Isolation, Immunophenotyping and Lymphocyte Suppressive Properties of Equine Cord Blood-Derived Mesenchymal Stromal Cells

by

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in
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ABSTRACT

Isolation, immunophenotyping and lymphocyte suppressive properties of equine cord blood-derived mesenchymal stromal cells

Laurence Tessier
University of Guelph, 2013

Advisor:
Professor T. G. Koch

Multipotent mesenchymal stromal cells (MSC) have attracted interest for their potential in allogeneic cytotherapy, partly due to their ability to secrete immnosuppressive factors. However, development of efficacious and reproducible therapies is hampered by a knowledge-gap in equine MSC characterization. I hypothesized that equine MSC can be consistently isolated from cord blood, have unique and reproducible marker expression, which includes cytoplasmic Toll-like receptor (TLR) 3 and TLR4, and in vitro suppress lymphoproliferation. I aimed to establish the immunophenotype of cord blood-derived- (CB)-MSC before and after cryopreservation and confirm their ability to suppress in vitro lymphoproliferation. Cultured CB-MSC expressed CD29, CD44, CD90, and not MHC I, MHC II, CD4, CD8, CD11a/18, and CD73 before and after cryopreservation. CB-MSC suppressed in vitro lymphoproliferation and constitutively expressed TLR4. These findings suggest anti-inflammatory properties of CB-MSC. Lack of MHC I expression suggests reduced risk of allorejection. The relationship between TLR4 and lymphocyte function requires further investigation.
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DECLARATION OF WORK PERFORMED

I declare that all work submitted for assessment of this thesis is my own work with the exception of the items indicated below.

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AGGREGAN</td>
<td>Cartilage-specific proteoglycan core protein</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>AT-MSC</td>
<td>Adipose tissue-derived MSC</td>
</tr>
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<td>B2M</td>
<td>Beta-2-microglobulin</td>
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<tr>
<td>BLAST</td>
<td>Basic local alignment search tool</td>
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<td>BM-MSC</td>
<td>Bone marrow-derived MSC</td>
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<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>CB-MSC</td>
<td>Cord blood-derived MSC</td>
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<td>CCL</td>
<td>Chemokine ligand</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
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<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony-forming-unit–fibroblast</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CIITA</td>
<td>Class II major histocompatibility complex transactivator</td>
</tr>
<tr>
<td>c-Myc</td>
<td>v-myc myelocytomatosis viral oncogene homolog</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3′-diaminobenzidine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
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<td>Full Form</td>
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<tr>
<td>HES</td>
<td>Hydroxyethyl starch</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
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<tr>
<td>HPRT1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
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<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>ICC</td>
<td>Immunocytochemistry</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine-2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL1RA</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IκB</td>
<td>Inhibitor of nuclear factor-kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of nuclear factor-kappa-light-chain-enhancer of activated B cell kinase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRAK</td>
<td>Interleukin-1 receptor-associated kinase</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MIQE</td>
<td>Minimum information for publication of quantitative real-time polymerase chain reaction experiments</td>
</tr>
<tr>
<td>MNC</td>
<td>Mononuclear cells</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSC</td>
<td>Multipotent mesenchymal stromal cell</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary-response protein 88</td>
</tr>
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<td>Abbreviation</td>
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<tr>
<td>NANOG</td>
<td>Embryonic stem cell specific homeobox protein</td>
</tr>
<tr>
<td>NCBI</td>
<td>National center for biotechnology information</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>OCT4</td>
<td>Octamer-binding transcription factor 4</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEQ</td>
<td>PrepaCyte-EQ</td>
</tr>
<tr>
<td>PGE-2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>Poly (I:C)</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PRDM14</td>
<td>PR domain zinc finger protein 14</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>SSEA</td>
<td>Stage-specific embryonic antigen</td>
</tr>
<tr>
<td>STRO-1</td>
<td>Stromal cell surface marker-1</td>
</tr>
<tr>
<td>TAB</td>
<td>Transforming growth factor- β-activated kinase -1-binding protein</td>
</tr>
<tr>
<td>TAK</td>
<td>Transforming growth factor- β-activated kinase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/interleukin-1 receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>Toll/interleukin-1 receptor domain-containing adapter protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNAIP6</td>
<td>Tumor necrosis factor-alpha-induced protein 6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TRAM</td>
<td>Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β-related adaptor molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>SAS</td>
<td>Statistical Analysis System</td>
</tr>
<tr>
<td>TRIF</td>
<td>Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>UEV1A</td>
<td>Ubiquitin-conjugating enzyme E2 variant 1</td>
</tr>
</tbody>
</table>
INTRODUCTION

Osteoarthritis (OA) and post-traumatic OA (PTOA) are some of the most common causes of equine lameness (Frisbie et al., 2005). Multipotent mesenchymal stromal cells (MSC) have been suggested as potential therapeutic candidates for joint cartilage and OA in the horse (Frisbie et al., 2009). Stem cells therapies in veterinary medicine are becoming increasingly popular for a variety of conditions (Cyranoski, 2013). However, the stem cell area is unregulated and most therapies have almost no research to justify their use, which has prompted oversight bodies in several countries to carefully follow the application of veterinary cell-based therapies (Cyranoski, 2013). Investigation on the use of stem cells in equine veterinary medicine is of particular importance as the horse industry contributes up to $19 billion to the Canadian economy every year. Horse racing by itself contributes up to $300 million in provincial and federal taxes. In Canada 963,500 horses reside on 145,000 properties (Equine Canada, 2010). Further investigation of equine stem cell therapies to ensure safe and efficacious treatments is therefore crucial.

MSC are plastic adherent multipotent cells possessing trilineage differentiation potency toward adipogenic, chondrogenic and osteogenic cell fates (Dominici et al., 2006). In addition, undifferentiated MSC are attractive cyotherapy candidates as they are capable of secreting immunosuppressive factors (Caplan et al., 2006). For this reason MSC have been suggested as potential therapeutic candidates for joint cartilage and OA in the horse (Frisbie et al., 2009). In support of this anti-inflammatory hypothesis, MSC has convincingly demonstrated immunosuppressive properties in the treatment of human graft-versus-host disease (GVHD) where the intravenous infusion of bone marrow-derived (BM) MSC is often curative of steroid refractory GVHD (Ringden et al., 2006).

No complete and consistent phenotype has been reported for equine CB-MSC with regard to antigen and gene expression. Although MSC immunosuppressive properties have attracted much interest, there is no
identified specific predictive MSC marker. MSC immunomodulation phenotype have recently been associated with TLR expression, specifically TLR3 and TLR4 (Waterman et al., 2010). However, equine CB-MSC characterization for these markers as well as their link to \textit{in vitro} lymphocyte suppression and \textit{in vivo} immunosuppression has yet to be established.

Better characterization of equine CB-MSC may allow for more reproducible and effective therapies through selection of cells with known properties. In this study, equine MSC were derived from cord blood samples in a consistent manner, and cultured over multiple passages. MSC were cryopreserved and recovered for additional phenotypic and functional studies. MSC were characterized by flow cytometric interrogation with a panel of antibodies validated for binding specifically to equine antigens. Furthermore, for each protein assessed flow cytometrically, a qPCR assay was designed and gene expression was determined. The ability of MSC to suppress allogeneic lymphoproliferation was determined, and expression of TLR3 and 4 was investigated by qPCR and ICC. Results suggest consistent phenotype and function of equine CB-MSC, and expression of TLR4 but not TLR3. These findings contribute to fundamental knowledge of equine stem cells, and will enable future therapeutic applications.
LITERATURE REVIEW

Characteristics and phenotype of multipotent mesenchymal stromal cells

The first isolation of multipotent mesenchymal stromal cells (MSC) from bone marrow was published in 1968 and involved isolation of a hematopoietic precursor population with plastic adherence properties and fibroblast-like morphology (Friedenstein et al., 1968). MSC have now been isolated from several sources such as adipose tissue (Zuk et al., 2001), synovial membrane (De Bari et al., 2001), skeletal muscle (Williams et al., 1999), dental pulp (d’Aquino et al., 2007), perinatal tissues and placenta (In ’t Anker et al., 2004), amniotic fluid (In ’t Anker et al., 2003), umbilical cord blood (Rice et al., 2001), liver, lung, spleen (In ’t Anker et al., 2003) and several other organs (Young et al., 1995).

General characteristics and phenotype

Minimal criteria to define human MSC were established by the International Society for Cellular Therapy (ISCT) in 2005 and 2006 (Dominici et al., 2006; Horwitz et al., 2005). These included multipotency, plastic adherence and surface expression of CD73, CD90 and CD105 and absence of expression of CD11b or CD14, CD45, CD34, CD79a or CD19, and human leukocyte antigen-DR (HLA-DR) (Dominici et al., 2006). Most studies regarding animal MSC have only addressed the first two criteria due to lack of antibodies with cross-reactivity to equine epitopes. No consistent surface marker panel has been established yet for animal MSC. Investigations of equine MSC suggested high expression of CD90, and low or absent expression of CD73 and CD105 (Iacono et al., 2012; Lange-Consiglio et al., 2013; De Schauwer et al., 2012; De Mattos Carvalho et al., 2009; Ranera et al., 2011; Braun et al., 2010; Ranera et al., 2012; Maia et al., 2013; Radtke et al., 2013; Pascucci et al., 2011; Raabe et al., 2011; Guest et al., 2008; Carrade et al., 2012; Lovati et al., 2011; Arnhold et al., 2007). Discrepancy was observed for CD44 and CD73 expression based on assessment of antigen or gene (Ranera et al., 2011), potentially explained by lack of cross-reactivity of antibodies, as also suggested by the authors. A
consistent surface marker phenotype for antigen and gene expression of equine MSC is still lacking. Findings on equine MSC surface marker expression published to date are summarized in Table 1.

Table 1. Studies of equine MSC surface marker expression

<table>
<thead>
<tr>
<th>Marker</th>
<th>Bone marrow</th>
<th></th>
<th>Adipose tissue</th>
<th></th>
<th>Cord blood</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface antigen expression</td>
<td>Gene expression</td>
<td>Surface antigen expression</td>
<td>Gene expression</td>
<td>Surface antigen expression</td>
<td>Gene expression</td>
</tr>
<tr>
<td>MHC I</td>
<td>++ to ++++ (14)(15)</td>
<td>+ (1)(13)</td>
<td>++ to ++++ (14)</td>
<td>+ to ++++ (14)(15)</td>
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<tr>
<td>MHC II</td>
<td>- (14)(15)</td>
<td>Variable (1)(13)</td>
<td>- (14)</td>
<td>- to -/+ (2)(14)(15)</td>
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<tr>
<td>CD13</td>
<td>+ (3)</td>
<td>+/- to + (4)</td>
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<td></td>
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</tr>
<tr>
<td>CD14</td>
<td>- (15)</td>
<td>+ (5)</td>
<td>+ ( % not specified) (5)</td>
<td>-to +/- (6)(15)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td>- (3)(7)</td>
<td></td>
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<tr>
<td>CD34</td>
<td>+/- (3)(8)(9)</td>
<td>- (3)(5)(7)(10)</td>
<td>+/- to + (3)(5)(9)</td>
<td>+ (3)(7)</td>
<td>+ (6)</td>
<td>- (13)</td>
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<td>+/- (3)(9)</td>
<td>- (3)(7)(5)</td>
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<td>-/+ to + (2)(6)</td>
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<td>CD49d</td>
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<td>CD73</td>
<td>-</td>
<td>+</td>
<td>- (%) not specified</td>
<td>+</td>
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<td>(3)(7)</td>
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<td></td>
<td></td>
<td>(11)</td>
<td>(11)</td>
<td></td>
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</tbody>
</table>

Surface antigen expression: (-): negative, (+/-): <5%, (+): 5-25%, (++): 25-50%, (+++): 50-75%, (++++): 75-100%

Gene expression: (-) transcript detected, (+) transcript not detected

References: (1) Lange-Consiglio et al., 2013; (2) De Schauwer et al., 2012; (3) Ranera et al., 2011; (4) De Mattos Carvalho et al., 2009; (5) Braun et al., 2010; (6) Iacono et al., 2012; (7) Ranera et al., 2012; (8) Maia et al., 2013; (9) Radtke et al., 2013; (10) Violini et al., 2009; (11) Pascucci et al., 2011; (12) Raabe et al., 2011; (13) Lovati et al., 2011; (14) Carrade et al., 2012; (15) Guest et al., 2008; (16) Arnhold et al. (2007).
Flow cytometry

Flow cytometry is a very powerful technique allowing single cell analysis of multiple parameters within a heterogeneous cell population. However, it is prone to artefact from non-specific antibody binding, and cells with high autofluorescence, as reported for human macrophages, may yield false positive signals (Li et al., 2012). Rigorous controls and confirmation methods should be used, particularly when looking at intra-cellular epitopes or weak fluorescence, or when using low-affinity antibodies (Li et al., 2012). Quantitative flow cytometry data should be confirmed with other qualitative or quantitative assays such as qPCR, Western blot (Li et al., 2012) or confocal microscopy (De Schauwer et al., 2012). A protocol for equine cells phenotyping incorporating antibody-binding assessment on positive control cells and equine cells by flow cytometry and confocal microscopy was recently reported (De Schauwer et al., 2012). Proper assessment is becoming increasingly necessary, in particular with quantitative multi-color flow cytometry, since manual compensation and precise population gating are major sources of variability, even when working with standardized protocols (Maecker et al., 2005).

Antibody-based characterization of equine MSC

A major challenge for equine MSC characterization is the lack of cross-reactive antibodies (Ibrahim et al., 2007), likely contributing to discrepancies across reports as a consequence of false-positive and false-negative results. Ranera et al. (2012) found gene expression with qPCR for CD44, CD73 and CD105, but no antigen expression based on flow cytometry on bone marrow (BM)- and adipose tissue-(AT)- derived MSC. The authors attributed this discrepancy to lack of antibody cross-reactivity. In addition, it is important to account for variability in reagents, such as use of directly or indirectly labelled antibodies, different epitope specificity, and variable lots of antibody, as demonstrated by Radcliffe et al. (2010). In that study, a CD45RB antibody, previously validated for equine species, yielded inconsistent flow cytometry results and had non-specific reactivity on Western blot analysis. These results were validated with gene expression.
The use of trypsin/ethylenediaminetetraacetic acid (EDTA) for cell detachment is common practice for sub-culture of adherent cells. To allow for detachment, proteolytic activity of trypsin causes the cleavage of surface proteins. This was reported to potentially affect surface expression as detected by flow cytometry (Corver et al., 1995), inducing additional false-negative results. Generalized proteome disruption was also reported following sub-culture with 0.05% trypsin-EDTA (Huang et al., 2010). Alternatives to trypsin such as StemPro® Accutase® and Accumax®, marine-origin enzymes with proteolytic and collagenolytic activity, were reported as more suitable for analysis of surface expression of CD133 on central nervous system (CNS) cells as compared to trypsin (Panchision et al., 2007; Singh et al., 2004). Accutase and Accumax were reported to be gentler than trypsin, but the exact composition of this enzyme mixture is proprietary.

Antigen versus gene expression

Ranera et al. (2012) suggested the lack of antibody-based characterization of equine MSC could be circumvented by assessing gene expression alone. Gene expression analysis is a very useful tool, but use as a proxy for protein expression with no additional validation might be inappropriate as many regulatory processes are involved in the production of a protein from the initial gene transcript. In some cases, only 40% of protein abundance was accounted for by messenger RNA (mRNA) analysis, while the remaining 60% were considered due to post-transcriptional regulatory processes (Maier et al., 2009; Vogel et al., 2013). Estimation of protein levels from mRNA expression, based on the assumption that mRNA abundance is the main determinant of protein concentration, might therefore be unreliable (Vogel et al., 2013). In addition, the importance of validating quantitative gene expression assays is still underestimated (Bar et al., 2012). This review does not cover detailed description of PCR, however inaccurate estimation of enzyme kinetics, efficiency (Bar et al., 2012; Rutledge et al., 2008) and thresholds (Ruijter et al., 2009) can profoundly affect robustness of qPCR data.
Effects of cryopreservation on MSC phenotype

Cryopreservation allows long-term storage and is a critical step as cryopreserved cells are often used as a proxy for \textit{in vivo} experiments. Cell viability and attachment are proposed criteria for indicating successful cryopreservation of human MSC (Xu et al., 2012). Loss of ability to suppress \textit{in vitro} lymphoproliferation immediately after thawing has been reported (François\textsuperscript{1} et al., 2012). Ability, however, recovered following a seven-day culture period, suggesting that modifications induced in the cryopreservation process might be reversible. Conserved CD expression pattern, morphology, differentiation abilities, alkaline phosphatase activity, telomerase activity, karyotype profile and proliferation rate, were also reported after cryopreservation (Martinello et al., 2010). A freezing rate of 1°C/min was suggested to preserve spindle shape morphology, in contrast to 5°C/min and 10°C/min freezing rates (Xu et al., 2012). Cells cryopreserved at a cooling rate of 10°C/min had a more acidic intra-cellular pH and aggregated mitochondria immediately after cryopreservation (Xu et al., 2012).

Cryo-protectants can be separated into two categories: penetrating cryo-protectants such as dimethyl sulfoxide (DMSO), and non-penetrating cryo-protectants, such as hydroxyethyl starch (HES). DMSO enters the cell removing molecules of water, while HES binds to extracellular molecules of water, creating a gradient during which intra-cellular water will leave the cell (Stolzing et al., 2012). DMSO is known to have some toxicity depending on exposure time, temperature, and concentration (Wang et al., 2007). A concentration of 10% DMSO as compared to 15% and 20% yielded maximum viability post-cryopreservation for dermal fibroblasts (Wang et al., 2007). HES used alone yielded lower viability post-thawing as compared to DMSO (Naaldijk et al., 2012). Combination of DMSO and HES up to 10% concentration resulted in reduced viability when DMSO concentrations were below 4%. Therefore, even in combination with HES, the DMSO concentration should not be less than 5% of a total of 10% cryo-protectant volume (Naaldijk et al., 2012).
Temporal phenotype

The Hayflick model describes cellular senescence as the number of cell doublings occurring before cell death by senescence (Hayflick, 1965). This applies to MSC similarly as to other somatic cells. However, specific mechanisms involved are still poorly understood. In vitro expansion of MSC to increase cell number is common practice, inducing dynamic changes with time and cell doubling number (Wagner et al., 2008), ultimately leading to cellular senescence (Hayflick, 1965). Phenotypic drift over time in vitro was reported (Wagner et al., 2008; Otte et al., 2013; Bonab et al., 2006; Vacanti et al., 2005). Radcliffe et al. (2010) reported that adherent equine bone marrow-derived MSC after 2 hour in culture were CD44 hi, CD29 hi, CD90 lo, CD11a/CD18 hi, and CD45RB lo. Over time, phenotypic drift was noted and at day 14 the cells were CD44 hi, CD29 hi, and CD90 hi, CD11a/CD18 neg, and CD45RB neg. While CD29 and CD44 expression remained homogeneous, CD90 varied in expression and heterogeneity. This is potentially explained by MSC up-regulation of CD90 or selection for CD90+ cells in culture. MSC were also suggested to undergo maturation once plated onto plastic with up-regulation of several markers (Radcliffe et al., 2010). Further cell expansion was not associated with phenotypic changes.

Influence of donor age: impact on adult tissue-derived MSC characteristics

Specifically, aging characteristics of adult tissue-derived MSC, when isolated from donors of different ages, are not well characterized. Morphological changes were reported after long-term cultures reaching the maximal lifespan (Stenderup et al., 2003; Baxter et al., 2004) but not for shorter term cultures (Lund et al., 2010). No differences were observed after 21-28 days of hypoxic culture regarding differentiation potential, surface marker expression, ability to handle oxidative stress, migratory ability, telomere length, expression of inducible nitric oxide synthase (iNOS) and production of prostaglandin-E2 (PGE-2) (Lund et al., 2010). When assessed immediately following isolation, or after one passage in vitro, no differences were reported relative to donor age for expression of octamer-binding transcription factor 4 (OCT4), PR domain zinc finger protein 14 (PRDM14) and embryonic stem cell specific homeobox
protein (NANOG), or for differentiation abilities of BM-MSC, suggesting similar differentiation potency. Similarly, in a mouse model, expression of pluripotency markers such as v-myc myelocytomatosis viral oncogene homolog (c-Myc) and NANOG was observed for every donor independently of age (Katsara et al., 2011). Doubling time and colony-forming unit-fibroblast (CFU-F) were, however, reported to vary significantly according to donor age (Siegel et al., 2013). Molecular changes related to donor age remain to be characterized. Attempts to establish a gene expression profile for aging in MSC found no correlation in association with cellular senescence (Alves et al., 2012). Changes following in vitro expansion will require further characterization.

Embryonic, perinatal and adult sources

MSC variability according to cell source is incompletely characterized. First-trimester fetal MSC from blood, liver, and bone marrow were found to express pluripotency markers such as OCT4, NANOG, and stage-specific embryonic antigen (SSEA)-3 and -4 in contrast to adult bone marrow MSC (Guillot et al., 2007). Additionally, fetal MSC demonstrated more rapid growth, longer telomeres, higher telomerase activity and myogenic differentiation ability (Chan et al., 2006). Rhesus monkey fetal-derived MSC demonstrated higher trilineage potential (adipo-, chondro-, and osteo-ogenesis) and higher growth rates (Lee et al., 2006). Perinatal tissues were also suggested to contain cell populations expressing embryonic markers as reported in human cord blood (Baal et al., 2004; McGuckin et al., 2005). In terms of immunoreactivity, fetal tissue-derived MSC were reported to have greater ability to suppress lymphoproliferation in vitro as compared to adult-derived MSC (Chan et al., 2012). Contrasting results have been reported in horses (Koch et al., unpublished results; Carrade et al., 2012; Yoo et al., 2009).

MSC immunomodulatory properties

MSC are receiving intense investigation because of their possible immunomodulatory properties. MSC are hypothesized to act in two different ways once injected or grafted in vivo. They may directly
integrate into functional tissue or secrete trophic and immunomodulating factors which lead to tissue regeneration (Caplan et al., 2006). The publications concerning the cytokine secretory profile of BM-MSC by Haynesworth et al. (1996) and one of the first reports on the ability of MSC to suppress lymphocyte proliferation \emph{in vitro} in 2002 by Di Nicola et al. laid the basis for use of MSC in various conditions.

\textbf{Selected examples of \textit{in vivo} MSC immunomodulatory effects}

\textbf{Osteoarthritis}

Osteoarthritis (OA) is a multifactorial condition that often is subdivided into idiopathic OA or degenerative joint disease (in older individuals), post-traumatic OA (occurring secondary to a joint injury) and rheumatoid arthritis (Anderson et al., 2011; Nesic et al., 2006; Carrington et al., 2006; Sacitharan, 2012). Inflammation or inflammatory cytokines such as of IL-1β and tumor necrosis factor-alpha (TNF-α) (Kapoor et al., 2011; Lawrence et al., 2011) are central agents in the pathogenesis of idiopathic OA as well as PTOA. Corticosteroids as OA treatments have been reported as beneficial (Leung et al., 2011) suggesting inflammation as target for treatment and evidences for therapeutic targeting of IL-1β and TNF-α for idiopathic OA and PTOA are accumulating (Pelletier et al., 1997; Kobayashi et al., 2005; Westacott et al., 2000). In a dog study, treatment of OA with AT-MSC improved lameness (Black et al., 2007). In horses, OA arthroscopically treated with BM-MSC had decreased concentration of PGE-2 in synovial fluid as compared to the control group. However, no other significant improvement was noted (Frisbie et al., 2009). In another study of horses, the effect of autologous BM-MSC together with micro-fracture or microfracture along was evaluated in arthroscopically created joint defects (McIlwraith et al., 2011). Increased accumulation of cartilage-associated proteoglycan core protein (AGGRECAN) (Kiani et al., 2002) and greater cartilage firmness were observed in the joint treated with BM-MSC (McIlwraith et al., 2011). A recent pilot study looked at the effect of intra-articular injection of autologous BM-MSC in 12 human patients with radiologic
evidence of OA (Orozco et al., 2013). The authors reported reduced pain and a decrease in the percentage of poor cartilage for 11 out of 12 patients after 12 months of follow-up (Orozco et al., 2013). These findings support continued investigations into the overall therapeutic effects, dosage and route of administration of MSC for the treatment of OA.

Graft-versus-host disease

The first clinical trial using autologous MSC *in vivo* was reported in 1995 by Lazarus et al. (1995) and the first report on the use of third party haploidentical MSC for treatment of graft-versus-host disease (GVHD) was report in 2004 by Le Blanc et al. In the latter study, a 9 year old boy with severe treatment-resistant grade IV acute GVHD of the gut and liver received MSC. The striking benefit from MSC treatment suggested high immunosuppressive potency *in vivo*, and established MSC as potential treatment for GVHD (Le Blanc et al., 2004). This finding also introduced the use of MSC to treat steroid-resistant GVHD, and several successful attempts have been reported so far (reviewed in McGuirk et al., 2011). As this treatment has become more used, observation of failed treatment and differential response according to different patient cohorts has been reported (Galipeau, 2013). Even though most reports used BM-MSC, adipose tissue-derived MSC were also applied with similar results. As well, similar results have been reported for fresh and cryopreserved MSC (McGuirk et al., 2011). As discussed earlier, perinatal sources offer great advantages, but more studies are required regarding their use *in vivo*.

MSC offer promising alternatives for treatment of immune-related diseases. However, incomplete characterization in humans and animal models remains a big problem for application. MSC are heterogeneous and characterized as a cell population rather than as individual cells, meaning that the characteristics applied to the population cannot be assumed to apply to each individual cell. Differences in cell source, donor species and culture conditions also contribute to variation.
**In vitro MSC properties**

MSC secreted soluble factors

MSC secrete a broad range of soluble factors such as hepatocyte growth factor (HGF), PGE-2, indoleamine-2,3-dioxygenase (IDO), nitric oxide (NO), heme oxygenase-1 (HO-1), IL-10, IL-6, HLA-G5, leukemia inhibitory factor (LIF) and several chemokines such as chemokine ligands (CCL) 2 and 5 (reviewed in Bassi et al., 2012). These factors act on several leukocyte subsets to promote and/or suppress inflammation. A summary of current knowledge on immunosuppressive cytokines is shown in Figure 1.

Figure 1. Immunomodulatory effects of MSC secreted soluble factors on leukocyte subsets. (Bassi et al., 2012).
MSC effect on different leukocyte subsets

Lymphocytes

Subsets of lymphocytes affected by MSC include CD8+ cytotoxic T-lymphocytes (CTL) and CD4+ helper T-cells (Th), B lymphocytes, regulatory T-cells (Treg), gamma delta T-cells and natural killer (NK) cells (Wang et al., 2012). The ability of MSC to suppress lymphocyte proliferation in vitro has been observed consistently (Le Blanc et al., 2007; Ripoll et al., 2011; Le Blanc et al., 2003; Ennis et al., 2008), and is thought to involve soluble factors such as HGF, IDO, transforming growth factor (TGF)-β, PGE-2, IL-10, and NO (Kim et al., 2013; Ringden et al., 2011).

T-cells, B-cells, Tregs

Th lymphocyte phenotypes can be divided in two major groups: Th1 pro-inflammatory phenotype and Th2 anti-inflammatory phenotype with their respective secretion of pro-inflammatory and anti-inflammatory cytokines (Berger, 2000). MSC are suggested to induce differentiation of naïve T cells into a Th2 rather than Th1 phenotype, as demonstrated by Aggarwal et al. (2005). When co-cultured with MSC, significant increase in IL-4 secretion along with significant decrease of IFN-γ was observed (Aggarwal et al., 2005) suggesting a T-cell phenotypic switch from Th1 to Th2 (Mosmann et al., 1989). MSC were also reported to inhibit lymphocyte differentiation into a CD8+ cytotoxic phenotype (Rasmusson et al., 2003) to induce T cell differentiation into Treg as demonstrated by Maccario et al. (2005) and Aggarwal et al. (2005). Treg are a distinct CD4+ T-cell subset, expressing IL-2 receptor α-chain (CD25) and promoting immunosuppression. MSC also affect Th17 T-cells characterized by IL-17 cytokine secretion (Ghannam et al., 2012). IL-17 was suggested to play a major role in auto-immune diseases while its deficiency reduced the incidence of joint inflammation and bone erosion in experimental arthritis, and destructive synovitis (Koender et al., 2005; Lubberts et al., 2005). MSC inhibited T-cell differentiation into Th17 phenotype in vitro, but also inhibited pro-inflammatory
cytokine production of differentiated Th17 T-cells when co-cultured, as demonstrated by down-regulation of IL-17 and IL-22 along with up-regulation of IL-10 (Ghannam et al., 2010).

MSC arrest B lymphocytes in the G0/G1 phase of the cell cycle (Tabera et al., 2008). This was supported by the observation that co-culture with MSC did not decrease B cell viability but down-regulated expression of the proliferation marker Ki67. Proliferation inhibition, increased immunoglobulin production and increased chemokine receptor expression were also observed in a transwell system, suggesting soluble factor-mediated interactions (Corcione et al., 2006).

**Natural killer cells**

The immune system is thought to have evolved around the idea of recognition of “self” as opposed to “non-self” with associated immune response. The main molecules involved in this mechanism are the MHC I and II molecules (reviewed in Yin et al., 2012). Cells that do not express surface MHC I were expected to be ignored by the immune system, even in an allogeneic context. This concept was challenged regarding NK cell interactions. NK cells are effector lymphocytes of the innate immune system expressing a broad range of surface markers that control activation, function and proliferation (reviewed in Lanier et al., 2009). The “Missing Self” theory proposed by Ljunggren et al. (1990) suggested that NK cells protect the organism by lysing cells lacking MHC I expression as they are not identified as part of self. Cells with low or absent MHC I expression are then more likely to be targeted for NK-mediated cell lysis. MHC I expression is typical of MSC. Interestingly, an in vitro study showed that interferon-gamma (IFN-γ) stimulation of MSC spared them from NK-mediated cell lysis through increased MHC I expression (Spaggiari et al., 2006). Further studies are required to understand the interactions between MSC and NK cells in vivo and the potential consequences.

**Dendritic cells**
Dendritic cells (DCs) are derived from CD34+ hematopoietic progenitor cells (Frelinger et al., 1979) and act as antigen presenting cells (APC). They play a crucial role in antigen specific T-cell activation (reviewed by Villadangos et al., 2005 and Spaggiari et al., 2013). MSC interactions with DC result in indirect suppression of T-cells. The mechanisms of suppression of DCs by MSC are controversial, but it is thought to involve suppression of precursor maturation, impairment of antigen presenting functions and decrease of inflammatory abilities.

**MSC polarization paradigm**

In 2006, a study of human macrophages suggested that there are also two distinct phenotypes: pro-inflammatory and anti-inflammatory (Verreck et al., 2006). MSC have been widely recognized as demonstrating an immunosuppressive behavior when exposed to inflammatory environment, but the idea of a single phenotype was recently challenged by a study which suggested that MSC, similarly to macrophages, might be classified into a MSC-1-like phenotype with pro-inflammatory features, and a MSC-2-like phenotype with anti-inflammatory properties (Waterman et al., 2010). TLR3 and TLR4 were suggested to characterize MSC-1 pro-inflammatory and MSC-2 anti-inflammatory phenotypes, respectively, with each phenotype demonstrating specific cytokine secretion patterns.

**Toll-like receptors**

TLR are type I transmembrane proteins that play a major role in immunity by recognition of pathogen-associated molecular patterns (PAMPs) through ectodomains containing leucine-rich repeats (Kawai et al., 2010). PAMPs include microbe-derived lipids, lipoproteins, proteins and nucleic acids (Kawai et al., 2010; Akira et al., 2006).
Each TLR has been associated with a specific ligand (Figure 2). TLR2 is known to recognize lipoproteins or lipopeptides when forming heterodimers with TLR1 or TLR6. TLR3 recognizes viral double-stranded RNA, TLR4 recognizes lipopolysaccharide (LPS) and TLR5 recognizes flagellin. TLR7 and TLR8 recognize single stranded RNA, and TLR9 recognizes microbial DNA (reviewed in Uematsu et al., 2008). While TLR1, 2, 4, 5, 6 and 11 are expressed on the cell surface, TLR3, 7, 8 and 9 are intracellular and bind mostly to nucleic acids (Figure 3, Kawai et al., 2010; Akira et al., 2006; Takeda et al., 2005).

Once bound to a ligand, TLRs dimerize and recruit adaptor proteins, which activate downstream signalling. There are several intra-cellular adaptor proteins. The best known are myeloid differentiation primary-response protein 88 (MyD88), Toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM) and the TIR domain-containing adapter protein (TIRAP). MyD88 was the first identified adaptor protein and is involved in signalling for 9 of 10 TLRs. MyD88 signalling (Figure 3) activates the well characterized nuclear factor kappa-light-chain-
enhancer of activated B cells (NF-κB) pathway. MyD88 primarily binds to the intracellular domain of TLRs, triggering downstream molecule signalling, leading to NF-κB translocation to the nucleus where it acts as a transcription factor. The MyD88-NF-kB signalling pathway is further summarized in Figure 3 (reviewed by Akira et al., 2004).

Figure 3. Human TLR signalling. (1) Stimulated TLR bind to MyD88 adaptor protein. MyD88 then recruits IL-1R-associated kinase-(IRAK)-4, creating a complex with IRAK1 and tumor-necrosis-factor receptor-associated factor-(TRAF)-6. (2) Formation of a protein complex with IRAK1, TRAF6, transforming growth factor-β-activated kinase-(TAK)-1, TAK1-binding protein (TAB)-1 and TAB2. IRAK1 is subsequently degraded and the complex associates with the ubiquitin ligases ubiquitin conjugating enzyme-(UBC)-13 and ubiquitin-conjugating enzyme E2 variant 1 (UEV1A). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. (3) TAK1 then phosphorylates the
mitogen-activated protein (MAP) kinases (4) and the inhibitor of nuclear factor-κB (IκB)-kinase (IKK) complex. (5) IKK complexe then phosphorylates IκB leading to its degradation, (6) allowing the NF-κB to translocate to the nucleus and act as a transcription factor (Modified from: Akira et al., 2004).

**MyD88-dependent and independent pathways**

MyD88 was first identified as an adaptor protein in TLR signalling pathways (Wesche et al., 1997) and suggested as mandatory for activation of NF-κB (Adachi et al., 1998). However, this was later proven incorrect as MyD88-deficient mice were still responsive to LPS (Kaisho et al., 2001) and Polynosinic:polycytidylic acid (poly [I:C]) (Alexopoulou et al., 2001). An alternative pathway, known as MyD88-independent pathway, exists for TLR3 and TLR4 and involves TRIF and TRAM adaptor proteins (Figure 4). In 2001, a study with MyD88-deficient mice demonstrated that LPS-induced IFN regulatory factor (IRF)-3 and IFN-inducible genes in a MyD88-independent manner, which was abrogated when inactivating TRIF (Kawai et al., 2001). This suggested signalling of TLR4 through two specific pathways: MyD88-dependent and independent pathways, the latter being also TRIF-dependent (Figure 4). MyD88-independent pathway downstream signalling is summarized in Figure 4 for TLR3 and TLR4.
Figure 4. TLR4 (A) and TLR3 (B) intra-cellular signalling. Stimulation of TLR4 leads to activation of two pathways: 1) MyD88-dependant pathway leading to inflammatory cytokines activation through NF-κB early phase response, and 2) MyD88-independent pathway leading to IFN-β production and IFN-inducible gene expression through NF-κB late phase response. (Akira et al., 2004).

**TLR expression**

MSC from different sources were reported to express different TLRs at gene and antigen levels, and expression of these TLRs was suggested to be correlated with their immunomodulatory properties, particularly TLR3 and TLR4 (Waterman et al., 2010). TLRs expression in human cells is summarized in Table 2.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Bone marrow Antigen</th>
<th>Gene expression</th>
<th>Adipose tissue Antigen</th>
<th>Gene expression</th>
<th>Cord blood Antigen</th>
<th>Gene expression</th>
<th>Wharton’s jelly Antigen</th>
<th>Gene expression</th>
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<tr>
<td>TLR1</td>
<td>+</td>
<td>(1) (2) (5)(6)(8)</td>
<td>+</td>
<td>(5) (8)</td>
<td>+</td>
<td>(9)</td>
<td>+</td>
<td>(8)</td>
</tr>
<tr>
<td>TLR2</td>
<td>+</td>
<td>(1) (6)</td>
<td>+</td>
<td>(5) (8)</td>
<td>-</td>
<td>(9)</td>
<td>+</td>
<td>(8)</td>
</tr>
<tr>
<td>TLR3</td>
<td>+</td>
<td>(1) (6) (8)</td>
<td>+</td>
<td>(5) (8)</td>
<td>+</td>
<td>(9)</td>
<td>+</td>
<td>(8)</td>
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<tr>
<td>TLR4</td>
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<td>+</td>
<td>(9)</td>
<td>+</td>
<td>(8)</td>
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<tr>
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<td>(9)</td>
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<td>(8)</td>
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<tr>
<td>TLR6</td>
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*Table 2. Findings on TLR expression in human MSC.*
## Antigen expression level: 
- (·) = antigen expression not detected, (+) = antigen detected.

## mRNA expression level: 
- (·) = transcript not detected, (+) = transcript detected.

### References

#### MSC pro-inflammatory and immunosuppressive phenotypes

Pro-inflammatory (MSC1-like) and immunosuppressive (MSC2-like) phenotypes are thought to be correlated respectively with TLR4 and TLR3 expression (Figure 5).
Figure 5. MSC polarization paradigm. MSC are suggested to present two distinct immunomodulatory phenotypes: pro-inflammatory (MSC1) driven by TLR4 expression and primed in vitro by LPS, and immunosuppressive (MSC2) driven by TLR3 expression and primed in vitro by poly(I:C). (Modified from Bunnell et al., 2010).

This hypothesis was further supported by findings of Waterman et al. (2010) where poly (I:C)-treated cells demonstrated higher ability to suppress \textit{in vitro} lymphoproliferation as compared to untreated and LPS-treated cells. Similarly, LPS-treated cells had reduced ability to suppress \textit{in vitro} proliferation as compared to untreated and poly (I:C) treated cells.

In a recent review it was attempted to classify cytokines expressed by MSC according to their immunosuppressive or pro-inflammatory role: pro-inflammatory cytokines included IL-1\textbeta, IL-6, IL-8, IL-12, type I IFNs and TNF-\textalpha while immunosuppressive cytokines included IDO, iNOS, PGE-2, TGF-\textbeta1, HLA-G, HGF, LIF, IL-1 receptor antagonist (IL1RA), CCL2, galectin-3, galectin-1 and semaphorin-3A and TNF-\textalpha-induced protein 6 (TNAIP6) (Bunnell et al., 2010).

In an attempt to characterize each phenotype according to secretion pattern, Waterman et al (2010) used LPS and poly (I:C) to prime TLR4 and TLR3 expression, respectively. As a result, “TLR3-primed cells” had increased gene expression of chemokine (C-X-C motif) ligand-(CXCL)-10, CCL5, and IL10 as compared to untreated and LPS-treated cells. This is in accordance with another study where MSC treatment with poly (I:C) caused up-regulation of gene expression for CCL2 and CXCL10, but also of IL-6, IL-8 and IFN1\textbeta (Tomchuck et al., 2008). In that study levels of IDO and PGE-2 were more elevated in TLR3-primed cells than TLR4-primed cells. Tomchuck al. (2010) reported that LPS stimulation to induce TLR4 expression up-regulated gene expression of CXCL10 (IP10), IL-6, IL-8, IFN1\textbeta and NF-\textkappaB. Cytokine antibody arrays were subsequently used to test for presence of proteins, which detected the up-regulation of CXCL10 (IP10), IL-6, IL-8 and TNF-\textalpha.
There are discrepancies between studies regarding TLR expression patterns, which may, in part, be due to inconsistent validation of the methods applied. One of the first reports to thoroughly characterize MSC for TLR expression, and the effect of specific ligand binding, was published in 2008 (Tomchuck et al., 2008). One finding was the inhibited migration following anti-TLR3 neutralizing antibody treatment. This suggested involvement of TLR3 in MSC effector functions, as *in vivo* danger signals are a known trigger of human MSC migration, invasion, and engraftment into damaged and inflamed tissues (Tomchuck et al., 2008). However, this report also illustrated the complexity of TLR expression. Authors reported up-regulation of several TLRs and downstream pathway molecules following incubation with individual ligand, including LPS and poly (I:C), as assessed by qPCR, as well as a dynamic pattern of protein localization on immunocytochemical analysis. Donor-donor variation, referring to variation between individual donors, in response to TLR ligands was also reported (Tomchuck et al., 2008).

Based on Tomchuck et al. (2008), Waterman et al. (2010) attempted to characterize two distinct MSC phenotypes following incubation with poly (I:C) and LPS as ligands for TLR3 and 4 respectively. The two cell phenotypes, designed as “TLR3-primed” and “TLR4-primed”, were suggested to respectively demonstrate immunosuppressive and pro-inflammatory abilities along with a specific pattern of cytokine expression. The authors based their induction protocol on the Tomchuck et al. (2008) study, with no further validation of TLR gene or protein expression, despite reported donor-donor variation. However, to validate the findings, the study included dominant-negative TLR3 and 4 expression vectors (Waterman et al., 2010). Therefore, changes regarding CXCL10, CCL5, and IL10 following poly (I:C) treatment could be attributed to TLR3 (Waterman et al., 2010), which further supported the role of TLR3 in immunosuppression by MSC.

However, considering the complex changes occurring following incubation with ligands, it seems incorrect to attribute any change of function to a specific TLR, unless it is specifically inhibited. In
addition, designating each group as “TLR3-primed” and “TLR4-primed” when TLRs expression was not determined seems inappropriate. Mastri et al. (2012) reported that the activation of TLR3 resulted in enhanced cardiac repair. Different concentrations of poly (I:C) at different time points were used, but a single “representative” qPCR result for TLR3 expression was shown due to presumed similar expression throughout the experiment. Such consistent expression would be surprising considering the reported transient changes following poly (I:C) incubation (Tomchuck et al., 2008) as well as donor-related variation (Tomchuck et al., 2008; Waterman et al., 2010). Furthermore, validation of other TLRs was not performed. It therefore seems erroneous to attribute the observed functions to TLR3 without further exploratory experiments such as TLR3-knock-down, and similarly evaluating the role of other TLRs. Hence, available data on MSC immunomodulatory properties should be carefully analyzed to avoid misinterpretation.
RATIONAL

Osteoarthritis (OA) and post-traumatic OA (PTOA) are some of the most common causes of equine lameness (Frisbie et al., 2005). Undifferentiated MSC are attractive cytotherapy candidates as they are capable of secreting immunosuppressive factors (Caplan et al., 2006). For this reason MSC have been suggested as potential therapeutic candidates for OA in the horse (Frisbie et al., 2009). Advantages of MSC from cord blood include a non-invasive collection method, high proliferation potential and differentiation potency, and long-term cryogenic storage (Kern et al., 2006; Kogler et al., 2006; Koch et al., 2007). Previous inconsistent MSC isolation success from cord blood caused bone marrow and adipose tissue to be the favoured equine MSC sources. However, an improved CB-MSC protocol was recently described reporting 100% isolation success for 5 samples (Koch et al., 2009). No consistent surface marker panel has been established for equine MSC. Better MSC characterization may allow for more reproducible and effective therapies. Suppressed lymphoproliferation by MSC in vitro is thought to occur through modulation of T-cell, B-cell, dendritic cell, and NK cell (Le Blanc et al., 2005) function by secretion of immunosuppressive factors and cell-to-cell contact (Caplan et al., 2006; Caplan, 2009; Ghannam et al., 2008). It was recently suggested that the human MSC anti-inflammatory phenotype might be correlated with expression of TLR3 and the pro-inflammatory phenotype with TLR4 (Waterman et al., 2010).

HYPOTHESIS

Equine MSC can be consistently isolated from cord blood, have unique and reproducible marker expression, which includes cytoplasmic TLR3 and TLR4, and in vitro suppress lymphoproliferation.

OBJECTIVES

1) To establish the immunophenotype of CB-MSC before and after cryopreservation.
2) To determine the in vitro effect on lymphoproliferation of CB-MSC.
3) To determine TLR3 and TLR4 expression of CB-MSC.
MATERIALS AND METHODS

Experimental design

Figure 1. Experimental design. CB-MSC cultures were established from nine individual samples and analyzed for phenotype, gene expression and functional properties as outlined.

Cord-blood collection and shipping

Umbilical cord blood was collected from nine foals immediately after foaling, as previously described (Koch et al., 2007). Venipuncture of the umbilical vein was performed with a 16G hypodermic needle attached to a 450 mL blood transfusion collection bag (Fenwal, Baxter, Deerfield, IL) containing citrate phosphate dextrose adenine as the anticoagulant solution. The blood was stored and transported overnight by courier at 4-8°C until processed. A Greenbox system (Greenbox system Ltd., ThermoSafe
Brands; Arlington Heights, VA) was used for shipping and efficient temperature control for up to 48 hours.

**CB-MSC isolation and culture**

CB-MSC isolation and culture methods were adapted from a previously described protocol (Koch et al., 2009). In brief, the NC fraction was isolated using PrepaCyte-EQ (PEQ) medium (BioE Inc., St Paul, MN). Whole cord-blood was mixed 1:1 with PEQ medium in 50 mL BD Falcon™ conical tubes (BD Biosciences, Mississauga, ON), mixed for 5 minutes and incubated for 25 minutes at room temperature (RT). Supernatant was collected, pooled and spun at 400g for 10 minutes at RT. Pellets were suspended in isolation medium (IM) consisting of Dulbecco’s modified eagle medium (DMEM)-low glucose (1 g/L; Lonza, Wakersville, MD), fetal bovine serum (FBS, 30%; Invitrogen, Burlington, ON), penicillin (100 IU/mL; Invitrogen), streptomycin (0.1 mg/mL; Invitrogen), L-glutamine (2 mM; Sigma-Aldrich, St. Louis, MO) and dexamethasone (10^{-7}M; Sigma-Aldrich) and incubated at 5% CO₂ at 38°C in a humidified atmosphere as previously described (Koch et al., 2009). Live and dead cell counts were determined using an automated cell counter (NucleoCounter NC-100, Mandel Scientific, Guelph, ON) and plated at 1x10^6 live cells/cm² in 75 cm² polystyrene cell culture flasks (Corning® Flask, Sigma-Aldrich). The primary colonies were detached using trypsin/ EDTA (0.04%/0.03%; Sigma-Aldrich) and hereafter expanded in the same culture medium without dexamethasone (expansion medium, EM). During expansion, cells were seeded at 5,000/cm². Cells were cryopreserved in EM with 10% DMSO (Sigma-Aldrich) using a slow cooling device (Invitrogen) for controlled rate freezing at -80°C for 24 hours, prior to long-term storage in liquid nitrogen. Cell concentration was 1 to 2x10^6/mL.
Colony counting, MSC progenitor frequency, population doubling time and cell morphology

After eight days of incubation, cultures were inspected daily for presence of colonies. Confluent colonies were detached using trypsin/EDTA (0.04%/0.03%; Sigma-Aldrich) and seeded in one T175 culture flask. To document cell morphology, digital images were obtained prior to detachment at passage 1, 3, 4 and post-thawing passage 5 using phase-contrast microscopy and Q-Capture software (Q-Imaging, Surrey, BC). MSC progenitor frequency (PF) was calculated as:

\[
\text{Progenitor frequency (PF)} = \frac{\text{Colony number}}{\text{Number of initial nucleated cells}} \times 100
\]

Population doubling times were calculated according to:

1) Cell-doubling number (CD) = \frac{\ln(N_f/N_i)}{\ln(2)}

\[\begin{align*}
\ln & = \text{natural logarithm} \\
N_f & = \text{final cell count} \\
N_i & = \text{initial cell count}
\end{align*}\]

2) Cell-doubling time (DT) = \frac{\text{CT}}{\text{CD}}

\[\begin{align*}
\text{CT} & = \text{cell culture time}
\end{align*}\]

Flow cytometric analysis

Leukocytes (NC fraction) and CB-MSC cultures at passage 2, 3 and 5 were analyzed with flow cytometry in a FACScan flow cytometer (Becton Dickinson, Mississauga, ON) for the following antigens: CD4 (clone: CVS4, abdSerotec, Raleigh, NC), CD8 (clone: HTI4A, abdSerotec), CD11a/18 (clone: 116.2D11B10, abdSerotec), CD24 (clone: 4B4-Fluorescein isothiocyanate (FITC), Beckman Coulter Canada, LP, Mississauga, ON), CD44 (clone: CVS18, abdSerotec, Raleigh, NC), CD45 clone: DH16A; VMRD), CD73 (clone: 10f1; Abcam), CD90 (clone: DH24A; VMRD, Pullman, WA), MHC I (clone: 117.1B12C11, abdSerotec) and MHC II (clone: 130.8E8C4, abdSerotec). Incubations were at 4°C in the dark for 15 minutes, followed by a wash and secondary antibody incubation at 4°C for 15 minutes in the dark. Rat anti-mouse IgM-FITC or goat anti-mouse IgG1-FITC (both Abcam, Toronto,
ON) for CD90 or remaining unconjugated primary antibodies respectively were used as secondary antibodies. Prior to staining, ammonium chloride (Sigma-Aldrich) red blood cell lysis was performed on peripheral and cord blood for leukocyte isolation followed by a wash with flow buffer (phosphate buffer saline (PBS; Sigma Aldrich), 5mM EDTA, 1% horse serum (HS), and 0.1% sodium azide. Similarly, prior to staining, cultured CB-MSC were chemically detached with Accumax (STEMCELL Technologies Inc., Vancouver, BC) and washed with flow buffer (PBS, 5mM EDTA, 1% HS, and 0.1% sodium azide). Leukocytes and MSC at passage 2, 3 and 5 were stained for CD4, CD8, CD11a/18, CD45, CD90, MHC I and MHC II surface expression. MSC at passage 5 were additionally assessed for CD24, CD44 and CD73. Cells incubated with only secondary antibodies were included in each experiment. Cells incubated with isotype-matched non-binding primary antibody and fluorescent secondary antibody was included in each experiment for IgM and at passage 2, 3 and 5 for IgG isotype. CD45 and MHC II were respectively used as IgM and IgG isotype-matched non-binding primary antibody. A minimum of 10,000 events were acquired for each antibody with CellQuest software (Becton Dickinson) and data were analyzed with FlowJo software (Tree Star Inc., Ashland, OR, USA). Gates to identify leukocytes or MSC populations were maintained consistent throughout all experiments.

Monoclonal antibody for CD73, previously suggested to cross-react with equine cells (De Schauwer et al., 2012), was initially tested by flow cytometry using human leukocytes as positive control. This was followed by analysis of equine leukocytes. Samples from humans were collected under University of Guelph Research Ethics Board (REB; protocol #12FE008). All samples collected from horses were approved by the University of Guelph Animal Care Committee (protocol #11R034).

Real-time polymerase chain reaction relative quantification

Table 1. Nucleotide sequence of primers.
<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4</td>
<td>5′ CCAGACTGACCAGACTGCAA 3′</td>
</tr>
<tr>
<td></td>
<td>5′ TTGGATTTCCAGCAGGACTTT 3′</td>
</tr>
<tr>
<td>CD8</td>
<td>5′ AGTGGGCTGGACTCGACTGT 3′</td>
</tr>
<tr>
<td></td>
<td>5′ CAACACGTCTTTTGGTGTGTCCT 3′</td>
</tr>
<tr>
<td>CD11a/18</td>
<td>5′ TTCAGCCAGCAACAAGAAGA 3′</td>
</tr>
<tr>
<td></td>
<td>5′ GACAGCTGTGTTCCCACTGA 3′</td>
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<td>CD29</td>
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<td></td>
<td>5′ TCTGCTTTTCTTGGTGCTAGC 3′</td>
</tr>
<tr>
<td>CD73</td>
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<td>B2M*</td>
<td>5′ TCGGGCTACTCTCCCTGACT 3′</td>
</tr>
<tr>
<td></td>
<td>5′ ATTCACGTCACGGCGATG 3′</td>
</tr>
<tr>
<td>CIITA**</td>
<td>5′ GGTGCACTACTCTCCGCTTGACT 3′</td>
</tr>
<tr>
<td></td>
<td>5′ TGGCCACGACTCAGCAATGGCCTTCG 3′</td>
</tr>
<tr>
<td>TLR4</td>
<td>5′ CCCACATCAAACAGGAACCT 3′</td>
</tr>
<tr>
<td></td>
<td>5′ ATGGCTGAGGGCCCTGATAG 3′</td>
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<tr>
<td>TLR3***</td>
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<tr>
<td>S18</td>
<td>5′ ACTGAGGATGAGGTCGAACG 3′</td>
</tr>
<tr>
<td></td>
<td>5′ GCCCGTATCTTCTTCAGTCG 3′</td>
</tr>
</tbody>
</table>

*B2M sequence was used for gene expression detection of MHC I
**CIITA sequence was used for gene expression detection of MHC II
***Previously published sequence in: Figueiredo et al. (2009).

Total RNA was extracted from leukocytes and CB-MSC at passage 3, 4, and 5 using the mirVana miRNA Isolation Kit (Ambion, Life Technologies, Burlington, ON) following the manufacturer’s instructions. RNA was quantified using NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA) spectrophotometer, aliquoted and stored at -80°C for subsequent reverse transcription. For complementary DNA (cDNA) synthesis, RNA was thawed and any potential residual genomic DNA was
digested using DNase I amplification grade treatment (Invitrogen), directly followed by reverse transcription using the SuperScript II Reverse Transcriptase kit (Invitrogen) and random priming of a constant amount of RNA throughout the experiment. RNA degradation from freezing or thawing was tested with Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON). RNA integrity numbers (RIN) were consistently > 9/10 in samples tested.

Relative quantification was performed in a CFX Real-Time PCR Detection System (Bio-Rad, Kitchener, ON) by two-step real-time qPCR. Primers (Table 1) were designed using Invitrogen OligoPerfect Designer or the national center for biotechnology information (NCBI) primer-BLAST software or adapted from previously published sequence (Figueiredo et al, 2009). Beta-2-microglobulin (B2M) and class II MHC transactivator (CIITA) sequences were used for gene expression detection of MHC I and II, respectively. For all genes, amplicons were separated by 2% agarose gel electrophoresis, extracted and purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). DNA was sequenced and sequences analyzed using the NCBI basic local alignment search tool (BLAST) tool against equine sequence. Minimal identity cut-off was set at 96%. CD4 and CD73 primers did not include an intron-exon spanning junction due to amplification difficulties. Otherwise, all primers were designed to span at least one intron-exon junction to reduce the likelihood of amplifying genomic DNA. To ensure specificity of all primers, a sample without reverse transcription (RT-control) and a sample with buffer but without template (NT-control) was included in each run. PCR assays were performed in triplicate in a 10 µL total volume consisting of 5 µL of PCR SsoFast EvaGreen Supermix; (Bio-Rad), 0.2 µM of primer mix, and cDNA template. After an initial incubation at 95 °C for 3 min, reactions were cycled 40 times with denaturation at 95 °C for 5 s, annealing for 5 s at temperatures specific for each primer pair, and extension at 72 °C for 15 s. Amplification specificity was determined with melting-curve analysis whereby each amplicon was heated from 65 to 95 °C. Efficiency for each primer pair was determined from a standard curve generated from peripheral blood leukocytes or cDNA of target samples. qPCR
data were analyzed by the delta-delta Ct method (ΔΔCt) (Livak et al., 2001) using two endogenous reference genes chosen following the minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines (Bustin et al., 2009). Expression stability analysis of the reference genes was performed with GeNorm software. Initial selection of housekeeping genes was based on review of current literature (Radcliffe et al., 2010; Ranera et al., 2012) and availability of equine sequence data and thus comprised of, β-actin, S18, B2M, and hypoxanthine phosphoribosyltransferase 1 (HPRT1). Based on GeNorm analysis, only two housekeeping genes were selected based on the lowest variability in expression across samples. Assessment of variability in expression for the different genes was based on the M-value calculated as the average pairwise variation of a gene compared to the other control genes (Nailis et al., 2006). The initial cut-off M-value was < 1.5, and S18 and β-actin were selected to be used together as reference genes for subsequent relative quantification.

Lymphoproliferation

Blood was obtained from the jugular vein of five adult horses of variable breeds and sex with an 18G hypodermic needle attached to a 500 to 1000 mL blood transfusion collection bag (Fenwal, Baxter, Deerfield, IL). Mononuclear cells (MNC) were isolated using Ficoll-density gradient. At RT 15 mL of Ficoll-Paque Plus (density 1.078 g/mL, STEMCELL Technologies) was loaded in a 50 mL tube with 35 mL of whole blood. Gradients was centrifuged at 300g for 30 min at RT with no brake. Interphases containing the MNC fraction was carefully removed, pooled and washed with PBS. Supernatant was then removed and pellets were washed again with 10 mL of PBS. Pellets were then resuspended in 10 mL of RMPI 1640 medium supplemented with penicillin (100 IU/mL; Invitrogen), streptomycin (0.1 mg/mL; Invitrogen) and 10% FBS (Invitrogen), and a live cell count was performed with an automatic cell counter (NC-100, Chemometec). Cells were resuspended in freshly prepared cryomedium consisting of Roswell park memorial institute (RPMI) 1640 media supplemented with penicillin (100 IU/mL) (Invitrogen), streptomycin (0.1 mg/mL; Invitrogen), 10% FBS (Invitrogen) and 10% DMSO (Sigma-
Aldrich) at a concentration of 6x10⁶ cells/mL. One mL of cell suspension was placed in a 2 mL cryovial (Corning® Flask, Sigma-Aldrich) on ice for 30 minutes before gradual freezing over 12-18 hours, and storage in liquid nitrogen.

For assessing lymphocyte suppression, triplicate cultures of 1x10⁵ responder MNC were incubated with either 10,000 irradiated autologous MNC or 10,000 irradiated allogeneic MNC (pooled from three horses), yielding 10:1 responder:stimulator cell ratios. These cultures served, as negative and positive controls, respectively. The test wells were comprised of 1x10⁵ MNC unrelated to MSC cultures, 10,000 irradiated pooled allogeneic MNC and 10,000 irradiated MSC. Cells were cultured in RPMI 1640 media supplemented as above. Reactions were incubated for five days in round-bottom 96 well plates in 250-300 µL volume. Bromodeoxyuridine (BrdU) staining was performed with a FITC BrdU Flow Kit (BD Biosciences). Parameters for flow cytometry were set according to the manufacturer’s instructions. One mM of BrdU was added to MNC cultures on day five and cells were incubated for 24 hours. Cells were then fixed and permeabilized according to the manufacturer’s instructions. FITC-conjugated antibody to BrdU was incubated with cells for 20 minutes at RT. Subsequently, 7-AAD was added for 5 minutes, and cells were analyzed. All data were analyzed using the same settings as above with 5,000 events acquired per triplicate sample. Appropriate negative samples were included in each experiment.

**Immunocytochemistry**

For ICC staining, 5,000 MSC were cultured in 250 µL of EM in 8-well permanox chamber slides (Thermo Fisher Scientific) until 60-80% confluency. Cells were exposed to 1 µg/mL of poly (I:C) (Sigma-Aldrich) and 10 ng/mL of LPS (Sigma-Aldrich) for TLR3 and TLR4 induction for one hour. For ICC, cells were first washed thrice with PBS and fixed in situ for 5 minutes with 4% paraformaldehyde (PFA; Invitrogen), washed thrice with PBS and permeabilized with 0.1% Triton for 15 minutes. Cells were then washed again and treated with 3% hydrogen peroxide for 15 minutes. Non-specific antibody
binding was blocked by incubation with 5% FBS for 10 minutes followed by a one hour exposure to polyclonal primary antibody for TLR3 or TLR4 (both IMGENEX, San Diego, CA). Cells were then washed three times with PBS and incubated for one hour with goat anti-rabbit secondary antibody (Abcam). Bound antibody linked to horseradish peroxidase (HRP) was detected using 3,3'-diaminobenzidine (DAB) chromogen (DAKO, Burlington, ON). Cells were counterstained with Harris hematoxylin to visualize nuclei and images were acquired on a phase contrast microscope.

**Statistical analysis**

All data were analysed with SAS software (SAS Institute, Cary, NC). Effects were considered significant at $P \leq 0.05$. Because flow cytometry data were recorded as percentage values, a logit transform with a bias correction term was applied ($\text{logit} = \log[(r+k)/(n-r+k)]$ where $r$ cells, out of $n$ cells counted, were positive and $k$ is the bias correction term, 0.25). The MSC marker panel of MHC I, MHC II, CD4, CD8, CD11a/18, CD90 and the mixed lymphocyte reaction data were analyzed using a three-factor factorial analysis of variance (ANOVA) without replication with covariate (negative control). The MSC marker panel of CD29, CD44, CD73 and CD90 was analyzed as single assays using a two-factor factorial ANOVA and Pmax as a covariate. Data were back-transformed and plotted as percentage of positive staining with 95% confidence intervals (CI); back-transforms of the differences are odds ratios, with appropriate CIs. All possible two-way interactions among factors were assessed as well as factor by covariate interactions and the covariate quadratics. All non-significant terms were removed. Relative quantification of all qPCR data were analyzed using a two-factor factorial in a randomized block with sub-sampling, accommodating unequal variances amongst targets to meet ANOVA assumption. Multiple comparisons were subsequently adjusted using Tukey’s HSD method. For CD8 and CIITA, transcripts were detected only once precluding statistical analysis. Analysis of residuals was performed to assess the ANOVA assumption. Residuals were plotted against the predicted values and explanatory variables used in the model. The residuals were tested for normality using the four tests offered by SAS: Shapiro-Wilk,
Kolmogorov-Smirnov, Cramer-von Mises, and Anderson-Darling. Residual analyses may identify potential outliers, bimodal distributions, unequal variances, the need for data transformations or other problems that need addressing.
RESULTS

MSC progenitor frequency population doubling time and cell morphology assessment

Nine out of nine cord blood samples yielded colonies with classic spindle-shaped fibroblast-like morphology (not shown) giving an isolation success rate of a 100%. The number of colonies for each sample varied between 1 and 14, with a progenitor frequency between 4.44 x 10^{-7} % to 6.22 x 10^{-6} %, e.g. one MSC per 1.61x10^5 - 2.25x10^6 NC. All the CB-MSC cultures were expandable to the point of cryopreservation in the range of 1.1x10^6 – 13.8x10^6 at the end of passage 2. The cell-doubling times (DT) were as follows: passage 2 to 3 (n=9): 1.67 ± 0.15, passage 3 to 4 (n=9): 1.68± 0.15, passage 4 to 5 (n=5): 1.89 ± 0.58.

Cells also demonstrated spindle-shaped fibroblast-like morphology at passage 1 (Fig. 2A), 3 (Fig. 2B), 4 (Fig. 2C) and post-cryopreservation passage 5 (Fig. 2D).

Morphology was preserved through passaging.

Figure 2. CB-MSC morphology. CB-MSC have fibroblast-like morphology which is preserved throughout passage 1 to 5 (A-D); a = 40x, b = 100x.

Images were adjusted for black and white balance, brightness and contrast.

Expression of CD90 but loss of leukocyte markers after serial passage and cryopreservation of MSC

Following red blood cell lysis, leukocyte population was MHC I ^+, MHC II ^+, CD4^+, CD8^+, CD11a/18^+ and CD90^+ at antigen (Fig. 3, 4B) and gene expression level (Fig. 4A). Flow cytometry histograms (Fig. 3) demonstrate decreased expression for all markers at passage 2 except for CD90. For the leukocyte
population, CD90 staining appears as two distinct peaks, which progressively became the single peak observed at passage 5, suggesting increased homogeneity of CD90 expression with time in culture. Expression of other markers remained similar for subsequent passages.

**Figure 3.** Surface marker expression on leukocytes and CB-MSC. Histograms represent leukocytes, and CB-MSC at passage 2, 3 and 5 in gate 1: cells incubated with antibody (blue line) compared to control antibody (black line) for a representative sample. Results were similar for samples from 5 animals. Numbers in each plot represent the mean percent positive cells for each marker, and the confidence interval.
Surface antigen expression presented in Fig. 3 is also reported in Fig. 4B in combination with gene expression (Fig. 4A). Antigen (Fig. 4B) and gene expression (Fig. 4A) significantly decreased for all markers after culture except for CD90, for which expression significantly increased. Between passage 2 and 3, no significant change was observed for CD90, MHC I and MHC II expression. Slight but significant increases in protein expression was observed for CD8 and CD11a/18 and a significant increase of gene and protein expression was noted for CD4. Transcripts for CIITA and CD8 were detectable only for the leukocyte population.

Figure 4. Gene and protein expression of markers on leukocytes and CB-MSC. Gene (A) and protein (B) expression were assessed with qPCR and flow cytometry, respectively, on leukocytes, and on CB-MSC at passage 2 and 3. A. Gene expression was normalized to S18 and β-actin, and is shown relative to the lowest average CT. B. Surface protein expression presented as percentage of positive cells. Bars represent CI. Significance set at p<0.05 and different letters indicate significant differences between groups. Protein and gene expression of all markers except CD90 was significantly down-regulated in culture. There was significant up-regulation of CD90 surface expression at passage 2, slight but significant increase of CD8 and CD11a/18 surface expression at passage 2 and 3, and significant up-regulation of CD4 gene and
surface expression at passage 2 and 3. Transcripts for MHC II and CD8 were detected only in the leukocyte samples.

**CD29, CD44 and CD90 are highly and consistently expressed on CB-MSC at passage 5**

At passage 5 following cryopreservation (Fig. 5), MHC I, MHC II, CD4, CD8, CD11a/18 demonstrated low antigen (Fig. 5A) and gene (Fig. 5B) expression as compared to high expression for CD90. However, due to the lack of a reliable inter-run calibrator, it is impossible to compare expression levels before and after cryopreservation.

---

**Figure 5.** High CD90 expression at passage 5. Protein (A) and gene (B) expression were assessed with qPCR and flow cytometry, respectively, for CB-MSC at passage 5.  **A.** The percent positive cells.  **B.** Relative mRNA expression.
Gene expression normalized to S18 and β-actin and relative to the lowest average CT. Error bars represent CI. Protein and gene expression for all markers except CD90 was significantly down-regulation in culture. There was high CD90 protein and gene expression for CB-MSC at passage 5 compared to MHC I, MHC II, CD4, CD8 and CD11a/18.

**CD29, CD44 and CD90 are highly and consistently expressed on CB-MSC at passage 5**

CB-MSC were assessed post-cryopreservation at passage 5. Flow cytometry histograms demonstrated a single peak (Fig. 6A), suggesting homogeneity in expression. Antigen expression is also reported in Fig. 6B in combination with gene expression (Fig. 6C). Fig. 6B demonstrates similar high expression of CD29, CD44, and CD90, but significantly lower expression of CD73 for antigen expression. For gene expression (Fig. 6C), high expression of MSC markers CD29, CD44 was observed, with significantly lower expression of CD90 as compared to CD29 and CD44.
Figure 6. CD29, CD44 and CD90 are highly and consistently expressed on CB-MSC at passage 5. A. Histograms represent the percent of stained (blue line) CB-MSC at passage 5 in gate 1 relative to negative control (black line). Results are representative of 5 experiments. Numbers in each plot represent the mean percent positive cells for each marker, and the confidence interval. B. Graphical representation of flow cytometry results from 5 experiments. C. Relative gene expression, error bars represent CI. Significance set at p<0.05 and different letters indicate significant differences between groups. CB-MSC at passage 5 had consistently high expression of CD29, CD44 and CD90 relative to CD73. CD29 and CD44 had significantly higher protein expression than CD90.

CB-MSC decrease lymphocyte proliferation in mixed lymphocyte reactions

All CB-MSC cultures tested demonstrated significant suppression of lymphocyte proliferation and 3 out of 4 groups showed proliferation significantly lower than the negative control (autologous) group (Fig. 7).
CB-MSC constitutively express TLR4 with minimal up-regulation after LPS stimulation

TLR3 and TLR4 were assessed for gene expression with qPCR (Fig. 8A, B) and protein expression with ICC (Fig. 8C, D) before and after incubation with poly (I:C) for and LPS respectively. TLR4 was constitutively expressed at antigen (Fig. 8D) and gene expression (8B) levels and expression was not significantly changed after treatment with LPS. TLR3 gene expression was low (<35 Ct) (Fig. 8A) and correlated with low protein expression (Fig. 8C). Statistical analysis suggested significant up-regulation following treatment with poly (I:C), however, expression remained over 35 Ct.
A

**TLR3**

- **Ct>35**
  - Control
  - Poly(T/C)

B

**TLR4**

- **Ct<35**
  - Control
  - LPS

**Relative gene expression**

**Ct>35**

**Ct<35**

**Relative mRNA expression**
Figure 8. CB-MSC constitutively express TLR4 with minimal up-regulation after LPS stimulation. Gene expression of TLR3 (A) and TLR4 (B) was normalized to S18 and β-actin and expressed relative to untreated samples. Bars indicate CI. Significance set at p<0.05 and different letters indicate significant differences between groups. B. ICC for TLR3 (C) and TLR4 (D). Negative secondary antibody control for (a), staining of untreated CB-MSC (b), and staining of CB-MSC after treatment with poly (I:C) or LPS (c). TLR4 antigen and gene expression was detected in CB-MSC, and showed minimal response to LPS stimulation. There was significant TLR3 gene up-regulation after poly (I:C) stimulation, but expression remained very low and inconsistently detectable by ICC.
I report a 100% success rate of MSC isolation from equine cord blood. MSC isolation success of 100% from cord blood was first reported in the horse (Koch et al., 2009), and confirmed in this study with a greater number of samples. This is an important finding since it confirms that this method is robust for the isolation of equine CB-MSC and not operator-dependent. A major advantage of CB-MSC compared to adult MSC from bone marrow and adipose tissue is that a highly differentiation potent population of MSC can be obtained non-invasively and be cryopreserved for later use (Kern et al., 2006; Kogler et al., 2006; Koch et al., 2007). However, the number of human cord blood samples successfully yielding MSC was generally reported to be low as no MSC progenitor could be isolated in a number of samples (Bieback et al., 2004). In equine medicine the use of autologous CB-MSC is now only challenged by the risk of cord blood collection failure at the time of foaling. Once cord blood is collected CB-MSC can be reliably isolated.

I report, for the first time, a consistent constellation of antigens expressed on CB-MSC consisting of CD29, CD44, CD90, along with lack of surface MHC I, MHC II, CD4, CD8, CD11a/18, and CD73. Consistent with my results, De Schauwer et al. (2012) found no major differences in phenotype before and after cryopreservation, except for CD73, which demonstrated less variation (De Schauwer et al., 2012). Findings regarding presence of transcripts detection for CD29, CD44 and MHC I (B2M) and absence of transcripts for MHC II (CIITA in my study) after culture and cryopreservation are in agreement with a previous report by Lovati et al. (2011). As expected, following the initial leukocyte isolation, surface antigen expression was; MHC I^{high}, MHC II^{high}, CD4^{high}, CD8^{high}, CD11a/18^{high} and CD90^{high}. Monolayer culture expansion subsequently negatively selected for these antigens with the exception of CD90 which expression up-regulated significantly. Phenotype as of passage 2 was then MHC I^{low/neg}, MHC II^{low/neg}, CD4^{low/neg}, CD8^{low/neg}, CD11a/18^{low/neg} and CD90^{high}. Between passage 2 and
3, antigen and gene expression for CD4, and antigen expression for CD8 and CD11a/18 significantly increased. However, it was only a slight increase and expression remained very low. As reviewed earlier, CD90 was expected to be highly expressed as it is known as positive on equine MSC. This result is also in accordance with a previous study looking at temporal phenotype of equine BM-MSC reported CD44$^{hi}$, CD29$^{hi}$, CD90$^{lo}$, CD11a/CD18$^{hi}$, and CD45RB$^{lo}$ expression 2 hours after culture, which became CD44$^{hi}$, CD29$^{hi}$, and CD90$^{hi}$, CD11a/CD18$^{ne}$, and CD45RB$^{ne}$ after 14 days in culture (Radcliffe et al., 2010). My results showed a similar pattern of expression. Expression of the lymphocyte markers CD4 and CD8 has not previously been reported in equine MSC, but my results are consistent with the observation that CD4 and CD8 positive lymphocytes do not adhere to plastic. These cells were therefore likely removed through media changes and sub-culturing.

The consistently low CB-MSC MHC I antigen expression following culture contrasts the findings of a previous study on equine CB-MSC that found surface expression of MHC I (49-95%) (Carrade et al., 2012). In the same study, similar expression was also reported for equine BM-, AT- and CB-MSC (Carrade et al., 2012). In this thesis, all surface antigens detected were also interrogated with qPCR-assays for gene expression, hence, MHC I expression can be regarded as a robust finding. Differences in surface expression between two independent studies can be influenced by several factors such as culture conditions and variability in reagents. Variation can occur between different antibody sources, clones or lot numbers. However, even if the antibody I used was different than the one used by Carrade et al., it does not explain the observed discrepancy, since I also found consistent low B2M gene expression. Since B2M expression is required for surface expression of MHC I molecules (Otten et al., 1992), this supports the MHC I result and suggests the low MHC I expression is real. A notable difference between my culture method and that of Carrade et al. (2012) is the presence of dexamethasone in my isolation medium. The possible influence of dexamethasone on MHC I expression was not investigated. However,
unrestricted somatic stem cells (USSC), pluripotent cells present in cord blood, are also isolated in presence of dexamethasone and express surface MHC I (Kögler et al., 2004).

An immunoprivileged phenotype of these equine CB-MSC is suggested by the low MHC I expression (Hass et al., 2011). Neural stem cells negative for MHC I were reported to not be recognized by cytotoxic T lymphocytes and NK cell in vitro in a mouse model (Mammolenti et al., 2004). However, more work is needed to determine if MHC I high or low expression is related to different culture conditions, and whether this phenotype is stable in time. The immunoprivileged phenotype has been suggested to be unstable and equine CB-MSC MHC I expression has been found to be up-regulated following inflammation (Carrade et al., 2012). In addition, MSC differentiation has been associated with increased immunoreactivity, which may be due to altered MHC expression. For example, adipo-, osteo- and chondro-genic differentiation of human MSC increased MHC I expression, but not MHC II in vitro (Le Blanc et al., 2003). However, the same report suggests efficient in vitro suppression of lymphoproliferation by MSC even after 2 weeks in differentiation medium (Le Blanc et al., 2003). Transition from an immunoprivileged to an immunoreactive phenotype is suggested to occur gradually. Injection of allogeneic MSC into rat infarcted myocardium did not elicit an immune response after 1 week, but did after 5 weeks as evaluated by cytokine expression in the heart and presence of alloantibody in the recipient circulation. Allogeneic, but not syngeneic, MSC failed to restore cardiac function 6 months after induced myocardium infarct (Huang et al., 2010), suggesting involvement of MHC I and II in the reported failure. Thus, long-term transition of MSC to an alloreactive phenotype remains an important area of research in order to determine the key factors that limit or facilitate successful MSC allogeneic treatments.

In addition, I report consistent ability of equine MSC to suppress lymphocyte proliferation in vitro, consistent with similar report on equine cord blood-derived MSC (Carrade et al., 2012). I also report
constitutive TLR4 expression and low/absent TLR3 expression. This is in contrast with my hypothesis based on human reports. This is the first report regarding TLR3 and TLR4 expression on equine CB-MSC, and suggests potential inter-species differences. *In vitro* suppression of lymphoproliferation is used as a proxy for possible anti-inflammatory effect of MSC *in vivo*. The mechanisms by which MSC exert their immunomodulation abilities is still largely unknown, but it was recently suggested that expression of TLRs might be important determinants. TLR3 has been suggested as involved in the MSC immunomodulation abilities, while TLR4 associated with a pro-inflammatory phenotype (Tomchuck et al., 2008; Waterman et al., 2010). Interestingly, I observed constitutive expression of TLR4 at both antigen and gene expression level as well as very low and inconsistent TLR3 mRNA expression along with lack of consistent ICC staining. Similar to my results, constitutive TLR4 expression has been reported in human BM-, AT-, and CB-MSC as well as in mouse BM-derived MSC. However, contrary to my results, some reports also demonstrated constitutive expression of TLR3 (Raicevic et al., 2010; Liotta et al., 2008; Hwa Cho et al., 2006; Tomchuck et al., 2007; Pevsner-Fischer et al., 2007; Raicevic et al., 2011; van den Berk et al., 2009). More investigation into the relation of TLR3 and TLR4 expression with MSC immunomodulation properties are required. Knock-down of TLR3 and TLR4 separately with subsequent assessment in mixed lymphocyte reaction is suggested to test TLR3 and 4 involvements in equine CB-MSC ability to suppress *in vitro* lymphoproliferation. TLR3- and TLR4-knocked-out cells reactivity to IFN-γ treatment should also be tested. Similar assessment could be performed with MyD88- and TRIF-knocked-out cells to test whether or not MyD88 involvement is more decisive of immune abilities than an individual TLR.

In addition, based on previous reports, it was unexpected that TLR4 and TLR3 mRNA and protein expression was not upregulated following stimulation with LPS and poly (I:C), respectively. Only one concentration and assessment time point was used. Dose titration and temporal assessment may reveal
differences. At this point in time, no consensus protocol exists for TLR up-regulation using specific ligands, as reports on the responses are conflicting. It appears to depend on several factors, such as cell source and species as reported by Waterman et al. (2010). Both TLR3 and TLR4 are also specifically known to exert inter-species differences (Lundberg et al., 2007; Ferwerda et al., 2008).

From a clinical point of view, identification of a specific cell population by detection of a surface marker would be desirable, since it might allow cell sorting. However, no constitutively expressed markers have been correlated with immunosuppressive abilities of MSC. Responsiveness to IFN-γ would currently not allow single cell analysis and marker-based cell isolation, but would be very useful to assess the general immune abilities of MSC-derived from a particular donor. MSC from individuals yielding higher immunosuppressive abilities could then be used in priority for allogeneic cytotherapy, as suggested by Galipeau (2013) regarding human donors. Human MSC abilities to suppress lymphocyte proliferation were suggested to correlate to IDO responsiveness after IFN-γ treatment (François et al., 2012). However, this possible surrogate marker to quantify the response seems variable across species, as indicated by variability between humans and mice (Ren et al., 2008). In horses, no report specifically answered this question. However, a report by Carrade et al. (2012) suggests IL-6 and PGE-2 as potential candidates rather than IDO or iNOS. I also report up-regulation of IL-6 gene expression in response to IL-1β (Appendix III). Measurement of IDO, iNOS, IL-6 and PGE-2 quantitative expression following incubation with IFN-γ in vitro with qPCR and western blots is suggested. Subsequently, each cytokine could be separately knocked-down and knocked-out CB-MSC assessed in a mixed lymphocyte reaction to assess potential lost of immunomodulatory abilities.

In conclusion, CB-MSC can consistently be isolated and possess specific surface phenotype post-cryopreservation. The CB-MSC phenotype of CD90<sup>high</sup>, and MHC I<sup>low/absent</sup>, MHC II<sup>low/absent</sup>, CD4
CD8\textsuperscript{low/absent} and CD11a/\textsuperscript{18} \textsuperscript{low/absent} was unaffected by cryopreservation. In addition, CB-MSC at passage 5 post-cryopreservation highly expressed CD29 and CD44 with low/absent expression of CD73. In contrast to my hypothesis, I found constitutive expression of TLR4 on equine CB-MSC along with lack of TLR3 expression. Further studies are required to characterize TLR expression on CB-MSC as well as their functional abilities, and involvement in immunomodulatory phenotype. Low MHC I expression suggests a reduced risk of CB-MSC being immediately rejected \textit{in vivo}. Further characterization of mechanisms by which MSC exert their immunomodulatory properties is required. The work presented is an important step toward clinical use of CB-MSC and further investigation of the role of TLR3 and TLR4 in equine MSC immunomodulatory mechanism.
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APPENDIX I - Immune markers and cytokine expression for MSC before and after IL-1B and IL-6 treatment

CB-MSC were assessed for gene expression of markers potentially correlated with immunomodulation abilities. Expression was measured before and after incubation with IL-1B or IL-6.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Untreated MSC</th>
<th>IL-1B treatment</th>
<th>IL-6 treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TAP1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CIITA</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
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<td>+/-</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>TLR4</td>
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<td>+</td>
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</tr>
<tr>
<td>MyD88</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TRIF</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TRAM</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>IL-1B</td>
<td>-</td>
<td>-</td>
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<tr>
<td>IL-6</td>
<td>+/-</td>
<td>++</td>
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<tr>
<td>TGFB1</td>
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<tr>
<td>HO-1</td>
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<tr>
<td>CXCL10</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Fe-H</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>TFR1</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Gene expression:
(-): transcript not detected
(+/-): transcript detected >35 Ct
+: transcript detected 25 >Ct < 35
++: transcript detected < 25 Ct

CB-MSC were separated in three groups and assessed separately: untreated cells, IL-1B treated cells and IL-6 treated cells. CB-MSC were cultured in EM until 60-70% confluency and subsequently incubated for 24h in: EM, EM supplemented with 10ng/mL of recombinant equine IL-1B or 20ng/ml of recombinant equine IL-6. Gene expression was analyzed with qPCR. Potential changes in expression were observed for IL-6, TGFB1, Fe-H, CXCL10.