CNS DISEASE DIMINISHES THE THERAPEUTIC FUNCTIONALITY OF MESENCHYMAL STEM CELLS

BY

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CNS Disease Diminishes the Therapeutic Functionality of

Mesenchymal Stem Cells

Abstract

By

ALEX GREGORY SARGENT

Mesenchymal stem cells (MSCs) have recently emerged as a potentially powerful cellular therapy for both inflammatory and neurological diseases such as multiple sclerosis (MS). Studies in animal models of MS, particularly experimental autoimmune encephalomyelitis (EAE), have demonstrated a remarkable ability of systemically delivered MSCs to suppress inflammation and improve neurological and functional recovery. Based on these preclinical studies, over twenty clinical trials have been initiated to evaluate the potential of MSCs to treat MS. Most of these trials use autologous MSCs (from the MS patients), in contrast to previous work done in the EAE model that used MSCs from healthy animals or donors. This raises a serious and unresolved issue about whether autologous MSCs are equivalent to healthy MSCs in their potential to treat diseases like MS.

To determine if and how inflammatory diseases like MS affect MSC functionality, we cultured MSCs from the bone marrow of MOG_{35-55} induced EAE mice at different phases of disease and compared them to naïve MSCs derived from healthy donors in their therapeutic potential. We found that EAE-MSCs have less therapeutic efficacy compared to naïve MSCs in their ability to ameliorate EAE. The

lack of therapeutic efficacy in EAE-MSCs reflected changes in their secretion of paracrine inflammatory cytokines, which EAE-MSCs up regulate at both the protein and gene level. Co-culture studies showed that conditioned medium from EAE-MSCs has differential effects on immune cell activation and neural cell development compared to naïve MSCs. Furthermore, we found that bone marrow MSCs derived from MS patients show similar changes to EAE-MSCs and also lack therapeutic efficacy in treating EAE. Collectively, our data shows that disease diminishes the therapeutic functionality of MSCs, raising concern about the continued use of autologous MSCs as a potential therapy for MS.

Chapter I:

General Introduction

1.1: Mesenchymal stem cells (MSCs)

The term "mesenchymal stem cell" was proposed by Arnold Caplan in 1991 to describe a unique class of bone marrow stem cell biologically distinct from the hematopoietic stem cells (HSCs) that also reside in this niche[1]. Mesenchymal stem cells (MSCs) are unlike HSCs in that they did not differentiate into red or white blood cells, but rather into other types of connective tissue such as bone, fat, and cartilage[2]. Since their initial isolation and characterization from human and murine bone marrow samples over twenty-five years ago, other terms of have been proposed to describe this specialized stem cell, including "mesenchymal stromal cell" and "multipotent stem cell," but "mesenchymal stem cell" remains the common terminology in the published literature[3]. MSCs are defined by a specific set of functional criteria established by a consensus statement from the International Society for Stem Cell Research (ISSCR)[4]. These criteria apply to MSCs isolated from different tissues and across different species, including humans (*Homo sapiens*) and research animals such as mice (*Mus musculus*) and rats (*Rattus novicus*).

Defining and culturing MSCs

One defining characteristic of MSCs provides the basis for their isolation and purification from other cell types. MSCs are strongly plastic adherent, and must adhere to common plastic surfaces (such as polystyrene) in-vitro to be classified as MSCs[4]. The fact that MSCs readily adhere to plastic whereas other bone marrow cells (particularly HSCs) do not is commonly exploited as a means of isolating MSCs from other cell populations[5].

Currently plastic adherence is one of the most common methods for selecting and purifying MSCs from the bone marrow[6]. When primary cultures of bone marrow cells are seeded into plastic culture dishes, MSCs will strongly adhere to the bottom of the dish, whereas HSCs and other bone marrow cells remain in suspension or only weakly attach and can be removed with successive washes. Adherent MSCs generally have a spindle-shaped, fibroblast-like morphology, and grow in small colonies on the bottom of the dish until reaching confluency as an adherent monolayer of cells[7]. The adherent properties of MSCs can vary considerably between species. For example, human and rat MSCs usually adhere much more strongly and are easier to purify than mouse MSCs, but over one or two passages and subsequent washings a purified population of murine MSCs can be readily obtained[8,9].

The second criterion that defines MSCs is their expression or lack thereof of specific cellular markers. MSCs are a non-hematopoietic type of stem cell thought to comprise a distinct lineage from HSCs[2,10]. Thus when analyzed via flow cytometry or immunocytochemistry, MSCs must lack expression of HSC lineage markers, including CD11b, CD34, and CD45[4,11]. Furthermore, MSCs should express certain stem cell or mesenchyme tissue markers, including but not limited to: STRO-1, Sca-1, CD105, CD90, and CD44[12]. It is important to note that lack of expression of any one of these markers does not exclude a cell from being an MSC. Expression of each of these markers can vary in MSCs depending on culture conditions or, in the case of murine MSCs, what strain of animal the MSCs are derived from[12,13]. Currently there is no reliable or specific cellular marker for MSCs[12]. Instead MSCs are identified by

their lack of expression of specific HSC markers, and by their common expression of a panel of non-specific mesenchyme markers[13].

The third defining characteristic of MSCs is their ability to differentiate into different types of stromal cells and tissue, including osteocytes (bone), adipocytes (fat), and chondrocytes (cartilage)[4]. MSCs readily differentiate into each of these cell types in-vitro under suitable culture conditions[14]. It's unclear though whether MSCs give rise to these cell types in-vivo during tissue development or repair, as there is currently no specific cellular marker that can be used for fate mapping MSCs[14,15]. Although MSCs must be able to differentiate into the above mentioned stromal cell types to be classified as MSCs, they also have the potential to give rise to other cell types in-vitro[16]. For example, MSCs can differentiate into other connective tissue cells under appropriate culture conditions, including tendinocytes and myocytes[3,17]. MSCs can also differentiate in-vitro into vasculature cells such as endothelial cells and pericytes[18,19]. It has been proposed that pericytes may even represent a specialized subpopulation of MSCs[20,21], as these two cell types share many similarities in-vitro, although direct in-vivo evidence for this is lacking[22].

Cultured MSCs can also give rise to different types of neural cells. Several independent laboratories have shown MSCs can differentiate into glial cells and functional neurons under specific culture conditions[23-25]. It is not known whether MSCs directly differentiate into glial cells and/or neurons, or first de-differentiate into a more primitive, multipotent cell type that can then give rise to neural cells[23]. Thus it is unclear whether MSCs have an intrinsic capacity to give rise to neural

cells, or if their differentiation into neural cells is a consequence of special in-vitro manipulations[26].

Sources of MSCs

Although first isolated from the bone marrow, MSCs can be cultured from other tissues and organs. In particular, MSCs can be derived and cultured in large numbers from tissues including adipose (fat), skeletal muscle, dental pulp, the umbilical cord, and the placenta[27]. Although these MSCs share the defining characteristics of bone marrow MSCs (BM-MSCs) and are commonly isolated by their plastic adherent properties[27], studies comparing BM-MSCs to MSCs derived adipose tissue, dental pulp, and umbilical cord suggests there are key differences between these cell types in gene expression and cellular characteristics such as their proliferative capacity[28-30]. It is not clearly understood how MSCs from other sources may differ from each other on a functional level, particularly in regards to which type of MSCs may be best suited for therapeutic applications[31].

MSCs can be isolated in much smaller numbers from most other postnatal organs and tissues, including the heart, lungs, liver, kidney, blood, brain, and spinal cord[32]. While low numbers of MSCs can be cultured from each of these tissues, it is unclear if MSCs actually reside in any of these tissues in-vivo[15]. MSCs derived from these tissues may in fact represent de-differentiated stromal cells[20]. For example, MSCs derived from the CNS and peripheral organs may actually be pericytes that have de-differentiated back into MSCs in-vitro.

It is also unclear if MSCs derived from different tissues have different development origins. MSCs arise from at least two different primary germ layers: the ectoderm and mesoderm, however it is not known if these different populations of MSCs come to reside in different tissues or serve different functions[33-35]. Because MSCs are only defined by broad characteristics in-vitro, it is possible that MSCs comprise a heterogeneous population of stem cells indistinguishable from each other by current criteria [36]. Due to the lack of specific cellular markers for MSCs, our understanding of the origin, fate, and functionality of MSCs in-vivo remains largely incomplete[35].

MSCs as a cellular therapy for disease

Although the biology of endogenous MSCs is still not clearly understood, MSCs have moved rapidly in recent years from the laboratory to the clinic as a novel therapy for disease. Mesenchymal stem cells possess unique properties that make them well suited as a potential cellular therapy. MSCs are relatively easy to obtain, purify, and grow in large numbers from sources like the bone marrow from human donors[37]. Also, MSCs express relatively low levels of major histocompatibility antigens (MHC) and are hypo-immunogenic[38]; when MSCs are transplanted into a host they have a very low risk of rejection compared to other cell types[37]. To minimize the risk of rejection even further, autologous MSCs can be derived from sources such as the bone marrow, expanded in-vitro, and transplanted back into the donor for therapeutic purposes.

Perhaps most importantly, MSCs have innate immunomodulatory and pro-regenerative properties that make them a potentially powerful cellular therapy for disease. MSCs exhibit strong immunosuppressive functionality both in-vitro and in-vivo when transplanted into different inflammatory disease settings[39]. Although the precise mechanisms by which MSCs influence immune cells are still incompletely understood, it is becoming increasingly clear that MSCs possess an intrinsic capacity to modulate the immune response at the cellular and molecular level.

1.2: Immunomodulatory properties of MSCs

How MSCs regulate immune cells

Multiple in-vitro studies have demonstrated the ability of MSCs to directly regulate the activation and function of different types of immune cells. For example, MSCs suppress the proliferation and activation of T-cells stimulated by allogeneic cells (mixed lymphocyte cultures) or various mitogens and antigens[40-42]. MSCs also affect T-cell survival, inducing apoptosis of activated T-cells and enhancing the survival of immature or naïve T-cells in-vitro [43,44]. In addition, MSCs can also bias T-cell polarization, as they inhibit the differentiation of naïve T-cells into pro-inflammatory Th1 or Th17 cells and promote their differentiation into anti-inflammatory Th2 or regulatory T-cells[45,46].

MSCs similarly affect the activation and maturation of other types of adaptive immune cells, including B-cells, dendritic cells, and macrophages. MSCs suppress B-cell proliferation, IgG antibody secretion, and B-cell differentiation into mature plasma cells[47,48]. MSCs also inhibit the maturation of dendritic cells in-vitro and

induce mature dendritic cells into a regulatory phenotype with lowered immunogenicity[49,50]. Mesenchymal stem cells influence monocyte proliferation and macrophage polarization, biasing macrophages away from a pro-inflammatory (M1) phenotype and towards a more anti-inflammatory (M2) phenotype[51,52].

The immunomodulatory capacity of MSCs is derived in large part from their secretion of a spectrum of anti-inflammatory cytokines, chemokines, and growth factors that act on a diverse array of cellular targets[53,54]. Proteomic analysis of conditioned medium (CM) from cultured MSCs shows that they secrete hundreds to thousands of different soluble factors[55,56]. This has led to MSCs being described as cellular "drug stores", with the prevailing concept emerging that the immunosuppressive and therapeutic potential of MSCs stems from the multitude of factors they secrete[35,57,58].

Evidence that secreted factors mediate MSC immunomodulation comes from studies showing conditioned medium (CM) from MSCs inhibits the activation of T-cells, B-cells, and macrophages[45,48,59]. To date, multiple paracrine factors have been implicated in mediating the immunosuppressive effects of MSCs. For example, MSCs secrete prototypical anti-inflammatory cytokines including transforming growth factor beta 1 (TGFβ1), interleukin 4 (IL-4), and interleukin 10 (IL-10)[60]. TGFβ1 partially mediates the inhibitory effects of MSCs and MSC CM on T-cell proliferation in-vitro[61,62], whereas IL-10 mediates MSC-induced differentiation of naïve T-cells into Th2 and T-regulatory cells[45]. Another important effector molecule in MSC immunomodulation is prostaglandin E2 (PGE2). MSC-secreted PGE2 inhibits T-cell proliferation in-vitro[62,63], and PGE2 also inhibits the differentiation of naïve T-cells

into pro-inflammatory Th1 or Th17 cells[64]. Furthermore, PGE2 mediates MSC-induced inhibition of dendritic cells[65], and plays a role in MSC-induced polarization of macrophages into an anti-inflammatory (M2) phenotype[59].

Other secreted factors, including indoleamine 2,3 dioxygenase (IDO), human leukocyte antigen G (HLA-G), tumor necrosis factor inducible gene protein 6 (TSG6), interleukin 1 (IL-1) receptor antagonist and an antagonistic variant of CCL2 have also been associated with the immunosuppressive functionality of MSCs[66-70]. It is important to note though that all of these factors currently identified as having a role in MSC immunomodulation represent only a small fraction of the hundreds to thousands of proteins MSCs secrete. Thus a complete understanding of the molecules and mechanisms mediating MSC immunomodulation is lacking [71]. Nevertheless, MSCs consistently display strong immunosuppressive properties in-vitro, providing a rationale for their use as a cellular therapy to treat chronic inflammation in-vivo.

MSCs as a therapy for inflammatory disease

Many of the insights into the immunosuppressive functionality of MSCs have come from analyses of their ability to modulate inflammatory disease. MSCs are currently being evaluated in clinical trials as a cellular therapy for a number of different inflammatory conditions, including graft versus host disease, Crohn's disease, rheumatoid arthritis, diabetes mellitus (type 1 and 2), lupus, and multiple sclerosis[72,73]. While BM-MSCs are currently more widely used in clinical trials and studied more in preclinical animal models, it should be noted that MSCs derived

from other tissues have similar anti-inflammatory functionality when transplanted-vivo[39,74]. To illustrate the immunosuppressive and therapeutic functions of transplanted MSCs, their use in two different inflammatory conditions, graft versus host disease and rheumatoid arthritis, will be briefly discussed.

MSCs in the treatment of graft versus host disease

Acute graft versus host disease (GVHD) is an inflammatory condition that follows allogeneic tissue transplantation. GVHD is most commonly associated with allogeneic bone marrow transplantation, especially hematopoietic stem cell transplantation (HSCT), but it can also occur following blood transfusion and solid organ transplantation[75]. Because allogeneic bone marrow transplantation is widely used to treat malignant cancers such as leukemia, lymphoma, and multiple myeloma, there is a crucial need for therapies that reduce or eliminate the risk of GVHD[75]. While HLA matching between donors and transplant patients and the use of immunosuppressant drugs can help reduce the risk of GVHD, the disease remains a common complication following allogeneic bone marrow transplantation, with an incidence rate of between 30%-50% and a mortality rate between 20%-40%[76].

GVHD is an immune-mediated disease in which the transplanted tissue graft rejects the host[77]. GVHD is triggered by the activation of immune cells present in the transplant graft, especially donor T-cells which up-regulate pro-inflammatory cytokines and mediate destruction of host tissues[78]. Both donor and host antigen presenting cells (APCs) recognize and present host antigens, including HLA proteins (MHC proteins in mice) to donor T-cells, thereby stimulating their proliferation and

differentiation into pro-inflammatory subtypes[79]. While HLA antigens are thought to be a crucial trigger, GVHD can also occur in HLA-identical donors, suggesting other antigens play a role in disease etiology[80].

GVHD is one of the first diseases in which MSCs were tested in clinical trials[81]. Multiple clinical trials have reported that transplantation of allogeneic bone marrow MSCs into patients with acute GVHD improves disease symptoms and increases patient survival[82-85]. MSC transplantation into patients with GVHD is associated with immunosuppressive effects, such as decreased levels of activated lymphocytes and pro-inflammatory cytokines in blood serum [85].

Insight into how MSCs mediate recovery in GVHD has come from studies of MSC transplantation into animal models of the disease. In mice or rats with acute GVHD, systemic infusion of human bone marrow MSCs attenuates disease severity and reduces blood serum levels of pro-inflammatory cytokines[86-88]. MSCs are observed in peripheral immune organs such as the spleen up to 5 days post-transplantation, with MSC-treated mice having lower numbers of activated T-cells in their spleen and lymph nodes[87,88]. Proliferation of T-cells derived from GVHD animals is inhibited by co-culture with MSCs or MSC CM[87,88]. Inhibitors to PGE2 reduce this effect, suggesting a role for soluble PGE2 in mediating MSC immunosuppression in GVHD[88].

MSCs in the treatment of rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects the joints[89]. This inflammatory response results in tissue damage and secondary

progressive inflammation that result in damage or loss to different tissues including cartilage and bone[89]. The stimulus that initiates the immune attack in RA is not well defined and may be antigenically restricted, however there is evidence that as the disease progresses the spectrum of immunogenic epitopes spreads or increases and the exposure to additional epitopes contributes to ongoing disease[90]. While multiple cell types including neutrophils, macrophages, and fibroblast-like synoviocytes (FLS) contribute to tissue damage, several lines of evidence suggest that T-cells are the major effectors of disease in RA[90]. Large numbers of T-cells, of which the majority have a Th1 phenotype, are found in inflamed joints, and are recruited through the local expression of an array of cytokines and chemokines[91]. The infiltrating T-cells are thought to stimulate other immune cells including mononuclear cells, macrophages and FLS that contribute to further tissue damage.

Multiple pro-inflammatory cytokines are elevated in RA[92], including tumor necrosis factor alpha (TNFα), which plays a major role in disease pathogenesis[93]. Indeed, anti-TNFα treatments have demonstrated significant benefit in reducing inflammation in RA patients and neutralizing TNFα with humanized antibodies reduces disease progression[94,95].

Rheumatoid arthritis represents an interesting target for the application of MSCs. MSCs are highly immunomodulatory, and can inhibit the proliferation and activation of a variety of immune cells including T-cells, which are thought to be major drivers of tissue damage and disease pathology in RA[90]. Several studies have shown that MSCs can modulate T- cells in animal models of RA and can improve clinical recovery[96-98]. Systemically transplanting MSCs into rodents with the

collagen-induced arthritic (CIA) model of RA leads to a decrease in the number of circulating Th1-polarized T-cells and an increase in the number of Th2-polarized regulatory T-cells[96-98]. This modulation of T-cells by MSCs in CIA mice is paralleled by improvements in tissue integrity and clinical recovery[97,98]. How MSCs might influence other types of immune cells such as macrophages or FLS in CIA is unclear, but these studies demonstrate that MSCs can improve recovery in animal models of RA by apparently modulating T-cells and the immune response.

Not only are MSCs immunoregulatory and thus able to influence the pathogenic process in RA, but they may also be capable of differentiating into cells of the tissues damaged by the disease (such as bone and cartilage) and could thereby directly contribute to tissue regeneration. As a result, several studies have attempted to deliver MSCs directly into the damaged area with the hope that they will directly facilitate repair. It seems likely that in order for such an approach to be effective the transplanted MSCs have to be retained in the location of the inflamed joint. One approach that has been effective in animal models is to use a scaffold composed of biodegradable nanofibers to localize MSCs[99,100]. Such an approach results in greater retention of the cells in the target joint and reduction in the inflammatory response, but evidence for a direct contribution of the transplanted MSCs or their progeny in tissue repair is still lacking[100].

1.3: Regenerative Properties of MSCs

The trophic potential of MSCs

Beyond their immunosuppressive properties, MSCs also have the capacity to promote cell survival and tissue regeneration. The regenerative potential of MSCs seems to be derived from their ability to secrete a large number of different trophic molecules and growth factors[57]. These include pro-angiogenic factors such as endothelial growth factor (EGF) and vascular endothelial growth factor (VEGF)[101], as well as factors that promote cell survival and differentiation, including fibroblast growth factors (FGFs), Insulin-like growth factor 1 (IGF-1) and stromal cell-derived factor 1 (SDF1) [102-104].

By secreting a diverse array of growth factors and trophic molecules, MSCs can influence cell survival and regeneration in many different tissues[57]. Notably, MSCs are a potential cellular therapy to augment neuroprotection and nerve regeneration in the CNS after injury or disease[105]. MSCs secrete a variety of neurotrophic factors including brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), and ciliary neurotrophic factor (CNTF) [106-108]. These in turn can act with other growth factors secreted by MSCs to influence the proliferation, fate, and survival of both neurons and glial cells.

How MSCs regulate neural cells

Multiple in-vitro studies have shown that MSCs have strong neuroprotective properties. For example, MSCs and MSC CM enhances neuron survival and neurite outgrowth in human neural cell lines and primary cultures of rodent neural cells[109-

[112]. MSCs and MSC CM also enhances the survival of neural stem cells (NSCs) and their differentiation into neurons in-vitro[113,114], and MSC CM enhances axon growth in cultured neurons[115]. Multiple factors have been implicated in contributing to the neuromodulatory effects of MSCs in-vitro, including BDNF, NGF, IGF1 and CNTF[106,110,115].

MSCs can also influence the development and maturation of glial cells. For example, MSC CM biases NSCs away from astroglial fate and promotes their differentiation into oligodendrocytes and/or neurons[116,117]. MSC CM also enhances the differentiation of oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes in-vitro[118], and MSCs promote oligodendrocyte differentiation and myelination in hippocampal slice cultures[119]. Hepatocyte growth factor (HGF) appears to be an important mediator of MSC induced oligodendrogenesis[114], although it is likely other secreted factors contribute to the ability of MSCs to modulate glial cells[120].

MSCs as a cellular therapy for neurodegenerative disease

Based on their demonstrated safety and success in treating inflammatory diseases and their capacity to modulate neural cells in-vitro, MSCs have rapidly emerged as a possible cellular therapy for neurodegenerative disease[121]. Currently MSCs are in clinical trials as a potential therapy for neurological conditions such as stroke, spinal cord injury, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Parkinson's disease, Huntington's disease, and multiple sclerosis[105,122]. To illustrate the therapeutic function of MSCs and how they might modulate neural cells when

transplanted in-vivo, their use as a treatment for spinal cord injury (SCI) will be briefly discussed.

MSCs in the treatment of spinal cord injury (SCI)

Multiple studies have reported MSC transplantation into mice or rats with a spinal cord injury (SCI) improves functional and neurological recovery[123-127]. Most of these studies directly injected bone marrow MSCs (human or rat) into the lesion cavity or cerebrospinal fluid (CSF) of animals within 1 week after SCI[123-125], although some studies report functional improvements in SCI animals receiving intravenous injections of MSCs[126,127]. MSCs directly injected into the CSF or lesion cavity migrate into the lesion penumbra and other surrounding spinal cord tissue shortly after transplantation[123,124]. Cell tracking studies show MSCs are present in the spinal cord weeks after transplantation, although the number of cells in the CNS parenchyma decreases rapidly over time[123,124]. These MSCs do not appear to differentiate into neural cells, as evident by their lack of expression of specific neuron or glial cell markers[123,124,127].

The therapeutic benefit of transplanted MSCs in SCI appears to be derived from the immunosuppressive and potentially neuroprotective properties of MSCs[128]. After SCI, axonal dieback and degeneration is mediated by microglia and pro-inflammatory macrophages[129]. Intravenous infusion of MSCs and/or direct injection into the CNS after SCI reduces the number of activated microglia and macrophages in the lesion cavity and adjacent spinal cord[125,127]. MSCs also seem to modulate the peripheral immune response in SCI, as intravenous infusion of MSCs

reduces levels of pro-inflammatory cytokines in blood serum and the number of proliferating leukocytes in the spleen of SCI animals[126,127].

Transplanted MSCs also appear to have a neuroprotective effect in SCI. There are more axons passing through the lesion in SCI animals treated with MSCs, and a decrease in the number of apoptotic neurons and oligodendrocytes near the lesion site[123,124,130]. It is difficult to assess whether such improvement in neuropathology are a consequence of MSCs directly enhancing neural repair after SCI or an indirect effect of MSCs suppressing inflammation that would otherwise damage axons and neurons[128]. Several lines of evidence suggest transplanted MSCs may directly contribute to neuron survival and axon regeneration after SCI. First, levels of neurotrophins secreted by MSC, including BDNF and NGF, are significantly elevated near the lesion in SCI animals treated with MSCs[126,131]. Second, MSCs and MSC CM significantly improved neuronal survival and axon outgrowth in dorsal root ganglion explant cultures derived from SCI animals [123].

Distinguishing between the anti-inflammatory versus neuroprotective effects of MSCs transplanted in-vivo is a common challenge in evaluating how MSCs mediate recovery in neurodegenerative injury or disease[121]. Although the precise cellular and molecular mechanisms are not well defined, studies of MSCs in different inflammatory diseases and neurological conditions have revealed that these cells possess considerable therapeutic potential. The immunosuppressive and pro-regenerative functionality of MSCs makes them a particularly attractive cellular therapy for diseases with both inflammatory and neurodegenerative components, such as multiple sclerosis.

1.4: MSCs as a treatment for multiple sclerosis

Overview: pathology of multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune disease of the CNS and is a leading cause of neurological disability in young adults[132]. An estimated 2.5 million people suffer from MS worldwide, with the average age of symptom onset being between 25-35 years of age[133].

Symptoms of MS can be relatively mild, such as numbness or muscle weakness, or severe, including spasticity, paralysis, and vision loss[133]. Approximately 80%-90% of patients with MS begin with a relapsing remitting course of the disease (relapsing-remitting MS)[134]. Relapses in the disease are characterized by active inflammation in CNS white matter and the formation of multiple sclerotic lesions or plaques[135]. The CNS lesions in MS are characterized by a loss of oligodendrocytes and their myelin sheaths[136]. Since myelination is required for the rapid and efficient conduction of information along axons, its loss results in impaired conduction in the affected axons, leading to functional deficits in MS patients[136]. The location of the demyelinated lesions in the CNS correlates with the symptoms in MS. For example, lesions in the optic nerve or optic tract result in loss of visual acuity, while damage to the spinal cord white matter results in muscle weakness or paralysis[137].

In relapsing-remitting MS (RRMS), periods of inflammatory demyelination (relapse) appear to be followed by stages of limited remyelination and recovery (remittance), where adult OPCs differentiate into new oligodendrocytes to replace those lost to disease[138,139]. Unfortunately, remyelination in MS is often insufficient, and over subsequent relapses and progression of the disease cannot compensate for

continual loss of oligodendrocytes and myelin[140,141]. It is also clear that there is a chronic neurodegenerative component to MS, as CNS inflammation and demyelination seems to drive progressive axonal degeneration and neuronal death during disease[142].

Although the pathology of MS is relatively well characterized, its underlying cause is unknown[134]. Current therapies for MS broadly target immune system activation with the aim of reducing CNS inflammation[143]. Although these can reduce the number of relapses and slow the progression of MS, they fail to treat the underlying cause or neurological symptoms of the disease. Even when treated with current immunosuppressive therapies, most RRMS patients eventually progress to a secondary progressive phase of disease that fails to respond to treatment[134,143]. There is also an unmet need for neuroprotective therapies in MS that can enhance remyelination and improve functional recovery in patients[141,142].

In order to better treat MS, new therapies are needed that provide long term immunosuppression and/or enhance myelin repair and axonal survival. In this regard, MSCs represent an attractive cellular therapy for the treatment MS. Studies in animal models of MS have helped to provide a rationale for using MSCs as potential cellular therapy to treat inflammation and demyelination in MS patients.

MSCs in animal models of MS: experimental autoimmune encephalomyelitis (EAE)

The most commonly used animal model of MS is experimental autoimmune encephalomyelitis (EAE)[144]. In appropriate strains of mice, immunization with specific peptides of myelin proteins results in a CNS directed inflammatory

response. For example, immunization of C57/BL6 mice with a peptide of myelin-oligodendrocyte glycoprotein (MOG peptide containing amino acids 35-55) results in an inflammatory demyelinating disease with a chronic course, whereas immunization of SJL mice with a peptide of proteolipid protein 1 (PLP1 peptide containing peptide of amino acids 139-151) results in inflammatory demyelinating disease with a relapsing-remitting course[145]. The demyelination in both models is a result of infiltration of T-cells targeted against myelin and oligodendrocytes[145]. The spinal cord is severely affected in these animals and they develop functional motor deficits that can be easily scored.

Multiple studies have demonstrated that intravenous infusion of MSCs into chronic MOG_{35-55} or relapsing-remitting $PLP_{139-151}$ EAE mice prior to disease onset blocks disease development and MSC infusion at the peak of disease significantly reduces disease severity[117,146-149]. These findings have been replicated by several independent laboratories and provide compelling evidence that MSC transplantation can effectively modulate disease progression in EAE. However the underlying mechanisms that contribute to the MSC-mediated reduction in disease burden are not clearly defined.

One general finding is that MSC treatment modulates the immune response in EAE[150]. EAE mice treated with MSCs have significantly lower numbers of peripheral immune cells and T-cells in their spinal cord, a reduction in the number of activated T-cells in their lymph nodes, and reduced expression of pro-inflammatory cytokines in their blood serum[117,146,148]. Infusion of MSCs into EAE mice seems to bias the immune response from a predominantly pro-inflammatory

TH1 profile to a more anti-inflammatory TH2 profile[117,148]. The ability of MSCs to modulate disease progression in EAE also appears to be a general phenomenon that crosses species. For example, when human bone marrow derived MSCs are injected intravenously into mice with EAE there is rapid improvement in functional recovery and a reduction in CNS inflammation that is sustained for an extended period[117,149].

MSCs also attenuate myelin loss and improve neuropathology in EAE mice. EAE mice treated with MSCs have less demyelination and higher overall numbers of oligodendrocytes in their spinal cords[146,147,149]. Infusion of MSCs into EAE mice also reduces axonal loss and increases the number of myelinated axons[117,147]. It is unclear however if this improvement in neuropathology is because MSCs directly enhance remyelination and/or axonal survival in EAE or if it is an indirect effect of MSCs suppressing the immune system and CNS inflammation[151].

Much of the uncertainty about whether MSCs are directly neuroprotective in EAE comes from conflicting reports about whether intravenously transplanted MSCs cross the blood brain barrier and migrate into the CNS. Most studies agree that large numbers of MSCs migrate into the spleen, lymph nodes, and lungs after intravenous infusion into EAE mice, although there are differing reports about how long MSC engraft in these tissues[146,148,152]. Some studies report that a much smaller number of infused MSCs (labeled with human specific antibodies or fluorescent dyes) cross the blood brain barrier and engraft into the spinal cords of EAE mice near demyelinated lesions[117,152]. However, other studies using MSCs virally transduced with reporter genes report no MSCs are found in the CNS parenchyma of EAE mice up to one-month post infusion[146,148,153]. The different outcomes in these cell-tracking studies

of MSCs infused into EAE mice can be attributed to many factors. For example, differences in how MSCs are cultured, the strategy used to label MSCs, differences in the number of MSCs given, and differences in the induction and severity of EAE (which can affect blood brain permeability) could all potentially influence where MSCs engraft into EAE mice.

MSCs in other animal models of MS: the cuprizone model of demyelination

While it is difficult to distinguish between the immunosuppressive and neuroprotective functions of MSCs in EAE, studies in other animal models of demyelination suggest MSCs are capable of directly enhancing myelin repair. In the cuprizone mouse model, chemical demyelination is induced in adult mice by feeding them food supplemented with a copper-chelating agent (cuprizone) for several weeks[145]. In one study, intraventricular injection of bone marrow MSCs increased the number of oligodendrocytes and enhanced remyelination in the forebrain of chronically demyelinated cuprizone mice[154]. While very few transplanted MSCs (labeled with iron nanoparticles) migrated into the CNS parenchyma, MSCs were observed within and adjacent to the ventricles of cuprizone-fed mice up to three months post transplantation[154]. In another study where fluorescently labeled MSCs were intravenously infused into cuprizone-fed mice, MSCs were reported to cross the blood brain barrier and preferentially home to demyelinated lesions, where they subsequently increased remyelination and the number of oligodendrocytes while also decreasing the number of reactive astrocytes and macrophages[155]. These results parallel other studies reporting MSCs and MSC CM enhances oligodendrocyte

maturation in-vitro, and promotes developmental myelination in hippocampal slice cultures[118,119].

The ability of MSCs to enhance remyelination in the cuprizone mouse model and promote oligodendrogenesis in-vivo and in-vitro underscores the capacity of MSCs to influence neural cell fate and remyelination. Thus MSCs can promote recovery in EAE by modulating the peripheral immune response and possibly also by enhancing neural repair[156]. While the precise cellular interactions mediating this phenomenon are poorly defined in-vivo, recent studies have identified several secreted factors that contribute to the therapeutic effects of MSCs in EAE.

How MSCs mediate recovery in EAE

Many of the effects of MSCs in alleviating disease progression in EAE appear to be mediated by secreted factors rather than by cell-cell contact. For example, conditioned medium (CM) collected from bone marrow MSCs is sufficient to reduce disease burden when injected intravenously into MOG $_{35-55}$ EAE mice[114,157]. As with cell delivery, the effects of treatment with MSC CM are long lasting, suggesting there is a physiological change in the host as a consequence of exposure to MSC CM. MSC CM also inhibits MOG-induced restimulation of leukocytes cultured from EAE mice, and suppresses the proliferation of MOG-specific T-cells in-vitro[146,148]. MSC-CM can also influence the functionality of neural cells, as it promotes oligodendrocyte differentiation from NSCs and OPCs in-vitro[117,118].

To date, several secreted factors are directly associated with MSC-induced recovery in EAE. The first is an antagonistic variant of the monocyte

chemoattractant protein CCL2. MSC secretion of this CCL2 variant inhibits proliferation of MOG specific T-cells in-vitro[70]. Bone marrow MSCs derived from CCL2 null mice fail to secrete this antagonistic CCL2 variant, and have reduced therapeutic efficacy when transplanted into EAE mice[70]. Additionally, the anti-inflammatory cytokine prostaglandin e2 contributes to MSC-induced recovery in EAE. Inhibitors to PGE2 block MSC-mediated inhibition of Th1 cells in-vitro[64], and bone marrow MSCs treated with a PGE2 inhibitor prior to transplantation have less therapeutic efficacy in EAE mice[158].

Another important effector in MSC-induced recovery in EAE is hepatocyte growth factor (HGF). HGF is present at high levels in MSC CM, and antibodies to the HGF receptor (C-met) block functional recovery mediated by MSC CM in EAE mice[114]. Furthermore, intravenous delivery of HGF into EAE mice recapitulates the therapeutic benefits of infusing MSCs or MSC CM[114]. The precise cellular target of HGF in EAE remains unclear, although HGF has potent immunomodulatory and neuroprotective properties. For example, HGF inhibits T-cell proliferation and dendritic cell maturation in-vitro[159,160]. Overexpression of HGF in-vivo results in the induction of regulatory T-cells and tolerogenic dendritic cells that make the animals refractory to the induction of EAE[161]. HGF also increase differentiation of NSCs and OPCs into mature oligodendrocytes, and intravenous infusion of HGF enhances remyelination in rats whose spinal cords were lesioned with the demyelinating agent lysolecithin[114,118].

1.5: From bench to bedside: MSCs in clinical trials for MS

Preliminary results from clinical trials

Therapies like MSC infusion that could potentially target both a reduction in inflammation and an enhancement in myelin repair are an attractive candidate for the treatment of MS[162]. The application of MSCs to the clinical treatment of MS has moved rapidly in recent years and there are multiple ongoing clinical trials[162,163]. Most of these trials utilize bone marrow derived MSCs, however adipose derived MSCs are gaining prevalence in clinical applications for MS[163]. MSC transplantation is targeted towards progressive forms of MS (primary or secondary) in a majority of clinical trials, although several trials are looking at MSCs as a cellular therapy for RRMS[163]. While the outcomes of most of these trials are not yet complete, trends are emerging.

In general, MSC therapy appears safe and there have been few reports of adverse effects[164-168]. Although some trials have reported modest improvement in MS patients, compared to the outcomes seen in animal models, the efficacy of MSC treatment has been somewhat disappointing. For example, a pilot study where autologous BM-MSCs were intrathecally delivered into progressive MS patients reported an improvement in EDSS (Expanded Disability Status Scale) in only one patient[165]. By contrast, five patients showed a worsening in EDSS scores, and four patients showed no change[165]. Other pilot studies report only transient improvements in MS patients receiving MSCs. In two studies where autologous BM-MSCs were intrathecally injected into progressive MS patients, patients showed improvement in EDSS score and a reduction in the size of brain lesions (assessed by

MRI) at 3 months post treatment, but no significant improvement 6 months after MSC transplantation[166,168]. And in a study that measured visual acuity and axonal conductance in a small cohort of MS patients receiving autologous MSCs, a significant improvement was detected in patients several months after intravenous MSC infusion but no improvement was found one-year post treatment[164]. Although interpretations of these studies must be guarded as they measure clinical outcomes in a very small number of patients, overall they suggest current MSC treatment paradigms may be ineffective at significantly modulating disease progression in chronic MS patients.

Autologous versus allogeneic MSCs to treat MS

Several reasons may account for the apparent lack of efficacy of MSCs in the treatment of MS. For example, the route of delivery in human clinical trials (intrathecal versus intravenous delivery) likely influences the cellular targets and overall effectiveness of the transplanted MSCs. The capacity of MSCs to promote functional recovery in EAE animal models appears to reflect their ability to modulate the peripheral immune response [117,146,148], and intravenous delivery of MSCs (over intrathecal injections) may allow MSCs to better migrate to peripheral immune organs[162]. Other factors that could affect the efficacy of transplanted MSCs include the source of the cells, conditions of expansion, and dosage[162,163]. Another important issue is the host the MSCs are derived from. Previous studies evaluating MSCs in EAE animal models used "naïve" MSCs derived from healthy animals or human donors[117,146-149]. In contrast, the majority of clinical trials use autologous

MSCs (from the MS patient) in order to reduce the risk of adverse reactions or transplant rejection[162,163]. This raises concern that diseases like MS could alter MSCs and reduce their therapeutic potential.

While most clinical trials use autologous MSCs, there has been relatively little investigation into how MS might impact the functionality of MSCs. Two studies reported cultured bone marrow MSCs from MS patients (MS-MSCs) are similar to naïve MSCs in proliferation, differentiation, and expression of common MSC cell surface markers, although one these studies did report MS-MSCs secrete higher levels of the immunomodulatory cytokine CXCL10[169,170]. By contrast, another more recent study reported bone marrow MS-MSCs have decreased proliferative capacity and higher rates of cellular senescence in-vitro when compared to naïve MSCs[171]. Another study that compared gene expression profiles of bone marrow MS-MSCs to naïve MSCs reported MS-MSCs up-regulate many pro-inflammatory genes[172]. However, there has been no direct comparison of the therapeutic efficacy of MS-MSCs to naïve MSCs when each is transplanted into a disease model like EAE.

Studies analyzing MSCs derived from EAE mice (EAE-MSCs) report conflicting results about whether inflammatory disease alters MSCs. One study reported BM-MSCs from MOG-induced EAE mice are similar to naïve MSCs in proliferation and differentiation potential in-vitro, and are just as effective as naïve MSCs in ameliorating disease when infused into EAE mice[173]. Another more recent study that isolated BM-MSCs from MOG EAE mice later on in disease, when symptoms are more severe, found EAE-MSCs are different from naïve MSCs in terms of growth rate, differentiation potential, and expression levels of important histone-

modifying genes[174]. Yet this study did not compare EAE-MSCs to naïve MSCs in terms of their therapeutic efficacy.

1.6: Focus of Thesis

To determine if and how inflammatory diseases like MS affect MSC functionality, we cultured MSCs from the bone marrow of MOG_{35-55} induced EAE mice at different phases of disease and compared them to MSCs derived from healthy donors (naïve MSCs) in their therapeutic potential. We found that EAE-MSCs lack therapeutic efficacy compared to naïve MSCs in their ability to ameliorate EAE. Our data suggests this lack of therapeutic efficacy is due to differences in paracrine factors that EAE-MSCs secrete. EAE-MSCs up regulate a spectrum of pro-inflammatory cytokines at both the protein and gene level, and co-culture studies show that conditioned medium from EAE-MSCs differentially modulates immune cell activation and neural cell development. Similarly, bone marrow MSCs derived from MS patients up regulate secretion of some of the same pro-inflammatory cytokines and have less therapeutic efficacy than naïve MSCs in their ability to treat EAE. Collectively, our data shows that disease reduces the therapeutic functionality of MSCs, and raises new concerns about the efficacy of using autologous MSCs to treat MS.

Chapter II:

CNS disease diminishes the therapeutic functionality of bone marrow mesenchymal stem cells

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2.1: Abstract

Mesenchymal stem cells (MSCs) have emerged as a potentially powerful cellular therapy for autoimmune diseases including multiple sclerosis (MS). Based on their success in treating animal models of MS like experimental autoimmune encephalomyelitis (EAE), MSCs have moved rapidly into clinical trials for MS. The majority of these trials use autologous MSCs derived from MS patients, although it remains unclear how CNS disease may affect these cells. Here, we report that bone marrow MSCs derived from EAE mice lack therapeutic efficacy compared to naïve MSCs in their ability to ameliorate EAE. Treatment with conditioned medium from EAE-MSCs also fails to modulate EAE, and EAE-MSCs secrete higher levels of many pro-inflammatory cytokines compared to naïve MSCs. Similarly, MSCs derived from MS patients have less therapeutic efficacy than naïve MSCs in treating EAE and secrete higher levels of some of the same pro-inflammatory cytokines. Thus diseases like EAE and MS diminish the therapeutic functionality of bone marrow MSCs, prompting reevaluation about the ongoing use of autologous MSCs as a treatment for MS.

2.2: Introduction

Transplantation of mesenchymal stem cells (MSCs) has recently emerged as a promising new therapeutic approach for the treatment of both autoimmune and neurological diseases including multiple sclerosis (MS)[162]. MSCs are a multipotent, non-hematopoietic class of stem cell that can be isolated from a variety of tissues, including the bone marrow[2]. MSCs possess strong immunomodulatory and

regenerative properties that are derived from their ability to secrete a wide array of diverse chemokines, cytokines, and trophic factors[57,175], and have been shown to be effective in modulating disease progression in a number of different conditions, including graft vs host disease and rheumatoid arthritis [176,177].

Based on their immunomodulatory properties and success in treating other diseases, bone marrow-derived MSCs (BM-MSCs) were seen as a strong candidate for a cell-based therapy for MS[156], and have been tested in a number of animal models of MS, including experimental autoimmune encephalomyelitis (EAE). Several independent laboratories have shown that when transplanted systemically into mice with ongoing EAE, BM-MSCs rapidly halt disease progression and improve recovery [117,146,149]. BM-MSCs appear to mediate recovery in EAE not by replacing cells lost to disease but rather by suppressing the immune response and inhibiting CNS inflammation while also promoting remyelination and neural repair [178]. At the cellular level, transplanted BM-MSCs are thought to modulate disease progression by secreting multiple factors that inhibit inflammation and/or promote tissue repair [150], including immunomodulatory cytokines like prostaglandin e2 and TGFB and trophic factors like hepatocyte growth factor (HGF) [114,158,179].

Due to their success in animal models of MS, BM-MSCs have moved into clinical trials in MS patients. The majority of these trials utilize autologous MSCs (derived from MS patients) [180], in contrast to previous work in animal models that utilized BM-MSCs from healthy animal or human donors. While preliminary clinical trials using autologous MSCs report good safety data most report limited therapeutic efficacy [164-167]. One possible explanation for the limited effects of

autologous BM-MSCs in clinical trials is that autoimmune diseases like MS alter the functionality of MSCs and reduce their therapeutic potential.

In order to determine if inflammatory diseases like MS might alter MSC functionality, we cultured MSCs from the bone marrow of MOG_{35-55} induced EAE mice at different phases of the disease and compared them to naïve MSCs in terms of their therapeutic efficacy. We found that EAE-MSCs fail to improve recovery when transplanted into EAE mice, in contrast to the strong therapeutic effect observed following transplantation of naïve MSCs. Our data suggests this lack of therapeutic functionality of EAE-MSCs stems from differences in the paracrine factors they secrete relative to naïve MSCs, as EAE-MSCs secrete higher levels of many pro-inflammatory cytokines, including IL-6 and CCL2. Similarly, we found that MSCs derived from patients with MS (MS-MSCs) also lack therapeutic efficacy in treating EAE and secrete higher levels of the same pro-inflammatory cytokines. Our results show that diseases like EAE and MS diminish the therapeutic functionality of BM-MSCs, raising concern about the continued use of autologous BM-MSCs in the treatment of MS.

2.3: Methods

EAE induction and scoring

EAE was induced in 10-12 week-old female C57BL/6 mice (Jackson Laboratory: 000664) using Hooke Labs MOG_{35-55} EAE Induction kits according to the manufacturer's protocol. Briefly, mice were immunized via subcutaneous injection of 200ul of MOG_{35-55} peptide in complete Freund's adjuvant. Pertussis toxin (250 ng)

was injected intraperitoneally at 2 and 24 hours post immunization. Animals began showing signs of paralysis 9-11 days post immunization, and were graded by blinded observers according to a previously described clinical index [114]: 1 = limp tail, 2 = hind limb weakness, 3 = plegia of one limb, 4 = plegia of two limbs, 5 = moribund or dead.

MSC culture and treatment protocols

Mouse MSC isolation and culture: Mouse mesenchymal stem cells were isolated and cultured from the bone marrow of MOG_{35-55} – induced EAE mice at either 14 days (peak EAE-MSCs) or 28 days (chronic EAE-MSCs) after immunization, with the animals having a clinical score of 4 or higher. Naïve MSCs were cultured from non-immunized, age-matched C57BL/6 mice. Each culture preparation consisted of MSCs derived from 4-6 mice, and a different culture preparation was used for each experiment. Growth medium for all mouse cultures consisted of α-MEM with GLUTAMAX (Gibco) supplemented with 10% MSC-qualified fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco).

MSCs were isolated according to a previously published protocol [9]. Briefly, bone marrow from the tibias and fibulas was collected by flushing out each bone's central canal with a 26 x g syringe containing fresh growth medium. Bone marrow cells were then seeded in P75 flasks (Corning) at a concentration of 2 X 10^5 cells/cm^2, with cells grown in a 37°C with 5% CO2. Flasks were washed twice with media 48 hours later to remove non-adherent cells, with medium changed every 2-3 days. Cells were then passaged using 0.25% Trypsin/EDTA (Gibco) for 2 minutes at

37°C and re-plated in P75 flasks at a concentration of 1 x 10^4 cells/cm^2. Cells derived from both naïve and EAE mice were identified through immunolabeling as a purified population of MSCs (Figure 5), and were subsequently expanded with cells from passages 2-4 (21-30 days in-vitro) used for all experiments. For cell transplantation experiments, 0.8 x 10^6 MSCs in saline were delivered intravenously into EAE mice via tail vein injection at 15 days post immunization. In experiments where MSCs were first labeled with CMTPX (ThermoFisher), cells were incubated with 20 uM dye for 2 hours immediately prior to infusion according to the manufacturer's protocol.

Human MSC isolation and culture: For functional studies, bone marrow aspirates were obtained from the iliac crest of patients at the Southwest Hospital of the Third Military Medical University. For molecular studies, marrow aspirates were obtained from the posterior superior iliac crest of patients at University Hospitals Case Medical Center. MS-MSCs were obtained from bone marrow aspirates derived from 3 separate female donors diagnosed with relapsing-remitting MS, while naïve MSCs were obtained from bone marrow aspirates from female age-matched donors**.** Both MS-MSCs and naïve MSCs were isolated from the bone marrow aspirates and expanded under identical culture conditions at Case Western Reserve School of Medicine according to standard protocols used in the Cellular Therapy Laboratory at Case Western Reserve University [181]. Growth medium consisted of low glucose DMEM (Invitrogen), 10% fetal bovine serum [FBS] (Gibco), 1% antibiotic/antimycotic, 1% Glutamax (Gibco), and 10 ng/ml fibroblast growth factor (Peprotech). After being expanded for 2-3 passages, 4×10^6 hMSCs per milliliter

were frozen in cryovials using Plasma-Lyte A, containing 10% dimethyl sulfoxide and 5% human serum albumin as the freezing medium.

Prior to transplantation or collection of conditioned medium, hMSCs were thawed, reseeded into P75 flasks at a concentration of 7.5x10^3 cells / cm^2, and grown for 1-2 additional passages (4-7 days) in complete growth medium. 1x10^6 hMSCs in saline were then intravenously infused via the tail vein into MOG_{35-55} – induced EAE mice at 14 days post immunization.

Tissue processing & histopathology

For immunohistochemistry, mice were perfused transcardially, and tissue was dissected out and processed as previously described [114]. Antibodies used including rat anti-CD45 (BioLegend; 103101) and rat anti-CD3 (BioLegend; 100201).

To assess the amount of myelin loss, sections were also stained for solochrome cyanine using a previously published protocol [182]. Lesion load was calculated from six serial sections of the lumbar spinal cord by measuring lesion area and dividing it by the total area of white matter. Observers blinded to sample treatment performed cell counting and lesion load analysis. For electron microscopy, animals were perfused with 2.5% glutaraldehyde (Electron Microscopy Sciences). Spinal cords were then post-fixed in 1% osmium tetroxide (Sigma) for 1 hour, dehydrated and embedded in epoxy resin. One-micrometer epoxy sections were stained with toluidine blue and examined by light microscopy.

Collection and analysis of MSC CM

Conditioned medium was collected from naïve or EAE MSCs grown in P75 flasks that contained 1.5 x 10^6 cells; medium was collected between 21 and 24 days in-vitro, with unconditioned medium collected as a control. Conditioned medium was collected from a different culture preparation for each experiment. Conditioned medium was likewise collected from passage 3 MS-MSCs and DN-MSC between 21-25 DIV. Analysis of murine MSC conditioned medium was performed using the RayBio Mouse Inflammation Antibody Array C1 (Raybiotech), while analysis of human MSC conditioned medium was performed using the RayBio Human Cytokine Antibody Array C5. Arrays were treated and analyzed according to the manufacturer's instructions. Densitometric quantification of spot intensities for each cytokine was performed using ImageJ, with spot intensity values normalized to cell number. To account for any signal in the arrays that may have been due to serum proteins present in the growth medium, arrays of unconditioned medium were also analyzed and any spot intensity values observed were subtracted from values obtained for MSC conditioned medium. HGF levels were quantified via ELISA (R&D Systems).

For experiments where mice were treated with MSC conditioned medium, aliquots were thawed and the total amount of protein was quantified using a Pierce Bradford Protein Assay Kit (Thermofisher Scientific) according to the manufacturer's instructions. Then 0.5 mg of total protein (approximately 100ul of medium) was delivered intravenously into EAE mice via tail vein injection at 16 days post immunization.

Splenocyte culture and recall assay

Mouse splenocytes were cultured from MOG_{35-55} induced EAE mice at 14 days post induction using a previous protocol [146]. Briefly, spleens were dissected out and manually dissociated in culture medium (10% FBS, 90% RPMI 1640 medium, 1% sodium pyruvate, 1%, non-essential amino acids, 1% penicillin/streptomycin), and passed through a 70 um cell strainer to generate a single cell suspension. After centrifugation cells were re-suspended in 2 mL BD Pharm Lyse Solution (BD Biosciences) for 2 minutes to lyse out red blood cells, and the purified splenocytes were re-suspended in fresh culture medium before being plated in 96 well plates, 2x10^5 cells per well.

To test the effects of MSC CM on MOG_{35-55} induced recall, cells were cultured in medium that contained 50ul splenocyte culture medium and 50ul MSC CM or unconditioned medium (controls). MOG_{35-55} peptide dissolved in culture medium (Hooke Labs) was then added to each well to a final concentration 20uM MOG_{35-55} per well. Twenty-four hours later, BrdU reagent (10uM) was added to each well, and twenty-four hours after that the plates were fixed and processed according to the manufacture' protocol for BrdU Elisa (Abcam). The Stimulation Index (SI) for each treatment was calculated as SI = (Absorbance value of MOG stimulated cells) / (Absorbance value of un-stimulated cells). Experiments were replicated three times.

MSC characterization

For characterization of mouse MSCs, cells were stained for surface markers using a previously described protocol [117]. Antibodies used include: anti-CD45

(Abcam: ab25386), anti-CD44 (Abcam: ab25340), anti-CD90 (BioLegend: 206101), and anti Sca-1 (BioLegend: 108101). For characterization of human MSCs, cells were stained and analyzed via flow cytometry as previously described [117], with antibodies used including anti-CD90 (Abcam: ab11155), anti-CD105 (Abcam: ab2529), and anti-STRO1 (Abcam: ab190282). Differentiation of MSCs was carried out using a MSC Functional Identification Kit (R&D Systems) according to the manufacturer's instructions. Coverslips were stained with a 0.5% Oil-Red O solution (Sigma) to assess differentiation into adipocytes or stained with an Alkaline Phosphatase detection kit (EMD Millipore) to assess differentiation into osteoblasts. To assess proliferation, MSC treated with 10 uM BrdU (Sigma) for sixteen hours. Coverslips were then fixed for 10 minutes with 4% PFA and stained for rat anti-BrdU (Abcam: ab6326) using a previously described protocol [174].

Statistical Analysis

All statistical tests were performed using GraphPad Prism 6. All statistical tests are indicated in text or figure legends, with Dunnett's multiple comparison tests performed post-hoc for one-way ANOVAs. *P* values of ≤0.05 were considered statistically significant.

Study Approval

All animal experiments were approved by Case Western Reserve University School of Medicine's IACUC with adherence to the NIH Guide for the Care and Use of Laboratory Animals. Human bone marrow cells were obtained after informed

written consent from patients in accordance with a protocol approved by the institutional review board at the Cleveland Clinic or Third Military Medical University.

2.4: Results

Transplanted EAE-MSCs fail to significantly improve recovery in EAE

In order to determine if disease affects the therapeutic functionality of BM-MSCs, and to better model transplantation of autologous MSCs as a therapy for MS, MSCs were derived from the bone marrow of MOG_{35-55} EAE mice at two distinct phases of the disease: at 14 days post induction during the peak of the disease ("peak EAE-MSCs"), and later at 28 days post induction during the chronic phase of EAE ("chronic EAE-MSCs"). Both peak and chronic EAE-MSCs derived from multiple donors were then expanded and systemically delivered into cohorts of MOG_{35-55}-induced EAE mice and compared to naïve MSCs in their therapeutic efficacy.

While intravenous infusion of naïve MSCs improved functional recovery in EAE mice, infusion of EAE-MSCs produced no significant improvement in clinical recovery. Infusion of naïve MSCs (0.8x10^6) into MOG_{35-55} EAE mice 15 days after EAE induction resulted in a rapid improvement in clinical score. The improvement in functional recovery in mice treated with naïve MSCs was sustained for up to 1 month after infusion (Figure 2.1A). Cumulative disease score after 30 days (measured as area under the curve) was significantly lower in EAE mice treated with naïve MSCs relative to controls (116 ± 6 naïve MSCs: 190 ± 9 controls; $p<0.001$, n =12 mice per group from 3 independent experiments, one-way ANOVA). By

contrast, infusion of peak or chronic EAE-MSCs (0.8x10^6) into MOG_{35-55} EAE mice 15 days after EAE induction failed to produce comparable improvements in functional recovery. Though infusion of peak EAE-MSCs resulted in a small improvement in average clinical score 6 days after treatment (2.8 ± 0.28 compared to 3.3 ± 0.28 for controls), this improvement was transient, as the average clinical score returned to that of controls within a few days (Figure 2.1A). Consistent with these observations, mice treated with peak EAE-MSCs showed no significant difference from controls in cumulative disease score (183 ± 13 compared to 190 ± 9 for controls; $p>0.05$, n =12 mice per group from 3 independent experiments, one-way ANOVA). Infusion of chronic EAE-MSCs failed to produce any improvement in functional recovery (Figure 2.1A), with mice receiving chronic EAE-MSCs showing no significant difference from controls in cumulative disease score after 30 days of treatment (194 ± 5 compared to 190 ± 9 for controls; $p>0.05$, n =12 mice per group from 3 independent experiments, one-way ANOVA).

This lack of functional recovery in mice treated with EAE-MSCs also correlated with a lack of improvement in CNS histology. Solochrome cyanine staining of spinal cord sections showed that mice infused with naïve MSCs had less white matter loss at 30 days post treatment compared to saline treated controls, whereas mice treated with peak or chronic EAE-MSCs had higher levels of myelin loss similar to controls (Figure 2.1B). Quantification of lesion load from solochrome cyanine stained spinal cord sections 30 days after treatment demonstrated that mice treated with naïve MSCs had a significantly lower lesion load compared to controls, while mice treated with peak or chronic EAE-MSCs had no significant

difference from controls (Figure 2.1C). Toluidine blue staining of spinal cord sections from EAE mice 30 days after treatment confirmed that mice treated with naïve MSCs had more myelinated axons and less inflammatory infiltrates compared to controls, in contrast to mice treated with peak or chronic EAE-MSCs, which showed no appreciable difference (Figure 2.1D). There was a significant reduction in the number of CD45 positive immune cells and CD3 positive T-cells in spinal cord sections of EAE mice treated with naïve MSCs, while mice treated with either peak or chronic EAE-MSCs had higher numbers of inflammatory cells and T-cells similar to controls (Figure 2.1E).

Overall, mice treated with EAE-MSCs failed to show any substantial or sustained improvement in clinical recovery or neuropathology, in contrast to mice treated with naïve MSCs. One possible explanation for these results is that EAE-MSCs might fail to migrate and engraft into their hosts at equivalent levels to naïve MSCs. To test this hypothesis, peak EAE-MSCs, chronic EAE-MSCs, and naïve MSCs were labeled with the fluorescent cell tracking dye CMTPX (Supplemental Figure 2.1A). CMTPX-labeled MSCs were then intravenously infused into MOG_{35-55} EAE mice (0.5x10^6 cells/animal) at 18 days after EAE induction, to assess if EAE-MSCs were any different than naïve MSCs in where they engrafted. One day after infusion, naïve MSCs and EAE-MSCs had migrated into the liver, spleen, and lungs (Supplemental Figure 2.1B). No CMTPX-labeled MSCs were observed in the heart, kidney, brain or spinal cord (data not shown), consistent with previous studies demonstrating that MSCs do not engraft into the CNS of EAE mice [146,148]. No difference in the number of peak or chronic EAE-MSCs compared to the number of naïve MSCs was observed in

any of these tissues at one or seven days after infusion (Supplemental Figure 2.1D), suggesting the lack of therapeutic efficacy observed in transplanted EAE-MSCs is not due to lack of cell engraftment or survival.

Conditioned medium from EAE-MSCs fails to ameliorate EAE

Previous studies suggest that naïve MSCs mediate recovery in EAE by secreting various cytokines and growth factors that suppress inflammation and promote remyelination and neural repair [178]. Much of the therapeutic benefits of transplanting naïve MSCs into EAE mice can be recapitulated by treating mice with conditioned medium (CM) from MSCs [114]. To determine if the diminished therapeutic functionality of EAE-MSCs was due to differences in paracrine factors they secreted relative to naïve MSCs, conditioned medium was collected from naïve MSCs (naïve MSC CM), peak EAE-MSCs (peak EAE-MSC CM), and chronic EAE-MSCs (chronic EAE-MSC CM), and intravenously infused into MOG_{35-55} - induced EAE mice.

While treatment with conditioned medium from naïve MSCs improved functional recovery in EAE mice, treatment with CM from EAE-MSCs produced little or no improvement in clinical recovery. Mice treated with naïve MSC CM (0.5 mg total protein, approximately 0.1 mL) at 16 days post EAE induction showed substantial improvement in clinical score compared to control mice treated with unconditioned medium (Figure 2.2A). This improvement in functional recovery persisted for approximately one week, with the cumulative disease score of naïve MSC CM treated mice being significantly lower than controls (61 ± 2 compared to 89 ± 4 for controls; $p<0.01$, n=11-12 mice per group from 3 independent experiments,

one-way ANOVA). By contrast, mice treated with CM from EAE-MSCs failed to show comparable improvements in functional recovery. While mice treated with peak EAE-MSC CM did show a small improvement in average clinical score starting at 11 days post treatment (3.0 ± 0.23 compared to 3.5 ± 0.26 for controls), this persisted for only a few days (Figure 2.2A), and the cumulative disease score of mice that received peak EAE-MSC CM was not significantly different from controls (84 ± 4 compared to 89 ± 4 for controls; $p>0.05$, n=11-12 mice per group from 3 independent experiments, one-way ANOVA). Mice infused with CM from chronic EAE-MSCs showed no improvement in clinical score versus controls at any time up to two weeks post-treatment, and had no significant difference in cumulative disease score versus controls (92 ± 4 compared to 89 ± 4 for controls; $p>0.05$, n=11-12 mice per group from 3 independent experiments, one-way ANOVA).

The lack of improvement in functional recovery in mice treated with EAE-MSC CM was paralleled by a lack of improvement in CNS histopathology. Solochrome cyanine staining of spinal cord sections from mice treated with naïve MSC CM had less white matter loss and a significantly lower lesion load compared to controls at 14 days post treatment, whereas mice treated with either peak or chronic EAE-MSC CM had no significant difference in demyelination or lesion load compared to controls (Figure 2.2B and 2.2C). Likewise the number of CD45-positive immune cells and CD3-positive T-cells was significantly decreased in spinal cord sections from mice treated with naïve MSC CM compared to controls, but there was no such reduction in the number of inflammatory cells or T-cells in mice treated with peak or chronic EAE-MSC CM (Figure 2.2E).

EAE-MSCs secrete higher levels of pro-inflammatory cytokines

These results suggest the lack of therapeutic functionality observed in EAE-MSCs relative to naïve MSCs is due to differences in their secretion of paracrine factors. To identify factors that are differentially secreted by EAE-MSCs, an antibody array was used to detect and compare expression levels of 40 different candidate inflammatory proteins in the CM of EAE-MSCs versus the CM of naïve MSCs. This array profiles both pro-inflammatory and anti-inflammatory factors important in immune response and EAE pathogenesis, and many of these factors, including TGFB, IL-4, IL-6, IL-10, and others, have previously described roles in MSC immunomodulation in EAE or other diseases [60].

Conditioned medium from EAE-MSCs had considerably higher levels of many pro-inflammatory cytokines compared to conditioned medium from naïve MSCs. Comparison of spot intensity values on respective arrays showed that peak EAE-MSCs secreted 2 to 4 fold higher levels of the pro-inflammatory cytokines IL-6, CCL2, and CCL9 compared to naïve MSCs (Figure 2.3A and 2.3B). Chronic EAE-MSCs also secreted higher levels of these three cytokines, but at an even greater magnitude. Conditioned medium from chronic EAE-MSCs had 16 fold higher levels of IL-6 and CCL2, and nearly 5 hold higher levels of CCL9. Also, chronic EAE-MSC CM had a 16-fold increase in the pro-inflammatory cytokines CXCL1 and CXCL5 (Figure 2.3A and 2.3B). In total, the antibody array detected 21 out of 40 proteins profiled in the MSC conditioned medium samples; 4 of these proteins (IL-6, CCL2, CCL9, and sTNFR1) were higher in peak EAE-MSC CM compared to naïve MSC CM and 8 of these proteins (IL-6, CCL2, CCL9, sTNFR1, sTNFR2, CXCL1, CXCL5, and

TIMP1) were higher in chronic EAE-MSC CM compared to naïve MSC CM. The array found no proteins that were down regulated in EAE-MSC CM relative to naïve MSC CM.

In a previous study, hepatocyte growth factor (HGF) was found to be an important mediator of MSC-induced recovery in EAE [114]. Because HGF was not profiled in the antibody arrays, quantitative ELISA was used to compare HGF levels in naive MSC CM versus EAE-MSC CM. Unexpectedly, peak EAE-MSC CM had significantly higher levels of HGF (approximately a 2 fold increase) compared to naïve MSC CM (Supplemental Figure 2.2). In contrast, CM from chronic EAE-MSCs had significantly lower levels of HGF (approximately 50%) compared to CM from naïve MSCs (Supplemental Figure 2.2).

Conditioned medium from chronic EAE-MSCs lacks immunosuppressive functionality in-vitro

The elevated levels of pro-inflammatory cytokines in EAE-MSC CM suggests that these cells may no longer have the immunosuppressive, anti-inflammatory functionality commonly associated with naïve MSCs [146]. To test this hypothesis, a splenocyte restimulation assay was employed, in which spleenic lymphocytes were cultured from MOG_{35-55} induced EAE mice and subsequently restimulated by 20uM MOG_{35-55} antigen in-vitro, driving lymphocyte activation and increased proliferation.

Consistent with previous studies [146,148], conditioned medium from naïve MSCs effectively suppressed MOG_{35-55} induced restimulation of splenocytes in-vitro. As measured by BrdU incorporation, proliferation was significantly decreased in

MOG stimulated splenocytes treated with naïve MSC CM compared to control cultures that were treated with unconditioned medium (Figure 2.4). Surprisingly, conditioned medium from peak EAE-MSCs significantly reduced proliferation of MOG restimulated splenocytes (Figure 2.4). By contrast, conditioned medium from chronic EAE-MSCs failed to suppress splenocyte response to MOG, as MOG stimulated cultures treated with chronic EAE-MSC CM showed no significant difference in proliferation compared to controls (Figure 2.4).

EAE-MSCs differ from naïve MSCs in differentiation and proliferation

EAE-MSCs had similar cellular characteristics that classically define naïve MSCs. For example, EAE-MSCs had a similar morphology to naïve MSCs (Figure 2.5A), and expressed common mesenchyme and stem cell markers used to identify MSCs in-vitro (Figure 2.5B, Supplemental Figure 2.3). EAE-MSCs could also be differentiated along common mesenchymal lineages, into adipocytes and osteoblasts under appropriate culture conditions. However, EAE-MSCs showed significant differences in differentiation potential compared to naïve MSCs (Figure 2.5C). For instance, when cultured under identical conditions, fewer peak EAE-MSCs differentiated into Oil-Red O positive adipocytes compared to naïve MSCs. Also, significantly more chronic EAE-MSCs differentiated into Alizarin Red positive osteoblasts/osteocytes compared to naïve MSCs, even though all cells were cultured under identical conditions (Figure 2.5C).

EAE-MSCs proliferated at a significantly higher rate than naïve MSCs in-vitro. As measured by BrdU pulse (10 uM for 16 hours) and subsequent immunolabeling,

the proportion of both peak and chronic EAE-MSCs that were BrdU positive was significantly higher versus the percentage of naïve MSCs (Figure 2.5D). Collectively, our data suggests that while EAE-MSCs have the same functional characteristics used to define naïve MSCs in-vitro, they also possess intrinsic differences in properties like growth rate and differentiation potential, in addition to important differences in cytokine expression and therapeutic functionality.

MSCs derived from MS patients lack therapeutic efficacy in modulating EAE

We next asked whether the diminished therapeutic functionality observed in EAE-MSCs was in fact paralleled by human MSCs derived from MS patients (MS-MSCs). To address this question, BM-MSCs were obtained from 3 different donors diagnosed with relapsing-remitting MS and expanded in-vitro. These MS-MSCs were phenotypically similar to naïve MSCs in their expression of common MSC markers (Supplemental Figure 2.4). MS-MSCs were then intravenously infused (1x10^6 cells/animal) into MOG_{35-55} induced EAE mice 14 days after EAE induction to compare them against naïve MSCs (from age and sex matched healthy donors) in their therapeutic potential.

For each of the respective donors, MS-MSCs were less effective at improving functional recovery in MOG - induced EAE mice compared to naïve MSCs. Although differences in therapeutic potential between MS-MSCs derived from different donors were observed. For instance, EAE mice treated with MS-MSCs derived from Donor 1 showed a limited improvement (about 1 point) in functional recovery compared to saline-treated controls for about 7 days after infusion, but this

improvement rapidly reversed (Figure 2.6A). In contrast, MS-MSCs derived from Donor 2 promoted a more sustained improvement in functional recovery compared to the MS-MSCs derived from Donor 1, although these cells were still less effective than naïve MSCs (Figure 2.6A). Overall, only EAE mice treated with naïve MSCs showed a significant reduction in cumulative disease score (Figure 2.6B).

Since MS-MSCs and EAE-MSCs both lacked therapeutic efficacy in treating EAE, we next asked if MS-MSCs secreted higher levels of pro-inflammatory factors like EAE-MSCs. An antibody array was used to profile and compare CM from MS-MSCs to CM from naïve MSCs. While these arrays detected a relatively small number of proteins in human MSC CM (12 factors detected out of 80 targets profiled), this approach nevertheless identified several pro-inflammatory cytokines that were elevated in MS-MSC CM. Conditioned medium from MS-MSCs contained higher levels of both IL-6 and CCL2 compared to CM from naïve MSCs (Figure 2.6C), paralleling the increase observed in CM from EAE-MSCs (Figure 2.3). Additionally, MS-MSC CM had higher levels of IL-8 and Timp1 compared to naïve MSC CM. MS-MSC CM also had much higher levels of HGF compared to naïve MSC CM (Figure 2.6C), although this increase parallels the increase in HGF levels observed in CM from peak EAE-MSCs.

2.5: Discussion

Here, we demonstrate for the first time that bone marrow MSCs isolated from EAE mice and MS patients have reduced therapeutic efficacy compared to naïve MSCs in modulating EAE. These results have important clinical implications,

as most clinical trials evaluating BM-MSCs to treat MS utilize autologous MSCs [180]. Indeed, results from completed trials report good safety but little or no therapeutic efficacy in systemically transplanting autologous BM-MSCs into MS patients [164-167]. Our results show that autologous EAE-MSCs and MS-MSCs likewise have little therapeutic effect when transplanted into MOG EAE mice, and are comparatively worse than naïve MSCs in modulating EAE.

Despite the fact that most clinical trials utilize autologous BM-MSCs to treat MS, few previous studies have compared autologous diseased MSCs to naïve cells. Two studies reported cultured MS-MSCs were similar to naïve MSCs in proliferation, differentiation, and expression of common MSC cell surface markers, although one of these studies did report MS-MSCs secreted higher levels of immunomodulatory cytokines including CXCL10[169,170]. A more recent study that compared gene expression profiles of bone marrow MSCs derived from MS patients compared to those from healthy controls found that MS-MSCs down-regulated anti-inflammatory genes like IL-10 and up-regulated pro-inflammatory genes like IL-6 [172].

Studies comparing bone marrow EAE-MSCs to naïve MSCs report conflicting results. One study found BM-MSCs isolated from MOG induced EAE mice are no different than naïve MSCs in terms of growth rate or differentiation, and are just as effective as naïve MSCs in ameliorating disease when transplanted systemically into EAE mice [173]. This study isolated MSCs from EAE mice early on in the disease, when the mice showed clinical symptoms such as tail paralysis or hind limb weakness. In contrast, a more recent study compared BM-MSCs isolated from MOG EAE mice later in disease, when symptoms are more severe, and found that these EAE-MSCs were

different from naïve MSCs in terms of growth rate, differentiation potential, and mRNA expression levels of important histone-modifying genes [174]. This later study did not compare EAE-MSCs to naïve MSCs in terms of therapeutic efficacy. Our results show that EAE-MSCs isolated from mice later in disease when symptoms are more severe (full hind limb paralysis) lack therapeutic efficacy compared to naïve MSCs and have distinct differences in growth rate and differentiation potential.

While a number of factors, such as differences in MSC isolation protocol or culture conditions, might explain these conflicting results, one intriguing possibility for these different findings is that changes in the functionality to BM-MSCs in EAE is dependent on the severity and stage of the disease. Our data lends support to this hypothesis, as we found that the progression of disease in EAE seems to correlate to loss of functionality in BM-MSCs. While both peak EAE-MSCs and chronic EAE-MSCs lacked therapeutic efficacy in modulating EAE compared to naïve MSCs, mice that received peak EAE-MSCs did show small, albeit temporary, improvements in functional recovery. Furthermore, while conditioned medium from chronic EAE-MSCs failed to inhibit proliferation of MOG-stimulated splenocytes in-vitro, conditioned medium from peak EAE-MSCs effectively suppressed proliferation in a manner similar to naïve MSC CM. Thus, while lacking therapeutic efficacy compared to naïve MSCs, peak EAE-MSCs still seem to have some limited therapeutic effect in-vivo and immunosuppressive functionality in-vitro.

One of the striking differences observed between peak and chronic EAE-MSCs was in their secretion of immunomodulatory cytokines and growth factors. Analysis of conditioned medium from peak EAE-MSCs showed they secrete higher

levels of IL-6, CCL2, and CCL9 compared to naïve MSCs. Chronic EAE-MSCs also secrete higher levels of the same proteins relative to naïve MSCs, but at a much greater magnitude. For example, peak EAE-MSC CM had an approximately 2.5-fold increase in IL-6 versus naïve MSC CM, while chronic EAE-MSC CM had 16-fold higher IL-6 levels. In addition, chronic EAE-MSCs also secreted much higher levels of two other cytokines: CXCL1 and CXCL5. There were also differences between peak and chronic EAE MSCs in their secretion of HGF, which has anti-inflammatory and pro-regenerative properties and has been implicated as an important mediator of MSC-induced recovery in EAE [114]. Peak EAE-MSC CM actually had higher levels of HGF compared to naïve MSC CM, whereas chronic EAE-MSC CM had significantly lower levels of HGF. The findings that peak EAE-MSC CM contains higher levels of HGF may contribute to its ability to inhibit MOG induced lymphocyte proliferation in-vitro, whereas chronic EAE-MSC CM has no effect. Independent of HGF, peak EAE-MSCs secrete higher levels of pro-inflammatory cytokines relative to naïve MSCs, and this might account for their reduced therapeutic efficacy compared to naïve MSCs when transplanted in-vivo.

Results from experiments in which EAE mice were treated with conditioned medium from naïve MSCs or EAE-MSCs suggest the lack of therapeutic efficacy in EAE-MSCs is due to differences in factors they secrete relative to naïve MSCs. Previous studies have shown naïve MSCs mediate recovery in EAE by secreting various anti-inflammatory cytokines, chemokines, and trophic factors [114,150,158]. These in turn act to suppress auto-reactive immune cells and inhibit inflammation while also promoting remyelination and neural repair [178]. In agreement with these

studies, we found that conditioned medium (CM) from naïve MSCs improves both functional recovery and neuropathology in EAE mice, reducing lesion load and the number of immune and T-cells in the CNS. The improvement in functional recovery with naïve MSC CM seemed more transient than the benefits observed upon directly injecting the cells, as mice treated with a single injection of naïve MSC CM appeared comparable to controls in disease burden at later time points (unpublished observations). While infusion of CM from peak EAE-MSCs into EAE mice results in a small improvement in functional recovery, this effect was very limited and only temporary, and so overall peak EAE-MSC CM failed to significantly improve clinical recovery or neuropathology. Similarly, infusion of CM from chronic EAE-MSCs did not improve functional or histological recovery.

While a number of different cytokines are up regulated in the CM of both peak and chronic EAE-MSCs, it is unclear which, if any, of these factors might account for the loss of therapeutic efficacy observed in EAE-MSCs. All of the cytokines identified have pro-inflammatory functionality and are known mediators of inflammation in EAE. IL-6 in particular is thought to be an important driver of T-cell activity in diseases like MS and EAE [183], as it biases the development of immature or regulatory T-cells into pro-inflammatory Th17 cells [184]. Both CCL2 and CCL9 are known chemokines for monocytes and inflammatory macrophages [185], with CCL2 recruiting inflammatory immune cells into the CNS of EAE mice [186]. Both CXCL1 and CXCL5 are chemokines important in the recruitment of neutrophils to sites of inflammation [187,188], and both are systemically up regulated in MS patients during active lesion formation [189].

Conditioned medium from MS-MSCs had higher levels of some of the same pro-inflammatory cytokines, including IL-6 and CCL2. MS-MSCs were also similar to peak EAE-MSCs in that they secreted higher levels of HGF compared to naïve MSCs. One question raised by these findings is whether secretion of HGF or other immunomodulatory factors by MS-MSCs is affected by severity or duration of disease. For instance, are BM-MSCs from MS patients different during relapse versus remittance, or are MSCs from relapsing-remitting MS patients different from MSCs from progressive MS patients? While MSCs can be isolated from EAE mice at different phases of disease, it is more challenging to isolate and compare MSCs from MS patients during specific periods of disease or control for variables like disease duration, severity of disease, or differences in medications donors were receiving at the time their bone marrow was collected. Future work including both prospective and retrospective studies comparing MS-MSCs to naïve MSCs are required to better document the effect of such factors as well as genetic and environmental variables that could also influence the functionality of bone marrow MSCs. The benefits of autologous MSC therapy in MS will likely be unrealized until there is a better understanding of how disease status and severity correlate to loss of therapeutic functionality in these cells.

The demonstration of differences in the functionality of bone marrow derived MSCs following CNS inflammation and demyelination is consistent with the observation of changes in bone physiology in patients with MS. Osteoporosis and osteopenia are commonly associated with MS, with MS patients having a higher risk of both conditions, but the mechanisms underlying bone degradation in MS are

currently unclear [190,191]. It has been proposed that various factors contribute to reduced bone density in patients with MS, including paralysis and musculoskeletal atrophy, vitamin D deficiency, and inflammation that directly damages bone structure [192]. Our data shows that diseases like EAE and MS can dramatically alter the functionality of bone marrow MSCs, including aspects of their proliferation and differentiation, raising the possibility that changes to bone marrow MSCs in MS could contribute to changes in bone density observed in MS patients.

2.6: Conclusions

The pronounced lack of therapeutic efficacy of both EAE and MS-MSCs in an experimental model of MS suggests that autologous MSCs may be a poor candidate for cell-based therapies to treat MS. The present study thus supports the advancement of allogeneic MSC therapy over autologous MSC therapy for the treatment of MS, and raises important concerns over the efficacy of using autologous BM-MSCs in clinical trials.

2.7: Acknowledgements

This work was supported by NS30800 (NIH) and the Myelin Repair Foundation. The authors thank the Southwest Hospital of the Third Military Medical University for providing human bone marrow samples, and the CWRU stem cell core and GWU imaging core for assistance.

Figure 2.1

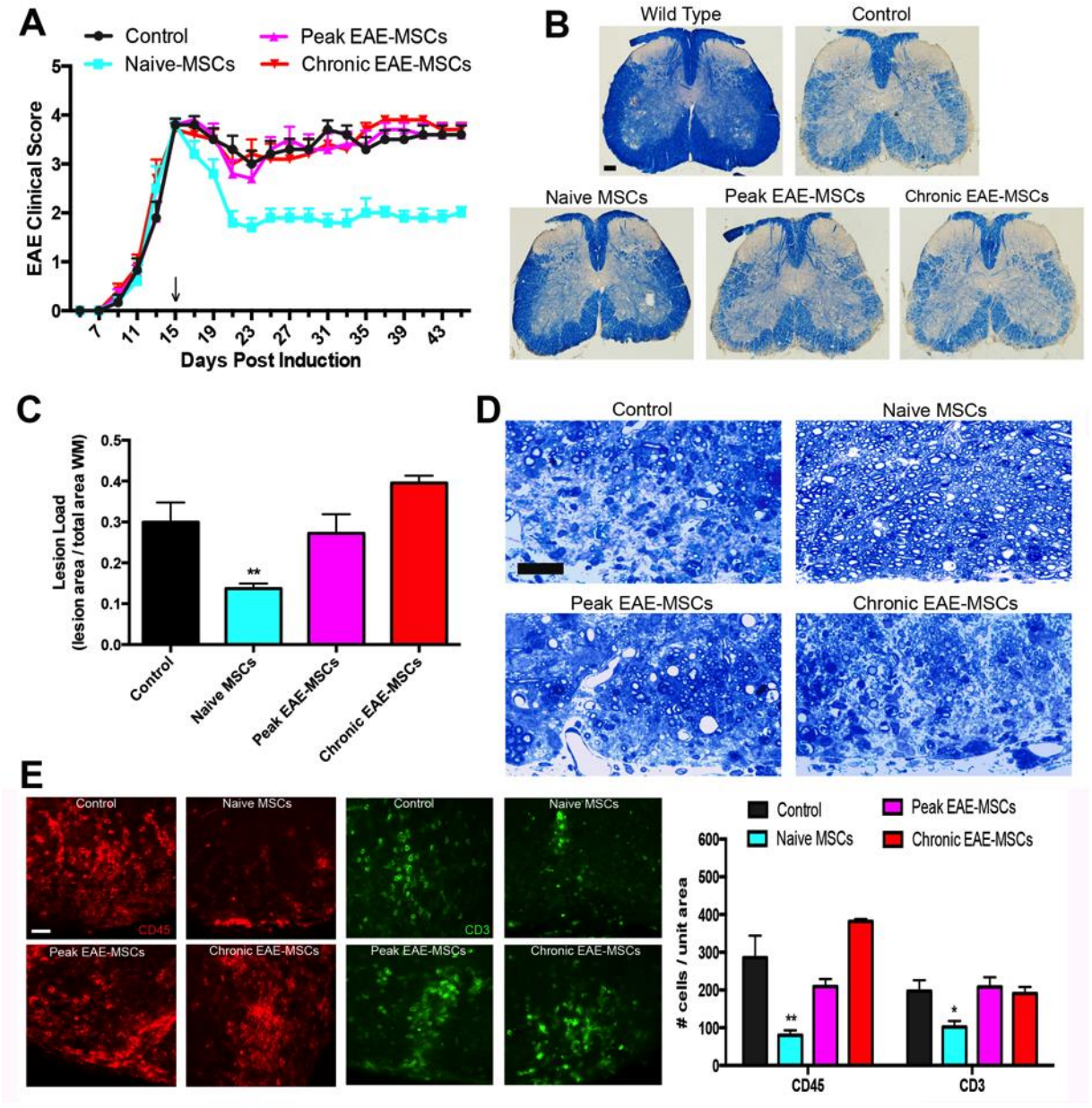

A
Control
Naive-MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
EAE Clinical Score
Days Post Induction
B
Wild Type
Control
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
C
Lesion Load
(lesion area / total area WM)
Control
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
D
Control
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
E
Control
Naive MSCs
CD45
CD3
Peak EAE-MSCs
Chronic EAE-MSCs
cells / unit area
CD45
CD3

Figure 2.1: Transplanted EAE-MSCs fail to improve functional recovery or CNS pathology in EAE mice. (A) Infusion of naïve MSCs leads to improved functional recovery in MOG_{35-55} EAE-mice, while treatment with peak or chronic EAE-MSCs does not improve functional recovery versus saline-treated controls. 0.8 x 10^6 MSCs were intravenously injected into mice 15 days after EAE induction (arrow). Data shown represents mean + SEM, n=12 mice per group, from 3 independent experiments. **(B)** Representative images of solochrome cyanine stained spinal cord sections showing myelin loss in EAE mice 30 days after treatment with naïve MSCs versus peak or chronic EAE-MSCs. **(C)** EAE mice treated with naïve MSCs have less myelin loss and lower lesion load, while mice treated with EAE-MSCs have a higher lesion load that is comparable to controls. Data shown = mean + SEM, with lesion load quantified from 6 solochrome cyanine stained sections per mouse, 3 mice per group; **P<0.01, One-way ANOVA. **(D)** Representative images of Toluidine blue stained spinal cord sections from EAE mice 30 days after treatment confirms that mice treated with naïve MSCs have more myelinated axons and less inflammatory infiltrates than controls. By contrast, animals treated with peak or chronic EAE-MSCs show no appreciable difference from controls. **(E)** EAE-mice treated with naïve MSCs have significantly lower numbers of CD45 positive inflammatory cells (red) and CD3 positive T-cells (green) in their spinal cords 30 days after treatment, while mice treated with peak or chronic EAE-MSCs show no significant difference from controls. Data shown in graph = mean + SEM, quantified from 4 sections per animal, 3 animals per group. *P<0.05, **P<0.01, One-way ANOVA. Scale bars in **(B)** = 500um, **(D)** = 25 um, **(E)** = 20um.

Figure 2.2

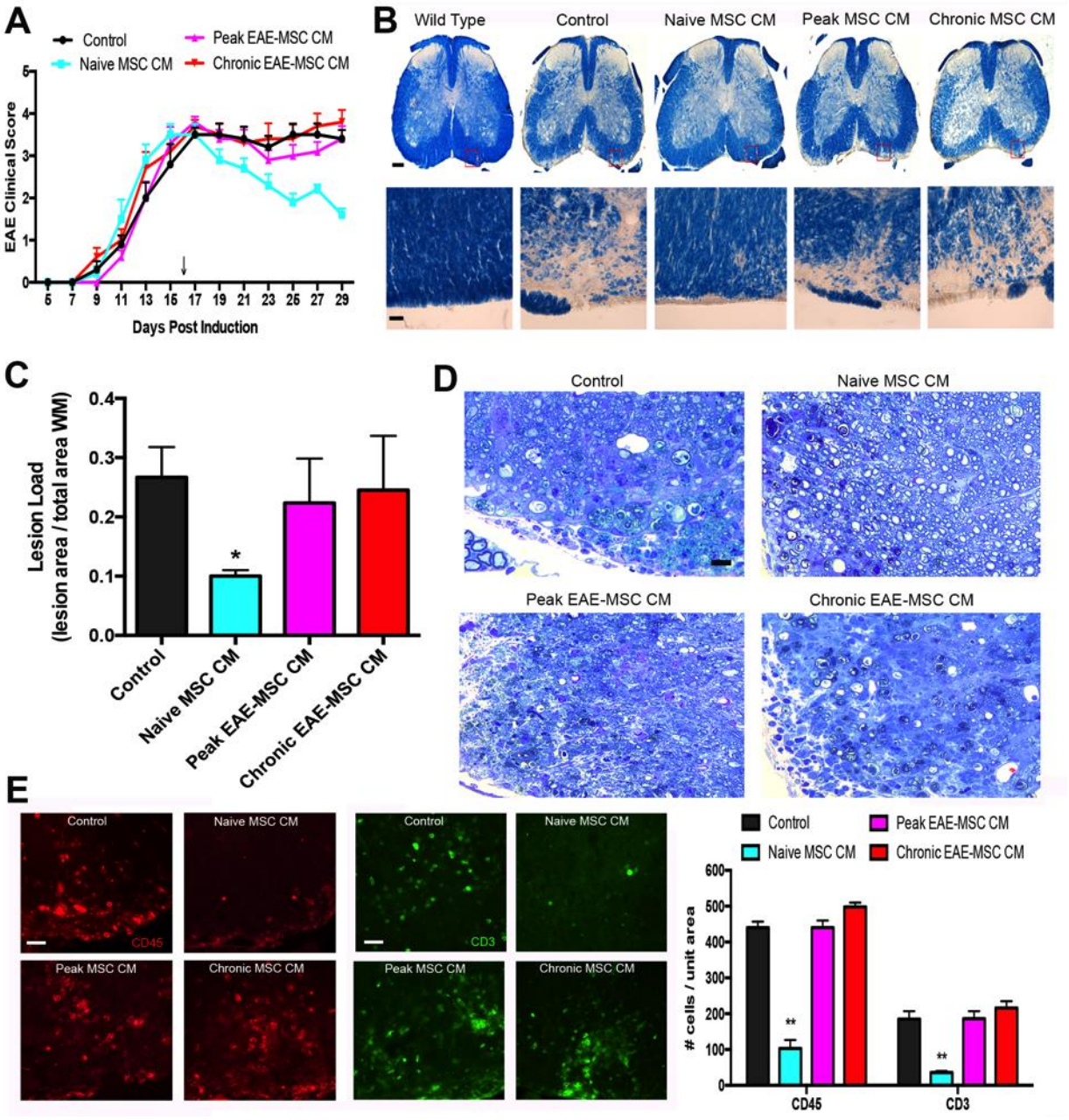
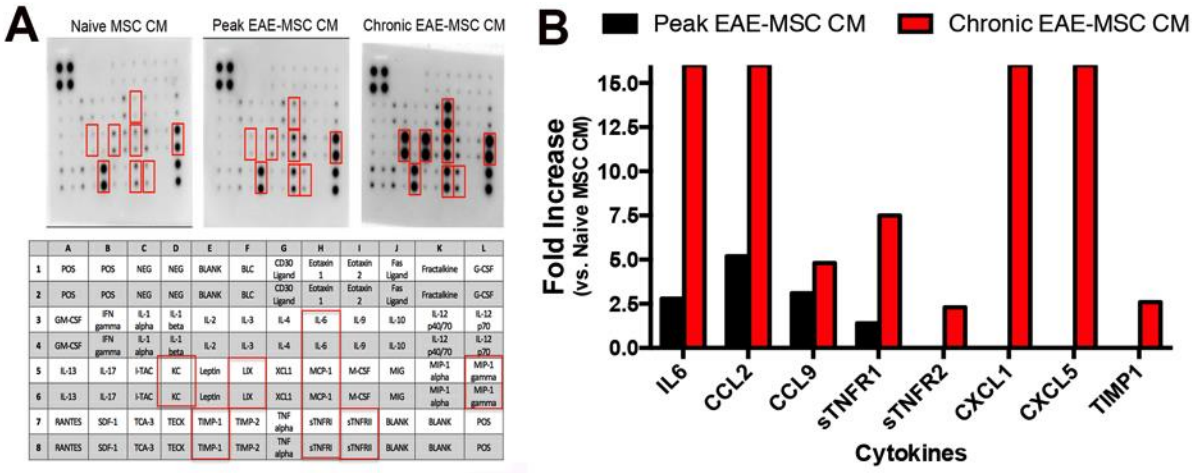

A
Control
Naive MSC CM
Peak EAE-MSC CM
Chronic EAE-MSC CM
EAE Clinical Score
Days Post Induction
B
Wild Type
Control
Naive MSC CM
Peak MSC CM
Chronic MSC CM
C
Lesion Load
(lesion area / total area WM)
Control
Naive MSC CM
Peak EAE-MSC CM
Chronic EAE-MSC CM
D
Control
Naive MSC CM
Peak EAE-MSC CM
Chronic EAE-MSC CM
E
CD45
CD3
cells / unit area

Figure 2.2: Conditioned medium (CM) from EAE-MSCs fails to improve functional recovery or CNS pathology in EAE mice. (A) Intravenous infusion of conditioned medium (CM) from naïve MSCs leads to improved functional recovery in MOG_{35-55} EAE-mice, while infusion of CM from either peak or chronic EAE-MSCs does not improve functional recovery relative to controls (EAE mice that received unconditioned medium). 0.5 mg conditioned medium was given 16 days after EAE induction (arrow), data shown represents mean + SEM, n=11-12 mice per group, from 3 independent experiments. **(B)** Representative images of solochrome cyanine stained spinal cord sections showing myelin loss in EAE mice 14 days after they received CM from naïve MSCs versus peak or chronic EAE-MSCs. **(C)** EAE mice treated with naïve MSC CM have less myelin loss and lower lesion load, while mice treated with EAE-MSC CM have a higher lesion load comparable to controls. Data shown = mean + SEM, with lesion load quantified from 6 solochrome cyanine stained sections per mouse, 3 mice per group; *P<0.05, One-way ANOVA. **(D)** Toluidine blue staining of spinal cord sections in EAE mice 14 days after treatment shows more myelinated axons in mice that received naïve MSC CM, while mice receiving EAE-MSC CM have less myelinated axons and more inflammatory infiltrates similar to controls. **(D)** EAE-mice treated with naïve MSC-CM have significantly lower numbers of CD45 positive inflammatory cells (red) and CD3 positive T-cells (green) in their spinal cords 14 days after treatment, whereas mice treated with EAE-MSC CM show no significant difference from controls. Data shown in graph = mean + SEM, quantified from 5 sections per animal, 3 animals per group.

P<0.01, One-way ANOVA. Scale bars in **(B) *(Top)*= 500um, *(Bottom) = 25um* **(D)** = 25 um, **(E)** = 20um.

Figure 2.3



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2	POS	POS	NEG	NEG	BLANK	BLC	CD30 Ligand	Eotaxin 1	Eotaxin 2	Fas Ligand	Fractalkine	G-CSF
3	GM-CSF	IFN gamma	IL-1 alpha	IL-1 beta	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12 p40/70	IL-12 p70
4	GM-CSF	IFN gamma	IL-1 alpha	IL-1 beta	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12 p40/70	IL-12 p70
5	IL-13	IL-17	I-TAC	KC	Leptin	LIX	XCL1	MCP-1	M-CSF	MIG	MIP-1 alpha	MIP-1 gamma
6	IL-13	IL-17	I-TAC	KC	Leptin	LIX	XCL1	MCP-1	M-CSF	MIG	MIP-1 alpha	MIP-1 gamma
7	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF alpha	sTNFRI	sTNFRII	BLANK	BLANK	POS
8	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF alpha	sTNFRI	sTNFRII	BLANK	BLANK	POS

Figure 2.3: EAE-MSCs secrete higher levels of pro-inflammatory cytokines. **(A)** Representative images of antibody arrays treated with conditioned medium (CM) from naïve MSCs, peak EAE-MSCs, or chronic EAE-MSCs. Cytokines found to be up-regulated in the arrays are indicated by red boxes and identified in the array diagram depicted below; CCL2 = MCP-1, CCL9 = MIP-1 gamma, CXCL1 = KC, and CXCL5 = LIX. **(B)** Fold changes of proteins increased in conditioned medium from peak or chronic EAE-MSCs relative to naïve MSCs; note conditioned medium from EAE-MSCs contains higher levels of pro-inflammatory cytokines, including IL6, CCL2, and CCL9. Fold changes were calculated by densitometric quantification of spot intensity values from 3 separate antibody arrays per group, n = 3 experiments. Fold changes were capped at 16, with no fold decreases of any cytokines tested observed in the arrays.

Figure 2.4

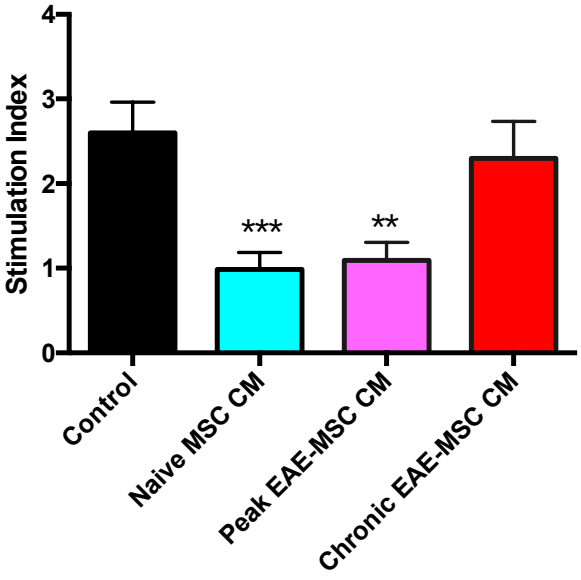

Stimulation Index
4
3
2
1
0
***
**
Control
Naive MSC CM
Peak EAE-MSC CM
Chronic EAE-MSC CM

Figure 2.4: Effects of EAE versus naïve MSC CM on MOG-stimulated splenocytes. Conditioned medium from naïve MSCs or peak EAE-MSCs suppresses MOG induced restimulation of splenocytes *in-vitro*, whereas conditioned medium from chronic EAE-MSCs fails to significantly inhibit splenocyte proliferation. Proliferation was assessed 48 hours after MOG stimulation (20uM) via BrdU Elisa. Data shown = mean + SEM, n = 3 experiments, **P<0.01, ***P<0.005, One-way ANOVA.

Figure 2.5

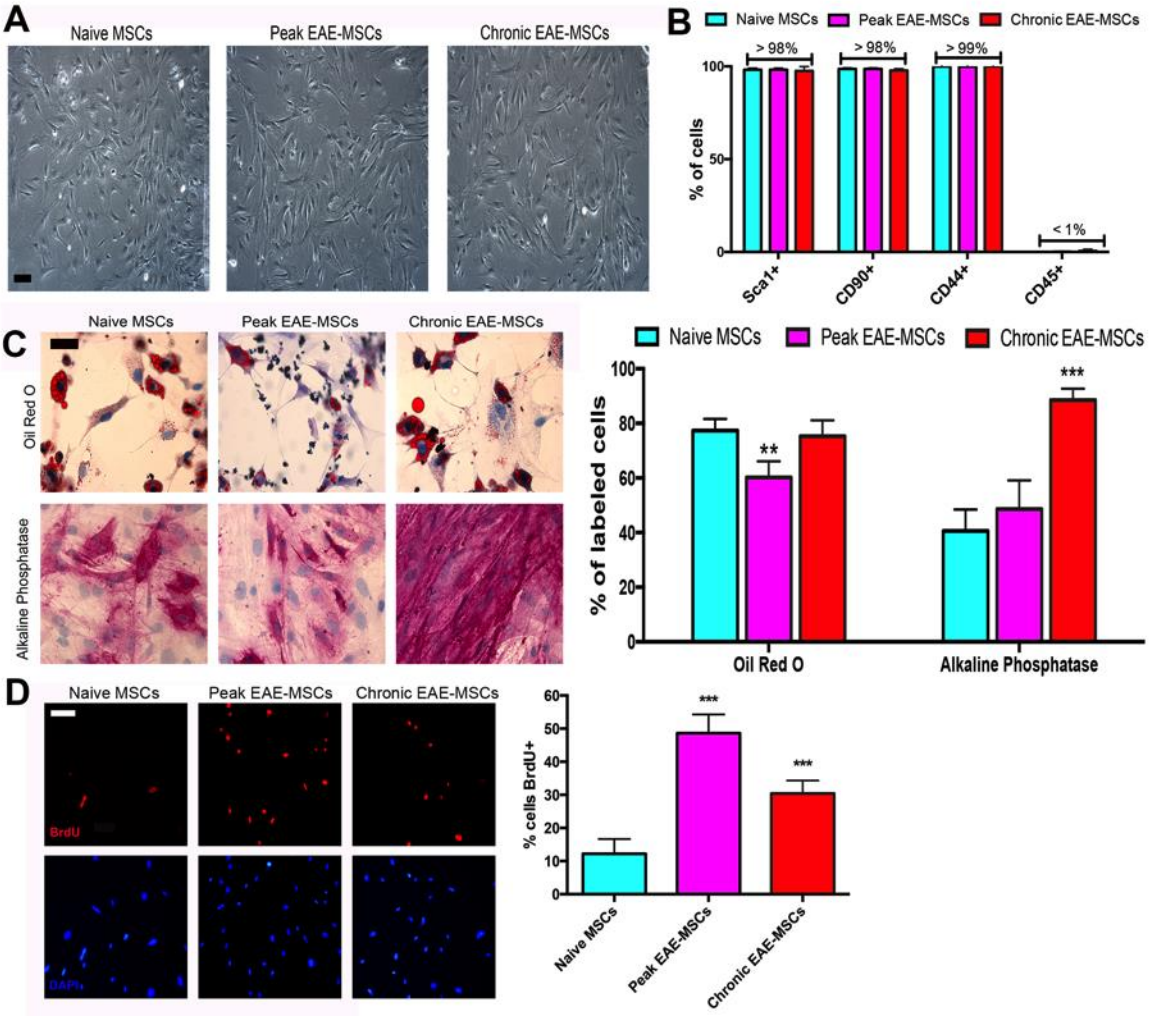

A
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
B
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
> 98%
> 98%
> 99%
< 1%
% of cells
100
50
0
Sca1+
CD90+
CD44+
CD45+
C
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
Oil Red O
Alkaline Phosphatase
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
% of labeled cells
100
80
60
40
20
0
**
***
Oil Red O
Alkaline Phosphatase
D
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs
BrdU
DAPI
% cells BrdU+
60
50
40
30
20
10
0
***
***
Naive MSCs
Peak EAE-MSCs
Chronic EAE-MSCs

Figure 2.5: Comparison of cultured EAE-MSCs with naïve MSCs. (A) Phase contrast images of passage 3 naïve MSCs versus peak and chronic EAE-MSCs showing cells have similar morphologies. **(B)** Both peak and chronic EAE-MSCs express common MSC markers and are CD45 negative like naïve MSCs. Data shown = mean + SEM, n = 3 experiments. **(C)** *(Left)* Representative images of naïve and EAE-MSCs differentiated into Oil-Red O positive adipocytes and Alkaline Phosphatase (ALP) positive osteoblasts. *(Right)* Slightly fewer peak EAE-MSCs differentiate into Oil-Red O stained adipocytes compared to naïve or chronic EAE MSCs, whereas differentiation of chronic EAE-MSCs into ALP-positive osteoblasts is significantly increased. Data shown = mean + SEM, n = 2 experiments. **P<0.01, ***P<0.005, One-way ANOVA. **(D)** *(Left)* Representative images of BrdU labeled naïve MSCs versus peak and chronic EAE-MSCs after a 16 hour BrdU pulse. *(Right*) The proportion of cells labeled with BrdU (16 hour pulse) is significantly higher for peak and chronic EAE-MSCs versus naïve MSCs. Data shown = mean + SEM, n = 3 experiments, ***P<0.005, One-way ANOVA. Scale bar in **(A)** = 50um, **(C)** and **(D)** = 20 um.

Figure 2.6

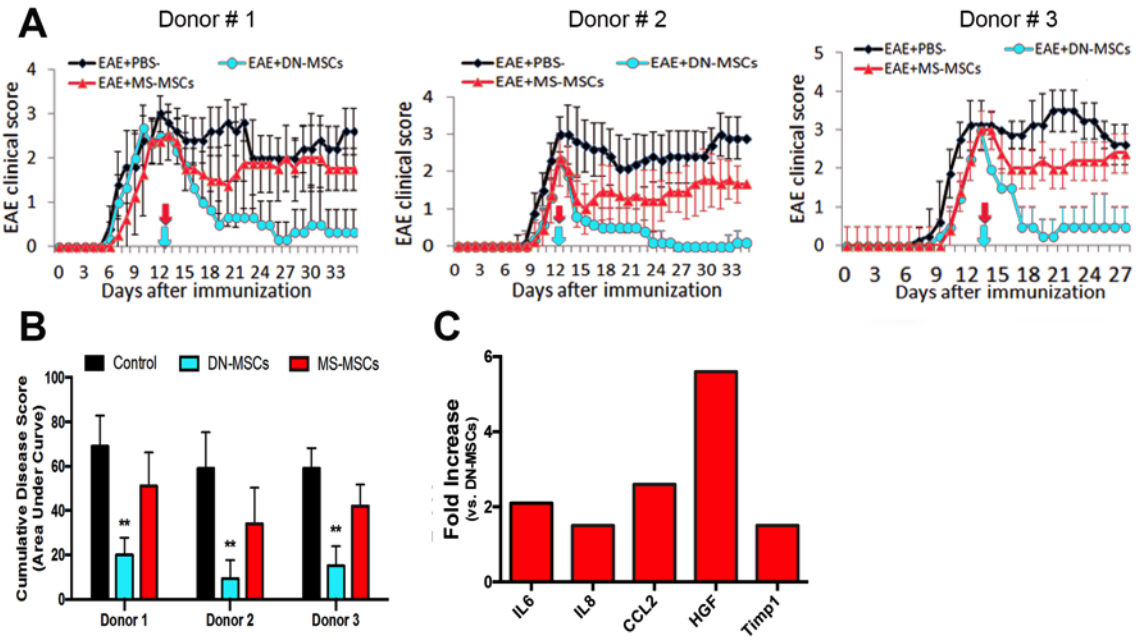
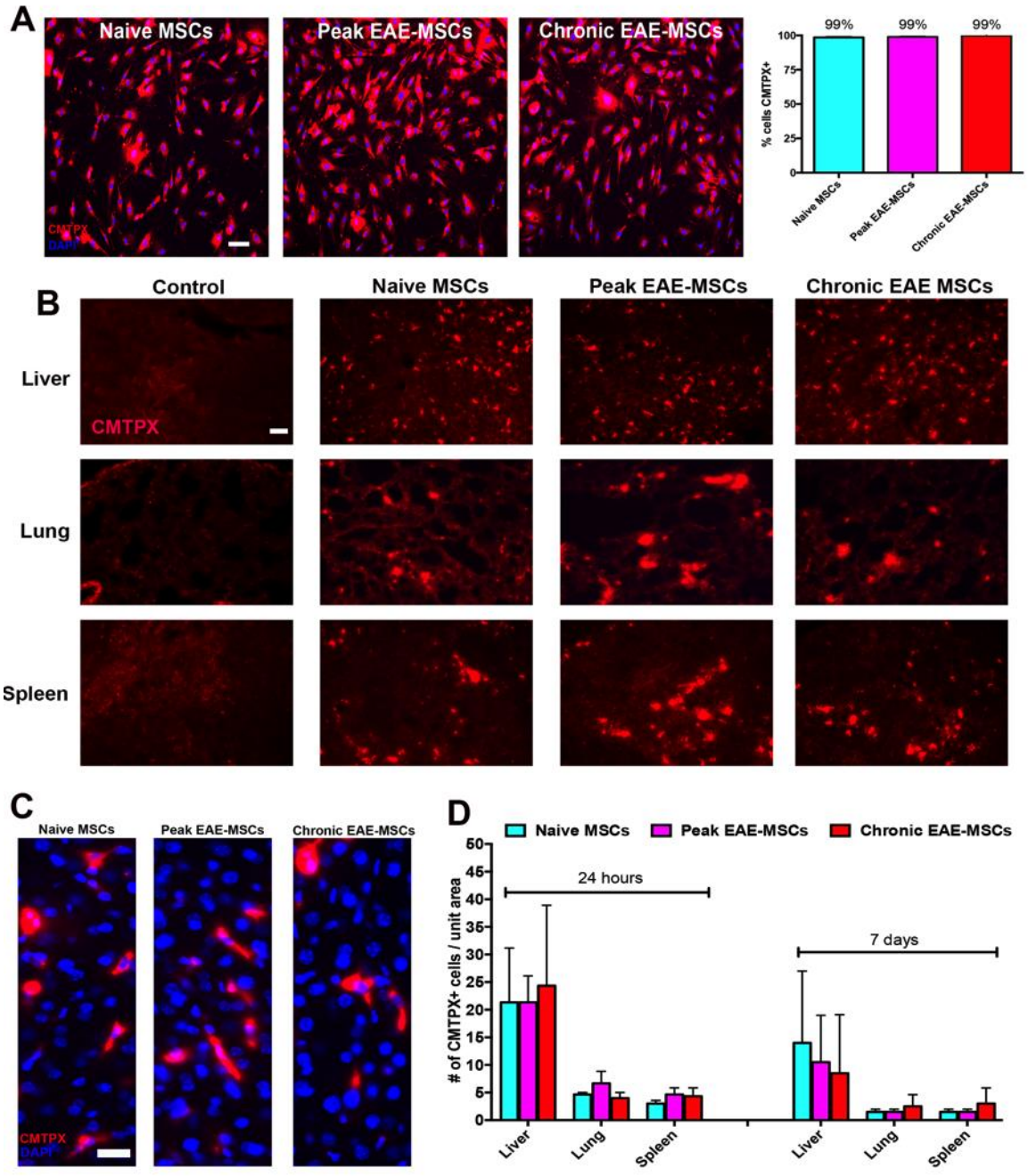

A
Donor # 1
Donor # 2
Donor # 3
EAE+PBS-
EAE+DN-MSCs
EAE+MS-MSCs
EAE clinical score
Days after immunization
B
Control
DN-MSCs
MS-MSCs
Cumulative Disease Score (Area Under Curve)
Donor 1
Donor 2
Donor 3
C
Fold Increase (vs. DN-MSCs)
IL6
IL8
CCL2
HGF
Timp1

Figure 2.6: MS-MSCs lack therapeutic efficacy compared to naïve MSCs in modulating EAE. (A) MSCs derived from MS patients (MS-MSCs) are less effective at improving functional recovery when transplanted into EAE mice compared to naïve MSCs (DN-MSCs) derived from healthy donors. MOG_{35-55} induced EAE mice were infused with 1x10^6 MSCs at 14 days post EAE induction (arrows), with MS-MSCs derived from 3 separate donors diagnosed with relapsing-remitting MS and DN-MSCs derived from corresponding sex and age-matched healthy donors. Experiments were carried out by Lianhua Bai (Case Western Reserve University); data shown = mean ± SEM, n = 11-13 mice per group. **(B)** EAE mice treated with DN-MSCs from each of the respective donors show a significant reduction in cumulative disease scores (area under the curve), whereas EAE mice treated with MS-MSCs show no significant difference versus saline treated controls. Data shown = mean + SEM, **P<0.01, One-way ANOVA. **(C)** Fold changes of proteins increased in conditioned medium from MS-MSCs relative to naïve MSCs; note conditioned medium from MS-MSCs contains higher levels of pro-inflammatory cytokines, including IL-6, IL-8, and CCL2. Fold changes were calculated by comparing spot intensity values from 2 separate antibody arrays per group, with each array treated with an independent sample. No fold decreases of any cytokine tested were observed in the arrays.

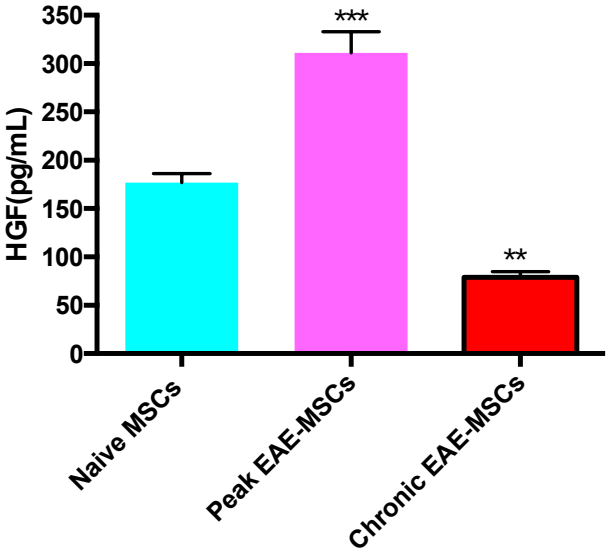
Supplemental Figure 2.1



Supplemental Figure 2.1: EAE-MSCs migrate and engraft into host tissues like naïve MSCs. (A) *(Left)* MSCs *in-vitro* 2 days after being labeled with the fluorescent cell tracking dye CMTPX; *(Right)* virtually all MSCs are CMTPX positive 2 days after labeling. Data shown at right = mean+ SEM, n= 2 experiments.

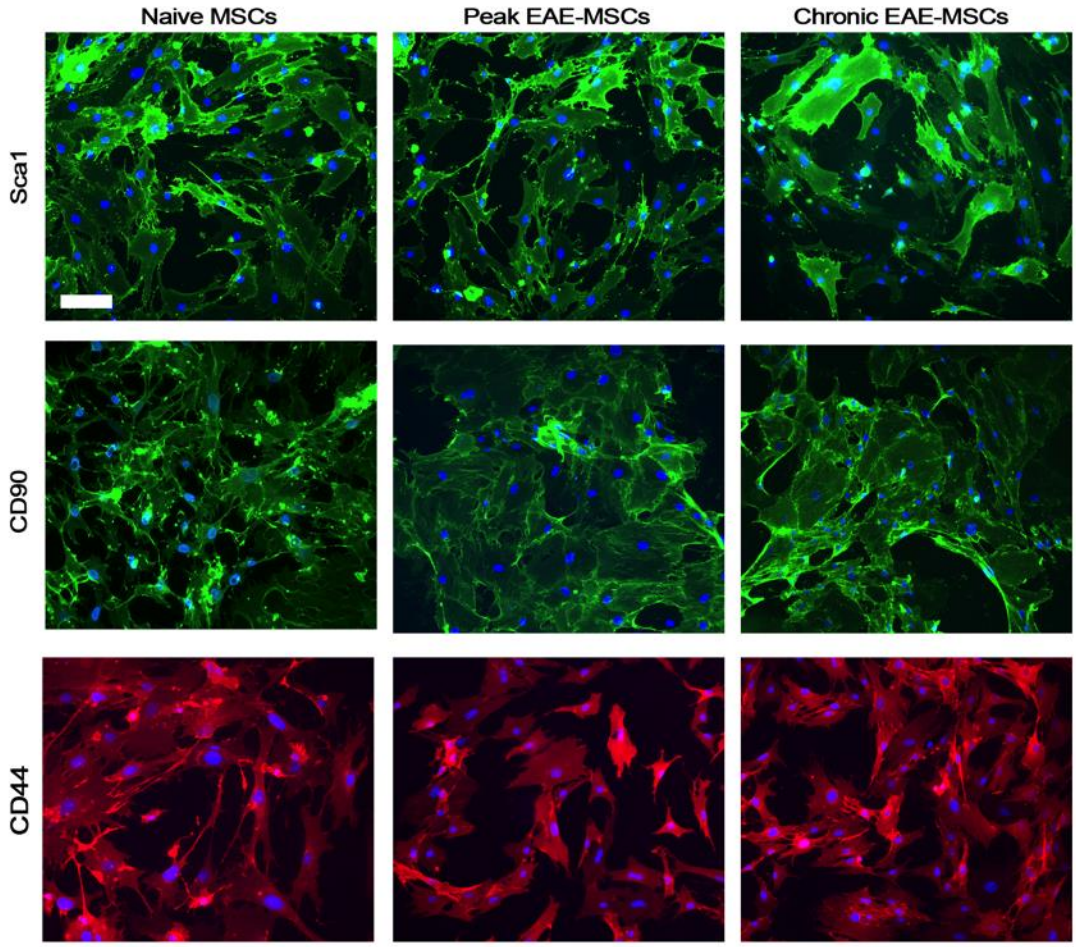
(B) Representative images of CMTPX positive MSCs in different tissues 24 hours after 0.5 x 10^6 labeled cells were infused into MOG_{35-55} EAE mice (control mice received saline). **(C)** Higher power images of CMTPX labeled MSCs co-localized with the nuclear marker DAPI in liver sections of EAE mice 24 hours after infusion. **(D)** No difference was found between the number of naïve MSCs versus the number of peak or chronic EAE-MSCs in each tissue either 24 hours or 7 days after infusion into EAE mice. Data shown = mean + SEM, quantified from 3 sections per mouse, n = 2-3 mice per group from 2 independent experiments. Scale bar in **(A) (B)** = 50 um, **(C)** = 20 um.

Supplemental Figure 2.2



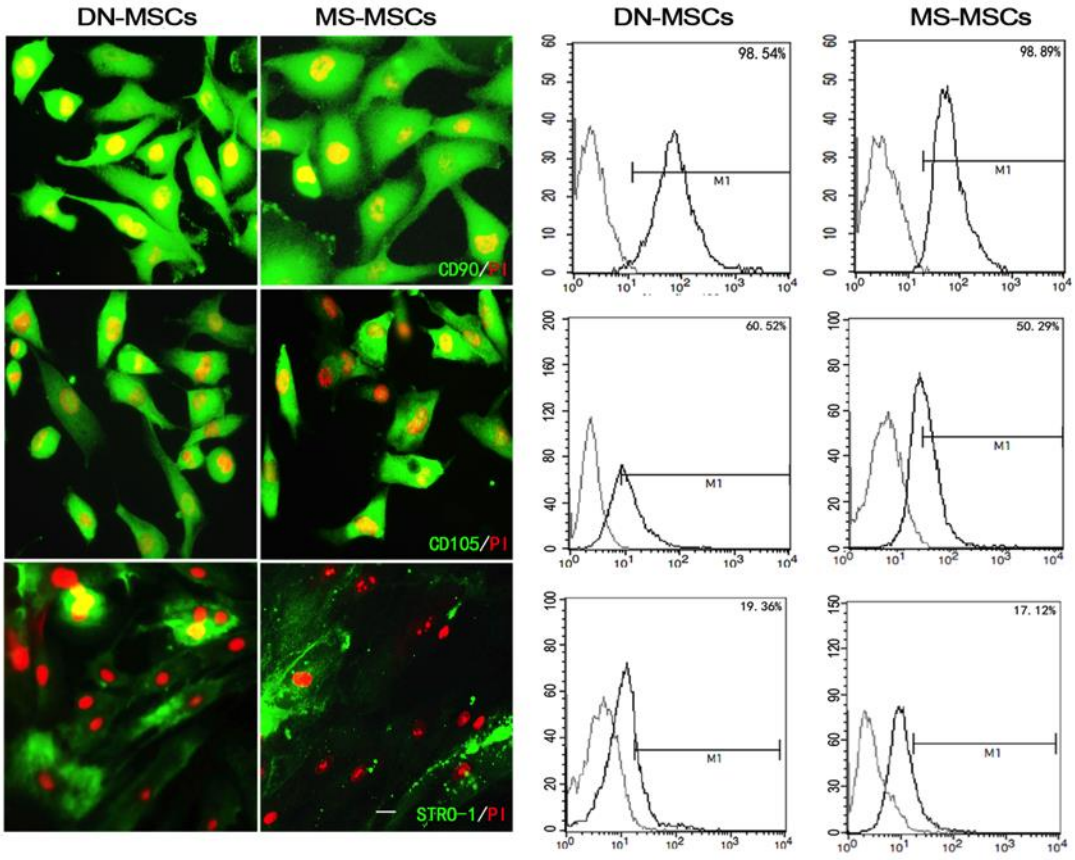
Supplemental Figure 2.2: EAE-MSCs differ from naïve MSCs in HGF secretion. Compared to naïve MSC CM, peak EAE-MSC CM has significantly higher levels of HGF, while chronic EAE-MSC CM has significantly lower HGF levels. Data shown = mean + SEM, n = 3 experiments, with HGF levels quantified via ELISA from 3 independent samples per group. **P<0.01, ***P<0.005, One-way ANOVA.

Supplemental Figure 2.3



Supplemental Figure 2.3: Immunocytochemistry of cultured EAE and naïve MSCs. Cultured EAE MSCs are like naïve MSCs in that they express common MSC markers, including Sca1, CD90, and CD44. Scale bar = 50 um.

Supplemental Figure 2.4



Supplemental Figure 2.4: MS-MSCs express common MSC markers like naïve MSCs. Expression of common MSC markers (CD90, CD105, and STRO-1) is similar for DN-MSCs and MS-MSCs. PI= propidium iodide, scale bar = 20um.

Chapter III:

Transcriptional profiling of mesenchymal stem cells identifies distinct neuroimmune pathways altered by CNS disease

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3.1: Abstract

Bone marrow mesenchymal stem cells (BM-MSCs) are an attractive cell based therapy in the treatment of CNS demyelinating diseases such as multiple sclerosis (MS). Preclinical studies in animal models demonstrate that rodent and human BM-MSCs can be highly effective at reducing clinical burden and enhancing recovery in experimental allergic encephalitis (EAE), a commonly used model of MS. Translation to the clinic using autologous BM-MSCs to treat MS patients has been disappointing however, as a number of recent clinical trials have not shown significant functional benefit following BM-MSC infusion. One possibility for the discrepancy between animal and human studies is the source of the cells. Recent studies suggest that BM-MSCs derived from MS patients or animals with EAE lack reparative efficacy compared to naïve cells. To define the differences between diseased and naïve MSCs, we have utilized RNA Seq to assess changes in gene expression between BM-MSCs derived from animals with EAE and those derived from healthy controls. We show that EAE alters the expression of a large number of genes in BM-MSCs and that changes in gene expression are more pronounced in chronic versus acute disease. Bioinformatic analysis revealed extensive perturbations in BM-MSCs in pathways related to inflammation and the regulation of neural cell development. These changes suggest that signals from EAE derived BM-MSCs inhibit rather than enhance remyelination, and in-vitro studies showed that conditioned medium from EAE MSCs fails to support the development of mature oligodendrocytes, the myelinating cells of the CNS. Together our data

provide insight into the failure of autologous BM-MSCs to promote recovery in MS and support the concept of utilizing non-autologous MSCs in future clinical trials.

3.2: Introduction

Mesenchymal stem cells (MSCs) are a multipotent class of stem cell with potential as a cell-based therapy for both autoimmune and neurological diseases[54,121]. While MSCs can be derived from a variety of tissues, one of the most common and readily available sources of MSCs for clinical applications is the bone marrow[27]. Bone marrow derived MSCs (BM-MSCs) are a non-hematopoietic type of stem cell that can be collected and expanded in-vitro from both human donors and experimental animal models[2].

BM-MSCs possess a remarkable capacity to modulate the responses of different immune and neural cell types[193]. For example, BM-MSCs have strong immunosuppressive functionality[177], and can inhibit the proliferation and activation of a range of immune cells including T-cells, B-cells, and macrophages[35,71]. These immunosuppressive capabilities appear to be mediated in large part by the secretion of a broad spectrum of anti-inflammatory chemokines and cytokines by BM-MSCs [39,175]. In addition to their immunomodulatory capabilities BM-MSCs are also neuroprotective and may influence the development of distinct classes of neural cells in the CNS. For example, BM-MSCs as well as conditioned medium from BM-MSCs biases neural stem cell fate away from an astroglial fate and promotes neural stem cell differentiation into oligodendrocytes and/or neurons[116,117]. In addition, BM-MSCs and BM-MSC conditioned medium enhances differentiation of

oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes[118]. The effects of BM-MSCs on neural cells also appears to be mediated by secreted factors, including growth factors like hepatocyte growth factor (HGF)[114].

Due to their immunosuppressive and neuromodulatory properties, BM-MSCs are considered to be a potentially powerful cellular therapy for inflammatory and neurodegenerative diseases of the CNS such as multiple sclerosis (MS) [156,194]. Experimental autoimmune encephalomyelitis (EAE) is a commonly used model of MS that results in a predictable onset of functional deficits that correlate with immune mediated CNS demyelination[195]. When BM-MSCs are transplanted intravenously into EAE they rapidly halt disease progression and improve motor recovery and function[117,146,149]. Although the mechanisms mediating this functional improvement are not well defined, transplanted BM-MSCs appear to suppress the immune response as well as the activation and migration of peripheral immune cells into the CNS of EAE mice. In addition, BM-MSCs attenuate demyelination and possibly promote remyelination and axonal survival[117,146,147]. The therapeutic benefits of BM-MSCs in EAE appear to be mediated by their secretion of anti-inflammatory and pro-regenerative factors[114,150].

Based on their success in treating animal models of MS like EAE, BM-MSCs have moved into clinical trials for MS patients. The majority of these trials use autologous MSCs (derived from MS patients)[180], in contrast to most previous studies in EAE animal models, which used MSCs from healthy human donors or healthy animals ("naïve MSCs") [117,146,147,149]. New evidence suggests however that inflammatory disease can alter the therapeutic functionality of BM-MSCs[58]. In our

recent study, BM-MSCs derived from EAE mice and MS patients were shown to have reduced therapeutic efficacy compared to naïve MSCs in their ability to ameliorate disease progression in EAE (Chapter II). The MSCs derived from donors with ongoing disease secreted higher levels of many pro-inflammatory cytokines, and demonstrated less immunosuppressive potential in-vitro (Chapter II).

Defining how diseases like MS affect the functionality of BM-MSCs is crucial in evaluating whether autologous MSCs represent a viable strategy for treating MS and other inflammatory diseases. The current study was undertaken to gain insight into how disease affects BM-MSCs at a transcriptional level though RNA-Sequencing experiments to compare gene expression profiles between naïve MSCs and MSCs derived from EAE mice (EAE-MSCs). Alternations in the expression levels of a large number of genes between EAE-MSCs and naïve MSCs were detected using this approach, with bioinformatic analysis revealing significant perturbations in pathways related to inflammation and immune cell regulation as well as myelination and neural cell fate. Such changes in gene expression in EAE-MSCs correlated with important functional consequences, as conditioned medium from EAE-MSCs failed to promote oligodendrogenesis in vitro and instead favored astroglial expansion. Collectively, our data underscores how inflammatory diseases like EAE dramatically alter BM-MSCs at a transcriptional and functional level, raising concern about the efficacy of using autologous MSCs as a cellular therapy for MS.

3.3: Methods

EAE induction and scoring

EAE was induced in 10 week-old female C57BL/6 mice (Jackson Laboratory: 000664) using Hooke Labs MOG_{35-55} EAE Induction kits according to the manufacturer's protocol. Briefly, mice were immunized via subcutaneous injection of 200ul of MOG_{35-55} peptide in complete Freund's adjuvant. Pertussis toxin (250 ng) was injected intraperitoneally at 2 and 24 hours post immunization. Animals began showing signs of paralysis 9-11 days post immunization, and were graded by blinded observers according to a previously described clinical index[114]: 1 = limp tail, 2 = hind limb weakness, 3 = plegia of one limb, 4 = plegia of two limbs, 5 = moribund or dead.

MSC culture and RNA isolation

Mesenchymal stem cells were isolated and cultured from the bone marrow of MOG_{35-55} – induced EAE mice at 14 days (peak EAE-MSCs) or 28 days (chronic EAE-MSCs) after immunization, with the animals having a clinical score of 4. Naïve MSCs were cultured from non-immunized, age-matched C57BL/6 mice. Growth medium for all cultures consisted of α-MEM with GLUTAMAX (Gibco) supplemented with 10% MSC-qualified fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco).

BM-MSCs were isolated as previously described (see *Methods* Chapter II). Briefly, bone marrow from the tibias and fibulas was collected by flushing the central canal of the bone with a 26 x g syringe containing fresh growth medium.

Bone marrow cells were then seeded in P75 flasks (Corning) at a concentration of 2 X 10^5 cells/cm^2 and grown at 37°C with 5% CO2. Flasks were washed twice with media 48 hours later to remove non-adherent cells, and the medium was changed every 2-3 days. Cells were passaged using 0.25% Trypsin/EDTA (Gibco) for 2 minutes at 37°C and re-plated in P75 flasks at a concentration of 1 x 10^4 cells/cm^2.

At the third passage (21 to 28 days in-vitro), 1 x10^6 MSCs were collected for RNA extraction using RNeasy Mini kits (Qiagen) according to the manufacturer ‘s protocol. An independent culture preparation consisting of MSCs derived from 2-3 mice was used for each RNA sample. Conditioned medium (CM) was likewise collected from passage 3 naïve and EAE-MSCs between 21 and 25 days in vitro, with unconditioned medium collected as a control. All CM samples were filtered through a 0.22 um filter, centrifuged at 10,000 x g for 10 minutes, then frozen at -80 C. Conditioned medium was collected from a different culture preparation for each experiment.

RNA Sequencing

RNA libraries were prepared using TruSeq Stranded Total RNA Library Prep kits with Ribo-Zero Gold according to the manufacturer’s protocol. Samples were analyzed for whole-transcriptome analysis using Illumina HiSeq 2500 v2 (RAPID RUN), with a read length of 1x50 bp for gene expression analysis.

Mapping of reads and differential expression analysis

Mapping of reads and differential expression analysis was done in BaseSpace using the RNA Express Legacy application (Version 1.0.0). Reads were mapped to the *Mus musculus* – UCSC (build mm10) reference genome. Criteria for differentially expressed genes included a fold change of 1.5 or greater, q-value ≤ 0.05, and a normalized mean expression count ≥ 10.

Gene ontology and pathway analysis

Gene ontology analysis of the top 2000 differentially expressed genes (ranked by greatest magnitude fold change) was performed using DAVID (Version 6.7). Further gene pathway analysis of all differentially expressed genes was performed using MetaCore. Heat maps were constructed using OriginLab Pro (version 2017) with genes mapped according to their respective z-score.

Neural cell culture and immunocytochemistry

Dissociated neural cell cultures were prepared as previously described[117]. Briefly, spinal cords from P3 C57/B6 mice were dissected manually and chemically dissociated in 0.1% Trypsin/EDTA for 20 minutes and passed through a 30 um cell strainer to generate single cell suspension of neural cells. These cells were then plated onto poly-L-lysine coated glass coverslips and grown in neural cell culture medium (DMEM supplemented with 10% FBS and 1% antibiotic).

To test the effects of MSC CM of neural cell development and differentiation, cells were cultured in medium that contained 50% neural cell culture media and

50% MSC CM or unconditioned medium (controls) for 4 days. Coverslips were fixed for 10 minutes in 4% paraformaldehyde and labeled for respective cell markers using a previously published protocol[117]. Antibodies used include: rat anti-MBP (Abcam: ab7349 at 1:100), rabbit anti-GFAP (Millipore: ab5804 at 1:800), and rabbit anti-NG2 (Millipore: ab5320 at 1:200). To quantify the percentage of cells labeled with each respective marker, 6 fields per coverslip were counted, with 2-3 coverslips per experiment over 2 independent experiments.

Statistical Analysis

For RNA sequencing and expression experiments, statistical analysis was performed via the RNA express app in BaseSpace, with q-values (multiplicity adjusted p-values) of ≤ 0.05 considered statistically significant. For cell counting experiments, statistical tests were performed using GraphPad Prism 6, with Dunnett's multiple comparison tests performed post-hoc for one-way ANOVAs and p-values of ≤ 0.05 considered statistically significant.

3.4: Results

RNA-Seq reveals disease-related gene expression changes in BM-MSCs in EAE

RNA-Seq analysis was conducted on MSCs derived from the bone marrow of MOG_{35-55}-induced EAE mice at two distinct phases of disease: at 14 days post induction during the peak of the disease ("peak EAE-MSCs") and at 28 days post induction during the chronic phase of the disease ("chronic EAE-MSCs"). These specific time points were selected based on a previous study showing BM-MSCs

derived from MOG-induced EAE mice at these stages of disease have a diminished capacity to suppress inflammation and ameliorate EAE when compared to MSCs derived from non-diseased donors (Chapter II). To identify transcriptional changes underlying the reduced therapeutic functionality in EAE-MSCs, gene expression profiles of both peak and chronic EAE-MSCs were compared to expression profiles of naïve MSCs derived from healthy, sex-matched littermate controls.

To ensure that control MSCs were appropriately age-matched to their respective disease counterparts, different cultures of naïve MSCs were derived from healthy littermates at the time of peak EAE and two weeks later for chronic EAE. At least three biological replicates were used for gene expression comparisons and analysis. A different primary culture preparation, consisting of 1x10^6 BM-MSCs expanded for 3 passages (21-28 days in-vitro) and derived from 2-3 mice, was used for each replicate.

RNA-Seq analysis revealed widespread changes in gene expression patterns in EAE-MSCs that correlated with disease progression. Differentially expressed genes (DEGs) between EAE-MSCs and naïve MSCs were defined by a magnitude fold change of 1.5 or greater, q-value ≤ 0.05, and a normalized mean expression count ≥ 10. Using these criteria, RNA-Seq analysis identified 2,337 DEGs between peak EAE-MSCs and naïve MSCs (Figure 3.1A). Chronic EAE-MSCs had more pronounced changes in gene expression, with 5, 837 DEGs between chronic EAE-MSCs and naïve MSCs (Figure 3.1A). The magnitude of individual gene expression changes in chronic EAE-MSCs also tended to be higher than the magnitude of gene expression changes in peak EAE-MSCs (Figure 3.1A). For example, when comparing chronic EAE-MSCs

to naïve MSCs, 34% of DEGs showed a fold change of 3 or greater, whereas only 12% of DEGs showed a fold change of 3 or greater when comparing peak EAE-MSCs to naïve MSCs. Unsupervised hierarchical clustering showed peak EAE-MSC and chronic EAE-MSC replicates clustered together independently of naïve MSC replicates (Figure 3.1B). Our data indicates that both peak EAE-MSCs and chronic EAE-MSCs have distinguishable and reproducible changes in gene expression patterns compared to naïve MSCs.

EAE-MSCs up-regulate genes linked to inflammation and immune activation

The immunomodulatory and regenerative capacity of BM-MSCs in models of MS is derived in large part from their ability to secrete a wide array of diverse chemokines, cytokines, and trophic factors that may act on a act on a variety of cellular targets, including adaptive and innate immune cells involved in inflammation [39]. To identify cellular and biological processes associated with the gene expression changes in EAE-MSCs, gene ontology (GO) enrichment analysis was performed on the DEGs found between naïve and EAE MSCs.

Gene ontology analysis suggested many DEGs are secreted factors or linked to the secretory pathway. For example, comparison of the distribution of DEGs amongst different cellular compartments revealed a large number of DEGs for both peak and chronic EAE-MSCs were associated with vesicles or the endoplasmic reticulum (ER) and Golgi apparatus (Figure 3.2). Out of the top five specific cellular compartments DEGs mapped to, vesicles and the ER/Golgi apparatus were third and fourth respectively for both peak EAE-MSCs and chronic EAE-MSCs. The only

compartments with a higher level of associated DEGs were the nucleus and cell membrane (Figure 3.2).

Gene ontology enrichment analysis revealed many DEGs in EAE-MSCs were linked to immune activation and inflammation. For example, analysis of the DEGs for both peak EAE-MSCs and chronic EAE-MSCs showed that the third most enriched term (ranked by p-value) was "immune response" (Figure 3.3A). Many of the GO terms associated with DEGs for both peak and chronic EAE-MSCs related to aspects of cell-mediated immunity or inflammatory response, including terms like "lymphocyte mediated immunity, " "leukocyte chemotaxis", "positive regulation of immune response" and "activation of immune response," (Figure 3.3A).

RNA-Seq analysis revealed an up-regulation of many immune and pro-inflammatory genes in EAE-MSCs compared to naïve MSCs. These included a number of inflammatory chemokines, cytokines, and other factors previously shown to be important in mediating MSC immunomodulation. For example, peak and chronic EAE-MSCs showed an up-regulation of multiple pro-inflammatory chemokines including Cxcl1 and Cxcl5, as well as interleukins important in T-cell activation such as IL-6 (Figure 3.3B)[184]. This data is consistent with a previous study showing that EAE-MSCs have elevated expression of each of these pro-inflammatory cytokines at the protein level and secrete higher levels of these factors compared to naïve MSCs (Chapter II). Previous studies have demonstrated that MSC-induced activation of the complement system after transplantation may reduce MSC viability and compromise their therapeutic efficacy[196]. RNA-Seq analysis showed higher gene expression of many complement system factors in EAE-MSCs compared to naïve

MSCs, suggesting enhanced complement activation by EAE-MSCs (Figure 3.3B). The therapeutic efficacy of BM-MSCs is also influenced by Toll-like receptor (TLR) signaling that can affect the immunomodulatory characteristics of MSCs[197,198]. The expression of many TLR genes was increased in EAE-MSCs compared to naïve MSCs (Figure 3.3B), which may contribute to the functional differences between the two cell populations.

Further analysis of DEGs was performed using MetaCore to identify specific biological pathways associated with the gene expression changes observed in EAE-MSCs. MetaCore analysis revealed many DEGs in EAE-MSCs were associated with pathways important in immune cell signaling and immune response. For example, gene expression changes in peak EAE-MSCs and chronic EAE-MSCs were linked to the regulation of T-cell differentiation and activation, and to signaling pathways important in T-cell polarization (Figure 3.4A). These results are consistent with previous findings from GO enrichment analysis of the DEGs in EAE-MSCs that found immune and inflammatory processes were significantly over-represented. Together the comparative analysis of gene expression between naïve and EAE BM-MSCs reveals an extensive change in immune associated genes. Such data is consistent with the notion that EAE-MSCs are more pro-inflammatory than naïve MSCs and have reduced immunosuppressive functionality compared to naïve MSCs both in-vitro and in-vivo after transplantation into EAE mice (Chapter II).

EAE-MSCs differentially regulate neural cells and oligodendrogenesis

In addition to being immunomodulatory, previous studies have suggested that naïve MSCs have the capacity to influence neural cell fate[194]. Metacore analysis of the DEGs between EAE-MSCs and naïve MSCs identified changes in pathways important in regulating neural cell development. In both peak and chronic EAE-MSCs, multiple DEGs were associated with "oligodendrocyte differentiation," and expression changes in peak or chronic EAE-MSCs were linked to "neural stem cell lineage commitment," "astrocyte differentiation," and "neurogenesis" (Figure 3.4A). Signaling pathways important in neural cell differentiation and development, including WNT, EGFR, and PDGF signaling[199], were also associated with gene expression changes in peak and chronic EAE-MSCs (Figure 3.4A).

EAE-MSCs showed changes in gene expression for multiple factors known to regulate neural cell development and oligodendrocyte differentiation. For example, both peak EAE-MSCs and chronic EAE-MSCs up-regulated expression of Fgf and Bmp genes, including Fgf2, Bmp4, and Bmp6 (Figure 3.4B). FGF and BMP proteins are important in influencing neural cell fate, as they favor the differentiation of neural stem cells (NSCs) and oligodendrocyte precursor cells (OPCs) into astrocytes versus their differentiation into oligodendrocytes [200,201]. Chronic EAE-MSCs also down regulated expression of genes like Tgfb1 and Igf1, which code for growth factors that promote oligodendrocyte maturation and differentiation[202,203].

Such changes in gene expression suggest EAE-MSCs may differentially influence neural cell fate compared to naïve MSCs. To test this hypothesis,

dissociated cultures of spinal cord neural cells were treated with conditioned medium (CM) from naïve MSCs or EAE-MSCs. Cultures were treated for 4 days (with unconditioned medium used as a control) and the proportion of GFAP+ astrocytes and MBP+ oligodendrocytes was assayed to determine whether factors secreted by EAE-MSCs differentially bias neural cell development.

While cultures grown in CM from naïve MSCs had increased numbers of mature oligodendrocyte compared to controls, cultures grown in CM from chronic EAE-MSCs had reduced numbers of mature oligodendrocytes and increased numbers of astrocytes (Figure 3.4C). Parallel studies using CM from Peak EAE-MSCs showed a less significant effect with a slight increase in the number of astrocytes and no reduction in the number of oligodendrocytes (Figure 3.4C). Changes in the relative number of oligodendrocytes in cultures treated with CM from chronic EAE-MSCs appeared to reflect changes in oligodendrocyte maturation since no significant effect on the number of NG2+ oligodendrocyte precursors was seen compared to controls (Figure 3.4C). No changes were seen in the relative number of neurons, which at less than 3% accounted for a very small percentage of cultured cells (data not shown). Our data suggests that the failure of EAE-MSCs to promote recovery in animal models of CNS demyelination may be associated in part with their reduced capacity to promote the development of mature oligodendrocytes.

Changes in gene expression in BM-MSCs during EAE correlate with disease progression

RNA-Seq analysis suggests that gene expression changes in BM-MSCs during EAE evolve with disease progression. For example, the number of DEGs is much higher when comparing chronic EAE-MSCs to naïve MSCs versus comparing peak EAE-MSCs to naïve MSCs. To investigate what genes are differentially expressed between peak and chronic EAE-MSCs, expression profiles of peak EAE-MSCs and chronic EAE-MSCs were compared to each other using previous criteria for DEGs: magnitude fold change of 1.5 or greater, q-value ≤ 0.05, and a normalized mean expression count ≥ 10.

RNA-Seq analysis revealed BM-MSCs show continual changes in gene expression patterns throughout EAE progression. There were 3, 736 DEGs between peak EAE-MSCs and chronic EAE-MSCs (Figure 3.5A). Consistent with comparative studies between naïve and EAE-MSCs, GO analysis identified many of these genes as being associated with immune or inflammatory processes (Figure 3.5B). Also, GO analysis suggested many of these DEGs are associated with processes involved in cell uptake and secretion, as "phagocytosis," "membrane invagination," and "endocytosis" were among top GO terms (Figure 3.5B).

Many of the DEGs found between peak and chronic EAE-MSCs were immune or inflammatory genes. These include some of the same chemokine and cytokine genes previously found elevated in EAE-MSCs in comparison to naïve MSCs. For example, chronic EAE-MSCs had elevated levels of expression of pro-inflammatory cytokine genes such as Cxcl1, Cxcl5, and Cx3cl1 compared to peak EAE-MSCs (Figure

3.5C). Not all gene expression was elevated in chronic EAE-MScs. Several Toll-like receptor genes and compliment component genes were down regulated in chronic EAE-MSCs compared to naïve MSCs (Figure 3.5C).

Overall, there was considerable consistency in the genes whose expression changed in BM-MSCs during EAE. For example, of the 2,337 DEGs identified between peak EAE-MSCs and naïve MSCs, 65% of these (1,498 / 2,337) were also differentially expressed between chronic EAE-MSCs and naïve MSCs (Figure 3.5D). Furthermore of the 5,837 DEGS found between chronic EAE-MSCs and naïve MSCs, 80% of these genes (2,979/3,736) were differentially expressed when comparing peak EAE-MSCs to chronic EAE-MSCs. In total, there were 988 common DEGs in BM-MSCs throughout all time points analyzed during EAE (Figure 3.5D).

Bioinformatic analysis revealed a high degree of conservation in the transcriptional regulators associated with the DEGs identified in EAE-MSCs. For example, the transcription factor SP1 was most significantly associated with the DEGs identified in both peak EAE and chronic EAE-MSCs, and both peak EAE-MSCs and chronic EAE-MSCs showed a high degree of similarity in other transcription factors linked to their respective DEGs (Figure 3.5E). Overall the current bioinformatic analysis suggests the changes in gene expression by BM-MSCs during EAE and the transcriptional regulators associated with these changes are highly conserved.

3.5: Discussion

Utilizing RNA-Sequencing, genome-wide expression profiles of BM-MSCs derived from healthy and EAE mice have been analyzed and compared for the first time to provide a better understanding of the transcriptional and functional changes BM-MSCs undergo during inflammatory disease. Widespread gene expression changes were detected by RNA-Seq analysis in BM-MSCs derived from animals with EAE compared to naive cells derived from non-diseased animals. The degree of altered gene expression correlated with disease progression. Many of the most pronounced increases in gene expression were in genes involved in the regulation of the immune system and inflammatory processes. Bioinformatic analysis also identified important changes in EAE-MSCs in genes associated with pathways that influence neural cell fate and antagonize oligodendrocyte differentiation. Consistent with these changes in gene expression, conditioned medium from EAE BM-MSCs reduced the number of mature oligodendrocytes that developed in cultures of neonatal spinal cord, while conditioned medium from naïve BM-MSCs increased the number of oligodendrocytes in parallel cultures.

Mesenchymal stem cells have many characteristics that make them suitable for clinical application in a number of diseases[204]. In order to ensure maximal benefit of such trials it is important that the most appropriate cells are used and the cells should be well defined as effectively as possibly. The gene expression changes identified between naive BM-MSCs and MSCs derived from an animal model of multiple sclerosis (EAE) are potentially important given the majority of clinical trials for MS patients use autologous MSCs that are likely more equivalent to EAE

derived MSCs. While preliminary results from these clinical trials report good safety data in transplanting autologous MSCs into MS patients, most report limited therapeutic efficacy[164,166,167,205] prompting questions about whether autologous MSCs from MS patients (MS-MSCs) are equivalent to allogeneic MSCs from healthy patients (naïve MSCs) in their functional characteristics and therapeutic potential[163].

Comparisons of the biological and functional characteristics of naïve and MS-MSCs have failed to reach consensus as to whether MS-MSCs show differences in characteristics like proliferation or cytokines secretion compared to naïve MSCs[169-171]. One recent study reported MS-MSCs have less immunosuppressive functionality compared to naïve MSCs in-vitro, and utilized microarrays to profile and compare gene expression in MS-MSCs and naïve MSCs[172]. Although there was a significant up-regulation of inflammatory genes in MS-MSCs, consistent with the RNA-Seq analysis of EAE-MSCs in the current study, the magnitude of changes was more limited. For example, less than 700 DEGs were seen in the MS study, compared to the greater than 2000 DEGs seen in EAE [172]. Interpreting genomic or functional data that compares MS-MSCs to naïve MSCs can be complicated as there are a range of variable that may alter the data and are difficult to control for. Factors such as genetic background or donor age may influence the functionality of BM-MSCs [206]. Likewise, current or previous medication regimes, disease duration, and disease severity may affect gene expression levels in autologous BM-MSCs. Together such variables may make defining significant functional or gene expression changes in BM-MSCs derived from patients with diseases such as MS difficult. The utilization of

a mouse model of disease such as EAE eliminates many of these variables, including genetic traits and prior disease history, and can provide clearer insight into the effects of disease on BM-MSCs.

Our study comparing BM-MSCs derived from MOG EAE mice to naïve MSCs from healthy, age-matched mice demonstrated that the EAE-MSCs had a reduced ability to ameliorate functional deficits caused by EAE (Chapter II). This lack of therapeutic efficacy of EAE-MSCs was correlated with increases in the relative levels of pro-inflammatory cytokines and decreases in the levels of anti-inflammatory growth factors EAE-MSCs secreted relative to naïve MSCs. This cytokine expression data is consistent with the present RNA-Seq analysis showing EAE-MSCs up-regulate a wide variety of inflammatory genes associated with immune response and immune cell activation. The up regulation of gene expression for Cxcl1, Cxcl5, and IL-6 correlates directly with the increased expression of these cytokines in conditioned medium from EAE-MSCs (Chapter II).

The duration of disease appears to directly influence BM-MSC gene expression. For example, the number of DEGs was significantly increased in chronic EAE-MSCs versus naïve MSCs, and the magnitude of the gene expression changes tended to be higher in chronic EAE-MSCs compared to naïve MSCs. Increases in gene expression were particularly evident with inflammatory genes. These results are consistent with previous findings that BM-MSCs isolated from MOG-induced EAE mice during the chronic phase of the disease express elevated levels of inflammatory cytokines and have less immunosuppressive potential than BM-MSCs isolated from EAE mice during the peak of the disease (Chapter II).

In addition to modulating the immune system, MSCs have been suggested to influence neural cell fate. Several studies demonstrate that naïve MSCs have the capacity to enhance oligodendrocyte differentiation and maturation in-vitro and remyelination in-vivo in demyelinating animal models[114,117,118]. It is currently unclear whether MSCs alter the fate of multipotent neural progenitor cells or directly promote the differentiation of oligodendrocyte precursors when transplanted in-vivo. Furthermore, the relative contributions of MSC mediated recovery in EAE from their capacity to influence neural cell fate and remyelination versus the ability of MSCs to suppress inflammation and the immune response is currently unclear[178,207]. RNA-Seq analysis revealed a significant up regulation in gene expression of a number of TGFβ family members including BMPs in EAE-MSCs. Developmental studies have defined a central role for BMP signaling in inhibiting the development of mature oligodendrocytes from their precursors[208], and here we show that conditioned medium from EAE-MSCs no longer promotes the appearance of mature oligodendrocytes. These observations raise the possibility that the reduced therapeutic functionality of EAE-MSCs may be due in part to the diminished ability of these cells to promote oligodendrocyte maturation and subsequent remyelination. Future studies are required to better address how EAE alters the capacity of BM-MSCs to modulate neural cells and promote myelin repair, and to determine whether the loss of therapeutic functionality in EAE-MSCs stems from changes in their immunosuppressive versus neuroprotective potential.

3.6: Conclusion

RNA-Seq analysis of BM-MSCs in EAE helps to support newly emerging evidence that disease alters the functional characteristics and therapeutic capacity of MSCs. EAE-MSCs show clear changes in gene expression patterns relative to naïve MSCs, including up-regulation of inflammatory genes. In addition, EAE-MSCs differentially modulate neural cell development and fail to promote the formation of mature oligodendrocytes in-vitro. We show that inflammatory disease dramatically alters BM-MSCs at both a transcriptional and functional level, raising concern about the efficacy of using autologous MSCs to treat MS.

3.7: Acknowledgements

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Figure 3.1

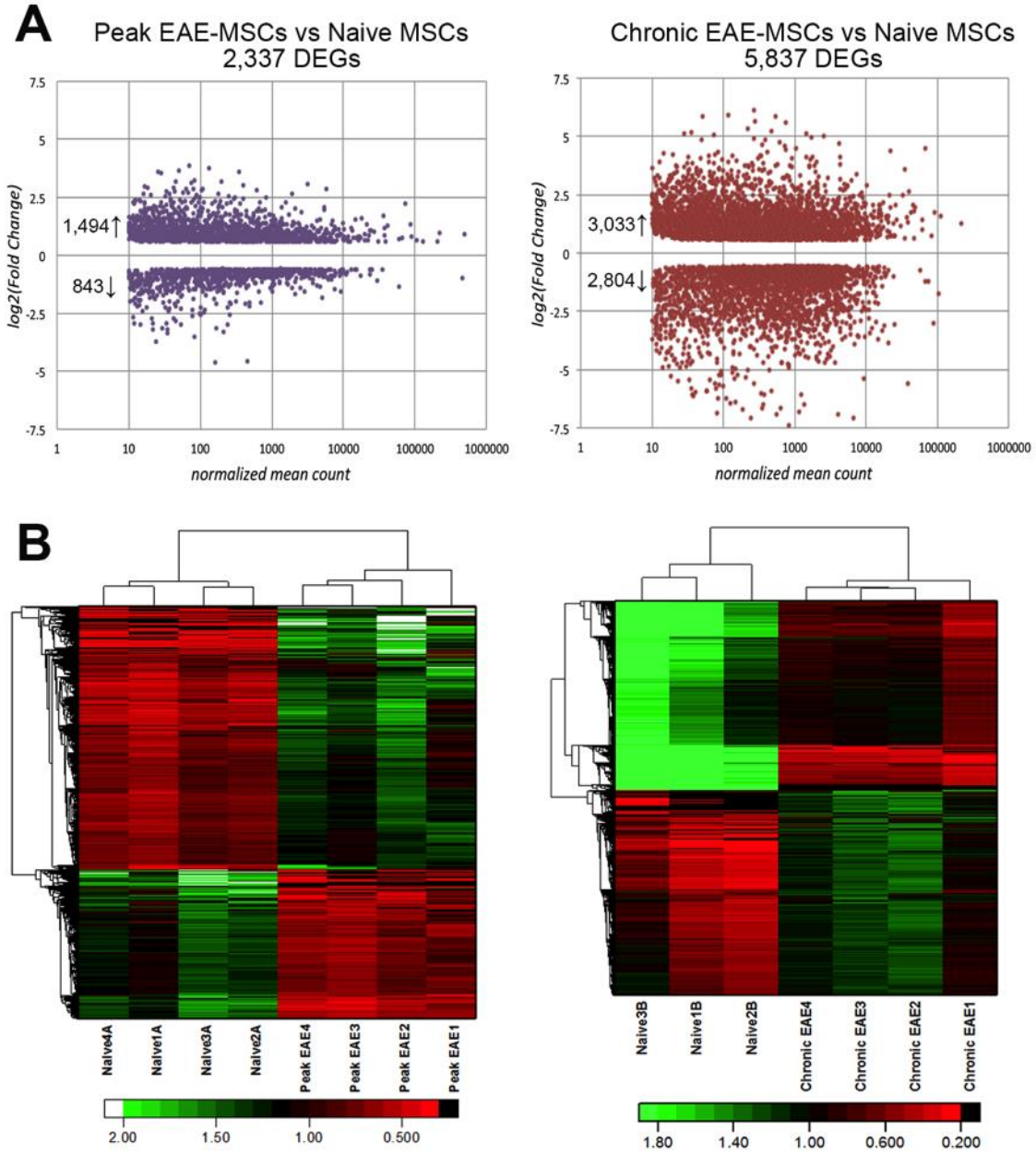
A
Peak EAE-MSCs vs Naive MSCs
2,337 DEGs
1,494↑
843↓
log2(Fold Change)
normalized mean count
Chronic EAE-MSCs vs Naive MSCs
5,837 DEGs
3,033↑
2,804↓
log2(Fold Change)
normalized mean count
B
Naive4A
Naive1A
Naive3A
Naive2A
Peak EAE4
Peak EAE3
Peak EAE2
Peak EAE1
2.00
1.50
1.00
0.500
Naive3B
Naive1B
Naive2B
Chronic EAE4
Chronic EAE3
Chronic EAE2
Chronic EAE1
1.80
1.40
1.00
0.600
0.200

Figure 3.1: RNA-Seq analysis of gene expression changes in BM-MSCs in EAE.

(A) Volcano plots depicting differentially expressed genes (DEGs) measured by RNA-Seq analysis of peak EAE-MSCs versus naïve MSCs (left) or chronic EAE-MSCs versus naïve MSCs (right). DEGs were defined by a magnitude fold change of 1.5 or greater, q-value ≤ 0.05, and a normalized mean expression count ≥ 10.

(B) Unsupervised hierarchical clustering of samples using Pearson correlation with average linkage, along with corresponding dendrogram and heatmap of DEGs. Peak EAE-MSC and chronic EAE-MSC replicates cluster together independently of naïve MSC replicates.

Figure 3.2

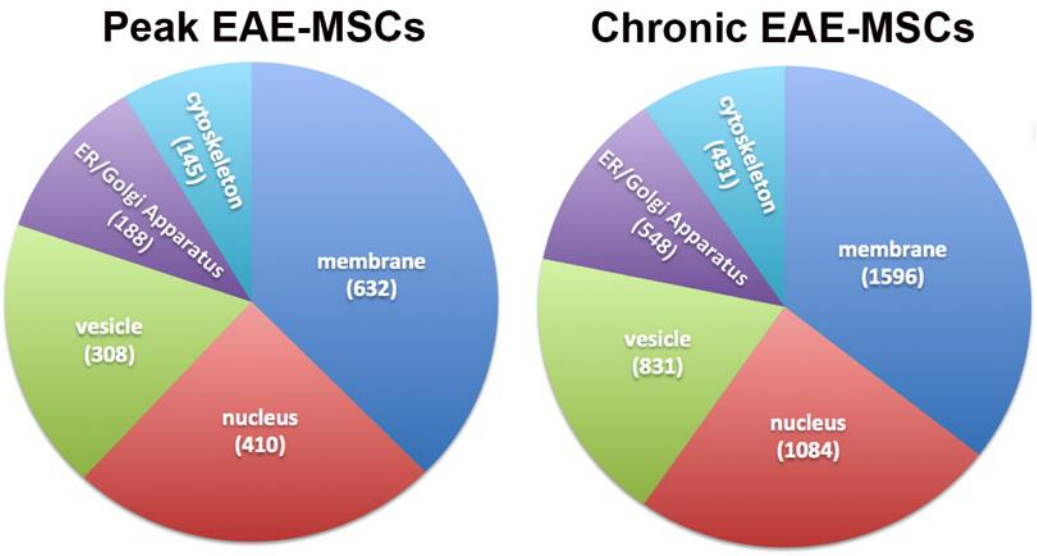
Peak EAE-MSCs
cytoskeleton (145)
ER/Golgi Apparatus (188)
membrane (632)
vesicle (308)
nucleus (410)
Chronic EAE-MSCs
cytoskeleton (431)
ER/Golgi Apparatus (548)
membrane (1596)
vesicle (831)
nucleus (1084)

Figure 3.2: Distribution of DEGs in different cellular locations. Top five specific cellular locations associated with the DEGs found between peak EAE-MSCs and naïve MSCs (left) or chronic EAE-MSCs and naïve MSCs (right). The number of DEGs associated with that location is given in parenthesis.

Figure 3.3

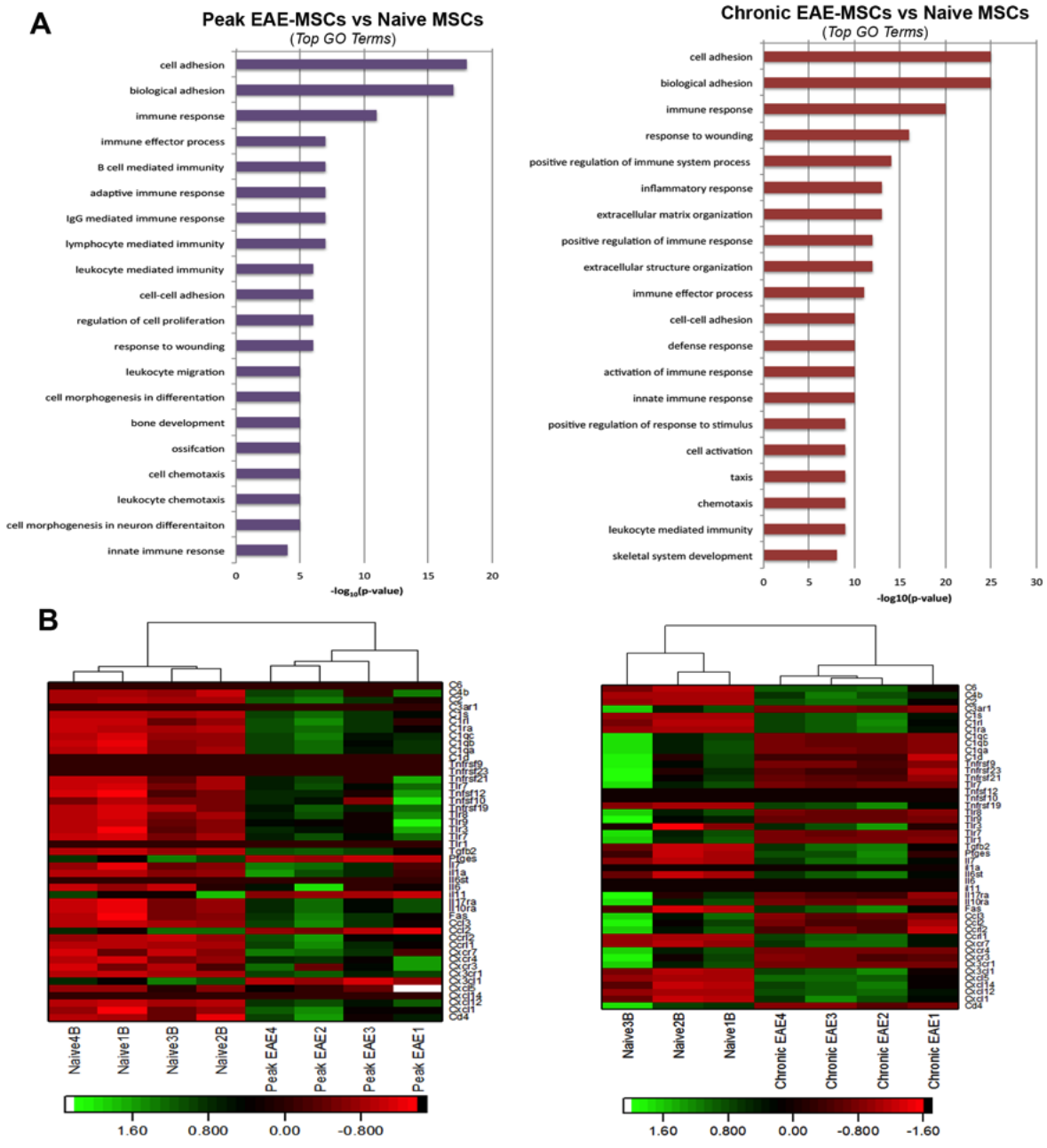
A
Peak EAE-MSCs vs Naive MSCs
(Top GO Terms)
cell adhesion
biological adhesion
immune response
immune effector process
B cell mediated immunity
adaptive immune response
IgG mediated immune response
lymphocyte mediated immunity
leukocyte mediated immunity
cell-cell adhesion
regulation of cell proliferation
response to wounding
leukocyte migration
cell morphogenesis in differentation
bone development
ossifcation
cell chemotaxis
leukocyte chemotaxis
cell morphogenesis in neuron differentaiton
innate immune resonse
0 5 10 15 20
-log10(p-value)
Chronic EAE-MSCs vs Naive MSCs
(Top GO Terms)
cell adhesion
biological adhesion
immune response
response to wounding
positive regulation of immune system process
inflammatory response
extracellular matrix organization
positive regulation of immune response
extracellular structure organization
immune effector process
cell-cell adhesion
defense response
activation of immune response
innate immune response
positive regulation of response to stimulus
cell activation
taxis
chemotaxis
leukocyte mediated immunity
skeletal system development
0 5 10 15 20 25 30
-log10(p-value)
B
Naive4B
Naive1B
Naive3B
Naive2B
Peak EAE4
Peak EAE2
Peak EAE3
Peak EAE1
1.60 0.800 0.00 -0.800
Naive3B
Naive2B
Naive1B
Chronic EAE4
Chronic EAE3
Chronic EAE2
Chronic EAE1
1.60 0.800 0.00 -0.800 -1.60

Figure 3.3: EAE-MSCs show changes in genes associated with inflammation and immune activation. (A) Top 20 gene ontology terms (ranked by p-value) for DEGs found between peak EAE-MSCs and naïve MSCs (left) or chronic EAE-MSCs and naïve MSCs (right). Most GO terms relate to immune processes, particularly immune activation and immune response. **(B)** Heatmap showing immune genes differentially expressed between peak EAE-MSCs and naïve MSCs (left) or chronic EAE-MSCs and naïve MSCs (right). Many inflammatory genes, including chemokines, cytokines and elements of the complement system, are up regulated in EAE-MSCs.

Figure 3.4

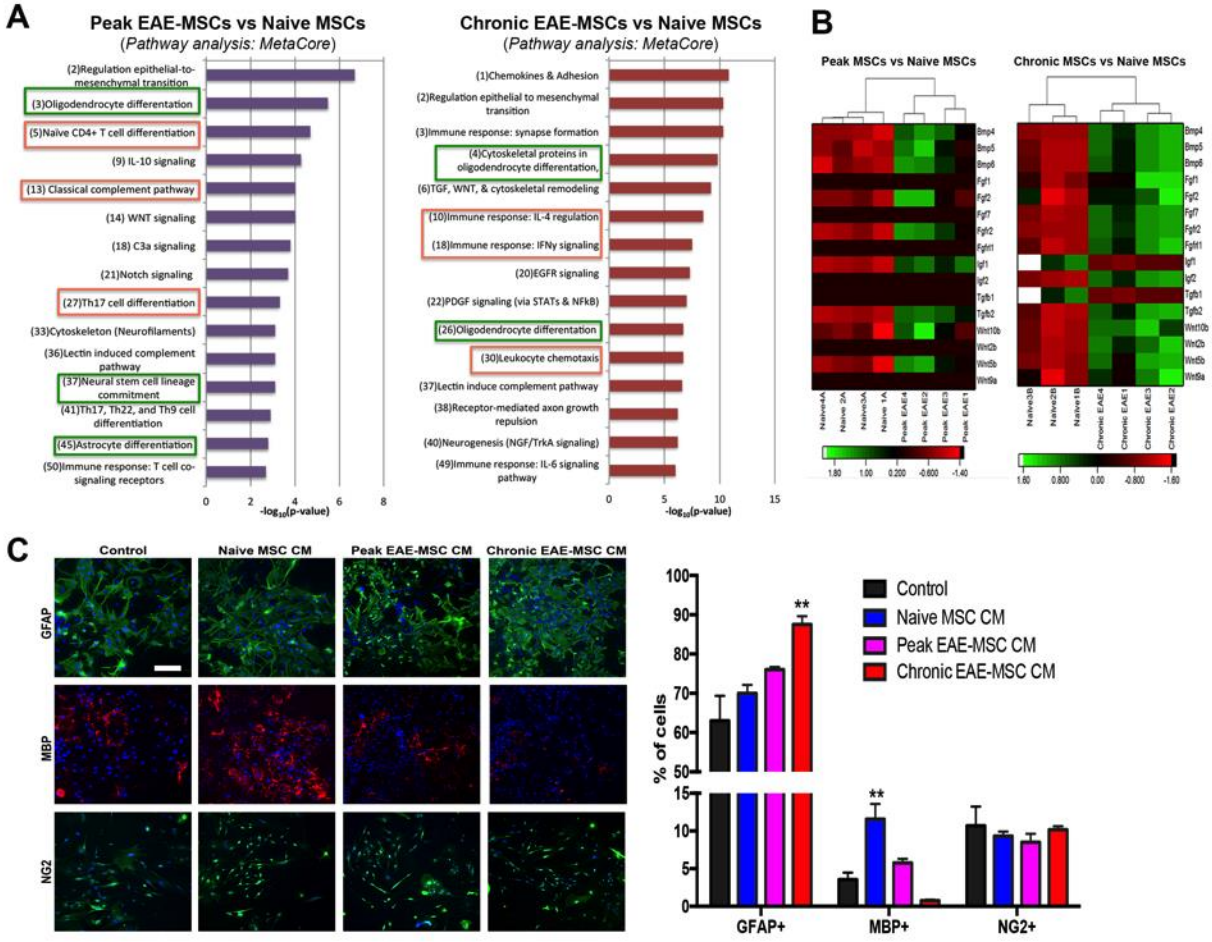
A
Peak EAE-MSCs vs Naive MSCs
(Pathway analysis: MetaCore)
(2)Regulation epithelial-to-mesenchymal transition
(3)Oligodendrocyte differentation
(5)Naive CD4+ T cell differentiation
(9) IL-10 signaling
(13) Classical complement pathway
(14) WNT signaling
(18) C3a signaling
(21)Notch signaling
(27)Th17 cell differentiation
(33)Cytoskeleton (Neurofilaments)
(36)Lectin induced complement pathway
(37)Neural stem cell lineage commitment
(41)Th17, Th22, and Th9 cell differentiation
(45)Astrocyte differentiation
(50)Immune response: T cell co-signaling receptors
-log10(p-value)
Chronic EAE-MSCs vs Naive MSCs
(Pathway analysis: MetaCore)
(1)Chemokines & Adhesion
(2)Regulation epithelial to mesenchymal transition
(3)Immune response: synapse formation
(4)Cytoskeletal proteins in oligodendrocyte differentation,
(6)TGF, WNT, & cytoskeletal remodeling
(10)Immune response: IL-4 regulation
(18)Immune response: IFNγ signaling
(20)EGFR signaling
(22)PDGF signaling (via STATs & NFkB)
(26)Oligodendrocyte differentation
(30)Leukocyte chemotaxis
(37)Lectin induce complement pathway
(38)Receptor-mediated axon growth repulsion
(40)Neurogenesis (NGF/TrkA signaling)
(49)Immune response: IL-6 signaling pathway
-log10(p-value)
B
Peak MSCs vs Naive MSCs
Chronic MSCs vs Naive MSCs
C
Control
Naive MSC CM
Peak EAE-MSC CM
Chronic EAE-MSC CM
GFAP
MBP
NG2
% of cells
GFAP+
MBP+
NG2+

Figure 3.4: EAE-MSCs differentially regulate neural cell development and oligodendrocyte formation. (A) Top pathways (ranked by p-value) associated with the DEGs found between peak EAE-MSCs and naïve MSCs (left) or chronic EAE-MSCs and naïve MSCs (right). The number in parenthesis next to the pathway identifies statistical ranking assigned by MetaCore. Notable immune and neural cell pathways have been highlighted in green and red respectively. **(B)** Heatmap showing expression changes between peak EAE-MSCs and naïve MSCs (left) or chronic EAE-MSCs and naïve MSCs (right) for genes important in regulating neural cell development. **(C)** Neural cell cultures treated with conditioned medium (CM) from naïve MSCs had significantly higher number of MBP+ oligodendrocytes compared to control cultures treated with unconditioned medium, whereas cultures treated with EAE-MSC CM had a higher number of GFAP+ astrocytes relative to controls. Scale bar = 30 um, data shown in graph = mean + SEM, **P<0.01, One-way ANOVA.

Figure 3.5

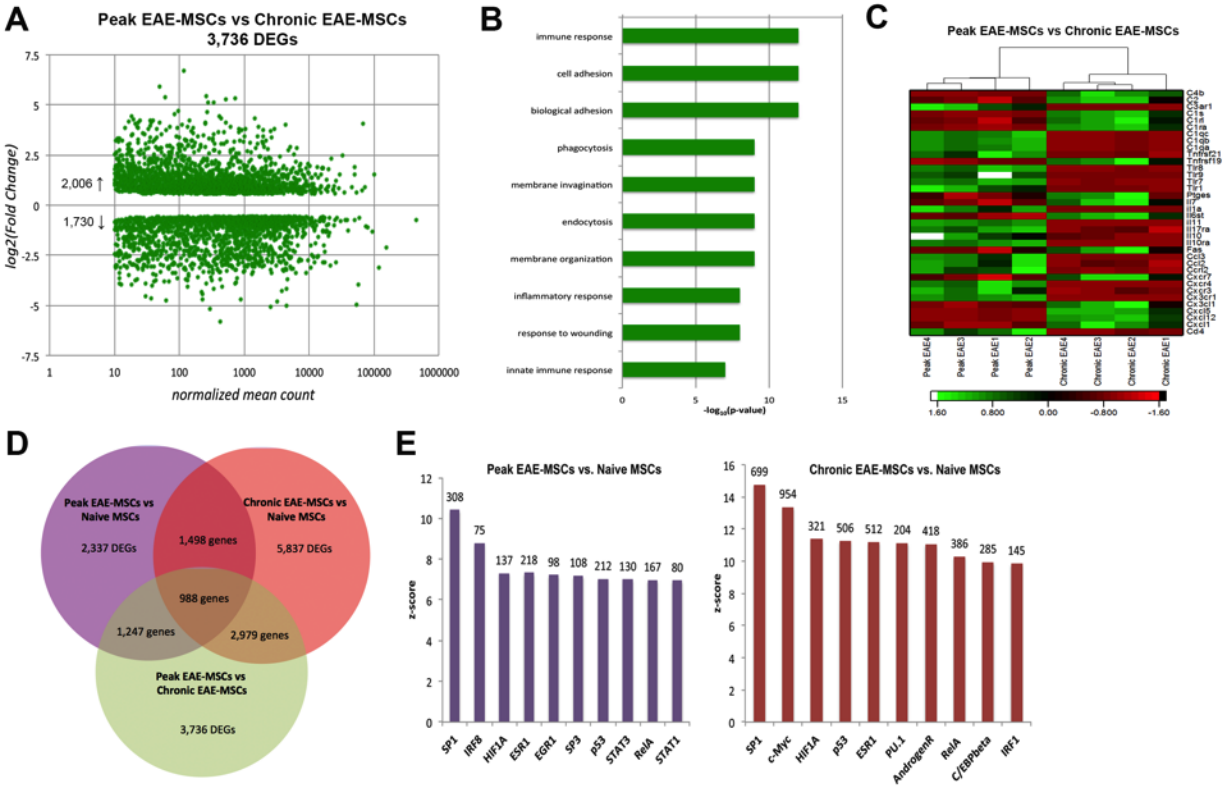
A
Peak EAE-MSCs vs Chronic EAE-MSCs
3,736 DEGs
log2(Fold Change)
normalized mean count
2,006 ↑
1,730 ↓
B
immune response
cell adhesion
biological adhesion
phagocytosis
membrane invagination
endocytosis
membrane organization
inflammatory response
response to wounding
innate immune response
C
Peak EAE-MSCs vs Chronic EAE-MSCs
D
Peak EAE-MSCs vs Naive MSCs
2,337 DEGs
Chronic EAE-MSCs vs Naive MSCs
5,837 DEGs
1,498 genes
988 genes
1,247 genes
2,979 genes
Peak EAE-MSCs vs Chronic EAE-MSCs
3,736 DEGs
E
Peak EAE-MSCs vs. Naive MSCs
z-score
308
75
137
218
98
108
212
130
167
80
SP1
IRF8
HIF1A
ESR1
EGR1
SP3
p53
STAT3
RelA
STAT1
Chronic EAE-MSCs vs. Naive MSCs
699
954
321
506
512
204
418
386
285
145
SP1
c-Myc
HIF1A
p53
ESR1
PU.1
AndrogenR
RelA
C/EBPbeta
IRF1

Figure 3.5: Changes in gene expression in BM-MSCs throughout EAE.

(A) Volcano plot depicting differentially expressed genes (DEGs) measured by RNA-Seq analysis of peak EAE-MSCs versus chronic EAE-MSCs. **(B)** Top 10 gene ontology (GO) terms ranked by p-value associated with the DEGs found between peak and chronic EAE-MSCs. **(C)** Heatmap showing immune genes differentially expressed between peak EAE-MSCs and chronic EAE-MSCs. Many inflammatory genes are up regulated in chronic EAE-MSCs compared to peak EAE-MSCs. **(D)** Three – way Venn diagram illustrating similarities in DEGs found between naïve, peak, and chronic EAE-MSCs. **(E)** Top 10 transcription regulators (identified through MetaCore) associated with the DEGs found between peak EAE-MSCs and naïve MSCs (left) or chronic EAE-MSCs and naïve MSCs (right). Transcription factors (TF) are ranked according to their respective z-score (the level of connectivity of the TF to the DEG list), with the number of DEGs associated with that TF indicated above each bar.

Chapter IV:

General Discussion

4.1: Implications: autologous MSCs for the treatment of MS and other inflammatory diseases

Over twenty NIH-registered clinical trials are currently underway to evaluate MSCs as a potential cellular therapy for MS[163]. These trials follow evidence of MSC safety and clinical efficacy in a variety of other inflammatory conditions[72,73], as well as studies in animal models of MS like EAE that demonstrated MSC infusion effectively reduces disease burden and improves functional recovery[163]. However, the majority or previous studies evaluating MSCs in the EAE model utilized naïve MSCs derived from healthy animals or human donors[117,146-149]. In contrast, most clinical trials in MS patients utilize autologous MSCs in order to minimize the risk of cell rejection or adverse reactions[163]. This raises a serious question about whether autologous MS-MSCs are equivalent to naïve MSCs in their ability to modulate disease, or whether disease alter the functionality of these cells and compromise their therapeutic capacity.

While previous studies have compared MS-MSCs to naïve MSCs in-vitro[169-171], no study before ours has looked at how these different cell populations modulate disease in-vivo when transplanted into an MS animal model such as EAE. Only one previous study has directly compared the therapeutic efficacy of bone marrow derived EAE-MSCs to naïve MSCs when each is transplanted into EAE mice[173]. While this study reported EAE-MSCs are no different than naïve MSCs in their cellular characteristics and ability to ameliorate EAE, it only isolated MSCs from mice early on in EAE progression when the animals began showing physical signs of disease[173]. This paradigm does not accurately represent the use of autologous MSCs in most clinical trials though, as these cells are typically isolated from patients that haven

diagnosed with MS (usually progressive MS) for many years. A more recent study reported that bone marrow MSCs isolated from EAE mice later during disease (when symptoms are more severe) were different than naïve MSCs in their proliferation and differentiation potential, but did not compare these two cell populations in terms of their therapeutic capacity[174].

Here, we show for the first time that bone marrow MSCs derived from MOG-induced EAE mice and from patients with relapsing-remitting MS have reduced therapeutic efficacy compared to naïve MSCs derived from healthy donors. Both EAE-MSCs and MS-MSCs fail to significantly improve functional recovery when intravenously infused into MOG-induced EAE mice, in contrast to the therapeutic benefits observed in infusing naïve MSCs. There were important differences however between EAE-MSCs and MS-MSCs in their ability to promote functional recovery in the MOG EAE model. Infusion of MS-MSCs into EAE mice did cause some improvement in functional recovery, although this improvement was often transient and largely donor dependent. For each of the three respective donors overall, MS-MSCs lacked therapeutic efficacy compared to naïve MSCs and failed to significantly improve cumulative disease score.

There were also functional differences in MSCs derived from MOG-induced EAE mice during the peak of disease (peak EAE-MSCs) versus those derived from EAE mice later in disease progression during the chronic phase of EAE (chronic EAE-MSCs). While both peak and chronic EAE-MSCs lacked therapeutic efficacy compared to naïve MSCs, infusion of peak EAE-MSCs into EAE mice did promote a small, transient improvement in functional recovery for a few days. However this

rapidly reversed, and overall mice that received peak EAE-MSCs or chronic EAE-MSCs showed no significant improvement in cumulative disease score relative to controls.

Overall, our data suggests that disease compromises the therapeutic capacity of MSCs. However, changes in the therapeutic functionality of MSCs seem to directly correlate with progression of disease, at least in the MOG EAE model. Chronic EAE-MSCs had more pronounced changes in their expression of inflammatory genes and their secretion of pro-inflammatory factors compared to peak EAE-MSCs. Whereas conditioned medium (CM) from chronic EAE-MSCs failed to suppress MOG-induced restimulation of lymphocytes in-vitro, CM from both peak EAE-MSCs and naïve MSCs significantly reduced lymphocyte proliferation. The disparate effects between naïve, peak EAE-MSCs, and chronic EAE-MSCs were also observed in co-culture experiments with primary mouse neural cells. While CM from naïve MSCs significantly increased the formation of mature oligodendrocytes in neural cell cultures, CM from peak EAE-MSCs had no effect on oligodendrogenesis, although it did slightly increase the number of astrocytes. In contrast, CM from chronic EAE-MSCs decreased the number of mature oligodendrocytes, and significantly increased the number of astrocytes.

Future studies will need to better examine the association between disease severity and MSC functionality in different EAE models and in MS patients. For example, in a relapsing remitting model of EAE, do MSCs derived from mice during relapse have less therapeutic efficacy than those derived from mice during remittance? Similarly, are there differences in the therapeutic functionality of MSCs

from relapsing-remitting MS patients during different periods of disease, or do MSCs from relapsing-remitting MS patients differ from those derived from progressive MS patients?

Understanding precisely how disease status and severity correlate to changes in the therapeutic functionality of MSCs will be critical in better utilizing MSCs as a cellular therapy for MS. Preliminary data from clinical trials evaluating autologous bone marrow MSCs as a treatment for MS report good safety but little overall therapeutic efficacy[164-166,168,205]. Notably, several pilot trials report autologous MS-MSCs provide only transient benefit in functional recovery and/or lesion pathology (assessed by MRI) in MS patients[164,168,205]. Although interpretation of these results must be guarded since these trials evaluate a very small number of patients, these results parallel our own findings that MS-MSCs and EAE-MSCs lack therapeutic efficacy compared to naïve MSCs, and provide only a small, transient benefit at most.

These findings have important implications concerning the design of future clinical trials evaluating MSCs as a treatment for MS and perhaps other inflammatory diseases. The lack of therapeutic efficacy of both EAE and MS MSCs compared to naïve MSCs in an MS animal model suggests autologous MSCs are a poor candidate for use in clinical trials and supports the advancement of allogeneic MSCs in future clinical applications. Although allogeneic cells generally pose a higher risk of transplant rejection compared to autologous cells, allogeneic MSCs are considered to be immune privileged or at least hypo-immunogenic and have a very low risk of rejection[209]. Multiple clinical trials have reported allogeneic MSCs are

safe and effective in treating other inflammatory conditions such as graft versus host disease[81,85].

Another possible strategy is to collect autologous MSCs from MS patients very early on in the disease, as our results suggest the functionality of MSCs diminishes over time. Thus MSCs derived from MS patients early on in the disease may still be therapeutically relevant in treating MS once it becomes more severe. These cells could be frozen down and used as a potential therapy when the disease advances to a progressive stage or no longer responds to other treatments. Such a strategy would require a more precise understanding of when the functionality of MSCs changes during MS. Future studies examining the effectiveness and/or functionality of MSCs as it relates to disease progression or severity in MS will be crucial to understanding when autologous MSCs may be better suited for clinical applications.

One question raised by our results is whether changes in the therapeutic functionality of MSCs in diseases like EAE and MS extend to other inflammatory conditions. This question is especially relevant given autologous MSCs are being used as a potential cellular therapy for a wide range of other inflammatory and neurological diseases. In one study, bone marrow MSCs derived from the collagen-induced arthritic (CIA) mouse model of rheumatoid arthritis had significant changes in their proliferative capacity and differential potential compared to naïve MSCs[210]. MSCs from CIA mice also had less immunosuppressive functionality in-vitro compared to naïve MSCs, and produced higher levels of pro-inflammatory cytokines such as IL-6[211]. These changes in MSCs in the CIA model appear to be paralleled in

patients with rheumatoid arthritis (RA), as one study reported bone marrow MSCs derived from RA patients fail to inhibit the proliferation and polarization of pro-inflammatory T-cells as effectively as naïve MSCs derived from healthy donors[212].

4.2: Potential mechanisms mediating changes in MSC functionality

Although our work and other studies show inflammatory disease can alter the functionality of bone marrow MSCs, it is unclear if there is a common mechanism mediating these effects. One important question concerns the identity of the extracellular signal(s) that alter the functionality of BM-MSCs in EAE and MS and the source from which those signal(s) arise. Bone marrow is a complex cellular niche filled with different types of immune cells, making it possible that the loss of functionality in BM-MSCs might be mediated from cellular signals in their local environment[213]. Alternatively, the signals could be more systemic. For instance, previous studies have shown that the biology of MSCs is regulated by noradrenergic innervation to the bone marrow[214,215]. Also, blood borne factors could possibly influence the functionality of BM-MSCs[35]. However, previous studies have shown that typical pro-inflammatory signals actually promote the immunosuppressive function of MSCs. Treating bone marrow MSCs with pro-inflammatory factors, including interferon-gamma (IFN-γ), interleukin-17 (IL-17), and tumor necrosis factor alpha (TNFα), causes them to up-regulate secretion of anti-inflammatory factors such as HGF and PGE2 and enhances their capacity to inhibit T-cell proliferation[216-219].

Multiple studies have found that Toll-like receptor (TLR) signaling can affect the functionality of bone marrow MSCs[181,197,220]. While treating MSCs with exogenous ligands to TLR3 enhances their immunosuppressive capacity, treating MSCs with ligands to TLR4 seems to polarize MSCs to a more pro-inflammatory phenotype[197,220]. MSCs treated with TLR4 ligands down-regulate secretion of PGE2, VEGF, and HGF, and promote T-cell proliferation in-vitro[181]. In a recent study, BM-MSCs treated with the TLR4 agonist lipopolysaccharide (LPS) immediately prior to infusion into EAE mice subsequently lacked therapeutic efficacy compared to untreated MSCs[198]. Based on these studies, TLR signaling may be an important effector underlying the diminished the therapeutic functionality of MSCs in EAE. RNA-Seq analysis revealed many TLR genes are differentially expressed in EAE-MSCs compared to naïve MSCs. TLRs can be activated by a number of endogenous ligands collectively referred to as DAMPs (Damage-associated molecular patterns) that are released from dying cells[221]. Increased serum levels of multiple DAMPS have been reported in many inflammatory diseases, including EAE and MS[221,222]. Whether such signaling cascades actually mediate the loss of efficacy in autologous bone marrow MSCs from MS patients or EAE mice is unclear.

Our RNA-Seq analysis revealed that EAE-MSCs differ remarkably from naïve MSCs in their gene expression profiles, consistent with a previous study showing MS-MSCs have similar changes in gene expression compared to naïve MSCs[172]. This suggests that the loss of functionality in diseased MSCs is mediated by transcriptional mechanisms. The fact that there are significant differences in EAE-MSCs and MS-MSCs after they have been isolated and grown in culture for several

weeks strongly implies the phenotype observed in diseased MSCs is maintained at a transcriptional level. Bioinformatic analysis of our RNA-Seq data revealed a high degree of conservation in the transcriptional regulators associated with the differentially expressed genes (DEGs) identified in EAE-MSCs. For example, the transcription factor hypoxia inducible factor 1 alpha (HIF1α) was highly associated with the DEGs in both peak and chronic EAE-MSCs. Inhibiting HIFα attenuates inflammation in a variety of biological systems and disease settings, suggesting HIF1α is key regulator of inflammation and immunity[223]. Targeting HIF1α and/or other transcription factors associated with the gene expression changes observed in EAE-MSCs may represent one approach to restoring the therapeutic functionality of diseased BM-MSCs.

Future studies will need to explore both the extracellular signals and internal transcriptional mechanisms underlying the changes in bone marrow MSCs in EAE and MS. Until the mechanisms mediating the loss of therapeutic functionality in diseased MSCs can be better identified and corrected, the benefits of autologous MSC therapy in MS will likely be unrealized. Understanding how MS alters MSC functionality as part of disease progression may also reveal new insights into MS pathogenesis. Although biology and function of endogenous MSCs is poorly understood, these cells may act as key regulators of immune cells in lymphoid organs such as the bone marrow[35]. Changes to the functionality of MSCs in EAE and MS may therefore contribute to autoimmunity and inflammation in these diseases. A recent study found that the number of MSCs in the bone marrow of MOG-induced EAE mice was inversely correlated with the number of activated T-cells also present

in the bone marrow[224]. Thus these two cell populations may influence each other during the progression of EAE, and so changes to the immunosuppressive functionality of bone marrow MSCs may also contribute to the immunopathology of disease.

4.3: MSCs as a therapeutic in disease: immunomodulation versus neuroprotection

It is currently unclear whether naïve MSCs promote recovery in EAE by directly influencing neural cells and remyelination, or whether their therapeutic benefit is derived solely from their ability to suppress inflammation and the immune response[193]. Multiple studies have demonstrated that naïve MSCs infused into EAE mice are potently immunosuppressive[60]. Transplanted MSCs migrate and engraft into immune organs such as the spleen and lymph nodes, where they appear to bias the systemic immune response from a pro-inflammatory (Th1) profile to a more anti-inflammatory (Th2) profile and inhibit the activation and migration of peripheral immune cells into the CNS[117,146,148]. However, MSCs can also influence the function of neural cells and can promote neural repair. Co-culture studies show naïve MSCs promote the differentiation of neural stem cells (NSCs) and oligodendrocyte precursor cells (OPCs) into oligodendrocytes and/or neurons [116-118]. And in non-immune mediated model of demyelination such as the cuprizone model, transplanted MSCs can promote oligodendrocyte formation and enhance myelin repair [154,155]. Distinguishing between immunosuppression versus enhanced

remyelination in the EAE model is difficult however, and so it is unclear whether MSCs have similar effects when infused into EAE mice.

Our results suggest the diminished therapeutic functionality of EAE-MSCs may stem from differences in both their immunosuppressive and neuroprotective functionality. Infusion of naïve MSCs or naïve MSC CM into EAE mice significantly reduced the number of peripheral immune cells and T-cells in the spinal cord. In contrast, infusion of peak EAE-MSCs or chronic EAE-MSCs and their respective CM failed to attenuate the number of inflammatory cells in the spinal cord. In line with these observations, CM from EAE-MSCs contained higher levels of multiple pro-inflammatory cytokines. CM from chronic EAE-MSCs also failed to suppress MOG-induced restimulation of lymphocytes in-vitro in a manner similar to naïve MSC CM, and transcriptional profiling of EAE-MSCs revealed an up regulation of many pro-inflammatory genes associated with immune cell activation and immune response. Collectively, our data shows that the immunomodulatory capacity of bone marrow MSCs is altered during EAE, and this likely contributes to the diminished therapeutic functionality of EAE-MSCs.

In our cell tracking experiments, we found no evidence that either naïve MSCs or EAE-MSCs migrate and engraft into the CNS of EAE mice within the first week after transplantation. Our data initially suggested that transplanted MSCs do not directly modulate remyelination or neural repair in EAE mice, and thus the lack of therapeutic efficacy of EAE-MSCs is not a consequence of their diminished neuroprotective potential. However, RNA-Seq analysis revealed a number of genes and pathways altered in EAE-MSCs that affect oligodendrocyte differentiation and

neural cell development. Consistent with these observations, CM from EAE MSCs had differential effects on neural cell development in-vitro. Whereas CM from naïve MSCs promoted the formation of mature oligodendrocytes, CM from peak EAE-MSCs had no significant effect on oligodendrogenesis, although it did show a trend towards increasing the number of astrocytes. In contrast, CM from chronic EAE-MSCs reduced the number of mature oligodendrocytes, and significantly increased the proportion of astrocytes.

These observations raise the possibility that the reduced therapeutic functionality of EAE-MSCs may be due in part to the diminished ability of these cells to promote oligodendrocyte maturation and subsequent remyelination. At first glance, our current findings that MSCs do not migrate and engraft into the CNS may seem to contradict this possibility. However, there are several important considerations to take into account. First, we only examined whether MSCs migrate and engraft into the CNS with the first week post-transplantation. It is possible that MSCs may not come to reside in the CNS until later on after infusion. Indeed, in a study by Zappia et al., GFP expressing MSCs were not found in the CNS of EAE mice until over one month post infusion[146]. Second, while transplanted MSCs may not engraft into the CNS parenchyma, it is possible they may reside in areas like the subarachnoid space or ventricular system where they could in turn secrete factors that influence neural cell development and remyelination. In a study where MSCs were intreventricularly injected into cuprizone demyelinated mice, very few MSCs actually migrated out of the ventricles and into the brain; however, these MSCs were found within the ventricles and ventricular wall for up to 3 months after injection[154].

Transplanted MSCs were associated with an increase in oligodendrocyte differentiation from neural stem cells in the subventricular zone, as well as enhanced forebrain remyelination[154]. Third, our results show that MSC secreted factors can act in a non-localized, systemic fashion to modulate disease. A single intravenous injection of CM from naïve MSCs is sufficient to promote functional recovery in MOG-induced EAE mice. Therefore, MSCs that migrate and engraft into different organs and tissues, such as the liver or lungs, could conceivably secrete paracrine factors into the blood stream that modulate repair in other tissues.

Collectively, our results highlight a potential dual mechanism by which disease diminishes the therapeutic capacity of MSCs. One, EAE-MSCs may have less immunosuppressive potential than naïve MSCs, and fail to effectively inhibit the activation of immune cells such as T-cells. Two, EAE-MSCs have less neuroprotective potential than naïve MSCs, and fail to effectively promote oligodendrocyte maturation and remyelination. Future studies are required to determine whether the loss of therapeutic functionality in EAE-MSCs stems from changes in their immunosuppressive versus neuroprotective potential. For example, examining how diseased MSCs influence neural repair in other non-immune mediated models of demyelination may provide insight how these cells differentially modulate neural cells and remyelination.

4.4: Concluding Remarks

In summary, we show that CNS disease diminishes the therapeutic functionality of MSCs. Bone marrow MSCs derived from EAE mice and MS patients

lack therapeutic efficacy compared to naïve MSCs in their ability to ameliorate EAE. Our data suggests that the reduced therapeutic efficacy in diseased MSCs is derived from differences in the paracrine factors these cells secrete, which differentially modulate immune cell activation and neural cell development. These results have important clinical implications, as they raise serious concern about the efficacy of using autologous MSCs to treat MS and possibly other inflammatory diseases.

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