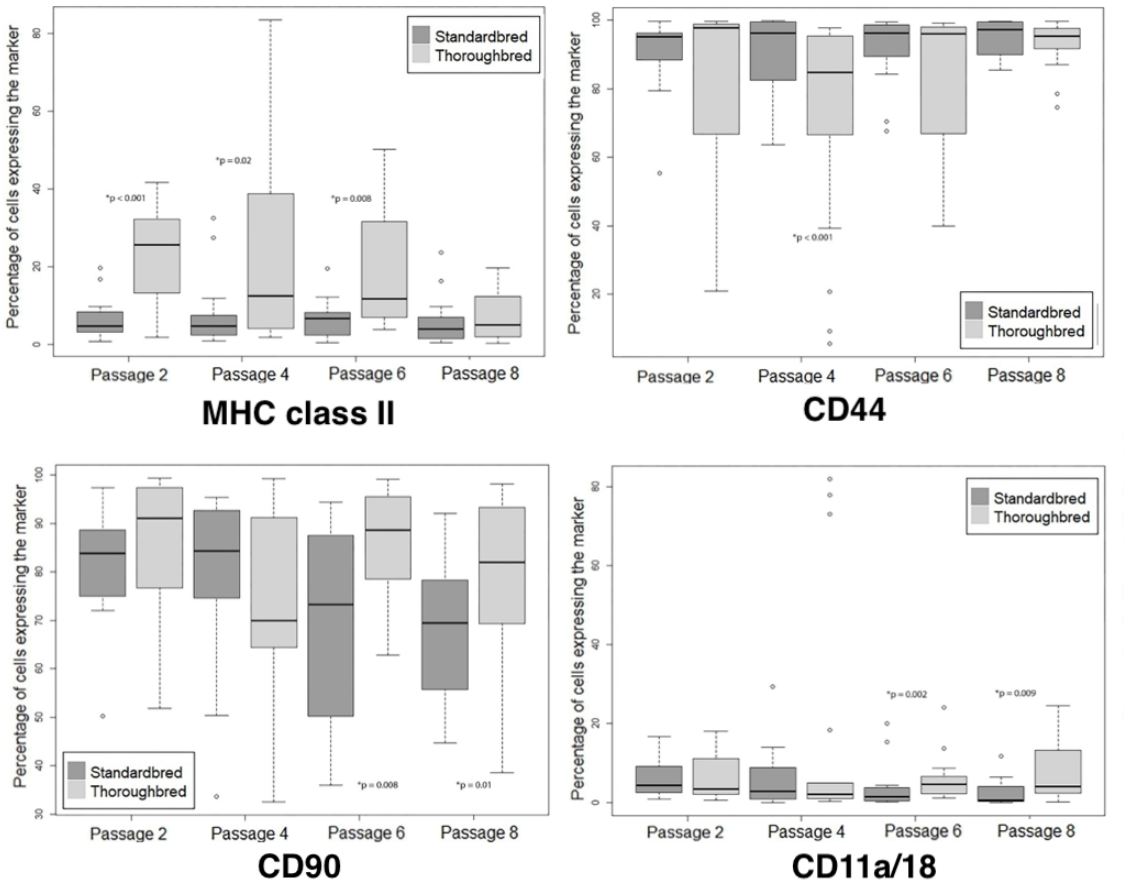

Figure 7. Marker expression by breed.

These graphs show the breed differences in marker expression over the time points (passages). Median marker expression is represented in each of the graphs as a percent of cells that show the marker as compared to the total gated cell population. The IQR is shown as the top and bottom of the box. Extreme values are shown with the error bar. Excluded data points are listed with a bullet. Passages with significantly different expression between the Thoroughbred and Standardbred populations are indicated by an asterisk.

CD11a/18 expression was also low through all passages with the median not exceeding 5% at any passage number (Figure 7). CD11a/18 expression was significantly higher in Thoroughbreds over all time points (p=0.001), especially at the later passages (p=0.002 at passage 6; p=0.009 at passage 8) (Figure 7). CD44 expression was high through all passages with the mean > 80% in both groups. Its

expression was significantly higher in the Standardbred population over all time points ($p=0.002$) and most impressively at passage 4 as compared to Thoroughbreds ($p<0.001$). CD90 expression was also high through all passages with > 70% of MSCs expressing CD90 in both groups. CD90 was expressed significantly more often in Thoroughbred MSCs over all time points ($p<0.001$) and, in particular, at passages 6 ($p=0.008$) and 8 ($p=0.01$) as compared to Standardbred MSCs.

Comparing changes in expression with passage, within the Thoroughbred group MHCII expression differed significantly between passages 2 ($p<0.001$), 4 ($p= 0.007$), 6 ($p= 0.005$) and passage 8. It did not significantly differ among passages within the Standardbred group. CD11a/18 expression did not significantly differ among passages for the Thoroughbred group. CD11a/18 expression in Standardbreds differed significantly between passages 2 ($p=0.01$), 4 ($p=0.013$) and passage 8, and between passages 4 and 6 ($p= 0.047$). There were no significant differences in CD44 expression among passages within the Thoroughbred or Standardbred groups. CD90 expression within the Thoroughbred group differed significantly between passages 2 and 4 ($p= 0.034$), and between passages 4 and 6 ($p= 0.01$). CD90 expression within the Standardbred group differed significantly between passages 2 and 8 ($p= 0.002$), and between passages 4 and 8 ($p= 0.002$).

Analysis of marker expression by blood type shows significant difference between universal blood donor non-blood donor MSCs

When the 8 universal blood donor horses (all were Standardbreds) were compared to the 10 non-blood donor Standardbred horses, there were significant differences in MSC expression of CD11a/18, CD44, and CD90 molecules (Figure 8). Expression of MHC class II was not significantly different between the non-blood donor horses as compared to the universal blood donor horses ($p=0.72$). Expression of CD11a/18 was lower in the non-blood donor horses at passages 4 ($p=0.020$) and 6 ($p=0.007$). CD44 expression was consistently high with a median of > 80% for both groups. Non-blood donor horses had significantly higher CD44 expression compared to blood universal donor horses at passages 6 ($p=0.040$) and 8 (0.040). CD90 expression was significantly higher in universal blood donor MSCs at passages 2 ($p=0.040$) and 4 ($p=0.020$).

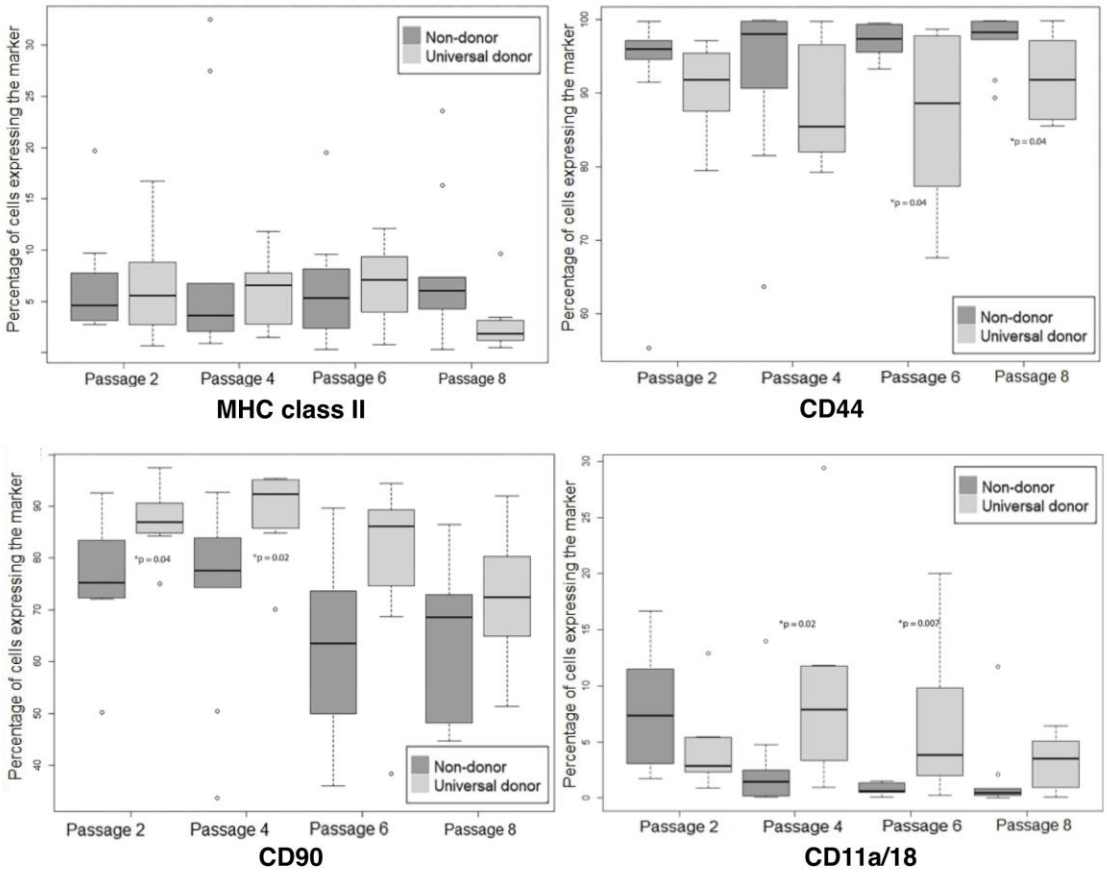

Figure 8. Marker expression by blood donor status.

These graphs show the differences in marker expression between blood donor horses and non-blood donor horses over successive passages. Median marker expression is represented in each of the graphs as a percent of cells that show the marker as compared to the total gated cell population. The IQR is shown as the top and bottom of the box. Extreme values are shown with the error bar. Excluded data points are listed with a bullet. Passages with significantly different expression between the Thoroughbred and Standardbred populations are indicated by an asterisk.

Correlation among cell markers shows MHC class II, CD90 and CD11a/18 are expressed similarly

When all groups of horses and all passages were analysed, cell markers showed correlation in their expression with the other measured markers (Table 3.)

CD11a/18 expression was positively correlated with CD90 and MHC class II ($p<0.001$, Table 3). Expression of CD90 and MHC class II were positively correlated ($p<0.001$, Table 3). CD44 expression was not correlated with that of any other cell marker.

Table 3. Correlation of Marker Expression.

	CD11a/18	**CD44**	**CD90**	**MHC class II**
CD11a/18	1	0.0647 (0.483)	**0.547 (<.0001)**	**0.655 (<.0001)**
CD44	0.0647 (0.483)	1	-0.0571 (0.535)	-0.0741 (0.421)
CD90	**0.547 (<.0001)**	-0.0571 (0.535)	1	**0.393 (<.0001)**
MHC class II	**0.655 (<.0001)**	-0.0741 (0.421)	**0.393 (<.0001)**	1

Marker expression correlation is listed for all data through all time points. The degree of correlation (R value) is listed followed by the p-value (in parentheses). Bold values show significant correlations.

2.6 Discussion

There are several breed and blood donor-status effects on MSC marker expression, influenced by the passage number. Bone marrow-derived MSCs from Standardbreds showed significantly less MHC class II expression at early passages as compared to Thoroughbreds. Evidence of breed associated differences in cell surface expression may explain, in part, why such large differences in the literature exist for MHC class II expression by equine MSCs.

Paebst *et al. (*2014) showed the mean percent of MSCs from Warmblood horses expressing MHC class II to be 0.25% at passage 3 (Paebst *et al.* 2014). Schnabel *et al.* (2014) reported that the mean percent of MSCs from Thoroughbred horses expressing MHC class II at passage 2 was 59.0% ± 26.3 and at passage 4 was 46.8% ± 36.2 (9). In comparison with the expression data from Schnabel *et al.* (2014), our Thoroughbred horse data showed a decreased median MHC class II expression at 18.5% and 12.5% for passages 2 and 4, respectively. It is possible that this difference in Thoroughbred expression between studies is due to breed variation secondary to gene flow as New Zealand based Thoroughbreds were used for the current study (He *et al.* 2017, Talbot *et al.* 2017).

The effect of blood donor status on MSC phenotype has not previously been studied in horses. It appears that the lack of immunogenic antigens on the surface of the erythrocyte (blood donor-status) does not correlate with a lack of MHC class II on the MSC surface as there was no significant difference in MHC class II expression between blood donors and non-blood donor horses. This observation was limited to Standardbreds, as MSC samples from universal donor-type Thoroughbreds were not identified during the screening process for recruitment to the study. The use of other blood donors of other breeds would have assisted our analysis.

One finding in the current study and in those previously published is that some horses with high initial MHC class II expression show a decreased expression over time (Schnabel *et al.* 2014). Five of 11 highly expressing samples in Schnabel *et al.* (2014) decreased to less than 2% of cells expressing MHC class

II (Schnabel *et al.* 2014). Six of 14 horses in our study with higher MHC class II expression at passage 2 decreased to less than 5% by passage 8. While decreased expression may be beneficial insofar as these cells are less likely to stimulate immune responses in the recipient than MHC class II high cells, MSCs at these late passages have deficits as compared to their younger relatives (Kundrotas *et al.* 2016). More highly passaged MSCs show an altered phenotype (decreased expression of MSC markers), have decreased proliferation rates, and develop an altered morphology (Bonab *et al.* 2006, Kundrotas *et al.* 2016, Esteves *et al.* 2017). For these reasons, older MSCs may be considered less desirable for treatment of disease.

MSCs were consistently positive for CD44 in this study, and this marker was highly expressed in all of the MSC populations examined. This consistent high expression in MSCs is in agreement with previously published studies (De Schauwer *et al.* 2012, Schnabel *et al.* 2014, Barrachina *et al.* 2016, Zahedi *et al.* 2017).

In the current study, the percent of MSCs positive for CD90 was high through all passages. Ranera *et al.* (2011) found 90% positive expression of CD90 at passage 3 in equine bone marrow-derived MSC sample, which is comparable to the results attained in our study (Ranera *et al.* 2011). Universal blood donor-type Standardbreds had significantly higher CD90 expression than non-blood donor Standardbreds over all time points ($p<0.001$). CD90 is known to be involved in cell proliferation, survival, migration and regulating differentiation (Campioni *et al.* 2008, Moraes *et al.* 2017). When CD90 gene expression is suppressed using

interfering RNA, cells move towards differentiation (Moraes *et al.* 2017). A high level of CD90 expression in the MSC population appears important for maintaining pluripotency (Campioni *et al.* 2008, Favi *et al.* 2013, Moraes *et al.* 2016). Therefore, the universal blood donor-type Standardbred may provide superior MSCs than the non-blood donor Standardbreds.

CD11a/18, an adhesion protein used by leukocytes to adhere to endothelium, was used in this assay to identify contaminating cells (van Kooyk *et al.* 1993, Zhao *et al.* 2014). CD11a/18 expression was significantly lower in Standardbreds as compared to Thoroughbreds and in non-blood donor horses as compared to universal blood donors. The cause of increased leukocyte contamination in some groups is unclear as the MSC isolation regimes were identical. The evidence of higher leukocyte contamination may be related to a difference in the number and ratio of myeloid cells in the bone marrow in one breed as compared to the other, though no studies have been performed to corroborate this hypothesis. Most importantly, CD11a/18 expression was low in all groups (Figures 4 and 5).

Expression of each marker was compared to one another to determine if there were significant correlations of expression (Table 1). Most interestingly, there was no correlation between CD44 and CD90 ($r=-0.0571$, $p=0.535$). Both of these antigens are commonly found on cultured bone marrow-derived equine MSCs (Radcliffe *et al.* 2010, Schnabel *et al.* 2014). Although these markers were both consistently highly expressed on our MSCs, based on findings in the current study, their cell functions do not appear to be linked. Their expression has seldom been

linked in previous MSC marker expression studies (Moraes *et al.* 2016). The correlation of expression in CD11a/18, CD90 and MHC class II may due to their expression on a small number of contaminating non-MSCs that represented by the total CD11a/18 positive cell population.

Studies define a cell population expressing a marker ≥90% of the time as “positive” for the marker while a cell population expressing a marker ≤10% of the time are “negative” for the marker (Kisselbach *et al.* 2009). Overall, our MSCs are CD11a/18 negative, CD44 and CD 90 positive, and MHC class II heterogenous.

In conclusion, universal blood donor-type Standardbred horses appear less likely to cause an MHC class II driven immune reaction and have high levels of bone marrow-derived MSC markers. As bone marrow-derived MSCs express MHC class I, further testing will be needed to determine whether these early passage universal blood donor-type Standardbred MSCs can be used in an allogeneic manner or if haplotyping will be necessary.

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2.8 Supplemental Information

Antibody Clone	Distributer, Catalog number	Conjugated fluorochrome or Kit used for labeling antibody	Host Species	Target Species	Ig Type	Dilution	Positive equine cell populations (publication reported)	Negative equine cell populations (publication reported)
CD 11a/18 CVS9	Bio-Rad, MCA1081PE	PE	Mouse	Equine	IgG1	1:50	Leukocytes [21]	Erythrocytes, MSCs [23]
CD44 CVS18	Bio-Rad, MCA1082GA	Biotium Mix-N-Stain CF647 Antibody Labelling Kit	Mouse	Equine	IgG1	1:200	Leukocytes, MSCs [21, 23]	Erythrocytes [23]
CD90 Thy-1	Washington State University Monoclonal Antibody Center, DH24A	LYNX Rapid PerCP-Cy5.5 Antibody Conjugation Kit	Mouse	Equine	IgM	1:100	Granulocytes, MSCs [21, 23]	Lymphocytes [23]
MHC class II CVS20	Bio-Rad, MCA1085F	FITC	Mouse	Equine	IgG1	1:100	Lymphocytes [21]	Granulocytes [23]

Table 4. Antibodies used for flow cytometry assays.

Antibodies showed high fluorescence in the appropriate positive cell population and negative to poor fluorescence on the negative cell population. The optimal dilution according to the stain index is listed.

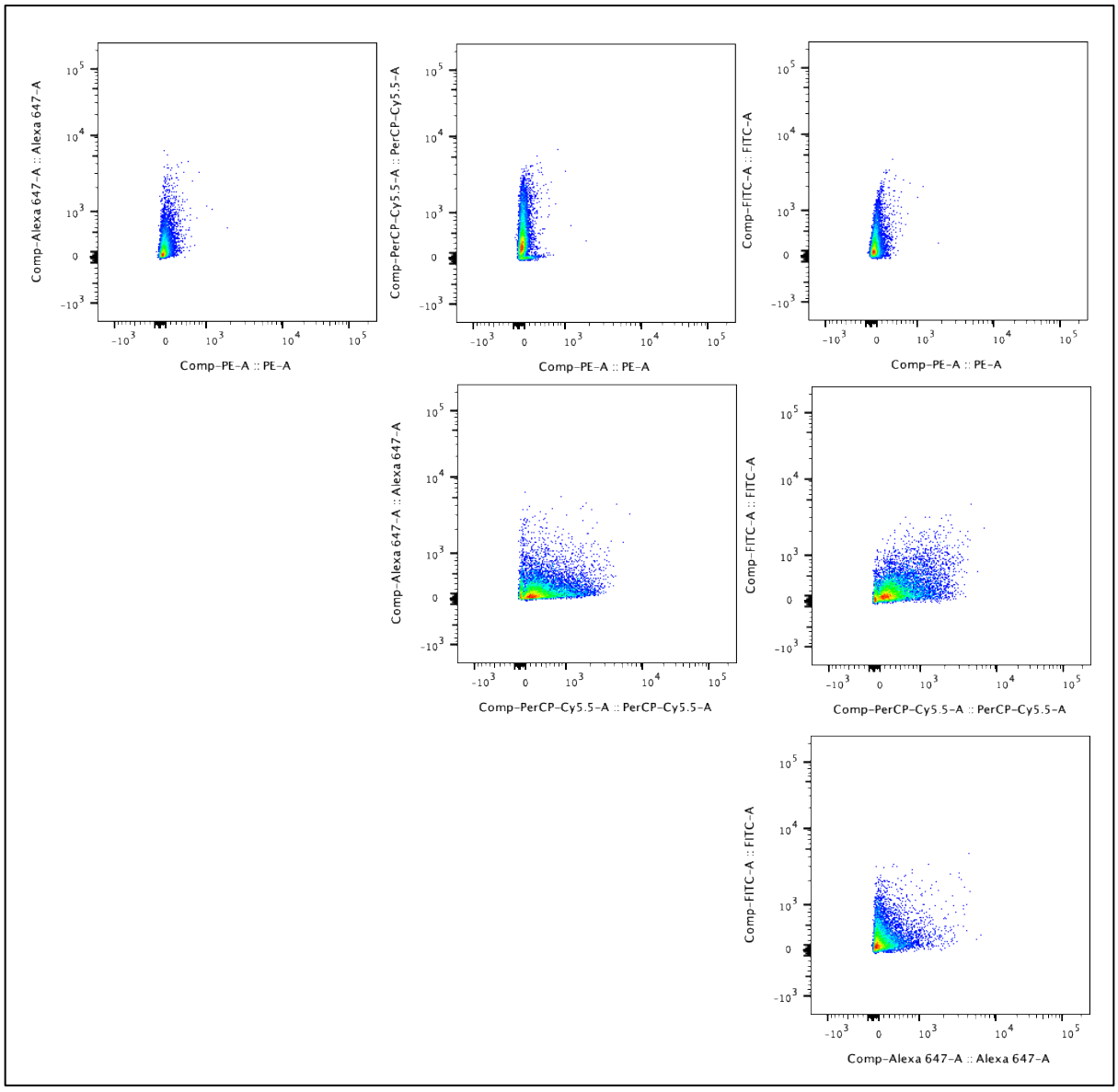

Figure 9. Antibody combinations included in flow cytometry assays.

The PE fluorochrome is conjugated to the CD11a/18 antibody, CF647 labels the CD44 antibody, PerCP-CY5.5 fluorochrome labels the CD90 antibody, and FITC is conjugated to the MHC II antibody.

2.9 Epilogue

Summarising Comments

The MSC expression information presented in this chapter served as a base for all further investigation. The fact that there was a breed difference in MHC II expression allowed us to then research whether this difference was immunologically relevant. This data confirmed that we were culturing a relatively pure population of MSCs and were justified in further testing their immunomodulatory and immunogenic effects.

Additional Discussion

Further discussion is provided here to investigate topics that were not fully addressed previously.

Trilineage images assessed using ImageJ software were blinded to the investigator (LK). Color thresholds for Oil Red O (adipogenesis), alizarin red (osteogenesis), and alcian blue (chondrogenesis) were set such that each image was assessed using the same set of thresholds. Staining for each of the three lineages is shown in Figure 4. In the adipogenesis lineage, Oil Red O stains lipid droplets a red color. In the osteogenesis lineage, alizarin stains calcium red. In the chondrogenesis lineage, alcian blue stains GAGs and glycoproteins a dark blue to violet color. Areas of positive stain uptake were identified using an initial image and selected as the threshold color for selection. This color was then used to assess the remaining slides. Computation of the area of staining was performed by the ImageJ software.

Radcliffe *et al.* (2010) performed antibody analysis to identify markers of equine MSCs. Erythrocytes were included in the analyses in order the illustrate the appropriate lack of their binding to several antibodies. The erythrocytes served as a negative control. In order to prevent nonspecific binding, Radcliffe *et al.* (2010) performed pre-blocking with serum and fluorochrome-specific non-binding control antibodies.

In Figure 7, three samples showed extremely elevated levels of CD11a/18 on the surface of the cells. These samples were removed as outliers due to their values being greater than 1.5 times the IQR above the 75^{th} quartile. We do not believe the results to be accurate as previous and later samples from these same horses showed low CD11a/18 marker levels. Additionally, the cells adhered to the bottom of the flask and had the histologic appearance of MSCs. For these reasons, we believe these cells to not be CD11a/18 positive and therefore removed them from statistical analysis.

Chapter 3. CellTrace Violet inhibits equine lymphocyte proliferation

3.1 Prelude

Chapter 2 provided us with three distinct groups of MSCs to be used for comparison in upcoming assays. We then needed to compare the interactions of the allogeneic MSC groups with leukocytes and complement. In the validation of our immune assays, we found that one commonly used lymphocyte proliferation dye for flow cytometry did not perform as expected with equine lymphocytes. This was interesting in that, although this dye had not been used in published assays using equine cells, a similar dye with potentially greater cytotoxic effects had been used in published assays. This brought into question whether these dyes were useful when using equine cells. We completed the following assays to determine whether we could use proliferation dyes for flow cytometric evaluation of lymphocyte activation in horses.

CellTrace Violet™ inhibits equine lymphocyte proliferation

Published in Veterinary Immunology and Immunopathology (2020 May;223:110037).

3.2 Abstract

CellTrace Violet™ is a commonly used fluorescent dye used with flow cytometry to identify cell proliferation. Activated equine lymphocytes were examined using flow cytometry, microscopy and tritiated thymidine proliferation assays. CellTrace Violet™ was incorporated into the equine lymphocytes effectively. Equine lymphocytes proliferated when activated with pokeweed mitogen, but did not proliferate when previously stained with CellTrace Violet™. Serial dilutions of CellTrace Violet™ did not moderate the inhibition of activated lymphocytes. Equine lymphocyte viability was greater than 90% for both stained and unstained cells. Based on these data, CellTrace Violet™ is not recommended for the assessment of lymphocyte proliferation in equine cells. The mechanism of inhibition of equine lymphocyte proliferation by CellTrace Violet™ is unknown.

3.3 Introduction

Cell proliferation dyes are used in flow cytometry to assess successive rounds of cell division. Fluorescent dyes are incorporated into cells, and when these cells divide, an equal amount of dye is passed on to daughter cells (Filby *et al*. 2015). When detected using a flow cytometer, a fluorescent peak is seen for

the parent cell, and in the case of two daughter cells, for example, a fluorescent peak of approximately half the fluorescence intensity is created by each (Filby *et al*. 2015). By examining a fluorescence histogram, the magnitude of the rounds of cell division can be quantified and thereby the degree of cell proliferation determined (Quah *et al*. 2012, Filby *et al.* 2015).

The original cell proliferation dye utilized for flow cytometry is 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Lyons and Parish 1994). CFSE has been used in large animal and equine research to study the proliferation of lymphocytes (Waters and Sacco 2007, Schnabel *et al.* 2014, Colbath *et al.* 2016).

CellTrace™ dyes (Molecular Probes™, Thermo Fisher Scientific, Waltham, MA, USA) are more recently available and have been advertised as less cytotoxic than other commonly used cell proliferation dyes including CFSE (www.thermofisher.com/nz/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-assays-reagents/cell-proliferation-flow-cytometry/improved-cfse-alternatives-cell-proliferation.html). Numerous publications have shown that CellTrace™ dyes have low cytotoxicity in human and murine lymphocytes (Quah *et al.* 2012, Filby *et al.* 2015). To the authors' knowledge, no publications have described using CellTrace™ dyes for the assessment of equine cells.

The goal of this project was to determine the effectiveness of a CellTrace™ dye to study equine lymphocyte proliferation. Viability studies were performed on

stimulated lymphocytes to determine if CellTrace™ Violet (CTV) caused viability loss. Further testing was carried out to determine if cell division could be measured and enumerated using flow cytometry following CTV labelling.

3.4 Materials and Methods

Lymphocyte harvest and culture

Following ethics approval by the Massey University Animal Ethics Committee (MUAEC Protocol 18/06), blood was aspirated aseptically from the left jugular vein of Standardbred horses (n= 1 to 3 per experiment, 10 horses used in total, as detailed in the 'Results'). Blood was placed in heparinized blood tubes (BD Vacutainer®, San Jose, CA, USA) for lymphocyte collection and subsequently processed using Lymphoprep™ (Alere Technologies AS, Norway). Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS) (Gibco™, Thermo Fisher®). Fifteen ml of Lymphoprep™ was placed in a centrifuge tube, and 30 ml of diluted blood was placed on top of the Lymphoprep™. The tube was then centrifuged for 25 minutes at 1125 x g at low acceleration and without braking, thereby forming a density gradient. The lymphocyte rich layer at the interface of the serum and the gradient agent was recovered and washed three times with PBS.

CellTrace™ Violet incorporation

CellTrace™ Violet (Molecular Probes™ Thermo Fisher Scientific, CellTrace™ Violet Cell Proliferation Kit, Waltham, MA, USA) was tested at a 5 µM concentration (as instructed by the manufacturer) and two-fold dilutions there-of. CTV was diluted in PBS and incubated with lymphocytes according to the

manufacturer's instructions. Briefly, 1 x 10^6 lymphocytes were incubated with the desired dilution of CTV in PBS for 20 minutes at 37°C. Samples were quenched with RPMI 1640 media (Gibco™, Thermo Fisher®, Waltham, MA, USA) with 10% autologous equine serum, for 5 minutes. Cells were then washed in excess PBS and cultured as described below.

Additionally, CTV incorporation was assessed following a modified method proposed by Quah *et al*. (2007). Equine lymphocytes were stained with CTV (5µM) in RPMI 1640 media with 10% serum. The addition of serum to the staining solution is intended to decrease cytotoxicity of the CTV. Using a concentration of 5 µM CTV, little to no CTV incorporation into the lymphocytes was seen when following this protocol (data not shown). CTV was incorporated using PBS as the staining diluent for all of the remaining assays.

Lymphocyte stimulation

The activating agents used in this study were Pokeweed mitogen (PWM) (Sigma®, St Louis USA) (1 µg/ml, 2.5 µg/ml and 10 µg/ml) and Concanavalin A (ConA, eBioscences™, Thermo Fisher®, Waltham, MA, USA) (1 µg/ml and 10 µg/ml).

Cultures were established on multi-well plates (Greiner Bio-One) using RPMI 1610 media with 10% autologous equine serum or fetal bovine serum (FBS, Gibco™, Thermo Fisher®), penicillin-streptomycin (100 ug/ml) (Sigma-Aldrich®, St Louis, MO, USA), 2-Mercaptoethanol (0.1mM) (Gibco™, Thermo Fisher®), +/-

activation agent as indicated. Cells were plated at a concentration of 3 x 10^5 cells/cm^3.

Tritiated thymidine incorporation assay to assess proliferation

Tritiated thymidine assays were performed using 1 x 10^5 cells per well in a 96 well plate (Greiner Bio-One, Austria). Cells were incubated in media with or without activating mitogens as indicated. Cultures were left for a period of three to seven days prior to harvest for data collection as indicated. Cells were stained with CTV as described previously or left without stain as indicated. One µCi of [methyl-^{3}H]-Thymidine (Perkin-Elmer, MA, USA) was added per well and cells were incubated for a further 18 hours. Cells were harvested onto glass fibre mats (Tomtec, USA) and cell-incorporated radioactivity was measured using a scintillation counter (Wallac, Finland) and reported as counts per minute (cpm).

Imaging of lymphocytes in culture

An inverted phase contrast microscope (Olympus CK2™, Olympus Corporation, San Jose CA, USA) was used to assess lymphocyte activation at 100X magnification three days following culture with 2.5 µg/ml PWM. Three horses' lymphocytes were tested each in triplicate. Activation was evaluated by determining the degree of cell clumping as described by Teague *et al*. (1993). Clumping was graded: 0- no clumping, 1- moderate clumping, and 2- significant clumping.

Flow cytometry

Fixable viability dye (Efluor 780™, eBioscences™ , Thermo Fisher®, Waltham, MA, USA) was used to assess cell viability in flow cytometry assays in conjunction with CTV uptake. Samples were measured using a BD FACSVerse™ (San Jose, CA, USA). Ten thousand events in a lymphocyte gate were recorded. The lymphocyte population was characterised using the gating hierarchy as shown in Supplementary Information using flow cytometry analysis software Flowjo (Flowjo LLC, Oregon, USA).

Statistics

Thymidine incorporation data were assessed using R software (R, Version 3.4.3, R Core Development Team). Data were not normally distributed as determined by Shapiro-Wilk's testing. Data transformation did not produce normally distributed data. Beta regression was performed to measure proliferation for the tritiated thymidine incorporation assay. Summary statistics for thymidine incorporation and viability are expressed as median (range). A Kruskal-Wallis test was performed to determine significance in the activation assay. To account for repeated measures in the time trial analysis, a Freidman's Two way ANOVA was performed. Significance was identified at $p<0.05$.

3.5 Results

Pokeweed mitogen shows the greatest level of lymphocyte activation

The activating agents Pokeweed mitogen (PWM) (Sigma®, St Louis USA) (1 µg/ml, 2.5 µg/ml and 10 µg/ml) and Concanavalin A (ConA, eBioscences™,

Thermo Fisher®, Waltham, MA, USA) (1 µg/ml and 10 µg/ml) were compared in their ability to stimulate lymphocyte proliferation. Cultures were assessed after three days of activation using tritiated thymidine. Tests were carried out in duplicate on one horse.

PWM at 2.5 ug/ml in media with autologous serum showed the highest level of proliferation as determined by thymidine incorporation (Figure 10). The use of FBS with low concentration PWM (1 ug/ml) greatly lowered its effect on the lymphocytes.

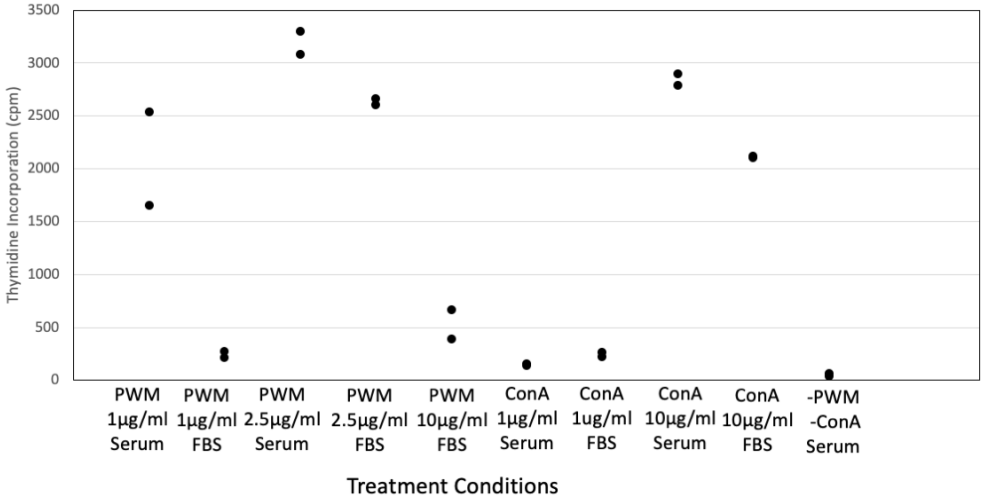

Figure 10. Comparison of activating agents in their ability to cause lymphocyte proliferation.

PWM and ConA were compared in their ability to promote lymphocytes to multiply after three days of activation using a thymidine incorporation assay. PWM at 1 µg/ml, 2.5 µg/ml, and 10 µg/ml were compared with ConA at 1 µg/ml and 10 µg/ml. Duplicates are shown from one horse.

Time points three days, five days and seven days of lymphocyte activation in culture were compared using tritiated thymidine incorporation in one horse's lymphocytes and testing was carried out in triplicate. Activated lymphocytes (2.5 µg/ml PWM) at time point three days showed significantly higher proliferation than the other time points (median ^{3}H-thymidine (range)), Day 3: 37074 (36196-46133), Day 5: 28403 (22757-28569), Day 7: 9073 (7378-9808), p=0.049). The time point of three days was used for the following experiments.

CTV loaded lymphocytes fail to proliferate in response to PWM nor ConA

Two flow cytometry assays were completed. First, lymphocyte proliferation after CTV staining was assessed when two different lymphocyte activators (PWM at 2.5 µg/ml and Concanavalin A at 10 µg/ml) were added to the media three days prior to analysis. Three horses were used for this portion of the study (the same animals that were used for microscopic evaluation). All horses were sampled in triplicate. Lymphocytes labelled with CTV (5 µM) and activated with PWM (2.5 µg/ml) or ConA (10 µg/ml) showed no proliferation as determined by flow cytometry (Figure 11). The presence of only one, high fluorescence intensity peak of signal from within the lymphocyte population, unchanged from that of the non-stimulated lymphocytes, signifies a single generation of lymphocytes and no cell division (Figure 11).

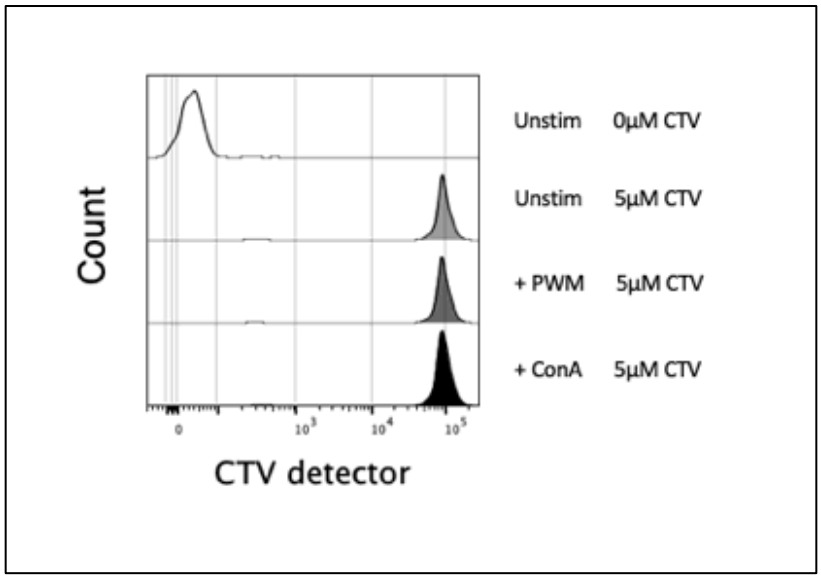

Figure 11. CTV labelled lymphocytes stimulated with PWM or ConA fail to proliferate.

Representative data from lymphoctes from one horse cultured in triplicate for three days in the presence of PWM (2.5 µg/ml) or ConA (10 µg/ml) show no loss of CTV signal, indicating lack of proliferation. Lymphocytes from three horses were assayed with the same result.

A CTV concentration study was then performed. Lymphocytes from one horse were incubated with the manufacturer's recommended concentration of CTV (5µM) and in serial dilutions of CTV. Lymphocytes were then treated with activating antigen (2.5 µg/ml PWM). Cell proliferation was assessed in triplicate after 3 days using the methodology above.

Lymphocyte analysis showed only one population of cells at a concentration-dependent emission intensity for each CTV dilution (Figure 12) with no evidence of proliferation at any CTV concentration tested. As CTV concentration decreased, the fluorescence intensity decreased (Figure 12). The fluorescence intensity of the most diluted concentration (0.3125 µM) has a

fluorescence peak that nears the CTV negative peak so that this dilution and further dilutions of the CTV would likely make resolution of daughter peaks unrecognizable. Lymphocyte survival was greater than 90% for all concentrations of CTV examined (Table 5).

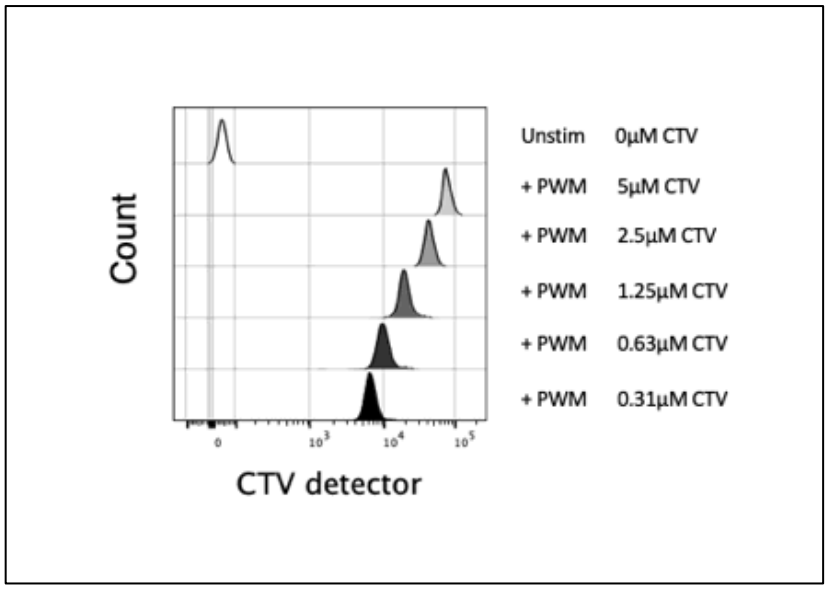

Figure 12. Reducing CTV loading concentration fails to recover lymphocyte proliferation.

Lymphocytes were labelled with CTV at concentrations as indicated and then activated with PWM (2.5 μg/ml) for three days prior to assay. The most dilute concentration (0.31 μM) has a fluorescence peak that approaches the CTV negative peak so that further dilution of the CTV would likely make daughter peaks challenging to distinguish. Representative data from one series of triplicates is shown.

Table 5. Survival of lymphocytes as determined by flow cytometry.

POPULATION	MEDIAN LIVE CELL (%) (RANGE)
LYMPHOCYTES (NO CTV)	96.0 (95.1-98.0)
LYMPHOCYTES WITH 0.3125µM CTV	94.0 (92.2-95.3)
LYMPHOCYTES WITH 0.625 µM CTV	92.9 (89.0-94.9)
LYMPHOCYTES WITH 1.25 µM CTV	94.3 (93.2-97.2)
LYMPHOCYTES WITH 2.5 µM CTV	93.8 (93.7-94.2)
LYMPHOCYTES WITH CTV AT 5 µM DILUTION	94.0 (93.3-94.0)

Lymphocytes treated with CTV showed a high level of viability. Lymphocytes were labelled with CTV at the concentrations indicated and then activated with PWM (2.5 µg/ml) for three days prior to assay.

Cell clumping score of stimulated lymphocytes is reduced to zero in presence of CTV

Lymphocytes activated with PWM (2.5 µg/ml) and either labelled with CTV (5 µM concentration) or left unlabelled, were visualized in culture using phase contrast microscopy at 10X (Figure 13). Lymphocytes from three horses were used and examined in triplicate. All samples using unlabelled lymphocytes (no CTV added) were scored as grade 2 for cell clumping (Teague *et al.* 1993). All samples of lymphocytes labelled with CTV showed little to no cell clumping (grade 0).

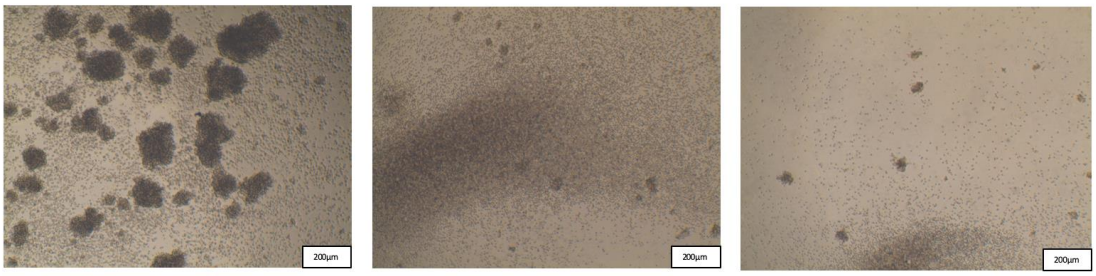

Figure 13. PWM stimulated lymphocytes do not clump in culture when loaded with CTV.

Extensive clumping of lymphocytes is seen in this image of lymphocytes treated with PWM in the absence of CTV. No clumping of the lymphocytes is seen when treated with PWM and CTV is added at the manufacturer's recommended concentration (5µM) nor when no PWM nor CTV is added. One representative image showing the area of the culture plate with the most clumping from each treatment group is shown.

CTV loaded lymphocytes fail to respond to PWM in thymidine incorporation assay

After identifying failure to proliferate via flow cytometry and microscopy, the authors hypothesised that the CTV loaded lymphocytes would be impeded from proliferating in the thymidine incorporation assay. To test this, lymphocytes were loaded with a dilution series of CTV and stimulated with PWM at 2.5 µg/ml for three days prior to addition of tritiated thymidine. The lymphocytes from three horses were used for this assay and results were repeated in triplicate. Results for this assay are shown in Figure 14. All dilutions showed inhibition of proliferation of lymphocytes as compared to lymphocytes and activator alone. Across all horses, inhibition of proliferation was significant at the manufacturer's recommended concentration (5 µM) and at half the manufacturer's recommended concentration

(2.5 µM) ($p<0.05$). Inhibition of proliferation trended toward significance for one quarter and one eighth of the manufacturer's recommended concentration (1.25 µM and 0.625 µM) ($p<0.10$). Inhibition of proliferation was not significant at one sixteenth of the manufacturer's recommended concentration (0.3125 µM) ($p=0.137$).

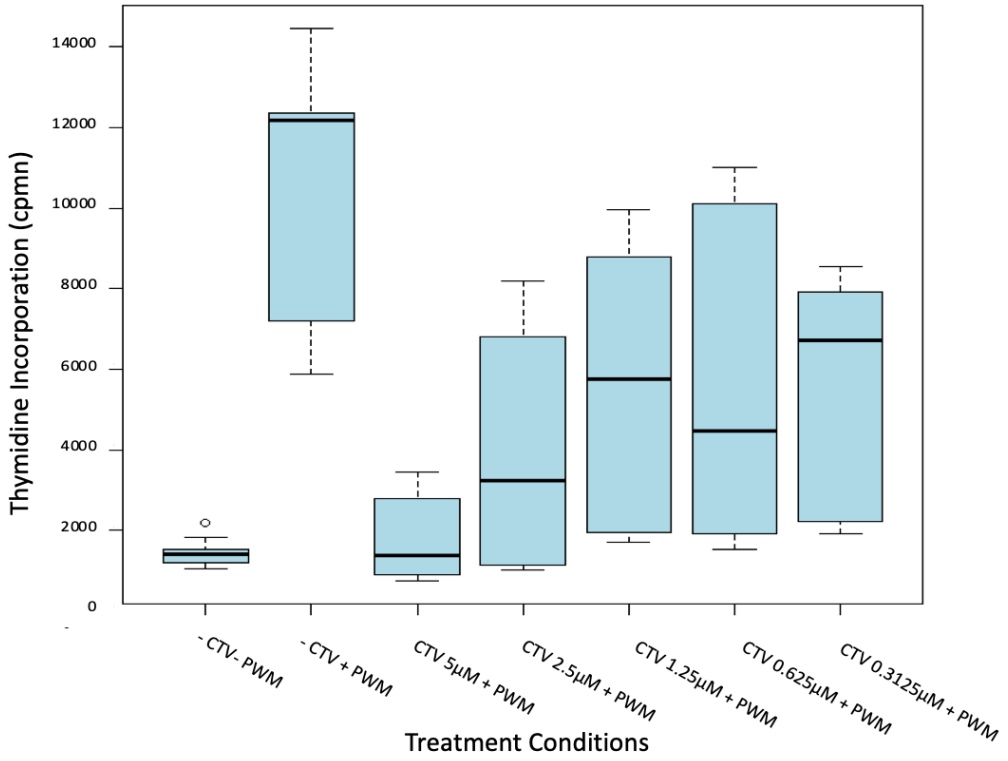

Figure 14. CTV loaded lymphocytes show impaired proliferation in thymidine uptake assay.

PWM stimulated lymphocytes that had been pre-loaded with CTV at two-fold dilutions are shown alongside positive (-CTV, +PWM) and negative (-CTV, -PWM) controls. Three horses' lymphocytes were tested in triplicate. Results from all three horses are shown as a box and whisker plot with minimum, first quartile, median, third quartile, and maximum values shown in ascending order. PWM stimulated lymphocytes, and lymphocytes alone were cultured for three days, pulsed with thymidine for 18 hours, and assessed for thymidine uptake to indicate proliferation. All CTV concentrations inhibited lymphocyte proliferation.

3.6 Discussion

Although many studies have been published which illustrate ample proliferation of human and murine lymphocytes labelled with CTV (Quah and Parish 2012, Zolnierowicz *et al*. 2013, Tempany *et al*. 1993), this study has shown that proliferation of equine lymphocytes is severely inhibited by staining with CTV. CellTrace™ dyes are purported to be less cytotoxic than CFSE (www.thermofisher.com/nz/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometry-assays-reagents/cell-proliferation-flow-cytometry/improved-cfse-alternatives-cell-proliferation.html). Publications using human and murine cells have shown CTV to have a similarly low level of toxicity as CFSE (Quah and Parish 2012, Filby *et al.* 2015). The observation that CTV does not allow for proliferation in equine lymphocytes (our data) while CFSE does allow for proliferation in equine lymphocytes (Schnabel *et al.* 2014, Colbath *et al.* 2016) indicates that equine cells respond differently to the dyes than human and murine derived cells. We did not test whether CFSE limited the proliferation of our equine lymphocytes as this was not the objective of our assays.

A better understanding of why CTV may impede proliferation can be gained by knowledge of the label's method of action. The CellTrace™ reagent covalently binds to the amine group of intracellular proteins, and becomes fluorescent when cleaved by intracellular esterases (Filby *et al.* 2015). With cell division, equal amounts of the reagent and fluorochrome are passed on to daughter cells. It has been shown that different fluorochromes linked to the CellTrace™ molecule cause differing levels of cytotoxicity (Tempany *et al.* 1993). CTV was not toxic in murine

cells at the manufacturer's concentration (5μM) but was toxic at 7.5μM while CellTrace™ Yellow was not toxic at concentrations up to 20μM (Tempany *et al*.1993) Therefore it may be the fluorochrome linked to the reagent rather than the CellTrace™ reagent itself that causes decreased proliferation in equine cells. Future studies into alternative CellTrace™ dyes are needed.

Dilution of the cell proliferation dye is important to determine the least toxic dye concentration that allows for appropriate separation of cell division peaks. In our current work, dilutions as low as one sixteenth of the manufacturer's recommended concentration (5μM) were used (Invitrogen CellTrace Cell Proliferation User Guide). Lowering the concentration to this level reduced the intensity of fluorescence of undivided cells thus compromising the available dynamic range for detection of reduced fluorescence in divided daughter cells (Figure 12).

It is unclear to the authors if the lack of proliferation seen in these equine lymphocytes caused by CTV was due to toxicity, a limitation in one of the steps in lymphocyte proliferation, or impedance of activation of the lymphocytes. If cell toxicity were seen, lymphocyte viability would be expected to decrease in the CTV treated population. This was not seen to any significant extent. The viability of lymphocytes without CTV was a median of 96% while the viability of the most concentrated CTV sample was 94% (Table 5).

3.7 Conclusion

Based on the findings of this study, the use of CTV to measure proliferation in equine lymphocytes is not recommended. Proliferation dyes must be tested in each different species as significant variability in how cells will respond to these dyes may be encountered.

3. 8 References

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3.9 Supplemental information

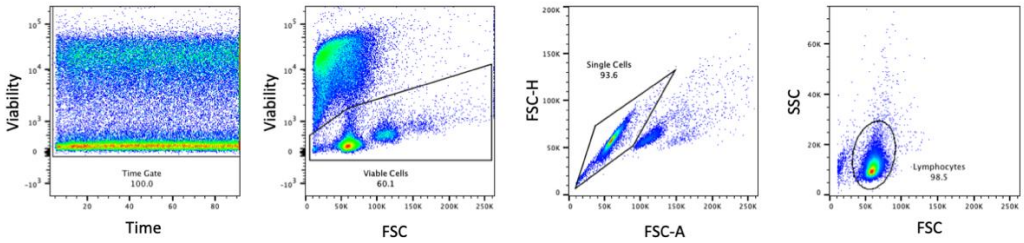

Figure 15. Gating hierarchy for lymphocyte gating in CTV studies.

3.10 Epilogue

Summarising Comments

This information was gained during the validation of our assays for testing the immunogenicity of equine MSCs. The use of CellTrace Violet was deemed inappropriate for testing the proliferation of equine lymphocytes. Alternative lymphocyte proliferation assays were then tested in order to find the best method of assessing lymphocyte activation.

Additional Discussion

Further discussion is provided here to investigate topics that were not fully addressed previously.

The term ‘clumping’ was used in this manuscript to refer to the histological formation of lymphocyte clusters in solution as seen on microscopy after activation. This term had been used in referenced manuscript (Teague *et al.* 1993). Aggregation would be another general term that could be used to describe the histological characteristics of clumping of cells in a solution. Agglutination is a term which defines cells which adhere to one another via antibody interactions which was not assessed in our analysis. Clonal expansion is proliferation of a specific cell type. Clonal expansion can not be determined without cytometric analysis which was not performed in our assays.

The activating agents Pokeweed mitogen and Concanavalin A were compared in their ability to stimulate lymphocyte proliferation. These activating

agents were chosen due to their successful use in previous equine lymphocyte proliferation studies.

DMSO was used in the dilution of the concentrated CTV dye as directed in the user guide (Invitrogen CellTrace Cell Proliferation User Guide, 2017). DMSO is an anti-inflammatory molecule known to significant decrease proliferation in human lymphocytes at concentrations of 1%v/v and greater (de Abrue Costa *et al.* 2017). Concentrations of 0.5%v/v did not impede proliferation (de Abrue Costa *et al.* 2017). At the highest concentration, we used 0.1%v/v DMSO in our CTV and cell samples. The DMSO concentration was decreased further in correlation with the concentration of CTV in our dilution assays. It is possible that the DMSO in the CTV dying mixture may have impeded the proliferation of equine lymphocytes. As we know from our CTV study, equine lymphocyte proliferation behaviours vary from human lymphocytes. A DMSO sample not containing CTV would have been beneficial to assess the effect of the DMSO on the proliferation as compared to the CTV and DMSO mixture. In saying this, the fact that the CTV must be diluted with DMSO, the same results would be derived in that CTV (as described for use in the User Guide) inhibits proliferation in equine lymphocytes.

Chapter 4. Immune response to allogeneic equine mesenchymal stromal cells

4.1 Prelude

Using the information gained by the assays utilized in Chapter 3, we determined that CellTrace violet could not be used in our assays. After inspecting the previously published data on carboxyfluorescein diacetate succinimidyl ester (CFSE) we decided that this dye may prevent the proliferation of equine lymphocytes as well. We turned to the tritiated thymidine proliferation test as we had used that in the testing of CellTrace Violet and had found it to allow for ample proliferation of equine lymphocytes similar to the activation seen when only an activating agent was added to lymphocyte cultures. We then utilized the tritiated thymidine test when accessing our allogeneic and autologous MSC groups for activation of lymphocytes. We used this assay in addition to flow cytometry and gene expression analysis to determine the behaviours of our allogeneic MSCs when placed in co-culture with recipient leukocytes and neutrophils. These assays allowed us to test our hypothesis that MHC expression would affect the immunogenicity of MSCs when used in an allogeneic manner.

Kamm JL, Riley CB, Parlane NA, Gee EK, McIlwraith CW. Immune response to allogeneic equine mesenchymal stromal cells. Stem Cell Res Ther. 2021;12(1):570.

4.2 Abstract

Background- Mesenchymal stromal cells (MSCs) are believed to be hypoimmunogenic with potential use for allogeneic administration.

Methods- Bone marrow was harvested from Connemara (n=1), Standardbred (n=6), and Thoroughbred (n=3) horses. MSCs were grouped by their level of expression of major histocompatibility factor II (MHC II). MSCs were then sub-grouped by those MSCs derived from universal blood donor horses. MSCs were isolated and cultured using media containing fetal bovine serum until adequate numbers were acquired. The MSCs were cultured in xenogen-free media for 48 hours prior to use and during all assays. Autologous and allogeneic MSCs were then directly co-cultured with responder leukocytes from the Connemara horse in varying concentrations of MSCs to leukocytes (1:1, 1:10, and 1:100). MSCs were also cultured with complement present and heat-inactivated complement to determine if complement alone would decrease MSC viability. MSCs underwent haplotyping of their equine leukocyte antigen (ELA) to determine whether the MHC factors were matched or mis-matched between the donor MSCs and the responder leukocytes.

Results- All allogeneic MSCs were found to be ELA mis-matched with the responder leukocytes. MHC II-low and universal blood donor MSCs caused no peripheral blood mononuclear cell (PBMC) proliferation, no increase in B cells, and no activation of CD8 lymphocytes. Universal blood donor MSCs stimulated a significant increase in the number of T regulatory cells. Neutrophil interaction with MSCs showed that universal blood donor and MHC II-high allogeneic MSCs at the 6h time point in co-culture caused greater neutrophil activation than the other co-culture groups. Complement-mediated cytotoxicity did not consistently cause MSC death in cultures with active complement as compared to those with inactivated

complement. Gene expression assays revealed that the universal blood donor group and the MHC II-low MSCs were more metabolically active both in the anabolic and catabolic gene categories when cultured with allogeneic lymphocytes as compared to the other co-cultures. These upregulated genes included CD59, FGF-2, HGF, IDO, IL-10, IL-RA, IL-2, SOX2, TGF-β1, ADAMSTS-4, ADAMSTS-5, CCL2, CXCLB/IL-8, IFNγ, IL-1β, and TNFα.

Conclusions- MHC II-low MSCs are the most appropriate type of allogeneic MSC to prevent activation of the innate and cell-mediated component of the adaptive immune systems and have increased gene expression as compared to other allogeneic MSCs.

4.3 Introduction

The interaction of the immune system with foreign antigens initiates inflammation and allorecognition. When the foreign source of antigenic stimulation is a therapy such as allogeneic mesenchymal stem cells (MSCs), the immune reaction can be detrimental to the survival of the donor cells and, consequently, may impair the intended health benefits for the recipient. MSCs are commonly believed to have innate immunosuppressive properties (Duffy *et al*. 2011, Duffy *et al*. 2011(2), Griffen *et al.* 2013, Gnecchi and Cervio 2013, Consentius *et al.* 2015, Contreras-Kallens *et al.* 2018, Zhou *et al.* 2020). Human studies have repeatedly shown that MSCs have immunosuppressive effects via T regulatory (Treg) and B regulatory cell upregulation leading to decreased activation of T lymphocytes and B cells, respectively (Wang *et al.* 2009, Carreras-Planella *et al*. 2019).

Immunosuppression within the recipient site by MSCs is necessary as allogeneic MSCs may be rejected due to their expression of foreign surface

antigens. The presence of major histocompatibility class I and II (MHC I and II) surface antigens on equine MSCs (and their specific equine leukocyte antigen (ELA) haplotype), facilitates immune recognition by lymphocytes (Pezzanite *et al.* 2015, Alagesan *et al.* 2018). Equine bone marrow-derived MSCs express MHC I and variably express MHC II (Schnabel *et al.* 2013, Kamm *et al.* 2019). Mis-matched ELA haplotype donor MSCs have been shown to induce greater lymphocyte activation *in vitro* as compared to matched donor MSCs (Schnabel *et al.* 2014). When given as a repeat treatment mis-matched MSCs may activate an alloantibody response which can target the MSCs for destruction prior to exerting their therapeutic effects (Rasmussen *et al.* 2007, Griffen *et al.* 2013, Pezzanite *et al.* 2015, Berglund *et al* 2017, Barrachina *et al.* 2020).

In order to find an MSC that would defer immune recognition, we studied various groups of horses with unique MSC types. In a previous study, we found two groups of horses, one with high levels of MHC II expression on the surface of their MSCs and one with low levels of MHC II expression (Kamm *et al.* 2019). Our previous study contained a subset of horses with low expressing MHC II MSCs who were also known to be universal blood donors (Aa, Ca, and Qa erythrocyte antigen negative) (Proverbio *et al.* 2020). None of the universal donor horses had MSCs with high levels of MHC II expression (Kamm *et al.* 2019). For this reason, we sought to determine if there was a link between being a universal blood donor and having MSCs with low antigen expression which may make them more immune privileged than a non-blood donor.

The broad aim of this study was to determine the behavior and effect of MSC interaction with ELA mis-matched responder leukocytes in an unactivated environment. The use of unactivated leukocytes would best demonstrate the degree of immune activation of leukocytes when they come into contact with allogeneic MSCs. We hypothesize that there will be significant differences in the interactions between our different MSC groups (MHC II - low expressing MSCs, MHC II- high expressing MSCs and universal blood donor MSCs) and the responder leukocytes.

4.4 Methods

Animals, blood-typing, and sample groups

Equine bone marrow was harvested from the sternebrae of Standardbred (n=18) and Thoroughbred (n=18) horses, and a Connemara (n=1) pony following ethics approval by the Massey University Animal Ethics Committee (MUAEC Protocol 15/13) as described in Kamm *et al.* 2019. All horses had no previous history of foreign cell administration including blood transfusion nor allogeneic MSC therapy. The horses were either owned independently or by Massey University, and informed consent for their use was granted by all parties.

All horses were blood typed for Aa, Ca, and Qa antigens. Five mL of blood was collected in anticoagulant tubes (ACD Tube, BD Vacutainer®, San Jose, CA, USA) for blood typing at the Equine Parentage and Animal Services Centre at

Massey University. A horse was considered a universal blood donor if it was negative for Aa, Ca, and Qa antigens (Hardy 2009, Snyder *et al.* 2012).

Only 10 horses of the original 37 that best fit the following criteria were utilized in further assays. MSCs were selected from the Standardbred and Thoroughbred groups according to their MHC class II expression and blood-type. The MHC II expression was determined and described in a previous study (Kamm *et al.* 2019) Three horses with the lowest MHC class II expression that were not universal blood-types were chosen to create an 'MHC class II-low' group. These MSCs were from Standardbreds (age 2-9 years). Three horses with the highest MHC class II expression that were not universal blood-donor types were chosen to create an 'MHC class II-high' group. These MSCs were from Thoroughbreds (age 4-6 years). Three samples of MSCs were randomly selected from the universal blood donor horses; all were Standardbreds (age 12-21 years). MSCs from one Connemara pony (age 21 years) were used. This horse also had peripheral blood taken for mononuclear, neutrophil and serum isolation as this breed is likely to have a different ELA haplotype than Thoroughbreds (Ranera *et al.* 2016). All horses' MSCs were tested in triplicate in each of the assays. Control assays (MSCs or immune cells cultured alone) were also performed in triplicate.

MSC isolation and culture

MSCs were isolated and cultured from bone marrow as described in Kamm *et al*. 2019. Passage 3 MSCs from the 10 horses chosen as samples (see 'sample groups' above) were plated in 48 or 96 well-plates (Greiner Bio-One, Austria)

(dependent on the assay) with MSC proliferation media containing alpha modification of Eagle's medium with 10% equine serum (Horse serum, Thermo Fisher®), 1% penicillin/streptomycin/amphotericin B and 2.5% 1M HEPES buffer. These MSCs were cultured for 48 hours prior to fresh media with leukocytes being added (see below). The MSCs were grown without fetal bovine serum (FBS) to minimize any immune reaction to xeno-contaminants as has previously been seen (Joswig *et al.* 2017).

The MSCs that we used in our studies had been confirmed as being a pure population of MSCs via marker expression analysis and trilineage testing (Kamm *et al.* 2019).

MSC haplotyping using microsatellite analysis

Haplotype analysis was performed as described previously (Holmes *et al.* 2019). DNA was isolated from each MSC donor using DNA isolation kits (DNeasy Blood and Tissue, Qiagen, Germantown, MD, USA).

6FAM or NED fluorescently labelled PCR primers for 12 horse intra-MHC microsatellite markers were amplified in six PCR reactions then pooled into four groups for fragment analysis. PCR products were combined with GeneScan Liz-500 size standard and electrophoresed on an ABI3700 instrument at the Cornell BioResource Center.

Fragment lengths were analyzed using GeneMarker v3.0.1 software (Softgenetics, State College, PA, USA) and exported into Excel for phasing.

Isolation of peripheral blood mononuclear cells

Following ethics approval by the Massey University Animal Ethics Committee (MUAEC Protocol 18/06), blood was aspirated aseptically from the left jugular vein of one Connemara pony. This was the same pony used for MSC isolation. Blood was placed in heparinized blood tubes (BD Vacutainer®, California, USA) for lymphocyte collection and subsequently processed using Lymphoprep™ (Density 1.077 g/mL, Alere Technologies AS, Norway). Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS) (Gibco™, Thermo Fisher®). Fifteen ml of Lymphoprep™ was placed in a centrifuge tube, and 30 ml of diluted blood was placed on top of the Lymphoprep™. The tube was centrifuged for 25 minutes at 1125 x g at low acceleration and without braking, thereby forming a density gradient. The lymphocyte- rich layer at the interface of the serum and the gradient agent was recovered and the neutrophil-rich pellet then used.

The peripheral blood mononuclear cell (PBMC) rich layer was washed with PBS. The PBMCs were then diluted in PBMC media composed of RPMI 1610 media (Gibco™, Thermo Fisher®) with 10% autologous equine serum, penicillin-streptomycin (100 ug/ml) (Sigma-Aldrich®, St Louis, MO, USA), and 2-Mercaptoethanol (0.1mM) (Gibco™, Thermo Fisher®).

Neutrophil isolation

A density gradient using fresh blood from a Connemara pony was performed in the presence of Lymphoprep™ (as described previously). The pellet from the density gradient was used to isolate neutrophils. Thirty-five mL of sterile water was added to the pellet. The centrifuge tube was inverted twice for mixing. Five mL of concentrated PBS (10X) (Gibco™, Thermo Fisher®) was then added, and the tube centrifuged for 10 minutes at 1000g. The supernatant was discarded and the neutrophil-rich pellet was washed in PBS. The neutrophils were cultured in media with alpha modification of Eagle's medium with 10% autologous equine serum, 1% penicillin/streptomycin/amphotericin B and 2.5% 1M HEPES buffer.

Serum collection

Blood from the Connemara pony was also collected into clot activating tubes (CAT BD Vacutainer®, San Jose, CA, USA). The tubes were incubated at 37°C for 1 hour prior to centrifugation at 3220g for 15 minutes (Bergseth *et al.* 2013). The serum was harvested and used in co-culture media within 90 minutes of harvest (active serum). Twenty ml of the serum was inactivated by heating to 56°C for 30 minutes (inactive serum) and used only in the complement assay.

MSC and PBMC co-culture

After the MSC were incubated for 48 hours media containing equine serum, the media was removed and PBMCs in PBMC media were added to the MSC wells. PBMCs were added in three different ratios of MSCs to PBMCs: 1:1, 1:10,

and 1:100. These ratios are based on published values typical for an equine joint during its normal cycle of reaction to an intra-articular MSC injection (de Grauw *et al* 2009, Ardanaz *et al.* 2016). PBMCs without MSCs +/- 2.5µg/ml of pokeweed mitogen as an activation agent (PWM; Sigma-Aldrich, Missouri USA) served as controls.

PBMCs and MSCs co-cultured at 37^0C with 5%CO_2 for 3 or 5 days prior to analysis.

Tritiated thymidine incorporation assay to assess lymphocyte proliferation

PBMCs and MSCs were co-cultured in triplicate for 3 or 5 days in a 96 well plate (Greiner Bio-One, Monroe, NC, USA) prior the addition of tritiated thymidine in order to determine if there was lymphocyte proliferation subsequent to MSC co-culture. Tritiated thymidine assays were performed using 1 x 10^5 cells per well in a 96 well plate (Greiner Bio-One, Monroe, NC, USA). One µCi of [methyl-^{3}H]-Thymidine (Perkin-Elmer, MA, USA) was added per well and cells were incubated for a further 18 hours. Cells were harvested onto glass fibre mats (Tomtec Harvester, Connecticut, USA) and cell-incorporated radioactivity measured using a scintillation counter (Wallac TriLux MicroBeta 1450, Finland) and reported as counts per minute (cpm).

Flow cytometry on PBMCs and MSCs

Flow cytometry was performed to assess changes in lymphocyte sub-populations and MSC antigen expression after co-culture. PBMCs and MSCs were tested just prior to co-culture (Day 0) and on Days 3 and 5 of co-culture. Cells were separated using a previously validated method of separation. The media was aspirated and placed in a vial for PBMC assessment. A detachment solution (StemPro® Accutase®, GibcoTM, Thermo Fisher®) was placed in the wells for 30 seconds. Then the detachment solution and loosely adherent cells were then aspirated and added to the PBMC sample. Cells that were identified as MSCs as seen by marker expression and FSC and SSC scatter were removed from analysis in the PBMC flow cytometry panel via gating schemes. The detachment solution was then applied and incubated for 10 minutes at 37°C. This sample was then aspirated and utilized for the MSC flow cytometry panel. Cells that were PBMCs as seen by marker expression and FSC and SSC scatter were removed from analysis in the MSC flow cytometry panel.

For lymphocyte analysis, antibodies against extracellular CD4 (CVS4, US Biological, Salem, MA, USA) (Hamza *et al*. 2011), CD8 (CVS8, BioRad, Hercules, CA, USA) (Robbin *et al*. 2011), CD21 (CA2.1D6, AbCam, Cambridge, UK) (Arzi *et al*. 2017), and CD25 (RND Systems, Minneapolis, MN, USA) (Hamza *et al*. 2011) were used in accordance with previous publications. Following permeabilization (FoxP3 Transcription Factor Staining Buffer, eBiosciences, San Diego, CA, USA), an intracellular antibody for FOXP3 (FJK-16s , eBiosciences, San Diego, CA, USA) (Hamza *et al*. 2011) was then used (Supplementary information).

MSCs were stained for MHC I (CVS22, BioRad, Hercules, CA, USA) and MHC II (CVS20, BioRad, Hercules, CA, USA) using antibodies used in previous publications (Carrade *et al*. 2011, Kamm *et al*. 2019). Dilution and conjugation for all antibodies are shown in the supplementary information.

Fixable viability dye (Efluor 780™, eBioscences™ , Thermo Fisher) was used to assess cell viability in flow cytometry assays (Kamm *et al*. 2019). Samples were measured using a flow cytometer (BD FACSVerse™, BD Biosciences, San Jose, CA, USA). All events in the sample were recorded for the leukocyte population and for the MSC population separately. The MSC and leukocyte populations were characterised using the gating hierarchy as shown in Supplementary Information using flow cytometry analysis software (Flowjo LLC, Oregon, USA).

MSC and neutrophil co-culture

Neutrophils and MSCs were co-cultured to determine the degree of neutrophil activation subsequent to their interaction. Following 48 hours incubation to allow for MSCs adherence to the plate with media containing equine serum, the media was removed, and fresh neutrophils (less than 2 hours post-blood draw) in neutrophil media (described above) were added to the MSC wells. Neutrophils were added in the same ratios described for PBMCs. Neutrophils alone +/- 2.5uM activation agent phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, Missouri, USA) (were cultured to serve as controls.

Neutrophils and MSCs were co-cultured at 37^0C with 5%CO_2 for 6 hours or 12 hours prior to analysis with flow cytometry.

Flow cytometry on neutrophils

Neutrophil activation was assessed after co-culture with autologous or allogeneic MSCs. After 6 or 12h of co-culture, 123-dihydrorhodamine (0.25ug/sample) was added to each well and incubated for 20 minutes in the dark at 37°C. The wells were the placed on ice for 10 minutes. The cells were then stained with viability dye (Efluor 780™). The entire sample was utilized for flow cytometric analysis. Gating was then used to separate MSCs from neutrophils (Supplmentary information) and fluorescence used for statistical analysis.

MSC and complement culture

Complement and the MSC samples were incubated together to determine if the complement had a cytotoxic effect on the autologous or allogeneic MSCs. Following 48 hours incubation to allow for MSCs adherence to the plate with media containing inactivated equine serum (Horse serum, Thermo Fisher), the media was removed and MSC proliferation media containing 30% active or inactivated serum was added. After 1 hour, MSCs were stained as described for flow cytometry.

Flow cytometry on MSCs cultured with complement

Flow cytometry was performed to assess changes in MSC viability after culture with complement. MSCs were tested after 1 hour of culture with active or inactivated complement. Fixable viability dye (Efluor 780™, eBioscences™ , Thermo Fisher) was used to assess cell viability in flow cytometry assays. Samples were measured using a BD FACSVerse™ (San Jose, CA, USA). Ten thousand events in a leukocyte gate were recorded.

Gene expression assay

Transcriptional analysis was performed on PBMCs and MSCs after 0, 3 or 5 days of co-culture using the nCounter Analysis System (NanoString, Seattle, WA, USA). Anabolic genes assessed were: transforming growth factor (TGF)-β1 protein, fibroblast growth factor (FGF), interleukin 1 receptor antagonist (IL-1RA), Indoleamine-pyrrole 2,3-dioxygenase (IDO1), CD59, hepatocyte growth factor (HGF), IL-10, IL-2, vascular endothelial growth factor 2 (VEGF2) and SOX2. Catabolic genes assessed were: tumor necrosis factor α (TNF-α), IL-1β, aggrecanases (ADAMSTS-4, ADAMSTS-5), matrix metalloproteinase (MMP)-13, chemokine ligand 2 (CCL2), C-X-C motif chemokine ligand 8 (CXCL8/IL-8), interferon γ (IFNγ), cyclooxygenase-2 (PTGS-2/ COX-2), and IL6. Two sets of gene-specific probes (along with a reporter probe and a capture probe) were designed by NanoString and their accession numbers are listed in the Supplementary Information. Total RNA (85±59 ng per sample) was hybridised using nCounter PlexSet-24 Reagent Pack according to the PlexSet™ Reagents User Manual. After hybridisation, samples were vertically pooled and were placed

on the automated nCounter Prep Station (NanoString) for purification and were immobilised in the cartridge. This cartridge was then transferred to the nCounter Digital Analyzer for data collection. Data analysis was performed with nSolver™ 4.0 Analysis Software according to user manual. All samples passed the quality control. Positive control normalization was carried out by using the geometric mean of the highest three positive counts. Reference gene normalization was calculated using the geometric mean of counts for the reference genes GUSB, PPIA, TBP, YWHAZ (Ragni *et al.* 2019).

Statistics

Summative and comparative statistical analyses were performed using statistical software R software (R, Version 3.4.3, R Core Development Team). PBMC population analysis, MSC markers, PBMC proliferation, neutrophil activation, MSC survival, and gene expression data were not normally distributed as determined by Shapiro-Wilk's testing. Data transformation did not produce normally distributed data. MHC I and MHC II marker expression MSCs for the autologous sample and the universal blood donor, MHCII high and MHC II low groups were compared for each of the time points and at each of the three different ratios of MSCs to PBMCs (1:1, 1:10, and 1:100) by Kruskal-Wallis ANOVA by ranks. If a significant difference was identified, post-hoc comparisons were then performed using the Benjamini-Hochberg method. Significance was identified at $p<0.05$.

4.5 Results

MSC Haplotyping

The Connemara pony was of a different ELA haplotype than all of the other horses utilized in this study (Supplemental information). Therefore, all allogeneic co-cultures were ELA mis-matched.

MSC and PBMC Co-culture

MHC I expression was consistently high on all MSC samples while MHC II expression varied.

The median value of MHC I expression was greater than 90% for each of the sample groups at Day 0, 3 and 5 of culture (Table 6, Figure 16a, b and c). There were significant differences between the groups as shown in Figure 16a, b, and c, but due to all the MSCs expressing a high level of MHC I, this won't be discussed further.

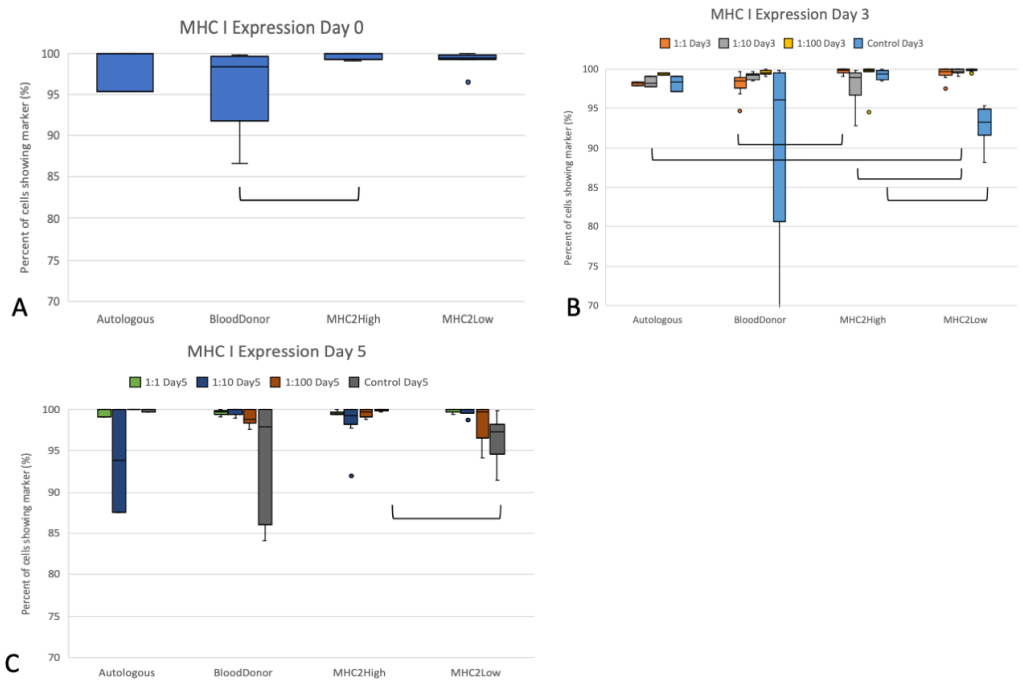

Figure 16. Analysis of MHC I expression on MSCs co-cultured with PBMCs.

MHC I expression on MSCs (a) is shown prior to co-culture, (b) after 3 days in co-culture, and (c) at 5 days of co-culture. MHC I expression increased on MSCs in co-culture as compared to their control (non-co-cultured) value (14b and c). Autologous, autologous MSCs (n=1); blood donor, universal blood donor MSCs (n=3); MHC II high, MHC II-high MSCs (n=3), MHC II Low, MHC II-low MSCs (n=3). All tests performed in triplicate.

Table 6. MHC I expression on MSCs prior to and during co-culture with PBMCs.

	DAY 0	**DAY 3**				**DAY 5**			
	Control	1:1	1:10	1:100	Control	1:1	1:10	1:100	Control
Autologous	100 (2.3)[ab]	98.1 (0.2)[a]	98.2 (0.7)[a,b]	99.4 (0.2)[a]	98.3 (1.0)[a,b]	100 (0.5)[a]	93.8 (6.3)[a]	100 (0)[a]	99.9 (0.2)[a,b]
Blood donor	98.3 (6.1)[a]	98.5 (0.5)[a]	99.2 (0.5)[a,b,c]	99.5 (0.5)[a]	96.1 (18.2)[a,b]	99.6 (0.3)[a]	100 (0.4)[a]	98.8 (1.4)[a]	97.9 (12.0)[a,b]
MHC II-high	100 (0.6)[b]	99.8 (0.2)[a]	98.9 (2.4)[a,b]	99.8 (0.2)[a]	99.4 (1.1)[a]	99.5 (0.4)[a]	99.2 (1.4)[a]	99.7 (0.8)[a]	100 (0.2)[a]

MHC II-low	99.5 (0.6)[ab]	99.7 (0.5)[a]	99.7 (0.5)[c]	99.8 (0.1)[a]	93.2 (3.0)[b]	99.9 (0.2)[a]	100 (0.5)[a]	99.7 (2.7)[a]	97.2 (1.6)[b]

Median percent of MSCs expressing MHC I and the interquartile range (IQR) are shown for control cultures (no PBMCs) and for co-cultures with the ratio of MSC:PBMC. Values within each column which have different letters are significantly different ($p<0.05$). Autologous, autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3), MHC II Low, MHC II-low MSC co-culture (n=3). Tests performed in triplicate.

MHC II expression was variable at time 0 with the MHC II-high group expressing a significantly greater amount of MHC II antigen ($p<0.05$) on their surface as compared to the other MSC groups (Figure 17, Table 7). In co-culture with PBMCs at day 3 and 5, MSC MHC II expression increased greatly for the universal blood donor and MHC II-low MSC co-cultures. MHC II expression was significantly higher on the blood donor MSCs as compared to the autologous and MHC II-high groups when co-cultured with PBMCs ($p<0.05$ for both comparisons across all ratios) (Figure 17, Table 7). The MHC II-low group had significantly greater MHC II expression in co-culture as compared to the autologous samples ($p<0.02$ for both day 3 and day 5) and the MHC II-high group at day 3 ($p<0.001$). Additionally, for both the blood donor and the MHC II-low co-culture groups, MHC II expression was significantly higher when the MSCs were co-cultured with PBMCs as compared to their control (MSCs cultured alone) values at day 3 and 5 ($p<0.001$) (Figure 17, Table 7). MHC II expression was not significantly different between the control and co-culture MSCs for the MHC II-high and autologous samples.

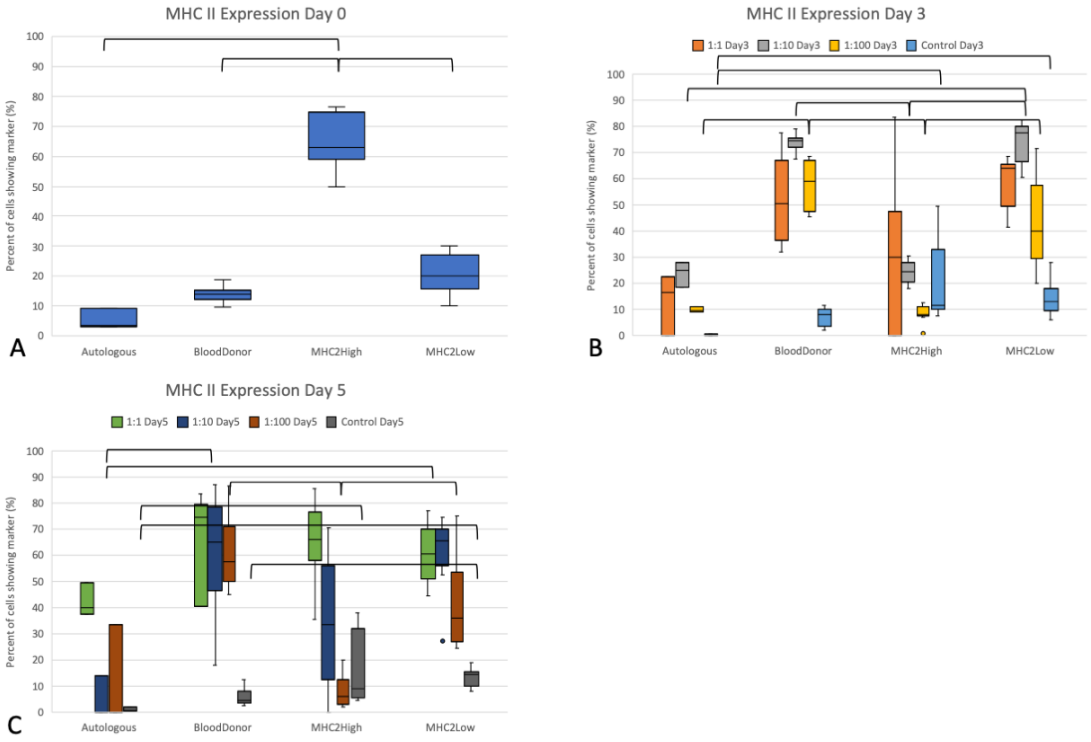

Figure 17. Analysis of MHC II expression on MSCs co-cultured with PBMCs.

MHC II expression on MSCs is shown prior to co-culture (a), after 3 days in co-culture (b), and at 5 days of co-culture (c). Increased levels of MHC II expression were seen when MSCs and lymphocytes were co-cultured at low ratios. Autologous, autologous MSCs (n=1); blood donor, universal blood donor MSCs (n=3); MHC II high, MHC II-high MSCs (n=3), MHC II Low, MHC II-low MSCs (n=3). Tests performed in triplicate.

Table 7. MHC II expression on MSCs prior to and during co-culture with PBMCs.

	DAY O	**DAY 3**				**DAY 5**			
	Control	1:1	1:10	1:100	Control	1:1	1:10	1:100	Control
Autologous	3.6 (3.2) [a]	16.8 (11.3) [a]	25.0 (4.8) [a,b]	9.7 (1.1) [a,c]	0.42 (0.28) [a]	40.0 (6.1) [a]	0 (7.2) [a]	0 (16.6) [a,b]	0.7 (0.8) [a]
Blood donor	14.2 (2.8) [a]	50.7 (28.5) [a]	74.4 (2.2) [b,c]	59.1 (18.0) [b]	8.0 (5.2) [a,b]	74.2 (37.4) [a]	65.1 (27.9) [b]	57.3 (10.2) [a]	4.6 (3.4) [a,b]
MHC II-high	62.7 (0.6) [b]	30.1 (42.1) [a]	24.3 (7.1) [a]	8.3 (2.9) [a]	11.4 (10.8) [b]	66.1 (14.0) [a]	33.3 (24.5) [a,b]	6.0 (7.4) [b]	9.0 (21.5) [b,c]

MHC II-low	20.2 (7.2) [a]	63.7 (11.3) [a]	77.2 (8.7)[c]	40.0 (19.6)[b,c]	13.0 (7.9)[b]	60.7 (13.6) [a]	65.6 (8.0)[b]	36.2 (21.3)[a]	14.5 (4.8)[c]

Median percent of MSCs expressing MHC II and the IQR is shown for control cultures (no PBMCs) and for co-cultures with the ratio of MSC:PBMC. Values within each column which have different letters are significantly different ($p<0.05$). Autologous, autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3), MHC II Low, MHC II-low MSC co-culture (n=3).

Lymphocyte activation was greater in the presence of MHC class II-high MSCs and lower in MHC class II-low MSCs as compared to autologous MSC co-cultures.

When MSCs and PMBCs were co-cultured in the presence of tritiated thymidine to assess the level of lymphocyte proliferation, significant differences were observed between MSC groups (Figure 18, Table 8). At Day 3 of co-culture, autologous MSCs had less tritiated thymidine incorporation linked to less PBMC proliferation than the MHC II-high co-culture when all ratios were combined ($p=0.028$) and at ratio 1:10 ($p=0.016$).

At Day 5 of co-culture, MHC class II-low MSCs were associated with significantly less PBMC proliferation than the autologous MSCs across all ratios ($p=0.029$) and was significantly less at ratio 1:1 ($p=0.041$) (Table 8). MHC class II-low MSCs had an activation level similar to PBMCs alone and was not associated with a significantly greater amount of proliferation at any ratio at Day 3 nor Day 5. The other two allogeneic groups, and the autologous MSCs had significantly greater activation as compared to PBMCs alone at least one time point/ratio combination.

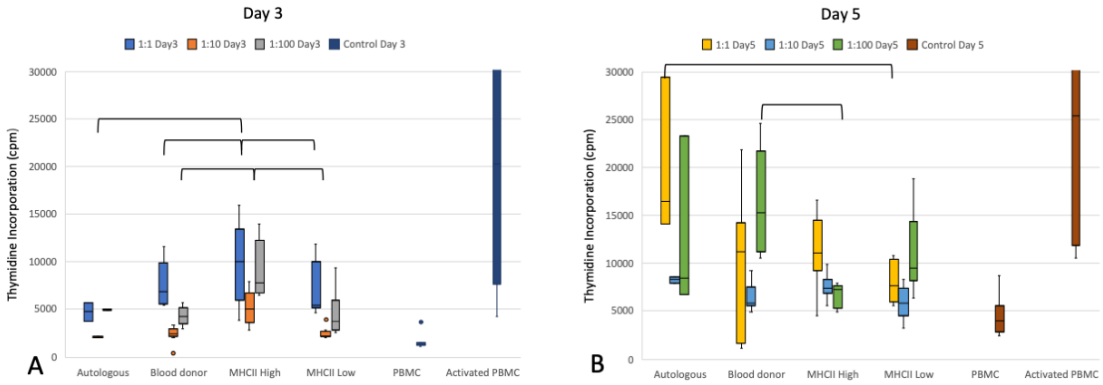

Figure 18. PBMC proliferation in co-culture with MSCs at day 3 (a) and day 5 (b).

MHC II-high MSCs caused significantly greater PBMC proliferation on day 3 than the autologous group. Autologous MSCs caused significantly greater PBMC proliferation than the MHC II-low co-cultures at day 5. Significant differences between the co-culture groups only are shown with brackets. Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3); PBMC, PBMCs alone (n=3); activated PBMC, PBMCs activated with 2.5µg/ml of PWM (n=3). All tests were performed in triplicate.

Table 8. Lymphocyte activation during co-culture with MSCs.

	Day 3				Day 5			
	1:1	1:10	1:100	Control	1:1	1:10	1:100	Control
Autologous	4795 (986)[a]	2092 (109)[a]	4973 (86)[a,b]	-	16435 (7688)[a,#]	8349 (272)[a,#]	8473 (8292) [a,b]	-
Blood donor	6882 (3570)[a,#]	2449 (494)[a]	4319 (1162)[a]	-	11156 (12090)[a,b]	5818 (1572)[a]	15215 (7230)[a,#]	-
MHC II-high	9986 (6166)[a,#]	4996 (2114)[b]	7805 (5145)[b,#]	-	11135 (3348)[a,b]	7416 (1110)[a,#]	7241 (2015)[b]	-
MHC II-low	5380 (4573) [a]	2202 (317)[a]	3800 (2815)[a]	-	7686 (3961)[b]	5787 (1819)[a]	9493 (5314) [a,b]	-
PBMC	-	-	-	1251 (226)	-	-	-	4048 (2513)
Activated PBMC	-	-	-	20237 (27350)	-	-	-	25342 (29759)

Median percent of lymphocytes activated and IQR is shown for co-cultures with the ratio of MSC:PBMC and for control cultures with PBMCs cultured alone with or without activating agent. Values within each column which have different letters

are significantly different ($p<0.05$). Values that are significantly greater than the PBMC alone are shown with a #. Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3); lymphocyte, lymphocytes alone (n=3); activated lymphocyte, lymphocytes activated with 2.5µg/ml of PWM (n=3). All tests were performed in triplicate.

CD4 lymphocyte counts decreased over time in co-culture

Flow cytometric analysis of the PBMCs at Day 3 and Day 5 of co-culture showed a decrease in CD4 populations over time in co-culture while this count rose in lymphocyte only controls (Figure 19). There were significantly less CD4 lymphocytes in all of the allogeneic MSC co-cultures at day 5 as compared to lymphocytes cultured alone ($p<0.05$).

When the co-culture groups were compared, there were significantly less CD4 lymphocytes in universal blood donor and MHC II-low co-cultures as compared to MHC II-high co-cultures at day 3 in the 1:10 ratio ($p=0.005$ and $p<0.001$, respectively). The decrease in CD4 lymphocytes was also seen at the 1:100 ratio for universal blood donor MSC co-cultures as compared to MHC II-high ($p=0.013$). Less CD4 lymphocytes were seen in MHC II-low co-cultures as compared to autologous cultures at day 3 at ratio 1:10 ($p<0.001$). Similarly, there were significantly less CD4 lymphocytes in blood donor and MHC II-low co-cultures as compared to MHC II-high co-cultures at day 5 in the 1:10 ratio and 1:100 ratios ($p=0.034$ and $p=0.037$ at 1:10 and $p=0.035$ and $p=0.005$ at 1:100, respectively).

Activation of CD4 lymphocytes as seen by expression of CD25 was similar for most groups of allogeneic MSCs, the autologous co-cultures, and for

lymphocytes cultured alone. This was true except for the universal blood donor co-cultures which contained significantly more activated CD4 lymphocytes than MHC II-high, MHC II-low (ratio 1:1 on day 3, p=0.021 and p=0.006, respectively), and autologous MSC co-cultures (day 3 at 1:100, p=0.034; day 5 at 1:1, p=0.028) (Figure 19).

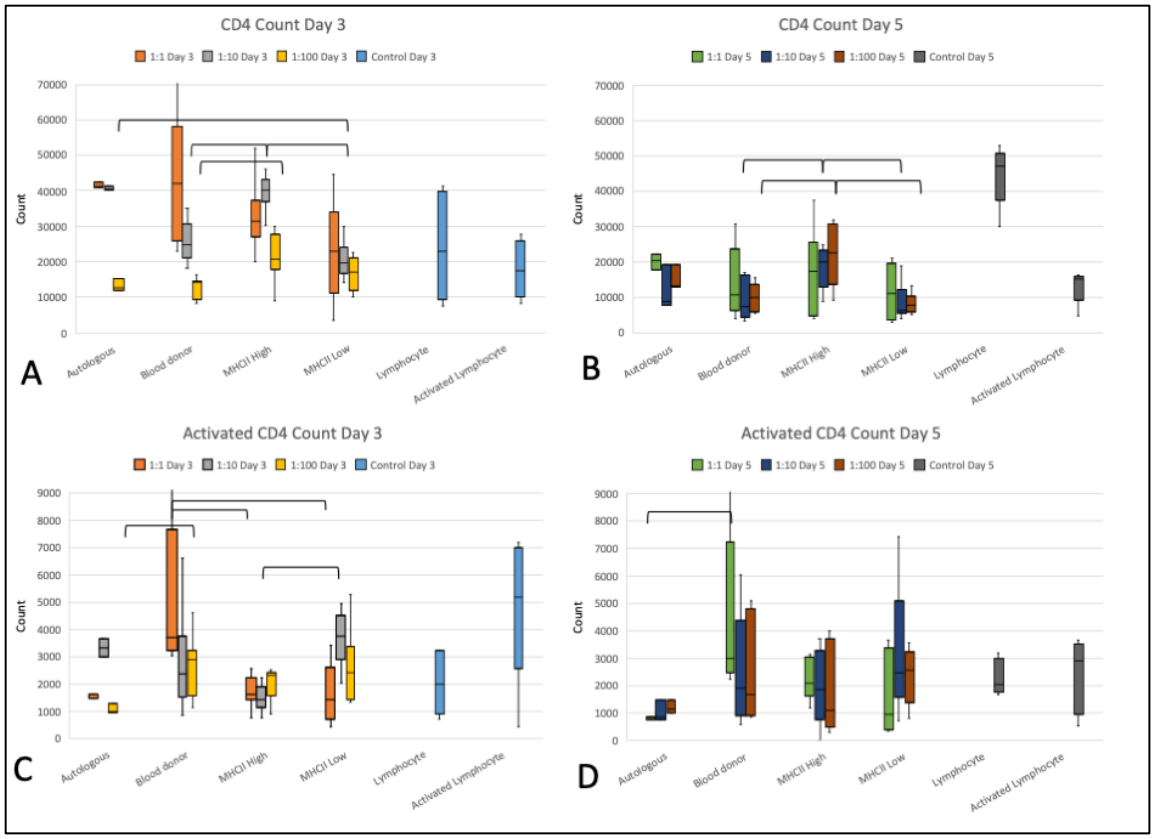

Figure 19. Total CD4 lymphocyte counts.

Total CD4 lymphocyte counts.at day 3 (a) and day 5 (b). Activated CD4 lymphocyte count is shown at day 3 (c) and day 5 (d). Significant differences between the co-culture groups are shown with brackets. Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3); lymphocyte, lymphocytes alone (n=3); activated lymphocyte, lymphocytes activated with 2.5µg/ml of PWM (n=3). All tests were performed in triplicate.

CD8 lymphocyte counts in co-culture decreased over time

Median CD8 lymphocyte counts decreased from day 3 to 5 when co-cultured with autologous or allogeneic MSCs (Figure 20). At day 3, co-culture with MHC II-low MSCs led to a significantly lower number of CD8 lymphocytes as compared to MHC II-high co-culture groups at ratios 1:10 and 1:100 ($p<0.001$ and $p=0.045$, respectively).

Activation of CD8 lymphocytes as shown by expression of CD25 was generally low and similar to lymphocytes cultured alone. Some significant differences were seen between the groups as shown in Figure 5, though none of these differences were consistent from one time point to the next nor across more than one ratio of MSC:PBMC.

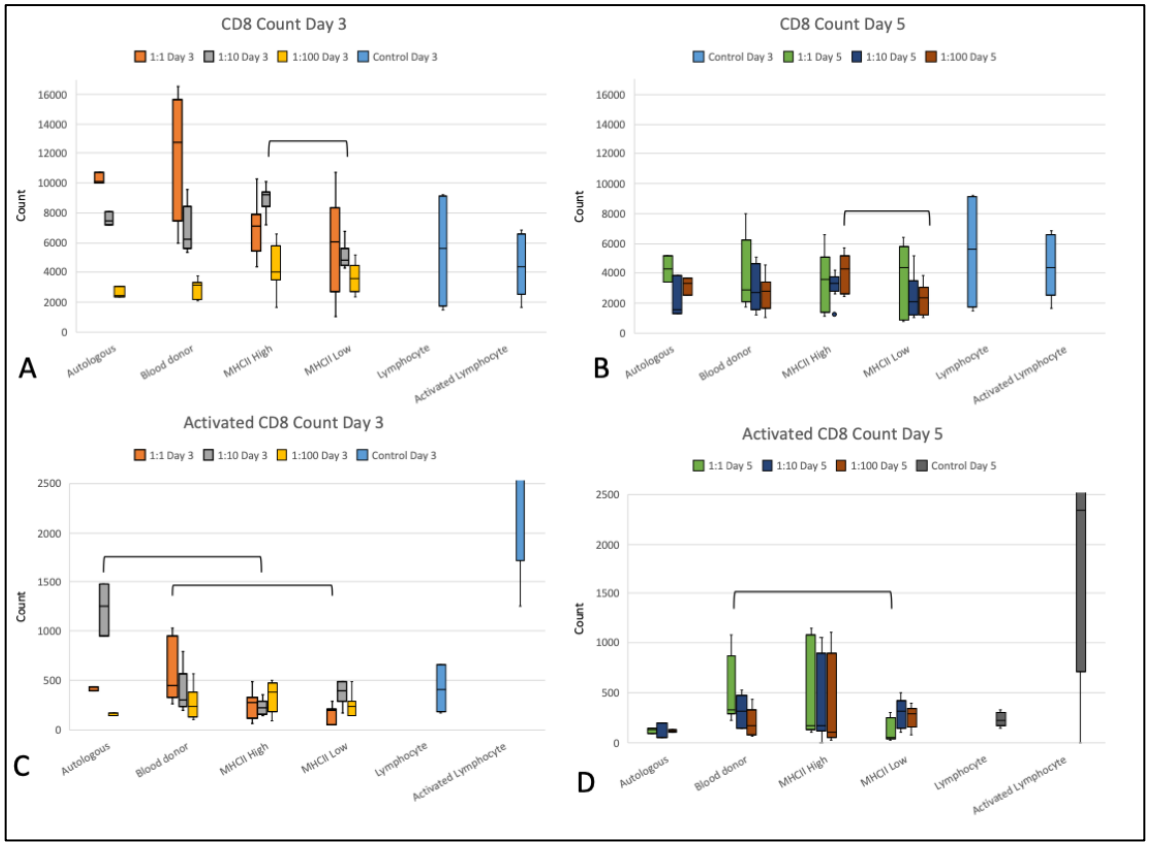

Figure 20. Total count of CD8+ lymphocytes.

Total CD8+ lymphocyte counts are shown at day 3 (a) and day 5 (b) of co-culture. Activated CD8+ lymphocytes in co-culture with MSCs are shown at day 3 (c) and day 5 (d). Data for co-cultures, activated lymphocytes, and lymphocytes alone is shown at Day 3 and 5. Significant differences between the co-culture groups o are shown with brackets. Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3); lymphocyte, lymphocytes alone (n=3); activated lymphocyte, lymphocytes activated with 2.5µg/ml of PWM (n=3). All tests were performed in triplicate.

B cells in co-culture were consistent over the culture period

Flow cytometric evaluation of B cell populations as shown by CD21 marker expression, showed that the total number of B lymphocytes remained relatively constant between day 3 and day 5 in co-culture with MSCs while control B cells (cultured without MSCs), showed an increase from day 3 to day 5 (Figure 21). Activation of the lymphocytes with PWM did not lead to B cell proliferation (Figure 21). There were a greater number of B cells when co-culture ratios were low as the 1:1 ratio across all co-cultures had a significantly greater number of B cells than the 1:10 ratio at day 3 and day 5 ($p<0.001$) and a significantly greater number of B cells than the 1:100 ratio at day 3 ($p<0.001$).

MHC class-high co-cultures had significantly greater B cell counts at day 3, (ratio 1:10 than all other co-culture groups; $p=0.029$, $p=0.012$, $p=0.026$, for autologous, universal blood donor and MHC II-low, respectively). At day 3 ratio 1:100, MHC II-low co-cultures caused greater B cell count than autologous and MHC II-high co-cultures ($p=0.032$ and $p=0.020$ respectively). There were no significant differences between co-culture groups at day 5.

Treg lymphocytes increase in universal blood donor and MHC class II-low MSC co-culture with PBMCs

Regulatory T lymphocytes (Tregs) were identified using cell surface antibodies for CD4 and CD25 and intracellular antibodies for FOXP3. Tregs were low in PBMC only cultures (median was <1% of CD4 cells at day 3 and day 5 of culture) and high in PWM activated cultures (median >60% of CD4 cells at day 3 and day 5 of culture) (Figure 19). The lowest ratio of MSCs:lymphocytes across all co-culture groups was consistently associated with a greater number of Tregs as compared to those co-cultures containing fewer MSCs as compared to lymphocytes ($p<0.001$ for 1:1 to 1:10 and 1:1 to 1:100 at day 3) ($p=0.002$ for 1:1 to 1:10 and $p=0.013$ for 1:1 to 1:100 at day 5).

Universal blood donor MSCs were associated with a significant increase in the Treg population as compared to other co-cultures (MHC class II-high MSCs at day 3, ratio 1:10, $p<0.001$; autologous MSCs at day 5, ratio 1:100, $p=0.03$) (Figure 21). MHC II-high co-cultures caused a significantly greater number of Tregs as compared to autologous MSCs at day 3, ratio 1:100 ($p=0.003$).

Figure 22 shows a summary of the population dynamics of the CD4, CD8, B cell and Treg lymphocytes when co-cultured with the MSC groups.

CD8moderate/ CD25+ lymphocytes may be a gamma delta T lymphocytes

A subset of cells not characterizable as CD4 or CD8 T lymphocytes, B cells or Tregs were apparent upon analysis using antibodies for CD8 and CD25. These cells were moderate in their expression of CD8 and strong in their expression of CD25 and likely fit the description of gamma delta (γδ) T cells (Figure 21). The absolute number of these cells was high on day 0 (median across all cultures was 31900 cells/well) but decreased by day 3 to less than 3500 across all cultures (Figure 21). At day 3 of co-culture, MHC class II-high co-cultures had significantly greater numbers of these possible γδ T lymphocytes as compared to universal blood donor and MHC class II-low co-cultures across more than one MSC:PBMC ratio (Figure 21). At day 5, MHC class II-high co-cultures had significantly greater numbers of these lymphocytes than universal blood donor and MHC II-low co-cultures at ratio 1:100 (Figure 21).

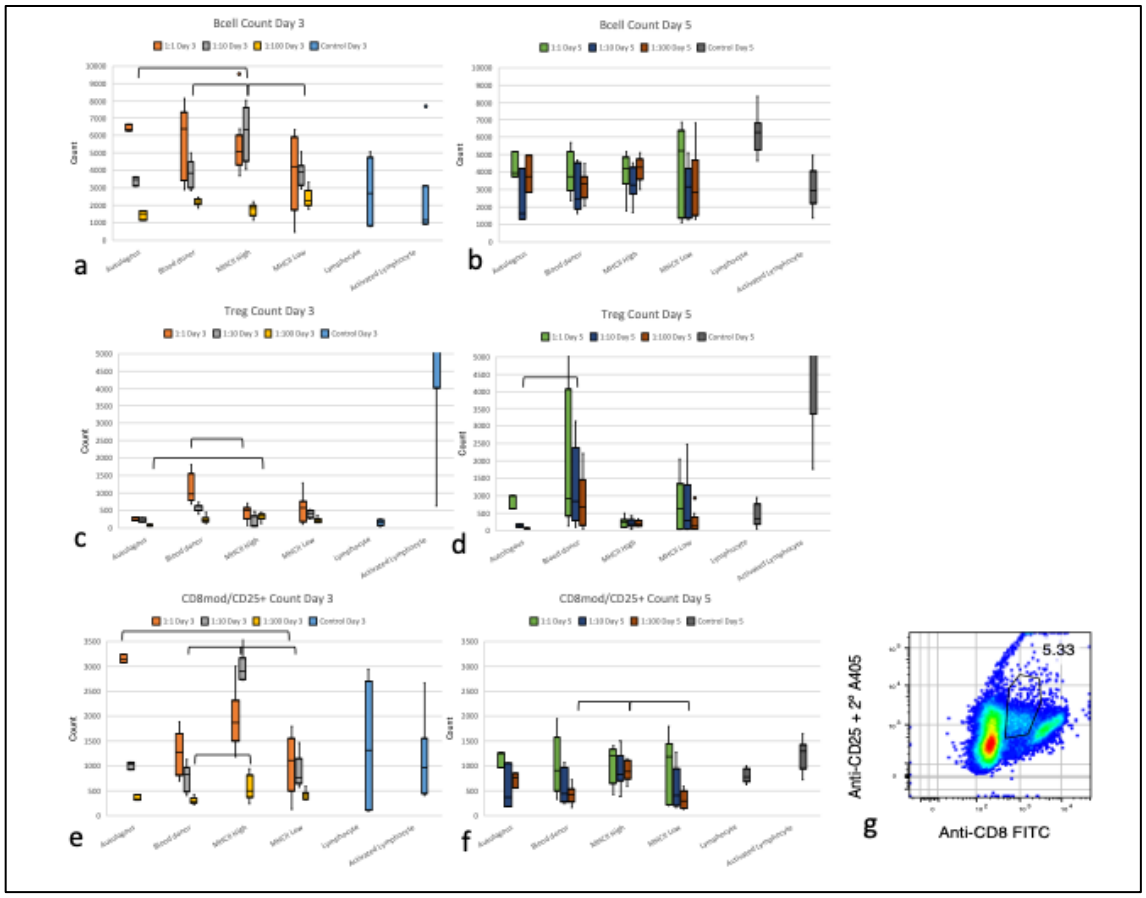

Figure 21. B cell, T cell and CD8moderate/ CD25+ lymphocyte counts

B cell, T cell and CD8moderate/ CD25+ lymphocyte counts are shown in co-culture at day 3 (a,c,e) and day 5 (b,d,f). B cell counts increased in PBMC only wells over time. Lower ratios of MSCs to PBMCs caused greater B cell counts. Significant differences between the co-culture groups are shown with brackets. Tregs were increased in 1:1 MSC:PBMC co-cultures as compared to cultures with a greater ratio of PBMCs. Blood donor co-cultures had consistently higher Tregs than other co-cultures. Total CD8moderate/ CD25+ lymphocyte count illustrates elevated counts in MHC II-high co-cultures at day 3 (e) and 5 (f). A representative sample of CD8moderate/ CD25+ lymphocytes is shown as the gated sample (black circle) (g). Significant differences between the co-culture groups are shown with brackets. Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3); lymphocyte, lymphocytes alone (n=3); activated lymphocyte, lymphocytes activated with 2.5µg/ml of PWM (n=3). All tests were performed in triplicate.

Co-culture MSC group	Lymphocyte Proliferation	CD4	Activated CD4	CD8	Activated CD8	Bcell	Treg
Blood donor	-	-		-	-	-	
MHC-II High			-	-			
MHC-II Low			-	-	-	-	-

Figure 22. Lymphocyte population dynamics when co-cultured with MSCs.

Red arrows show significant increases in inflammatory-type lymphocytes. Green arrows show significant decreases in inflammatory-type lymphocytes or significant increases in anti-inflammatory-type lymphocytes. Black dashes show no significant change as compared to autologous MSC co-cultures.

CD4-/CD8-/CD21-/CD25- PBMCs comprise approximately 10% of the population

A final group of PBMCs was consistently identified. These were negative to all of the antibodies used in our panel. These unbound cells may be natural killer cells which are known to lack expression of the antibodies in our panel. There were no significant differences between the co-culture groups at day 3 and 5 (Supplementary information).

Neutrophil and MSC co-culture

Neutrophil activation was seen in co-cultures with allogeneic MSCs at time point 6 hours but had dissipated by time point 12h

Some allogeneic MSC groups caused significant activation of neutrophils as compared to autologous MSC co-cultures at 6 hours of co-culture (Figure 23).

Significant differences are shown in Figure 23 and Table 9. At the 6 hour time point, median levels of activation were low for all groups with a median percent of neutrophils that were activated at less that 6%. Both the universal blood donor and the MHC II-high group showed significant increases in neutrophil activation over the other groups at one of the MSC:Neutrophil ratios (Figure 23, Table 9). At the 12 hour co-culture time point, median neutrophil activation levels increased for most co-cultures and for the neutrophils cultured alone. MHC II-high co-cultures showed increased activation as compared to the universal blood donor co-cultures (Figure 23, Table 9).

Table 9. Neutrophil activation in co-culture with MSCs.

	6 hours				12 hours			
	1:1	1:10	1:100	Control	1:1	1:10	1:100	Control
Autologous	0.53 (0.09)[a]	0.50 (0.05)[a]	0.66 (0.17)[a]	-	10.3 (2.4)[a,b]	4.5 (0.4)[a,b]	5.1 (0.4)[a,b]	-
Blood donor	5.76 (3.17)[b]	2.40 (1.75)[b]	1.00 (0.34)[a]	-	5.1 (1.5)[a]	1.0 (0.6)[a]	3.2 (2.4)[a]	-
MHC II-high	3.04 (0.08)[b,c]	0.92 (0.30)[a,b]	1.42 (0.31)[b]	-	12.5 (12.9)[b]	6.4 (6.0)[b]	9.3 (2.3)[b]	-
MHC II-low	1.78 (0.82)[a,c]	0.99 (0.41)[a,b]	0.99 (0.37)[a]	-	6.4 (3.7)[a,b]	3.2 (1.7)[a,b]	5.4 (2.8)[a,b]	-
PBMC	-	-	-	0.21 (0.20)	-	-	-	9.3 (9.8)
Activated PBMC	-	-	-	99.9 (0.1)	-	-	-	99.5 (1.6)

Median percent of neutrophils activated and the IQR is shown for co-cultures with the ratio of MSCs:Neutrophils and for neutrophils cultured alone. Values within each column which have different letters are significantly different ($p<0.05$). Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3); Neutrophils, Neutrophils alone; Activated neutrophils, Neutrophils activated with 2.5uM phorbol myristate acetate (PMA).

MSC survival had a median of >95% when co-cultured with neutrophils

MSC survival was assessed using flow cytometry at time 6h and 12h of co-culture with neutrophils (Figure 23). At time point 6h, no MSC co-cultures had significant loss of viability as compared to those MSCs cultured without neutrophils. At 12h, only the MHC class II-high groups had a significant loss of viability compared to cultures without neutrophils. The median (IQR) for 1:1 ratio, 1:100 ratio, and MSCs cultured without neutrophils was 96.7% (1.1), 95.1% (0.5), 98.1% (0.8), respectively.

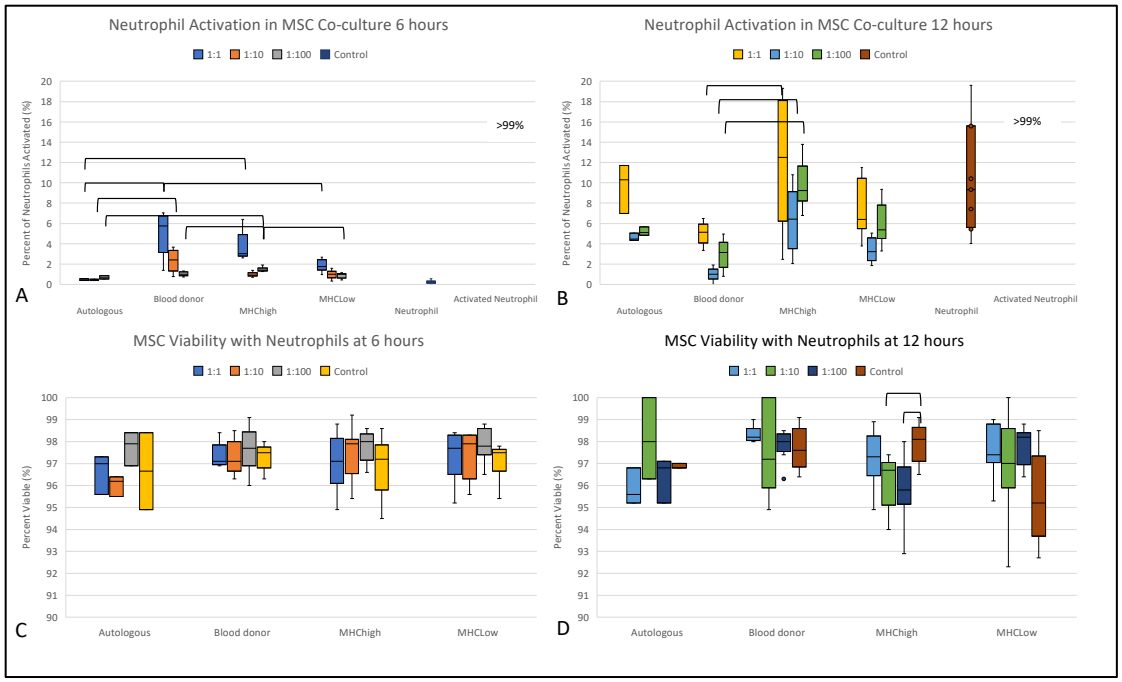

Figure 23. Neutrophil activation and MSC survival in co-culture with neutrophils.

Neutrophil activation and MSC survival in co-culture with neutrophils is shown at time points 6h (a,c) and 12h (b,d). Ratios of 1 MSC:1 Neutrophil, 1MSC:10 Neutrophils and 1 MSC:100 Neutrophils and control MSCs (no neutrophils) are shown. At time 6h, universal blood donor and MHC II-high co-cultures had ratios where activation was significantly greater than the autologous co-culture. At time 12h, only the MCH II-high co-cultures had greater activation as compared to the universal blood donor (BD) co-cultures. Median MSC survival was greater than 95% when co-cultured with neutrophils at time point 6h (c) and 12h (d). The only

group with significant viability loss with neutrophils as compared to MSCs cultured alone was the MHC II-high group at 12h. Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3); neutrophil, neutrophils alone (n=3); activated neutrophil, neutrophils activated with 2.5uM PMA (n=3). All tests were performed in triplicate.

Complement-mediated effects on MSCs

Complement mediated MSC death was minimal for both the autologous and allogeneic MSCs.

Complement had little effect on MSC viability after one hour in co-culture. Only the MHC II-high MSCs had a significant loss of viability when cultured with complement as compared to those MSCs cultured without active complement (Supplementary information). The median (IQR) percent survival for MHC II-high MSCs cultured with complement and the MHC II-high MSCs cultured with inactivated complement was 88.3% (3.3) and 90.5% (2.2), respectively. (Supplementary information).

Gene expression in MSC and PBMC co-culture

Higher anabolic and anti-inflammatory gene expression is seen in MHC class II-low and universal blood donor MSCs when co-cultured with PBMCs

Gene expression for 10 anti-inflammatory or anabolic genes was measured at day 3 and 5. MHC class II-low and universal blood donor MSCs were consistently higher in their gene expression as compared to autologous and MHC class II-high MSCs. MHC class II-low MSCs had significantly greater gene expression than autologous and MHC II-high MSCs for the genes encoding CD59,

FGF-2, HGF, IDO, IL-10, IL-RA, IL-2, SOX2, and TGF-β1. Universal blood donor MSCs had significantly greater gene expression than autologous and MHC II-high MSCs for the genes encoding FGF-2, HGF, IDO, IL-10, IL-RA, SOX2, and TGF-β1 (Table 10).

Table 10. Anabolic gene expression in MSCs co-culture with PBMCs.

		Autologous	Blood donor	MHC II-high	MHC II-low
CD59	1:1	3.53 (0.69)[a]	4.24 (3.08)[a]	5.42 (2.69)[a,b]	14.5 (10.4)[b]
Day 3	Control	3.68 (0.98)	4.55 (4.52)	7.75 (7.03)	2.31 (1.08)
CD59	1:1	9.54 (27.5)	11.5 (10.1)	5.18 (7.53)	9.08 (4.61)
Day 5	Control	4.41 (0.77)	2.60 (1.90)	4.74 (2.69)	4.01 (2.08)
FGF-2	1:1	852 (21.8) [a,c]	5910 (5300)[b]	450 (112)[a]	2470(2150)[b,c]
Day 3	Control	919 (192)	5100 (3690)	955 (1110)	4150 (5550)
FGF-2	1:1	1450 (307) [a]	1390 (1260)[a]	473 (397)[b]	1100 (451)[a,c]
Day 5	Control	1100 (1040)	3570 (3590)	1820 (1520)	2850 (6170)
HGF	1:1	41.2 (3.55)[a]	307 (137)[b]	76.4 (64.4)[c]	386 (184)[b]
Day 3	Control	166 (43.4)	263 (391)	136 (48.9)	572 (440)
HGF	1:1	85.8 (17.5)	132 (55.0)	113 (52.3)	119 (14.9)
Day 5	Control	189 (80.5)	296 (236)	356 (172)	1050 (519)
IDO	1:1	123 (26.8)[a]	3850 (1220)[b]	207 (154)[a]	3510 (2170)[b]
Day3	Control	3.90 (1.41)	8.01 (5.27)	45.7 (51.1)	9.90 (11.3)
IDO	1:1	485 (222)[a]	1420 (1100)[b]	205 (225)[a]	2050 (1900)[b]
Day 5	Control	2.16 (1.42)	4.55 (8.86)	5.08 (4.62)	3.29 (2.22)
IL-10	1:1	17.0 (1.45)[a]	57.1 (88.4)[b]	32.0 (7.3)[a]	66.4 (45.5)[b]
Day 3	Control	1.71 (1.45)	4.32 (1.55)	16.5 (12.9)	24.7 (22.1)
IL-10	1:1	29.7 (13.5)	258 (214)	62.4 (17.6)	207 (230)
Day 5	Control	5.83 (2.50)	6.71 (3.10)	5.74 (4.76)	7.15 (4.08)
IL-ra	1:1	27.0 (3.0) [a,b]	76.0 (353)[b,c]	24.4 (14.5)[a]	725 (500)[c]
Day 3	Control	2.0 (6.5)	5.7 (3.9)	14.9 (39.3)	18.5 (21.2)
IL-ra	1:1	78.4 (33.6) [a,b]	65.6 (39.2) [a,b]	29.6 (14.6) [a]	106 (53.9) [b]
Day 5	Control	3.2 (2.1)	6.4 (2.3)	5.1 (7.9)	3.2 (2.6)
IL-2	1:1	4.7 (0.4) [a]	25.5 (12.3) [a,b]	10.7 (15.9) [a]	38.4 (11.6) [b]
Day 3	Control	2.3 (0.5)	3.3 (1.1)	7.8 (1.6)	5.9 (1.3)
IL-2	1:1	4.6 (5.2)	11.3 (9.2)	18.5 (4.7)	11.9 (5.6)
Day 5	Control	2.2 (0.7)	2.5 (3.0)	1.9 (1.4)	6.0 (4.5)
Sox2	1:1	1.0 (0.1) [a,c]	3.6 (2.9) [b]	1.0 (0) [c]	2.5 (2.7) [a,b]
Day 3	Control	1.0 (0.2)	1.2 (0.3)	3.7 (10.6)	1.1 (0.2)
Sox2	1:1	5.7 (9.7) [a,b]	1.0 (0.6) [a,b]	1.9 (2.4) [a]	1.0 (0) [b]
Day 5	Control	1.0 (0)	1.0 (0)	1.0 (0)	1.0 (0)
TGFB1	1:1	299 (20) [a]	625 (86) [b]	334 (89) [a]	556 (294) [b]
Day 3	Control	422 (71)	255 (54)	283 (112)	249 (46)
TGFB1	1:1	411 (101)	601 (320)	460 (213)	669 (384)

Day 5	Control	575 (38)	428 (47)	573 (174)	409 (36)
VEGF	1:1	19900 (500)	20700 (19700)	26100 (6600)	21600 (4460)
Day 3	Control	2880 (550)	6030 (3380)	3200 (4900)	8170 (7780)
VEGF	1:1	18200 (4600)	18100 (14600)	23000 (6800)	14900 (3570)
Day 5	Control	4130 (406)	4410 (1650)	5520 (780)	3930 (5490)

Median RNA copy number and IQR is shown for co-cultures with PBMCs and for MSCs cultured alone (control) at day 3 and 5 of co-culture. Values for co-cultured MSC gene expression (within each row) which are significantly different are marked with different letters ($p<0.05$). Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II low, MHC II-low MSC co-culture (n=3). All samples were performed in triplicate.

Inflammatory gene expression was generally higher for universal blood donor and MHC class II-low MSCs when co-cultured with PBMCs

Gene expression for ten inflammatory or catabolic genes were assessed at day 3 and 5 of co-culture. Autologous cells were consistently lower in inflammatory gene expression except for ADAMSTS-5 and MMP-13 at Day 5 (Table 11). MHC class II-high MSCs were generally low in expression of these genes except for ADAMSTS-4 at day 5. Universal blood donor MSC gene expression was significantly higher than the autologous MSCs for its expression of ADAMSTS-4, ADAMSTS-5, CCL2, CXCLB/IL-8, IFNγ, IL-1β, and TNFα. MHC class II-low gene expression was significantly higher than other MSC groups for its expression of ADAMSTS-4, CCL2, CXCLB/IL-8, IFNγ, IL-1b, and TNFα (Table 11).

Table 11. Catabolic gene expression in MSCs co-culture with PBMCs.

		Autologous	Blood donor	MHC II-high	MHC II-low
ADAMTS-4	1:1	179 (13)[a]	924 (997)[b,c]	371 (137)[a,c]	526 (282)[c]
Day 3	Control	66 (30)	28 (20)	90 (44)	34 (8)
ADAMTS-4	1:1	253 (50)[a]	882 (444)[b,c]	1087 (805)[b]	671 (606)[a,c]
Day 5	Control	108 (27)	85 (21)	117 (183)	50 (25)
ADAMTS-5	1:1	2050 (220)[a]	5110 (3940)[b]	2420 (610)[a]	4000 (2200)[a,b]
Day 3	Control	1150 (150)	2200 (780)	1390 (1240)	1260 (910)
ADAMTS-	1:1	6990 (1450)[a]	2490 (3840)[a,b]	2950 (2040)[a]	1540 (110)[b]

Day 5	Control	1210 (260)	2230 (680)	1680 (1270)	835 (1170)
CCL2	1:1	14200 (1300) [a]	59900 (25400) [b]	15300 (8800) [a]	76600 (60000) [b]
Day 3	Control	2110 (330)	4080 (4230)	2840 (1140)	4110 (3470)
CCL2	1:1	10100 (1700) [a,c]	38200 (11400) [b]	3440 (4060) [a]	36900 (19300) [a,c]
Day 5	Control	1820 (100)	2880 (2230)	2190 (830)	2760 (1860)
IL-8	1:1	860 (66) [a]	6570 (22200) [b,c]	2770 (1460) [a,c]	12800 (17800) [b,c]
Day3	Control	10.9 (5.4)	146 (234)	9.88 (10.0)	32.2 (30.7)
IL-8	1:1	66.2 (95.3) [a]	2370 (3930) [b]	176 (472) [a]	3140 (8670) [b]
Day 5	Control	2.64 (1.38)	97.7 (70.1)	9.6 (6.5)	6.31 (12.7)
IFNγ	1:1	9.77 (3.88) [a]	392 (164) [b]	6.29 (10.8) [a]	492 (359) [b]
Day 3	Control	2.20 (0.53)	5.66 (6.84)	20.8 (28.2)	3.22 (1.27)
IFNγ	1:1	21.5 (21.0) [a,c]	546 (618) [b]	13.4 (10.5) [a]	640 (680) [a,c]
Day 5	Control	2.41 (3.20)	2.78 (2.59)	2.25 (1.35)	3.93 (3.53)
IL-1b	1:1	12.4 (6.3) [a]	255 (1050) [b,c]	47.1 (42.2) [a,b]	460 (1457) [c]
Day 3	Control	3.95 (1.53)	11.9 (3.5)	5.3 (11.4)	12.6 (9.8)
IL-1b	1:1	32.1 (23.0) [a]	230 (279) [b]	22.4 (11.7) [a]	352 (1016) [b]
Day 5	Control	11.8 (5.0)	13.8 (4.4)	8.15 (9.35)	6.15 (4.16)
IL-6	1:1	16200 (800) [a,b]	50200(24400) [a]	6240 (3310) [b]	47500 (56200) [b]
Day 3	Control	48.9 (14.6)	1360 (1100)	40.5 (52.2)	234 (460)
IL-6	1:1	4750 (1520) [a,b]	19500 (9200) [a]	1010 (2090) [b]	13400 (6000) [a]
Day 5	Control	33.6 (31.8)	541 (111)	24.1 (17.0	79.1 (283)
MMP-13	1:1	315 (16) [a,b]	92.7 (6945) [a]	855 (372) [b]	140 (47) [a]
Day 3	Control	18.1 (6.9)	181 (195)	1010 (8550)	368 (352)
MMP-13	1:1	687 (181) [a]	136 (872) [a]	935 (607) [a]	62.8 (70.8) [b]
Day 5	Control	90.3 (19.2)	242 (225)	2190 (14500)	1010 (1040)
COX2	1:1	9400 (810) [a,b]	16400 (7900) [a]	7610 (2820) [b]	9440 (18700) [a]
Day 3	Control	71.5 (10.3)	3560 (4100)	344 (1350)	1690 (1520)
COX2	1:1	8930 (4150)	10300 (8900)	6290 (26700)	4990 (1850)
Day 5	Control	186 (1220)	6830 (6520)	558 (1280)	612 (1690)
TNFa	1:1	8.29 (3.65) [a]	48.9 (17.5) [b]	15.1 (8.5) [a]	51.8 (42.1) [b]
Day 3	Control	2.17 (1.27	6.22 (1.97)	15.2 (10.8)	14.2 (3.0)
TNFa	1:1	29.8 (31.9)	52.1 (44.3)	12.2 (23.9)	63.3 (35.5)
Day 5	Control	1.77 (0.71)	3.65 (0.99)	5.45 (2.32)	4.61 (1.19)

Median RNA copy number and IQR is shown for co-cultures with PBMCs and for MSCs cultured alone (control) at day 3 and 5 of co-culture. Values for co-cultured MSC gene expression (within each row) which are significantly different are marked with different letters ($p<0.05$). Autologous, Autologous MSC co-culture (n=1); blood donor, universal blood donor MSC co-culture (n=3); MHC II high, MHC II-high MSC co-culture (n=3); MHC II Low, MHC II-low MSC co-culture (n=3).

PBMC and MSC separation

Gene expression was also analyzed on the PBMCs that were co-cultured with the MSC groups. When comparing MSC and PBMC expression in each group, gene expression between these two types of cells varied (Supplementary information). This illustrates that our cell separation method was adequate to remove the PBMCs from the MSCs in each well.

Inflammatory gene expression of PBMCs was increased when co-cultured with universal blood donor or MHC class II-high MSCs

Gene expression for the same ten inflammatory genes was analyzed on the PBMCs in co-culture. PBMCs co-cultured with universal blood donor MSCs showed significantly greater expression of the inflammatory genes ADAMSTS-4, ADAMTS-5, CXCL8/IL-8, IL-6, and PTGS2/COX-2 as compared to those cultured with autologous MSCs (Supplementary information). PBMCs co-cultured with MHC class II-low MSCs showed significantly greater expression of the inflammatory genes ADAMTS-5 and CXCL8/IL-8 as compared to those cultured with autologous MSCs (Supplementary information). PBMCs co-cultured with MHC II-low MSCs had significantly lower MMP-13 expression as compared to those cultured with autologous MSCs.

4.6 Discussion

This study was a first of its kind in equine medicine to monitor multiple types of leukocytes in their interaction with MSCs without the presence of external

activators. The lack of activation of the leukocytes would allow the immune cells to respond to the MSCs without other contributing factors of inflammation. Although this method may not appropriately represent injured tissues which contain inflammation, our research focused on the basic interaction of the MSCs and leukocytes.

The approach was to use a single leukocyte population to determine variation in reactions across ten different MSC populations. Although the use of one leukocyte population is intrinsically limiting on universality of this data, the number of MSC types and great breadth of assays completed provides us a broad understanding of the interactions occurring between the leukocytes and the MSCs.

Haplotyping revealed that the leukocytes utilized from the Connemara horse were ELA mis-matched from all of the allogenic MSCs used in co-culture. Therefore, each of the allogeneic MSCs were equally susceptible to an immune response by the recipient leukocytes, and none of the MSC groups had haplotype matching which may make them less recognizable to the leukocytes in co-culture. It has been hypothesized that the use of haplotype-matched donor MSCs may be the future of allogeneic regenerative medicine when repeat therapy is necessary (Rowland *et al.* 2021). As there are greater than 300 ELA subtypes identified (Ranera *et al.* 2016), finding a matched donor-recipient pair may prove difficult. For this reason, we sought to identify minimally immunogenic MSCs that may be used as a one-time therapy or potentially be utilized repeatedly by rotating the haplotype of the donor MSC. Rotation of allogeneic MSC haplotypes may prevent antibodies being present in the recipient at the time of administration.

The PBMC proliferation rates and cell surface antigens evaluated in this study illustrate that there were no signs of severe negative reactions of PBMCs when cultured with allogeneic MSC. Activation rates of CD4 and CD8 lymphocytes were consistent with those co-cultured with autologous cells (Figures 19, 20). The only exception to this was the universal blood donor MSC group which caused greater activation of CD4 lymphocytes at some concentrations.

B lymphocyte numbers were not consistently increased in the face of allogeneic MSCs (Figure 21). B lymphocyte numbers increased over time when PBMCs were cultured alone, but this was not seen in co-cultures. Antibody production is a common concern for successive allogeneic MSC treatments (Pezzanite *et al* 2016, Owen *et al*. 2016), and it has previously been reported that B cells create antibodies against ELA mis-matched allogeneic MSCs which leads to MSC destruction (Berglund *et al* 2018, Barrachina *et al.* 2020). It is interesting that in our study B cells were not stimulated leading to proliferation when faced with allogeneic MSCs. This may be an indication of B cell suppression by MSCs that will help to provide MSCs with some degree of persistent alloimmunity. There is widespread evidence that human MSCs can also suppress activated B cell responses (Comoli *et al*. 2008, Asari 2009, Ge 2009, Franquesa 2012).

MSC-mediated immunosuppression is caused in large part due to an increase in regulatory T lymphocytes which serve to dampen the adaptive immune response and can prevent rejection of foreign cells by the host (Wang *et al*. 2009). In our study, Tregs cells were consistently increased in co-cultures at low MSC: PBMC ratios where MSCs would potentially have the greatest interaction with

lymphocytes (Figure 21). Blood donor MSCs caused a significant increase in Tregs as compared to other MSC groups at both day 3 and 5, and MHC II-low MSCs showed a somewhat lesser increase though this was not significant (Figure 21). The increase in Tregs when cultured with MSCs is consistent with previously published human studies (Duffy *et al*. 2011, Duffy *et al*. 2011(2), Griffen *et al*. 2013).

In addition to the already mentioned populations of PBMCs, there were 2 other distinct groups identified on flow cytometry. A CD8moderate/CD25+/CD4- population may represent gamma delta (γδ) T cells, as these cells are known to be negative for CD4, but can be variable in their CD8 expression (Ahamad *et al*. 2005) (Figure 21). No previous flow cytometry studies have been published on this type of cell in the horse and further assessment of the significance of the loss of these cells in equine co-cultures is required. A subpopulation of CD4-/CD8-/CD21-/CD25-cells was identified (Supplemental information). This population represents approximately 10% of PBMCs and may constitute a population of NK cells based on its lack of marker expression and approximate percent contribution of cells to the PBMC population (Merkt *et al*. 2015). Further research and an expanded number of antibodies are needed for appropriate identification.

Neutrophils are often the first line of defense against foreign antigens (Li *et al.* 2019, Joel *et al.* 2019) and therefore would potentially be an initial impediment against the use of allogeneic MSCs. Many studies have shown no increase in activation of neutrophils in the presence of allogeneic MSCs and have shown instead that allogeneic MSCs serve to decrease oxidation and preserve neutrophil

viability (Raffaghello *et al.* 2008, Mumaw *et al.* 2015). Some concern exists when MSCs are used intra-articularly in that a neutrophil influx occurs following MSC administration (Ardanaz *et al.* 2016, Colbath *et al.* 2020). In our study, neutrophils were activated upon interaction with allogeneic MSCs, but this activation was minimal and short-lived for the MHC II-low MSC co-cultures. Neutrophil interaction with MSCs showed that the universal blood donor and the MHC II-high allogeneic MSCs at the 6h time point in co-culture caused greater neutrophil activation than other co-cultures. MHC II-high MSCs consistently caused the highest levels of neutrophil activation (median of all ratios 1.43% at 6h, 8.9% at 12h, Figure 23). This did not have an effect on MSC survival except at high ratios of MHC II-high MSCs to neutrophils at 12h of co-culture which had a median decreased survival of <3% (median 95.8% (0.5)) as compared to MHC II-high MSCs cultured alone (median 98.1% (0.8), Figure 23). *In vivo* work in the horse found that MHC II-high MSCs caused no greater neutrophil infiltration after an intra-articular injection of allogeneic MSCs as compared to autologous or MHC II-low MSCs (Joswig *et al.* 2017). Another study found no difference in neutrophil infiltration in joints treated with autologous MSCs as compared to allogeneic MSCs (Ardanaz *et al.* 2016). It is possible that the significantly increased neutrophil activation seen in the allogeneic co-cultures is so mild and transient that the activation of neutrophils is not clinically significant.

Complement has been considered to be another impediment to allogeneic MSC use as complement can flag the foreign material for phagocytosis or cause cell lysis by forming a membrane attack complex (Murphy 2007). One group

studied human MSC survival in the presence of complement and found >40% of the MSCs were damaged upon incubation with complement (Li and Lin 2012, Li *et al.* 2016). In contrast, we found complement-mediated cytotoxicity did not cause consistent MSC death in cultures with active complement as compared to inactive complement. Even in the only MSC culture that showed any significant loss of MSC viability, the MHC II-high MSC culture, this loss of viability was only 2.2% of MSCs (Supplemental information).

Gene regulation of allogeneic MSCs in co-culture is highly variable and appears to relate to the level of MHC II expression of the MSCs. MSCs are known to deliver anabolic factors such as TGF-β1, FGF, and G-CSF; anti-inflammatory factors such as IL-1RA and IDO1; and immunomodulating factors such as CXCLB/IL8 and IFN- γ (Di Nicola *et al.* 2002, Amable *et al.* 2014, Cassano *et al.* 2018). Two groups of MSCs, the blood donor and the MSC II-low groups, increased their gene expression of these anabolic genes.

Several of the genes expressed in greater amounts in the blood donor and MHC II-low groups were genes aimed at suppressing the immune system. Indoleamine-pyrrole 2,3-dioxygenase (IDO1) quells T lymphocyte responses and leads to immune tolerance whose effect alone can determine the difference between organ rejection and acceptance (Ge *et al.* 2010). IL-2 binding directly to T lymphocytes causes upregulation of regulatory T cells (Tregs), and increases activation-induced cell death for lymphocytes. Regulatory T cells, cells crucial to the immunosuppressive ability of MSCs, were consistently elevated in lymphocyte co-cultures with universal blood donor MSCs (Figure 21). Interestingly, this group

had a higher level of anabolic and catabolic gene expression, including TGFβ and IFNγ. This is line with the findings of Zhang *et al.* 2018 who found that pretreatment of MSCs with TGFβ and IFNγ resulted in MSCs that had a greater capability of forming Tregs. MSCs appear to need some sort of activation of their own to assist in their ability to implement their immunosuppressive effects (Klinker *et al.* 2019).

Catabolic molecules such as TNF- α, IL1β, aggrecanases, and MMP-13 are commonly upregulated in the face of inflammation (Kamm *et al.* 2010, Nguyen *et al.* 2017). In our gene expression assay, several catabolic factors were increased in blood donor and the MSC II-low co-cultures. This seems to contradict our other data showing decreased inflammation and leukocyte activation when leukocytes were co-cultured with universal blood donor and the MSC II-low MSCs.

The expression of IFNγ in our universal blood donor and MHC II-low co-cultures is especially interesting for two reasons. First, IFNγ can cause increased expression of immunosuppressive genes such as IDO1, HGF and PGE2/COX2 (Prasanna *et al.* 2010, Klinker *et al.* 2019). In our cultures where IFNγ was increased, these genes were significantly upregulated (Table 10 and 11). A previous equine MSC study has shown inflammatory licensing by pretreatment of MSCs with IFNγ had superior immunosuppressive effects as compared to non-pre-treated MSCs (Cassano *et al.* 2018). Secondly, in both the universal blood donor and MHC II-low groups, where IFNγ gene expression was increased in both the MSCs and the lymphocytes, a significant increase in MHC II expression was seen on the surface of the MSCs as compared to those cells cultured alone. This

is consistent with previous work which showed the treatment of MSCs with IFNγ caused increased MHC II expression (Hill *et al*. 2016).

From the gene expression data, it is clear that the universal blood donor group and the MHC II-low MSCs were more metabolically active in both the anabolic and catabolic gene categories. Researchers must determine if the more metabolically active MSCs would be more beneficial as a therapy as compared to the less metabolically active groups, the MHC II-high and autologous MSCs. To better achieve this goal, the alterations to the co-cultured leukocyte population must be examined. As previously discussed, there was no significant neutrophil activation and no decrease in complement-mediated viability in the MHC-low and universal blood donor MSC co-cultures. When considering the interaction of our MSCs with lymphocytes in the current study, MHC II-low MSCs showed only beneficial decreases in lymphocyte proliferation and total CD4 lymphocyte count as compared to autologous MSC co-cultures (Figures 18 and 19). Universal blood donor MSC co-cultures had an elevated activated CD4 lymphocyte count, but this included increased numbers of Tregs which would serve to decrease an immune response. Only the MHC II-high MSCs repeatedly showed increased lymphocyte activation.

MSCs have been shown to go through a phenotypic and genotypic metamorphosis when they interact with the immune system (Cassano *et al.* 2018). For MHC II-low MSCs, this change in structure and expression appears to affect the cells in a manner that may be preferential when used as an allogeneic treatment. The influence of the origin of these cells from universal blood donor

horses or non-blood donor horses does not appear to be markedly significant. MHC II-low MSCs prevent proliferation of PBMCs, increase expression of both anabolic and catabolic genes, decrease activation of neutrophils, and maintain viability when exposed to complement. There were minimal differences between autologous and allogeneic MSCs in their effects on the activation and differentiation of lymphocytes. MHC II-high allogeneic MSCs were the only group of allogeneic MSCs that repeatedly showed increased lymphocyte activation. Some inflammatory gene expression increased in MHC II-low co-cultures, but a reciprocal anti-inflammatory gene response was also seen. These MHC II-low MSCs appear to be activated in the recipient environment to perform immunosuppressive and anabolic functions.

4.7 Conclusion

From the results of this body of *in vitro* work, we conclude that bone marrow-derived, low passage number MHC II-low MSCs from healthy donors have minimal negative effects on an allogeneic leukocyte population *in vitro*. This includes the lack of lymphocyte and neutrophil activation and a lack of B cell proliferation. Allogeneic MSCs maintained a high level of viability through all testing, and the MHC II-low MSCs upregulated both their inflammatory and catabolic gene profiles in response to lymphocyte co-culture.

List of Abbreviations

Chemokine ligand (CCL), C-X-C motif chemokine ligand 8 (CXCL8); cluster of differentiation (CD); equine lymphocyte antigen (ELA); fibroblast growth factor

(FGF); hepatocyte growth factor (HGF); interleukin (IL); interleukin receptor antagonist (IL-RA); interferon (IFN); indoleamine-pyrrole 2,3-dioxygenase (IDO1); major histocompatibility class (MHC); matrix metalloproteinase (MMP); mesenchymal stromal cell (MSC), cyclooxygenase-2 (PTGS-2/ COX-2), T regulatory cells (Tregs); tumor necrosis factor (TNF); thyroid growth factor (TGF), vascular endothelial growth factor 2.

4.8 References

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4.9 Supplemental information

Antibody Clone	Distributer, Catalog number	Conjugated fluorochrome or secondary antibody	Host Species	Ig Type	Dilution
CD4 CVS4	US Biological, 227417-ML405	MaxLight650	Mouse	IgG1	1:700
CD8 CVS8	BioRad, MCA2385F	FITC	Mouse	IgG1	1:200
CD21 CA2.1D6	AbCam, ab34124	PE	Mouse	IgM	1:5
CD25/IL-2 R alpha	RND Systems, AF-223-NA	Donkey Anti-Goat IgG H&L (Alexa Fluor® 405)	Goat	IgG	1:10
FOXP3 FJK-16s	eBioscience, 17-5773-82	PE-Cyanine7	Rat	IgG2a	1:100
MHC class I CVS22	BioRad, MCA1086	RPE	Mouse	IgG	1:10
MHC class II CVS20	Bio-Rad, MCA1085F	FITC	Mouse	IgG1	1:100

Table 12. Antibodies used for flow cytometry assays.

			I	I	I	I	III	III	II	II	II	II	II	II	
			UMNJH-38	COR110	305-93	CZM002	ABGe9019	UMNe65	ABGe9030	EQMHC1	COR112	COR113	UM011	COR114	
	Acc #	Breed	28,905,580	29,232,117	29,289,063	30,013,021	31,385,172	31,474,974	32,544,656	32,689,801	33,282,568	33,480,869	33,510,218	33,516,405	Haplotype
1	LK_H1	TB	156	211	343	259	299	257	207	190	237	266	179	241	Novel
			163	207	343	251	312	261	211	192	262	268	176	247	ELA-A3b
2	LK_H2	TB	156	211	343	249	301	259	209	192	262	268	174	234	ELA-A2
			163	207	343	251	312	261	211	192	262	268	176	247	ELA-A3b
3	LK_H3	TB	156	217	336	249	301	259	209	192	262	268	174	234	Novel
			163	207	343	251	312	261	211	192	262	268	176	247	ELA-A3b
4	LK_L1	Stbd	156	217	336	249	312	259	215	190	262	274	169	247	Novel
			156	221	340	261	299	257	212	190	254	260	172	243	ELA-A5a
5	LK_L2	Stbd	156	211	346	230	299	257	207	190	237	266	179	241	Unphased
			156	221	342	230	299	257	207	190	237	266	179	241	Unphased
6	LK_L3	Stbd	156	211	346	230	299	257	207	190	237	266	179	241	Novel
			156	217	336	249	312	259	215	190	262	274	169	247	ELA-A4c
7	LK_BD1	Stbd	156	205	345	249	314	259	207	190	237	266	179	241	Novel
			156	221	342	259	312	261	207	190	237	266	179	241	Stbd_01
8	LK_BD2	Stbd	156	205	345	249	314	259	205	194	256	270	172	249	ELA-A7
			156	217	336	249	312	259	215	190	262	274	169	247	ELA-A4c
9	LK_BD3	Stbd	156	221	342	259	312	261	207	190	237	264	180	243	ELA-A10a
			156	221	340	261	299	257	212	190	254	260	172	243	ELA-A5a
10	LK_A	Connemara	156	209	343	253	314	257	211	184	252	274	165	236	Unphased
			156	215	340	261	316	249	215	190	258	280	172	247	Unphased

Table 13. ELA haplotype analysis.

The list of haplotypes shows mis-matched haplotypes between the Connemara (horse 10) and the other animals.

Gene	RefSeq Accession number
TGF-B1	NM_001081849
IL-1RA	NM_001082525.2
CD-59	XM_023653832.1
FGF-2	NM_001195221.1
IDO-1	XM_014736538.2
IL-10	NM_001082490.1
VEGF-a	NM_001081821.1
SOX2	XM_023623361.1
PTGS-2/ COX-2	NM_001081775.2
IL-1b	XM_001495926.5
IL-2	NM_001085433.2
TNFa	NM_001081819.2
Hepatocyte GF	XM_014739139.2
IFNgamma	NM_001081949.1
CXCL8/IL-8	NM_001083951.2
IL-6	NM_001082496
MMP-13	NM_001081804.1
ADAMTS-4	NM_001111299.2
ADAMTS-5	XM_003364218
CCL2	NM_001081931.2
TBP	XM_014738168
GUSB	XM_023655543
PPIA	XM_001496943.5
YWHAZ	XM_014728222.2

Table 14. Accession numbers for genes used in NanoString assays.

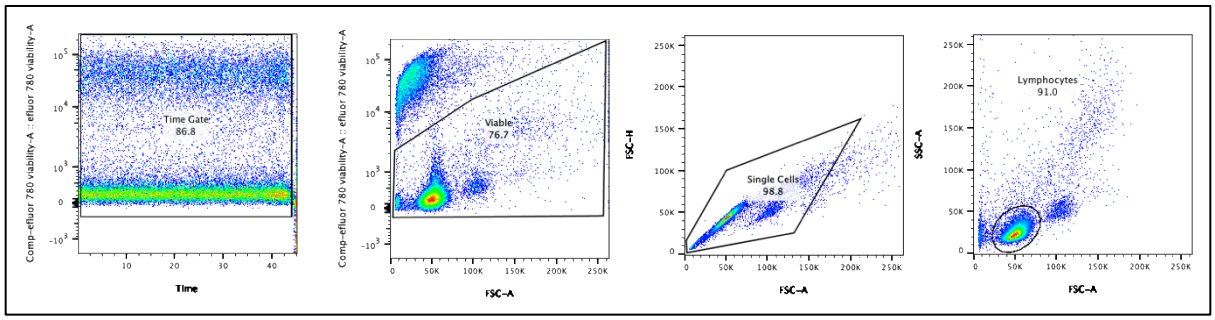

Figure 24. Flow cytometry gating scheme for lymphocytes.

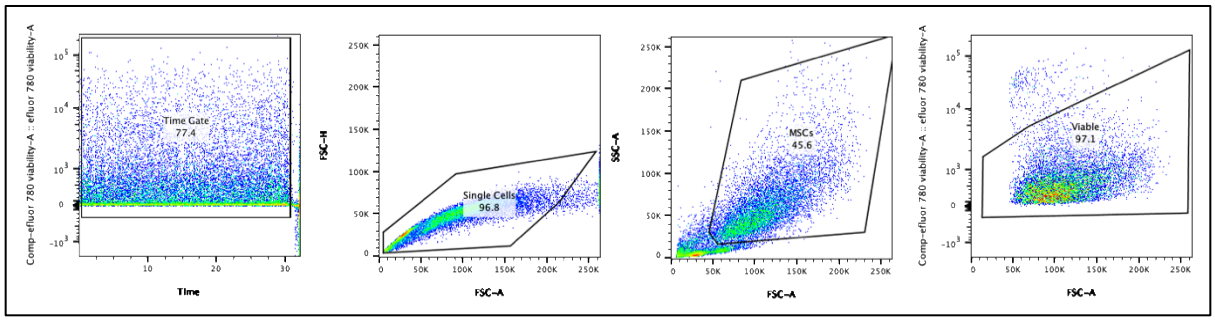

Figure 25. Flow cytometry gating scheme for MSCs with PBMCs.

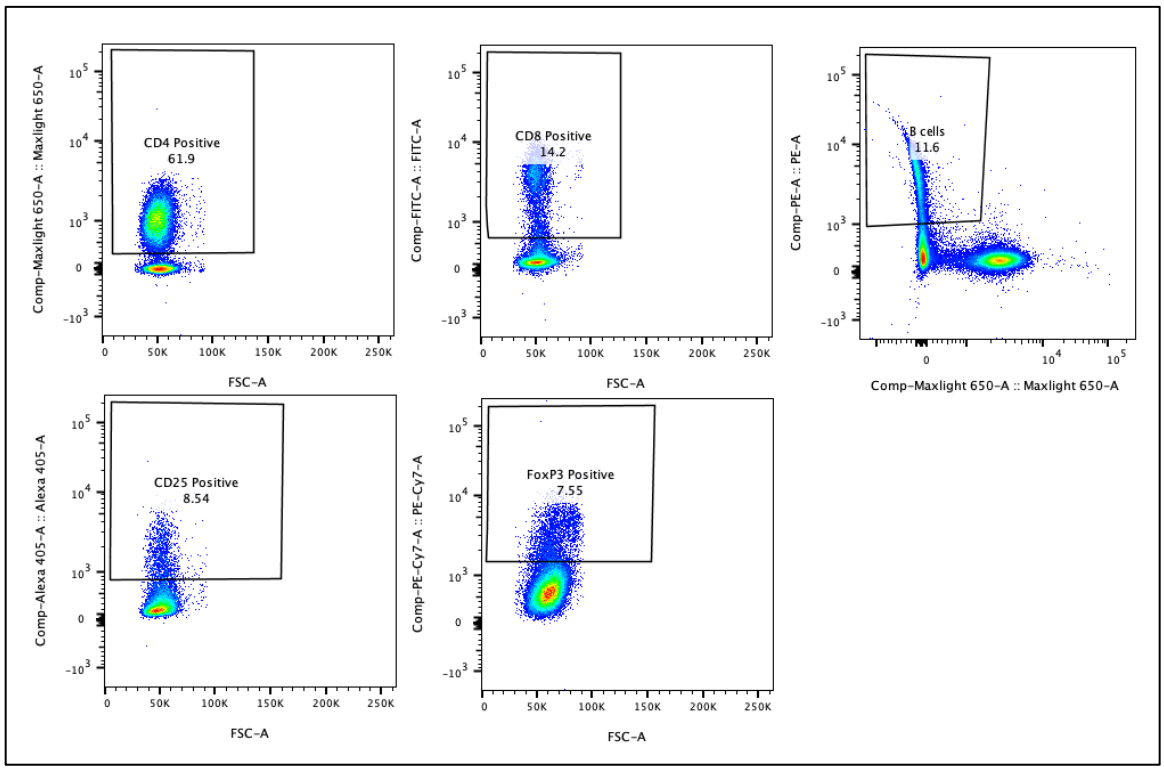

Figure 26. Gating scheme for PBMC antibodies.

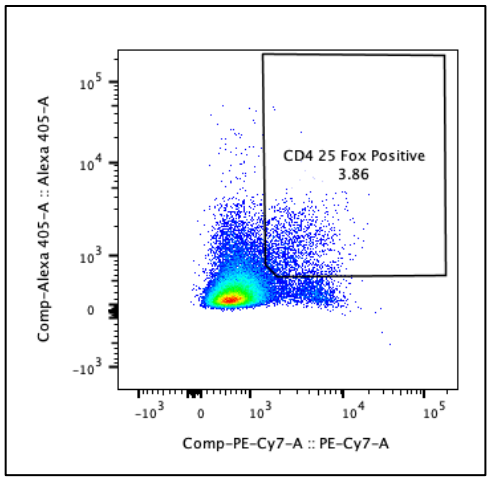

Figure 27. Representative sample of CD4+/CD25+/FoxP3+ lymphocytes. These may be T regulatory lymphocytes or activated CD4 lymphocytes.

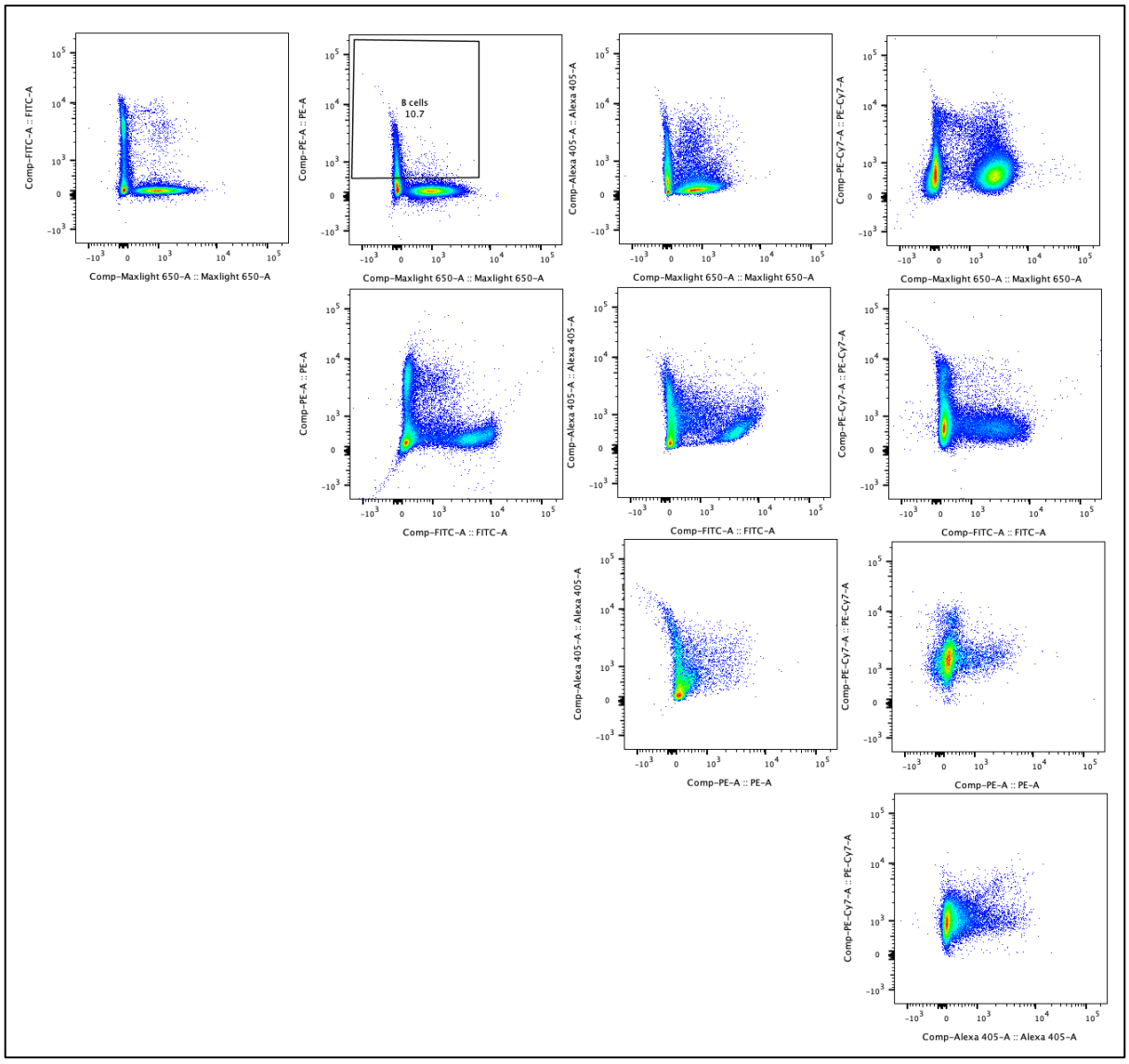

Figure 28. Antibody binding comparison across all PBMC antibodies.

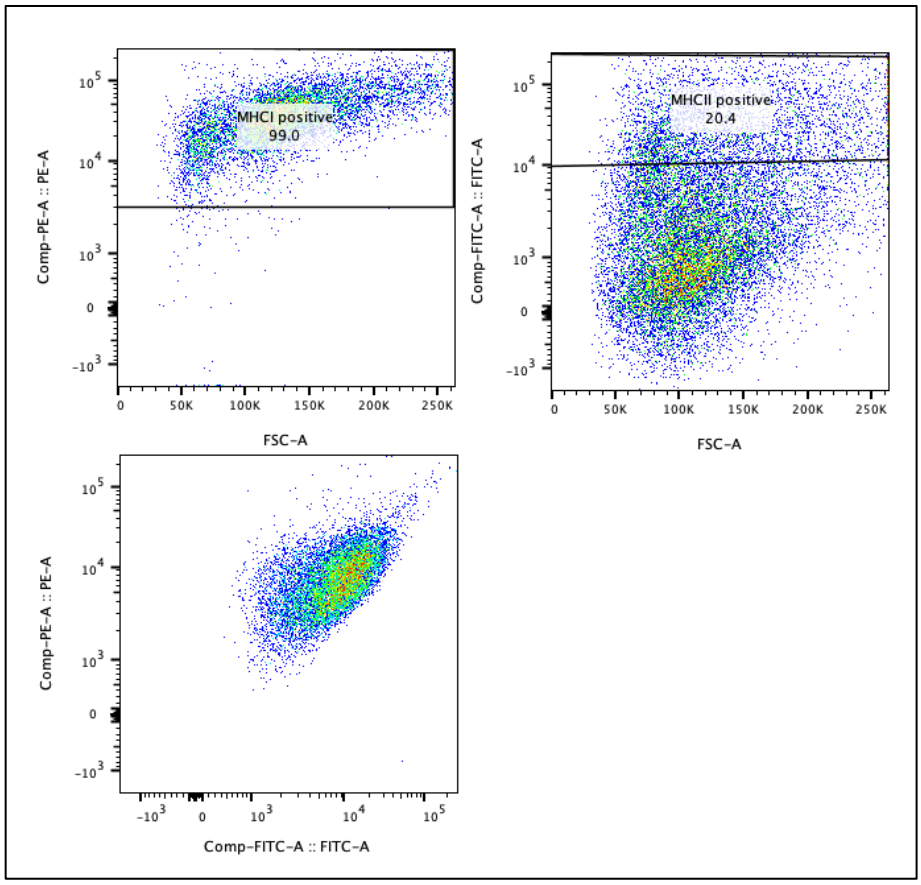

Figure 29. Gating scheme and comparison for MSC antibodies.

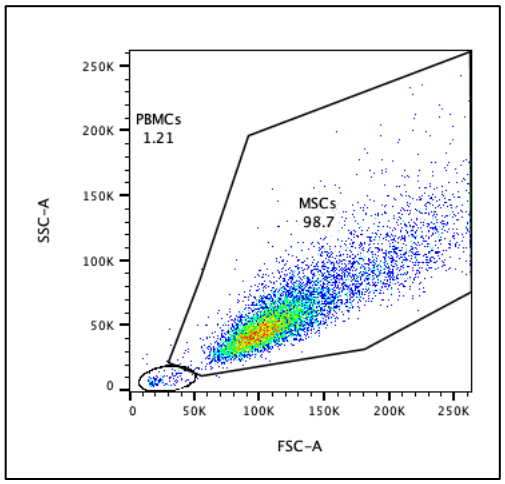

Figure 30. MSC and PBMC separation in the MSC sample.

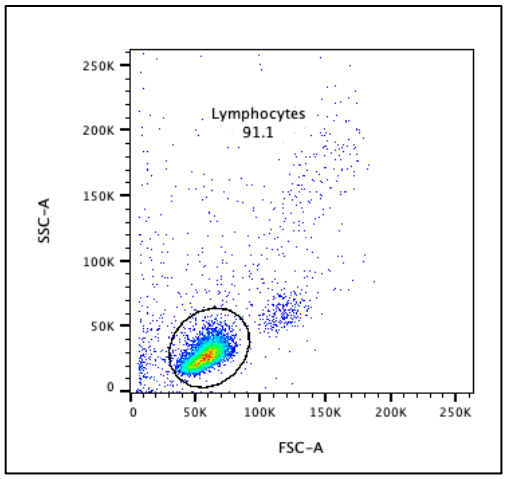

Figure 31. PBMC separation in the PBMC sample.

PBMCs that were separated from co-culture with MSCs. Note a small amount of larger cells likely to be MSCs are seen in the upper right quadrant.

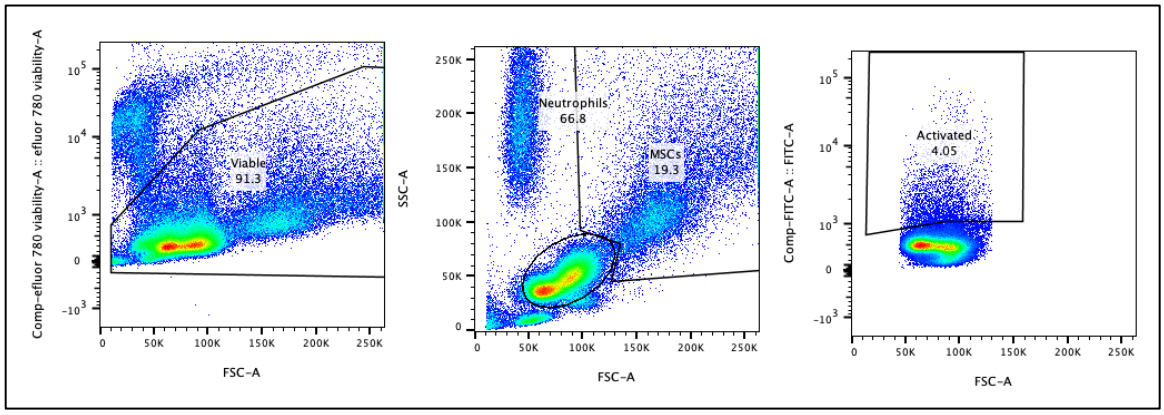

Figure 32. Neutrophil and MSC flow cytometric analysis for neutrophil activation.

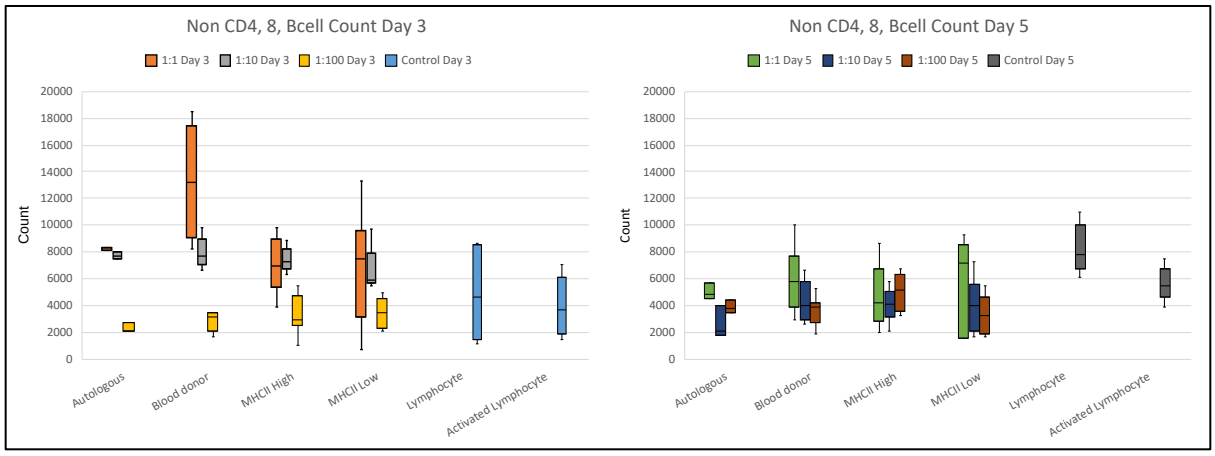

Figure 33. CD4-/CD8-/CD21-/CD25- PBMCs are shown at days 3 and 5 of co-culture with MSCs.

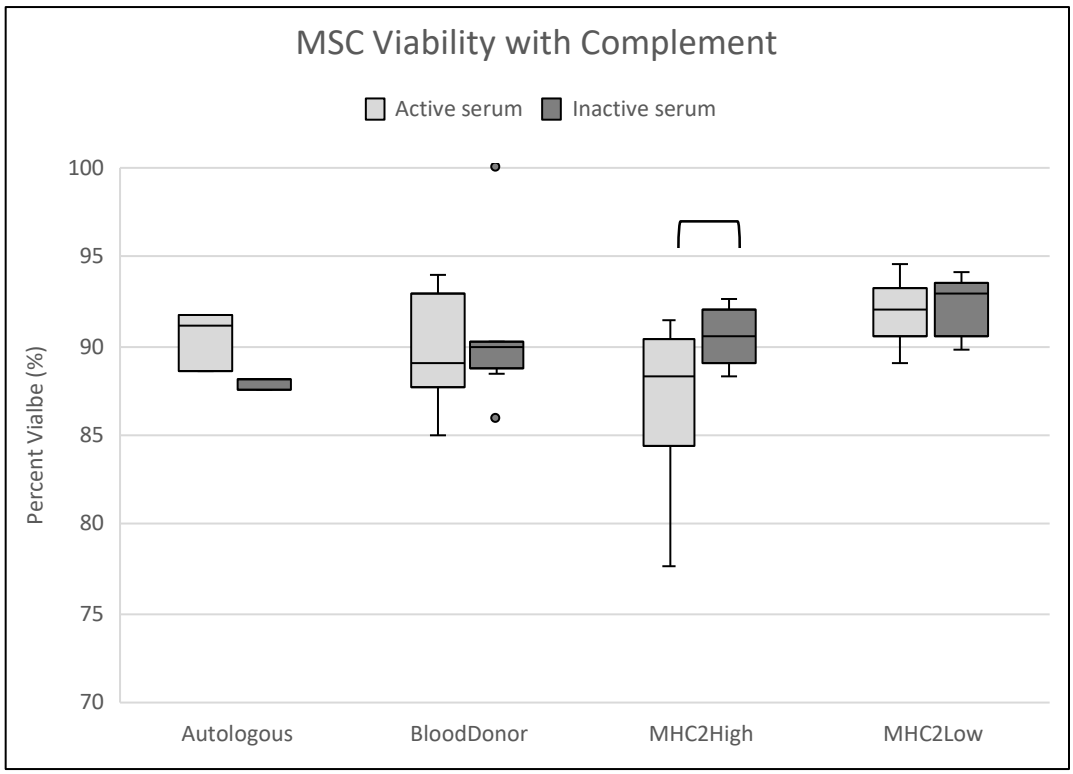

Figure 34. MSC survival with complement.

MSC survival was not significantly different between cells cultured in active or inactivated complement except for the MHC II-high MSC group which showed a 3% decrease in viability with active complement as compared to inactive complement.

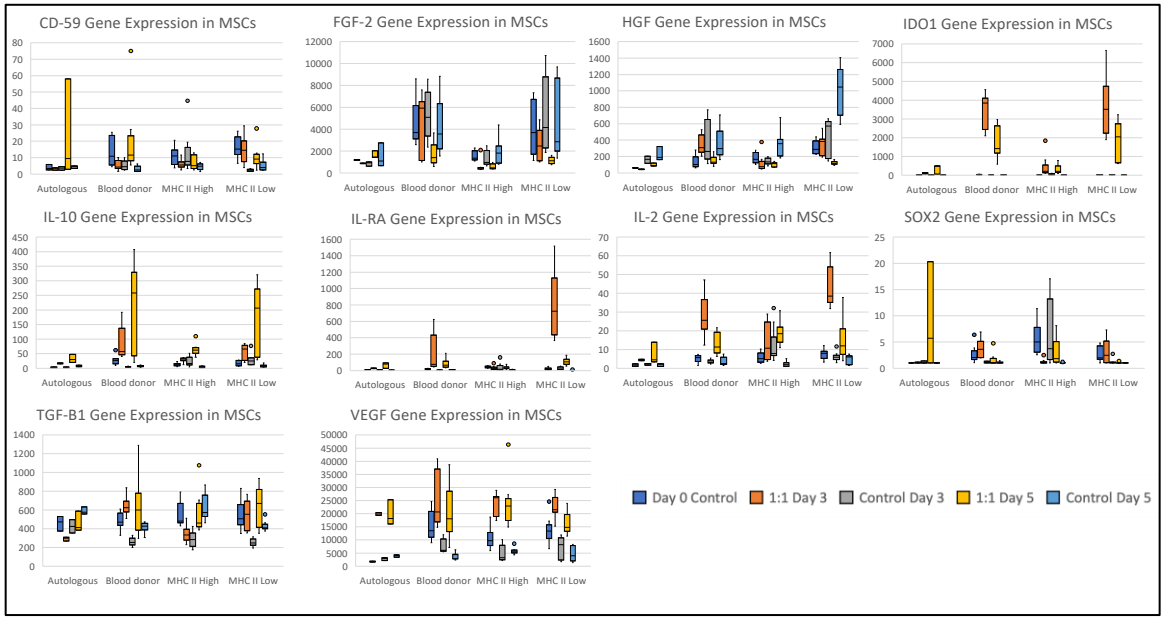

Figure 35. MSC gene expression for anti-inflammatory genes on Day 0, Day 3 and Day 5.

The median is shown in the box and whisker plot.

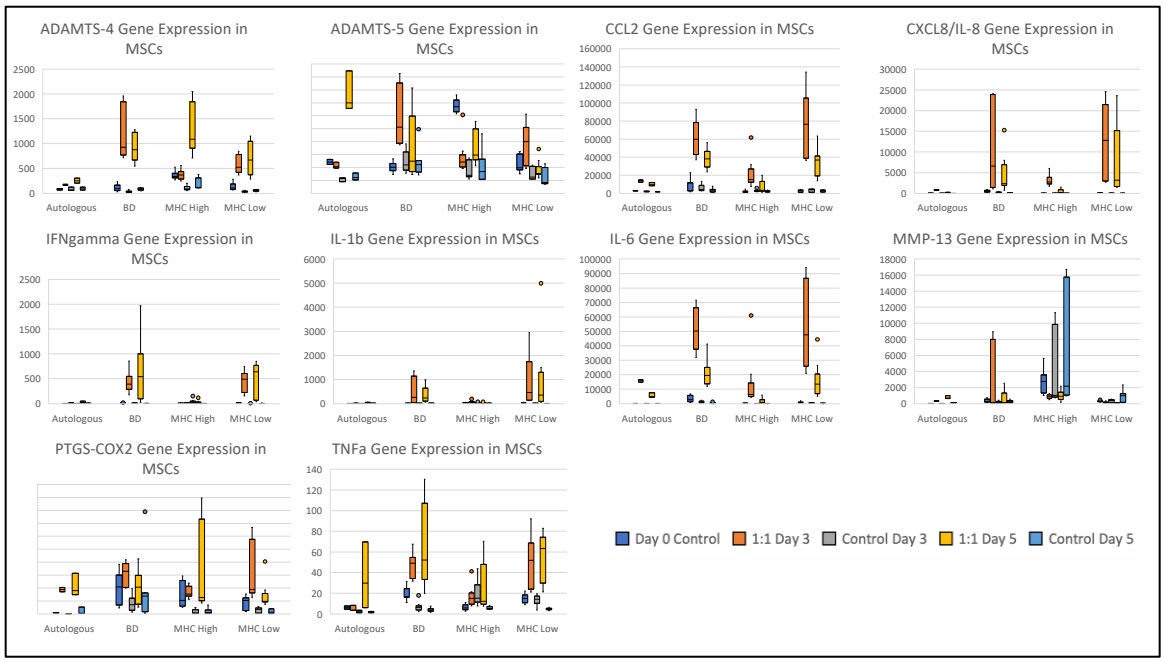

Figure 36. MSC gene expression for inflammatory genes on Day 0, Day 3 and Day 5.

The median is shown in the box and whisker plot.

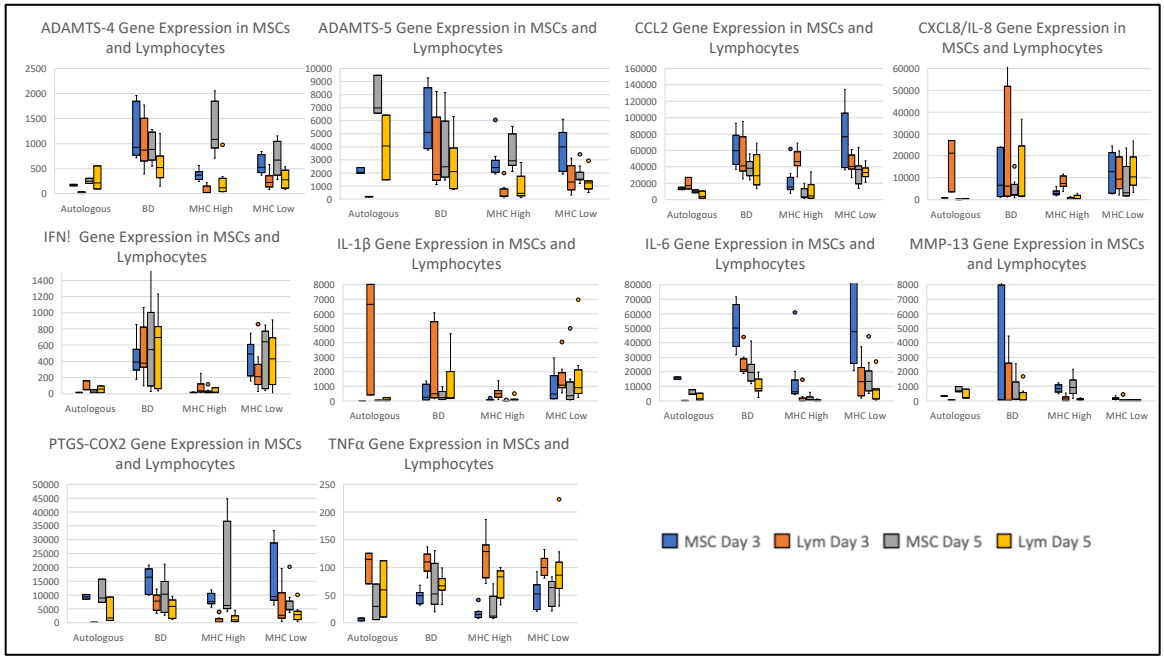

Figure 37. Day 3 and Day 5 catabolic gene expression in MSC and PBMC co-cultures.

Mean MSC RNA copy number is listed in blue. Mean PBMC RNA copy number is listed in orange.

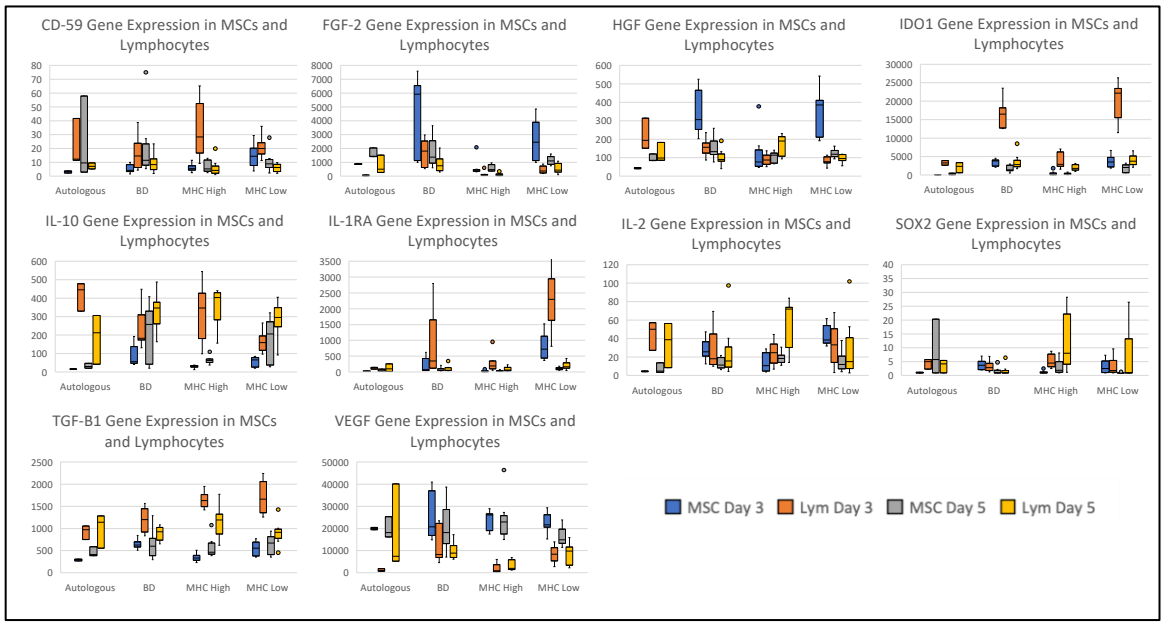

Figure 38. Day 3 and Day 5 anti-inflammatory/ anabolic gene expression in MSC and PBMC co-cultures.

Median MSC RNA copy number is listed in blue. Median PBMC RNA copy number is listed in orange.

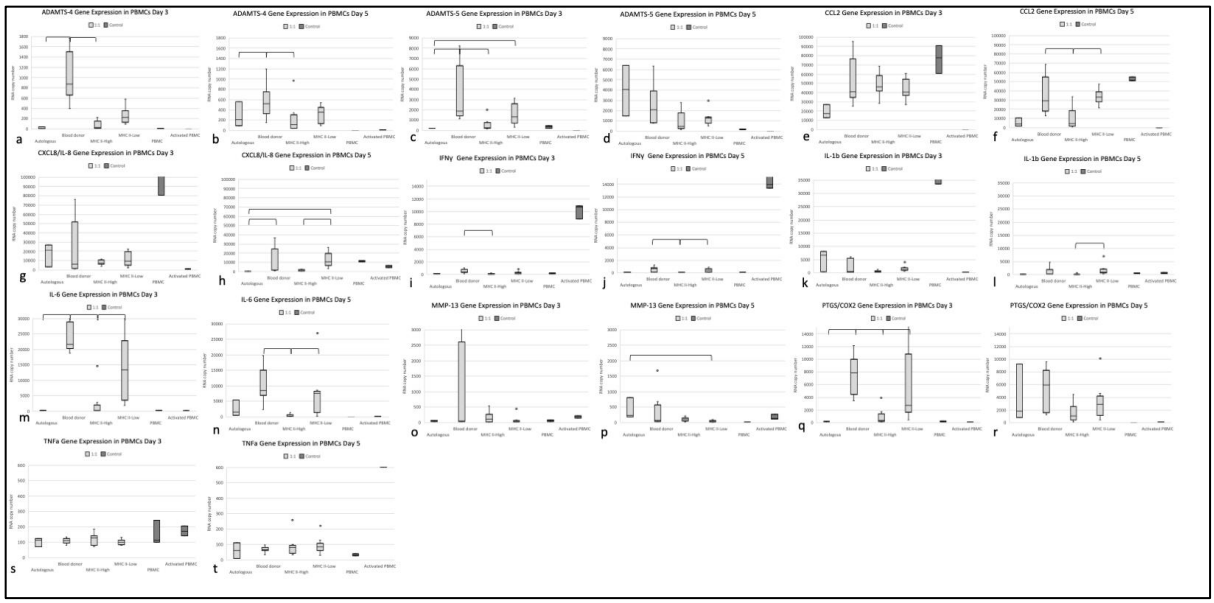

Figure 39. Inflammatory gene expression for PBMCs.

PBMCs in culture with MSCs, PBMCs alone, or PBMCs with activation media is shown. Median RNA copy number of inflammatory genes expressed by PBMCs in shown**.** Cultures of 1 MSC:1 Lymphocyte and control MSCs (no lymphocytes) are shown. PBMCs cultured with universal blood donor MSCs had higher levels of inflammatory gene expression as compared to those cultured with autologous MSCs in 5 of 10 genes examined.

Assay	Ratio MSC: WBC	Number of MSCs	Number of leukocytes
Thymidine	1:1	1×10^4	1×10^4
	1:10	1×10^4	1×10^5
	1:100	1×10^4	1×10^6
Flow Cytometry	1:1	5×10^5	5×10^5
	1:10	1×10^5	1×10^6
	1:100	1×10^4	1×10^6
Gene expression	1:1	5×10^5	5×10^5

Table 15. Number of cells utilized for each assay and ratios of MSC:WBC used.

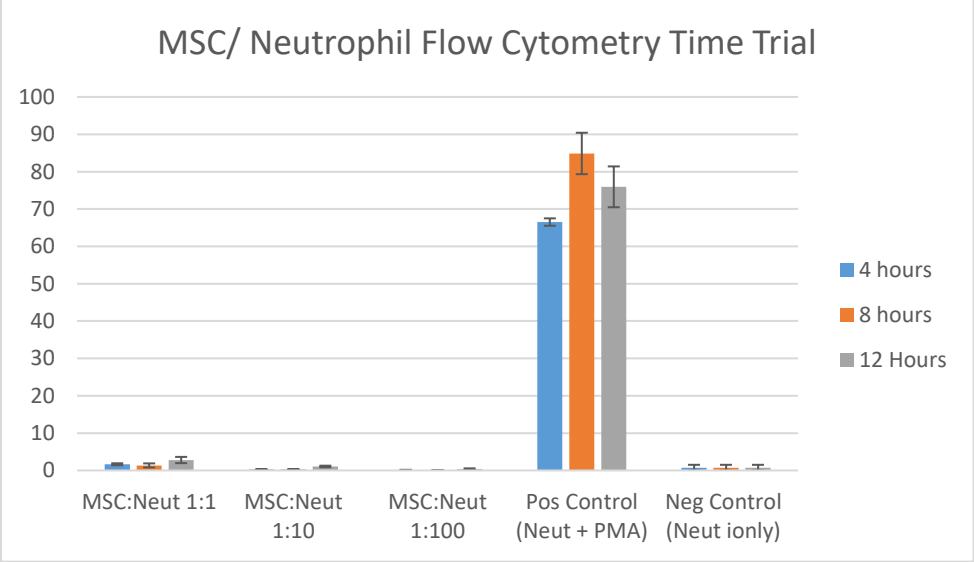

Figure 40. Neutrophil time trial for validation of neutrophil co-culture assay.

4.10 Epilogue

Summarising Comments

The information presented in this chapter represents the culmination of our research. Leukocytes from our allogeneic donor behaved differently depending on the origin of the equine MSCs. The least immunogenic MSC donor with high levels of anabolic gene expression was identified, and the future clinical use of these cells in an allogeneic manner was discussed.

Additional Discussion

Further discussion is provided here to investigate topics that were not fully addressed previously.

Cells in culture grow and proliferate under a standard cell growth curve in culture which includes a beginning lag phase as the cells become acclimated to the culture conditions, a log phase of growth with sufficient nutrients available in the media, and a stationary phase where the cells become stressed (Pereira *et al.* 2020). Exhaustion of a nutrient or a decrease in pH are common causes of cells in culture reaching the plateau phase (Quinlan 1986). These stressors can lead to a decrease in cell number over time (Quinlan 1986).

Consideration of this cell culture curve may be utilized in determining the cause for the general decline in the number of viable lymphocytes seen in co-culture on flow cytometry between Day 3 to 5 in our assays. This would lead one to believe the cells would be in the stationary phase of growth from Day 3 to 5. It

is possible that the stressing of the cells may cause a lack of proliferation when they may be one under proliferative conditions.

The cause for the decrease in lymphocyte count from Day 3 to Day 5 may be concerning, but a decrease in cell number is common in equine lymphocyte assays over time (Remacha *et al.* 2015). The thymidine incorporation and therefore cell proliferation levels in our study appeared to increase on day 5 as compared to day 3. One would assume that with this proliferation, a higher number of lymphocytes would be present for analysis. The most likely cause for the decrease in lymphocyte count is cell death. A decrease in the percent of viable lymphocytes was seen on day 5 as compared to day 3 as nonviable cells were removed from flow cytometric analysis. Some degree of this cell death is due to natural apoptosis, though it is known that lymphocytes can survive in culture for much longer than 5 days. Lymphocyte media changes are commonly done every 5 days (Rosado-Sanchez *et al.* 2018) which would be in line with our end period of the assay being 5 days. The cell media appeared of normal color showing no acid-base abnormalities using phenol red. Thymidine incorporation analysis was high in the activated lymphocyte positive control samples on day 5, showing no severe loss of nutrients that would cause decreased cell mitosis. The cause for the decrease in lymphocyte count is poorly understood, but there is little evidence that would put into doubt the day 5 results of this study.

The lymphocyte proliferation assay using tritiated thymidine illustrated both MSC proliferation and lymphocyte proliferation. This lack of clarity was considered prior to use of this assay and attempts were made to utilize other assays. After

validation, we found our use of control samples where only MSCs or lymphocytes were also assessed would allow us to compare proliferation rates in lymphocytes across each of the MSC co-culture groups. The MSC proliferation rates were consistent between samples and between MSC groups. Alterations of our protocol could have been performed to prevent MSC proliferation through the use of irradiation or a mitotic inhibitor.

Cell separation methods for MSCs and lymphocytes were performed for flow cytometry and gene expression assays. The methods for cell separation were validated prior to use in this assay so that the target cell populations were approximately 90% or greater of the total cell population (see gating regime in Supplemental Information). The method of cell separation is described in Appendix C: Compiled Methods. Ideally, we would have a 100% pure cell population for our studies. A cell separation column with antibodies would have been needed in order to improve upon our cell purification methods. We did not pursue cell purification any further due to the consistent 90% or greater level of cell purification and our ability to separate the MSCs from lymphocytes in our flow cytometry assays. See further discussion in Chapter 5, ‘Limitations.’

The gene expression assay utilized PWM as an activator of the lymphocyte genes. This was the same lymphocyte activating agent as used in the flow cytometry assays. In retrospect, a validation study of the activator used in the gene expression assay should have been performed. A thorough validation study to find the best activator was performed for the flow cytometry assay (Chapter 3). We utilized these results for our gene expression assay believe there would be

sufficient cross-over. Perhaps another activator may have caused upregulation of more of the inflammatory genes.

Our neutrophil activation assay was studied and validated prior to its completion. Neutrophils are known to have a half life of 13-16 hours in circulation (Lahoz-Beneytez *et al.* 2016). Assays utilizing potent neutrophil activators examine the neutrophils within 1 hour of the addition of the activating agent as reactive oxygen species peak within 60 minutes (Kirchner *et al.* 2012). Less potent additives likely require longer to activate neutrophils (see our results below). One neutrophil activation assay using MSCs utilized the 4 hour time point (Jiang *et al.* 2016). One assay utilized a 24 hour time point when determining if MSCs delayed natural neutrophil apoptosis (Khan *et al.* 2015). Validation assays of utilizing various time points of neutrophil activation were performed prior to performing our published neutrophil assay. Time points 4, 8 and 12 hours were utilized as we believed it would take time for the neutrophil-MSC interaction to take place and activation levels were likely to be subtle. Immediate testing as is performed with some neutrophil activating agents would be unlikely to illustrate activation of the neutrophils when these cells were inactivated and placed in co-culture with MSCs. The results of our validation assays showed that all time points had appropriate activation of the neutrophils when PMA was added as a positive control (Supplemental information). When MSCs were co-cultured with the neutrophils, time points 4, 6 and 8 hours showed very low levels of neutrophil activation. Time point 12 hours had the highest level of neutrophil activation, though this was still quite low. Although we know neutrophils only survive less than 24 hours in the

blood stream (McKraken *et at.* 2014, Khan *et al.* 2015), we found the 12 hour time point to be important in that it would show the greatest degree of neutrophil activation if the allogeneic MSCs were to cause neutrophil activation. Some neutrophils were apoptotic at the 12 hour time point, and these were removed from analysis by flow cytometry.

Our complement assay showed minimal complement mediated MSC death. Previous reports found >40% of the MSCs were damaged upon incubation with complement (Li and Lin 2012, Li *et al.* 2016). It is possible that the variation in our results originated from the assays utilized to determine the level of MSC viability. Li and Lin 2012 used cell leakage assays while our study utilized viability stain uptake of a normally impermeable molecule. Additionally, our study lacked controls that were included in previous assays including the use of specific complement-component depleted serum (Li and Lin 2012) or enzyme-linked immunosorbent assays to quantify the complement components (Yang *et al.* 2015). No purified complement components were utilized in our assay nor the addition of an anti-equine MSC antibody as we wanted to determine and compare the level of naturally-occurring complement-mediated MSC death. In the future, a positive control should be utilized to confirm the presence and potency of the complement components in equine serum.

Fetal bovine serum was used in the MSC cultures during the MSC expansion period. The media was rinsed and changed to FBS-free media 48 hours prior to the addition of leukocytes. This is important in that equids are known to have anti-bovine antibodies in circulation likely due to being immunized with

vaccines with bovine products (Rowland *et al.* 2021). For this reason, even a small amount of FBS in the MSCs can cause an antibody-mediated immune response. Joswig *et al.* 2017 stated that greater than 95% of the FBS particles were removed with this wash out period. Without removal of the FBS, an immune response is generated against the MSCs when used *in vivo* (Joswig *et al.* 2017). An increase in the immune response was not seen in those MSCs that were allowed a 48 hour FBS-depletion period (Joswig *et al.* 2017). Still, some FBS remained in the cells that can cause an immune response. All autologous and allogeneic MSCs were treated similarly, and the same leukocytes were used for all samples. For this reason, a similar immune response should be generated. Therefore, in comparing the autologous and allogeneic MSCs, the differences in immune response between the groups were likely due to the MSCs rather than the anti-bovine response.

T regulatory cells play an integral role in immunosuppression of early stage inflammation. Tregs dampen the immune response through the use of cytotoxic T lymphocyte antigen 4 (CTLA-4) (and some less important co-stimulatory molecules such as LAG3 and IL-10) on the Treg cell surface (Read *et al.* 2000). This molecule binds to CD80 and CD86 on the surface of antigen presenting cells. This prevents these molecules from stimulating conventional CD4+ and CD8+ lymphocytes (Wing *et al.* 2019). This can lead to apoptosis of those CD4+ and CD8+ cells, thus dramatically decreasing the immune response (Wing *et al.* 2019). Tregs also utilize immunosuppressive cytokines that can inhibit lymphocyte

activation and function. These include IL-10, TGF-β, IL-35, TIGIT, CD39, and CD73 (Wing *et al.* 2019).

When considering the identification of Treg cells in our MSC/ PBMC co-cultures, the CD4/25/FoxP3 positive cells identified in Chapter 4 may not have been properly identified as Tregs. CD4/25/FoxP3 positive cells can be categorized as either activated CD4 cells or regulatory T cells. Activated T cells go through a transient period of becoming FoxP3 positive prior to returning to their FoxP3 negative phenotype (Pillai et al. 2006). During this time, the activated T cells expressing FoxP3 inhibit proliferation of CD4 lymphocytes in a similar manner as Tregs (Pillai et al. 2006). But unlike Tregs, these activated CD4 cells go on to potentiate the immunoactivation that caused their activation (Pillai *et al.* 2006).

In regards to the results from Chapter 4, the cells labelled as Tregs may also be transiently Foxp3 expressing activated T cells. Without functional assays, we cannot fully identify these lymphocytes. There are 2 co-culture groups that are labelled to have significantly more Tregs than other groups (Figure 19). These are ‘blood donor MSCs’ at ratio 1:1 and ‘MHC II-high MSCs’ at ratio 1:100. The co-culture containing blood donor MSCs also had a high number of activated CD4 lymphocytes (CD4+/CD25+/FoxP3-). This may suggest that the cells labelled Treg in this group, may be transient activated CD4 lymphocytes instead. As for the other co-culture with a high number of Tregs, the MHC II-high MSCs co-culture, this culture had a low level of activated lymphocytes (CD4+/CD25+/FoxP3-), so likely these cells are truly Tregs.

In light of these findings, the blood donor MSC co-culture group may not have a higher number of Tregs as compared to other co-cultures and these CD4+/CD25+/FoxP3+ cells may simply be activated CD4 lymphocytes with transient FoxP3 expression.

Further clarification is needed for the actions of IL-2 *in vivo* as it is a critical cytokine in immunologic activation and dampening. IL-2 serves to regulate immune responses by increasing differentiation or preventing differentiation depending on the CD4+ lymphocyte subsets and the antigen and other cytokines present (Spolski *et al.* 2018). Without the presence of IL-2, lymphocytes would proliferate uncontrollably and severe autoimmunity would occur (Mizui 2019). IL-2 binds directly to CD4+ lymphocytes leading to immunoactivation or immunosuppression. Binding of IL-2 to CD4+ can drive the cells to differentiate into Thelper (T_H1 and T_H2 cells) and memory T helper cells which creates a prolonged immune response when the body is faced with a repeat infection. IL-2 inhibits lymphocyte differentiation into T_H17 lymphocytes but then promotes its proliferation once the cell has differentiated (Spolski *et al.* 2018). Therefore, IL-2 serves to dampen responses to extracellular infectious materials and auto-immune diseases at their initial stages and increases lymphocyte responses later in the disease process.

IL-2's effects on Treg lymphocytes were discussed briefly in the manuscript but are further explained here. Regulatory T cells have high affinity for IL-2 (Mizui 2019). In the presence of IL-2, Treg cells proliferate and their survival is enhanced. Opposingly, CD8+ cells have a lower affinity for IL-2. Therefore, when low levels of IL-2 are present, Treg cells cause immunosuppression. Later in the disease

process when the immune system needs to ramp up, higher levels of IL-2 are present leading to CD8+ lymphocyte proliferation (Mizui 2019).

All of these IL-2/lymphocyte interactions culminate to cause an initial downregulation of lymphocyte activation early in a disease process. T_{reg} proliferation prevents activation of effector and memory lymphocyte subtypes (Abbas *et al.* 2018). In the face of increasing inflammation, IL-2 increases in concentration and induces IFNγ leading to increased activation of CD4+ and CD8+ lymphocytes (Spolski *et al.* 2018).

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Chapter 5. General Discussion

5.1 Aims and objectives

This research was performed to test the hypothesis that there exists a phenotype of equine BM-MSC that would be ideal for use as a donor MSC for treatment of equine disease. An ideal donor MSC is one that is immunopriveledged in the recipient so that it may provide anabolic effects that would lead the improvement of disease. In order to test our hypothesis, we had to identify groups of donors with similar MSC phenotypes. We then had to test these MSCs in an allogeneic environment to determine if there was a link between MSC phenotype and immunosuppressive and, potentially, therapeutic ability.

5.2 Phenotypic variation by breed and blood-donor status

Through this investigation of BM-MSCs, we were able to characterise phenotypic differences between donor MSC groups. There were several breed and blood donor-status effects on MSC marker expression and these, in turn were influenced by the passage number. Bone marrow-derived MSCs from Standardbreds showed significantly less MHC class II expression at early passages as compared to Thoroughbreds whose MHC class II expression at early passages particularly was elevated. The MSC markers, CD44 and CD90, were both expressed at high levels on both Standardbred and Thoroughbred MSCs.

The large difference in MHC II expression between Standardbreds and Thoroughbreds helps to explain the inconsistency in findings described in

published studies on the MHC II status of MSCs in horses (Paebst *et al.* 2014, Schnabel *et al.* 2014). Some horses' MSCs express very low levels of MHC II while others express high levels, especially at early passages. MHC II expression declines with passage, but as the early passage MSCs are the most prolific, metabolically active and, therefore useful clinically, the early stage expression is most crucial.

Universal blood donor MSCs had little phenotypic variation from their parent breed of Standardbreds as these horses were consistently low in their expression of MHC II and high in their expression of the MSC marker CD44. Though these horses had a phenotype that was different on their erythrocytes, this contrast did not carry over to their MSCs. The only significant variation was that CD90 was expressed more highly on universal blood donor MSCs as compared to non-blood donor Standardbred MSCs. A high level of CD90 expression is desirable in that this MSC marker appears important for maintaining pluripotency. The conclusion from stage one of our analysis was that universal blood donor-type Standardbred horses appear less likely to cause an MHC II driven immune reaction and have high levels of bone marrow-derived MSC markers.

5.3 MSC immunosuppression and immunoactivation

When we applied each group of MSCs to co-culture with leukocytes, MHC II-low MSCs were superior at preventing immune activation and upregulated their production of anabolic and anti-inflammatory RNA. The origin of these cells from universal blood donor horses or non-blood donor horses only appeared significant at the level of CD4 and CD8 activation where universal blood donor MSCs were

inferior to non-blood donor MSCs. MHC II-low MSCs prevented proliferation of activated PBMCs, increased expression of both anabolic and catabolic genes, decreased activation of neutrophils, and maintained viability when exposed to complement. Some inflammatory gene expression did increase with these cells, but a reciprocal anti-inflammatory gene response was also seen. These MHC II-low MSCs were activated in the recipient environment to perform immunosuppressive and anabolic functions. We found these cells to be the most ideal donor allogeneic MSC of the phenotypes tested.

5.4 Limitations

The primary limitation with our immune assays was that only a single recipient leukocyte population could be used to test the ten donor MSCs in our final co-culture assay to determine the most ideal allogeneic MSC phenotype. The number of assays and samples required made the use of multiple recipient leukocytes impossible.

Another limitation of our methodologies was the lack of use of a blocking buffer to prevent nonspecific binding to Fc receptors. A blocking buffer was not used for several reasons. First, we believed there would be minimal contamination of our PBMC sample with cells with Fc receptors. The B cell component of the cells included in our PBMC gate was likely the most populous cell that carries the Fc receptor. The average B cell component was <10% of our total PBMC population. We used a fixable viability dye which does not allow the use of protein in the staining buffer. Our media used on the cells included 10% FBS and there was likely some residual protein with the cells thought they were washed with PBS. Most

importantly, during our validation assays, the appropriate co-staining in appropriate proportions known to be in equine blood was seen on dot plots which made nonspecific binding appear to be a negligible issue.

During our CellTrace Violet assays, we utilized DMSO as a carrier agent for the concentrated dye as directed by the kit instructions. DMSO is an anti-inflammatory molecule known to significant decrease proliferation in human lymphocytes at concentrations of 1%v/v and greater (de Abrue Costa *et al.* 2017). Concentrations of 0.5%v/v did not impede proliferation (de Abrue Costa *et al.* 2017). At the highest concentration, we used 0.1%v/v DMSO in our CTV and cell samples. The DMSO concentration was decreased further in correlation with the concentration of CTV in our dilution assays. It is possible that the DMSO in the CTV dying mixture may have impeded the proliferation of equine lymphocytes. As we know from our CTV study, equine lymphocyte proliferation behaviours vary from human lymphocytes. A DMSO sample not containing CTV would have been beneficial to assess the effect of the DMSO on the proliferation as compared to the CTV and DMSO mixture. Our results would be better stated as: CTV diluted with DMSO according to the manufacturer's instructions inhibits proliferation in equine lymphocytes.

Cell separation methods for MSCs and lymphocytes were performed in our co-culture assays for flow cytometry and gene expression analysis. The methods for cell separation were validated prior to use in this assay so that the cell populations were approximately 90% or greater the cell population specified (see gating regime in Supplemental Information, Chapter 4). The method of cell

separation is described in Appendix C: Compiled Methods. Ideally, we would have a 100% pure cell population for our studies. A cell separation column with antibodies would have been needed in order to improve upon our cell purification methods. We did not pursue the cell purification due to the consistent 90% or greater level of cell purification and our ability to separate the MSCs from lymphocytes in our flow cytometry assays.

This lack of a pure population of cells may have distorted the results of the gene expression analysis by allowing some MSCs or lymphocytes be present in the gene analysis for anabolic and inflammatory molecules. The distortion would make either the MSCs or the lymphocytes appear to be expressing a gene that the other cell type was expressing. Therefore, the assessment between each of the autologous and allogeneic MSC types would not have changed, only the origin of the increased gene expression. We believe that this does not change our understanding of the comparison between the MSC groups, which is the primary focus of our analysis. The genes that are upregulated would be produced in the co-culture no matter whether they were originating from the MSC or the lymphocyte. Furthermore, as we had greater than a 90% cell purification rate as seen on flow cytometry, there would only be a 10% variation from the pure population. When side-by-side analysis is performed as was done in the Supplemental Information for Chapter 4, we can see that for each MSC group, there are genes with high variation between the MSC and lymphocyte expression levels. As the same samples was utilized for analysis of all of the genes, the appearance of a high variation between MSC and lymphocyte even in the

expression of a single gene illustrates adequate cell separation methods. For those genes that show a low expression in one cell type and a higher expression in the other cell type, it is possible that the lower expressing cell type does not express the gene. This would be from the small amount of cell impurity from the other cell type that does express the gene.

Another limitation of our methodology was the lack of appropriate controls for our complement-mediated MSC death assay. Such controls include the use of specific complement-component depleted serum (Li and Lin 2012) or enzyme-linked immunosorbent assays to quantify the complement components (Yang *et al.* 2015). No purified complement components were utilized in our assay nor the addition of an anti-equine MSC antibody as we wanted to determine and compare the level of naturally-occurring complement-mediated MSC death. In the future, a positive control should be utilized to confirm the presence and potency of the complement components in equine serum.

Finally, we must assess the applicability of our assays to the natural world. *In vitro* studies in themselves are a limitation as the true environment in the animal is never perfectly simulated. In saying this, we believed *in vitro* trails were crucial in allowing us to control for variation and test specific leukocyte interactions. The fact that there is no bone marrow-derived MSC available that does not express MHC I assures that even a MHC II-negative MSC will be identified in the recipient immune system as a foreign cell. Our goal was not to negate this fact. Our goal was to determine if the variation in MHC II expression on the naïve MSC would lead to variations in immune response when placed in co-culture.

5.5 Conclusion

If we combine our results all of our studies, we come to two important conclusions. First, allogeneic MSCs caused impressively little activation of leukocytes and behaved, overall, similarly as autologous MSCs. The MSCs and leukocytes were mismatched in their ELA haplotypes which would lead to maximal leukocyte activation when compared to matched cells. Mismatched lymphocytes consistently cause lymphocyte activation as seen in previous equine mixed leukocyte reaction assays (Carrade 2012, Schnabel 2014). Clearly MSCs cause some degree of immunosuppression when ELA mismatched cells are co-cultured.

Our second finding was that MHC II-low MSCs were the ideal candidate for allogeneic use. Although universal blood donor MSCs retained their differentiation ability better than non-blood donor MSCs, the non-blood donor MHC II-low MSCs were better able to prevent immunoactivation and promote immunosuppression via their effect on T lymphocytes when used in an allogeneic manner. Both types of MHC II-low MSCs had gene upregulation. Both MHC II-low MSCs caused minimal neutrophil activation and maintained viability when cultured with neutrophils or complement. We know from previous research that lower passage MSCs have preferred physiologic properties included a reduced population doubling time and increased metabolic activity (Bakopoulou *et al.* 2017). For these reasons, passage 2-4 non-blood donor MHC II-low MSCs should be preferred for use in allogeneic therapeutic testing.

5.7 Future Work

Research in allogeneic MSCs is moving in the direction of major histocompatibility complex (ELA) haplotyping of the MSC donor and recipient (Rowland *et al.* 2020). This is especially important in cases requiring repeat treatment as antibodies would be present in the recipient that could prevent MSC survival (Berglund *et al.* 2017). This would prevent immune recognition and therefore prevent reactions which may damage the recipient tissue or decrease viability of the donor MSCs. Unfortunately, there are hundreds of different ELA haplotypes originating from 12 loci on the major histocompatibility complex (Holmes *et al.* 2019). This could make finding a matched donor difficult, time intensive and expensive.

Instead of using matched ELA donors for MSC therapy, a more realistic option at this time would be to rotate the MHC haplotype of the donor MSC if repeat MSC treatments were needed. This, along with using the appropriate MHC II-low phenotype MSC for all treatments given, would likely be the best cost-to-benefit ratio for treatment of equine disease.

In moving ahead with the results, it would be ideal to apply the MHC II-low MSCs to an *in vivo* model using tracking dyes to observe the survival of these cells as it is hypothesized that the greater survival of the MSCs would provide a stronger therapeutic effect. These cells could be applied to a disease model, and, using histology or *in vivo* fluorescence marker techniques, they could be monitored for viability, proliferation, and their effects on disease. MHC II-low MSCs could be used in a model of repeat treatment where the ELA haplotype of the donor MSC

is different for each of the successive treatments. Tracking and histologic analysis could be used to determine whether the MSCs are capable of long-term survival when used in a repeat treatment, and most importantly, whether repeat treatment is effective at improving disease state.

5.8 References

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Appendices

Appendix A: Efficiency of MSC isolation procedures

Introduction

As discussed in chapter 1.3, MSC isolation is performed by separating the components of bone marrow. Separation can be performed using one of three general methods: centrifugation, erythrocyte lysis, or the use of a density gradient (Bourzac *et al.* 2010, Zhang *et al*. 2014). More advanced cell separation methods such as magnetic bead and fluorescence-activated cell separation can be performed but have a low yield and therefore are less commonly used for MSC isolation from equine bone marrow as a large number of cells are needed (Freshney 2010).

The intention of the bone marrow separation is to separate the mononuclear cells including the MSCs from the other components of the bone marrow in order to get a high yield of MSCs at the end of the culturing procedure.

Methods

Three commonly used methods for MSC isolation were compared. Fifteen ml of heparinized bone marrow was harvested from four horses. The marrow was mixed completely prior separating into 3 aliquots of 5ml bone marrow. Five ml of bone marrow was used for each of the three methods. Cells were plated on polystyrene tissue culture flasks (CellStar®, Greiner Bio-one, Monroe, NC, USA) and incubated at 37°C in 5% CO2. Mesenchymal stromal cell media was changed 24 hours post-plating and then every 3 days until 13 days post-initial plating. Cells

were then lifted from the plates using trypsin (GibcoTM, Thermo Fisher®) and stained with trypan blue. The cells were then counted using a haemocytometer. Results for the total live cells for each method of isolation were compared using the mean across all 4 horses. No statistics were performed.

The high speed centrifugation method consisted of centrifugation of the bone marrow at 1000g for 15 minutes (Bourzac et al. 2010). The supernatant was removed and the bottom 1ml was taken for cell counting and plating. Cells were plated at 1.2×10^6 cells/cm^3.

The low speed centrifugation method consisted of centrifugation of the bone marrow at 100g for 5 minutes (Kisiday et al. 2013). The supernatant was then removed and centrifuged at 1000g for 10 minutes. The pellet was used for counting and the cells were plated at 0.267×10^6 cells/cm^3.

The erythrocyte lysis method used 15mM buffered ammonium chloride solution (Cherneyshev *et al.* 2008). The 5ml bone marrow was mixed with 5ml of 30mM ammonium and the tube was gently inverted prior to a 10 minute incubation at room temperature. The solution was then centrifuged at 600g for 5 minutes. The pellet was taken for cell counting and the cells were plated at 0.267×10^6 cells/cm^3.

Results

All methods provided ample MSCs after the 13 day incubation period. The low speed centrifugation method and the erythrocyte lysis method gave relatively similar results while the high speed centrifugation method provided less MSCs at

the end of the culture period (7.95+/-5.3 x 10^6 cells, 7.99 +/- 5.8 3 x 10^6 cells, and 4.59 +/- 5.0 x 106 cells, respectively).

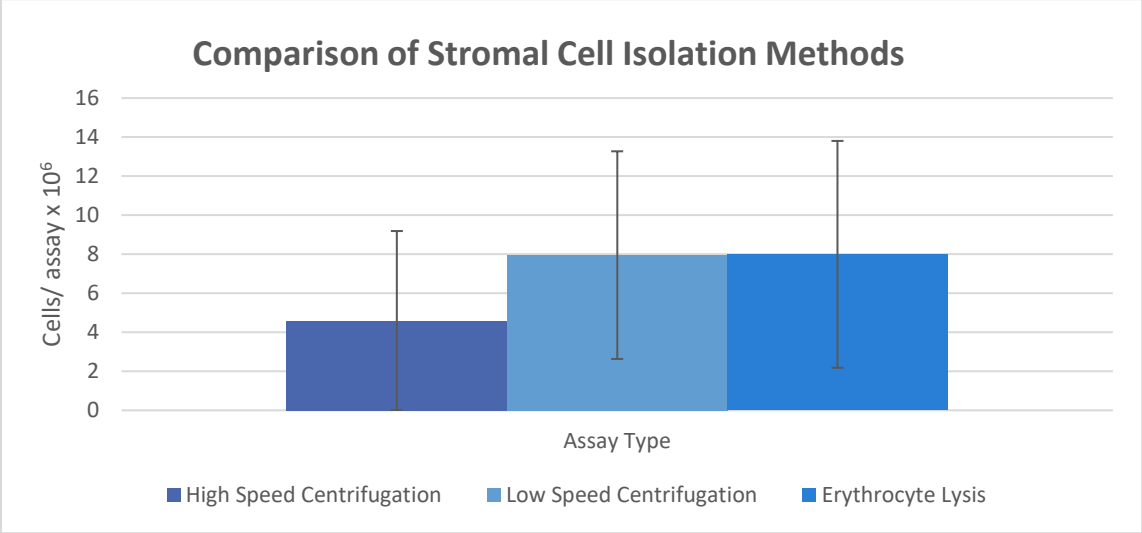

Figure 1. Low speed centrifugation and the erythrocyte lysis method provided the most efficient methods of MSC isolation.

Conclusion

Low speed centrifugation proved to be the most efficient method of MSC isolation as it consistently provided high numbers of MSCs. What makes it preferable to the erythrocyte lysis method is that it no additional reagents needed to be added to the marrow. Reagents can serve a source of contamination.

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Appendix B. Methods to provide optimal viability post-cryopreservation

Introduction

Cryopreservation is the technique of freezing a live cell or tissue in order to allow it to maintain viability during and after the freezing period. This involves a gradual cooling process using appropriate media to prevent cell death. Here we test several methods of freezing using different concentrations of cryopreservation media in order to find the technique that provides the highest viability after thawing the cells.

Methods

One horse's passage 3 MSCs were grown to sufficient numbers to allow for 2 x 10^6 cells to be frozen in 200ul freeze media. Freeze media consisted of autologous serum and DMSO. The amount of DMSO varied from 0-15%. Cells were placed in a cryovial and then a cooling device to allow for gradual cooling (Mr Frosty™, Thermo Fisher®). Cells were placed in the temperature as detailed in the results. Cells were held at each temperature for 72hours prior to transfer to the next temperature or prior to analysis (depending on the sample). The cryovials were placed in a warm water bath to allow the cells to quickly return to 38C prior to analysis. The cells were stained with trypan blue and then counted using a haemocytometer. Samples were reported as the percent of viable cells. One sample was used per data point.

Results

Cells that were kept at 4C for 72 hours without DMSO had good survival (85.2%). Cells placed in -20C for 72 hours had adequate survival (67.7%, 92%, and 66.6% at DMSO concentrations of 5%, 10%, and 15% respectively). Cells taken from -20 (72 hours) and then to liquid nitrogen (-196C) showed poor survival (19.3% at 10% DMSO). Cells frozen to -80C for 72 hours had adequate survival (65.9% and 83.3% at DMSO concentrations of 5% and 10% respectively). Cells frozen to -80 for 72 hours and then transferred to -196C for 72 hours had adequate survival (65.4% and 79.7% at DMSO concentrations of 5% and 10% respectively).

The 10% DMSO concentration consistently showed improved viability as compared to 5 or 15% DMSO.

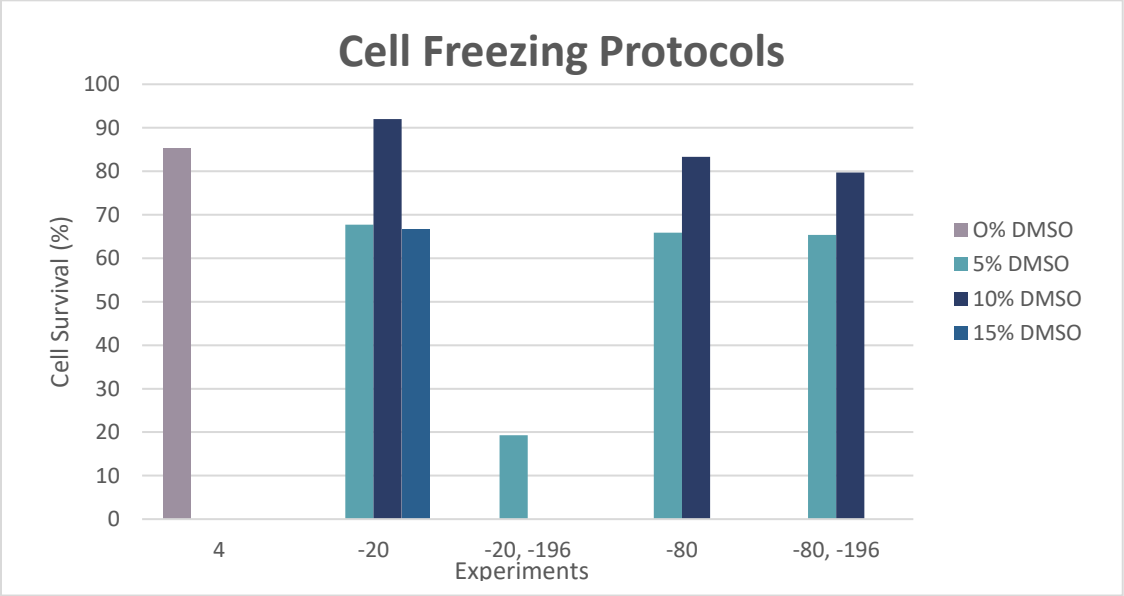

Figure 1. Optimal survival of MSCs was seen when cells were cooled to 4C or frozen to -20C or -80C or frozen from -80C to -196C and kept in 10% DMSO. Cells moved directly from -20C to -196C had poor survival.

Conclusion

For long term freezing, cells should be gradually frozen to -80C and then transferred to liquid nitrogen. The optimal freeze media contained serum and 10% DMSO. Cells can be kept at 4C in serum for 72 hours with good survival.

Appendix C. Compiled methods

Bone marrow harvest, isolation and culture

Following ethics approval by the Massey University Animal Ethics Committee (MUAEC Protocol 15/13), MSCs were harvested from the sternum of all 36 horses. In brief, 15 mL of bone marrow was aseptically harvested and added to 3 mL of 1000 IU/mL heparin (Pfizer®, New York, NY, USA). Blood (25 mL) was collected via the jugular vein and placed in blood tubes (Rapid Serum Tube, BD Vacutainer®, San Jose, CA, USA) for serum collection. The bone marrow aspirates and blood tubes were transported to the laboratory on cold saline bags (3-5°C).

MSCs were isolated within 12 hours of harvest. Bone marrow aspirates were centrifuged at 200 X g at room temperature for 2 minutes. The supernatant was centrifuged at 1,000 X g for 10 minutes to pellet the nucleated cells. The supernatant was discarded and the pellet suspended in low-glucose Dulbecco modified Eagle's medium (DMEM, Gibco, Thermo Fisher®, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Gibco, Thermo Fisher®), penicillin (100 IU/ml), streptomycin (100 ug/ml) and amphotericin B (0.25ug/ml) (Sigma-Aldrich®, St Louis, MO, USA) and 2.5% 1M HEPES buffer (Gibco, Thermo Fisher®). The same FBS batch was used throughout the study. Polystyrene tissue culture flasks (CellStar®, Greiner Bio-one, Monroe, NC, USA) were plated at a concentration of 0.267 x 106 cells/cm2 and incubated at 37°C in 5% CO2. The culture media was completely replaced after 24 hours. Once MSC colonies had formed, the cells were lifted from the flasks using Accutase (StemPro®, Thermo Fisher®) and plated onto

new flasks. Cells were then fed with MSC proliferation media comprised of Alpha modification of Eagle's medium (AMEM, Gibco, Thermo Fisher®) with 10% FBS, 1% penicillin/streptomycin/amphotericin B and 2.5% 1M HEPES buffer.

Following passaging, cells were grown in culture flasks to 80% confluence. Cells from passages 2, 4, 6, and 8 were frozen at a concentration of 107 cells/mL in freezing media (autologous equine serum and 10% dimethylsulfoxide (Molecular Probes, Eugene, OR, USA). Cryovials (2mL, Greiner Bio-one, Monroe, NC, USA) were cooled to -80°C using a slow-cooling container (Mr Frosty™, Thermo Fisher®) followed by storage in liquid nitrogen.

Trilineage potential

MSCs from passage 4 of four horses from the Standardbred, Thoroughbred and blood donor groups were assessed for trilineage potential. Each horse's cells were sampled in triplicate. The potential for adipogenic, osteogenic and chondrogenic differentiation was determined for the MSCs samples through cell expansion according to the manufacturer's instructions. Briefly, MSCs were plated on chamber slides (Lab-Tek, Thermo Fisher®) at 1 x 104 cells/cm2 for the evaluation of adipogenesis, and at 5 x 103 cells/cm2 for the determination of osteogenic potential. The chondrogenesis assay used 0.25 x 106 cells that were centrifuged at 1000 x g for 5 minutes to form a cell pellet. After 24 hours of growth in proliferation media, MSCs were grown using specialized media (StemPro® Adipogenesis, Osteogenesis, and Chondrogenesis Differentiation Kits, Thermo Fisher®). The cells were grown in the differentiation media in monolayer for 14

days for adipogenic and osteogenic lineage assays. Cells were grown in pellet culture for 21 days for the chondrogenic lineage assay.

An additional set of cells was made by combining the Thoroughbred, Standardbred and blood donor cells in equal proportions. These cells were used as a control. A control sample was made for each lineage (adipogenic, osteogenic, and chondrogenic). These cells were cultured and treated in a similar manner as the trilineage groups except that only proliferation media was used (no induction media).

All cells were fixed in 4% formaldehyde at the end of the culture periods and stained as described for the respective differentiation protocols. Adipogenic cells were stained with Oil Red O. Osteogenic cells were stained with Alizarin red S. Chondrogenic pellets were embedded in paraffin and stained with alcian blue and counterstained with hematoxylin and eosin. Five randomly selected regions of each of the samples were assessed, providing 45 images to be used for evaluation of each of the Standardbred, Thoroughbred, and blood donor groups. The presence or absence of differentiation was evaluated using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). Adipogenesis was determined by percentage Oil Red O staining over total area of cell coverage. Osteogenesis was measured as percentage of alizarin red-positive area over total area. Chondrogenesis was measured as percentage of alcian blue-positive area over total area of cell coverage.

Blood typing

Five mL of blood was collected in heparinized tubes (Heparin Tube, BD Vacutainer®, San Jose, CA, USA) for blood typing at the Equine Parentage and Animal Services Centre at Massey University. Blood was screened for Aa Ca and Qa antigens as horses that are used for blood donation (universal donors).

Flow cytometry for MSC markers

The methods and the efficacy of the selected cell markers were first validated in a pilot study prior to use on the study population. All antibodies used in the main assay were first validated in the pilot study. Flow cytometry assays for CD 11a/18, CD 44, CD 90, and MHC class II antigens were validated using bone marrow-derived MSCs or leukocytes. The specific antibodies used are included in the supporting information. Erythrocytes were added to exclude non-specific binding. Erythrocytes autofluorescence did not cause these cells to appear positive for the fluorochromes as has been seen in other studies. Samples from three horses were used for each antigen for validation assays. MHC class I molecules were not tested as they are consistently expressed at high levels in equine bone-marrow derived MSCs. Antibodies used were those previously reported and listed in the supporting information. All of the antibodies used were fluorescence conjugated for direct immunofluorescence. Those antibodies that were distributed without a conjugated fluorescing label were conjugated using an antibody labelling system (Mix-n-Stain™ Dye Antibody Labelling Kit, Biotium, Fremont, CA, USA; LYNX Rapid Antibody Conjugation Kit, Bio-Rad Laboratories, Hercules, CA, USA) (see supporting information). Antibody titration was performed to assure the

optimal dilution was used. Antibody concentrations of 1:10, 1:50, 1:100, and 1:200 were compared using the stain index equation. The dilution with the highest stain index was used. The most appropriate dilutions identified are listed in the supporting information, and these dilutions were used in subsequent assays.

For the validation study, aliquots of MSCs or leukocytes were suspended in phosphate-buffered saline (PBS) to obtain a concentration of 25 x 10^3 cells/µL. A 40 µL aliquot (1 x 10^6 cells) was used for each flow cytometry assay. The cells were incubated with a viability stain (1ul/500ul cells, Efluor 780™, eBioscience™, San Diego, CA, USA) for 30 minutes on ice and protected from visible light. The cells were then washed with PBS and the diluted antibodies for CD11a/18, CD44, CD90, and MHC class II molecules added were added at the same time. The mixture was incubated on ice and protected from visible light for 30 minutes. The samples were then washed with 2mL PBS to remove excess (non-bound) antibody and fixed in 3% paraformaldehyde for 20 minutes. After a final wash and dilution in 1mL PBS, the cells were evaluated on a flow cytometer (BD FACSVerseTM, San Jose, CA, USA). Data were collected on 1 x 10^4 large cell events (small debris was ungated) for each sample.

All data were compensated and corrected for autofluorescence using cytometric capture beads (BD™ CompBeads, San Jose, CA, USA), single stains, and all-fluorochromes-minus-one compensation tubes. Compensation for any spectral overlap between fluorochromes and data evaluation was performed using specialized flow cytometry software (FlowJo®, Ashland, OR, USA).

Gating was performed on a hierarchy format with, first, cells being isolated over a time frame that provided consistent cell acquisition data. Then viable cells were selected according to their low viability stain uptake. A mononuclear cell subset was selected by graphing on forward cell scatter area and height. Finally a large cell population was selected. This gated cell population was used to determine cell marker expression.

After initial gating to identify an appropriate cell population for further analysis, these cells were gated to identify populations of cells positive and negative for each of the markers. The populations were gated using both unstained cells and stained cells known to be negative or positive for the marker. Data were reported as the percent of cells in this population that showed fluorescence for a specific marker. Both LK and JR (acknowledgements) performed independent data analysis prior to finalizing the results.

After antibody validation, a sample of 1×10^6 equine MSCs in the fourth passage was used to compare expression levels from MSCs immediately removed from culture and those that had been cryopreserved 24 hours prior. Samples from three horses were used in this part of the study. Expression of the cell markers were compared using a Chi-Square test for proportional populations. This pilot study was performed to confirm that cryopreserved cells could be used to accurately depict the cell marker expression.

After these validation steps were performed, MSCs derived from bone marrow samples of the 36 test horses were examined (Chapter 2). Cell surface expression of CD11a/18, CD44, CD90, and MCH class II molecules at culture

passages 2, 4, 6 and 8 were analysed for each sample. These passages were selected to give an overview of marker expression during the early culture period, when MSCs are commonly utilized for therapy because they are more proliferative and therefore provide sufficient numbers for treatment, and have a greater potential for differentiation than later stage passages.

Isolation of peripheral blood mononuclear cells and neutrophils

Following ethics approval by the Massey University Animal Ethics Committee (MUAEC Protocol 18/06), blood was aspirated aseptically from the left jugular vein of one Connemara horse. This was the same horse that had been used for MSC isolation. Blood was placed in heparinized blood tubes (BD Vacutainer®, California, USA) for lymphocyte collection and subsequently processed using Lymphoprep™ (Alere Technologies AS, Norway). Briefly, blood was diluted 1:1 with phosphate buffered saline (PBS) (Gibco, Thermo Fisher®). Fifteen ml of Lymphoprep™ was placed in a centrifuge tube, and 30 ml of diluted blood was placed on top of the Lymphoprep™. The tube was then centrifuged for 25 minutes at 1125 x g at low acceleration and without braking, thereby forming a density gradient. The lymphocyte- rich layer at the interface of the serum and the gradient agent was recovered and the neutrophil- rich pellet were then used.

The PBMC rich layer was washed three times with PBS. The PBMCs were then diluted in PBMC media which was composed of RPMI 1610 media with 10% active equine serum, penicillin-streptomycin (100 ug/ml) (Sigma-Aldrich®, St Louis, MO, USA), 2-Mercaptoethanol (0.1mM) (Gibco, Thermo Fisher®).

The pellet from the density gradient was then used to isolate neutrophils. Thirty-five mL of sterile water was added to the pellet. The centrifuge tube was inverted two times to allow for mixing. Five mL of PBS (10X) was then added to neutralize the osmolality. The tube was centrifuged for 10 minutes at 1000g. The supernatant was discarded and the neutrophil-rich pellet was washed in PBS. The neutrophils were cultured in media with Alpha modification of Eagle's medium with 10% active equine serum, 1% penicillin/streptomycin/amphotericin B and 2.5% 1M HEPES buffer.

Serum collection

Serum was also collected using clot activating tubes (CAT BD Vacutainer®, San Jose, CA, USA). The tubes were allowed to sit for 1 hour prior to centrifugation at 3220g for 15 minutes (Bergseth *et al.* 2013). The serum was used in co-culture media within 90 minutes of harvest (active serum). A separate aliquot of serum was inactivated by heating to 56°C for 30 minutes (inactive serum) and used only in the complement assay.

MSC and PBMC co-culture

Following a 48 hour incubation period allowing the MSCs to adhere to the base of the plate with media containing equine serum, the media was removed and PBMCs in PBMC media (see above) were added to the MSC wells. PBMCs were added as a ratio of MSCs to PBMCs so that there were equal numbers (ratio 1:1), ten times the number of PBMCs as MSCs (ratio 1:10), and 100 times the number of PBMCs as MSCs (ratio 1:100). These numbers can be considered to be typical for an equine joint during its normal cycle of reaction to an intra-articular

MSC injection (de Grauw *et al.* 2009, Ardanaz *et al.* 2016). PBMCs without MSCs +/- activation agent (Pokeweed mitogen (PWM) (Sigma-Aldrich, Missouri USA) (2.5µg/ml) were cultured to serve as controls.

PBMCs and MSCs were placed in co-culture at 37C with 5%CO2 for 3 to 5 days prior to analysis.

MSC and neutrophil co-culture

Neutrophils and MSCs were co-cultured to determine the degree of neutrophil activation subsequent to their interaction. Following 48 hours incubation to allow for MSCs adherence to the plate with media containing equine serum, the media was removed, and fresh neutrophils (less than 2 hours post-blood draw) in neutrophil media (described above) were added to the MSC wells. Neutrophils were added in similar ratios as that of lymphocytes. Neutrophils alone +/- activation agent Phorbol myristate acetate (PMA, Sigma-Aldrich, St Louis, Missouri, USA) (2.5uM) were cultured to serve as controls.

Neutrophils and MSCs were placed in co-culture at 37C with 5%CO2 for 6 hours or 12 hours prior to analysis with flow cytometry.

MSC and complement co-culture

Following a 48 hour incubation period allowing the MSCs to adhere to the base of the plate with media containing inactivated equine serum, the media was removed and MSC proliferation media containing 30% active or inactivated serum was added. After 1 hour, MSCs were stained as described in the flow cytometry section.

Tritiated thymidine incorporation assay to assess proliferation in co-culture

PBMCs and MSCs were co-cultured for 3 or 5 days prior the addition of tritiated thymidine in order to determine if there was lymphocyte proliferation subsequent to MSC co-culture. Tritiated thymidine assays were performed using 1 x 10^4 MSCs and the corresponding ratio of 1:1, 1:10 and 1:100 MSCs:Lymphocytes per well in a 96 well plate (Greiner Bio-One, Monroe, NC, USA). One µCi of [methyl-3H]-Thymidine (Perkin-Elmer, MA, USA) was added per well and cells were incubated for a further 18 hours. Cells were harvested onto glass fibre mats (Tomtec Harvester, Connecticut, USA) and cell-incorporated radioactivity was measured using a scintillation counter (Wallac TriLux MicroBeta 1450, Finland) and reported as counts per minute (cpm).

MSC and PBMC separation

MSCs were separated from the PBMCs for flow cytometry and gene expression analysis. Validation assays were used to verify these methods so that >90% of MSCs and lymphocytes were appropriately separated as determined by flow cytometry. First, the media containing the PBMCs was removed. Then the remaining adhered cells were incubated for 1 minute with Accutase (StemPro, ThermoFisher, Massachusetts, USA) at room temperature to allow loosely adhered cells to be removed from the plate. These were added to the PBMC sample. The remaining cells were incubated with Accutase for 10 minutes or until all cells were no longer adhered to the base of the plate. These cells were kept for an MSC sample. All cells were washed twice with DPBS prior to flow cytometry.

Flow cytometry for PBMCs and MSCs

Flow cytometry was performed to assess changes in lymphocyte sub-populations and MSC antigen expression after co-culture. PBMCs and MSCs were tested just prior to co-culture (Day 0) and on Days 3 and 5 of co-culture. Fixable viability dye (Efluor 780, eBioscences, Thermo Fisher) was used to assess cell viability in flow cytometry assays. Samples were measured using a BD FACSVerseTM (San Jose, CA, USA). All events in the sample were recorded for the leukocyte population and for the MSC population separately. The MSC and leukocyte populations were characterised using the gating hierarchy as shown in Supplementary Information using flow cytometry analysis software Flowjo (Flowjo LLC, Oregon, USA).

For lymphocyte analysis, antibodies against extracellular CD4 (CVS4, US Biological, Salem, MA, USA) (Hamza *et al*. 2011), CD8 (CVS8, BioRad, Hercules, CA, USA) (Robbin *et al*. 2011), CD21 (CA2.1D6, AbCam, Cambridge, UK) (Arzi *et al*. 2017), and CD25 (RND Systems, Minneapolis, MN, USA) (Hamza *et al*. 2011) were used in accordance with previous publications. Following permeabilization (FoxP3 Transcription Factor Staining Buffer, eBiosciences, San Diego, CA, USA), an intracellular antibody for FOXP3 (FJK-16s , eBiosciences, San Diego, CA, USA) (Hamza *et al*. 2011) was then used (Table 1).

MSCs were stained for MHC I and II (Table 1).

Antibody Clone	Distributer, Catalog number	Conjugated fluorochrome or secondary antibody	Host Species	Ig Type	Dilution
CD4 CVS4	US Biological, 227417-ML405	MaxLight650	Mouse	IgG1	1:700
CD8 CVS8	BioRad, MCA2385F	FITC	Mouse	IgG1	1:200
CD21 CA2.1D6	AbCam, ab34124	PE	Mouse	IgM	1:5
CD25/IL-2 R alpha	RND Sytsems, AF-223-NA	Donkey Anti-Goat IgG H&L (Alexa Fluor® 405)	Goat	IgG	1:10
FOXP3 FJK-16s	eBioscience, 17-5773-82	PE-Cyanine7	Rat	IgG2a	1:100
MHC class I CVS22	BioRad, MCA1086	RPE	Mouse	IgG	1:10
MHC class II CVS20	Bio-Rad, MCA1085F	FITC	Mouse	IgG1	1:100

Appendix C Table 1. Antibodies used for co-culture flow cytometry assays.

Flow cytometry on neutrophils

For neutrophil analysis, 123-Dihydrorhodamine (0.25ug/sample) was added to each well for a 20 minute incubation period in the dark at 37°C. The wells were the placed on ice for 10 minutes. The cells were then stained with viability dye (Efluor 780). All events in the sample were recorded and their fluorescence used for statistical analysis.

Gene expression assay

Following co-culture, plates for gene analysis were frozen at -20°C until RNA was isolated. RNA was isolated from frozen samples using an RNeasy Kit (Qiagen, Hilden, Germany). Transcriptional analysis was performed using the nCounter Analysis System (NanoString, Seattle, WA, USA). Two sets of gene-

specific probes (along with a reporter probe and a capture probe) were designed by NanoString and their accession numbers are listed in the Supplementary Information. Total RNA (85±59 ng per sample) was hybridised using nCounter PlexSet-24 Reagent Pack according to the PlexSet™ Reagents User Manual. After hybridisation, samples were vertically pooled and were placed on the automated nCounter Prep Station (NanoString) for purification and were immobilised in the cartridge. This cartridge was then transferred to the nCounter Digital Analyzer for data collection. Data analysis was performed with nSolver™ 4.0 Analysis Software according to user manual. All samples passed the quality control. Positive control normalization was carried out by using the geometric mean of the highest three positive counts. Reference gene normalization was calculated using the geometric mean of counts for the three reference genes GUSB, PPIA, TBP, YWHAZ.

CellTrace Violet incorporation

CellTrace Violet (Molecular Probes™ Thermo Fisher Scientific, CellTrace™ Violet Cell Proliferation Kit, Waltham, MA, USA) was tested at a 5 µM concentration (as instructed by the manufacturer) and two-fold dilutions there-of. CTV was diluted in PBS and incubated with lymphocytes according to the manufacturer's instructions. Briefly, 1 x 106 lymphocytes were incubated with the desired dilution of CTV in PBS for 20 minutes at 37°C. Samples were quenched with RPMI 1640 media (Gibco, Thermo Fisher®, Waltham, MA, USA) with 10% autologous equine serum, for 5 minutes. Cells were then washed in excess PBS and cultured as described below.

Additionally, CTV incorporation was assessed following a modified method proposed by Quah et al. (2007). Equine lymphocytes were stained with CTV (5µM) in RPMI 1640 media with 10% serum. The addition of serum to the staining solution is intended to decrease cytotoxicity of the CTV. Using a concentration of 5µM CTV, little to no CTV incorporation into the lymphocytes was seen when following this protocol (data not shown). CTV was incorporated using PBS as the staining diluent for all of the remaining assays.

Lymphocyte stimulation

The activating agents used in Chapter 3 were Pokeweed mitogen (PWM) (Sigma-Aldrich, St Louis USA) (1 µg/ml, 2.5 µg/ml and 10 µg/ml) and Concanavalin A (ConA, eBioscences, Thermo Fisher®, Waltham, MA, USA) (1µg/ml and 10µg/ml).

Cultures were established on multi-well plates (Greiner Bio-One) using RPMI 1610 media with 10% autologous equine serum or fetal bovine serum (FBS, Gibco, Thermo Fisher®), penicillin-streptomycin (100 ug/ml) (Sigma-Aldrich®, St Louis, MO, USA), 2-Mercaptoethanol (0.1mM) (Gibco, Thermo Fisher®), +/- activation agent as indicated. Cells were plated at a concentration of 3 x 105 cells/cm3.

Imaging of lymphocytes in culture with or without CellTrace Violet

An inverted phase contrast microscope (Olympus CK2, Olympus Corporation, San Jose CA, USA) was used to assess lymphocyte activation at 100X magnification three days following culture with 2.5 µg/ml PWM. Three horses'

lymphocytes were tested each in triplicate. Activation was evaluated by determining the degree of cell clumping as described by Teague et al. (1993). Clumping was graded: 0- no clumping, 1- moderate clumping, and 2- significant clumping.

Flow cytometry for CellTrace Violet

Fixable viability dye (Efluor 780, eBioscences, Thermo Fisher®, Waltham, MA, USA) was used to assess cell viability in flow cytometry assays in conjunction with CTV uptake. Samples were measured using a BD FACSVerse (San Jose, CA, USA). Ten thousand events in a lymphocyte gate were recorded. The lymphocyte population was characterised using the gating hierarchy as shown in Supplementary Information using flow cytometry analysis software Flowjo (Flowjo LLC, Oregon, USA).

Appendix D: Compiled References

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Appendix E: DRC16 Statements

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Lacy Kamm
Name/title of Primary Supervisor:	Chris Riley - Professor
Name of Research Output and full reference:	
Kamm JL and McIlwraith CW. Mesenchymal stem cell treatment for equine musculoskeletal disease. Eq Vet Practitioner. July 2016.	
In which Chapter is the Manuscript /Published work:	1.3.1
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	95%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Research, writing and submission of manuscript.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Lacy Kamm Digitally signed by Lacy Kamm Date: 2020.12.15 11:45:09 +13'00'
Date:	12-15-20
Primary Supervisor's Signature:	
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Name of candidate:	J Lacy Kamm
Name/title of Primary Supervisor:	Chris Riley/ Professor
Name of Research Output and full reference:	
Kamm JL, Riley CB, Pariano NP, Gee EK, Mitchell CH. Interactions between allogeneic mesenchymal stromal cells and the recipient immune system: A comparative review with relevance to equine outcomes. Frontiers in Veterinary Science. Dec 202	
In which Chapter is the Manuscript /Published work:	1.3.5
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Literature review, manuscript compostiion	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Lacy Kamm Digitally signed by Lacy Kamm Date: 2020.12.15 11:40:05 +13'00'
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Name/title of Primary Supervisor:	Chris Riley - Professor
Name of Research Output and full reference:	
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In which Chapter is the Manuscript /Published work:	2
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Name of candidate:	Lacy Kamm
Name/title of Primary Supervisor:	Chris Riley - Professor
Name of Research Output and full reference:	
Kamm JL, Parlane HA, Riley CB, Gee EK, Roberts JM, McIlwraith CW. CellTrace Violet™ inhibits equine lymphocyte proliferation. Vet Immunol Immunopathol. 2020 May;223:110037. doi: 10.1016/j.vetimm.2020.110037.	
In which Chapter is the Manuscript /Published work:	3
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90%
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Name of candidate:	J Lacy Kamm
Name/title of Primary Supervisor:	Chris Riley
Name of Research Output and full reference:	
Kamm JL, Riley CB, Perlaine NA, Gee EK, McIlwraith CW. Immune response to allogeneic equine mesenchymal stromal cells. Stem Cell Res Ther. 2021 Nov 12;12(1):570.	
In which Chapter is the Manuscript /Published work:	4
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	90%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Assay completion, background research, writing and submission of manuscript.	
For manuscripts intended for publication please indicate target journal:	
Candidate's Signature:	Lacy Kamm
Date:	29-11-21
Primary Supervisor's Signature:	
Date:	7/12/21

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