ABSTRACT

BERGLUND, ALIX KAY. Investigating the Use of TGF-β2 to Downregulate MHC Expression and Reduce the Immunogenicity of Equine Bone Marrow-derived Mesenchymal Stem Cells. (Under the direction of Lauren V. Schnabel and Samuel L. Jones).

Allogeneic bone marrow-derived mesenchymal stem cells (MSCs) are a promising cell therapy for effective and efficient treatment of various inflammatory and immune-mediated diseases. While the prevailing dogma has been that MSCs are immune privileged, very few studies have controlled for MHC haplotype or adequately measured MSC immunogenicity in vitro or in vivo. Controlled studies have found that major histocompatibility complex (MHC)-mismatched MSCs evoke both cell-mediated and humoral immune responses in vivo. Microcytotoxicity assays were used to show that horses injected with MHC-mismatched MSCs generate cytotoxic alloantibodies capable of killing MSCs as early as 7-days post-transplantation. Rejection of MSCs likely leads to reduced therapeutic efficacy and the development of strategies to avoid allorecognition and rejection are necessary to provide safe and efficacious allogeneic therapy.

Downregulation of MHC expression allows cells to avoid immune surveillance and may enhance the ability of MSCs to avoid allorecognition and rejection. Transforming growth factor-β2 (TGF-β2) has been shown to downregulate MHC surface expression in various cell types. In agreement with what has been demonstrated in other cell types, TGF-β2 treatment significantly reduced constitutive MHC I and MHC II surface expression and partially blocked IFN-γ-induced MHC expression on equine MSCs. TGF-β2 treatment did not significantly affect the morphology, cell surface markers, viability, or secretion of TGF-β1 and TGF-β2, but did increase the cell yield from cultures. This data indicates that TGF-β2

may reduce MSC immunogenicity without altering the immunomodulatory properties of the cells.

The immunomodulatory capabilities of TGF-β2-treated MSCs were analyzed in modified one-way mixed leukocyte reactions and ELISAs. Naïve and TGF-β2-treated MSCs both significantly reduced T cell proliferation as measured by the relative division index and relative CFSE geometric mean fluorescent intensity attenuation. Similar amounts of PGE2 and TGF-β2 were also measured in the supernatant of MLRs with naïve and TGF-β2-treated MSCs. This supports that TGF-β2 treatment does not negatively affect the immunomodulatory properties of equine MSCs, which are critical for therapeutic function and evading immune responses in vivo.

In conclusion, although MHC-mismatched equine MSCs are immunogenic in vivo, MHC I and MHC II surface expression can be manipulated by treating cells with TGF-β2 in vitro. Downregulate of MHC surface expression is a promising strategy for enhancing the ability of MSCs to evade immune responses allowing for allogenic use clinically without the risk of immune rejection. The ability of TGF-β2-treated MSCs to avoid immune rejection should continue to be investigated in vitro and in vivo along with the mechanism by which TGF-β2 downregulates MHC expression.

Investigating the Use of TGF-β2 to Downregulate MHC Expression and Reduce the Immunogenicity of Equine Bone Marrow-derived Mesenchymal Stem Cells.

by
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DEDICATION

This thesis is dedicated to my family, for their unwavering support, and to Oliver and Leopold for their patience.

BIOGRAPHY

Alix Kay Berglund was born and raised in Auburn, Washington with her sister, Lindsay, by her parents, Dan and Mary Berglund. Alix was born with a strong love of animals, especially horses, and decided to become a veterinarian in the first grade. After second grade, she convinced her parents to spend their family summer vacation visiting Washington State University and tour the new College of Veterinary Medicine Teaching Hospital. After that, she never once changed her mind about becoming a veterinarian.

She obtained her Bachelor of Science in Zoology in 2010 and Doctorate of Veterinary Medicine in 2014 both from Washington State University. During vet school, Alix had the privilege of working with Drs. Donald Knowles and Margaret Highland and a herd of bighorn sheep, which helped convince her that her true calling was in veterinary research. Sick of the cold eastern Washington winters, Alix headed south to North Carolina State University to begin her PhD in the Comparative Biomedical Sciences with a concentration in immunology. She began her graduate studies in the Dr. Lauren Schnabel's Laboratory of Equine Sports Medicine, which allowed her the opportunity to combine her love of horses and immunology. The focus of her graduate research was the immunogenicity of allogeneic equine mesenchymal stem cells, which is the work presented here.

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INTRODUCTION

Mesenchymal stem cells (MSCs) are fibroblast-like cells found in tissues like bone marrow, adipose, and cord blood. MSCs show significant promise for treating inflammatory, immune-mediated, and degenerative diseases (Squillaro, Peluso, & Galderisi, 2016) and are now considered the standard of care for treating tendonitis in horses (Godwin, Young, Dudhia, Beamish, & Smith, 2012). Although, MSCs are capable of differentiating into bone, cartilage, and fat in vitro (Caplan, 1991), there is little evidence to suggest that their primary function in vivo is to engraft, differentiate, and form new tissue. Rather, the secretion of paracrine factors that attenuate immune responses, stimulate angiogenesis, reduce apoptosis, and promote the differentiation of local progenitor cells appears to be the primary mechanism by which MSCs support regeneration of injured tissue (da Silva Meirelles, Fontes, Covas, & Caplan, 2009).

Researchers initially concluded MSCs were immune privileged because of their strong immunomodulatory properties in vitro and could be used allogeneically without major histocompatibility complex (MHC) matching donors and recipients (Le Blanc, Tammik, Rosendahl, Zetterberg, & Ringdén, 2003). However, subsequent in vivo studies with MHC-matched and mismatched controls have found that MHC-mismatched MSCs do induce both cell-mediated and cytotoxic humoral immune responses leading to rejection of MSCs (Eliopoulos & Stagg, 2005; Nauta et al., 2006; Pezzanite et al., 2015; Zangi et al., 2009). Despite this clear evidence of in vivo immunogenicity, investigators have been reluctant to concede that MSCs can be rejected in vivo. The persisting dogma of immune privilege has

also limited investigation into preventing immune rejection of MHC-mismatched MSCs in vivo and further promoting the immune evasive properties of the cells.

The specific aims of this research were to 1) determine if MHC-mismatched equine MSCs could be killed by cytotoxic antibodies, 2) develop an in vitro method to manipulate MHC expression, and 3) characterize the immunomodulatory properties of MHC manipulated MSCs. Completion of these aims will further support development of methods to allow MHC-mismatched MSCs to evade recipient immune responses in vivo leading to safe and efficacious clinical therapy.

CHAPTER 1: Immunogenicity of Allogeneic Mesenchymal Stem Cells

Literature Review: Immune privileged no more: measuring the immunogenicity of allogeneic adult mesenchymal stem cells.

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Abstract

Autologous and allogeneic adult mesenchymal stem/stromal cells (MSCs) are increasingly being investigated for treating a wide range of clinical diseases. Allogeneic MSCs are especially attractive due to their potential to provide immediate care at the time of injury of disease diagnosis. The prevailing dogma has been that allogeneic MSCs are immune privileged, but there have been very few studies that control for matched or mismatched major histocompatibility complex (MHC) molecule expression and that examine

immunogenicity in vivo. Studies that control for MHC expression have reported both cell-mediated and humoral immune responses to MHC-mismatched MSCs. The clinical implications of immune response to MHC-mismatched MSCs are still unknown. Pre-clinical and clinical studies that document the MHC haplotype of donors and recipients and measure immune responses following MSC treatment are necessary to answer this critical question. This review details what is currently known about the immunogenicity of allogeneic MSCs and suggests contemporary assays that could be utilized in future studies to appropriately identify and measure immune responses to MHC-mismatched MSCs.

Background

Mesenchymal stem cells (MSCs) are currently defined as plastic-adherent cells with a fibroblast-like morphology that are capable of differentiating into bone, cartilage, and fat in vitro and that express a defined set of surface markers, which vary slightly by species (Calloni, Viegas, Türck, Bonatto, & Pegas Henriques, 2014; Dominici et al., 2006). The origin of MSCs in vivo is controversial, but there is evidence to support that MSCs are a type of pericyte or adventitial cell (Caplan, 2016; Souza, Malta, Kashima Haddad, & Covas, 2016). The multipotent properties of MSCs led to initial conclusions that these cells could be used clinically to repair or regenerate injured tissues (Caplan, 1991), and animal studies supported that MSCs provided a therapeutic benefit (Awad et al., 1999). However, MSCs have poor engraftment rates (Eggenhofer et al., 2012; Tögel, Hu, & Weiss, 2005) and there is little evidence to suggest that the primary function of MSCs is to differentiate into new tissue in vivo (Caplan, 2017), questioning the relevance of differentiation to the therapeutic

properties of MSCs when injected in a naive state. Tri-lineage differentiation assays may still be important in some cases for confirming that the cells used in studies are MSCs, since MSCs and fibroblasts have similar morphology and phenotype (Alt et al., 2011).

Secretion of paracrine factors is now recognized as the primary mechanism by which MSCs promote a regenerative environment conducive to healing with healthy tissue (M. B. Murphy, Moncivais, & Caplan, 2013), although cell-to-cell contact has also been shown to be important under some conditions (De Windt et al., 2015; English et al., 2009). MSCs home to sites of inflammation where they secrete a variety of soluble factors including growth factors, cytokines, and chemokines (da Silva Meirelles, Fontes, Covas, & Caplan, 2009). In-vivo studies have demonstrated that MSC therapy promotes angiogenesis and growth and differentiation of local progenitor cells, prevents fibrosis and apoptosis, attracts immune cells to the site of injury, and modulates immune responses (L. Chen, Tredget, Wu, Wu, & Wu, 2008; Y.-T. Chen et al., 2011; da Silva Meirelles et al., 2009; Wu et al., 2014). As engraftment appears to be unnecessary for the therapeutic effect, exogenous MSCs likely need to persist through the initial inflammatory phase and into the repair and remodeling phase of tissue healing to have a full therapeutic effect. Adult MSCs, which are obtained from the bone marrow, peripheral blood, or adipose tissue of patients, are currently being investigated in over 450 clinical trials to treat numerous diseases including musculoskeletal diseases, degenerative and traumatic neurological diseases, and immune-mediated diseases (Squillaro, Peluso, & Galderisi, 2016). MSC therapy has been effective at treating several animal models of disease (Okazaki et al., 2008; Zappia et al., 2008) and shown success in human clinical trials (Squillaro et al., 2016). The therapeutic benefits of MSC therapy

demonstrated in preclinical trials has not translated to success in every human clinical trial, however, and the use of allogeneic versus autologous MSC therapy is one factor that may contribute to the differences in efficacy seen in some clinical trials (Galipeau et al., 2015; Wang et al., 2016).

In-vitro expansion of MSCs prior to clinical use can take several weeks to obtain enough cells for administration, resulting in loss of stemness; the age and disease state of the patient can also negatively affect the quality of the cells (Choudhery et al., 2012; Nie, Lau, Lie, Chan, & Mok, 2010). Adult allogeneic MSC therapy is particularly attractive as it allows for immediate treatment with quality cells at the time of injury or diagnosis. In early studies, researchers discovered that allogeneic MSCs were capable of inhibiting the proliferation of major histocompatibility complex (MHC)-mismatched lymphocytes in mixed leukocyte reactions (MLR) in vitro (Le Blanc, Tammik, Rosendahl, Zetterberg, & Ringdén, 2003). MSCs produce a variety of immunomodulatory cytokines including transforming growth factor-β1, indoleamine 2,3-dioxygenase, inducible nitric oxide synthase, and prostaglandin E_2, which contribute to the ability of MSCs to modulate immune responses (da Silva Meirelles et al., 2009). This discovery initially indicated that MSCs were "immunoprivileged" and were subsequently promoted as safe to use in allogeneic settings without concern for immune rejection (Le Blanc et al., 2003).

Although allogeneic MSC therapy is generally regarded as safe (Lalu et al., 2012), there have been several reports of adverse clinical events including increased synovial cellularity and total nucleated cell counts following intra-articular injection of allogeneic MSCs in equine models (Joswig et al., 2017; Pigott, Ishihara, Wellman, Russell, & Bertone,

2013a). Most studies do not characterize if allogeneic donor MSCs and recipients are MHC-matched or MHC-mismatched, nor do they investigate if the MSCs induce immune responses and are rejected. Furthermore, few studies have compared allogeneic versus autologous MSC therapy using cells of comparable quality to determine if there is a difference in efficacy for tissue healing or disease outcome and if those differences correlate or not with immune rejection of allogeneic MSCs. In order to fully understand the potential of allogeneic MSC therapy, further investigation into the immune responses towards allogeneic cells and if immune responses affect the therapeutic outcome of MSC therapy are warranted.

The purposes of this review are to outline what is currently understood about immune responses to adult allogeneic MSCs and to describe contemporary assays that could be utilized in future preclinical studies and clinical trials to appropriately identify and measure immune responses to allogeneic MSCs. By gaining a better understanding of how and under what circumstances a recipient immune system responds to allogeneic MSCs, researchers can develop strategies to improve allogeneic MSC efficacy and ensure safety.

In vivo immunogenicity of allogeneic MSCs

The few published studies that controlled for MHC haplotype of donors and recipients and assessed immune responses following injection of MSCs support that adult MHC-mismatched MSCs are not immune privileged. In multiple animal models, bone marrow-derived MHC-mismatched MSCs induced both cell-mediated and humoral immune responses in vivo and were subsequently rejected (Table 1) (Badillo, Beggs, Javazon, Tebbets, & Flake, 2007; Eliopoulos & Stagg, 2005; Isakova et al., 2014; Nauta et al., 2006;

Pezzanite et al., 2015; Poncelet, Vercruysse, Saliez, & Gianello, 2007; Zangi et al., 2009). These studies provided valuable information about how the immune system responds to MHC-mismatched MSCs, although the clinical implications of MSC rejection by the recipient immune system are still not entirely clear.

Cell-mediated responses to MSCs are induced when T cells become activated following recognition of foreign donor MHC molecules expressed on the surface of the MSCs. Significant increases in circulating T cells and natural killer cells were detected in rhesus macaques as early as 10 days after intracranial injection with MHC-mismatched MSCs, but not those injected with autologous MSCs (Isakova et al., 2014). Cytotoxic peripheral blood leukocytes (PBLs) capable of lysing donor MSCs were also found in macaques that received MHC-mismatched MSCs but not macaques that received autologous MSCs. In this study, the degree of MHC I and MHC II mismatch between donor and recipient correlated with the magnitude of the immune response, supporting that the immune response against donor MSCs was MHC-specific. This study also demonstrated that injection of MHC-mismatched MSCs into a relatively immune privileged area like the central nervous system (CNS) did not prevent immune responses against the cells. Studies in mice and pigs have established that MHC-specific memory lymphocytes are generated in response to MHC-mismatched MSCs (Badillo et al., 2007; Eliopoulos & Stagg, 2005; Nauta et al., 2006; Poncelet et al., 2007). Mice injected intravascularly with MHC-mismatched MSCs had significant increases in $CD4^+$ and $CD8^+$ splenocytes with a memory phenotype ($CD122^+CD44^+CD62L^{low}$), but not mice injected with MHC-matched MSCs (Zangi et al., 2009). In separate studies where mice were injected intraperitoneally and a pig injected

intracardiacally with MHC-mismatched MSCs, responder lymphocytes showed accelerated proliferation in an ex-vivo MLR when exposed to stimulator cells of the same MHC haplotype as donors, demonstrating the presence of MHC-specific memory lymphocytes (Badillo et al., 2007; Poncelet et al., 2007). The formation of memory immune cells in recipients of MHC-mismatched MSCs is important since immunologic memory can lead to accelerated rejection of allogeneic cells upon reinjection. Collectively, these studies indicate that, regardless of the species or route of administration, recipient lymphocytes are sensitized to mismatched MHC molecules expressed by donor MSCs and differentiate into MHC-specific effector and memory cells.

Pre-existing antibodies crossreactive for donor MHC molecules or alloantibodies produced following activation of B cells by cognate alloantigens can also contribute to rejection of allogeneic cells. A significant increase in total serum immunoglobulin (Ig)G was reported in rhesus macaques injected with MHC-mismatched MSCs, but not in macaques injected with autologous MSCs (Isakova et al., 2014). Alloantibodies have also been detected in mice, pigs, and horses injected with MHC-mismatched MSCs (Badillo et al., 2007; Pezzanite et al., 2015; Poncelet et al., 2007). Horses injected intradermally with MHC-mismatched MSCs generated cytotoxic anti-MHC I alloantibodies as early as 7 days postinjection, while a control horse injected with MHC-matched MSCs did not (A K Berglund & Schnabel, 2017; Pezzanite et al., 2015). Anti-MHC antibodies and alloreactive T cells have been detected following exposure to unrelated proteins (Amir et al., 2010; Hirata & Terasaki, 1970; Pantenburg, Heinzel, Das, Heeger, & Valujskikh, 2002) so it is possible for recipients to be primed against allo-MHC molecules and mount antibody responses

quickly against allogeneic MSCs after a single injection. Two recent human MSC clinical trials monitored patients for alloantibody production and found that while the majority of patients do not develop significant alloantibody after injection with allogeneic MSCs, a minority of patients do develop alloantibodies (Golpanian et al., 2017; Hare et al., 2017). It is possible that induction of alloantibodies by allogeneic MSC therapy is correlated to the degree of MHC-mismatch between donor and recipient and further supports that MHC haplotyping of donors and recipients be performed. The health and immune status of recipients may also be important and should be fully disclosed in future clinical trials. Hyperacute rejection-like symptoms have not been reported in human patients who receive allogeneic MSC therapy, but further investigation into alloantibodies induced by MSCs is warranted to protect patients who may receive multiple injections of allogeneic MSCs (Ankrum, Ong, & Karp, 2014) or patients who may have been previously sensitized to human leukocyte antigens (HLAs) from a pregnancy, blood transfusion, or an organ transplantation.

In-vivo rejection of MSCs has been measured both directly using bioluminescent imaging and in-vivo cytotoxicity assays and indirectly by measuring hematocrit following injection with erythropoietin-expressing MSCs. In each of these studies, MHC-mismatched MSCs survived for a significantly shorter period of time than MHC-matched MSCs in immunocompetent mice and were rejected more quickly in previously sensitized animals (Eliopoulos & Stagg, 2005; Nauta et al., 2006; Zangi et al., 2009). MHC-mismatched MSCs did persist longer than MHC-mismatched fibroblasts, however, supporting that they are still immunomodulatory in vivo (Zangi et al., 2009). While the immunomodulatory properties of

MSCs may improve survival compared to non-immunomodulatory cells such as fibroblasts, recipient immune responses appear to limit survival of MHC-mismatched MSCs compared to MHC-matched MSCs. Although large animal studies in macaques, horses, and pigs have measured immune responses that could potentially lead to the rejection of MSCs, in-vivo rejection has currently only been measured using mouse models. While it is likely that a similar phenomenon may occur in large animals and humans, it is currently unknown in these species how long MHC-mismatched MSCs survive following injection, how quickly they are rejected, or if rejection is primarily due to cell-mediated or humoral immune responses. The answers to these questions may help with the development of targeted strategies to limit the rejection and retain the therapeutic window of efficacy for MHC-mismatched MSCs in vivo.

Methods to measure immune responses

A number of standard immunological assays and techniques are available to measure the immunogenicity of MSCs. For these assays, the MHC haplotype of donors, recipients, stimulators, and responders should be determined to understand if donor or stimulator MSCs are full or partial mismatches to recipients or responder cells. Control cells should include donor or target cells of the same MHC haplotype as recipients or responders to control for MHC-specific immune responses. Modified one-way in-vitro MLRs, where responder splenocytes or PBLs are cocultured with stimulator allogeneic MSCs, have traditionally been used to measure the immunogenicity of MSCs, but several studies have demonstrated that in-vitro MLR assays are poor predictors of in-vivo immunogenicity (Nauta et al., 2006; Pezzanite et al., 2015; Poncelet et al., 2007; Schnabel, Pezzanite, Antczak, Felippe, &

Fortier, 2014). It is likely that the high cell concentrations and cytokine levels relative to physiologic levels in an MLR account for discrepancies in MSC in-vitro and in-vivo immunogenicity. The immunomodulatory functions of MSCs can be measured using traditional MLRs, where responder and stimulator splenocytes or PBLs are cocultured with MSCs, but the ability of allogeneic MSCs to suppress T-cell proliferation does not correlate with the in-vivo immunogenicity of allogeneic MSCs either (Badillo et al., 2007; Eliopoulos & Stagg, 2005; Isakova et al., 2014; Zangi et al., 2009).

Measuring heat, swelling, or the infiltration of immune cells or lack thereof into the site where MSCs were injected or the tissue of interest is also not sufficient to determine if donor MSCs have induced an immune response. The absence of a local immune response does not rule out a systemic response (for example, in the spleen where MSCs may home following injection (Leibacher & Henschler, 2016)) and does not measure if there is an MHC-specific response. Overall changes in peripheral blood lymphocyte counts also do not indicate if there is a targeted immune response to MHC-mismatched MSCs. Similarly, overt clinical signs such as fever or anaphylaxis have not been found to correlate with immune responses or rejection of allogeneic MSCs (Owens, Kol, Walker, & Borjesson, 2016; Pezzanite et al., 2015). When possible, functional assays should be performed to determine the type of immune responses and evaluate the potential implications for clinical therapy. The in-vivo immune response to MHC-mismatched MSCs and appropriate assays for detecting each response are depicted in Fig. 1. When testing in-vivo immunogenicity, assays should be performed prior to injection of donor MSCs in humans and large animals or in untreated control animals to measure baseline immune responses. Testing at multiple time

points after administration is also preferable for measuring the kinetics of the immune response. Appropriate assays for detecting immune responses against MSCs are summarized in Table 2.

Cell-mediated functional assays

Ex-vivo MLRs can be useful for estimating sensitization of recipient T cells to donor MHC molecules postadministration with allogeneic MSCs. Splenocytes or PBLs from recipients are collected and used as responders in a standard one-way MLR using stimulator splenocytes or PBLs of the same MHC haplotype as the MSC donor (Badillo et al., 2007). Proliferation of responder PBLs indicates immune cell recognition of allo-MHC molecules and subsequent activation. Accelerated proliferative responses in an ex-vivo MLR following administration of MSCs demonstrate differentiation and activation of donor MHC-specific memory lymphocytes.

Interferon (IFN)-γ and interleukin (IL)-4 enzyme-linked immunospots (ELISPOTs) can measure functional responses of T cells upon restimulation with donor MHC molecules as well as predict the in-vivo immunogenicity. Similar to the ex-vivo MLR, following in-vivo administration of MSCs, recipient splenocytes or PBLs can be isolated and restimulated using splenocytes or PBLs of the same MHC haplotype as donor MSCs. Secretion of IFN-γ or IL-4 above baseline indicates expansion of $CD8^+$ or $CD4^+$ effector and memory cells against donor MSCs (Benichou, Valujskikh, & Heeger, 1999). Preformed T-cell responses measured using ELISPOTs accurately predict graft rejection in organ transplantation cases (Crespo et al., 2015) and may be able to predict the in-vivo cell-mediated immunogenicity of donor MSCs. Enzyme-linked immunosorbent assays (ELISAs) can also measure changes in

cytokine secretion from restimulated recipient splenocytes or PBLs, but cannot measure the frequency of MHC-specific immune cells.

Cytotoxicity assays can be used to measure direct lysis of MSCs by MHC-specific cytotoxic T lymphocytes (CTLs). As MSCs inhibit formation of CTLs in MLRs (Rasmusson, Ringdén, Sundberg, & Le Blanc, 2003), effector cells should be induced in a standard MLR with splenocyte or PBL stimulator cells or in vivo (Wonderlich, Shearer, Livingstone, & Brooks, 2006). If MSCs are administered in vivo the cytotoxicity of MSCs of the same MHC haplotype as the donor by splenocytes or PBLs can be compared against baseline cytotoxicity to measure expansion of CTLs. Increases in cytotoxicity above baseline indicates that differentiation of T cells into MHC-specific effector and memory CTLs has occurred and that the immune system is capable of rejecting the donor MSCs. In-vitro cytotoxicity assays can be performed using a standard chromium 51 assay (Wonderlich et al., 2006) or newer flow cytometry-based assays (Kim, Donnenberg, Donnenberg, Gooding, & Whiteside, 2007). In-vivo cytotoxicity assays have also been described and can be utilized in small animal models (Durward, Harms, & Splitter, 2010).

Humoral assays

Several assays are available for detecting alloantibodies and identifying the specificity and function of alloantibodies. Microcytotoxicity assays, also called lymphocytotoxicity assays, were originally developed for tissue typing, but can also be used to detect cytotoxic anti-MHC alloantibodies in serum. Standard one- or two-stage microcytotoxicity assays use eosin or fluorescent dye to detect antibody-mediated complement-dependent cytotoxicity (CDC) following incubation of sera from animals

injected with MHC-matched or MHC-mismatched MSCs with donor PBLs or MSCs and rabbit complement (McCloskey, DJ, Brown J, 1993; Pezzanite et al., 2015). Flow cytometry-based CDC assays have also been utilized with MSCs (Poncelet et al., 2007). Due to the simplicity of these assays, CDC assays can be performed using serum and target cells from nontraditional model organisms that lack the commercial reagents available for humans.

Donor MSC-specific antibodies can also be detected by incubating donor MSCs or PBLs of the same MHC haplotype with sera from recipients and staining with anti-IgG or anti-IgM secondary antibodies (Badillo et al., 2007). Single antigen bead (SAB) assays, ELISPOTs, and HLA-tetramers have also been used to screen human sera for MHC-specific alloantibodies (Karahan, Claas, & Heidt, 2015), and commercial kits are readily available. However, these assays do not determine the functionality of the alloantibodies.

Imaging

In-vivo imaging can also be used to track the survival of injected MSCs. MSCs from transgenic mice that constitutively express luciferase or fluorescent proteins allow for long-term tracking of cells and estimations of survival in vivo (Zangi et al., 2009). For larger animal models, where MSCs cannot be imaged in vivo by bioluminescence, iron oxide-labeled MSCs have been tracked via magnetic resonance imaging (MRI) (Geburek et al., 2016). The disadvantage to labeling cells with iron oxide is that the signal will persist even after the MSCs have died or have been phagocytosed (Amsalem et al., 2007). Labeling of cells with membrane dyes also allows for identifying remaining transplanted MSCs on histology, but the cells cannot be tracked over time (Geburek et al., 2016). Imaging alone cannot detect or assess immune responses, but when used with the other functional assays

described above it can help determine the effects and kinetics of an immune response on MSC survival.

Conclusions

Allogeneic MSC therapy holds significant promise for treating numerous diseases, but further studies are needed to assess the potential of allogeneic MSCs for widespread clinical use. In-vitro and in-vivo studies designed with appropriate MHC controls and thorough immune response analyses will help answer under what conditions immune responses to allogeneic MSCs occur and if these immune responses affect the safety and efficacy of MSC therapy. Additionally, as animal studies support that allogeneic MSCs are rejected in vivo, strategies to reduce the immunogenicity and increase the ability of MSCs to avoid immune responses should be investigated to enhance the survival of allogeneic MSCs.

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The authors would like to thank Alice Harvey for the graphic used in Fig. 1

Table 1 In-vivo studies with MHC controls and immune response analysis

Author	Species	Cell-mediated	Humoral	In vivo rejection	Methods used
Eliopoulos and Stagg, 2005 [29]	Mouse	+		+	In-vivo cytotoxicity
Nauta et al., 2006 [30]	Mouse	+		+	In-vivo cytotoxicity
Badillo et al., 2007 [31]	Mouse	+	+		Ex-vivo MLR, allograft rejection
Poncelet et al., 2007 [32]	Pig	+	+		Ex-vivo MLR, CDC
Zangi et al., 2009 [33]	Mouse	+	+	+	Allograft rejection, in-vivo imaging
Isakova et al., 2014 [34]	Rhesus Macaques	+			In-vitro cytotoxicity
Pezzanite et al., 2015 [35]	Horse		+		CDC

CDC complement-dependent cytotoxicity, *MHC* major histocompatibility complex, *MLR* mixed leukocyte reaction

Table 2 Assays for measuring cell-mediated and humoral immune responses against allogeneic MSCs

Assay	Effector cell	Target cell	Immune function measured	Outcome measurement
Ex-vivo MLR	Lymphocytes (primarily $CD4^+$)	Splenocytes/lymphocytes	Cell-mediated	T-cell proliferation
Cytotoxicity	Cytotoxic T lymphocytes	MSCs	Cell-mediated	Target cell death
ELISPOT	T or B cell	Splenocytes/lymphocytes	Cell-mediated or humoral	Cytokine secretion
Antibody-dependent CDC	B cell	Lymphocytes or MSCs	Humoral	Target cell death
In vivo imaging		MSCs		MSC survival

CDC complement-dependent cytotoxicity, *ELISPOT* enzyme-linked immunospot, *MLR* mixed leukocyte reaction, *MSC* mesenchymal stem cell

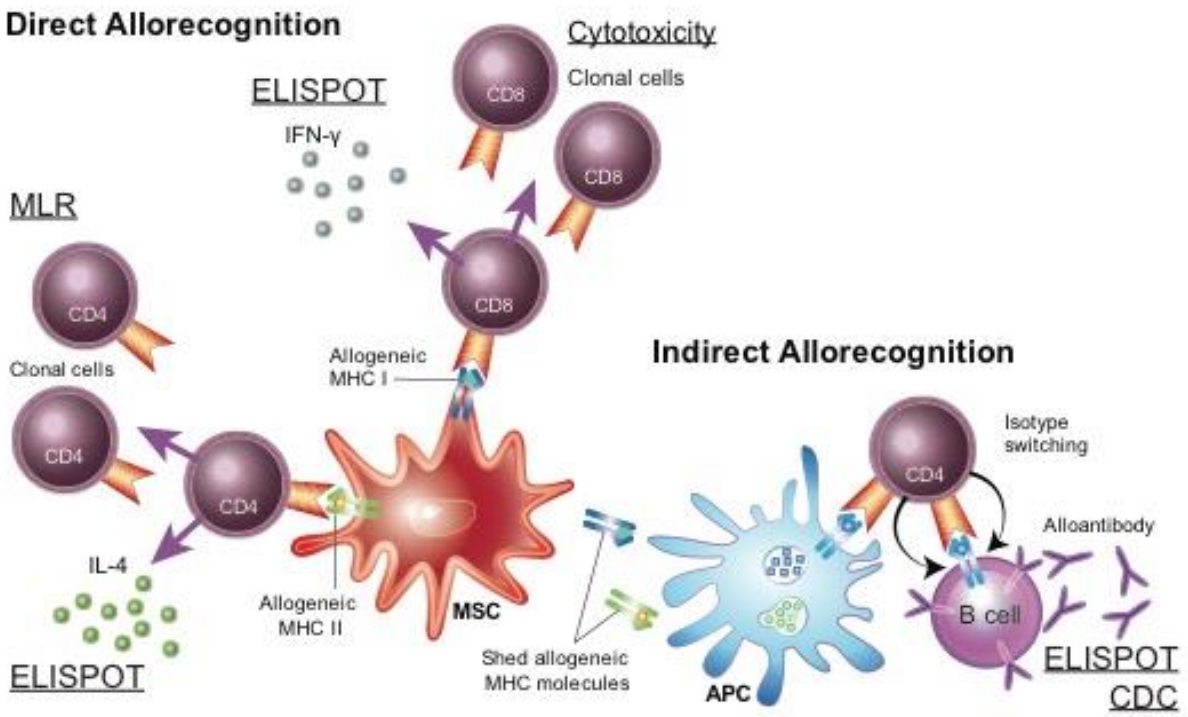

Figure 1. In vivo immune responses to MHC-mismatched MSCs and corresponding assays. Following injection of MHC-mismatched MSCs in vivo, allogeneic MHC I molecules are directly recognized by alloreactive $CD8^+$ T cells, which induces secretion of interferon (IFN)-γ and clonal expansion of cytotoxic T cells. IFN-γ secretion by T cells restimulated with donor allogeneic MHC I molecules can be measured using an ELISPOT. Effector function of cytotoxic T cells specific for donor allogeneic MHC I molecules can be measured using cytotoxicity assays. Allogeneic MHC II molecules are directly recognized by alloreactive $CD4^+$ T cells, which induces secretion of IL-4 or clonal expansion of helper T cells. IL-4 secretion by T cells restimulated with donor allogeneic MHC II molecules can be measured by ELISPOT. Expansion of MHC-specific $CD4^+$ T cells can be detected using an ex-vivo MLR. Allogeneic MHC molecules can be shed into the environment where they are processed and presented to lymphocytes by APCs. Following activation by allogeneic MHC peptides, B cells can produce alloantibodies with the support of CD4 T cells activated by indirect allorecognition. Alloantibodies can be detected by ELISPOT or complement-dependent cytotoxicity assays. *APC* antigen presenting cell, *CDC* complement-dependent cytotoxicity, *ELISPOT* enzyme-linked immunospot, *IL-4* interleukin-4, *MHC* major histocompatibility complex, *MLR* mixed leukocyte reaction, *MSC* mesenchymal stem cell

CHAPTER 2: Humoral Immune Responses to Allogeneic Mesenchymal Stem Cells

Allogeneic major histocompatibility complex-mismatched bone marrow-derived mesenchymal stem cells are targeted for death by cytotoxic anti-major histocompatibility complex antibodies.

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Summary

Background: Allogeneic mesenchymal stem cells (MSCs) are a promising cell source for treating musculoskeletal injuries in horses. Controversy exists, however, over whether major histocompatibility complex (MHC)-mismatched MSCs are recognised by the recipient immune system and targeted for death by a cytotoxic antibody response.

Objectives: To determine if cytotoxic anti-MHC antibodies generated in vivo following MHC-mismatched MSC injections are capable of initiating complement-dependent cytotoxicity of MSCs.

Study design: Experimental controlled study.

Methods: Antisera previously collected at Days 0, 7, 14 and 21 post-injection from 4 horses injected with donor MHC-mismatched equine leucocyte antigen (ELA)-A2 haplotype

MSCs and one control horse injected with donor MHC-matched ELA-A2 MSCs were utilised in this study. Antisera were incubated with ELA-A2 MSCs before adding complement in microcytotoxicity assays and cell death was analysed via eosin dye exclusion. ELA-A2 peripheral blood leucocytes (PBLs) were used in the assays as a positive control.

Results: Antisera from all 4 horses injected with MHC-mismatched MSCs contained antibodies that caused the death of ELA-A2 haplotype MSCs in the microcytotoxicity assays. In 2 of the 4 horses, antibodies were present as early as Day 7 post-injection. MSC death was consistently equivalent to that of ELA-A2 haplotype PBL death at all time points and antisera dilutions. Antisera from the control horse that was injected with MHC-matched MSCs did not contain cytotoxic ELA-A2 antibodies at any of the time points examined.

Main limitations: This study examined MSC death in vitro only and utilized antisera from a small number of horses.

Conclusions: The cytotoxic antibody response induced in recipient horses following injection with donor MHC-mismatched MSCs is capable of killing donor MSCs in vitro. These results suggest that the use of allogeneic MHC-mismatched MSCs must be cautioned against, not only for potential adverse events, but also for reduced therapeutic efficacy due to targeted MSC death.

Introduction

Initial studies evaluating the efficacy of equine mesenchymal stem cells (MSCs) from mature horses for the treatment of musculoskeletal injuries used autologous cells (Caniglia, Schramme, & Smith, 2012; Conze et al., 2014; Crovace, Lacitignola, Rossi, & Francioso,

2010; Ferris et al., 2014; Pacini et al., 2007; Schnabel et al., 2009; Wilke, Nydam, & Nixon, 2007), but more recently, numerous studies have been performed to evaluate the safety of allogeneic, mature equine MSCs in the hope of replacing autologous cells with allogeneic cells (Ardanaz et al., 2016; Carrade et al., 2012; Guest, Smith, & Allen, 2010; Kol et al., 2015; Paterson, Rash, Garvican, Paillot, & Guest, 2014; Pezzanite et al., 2015; Pigott et al., 2013a; Pigott, Ishihara, Wellman, Russell, & Bertone, 2013b; Ricco et al., 2013; Schnabel et al., 2014). Allogeneic MSCs would allow for the immediate treatment of acute injuries with cells of known quality and sufficient quantity and eliminate the need for either bone marrow aspirate or adipose biopsy from the patient. Controversy exists, however, over the immunogenicity of these MSCs in vivo, probably due to variability in the way the term allogeneic is defined and how the immune response is examined.

While all previous studies used the term allogeneic to describe MSCs from a different animal of the same species, most do not clarify whether or not the MSCs were major histocompatibility complex (MHC)-matched or mismatched (Ardanaz et al., 2016; Carrade et al., 2012; Kol et al., 2015; Paterson et al., 2014; Pigott et al., 2013a, 2013b; Ricco et al., 2013). This information is critical as previous studies have shown that MHC-mismatched MSCs induce both recipient humoral and memory T cell responses in vivo (Badillo et al., 2007; Nauta et al., 2006; Pezzanite et al., 2015; Schnabel et al., 2014). Currently, only one equine study has evaluated MSC recipients for an antibody response (Pezzanite et al., 2015): injection of recipient horses with MHC-mismatched donor MSCs resulted in a detectable antibody against the donor MHC haplotype in all 6 recipients as determined by complement-dependent cytotoxicity of donor peripheral blood leucocytes (PBLs). Although none of the

recipients in that study had a detectable systemic inflammatory response, the antibody response findings led to major concerns over the safety of MSC injections following recipient immune priming and the viability and efficacy of donor MSCs once transplanted.

There is evidence from other species that donor allogeneic MHC- mismatched MSCs are targeted for destruction by the recipient immune system and do not persist as long as allogeneic MHC-matched or autologous MSCs (Eliopoulos & Stagg, 2005; Zangi et al., 2009). Even though most adult MSCs are used for their paracrine signalling effects rather than for expected differentiation or engraftment into host tissue, they still need to persist throughout the inflammatory phase and into the remodelling phase for maximal therapeutic benefit. The purpose of this study was to determine if recipient antisera containing antibodies against the MHC haplotype of the donor would cause the death of donor MSCs and at what time points following MSC injection. Our hypothesis was that MSCs would be targeted for death by cytotoxic antibodies with similar cytotoxicity as PBLs.

Materials and Methods

A schematic of the study design is shown in Figure 1.

Antisera against the equine leukocyte antigen (ELA)-A2 haplotype

This study used serum samples obtained from a previous experiment conducted by the corresponding author and colleagues at Cornell University that is described above in the introduction (Pezzanite et al., 2015). Briefly, bone marrow-derived MSCs from female donor horses of the ELA-A2 haplotype were injected intradermally in the neck of MHC-mismatched recipient horses identified as non-ELA-A2 haplotype by microsatellite typing. In

addition, one ELA-A2 haplotype homozygote horse was the recipient of female donor ELA-A2 MSCs as a MHC-matched (negative) control. Blood samples from experimental and control horses were collected preinjection, at time of injection, and every 48 h for 4 weeks following injection. Each sample was processed to collect the serum, which was then aliquoted and frozen at 20°C for later use. First, aliquots were used in microcytotoxicity assays to determine cytotoxic antibody titres for each recipient horse against donor ELA-A2 PBLs as previously described (Pezzanite et al., 2015). Next, antisera from the 4 recipients that were found in those assays to have strong antibody responses as determined by donor ELA-A2 PBL death were selected to be used in this study to determine if those same antibody responses would cause the death of ELA-A2 MSCs in vitro. Sera from the control ELA-A2 recipient that was confirmed to have no anti-ELA antibody response in the previous study was used again as a negative control in this study. In addition, antisera from the strongest responder in the previous study that was found to be cross-reactive against the ELA-A3 haplotype was used against ELA-A3 MSCs.

Mesenchymal stem cell culture

Frozen passage 4 bone marrow-derived MSCs from 2 female horses of the ELA-A2 haplotype and one male horse of the ELA-A3 haplotype were thawed and expanded in culture over 5 days. All MSCs had been previously validated by a panel of positive (MHC I, CD44, CD29, CD90) and negative (CD11a/CD18, CD45RB) markers (Schnabel et al., 2014). MSCs were plated at a density of 1×10^4 cells/cm^2 and cultured for 24 h in standard media containing low glucose (10 g/l) DMEM, 10% fetal bovine serum, 2 mol/l L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml), and 1 ng/ml basic fibroblast growth

factor. Media was then changed so that the serum component consisted of 5% fetal bovine serum and 5% equine serum of the same ELA haplotype as the MSCs for the next 48 h followed by another media change so that the serum component consisted solely of 10% equine serum for the 48 h just prior to the microcytotoxicity assay. MSCs were lifted from tissue culture plates using Accumax cell-dissociation solution, washed three times with phosphate-buffered saline (PBS), and counted using a Cellometer Auto 2000 cell counter and ViaStain AOPI Staining Solution. MSCs were diluted to 1 x 10^9 live cells/l in PBS and used immediately in the microcytotoxicity assays.

Peripheral blood leucocyte isolation

Blood was collected via jugular venipuncture from one ELA-A2 haplotype horse and one ELA-A3 haplotype horse into sterile blood collection tubes containing 158 units of lithium heparin. Plasma was allowed to separate in each tube for 20 min at room temperature. PBLs were then isolated from the plasma via carbonyl iron granulocyte depletion and Ficoll–Paque Plus gradient centrifugation. Isolated PBLs were counted using the Cellometer cell counter and ViaStain AOPI staining solution and diluted in PBS to 3 x 10^9 live cells/l to be used immediately in the microcytoxicity assays.

Microcytotoxicity assays

The standard two-stage microcytotoxicity dye exclusion test was used to detect cytotoxic antibodies as previously described (Adams & Antczak, 2001; Antczak, Bright, Remick, & Bauman, 1982), but with MSCs as target cells in addition to traditional PBLs. Briefly, PBLs and MSCs were tested against diluted antisera (neat, 1:2, and 1:16) from the four known MHC-mismatched recipients with the strongest immune responses from the

previous study as described above and the one MHC-matched (control) recipient. One microliter of diluted antisera and 1 μl of PBL or MSC suspension was incubated for 30 min at room temperature under oil in wells of Terasaki plates. Five microliters of rabbit complement was then added and plates incubated for an additional hour at room temperature. The wells were then stained with 2 μl of 5% eosin dye and fixed with 5 μl of 10% buffered formalin (pH between 7.2 and 7.4). All experiments were run in duplicate and target cell death assessed by the two authors. Results are expressed as the average cytotoxicity score by microscopic evaluation of the percentage of dead cells using a modified National Institute of Heath cytotoxicity scoring system as shown: score 1: <10%, score 2: 10–19%, 4:20–49%, 6: 50–80%, 8: 81–100%. Scores of 6 or greater are considered positive for alloantibodies in sera. Images of wells were taken at 109 using an IX83 inverted microscope and cellSens software.

Data analysis

Microcytotoxicity scores for MHC-mismatched PBLs and MSCs at each dilution of antisera were compared over the antisera collection times of 0, 7, 14 and 21 days using 2-way repeated measures ANOVAs and Holm–Sidak all pairwise multiple comparison procedures. All analyses were performed using SigmaPlot Version 13l and significance set at $P<0.05$.

For each dilution and collection time, the cytotoxicity scores of the two lines of ELA-A2 MSC target cells were compared using multiple *t* tests and the Holm–Sidak method. All analyses were performed using Prism Version 7 and significance set at $P\leq0.05$.

Results

Cell viability prior to microcytotoxicity assay

Peripheral blood leucocyte viability was >95% after carbonyl iron granulocyte depletion and Ficoll–Paque Plus gradient centrifugation isolation. MSC viability following culture expansion and enzymatic dissociation from tissue culture plates was >92%.

Microcytoxicity assays

The antisera from the 4 horses that received a single injection of MHC-mismatched MSCs and the one control horse that received MHC-matched MSCs were tested in microcytotoxicity assays against PBLs from one ELA-A2 haplotype horse and MSC target cells from 2 ELA-A2 haplotype horses. Eosin dye exclusion was used to estimate the cytotoxicity score of the antisera following incubation of the antisera with target cells and rabbit complement. Target cells that appeared round and refractile with a clear centre were estimated to be alive, while flat, uniformly dark cells were counted as dead (Fig 2).

Incubation of antisera from the control horse with target cells did not result in significant cell death (<20% cell death) at any time point or at any dilution (Fig 3) indicating the absence of ELA-A2 antibodies. None of the experimental horses had significant levels of pre-existing ELA-A2 antibodies prior to injection with ELA-A2 MSCs as shown by the lack of significant target cell death following incubation with Day 0 antisera. By Day 7, 2 of the 4 experimental horses had cytotoxic ELA-A2 antibodies present at concentrations capable of killing at least 50% of PBL and MSC target cells at the neat antisera concentration. By Day 14, all 4 of the recipient horses had >50% cell death of PBL and MSC target cells for neat antisera. Similar results were seen for antisera from Day 21. A comparable time-dependent

trend was seen with 1:2 and 1:16 diluted antisera, but with reduced cytotoxicity. There was a significant time-dependent effect on cytotoxicity score from Day 0 to Day 14 and 21 for both PBL and MSC target cells at all dilutions as well as Day 7 compared with Day 14 and 21 cytotoxicity scores. There was a large amount of variation in cytotoxicity of antisera between horses at Day 7, but the median cytotoxicity scores for PBLs and MSCs were not significant ($P = 0.061$) compared with Day 0 scores. There was no significant difference between cytotoxicity of PBLs and MSCs at any time point (neat $P = 0.9$; 1:2 $P = 0.3$; 1:16 $P = 0.3$) and no significant difference between cytotoxicity of the 2 lines of ELA-A2 MSC target cells used at any time point or dilution.

Antisera from the one non-ELA-A2 experimental horse determined to have developed anti-ELA-A3 antibodies in the previous study was also tested against PBL and MSC target cells from an ELA-A3 haplotype horse (Fig 2). Incubation with Day 0 antisera did not result in significant cell death of PBLs or MSCs demonstrating a lack of pre-existing ELA-A3 antibodies prior to MSC injection. The cytotoxicity of the ELA-A3 target cells followed a similar time-dependent effect as the ELA-A2 target cells (Fig 4). Incubation with Day 7 and 14 neat antisera resulted in >50% cell death of both PBL and MSC target cells and >80% cell death of both PBL and MSC target cells following incubation with Day 21 neat antisera with similar trends seen for antisera diluted 1:2 and 1:16.

Discussion

In a previous study, it was demonstrated that cytotoxic anti-MHC antibodies were produced following injection of MHC-mismatched MSCs (Pezzanite et al., 2015). The aim of

the current study was to determine if these anti-MHC antibodies could initiate complement-dependent cytotoxicity of equine MSCs, as they had for PBLs. Our data demonstrates that incubation of ELA-A2 haplotype PBL and MSC target cells with antisera containing anti-ELA-A2 antibodies and complement resulted in target cell death. There was no significant difference between the cytotoxicity of PBLs and MSCs indicating that the immunosuppressive properties of MSCs are not capable of protecting the cells from a targeted humoral immune response against allogeneic MHC molecules and that any cell expressing allogeneic MHC surface molecules can potentially be killed by a targeted humoral immune response. All of the horses tested in the current study were strongly positive for alloantibodies by Day 14 despite having no significant amounts of pre-existing alloantibodies. In human transplant studies, the presence of complement-binding donor-specific anti-HLA antibodies post- transplantation correlates with rejection and poor graft outcome even with immunosuppressive therapy (Loupy et al., 2013). This evidence from the human literature supports that the alloantibodies induced post-transplantation in horses are likely to contribute to targeted destruction of MSCs in vivo, similar to cells in solid organ grafts.

Importantly, anti-ELA-A2 antibodies were present in the serum as early as Day 7 in 2 of the horses indicating that memory T and B cells may exist in some horses that are capable of inducing a cytotoxic humoral immune response against MSCs. Even if a case has no known history of a sensitising event such as pregnancy or a blood transfusion, it is possible for animals to develop anti-MHC I antibodies due to cross- reactive epitopes on microorganisms, allergens, or ingested proteins (Amir et al., 2010; Hirata & Terasaki, 1970; Pantenburg et al., 2002). Any animal could therefore be primed and quickly mount an antibody response against MSCs even

after a single injection. Repeated injections of MSCs after the initial sensitisation would result in accelerated rejection of the cells further limiting their beneficial effects and increasing the potential for adverse events. It is currently unknown how the site of transplantation or the cell dosage affect the outcome or kinetics of an alloantibody response and should be investigated in future studies.

In addition to a targeted humoral immune response, there is evidence in the literature that cell-mediated alloimmune responses can limit the persistence of MSCs in vivo (Badillo et al., 2007; Eliopoulos & Stagg, 2005; Nauta et al., 2006; Zangi et al., 2009). While it is possible that injection of donor MSCs into less vascularised tissues like tendons and joints may result in different responses than intradermal or intravascular administration, during injury even tendons and joints are infiltrated with immune cells (Haynes, Hume, & Smith, 2002; Spurlock, Spurlock, & Parker, 1989) that can contribute to allorecognition and subsequent immune rejection of MSCs. Inflammatory cytokines present in injured tissue such as interferon-γ are known to upregulate MHC expression on equine MSCs (Schnabel et al., 2014), which may make MSCs more likely to be recognised and rejected by a cell-mediated response. The health status, genetic background, immune cell repertoire and other individual variables of the recipient may all affect the strength and specificity of an alloimmune response. To complete these gaps in knowledge, any future allogeneic MSC clinical trial in horses should include analysis of both the cell-mediated and humoral immune response following MSC therapy in addition to MHC haplotyping donors and recipients.

MSC therapies in horses can improve the outcome of potentially career- and life-ending musculoskeletal injuries. While previous studies in horses have found that repeat injections of

allogeneic MSCs do not cause overt clinical symptoms of transplant rejection (Ardanaz et al., 2016; Kol et al., 2015), these studies did not thoroughly investigate whether a cell-mediated or humoral immune response was induced or whether this limited the persistence of the cells in vivo. As the likely therapeutic benefits of MSCs appear to be largely due to the secretion of paracrine factors that promote healing of healthy tissue (da Silva Meirelles et al., 2009; Gnecchi, Zhang, Ni, & Dzau, 2008), it is necessary for the cells to persist throughout the initial inflammatory and healing period. Targeted destruction of allogeneic, MHC-mismatched MSCs shortly after transplant would therefore limit their therapeutic potential. Until the in vivo immune response against allogeneic MSCs is better understood and strategies are developed to prevent rejection, allogeneic MSC therapy should be strongly cautioned against. Further investigation into cell-mediated and humoral immune responses against MHC-mismatched, allogeneic MSCs in vitro and in vivo are necessary to determine if safe and efficacious allogeneic MSC therapy is an obtainable goal.

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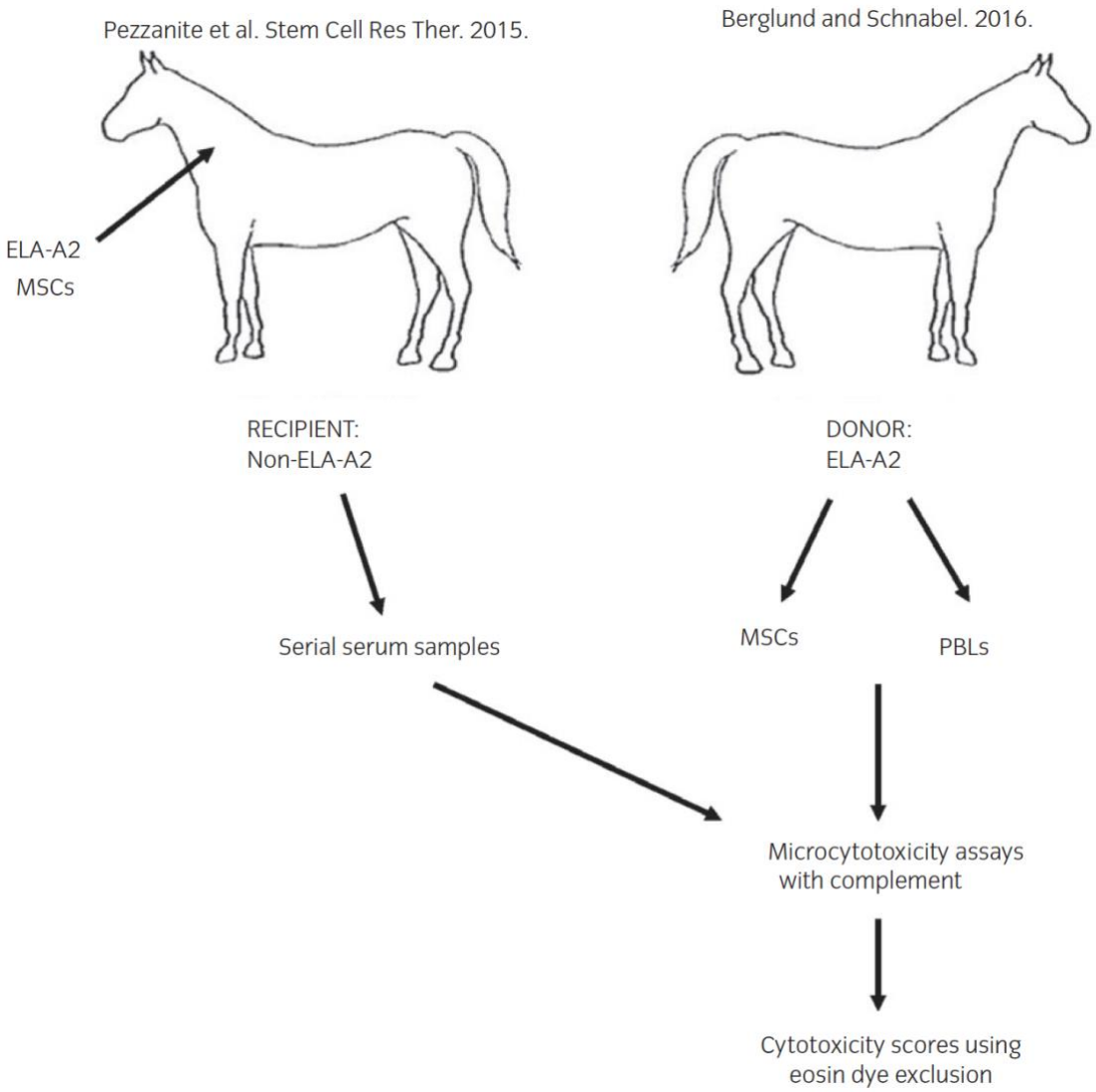

Figure 1: Schematic of study design. Antisera obtained in the study by Pezzanite et al, was incubated with mesenchymal stem cells (MSCs) and peripheral blood leucocyte (PBL) target cells collected from equine leucocyte antigen (ELA)-A2 donor horses and complement in microcytotoxicity assays. Eosin dye exclusion was used to estimate percent cytotoxicity of target cells.

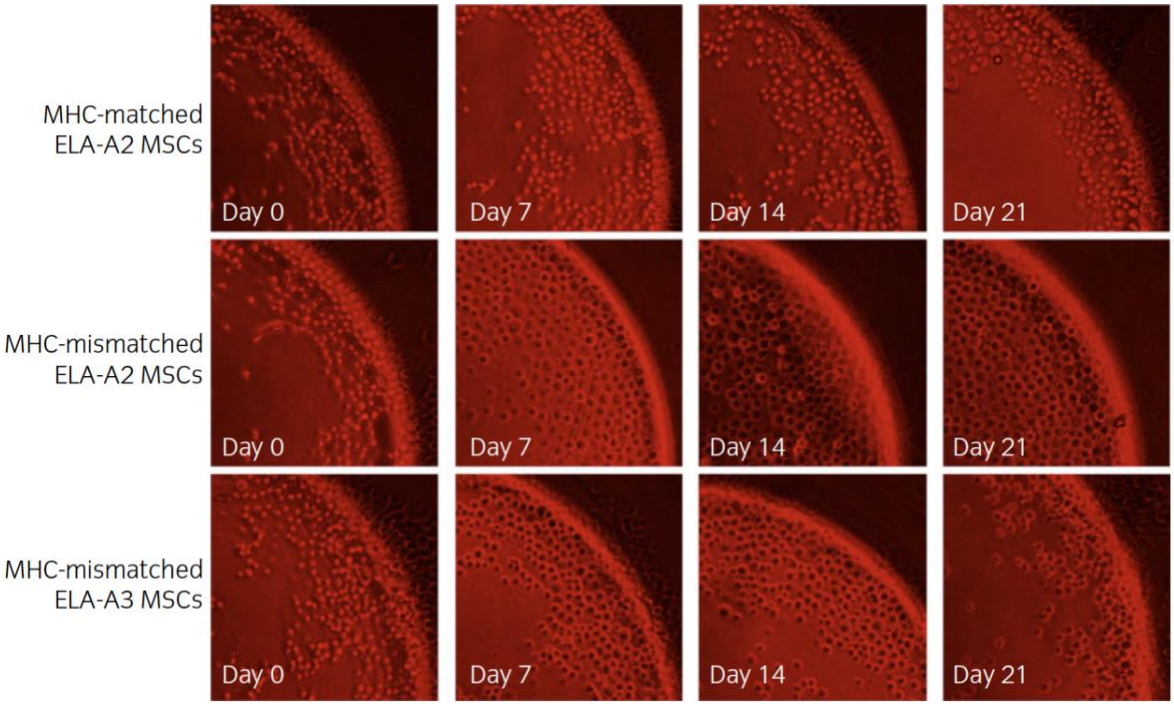

Figure 2: 10X images from Terasaki plate wells used for microcytotoxicity assays containing equine leucocyte antigen (ELA)-A2 mesenchymal stem cells (MSCs) or ELA-A3 MSCs and neat antisera collected on days 0, 7, 14, 21 post-injection with either major histocompatibility complex (MHC)-matched or MHC-mismatched MSCs. Live cells appear round with a clear centre. Dead cells appear flat with a dark centre. Cell death was estimated to be <10% for MHC-matched wells on all days and for MHC-mismatched wells on Day 0 as shown in this figure. Cell death was estimated to be >80% for all MHC-mismatched well on Days 7-21 as shown in this figure.

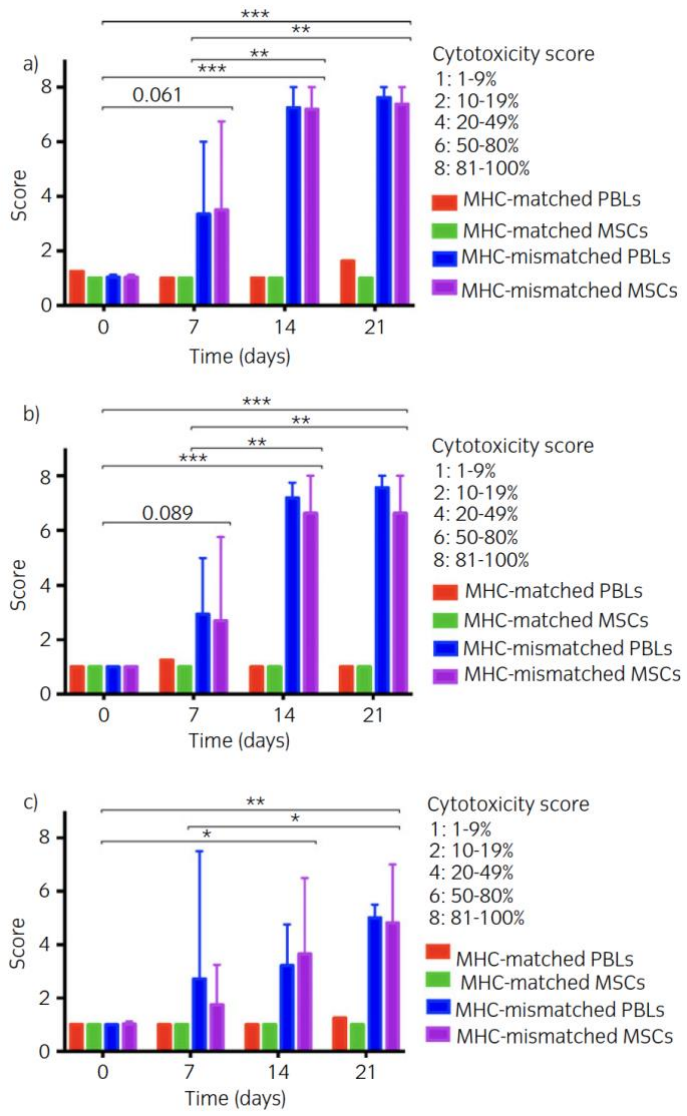

Figure 3: Cytotoxicity scores of peripheral blood leucocytes (PBLs) and mesenchymal stem cells (MSCs) from the equine leucocyte antigen (ELA)-A2 microcytotoxicity assays. A standard two-stage microcytotoxicity assay was used to compare the cytotoxicity of ELA-A2 haplotype PBLs and MSCs following incubation with antisera. Mean cytotoxicity scores and range for ELA-A2 haplotype PBLs and MSC target cells following microcytotoxicity assay with n = 4 major histocompatibility complex (MHC)-mismatched antisera neat a), 1:2 b), and 1:16 c) dilutions. * P<0.001 using repeated measures ANOVA and Holm-Sidak all pairwise multiple comparisons procedures. Scores from the microcytotoxicity assay with MHC-matched antisera are included as reference. Error bars indicate the range of scores above the mean.

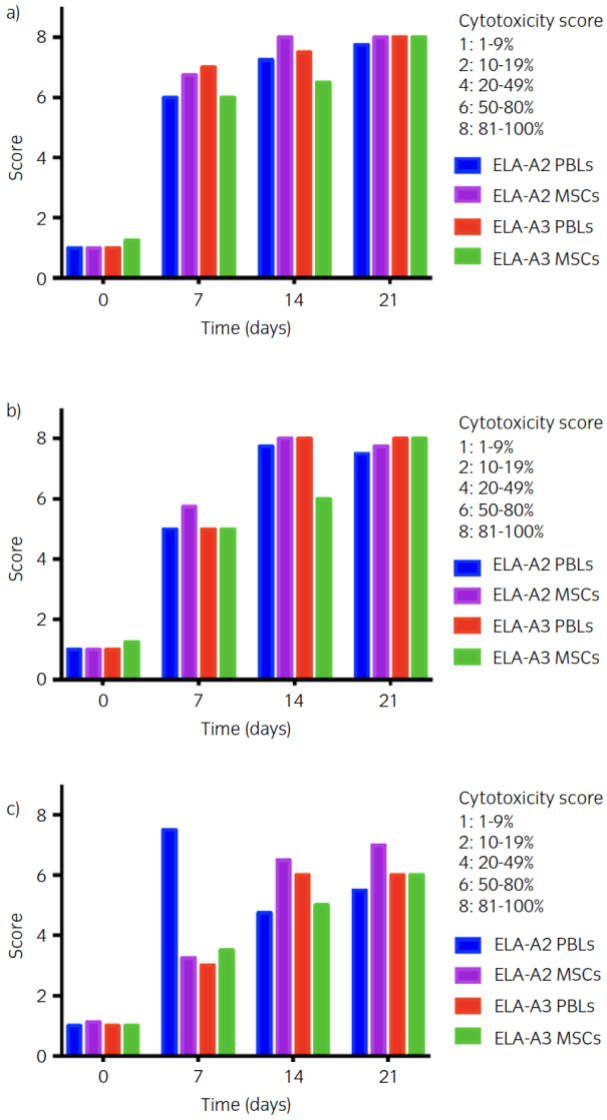

Figure 4: Cytotoxicity scores of peripheral blood leucocytes (PBLs) and mesenchymal stem cells (MSCs) from the equine leucocyte antigen (ELA)-A3 microcytotoxicity assays. A standard two-stage microcytotoxicity assay was used to compare the cytotoxicity of ELA-A3 haplotype PBLs and MSCs following incubation with antisera. Mean cytotoxicity scores and range for ELA-A2 haplotype PBLs and MSC target cells following microcytotoxicity assay with n = 1 major histocompatibility complex (MHC)-mismatched antisera neat a), 1:2 b), and 1:16 c) dilutions. * $P<0.001$ using repeated measures ANOVA and Holm-Sidak all pairwise multiple comparisons procedures. Scores from the microcytotoxicity assay with MHC-matched antisera are included as reference.

CHAPTER 3: Manipulation of MHC Expression on Mesenchymal Stem Cells

Transforming growth factor-β2 downregulates major histocompatibility complex (MHC) I and MHC II surface expression on equine bone marrow-derived mesenchymal stem cells without altering other phenotypic cell surface markers.

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Abstract

Allogeneic mesenchymal stem cells (MSCs) are a promising cell source for treating musculoskeletal injuries in horses. Effective and safe allogeneic therapy may be hindered, however, by recipient immune recognition and rejection of major histocompatibility complex (MHC)-mismatched MSCs. Development of strategies to prevent immune rejection of MHC-mismatched MSCs *in vivo* is necessary to enhance cell survival and potentially increase the efficacy and safety of allogeneic MSC therapy. The purposes of this study were to evaluate if

transforming growth factor-β2 (TGF-β2) downregulated MHC expression on equine MSCs and to determine if TGF-β2 treatment altered the phenotype of MSCs. Equine bone marrow-derived MSCs from 12 horses were treated with 1, 5, or 10 ng/ml TGF-β2 from initial isolation until MHC expression analysis. TGF-β2-treated MSCs had reduced MHC I and MHC II surface expression compared to untreated controls. TGF-β2 treatment also partially blocked IFN-γ-induced upregulation of MHC I and MHC II. Constitutive and IFN-γ-induced MHC I and MHC II expression on equine MSCs was dynamic and highly variable, and the effect of TGF-β2 was significantly dependent on the donor animal and baseline MHC expression. TGF-β2 treatment did not appear to change morphology, surface marker expression, MSC viability, or secretion of TGF-β1, but did significantly increase the number of cells obtained from culture. These results indicate that TGF-β2 treatment has promise for regulating MHC expression on MSCs to facilitate allogeneic therapy, but further work is needed to maintain MHC stability when exposed to an inflammatory stimulus.

Introduction

Bone marrow-derived mesenchymal stem cell (MSC) therapy has shown significant promise for decreasing healing time and reducing reinjury rates in horses with musculoskeletal injuries (Godwin, Young, Dudhia, Beamish, & Smith, 2012). Although MSCs are capable of differentiating *in vitro*, the therapeutic properties of MSCs are derived primarily from immunomodulatory and trophic factors secreted by the cells (Caplan & Sorrell, 2015; Meirelles Lda, Nardi, Meirelles, & Nardi, 2003; K. C. Murphy, Fang, & Leach, 2014). Allogeneic MSC therapy is particularly attractive because quality donor cells

could be used at the time of injury. Currently, controversy exists over the use of autologous versus allogeneic MSCs. MSCs were originally described in early literature as immune-privileged due to their strong immunomodulatory properties (Ryan, Barry, Murphy, & Mahon, 2005). Analysis of alloimmune responses following *in vivo* transplantation have shown, however, that major histocompatibility complex (MHC)-mismatched MSCs do evoke both cell-mediated and humoral immune responses and do not persist as long as autologous MSCs (Badillo et al., 2007; Eliopoulos & Stagg, 2005; Isakova et al., 2014; Poncelet et al., 2007; Zangi et al., 2009). A recent study found that repeated intra-articular injections of allogeneic equine MSCs, but not autologous MSCs, resulted in adverse clinical signs, indicating an immune response against the allogeneic MSCs (Joswig et al., 2017). We have also recently shown that horses injected with MHC-mismatched MSCs produce anti-MHC antibodies that are cytotoxic to MSCs as early as 7–14 days post transplantation (AK Berglund & Schnabel, 2017; Pezzanite et al., 2015). Recipient immune recognition of mismatched MHC molecules on the surface of donor cells, termed allorecognition, and subsequent rejection of MSCs likely leads to decreased efficacy of therapy and an increase in the likelihood of adverse events.

Allorecognition of both MHC I and MHC II surface molecules on donor MSCs can contribute to *in vivo* rejection. MHC I molecules can be directly recognized by $CD8^+$ T cells resulting in direct cytotoxicity of the foreign cell (Halamay et al., 2002). $CD4^+$ T cells can directly recognize MHC II surface molecules and enhance either cytotoxic or humoral immune responses. MHC molecules can also be shed by the donor cell and then internalized by antigen-presenting cells and presented to B or T cells, a process known as indirect

recognition. Indirect recognition of MHC peptides is critical for $CD4^+$ T cells to initiate class switching and alloantibody production in B cells (Sauvé, Baratin, Leduc, Bonin, & Daniel, 2004; Steele et al., 1996). Dendritic cells have been shown to present intact MHC I peptides to B cells, which leads to alloantibody generation (Curry et al., 2007). It is therefore critical to limit allorecognition of both MHC I and MHC II in order to prevent rejection of MHC-mismatched MSCs.

Development of strategies to limit allorecognition may help facilitate the use of allogeneic MSCs clinically. Downregulation of MHC surface expression is a common tactic utilized by viruses, neoplastic cells, and cells in immune-privileged tissues to avoid immune surveillance (Cromme et al., 1995; Niederkorn, 2003; Rinaldo, 1994). In immune-privileged tissues like the brain, transforming growth factor-β2 (TGF-β2) is highly expressed and appears to help prevent autoantigen presentation and subsequent immune responses (Siglienti et al., 2007). TGF-β2 has been shown to downregulate constitutive MHC I surface expression on a variety of cell types including melanoma cells, intestinal epithelial cells, and astrocytes as well as block IFN-γ-induced MHC expression (Donnet-Hughes, Schiffrin, & Huggett, 1995; Ma & Niederkorn, 1995; Schluesener, 1990). The TGF-β2 isoform has previously been shown to be more effective than TGF-β1 at blocking IFN-γ-induced MHC expression (Y. J. Lee et al., 1997). All equine MSCs express MHC I and can be heterogeneous for MHC II, but IFN-γ can strongly upregulate expression of MHC I and MHC II (Schnabel et al., 2014). Other inflammatory cytokines including IL-1β and TNF-α can alter MHC expression, but only IFN-γ upregulates both MHC I and MHC II surface expression (Hill, Cassano, Goodale, & Fortier, 2017). Because MSCs are typically injected

directly into an inflammatory environment where IFN-γ may be present, it is particularly important to block IFN-γ-induced expression in order to limit allorecognition.

The aim of this study was to characterize the effects of TGF-β2 on constitutive and IFN-γ-induced MHC surface expression on equine bone marrow-derived MSCs. We also examined the morphology, cell surface marker expression, and TGF-β isoform secretion, which we define as the MSC phenotype, to determine if these were altered by TGF-β2 treatment.

Materials and Methods

Horses

A total of 12 horses were used for this study. All animals were between the ages of 5 and 17 years, free of systemic disease as determined by routine physical examinations and bloodwork, free of medication for 48 h prior to use, and non-pregnant. The Institutional Animal Care and Use Committee of North Carolina State University approved the use of horses in these studies.

MSC isolation and culture

Bone marrow aspirates were collected aseptically from the sternum of 12 horses by using 11-gauge Jamshidi bone marrow biopsy needles under standing sedation with local anesthesia. For each harvest, a total of 120 ml of aspirate was collected into 60-ml syringes containing 25,000 U of heparin each. Bone marrow aspirates were purified *via* Ficoll-Paque Plus (GE Healthcare, Chicago, IL, USA) gradient centrifugation, as previously described

(Radcliffe, Flaminio, & Fortier, 2010). Isolated cells from each horse were evenly divided onto 100-mm tissue-culture plates containing the appropriate media for each treatment group. MSC base media used for the negative control group consisted of low glucose (1 g/dl) DMEM media containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA), 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml). Standard MSC media used for the traditional control group consisted of MSC base media with the addition of basic fibroblastic growth factor (bFGF, 1 ng/ml) (Corning, Inc., Corning, NY, USA), which is a normal addition to media for equine MSC culture (Pezzanite et al., 2015; Schnabel et al., 2014). TGF-β2 treatment group media consisted of standard MSC media containing human recombinant TGF-β2 at concentrations of 1, 5, or 10 ng/ ml (BioLegend, San Diego, CA, USA).

Media were exchanged every 48 h. Cells were passaged 1:3 at approximately 80% subconfluency by using Accumax cell-dissociation solution (Innovative Cell Technologies, Inc., San Diego, CA, USA) and plated at a density of approximately 1×10^4 cells/cm^2. Cell counts and viability at each passage were determined using a Cellometer® Auto 2000 and ViaStain™ AOPI Staining Solution (Nexcelom Bioscience LLC, Lawrence, MA, USA). Cell cultures were imaged using an IX83 inverted microscope and cellSens™ software (Olympus Corporation, Center Valley, PA, USA). Cells to be cryopreserved were pelleted after dissociation and resuspended in freeze media (base or standard MSC medium with 10% FBS and 10% dimethyl sulfoxide).

Immunophenotyping of MSCs

Mesenchymal stem cells were immunophenotyped at passage 2 (P2) for expression levels of MHC class I and MHC class II using FACS analysis. The MHC I and MHC II antibodies (clones cz3 and cz11, respectively, Antczak Laboratory) used in this study were previously validated for the horse and used at dilutions of 1:10 according to previous experience (Radcliffe et al., 2010; Schnabel et al., 2014). Cells were pelleted in aliquots containing approximately 1×10^6 cells on 96-well V-bottom plates and treated with a 20-min blocking step by using 10% normal goat serum in phosphate-buffered saline. The cells were pelleted and resuspended in unconjugated primary monoclonal antibody and incubated for 45 min at 4°C. MSCs were then washed and resuspended in a secondary allophycocyanin-conjugated goat anti-mouse IgG antibody (BD Biosciences, San Jose, CA, USA), and incubated for an additional 45 min at 4°C. Cells were analyzed on a LSRII (BD Biosciences) flow cytometer equipped with FACSDiva analysis software (BD Biosciences). MSCs stained with the secondary antibody alone were used as negative controls. Cells were gated as previously described and data were collected on a minimum of 1×10^4 cells for each sample (26).

MHC I quantification

The QIFIKIT® (Agilent Technologies, Inc., Carpinteria, CA, USA) was used to quantify MHC I surface expression in MSCs and equine fetal fibroblasts, which were isolated from a portion of the body wall of day 34 equine conceptuses collected by uterine lavage and grown to confluency. A F(ab′)2 fragment of FITC-conjugated goat anti-mouse IgG antibody from the kit was used as the secondary antibody for the setup beads, calibration beads,

MSCs, and fetal fibroblasts. On an LSRII, the setup beads were used to set voltages and then the geometric mean fluorescence intensity (GMFI) was recorded for the five populations of calibration beads and MSCs. From the GMFIs of the calibration beads, a linear regression of the calibration curve was generated using using Prism 6 (GraphPad, La Jolla, CA, USA). Calculation of antigen binding capacity was completed from the linear regression according to the manufacturer's instruction as a means of determining the average number of MHC I surface molecules for each MSC and fetal fibroblast population.

IFN-γ stimulation

For immunophenotyping of IFN-γ-stimulated MSCs, P3 MSCs were divided into the following treatment groups: untreated MSCs (−/− TGF-β2), TGF-β2-pretreated MSCs (+/− TGF-β2), and continuously treated MSCs (+/+ TGF-β2) (**Figure 1**). Pretreated MSCs were cultured in TGF-β2 from initial isolation to just prior to IFN-γ stimulation. MSCs were stimulated by replacing cell culture media with standard media containing 1 ng/ml recombinant equine IFN-γ (R&D Systems, Minneapolis, MN, USA) at 0 and 48 h as shown in **Figure 1**. Untreated and pretreated MSCs not stimulated with IFN-γ were used as controls. Baseline MSC expression was determined by harvesting MSCs prior to IFN-γ stimulation. All 72 h MSC MHC I expression level, results were reported as fold change from that of the untreated (−/− TGF-β2) MSC group cultured without IFN-γ.

For IFN-γ-induced MHC expression kinetics, untreated and TGF-β2-pretreated P3 MSCs were stimulated with IFN-γ as described above. MSCs were harvested at baseline, 24, 48, and 72 h and frozen until FACS analysis.

MSC surface markers

Cryopreserved TGF-β2-treated and untreated P2 and P3 MSCs were expanded for one passage before staining for surface markers as previously described (Radcliffe et al., 2010). The following antibodies were used to confirm MSC phenotype: LFA-1 (cz3.2 Antczak lab), CD29 (TDM29 EMD Millipore), CD44 (CVS18 BioRad), CD45RB (DH16A Washington State University), and CD90 (DH24A Washington State University). LFA-1 was used neat, while dilutions of 1:10 (CD45RB), 1:100 (CD29, CD44), and 1:200 (CD90) were used, according to manufacturer's directions and according to previous experience.

Cytokine analysis

P3 MSCs were divided into untreated and pretreated groups and stimulated with 1 ng/ml IFN-γ as described under IFN-γ stimulation. All groups were washed at 72 h and media replaced with standard media containing no IFN-γ or TGF-β2. Supernatant was collected 24 h later for analysis of TGF-β1, 2, and 3 using a commercially available multiplexed cytokine/chemokine magnetic bead kit (EMD Millipore, Billerica, MA, USA). All samples were analyzed in duplicate using a 96-well platform performed per manufacturer's instructions. Sample volume used was 25 µl and all samples were run at a 1:5 dilution. Plates were read using a Luminex MagPix instrumentation (Luminex Corporation, Austin, TX, USA). A minimum bead count of 50 for each cytokine was acquired for data analysis. Data were analyzed using Milliplex Analyst 5.1 software (Luminex Corporation, Austin, TX, USA).

Statistical analyses

Immunophenotyping and IFN-γ stimulation data were normalized by log transformation and analyzed by analysis of covariance (ANCOVA) with horse as covariate, followed by the Tukey's test for multiple comparisons. TGF-β concentrations in supernatant were analyzed by ANCOVA with horse as covariate, followed by the Tukey's test for multiple comparisons. Baseline MHC data for the IFN-γ stimulation experiments, cell yield, and viability were analyzed using a two-tailed *t*-test. Analyses were performed using JMP® Pro11 (SAS Institute Inc., Cary, NC, USA) and significance set at $p < 0.05$.

Results

MSC immunophenotyping

To first determine the effects of TGF-β2 treatment on MSC immunophenotype, MSCs from the same bone marrow aspirate were cultured with 1, 5, or 10 ng/ml TGF-β2. Untreated MSCs cultured in standard media with or without bFGF were used as controls. GMFI was used to assess expression levels of MHC I because all MSCs should be positive for MHC I with a fairly normal distribution of expression. MSCs cultured in 1, 5, and 10 ng/ml TGF-β2 all had lower MHC I surface expression compared with either control group (**Figure 2A**) and had a significantly lower fold change in GMFI compared to the traditional control group (**Figure 2B**). There was no significant difference in MHC I expression between any of the TGF-β2 concentrations. Negative control MSCs (0 ng/ml bFGF) had the largest variation in MHC I expression, but the relative GMFI was not significantly different from the

traditional control group (1 ng/ml bFGF) indicating that bFGF may have some stabilizing effect on MHC I expression.

Major histocompatibility complex II expression was reported as the average percent of cells positive for each control and treatment group as expression can be heterogeneous and not accurately represented by any measure of MFI. MSCs from two of the eight horses sampled for this experiment were positive for MHC II. Treatment with all concentrations of TGF-β2 strongly reduced the proportion of P2 MSCs positive for MHC II, but statistical significance could not be determined due to the low sample size (**Figures 2C–D**).

MHC I quantification

Average MHC I surface expression was quantified for each treat- ment group using the Dako QIFIKIT® to determine the number of surface molecules expressed by MSCs. Quantification of MHC I allows for direct comparison of expression to fetal fibroblasts, which are considered non-immunogenic in part due to low MHC I surface expression (Zuliani, Saiagh, Knol, Esbelin, & Dréno, 2013). The GMFI of each calibration bead population was obtained (**Figure 3A**) and used to generate a linear regression equation for antigen binding capacity. The average MHC I GMFI of each MSC treatment group for each horse was then used to calculate the average antigen binding capacity, which would correlate in this experiment to the number of MHC I surface molecules (**Figure 3B**). The assay confirmed that P2 MSCs treated with TGF-β2 have significantly fewer MHC I surface molecules than MSCs from either control group (**Figure 3C**). MHC I expression levels of treated cells were also more similar to equine fetal fibroblasts than control groups and had less variation in expression between horses (**Figure 3C**). Due to the small number of fetal

fibroblast samples (n = 3), fetal fibroblast MHC I expression was not included in the statistical analysis.

As there were no significant differences between MHC I expression for the three TGF-β2 concentrations, the lowest concentration, 1 ng/ml, was used for all subsequent experiments. The traditional control group, which was cultured with bFGF, was also used as the untreated control for all other experiments.

IFN-γ stimulation

As MSCs are typically injected directly into sites of active inflammation, we measured the stability of MHC I and MHC II surface expression on untreated, TGF-β2-pretreated, and TGF-β2 continuously treated MSCs following stimulation with IFN-γ. IFN-γ stimulation induced upregulation of MHC I surface expression on untreated and TGF-β2-treated cells as compared to the unstimulated controls (**Figure 4A**). Relative to the unstimulated and untreated control group, there was no significant difference in MHC I expression in any of the treatment groups following IFN-γ stimulation, although the pretreated and continuously treated MSCs did trend toward having lower MHC I expression (**Figure 4B**). However, the continuously treated TGF-β2 MSCs did have significantly lower MHC I expression than the untreated controls following IFN-γ stimulation, indicating that TGF-β2 is able to partially block IFN-γ-induced MHC I expression under these conditions. There was a significant variation in MHC I expression between horses that affected how strongly the cells upregulated MHC I with IFN-γ stimulation. Individual horses could be divided into two categories, MHC I low and MHC I high, depending on the fold change in MHC expression following IFN-γ stimulation. Horses that had at least a 1.8-fold change

increase in expression following IFN-γ stimulation and also had the lowest MHC I expression (**Figure 4C**). Horses that already expressed high levels of MHC I had less than a 1.4-fold change increase in expression (**Figure 4D**). Over the 72 h without TGF-β2, MHC I expression of pretreated MSCs not stimulated with IFN-γ was not significantly different from baseline expression and still significantly lower than untreated MSCs (**Figure 4E**).

For MHC II, horses were characterized as MHC II negative or MHC II positive depending on baseline expression. Untreated MSCs from four horses were negative for MHC II and untreated MSCs from three horses were positive for MHC II at the beginning of the IFN-γ stimulation. For MHC II negative MSCs, neither pretreatment nor continuous treatment significantly prevented IFN-γ-induced expression of MHC II (**Figures 5A,B**), although like MHC I, treated MSCs tended to have a lower number of MSCs positive for MHC II. For MSCs that were MHC II positive prior to IFN-γ challenge, only the MSCs continuously treated with 1 ng/ml TGF-β2 had significantly fewer cells with MHC II expression compared with untreated MSCs (**Figures 5C,D**). Significant upregulation still occurred, however, compared with TGF-β2-treated MSCs that were not challenged with IFN-γ again indicating partial, but incomplete, blocking of IFN-γ-induced MHC expression.

IFN-γ-induced MHC kinetics

Next, we analyzed if TGF-β2 treatment changed the rate of IFN-γ-induced MHC I and MHC II upregulation. MSCs from two horses were compared in this assay. On MSCs from Horse 1, MHC I expression peaked at 24 h on both untreated and pretreated MSCs, then declined over the next 48 h (**Figure 6A**). Pretreated MSCs had lower MHC I expression at 0, 24, and 48 h, but slightly higher expression at 72 h. Horse 2 had very high MHC I baseline

expression on untreated MSCs and expression decreased over the 72 h (**Figure 6A**). Horse 1 was baseline MHC II negative and Horse 2 was baseline MHC II positive. MHC II expression on MSCs from both horses gradually increased throughout the 72 h of the assay in both untreated and pretreated MSCs (**Figure 6B**). MHC II expression on pretreated MSCs remained lower than untreated MSCs throughout the assay. The MHC I expression patterns at 72 h were different in these two individual horses than what was seen overall in the previous IFN-γ stimulation assay further demonstrating that MHC I expression on MSCs is dynamic and dependent on baseline expression and the individual MSC donor.

MSC morphology and surface markers

Morphology of P2 untreated and TGF-β2-treated MSCs was visually compared using phase contrast microscopy. Untreated and TGF-β2-treated MSCs were both spindle shaped with similar width and length (**Figure 7A**). Expression of standard MSC surface markers was measured to determine if 1 ng/ml TGF-β2 treatment altered equine MSC surface marker expression. Both untreated and TGF-β2-treated MSCs were positive for CD29, CD44, and CD90 and negative for CD45RB and LFA-1 (**Figure 7B**). Overall, there were not significant differences between the expression for treated or untreated MSCs for any of the positive or negative surface markers, except CD90 expression was slightly downregulated on TGF-β2-treated MSCs in four of the five horses. Unlike MHC surface expression, expression levels of CD29, CD44, and CD90 were very similar between individual horses.

Cell yield and viability

Transforming growth factor-β2 is well known as a negative regulator of the cell cycle and can induce apoptosis in certain cell types (Datto et al., 1995; Moustakas & Heldin,

2005). To determine effects of TGF-β2 treatment on MSC proliferation capacity, the number and viability of cells obtained from P0 to P2 was compared between untreated and TGF-β2-treated MSCs. For P0–P2, TGF-β2-treated MSCs had significantly increased cell yields compared to untreated, despite the fact that the same number of cells were plated at the beginning of each passage and the groups were grown for the same amount of time (**Figure 8A**). The relative difference between cell yield appeared to diminish with each passage, but there was still a significantly higher cell yield at P2 in the TGF-β2-treated group. The increase in cell yield appears to be dependent on coculture with bFGF as MSCs grown with only TGF-β2 had significantly reduced cell yield relative to cells cultured with bFGF and TGF-β2 ($p < 0.0001$) (Figure S1 in Supplementary Material). There was no significant difference between the viability of untreated or treated MSCs from P0 to P2 (P0: $p = 0.257$, P1: $p = 0.313$, P2: $p = 0.100$) (**Figure 8B**).

TGF-β isoform production

Constitutive secretion of TGF-β1, but not TGF-β2, has been previously reported from equine MSCs (Carrade et al., 2012). Concentrations of TGF-β1 and TGF-β2 were measured in the supernatant of unstimulated and IFN-γ-stimulated MSC cultures to determine if treatment with TGF-β2 affected production of either TGF-β isoform. Unstimulated and IFN-γ-stimulated MSCs from both untreated and TGF-β2-pretreated groups secreted TGF-β1 as well as TGF-β2 (**Figures 9A,B**). There was no significant difference between the concentrations of TGF-β1 secreted by untreated or TGF-β2-pretreated MSCs or between unstimulated and stimulated MSCs ($p = 0.102$) (**Figure 9A**), but unstimulated TGF-β2-pretreated MSCs did secrete significantly more TGF-β2 than untreated MSCs (**Figure 9B**).

Expression of TGF-β1 was significantly different between individual horses ($p = 0.011$). TGF-β3 could not be detected in any of the samples (data not shown).

Discussion

The aims of this study were to determine the effects of TGF-β2 on MHC expression, phenotype, and TGF-β isoform secretion of equine bone marrow-derived MSCs. The data obtained in this study are consistent with these previous findings in other cell types that TGF-β2 is capable of downregulating MHC I and II expression and partially blocking IFN-γ-induced expression (Donnet-Hughes et al., 1995; Y. J. Lee et al., 1997; Ma & Niederkorn, 1995; Schluesener, 1990). Our studies also confirm that MSCs treated with TGF-β2 retain their phenotype and are able to secrete high levels of the immunomodulatory cytokine TGF-β1 as well as TGF-β2.

As shown in our initial immunophenotyping, although all MSCs are positive for MHC I expression, the degree of MHC I expression on untreated bone marrow-derived MSCs is highly variable between individual horses. MSCs treated with TGF-β2 in this study had consistently lower MHC I surface expression than untreated MSCs, less variation in MHC I expression levels than untreated MSCs, and similar expression levels as fetal fibroblasts. It is important to note that the TGF-β2-treated cells are still positive for MHC I, as absent MHC I expression would result in natural killer cell-mediated lysis (Ljunggren & Kärre, 1990). As shown in the MHC I quantification assay, constitutive MHC I expression levels on MSCs are highly variable between horses, but in our experience, individual horses tend to have consistent MHC I expression levels between bone marrow aspirates. This

variation between individuals has also been documented in humans and chickens and is influenced by MHC haplotype in chickens (Chappell et al., 2015; Hobbs, Rees, Heyderman, Birchall, & Bailey, 2006). The immunophenotyping results for MHC II expression on untreated MSCs are consistent with previous findings that equine bone marrow-derived MSCs are heterogeneous for MHC II expression (Schnabel et al., 2014). Untreated MSCs from two of the initial eight horses used for testing the three TGF-β2 concentrations were positive for MHC II, but treatment with TGF-β2 was able to dramatically reduce MHC II expression on unstimulated MSCs to nearly undetectable levels. Further research on individual variance in MHC I and MHC II expression levels may be helpful for identifying ideal donor horses for allogeneic MHC therapy and for developing further strategies to regulate MHC expression.

Similar to a previous study on intestinal epithelial cells (Donnet-Hughes et al., 1995), IFN-γ-induced upregulation of MHC I on MSCs in this study was attenuated by TGF-β2 treatment, although the significance was dependent on if the donor horse was MHC I baseline high or low. The degree to which IFN-γ was able to upregulate MHC I and TGF-β2 to block IFN-γ-induced MHC I expression appeared to be highly dependent on the individual horse from which the MSCs were obtained. It is still unclear if these differences in response to IFN-γ and TGF-β2 between horses are influenced by MHC haplotype or the health status of the animal, although all horses in this study were systemically healthy. TGF-β2 treatment also reduced the proportion of MSCs positive for MHC II following IFN-γ challenge, but this change was only significant for MSC populations that were already positive for MHC II at baseline measurement. Continuous treatment with TGF-β2 was shown to be the most

effective at blocking IFN-γ-induced MHC I and MHC II expression in MSCs and co-delivery of TGF-β2 *via* encapsulation in hydrogels or scaffolds with MSCs may be possible for *in vivo* therapy (K. Lee, Silva, & Mooney, 2011). Other methods that can be used with TGF-β2 treatment expression, like 3D culture (Guo, Zhou, Wang, & Wu, 2014) or manipulation of expression of the class II transactivator (Waldburger, Suter, Fontana, Acha-Orbea, & Reith, 2001), should also be explored to stabilize MHC expression on MSCs.

In comparison to the changes seen in MHC expression, TGF- β2 treatment did not appear to significantly change the morphology, surface markers, or viability of MSCs. TGF-β2-treated MSCs were still positive for MSC surface markers CD29, CD44, and CD90 and negative for hematopoietic markers CD45RB and LFA-1. Four of the five horses had reduced CD90 expression, but CD90 has previously been shown to have no effect on the immunomodulatory properties of human MSCs (Moraes et al., 2016). TGF-β2 treatment also had unexpected, yet positive effects on the proliferative capacity of MSCs. TGF-β isoforms are well understood to negatively regulate of cell cycle progression from G1 to S phase (Datto et al., 1995), but treatment of MSC cultures with 1 ng/ml TGF-β2 significantly increased the cell yield of early passage MSCs in this study. TGF-β has been shown to increase production of connective tissue growth factor, a mitogenic peptide, in fibroblasts (Grotendorst, 1997) and may have similar effects on production in MSCs. MSCs from older patients may not proliferate as well as younger and healthier patients (Fafián-Labora et al., 2015; Kretlow et al., 2008), so TGF-β2 treatment may be a viable option for increasing autologous MSC cell yield from older horses or horses with poorly proliferating MSCs. It is important to note that MSCs treated with TGF-β2 in the absence of bFGF have reduced

proliferative abilities compared to MSCs cultured with both indicating that cotreatment with bFGF is necessary. The difference in proliferative capabilities depending on the presence of bFGF may be due to changes in cell cycle progression and should be explored in future studies. The effects of TGF-β2 treatment on the *in vitro* differentiation capabilities or other paracrine factors were not explored in this study, but may be relevant for future therapies. As TGF-β isoforms activate transcription factors with broad cellular effects (Morikawa, Koinuma, Miyazono, & Heldin, 2012), it is possible that treatment may alter the potency or therapeutic properties of the cells (Watabe & Miyazono, 2009).

We also report that equine MSCs constitutively express TGF-β1 and TGF-β2 and that TGF-β2 treatment does not negatively impact expression of either of these. *In vitro* assays like mixed leukocyte reactions and cytotoxicity assays are needed to determine if the modest increases in TGF-β2 expression seen in TGF-β2-treated MSCs change the immunomodulatory properties of the cells and immune recognition. There was also significant variation in concentrations of TGF-β1 and TGF-β2 secreted by individual horses further supporting that some animals may make more ideal donors based on the MHC expression and cytokine profiles of their cells.

In conclusion, TGF-β2 downregulates constitutive MHC I and MHC II surface expression, partially blocks the effects of IFN-γ on MHC expression, and does not significantly alter the morphology, cell surface marker expression, or secretion of TGF-β1 in equine bone marrow-derived MSCs. These findings warrant further investigation into the *in vitro* and *in vivo* cell-mediated immunogenicity of TGF-β2-treated MSCs to determine if

downregulation of MHC I and MHC II expression is sufficient to prevent immune rejection of MHC-mismatched MSCs and to improve clinical applications.

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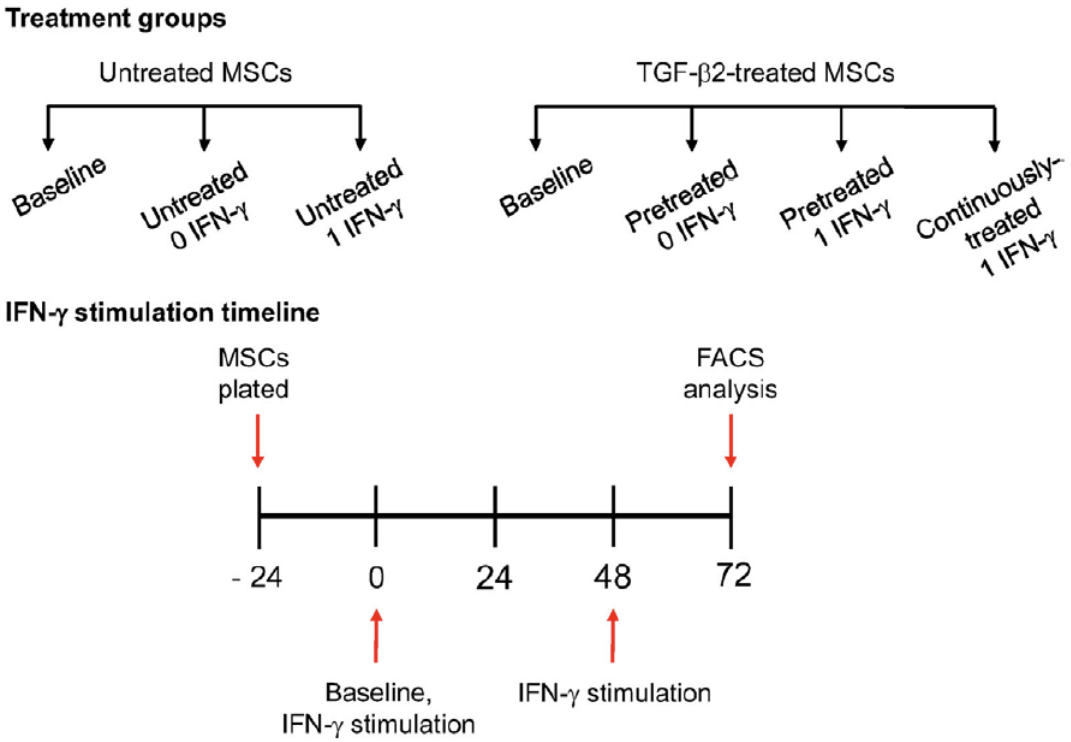


Figure 1. IFN-γ stimulation methods. P3 untreated [–/– transforming growth factor-β2 (TGF-β2)], pretreated (+/– TGF-β2), and continuously treated (+/+ TGF-β2) mesenchymal stem cells (MSCs) were stimulated with 1 ng/ml equine IFN-γ over a 72 h period. Untreated and pretreated MSCs not stimulated with IFN-γ were used as controls. Major histocompatibility complex (MHC) I and MHC II expression was measured via FACS following stimulation. Baseline MHC expression was obtained by freezing cells from each treatment group just prior to IFN-γ stimulation.

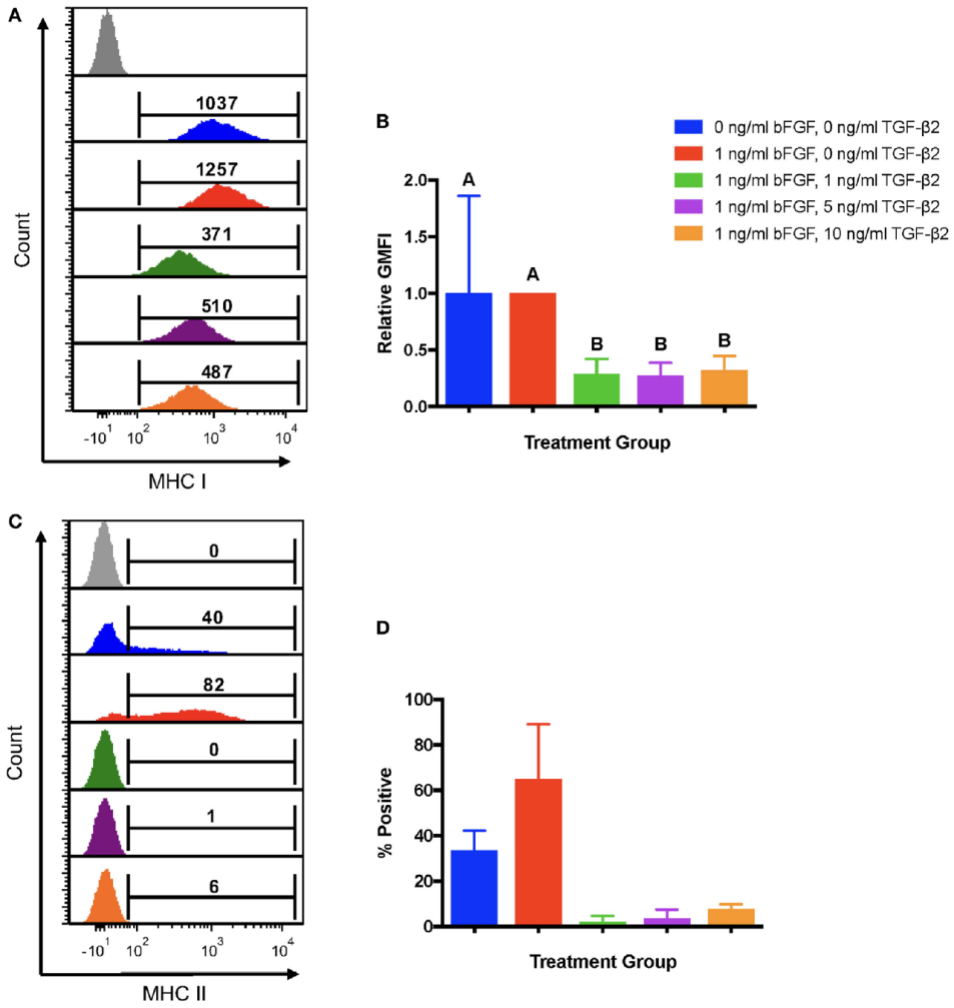


Figure 2. Major histocompatibility complex (MHC) I and MHC II surface expression on untreated and transforming growth factor-β2-treated mesenchymal stem cells. Equine MSCs were cultured in media containing 0 ng/ml basic fibroblast growth factor (bFGF) and 0 ng/ml TGF-β2 (negative control group), 1 ng/ml bFGF and 0 ng/ml TGF-β2 (traditional control group) or 1 ng/ml bFGF and 1, 5, or 10 ng/ml TGF-β2 from initial isolation to P2. MHC I and MHC II expression was measured via FACS analysis. Superscript letters indicate significant differences between groups. **(A)** Representative histograms from one horses of MHC I expression for all treatment groups. Numbers above gate represent population geometric mean fluorescent intensity (GMFI) compared to MHC I negative control (gray). **(B)** MHC I expression shown as average fold change in GMFI relative to the traditional control group. Data shown are mean +/- SD of n = 8, $p < 0.0001$. **(C)** Representative histograms from one horse of MHC II expression for all treatment groups. Numbers above gates represent the percent of the parent population positive for MHC II compared to the negative control. **(D)** MHC II expression shown as the average percent of MSCs positive for MHC II. Data shown are mean +/- SD of n = 2.

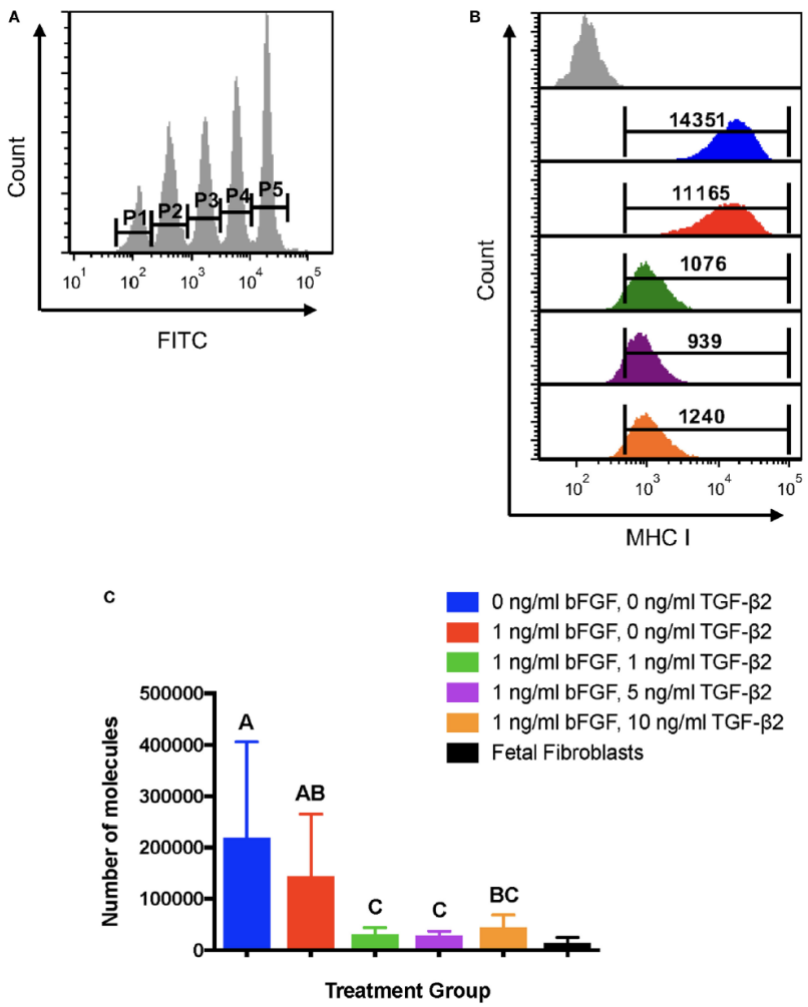
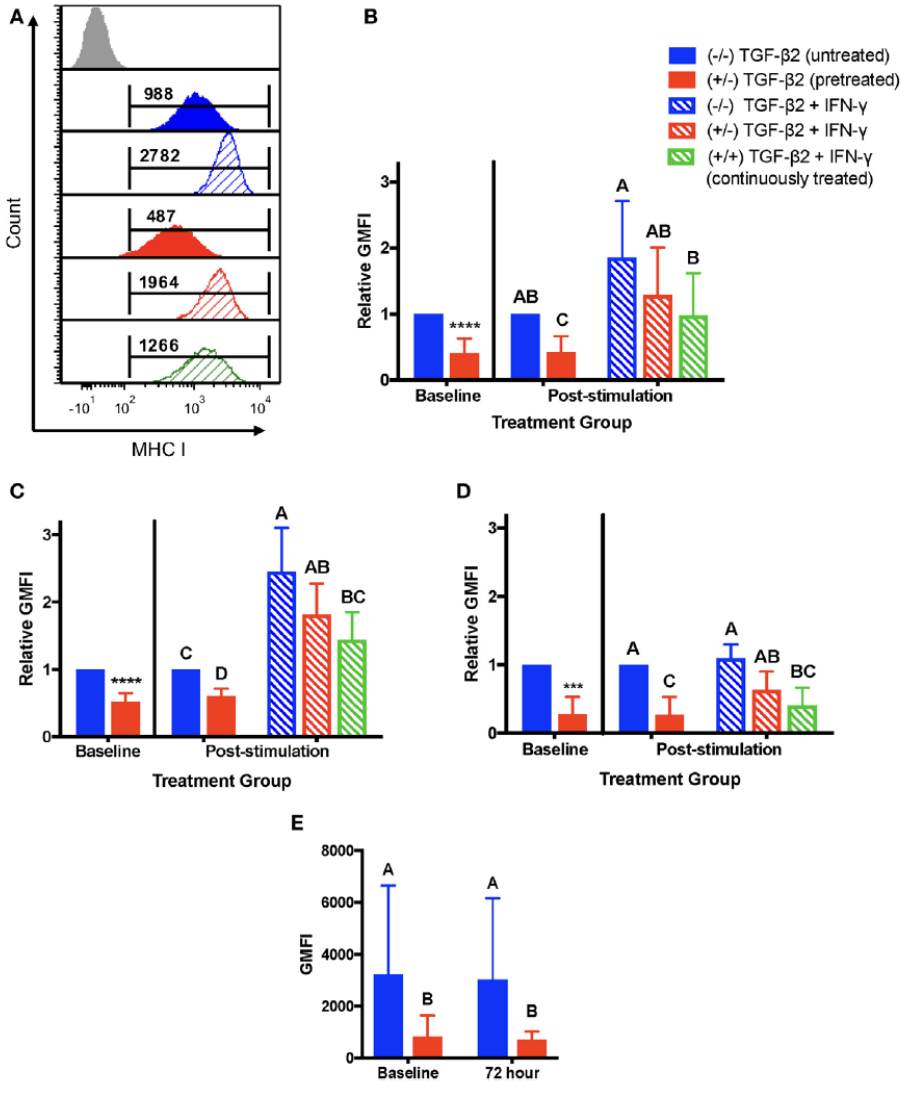


Figure 3. Quantification of major histocompatibility complex (MHC) I surface expression on untreated and transforming growth factor-β2-treated mesenchymal stem cells (MSCs). The QIFIKIT assay was used to quantify the average number of MHC I surface molecules on passage 2 MSCs based on the population geometric mean fluorescent intensity (GMFI). Superscript letters indicate significant difference between groups. **(A)** Calibration bead populations were gated to obtain GMFI for linear regression analysis. **(B)** Representative histograms from one horse showing MHC I expression and population GMFI for all treatment groups. **(C)** Average number of MHC I molecules as determined by linear regression analysis for all treatment groups. Data shown are mean +/- SD of n = 7, p = 0.0003. Fetal fibroblasts are included as a MHC I^{low} reference cell.

Figure 4. Major histocompatibility complex (MHC) I surface expression on untreated and transforming growth factor-β2 (TGF-β2)-treated mesenchymal stem cells (MSCs) following IFN-γ stimulation. P3 untreated (–/–), TGF-β2-pretreated (+/–), and TGF-β2 continuously treated (+/+) MSCs were stimulated with 1 ng/ml IFN-γ for 72 h before MHC I expression analysis via FACS. Untreated and pretreated MSCs not stimulated with IFN-γ were used as controls. Superscript letters indicate significant differences between groups. **(A)** Representative histograms from one horse of MHC I expression for all treatment groups. Numbers above gates represent population geometric mean fluorescent intensity (GMFI) compared to MHC I negative control (gray). **(B)** MHC I expression shown as the average fold change in GMFI relative to the control group (–/– TGF-β2 untreated, solid blue bar). Data shown are mean +/- SD of n = 9, *p < 0.0001 by t-test, p < 0.0001 by analysis of covariance (ANCOVA). **(C)** MHC I expression for MHC I low horses shown as the average fold change in GMFI relative to the control (–/– TGF-β2 untreated, solid blue bar). Data shown are mean +/- SD of n = 5, * p <0.0001 by t-test, p < 0.0001 by ANCOVA. **(D)** MHC I expression for MHC I high horses shown as the average fold change in GMFI relative to the control group (–/– TGF-β2 untreated, solid blue bar). Data shown are mean +/- SD of n = 4, * p = 0.0049 by t-test, p < 0.0001 by ANCOVA. **(E)** MHC I GMFI of baseline and unstimulated MSCs treatment groups. Data shown are mean +/- SD of n = 9, p < 0.0001.


A
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988
2782
487
1964
1266
MHC I
B
(-/-) TGF-β2 (untreated)
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(-/-) TGF-β2 + IFN-γ
(+/-) TGF-β2 + IFN-γ
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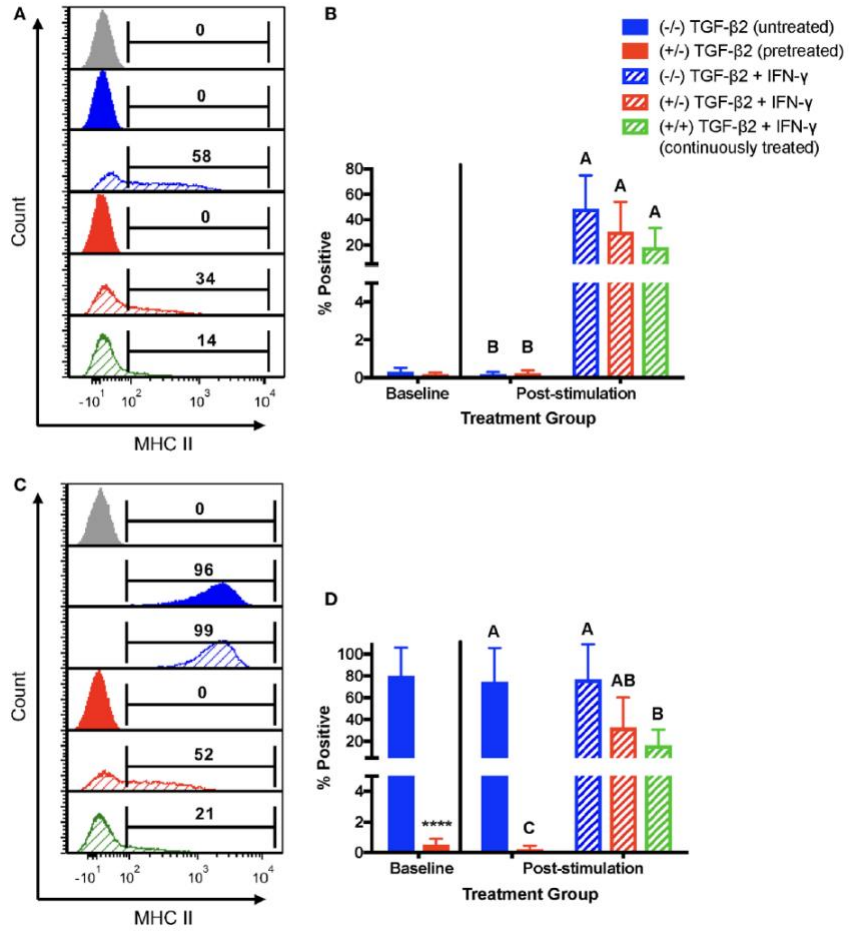


Figure 5. Major histocompatibility complex (MHC) II surface expression on untreated and transforming growth factor-β2 (TGF-β2)-treated mesenchymal stem cells (MSCs) following IFN-γ stimulation. P3 untreated (–/–), TGF-β2-pretreated (+/–), and TGF-β2 continuously treated (+/+) MSCs were stimulated with 1 ng/ml IFN-γ for 72 h before MHC II expression analysis via FACS. Untreated and pretreated MSCs not stimulated with IFN-γ were used as controls. Superscript letters indicate significant differences between groups. **(A)** Representative histograms from one MHC II baseline negative horse showing MHC II expression for all treatment groups. Numbers above gates represent the percent of the parent population positive for MHC II compared to the negative control (gray). **(B)** MHC II expression shown as the average percent of the parent population positive for MHC II relative to the control group (–/– TGF-β2 untreated, solid blue bar). Data shown are mean +/- SD of n = 6, $p < 0.0001$. **(C)** Representative histograms from one MHC II baseline positive horse showing MHC II expression for all treatment groups. Numbers above gates represent the percent of the parent population positive for MHC II compared to the negative control (gray). **(D)** MHC II expression shown as the average percent of the parent population positive for MHC II relative to the control group (–/– TGF-β2 untreated, solid blue bar). Data shown are mean +/- SD of n = 3, * $p < 0.0001$ by t-test, $p < 0.0007$ by analysis of covariance.

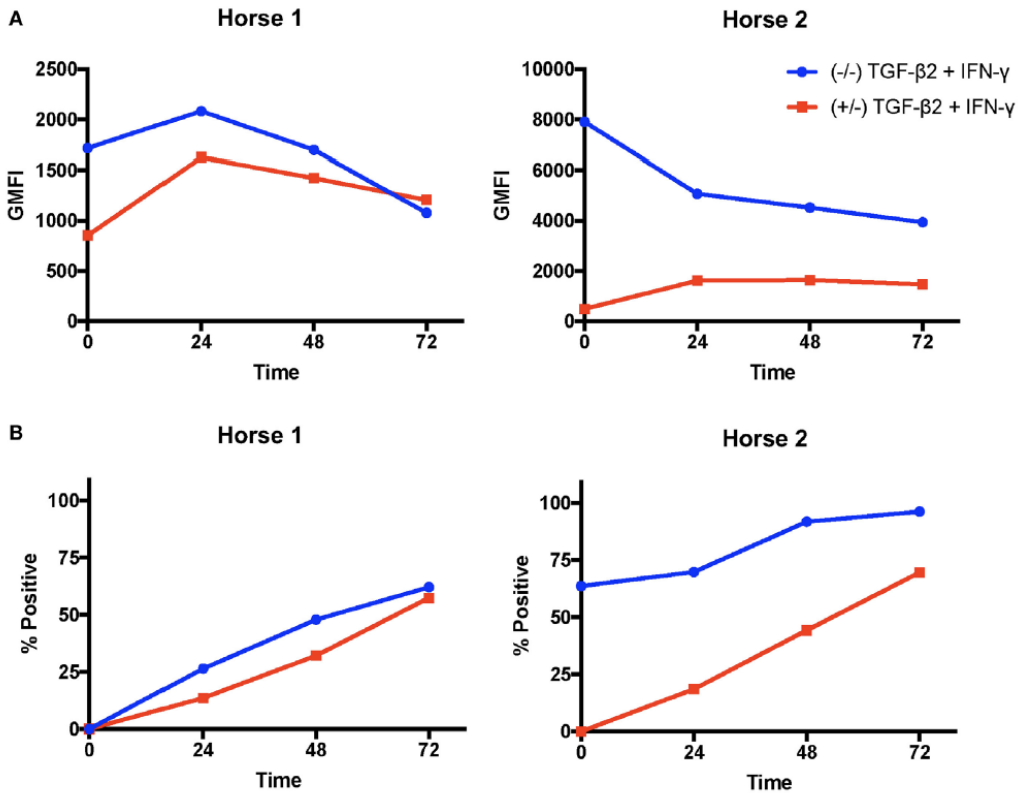
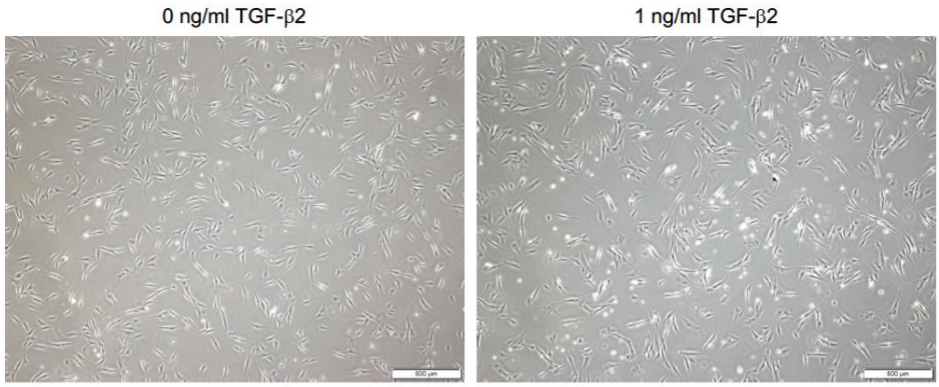


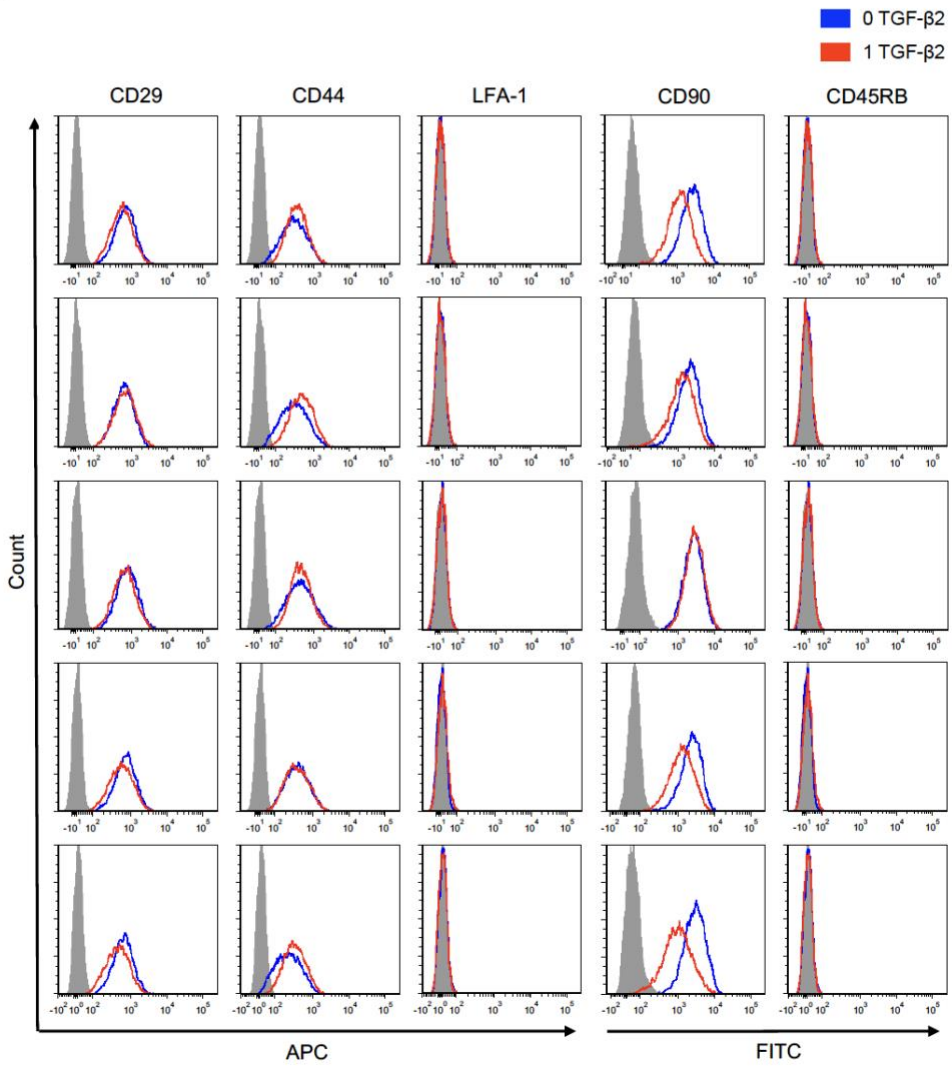
Figure 6. Major histocompatibility complex (MHC) I and MHC II surface expression kinetics on untreated and transforming growth factor-β2 (TGF-β2)-pretreated mesenchymal stem cells (MSCs) following IFN-γ stimulation. MHC I and MHC II expression were measured on untreated and TGF-β2-pretreated MSCs from two horses via FACS every 24 h for 72 h following IFN-γ stimulation. **(A)** MHC I expression shown as the geometric mean fluorescent intensity (GMFI) of MSCs in each treatment group over time. **(B)** MHC II expression shown as the percent of the parent MSC population positive for MHC II in each treatment group over time.

Figure 7. Phenotype of untreated and transforming growth factor-β2 (TGF-β2)-treated mesenchymal stem cells (MSCs). **(A)** P2 MSCs were imaged via phase microscopy, bar = 500 μM. **(B)** P3 untreated and TGF-β2-treated MSCs were stained for positive MSC surface markers CD29, CD44, and CD90 and negative surface markers LFA-1 and CD45RB. Expression is shown as FACS histogram from five horses.

A


0 ng/ml TGF-β2
1 ng/ml TGF-β2

B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1 TGF-β2
CD29
CD44
LFA-1
CD90
CD45RB
Count
APC
FITC

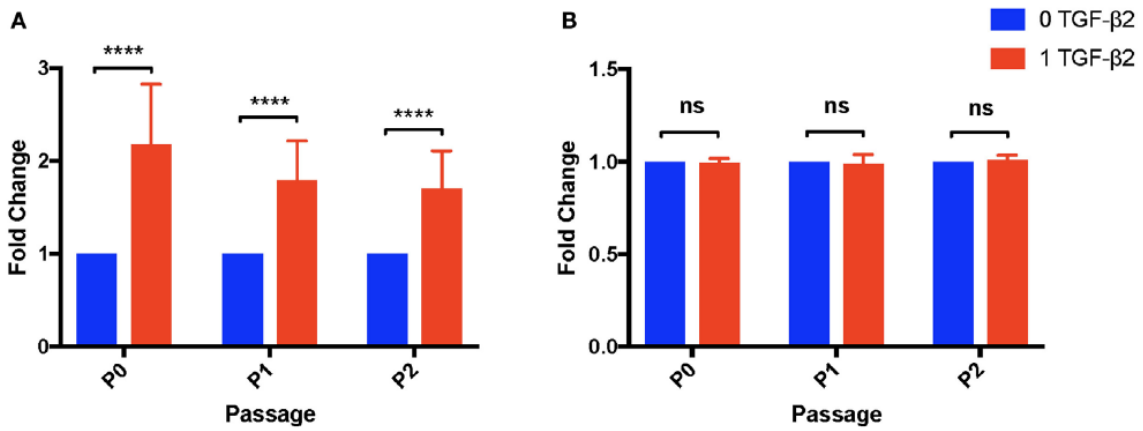

Figure 8. Cell yield and viability of untreated and transforming growth factor-β2 (TGF-β2)-treated mesenchymal stem cells. Cell yield and viability were determined at each passage using a Cellometer Auto 2000 and ViaStain AOPI Staining Solution. **(A)** Cell yield is displayed as the average fold change in cell counts relative to untreated MSCs. Data shown are mean +/- SD of n = 8, ****p<0.0001 by t-test. **(B)** Viability is displayed as the average fold change in percent viability of MSCs relative to untreated MSCs. Data shown are mean +/- SD of n = 8.

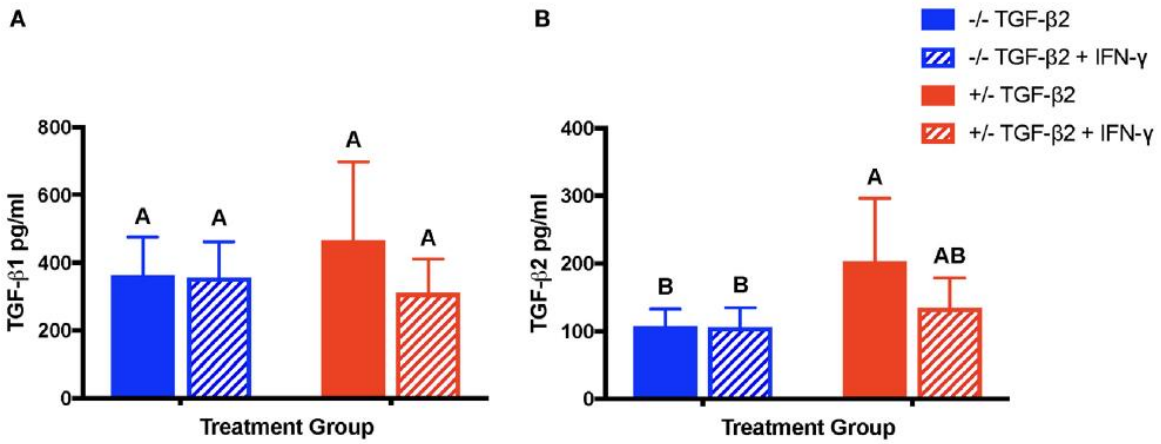

Figure 9. Production of transforming growth factor-β1 (TGF-β1) and TGF-β2 by untreated and TGF-β2-treated mesenchymal stem cells. TGF-β1 and TGF-β2 concentrations were measured in the supernatant of untreated, untreated and IFN-γ stimulated, TGF-b2-pretreated, and TGF-β2-pretreated and IFN-γ stimulated MSCs using a TGF-β multiplex assay. Superscript letters indicate significant differences between groups. (A) TGF-β1 is displayed as the mean concentration +/- SD of n = 7. (B) TGF-β2 is displayed as the mean concentration +/- SD of n = 7, p = 0.0045 by analysis of covariance.

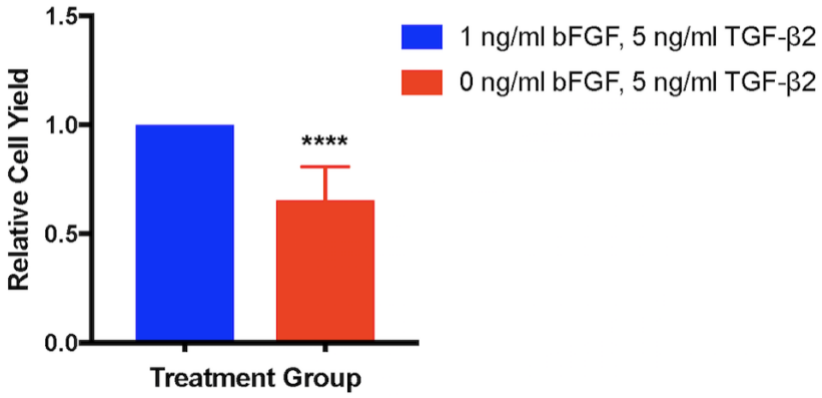

Figure S1. Effects of basic fibroblast growth factor (bFGF) and transforming growth factor-β2 (TGF-β2) on cell yield. Cell yield was determined by manual hand count. Cell yield is displayed as the average fold change in cell count relative to mesenchymal stem cells cultured with 1 ng/ml bFGF, 5 ng/ml TGF-β2. Data shown are mean +/- SD of n = 6, ****p < 0.0001 by t-test.

CHAPTER 4: Immunomodulatory Properties of TGF-β2-treated Mesenchymal Stem Cells

Untreated and transforming growth factor-β2-treated equine bone marrow-derived mesenchymal stem cells have similar immunomodulatory properties.

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Introduction

The use of mesenchymal stem cells (MSCs) is currently being investigated in clinical trials for the treatment of musculoskeletal, immune-mediated, and degenerative diseases (Squillaro et al., 2016). The primary mechanism by which MSCs exert their therapeutic effect appears to be through the secretion of immunomodulatory and growth factors, which inhibit immune responses and promote the healing of tissues (Caplan & Sorrell, 2015; da Silva Meirelles et al., 2009). MSCs are strongly immunomodulatory in vitro, which led investigators to initially conclude MSCs were immune privileged and could be used allogeneically without risk of immune rejection (Le Blanc et al., 2003). Subsequent in vivo studies have demonstrated, however, that MHC-mismatched MSCs are recognized and rejected by the recipient immune system (Berglund & Schnabel, 2017; Eliopoulos & Stagg, 2005; Nauta et al., 2006; Zangi et al., 2009). MSCs are rejected more slowly than non-

immunomodulatory cells like fibroblasts, though (Zangi et al., 2009), and are now considered to be immune evasive, rather than immune privileged (Ankrum et al., 2014).

Despite evidence that MSCs are not immune privileged, allogeneic MSC therapy is still attractive over autologous therapy as the age and health status of a donor can greatly affect the quality of the cells (Fafián-Labora et al., 2015; Nie et al., 2010). MHC-matching is a labor-intensive process and is not practical in most clinical settings. Manipulation of MHC surface expression on MSCs is a promising strategy for reducing the immunogenicity of MHC-mismatched MSCs and has been shown to promote persistence of allogeneic MSCs in mouse models (de la Garza-Rodea et al., 2011; Huang et al., 2016). We recently published that treatment of equine bone-marrow derived MSCs with 1 ng/ml transforming growth factor-β2 (TGF-β2) significantly downregulated MHC I and MHC II surface expression without altering cell phenotype or production of TGF-β1 (Berglund, Fisher, Cameron, Poole, & Schnabel, 2017).

Mixed leukocyte reactions have traditionally been used to measure MSC immunogenicity, but more recent studies have demonstrated that the ability to suppress leukocyte proliferation in vitro does not correlate with the ability to avoid immune responses in vivo (Nauta et al., 2006; Poncelet et al., 2007; Zangi et al., 2009). However, MLRs are a useful assay for measuring the immunosuppressive capabilities of MSCs and for confirming that MSCs manipulated in vitro are still immunomodulatory. As the immunomodulatory properties of MSCs are critical for the therapeutic potential and immune evasive nature of the cells, we sought to investigate if TGF-β2 treatment affected the ability of equine bone marrow-derived MSCs to modulate T cell responses. Modified one-way mixed leukocyte

reactions and ELISAs were used to compare the immunomodulatory abilities of untreated and TGF-β2-treated MSCs. We report that TGF-β2-treated MSCs prevent T cell proliferation and secrete significant quantities of TGF-β1 and PGE2 similar to untreated MSCs.

Materials and Methods

Horses and MHC-haplotyping

A total of six horses were used in this study. All animals were between the ages of 5 and 17 years of age, free of systemic disease as determined by routine physical examinations, free of medication for 48 hours prior to use, and non-pregnant. The MHC haplotype of each horse was determined by microsatellite testing as previously described (Tallmadge, Campbell, Miller, & Antczak, 2010; Tseng, Miller, Cassano, Bailey, & Antczak, 2010). The Institutional Animal Care and Use Committee of North Carolina State University approved the use of horses in these studies.

MSC Isolation and Culture

Bone marrow aspirates were collected aseptically from the sternum of horses by using 11-gauge Jamshidi bone marrow biopsy needles under standing sedation with local anesthesia. For each harvest, a total of 80 ml of aspirate was collected into 60-ml syringes containing 25,000 U of heparin each. Bone marrow aspirates were purified via Ficoll-Paque Plus (GE Healthcare, Chicago, IL, USA) gradient centrifugation, as previously described (Radcliffe et al., 2010). Isolated cells from each horse were evenly divided onto 100-mm tissue culture plates containing the appropriate media for each treatment group. Untreated MSCs were cultured in control media, which consisted of low glucose (1 g/dl) DMEM media containing 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA),

2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), and basic fibroblast growth factor (bFGF, 1 ng/ml) (Corning, Inc., Corning, NY, USA). TGF-β2-treated cells were cultured in control media with the addition of 1 ng/ml human recombinant TGF-β2 (BioLegend, San Diego, CA, USA).

Media were exchanged every 48 hours. Cells were passaged 1:3 at approximately 80% subconfluency by using Accutase cell-dissociation solution (Innovative Cell Technologies, Inc., San Diego, CA, USA) and plated at a density of approximately 1 x 10^4 cells/cm^2. Cell to be cryopreserved were pelleted after dissociation and re-suspened in freeze media (control MSC media with 20% FBS and 10% dimethyl sulfoxide). Cryopreserved cells were culture expanded for at least one passage before use.

Peripheral blood leukocyte isolation

Peripheral blood leukocytes (PBLs) were isolated from venous blood by Ficoll-Paque Plus gradient centrifugation. Isolated PBLs were re-suspended in lymphocyte media containing RPMI 1640, 10% FBS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 0.1 mM molecular grade 2-mercaptoethanol.

Mixed leukocyte reactions

Modified one-way mixed leukocyte reactions were performed in duplicate in using MHC-matched (autologous) and MHC-mismatched responder PBLs and stimulator MSCs as previously described (Schnabel et al., 2014). All MSCs used in this study were MHC II negative. MHC-matched stimulator PBLs were used as negative MLR controls (baseline T cell proliferation) and MHC-mismatched stimulator PBLs were used as positive controls.

Responder PBLs were labeled with 5 μM 5(6)-carboxyfluoroscein diacetate N-sucinimidyl ester (CFSE) (MilliporeSigma, St. Louis, MO, USA). Stimulator untreated and TGF-β2-treated MSCs were plated at 5 x 10^4 cells/well in a 24-well plate in duplicate in control or TGF-β2 MSC media 24 hours before the addition of responder PBLs. MSC wells were washed twice with PBS to remove any exogenous TGF-β2 from the MSC media prior to the addition of responder PBLs. Stimulator PBLs were irradiated with 9 Gy at 60 cGy/min using a Varian Novalis TX linear accelerator and plated at 1 x 10^6 cells/well immediately before the addition of responder PBLs. The proliferative ability of responder leukocytes was verified via mitogen stimulation with 5 μg/ml concanavalin A (ConA) (MilliporeSigma) for each assay. Cultures were maintained for 5 days in lymphocyte media at 37 °C and 5% CO_2.

After culture, PBLs were aspirated from the wells. Supernatant from each well was frozen at -80 °C and cells were stained with a primary mouse anti-horse CD3 antibody (clone UC-F6G, 1:20 dilution, Laboratory of Dr. J. Scott, University of California-Davis, Davis, CA, USA) and a secondary goat anti-mouse IgG-APC antibody (1:100 dilution, BD Pharmigen). 4',6-diamidino-2-phenylindole (DAPI) was added to each FACS tube at a concentration of 500 ng/ml 15 minutes prior to analysis. Proliferation was calculated using CFSE attenuation and the division index of live (DAPI negative), $CD3^+$ cells using FlowJo v7.6 (FlowJo LLC, Ashland, Oregon, USA).

Cytokine analysis

ELISAs for human TGF-β1 (Promega, Madison, WI, USA) and PGE2 (Enzo Life Sciences, Inc., Farmingdale, NY, USA) previously validated for the horse were performed

per manufacturers' instructions (Carrade et al., 2012; Scharf et al., 2016). For PGE2 analysis, supernatant from MLRs with PBL stimulator cells were diluted 1:2 and supernatant from MLRs with MSC stimulator cells were diluted 1:100 in reagent diluent. All supernatant was diluted 1:5 for the TGF-β1 assay.

Statistical Analysis

MLR and ELISA data was normalized by log transformation and analyzed with analysis of covariance (ANCOVA), with horse as covariate, followed by Tukey HSD test for multiple comparisons. All analyses were performed using JMP Pro 12 (SAS Institute Inc., Cary, NC, USA) with significance set at $p < 0.05$.

Results

MHC-haplotyping

The MHC haplotype of each horse was determined by microsatellite testing to establish which combinations of horses would be MHC-mismatches for MLRs. The MHC haplotype as characterized by microsatellite testing is reported in Table 1. The MHC haplotype of horse A did not match any previously classified equine leukocyte antigen (ELA) haplotype, but was unlike the haplotype of the other horses. Horse B was classified as an ELA-A3a homozygote, horse C as an ELA-A9a homozygote, and Horse F as an ELA-A3b homozygote. Horses D and E had identical haplotypes classified as heterozygotes with ELA-A2 and an unclassified haplotype.

Mixed leukocyte reactions

The ability of untreated and TGF-β2-treated MSCs to inhibit T cell proliferation was measured in modified one-way MLRs. MHC-mismatched MLRs were performed in the following combinations: Horse A vs. horse F, horse B vs. horse D, and horse C vs. horse E with each horse in the combination acting as a responder and MHC-mismatched stimulator in the MLRs. All results were reported as the log fold change relative to the negative control, MLR with MHC-matched (autologous) PBL stimulator cells. Fewer live, $CD3^+$ cells were consistently recovered from the MLR wells with MSC stimulator cells compared the MHC-mismatched PBL wells (Figure 1A). A small amount of baseline T cell proliferation was seen in the negative control wells by the presence of $CFSE^{low}$ $CD3^+$ cells (Figure 1B). An increased number of $CFSE^{low}$ $CD3^+$ cells were detected in the MHC-mismatched MLR wells with PBL stimulator cells, but there were fewer $CFSE^{low}$ $CD3^+$ cells in the MHC-matched and MHC-mismatched MLRs with MSC stimulator cells even compared to the negative control (Figure 1B). T cell proliferation was significantly reduced in all MSC MLRs compared to the MHC-mismatched PBL MLR as measured by both the relative division index ($p = 0.0025$, Figure 1C) and relative GMFI of proliferating cells ($p < 0.0001$, Figure 1D). T cell proliferation as measured by the relative division index and relative CFSE GMFI in all MSC MLRs was lower compared to the negative control indicating MSCs were able to inhibit even baseline T cell proliferation. There was no significant difference between the untreated or TGF-β2-treated MSC MLRs for either the relative division index or relative CFSE GMFI. These results demonstrate that untreated and TGF-β2-treated MSCs display similar abilities to inhibit proliferation in in vitro MLRs.

TGF-β1 and PGE2 analysis

As TGF-β1 and PGE2 are the major immunomodulatory cytokines secreted by equine MSCs (Carrade et al., 2012; Carrade Holt et al., 2014), the concentrations of TGF-β1 and PGE_2 in the MLR culture supernatant were measured via ELISA. There was no significant difference in the concentration of TGF-β1in the supernatant of any of the MLR treatment groups (Figure 2A). PGE2 concentrations were significantly higher in all MLRs with MSC stimulator cells than in wells with PBL stimulator cells ($p < 0.0001$) (Figure 2B). There was no significant difference between PGE2 concentrations in the supernatant of MLRs with untreated and TGF-β2-treated MSCs. These results support that untreated and TGF-β2-treated MSCs secrete similar amounts of TGF-β1 and PGE2.

Discussion

The in vivo immunogenicity of MHC-mismatched or allogeneic MSCs and in vivo rejection of the cells by the immune system limits their practicality in clinical use. Downregulation of MHC surface expression using TGF-β2 may enhance the ability of MSCs to avoid immune rejection. As the immunomodulatory properties are important for both the therapeutic function of the cells and the ability to limit allorecognition and immune rejection, it is critical that TGF-β2-treatment not impair these functions. In vitro MLRs have previously been used to analyze the immunomodulatory properties of MSCs and have shown that MSCs are potent inhibitors of T cell proliferation due to the secretion of immunosuppressive paracrine factors (Carrade et al., 2012; Rasmusson, Ringdén, Sundberg, & Le Blanc, 2005;

Schnabel et al., 2014). We previously published that untreated and TGF-β2-treated MSCs had similar TGF-β1 secretion and phenotypic marker expression and hypothesized that untreated and TGF-β2-treated MSCs would also have similar abilities to inhibit T cell proliferation.

Modified one-way MLRs with PBLs or MSCs as stimulator cells were used to measure responder T cell proliferation. ELISAs were used to measure the supernatant concentrations of PGE2 and TGF-β1 in each assay. We found that in contrast to MHC-mismatched PBL stimulator cells, MHC-mismatched untreated and TGF-β2-treated MSCs did not stimulate T cell proliferation. T cell proliferation in both MHC-matched and MHC-mismatched assays with MSC stimulator cells was less than baseline proliferation measured in the MHC-matched MLR with PBL stimulator cells. There was also no significant difference in the concentrations of PGE2 or TGF-β1 in the supernatant of MLRs with untreated and TGF-β2-treated MSC stimulator cells. Only PGE2 concentrations were significantly elevated in MLRs with MSC stimulator cells compared to PBL stimulator cell assays and the concentration of PGE2 was inversely correlated with T cell proliferation. This is consistent with previous findings that MHC-mismatched equine bone marrow-derived MSCs negative for MHC II fail to stimulate T cell proliferation in vitro (Clark et al., 2016; Schnabel et al., 2014) and that PGE2 is the primary immune suppressive factor secreted by equine bone marrow-derived MSCs (Carrade Holt et al., 2014). PGE2 is known to suppresses T cell activation and proliferation through inhibition of IL-2 production and transferrin

receptor expression (Chouaib, Welte, Mertelsmann, & Dupont, 1985) while TGF-β1 also inhibits IL-2 production (Brabletz et al., 1993).

One limitation of this study is we did not examine the source of PGE2 or TGF-β1 in the MLRs. It is likely that the stimulator MSCs and not the responder PBLs are the main source of PGE2 in modified MLRs due to the significant increase in concentrations over the MLRs with PBL stimulator cells, but treating MSCs with a PGE synthase inhibitor prior to the MLR would confirm this. It is less clear if MSCs or PBLs are the primary secretors of TGF-β1 as there is no significant difference between MLRs with PBL or and MSC stimulator cells. It is possible though that if MSCs are inhibiting T cell activation and function, there may be reduced TGF-β1 expression by responder T cells and the primary source of TGF-β1 is from the MSCs. Intracellular cytokine staining or gene expression could be used to measure TGF-β1 expression separately in responder PBLs and MSCs. Further analysis of the roles of PGE2 and TGF-β1 and other tertiary cytokines is warranted to fully understand the mechanism of in the equine MSC-induced suppression of T cell activation and proliferation.

Also of note is that fewer responder cells were recovered from MLRs with MSC stimulator cells. Naïve T cells require TCR and CD28 ligation or exogenous IL-4 and IL-7 to prevent spontaneous cell death in culture (Kishimoto & Sprent, 1999; Vella, Teague, Ihle, Kappler, & Marrack, 1997). Unlike equine PBLs, equine MSCs do not express CD86 (Clark et al., 2016) and are not known to secrete significant quantities IL-4, or IL-7 so they would be unable to stimulate or promote the survival of naïve cells. It is likely then, that there is increased death of naïve PBL responder cells in the MLRs with MSC stimulator cells leading to fewer live cells recovered for analysis.

In addition to our previous research showing that TGF-β2 treatment significantly downregulates constitutive MHC I surface expression without altering other phenotypic markers, the results of this study support that TGF-β2 treatment does not significantly alter the immunomodulatory properties of equine bone marrow-derived MSCs. Further investigation into the in vitro and in vivo immunogenicity of the TGF-β2-treated MSCs is now needed to understand if downregulation of MHC I expression is sufficient to allow MHC-mismatched MSCs to avoid immune rejection in vivo.

Acknowledgements

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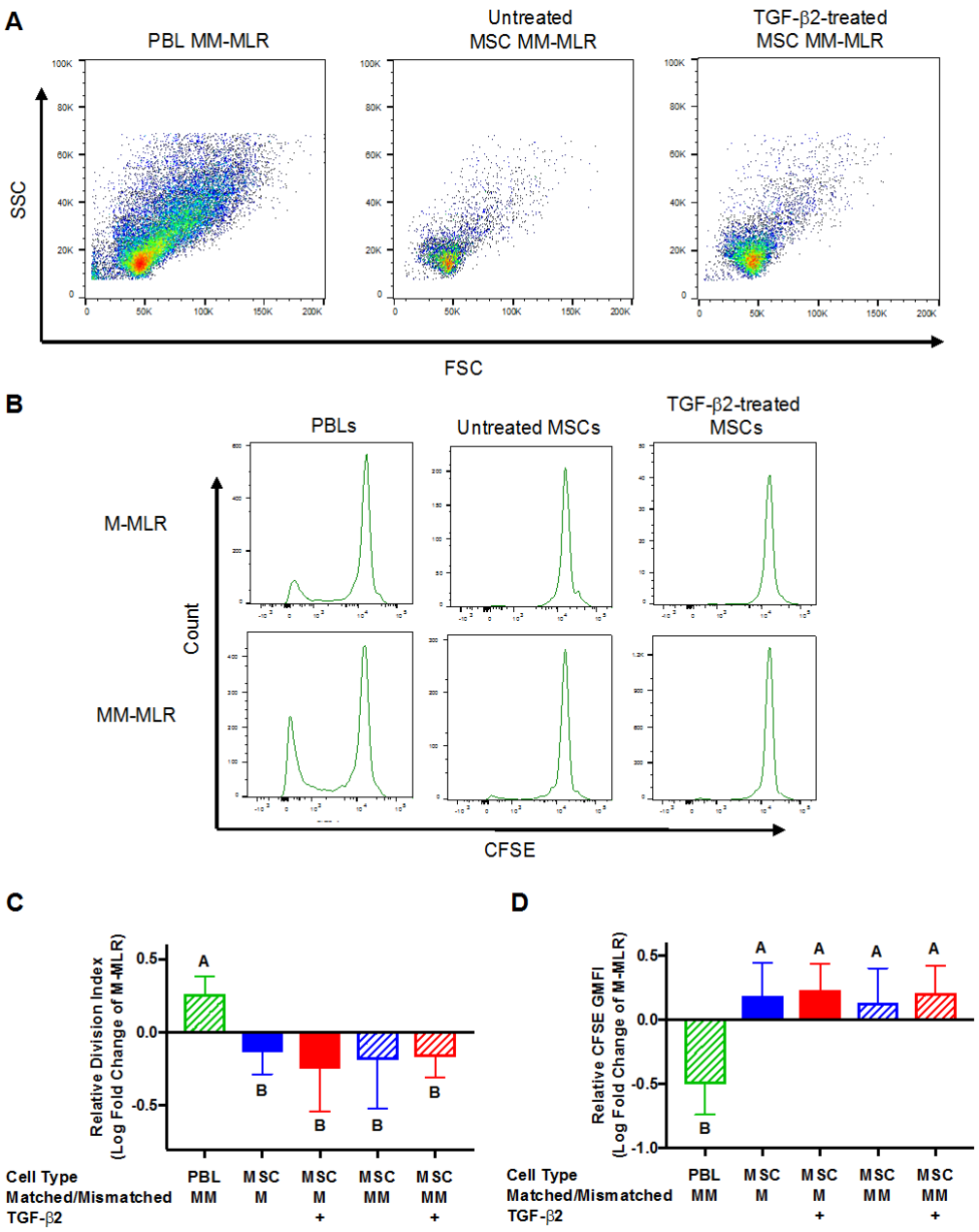
Table 1. MHC haplotypes of horses as determined by intra-MHC microsatellite testing.

		Intra-MHC microsatellite alleles										
		Class I		Class III					Class II			
Horse	Microsatellite haplotype	UMNJH-38	COR110	ABGe9019	UMNe65	ABGe9030	EQMHC1	TKY3324	COR112	COR113	UM011	COR114
A	Unclassified	156	221	299	257	206	192	258	243	270	172	249
	Unclassified	156	221	314	259	205	194	264	256	270	172	249
B	A3a	163	207	311		211		255	254	260	172	243
	A3a	163	207	311		211		255	254	260	172	243
C	A9a	156	217	307		215		Null	264	272	168	255
	A9a	156	217	307		215		Null	264	272	168	255
D	A2	156	211	301	259	209	192	269	262	268	174	234
	Unclassified	156	221	299	257	211	192	255	262	268	176	247
E	A2	156	211	301	259	209	192	269	262	268	174	234
	Unclassified	156	221	299	257	211	192	255	262	268	176	247
F	A3b	163	207	312	261	211	192	255	262	268	176	247
	A3b	163	207	312	261	211	192	255	262	268	176	247

Figure 1. Untreated and TGF-β2-treated MSCs suppress T cell proliferation.
CFSE stained responder leukocytes were cultured with MHC-matched (M-MLR) or MHC-mismatched (MM-MLR) PBLs or untreated MSCs or TGF-β2-treated MSCs for 5 days before FACS analysis. Superscript letters indicate significant differences between groups. **A.** Pseudocolor dot plots from MM-MLRs with PBL stimulator cells, untreated MSCs, and TGF-β2-treated MSCs. **B.** Histograms from one representative horse depicting CFSE attenuation in responder PBLs cultured with PBL, untreated MSC, and TGF-β2-treated MSC stimulator cells. **C.** T cell proliferation shown as the average log fold change in division index relative to the M-MLR with PBL stimulator cells. Data shown are mean +/- SD of n = 6, p = 0.0025. **D.** T cell proliferation shown as the average log fold change in CFSE GMFI relative to the M-MLR with PBL stimulator cells. Data shown are mean +/- SD of n = 6, p < 0.0001.


A
PBL MM-MLR
Untreated
MSC MM-MLR
TGF-β2-treated
MSC MM-MLR
SSC
FSC
B
PBLs
Untreated MSCs
TGF-β2-treated
MSCs
M-MLR
MM-MLR
Count
CFSE
C
Relative Division Index
(Log Fold Change of M-MLR)
Cell Type
Matched/Mismatched
TGF-β2
PBL MM
MSC M
MSC M +
MSC MM
MSC MM +
D
Relative CFSE GMFI
(Log Fold Change of M-MLR)
Cell Type
Matched/Mismatched
TGF-β2
PBL MM
MSC M
MSC M +
MSC MM
MSC MM +

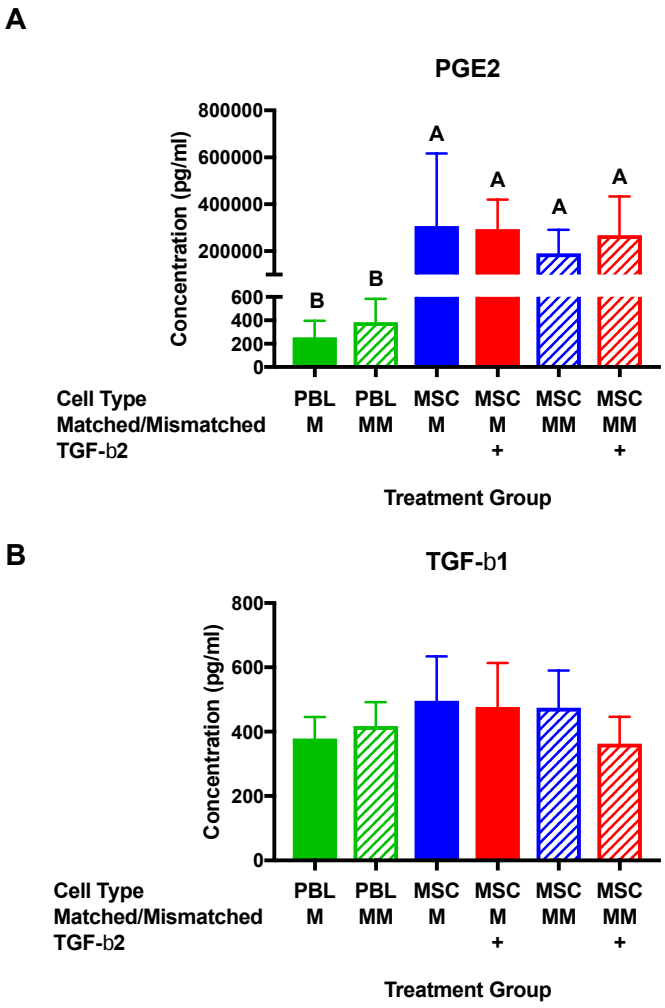


Figure 2. PGE2 and TGF-β1 are expressed in MLRs with PBL and MSC stimulator cells. Superscript letters indicate significant difference between groups. **A.** PGE2 concentrations in the supernatant of MHC-matched (M) and MHC-mismatched (MM) MLRs with PBL and untreated and TGF-β2-treated MSC stimulator cells by ELISA. Data shown are mean +/- SD of $n = 6$, $p < 0.0001$. **B.** TGF-β1 concentrations in MLR supernatant. Data shown are mean +/- SD of $n = 6$.

CHAPTER 5: Conclusions and Future Directions

The purposes of this work were to determine if MHC-mismatched mesenchymal stem cells (MSCs) could be targeted for death by the recipient immune system and if TGF-β2 treatment could downregulate MHC expression to prevent allorecognition and immune rejection. We found that anti-MHC I alloantibodies induced by MHC-mismatched MSCs in vivo were cytotoxic to MSCs in vitro. We also demonstrated that culturing MSCs with 1 ng/ml TGF-β2 significantly downregulated constitutive MHC I and MHC II surface expression and partially blocked IFN-γ-induced MHC expression. TGF-β2 treatment did not affect other phenotypic markers, cell viability, the ability of MSCs to inhibit of T cell proliferation, or secretion of TGF-β1 or PGE2. In order to fully reach the potential of allogeneic MSC therapy, further work is still needed to stabilize MHC expression on MSCs and to measure the immunogenicity of TGF-β2-treated MSCs.

TGF-β2-treated MSCs have significantly lower MHC I expression compared to untreated cells, but IFN-γ-induced MHC I and MHC II expression was only partially blocked even with continuous TGF-β2 treatment. It is currently unknown how TGF-β2 downregulates MHC I expression or why it only partially blocks IFN-γ-induced expression. If this mechanism can be characterized, it may be possible to develop a targeted strategy to completely block IFN-γ-induced MHC I and MHC II expression. Stabilizing MHC I expression and completely blocking MHC II expression may help limit allorecognition of MHC-mismatched MSCs and limit immunogenicity even in the presence of inflammation in vivo when MHC expression could be upregulated.

To determine if allogeneic TGF-β2-treated MSCs may be an option for future clinical therapies, the immunogenicity of TGF-β2-treated MSCs needs to be measured in vitro and in vivo. In vitro cytotoxicity assays, including Chromium-51 release assays, can be used to measure the ability of cytotoxic effector lymphocytes to kill TGF-β2-treated MSCs, or the lack thereof. In vivo assays are needed to determine the scope of immune responses to MHC-mismatched MSCs, if they are primarily cell-mediated or humoral responses, or if inflammation changes how the immune system responds and how quickly. While the work presented here is performed in the horse due to the horse's translational importance to regenerative musculoskeletal research, a lack of reagents and the size of horses is limiting for answering some of these mechanistic questions. We are currently in the process of developing two models to help answer these questions: an in vivo inflammatory mouse model to analyze immune responses to MHC-mismatched MSCs and a CRISPR-Cas9 system for inducing constitutive GFP expression in equine MSCs to enable in vivo tracking of cells in the horse. With these further studies and models we can move towards the development of non-immunogenic MSCs and an "off-the-shelf" method for delivering high-quality regenerative therapies to patients in an effective and cost-efficient manner.

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