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Functional Assessment of Placenta-derived Mesenchymal Stromal Cells and Secreted Extracellular-Vesicles: Therapeutic Applications for Central Nervous System Disorders

By

KAITLIN C CLARK
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry, Molecular, Cellular & Developmental Biology
Emphasizing in Translational Research

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2023

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“If you can't explain it simply, you don't understand it well enough.”
-Albert Einstein

Abstract

Multiple sclerosis (MS) is characterized by an immunological attack of the myelin sheath which leads to demyelination and axonal degradation. This debilitating disorder typically manifests as a progressive loss of motor function, with most cases involving relapsing flare-ups throughout disease progression. The underlying pathophysiology of adult-onset MS is similar to pediatric MS, however pediatric disease typically displays more acute inflammatory responses leading to degeneration. According to the MS society, in the US alone, there are 8,000-10,000 children diagnosed with MS that suffer from frequent relapses. Current therapies for MS reduce the incidence of flare-ups, but do not prevent progressive axonal and neurological degeneration. Cell-based therapies using mesenchymal stem/stromal cells (MSCs) have been under investigation in clinical trials for neurogenerative diseases including adult MS. The placenta has been suggested to be a unique source of MSCs that possess robust immunomodulatory properties and have been reported to be beneficial in graft verse host diseases mouse models. MSCs derived from the placenta (PMSCs) may be a more appropriate cell source for pediatric diseases, because during pregnancy the placenta demonstrates “fetomaternal tolerance”, which is attributed to the expression of human leukocyte antigen-G (HLA-G), a non-classical major histocompatibility complex (MHC) class I molecule that inhibits natural killer cell (NK) killing. Unlike bone marrow derived MSCs (BM-MSCs), PMSCs express HLA-G on their surface in response to interferon gamma (IFNγ), which is a key inflammatory mediator involved with the onset of MS. Therefore, the expression of HLA-G on PMSCs would make them a unique therapeutic cell source for the treatment of autoimmune diseases like pediatric MS. Currently, a clinical trial is underway using term placenta-derived PMSCs for adult MS and no paradoxical worsening of MS lesion counts was noted.

Notably cell-based therapies are limited by potential immune rejection of donor cells and other safety concerns. Increasingly, studies have shown that MSC survival and integration within the host after transplantation are usually poor and that MSCs exert their therapeutic functions

mainly via paracrine signaling mechanisms. Conditioned media of BM-MSCs is shown to protect neurons from apoptosis, activate macrophages and be pro-angiogenic. In particular, hepatocyte growth factor (HGF) secreted by MSCs into conditioned medium mediates recovery in a murine model of MS. Currently, the use of MSC conditioned media is limited in that the secreted protein factors are unstable, which creates technical difficulties for “off-the-shelf” clinical use. MSC derived extracellular vesicles (EVs) alternatively, are stable under long term storage conditions compared to freely secreted proteins and may serve as a superior source for cell-free therapy.

MSC-derived EVs readily cross the blood brain barrier (BBB) and deliver therapeutic cargo to reduce the effects of neuropathologic diseases, such as MS. It has been demonstrated that MSC-derived EVs exhibit systemic immunomodulatory effects and can facilitate neurological recovery *in vitro*. PMSC-EVs contain numerous proangiogenic, immunomodulatory, and neuroprotective proteins, including HGF. HGF is a pleiotropic factor shown to have neuronal and oligodendrocyte protective properties. In an experimental autoimmune encephalomyelitis (EAE) rodent model of MS, overexpression of HGF by neurons conferred neuroprotection by reducing inflammation in the CNS and activation of Tregs. HGF is secreted both in soluble form from MSCs and is also contained in exosomes; however, the effects of each form could lead to different cellular responses. It has been suggested that MSCs secrete multiple categories of exosomes, which are involved in differing cellular processes. Therefore, deciphering the molecular mechanism by which MSC-derived EVs alleviate the effects of neurodegenerative diseases is warranted. The goal of the current research project is to demonstrate the therapeutic potential of PMSC-EVs utilizing translational models of MS as well as to and to examine the role of HGF. These studies will be crucial as pre- investigational new drug data moving towards novel and bioengineered cell-free therapeutic treatments for MS patients.

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Chapter 1. The Molecular Mechanisms Through Which Placental Mesenchymal Stem Cell-Derived Extracellular Vesicles Promote Myelin Regeneration

1.1 Abstract

Multiple Sclerosis (MS) is a debilitating degenerative disease characterized by an immunological attack on the myelin sheath leading to demyelination and axon degeneration. Mesenchymal stem/stromal cells (MSCs) and secreted extracellular vesicles (EVs) have become attractive targets as therapies to treat neurodegenerative diseases such as MS due to their potent immunomodulatory and regenerative properties. The placenta is a unique source of MSCs (PMSCs), demonstrates 'fetomaternal' tolerance during pregnancy, and serves as a novel source of MSCs for the treatment of neurodegenerative diseases. PMSCs and PMSC-EVs have been shown to promote remyelination in animal models of MS, however the molecular mechanisms by which modulation of autoimmunity and promotion of myelination occurs has not been well elucidated. The current chapter will address the molecular mechanisms by which PMSC-EVs can promote remyelination in MS.

1.2 Introduction

Multiple sclerosis (MS) is a central nervous system (CNS) disorder that gives rise to chronic neurological deficits. MS is caused by immunological attack on the myelin sheath leading to demyelination, axonopathy and neurodegeneration. MS is an extremely heterogenous disease and typically manifests as a progressive loss of motor function, with most cases involving relapsing flare-ups throughout disease progression. An estimated 2.5 million individuals are affected by MS, with the disease more commonly presenting in young adults and females.[1] The etiology of MS is not fully understood, but several factors may contribute to disease onset including genetics, vitamin D, and Epstein-Barr virus infection.[2,3] Current treatments for MS typically involve long-term use of disease-modifying drugs (DMDs), with most aiming to suppress or modulate the inflammatory component of the disease.[4] Long-term administration of anti-

inflammatory medications can have detrimental effects to patients including risk of infection and malignancy from suppression of anti-microbial and anti-tumor immunity.[5] While these therapies can reduce the incidence of flare-ups, they do not prevent progressive axonal and neurological degeneration associated with MS pathology. Therefore cell-based therapies using mesenchymal stem/stromal cells (MSCs) have recently been under investigation in clinical trials for the treatment of neurogenerative diseases including MS.[6,7]

Therapeutically administered MSCs can migrate to sites of injury and interact with the inflammatory niche through cell-cell contact and/or paracrine signaling mechanisms.[8] Early gestational MSCs, including placenta-derived MSCs (PMSCs), preserve features from primitive embryonic layers and have been characterized by immunophenotyping and multipotency assays.[9] This unique cell subset has the potential to differentiate into various tissue types, has greater proliferative and immunomodulatory properties, and causes less immunogenicity than adult derived MSCs.[9] However, cell-based therapies are limited by several safety concerns including, teratoma formation and the possibility of eliciting immune responses or rejection of donor cells.[10] Increasingly, studies have shown that MSC survival and integration within the host after transplantation are usually poor, and that MSCs exert their therapeutic functions mainly via paracrine signaling mechanisms.[11] MSCs can release extracellular vesicles including exosomes, which are small nanovesicles that can carry protein, mRNA and microRNA and have been shown to play a key role in CNS inflammation.[12] Additionally, MSC-derived extracellular vesicles (EVs) are stable under long term storage conditions compared to freely secreted proteins and may serve as a superior source for cell-free therapy.[13]

MSC-derived EVs readily cross the BBB and deliver therapeutic cargo to reduce the effects of neuropathologic diseases such as MS.[14] It has been suggested that MSCs secrete multiple categories of EVs, which are involved in differing cellular processes.[15] A recent report demonstrated that MSC-derived EVs exhibit systemic immunomodulatory effects and can

facilitate neurological recovery *in vitro.*[16] PMSC-derived EVs (PMSC-EVs) contain numerous proangiogenic, immunomodulatory and neuroprotective proteins.[17,18] In an experimental autoimmune encephalomyelitis (EAE) model of MS, it was demonstrated that PMSCs and PMSC-EVs could mitigate motor deficits in treated animals, likely by promoting oligodendrocyte precursor cells (OPC) to a mature myelinating phenotype.[19] While this pre-liminary data is promising, the molecular mechanisms by which PMSC and PMSC-EVs elicit these effects on central nervous system damage is not fully characterized. The current review addresses the molecular mechanisms by which PMSC and PMSC-EVs can promote remyelination in MS.

1.3 Multiple Sclerosis Pathology

The primary characteristic of MS pathology is autoreactive lymphocytes crossing the BBB into the CNS. Activation of peripheral myelin-specific T cells home to the CNS, where they can become reactivated by antigen presenting cells (APCs). Within the CNS, resident immune cells will become activated and secrete pro-inflammatory mediators that lead to the degeneration of myelinated axons. It has been suggested that human leukocyte antigen (HLA)-DR15 modulates homing of autoreactive $CD4^+$ T cells, proliferation and infiltration of pro-inflammatory T helper cell (Th)-1 subsets into the CNS.[20] Integrin α4β1, also known as very late activation antigen (VLA)-4, has been implicated in mediating adhesion and migration of immune cells into the CNS through the interaction with integrin specific ligand vascular cell adhesion molecule (VCAM)-1 which in turn has been shown to be critical in mediating Th-1 cell migration in MS.[21] Activated peripheral T cells will enter circulating cerebral spinal fluid (CSF) through the subarachnoid space, which is mediated by constitutively expressed selectins and adhesion molecules.[22]

Evaluation of CSF obtained from MS patients showed presence of stimulated $CD4^+$ Th-1 cells that secrete cytokines including interferon gamma (IFNγ), interleukin (IL)-2 and tumor necrosis factor alpha (TNFα).[23] These pro-inflammatory cytokines create a feedback loop signaling pathway which will in turn activate microglia and further drive a pro-inflammatory M1-like

phenotype leading to upregulation of major histocompatibility complex (MHC)-II, driving reactivation of Th-1 cells.[24]

Th-17 cells can also mediate autoimmunity through expression of chemokine receptor (CCR)-6 which binds to chemokine ligand (CCL)-20 on vascular endothelium, allowing migration into the CNS.[25] Th-17 cells secrete IL-17A, which has been shown to inhibit proteins associated with tight junctions of the BBB, leading to increased permeability and entry of inflammatory cells and mediators into the CNS.[26] Th-1 and Th-17 cell responses will result in production of the inflammatory mediators IL-17 and IFNγ which can directly contribute to disease pathogenesis.[27] In the EAE model of MS, Th-17 cells producing IFNγ were shown to be enriched in myelin oligodendrocyte glycoprotein (MOG)-specific T cells, and can drive inflammatory responses in the CNS independent of Th-1 responses and have been shown to be effective in regulating astrocytic responses.[28] Astrocytes exist between the BBB and neurons, regulate interactions of the periphery with the CNS, and are responsible for neurogenesis and tissue repair. Astrocytes express an IL-17A receptor and can upregulate inflammatory cytokines and chemokines.[29] Therefore, reducing IL-17 signaling by astrocytes has been shown to ameliorate symptoms in the EAE model of MS.[29,30] Th-17 cells producing IL-17 have been shown to inhibit maturation and survival of oligodendrocyte lineage cells (OLs)[31] and cause apoptosis of myelinating cells.[32] Neurodegeneration and apoptosis of OLs is caused through direct cytotoxicity from antigen-specific T cells, autoantibodies, and T cell -mediated pro-inflammatory cytokines that activate resident microglia populations.[33] Overall, products of Th-17 cells, such as IFNγ and IL-17, can exacerbate inflammatory responses in astrocytes, oligodendrocytes and microglia. Regulating these inflammatory products that produce detrimental effects in CNS cells provides direction for future therapies.

T regulatory cells (Tregs) are another subset of T cells involved with MS pathology that can regulate Th-1, Th-17 and Th-2 responses. Tregs are involved in the maintenance of peripheral

immunotolerance and modulate CD4+CD25+ T cell subsets and can suppress effector T cell responses to maintain self-tolerance. It has been reported that Treg populations are reduced in MS patients but are not functionally impaired.[34] It has also been demonstrated that severity of EAE can be mitigated by transfer of Treg cells.[35] Tregs can inhibit proliferation and function of inflammatory T cell subsets and can decrease migration into target organs. Tregs have been shown to facilitate remyelination through the secretion of cellular communication network factor 3 (CCN3), which promotes oligodendrocyte differentiation and myelination in mice.[36] Tregs are typically classified as CD8+/CD25+/FOXP3+ cells however, CD8+ Tregs have been presented as a novel subset of cells that can regulate self-reactive CD4+ T cells, and disruption in these processes may lead to autoimmune response induction.[37] Effective Treg functions have been suggested to promote remyelination through direct mechanisms, but reduced Treg numbers in MS patients suggests the role of Tregs in the demyelinating pathology of MS.[40]

CD8+ effector T cells are typically referred to as cytotoxic T cells and target cells that present MHC I on their surface. Most resident CNS cells present MHC I in inflammatory conditions and thus can be targeted by CD8+ T cells. MHC I has been shown to be upregulated in macrophages in actively demyelinating lesions in early onset of MS, which suggests a direct role for activated macrophages and microglia in demyelination.[41] Additionally, the perivascular space around actively demyelinating MS lesions was found to contain CD8+ cells expressing IL-17.[42] It is unclear whether CD8+ T cells play a pathogenic or regulatory role in MS because it has been suggested that CD8+ T cells can be both pro and anti-inflammatory immune mediators.[44] CD8+ T cells can suppress activity of myelin-specific CD4+ T cells, and MS relapses have been shown to correlate with reduced CD8+ T cell numbers.[45,46] However, it has also been shown that increases in CD8+ T cells can correlate with axon damage,[47] and polarize near demyelinated axons.[48] Functional Tregs have been suggested to promote remyelination through direct mechanisms, but reduced Tregs numbers in MS patients suggests the role of Tregs in disease

pathology.[40] While the exact role of T cells is not fully understood, it appears that disruption of T cell subset expression and homeostasis leading to inflammatory responses is directly involved in the demyelinating component of MS pathology.[42] [43-48]

In MS, interactions between T lymphocytes and B lymphocytes are also likely disrupted. One study demonstrated that in 90% of patients, oligoclonal immunoglobulin (Ig) existed in CSF, suggesting a pathologic role of B cells in MS.[49] B cells can produce antibody responses that can target antigens including MOG and myelin basic protein (MBP), both of which are expressed in mature myelinating oligodendroglia cells.[50] Peripheral B cells may become autoreactive due to impaired or chronically exhausted Tregs, thus allowing B cells to interact with Th effector cells in lymphoid organs.[51] These interactions can lead to pathogenic cell infiltration through the BBB and allow activated $CD8^+$ T cells to become reactivated in the CNS.[51]

Local inflammatory responses in the CNS will lead to disruptions in homeostasis and cause resident immune cell subsets to become activated. Microglia are APCs that can promote myelin regeneration by expressing anti-inflammatory mediators, removing debris, and facilitating tissue repair. Chronic T cell and microglia activation can lead to accompanying demyelination and neurodegeneration associated with MS.[52] Chronic inflammation in the CNS can result in several pathogenic molecular processes including oxidative stress, mitochondrial injury and ion channel dysfunction.[53] T cells and microglia have been shown to co-localize in demyelinating sites in MS patients,[54] thus suggesting interactions between these two cell types could occur through cell-cell contact or through the secretion of bioactive factors. Microglia express MHC molecules and can secrete several pro-inflammatory and anti-inflammatory mediators including TNFα, IL-10, and co-stimulatory molecules ICAM-1, B7-1 and B7-2.[55] Activation of microglia to an M1 inflammatory phenotype can support T lymphocyte homing and reactivation in the CNS. It was demonstrated that TNF secreted by microglia was induced by T cells, in part through interactions between VCAM-1 on microglia and α4β1 on T cells.[56] The interactions of encephalitogenic T cells with

microglia can lead to further reactivation and inflammation, thus leading to cellular toxicity to neuronal cell populations leading to demyelination and degeneration. Clearly, interactions between activated microglia and T cells drive the immune component of MS pathology and require further mechanistic studies for understanding their role in molecular processes.

Micro-RNAs (miRNAs) have been proposed as key modulators of OPC maturation and differentiation. Mice lacking miRNA-processing enzyme Dicer demonstrated significant deficits in myelination.[57] Moreover, blocking studies demonstrated a critical role of miR-219 and miR-338 for oligodendrocyte differentiation and maturation.[58] In MS, deficits in OPC maturation have been observed.[59] OPCs express receptors for the pro-inflammatory mediators IFNγ,[60] IL-17[61] and TNFα,[62] all of which have been shown to be associated with MS pathology. Furthermore, IFNγ has been shown to induce a pro-inflammatory M1 microglia phenotype, and act on oligodendrocytes to cause endoplasmic reticulum stress, demyelination and degeneration.[60] It has been reported that OPC maturation can also be inhibited by effector T cell functions and overexpression of IFNγ.[63] In addition, IFNγ primed OPCs presented to $CD8^+$ T cells resulted in OPC death, suggesting that inhibiting inflammatory process can directly result in remyelination.[63]

Understanding the interaction between inflammatory responses and accompanying demyelination and neurodegeneration associated with MS pathology is critical in developing intervention strategies that can promote remyelination. The process of remyelination has been described as beginning with proliferation of OPCs, then OPC migration to demyelinating axons, followed by OPC maturation and then myelination of premature oligodendrocytes with axons.[64] Cellular therapeutics that can protect and promote myelinating oligodendrocytes, axons and neurons, in addition to targeting the inflammatory component of MS, will provide a novel approach to treat the complex and heterogenous nature of MS.

1.4 Placenta-derived mesenchymal stem/stromal cells novels are a novel source of MSCs for the treatment of MS

Cell-based therapies using MSCs have been recently investigated in clinical trials for neurogenerative diseases including MS.[7,65] MSCs display potent immunomodulatory and regenerative capabilities through the secretion of bioactive factors, such as proteins, cytokines, chemokines as well as the release of EVs. It has been reported that MSCs reduce B cell proliferation and maturation,[66] regulate natural killer (NK) cell activities[67] and direct macrophage polarization.[68] Moreover, MSCs have the potential to suppress activated $CD4^+$ and $CD8^+$ T lymphocyte proliferation and promote the induction of Tregs, all of which are directly involved with MS pathology.[69-71] This unique T cell subset can reduce inflammatory immune response and plays an important role in peripheral immunity.[69] These functional properties of MSCs make them ideal candidates for treating degenerative and inflammatory diseases, including MS.[69-71]

The placenta is a unique source of MSCs that possesses robust immunomodulatory properties and has been reported to be beneficial in graft verse host diseases mouse models.[8,65] PMSCs have been suggested to have advantages in terms of proliferation and plasticity as compared to adult derived tissue sources.[72] It has been reported that PMSCs have the capability to differentiate towards neural lineages including oligodendrocytes and neurons.[73] Compared to adult sources of MSCs, PMSCs have been shown to have superior doubling times, easily expanded,[74] and are more homogenous.[75] Additionally, homing of PMSCs to sites of injury may be superior due to higher expression of VLA-4 which aids endothelium adherence.[76] PMSCs exert their therapeutic functions through cell-cell contact coupled with paracrine signaling factors. In addition, PMSCs have been shown to express programmed death ligand (PDL)-1 which can directly interact with PD-1 inhibitory molecule on T cells, inducing apoptosis and modulating Th-1, Th-17 and Th-2 responses.[77] Overall, PMSCs are an attractive therapeutic approach in comparison to other adult sources of MSCs.

PMSCs may be a more appropriate cell source for pediatric diseases given during pregnancy the placenta demonstrates “fetomaternal tolerance”, which is attributed to the expression of human leukocyte antigen-G (HLA-G), a non-classical MHC class I molecule that inhibits natural killer cell (NK) function.[78] Unlike bone marrow derived MSCs (BM-MSCs), PMSCs express HLA-G on their surface in response to IFNγ,[79] which is a key inflammatory mediator involved with the onset of MS.[80] Currently, a clinical trial is underway using term PMSCs for adult MS and no paradoxical worsening of MS lesion counts has been noted.[81]

Interestingly, pregnancy in MS patients has been known to effectively attenuate disease activity.[82] This suggests a possible unique role the placenta may play in modulating MS symptoms. A study by Vukusic et al. assessed the role of pregnancy on immunosuppression in MS patients and compared $CD4^+$ and $CD8^+$ T cell subsets in peripheral blood and the decidua. This study demonstrated elevated $CD4^+$ T cells in peripheral blood of non-pregnant MS patients compared to non-pregnant healthy controls. $CD4^+$ T cell populations increased in pregnant healthy controls in the second trimester of pregnancy, but in MS patients there was no increase or changes noted, thusCD4+ T cell levels were then comparable to controls. No changes in CD8+ T cell populations were noted in the peripheral blood. Tregs have been shown to be reduced and/or impaired in MS patients, and this study also showed decreased Tregs in peripheral blood of non-pregnant MS patients compared to non-pregnant controls. Once pregnant, Treg levels declined in healthy women, but in MS patients Treg levels were not altered through gestation. This data suggests that pregnancy can alter the function of Tregs and allow for more immunological tolerance. Furthermore, there is physiological immune regulation that occurs at the fetal-maternal interface that can act regardless of the pathological features of MS.[83] Another group demonstrated that pregnancy allows for the expansion of Treg clonotypes that can recognize EAE-associated antigen and can regulate autoreactive T cells.[84] These findings

suggest that the placenta may have key features that innately modulate immune responses in MS, and suggest that the placenta is an advantageous source of MSCs for the treatment of MS.

Not only is the placenta a unique source of MSCs, but it has also been shown that placenta derived EVs are involved in biological processes during pregnancy. Placenta-EVs can interact with several cell types and can inhibit NK cell cytotoxicity,[85] inhibit T cell proliferation,[86] and drive monocyte and macrophage polarization[87]. Placenta-EVs can be detected in maternal circulation at 6 weeks of pregnancy.[88] Placenta-EVs have been shown to mediate immunosuppression via transfer of exosomal proteins to T cells, which lead to T cell apoptosis, inhibition of T cell proliferation, induction of Treg population and reduction of T cell cytotoxicity.[89] This data further demonstrates a unique role the placenta can play in mediating immune responses, and further shows a role for EVs in modulating inflammatory responses and demyelination associated with MS.

Cell-based therapies can be limited by potential immune rejection of donor cells and lack of cellular homing to sites of injury.[10] Increasingly, studies have shown that MSC survival and integration within the host after transplantation are usually poor and that MSCs exert their therapeutic functions mainly via paracrine signaling mechanisms.[11] Recently, conditioned media of BM-MSCs was shown to protect neurons from apoptosis, activate macrophages and be pro-angiogenic.[90] However, the use of MSC conditioned media is limited in that the secreted protein factors are unstable, which creates technical difficulties for “off-the-shelf” clinical use. MSC derived EVs alternatively, are stable under long term storage conditions compared to freely secreted proteins and may serve as a superior source for cell-free therapy.[13]

1.5 Potential mechanism of action by PMSC-EVs for myelin regeneration

Growing evidence suggests that MSCs exert their therapeutic functions through the secretion of EVs. EVs are small nanovesicles, which can play an important role in intercellular

communication by transporting various functional molecules, including proteins, lipids, microRNAs, and mRNAs, all of which can regulate the behaviors of cellular targets.[91] MSC-derived EVs readily cross the BBB and deliver therapeutic cargo to reduce the effects of neuropathologic diseases, such as MS.[14] Several reports have demonstrated that MSC-derived EVs from adult tissue sources exhibit systemic immunomodulatory effects and can facilitate neurological recovery *in vitro.*[16,92] The functional properties of EVs are largely dependent on the parent cells from which they are produced. EVs can be derived from various tissues and cell types including but not limited to urine, plasma, saliva, tissues, cerebrospinal fluid and synovial fluid under physiological or pathological conditions.[93] EVs interact with target cells through internalization by endocytosis, direct membrane fusion or receptor-based induction of intracellular signaling pathways.[94] Target cell regulation can occur through the presence of certain surface proteins on EV membranes, or EV cargo such as miRNA which can modulate transcriptional activity of genes associated with biologic activity. Soluble factors packaged in EVs can play an important role in cell-cell interactions that lead to immune modulation and neuronal regeneration.[95] Moreover, EVs can also regulate mitochondrial transfer. Morrison et al. demonstrated that MSC-EVs promote an inflammatory phenotype in a lung injury model by mediating mitochondrial transfer to macrophages and promoting oxidative phosphorylation.[96] EVs are not only versatile in their composition but also immensely diverse in their mechanism of action.

The role of miRNA content in MSC-EVs has been suggested to be key modulators of myelin regeneration. miRNAs are small non-coding RNAs that can silence gene expression by inhibiting post-transcriptional activity or by inducing mRNA degradation.[97] Therefore, miRNAs can regulate cellular proliferation, differentiation and apoptosis in target cells, and have been shown to modulate inflammatory immune responses.[98] Dysregulation of miRNAs can lead to disrupted immune responses resulting in disease pathology. MSC-EV miRNAs taken up into target cells

can regulate gene activity through gene silencing leading to downregulation of protein secretion by immune cell subsets. The miRNA content in MSC-EVs can also promote remyelination by modulating inflammatory responses or by directly promoting oligodendrocyte differentiation and maturation. Specifically, human BM-MSC-EVs stimulated by IFNγ have been shown to improve motor function scores in EAE animals, reduce demyelination and neuroinflammation, as well as upregulate Treg populations in the spinal cord of EAE mice. Priming MSCs with IFNγ led to the identification of miR-467f and miR-466q as modulators of inflammatory responses in microglia cultures as they downregulate TNF and IL-1β expression.[98] These were among several other differentially expressed miRNAs in MSC-derived EVs, indicating the role of MSC-EVs in affecting neuroinflammation in EAE mice. MSC-EVs enriched in the miR-17-92 cluster exhibited enhanced oligodendrogenesis, neurogenesis and neuron remodeling, indicating the role of native miR-17-92 in promoting these myelination-related processes.[99] MSC-EVs were also shown to transfer miRNA-133b, which lead to increased branch length and number to primary cultured astrocytes and neurons.[100] Additionally, miRNA-133b from human umbilical cord MSCs can augment trophoblast cell proliferation and migration, thus suggesting a critical role in trophoblast development.[101] Interestingly, the miRNA profile from MSCs derived from differing tissue sources including umbilical cord,[102] bone marrow and adipose tissues is altered.[103] These findings suggest that the source of MSC-EVs and miRNA transfer will have unique interactions on target cells and potentially different clinical impacts.[104] These MSC-EVs were found to reduce inflammatory responses by changing gene expression in activated macrophages. PMSCs-EVs also contain miRNA-138,[17] which has been suggested to play a key role in maturation of OPCs to OLs.[105] However, many of these findings were made in models of stroke and diabetes, and the role of miRNA content in MSC-EVs has not yet been explored in the context of MS. Altogether, these findings attribute the immunomodulatory and neurotrophic effects of MSC-EVs at least in part to their native miRNA content. Identification and subsequent enrichment of these miRNA

represent a viable research direction for treating MS through the reduction of demyelination and promotion of remyelination.

PMSCs also contain numerous proangiogenic, immunomodulatory and neuroprotective proteins, that can play a critical role in promoting myelination in CNS disorders. Hepatocyte growth factor (HGF) is a pleiotropic factor shown to have neuronal and oligodendrocyte protective properties.[17,18,106] In the EAE rodent model of MS, overexpression of HGF by neurons conferred neuroprotection by reducing inflammation in the CNS and activation of Tregs.[106] HGF is secreted both in soluble form from MSCs and is also contained in exosomes; however, the effects of each form could lead to different cellular responses. It has been suggested that MSCs secrete multiple categories of exosomes, which are involved in differing cellular processes.[15] BM-MSC-EVs have been shown to modulate inflammatory responses, by increasing IL-10, TGFβ, PGE_2 and IL-6 secretions and driving a Treg phenotype and thus switching to a Th-2 responses in a murine model of diabetes.[107] Engineered Tregs in the EAE model of MS effectively localized to brain tissue and were able to reduce IL-12 and IFNγ mRNAs in the brain and reduce inflammatory markers resulting in diminished symptoms in treated animals.[108] Furthermore, MSC-EVs have been shown to inhibit immunoglobulin production of B cells,[109] and co-cultures with various immune cell subsets demonstrated that B lymphocytes preferentially take up MSC-EVs.[110]

Galectin-1 (gal-1) is another soluble protein expressed in PMSC-EVs that may play a critical role in modulating inflammation and neurodegeneration.[17] Recent reports have suggested a critical role for gal-1 in chronic inflammatory diseases.[111] Gal-1 is a multifunctional ~14 kDa monomeric protein, which can dimerize and is involved in many cellular functions, such as cell growth and migration regulation, adhesion, angiogenesis and embryonic and adult tissue development.[112] Interestingly, this protein also plays many key functions in the immune system,[113,114] and has been shown to facilitate anti-inflammatory processes and modulate the adaptive immune system.[113] Gal-1 has the potential to activate T lymphocyte apoptosis and

induce the differentiation Tregs.[113,114] Moreover, studies have also reported that gal-1 can promote phenotypic and functional changes after binding to microglia *in vitro.*[115] The mammalian protein can downregulate M1 microglia activation via the regulation of p38-, CREB- and NF-κB-dependent pathways and induce differentiation toward an M2 phenotype.[114] Macrophages are also involved in MS pathology as they contribute to tissue damage via the production of pro-inflammatory cytokines (TNF-α, IL-6, and IL-23).[116] Although no studies have assessed the effect of gal-1 on macrophage polarization, many publications have shown that those leukocytes respond to the same signals as microglia since they also undergo polarization toward an M2 or M1 phenotype in the presence of IL-4/13 or IFNγ/ lipopolysaccharide (LPS) respectively.[115] Overall, gal-1 expression on MSCs is a mediator of inflammation as it binds to microglia and downregulates M1 microglia activation. Further studies are needed, namely as it pertains to secreted MSC-EVs that share the same surface protein expression profile as their parent cells.

In the EAE model of MS, it was shown that murine BM-MSC-EVs express gal-1, PD-L1, and TGFβ which lead to reduced lymphocyte proliferation and induced secretion of IL-10 and TGFβ.[99] Using the BV-2 microglia cell line, it has been shown that BM-MSC-EVs modulate microglia activation through the MAPK signaling pathway, which will lead to reduction in pro-inflammatory gene expression, thus modulating CNS inflammation.[109,117] It was also demonstrated that MSC-EVs can polarize microglia to an M2 phenotype and upregulate modulatory proteins including IL-10 and TGFβ in the EAE model, which lead to resolution of clinical scores.[118] Additionally, priming of MSCs can result in altered cargo loading or expression to EVs, and is a valuable tool to evaluate the translational applications of PMSC-EVs in a diseased environment. Priming MSCs with IFNγ led to the identification of miR-467f and miR-466q as modulators of pro-inflammatory responses in microglia cultures by downregulating TNF and Il-1β expression.[98] Lipopolysaccharide stimulated umbilical cord MSC-EVs immunomodulatory properties have been shown to be mediated by miRNA let-7b.[119] Human BM-MSC-EVs stimulated by IFNγ have been

shown to improve motor function scores in EAE animals, reduce demyelination and neuroinflammation, upregulated Treg cells in the spinal cord of EAE mice.[120] Additionally, this group showed that BM-MSC-EVs reduced activated peripheral blood monocular cell proliferation and reduced Th-1 and Th-17 cytokines in vitro.[120] These studies are critical for mimicking the disease environment and can provide insights into alterations of molecular signaling mechanisms by MSC-EVs when used for disease intervention.

MSC-EV mediated anti-inflammatory properties are largely mediated by immunoregulatory miRNAs and immunomodulatory protein delivery in inflammatory immune cells (M1 macrophages, dendritic cells (DCs), CD4+Th-1 and Th-17 cells), enabling their phenotypic conversion into immunosuppressive M2 macrophages, tolerogenic DCs, and Tregs, respectively.[5] The placenta represents a novel source of MSCs that have unique properties for clinical applications to MS. PMSC-EVs have been shown to be neuroprotective, immunomodulatory and pro-angiogenic.[17,18,121] It was recently demonstrated that PMSCs and PMSCs-EVs could lead to symptom improvements in the EAE model of MS, which was due to the promotion of remyelination by the maturation of OPCs to mature OLs.[19] The promotion of remyelination by PMSC-EVs is due to interactions with multiple cellular targets that can modulate inflammatory responses preventing further reactivation of immune cell subsets and damage to myelin, as well as interactions with myelinating cells promote differentiation into mature phenotypes. While the multifunctional properties of PMSC-EVs make them a promising therapeutic agent to promote remyelination in MS, limitations exist on the use of native EV sources. Namely, *in vivo* EV biodistribution studies revealed that after systemic administration, a significant amount of EVs accumulated in the spleen and liver, while very few were detected in the CNS.[122] Therefore, while PMSC-EVs are a promising new approach to address the inflammatory and neurodegenerative component of MS, additional, new strategies can be employed for superior targeting and delivery of EV cargo to pathological target cells to promote remyelination.

1.6 Bioengineering Approaches for EVs to promote remyelination for MS

Recent work has been done to apply engineering principles to the design of EVs for increased therapeutic potential. These techniques can be categorized broadly into three approaches: parent cell engineering, miRNA loading, and surface targeting modifications. The role for these strategies and current practices of engineering MSC-EVs as they apply to MS and CNS disorders, as well as highlighted potential avenues for future studies are discussed below.

1.6.1 Parent cell engineering approaches

A variety of methods have been applied to alter the state of parent cells to induce specific EV features through canonical EV biogenesis pathways. One such method involves gene editing of parent cells by transfection. A range of cell types (BV2 microglia, HEK293T) have been infected with lentiviral plasmids to overexpress either surface proteins or miRNAs internally, which are similarly expressed on EV surfaces as well internally.[123] This method of cell engineering takes advantage of natural EV biogenesis pathways to generate engineered EVs for various applications. For example, miR-219a-5p has been overexpressed in HEK293T cells to obtain EVs that induced OPC differentiation for remyelination applications.[123]

Another method to engineer EVs through modifications to their parent cells is preconditioning. Parent cells are subjected to modified culture conditions to promote or suppress the expression of certain proteins in the subsequently produced EVs. This was done in the context of ischemic stroke in which BV2 microglia were polarized using IL-4 to differentially express angiogenin and therefore promote angiogenesis in C57BL/J mice.[124] Currently, no studies have been performed to precondition cells to obtain EVs for treating other CNS disorders including MS and Alzheimer's. Microglia have been preconditioned with stimulants such as LPS for the study and treatment of CNS disorders through their secreted EVs, which are implicated to regulate many of their neuroinflammatory functions.[125] Hypoxia preconditioning has been used MSCs to stimulate secretion of EVs promoting bone fracture healing, indicating the feasibility of such an approach.[126] Additionally, hypoxic preconditioning of MSCs has shown to improve MSC-EV

immunomodulatory properties, which can promote an anti-inflammatory phenotype and in turn preserve or promote myelination.[123] This represents a potential direction for future work, where preconditioned cells can secrete EVs with increased myelinating or neuromodulatory capabilities.

1.6.2 EV surface targeting modification approaches

EV surface engineering has also been heavily studied to develop targeted drug delivery vehicles for the treatment of several diseases. For example, many groups have successfully used Click chemistry to conjugate functional ligands of interest onto EVs. Click chemistry refers to a class of covalent reactions used in bioconjugation that is both procedurally simple and high yield. It has previously been shown that conjugation of the integrin ligand LLP2A to an electrospun scaffold for the recruitment of PMSC-EVs improves vascularization.[127] In the context of CNS disorders, Click chemistry has been used to conjugate peptides onto MSC-derived EVs to treat cerebral ischemic stroke.[128] This type of surface modification generally allows for improved targeting and increased uptake by cells of interest. It has already been established that the autoimmune component of MS pathology involves several cell types including T cells, B cells, astrocytes, oligodendrocytes and microglia. Pathogenetic cell types all represent viable targets for engineering of targeting ligands to EV surfaces.

Glycosylation of EV surface represents another active area of study, especially in the context of CNS disorders. Glycoproteins naturally present on cell and EV membranes and facilitate a variety of functions, such as cell-cell interactions and the immune response. By altering the composition of glycoproteins on the cell or EV surface, biodistribution and targeting behaviors can be changed. For instance, modification of surface glycosylation through neuraminidase treatment resulted in increased accumulation of EVs in the brain.[129] This allows EVs to more efficiently perform their naturally functions maintaining homeostasis, or to promote remyelination through their engineered material. While parent cell engineering represents a top-down approach for generating EVs, taking advantage of natural EV biogenesis pathways to promote remyelination, synthetic EV production involves a bottom-up method of creating EVs. Essentially, components

are combined to create a well-defined synthetic EV, allowing for control over membrane protein expression. Microfluidic platforms have previously been used for characterization and isolation of EVs, but now have been shown to be viable platforms for the generation of synthetic EVs. PDMS microfluidic chips specifically have successfully created synthetic surface engineered EVs expressing tumor peptides as candidates for cancer immunotherapy.[131] These EVs lack the compositional complexity of native EVs, but can be decorated with specific targeting moieties and cargo to induce OPC differentiation or promote remyelination.

An alternate method for synthetically engineering EV surfaces is through fusion of distinct membranes. Combining two separate membranes provides the benefit of increased cellular uptake due to the unique expression of surface proteins on the fused membrane. Membrane fusion has been done via extrusion to create synthetic EVs with a hybrid lipid membrane structure. For example, a library of lipids was fused with EVs using a membrane extrusion technique to generate synthetic EVs with significantly increased cellular uptake to lung cancer cells with subsequent gene silencing.[132] This represents a novel approach in which multiple combinations of lipid and cellular membranes can be performed to produce EVs with increased targeting potential. This platform technology has the potential to promote downstream myelination processes for MS through improved EV biodistribution. A summary of these approaches is shown in Figure 1.1.

1.6.3 Cargo loading into EVs

In addition to and often alongside surface modification of EVs, loading of exogenous cargo represents an increasingly studied method for engineering EVs to improve therapeutic outcomes. Of the several types of cargo used for loading EVs, miRNAs stand out as a commonly researched approach with the potential to promote various molecular processes to modulate inflammation and remyelination.

Loading miRNA in EVs represents a promising approach for the treatment of neurodegenerative diseases such as MS. As previously mentioned, neuroinflammation is a

prominent component of MS pathology, and is mediated in part by microglia. Therefore, regulating the immune response via microglia modulation can potentially restore the immune cell homeostasis in the CNS. In one study, dendritic cell EVs were loaded with miR-124 and were able to reduce the expression of inflammatory markers by microglia.[133] miRNA was loaded into the EVs through incubation and lead to decreased microglial activation in mice.[133] Due to the role of neuroinflammation in driving MS disease progression, the neuromodulatory behavior of miRNA-loaded EVs demonstrates the potential of miRNA loading for treatment of MS.

To date, loading miRNA into EVs to treat MS has not been performed; however, studies have shown the effects of miRNA delivery in promoting myelination and other pathological processes. EVs loaded with miR-210 successfully promoted angiogenesis and increased mice survival rate in a model of cerebral ischemia.[58] Parent MSCs have been transfected with the miR-17-92 cluster to produce EVs that promote oligodendrogenesis and neurogenesis.[99] These EVs were shown in a rat model to promote neurite remodeling, neural plasticity, and functional recovery. Many other miRNAs have been identified to promote remyelination, representing future research avenues loading them into EVs for efficacy studies. These include miR-219 and miR-338, which have been found to promote both myelin repair and myelination in the CNS.[134] This avenue of research has not been investigated in the context of myelination and MS but can allow for an alternative technique for EV engineering.

As a result, current engineering techniques allow for modification of EV surfaces and the contents inside EVs. This results in a cell-free drug delivery platform that can specifically target certain cells or tissues. In the context of MS, PMSC-EVs can be modified to exhibit increased targeting and uptake by cell types of interest, such as activated T cells, microglia, and oligodendrocytes.

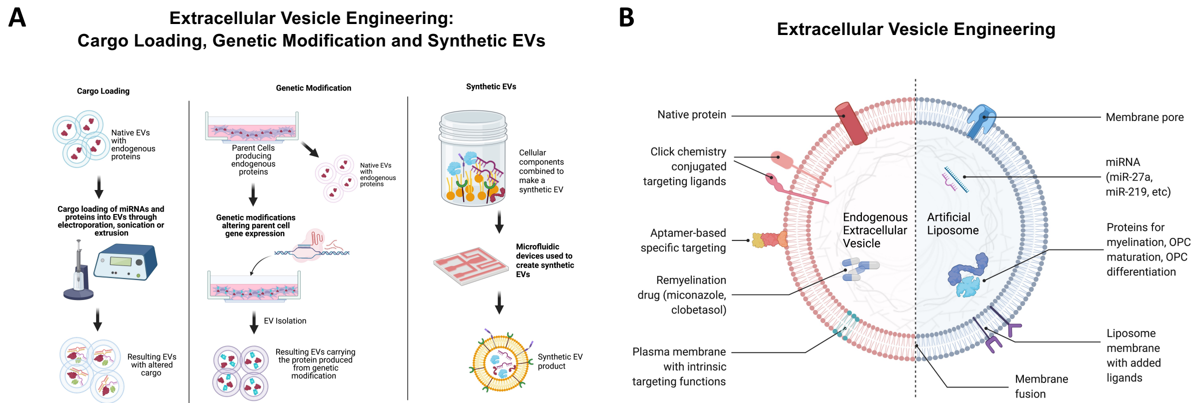

Figure 1.1. (A) Summary of bioengineering strategies to modify PMSC-EVs. One popular method of EV engineering is loading EVs with miRNA or protein cargo using techniques like electroporation, sonication or extrusion. Another approach is to genetically modify the parent cells to release customized EVs. Creating synthetic EVs using microfluidic devices is a more recent technique for engineering and customizing EVs. (B) EV modification approaches to promote remyelination. EV surfaces can be functionalized with biomolecules through membrane fusion or chemical modifications for targeting applications. Exogenous cargo, including remyelination drugs, miRNA and proteins can be loaded for remyelination applications.

1.7 Conclusion

In summary, MS is an autoimmune disorder of the CNS, largely characterized by an attack on the myelin sheath and subsequent loss of motor function. Recent studies have elucidated many of the processes contributing to and driving MS disease progression. As an autoimmune disease, lymphocytes have been heavily implicated in MS, specifically with increased pro-inflammatory T cell behavior, reduced T regulatory cell behavior, and disrupted T cell and B cell interactions. Leukocyte invasion and resident immune cell activation leads to reduced oligodendrocyte viability and ultimately the loss of protective myelin sheaths. Innate immune responses were found to be primarily driven by activated microglia, which in turn induce endoplasmic reticulum stress on oligodendrocytes to promote demyelination and degeneration.

In this immune-dominated environment, PMSCs represent a promising novel therapeutic agent because of their intrinsic immunomodulatory functions during fetal development.

Furthermore, secreted PMSC-EVs have been shown to play a significant role in neuroinflammation and myelin regeneration processes in the CNS. The mechanism of action has been proposed to involve specific components of secreted EVs, namely their surface protein expression and interior miRNA content. Not only have MSC-EVs been found to directly induce remyelinating processes such as neurogenesis and oligodendrogenesis, but both miRNA and proteins have been shown to be critical mediators of MSC-EVs inflammatory, neuroprotective and neuroregenerative properties. This involves driving an immuno-regulatory phenotype in immune cell subsets as well as promoting the secretion of anti-inflammatory cytokines. Recent studies have also shown strong potential for PMSC-EVs to be applied to MS research. Understanding the role of PMSC and native PMSC-EVs on CNS cell populations and the interface of oligodendroglia-axonal interactions and accompanying neuronal degeneration will be warranted in future studies. These studies demonstrate the potential of PMSC-EVs in both modulating upstream immune responses as well as promoting remyelinating processes.

Currently, many MSC and MSC-EV studies employ the intravenous administration strategy to treat MS. While optimization of intervention strategies is warranted, engineering approaches can serve as advantageous tools for superior targeting to pathologic cell types and lesions associated with neurodegenerative disease. Multiple engineering techniques previously established for EV engineering in different disease applications represent promising avenues of future research applying engineered PMSC-EVs for MS treatment. In tandem with the therapeutic properties of PMSCs and the native content of PMSC-EVs, bioengineered PMSC-EVs could allow for the development of novel MS treatments affecting a significant and therapy-lacking population.

1.8 References

[1] Dilokthornsakul, P. *et al.* Multiple sclerosis prevalence in the United States commercially insured population. *Neurology* **86**, 1014-1021, doi:10.1212/wnl.0000000000002469 (2016).

[2] Guan, Y., Jakimovski, D., Ramanathan, M., Weinstock-Guttman, B. & Zivadinov, R. The role of Epstein-Barr virus in multiple sclerosis: from molecular pathophysiology to in vivo imaging. *Neural Regen Res* **14**, 373-386, doi:10.4103/1673-5374.245462 (2019).

[3] Scazzone, C., Agnello, L., Bivona, G., Lo Sasso, B. & Ciaccio, M. Vitamin D and Genetic Susceptibility to Multiple Sclerosis. *Biochem Genet* **59**, 1-30, doi:10.1007/s10528-020-10010-1 (2021).

[4] Chisari, C. G. *et al.* An update on the safety of treating relapsing-remitting multiple sclerosis. *Expert Opin Drug Saf* **18**, 925-948, doi:10.1080/14740338.2019.1658741 (2019).

[5] Harrell, C. R., Jovicic, N., Djonov, V., Arsenijevic, N. & Volarevic, V. Mesenchymal Stem Cell-Derived Exosomes and Other Extracellular Vesicles as New Remedies in the Therapy of Inflammatory Diseases. *Cells* **8**, doi:10.3390/cells8121605 (2019).

[6] Cohen, J. A. *et al.* Pilot trial of intravenous autologous culture-expanded mesenchymal stem cell transplantation in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)*, 1352458517703802, doi:10.1177/1352458517703802 (2017).

[7] Yamout, B. *et al.* Bone marrow mesenchymal stem cell transplantation in patients with multiple sclerosis: a pilot study. *Journal of neuroimmunology* **227**, 185-189, doi:10.1016/j.jneuroim.2010.07.013 (2010).

[8] Abumaree, M. H., Abomaray, F. M., Alshabibi, M. A., AlAskar, A. S. & Kalionis, B. Immunomodulatory properties of human placental mesenchymal stem/stromal cells. *Placenta*, doi:10.1016/j.placenta.2017.04.003 (2017).

[9] Lee, J. M. *et al.* Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. *Int Immunopharmacol* **13**, 219-224, doi:10.1016/j.intimp.2012.03.024 (2012).

[10] Munir, H. & McGettrick, H. M. Mesenchymal Stem Cell Therapy for Autoimmune Disease: Risks and Rewards. *Stem Cells Dev* **24**, 2091-2100, doi:10.1089/scd.2015.0008 (2015).

[11] Kim, H. J., Lee, J. H. & Kim, S. H. Therapeutic effects of human mesenchymal stem cells on traumatic brain injury in rats: secretion of neurotrophic factors and inhibition of apoptosis. *Journal of neurotrauma* **27**, 131-138, doi:10.1089/neu.2008-0818 10.1089/neu.2008.0818 (2010).

[12] Selmaj, I., Mycko, M. P., Raine, C. S. & Selmaj, K. W. The role of exosomes in CNS inflammation and their involvement in multiple sclerosis. *Journal of neuroimmunology* **306**, 1-10, doi:10.1016/j.jneuroim.2017.02.002 (2017).

[13] Maumus, M., Rozier, P., Boulestreau, J., Jorgensen, C. & Noel, D. Mesenchymal Stem Cell-Derived Extracellular Vesicles: Opportunities and Challenges for Clinical Translation. *Front Bioeng Biotechnol* **8**, 997, doi:10.3389/fbioe.2020.00997 (2020).

[14] Andras, I. E. & Toborek, M. Extracellular vesicles of the blood-brain barrier. *Tissue Barriers* **4**, e1131804, doi:10.1080/21688370.2015.1131804 (2016).

[15] Beretti, F. *et al.* Amniotic fluid stem cell exosomes: Therapeutic perspective. *Biofactors* **44**, 158-167, doi:10.1002/biof.1407 (2018).

[16] Bonafede, R. *et al.* Exosome derived from murine adipose-derived stromal cells: Neuroprotective effect on in vitro model of amyotrophic lateral sclerosis. *Exp Cell Res* **340**, 150-158, doi:10.1016/j.yexcr.2015.12.009 (2016).

[17] Kumar, P. *et al.* Neuroprotective effect of placenta-derived mesenchymal stromal cells: role of exosomes. *FASEB J* **33**, 5836-5849, doi:10.1096/fj.201800972R (2019).

[18] Komaki, M. *et al.* Exosomes of human placenta-derived mesenchymal stem cells stimulate angiogenesis. *Stem Cell Res Ther* **8**, 219, doi:10.1186/s13287-017-0660-9 (2017).
[19] Clark, K. *et al.* Placental Mesenchymal Stem Cell-Derived Extracellular Vesicles Promote Myelin Regeneration in an Animal Model of Multiple Sclerosis. *Cells* **8**, doi:10.3390/cells8121497 (2019).
[20] Jelcic, I. *et al.* Memory B Cells Activate Brain-Homing, Autoreactive CD4(+) T Cells in Multiple Sclerosis. *Cell* **175**, 85-100 e123, doi:10.1016/j.cell.2018.08.011 (2018).
[21] Baiula, M., Spampinato, S., Gentilucci, L. & Tolomelli, A. Novel Ligands Targeting alpha4beta1 Integrin: Therapeutic Applications and Perspectives. *Front Chem* **7**, 489, doi:10.3389/fchem.2019.00489 (2019).
[22] Ransohoff, R. M., Kivisakk, P. & Kidd, G. Three or more routes for leukocyte migration into the central nervous system. *Nat Rev Immunol* **3**, 569-581, doi:10.1038/nri1130 (2003).
[23] Gutcher, I. & Becher, B. APC-derived cytokines and T cell polarization in autoimmune inflammation. *J Clin Invest* **117**, 1119-1127, doi:10.1172/JCI31720 (2007).
[24] Prajeeth, C. K. *et al.* Effector molecules released by Th1 but not Th17 cells drive an M1 response in microglia. *Brain Behav Immun* **37**, 248-259, doi:10.1016/j.bbi.2014.01.001 (2014).
[25] Reboldi, A. *et al.* C-C chemokine receptor 6-regulated entry of TH-17 cells into the CNS through the choroid plexus is required for the initiation of EAE. *Nat Immunol* **10**, 514-523, doi:10.1038/ni.1716 (2009).
[26] Kebir, H. *et al.* Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* **13**, 1173-1175, doi:10.1038/nm1651 (2007).
[27] Annunziato, F. *et al.* Phenotypic and functional features of human Th17 cells. *J Exp Med* **204**, 1849-1861, doi:10.1084/jem.20070663 (2007).
[28] Duhen, R. *et al.* Cutting edge: the pathogenicity of IFN-gamma-producing Th17 cells is independent of T-bet. *J Immunol* **190**, 4478-4482, doi:10.4049/jimmunol.1203172 (2013).
[29] Elain, G., Jeanneau, K., Rutkowska, A., Mir, A. K. & Dev, K. K. The selective anti-IL17A monoclonal antibody secukinumab (AIN457) attenuates IL17A-induced levels of IL6 in human astrocytes. *Glia* **62**, 725-735, doi:10.1002/glia.22637 (2014).
[30] Kang, Z. *et al.* Astrocyte-restricted ablation of interleukin-17-induced Act1-mediated signaling ameliorates autoimmune encephalomyelitis. *Immunity* **32**, 414-425, doi:10.1016/j.immuni.2010.03.004 (2010).
[31] Kang, Z. *et al.* Act1 mediates IL-17-induced EAE pathogenesis selectively in NG2+ glial cells. *Nature neuroscience* **16**, 1401-1408, doi:10.1038/nn.3505 (2013).
[32] Paintlia, M. K., Paintlia, A. S., Singh, A. K. & Singh, I. Synergistic activity of interleukin-17 and tumor necrosis factor-alpha enhances oxidative stress-mediated oligodendrocyte apoptosis. *J Neurochem* **116**, 508-521, doi:10.1111/j.1471-4159.2010.07136.x (2011).
[33] Dulamea, A. O. Role of Oligodendrocyte Dysfunction in Demyelination, Remyelination and Neurodegeneration in Multiple Sclerosis. *Adv Exp Med Biol* **958**, 91-127, doi:10.1007/978-3-319-47861-6_7 (2017).
[34] Venken, K. *et al.* Secondary progressive in contrast to relapsing-remitting multiple sclerosis patients show a normal CD4+CD25+ regulatory T-cell function and FOXP3 expression. *J Neurosci Res* **83**, 1432-1446, doi:10.1002/jnr.20852 (2006).
[35] Stephens, L. A., Malpass, K. H. & Anderton, S. M. Curing CNS autoimmune disease with myelin-reactive Foxp3+ Treg. *Eur J Immunol* **39**, 1108-1117, doi:10.1002/eji.200839073 (2009).
[36] Dombrowski, Y. *et al.* Regulatory T cells promote myelin regeneration in the central nervous system. *Nature neuroscience* **20**, 674-680, doi:10.1038/nn.4528 (2017).
[37] Correale, J. & Villa, A. Role of CD8+ CD25+ Foxp3+ regulatory T cells in multiple sclerosis. *Ann Neurol* **67**, 625-638, doi:10.1002/ana.21944 (2010).

[38] Jamshidian, A., Shaygannejad, V., Pourazar, A., Zarkesh-Esfahani, S. H. & Gharagozloo, M. Biased Treg/Th17 balance away from regulatory toward inflammatory phenotype in relapsed multiple sclerosis and its correlation with severity of symptoms. *Journal of neuroimmunology* **262**, 106-112, doi:10.1016/j.jneuroim.2013.06.007 (2013).

[39] Kimura, K. *et al.* Disrupted balance of T cells under natalizumab treatment in multiple sclerosis. *Neurol Neuroimmunol Neuroinflamm* **3**, e210, doi:10.1212/NXI.0000000000000210 (2016).

[40] Astier, A. L., Meiffren, G., Freeman, S. & Hafler, D. A. Alterations in CD46-mediated Tr1 regulatory T cells in patients with multiple sclerosis. *J Clin Invest* **116**, 3252-3257, doi:10.1172/JCI29251 (2006).

[41] Gobin, S. J. *et al.* Upregulation of transcription factors controlling MHC expression in multiple sclerosis lesions. *Glia* **36**, 68-77, doi:10.1002/glia.1096 (2001).

[42] Tzartos, J. S. *et al.* Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. *Am J Pathol* **172**, 146-155, doi:10.2353/ajpath.2008.070690 (2008).

[43] Elong Ngono, A. *et al.* Frequency of circulating autoreactive T cells committed to myelin determinants in relapsing-remitting multiple sclerosis patients. *Clin Immunol* **144**, 117-126, doi:10.1016/j.clim.2012.05.009 (2012).

[44] Crawford, M. P. *et al.* High prevalence of autoreactive, neuroantigen-specific CD8+ T cells in multiple sclerosis revealed by novel flow cytometric assay. *Blood* **103**, 4222-4231, doi:10.1182/blood-2003-11-4025 (2004).

[45] Baughman, E. J. *et al.* Neuroantigen-specific CD8+ regulatory T-cell function is deficient during acute exacerbation of multiple sclerosis. *J Autoimmun* **36**, 115-124, doi:10.1016/j.jaut.2010.12.003 (2011).

[46] Cunnusamy, K. *et al.* Disease exacerbation of multiple sclerosis is characterized by loss of terminally differentiated autoregulatory CD8+ T cells. *Clin Immunol* **152**, 115-126, doi:10.1016/j.clim.2014.03.005 (2014).

[47] Bitsch, A., Schuchardt, J., Bunkowski, S., Kuhlmann, T. & Bruck, W. Acute axonal injury in multiple sclerosis. Correlation with demyelination and inflammation. *Brain* **123 (Pt 6)**, 1174-1183, doi:10.1093/brain/123.6.1174 (2000).

[48] Neumann, H., Medana, I. M., Bauer, J. & Lassmann, H. Cytotoxic T lymphocytes in autoimmune and degenerative CNS diseases. *Trends Neurosci* **25**, 313-319, doi:10.1016/s0166-2236(02)02154-9 (2002).

[49] Disanto, G., Morahan, J. M., Barnett, M. H., Giovannoni, G. & Ramagopalan, S. V. The evidence for a role of B cells in multiple sclerosis. *Neurology* **78**, 823-832, doi:10.1212/WNL.0b013e318249f6f0 (2012).

[50] Kothur, K. *et al.* B Cell, Th17, and Neutrophil Related Cerebrospinal Fluid Cytokine/Chemokines Are Elevated in MOG Antibody Associated Demyelination. *PLoS One* **11**, e0149411, doi:10.1371/journal.pone.0149411 (2016).

[51] van Langelaar, J., Rijvers, L., Smolders, J. & van Luijn, M. M. B and T Cells Driving Multiple Sclerosis: Identity, Mechanisms and Potential Triggers. *Front Immunol* **11**, 760, doi:10.3389/fimmu.2020.00760 (2020).

[52] Ransohoff, R. M. How neuroinflammation contributes to neurodegeneration. *Science* **353**, 777-783, doi:10.1126/science.aag2590 (2016).

[53] Friese, M. A., Schattling, B. & Fugger, L. Mechanisms of neurodegeneration and axonal dysfunction in multiple sclerosis. *Nat Rev Neurol* **10**, 225-238, doi:10.1038/nrneurol.2014.37 (2014).

[54] Lucchinetti, C. F. *et al.* Inflammatory cortical demyelination in early multiple sclerosis. *N Engl J Med* **365**, 2188-2197, doi:10.1056/NEJMoa1100648 (2011).

[55] Ponomarev, E. D., Shriver, L. P., Maresz, K. & Dittel, B. N. Microglial cell activation and proliferation precedes the onset of CNS autoimmunity. *J Neurosci Res* **81**, 374-389, doi:10.1002/jnr.20488 (2005).

[56] Chabot, S., Williams, G. & Yong, V. W. Microglial production of TNF-alpha is induced by activated T lymphocytes. Involvement of VLA-4 and inhibition by interferonbeta-1b. *J Clin Invest* **100**, 604-612, doi:10.1172/JCI119571 (1997).

[57] Zhao, X. *et al.* MicroRNA-mediated control of oligodendrocyte differentiation. *Neuron* **65**, 612-626, doi:10.1016/j.neuron.2010.02.018 (2010).

[58] Zhang, H. *et al.* Exosome-mediated targeted delivery of miR-210 for angiogenic therapy after cerebral ischemia in mice. *J Nanobiotechnology* **17**, 29, doi:10.1186/s12951-019-0461-7 (2019).

[59] Kuhlmann, T. *et al.* Differentiation block of oligodendroglial progenitor cells as a cause for remyelination failure in chronic multiple sclerosis. *Brain* **131**, 1749-1758, doi:10.1093/brain/awn096 (2008).

[60] Tirotta, E., Kirby, L. A., Hatch, M. N. & Lane, T. E. IFN-gamma-induced apoptosis of human embryonic stem cell derived oligodendrocyte progenitor cells is restricted by CXCR2 signaling. *Stem Cell Res* **9**, 208-217, doi:10.1016/j.scr.2012.06.005 (2012).

[61] Wang, C. *et al.* IL-17 induced NOTCH1 activation in oligodendrocyte progenitor cells enhances proliferation and inflammatory gene expression. *Nat Commun* **8**, 15508, doi:10.1038/ncomms15508 (2017).

[62] Bonora, M. *et al.* Tumor necrosis factor-alpha impairs oligodendroglial differentiation through a mitochondria-dependent process. *Cell Death Differ* **21**, 1198-1208, doi:10.1038/cdd.2014.35 (2014).

[63] Kirby, L. *et al.* Oligodendrocyte precursor cells present antigen and are cytotoxic targets in inflammatory demyelination. *Nat Commun* **10**, 3887, doi:10.1038/s41467-019-11638-3 (2019).

[64] Prineas, J. W. & Connell, F. Remyelination in multiple sclerosis. *Ann Neurol* **5**, 22-31, doi:10.1002/ana.410050105 (1979).

[65] Jang, M. J. *et al.* Placenta-derived mesenchymal stem cells have an immunomodulatory effect that can control acute graft-versus-host disease in mice. *Acta haematologica* **129**, 197-206, doi:10.1159/000345267 (2013).

[66] Fan, L. *et al.* Interaction between Mesenchymal Stem Cells and B-Cells. *International journal of molecular sciences* **17**, doi:10.3390/ijms17050650 (2016).

[67] Petri, R. M. *et al.* Activated Tissue-Resident Mesenchymal Stromal Cells Regulate Natural Killer Cell Immune and Tissue-Regenerative Function. *Stem cell reports* **9**, 985-998, doi:10.1016/j.stemcr.2017.06.020 (2017).

[68] Shin, T. H. *et al.* Human umbilical cord blood-stem cells direct macrophage polarization and block inflammasome activation to alleviate rheumatoid arthritis. *Cell death & disease* **7**, e2524, doi:10.1038/cddis.2016.442 (2016).

[69] Marti, L. C., Ribeiro, A. A. & Hamerschlak, N. Immunomodulatory effect of mesenchymal stem cells. *Einstein (Sao Paulo, Brazil)* **9**, 224-228, doi:10.1590/s1679-45082011rw1843 (2011).

[70] Kovach, T. K., Dighe, A. S., Lobo, P. I. & Cui, Q. Interactions between MSCs and immune cells: implications for bone healing. *Journal of immunology research* **2015**, 752510, doi:10.1155/2015/752510 (2015).

[71] Yang, H. *et al.* Umbilical cord-derived mesenchymal stem cells reversed the suppressive deficiency of T regulatory cells from peripheral blood of patients with multiple sclerosis in a co-culture - a preliminary study. *Oncotarget* **7**, 72537-72545, doi:10.18632/oncotarget.12345 (2016).

[72] Parolini, O. *et al.* Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* **26**, 300-311, doi:10.1634/stemcells.2007-0594 (2008).
[73] Portmann-Lanz, C. B. *et al.* Turning placenta into brain: placental mesenchymal stem cells differentiate into neurons and oligodendrocytes. *Am J Obstet Gynecol* **202**, 294 e291-294 e211, doi:10.1016/j.ajog.2009.10.893 (2010).
[74] Heo, J. S., Choi, Y., Kim, H. S. & Kim, H. O. Comparison of molecular profiles of human mesenchymal stem cells derived from bone marrow, umbilical cord blood, placenta and adipose tissue. *Int J Mol Med* **37**, 115-125, doi:10.3892/ijmm.2015.2413 (2016).
[75] Barlow, S. *et al.* Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. *Stem Cells Dev* **17**, 1095-1107, doi:10.1089/scd.2007.0154 (2008).
[76] Karlsson, H. *et al.* Stromal cells from term fetal membrane are highly suppressive in allogeneic settings in vitro. *Clin Exp Immunol* **167**, 543-555, doi:10.1111/j.1365-2249.2011.04540.x (2012).
[77] D'Addio, F. *et al.* The link between the PDL1 costimulatory pathway and Th17 in fetomaternal tolerance. *J Immunol* **187**, 4530-4541, doi:10.4049/jimmunol.1002031 (2011).
[78] Alegre, E. & Rizzo, R. Some basic aspects of HLA-G biology. **2014**, 657625, doi:10.1155/2014/657625 (2014).
[79] Liu, K. J. *et al.* Surface expression of HLA-G is involved in mediating immunomodulatory effects of placenta-derived multipotent cells (PDMCs) towards natural killer lymphocytes. *Cell transplantation* **20**, 1721-1730, doi:10.3727/096368911x580590 (2011).
[80] Martino, G. & Hartung, H. P. Immunopathogenesis of multiple sclerosis: the role of T cells. *Current opinion in neurology* **12**, 309-321 (1999).
[81] Lublin, F. D. *et al.* Human placenta-derived cells (PDA-001) for the treatment of adults with multiple sclerosis: a randomized, placebo-controlled, multiple-dose study. *Mult Scler Relat Disord* **3**, 696-704, doi:10.1016/j.msard.2014.08.002 (2014).
[82] Vukusic, S. *et al.* Pregnancy and multiple sclerosis (the PRIMS study): clinical predictors of post-partum relapse. *Brain* **127**, 1353-1360, doi:10.1093/brain/awh152 (2004).
[83] Spadaro, M. *et al.* Immunomodulatory Effect of Pregnancy on Leukocyte Populations in Patients With Multiple Sclerosis: A Comparison of Peripheral Blood and Decidual Placental Tissue. *Front Immunol* **10**, 1935, doi:10.3389/fimmu.2019.01935 (2019).
[84] Engler, J. B., Heckmann, N. F., Jager, J., Gold, S. M. & Friese, M. A. Pregnancy Enables Expansion of Disease-Specific Regulatory T Cells in an Animal Model of Multiple Sclerosis. *J Immunol* **203**, 1743-1752, doi:10.4049/jimmunol.1900611 (2019).
[85] Mincheva-Nilsson, L. *et al.* Placenta-derived soluble MHC class I chain-related molecules down-regulate NKG2D receptor on peripheral blood mononuclear cells during human pregnancy: a possible novel immune escape mechanism for fetal survival. *J Immunol* **176**, 3585-3592, doi:10.4049/jimmunol.176.6.3585 (2006).
[86] Stenqvist, A. C., Nagaeva, O., Baranov, V. & Mincheva-Nilsson, L. Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells, suggesting exosome-mediated immune privilege of the fetus. *J Immunol* **191**, 5515-5523, doi:10.4049/jimmunol.1301885 (2013).
[87] Atay, S., Gercel-Taylor, C., Suttles, J., Mor, G. & Taylor, D. D. Trophoblast-derived exosomes mediate monocyte recruitment and differentiation. *Am J Reprod Immunol* **65**, 65-77, doi:10.1111/j.1600-0897.2010.00880.x (2011).
[88] Sarker, S. *et al.* Placenta-derived exosomes continuously increase in maternal circulation over the first trimester of pregnancy. *J Transl Med* **12**, 204, doi:10.1186/1479-5876-12-204 (2014).

[89] Bai, L. *et al.* Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nature neuroscience* **15**, 862-870, doi:10.1038/nn.3109 (2012).
[90] Cantinieaux, D. *et al.* Conditioned Medium from Bone Marrow-Derived Mesenchymal Stem Cells Improves Recovery after Spinal Cord Injury in Rats: An Original Strategy to Avoid Cell Transplantation. *PLOS ONE* **8**, e69515, doi:10.1371/journal.pone.0069515 (2013).
[91] Phan, J. *et al.* Engineering mesenchymal stem cells to improve their exosome efficacy and yield for cell-free therapy. *J Extracell Vesicles* **7**, 1522236, doi:10.1080/20013078.2018.1522236 (2018).
[92] Fathollahi, A. *et al.* Intranasal administration of small extracellular vesicles derived from mesenchymal stem cells ameliorated the experimental autoimmune encephalomyelitis. *Int Immunopharmacol* **90**, 107207, doi:10.1016/j.intimp.2020.107207 (2021).
[93] Street, J. M. *et al.* Identification and proteomic profiling of exosomes in human cerebrospinal fluid. *J Transl Med* **10**, 5, doi:10.1186/1479-5876-10-5 (2012).
[94] Kurian, N. K. & Modi, D. Extracellular vesicle mediated embryo-endometrial cross talk during implantation and in pregnancy. *Journal of assisted reproduction and genetics* **36**, 189-198, doi:10.1007/s10815-018-1343-x (2019).
[95] Fayazi, N., Sheykhhasan, M., Soleimani Asl, S. & Najafi, R. Stem Cell-Derived Exosomes: a New Strategy of Neurodegenerative Disease Treatment. *Mol Neurobiol*, doi:10.1007/s12035-021-02324-x (2021).
[96] Morrison, T. J. *et al.* Mesenchymal Stromal Cells Modulate Macrophages in Clinically Relevant Lung Injury Models by Extracellular Vesicle Mitochondrial Transfer. *Am J Respir Crit Care Med* **196**, 1275-1286, doi:10.1164/rccm.201701-0170OC (2017).
[97] Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233, doi:10.1016/j.cell.2009.01.002 (2009).
[98] Giunti, D. *et al.* Role of miRNAs shuttled by mesenchymal stem cell-derived small extracellular vesicles in modulating neuroinflammation. *Sci Rep* **11**, 1740, doi:10.1038/s41598-021-81039-4 (2021).
[99] Xin, H. *et al.* MicroRNA cluster miR-17-92 Cluster in Exosomes Enhance Neuroplasticity and Functional Recovery After Stroke in Rats. *Stroke* **48**, 747-753, doi:10.1161/STROKEAHA.116.015204 (2017).
[100] Qiu, G. *et al.* Mesenchymal stem cell-derived extracellular vesicles affect disease outcomes via transfer of microRNAs. *Stem Cell Res Ther* **9**, 320, doi:10.1186/s13287-018-1069-9 (2018).
[101] Wang, D., Na, Q., Song, G. Y. & Wang, L. Human umbilical cord mesenchymal stem cell-derived exosome-mediated transfer of microRNA-133b boosts trophoblast cell proliferation, migration and invasion in preeclampsia by restricting SGK1. *Cell Cycle* **19**, 1869-1883, doi:10.1080/15384101.2020.1769394 (2020).
[102] Fang, S. *et al.* Umbilical Cord-Derived Mesenchymal Stem Cell-Derived Exosomal MicroRNAs Suppress Myofibroblast Differentiation by Inhibiting the Transforming Growth Factor-beta/SMAD2 Pathway During Wound Healing. *Stem Cells Transl Med* **5**, 1425-1439, doi:10.5966/sctm.2015-0367 (2016).
[103] Baglio, S. R. *et al.* Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. *Stem Cell Res Ther* **6**, 127, doi:10.1186/s13287-015-0116-z (2015).
[104] Xu, H. K., Chen, L. J., Zhou, S. N., Li, Y. F. & Xiang, C. Multifunctional role of microRNAs in mesenchymal stem cell-derived exosomes in treatment of diseases. *World J Stem Cells* **12**, 1276-1294, doi:10.4252/wjsc.v12.i11.1276 (2020).

[105] Fitzpatrick, J. M., Anderson, R. C. & McDermott, K. W. MicroRNA: Key regulators of oligodendrocyte development and pathobiology. *Int J Biochem Cell Biol* **65**, 134-138, doi:10.1016/j.biocel.2015.05.021 (2015).

[106] Benkhoucha, M. *et al.* Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* **107**, 6424-6429, doi:10.1073/pnas.0912437107 (2010).

[107] Favaro, E. *et al.* Human mesenchymal stem cells and derived extracellular vesicles induce regulatory dendritic cells in type 1 diabetic patients. *Diabetologia* **59**, 325-333, doi:10.1007/s00125-015-3808-0 (2016).

[108] Fransson, M. *et al.* CAR/FoxP3-engineered T regulatory cells target the CNS and suppress EAE upon intranasal delivery. *J Neuroinflammation* **9**, 112, doi:10.1186/1742-2094-9-112 (2012).

[109] Chen, W. *et al.* Immunomodulatory effects of mesenchymal stromal cells-derived exosome. *Immunol Res* **64**, 831-840, doi:10.1007/s12026-016-8798-6 (2016).

[110] Di Trapani, M. *et al.* Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions. *Sci Rep* **6**, 24120, doi:10.1038/srep24120 (2016).

[111] Sundblad, V., Morosi, L. G., Geffner, J. R. & Rabinovich, G. A. Galectin-1: A Jack-of-All-Trades in the Resolution of Acute and Chronic Inflammation. *J Immunol* **199**, 3721-3730, doi:10.4049/jimmunol.1701172 (2017).

[112] Cedeno-Laurent, F. & Dimitroff, C. J. Galectin-1 research in T cell immunity: past, present and future. *Clin Immunol* **142**, 107-116, doi:10.1016/j.clim.2011.09.011 (2012).

[113] Camby, I., Le Mercier, M., Lefranc, F. & Kiss, R. Galectin-1: a small protein with major functions. *Glycobiology* **16**, 137r-157r, doi:10.1093/glycob/cwl025 (2006).

[114] Starossom, S. C. *et al.* Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. *Immunity* **37**, 249-263, doi:10.1016/j.immuni.2012.05.023 (2012).

[115] Nakagawa, Y. & Chiba, K. Role of microglial m1/m2 polarization in relapse and remission of psychiatric disorders and diseases. *Pharmaceuticals (Basel, Switzerland)* **7**, 1028-1048, doi:10.3390/ph7121028 (2014).

[116] Vogel, D. Y. *et al.* Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *J Neuroinflammation* **10**, 35, doi:10.1186/1742-2094-10-35 (2013).

[117] Jaimes, Y., Naaldijk, Y., Wenk, K., Leovsky, C. & Emmrich, F. Mesenchymal Stem Cell-Derived Microvesicles Modulate Lipopolysaccharides-Induced Inflammatory Responses to Microglia Cells. *Stem Cells* **35**, 812-823, doi:10.1002/stem.2541 (2017).

[118] Li, Z. *et al.* Exosomes derived from mesenchymal stem cells attenuate inflammation and demyelination of the central nervous system in EAE rats by regulating the polarization of microglia. *Int Immunopharmacol* **67**, 268-280, doi:10.1016/j.intimp.2018.12.001 (2019).

[119] Ti, D. *et al.* LPS-preconditioned mesenchymal stromal cells modify macrophage polarization for resolution of chronic inflammation via exosome-shuttled let-7b. *J Transl Med* **13**, 308, doi:10.1186/s12967-015-0642-6 (2015).

[120] Riazifar, M. *et al.* Stem Cell-Derived Exosomes as Nanotherapeutics for Autoimmune and Neurodegenerative Disorders. *ACS Nano* **13**, 6670-6688, doi:10.1021/acsnano.9b01004 (2019).

[121] Abumaree, M. H., Abomaray, F. M., Alshabibi, M. A., AlAskar, A. S. & Kalionis, B. Immunomodulatory properties of human placental mesenchymal stem/stromal cells. *Placenta* **59**, 87-95, doi:10.1016/j.placenta.2017.04.003 (2017).

[122] Wiklander, O. P. *et al.* Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *J Extracell Vesicles* **4**, 26316, doi:10.3402/jev.v4.26316 (2015).

[123] Andrews, S., Maughon, T., Marklein, R. & Stice, S. Priming of MSCs with inflammation-relevant signals affects extracellular vesicle biogenesis, surface markers, and modulation of T cell subsets. *Journal of Immunology and Regenerative Medicine* **13**, 100036, doi:https://doi.org/10.1016/j.regen.2020.100036 (2021).

[124] Tian, Y. *et al.* IL-4-polarized BV2 microglia cells promote angiogenesis by secreting exosomes. *Advances in clinical and experimental medicine : official organ Wroclaw Medical University* **28**, 421-430, doi:10.17219/acem/91826 (2019).

[125] Chen, Z. *et al.* Lipopolysaccharide-induced microglial activation and neuroprotection against experimental brain injury is independent of hematogenous TLR4. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **32**, 11706-11715, doi:10.1523/JNEUROSCI.0730-12.2012 (2012).

[126] Liu, W. *et al.* Hypoxic mesenchymal stem cell-derived exosomes promote bone fracture healing by the transfer of miR-126. *Acta Biomater* **103**, 196-212, doi:10.1016/j.actbio.2019.12.020 (2020).

[127] Hao, D. *et al.* Extracellular Matrix Mimicking Nanofibrous Scaffolds Modified With Mesenchymal Stem Cell-Derived Extracellular Vesicles for Improved Vascularization. *Front Bioeng Biotechnol* **8**, 633, doi:10.3389/fbioe.2020.00633 (2020).

[128] Tian, T. *et al.* Targeted delivery of neural progenitor cell-derived extracellular vesicles for anti-inflammation after cerebral ischemia. *Theranostics* **11**, 6507-6521, doi:10.7150/thno.56367 (2021).

[129] Royo, F., Cossío, U., Ruiz de Angulo, A., Llop, J. & Falcon-Perez, J. M. Modification of the glycosylation of extracellular vesicles alters their biodistribution in mice. *Nanoscale* **11**, 1531-1537, doi:10.1039/c8nr03900c (2019).

[130] Dusoswa, S. A. *et al.* Glycan modification of glioblastoma-derived extracellular vesicles enhances receptor-mediated targeting of dendritic cells. *J Extracell Vesicles* **8**, 1648995, doi:10.1080/20013078.2019.1648995 (2019).

[131] Zhao, Z., McGill, J., Gamero-Kubota, P. & He, M. Microfluidic on-demand engineering of exosomes towards cancer immunotherapy. *Lab on a chip* **19**, 1877-1886, doi:10.1039/c8lc01279b (2019).

[132] Jhan, Y. Y. *et al.* Engineered extracellular vesicles with synthetic lipids via membrane fusion to establish efficient gene delivery. *International journal of pharmaceutics* **573**, 118802, doi:10.1016/j.ijpharm.2019.118802 (2020).

[133] Chivero, E. T. *et al.* Engineered Extracellular Vesicles Loaded With miR-124 Attenuate Cocaine-Mediated Activation of Microglia. *Front Cell Dev Biol* **8**, 573, doi:10.3389/fcell.2020.00573 (2020).

[134] Tripathi, A. *et al.* Oligodendrocyte Intrinsic miR-27a Controls Myelination and Remyelination. *Cell Rep* **29**, 904-919 e909, doi:10.1016/j.celrep.2019.09.020 (2019).

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Chapter 2: Immunomodulatory Properties of Placenta Derived Mesenchymal Stem/Stromal Cell and Derived Extracellular Vesicles

2.1 Abstract:

Mesenchymal stem/stromal cells (MSCs) have potent immunomodulatory and regenerative properties that occur through the secretion of bioactive factors and the release of extracellular vesicles (EVs). These functional properties of MSCs make them attractive targets for the treatment of degenerative and inflammatory diseases, including multiple sclerosis (MS). MS is a complex and heterogenous disease that is typically characterized by autoreactive lymphocytes crossing the blood brain barrier (BBB) into the central nervous system (CNS) causing an inflammatory response that results in demyelination, gliosis and axonal loss[1]. Infiltration of autoreactive lymphocytes will release proinflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) into the CNS, which leads to accompanying neurodegeneration and disease pathology.

MSCs have been shown to migrate to sites of injury where they can interact with the inflammatory niche through paracrine signaling mechanisms[2]. Additionally, MSCs can release EVs which are small nanovesicles that can carry protein, mRNA and miRNA and have been shown to play a key role in CNS inflammation[3]. In particular, it has been shown that hepatocyte growth factor (HGF) secreted by MSCs in conditioned medium mediates recovery in a rodent model of MS[4]. HGF plays a critical role in angiogenesis and has been shown to have immunomodulatory activity through the stimulation of regulatory T cells (Tregs), which in turn mediate autoimmune responses[5]. HGF has also been shown to be present in EVs, and EVs derived from maternal and umbilical cord serum demonstrated that knock down of HGF in fetal exosomes played a more significant role in endothelial cell proliferation and migration[6].

This studies central hypothesis is that PMSC mediated immunomodulatory and neuroregenerative potential occurs through the secretion of HGF packaged in EVs. This study will investigate the role of fetal derived EVs for the treatment of MS. This will be achieved by utilizing an existing translational rodent model of MS to evaluate the clinical efficacy of PMSC-derived EVs (PMSC-EVs).

2.2 Background and Significance:

Pediatric MS is an autoimmune disease that gives rise to chronic neurologic deficits in children. It is characterized by an immunological attack of the myelin sheath which leads to demyelination and axonal degradation. According to the MS society in the US alone, there is an estimated 8,000-10,000 children diagnosed with MS that suffer from frequent relapses. Current medications can address inflammation associated with disease flare-ups; however, these therapies do not prevent progressive neuronal and axonal degeneration. Therefore, cell-based therapies using MSCs have been under investigation in clinical trials for neurogenerative diseases including adult MS[7,8]. The placenta has been suggested to be a unique source of MSCs that possess robust immunomodulatory properties and have been reported to be beneficial in graft verse host disease mouse models[2,9]. MSCs derived from the placenta may be a more appropriate cell source for pediatric diseases, because during pregnancy the placenta demonstrates "fetomaternal tolerance" which is attributed to the expression of HLA-G, a non-classical MHC class I molecule that inhibits natural killer cell (NK) killing[10]. Unlike bone marrow derived MSCs (BM-MSCs), PMSCs express HLA-G on their surface in response to IFNγ[11], which is a key inflammatory mediator involved with the onset of MS[1]. Therefore, the expression of HLA-G on PMSCs would make them a unique therapeutic cell source for the treatment of autoimmune diseases like pediatric MS. Currently, a clinical trial is underway using term placenta-derived PMSCs for adult MS with no paradoxical worsening of MS lesion counts was noted[12].

Cell-based therapies, however, are limited by potential immune rejection of donor cells and other safety concerns[13]. Increasing amounts of studies have shown that MSC survival and integration within the host after transplantation are usually poor and MSCs exert their therapeutic functions mainly via paracrine signaling mechanisms[14]. Conditioned media of BM-MSCs has been shown to protect neurons from apoptosis, activate macrophages and be pro-angiogenic[15]. In particular, it has been shown that HGF secreted by MSCs in conditioned medium mediates recovery in a mouse model of MS[4]. However, the use of MSC conditioned media is currently limited because the secreted protein factors are unstable, making storage and shipping a technical challenge and leading to batch-to-batch variability. In addition to freely secreted proteins these stem cells also release a significant amount of extracellular vesicles (EVs) including exosomes. EVs are small nanovesicles that carry protein, mRNA and miRNA and are shown to play a role in CNS inflammation[3]. They are also very stable with storage and hence an excellent candidate for cell-free therapy.

EVs secreted by MSCs may be able to cross the blood-brain barrier and deliver therapeutic cargo to reduce the effects of neuropathologic diseases, such as MS[16]. A recent report demonstrated MSC-derived EVs exhibit systemic immune modulatory effects and can facilitate neurological recovery[17]. PMSC-EVs have been shown to contain numerous proangiogenic, immunomodulatory, and neuroprotective proteins, including HGF[18,19]. HGF is a pleiotropic factor shown to have neuronal and oligodendrocyte protective properties[20]. In an experimental autoimmune encephalomyelitis (EAE) rodent model of MS, HGF conferred neuroprotection by reducing inflammation in the CNS and activation of Tregs[20]. HGF is secreted both in soluble form from MSCs and is also contained within EVs, however the effects of each form could lead to differing cellular responses. It has been suggested there are multiple categories of EVs secreted by MSCs which are involved in differing cellular processes[21]. Therefore, the molecular mechanism by which MSC-derived EVs alleviate the effects of neurodegenerative

diseases is warranted. The goal of the current research project is to demonstrate the therapeutic potential of PMSC-EVs in the context of pediatric MS. Furthermore, this study will investigate the effects of PMSC-EVs and the role of HGF in a translational model of MS.

2.3 Method and Materials

2.3.1 MSC Isolation and Expansion

PMSCs for this study were previously isolated from the chorionic villus tissue of de-identified discarded second trimester human placenta by explant culture[22]. Early passage (P2-P5) PMSCs were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM; GE Life Sciences, Pittsburgh, PA, USA) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologics, Flowery Branch, GA, USA), 20 ng/mL fibroblast growth factor basic (FGF basic; Advent Bio, Elk Grove Village, IL, USA), 20ng/mL epidermal growth factor (EGF; Advent Bio) and 1% penicillin/streptomycin (P/S; ThermoFisher Scientific, Pittsburgh, PA, USA). Human adult adipose derived MSCs (ASCs) were isolated and cultured as previously described[23]. Human bone marrow derived MSCs (BM-MSCs) were purchased and cultured according to manufacturer's instructions (ATCC). Adult MSCs were cultured in DMEM (GE Life Sciences) containing 10% FBS (Atlanta Biologics) and 1% P/S (ThermoFisher Scientific). Cells were cultured in tissue culture flasks at 37˚C, under 5% CO_2 as previously described[22].

2.3.2 Leukocyte Suppression Assay (LSA)

To evaluate immunosuppressive potential of PMSCs, leukocyte suppression assays (LSAs) were performed exactly as previously described[23]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by using an underlay method of Ficoll-Paque™ PLUS (GE healthcare, Uppsala, Sweden) and gradient centrifugation. PBMCs were collected from the buffy coat layer and stimulated with 5 µg/mL phytohemagglutin (PHA, Millipore-Sigma, St. Louis, MO, USA). PMSCs were irradiated (10Gy, Varian 2100C linear accelerator, Varian Medical Systems Inc., Palo Alto, CA, USA) and kept on ice before experimental setup. PBMCs and

PMSCs were co-cultured at a ratio of 5:1 in DMEM supplemented with 10% heat inactivated FBS, 1% P/S and L-tryptophan (600 μM, Millipore-Sigma).

To determine the role of cell contact, cells were plated in transwell dishes (Corning 0.4 μM polycarbonate membrane 24-well plate; Corning, NY, USA) with MSCs plated in the plate bottom and PBMCs in the insert. To determine the role of HGF in PMSC-mediated inhibition of lymphocyte proliferation, c-met blocking agents were used in LSA co-cultures. A human monoclonal c-Met receptor antibody (R&D Systems; Accession #P08581) was added to co-cultures at 1mg/mL or 10mgl/mL. Use of a monoclonal antibody will block binding of soluble HGF to c-Met Fc receptors. Additionally, Crizotinib (R&D Systems), a potent inhibitor of c-MET and anaplastic lymphoma kinase (ALK) (cell IC_{50} values are 8.0 and 20 nM respectively) and selective for c-MET and ALK against >120 different kinases, was added to co-cultures at 1nM or 10nM. To further asses the role of HGF in immune suppression, 5, 10 or 50 ng/mL of recombinant human HGF (R&D Systems) was added to PBMC cultures. To assess PMSC-EV immunomodulatory properties, LSAs were performed similarly however, PMSC-EVs were added to PBMC cultures at a ratio of 100,000:1 respectively.

After 3 days of co-culture, samples were treated with 1 mM Bromodeoxyuridine (BrdU, BD Biosciences). Twenty-four hours following BrdU treatment, leukocytes were collected, and cells were stained for LIVE/DEAD® Fixable Aqua Dead Cell to identify live cells, and CD3 (clone UCHT1, BD Bioscience) to identify lymphocyte populations. Expression of c-Met was also performed by using a monoclonal antibody directed against human c-Met-Ig fusion protein (Invitrogen; eBioclone 97). Cells were then stained for nuclear BrdU incorporation per manufacture directions (BrdU Flow Kit, BD Biosciences) and read by flow cytometry (Attune NxT Flow Cytometer, ThermoFisher Scientific). Flow cytometry data were analyzed using FlowJo software (Tree Star Inc.). Data was normalized and presented as a reduction of each respective PBMC donor.

2.3.3 Direct stimulation of PMSCs with interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα)

Cryopreserved PMSCs were thawed and expanded as previously described[24-26]. When cells were approximately 70% confluent, they were trypsinized and resuspended in DMEM (GE Healthcare) with 10% FBS (Atlanta Biologics,) and 1% P/S (ThermoFisher Scientific, Gibco,), supplemented with L-tryptophan (Sigma-Aldrich) to a final concentration of 600 μM. MSCs were seeded at $2x10^5$ per well of 24-well plate, with 1 mL total media volume per well for stimulation assays. MSC were stimulated with IFNγ (50ng/mL; human recombinant IFNγ, R&D) and TNFα (50ng/mL; human recombinant TNFα, R&D) for four days. Supernatants were collected, frozen and stored at -80°C for mediator quantification.

2.3.4 Quantification of Mediator Secretion

Frozen aliquots of supernatants collected from MSCs stimulated with IFNγ/TNFα (direct stimulation) and from LSA co-cultures (indirect stimulation) using two technical replicates from each assay were used to quantify brain derived neurotropic factor (BDNF), HGF, vascular endothelial growth factor (VEGF) and indoleamine 2,3 dioxygenase (IDO) activity. To assess IDO activity, a biochemical assay was performed on frozen supernatants as previously described[23] to quantify the conversion of tryptophan to N-formyl kynurenine mediated by IDO. In brief, culture media was treated with 30% trichloroacetic acid (Sigma), and Ehrlich's reagent (1% p-dimethylaminobenzaldehyde in glacial acetic acid, Sigma) was mixed and read at and absorbance of 490 nm on a Spectromax® i3 plate reader (Molecular Devices). Enzyme-linked immunosorbent assays (ELISAs) for BDNF (R&D Systems, Minneapolis, MN, USA), HGF (R&D Systems) and VEGF (R&D Systems) were performed per the manufacturer's instructions and read on a plate reader (SpectraMax Plate Readers, Molecular Devices).

2.3.5 PMSC-derived Extracellular Vesicle (EV) Isolation

PMSC-derived EVs were isolated as previously described[27]. In brief, EVs were first depleted from FBS by spinning FBS samples at 112,700 G using the L7 Ultracentrifuge (Beckman Coulter, Brea, CA, USA) and a SW28 rotor for 16 hours at 4˚C. Supernatants were collected, aliquoted and stored at -20˚C. PMSCs at passage 4 were seeded at 20,000 cells/cm^2 in T175 flasks (Corning Inc.) in 20mL of medium containing 5% EV-depleted FBS, 20 ng/mL FGF (Advent Bio), 20 ng/mL EGF (Advent Bio), and 1% P/S (ThermoFischer Scientific) for 48 hours at 37˚C, under 5% CO_2. For stimulation analysis, PMSC samples unstimulated or stimulated with 50ng/mL of IFNγ and TNFα were cultured for 72 hours, before conditioned medium was collected. Conditioned medium was collected and EVs were isolated by differential centrifugation exactly as previously described[27]. After the final centrifugation step, EV pellets were resuspended in 10 µL of triple-filtered PBS (GE, Life Sciences) per T175 flask used for the generation of the conditioned medium. EVs were aliquoted and stored at -80 ˚C.

2.3.6 Characterization of EVs by Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed to quantify EV yield and size distribution. The size, concentration, and zeta potential of the LRNVs were measured with nanoparticle tracking analysis (NTA) using the ZetaView (Particle Metrix). Samples were pre-diluted to an optimal concentration to allow for about 150 particles/frame (ZetaView v.8.05.12). For each measurement, 11 positions were scanned for two cycles using the following parameters: camera sensitivity: 92, shutter:150, frame rate: 30, cell temperature: 25°C. The analytical software determined EV size, mean, mode and standard deviation as well as the number of particles per frame and milliliter of sample.

2.3.7 Characterization of HGF in PMSC-EVs

2.3.7.1 ExoView Tetraspanin Kit Assay

ExoView Kits (NanoView Biosciences) were used to interrogate EVs. PMSC-EVs were added to plates as previously described[30]. ExoView detection was performed exactly as

previously described, with the current labeling panel including anti-CD63 (clone: H5C6), anti-CD9 (clone: HI9a) from the ExoView kit as well as anti-HGF (Bioss, Woburn, Massachusetts, Catalog# bs-1025R-A488). Following antibody labeling of PMSC-EVs on ExoView chips, the chips were scanned for interferometric and fluorescence imaging. During data analysis, fluorescence cut-offs were chosen by limiting the number of detected particles on MIgG capture spots to approximately 10 events. For tetraspanin antibodies, fluorescence cut-offs of 400 a.u. were used for the red and green channels and 600 a.u. for the blue channel in all experiments.

2.3.7.2 Super Resolution Microscopy

Super-resolution direct stochastic optical reconstruction microscopy (dSTORM) images of EVs were obtained using a temperature controlled Nanoimager S Mark II microscope from ONI (Oxford Nanoimaging, Oxford, UK) with a 100x objective. The samples were prepared using 10 µL of 0.01% Poly-L-Lysine (Sigma-Aldrich) placed on high-precision glass coverslips. Coated coverslips were placed at 37 °C in a humidifying chamber for 2 h. 1 µL of EVs resuspended in 9 µL of blocking solution (PBS-5% Bovine Serum Albumin, BSA) were incubated overnight at room temperature. The next day, samples were blocked for 30 min. Antibodies against CD9 (ONI, Oxford, UK), CD63 (ONI, Oxford, UK), and HGF (Bioss) were added to wells. Samples were incubated with 1 µL of each antibody, added into blocking buffer at a final dilution 1:10, under light protection, overnight at room temperature. The day after, samples were washed twice with PBS, and 10 µL of the mixed ONI B-Cubed Imaging Buffer (Alfatest, Rom, Italy) was added for amplifying the EV imaging. Two-channel (647 and 555) dSTORM data (5000 frames per channel) or three channels (2000 frames per channel) (647, 555 and 488) were acquired sequentially at 30 Hertz in total reflection fluorescence (TIRF) mode. Before each imaging session, bead slide calibration was performed to align fluorescent channels, achieving a channel mapping precision of less than 15 nm. All pictures were analyzed on CODI software (Oxford Nanoimaging). EVs

were identified through clustering of multi-channel localizations and quantified for HGF expression using the counting tool.

2.3.7.3 Single EV Flow Cytometrey

To evaluate surface expression of HGF on PMSC-EVs single EV flow cytometry was performed. PMSC-EVs were labeled with anti-HGF (Bioss) for 30 minutes protected from light. PBS with free anti-HGF antibody (Bioss), and unlabeled native PMSC-EVs were used as unstained controls. Following incubation, samples were run on the CytoFLEX flow cytometer (Beckamn Coulter). The cytometer we calibrated as previously described[30]. A 405 nm side scatter trigger channel was used. Flow cytometry data were analyzed using FlowJo software (Tree Star Inc.).

2.3.8 Proteomic Characterization of Native and Stimulated PMSC-EVs

Proteomic profiling of unstimulated and IFNγ/TNFα stimulated PMSC-EVs was performed by the University of California, Davis Proteomics Core Facility (Davis, CA). Samples were denatured, digested using the EasyPep sample preparation kit (ThermoFisher Scientific) and analyzed by liquid chromatography and mass spectrometry (LC-MS/MS) using the TimsToF Pro mass spectrometer (ThermoFisher Scientific). MS/MS acquisitions were searched against the most updated Uniprot Homo sapiens database with Sequest analysis workflow to identify proteins in the samples. Protein search and identification was done using Spectronaut (Biognosys AG) to obtain significantly expressed candidate proteins and relative protein expression levels. Gene ontology searches were performed using FunRich v.3.1.3, and KEGG pathway analysis was performed with Database for Annotation, Visualization, and Integrated Discovery (DAVID) v.6.7[31]. Data was visualized and plotted using both Microsoft Excel and R Studio.

2.3.9 Fluorescent Labeling of EVs

EVs isolated using differential ultracentrifugation were stained using 1μM of MemGlow™ 700 Fluorogenic Membrane Probe (Cytoskeleton) per manufacturer's instructions. In brief PBS control or EVs were incubated for 10 minutes at room temperature protected from light. To remove excess dye, stained solutions were brought to a volume of 150mL and added to qEV_{single} size exclusion chromatography columns (SEC) columns on the Izon AFC. Columns were rinsed using 0.2 μm-filtered PBS (4 mL). After rinsing, any remaining solution was removed from the top of column, 150 μL of sample was placed on top of the column and allowed to enter before adding filtered PBS. After void fraction elution, 2 fractions (0.2 mL each) were collected that were confirmed to contain EVs in prior experiments. Fractions were pooled were collected and imaged using the ChemiDoc XRS+ System (Bio-Rad) with Image Lab software to ensure free dye had been sufficiently removed.

2.3.10 Experimental Autoimmune Encephalomyelitis (EAE) Induction in a Murine Model

In order to mimic the pathology of human pediatric MS, EAE was induced using a murine model as previously described[32,33]. Six- to eight-week-old C57BL/6J mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 to induce EAE. In brief, 300 μg of rodent MOG peptide (amino acids 35–55, New England Peptides, Gardner, MA, USA) in Complete Freund's Adjuvant (CFA) containing 5 mg/mL killed Mycobacterium tuberculosis (Difco, ThermoFischer Scientific) was administered into the subcutaneous flank of mice at day 0. At day 0, each mouse received two subcutaneous injections of the MOG solution as well as a 100 μl dose of 2 ng/μl pertussis toxins and virulence factors (List Biological Laboratories Inc., Campbell, CA, USA) diluted in sterile PBS (Thermo Fisher Scientific). Pertussis toxins and virulence factors were administered again on day 2. Pertussis toxins allow an increase in the blood-brain barrier permeability in order to facilitate the incursion of the different treatments into the CNS.

In order to monitor disease progression, mice were weighed and scored daily. Neurological deficits were assessed on a five-point scale (limp tail or waddling gait = 1; limp tail

and waddling gait = 2; single limb paresis and ataxia = 2.5; double limb paresis = 3; single limb paralysis and paresis of second limb = 3.5; full paralysis of 2 limbs = 4; moribund = 4.5; and death = 5)[32].

2.3.11 Experimental Treatment of Healthy and EAE Animals

Daily motor quantification was performed for all EAE animals. Mouse treatment groups were randomized. For this study, animals were treated 14 days following MOG immunization. Age matched healthy controls were also used. On the day of treatment, PMSCs were detached from culture using TrypLE (ThermoFisher Scientific) and washed twice with PBS (ThermoFisher Scientific). PMSC-EVs were fluorescently labeled as described above prior to administration. Tail-vein injections were performed using 1mL syringes connected to PE 10 tubing and a 30G standard hypodermic needles (Covidien, Dublin, Republic of Ireland).

2.3.12 Live Animal Imgaing

MemGlow labeled PBS or EVs were injected into the animals per approved animal protocols. Mice were anesthetized using 2%-3% isoflurane (Piramal Critical Care, Bethlehem, Pennsylvania) and were imaged before injection and at ~15-, 30- and 60-minutes following injection. Following the one-hour time point, animals were sacrificed, and organs harvested for imaging. Healthy animal experiments were imaged with the IVIS Spectrum (PerkinElmer, Richmond, California) at the Genomic and Molecular Imaging Center (Davis, California) and EAE induced animals were imaged using Lago X Imaging System (Spectral Instruments Imaging, AZ).

2.3.13 Statistical Analyses

Results are expressed as mean and standard error. Imaging and *in vitro* data were analyzed using non-parametric Mann-Whitney-Wilcoxon t-tests (GraphPad Prism version 8.2.1 for macOS, La Jolla, CA, USA). Multiple comparisons were performed using a Kruskal-Wallis test, followed by Dunn's *post hoc* correction to determine which groups were significantly different

(GraphPad Prism version 8.2.1 for macOS). A p-value <0.05 was considered significantly different.

2.4 Results

2.4.1 PMSCs possess immunomodulatory properties

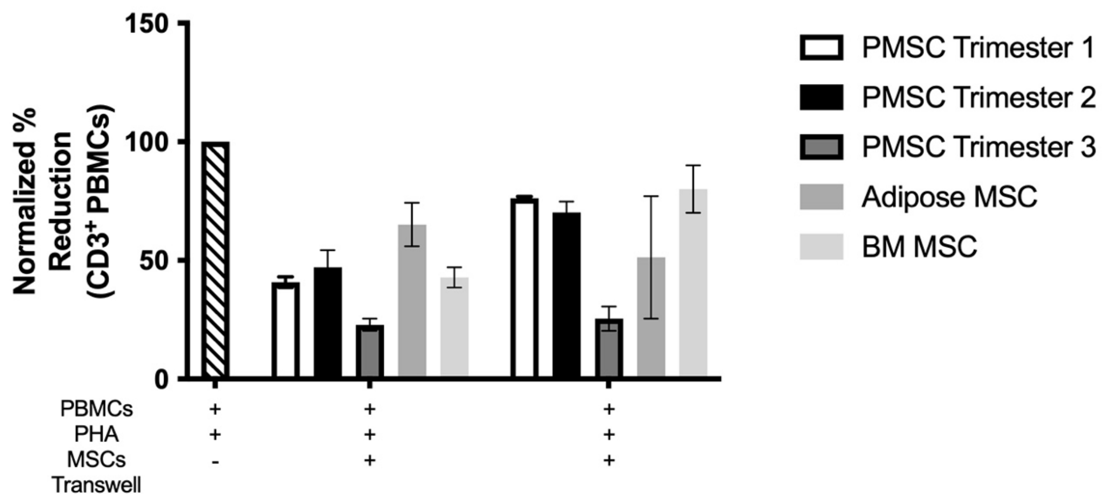


Figure 2.1. MSCs inhibit leukocyte proliferation. PMSCs inhibit leukocyte proliferation to comparable levels as adult derived tissue sources (adipose and bone marrow). Transwells were added to remove physical contact between MSCs, and mitogen (PHA) stimulated PBMCs. Early gestational PMSCs (first and second trimester) displayed a trend towards contact-dependent immunomodulation, while term and adult MSC sources trend towards contact-independent immunomodulation. Data presented as mean and standard error. PHA, phytohemagglutinin; LSA, leukocyte suppression assay; MSC, mesenchymal stem/stromal cell; PBMCs, peripheral blood mononuclear cells; PMSC (placenta derived MSC); BM (bone marrow).

To determine the functional properties of PMSCs, LSAs were performed to assess immunomodulatory potential. PBMCs from multiple donors were stimulated using the mitogen PHA and co-cultured with irradiated PMSCs isolated from first, second and term placentas as well as MSCs derived from adult tissue sources including ASCs and BM-MSCs. Variability of PBMC donor stimulation was observed, therefore data was normalized to a percent reduction with corresponding PBMC donors. All MSC sources reduced CD3 positive leukocyte proliferation for every donor screened (Figure 2.1). No significant differences were observed in the reduction of

CD3+ leukocytes by MSC sources, however sample size will need to be increased for future analysis. Additionally, LSA co-cultures performed with transwell inserts to remove direct MSC-PBMC cell contact resulted in a trend of rebounded PBMC proliferation in early gestation PMSCs but was not observed in term and adult MSC sources (Figure 2.1). Larger samples sizes will be required for further statistical analysis to be performed.

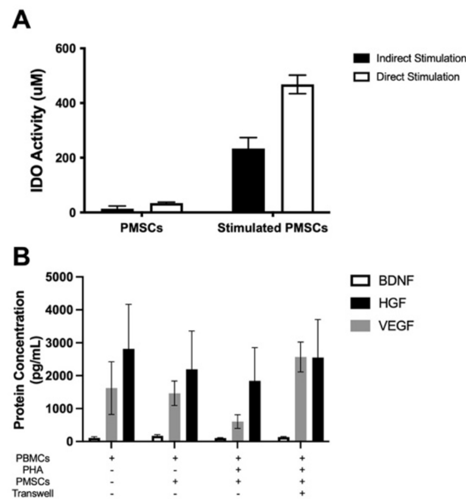


Figure 2.2. Secretome analysis of stimulated PMSCs. Secretome analysis was performed on supernatants from LSA cultures. Additionally, direct stimulation using recombinant human IFNγ, and TNFα was performed to evaluate differences in IDO activity via each stimulation modality. IDO activity was upregulated in PMSCs to comparable levels through indirect or direct stimulation. Furthermore, PMSCs secrete high levels of BDNF, HGF and VEGF regardless of cellular contact or stimulation. Data presented as mean and standard error. PHA, phytohemagglutinin; LSA, leukocyte suppression assay; PMSC, placenta derived mesenchymal stem/stromal cell; PBMCs, peripheral blood mononuclear cells; IDO, indoleamine 2,3 dioxygenase; BDNF, brain derived neurotropic factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma.

MSC immunomodulatory functions occur in part through the secretion of bioactive factors. While LSAs provide indirect stimulation of PMSCs via mitogen activation of leukocytes, donor variability from human PBMCs donors is observed. PMSCs were activated through direct stimulation to determine immunomodulatory properties using recombinant pro-inflammatory mediators known to be relevant in MS. Direct stimulation using recombinant IFNγ and TNFα was also performed to confirm stimulation of PMSCs by measuring IDO activity, a known factor to be

involved in MSC mediated immunomodulation. IDO activity was quantified from LSA supernatants as well IFNγ and TNFα stimulated PMSCs. Both indirect and direct stimulation of PMSCs resulted in comparable upregulation of IDO activity (Figure 2.2A). Further secretome analysis demonstrated that PMSCs co-cultured either in direct or indirect contact with PBMCs maintained high constitutive levels of BDNF, HGF and VEGF (Figure 2.2B)

2.4.2 Determining the role of HGF in immunomodulation of PMSCs

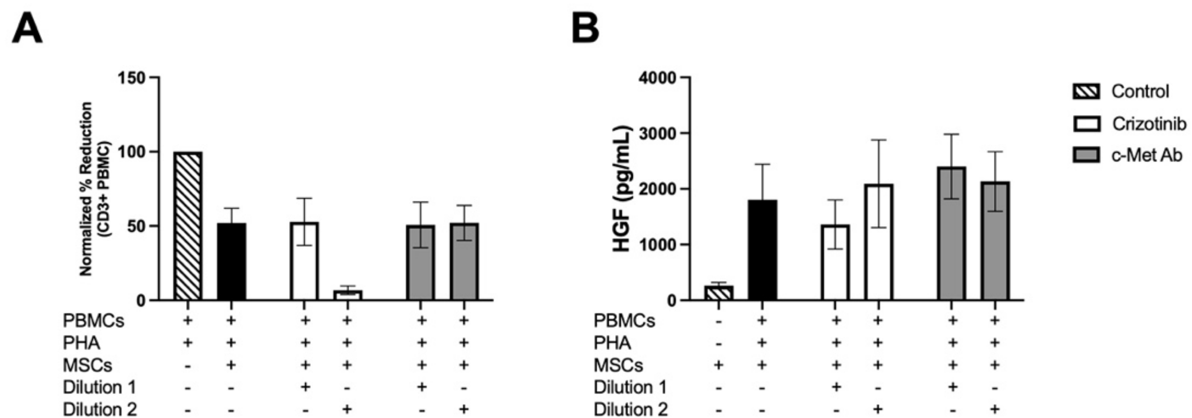


Figure 2.3. Evaluation of PMSC secreted HGF on leukocyte proliferation. To determine the role of PMSC secreted HGF on PBMC proliferation, inhibitors of c-Met were used to block HGF and receptor interaction. Blocking c-Met expression using Crizotinib or monoclonal c-Met antibody at high (dilution 1) and low (dilution 2) doses did not affect PMSC mediated immunomodulation (A). Furthermore, addition of c-Met inhibitors did not result in a reduction of HGF production by PBMCs as measured by ELISA (B). Data presented as mean and standard error. PHA, phytohemagglutinin; LSA, leukocyte suppression assay; PMSC, placenta derived mesenchymal stem/stromal cell; PBMCs, peripheral blood mononuclear cells; HGF, hepatocyte growth factor; ELISA, enzyme-linked immunosorbent assay.

HGF has been shown to be involved in key immunomodulatory and neuroprotective functional properties by MSCs and been implicated as a key mediator by which MSCs provide therapeutic benefits for MS[4]. To evaluate the role of HGF by PMSCs, inhibitors of c-Met receptors were utilized to block binding of soluble HGF. A monoclonal antibody against c-Met and Crizotinib were each added to LSA co-cultures at two dilutions (dilution 1=high dose, dilution 2 = low dose). Blockade of c-Met receptor binding did not result in any changes in suppression of PBMC

proliferation by PMSCs (Figure 2.3A). Furthermore, quantification of HGF from LSA supernatants was performed. High levels of HGF from all stimulated LSAs was observed regardless of c-Met blockaded (Figure 2.3B).

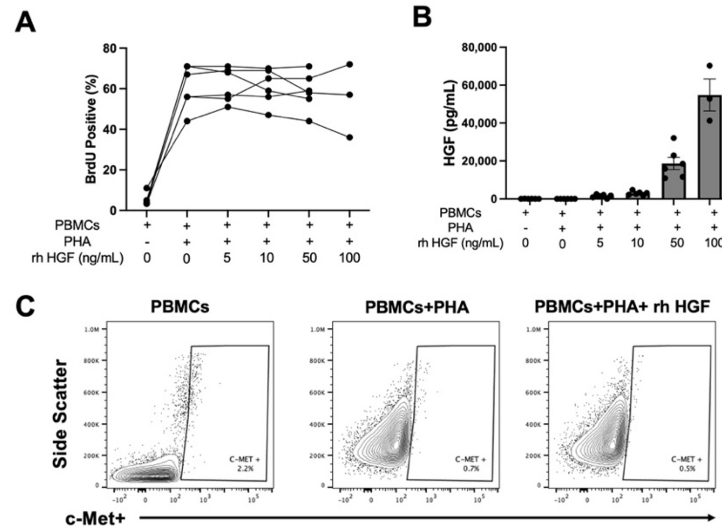


Figure 2.4. Evaluation of recombinant HGF on leukocyte proliferation. To determine the role of HGF on PBMC proliferation, recombinant HGF was added at varying concentrations to PHA stimulated PBMCs. Proliferation was quantified by BrdU incorporation. Addition of recombinant HGF did result in reduction of PBMC proliferation as measured by BrdU incorporation (A). Addition of recombinant HGF to PBMCs resulted in similar levels of HGF to supplemented concentrations as measured by ELISA (B). Expression of c-Met was quantified by flow cytometry. C-met expression was not observed in unstimulated or stimulated PBMCs or with the addition of recombinant HGF(C). Data presented as mean and standard error. PHA, phytohemagglutinin; LSA, leukocyte suppression assay; PMSC, placenta derived mesenchymal stem/stromal cell; PBMCs, peripheral blood mononuclear cells; HGF, hepatocyte growth factor; ELISA, enzyme-linked immunosorbent assay; BrdU, Bromodeoxyuridine.

To confirm bioactivity of HGF in LSAs, recombinant HGF was added to PBMCs to evaluate the effects of HGF on PBMC proliferation. Addition of recombinant HGF at various concentrations did not result in any changes in PBMC proliferation (Figure 2.4A). Quantification of HGF from PBMC supernatants supplement with recombinant HGF demonstrated active HGF in cultures at comparable levels to supplementation amounts, suggesting HGF was not up taken by PBMCs (Figure 2.3B). Confirmation of c-Met expression was next performed on PBMCs, and it was revealed that stimulation with PHA did not result in upregulation of c-Met expression (Figure

2.3C). These data suggest that to evaluate the role of HGF secreted by PMSCs on immunomodulation, alternative stimulation assays will need to be performed.

2.4.3 PMSC-EVs possess immunomodulatory properties

To determine if PMSC-EVs are the mechanism of action (MOA) by which PMSCs modulate immune response, PMSC-EVs were added to LSAs. PBMC to PMSC-EV ratios were determined to be 1:100,000 respectively, through titration assays (data not shown). PMSC-EVs were shown to inhibit CD3+ leukocyte proliferation to comparable levels as second trimester PMSCs (Figure 2.5). To evaluate the expression of HGF in PMSC-EVs, characterization of PMSC-EVs was performed using novel modalities including ExoView analysis, super resolution microscopy and single EV flow cytometry. HGF expression in PMSCs-EVs was confirmed via ExoView analysis (Figure 2.6A), and super resolution microcopy (Figure 2.6B). Single EV flow cytometry did not show HGF on the surface of PMSC-EVs, indicating HGF remains within the contents of PMSC-EVs (Figure 2.6C). Though the role of HGF by PMSCs has not been fully elucidated, confirmation of HGF expression within PMSC-EVs indicates a viable role that HGF may play on the therapeutic functions of PMSC-EVs namely for CNS disorders.

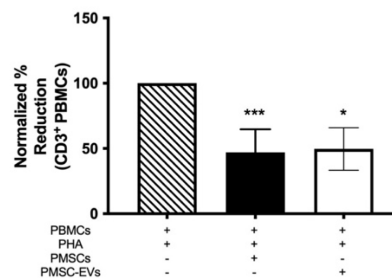


Figure 2.5. PMSC-EVs display immunomodulatory properties. PMSC-EVs inhibit leukocyte proliferation to comparable levels as PMSCs. PMSC-EVs added to stimulated PBMCs at a ratio of 100,000 to 1 respectively resulted in reduction of CD3+ leukocytes to comparable levels of PMSCs. (PMSC: PBMC, 1:5). Data presented as mean and standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. PHA, phytohemagglutinin; LSA, leukocyte suppression assay; Placenta derived mesenchymal stem/stromal cell; PBMCs, peripheral blood mononuclear cells; EV, extracellular vesicle.

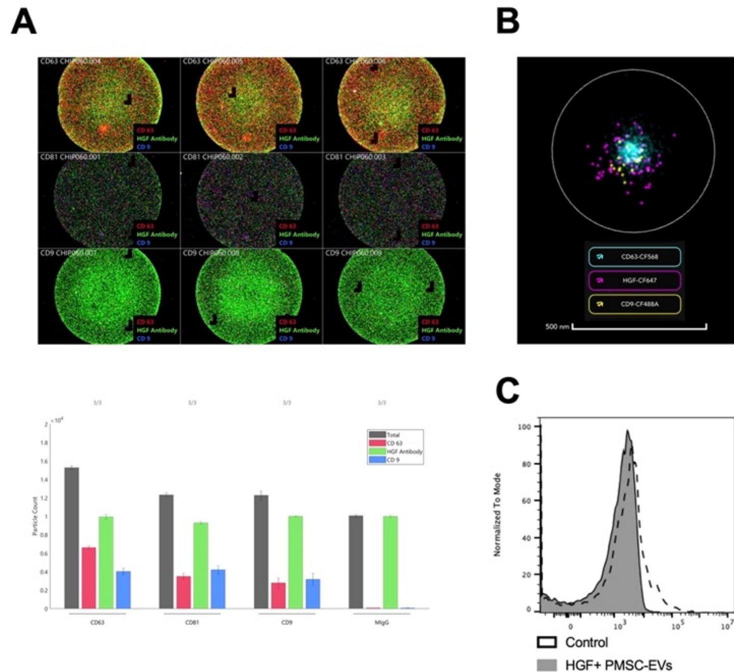


Figure 2.6. HGF is expressed in PMSC-EVs. Expression of HGF was confirmed via ExoView analysis (A) and super resolution microscopy (B). HGF was not shown to be expressed on the surface of PMSC-EVs using single EV flow cytometry techniques (C). Representative images are shown. HGF, hepatocyte growth factor; PMSC, placenta derived mesenchymal stem/stromal cell; PBMCs, peripheral blood mononuclear cells; EV, extracellular vesicle.

2.4.4 Proteomic analysis of stimulated PSMC-EVs

Protein composition of PMSC-EVs was characterized by proteomic analysis using tandem mass spectrometry. Proteomes of unstimulated and TNFα/ IFNγstimulated PMSC-EVs were compared from three matched PMSC donors (Figure 2.7). In total 2,363 proteins were identified with 2193 being conserved between the unstimulated and stimulated data sets (Figure 2.7A). Twenty-one unique proteins were identified and conserved amongst the stimulated PMSC-EV samples. Of the unstimulated donors (n=3), 1774 proteins were conserved (Figure 2.7B) and of the stimulated PMSC-EVs (n=3), 2119 proteins were conserved (Figure 2.7C). Analysis of the common proteins identified the presence of many growth factors involved in functional efficacy of MSC and MSC-EV mediated immunomodulation and neuroprotection were analyzed (Figure

2.7D). Galectin protein, transforming growth factors (TGF) and major histocompatibility complex expression were all identified (Figure 2.7D). Principal component analysis amongst unstimulated and stimulated PMSC-EV donors displayed conserved distinct grouping patterns (Figure 2.7E).

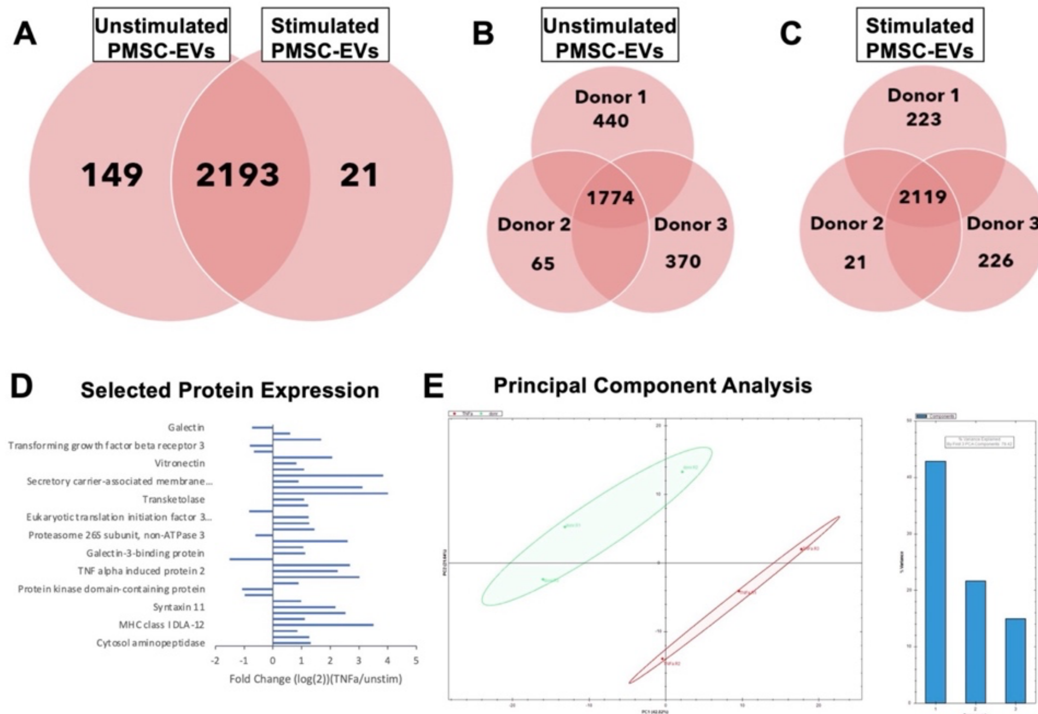


Figure 2.7. Proteomic analysis of unstimulated and TNFα/ IFNγ stimulated PMSC-EVs. Number of common proteins between unstimulated and stimulated PMSC-EV donors (A). Common proteins between unstimulated (B) and stimulated (C) PMSC-EV donors (n=3). The 2193 conserved proteins for all donors were analyzed for immunomodulatory and regenerative proteins (D). First and second principal component scores for protein's on PMSC-EVs and stimulated PMSC-EVs (E; n = 3 cell lines). Shapes represent the cluster membership at a 95% confidence interval (E). PMSC, placenta derived mesenchymal stem/stromal cell; EV, extracellular vesicle; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma.

Conserved proteins in the stimulated PMSC-EVs were then evaluated for functional enrichment and network analysis with gene ontology searches using FunRich software and Database for Annotation, Visualization, and Integrated Discovery (DAVID). Stimulated PMSC-EVs were found to be involved in several post-translational modifications processes including phosphoprotein and proteoglycans (Figure 2.8A). Furthermore, stimulated PMSC-EVs are

involved in biological process including translation, protein transport and antibody processing and presentation. KEGG pathway analyses for molecular functions revealed notable involvement in inflammatory diseases and immune processes (Figure 2.8C). These processes include, antigen processing and presentation, cell adhesion molecules, platelet activation, focal adhesion, complement and coagulation cascades, chemokine signaling pathways, leukocyte transendothelial migration and ECM-receptor interaction (Figure 2.8C). Further evaluation of cellular components (Figure 2.8D) and biological process (Figure 2.8E) analysis revealed notable involvement in cell-cell adhesion, immune response, neurotransmitter transport and programmed cell death.

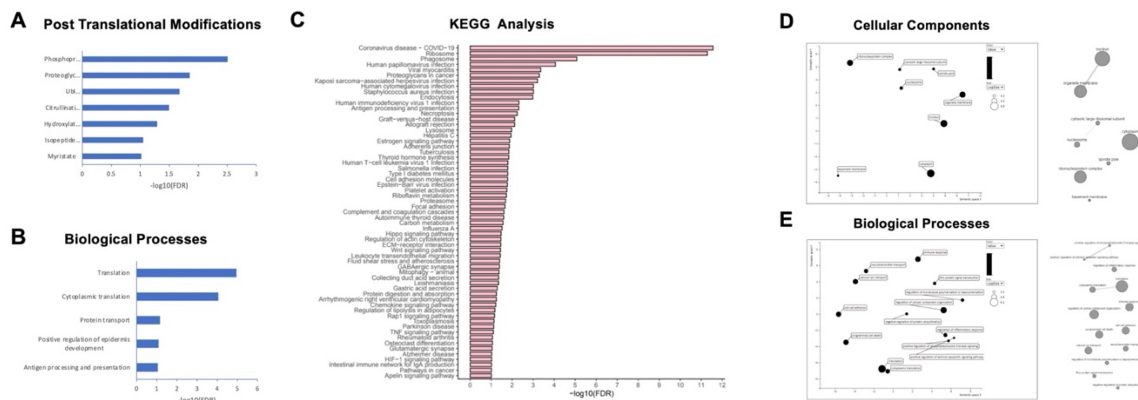


Figure 2.8. Gene ontology analysis of unstimulated and IFNγ/TNFα stimulated PMSC-EVs. Gene ontology searches for post-translational modifications (A), (biological processes B/E), KEGG pathway (C) and cellular components (D) analysis was conducted using FunRich software and DAVID. PMSC, placenta derived mesenchymal stem/stromal cell; EV, extracellular vesicle; TNFα, tumor necrosis factor alpha; IFNγ, interferon gamma.

2.4.5 In Vivo Biodistribution of PMSC-EVs

The molecular mechanism by which PMSCs confer therapeutic benefits for MS are largely unknown; however, these MSC functions have been shown to occur through paracrine signaling. A potential benefit to cell free therapeutics is an off the shelf product for diseases of the CNS and EVs may more readily cross the BBB. Therefore, to determine tissue homing of PMSC-EVs to

sites of injury *in vivo* biodistribution studies were performed. Saline or PMSC-EVs were labeled with MemGlow 700. Fluorescently labeled saline controls (free dye control) or PMSC-EVs were administered to healthy C57BL/6J or EAE mice via peripheral tail vein injection. Animals were imaged prior to experimental administration, and at 15m, 30m and 60m following injection. After the one-hour time point, animals were sacrificed, and organs dissected and imaged. In healthy mice, free dye control resulted in signal predominantly observed in the gastrointestinal tract (GI) of control mice (Figure 2.9A). Labeled PMSC-EVs were predominantly observed with signal in the liver and GI up to one hour following administration (Figure 2.9A). However, signal began to diminish at the one-hour point when imagining whole animals. Imaging of organs confirmed PMSC-EV uptake predominantly into the liver of healthy mice (Figure 2.9B). Quantification of regions of interest and increasing sample size is ongoing.

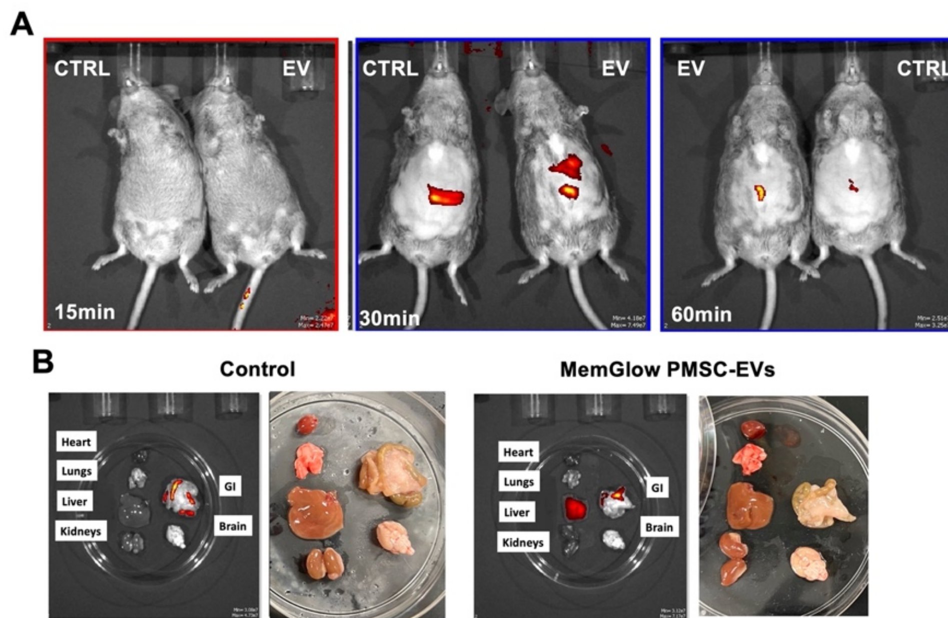


Figure 2.9. *In vivo* biodistribution of fluorescently labeled PMSC-EVs. Representative images of animals injected with free dye control saline or MemGlow labeled PMSC-EVs via tail vein injection at 15m, 30m and 60m (A). Representative images of organs from corresponding animals following one-hour whole animal imaging. Images were taken using the IVIS imaging system. PMSCs, placenta derived mesenchymal stem/stromal cells; EV, extracellular vesicle.

To determine if PMSC-EVs can cross a disrupted BBB which is associated with MS, an EAE murine model of MS was performed. Induction of EAE was achieved by immunizing C57BL/6J mice with MOG peptide 35–55 and pertussis toxin on day 0 and a secondary injection of pertussis toxin on day 2. Following MOG immunization, disease symptom onset typically peaks at 15–20 days. Animals were scored daily using an established motor scoring scale[34]. In the current pilot study, free dye control and labeled PMSC-EVs were administered at D14 following MOG immunization, despite animals not displaying motor deficits. Preliminary analysis showed signal most notably in isolated organ images. Representative images of EAE organs showed signal by PMSC-EVs in the CNS, suggesting that in model systems with CNS injury, PMSC-EVs may be able to pass through into the BBB. Whole animal live imaging will need to be optimized for CNS images, and sample size will need to be increased. Furthermore, timing of PMSC-EV administration will need to be optimized for effective evaluation of PMSC-EV distribution and effects on immune cell subsets.

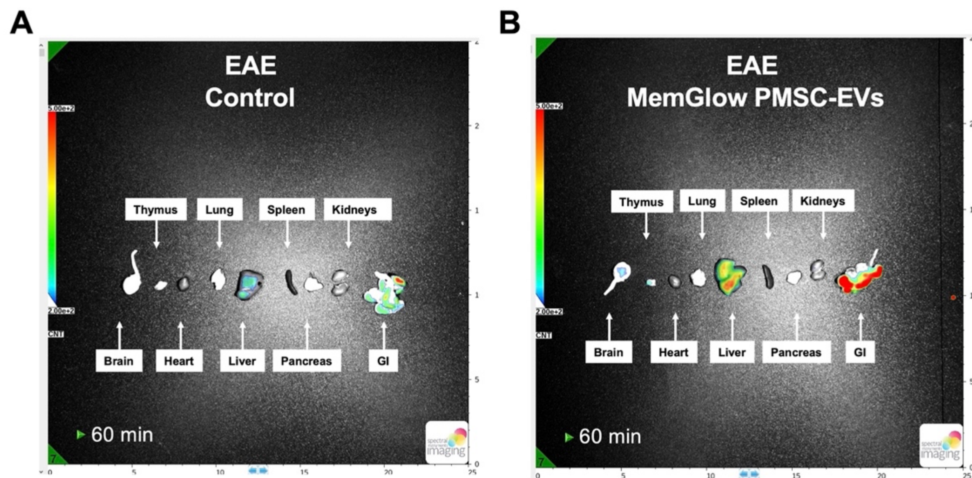


Figure 2.10. Organ biodistribution of fluorescently labeled PMSC-EVs in EAE animals. Representative images of dissected organs from EAE induced animals injected with free dye control saline or MemGlow labeled PMSC-EVs via tail vein injection. Images taken using the Lagos imaging system. PMSC, placenta derived mesenchymal stem/stromal cell; EV, extracellular vesicle; EAE, experimental autoimmune encephalomyelitis.

2.5 Discussion

The goal of the present study is to investigate the MOA by which a unique fetal derived MSC source modulates immune responses. MSCs have increasingly been utilized as a potential therapy for neurodegenerative diseases due to their potent neuroprotective, regenerative, and immunomodulatory properties. In most studies adult-derived sources of MSCs are utilized, however the placenta is a unique source of MSCs that maintains distinctive functional properties and may have significant clinical applications for pediatric diseases. Additionally, EVs have emerged as powerful cell-free therapeutics and can maintain functional properties such as tissue regeneration, neuroprotection and immunomodulation comparable to their parent cell sources of origin. PMSC-EVs can serve as mediators of intracellular communication and transporters of vital proteins, lipids, and nucleic acids, and may be the MOA by which PSMCs confer therapeutic benefits. HGF has been implicated as having a significant role by in neuroprotection in MS as well as potent immunoregulatory and pro-angiogenic properties[4,35]. Therefore, it is necessary to determine if PMSC-EVs can serve as an alternative cell-free therapy for the treatment of CNS disorders like MS.

PMSCs also contain numerous proangiogenic, immunomodulatory and neuroprotective proteins, that can play a critical role in promoting myelination in CNS disorders. To determine if PSMCs maintaining unique immunoregulatory functions, PMSCs isolated from first trimester, second trimester and term placentas were used in co-cultures with mitogen activated leukocytes. Addition of adult MSC sources including BM and adipose were also used to evaluate MSC mediated suppression of leukocyte proliferation. It was found that all MSCs regardless of tissue source inhibited PBMC proliferation and maintained immunomodulatory functions. A trend was observed that first and second trimester derived PMSCs inhibited PBMC proliferation in a cell-cell dependent fashion. Term PMSCs, ASCs and BM-MSCs displayed a trend of contact independent leukocyte suppression. Sample sizes for these assays will be increased to perform further

statistical analysis. These data suggest that differences in MOA may exist from early gestation PMSCs compared to adult MSC tissue sources. These findings are comparable to studies performed in the canine, which demonstrated PMSCs and ASCs both maintain immunomodulatory functional properties however, the MOA and bioactive molecule content differed[36]. Though MSCs from all sources display immunoregulatory properties, determination of MOA is highly important, and consideration of disease targets and MSC sources is crucial.

Secretome analysis was performed to investigate the bioactive mediators involved in the immunomodulatory potential of PMSCs. Second trimester PMSCs, activated via indirect stimulation in LSAs or direct stimulation using IFNγ and TNFα, resulted in upregulation of IDO activity to comparable levels. While IDO plays a critical role in alloreactivity by induction of T regulatory cells (Tregs), IDO activity by PMSCs in other species models does not appear to alter immunoregulatory properties through blocking study analysis[9]. Additionally, it has been reported that PMSCs have significantly increased basal secretion of HGF, BDNF and VEGF as compared to BM-MSCs[37]. It was demonstrated that following indirect stimulation of PMSCs, constitutive levels of BDNF, HGF and VEGF remain elevated. These mediators are involved in proangiogenic, immunomodulatory and neuroprotective functions by PMSCs, and can play critical roles in promoting myelination for CNS disorders.

HGF is a pleiotropic factor shown to have neuronal and oligodendrocyte protective properties[18-20]. It has been shown overexpression of HGF by neurons lead to reduction of inflammation in the CNS through activation of Tregs[20]. HGF is a potent angiogenic factor that has been shown to exert immunomodulatory effects through the stimulation of regulatory T cells, which in turn mediate autoimmune responses[5]. These factors may play a role in the protective and regenerative properties of PMSCs. HGF is secreted both in soluble form from MSCs and is also contained in EVs; however, the effects of each form could lead to different cellular responses. To further investigate the immunoregulatory role of HGF, LSAs were performed by blocking HGF binding to c-Met receptors to determine effects on leukocyte proliferation. In the current study,

blocking of soluble HGF to its target c-Met receptor led to no alterations in PMSC mediated immunoregulation. Addition of recombinant HGF resulted in similar effects. It was also determined that using a PHA stimulation protocol did not lead to c-Met receptor expression on PBMCs. One study found reported PHA stimulation increased expression of c-Met on PBMCs, alternative stimulation strategies lead to superior upregulation of the receptor[35]. While minimal effects on PBMCs were observed by c-Met receptor inhibition, alternative strategies to stimulate expression of c-Met on activated leukocytes will be necessary. Additionally, alternative strategies to block HGF may be employed to better decipher the role of this mediator in PMSC and PMSC-EV mediated immunoregulation. Preliminary data has also demonstrated PMSC-EVs express HGF via ExoView analysis and super resolution microscopy. These findings suggest a role of HGF by PMSC-EVs, however further studies investigating the role of soluble HGF by PMSCs will need to first be optimized.

Proteomic analysis revealed that between matched unstimulated and stimulated PMSC-EV donors, 2193 proteins were conserved with 21 unique proteins existing in stimulated PMSC-EVs. While heterogeneity has been reported in PMSC-EVs, screening modalities can be utilized to determine ideal MSC donor lines for banking of EVs for possible clinical use. PMSC-EVs expressed galectin proteins including Gal-1, which is a multifunctional protein involved in many cellular functions, such as cell growth and migration regulation, adhesion, angiogenesis and embryonic and adult tissue development[38]. Interestingly, this protein also plays many key functions in the immune system[39,40]. It has been demonstrated that Gal-1 facilitates anti-inflammatory processes and modulates the adaptive immune system[39]. Gal-1 has the potential to activate T-lymphocyte apoptosis and induce the differentiation of Tregs[39,40]. Moreover, studies have also reported that Gal-1 can promote phenotypic and functional changes after binding to microglia *in vitro*[41]. The mammalian protein can downregulate M1 microglia activation via the regulation of p38-, CREB- and NF-κB-dependent pathways and induce differentiation toward a M2 phenotype[40]. Macrophages are also involved in MS pathology as they contribute to tissue

damage via the production of pro-inflammatory cytokines (TNFα, IL-6, and IL-23)[42]. It has been shown that leukocytes respond to the same signals as microglia cells since they also undergo polarization toward the M2 or M1 phenotype in the presence of IL-4/13 or IFNγ/LPS respectively[41]. Though not reported in the current studies, galectin knockdown experiments have been performed. Galectin knockdown lead to MSC cell death, thus elucidating the role of this protein using these knockdown strategies is challenging. Further proteomic analysis also revealed key biological process involved with immune modulation, cellular adhesions, programmed cell death and many other processes. These functional pathways have significant clinical implications for neurologic diseases including Parkinson, Alzheimer's, and MS. Further proteomic analysis is pending; however, these data will be critical for understanding the therapeutic MOA by PMSC-EVs and can help direct future studies employing bioengineering strategies to improve therapeutic outcomes. The presence of these proteins and cellular components in PMSC-EVs also suggests these mediators may play an important role in neuroprotective, regenerative, and immunomodulatory properties of PMSCs.

It has been reported that MSC-EVs home to sites of injury, however uptake of EVs predominantly occurs in the liver in murine disease models and biodistribution studies[43]. To evaluate PMSC-EV biodistribution *in vivo,* healthy and MOG-peptide induced EAE C57BL/6 mice were intravenously injected with MemGlow labeled PMSC-EVs. MemGlow is a lipophilic fluorogenic probe that has high specificity, low background and is a self-quenching dye. Labeling techniques were optimized, and SEC columns were used to remove free dye from PBS controls and labeled PMSC-EVs. Fluorescent PMSC-EVs injected in the tail vein of healthy mice were imaged at 15m, 30m and 60min following PMSC-EV administration. Unsurprisingly, PMSC-EVs could be detected predominantly in the liver of healthy animals. The same approach was utilized in MOG-immunized EAE mice. Interestingly, animals did not display motor deficits, however leukocyte infiltration in the CNS was shown via flow cytometry. Signal from fluorescently labeled PMSC-EVs could be detected in the CNS of EAE animals. While preliminary, these data are

promising, and further optimization of administration modalities will need to be performed. Collectively, these data suggest PMSC-EVs can home to site of injury including the CNS and provides evidence PMSC-EVs can cross disrupted BBBs. Further studies will need to be performed to assess cellular uptake of EVs in the EAE model to evaluate immunological response following PMSC-EV administration.

These preliminary findings suggest that PMSC-EVs are a viable option for the treatment of neurodegenerative diseases and pose several advantages compared to cellular-based therapies. HGF may play a critical role in PMSC and PMSC-EV mediated immunomodulation, however further studies will be needed to elucidate this mechanism. Proteomic analysis will help direct future studies to decipher MOA or possibility guide loading or targeting bioengineering strategies to improve therapeutic efficacy of PMSC-EVs. These studies will be critical to development of a PMSC-EV therapeutic agent for the treatment of MS.

2.6 References

[1] Martino, G. & Hartung, H. P. Immunopathogenesis of multiple sclerosis: the role of T cells. *Current opinion in neurology* **12**, 309-321 (1999).

[2] Abumaree, M. H., Abomaray, F. M., Alshabibi, M. A., AlAskar, A. S. & Kalionis, B. Immunomodulatory properties of human placental mesenchymal stem/stromal cells. *Placenta*, doi:10.1016/j.placenta.2017.04.003 (2017).

[3] Selmaj, I., Mycko, M. P., Raine, C. S. & Selmaj, K. W. The role of exosomes in CNS inflammation and their involvement in multiple sclerosis. *Journal of neuroimmunology* **306**, 1-10, doi:10.1016/j.jneuroim.2017.02.002 (2017).

[4] Bai, L. *et al.* Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nature neuroscience* **15**, 862-870, doi:10.1038/nn.3109 (2012).

[5] Maraldi, T., Beretti, F., Guida, M., Zavatti, M. & De Pol, A. Role of hepatocyte growth factor in the immunomodulation potential of amniotic fluid stem cells. *Stem Cells Transl Med* **4**, 539-547, doi:10.5966/sctm.2014-0266 (2015).

[6] Jia, L. *et al.* Maternal and umbilical cord serum-derived exosomes enhance endothelial cell proliferation and migration. *FASEB J* **32**, 4534-4543, doi:10.1096/fj.201701337RR (2018).

[7] Cohen, J. A. *et al.* Pilot trial of intravenous autologous culture-expanded mesenchymal stem cell transplantation in multiple sclerosis. *Multiple sclerosis (Houndmills, Basingstoke, England)*, 1352458517703802, doi:10.1177/1352458517703802 (2017).

[8] Yamout, B. *et al.* Bone marrow mesenchymal stem cell transplantation in patients with multiple sclerosis: a pilot study. *Journal of neuroimmunology* **227**, 185-189, doi:10.1016/j.jneuroim.2010.07.013 (2010).

[9] Jang, M. J. *et al.* Placenta-derived mesenchymal stem cells have an immunomodulatory effect that can control acute graft-versus-host disease in mice. *Acta haematologica* **129**, 197-206, doi:10.1159/000345267 (2013).

[10] Alegre, E. & Rizzo, R. Some basic aspects of HLA-G biology. **2014**, 657625, doi:10.1155/2014/657625 (2014).

[11] Liu, K. J. *et al.* Surface expression of HLA-G is involved in mediating immunomodulatory effects of placenta-derived multipotent cells (PDMCs) towards natural killer lymphocytes. *Cell transplantation* **20**, 1721-1730, doi:10.3727/096368911x580590 (2011).

[12] Lublin, F. D. *et al.* Human placenta-derived cells (PDA-001) for the treatment of adults with multiple sclerosis: a randomized, placebo-controlled, multiple-dose study. *Multiple sclerosis and related disorders* **3**, 696-704, doi:10.1016/j.msard.2014.08.002 (2014).

[13] Munir, H. & McGettrick, H. M. Mesenchymal Stem Cell Therapy for Autoimmune Disease: Risks and Rewards. *Stem Cells Dev* **24**, 2091-2100, doi:10.1089/scd.2015.0008 (2015).

[14] Kim, H. J., Lee, J. H. & Kim, S. H. Therapeutic effects of human mesenchymal stem cells on traumatic brain injury in rats: secretion of neurotrophic factors and inhibition of apoptosis. *Journal of neurotrauma* **27**, 131-138, doi:10.1089/neu.2008-0818 10.1089/neu.2008.0818 (2010).

[15] Cantinieaux, D. *et al.* Conditioned Medium from Bone Marrow-Derived Mesenchymal Stem Cells Improves Recovery after Spinal Cord Injury in Rats: An Original Strategy to Avoid Cell Transplantation. *PLOS ONE* **8**, e69515, doi:10.1371/journal.pone.0069515 (2013).

[16] Andras, I. E. & Toborek, M. Extracellular vesicles of the blood-brain barrier. *Tissue Barriers* **4**, e1131804, doi:10.1080/21688370.2015.1131804 (2016).

[17] Bonafede, R. *et al.* Exosome derived from murine adipose-derived stromal cells: Neuroprotective effect on in vitro model of amyotrophic lateral sclerosis. *Exp Cell Res* **340**, 150-158, doi:10.1016/j.yexcr.2015.12.009 (2016).

[18] Kumar, P. *et al.* Neuroprotective effect of placenta-derived mesenchymal stromal cells: role of exosomes. *FASEB J* **33**, 5836-5849, doi:10.1096/fj.201800972R (2019).
[19] Komaki, M. *et al.* Exosomes of human placenta-derived mesenchymal stem cells stimulate angiogenesis. *Stem Cell Res Ther* **8**, 219, doi:10.1186/s13287-017-0660-9 (2017).
[20] Benkhoucha, M. *et al.* Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and CD25+Foxp3+ regulatory T cells. *Proc Natl Acad Sci U S A* **107**, 6424-6429, doi:10.1073/pnas.0912437107 (2010).
[21] Beretti, F. *et al.* Amniotic fluid stem cell exosomes: Therapeutic perspective. *Biofactors* **44**, 158-167, doi:10.1002/biof.1407 (2018).
[22] Lankford, L. *et al.* Manufacture and preparation of human placenta-derived mesenchymal stromal cells for local tissue delivery. *Cytotherapy* **19**, 680-688, doi:10.1016/j.jcyt.2017.03.003 (2017).
[23] Clark, K. C. *et al.* Human and feline adipose-derived mesenchymal stem cells have comparable phenotype, immunomodulatory functions, and transcriptome. *Stem Cell Res Ther* **8**, 69, doi:10.1186/s13287-017-0528-z (2017).
[24] Clark, K. C. *et al.* Canine and Equine Mesenchymal Stem Cells Grown in Serum Free Media Have Altered Immunophenotype. *Stem Cell Rev* **12**, 245-256, doi:10.1007/s12015-015-9638-0 (2016).
[25] Long, C. *et al.* Isolation and characterization of canine placenta-derived mesenchymal stromal cells for the treatment of neurological disorders in dogs. *Cytometry A*, doi:10.1002/cyto.a.23171 (2017).
[26] Long, C. *et al.* Isolation and characterization of canine placenta-derived mesenchymal stromal cells for the treatment of neurological disorders in dogs. *Cytometry A* **93**, 82-92, doi:10.1002/cyto.a.23171 (2018).
[27] Kumar, P. *et al.* Neuroprotective effect of placenta-derived mesenchymal stromal cells: role of exosomes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, fj201800972R, doi:10.1096/fj.201800972R (2019).
[28] Soo, C. Y. *et al.* Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology* **136**, 192-197, doi:10.1111/j.1365-2567.2012.03569.x (2012).
[29] Vestad, B. *et al.* Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study. *Journal of extracellular vesicles* **6**, 1344087, doi:10.1080/20013078.2017.1344087 (2017).
[30] Mizenko, R. R. *et al.* Tetraspanins are unevenly distributed across single extracellular vesicles and bias sensitivity to multiplexed cancer biomarkers. *J Nanobiotechnology* **19**, 250, doi:10.1186/s12951-021-00987-1 (2021).
[31] Ramasubramanian, L. *et al.* Development and Characterization of Bioinspired Lipid Raft Nanovesicles for Therapeutic Applications. *ACS Appl Mater Interfaces* **14**, 54458-54477, doi:10.1021/acsami.2c13868 (2022).
[32] Guo, F. *et al.* Macroglial plasticity and the origins of reactive astroglia in experimental autoimmune encephalomyelitis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**, 11914-11928, doi:10.1523/jneurosci.1759-11.2011 (2011).
[33] Soulika, A. M. *et al.* Initiation and progression of axonopathy in experimental autoimmune encephalomyelitis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 14965-14979, doi:10.1523/JNEUROSCI.3794-09.2009 (2009).
[34] Clark, K. *et al.* Placental Mesenchymal Stem Cell-Derived Extracellular Vesicles Promote Myelin Regeneration in an Animal Model of Multiple Sclerosis. *Cells* **8**, doi:10.3390/cells8121497 (2019).
[35] Benkhoucha, M., Senoner, I. & Lalive, P. H. c-Met is expressed by highly autoreactive encephalitogenic CD8+ cells. *J Neuroinflammation* **17**, 68, doi:10.1186/s12974-019-1676-0 (2020).

[36] Amorim, R. M. *et al.* Placenta-derived multipotent mesenchymal stromal cells: a promising potential cell-based therapy for canine inflammatory brain disease. *Stem Cell Res Ther* **11**, 304, doi:10.1186/s13287-020-01799-0 (2020).

[37] Wang, A. *et al.* Placental mesenchymal stromal cells rescue ambulation in ovine myelomeningocele. *Stem Cells Transl Med* **4**, 659-669, doi:10.5966/sctm.2014-0296 (2015).

[38] Cedeno-Laurent, F. & Dimitroff, C. J. Galectin-1 research in T cell immunity: past, present and future. *Clinical immunology (Orlando, Fla.)* **142**, 107-116, doi:10.1016/j.clim.2011.09.011 (2012).

[39] Camby, I., Le Mercier, M., Lefranc, F. & Kiss, R. Galectin-1: a small protein with major functions. *Glycobiology* **16**, 137r-157r, doi:10.1093/glycob/cwl025 (2006).

[40] Starossom, S. C. *et al.* Galectin-1 deactivates classically activated microglia and protects from inflammation-induced neurodegeneration. *Immunity* **37**, 249-263, doi:10.1016/j.immuni.2012.05.023 (2012).

[41] Nakagawa, Y. & Chiba, K. Role of microglial m1/m2 polarization in relapse and remission of psychiatric disorders and diseases. *Pharmaceuticals (Basel, Switzerland)* **7**, 1028-1048, doi:10.3390/ph7121028 (2014).

[42] Vogel, D. Y. *et al.* Macrophages in inflammatory multiple sclerosis lesions have an intermediate activation status. *Journal of neuroinflammation* **10**, 35, doi:10.1186/1742-2094-10-35 (2013).

[43] Tolomeo, A. M. *et al.* Biodistribution of Intratracheal, Intranasal, and Intravenous Injections of Human Mesenchymal Stromal Cell-Derived Extracellular Vesicles in a Mouse Model for Drug Delivery Studies. *Pharmaceutics* **15**, doi:10.3390/pharmaceutics15020548 (2023).

Chapter 3. Placental Mesenchymal Stem Cell-derived Extracellular Vesicles Promote Myelin Regeneration in an Animal Model of Multiple Sclerosis

3.1 Abstract

Mesenchymal stem/stromal cells (MSCs) display potent immunomodulatory and regenerative capabilities through the secretion of bioactive factors, such as proteins, cytokines, chemokines as well as the release of extracellular vesicles (EVs). These functional properties of MSCs make them ideal candidates for treating degenerative and inflammatory diseases, including multiple sclerosis (MS). MS is a heterogenous disease that is typically characterized by inflammation, demyelination, gliosis and axonal loss. In the current study, an induced experimental autoimmune encephalomyelitis (EAE) murine model of MS was utilized. At peak disease onset, animals were treated with saline, placenta-derived MSCs (PMSCs), as well as low and high doses of PMSC-EVs. Animals treated with PMSCs and high dose PMSC-EVs displayed improved motor function outcomes as compared to animals treated with saline. Symptom improvement by PMSCs and PMSC-EVs led to reduced DNA damage in oligodendroglia populations and increased myelination within the spinal cord of treated mice. In vitro data demonstrated that PMSC-EVs promote myelin regeneration by inducing endogenous oligodendrocyte precursor cells to differentiate into mature myelinating oligodendrocytes. These findings support that PMSCs mechanism of action is mediated by the secretion of EVs. Therefore PMSC-derived EVs are a feasible alternative to cellular based therapies for MS as demonstrated in an animal model of disease.

3.2 Introduction

Mesenchymal stem/stromal cells (MSCs) are highly proliferative, fibroblast-like cells that can be isolated from multiple tissue sources that possess potent regenerative, immunomodulatory, neuroprotective and proangiogenic properties[1]. MSCs have been widely studied to establish reliable and effective cures to numerous diseases, predominantly through the secretion of

bioactive factors[2-4]. The MSC secretome can include free proteins but also contains extracellular vesicles (EVs) including exosomes, which are nanosized particles that are produced by budding from the endosomal membrane. EVs act as messengers of intercellular communication and can contain bioactive factors including proteins, lipids and micro-RNAs[5]. EVs have also been proposed as a mechanism by which MSCs provide therapeutic benefits and have immunoregulatory and neuroprotective properties that are achieved through multiple molecular mechanisms[5,6]. Cell based therapies are limited by potential immune rejection of donor cells and also pose other safety concerns[7]. Increasingly, studies have shown that MSC survival and integration within the host after transplantation are usually poor and that MSCs exert their therapeutic functions mainly via paracrine signaling mechanisms[8]. Therefore, MSC-secreted EVs have promising cell-free based regenerative therapeutic potential; however, the exact molecular mechanism by which MSCs and secreted-EVs exert immunoregulatory, neuroprotective and proangiogenic properties is poorly understood.

MSC-derived EVs have also been shown to readily cross the blood brain barrier (BBB) and deliver therapeutic cargo to reduce the effects of neuropathological disease, including multiple sclerosis (MS)[9]. MS is a heterogenous disease that is characterized by demyelination and inflammation caused by immune cell infiltration of the central nervous system (CNS)[10]. MS results from an autoimmune response within the CNS, that can lead to neuronal degradation, inflammation and loss of axonal conductivity and gliosis in grey and white matter[11]. Myelin sheaths provide a supportive, insulating layer for axons and are produced by oligodendrocytes. Oligodendrocyte progenitor cells (OPCs) are an immature cell subset that can differentiate into mature myelinating oligodendrocytes (OLs)[12]. Loss of oligodendrocytes and accompanying demyelination is associated with progressive axonal degeneration and neurological decline[13]. Current therapies for MS target the immune component of the disease, but do not prevent the progressive axonal and neural degradation.

Cellular-based therapies utilizing MSCs are currently being used in clinical trials for the treatment of adult MS[11,14,15]. The placenta has been suggested to be a unique source of MSCs that possess robust immunomodulatory properties and have been reported to be beneficial in murine models of graft versus host disease[16,17]. MSCs derived from the placenta (PMSCs) may be a more appropriate cell source for pediatric diseases because the placenta demonstrates “fetomaternal tolerance” during pregnancy, which is attributed to the expression of human leukocyte antigen-G (HLA-G), a non-classical major histocompatibility complex (MHC) class I molecule that inhibits natural killer cell (NK) killing[18]. Unlike bone marrow-derived MSCs (BM-MSCs), PMSCs express HLA-G on their surface in response to interferon gamma (IFNγ)[19], which is a key inflammatory mediator involved with the onset of MS[10]. Therefore, the expression of HLA-G on PMSCs would make them a unique therapeutic cell source for the treatment of neurodegenerative diseases like MS. Currently, a clinical trial is underway using term PMSCs to treat adult MS and no paradoxical worsening of MS lesion counts was noted[20].

Our group has previously demonstrated that early-gestational chorionic villus-derived PMSCs can prevent hind limb paralysis in a surgically created lamb model of spina bifida (SB)[21]. The neuroprotective properties of PMSCs occur through paracrine signaling mechanisms. Thorough characterization of PMSC-derived EVs has been performed and it was demonstrated that this unique source of EVs has potent neuroprotective properties and contains key proteins and RNAs that contribute to neuronal survival[22]. The goal of the current study was to determine if PMSC-derived EVs provided regenerative effects in an experimental autoimmune encephalomyelitis (EAE) model of MS and their potential in promoting remyelination. It was hypothesized that PMSC-derived EV treatments would lead to motor function improvement in a dose-dependent manner by preventing oligodendroglia degradation and demyelination associated with MS pathology.

3.3 Materials and Methods

3.3.1 PMSC Isolation and Expansion

PMSCs for this study were previously isolated from the chorionic villus tissue of de-identified discarded second trimester human placenta by explant culture[23]. Early passage (P2-P5) PMSCs were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM; GE Life Sciences, Pittsburgh, PA, USA) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologics, Flowery Branch, GA, USA), 20 ng/mL fibroblast growth factor basic (FGF basic; Advent Bio, Elk Grove Village, IL, USA), 20ng/mL epidermal growth factor (EGF; Advent Bio) and 1% penicillin/streptomycin (P/S; ThermoFisher Scientific, Pittsburgh, PA, USA). Cells were cultured in T150 flasks (Corning Inc., Corning, NY, USA) at 37˚C, under 5% CO_2 as previously described[23].

3.3.2 PMSC Phenotype

PMSCs were detached using Accutase (Thermo Fisher Scientific) for immunophenotype analysis via flow cytometry. Single suspension PMSCs were first labeled with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Thermo Fischer Scientific) to detect dead cells. PMSCs were then washed and incubated with antibodies directed against CD44 (clone G44-26), CD90 (clone 5E10), CD73 (cloneAD2), CD29 (clone MAR4), CD34 (clone 563), CD31 (clone WM59), and CD45 (clone HI30). Appropriate isotype controls were used for each antibody as previously described[23]. All antibodies were purchased from BD Bioscience (San Jose, CA, USA). All samples were read by flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

3.3.3 Mixed Leukocyte Reaction (MLR)

To evaluate immunosuppressive potential of PMSCs, mixed leukocyte reactions (MLRs) were performed exactly as previously described[24]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by using an underlay method of Ficoll-Paque™ PLUS (GE healthcare, Uppsala, Sweden) and gradient centrifugation. PBMCs were collected from the

buffy coat layer and stimulated with 5 µg/mL phytohemagglutin (PHA, Millipore-Sigma, St. Louis, MO, USA). PMSCs were irradiated (10Gy, Varian 2100C linear accelerator, Varian Medical Systems Inc., Palo Alto, CA, USA) and kept on ice before experimental setup. PBMCs and PMSCs were co-cultured at a ratio of 5:1 in DMEM supplemented with 10% heat inactivated FBS, 1% P/S and L-tryptophan (600 µM, Millipore-Sigma).

After 3 days of co-culture, samples were treated with 1 mM Bromodeoxyuridine (BrdU, BD Biosciences). Twenty-four hours following BrdU treatment, leukocytes were collected, and cells were stained for LIVE/DEAD® Fixable Aqua Dead Cell to identify live cells, and CD3 (clone UCHT1, BD Bioscience) to identify lymphocyte populations. Cells were then stained for nuclear BrdU incorporation per manufacture directions (FITC BrdU Flow Kit, BD Biosciences) and read by flow cytometry (Attune NxT Flow Cytometer, Thermo Fisher Scientific). Flow cytometry data were analyzed using FlowJo software (Tree Star Inc.).

3.3.4 Enzyme-Linked Immunosorbent Assay (ELISA)

PMSCs were cultured for 24 hours and supernatants were collected for protein quantification via ELISA as previously described[23]. ELISAs for brain derived neurotropic factor (BDNF; R&D Systems, Minneapolis, MN, USA), hepatocyte growth factor (HGF; R&D Systems) and vascular endothelial growth factor (VEGF; R&D Systems) were performed per manufacturer instructions and read on a plate reader (SpectraMax Plate Readers, Molecular Devices, San Jose, CA, USA).

3.3.5 Neuroprotection Assay by Indirect Coculture

Neuroprotection assays were performed exactly as previously described[22]. In brief, the SH-SY5Y neuroblastoma cell line was cultured for 24 hours. PMSCs were indirectly cultured in hanging well inserts for 24 hours. To assess neuroprotective properties of PMSCs, SH-SY5Y cells were treated with 1 µM staurosporine to induce apoptosis. PMSC inserts were washed and inserts were co-cultured with apoptotic SH-SY5Y cells. After 96 hours, cells were washed with 2 µM

calcein AM (Thermo Fisher Scientific) and imaged using an Axio Observer D1 inverted microscope (Carl Zeiss). Images were processed with WimNeuron Image Analysis (Onimagin Technologies, Cordoba, Spain) for neurite outgrowth analysis.

3.3.6 PMSC-derived Extracellular Vesicle (EV) Isolation

PMSC-derived EVs were isolated as previously described[22]. In brief, EVs were first depleted from FBS by spinning FBS samples at 112,700 G using the L7 Ultracentrifuge (Beckman Coulter, Brea, CA, USA) and a SW28 rotor for 16 hours at 4˚C. Supernatants were collected, aliquoted and stored at -20˚C. PMSCs at passage 4 were seeded at 20,000 cells/cm^2 in T175 flasks (Corning Inc.) in 20mL of medium containing 5% EV-depleted FBS, 20 ng/mL FGF (Advent Bio), 20 ng/mL EGF (Advent Bio), and 1% P/S (Thermo Fischer Scientific) for 48 hours at 37˚C, under 5% CO_2. Conditioned medium was collected and EVs were isolated by differential centrifugation exactly as previously described[22]. After the final centrifugation step, EV pellets were resuspended in 10 µL of triple-filtered PBS (GE, Life Sciences) per T175 flask used for the generation of the conditioned medium. EVs were aliquoted and stored at -80 ˚C.

3.3.7 EV Characterization by Western Blot

To characterize EVs, Western blotting analysis was performed exactly as previously described[22]. In brief, EVs were treated with NuPAGE LDS Sample Buffer (Thermo Fisher Scientific) and heated to 90°C. The samples were run, transferred, probed with 1:500 dilution of primary antibodies against Alg-2 interacting protein X (ALIX; rabbit polyclonal, Millipore-Sigma), tumor susceptibility gene (TSG101; clone T5701; Millipore-Sigma), CD9 (clone MM2-57; Millipore-Sigma), calnexin (Clone C5C9, Cell Signaling Technology, Danvers, MA, USA), and CD63 (clone TS63; Thermo Fisher Scientific) in 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.5% Tween 20 (Millipore-Sigma). Blots were then probed against their respective secondary antibodies and developed using Chemidoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

3.3.8 Characterization of EVs by Nanoparticle Tracking Analysis

Nanoparticle Tracking Analysis (NTA) was performed to quantify EV yield and size distribution. The Nano Sight LM10 Nanoparticle Analysis System (Malvern Panalytical Ltd, Malvern, UK) and the NTA 3.1 Analytical Software (Malvern Panalytical Ltd) was used to characterize isolated EVs. A 5µl aliquot of stored EVs was diluted in 995µl of 0.2µm triple-filtered Milli-Q water (Millipore-Sigma) and injected into the NTA. A 1:200 dilution is necessary to obtain a concentration between 2×10^8 and 20×10^8 particle/mL, which is the suggested optimal range for NTA[25,26]. Three-30 second videos were recorded and analyzed by the software. The scientific complementary metal-oxide-semiconductor (sCMOS) camera collected 739 frames at 22.0˚C. A 404 nm laser, with a detection threshold of 5, determined the mean-square displacement of each nanoparticle based on its own Brownian motion. The analytical software determined EV size, mean, mode and standard deviation as well as the number of particles per frame and milliliter of sample.

3.3.9 Experimental Autoimmune Encephalomyelitis (EAE) Induction in a Murine Model

In order to mimic the pathology of human MS, EAE was induced using a murine model as previously described[27,28]. Three-month old C57BL/6J mice (male and female) were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 to induce EAE (IACUC #19014). In brief, 300 µg of rodent MOG peptide (amino acids 35–55, New England Peptides, Gardner, MA, USA) in Complete Freund's Adjuvant (CFA) containing 5 mg/mL killed Mycobacterium tuberculosis (Difco, Thermo Fischer Scientific) was administered into the subcutaneous flank of mice at day 0. At day 0, each mouse received two subcutaneous injections of the MOG solution as well as a 100 µl dose of 2 ng/µl pertussis toxins and virulence factors (List Biological Laboratories Inc., Campbell, CA, USA) diluted in sterile PBS (Thermo Fisher Scientific). Pertussis toxins and virulence factors were administered again on day 2. Pertussis toxins allow an increase

in the blood-brain barrier permeability in order to facilitate the incursion of the different treatments into the CNS.

In order to monitor disease progression, mice were weighed and scored daily. Neurological deficits were assessed on a five-point scale (limp tail or waddling gait = 1; limp tail and waddling gait = 2; single limb paresis and ataxia = 2.5; double limb paresis = 3; single limb paralysis and paresis of second limb = 3.5; full paralysis of 2 limbs = 4; moribund = 4.5; and death = 5)[27].

3.3.10 Experimental Treatment of EAE Animals

Daily motor quantification was performed. Mouse treatment groups were randomized in order to contain comparable numbers of males and females and an average score close to 3.5 to represent EAE onset. For this study disease onset and treatment occurred on day 19 post-MOG immunization. On the day of treatment, PMSCs were detached from culture using TrypLE (Thermo Fisher Scientific) and washed twice with PBS (Thermo Fisher Scientific). 1×10^6 PMSCs were resuspended in 200µl injectable saline and placed on ice prior to administration. Stored EVs were thawed and resuspended at either 1×10^7 (low dose) or 1×10^{10} (high dose) EVs in 200µl of injectable saline.

Tail-vein injections were performed using 1mL syringes connected to standard hypodermic needles (Covidien, Dublin, Republic of Ireland). EAE mouse scoring was repeated up to day 40 or 43 post-MOG immunization.

3.3.11 Tissue Preparation

Mice were euthanized by CO_2 asphyxiation and were perfused with ice-cold PBS (Thermo Fisher Scientific). Lumbar spinal cords were harvested, post-fixed in 4% paraformaldehyde (PFA) at room temperature for 2 hours, cryopreserved in 30% sucrose overnight, and embedded in OCT. Fourteen micrometer frozen transverse sections were cut on a Leica cryostat.

3.3.12 Quantification of Oligodendroglia Survival

Frozen sections were dried and blocked using PBS (Thermo Fisher Scientific) containing 0.1% Tween 20 (Millipore-Sigma) and 10% donkey serum (Thermo Fisher Scientific) for 1 hour at room temperature. Sections were incubated with primary antibodies directed against SOX-10 (clone EP268, Millipore-Sigma) at 4°C overnight, followed by 2-hour incubation at room temperature with secondary antibody. TUNEL staining (Terminal deoxynucleotidyl transferase dUTP nick end labeling) was performed on samples to quantify DNA damaged within SOX10 populations. TUNEL was performed using the In-Situ Cell Death Detection Kit (TMR Red, Millipore-Sigma) per manufacture instructions. DAPI (Thermo Fisher Scientific) was used to label nuclei, and the sections were mounted with Permount (Thermo Fisher Scientific) for microscopic analysis. Samples were imaged using a Carl Zeiss Axio Observer D1 inverted microscope and analyzed using NIH ImageJ software.

3.3.13 Quantification of Myelin Loss

In order to quantify loss of myelin in treated EAE mice, Luxol Fast Blue (LFB) was used to stain frozen sections. LFB is a copper phthalocyanine dye that binds to lipoproteins found within the myelin sheath. Frozen sections were dried and rehydrated using 95% EtOH. LFB staining was performed according to manufacturer instructions (IHC World, Woodstock, MD, USA). Sections were mounted with Permount (Thermo Fisher Scientific) and samples were imaged using a Carl Zeiss Axio Observer D1 inverted microscope. Images were analyzed using NIH ImageJ software. Myelin stains blue using LFB, therefore thresholds were standardized, and lack of LFB staining was quantified to denote the percent of myelin loss in each sample.

3.3.14 EV functions on Oligodendrocyte Precursor Cell (OPC) differentiation

Primary OPC culture and differentiation were conducted according to our previous protocol[29]. In brief, OPCs were isolated by immunopanning from neonatal mouse forebrain and maintained in growth medium for population expansion. To study the role of PMSC-EVs in OPC differentiation, 1 x 10^5 OPCs were switched to the chemically defined differentiation medium with

or without PMSC-EVs (2000 x 10^5). RNA was isolated from differentiating OPCs 24 hours after PMSC-EV treatment. RNA isolation was performed using the RNeasy Mini Kit's manufacturer protocol (QIAGEN, Germantown, MD, USA), and RNA concentration was measured with the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Sybr Green-based RT-qPCR was used to quantify the expression of OL differentiation markers oligodendrocyte-specific molecules (MOG), ectonucleotide pyrophosphatase/phosphodiesterase 6 (Enpp6) and myelin associated glycoprotein (MAG). Data was analyzed using the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and processed by the V2.3 StepOne software. For quantification, the mRNA expression level of interested genes in each sample was normalized to the internal control, housekeeping gene *Hsp90* and fold change in gene expression was calculated based on delta-delta Ct method as previously described[29,30].

3.3.15 Statistical Analyses

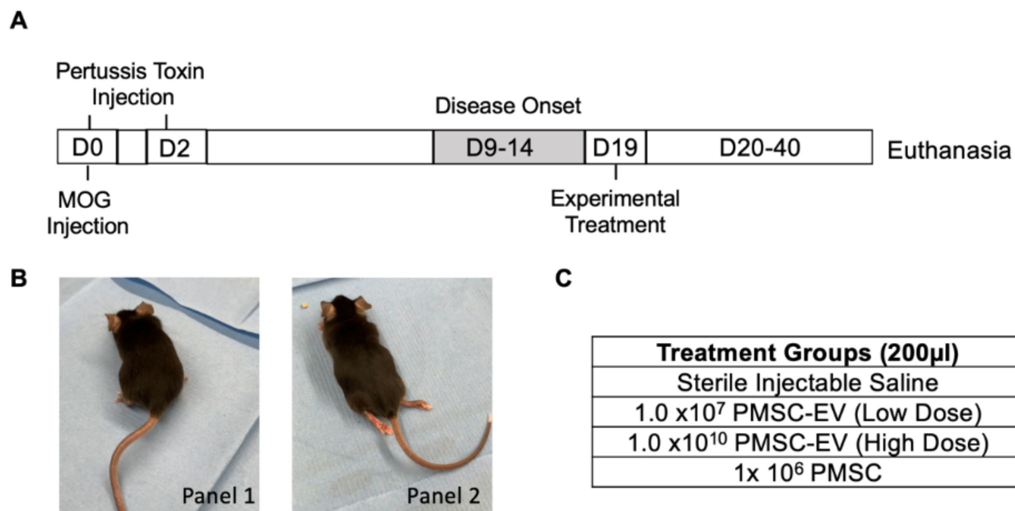
Results are expressed as mean and standard error. Imaging and *in vitro* data were analyzed using non-parametric Mann-Whitney-Wilcoxon t-tests (GraphPad Prism version 8.2.1 for macOS, La Jolla, CA, USA). Multiple comparisons were performed using a Kruskal-Wallis test, followed by Dunn's *post hoc* correction to determine which groups were significantly different (GraphPad Prism version 8.2.1 for macOS). A p-value <0.05 was considered significantly different.

3.4 Results

3.4.1 Treatment of EAE mice using EVs and PMSCs

The exact molecular mechanism by which PMSCs confer therapeutic benefits for MS are largely unknown, however these MSC functions have been shown to occur through paracrine signaling. To determine if PMSCs and secreted factors are suitable for the treatment of MS, an EAE model was utilized. An overview of the experimental design for the current study is summarized in Figure 3.1A. Induction of EAE was achieved by immunizing C57BL/6J mice with MOG peptide 35-55 and pertussis toxin on day 0 and a secondary injection of pertussis toxin on day 2. Following MOG

immunization, disease symptom onset typically peaks at 15-20 days. Motor function scoring was performed on mice daily, and peak motor deficiencies in this study were observed on day 19 following MOG immunization. Motor scoring was performed using a previously established scale[27]. Briefly, mice displaying loss of tail tension was scored as a 1, hind limb weakness was denoted as a score of 2 (Figure 3.1B, panel 1), hind limb paresis was denoted as a score of 3 and dual hind limb paralysis was denoted as a score of 4 (Figure 3.1B, panel 2). Comparable numbers of males and females were randomized into experimental groups, with each group averaging a score of about 3.5 prior to therapeutic intervention. Experimental treatment groups included sterile saline (negative control), $1{\times}10^7$ PMSC-EVs (low dose), 1×10^{10} PMSC-EVs (high dose) or 1×10^6 PMSCs (Figure 3.1C). Notably, many studies investigating the therapeutic utility of MSC-EVs from adult tissues do not provide absolute particle quantification of EV doses. Therefore, a low dose and high dose of EVs were administered to determine if outcomes occur in a dose-dependent manner. Motor scores were performed daily following treatment and mice were euthanized at day 40 or 43 post-MOG immunization.



Treatment Groups (200µl)
Sterile Injectable Saline
1.0 x10^7 PMSC-EV (Low Dose)
1.0 x10^{10} PMSC-EV (High Dose)
1x 10^6 PMSC

Figure 3.1: Experimental Design Overview. (A) Three-month old C57BL/6J mice (male and female) were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 to induce EAE on Day 0. MOG peptide in Complete Freud's Adjuvant (CFA), and pertussis toxin was

injected into mice on day 0. Pertussis toxin was administered again on day 2 following MOG immunization. Disease onset in EAE peaks 15-20 days following MOG administration. For this study peak disease onset occurred on day 19, which was determined using motor function scores. Treatments were administered on day 19 and motor function scores were recorded for 40-43 days following MOG immunization after which animals were sacrificed for further analysis. (B) Animals were scored on a 5-point scale, and groups were randomly assigned, averaging a score of 3.5. Animals with mild hind limb deficits, but still weight bearing were marked as a score of 2 (panel 1) and animals with complete hind limb paralysis were marked as a score of 4 (panel 2). (C) Treatment groups included saline (negative control), low dose PMSC-derived EVs, high dose PMSC-derived EVs and placenta-derived MSCs (PMSCs).

3.4.2 PMSC and PMSC-EV characterization

In the current study PMSCs were screened for typical phenotypic markers to identify MSC lineage. PMSCs were positive for the surface expression markers CD73, CD105, CD29, CD90 and CD44 (Figure 3.2A). Additionally, PMSCs were negative for hematopoietic markers CD31, CD34 and CD45 (Figure 3.2A). To assess the functional properties of PMSCs, mixed leukocyte reactions were performed to assess immunosuppressive potential of these cells. PBMCs from multiple donors were stimulated using the mitogen PHA and co-cultured with irradiated PMSCs. PMSCs reduced CD3 positive lymphocyte proliferation of every donor screened (Figure 3.2B). Additionally, PMSCs secrete high levels of BDNF, HGF and VEGF as measured by ELISA (Figure 3.2C). Neuroprotection assays were also performed with PMSCs, and it was shown these cells increase total branching points (fold change 2.01), circulatory length (fold change 1.52) and total segment counts (fold change 1.56) in SH-SY5Y apoptotic cells, which aligns with previous study findings[22]. EVs were then isolated from PMSCs that were shown to have both immunomodulatory and neuroprotective properties. Isolated PMSC-EVs express ALIX, CD9, CD63, TSG101 and are negative for calnexin (data not shown). Quantification of EVs for treatment was performed using NTA analysis, and the average nanoparticle size of PMSC-EVs used in this study was 124.6 +/- 4.1 nm (Figure 3.2D), which is within the reported size range of EVs (30-150 nm)[26].

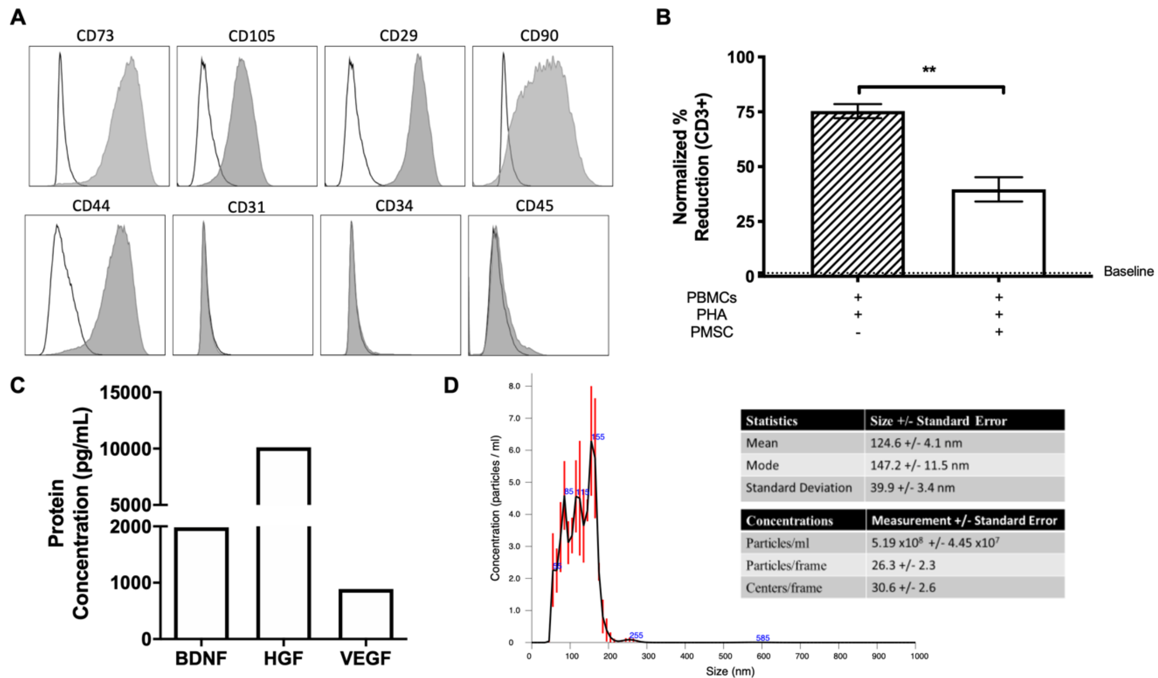


Figure 3.2: PMSC and PMSC-EV characterization. (A) PMSCs were analyzed using flow cytometry and were positive for typical MSC markers CD73, CD105, CD29, CD90 and CD44. Additionally, PMSCs were negative for the hematopoietic markers CD31, CD34 and CD45. Data are presented as median fluorescence intensity (MFI) overlays to respective negative isotypes. (B) Mixed lymphocyte reactions were performed to assess immunosuppressive potential of PMSCs. Peripheral blood mononuclear cells (PBMCs) from multiple donors (n=3) were stimulated with the mitogen Phytohemagglutinin (PHA) and co-cultured with irradiated PMSCs. Suppression of leukocyte proliferation was measured as a percentage of BrdU incorporation within CD3-positive PBMCs using flow cytometry. Unstimulated PBMC proliferation is denoted by a dashed line. (C) PMSCs secrete high levels of brain derived neurotropic factor (BDNF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF). (D) Extracellular vesicles (EVs) were collected from passage 4 PMSCs and used for further characterization analysis. Nanoparticle tracking analysis (NTA) measurements determined the size and concentration of PMSC-derived EVs. EV size had a mean of 124.6 +/- 4.1 nm. Data is represented as mean and standard error. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3.4.3 PMSC and PMSC-EVs Improve Motor Function Scores in EAE Mice

As compared to saline treated control mice, only high dose PMSC-EV and PMSC treated animals showed improved motor functions (Figure 3.3A). The high dose PMSC-EV treatment group had significantly improved motor function scores as compared to the saline only treatment group (treatment p-value=0.0002). Additionally, PMSCs also significantly improved motor function scores as compared to the saline control treatment group (treatment p-value=0.0002). No

significant differences were observed in low dose PMSC-EV treated animals as compared to saline controls (Figure 3.3A). Animals were sacrificed at 3 weeks following treatment administration to investigate responses to acute disease onset, mimicking therapeutic intervention during an active MS flare. Interestingly, sex differences in motor function outcomes were noted. In male mice, no changes were observed in low dose PMSC-EV treated animals (Figure 3.3B, panel 1), however high dose PMSC-EV (Figure 3.3B, panel 2) and PMSC (Figure 3.3B, panel 3) treated male mice were different compared to saline controls (treatment effect). However, no statistically significant changes were observed at any given time point following treatment administration (Figure 3.3B, panels 1-3). Similarly, in female mice no alterations in low dose PMSC-EV treated animals was observed (Figure 3C, panel 1). Significant changes in females was only noted in high dose PMSC-EV (Figure 3.3C, panel 2) and PMSC (Figure 3.3C, panel 3) groups, as observed in male mice. However, timelier responses were noted with female mice showing motor improvement at day 15 following high dose PMSC-EV (Figure 3.3C, panel 2) and PMSC treatment (Figure 3.3C, panel 3). Though sex differences were noted, mixed populations of male and female mice more accurately represent MS patient populations.

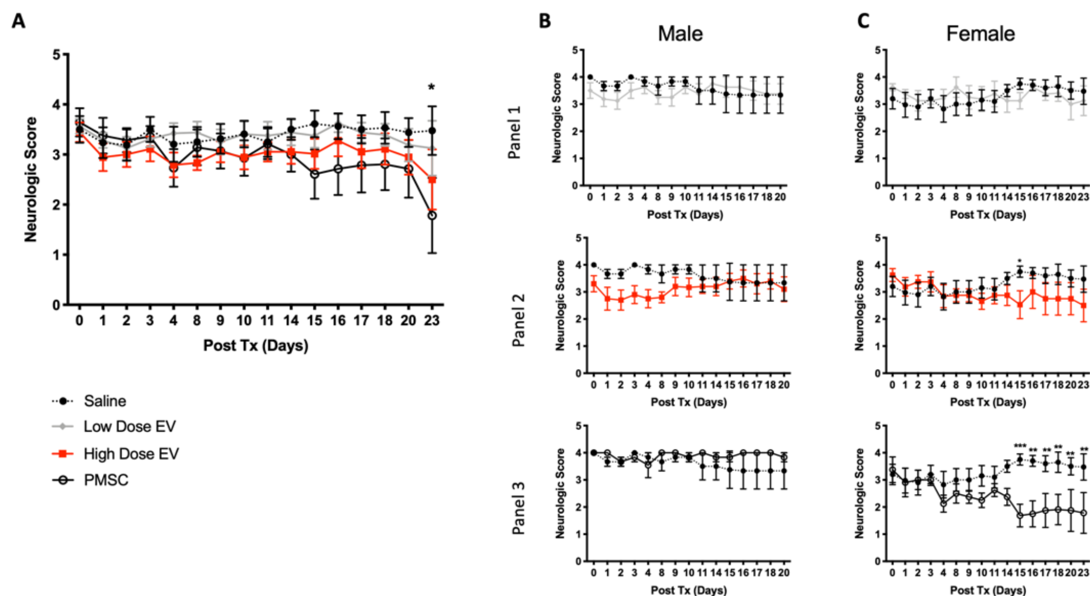


Figure 3.3. PMSC and High Dose PMSCs-EV Treatment Improves Motor Function in EAE mice. Animals were treated at peak disease onset following MOG immunization. (A) PMSC and high dose PMSC-EVs significantly improve motor function as compared to saline treated animals (treatment effect). Compared to baseline only high dose PMSC-EV and PMSC treated animals show motor improvement at day 23 following treatment administration. No observable changes were noted in low dose PMSC-EV treated animals. Interestingly, altering responses were noted in male and female mice and further analysis was performed on each sex. (B) Male mice displayed no significant alterations in the low dose PMSC-EV treated group (panel 1) as compared to saline controls, however both high dose PMSC-EV (panel 2) and PMSC (panel 3) treated animals had significant responses to treatment. These findings however did not reveal timewise alterations. (C) Female mice similarly displayed no changes in the low dose PMSC-EV treatment group (panel 1) compared to saline controls. Females did however display significant improvements in motor function in high dose PMSC-EV (panel 2) and PMSC (panel 3) treatment groups. Additionally, these improvements occur in a timelier manner beginning at day 15 following high dose PMSC-EV and PMSC administration as compared to saline mice. Data are presented as mean and standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.4.4 PMSCs and PMSC-EVs Protect Oligodendroglia Degeneration in EAE Mice

Motor function data demonstrated that only PMSC and high dose PMSC-EV treated animals showed significant improvement and therefore only these animals were used for immunohistological analysis. TUNEL (apoptotic cell marker) and SOX10 (oligodendrocyte marker) staining was performed to quantify damaged oligodendrocytes in spinal cord white matter. Representative images of a saline treated animal (Figure 3.4A), high dose PMSC-EV treated animal (Figure 3.4B), and a PMSC treated animal (Figure 3.4C) are shown. Quantification of SOX10+TUNEL+ cells was performed in both male and female mice. Double positive cells were most abundant in saline treated animals at lesion sites, therefore increased magnification of double positive cells is provided in Figure 3.4D. TUNEL staining in spinal cord tissue sections revealed a decrease in the expression of SOX10+TUNEL+ cells in EAE mice treated with high dose PMSC-EVs and PMSCs (Figure 3.4E).

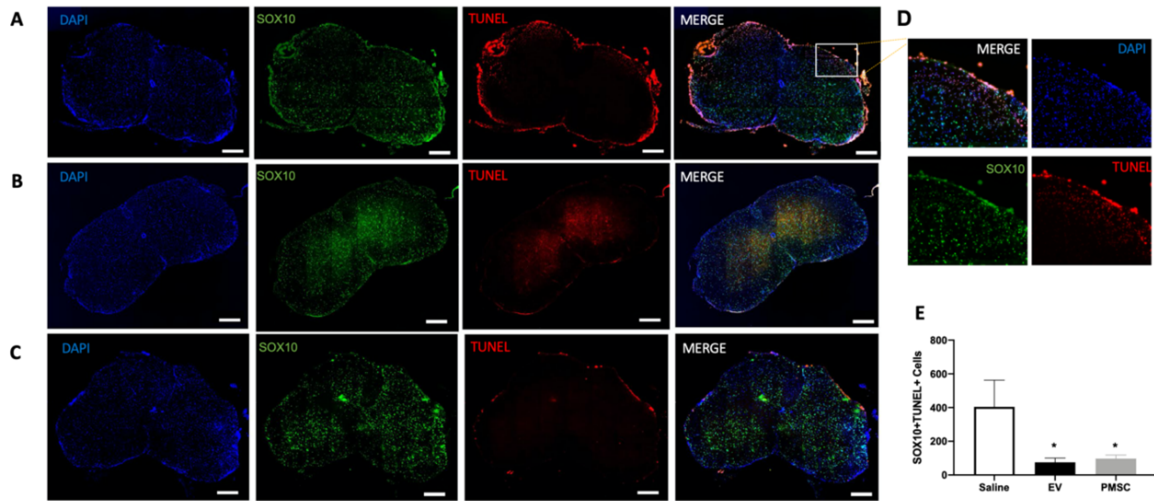


Figure 3.4. PMSC and PMSC-EV Treatment Both Reduce Oligodendrocyte Damage in EAE Mice. Representative images of spinal cords from EAE mice treated with (A) saline (control), (B) high dose PMSC-EVs or (C) PMSCs are shown at 20X magnification. Spinal cord sections were stained with SOX10 (green) to denote oligodendrocytes. TUNEL staining was also performed to identify DNA damage within oligodendrocyte populations. (D) Increased numbers of SOX10+TUNEL+ cells were observed in lesion sites within the white matter, most often in saline treated animals. Increased magnification of a lesion from a saline treated mouse is shown. (E) Compared to saline treated animals, high dose PMSC-EV and PMSC treated animals had a significant reduction of SOX10+TUNEL+ positive cells. Scale bar = 200μM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.4.5 PMSCs and PMSC-EVs Preserve Myelin in the Spinal Cord of EAE Mice

Myelin loss was quantified using Luxol Fast Blue staining, which binds to lipid membranes found within myelin. Percentage of negative staining was quantified within the white matter of the spinal cord. Myelin staining of representative images is shown for saline (Figure 3.5A), high dose PMSC-EV (Figure 3.5B) and PMSC (Figure 3.5C) treated animals. Demyelination occurred most often within spinal cord lesions, notably in saline treated animals. Myelin staining was greatly reduced in saline mice at lesions sites. These areas are highlighted by arrow indicators in Figure 3.5A. Quantification strategy for demyelination is shown in a saline treated mouse lesion (Figure 3.5D). Magnification of the lesion site within the white matter is shown in panel 1 of Figure 3.5D. Images were converted to black and white (Figure 3.5D, panel 2), and colors were inverted to highlight negative staining patterns (Figure 3.5D, panel 3). A standardized thresholding strategy was implemented, and negative black pixel intensity was quantified as a percent myelin loss in white

matter (Figure 3.5D, panel 4). Compared to saline treated EAE mice, high dose PMSC-EV and PMSC treated animals showed significant reduction of myelin present in the spinal cord (Figure 3.5D). No observable alterations were noted between high dose PMSC-EV and PMSC treated animals.

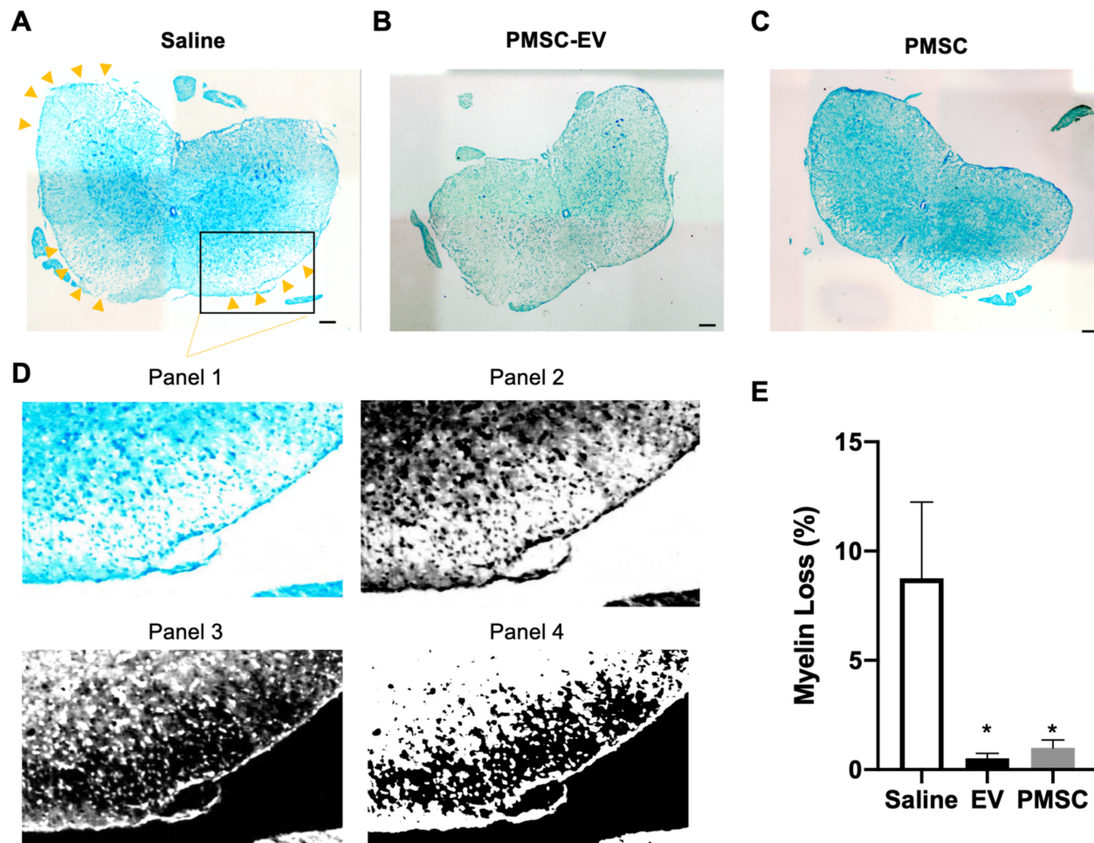


Figure 3.5. PMSC and PMSC-EV Treated EAE Mice Show Decreased Myelin Loss. Spinal cords from EAE mice treated with saline (control), high dose PMSC-EVs or PMSCs were stained with Luxol Fast Blue, a dye that binds to myelin. Representative images of (A) saline, (B) high dose PMSC-EV and (C) PMSC treated animals are shown at 10x magnification. (D) Magnification of a demyelinating lesion site in the saline control animal is highlighted in panel 1. Quantification of demyelination was performed by converting images to black and white (panel 2) and inverting colors resulting in demyelinating areas shown in black (panel 3). Standardized thresholding was applied (panel 4) and quantification was performed using pixel intensity of negatively stained areas in white matter. (E) Myelin loss within the white matter of mouse spinal cords was recorded. Compared to saline treated animals, high dose PMSC-EV and PMSC treated animals showed a significant reduction of myelin loss. Scale bar = 200μM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}P < 0.001$.

3.4.6 PMSC-EVs Promote OPC Differentiation In Vitro

To investigate whether PMSCs preserve oligodendrocyte populations or drive OPC differentiation to OLs, an OPC differentiation assay was performed with PMSC derived EVs. Primary OPCs isolated from neonate mice were treated with PMSC-EVs and oligodendrocyte differentiation markers MOG, Enpp6 and MAG were quantified using RT-PCR. Gene expression was compared between untreated OPCs and PMSC-EV treated OPCs. Both MOG (Figure 3.6A) and Enpp6 (Figure 3.6B) were significantly upregulated in the presence of PMSC-EVs. There was also a trend of increased expression of MAG (Figure 3.6C), however this was not statistically significant.

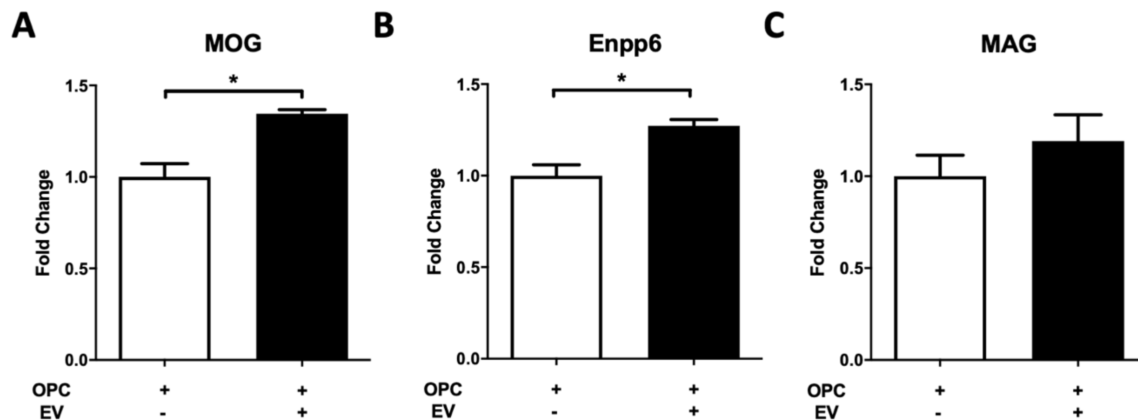


Figure 3.6. PMSC-EVs Drive Oligodendrocyte Precursor Cells (OPCs) to a Mature Lineage Phenotype. PMSC-EVs were added to primary murine OPC cultures. Expression of mature oligodendrocyte markers myelin oligodendrocyte protein (MOG), ectonucleotide pyrophosphatase/phosphodiesterase 6 (Enpp6) and myelin associated glycoprotein (MAG) were evaluated. PMSC-EVs promoted significant upregulation of (A) MOG and (B) Enpp6. Though not statically significantly a trend in increased expression of MAG was noted. Data is represented as mean and standard error. *p < 0.05, **p < 0.01, ***p < 0.001.

3.5 Discussion

The goal of the present study was to investigate the use of a novel early-gestational chorionic villus-derived MSC source for the treatment of MS. Furthermore, we aimed to investigate whether PMSC-derived EVs confer therapeutic benefits, and whether these particles can be used as a

cell-free treatment for neurodegenerative disease. Comparable to findings from other studies using adult derived MSCs[11], PMSCs achieved motor improvement in an EAE murine model of MS. Improvements in motor function were achieved in part through a neuroprotective mechanism. PMSC treated animals displayed less DNA damage within oligodendrocyte populations, and myelin was preserved in the spinal cords of these animals. Interestingly, in treatment group animals that did not show motor improvements, mild increases of DNA damage and demyelination were noted. Additionally, similar protective effects preserving oligodendroglia and myelination were also achieved by treatment with high dose PMSC-derived EVs. These effects were not noted in low dose PMSC-EV treatment groups, suggesting that the PMSC secretory mechanism of action occurs through EV signaling in a dose-dependent manner.

These findings demonstrate that the clinical benefits of PMSCs can be achieved by treatment with PMSC-EVs alone. Our group has previously demonstrated that PMSCs secrete high levels of BDNF, HGF and VEGF as compared to BM-MSCs[21]. Proteomic analysis of PMSC-EVs revealed the presence of HGF and VEGF in these nanoparticles. HGF, in particular, has been shown to be secreted in MSC-conditioned medium and can mediate motor recovery functions in an EAE model of MS[30]. HGF is a potent angiogenic factor that has been shown to exert immunomodulatory effects through the stimulation of regulatory T cells, which in turn mediate autoimmune responses[31]. These factors may play a role in the protective and regenerative properties of PMSCs. In this study, high dose PMSC-EV treatments lead to comparable responses to PMSC treatments. This suggests that PMSC mediated clinical improvements in the current EAE model occurs through an EV meditated mechanism. The presence of these factors in PMSC-EVs also suggests these mediators may play an important role in protective, regenerative and immunomodulatory properties of PMSCs. Additionally, PMSCs secrete higher levels of these proteins compared to BM-MSCs[21], thus this unique cell source may result in improved clinical outcomes compared to adult derived MSC sources.

Our *in vivo* data demonstrate increased myelin present in spinal cord white matter of PMSC and PMSC-EV treated EAE mice. In these animals, oligodendrocyte survival was increased as compared to saline treated controls. PMSC and high dose PMSC-EV treatments led to less DNA damage to oligodendroglia. Oligodendrocytes are myelin producing cells, therefore increased levels of oligodendrocytes correspond to increased myelin levels. To elucidate whether PMSC-EVs promote oligodendrocyte maturation, differentiation assays were performed. *In vitro* data demonstrate that PMSC-EVs drive OPCs to express OL differentiation markers, which suggests that PMSC-EVs are promoting maturation of myelinating oligodendroglia. These findings suggest that PMSCs and PMSC-EVs have both protective and regenerative properties exhibited by driving OPC differentiation. Additionally, interactions of oligodendrocytes and autoreactive T lymphocytes is not well understood, therefore investigations of these interactions in response to PMSC and PMSC-EV therapy is warranted.

Limitations of using an EAE model have been widely noted, and though the model is reliable to induce MS-like symptoms including inflammation and demyelination, variation in disease onset occurs even within genetically identical rodents[32]. Variability in disease onset was observed in this study; however, a standardized average score within each experimental group was used prior to experimental treatments to account for these alterations. This variability likely effected motor function and recovery data and could explain why some animals did not respond to treatment with PMSCs or high dose PMSC-EVs. Additionally, for the purposes of this study both male and female mice were used; however, a timelier improvement was noted in female mice. Sex differences have been noted in EAE onset, which reflects higher incidences of MS in human female populations[33]. These alterations may be due to effects of androgens on T lymphocytes, which are key mediators in the pathologic features of MS[34]. Sex differences in response to EAE onset methodologies can occur in multiple mouse strains, and it has been shown that male mice do not result in robust Th1 responses and instead shift to anti-inflammatory Th2 responses as compared

to females[33]. Sex differences are not typically noted by C57BL/6 mice however, in the current study EAE onset by males and females differed, but to recapitulate MS patient populations both sexes were included for analysis.

Myelin degeneration is a main pathologic feature of MS; however, autoimmune infiltration of Th1 pro-inflammatory cells into the CNS is a key contributor to the inflammatory and degenerative properties of MS[35]. T regulatory cells modulate Th1 and Th2 responses and have impaired functions in MS patients[36]. MSCs have been shown to induce T regulatory populations and drive shifts of Th1 to Th2 responses[36]. The induction of T regulatory responses by MSCs is a mechanism by which these cells modulate immune responses, and may have clinical utility for autoimmune diseases such as MS. Given male mice have a stronger tendency towards Th1 responses in EAE[33], this could explain the modest motor recovery responses observed in these animals to PMSC and PMSC-EV treatment. While the focus of the present study is on the myelin protective and regenerative properties of PMSCs and PMSC-EVs, future studies will investigate the immunoregulatory properties of these cells and nanovesicles within immune cell subsets.

Though PMSC and PMSC-EV treatment resulted in motor improvements following a single injection in mice in the acute phase of EAE, future studies are needed to address dosage timing and strategy. For this study, small volumes of diluted EVs and PMSCs were injected via tail veins of mice. However, it has been reported that PMSCs have poor homing and engraftment[2]. Additionally, nanoparticle treatments pose technical challenges because injections need to be precise to ensure proper and complete administration of therapeutic doses. In future studies, tracking cells and EVs, as well as evaluation of different administration modalities will be performed. Biodistribution studies will provide key insights to further improve clinical outcomes for MS patients.

The findings from this study demonstrate PMSC-EVs are a viable option for the treatment of neurodegenerative diseases, and pose several advantages compared to cellular-based therapies. EVs provide many benefits including immunotolerance, storage stability, heterogenous cargo and multiple therapeutic outcomes. This study provides key preliminary data that will facilitate future studies investigating the use of PMSC-EVs for the treatment of MS.

3.6 Conclusions

In an EAE rodent model of MS, PMSCs exert therapeutic benefits in part by preserving or driving oligodendroglia differentiation and myelination. Our data demonstrates that PMSC-EVs achieve similar clinical outcomes as PMSC treatments in a dose-dependent manner. The findings from this study provide evidence that PMSCs are a unique cell source for the treatment of neurodegenerative diseases, and that therapeutic benefits can be achieved using isolated EVs. Additionally, PMSCs may have superior clinical benefits in the context of pediatric neurodegenerative diseases. This study will be used as a platform for further investigation of the molecular mechanism and intracellular communication between cell subsets to improve potential treatment strategies for MS.

3.7 References

[1] Dominici, M. *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**, 315-317, doi:10.1080/14653240600855905 (2006).

[2] Chamberlain, G., Fox, J., Ashton, B. & Middleton, J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem cells (Dayton, Ohio)* **25**, 2739-2749, doi:10.1634/stemcells.2007-0197 (2007).

[3] Zhongju Shi, H. H., Shiqing Feng. Stem cell-based therapies to treat spinal cord injury: a review. *Journal of Neurorestoratology* **5**, 125-131, doi:10.2147/jn.S139677 (2017).

[4] Zhenrong Zhang, F. W., Mingjie Song. The cell repair research of spinal cord injury: a review of cell transplantation to treat spinal cord injury. *Journal of Neurorestoratology* **7**, 55-62, doi:10.26599/jnr.2019.9040011 (2019).

[5] Bobrie, A., Colombo, M., Raposo, G. & Thery, C. Exosome secretion: molecular mechanisms and roles in immune responses. *Traffic* **12**, 1659-1668, doi:10.1111/j.1600-0854.2011.01225.x (2011).

[6] Marino, J. *et al.* Donor exosomes rather than passenger leukocytes initiate alloreactive T cell responses after transplantation. *Sci Immunol* **1**, doi:10.1126/sciimmunol.aaf8759 (2016).

[7] Munir, H. & McGettrick, H. M. Mesenchymal Stem Cell Therapy for Autoimmune Disease: Risks and Rewards. *Stem Cells Dev* **24**, 2091-2100, doi:10.1089/scd.2015.0008 (2015).

[8] Kim, H. J. & Park, J. S. Usage of Human Mesenchymal Stem Cells in Cell-based Therapy: Advantages and Disadvantages. *Development & reproduction* **21**, 1-10, doi:10.12717/dr.2017.21.1.001 (2017).

[9] Andras, I. E. & Toborek, M. Extracellular vesicles of the blood-brain barrier. *Tissue Barriers* **4**, e1131804, doi:10.1080/21688370.2015.1131804 (2016).

[10] Martino, G. & Hartung, H. P. Immunopathogenesis of multiple sclerosis: the role of T cells. *Curr Opin Neurol* **12**, 309-321, doi:10.1097/00019052-199906000-00010 (1999).

[11] Cohen, J. A. Mesenchymal stem cell transplantation in multiple sclerosis. *Journal of the neurological sciences* **333**, 43-49, doi:10.1016/j.jns.2012.12.009 (2013).

[12] Magnus, T. *et al.* Evidence that nucleocytoplasmic Olig2 translocation mediates brain-injury-induced differentiation of glial precursors to astrocytes. *J Neurosci Res* **85**, 2126-2137, doi:10.1002/jnr.21368 (2007).

[13] Miron, V. E., Kuhlmann, T. & Antel, J. P. Cells of the oligodendroglial lineage, myelination, and remyelination. *Biochim Biophys Acta* **1812**, 184-193, doi:10.1016/j.bbadis.2010.09.010 (2011).

[14] Yamout, B. *et al.* Bone marrow mesenchymal stem cell transplantation in patients with multiple sclerosis: a pilot study. *Journal of neuroimmunology* **227**, 185-189, doi:10.1016/j.jneuroim.2010.07.013 (2010).

[15] Karussis, D. *et al.* Safety and immunological effects of mesenchymal stem cell transplantation in patients with multiple sclerosis and amyotrophic lateral sclerosis. *Arch Neurol* **67**, 1187-1194, doi:10.1001/archneurol.2010.248 (2010).

[16] Abumaree, M. H., Abomaray, F. M., Alshabibi, M. A., AlAskar, A. S. & Kalionis, B. Immunomodulatory properties of human placental mesenchymal stem/stromal cells. *Placenta*, doi:10.1016/j.placenta.2017.04.003 (2017).

[17] Jang, M. J. *et al.* Placenta-derived mesenchymal stem cells have an immunomodulatory effect that can control acute graft-versus-host disease in mice. *Acta haematologica* **129**, 197-206, doi:10.1159/000345267 (2013).

[18] Alegre, E. & Rizzo, R. Some basic aspects of HLA-G biology. **2014**, 657625, doi:10.1155/2014/657625 (2014).

[19] Liu, K. J. *et al.* Surface expression of HLA-G is involved in mediating immunomodulatory effects of placenta-derived multipotent cells (PDMCs) towards natural killer lymphocytes. *Cell transplantation* **20**, 1721-1730, doi:10.3727/096368911x580590 (2011).
[20] Lublin, F. D. *et al.* Human placenta-derived cells (PDA-001) for the treatment of adults with multiple sclerosis: a randomized, placebo-controlled, multiple-dose study. *Multiple sclerosis and related disorders* **3**, 696-704, doi:10.1016/j.msard.2014.08.002 (2014).
[21] Wang, A. *et al.* Placental mesenchymal stromal cells rescue ambulation in ovine myelomeningocele. *Stem cells translational medicine* **4**, 659-669, doi:10.5966/sctm.2014-0296 (2015).
[22] Kumar, P. *et al.* Neuroprotective effect of placenta-derived mesenchymal stromal cells: role of exosomes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, fj201800972R, doi:10.1096/fj.201800972R (2019).
[23] Lankford, L. *et al.* Manufacture and preparation of human placenta-derived mesenchymal stromal cells for local tissue delivery. *Cytotherapy* **19**, 680-688, doi:10.1016/j.jcyt.2017.03.003 (2017).
[24] Clark, K. C. *et al.* Human and feline adipose-derived mesenchymal stem cells have comparable phenotype, immunomodulatory functions, and transcriptome. *Stem cell research & therapy* **8**, 69, doi:10.1186/s13287-017-0528-z (2017).
[25] Soo, C. Y. *et al.* Nanoparticle tracking analysis monitors microvesicle and exosome secretion from immune cells. *Immunology* **136**, 192-197, doi:10.1111/j.1365-2567.2012.03569.x (2012).
[26] Vestad, B. *et al.* Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study. *Journal of extracellular vesicles* **6**, 1344087, doi:10.1080/20013078.2017.1344087 (2017).
[27] Guo, F. *et al.* Macroglial plasticity and the origins of reactive astroglia in experimental autoimmune encephalomyelitis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **31**, 11914-11928, doi:10.1523/jneurosci.1759-11.2011 (2011).
[28] Soulika, A. M. *et al.* Initiation and progression of axonopathy in experimental autoimmune encephalomyelitis. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **29**, 14965-14979, doi:10.1523/JNEUROSCI.3794-09.2009 (2009).
[29] Zhang, S. *et al.* Sox2 Is Essential for Oligodendroglial Proliferation and Differentiation during Postnatal Brain Myelination and CNS Remyelination. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **38**, 1802-1820, doi:10.1523/JNEUROSCI.1291-17.2018 (2018).
[30] Bai, L. *et al.* Hepatocyte growth factor mediates mesenchymal stem cell-induced recovery in multiple sclerosis models. *Nature neuroscience* **15**, 862-870, doi:10.1038/nn.3109 (2012).
[31] Maraldi, T., Beretti, F., Guida, M., Zavatti, M. & De Pol, A. Role of hepatocyte growth factor in the immunomodulation potential of amniotic fluid stem cells. *Stem cells translational medicine* **4**, 539-547, doi:10.5966/sctm.2014-0266 (2015).
[32] Constantinescu, C. S., Farooqi, N., O'Brien, K. & Gran, B. Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS). *British journal of pharmacology* **164**, 1079-1106, doi:10.1111/j.1476-5381.2011.01302.x (2011).
[33] Papenfuss, T. L. *et al.* Sex differences in experimental autoimmune encephalomyelitis in multiple murine strains. *Journal of neuroimmunology* **150**, 59-69, doi:10.1016/j.jneuroim.2004.01.018 (2004).
[34] Lai, J. J. *et al.* Androgen receptor influences on body defense system via modulation of innate and adaptive immune systems: lessons from conditional AR knockout mice. *Am J Pathol* **181**, 1504-1512, doi:10.1016/j.ajpath.2012.07.008 (2012).
[35] Bar-Or, A. *et al.* Immunopathophysiology of pediatric CNS inflammatory demyelinating diseases. *Neurology* **87**, S12-19, doi:10.1212/WNL.0000000000002821 (2016).

[36] Yang, H. *et al.* Umbilical cord-derived mesenchymal stem cells reversed the suppressive deficiency of T regulatory cells from peripheral blood of patients with multiple sclerosis in a co-culture - a preliminary study. *Oncotarget* **7**, 72537-72545, doi:10.18632/oncotarget.12345 (2016).

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Chapter 4. Convergence of human and veterinary medicine: Leveraging canine naturally occurring neurological disorders to develop regenerative treatments

4.1 Introduction

In recent years, large animal models of naturally occurring diseases have become increasingly studied, with the rationale that their disease attributes may better recapitulate the pathological features of corresponding human diseases as compared to induced disease models[1]. Of the available naturally occurring disease models, the canine is increasingly recognized as a valuable pre-clinical animal model in translational medicine for numerous human diseases, including cancer, respiratory disease, and inflammatory disease[1,2]. The canine also frequently suffers from central nervous system (CNS) disorders, such as brain and spinal cord injuries, neurodevelopmental diseases (e.g spina bifida (SB)) and neurodegenerative diseases (e.g inflammatory brain disease (IBD)) that have comparable pathological features to human CNS disorders[1,3]. Additionally, canines live in similar environmental conditions as humans and can receive long-term monitored medical care. While research-induced animal disease modeling systems are the standard *in vivo* approach to evaluate therapeutics, there are notable limitations, including inconsistency from natural disease in phenotypic heterogenicity, clinical and pathological features, and responsiveness to treatments. Furthermore, effective treatments are extremely underdeveloped for dogs with these conditions, and therefore many animals are left untreated or euthanized. The comparable features of canine CNS to human CNS disorders could allow the advancement of new standard care practices for companion animals, as well as provide critical insights for the development of regenerative medicine therapies for human clinical use.

Canine CNS Disorders:

Spinal Cord Injury (SCI):

Spinal cord injury (SCI) is a devastating condition that afflicts both human and canine patients. SCI can be acquired via acute injury or be the result of a congenital defect, most notably spina bifida (SB). SCI clinically presents as sensory, nerve and motor deficits, and in some cases complete paralysis. In the canine, SCI injuries typically result from acute injury including embolic myelopathy, disc herniation and trauma which can result in chronic injury leading to degeneration[4]. The pathology of SCI is generally regarded as a two-hit hypothesis, in which primary injury results from mechanical trauma to the spinal cord and leads to the secondary injury in which prolonged proinflammatory responses and astrogliosis that results in demyelination and neuronal injury[4]. Intervertebral disc disease (IVDD) is an example of SCI that occurs in both humans and canines and results in degeneration and loss of vascularization to intervertebral discs[1,4]. The pathological features of canine and human IVDD are comparable and the risk of secondary injury is also analogous. Unlike rodent models, canine disease can account for variability in clinical manifestations of SCI and the variability of lesion size and locale. Therefore, naturally occurring forms of SCI in the dog, including IVDD, may serve as superior models to evaluate therapeutic intervention strategies for the treatment of various forms of SCI.

Spina Bifida (SB):

SB is the most common cause of lifelong childhood paralysis and results from a congenital neural tube defect that exposes the spinal cord to chemical and mechanical trauma and results in paralysis and incontinence[3]. Canine SB clinically presents very similarly to human SB and occurs congenitally in dogs, making them a more relevant model of human SB, compared to induced disease models. English bulldogs, have the highest incidence of naturally occurring SB, making up a third of all canine cases due to the inbreeding[3]. There is currently no standard of care treatment available for dogs with SB, and many are euthanized at birth. Therefore, surgical intervention strategies for postnatal repair in canines are being explored. It has been shown that recovery from SCI is substantially higher in neonates compared to adult and young animals.[5,6]. One reason for this is that elevated adaptive plasticity is maintained in the spinal cord after birth, which has led to the introduction of plasticity-augmenting procedures into clinical trials on human SCI patients[6] . While this plasticity indicates the potential for a successful regenerative therapy

for SB, there are no existing animal models to study postnatal repair of SB. Additionally, for human SB, *in utero* intervention strategies are not applicable for all cases, creating a clinical need for improved postnatal treatment strategies. Therefore, the English bulldog serves as a unique novel model to study postnatal therapy to treat naturally occurring SB in human patients.

Inflammatory Brain Disease (IBD):

Canine inflammatory brain disease (IBD) has been shown to have a similar phenotype as multiple sclerosis (MS) in humans[7]. The two pathologic features of pediatric and adult MS are demyelination and inflammation caused by immune cell infiltration of the central nervous system (CNS)[8]. Mouse experimental autoimmune encephalitis (EAE) is one of the most widely used models for studying MS pathology and therapeutic intervention[8]. While this induced model strongly resembles the inflammatory and demyelinated state associated with MS, it does not capture all the features of the naturally occurring disease. Canine IBD can generically be referred to as meningoencephalomyelitis of unknown origin (MUO). Several forms of MUO can be characterized from histopathological findings into several categories including granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME) and necrotizing leukoencephalitis (NLE)[9]. MUO is presumed to be an autoimmune disease with a genetic predisposition[9]. It has been shown that there is a central role of MHC II positive cells, T-cells, and macrophages in disease onset of GME and NLE, much like what is seen in human forms of MS pathology[9]. Pugs specifically suffer from NME, which has been shown to have a strong association of dog leukocyte antigen (DLA) class II, similar to HLA in human MS[10]. NME in dogs has a strong autoimmune component and is an aggressive phenotype of disease that has early and acute onset, which may be more comparable to pediatric MS. Current treatment strategies for canine MUO and human MS manage the inflammatory component of the diseases but cannot address accompanying demyelination and degeneration. Though the heterogenous nature of MS pathology makes it a difficult disease to study using animal modeling strategies, a novel canine naturally occurring disease model would provide a meaningful approach to assess the function of therapeutics.

Mesenchymal stem/stromal cells (MSCs):

Mesenchymal stem/stromal cell (MSC) based regenerative therapy has become progressively popular for both human and veterinary patients[2,11]. MSCs are known to possess

potent immunomodulatory, anti-inflammatory, and regenerative properties through the secretion of bioactive mediators[11]. However, the mechanisms by which canine MSCs elicit these effects on CNS disorders are not yet fully characterized. MSCs may play a pivotal role in neuroregeneration by secretion of neuroprotective, anti-apoptotic and neurotrophic factors/cytokines as well as other extracellular elements. MSCs may also possess the ability to induce endogenous neuronal growth, promote neurogenesis, encourage synaptic connection from damaged neurons, recruit local oligodendrocyte precursors, stimulate angiogenesis, reduce demyelination and oxidative stress, and regulate neuroinflammation by modulating pathological T, B, natural killer (NK) and microglial cell responses[4,11]. In particular, placenta-derived MSCs (PMSCs) are an emerging therapy for disease treatment. The placenta is a unique, fetal-derived tissue that can be reliably obtained to generate PMSCs for the treatment of developmental and perinatal diseases. Compared to adult adipose derived MSCs, PSMCs have a distinctive secretome profile and have more robust immunomodulatory properties, modulating lymphocyte responses by inducing apoptosis[12]. Furthermore, the PMSC secretome includes extracellular vesicles (EVs), which are membrane-bound nanovesicles containing proteins and nucleic acids, and can be classified as apoptotic bodies, microvesicles, and exosomes[13].The PMSC secretome may additionally serve as a potential cell-free therapeutic to treat neurological disease. Collectively, these findings suggest that PMSCs represent a unique stem cell subset and hold significant promise for treatments of canine diseases and disorders.

MSCs therapy for canine CNS disorders:

MSCs have immense immunomodulatory and regenerative properties which have been demonstrated in several animal species research-induced disease modeling, using synthetic, genetic and technical manipulation provides critical preclinical insights for disease study, but has significant limitations modeling the complexity of native pathological features of biological disease. This likely contributes to the high failure rate of rodent disease models transitioning to human phase I clinical trials[2]. Therefore, naturally occurring companion animal disease may better recapitulate the complex and heterogenous features associated with human disease pathology.

Utilizing MSC therapy for the treatment of CNS disorders in the dog has become of high interest in recent years. The findings from these studies can provide critical insights to the use of MSCs and MSC derived EVs as potential drug candidates for human diseases as well. Clinical

trials have already been employed in the canine using adult tissue derived MSCs for several neurologic conditions including, IVDD and MUO[1]. It has previously been reported that canine MSCs derived from adult and fetal tissue have comparable functional properties feasible for the treatment of neurological disorders; however canine PMSCs have superior modulatory properties making them an ideal drug candidate. Investigating the therapeutic functions of canine PMSCs in treating canine neurological disorders will not only benefit canine patients but will also serve as preclinical data to accelerate the development of human counterpart PMSCs as a therapeutic for human patients. MSCs are also currently being evaluated for the treatment of MS, however the heterogenous nature of MS pathology poses a large challenge for development of intervention strategies. Utilizing naturally occurring canine SCI, SB and MUO may better direct treatment plans for future human clinical trials.

To assess the clinical feasibility of an MSC product for the treatment of canine neurologic diseases, clinical trials will be necessary. Studies utilizing canine MSCs to evaluate safety and efficacy in the form of a veterinary clinical trial would allow for a new standard of care for these patients. Determining outcomes of these studies will utilize analogous deficits observed in both canine and human patients. For many CNS disorders, ambulatory defects, incontinence, and impaired cognitive functions are typically observed in patients. The need for strategies to improve clinical outcomes for neurodegenerative diseases is warranted in both the veterinary and human medical fields. Large scale clinical trials evaluating the safety and efficacy of stem cell-based treatments long-term in canines provide unique opportunities to address clinical needs for both companion animals and their human counterparts. A schematic overview of studies utilizing *in vitro* models, research-induced disease models and naturally occurring veterinary disease models to develop regenerative treatments for both veterinary and human patients is shown in **Figure 4.1**. This approach will be a significant step toward translating this cellular therapy to human patients.

Conclusions:

Findings from these studies would be relevant to companion animal health as these results will provide clinical utility to several populations of dogs that do not have a treatment available in most cases. These animals will typically be euthanized due to the lack of standard care practices and

intensive care required for their survival. Due to the immunomodulatory and regenerative properties of stem cells, there are a wide range of pathologies these therapies can be utilized for. Placenta-derived MSCs are a unique subset of cells which may have efficacious therapeutic outcomes for neurodevelopmental disorders due to their potent immunomodulatory and neuroprotective functions. In conclusion the canine is becoming a significant translational model for human disease and may be better predictor of clinical outcomes in humans, compared to traditional research-induced disease models and will provide unique insights to translational therapies for human patients.

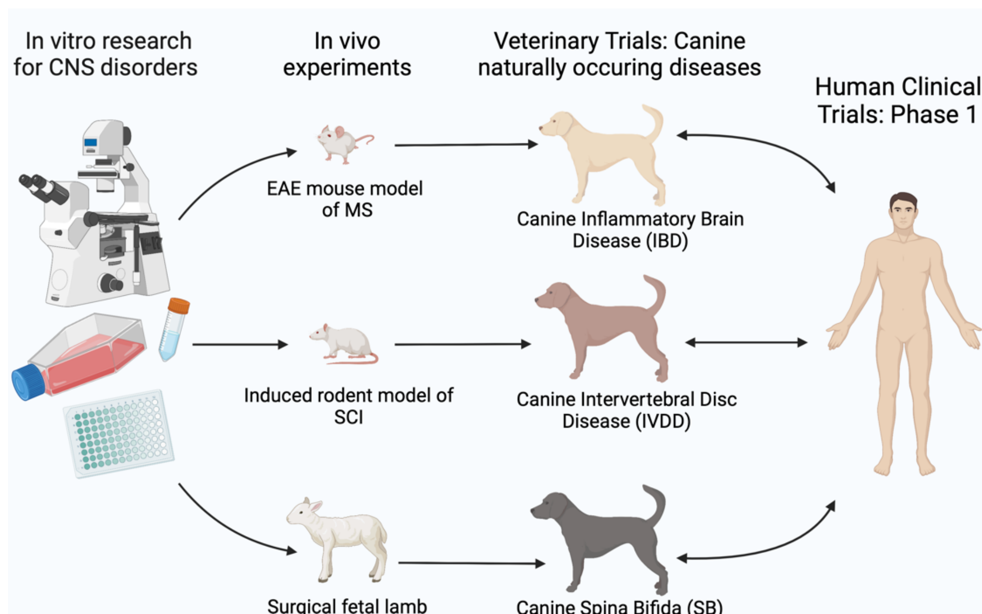

Figure 4.1. A schematic overview of studies utilizing *in vitro* models, research-induced disease models and naturally occurring canine disease models to develop regenerative treatments for veterinary and human patients. EAE: Experimental autoimmune encephalitis; IBD: inflammatory brain disease; IVDD: intervertebral disc disease; MS: multiple sclerosis; SB: spina bifida; SCI: spinal cord injury.

4.2 References

[1] Hoffman, A. M. & Dow, S. W. Concise Review: Stem Cell Trials Using Companion Animal Disease Models. *Stem Cells* **34**, 1709-1729, doi:10.1002/stem.2377 (2016).

[2] Kol, A. *et al.* Companion animals: Translational scientist's new best friends. *Sci Transl Med* **7**, 308ps321, doi:10.1126/scitranslmed.aaa9116 (2015).

[3] Song, R. B., Glass, E. N. & Kent, M. Spina Bifida, Meningomyelocele, and Meningocele. *Vet Clin North Am Small Anim Pract* **46**, 327-345, doi:10.1016/j.cvsm.2015.10.007 (2016).

[4] McMahill, B. G., Borjesson, D. L., Sieber-Blum, M., Nolta, J. A. & Sturges, B. K. Stem cells in canine spinal cord injury--promise for regenerative therapy in a large animal model of human disease. *Stem Cell Rev Rep* **11**, 180-193, doi:10.1007/s12015-014-9553-9 (2015).

[5] Zuchner, M., Kondratskaya, E., Sylte, C. B., Glover, J. C. & Boulland, J. L. Rapid recovery and altered neurochemical dependence of locomotor central pattern generation following lumbar neonatal spinal cord injury. *J Physiol* **596**, 281-303, doi:10.1113/jp274484 (2018).

[6] Boulland, J. L., Lambert, F. M., Zuchner, M., Strom, S. & Glover, J. C. A neonatal mouse spinal cord injury model for assessing post-injury adaptive plasticity and human stem cell integration. *PLoS One* **8**, e71701, doi:10.1371/journal.pone.0071701 (2013).

[7] Vitale, S. & Foss, K. Immune-Mediated Central Nervous System Disease-Current Knowledge and Recommendations. *Top Companion Anim Med* **34**, 22-29, doi:10.1053/j.tcam.2018.11.003 (2019).

[8] Bjelobaba, I., Begovic-Kupresanin, V., Pekovic, S. & Lavrnja, I. Animal models of multiple sclerosis: Focus on experimental autoimmune encephalomyelitis. *J Neurosci Res* **96**, 1021-1042, doi:10.1002/jnr.24224 (2018).

[9] Andersen-Ranberg, E., Berendt, M. & Gredal, H. Biomarkers of non-infectious inflammatory CNS diseases in dogs - Where are we now? Part I: Meningoencephalitis of unknown origin. *Vet J* **273**, 105678, doi:10.1016/j.tvjl.2021.105678 (2021).

[10] Greer, K. A. *et al.* Necrotizing meningoencephalitis of Pug dogs associates with dog leukocyte antigen class II and resembles acute variant forms of multiple sclerosis. *Tissue Antigens* **76**, 110-118, doi:10.1111/j.1399-0039.2010.01484.x (2010).

[11] Carrade, D. D. & Borjesson, D. L. Immunomodulation by mesenchymal stem cells in veterinary species. *Comp Med* **63**, 207-217 (2013).

[12] Amorim, R. M. *et al.* Placenta-derived multipotent mesenchymal stromal cells: a promising potential cell-based therapy for canine inflammatory brain disease. *Stem Cell Res Ther* **11**, 304, doi:10.1186/s13287-020-01799-0 (2020).

[13] Zakirova, E. Y., Aimaletdinov, A. M., Malanyeva, A. G., Rutland, C. S. & Rizvanov, A. A. Extracellular Vesicles: New Perspectives of Regenerative and Reproductive Veterinary Medicine. *Front Vet Sci* **7**, 594044, doi:10.3389/fvets.2020.594044 (2020).

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Chapter 5. Placenta-Derived Multipotent Mesenchymal Stromal Cells: A Promising Potential Cell-Based Therapy for Canine Inflammatory Brain Disease

5.1 Abstract

Background: Canine inflammatory brain disease (IBD) is a severe inflammatory disorder characterized by infiltration of activated immune cell subsets into the brain and spinal cord. Multipotent mesenchymal stromal/stem cells (MSCs) are a promising therapy for IBD, based on their potent pro-angiogenic, neuroprotective and immunomodulatory properties. The aim of this study was to compare the immunomodulatory attributes of canine adipose-derived MSCs (ASCs) and placenta-derived MSCs (PMSCs) *in vitro*. These data will serve as potency information to help inform the optimal MSC cell source to treat naturally occurring canine IBD. **Methods:** Indoleamine 2,3 dioxygenase (IDO) activity and prostaglandin E_2 (PGE_2) concentration at baseline and after stimulation with interferon gamma (IFNγ) and/or tumor necrosis factor alpha (TNFα) were measured from canine ASC and PMSC cultures. Leukocyte suppression assays (LSAs) were performed to compare the ability of ASCs and PMSCs to inhibit activated peripheral blood mononuclear cell (PBMC) proliferation. IDO activity and PGE_2, interleukin (IL)-2, IL-6, IL-8, TNFα, and vascular endothelial growth factor (VEGF) concentrations were also measured from co-culture supernatants. Cell cycle analysis was performed to determine how ASCs and PMSCs altered lymphocyte proliferation. **Results:** Activated canine MSCs from both tissue sources secreted high concentrations of IDO and PGE_2, after direct stimulation with IFNγ and TNFα, or in-direct stimulation by activated PBMCs. Both ASCs and PMSCs inhibited activated PBMC proliferation in LSA assays however, PMSCs inhibited PBMC proliferation significantly more than ASCs. Blocking PGE_2 and IDO in LSA assays determined that PGE_2 is important only for ASC inhibition of PBMC proliferation. Activated ASCs increased IL-6 and VEGF secretion and decreased TNFα secretion, while activated PMSCs increased IL-6, IL-8 and VEGF secretion. ASCs inhibited lymphocyte proliferation via cell cycle arrest in the G0/G1 and PMSCs inhibited lymphocyte

proliferation via induction of lymphocyte apoptosis. **Conclusion**: Our results demonstrate that ASCs and PMSCs have substantial *in vitro* potential as a cell-based therapy for IBD, however, PMSCs more potently inhibited lymphocyte proliferation by inducing apoptosis of activated lymphocytes. These data suggest that the mechanism by which ASCs and PMSCs downregulate PBMC proliferation differs. Additional studies may elucidate additional mechanisms by which canine MSCs modulate neuroinflammatory responses.

5.2 Introduction

Over the past decade, stem cell therapy has become a cornerstone in regenerative medicine therapies for many diseases. However, brain and spinal cord diseases represent a challenge in stem cell-based therapy, due to the multiplicity of cell types in the adult central nervous system (CNS) and the precision of cell interactions, in both space and time, required to enhance neuroregeneration [1]. Stem cells therapies for CNS injury are based on cell replacement, via the transplantation of neural progenitor cells, the stimulation of endogenous CNS stem cells, or on improvement of the microenvironment mediated by anti-inflammatory/immunomodulatory paracrine cell effects[2,3]. However, challenges arise due to a lack of standardization of therapeutic interventions, variability in animal models of disease, alterations in timing and modality of cell application as well as a lack of understanding of disease pathology[4].

Multipotent mesenchymal stromal cells (also known as mesenchymal stem cells; MSCs) derived from adult tissues, such as bone marrow and adipose tissues and from perinatal tissues, including umbilical cord, amniotic fluid and placenta, are the most common cell type investigated in cell-based therapy. Several reports have demonstrated positive effects of MSC therapy in a large number of disorders, including brain and spinal cord injuries, in laboratory animals, dogs and humans[5,6]. Dogs are increasingly recognized as important animal models for translational medicine because they have naturally-occurring brain and

spinal cord injuries, such as canine inflammatory brain disease (IBD), similar to multiple sclerosis (MS) in human beings[7-9].

Studies involving MSCs are increasing due to their immunomodulatory, anti-inflammatory and tissue regenerative properties including the secretion of numerous bioactive molecules leading to tissue regeneration[10,11]. However, the mechanisms by which MSCs elicit positive effects on the damaged nervous system are not fully characterized. Mechanisms that may play an important role in neuroregeneration include the secretion of growth factors, antiapoptotic factors, neurotrophic factors, cytokines and extracellular matrix proteins. The immunomodulatory and anti-inflammatory properties of MSCs are also implicated in their ability to protect and repair neurons. Together these factors promote endogenous neuronal growth, promote neuro/gliogenesis, encourage synaptic connection from damaged neurons, recruit local oligodendrocyte precursors, reduce demyelination, stimulate angiogenesis, decrease apoptosis, reduce oxidative stress, modulate microglial activation, and regulate inflammation by suppressing pathological T, B and Natural Killer (NK) cell responses [12-16]. Moreover, they can accelerate a shift from a predominance of pro-inflammatory Th1 cells toward an increase in the anti-inflammatory Th2 cells[17,18]. Although controversial, some studies have suggested that MSCs can also migrate into the CNS lesion and differentiate into neurons or astrocytes[19,20].

Canine adult and perinatal MSCs have been characterized by immunophenotyping and multipotency assays in many studies[21-27]. Perinatal MSCs, including placenta-derived stem cells, preserve some features of the primitive embryonic layers. They have the potential to differentiate into many tissues[28], have greater proliferative and immunomodulatory capacity and lesser immunogenicity than adult MSCs[28-30], and are neuroprotective [31,32]. These stem cells can also be easily harvested and expanded due to the availability of a large amount of tissue which is usually discarded at birth. There is no ethical conflict regarding placenta harvest making these stem cell sources attractive targets for banking and

regenerative therapies[28,30]. Nonetheless, a deeper understanding of their immunomodulatory function is still lacking.

IBD is a general term used to describe an idiopathic disorder that can be subdivided based on histopathology findings, i.e. granulomatous meningoencephalomyelitis (GME), necrotizing meningoencephalitis (NME) and necrotizing leukoencephalitis (NLE) [33,34]. Recently, the term meningoencephalomyelitis of unknown origin (MUO) has been proposed to encompass all of these diseases. MUO is presumed to be an autoimmune disease with a genetic predisposition[33]. Immunohistochemical phenotyping of the involved inflammatory cells demonstrates a pivotal role of MHC II+ cells, T cells, and macrophages in GME and NLE [35-37]. GME can be identified by CD3+ lymphocyte infiltration into sites of granulomatous inflammation as shown in a representative clinical case (Figure 5.1). The pattern of mRNA and protein expression of cytokines and chemokine receptors may be disease specific, such as interferon gamma (IFNγ) and CXCR3 in NME, and IL-17 and CCR2 in GME[34]. Cytokines

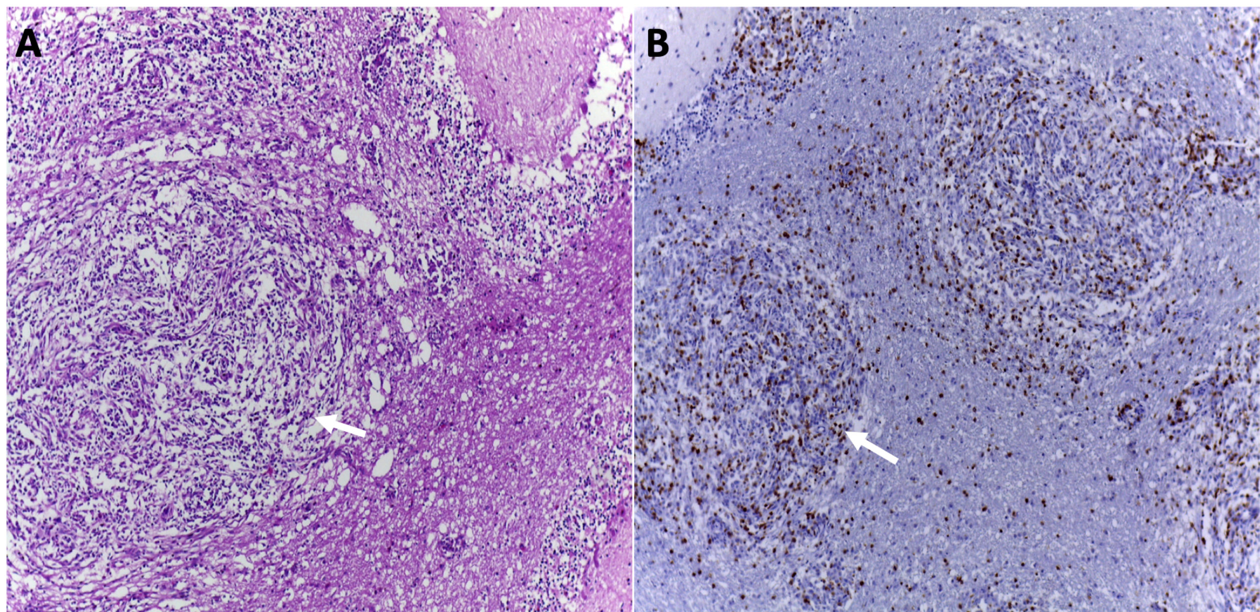

Figure 5.1. Canine Granulomatous Meningoencephalitis (GME) in a 2-year-old, female, Miniature Pinscher. (A) Arrow indicates area of granulomatous inflammation (Hematoxylin-eosin; 10X). (B) CD3 positive cells in the granulomatous inflammation are indicated (arrow) (Immunohistochemistry, DAB, Harris hematoxylin counter stain; 10X).

have also been implicated in the phenotype switching of microglia/macrophages from a classically activated pro-inflammatory type (M1) or into an alternatively activated anti-inflammatory phenotype (M2) in canine inflammatory CNS disease[37]. Thus, manipulating this inflammatory phenotype by cell-based therapy might represent a promising therapeutic approach for MUO[37].

Our goal was to compare the *in vitro* immunomodulatory ability of 2 types of canine MSCs focusing on secretion profiles and neuroreparative assays relevant for canine IBD. This study evaluated the *in vitro* potential of canine adipose tissue-derived multipotent mesenchymal stromal cells (ASCs) and placenta-derived multipotent mesenchymal stromal cells (PMSCs) to secrete anti-inflammatory cytokines and to inhibit lymphocyte proliferation, as well as the mechanisms involved in the canine MSC immunomodulatory process.

5.3 Methods

5.3.1 Canine MSC collection, isolation and culture

Adipose Tissue-derived Mesenchymal stromal cells (ASCs)

Canine low passage (P2-P5) ASCs were derived from falciform fat of 5 healthy dogs that presented to the UCD William R. Pritchard Veterinary Medical Teaching Hospital for routine abdominal surgery. Dogs ranged from 1-10 years of age and included 3 females and 2 males from different dog breeds, including mixed breeds. Fat was collected under an approved Institutional Animal Care and Use Committee and the Clinical Trials Review Board protocol at UCD (protocol number 19605). Fat was processed and canine ASCs were isolated, expanded, cryopreserved and phenotyped exactly as previously described[38,39]. Briefly, adipose tissue was minced and digested at 37 °C for 1-2 hours using 0.1% collagenase type I and 1% bovine-serum albumin (Worthington, Lakewood, NJ). Centrifugation was performed to remove the remaining lipid layer. The cell pellet was washed several times before being plated in standard medium [Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen,

Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT) and 10 µl/mL penicillin-streptomycin (ThermoFisher Scientific, Gibco, Pittsburgh, PA)].

Placenta-derived Mesenchymal Stromal Cells (PMSCs)

Canine low passage (P2-P5) placenta-derived MSCs from 5 dogs were obtained from Dr. Aijun Wang's laboratory cell bank, University of California Davis Medical Center. Isolation, culture and full phenotyping of these PMSC lines from discarded term canine placentas was previously described [40]. Briefly, at term canine placentas were processed by manual dissection and treated with 0.25% trypsin (ThermoFisher Scientific) solution for 30 min at 37°C. Cell pellets were washed and incubated with collagenase IA (1 mg/mL; Sigma-Aldrich, St. Louis, MO) for 45 min at 37°C. The cell pellet was resuspended and plated onto tissue culture flasks in standard medium [Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% FBS (HyClone, Logan, UT) and 100X penicillin-streptomycin (ThermoFisher Scientific).

5.3.2 Stimulation of Canine MSCs with IFNγ and tumor necrosis factor alpha (TNFα)

Cryopreserved MSCs were thawed and culture expanded as previously described [38,40,41]. When cells were approximately 70% confluent, they were trypsinized and resuspended in standard medium supplemented with L-tryptophan (Sigma-Aldrich) to a final concentration of 600 µM. These MSCs were seeded at $2x10^5$ per well of 24-well plate, with 0.75 mL total media volume per well for the stimulation assays. At the time of plating, MSC were stimulated with IFNγ (50ng/mL; canine recombinant IFNγ, Kingfisher, St.Paul, MN) or TNFα (50ng/mL; canine recombinant TNFα, Kingfisher) or a dual stimulation with both IFNγ (50ng/mL, Kingfisher) and TNFα (50ng/mL, Kingfisher). Stimulated MSCs were cultured for four days at which time supernatants were collected, frozen and stored at -80°C for mediator quantification.

5.3.3 Canine Leukocyte Suppression Assay (LSA)

Canine leukocyte suppression assay (LSAs) were performed exactly as previously

described [38]. In brief, peripheral blood was collected from healthy dogs 55 pounds or larger between 1-8 years old, into tubes containing sodium heparin (Vacutainer®, BD Biosciences) via jugular venipuncture. PBMCs were isolated using a discontinuous Ficoll gradient and were plated with irradiated (10 Gy, Varian 2100C linear accelerator, Varian Medical Systems, Inc., Palo Alto, CA) allogeneic canine ASCs or PMSCs in standard medium (DMEM with 10% FBS, 1% penicillin/streptomycin, supplemented with 600 µM L-tryptophan) [38]. PBMCs were activated with 5mg/mL concanavalin A (ConA; Sigma- Aldrich). PBMCs and irradiated MSCs were co-cocultured at a ratio of 5:1 in direct contact.

To determine the role of contact, cells were plated in transwell dishes (Corning 0.4 µM polycarbonate membrane 24-well plate; Corning, NY, USA) with MSCs plated in the plate bottom and PBMCs in the insert. To determine the role of IDO and PGE_2 in MSC mediated inhibition of lymphocyte proliferation, inhibitory agents were used in LSA co-cultures. Indomethacin, a cyclooxygenase (COX) inhibitor, was used to chemically block PGE_2 production. Alternatively, 1-methyl-DL-tryptophan (1-MT), an IDO competitive inhibitor, was used to partially inhibit IDO activity. Indomethacin was added to LSA assays during plating at a concentration of 10 µM (Sigma-Aldrich) as previously described[38] to determine the role of PGE_2 on MSC mediated immunosuppression. Additionally, at plating 1-MT (Sigma-Aldrich) was added at a concentration of 1mM as previously described[42] to determine the role of IDO on MSC mediated immunosuppression.

After 3 days of co-culture, wells were treated with 1 mM Bromodeoxyuridine (BrdU, BD Biosciences). Twenty-four hours post BrdU treatment, leukocytes were collected, and cells were stained with a viability dye (Fixable Viability Dye Fluor® 780; eBioscience, San Diego, CA) and anti-canine CD3 conjugated to Alexa Fluor® 488 (clone CA17.2A12; Leukocyte Antigen Biology Lab, UCD). Leukocytes were stained for nuclear BrdU incorporation (APC BrdU Flow Kit, BD Biosciences) per manufacturer directions and analyzed by flow cytometry

(Cytomics FC500). For cell cycle analysis, 7-aminoactinomycin D was added to cultures per manufacturer's instructions (APC BrdU Flow Kit; BD Biosciences) and analyzed by flow cytometry on days 1– 4. Flow cytometry data were analyzed using FlowJo flow cytometry software (Tree Star, Inc.). Data was normalized and presented as a reduction of each respective PBMC donor.

At the time of leukocyte collection, culture supernatant was collected, centrifuged and stored at −80 °C for the measurement of secreted mediators.

5.3.4 Quantification of mediator secretion

Frozen aliquots of supernatants collected from MSCs stimulated with IFNγ / TNFα and from LSA co-cultures were used to quantify PGE_2 and IDO activity. Canine PGE_2 was quantified using an ELISA kit per manufacturer directions (Prostaglandin E2 Express EIA kit (Monoclonal); Cayman Chemical Company, Ann Arbor, MI)[38]. To assess IDO activity a biochemical assay was performed on frozen supernatants as previously described[43] to quantify the conversion of tryptophan to *N*-formyl kynurenine mediated by IDO. In brief, culture media was treated with 30% trichloroacetic acid (Sigma), and Ehrlich's reagent (1% *p*-dimethylaminobenzaldehyde in glacial acetic acid, Sigma) was mixed and read at 490 nm on a microplate reader (Synergy HT Multi-Mode Gen5 software)[38].

Supernatants from LSAs co-cultures were used to measure concentrations of interleukin (IL)-2, IL-6, IL-8, vascular endothelial growth factor (VEGF) and TNFα. IL-2, IL-6, IL-8 and were quantified via Quantibody[VR] Canine Cytokine Array (RayBiotech cat# QAC-CYT-1). Negative controls were prepared from wells containing only media. The array was performed according to the manufacturer's instructions, and the resulting glass slide was scanned using a GenePix 4000B microarray scanner (Molecular Devices). Collected images were quantified using GenePix[VR] Pro 6 acquisition and analysis software, and further plotting of standard curves and analysis was performed using Microsoft Excel. TNFα was measured from supernatants using ELISA kits

(Canine TNFα DuoSet, R&D Systems) per manufactures instructions. All ELISA samples were read on a Synergy HT Multi-Mode microplate reader with Gen5 software (Biotek, Winooski, VT, USA).

5.3.5 Statistical Analysis

Results are presented as mean and standard error. Data were not normally distributed. As such, all data were analyzed using non-parametric Mann-Whitney-Wilcoxon t-test (GraphPad InStat version 3.06 for Windows, La Jolla, CA). $p < 0.05$ was considered statistically significant.

5.4 Results

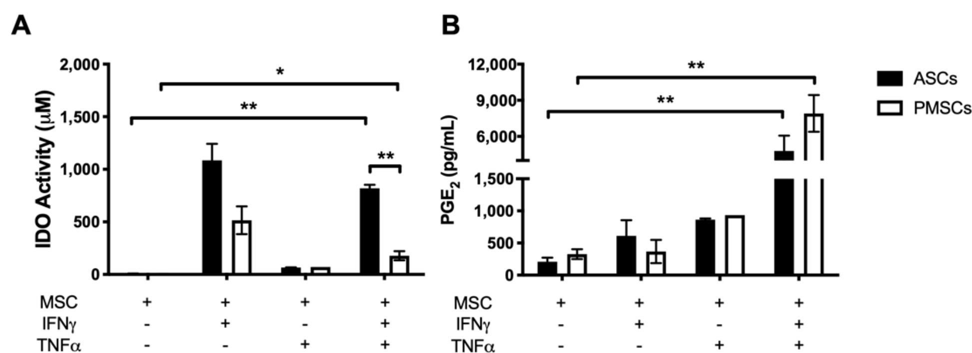

Figure 5.2. Direct stimulation of canine ASCs and PMSCs leads to production of IDO and PGE2. Canine adipose-derived MSCs (*ASCs*) and placenta-derived MSCs (*PMSCs*) secrete comparable levels of indoleamine 2,3 dioxygenase (IDO) activity and prostaglandin E_2 (PGE_2) in response to direct stimulation using recombinant interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα). (A) IDO activity is directly proportional to the conversion of tryptophan to *N*-formyl kynurenine. ASCs and PMSCs increase IDO activity in response to dual stimulation with IFNγ and TNFα. IFNγ is the main contributor to the production of IDO. IFNγ and TNFα stimulated ASCs promote significantly higher levels of IDO activity as compared to PMSCs. (B) Canine ASCs and PMSCs produce comparable levels of prostaglandin E_2 (PGE_2) in response to dual stimulation with IFNγ and TNFα. TNFα is the major contributor to MSC mediated PGE_2 production. Data presented as mean and standard error. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. *IDO* indoleamine 2,3 dioxygenase, *IFNγ* interferon gamma, *MSC* mesenchymal stromal cell, *PGE_2* prostaglandin E_2, *TNFα* tumor necrosis factor alpha.

5.4.1 Canine MSCs increase IDO and PGE_2 secretion in response to IFNγ and TNFα stimulation

MSC immunomodulatory functions occur in part through the secretion of bioactive factors. MSCs were activated through direct stimulation to determine the immunomodulatory potential using recombinant pro-inflammatory mediators known to be relevant in IBD.

Stimulation with IFNγ alone increased IDO activity, while stimulation with TNFα alone predominantly stimulated PGE_2 production by both ASCs and PMSCs (Figure 5.2A, B). The use of both stimulation agents resulted in a synergistic effect IDO activity and PGE_2 production, therefore dual stimulation using both IFNγ and TNFα was performed in canine ASC and PMSC cultures. Dual stimulation with both IFNγ and TNFα resulted in increased PGE_2 production and IDO activity (Figure 5.2A, B). Canine ASCs however, secreted significantly more IDO than PMSCs after 4 days of stimulation with canine recombinant IFNγ and TNFα (Figure 5.2A; p=0.0079). Dual stimulation of canine ASCs and PMSCs resulted in comparable increases in PGE_2 secretion (Figure 5.2B). IDO secretion was exclusively dependent on IFNγ with no synergistic increase after the addition of TNFα however PGE_2 secretion was augmented with dual stimulation.

5.4.2 Canine ASCs and PMSCs inhibit activated PBMC proliferation through distinct mechanisms in a contact-dependent manner

We have previously reported that canine ASCs reduce mitogen activated PBMC proliferation in LSA co-cultures [38]. Both canine ASCs and PMSCs in direct contact with activated PBMCs inhibited lymphocyte proliferation, however PMSCs more potently inhibited lymphocyte proliferation compared to ASCs (Figure 5.3A, p=0.0127). LSA co-cultures performed within a transwell to remove direct MSC-PBMC cell contact, resulted in marked restoration of PBMC proliferation regardless of MSC tissue source (Figure 5.3A). These data suggest that canine MSCs reduce activated lymphocyte proliferation in part via direct cell-cell contact.

MSC mediated immunomodulation in dogs also occurs through the secretion of bioactive factors [38,39,44,45]. PGE_2 and IDO have been implicated as crucial mechanisms by which MSCs downregulate inflammatory responses. To evaluate the role of PGE_2, the COX inhibitor indomethacin, was used to block PGE_2 synthesis and secretion. The competitive inhibitor 1-MT was used to block the functional properties of IDO. Blocking PGE_2 led to a

significant reduction of ASC mediated inhibition as compared to PMSCs (Figure 5.3B, p=0.0043). Blocking IDO however, lead to no alterations in ASC or PMSC mediated suppression of PBMC proliferation (Figure 5.3B). Representative photomicrographs of stimulated PBMCs and a PMSC LSA are shown in Figure 5.3C. No morphological changes were noted between canine ASC and PMSC LSA conditions.

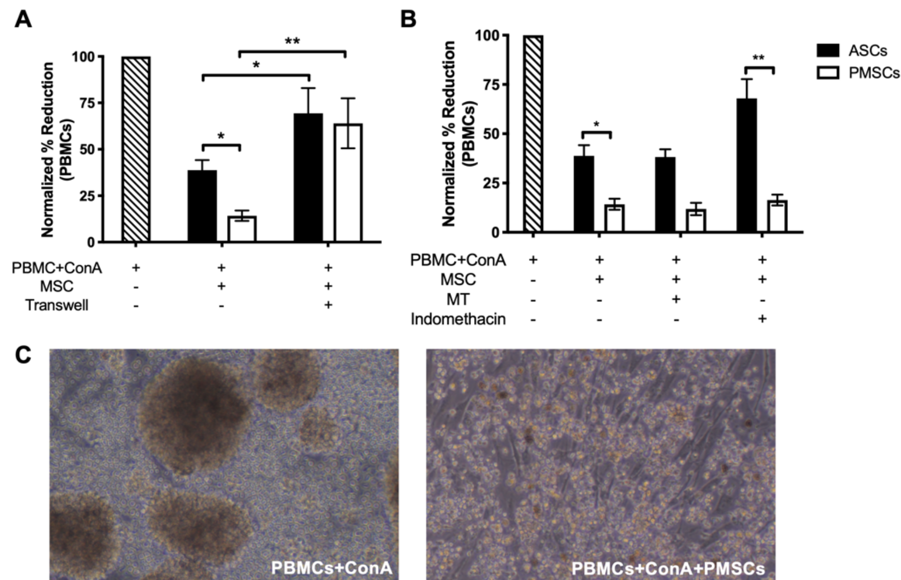

Figure 5.3. Canine ASCs and PMSCs inhibit lymphocyte proliferation in a contact dependent manner. Canine adipose-derived MSCs (*ASCs*) and placenta-derived MSCs (*PMSCs*) possess immune-suppressive functions. (A) Canine ASCs and PMSCs co-incubated with stimulated peripheral blood mononuclear cells (*PBMCs*) suppress lymphocyte proliferation. PMSCs significantly decrease lymphocyte proliferation more potently as compared to ASCs. Transwells were added to remove physical contact between MSCs, and mitogen (ConA) stimulated PBMCs. Lymphocyte proliferation increased when ASCs and PMSCs were not in direct contact with activated PBMCs. (B). To determine the role of indoleamine 2,3 dioxygenase (*IDO*) and prostaglandin E_2 (*PGE_2*) in MSC mediated immunosuppression 1-methyl-DL-tryptophan (1-MT) and indomethacin was added to co-cultures to block each respective mediator. Blocking of IDO activity using 1-MT resulted in no alterations in MSC mediated suppression of PBMC proliferation. Blocking PGE_2 using indomethacin resulted in a loss of lymphocyte inhibition by ASCs but no effects were observed by PMSCs. PBMC stimulation is normalized, and data is presented as a reduction to each respective donor. Representative photomicrographs of ConA stimulated PBMCs and PMSC LSA conditions (C). Data presented as mean and standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *1-MT* 1-methyl-DL-tryptophan, ConA concanavalin A*,* *IDO* indoleamine 2,3 dioxygenase*,* *LSA* leukocyte suppression assay, *MSC* mesenchymal stromal cell, *PBMCs* peripheral blood mononuclear cells, *PGE_2* prostaglandin E_2.

Secretion of PGE_2 and IDO in LSA supernatants was determined in standard LSA conditions (MSC-PBMC contact) and in transwell conditions (no cell contact). ASCs and PMSCs secreted comparable levels of IDO when co-cultured in direct contact with mitogen activated PBMCs (Figure 5.4A). Removing direct MSC-PBMC cell contact had no effect on

IDO secretion from either MSC source (Figure 5.4A). Both ASCs and PMSCs also secreted high levels of PGE_2 when co-cultured with stimulated PBMCs, however when direct PBMC cell contact was removed, only PMSCs secretion of PGE_2 significantly decreased (Figure 5.4B).

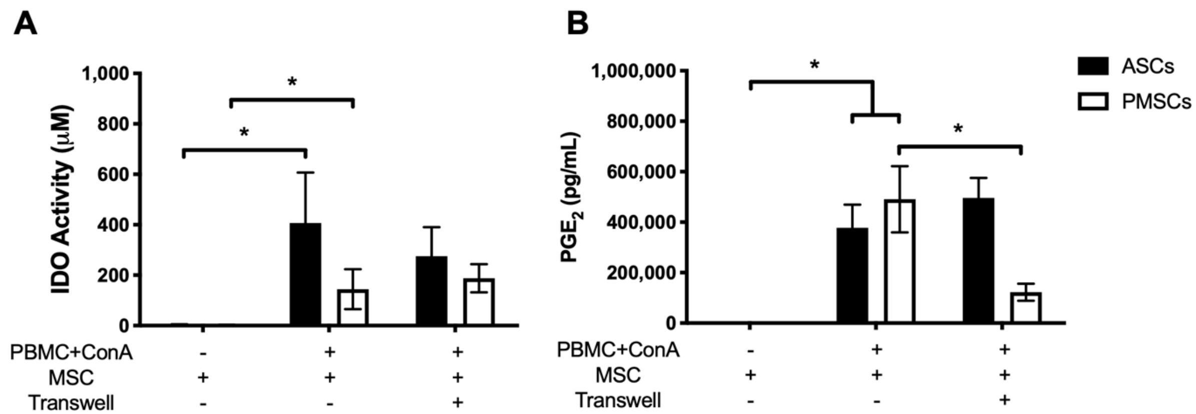

Figure 5.4. Indirect stimulation of canine ASCs and PMSCs in leukocyte suppression assay (LSAs) leads to production of IDO and PGE2. Canine adipose-derived MSCs (*ASCs*) and placenta-derived MSCs (*PMSCs*) produce similar secretory mediators when co-cultured in contact with activated peripheral blood mononuclear cells (PBMCs). (A) ASCs and PMSCs increase indoleamine 2,3 dioxygenase (*IDO*) activity in the presence of mitogen (ConA) activated PBMCs. Removal of direct cellular contact did not alter IDO activity by either ASCs or PMSCs. (B) Production of prostaglandin E_2 (*PGE_2*) occurred in both standard and transwell conditions. Canine PMSCs secrete significantly greater levels of PGE_2 however, when direct contact was removed using transwells only canine PMSCs drastically reduced PGE_2 production. No observable changes occur when contact was removed in ASC co-cultures. Data presented as mean and standard error. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *ConA* concanavalin A, *IDO* indoleamine 2,3 dioxygenase, *MSC* mesenchymal stromal cell, *PBMCs* peripheral blood mononuclear cells, *PGE_2* prostaglandin E_2.

5.4.3 Activated canine PMSC and ASC secretion profiles

Protein concentrations were measured to determine the mediators present in LSA supernatant after co-incubation of activated PBMCs with PMSCs and ASCs. Mediators implicated in the immunomodulatory functions of MSCs were measured and compared to basal levels from unstimulated MSCs. Statistical significance was not achieved however trends were observed suggesting that activated ASCs and PMSCs secrete VEGF (Figure 5.5A) and IL-6 (Figure 5.5B). Additionally, the mediator IL-8, which is secreted by both MSCs

and PBMCs was measured. Unstimulated MSCs did not secrete detectable levels of IL-8 (data not shown). Basal levels of IL-8 from activated PBMCs are comparable to concentrations when ASCs were added to co-culture, however PMSCs produced greater levels of IL-8 (Figure 5.5C). MSCs have also been shown to suppress inflammatory responses through the reduction of pro-inflammatory cytokines. Both ASCs and PMSCs displayed a trend of reduced IL-2 concentrations (Figure 5.5D), and both tissues sources reduced TNFα to similar concentrations (Figure 5.5E).

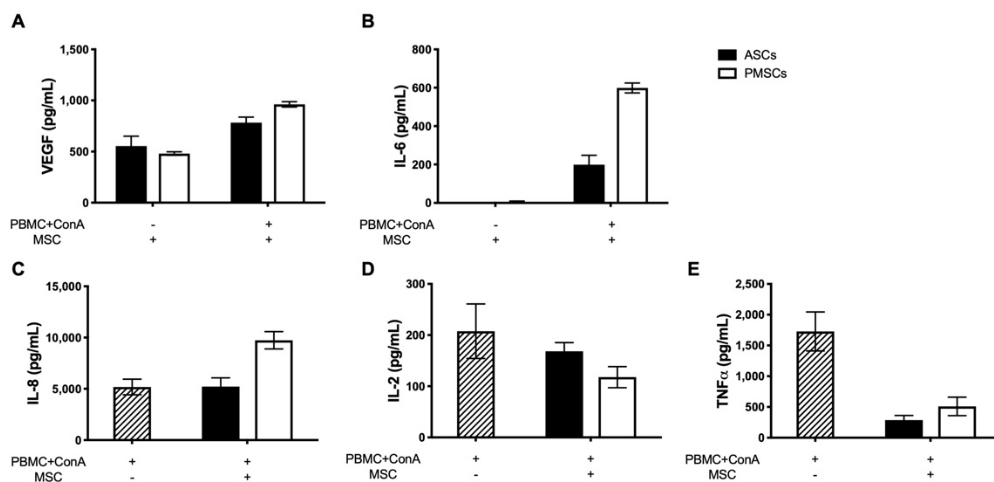

Figure 5.5. Bioactive factors associated with canine ASC and PMSC mediated immunosuppression differs. Canine adipose-derived MSCs (*ASCs*) and placenta-derived MSCs (*PMSCs*) increase production of vascular endothelial growth factor (VEGF) and IL-6. (A) Both canine ASCs and PMSCs mildly increased production of VEGF when co-cultured with mitogen (ConA) activated peripheral blood mononuclear cells (PBMCs). (B) Canine ASCs and PMSCs upregulated IL-6 production, however PMSCs secreted higher levels of IL-6 as compared to ASCs. (C) Production of IL-8 by canine ASCs was comparable to basal levels of IL-8 produced by stimulated PBMCs, however PMSCs increased levels of IL-8 greater than basal levels. Additionally, regulation of inflammatory mediators by canine ASCs and PMSCs was similar. (D) IL-2 production from stimulated PBMCs was mildly reduced by both canine ASCs and PMSCs. (E) Canine ASCs and PMSCs inhibit production of tumor necrosis factor alpha (*TNFα*) by stimulated PBMCs to comparable levels. Data presented as mean and standard error. *ConA* concanavalin A, *IL* interleukin, *MSC* mesenchymal stromal cell, *PBMCs* peripheral blood mononuclear cells, *TNFα* tumor necrosis factor alpha, *VEGF* vascular endothelial growth factor.

5.4.4 ASCs inhibit lymphocyte proliferation through cycle arrest, while PMSCs induce apoptosis

The inhibition of lymphocyte proliferation can be secondary to cell cycle arrest or the induction of apoptosis. Canine MSCs varied in how they inhibited lymphocyte proliferation,

depending on the tissue source (adipose or placenta). Canine PMSCs induced lymphocyte apoptosis (Figure 5.6A) while canine ASCs result in induced lymphocyte arrest in the G0/G1 phase of cell cycle (Figure 5.6B). Both tissue sources result in decreased lymphocyte entry into G2/M (Figure 5.6C) and S phase (Figure 5.6D) of the cell cycle, however each decreases entry through alternative mechanisms. Representative images of cell cycle flow scatter for unstimulated PBMCs (Figure 5.6E-F), ConA stimulated PBMCs (Figure 5.6E-F) and LSA conditions for canine ASCs (Figure 5.6E) and PMSCs (Figure 5.6F) are shown.

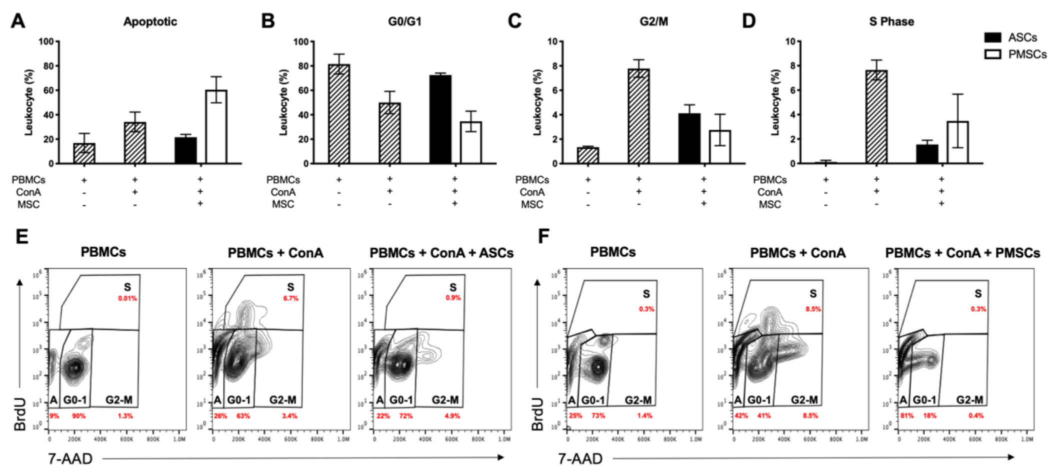

Figure 5.6. Inhibition of lymphocyte proliferation by canine ASCs and PMSCs occurs through different mechanisms. Cell cycle analysis was performed using peripheral blood mononuclear cells (PBMCs) and *BrdU* 5-bromo-2'-deoxyuridine and 7-aminoactinomycin D was measured. Unstimulated PBMCs and mitogen (ConA) activated PBMCs were used as controls. (A) Canine PMSCs inhibit lymphocyte proliferation by inducing apoptosis. Alternatively, canine ASCs caused cell cycle arrest which is demonstrated by PBMCs accumulating in G_0/G_1 (B) and hindering cells from entering G2/M (C) or DNA synthesis (S phase) (D). Representative images of cell cycle flow scatter plots and gating strategies for leukocyte DNA content (7-AAD) and proliferation via BrdU incorporation of PBMC controls (E-F) and co-incubations with canine ASCs (E) and PMSCs (F) are shown. *BrdU* 5-bromo-2'-deoxyuridine and 7-aminoactinomycin D, *ConA* concanavalin A, *LSA* leukocyte suppression assay, *MSC* mesenchymal stromal cell, *PBMCs* peripheral blood mononuclear cells.

5.5 Discussion

Companion animals are increasingly being utilized as naturally occurring large animal

disease models to evaluate the use stem cell-based therapies. Veterinary species suffer from many diseases that closely resemble the pathophysiology of human diseases, making them valuable translational models for preclinical data. The dog has been used to evaluate MSC therapy for the treatment of several inflammatory conditions including osteoarthritis, spinal cord injury, inflammatory bowel disease and graft-versus-host disease[19,46-48].

Murine Experimental Autoimmune Encephalomyelitis (EAE) is the most commonly used animal model to study MS. However, EAE does not reproduce all clinical, pathological or immunological features of human disease[49]. Canine MUO may be useful as naturally-occurring model of MS, given neuroimmunological similarities of these diseases, including the upregulation of IFNγ, IL-17 and MHC-II expression in the nervous system[5,7,34,50-52]. MS is suggested to be mediated by Th1 and Th17 lymphocytes, leading to demyelination and axonal injury [53,54]. Although the demyelination noted in MS is not present in NME, fulminant or non-prototypic acute variants of MS, such as Marburg variant, Balo's Concentric Sclerosis, and Acute Disseminated Encephalomyelitis closely resemble the pathological features of canine NME[7]. The focal and widespread forms of GME, consistent with a delayed hypersensitivity reaction, are also consistent with MS [5]. Cytokine expression in brain lesions of NME and GME display increased levels of interferon gamma (IFNγ) in NME and IL-4 and IL-17 in GME[34]. IL-17 and IFNγ production by T lymphocytes is also associated with active disease in MS patients[54]. In addition, CSF in dogs with MUO showed increased levels of CCL19 chemokine, also expressed in neuroinflammatory diseases such as MS / EAE, suggesting similar neuroimmunological events[55].

MSCs are an attractive target for neurodegenerative disease therapies due to their potent neuroprotective, regenerative and immunomodulatory properties. While most clinical studies utilize adult-derived sources of MSCs, the placenta is a unique source of MSCs that maintain unique functional properties for therapeutic use as compared to adult tissue derived MSCs. We found that MSCs from adult and perinatal tissue sources modulate PBMC proliferation through

multiple mechanisms. Both canine ASCs and PMSCs inhibit activated lymphocyte proliferation through a primarily cell-cell contact mediated mechanism. Notably, PMSCs more potently inhibited lymphocyte proliferation as compared to ASCs *in vitro*. Inhibition of PBMC proliferation by PMSCs occurred through the induction of apoptosis, while ASCs induced cell cycle arrest. Canine ASCs and PMSCs secreted high levels of both PGE_2 and IDO through direct stimulation with IFNγ and TNFα or through indirect stimulation in our mixed co-culture assay. For PMSCs, when cell-cell contact was removed a restoration of lymphocyte proliferation and a decrease of PGE_2 secretion was observed. Interestingly blocking PGE_2 production in PMSC cultures using a COX-inhibitor did not restore lymphocyte proliferation. These findings suggest that MSC-PBMC cellular contact is primarily responsible for the production of PGE_2 by PMSCs, however production of this mediator is not directly responsible for induction of lymphocyte apoptosis. Additionally, PMSCs produce IDO but regardless of removing cellular contact or blocking IDO via competitive inhibitor, no effect on PBMC proliferation was observed. Collectively this data highlights the importance of cellular contact for PMSC mediated immunosuppression and initiation of PBMC apoptosis.

Similarly, in ASCs when direct cellular contact was removed with PBMCs, lymphocyte proliferation was restored however this contact was not needed for PGE_2 secretion. Inhibiting PGE_2 production with indomethacin led to increased lymphocyte proliferation by ASCs. These data suggest that for canine ASCs there are two mechanisms leading to PBMC cell cycle arrest, one that is contact dependent and one that is PGE_2 dependent. As observed in PMSCs, inhibiting IDO with 1-MT showed no effect on ASC mediated immunosuppression. Removing MSC-PBMC contact did not alter IDO activity in canine ASC co-cultures. These findings suggest that IDO does not play a significant role in canine MSC mediated immunosuppression from either tissue source.

PGE_2 has been shown to play a key role in MSC mediated immunosuppression in humans, cats, dogs and horses[18,56]. PGE_2 inhibits production of IFNγ, IL-2 and induces T regulatory cells

[57]. Decidual stem cells also suppress alloreactivity through induction of T regulatory cells in a contact dependent manner, however involvement of programmed cell death 1 (PD-1), IDO, PGE_2, and IFNγ still play a role in this suppression[58]. These data suggest that our findings in canine PMSCs are comparable to human studies. Interestingly, we observed no alterations in immunosuppression by the secretion of IDO, which has been reported in both human adult and perinatal tissue sources. Here we report canine MSCs produce IDO when activated, however this does not seem to be a critical mediator in lymphocyte suppression.

Though not statistically significant, our data suggest that both ASCs and PMSCs secrete VEGF and IL-6. Additionally, PMSCs, trended towards secreting higher levels of IL-6 and IL-8 as compared to ASCs. There is also a trend towards a reduction of IL-2 production by both ASCs and PMSCs. Both tissue sources reduced the production of the pro-inflammatory mediator TNFα. Canine ASC mediators closely recapitulate studies in human ASCs, suggesting that the dog will serve as a useful translational model to evaluate therapeutic applications of MSCs[59]. The ability of canine MSCs from either tissue source to secrete immunomodulatory mediators and suppress inflammatory cytokines indicates these cells as attractive targets for canine IBD therapeutics.

MSC-immune cell contact, namely in the presence of IFNγ and TNFα, has been shown to upregulate PDL-1, vascular cell adhesion molecule 1 (VCAM-1) and inflammatory cytokine-induced adhesion molecule 1 (ICAM-1) and augments the secretion of soluble mediators [60-62]. Additionally, ICAM-1 by feline ASCs has been shown to play a critical role in ASC-T cell adhesion and mediates T cell proliferation[62]. Contact-dependent inhibition of immune cell function has been suggested to play a more important role in local immunosuppression[58]. It has also been shown that blockade of ICAM-1 and VCAM-1 ablates MSC mediated immunosuppression which highlights the potential mechanistic role of adhesion molecules by MSCs [61]. The role of ICAM-1/LFA ligand has been shown to play a critical role in feline ASC mediated immunosuppression through induction of G0-G1 cell cycle arrest[62]. Feline ASCs also induce cell cycle arrest by

utilizing PGE_2 which is comparable to our findings in dog MSCs, suggesting ICAM-1 may also play a significant role in canine MSC mediated immunosuppression. Therapies targeting adhesion molecules have been used for numerous diseases including MS, using a drug that targets $\alpha_4\beta_1$-integrin [63]. Our work has demonstrated the critical role of MSC-immune cell contact in immunoregulation, however the exact mechanism by which these interactions occur will need to be addressed in future studies. Additionally, a mechanistic comparison of canine and human PMSCs will need to be performed to fully establish the utility of this model.

Taken together, our data suggest that canine ASCs and PMSCs possess immunoregulatory properties. However, the mechanisms by which this immunoregulation occurs differs. Though both cell sources are immunosuppressive, PMSCs displayed more potent ability to decrease activated T cell proliferation. Additionally, PMSCs induce PBMC apoptosis while ASCs induce cell cycle arrest. This highlights the need to consider disease pathology when selecting MSC tissue sources for selected therapies. More studies are needed to understand the mechanistic differences between cells source and immune cell interactions. From our studies we suggest that PMSCs may be a novel therapeutic source for neurodevelopmental and neurodegenerative diseases.

5.6 Conclusions

The findings from this study demonstrate that canine ASCs and PMSCs have robust immunoregulatory potential. The mechanism of immune suppression by each cell source differs, in that PMSCs induce apoptosis of activated lymphocytes and ASCs induce cell cycle arrest. Secretome profiles of activated MSCs from each source also differed, however PMSCs notably more potently inhibit lymphocyte proliferation. While each tissue source holds great potential as a cell-based therapy for IBD, PMSCs may be an ideal tissue source for many neurodegenerative diseases in both animals and humans. Additional studies will be needed to further elucidate the mechanism by which canine MSCs modulate neuroinflammatory responses.

5.7 References

[1] Tanna, T. & Sachan, V. Mesenchymal stem cells: potential in treatment of neurodegenerative diseases. *Curr Stem Cell Res Ther* **9**, 513-521 (2014).

[2] Goldman, S. Stem and progenitor cell-based therapy of the human central nervous system. *Nat Biotechnol* **23**, 862-871, doi:10.1038/nbt1119 (2005).

[3] Lindvall, O. & Kokaia, Z. Stem cells for the treatment of neurological disorders. *Nature* **441**, 1094-1096, doi:10.1038/nature04960 (2006).

[4] Pearse, D. D. & Bunge, M. B. Designing cell- and gene-based regeneration strategies to repair the injured spinal cord. *J Neurotrauma* **23**, 438-452, doi:10.1089/neu.2006.23.437 (2006).

[5] Hoffman, A. M. & Dow, S. W. Concise Review: Stem Cell Trials Using Companion Animal Disease Models. *Stem Cells* **34**, 1709-1729, doi:10.1002/stem.2377 (2016).

[6] Momin, E. N., Mohyeldin, A., Zaidi, H. A., Vela, G. & Quinones-Hinojosa, A. Mesenchymal stem cells: new approaches for the treatment of neurological diseases. *Curr Stem Cell Res Ther* **5**, 326-344 (2010).

[7] Greer, K. A. *et al.* Necrotizing meningoencephalitis of Pug dogs associates with dog leukocyte antigen class II and resembles acute variant forms of multiple sclerosis. *Tissue Antigens* **76**, 110-118, doi:10.1111/j.1399-0039.2010.01484.x (2010).

[8] Kol, A. *et al.* Companion animals: Translational scientist's new best friends. *Sci Transl Med* **7**, 308ps321, doi:10.1126/scitranslmed.aaa9116 (2015).

[9] Penha, E. M. *et al.* Use of autologous mesenchymal stem cells derived from bone marrow for the treatment of naturally injured spinal cord in dogs. *Stem Cells Int* **2014**, 437521, doi:10.1155/2014/437521 (2014).

[10] Chamberlain, G., Fox, J., Ashton, B. & Middleton, J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* **25**, 2739-2749, doi:10.1634/stemcells.2007-0197 (2007).

[11] Carrade, D. D. *et al.* Comparative Analysis of the Immunomodulatory Properties of Equine Adult-Derived Mesenchymal Stem Cells(). *Cell Med* **4**, 1-11, doi:10.3727/215517912X647217 (2012).

[12] Hu, D. Z., Zhou, L. F., Zhu, J., Mao, Y. & Wu, X. H. Upregulated gene expression of local brain-derived neurotrophic factor and nerve growth factor after intracisternal administration of marrow stromal cells in rats with traumatic brain injury. *Chin J Traumatol* **8**, 23-26 (2005).

[13] Parr, A. M., Tator, C. H. & Keating, A. Bone marrow-derived mesenchymal stromal cells for the repair of central nervous system injury. *Bone Marrow Transplant* **40**, 609-619, doi:10.1038/sj.bmt.1705757 (2007).

[14] Maltman, D. J., Hardy, S. A. & Przyborski, S. A. Role of mesenchymal stem cells in neurogenesis and nervous system repair. *Neurochem Int* **59**, 347-356, doi:10.1016/j.neuint.2011.06.008 (2011).

[15] Uccelli, A., Benvenuto, F., Laroni, A. & Giunti, D. Neuroprotective features of mesenchymal stem cells. *Best Pract Res Clin Haematol* **24**, 59-64, doi:10.1016/j.beha.2011.01.004 (2011).

[16] Paul, G. & Anisimov, S. V. The secretome of mesenchymal stem cells: potential implications for neuroregeneration. *Biochimie* **95**, 2246-2256, doi:10.1016/j.biochi.2013.07.013 (2013).

[17] Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143-147 (1999).

[18] Aggarwal, S. & Pittenger, M. F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* **105**, 1815-1822, doi:10.1182/blood-2004-04-1559 (2005).

[19] Lee, J. *et al.* Migration and differentiation of nuclear fluorescence-labeled bone marrow stromal cells after transplantation into cerebral infarct and spinal cord injury in mice. *Neuropathology* **23**, 169-180 (2003).
[20] Yano, S. *et al.* In vivo fluorescence tracking of bone marrow stromal cells transplanted into a pneumatic injury model of rat spinal cord. *J Neurotrauma* **22**, 907-918, doi:10.1089/neu.2005.22.907 (2005).
[21] Spencer, N. D., Chun, R., Vidal, M. A., Gimble, J. M. & Lopez, M. J. In vitro expansion and differentiation of fresh and revitalized adult canine bone marrow-derived and adipose tissue-derived stromal cells. *Vet J* **191**, 231-239, doi:10.1016/j.tvjl.2010.12.030 (2012).
[22] Takemitsu, H. *et al.* Comparison of bone marrow and adipose tissue-derived canine mesenchymal stem cells. *BMC Vet Res* **8**, 150, doi:10.1186/1746-6148-8-150 (2012).
[23] Screven, R. *et al.* Immunophenotype and gene expression profile of mesenchymal stem cells derived from canine adipose tissue and bone marrow. *Vet Immunol Immunopathol* **161**, 21-31, doi:10.1016/j.vetimm.2014.06.002 (2014).
[24] Zucconi, E. *et al.* Mesenchymal stem cells derived from canine umbilical cord vein--a novel source for cell therapy studies. *Stem Cells Dev* **19**, 395-402, doi:10.1089/scd.2008.0314 (2010).
[25] Wenceslau, C. V. *et al.* Mesenchymal progenitor cells from canine fetal tissues: yolk sac, liver, and bone marrow. *Tissue Eng Part A* **17**, 2165-2176, doi:10.1089/ten.TEA.2010.0678 (2011).
[26] Vieira, N. M. *et al.* Isolation, characterization, and differentiation potential of canine adipose-derived stem cells. *Cell Transplant* **19**, 279-289, doi:10.3727/096368909X481764 (2010).
[27] Filioli Uranio, M. *et al.* Isolation, proliferation, cytogenetic, and molecular characterization and in vitro differentiation potency of canine stem cells from foetal adnexa: A comparative study of amniotic fluid, amnion, and umbilical cord matrix. *Molecular Reproduction and Development* **78**, 361-373, doi:10.1002/mrd.21311 (2011).
[28] Cremonesi, F., Corradetti, B. & Consiglio, A. L. Fetal adnexa derived stem cells from domestic animal: progress and perspectives. *Theriogenology* **75**, 1400-1415 (2011).
[29] Lee, J. M. *et al.* Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. *Int Immunopharmacol* **13**, 219-224, doi:10.1016/j.intimp.2012.03.024 (2012).
[30] Saulnier, N. *et al.* Canine placenta: A promising potential source of highly proliferative and immunomodulatory mesenchymal stromal cells? *Veterinary immunology and immunopathology* **171**, 47-55 (2016).
[31] Calzarossa, C. *et al.* Neurorescue effects and stem properties of chorionic villi and amniotic progenitor cells. *Neuroscience* **234**, 158-172, doi:10.1016/j.neuroscience.2012.12.038 (2013).
[32] Wang, A. *et al.* Placental mesenchymal stromal cells rescue ambulation in ovine myelomeningocele. *Stem Cells Transl Med* **4**, 659-669, doi:10.5966/sctm.2014-0296 (2015).
[33] Coates, J. R. & Jeffery, N. D. Perspectives on meningoencephalomyelitis of unknown origin. *Vet Clin North Am Small Anim Pract* **44**, 1157-1185, doi:10.1016/j.cvsm.2014.07.009 (2014).
[34] Park, E.-S., Uchida, K. & Nakayama, H. Th1-, Th2-, and Th17-Related Cytokine and Chemokine Receptor mRNA and Protein Expression in the Brain Tissues, T Cells, and Macrophages of Dogs With Necrotizing and Granulomatous Meningoencephalitis. *Veterinary Pathology* **50**, 1127-1134, doi:10.1177/0300985813488957 (2013).
[35] Kipar, A., Baumgartner, W., Vogl, C., Gaedke, K. & Wellman, M. Immunohistochemical characterization of inflammatory cells in brains of dogs with granulomatous meningoencephalitis. *Vet Pathol* **35**, 43-52, doi:10.1177/030098589803500104 (1998).

[36] Spitzbarth, I., Schenk, H. C., Tipold, A. & Beineke, A. Immunohistochemical characterization of inflammatory and glial responses in a case of necrotizing leucoencephalitis in a French bulldog. *J Comp Pathol* **142**, 235-241, doi:10.1016/j.jcpa.2009.08.158 (2010).

[37] Spitzbarth, I., Baumgartner, W. & Beineke, A. The role of pro- and anti-inflammatory cytokines in the pathogenesis of spontaneous canine CNS diseases. *Vet Immunol Immunopathol* **147**, 6-24, doi:10.1016/j.vetimm.2012.04.005 (2012).

[38] Clark, K. C. *et al.* Canine and Equine Mesenchymal Stem Cells Grown in Serum Free Media Have Altered Immunophenotype. *Stem Cell Rev* **12**, 245-256, doi:10.1007/s12015-015-9638-0 (2016).

[39] Kol, A. *et al.* Gastrointestinal microbes interact with canine adipose-derived mesenchymal stem cells in vitro and enhance immunomodulatory functions. *Stem Cells Dev* **23**, 1831-1843, doi:10.1089/scd.2014.0128 (2014).

[40] Long, C. *et al.* Isolation and characterization of canine placenta-derived mesenchymal stromal cells for the treatment of neurological disorders in dogs. *Cytometry A* **93**, 82-92, doi:10.1002/cyto.a.23171 (2018).

[41] Long, C. *et al.* Isolation and characterization of canine placenta-derived mesenchymal stromal cells for the treatment of neurological disorders in dogs. *Cytometry A*, doi:10.1002/cyto.a.23171 (2017).

[42] Hong, J. *et al.* Indoleamine 2,3-dioxygenase mediates inhibition of virus-specific CD8(+) T cell proliferation by human mesenchymal stromal cells. *Cytotherapy* **18**, 621-629, doi:10.1016/j.jcyt.2016.01.009 (2016).

[43] Clark, K. C. *et al.* Human and feline adipose-derived mesenchymal stem cells have comparable phenotype, immunomodulatory functions, and transcriptome. *Stem Cell Res Ther* **8**, 69, doi:10.1186/s13287-017-0528-z (2017).

[44] Chow, L., Johnson, V., Coy, J., Regan, D. & Dow, S. Mechanisms of Immune Suppression Utilized by Canine Adipose and Bone Marrow-Derived Mesenchymal Stem Cells. *Stem Cells Dev* **26**, 374-389, doi:10.1089/scd.2016.0207 (2017).

[45] Yang, H. M. *et al.* Canine mesenchymal stem cells treated with TNF-alpha and IFN-gamma enhance anti-inflammatory effects through the COX-2/PGE2 pathway. *Res Vet Sci* **119**, 19-26, doi:10.1016/j.rvsc.2018.05.011 (2018).

[46] Perez-Merino, E. M. *et al.* Safety and efficacy of allogeneic adipose tissue-derived mesenchymal stem cells for treatment of dogs with inflammatory bowel disease: Clinical and laboratory outcomes. *Vet J* **206**, 385-390, doi:10.1016/j.tvjl.2015.08.003 (2015).

[47] Black, L. L. *et al.* Effect of intraarticular injection of autologous adipose-derived mesenchymal stem and regenerative cells on clinical signs of chronic osteoarthritis of the elbow joint in dogs. *Vet Ther* **9**, 192-200 (2008).

[48] Mielcarek, M. *et al.* Mesenchymal stromal cells fail to prevent acute graft-versus-host disease and graft rejection after dog leukocyte antigen-haploidentical bone marrow transplantation. *Biol Blood Marrow Transplant* **17**, 214-225, doi:10.1016/j.bbmt.2010.08.015 (2011).

[49] Lassmann, H. & Bradl, M. Multiple sclerosis: experimental models and reality. *Acta Neuropathol* **133**, 223-244, doi:10.1007/s00401-016-1631-4 (2017).

[50] Park, S. S. *et al.* Functional recovery after spinal cord injury in dogs treated with a combination of Matrigel and neural-induced adipose-derived mesenchymal Stem cells. *Cytotherapy* **14**, 584-597, doi:10.3109/14653249.2012.658913 (2012).

[51] Moon, J. H. *et al.* A study of experimental autoimmune encephalomyelitis in dogs as a disease model for canine necrotizing encephalitis. *J Vet Sci* **16**, 203-211, doi:10.4142/jvs.2015.16.2.203 (2015).

[52] Jeffery, N. D. *et al.* The Association of Specific Constituents of the Fecal Microbiota with Immune-Mediated Brain Disease in Dogs. *PLoS One* **12**, e0170589, doi:10.1371/journal.pone.0170589 (2017).
[53] Sospedra, M. & Martin, R. Immunology of multiple sclerosis. *Annu Rev Immunol* **23**, 683-747, doi:10.1146/annurev.immunol.23.021704.115707 (2005).
[54] Atkins, H. L., Muraro, P. A., van Laar, J. M. & Pavletic, S. Z. Autologous hematopoietic stem cell transplantation for autoimmune disease--is it now ready for prime time? *Biol Blood Marrow Transplant* **18**, S177-183, doi:10.1016/j.bbmt.2011.11.020 (2012).
[55] Bartels, J. *et al.* MIP-3beta/CCL19 is associated with the intrathecal invasion of mononuclear cells in neuroinflammatory and non-neuroinflammatory CNS diseases in dogs. *BMC Vet Res* **10**, 157, doi:10.1186/1746-6148-10-157 (2014).
[56] Carrade, D. D. & Borjesson, D. L. Immunomodulation by mesenchymal stem cells in veterinary species. *Comp Med* **63**, 207-217 (2013).
[57] Kalinski, P. Regulation of immune responses by prostaglandin E2. *J Immunol* **188**, 21-28, doi:10.4049/jimmunol.1101029 (2012).
[58] Erkers, T., Nava, S., Yosef, J., Ringden, O. & Kaipe, H. Decidual stromal cells promote regulatory T cells and suppress alloreactivity in a cell contact-dependent manner. *Stem Cells Dev* **22**, 2596-2605, doi:10.1089/scd.2013.0079 (2013).
[59] Melief, S. M., Zwaginga, J. J., Fibbe, W. E. & Roelofs, H. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. *Stem Cells Transl Med* **2**, 455-463, doi:10.5966/sctm.2012-0184 (2013).
[60] Mohammadpour, H., Pourfathollah, A. A., Zarif, M. N. & Tahoori, M. T. TNF-alpha modulates the immunosuppressive effects of MSCs on dendritic cells and T cells. *Int Immunopharmacol* **28**, 1009-1017, doi:10.1016/j.intimp.2015.07.045 (2015).
[61] Ren, G. *et al.* Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. *J Immunol* **184**, 2321-2328, doi:10.4049/jimmunol.0902023 (2010).
[62] Taechangam, N., Iyer, S. S., Walker, N. J., Arzi, B. & Borjesson, D. L. Mechanisms utilized by feline adipose-derived mesenchymal stem cells to inhibit T lymphocyte proliferation. *Stem Cell Res Ther* **10**, 188, doi:10.1186/s13287-019-1300-3 (2019).
[63] Hutchinson, M. Natalizumab: A new treatment for relapsing remitting multiple sclerosis. *Ther Clin Risk Manag* **3**, 259-268, doi:10.2147/tcrm.2007.3.2.259 (2007).

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Chapter 6. Long Term Safety of Postnatal Stem Cell Repair of Spina Bifida in English Bulldogs: A Pilot Study

6.1 Abstract

Introduction: Placental mesenchymal stem/stromal cells are being investigated as an adjunct to prenatal repair of spina bifida (SB); however, similar treatments have not been explored for postnatal repair. English bulldogs are known to have a high incidence of naturally occurring SB and could serve as the first postnatal animal model of this devastating childhood disease.

Methods: Two 9-week-old English Bulldogs with clinical concern for SB underwent imaging and work-up to confirm and characterize corresponding defects. Each dog underwent a multi-segment laminectomy, followed by extracellular matrix (ECM) patch repair augmented with allogeneic canine placental mesenchymal stem/stromal cells (cPMSCs).

Results: Both dogs were initially ambulatory with notably abnormal gaits, as well as incontinent of urine and stool. Lumbar skin coverage was noted. Magnetic resonance imaging revealed L7-S1 defects of varying severity. One dog also showed a large syringohydromyelnia involving the lumbar spinal cord. Electrophysiologic testing showed low-normal conduction velocity for both motor and sensory hindlimb nerves. Intra-operatively, both lesions were found to have intact dural coverage. Following posterior laminectomy, patch repair was performed with an ECM patch seeded with allogeneic cPMSCs. Both dogs recovered uneventfully. Follow-up at 8 weeks showed subjective improved ambulation for both dogs during neurological exams. Repeat MRI showed only syrinx recurrence. No significant adverse events occurred in either dog by 5 months follow-up. Annual check in with current owner suggested no SB associated adverse events up to 5 years following cPMSC administration. Furthermore, no events of teratoma formation or oncological events were reported.

Conclusion: Postnatal patch repair of a naturally occurring canine model of SB augmented with allogeneic cPMSCs is feasible and appears safe. Further clinical studies are needed to evaluate efficacy, prior to translation to human postnatal repair.

6.2 Introduction

Spina bifida (SB) is a devastating congenital defect that can cause lifelong paralysis, bowel and bladder incontinence, musculoskeletal deformities, and cognitive and cranial nerve disabilities due to hindbrain herniation[1]. SB is the result of incomplete closure of the neural tube during the fourth week of gestation and in utero intervention has become the current standard of care for select patients[2]. Additionally, recent preclinical studies have shown that prenatal repair augmented with cell-based therapies results in improved functional outcomes in the well-established fetal lamb model of SB, and regenerative therapies for this disorder are in development[3-5]. Despite promising advances in the development of *prenatal* therapies, a large number of children are unable to undergo fetal repair. Therefore, postnatal repair remains the mainstay of treatment for the approximately 1500 children born every year with unrepaired SB[6]. It has been suggested pediatric patients retain elevated neural plasticity for some time after birth, and children typically demonstrate better recovery than adults following spinal cord injury[7]. Therefore, intervention strategies for early postnatal period for SB patients are still needed. Unfortunately, existing animal models for fetal intervention are difficult to survive after birth and are technically and financially challenging for the development of postnatal treatments[8].

Companion animals with naturally occurring illnesses are increasingly recognized as a promising alternative to experimental animals for translational studies[9]. English Bulldogs in particular are known to have a high incidence of naturally occurring SB with similar pathological and clinical features compared to human patients[10]. Currently, no standard of care exists for this canine patient population, and in most cases, these affected animals will be euthanized. Similar to human SB, canine SB varies in severity of the defect, which indicates that the underlying pathophysiology may be more comparable in humans and dogs than in any type of induced SB disease model (**Figure 6.1**). In the canine, SB is rarely diagnosed until after birth and these animals are typically euthanized which highlights the critical need for novel therapeutic approaches for these companion animals.

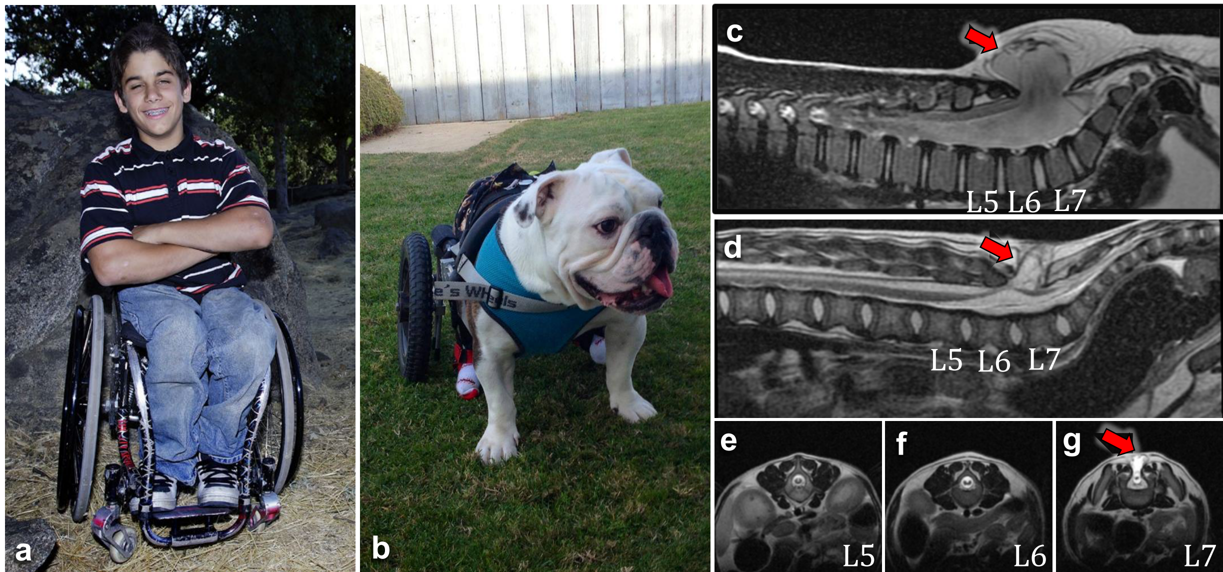

Figure 6.1: A Comparison of Human and Canine Spina Bifida. Humans and canines affected by SB share similar clinical symptoms including lower extremity/hind limb paralysis and bladder/bowel incontinence (a, b). Comparison of magnetic resonance imaging (MRI) also demonstrates similarities between human (c) and canine (d-g) SB lesions. These similarities include congenital absence of the lumbar spinous process and herniation of the spinal cord and meninges (indicated by red arrows in c, d, g). MRI sagittal sections (c, d); transverse sections (e-g). Image (c) was from a 3-year old human SB patient. Case courtesy of Dr. Mahmoud Yacout Alabd, Radiopaedia.org, rID: 39982. Images (d-g) were obtained from a 9-week old canine SB patient at the UC Davis Veterinary Medicine Teaching Hospital (VMTH).

Mesenchymal stem/stromal cell (MSC) therapy has become progressively popular for the treatment of naturally-occurring diseases in veterinary species[11]. Placenta-derived MSCs (PMSCs) are an emerging therapeutic agent for disease treatment. The placenta is a unique and fetal-derived tissue[12], that is reliably obtained and used for the treatment of developmental and perinatal diseases[13,14]. PMSCs display immunomodulatory capabilities[5,15,16], improve wound healing[17], and demonstrate neuroprotective effects[18-20]. PMSCs may also exhibit greater immunomodulatory properties and *ex vivo* expansion potential as compared to adult bone marrow derived MSCs[16,21]. Both fetal[22-26] and early postnatal[3,27-29] motor neuron apoptosis/loss and progression of motor function loss have been confirmed in animal models[3,25-27] and human patients [28,29]. As a pre-natal therapy, PMSCs have been shown to effectively cure paralysis in a sheep model of SB suggesting postnatal therapy using PMSC therapy will be safe[3]. Furthermore, it has also been demonstrated that canine PMSCs (cPMSCs) as compared to adult MSC sources

display more robust and unique immunomodulatory properties that may have clinical implications for perinatal diseases[30]. Therefore, a canine model of SB is feasible as a translational large animal model evaluate the safety and efficacy of cellular therapeutics including PMSCs.

The hypothesis of the current study is that the cell-based therapy successfully employed in the fetal lamb model could be adapted to postnatal repair of dogs with SB. In the current study, we demonstrate the feasibility and safety of postnatal repair augmented with allogeneic cPMSCs in young English bulldogs with SB.

6.3 Methods and Materials

6.3.1 Animal Recruitment

English Bulldogs, one male and one female, were recruited from a local animal rescue group. Both animals exhibited motor deficits and persistent incontinence; diagnostic workup to confirm a diagnosis of SB included a thorough physical and neurologic exam, magnetic resonance imaging (MRI) of the spinal cord, and electrophysiologic testing of both hindlimbs consisting of sensory and motor nerve conduction velocity and electromyography (EMG). Dogs with confirmed SB then underwent multi-segment posterior laminectomy, followed by repair with both cPMSC loaded hydrogel and SIS-ECM. Dogs were admitted to the Intensive Care Unit (ICU) post-operatively for close monitoring. After discharge, weekly follow-up by telephone was planned. Follow-up in clinical examinations were scheduled for 8 weeks after surgery with annual virtual check ins up up to five years following cPMSC administration. Care of all animals was in accordance with institutional guidelines.

6.3.2 Cell Isolation and Preparation

Allogeneic cPMSCs were isolated as described previously[31]. Briefly, term canine placentas were cultured using a combination enzymatic dissociation and explant culture. Canine placentas were processed by manual dissection and treated with 0.25% trypsin (ThermoFisher Scientific, Waltham, MA) solution for 30 min at 37 °C. Cell pellets were washed and incubated with collagenase IA (1 mg/mL; Sigma-Aldrich, St. Louis, MO) for 45 min at 37 °C. The cell pellet was

resuspended and plated onto tissue culture flasks in medium containing Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/mL penicillin and 100 μg/mL streptomycin (ThermoFisher Scientific).

6.3.3 cPMSC Phenotyping

After harvesting, cPMSCs expanded were washed and incubated with antibodies directed against CD44-AF647 (BioLegend; 103018), CD90-PE (BD Bioscience; 561970), CD29-PE (Biolegend; 303004), CD34-PE (BD Bioscience; 559369), CD31 (Bioss; bs-0548R), and CD45 (Peter Moore Laboratory, UC Davis School of Veterinary Medicine, Department of Pathology, Microbiology & Immunology[31]). cPMSCs were screened for typical canine MSC phenotype via flow cytometry as previously described[31].

6.3.4 Leukocyte Suppression Assay (LSA)

Leukocyte suppression assays (LSAs) in the canine were performed as previously described[32]. In brief, peripheral blood was collected into tubes containing sodium heparin (Vacutainer®, BD Biosciences) via jugular venipuncture. Peripheral blood mononuclear cells (PBMCs) were obtained using a discontinuous Ficoll gradient and were plated with allogeneic cPMSCs exactly as previously described[32]. PMBCs were activated with 5 μg/mL concanavalin A (Con-A, Sigma-Aldrich). After 3 days of co-culture, wells were treated with 1 mM Bromodeoxyuridine (BrdU, BD Biosciences). Twenty-four hours post BrdU treatment, leukocytes were collected, and cells were stained for nuclear BrdU incorporation per manufacturer directions (FITC BrdU Flow Kit, BD Biosciences) and read by flow cytometry.

6.3.5 Neuroprotection Assay

Neuroprotection assays were performed as previously described[31]. In brief, SH-SY5Y cells were cultured for 24h before media was changed to 5% FBS and treated with 1ul/mL of staurosporine to induce apoptosis for four hours. cPMSCs were added in transwell inserts with no cPMSCs serving as apoptotic controls and were cultured for 96 hours. At 96 h, the inserts

were removed, the cells were stained with 2 μM calcein AM (Thermo Fisher Scientific). Cells were then imaged at 6 randomized positions using a motorized Axio Observer D1 inverted microscope (Carl Zeiss). The images were subsequently processed by WimNeuron Image Analysis (Onimagin Technologies, Cordoba, Spain) for neurite outgrowth analysis.

6.3.6 Clinical Preparation of cPMSCs for Surgical Repair

One cPMSC line screened and shown to express typical MSC markers and demonstrate immunomodulatory properties was selected to treat both animals. Prior to clinical use, cPMSCs were assessed for the presence of mycoplasma and endotoxin as well as sterility and viability prior to treatment through the UC Davis School of Veterinary Medicine Clinical Laboratories.

A day prior to surgery, adherent cPMSCs were lifted using TrypLE Select (ThermoFisher Scientific). A thermosensitive collagen hydrogel was prepared by mixing ice cold 3.83 mg/mL rat tail collagen I solution (BD Biosciences) in DMEM containing cPMSCs, for a final concentration of 1 mg/mL collagen I and 5×10^5 cells/mL[3,33]. Clinical grade porcine small intestine submucosa extracellular matrix (SIS-ECM) was also seeded with cPMSCs at 3×10^5 cells/cm^2[34]. cPMSC and collagen/hydrogel suspension was incubated at 37 °C, 5% CO_2 for 90 minutes, after which culture media was added to cell culture plates until the following day. On the day of surgery, ECMs were washed twice with clinical grade saline before use. Representative images of cPMSCs cultured on hydrogels (Figure 6.2A) and SIS-ECM (Figure 2B), prior to washing are shown.

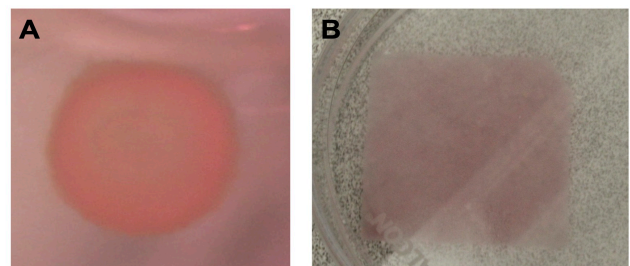

Figure 6.2. Representative images of cPMSCs cultured in hydrogel (A) and SIS-ECM (B) prior to administration.

6.3.7 Administration of cPMSCs

In animals receiving surgical treatment, reduction of the meningomyelocoele and spinal cord defect during open surgery using a routine approach to the canine spine and the protocol established in the fetal lamb model[35] for cPMSC implantation was performed. Briefly, incision in skin, exposing underlying fascia was performed to expose the paraspinal muscle (Figure 6.3 A-

B). The paraspinal muscles were then removed to expos the lumbar vertebrate which was then removed to expose the spinal cord (Figure 6.3 C-D). The dura matter was then excised, and application of SIS-extracellular matrix (SIS-ECM) scaffold seeded with cPMSCs directly above the defect site (Figure 6.3 E-F).

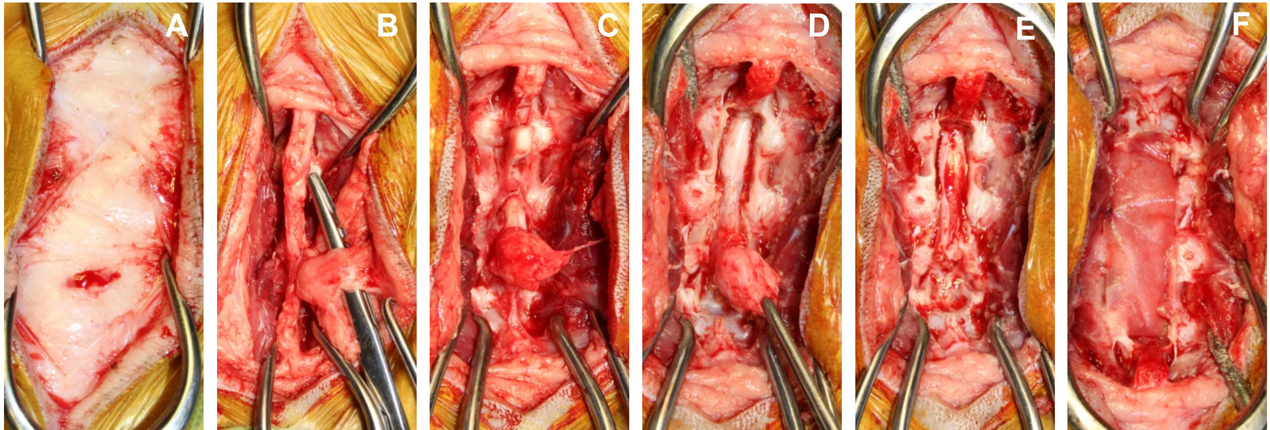

Figure 6.3. Representative Photographs of the Surgical Repair of Canine Spina Bifida. A) Incision in skin, exposing underlying fascia. B) Exposure of paraspinal muscles. C) Removal of paraspinal muscles, exposing lumbar vertebrae. D) Removal of lumbar vertebrae, exposing the spinal cord. E) Careful incision into the dura mater. F) Application of SIS-extracellular matrix (SIS-ECM) scaffold seeded with cPMSCs directly above the defect site.

6.4 Results

One cell line from established cPMSC cell banks was screened for typical PMSC lineage and functional properties. The selected cPMSC cell line to be administered to canine SB patients was shown to be positive for CD44, CD29 and CD90 and negative for CD31, CD34 or CD45 (Figure 6.4A). To assess the functional properties of cPMSCs, LSAs were performed to evaluate immunomodulatory potential as well as neural circularity assays were preformed to assess neuroprotective properties. PBMCs from multiple healthy canine donors were stimulated using the mitogen ConA and cultured with irradiated cPMSCs. The chosen donor line of cPMSCs

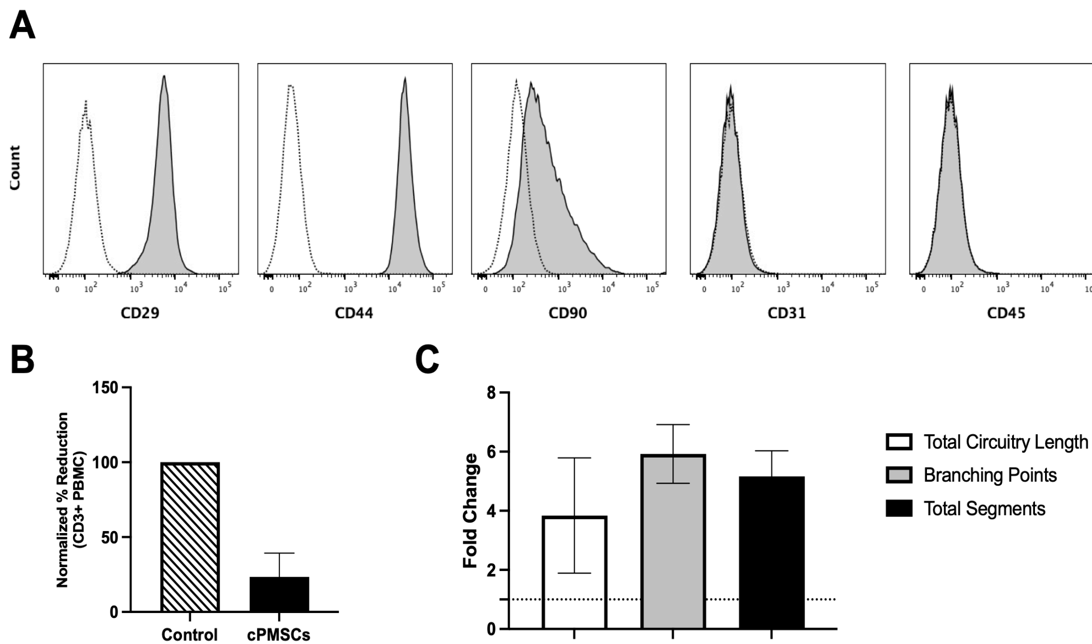

Figure 6.4. Characterization of cPMSC cell line for surgical repair of canine spina bifida. One cPMSC cell line was characterized and screened for functional properties prior to administration into canine patients. The cPMSC cell line used to treat both animals displayed typical MSC phenotypic markers and was positive for CD29, CD44 and CD90, and negative for CD31 and CD45 (A). In leukocyte suppression assays, stimulated peripheral blood mononuclear cells served as a control addition of cPMSCs reduced BrdU incorporation into CD3+ cells (B). Furthermore, in the neural circuitry network assay, cPMSCs added to apoptotic SH-SY5Y cells increased total circuitry length, branching points and total segments (C). Data presented as mean and standard error.

reduced CD3 positive leukocyte proliferation of all screened PBMC donors (Figure 6.4B). Furthermore, addition of cPMSCs into apoptotic SY-SH5Y cells in an established neuroprotection assay demonstrated increased fold change for circuitry length, total branching points and total segments (Figure 6.4C). Established cPMSC banks were used for administration into clinical patients, and prior to injection were confirmed to be void of mycoplasma and endotoxin.

Both animals were evaluated and confirmed to have SB. On exam, the skin of the lumbar back was noted to be intact with a small abnormal tuft of hair. Both puppies were ambulatory with abnormal gaits particularly affecting the bilateral hindlimbs (Figure 6.5). Both presented with bowel and bladder incontinence. MRI revealed defects involving the spinous processes of L7-S1 with protrusion of a lipomyelomeningocele. The male puppy was also found to have a large syringohydromyelia compressing the lumbar spinal cord (Figure 6.6). Electrophysiologic testing

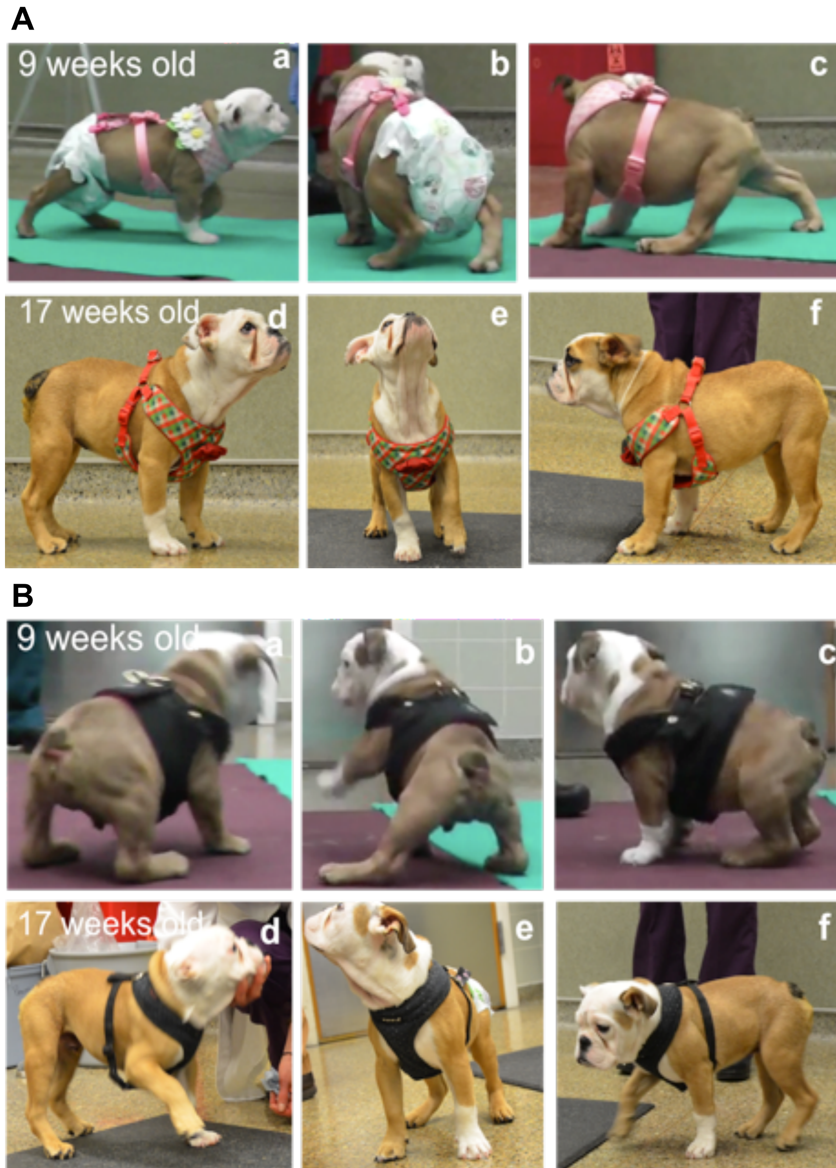

Figure 6.5: Ambulatory Characteristics of Bulldogs with spina Bifida Before and After Treatment. (A) Female bulldog pre-treatment at 9 weeks old (a-c) and post-treatment at 17 weeks old (d-f). The right hind limb of the female dog showed significant weakness in body support before surgery but seemed to function normally 8 weeks after surgery. (B) Male bulldog pre-treatment at 9 weeks old (a-c) and post-treatment at 17 weeks old (d-f). The male dog primarily used his hocks to walk and showed significantly impaired coordination before surgery but showed improved coordination and ambulation post-operatively.

was within normal limits for age for both sensory and motor nerve conduction velocities. On

electromyography, the male puppy had some mild changes in several hindlimb muscle groups, while the female had no abnormal findings.

Intra-operatively, bony defects were seen at the expected levels with protruding but intact dural sacs. Posterior laminectomy was performed on both puppies and the dura opened to expose the underlying cord. cPMSC loaded hydrogel was then placed into the dural space, followed by the cPMSC seeded ECM which was secured at each corner. The paraspinal muscles were re-approximated over the defect and the skin closed. Both puppies tolerated the procedure well. The

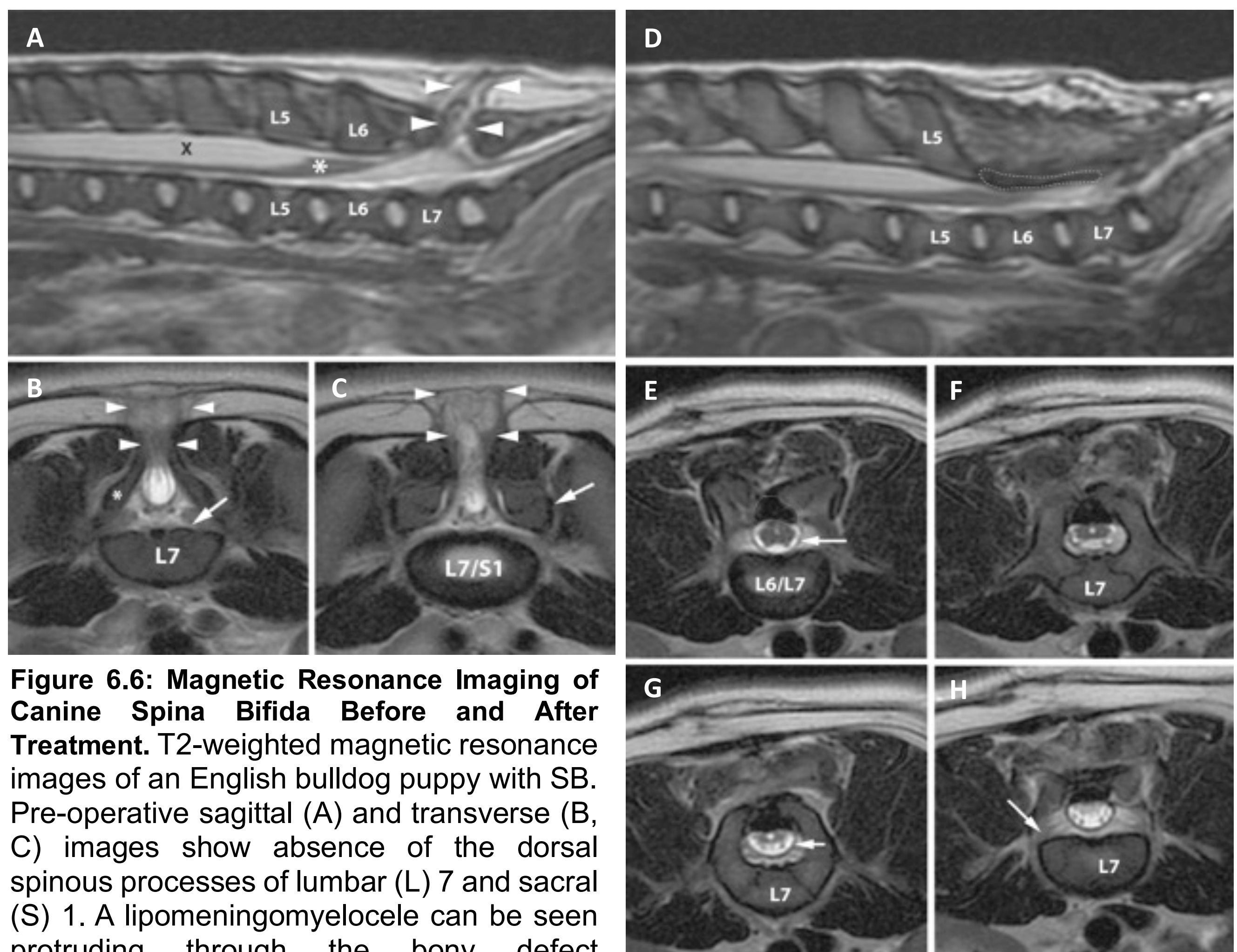

Figure 6.6: Magnetic Resonance Imaging of Canine Spina Bifida Before and After Treatment. T2-weighted magnetic resonance images of an English bulldog puppy with SB. Pre-operative sagittal (A) and transverse (B, C) images show absence of the dorsal spinous processes of lumbar (L) 7 and sacral (S) 1. A lipomeningomyelocele can be seen protruding through the bony defect (arrowheads) in the dorsal lamina. The terminal portion of the spinal cord (asterisk) is seen within the vertebral canal with suspected tethering. A syringomyelia (X) is also present. Post-operative sagittal (D) and transverse (E-H) images taken after 8 weeks. The dorsal spinous process of L6 has been removed and an extracellular matrix patch (dotted line) placed in the thecal sac. Midline musculature is visualized dorsal to the implant.

male puppy developed a fever post-operatively, however no infectious source was identified. Recovery was otherwise unremarkable and both puppies were discharged home with their caretaker.

Follow-up by telephone was performed weekly with no apparent complications in either puppy. Eight weeks after surgery, both puppies were seen in clinic with well-healed incisions. Both puppies were ambulating independently, though they remained incontinent. Repeat MRI was performed on the male puppy given his pre-operative syringomyelia, which was found to have re-accumulated. The dogs' caretaker reported no complications at telephone follow-up 5 months after surgery. Annual check ins with animals caretakes reported no adverse events or complications following cPMSC administration and surgical repair for up to 5-years post treatment.

6.5 Discussion

SB is the most common congenital defect of the central nervous system that is compatible with life, with a prevalence of approximately 3 per every 10,000 live births in the United States[36]. The landmark NIH-funded Management of Myelomeningocele Study (MOMS) showed that prenatal repair reduced the incidence and severity of hindbrain herniation, the need for ventriculoperitoneal shunting, and, for the first time, improvement in motor function with twice as many children able to walk independently or with assistive devices[2]. More recently, preclinical studies have shown that augmenting prenatal repair with PMSCs can further improve mobility in the widely used fetal sheep model[3].

However, the criteria for prenatal intervention are strict given the narrow window of time in which fetal surgery can be performed and the inherent risks to both the fetus and the mother. Unfortunately, development of a postnatal regenerative therapy cannot currently proceed as none of the current animal models of SB are suitable for long term survival after birth. Rodents with retinoic acid-induced SB do not survive after birth, while lambs with surgically created defects *in utero* are paraplegic and cannot be humanely survived due to their inability to nurse[37].

Naturally occurring SB in companion animals may be the solution to the current lack of suitable animal models. SB is known to occur in certain breeds of dogs, particularly English bulldogs, with symptoms similar to those seen in human patients[10]. These dogs often have extensive care needs and multiple rescue organizations exist to reduce the number of dogs that would otherwise be euthanized. The current pilot safety study was designed to test the feasibility of a postnatal regenerative therapy analogous to that previously used in the fetal sheep model[3]. This had the dual intent of adapting our regenerative therapy to a postnatal model for ultimate translation to human patients, as well as developing the first potential treatment for dogs with SB. To this end, canine PMSCs were isolated and characterized and found to have structural and functional similarity to human PMSCs[5,31]. These cells were then transplanted into the area of the defect to maximize local paracrine activity of these cells, which is believed to be the mechanism of action behind their immunomodulatory and neuroprotective effects[18,20,30,38].

There are several limitations to this study, including the small number of canine patients and the lack of functional outcome measures. Both puppies remained ambulatory after treatment, but the significance of this is unknown without a validated ambulation scale for comparison, as even normal bulldogs have an abnormal gait by standard measures. A larger clinical trial is underway, and a bulldog-specific ambulatory score is being developed to address these issues. While a naturally occurring, defect has certain benefits over chemically or surgically induced animal models, the presentation of SB in these bulldogs may differ from humans. In contrast to most forms of human SB, both dogs had complete skin coverage over the area of the defect. It is unknown whether open defects are uncommon in dogs or if these dogs are stillborn or simply euthanized at birth. Additionally, unlike human SB which is often diagnosed at the time of delivery, affected dogs may not be recognized until after 6-10 weeks of age, when most dogs would be expected to become ambulatory and continent[39]. This results in relatively late surgical intervention compared to human postnatal repair, which typically occurs within 48 hours after

delivery. Despite these limitations, the results of this pilot study are promising as both dogs successfully underwent postnatal repair augmented with allogeneic cPMSCs without any apparent complications.

6.6 Conclusions

Postnatal repair utilizing allogeneic cPMSCs is feasible and appears safe. Further clinical studies are needed to confirm safety and evaluate the efficacy of this approach, prior to translation to human postnatal treatment.

6.7 References

[1] Lee, S. Y., Papanna, R., Farmer, D. & Tsao, K. Fetal Repair of Neural Tube Defects. *Clin Perinatol* **49**, 835-848, doi:10.1016/j.clp.2022.06.004 (2022).

[2] Kuriyan, A. E. *et al.* Vision Loss after Intravitreal Injection of Autologous "Stem Cells" for AMD. *New England Journal of Medicine* **376**, 1047-1053, doi:doi:10.1056/NEJMoa1609583 (2017).

[3] Wang, A. *et al.* Placental mesenchymal stromal cells rescue ambulation in ovine myelomeningocele. *Stem cells translational medicine* **4**, 659-669, doi:10.5966/sctm.2014-0296 (2015).

[4] Fauza, D. O., Jennings, R. W., Teng, Y. D. & Snyder, E. Y. Neural stem cell delivery to the spinal cord in an ovine model of fetal surgery for spina bifida. *Surgery* **144**, 367-373, doi:10.1016/j.surg.2008.05.009 (2008).

[5] Lankford, L. *et al.* Early gestation chorionic villi-derived stromal cells for fetal tissue engineering. *World journal of stem cells* **7**, 195-207, doi:10.4252/wjsc.v7.i1.195 (2015).

[6] Parker, S. E. *et al.* Updated national birth prevalence estimates for selected birth defects in the United States, 2004–2006. *Birth Defects Research Part A: Clinical and Molecular Teratology* **88**, 1008-1016, doi:10.1002/bdra.20735 (2010).

[7] Wang, M. Y., Hoh, D. J., Leary, S. P., Griffith, P. & McComb, J. G. High rates of neurological improvement following severe traumatic pediatric spinal cord injury. *Spine* **29**, 1493-1497; discussion E1266 (2004).

[8] Guilbaud, L. *et al.* Limits of the surgically induced model of myelomeningocele in the fetal sheep. *Child's nervous system : ChNS : official journal of the International Society for Pediatric Neurosurgery* **30**, 1425-1429, doi:10.1007/s00381-014-2426-3 (2014).

[9] Kol, A. *et al.* Companion animals: Translational scientist's new best friends. *Science translational medicine* **7**, 308ps321, doi:10.1126/scitranslmed.aaa9116 (2015).

[10] Wilson, J. W., Kurtz, H. J., Leipold, H. W. & Lees, G. E. Spina bifida in the dog. *Veterinary pathology* **16**, 165-179, doi:10.1177/030098587901600202 (1979).

[11] Carrade, D. D. & Borjesson, D. L. Immunomodulation by mesenchymal stem cells in veterinary species. *Comp Med* **63**, 207-217 (2013).

[12] Murphy, S. V. & Atala, A. Amniotic fluid and placental membranes: unexpected sources of highly multipotent cells. *Semin Reprod Med* **31**, 62-68, doi:10.1055/s-0032-1331799 (2013).

[13] Portmann-Lanz, C. B. *et al.* Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. *Am J Obstet Gynecol* **194**, 664-673, doi:10.1016/j.ajog.2006.01.101 (2006).

[14] Roselli, E. A. *et al.* Fetal mesenchymal stromal cells from cryopreserved human chorionic villi: cytogenetic and molecular analysis of genome stability in long-term cultures. *Cytotherapy* **15**, 1340-1351, doi:10.1016/j.jcyt.2013.06.019 (2013).

[15] Vellasamy, S., Sandrasaigaran, P., Vidyadaran, S., George, E. & Ramasamy, R. Isolation and characterisation of mesenchymal stem cells derived from human placenta tissue. *World journal of stem cells* **4**, 53-61, doi:10.4252/wjsc.v4.i6.53 (2012).

[16] Lee, J. M. *et al.* Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. *Int Immunopharmacol* **13**, 219-224, doi:10.1016/j.intimp.2012.03.024 (2012).

[17] Jones, G. N. *et al.* Ontological differences in first compared to third trimester human fetal placental chorionic stem cells. *PLoS One* **7**, e43395, doi:10.1371/journal.pone.0043395 (2012).

[18] Calzarossa, C. *et al.* Neurorescue effects and stem properties of chorionic villi and amniotic progenitor cells. *Neuroscience* **234**, 158-172, doi:10.1016/j.neuroscience.2012.12.038 (2013).

[19] Hsieh, J. Y. *et al.* Mesenchymal stem cells from human umbilical cord express preferentially secreted factors related to neuroprotection, neurogenesis, and angiogenesis. *PLoS One* **8**, e72604, doi:10.1371/journal.pone.0072604 (2013).
[20] Yust-Katz, S. *et al.* Placental mesenchymal stromal cells induced into neurotrophic factor-producing cells protect neuronal cells from hypoxia and oxidative stress. *Cytotherapy* **14**, 45-55, doi:10.3109/14653249.2011.613928 (2012).
[21] Poloni, A. *et al.* Characterization and expansion of mesenchymal progenitor cells from first-trimester chorionic villi of human placenta. *Cytotherapy* **10**, 690-697, doi:10.1080/14653240802419310 (2008).
[22] Hutchins, G. M. *et al.* Acquired spinal cord injury in human fetuses with myelomeningocele. *Pediatric pathology & laboratory medicine : journal of the Society for Pediatric Pathology, affiliated with the International Paediatric Pathology Association* **16**, 701-712 (1996).
[23] Korenromp, M. J., van Gool, J. D., Bruinese, H. W. & Kriek, R. Early fetal leg movements in myelomeningocele. *Lancet (London, England)* **1**, 917-918 (1986).
[24] Sival, D. A. *et al.* Perinatal motor behaviour and neurological outcome in spina bifida aperta. *Early human development* **50**, 27-37 (1997).
[25] Stiefel, D., Copp, A. J. & Meuli, M. Fetal spina bifida in a mouse model: loss of neural function in utero. *Journal of neurosurgery* **106**, 213-221, doi:10.3171/ped.2007.106.3.213 (2007).
[26] Stiefel, D. & Meuli, M. Scanning electron microscopy of fetal murine myelomeningocele reveals growth and development of the spinal cord in early gestation and neural tissue destruction around birth. *Journal of pediatric surgery* **42**, 1561-1565, doi:10.1016/j.jpedsurg.2007.04.019 (2007).
[27] Khan, M. S. I. *et al.* Early neonatal loss of inhibitory synaptic input to the spinal motor neurons confers spina bifida-like leg dysfunction in a chicken model. *Disease models & mechanisms* **10**, 1421-1432, doi:10.1242/dmm.031054 (2017).
[28] Sival, D. A. *et al.* Neonatal loss of motor function in human spina bifida aperta. *Pediatrics* **114**, 427-434 (2004).
[29] Sival, D. A. *et al.* Spinal hemorrhages are associated with early neonatal motor function loss in human spina bifida aperta. *Early human development* **84**, 423-431, doi:10.1016/j.earlhumdev.2007.11.003 (2008).
[30] Amorim, R. M. *et al.* Placenta-derived multipotent mesenchymal stromal cells: a promising potential cell-based therapy for canine inflammatory brain disease. *Stem cell research & therapy* **11**, 304, doi:10.1186/s13287-020-01799-0 (2020).
[31] Long, C. *et al.* Isolation and characterization of canine placenta-derived mesenchymal stromal cells for the treatment of neurological disorders in dogs. *Cytometry. Part A : the journal of the International Society for Analytical Cytology*, doi:10.1002/cyto.a.23171 (2017).
[32] Clark, K. C. *et al.* Canine and Equine Mesenchymal Stem Cells Grown in Serum Free Media Have Altered Immunophenotype. *Stem cell reviews* **12**, 245-256, doi:10.1007/s12015-015-9638-0 (2016).
[33] Lankford, L. *et al.* Early gestation chorionic villi-derived stromal cells for fetal tissue engineering. *World journal of stem cells* **7**, 195-207, doi:10.4252/wjsc.v7.i1.195 (2015).
[34] Lankford, L. *et al.* Manufacture and preparation of human placenta-derived mesenchymal stromal cells for local tissue delivery. *Cytotherapy* **19**, 680-688, doi:10.1016/j.jcyt.2017.03.003 (2017).
[35] Brown, E. G. *et al.* Age Does Matter: A Pilot Comparison of Placenta-Derived Stromal Cells for in utero Repair of Myelomeningocele Using a Lamb Model. *Fetal diagnosis and therapy* **39**, 179-185, doi:10.1159/000433427 (2016).

[36] Canfield, M. A. *et al.* The Association Between Race/Ethnicity and Major Birth Defects in the United States, 1999–2007. *American Journal of Public Health* **104**, e14-e23, doi:10.2105/AJPH.2014.302098 (2014).
[37] Meuli, M. *et al.* Creation of myelomeningocele in utero: a model of functional damage from spinal cord exposure in fetal sheep. *Journal of pediatric surgery* **30**, 1028-1032; discussion 1032-1023 (1995).
[38] Lee, J. M. *et al.* Comparison of immunomodulatory effects of placenta mesenchymal stem cells with bone marrow and adipose mesenchymal stem cells. *International Immunopharmacology* **13**, 219-224, doi:10.1016/j.intimp.2012.03.024 (2012).
[39] Beaver, B. *Canine behavior: A guide for veterinarians*. (WB Saunders, 1999).