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Mesenchymal stem cells and inflammatory lung diseases

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Mesenchymal stem cells (MSCs) are emerging as a therapeuc modality in various inflammatory disease states. A numer of ongoing randomized Phase I/II clinical trials are evaluting the effects of allogeneic MSC infusion in patients with multiple sclerosis, graft-versus-host disease, Crohn's disease, nd severe chronic myocardial ischemia. MSCs are also being onsidered as a potential therapy in patients with inflammatory ung diseases. Several studies, including our own, have demontrated compelling benefits from the administration of MSCs n animal models of lung injury. These studies are leading to rowing interest in the therapeutic use of MSCs in inflammaory lung diseases. In this Review, we describe how the mmunoregulatory effects of MSCs can confer substantial proection in the setting of lung diseases such as acute lung injury, hronic obstructive pulmonary disease, asthma, and pulmonary ypertension. We also address potential pitfalls related to the herapeutic use of MSCs in fibrotic lung diseases such as idioathic pulmonary fibrosis. In addition, we identify emerging reas for MSC- based therapies in modulating oxidative stress nd in attenuating inflammation in alcohol-related acute lung njury.

KEY WORDS: Mesenchymal stem cells - Cytokines - Acute lung njury - Oxidative stress.

Inflammation, defined by Celsus around AD40 as "*rubor, calor, dolor, tumor"* (redness, heat, pain, nd swelling), is a highly evolved process that can rise in any tissue in response to pathogens, trauma, oxins, or autoimmune injury.[1] Deficiencies in cellular and humoral components of the inflammatory response lead to an increased risk of infection and death.[2] Thus, the ability to mount an inflammatory response is life-preserving. However, excessive inflammation can injure the lungs.

In patients with gram negative sepsis; a disregulated inflammatory response to bacterial endotoxin increases the risk for acute lung injury (ALI), which can lead to severe respiratory failure termed the acute respiratory distress syndrome (ARDS).[3] ALI and ARDS are associated with significant morbidity, and mortality rates of greater than 30%.[3] Because disregulated inflammation causes lung injury, strategies to attenuate the inflammatory response in ALI and ARDS are of considerable therapeutic interest.

Mesenchymal stem cells (MSC) are emerging as a therapeutic modality in various inflammatory diseases, including ALI. MSCs have the potential to differentiate to various connective tissue lineages which include adipose tissue, marrow stroma, cartilage, tendon, and bone.[4] MSCs have been isolated from multiple tissues including adipose tissue,[5] skeletal mus-

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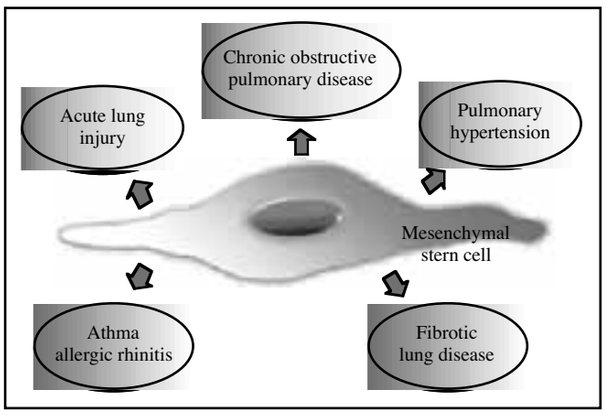

Figure 1.—Mesenchymal stem cells in inflammatory lung diseases. Mesenchymal stem cells are emerging as a therapeutic modality in various inflammatory lung diseases such as acute lung injury, chronic obstructive pulmonary disease, pulmonary hypertension and allergic diseases such as asthma. Due to their potential fibrotic effects *in vivo*, MSCs may not represent a therapeutic option in patients with fibrotic lung disorders such idiopathic pulmonary fibrosis.

cle,[6] synovium,[7] spleen, thymus,[8] blood, lung, fetal blood,[9] and amniotic fluid.[10] The most accessible and by far the best characterized source of MSCs is the bone marrow, and much of what we know about MSCs in lung inflammation is based on studies in bone marrow-derived mesenchymal stem cells (BMDMSC).[11, 12]

MSCs are present in the bone marrow in relatively small numbers with an estimate of about 10 MSC, for 1 million total bone marrow cells.[13] The low numbers of MSC necessitates their expansion *in vitro*. Enrichment of BMDMSCs from crude marrow suspensions is achieved by selection for a plastic-adherent cell population that expresses neither hematopoietic nor endothelial cell surface markers, but is positive for the expression of adhesion and stromal markers. Despite a wide array of markers used for the detection of BMDMSCs by flow cytometry, a defined panel of unambiguous markers distinguishing BMDMSCs is lacking. The absence of a standardized BMDMSC marker presents a challenge in the field and imposes additional criteria to distinguish BMDMSCs. A gold standard criterion for establishing BMDMSC phenotype is a trilineage differentiation assay where the plasticity of BMDMSCs is confirmed by their ability to differentiate into adipocytes, osteocytes, and chondrocytes, on stimulation. This plasticity makes BMDMSCs attractive tools for tissue regeneration and BMDMSCs have been used successfully in the clinic to treat osteogenesis imperfecta in children.[14]

In this review, the authors describe how the immunoregulatory effects of BMDMSCs can confer substantial protection in the setting of lung diseases such as acute lung injury (Figure 1). They also address potential pitfalls related to the therapeutic use of BMDMSCs in fibrotic lung diseases such as idiopathic pulmonary fibrosis. In addition, the authors identify emerging areas for BMDMSC based therapies in modulating oxidative stress and in attenuating inflammation in alcohol-related acute lung injury.

MSC and ALI

The ability of BMDMSC to create a tolerogenic niche by direct interaction with immune cells and by secretion of regulatory molecules makes them attractive therapeutic candidates in the regulation of the inflammatory response to infection and injury. Several studies, including publications from our group, have demonstrated compelling benefits from the administration of BMDMSC in animal models of lung injury. In a murine model of ALI, initiated by the administration of bacterial lipopolysaccharide (endotoxin/LPS), exogenous infusion of BMDMSC prevents the systemic inflammatory response to endotoxin, and attenuates lung injury. In humans, ALI is initiated by an acute inflammatory response to a physical trauma or infection,[3] most commonly sepsis,[15] and often leads to severe respiratory failure termed the acute respiratory distress syndrome.[3, 16] ALI is characterized by sequestration of neutrophils in the lung, lung edema, and upregulation of pro-inflammatory mediators systemically and locally in the lung. Administration of LPS to mice produces pathophysiologic changes similar to those seen in ALI in humans.

The authors of the current paper have recently shown that administration of BMDMSCs attenuates the massive inflammatory response to LPS and protects the lung from injury. They obtained BMDMSCs from syngeneic donors expressing GFP to facilitate *in vivo* tracking of donor-derived BMDMSCs. The cells were expanded *in vitro* and depleted of hematopoietic and macrophage markers (CD45 and CD11b, respectively), prior to administration. BMDMSCs were infused intravenously (i.v.) immediately after intraperitoneal challenge with 1mg LPS/kg body weight. This dose of

LPS causes structural alterations in the lung between 6 h and 48 h post-endotoxin and injury begins to resolve by 48 h.[17] BMDMSC infusion protected against pulmonary edema, a hallmark of ALI. Histological examination of lung sections demonstrated that infusion of BMDMSCs completely attenuated neutrophil infiltration in the lung between 6 h and 48 h. Plasma levels of pro-inflammatory cytokines, interleukin (IL)-1β, interferon (IFN)-γ, IL-6, and macrophage inflammatory protein (MIP)-1α were significantly decreased with BMDMSC infusion. Levels of IL-10 were maintained and granulocyte-colony stimulating factor (G-CSF) increased acutely.

Thus, BMDMSC altered the pattern of cytokine responses to LPS; (T helper 1) T_H1 responses were decreased and levels of T_H2 cytokines were maintained. The protection conferred by BMDMSC was not related to clearance of endotoxin, and appeared to be at least partly independent of BMDMSC engraftment into the lung. To further characterize the interaction between injured lung cells and BMDMSCs, the authors co-cultured lung cells obtained from LPS-treated animals, and from untreated animals with GFP^+ BMDMSCs. BMDMSCs, plated on the bottom of a chamber, were separated from lung cells by a transwell insert with a pore size large enough to allow BMDMSC migration. Significantly higher numbers of BMDMSCs migrated in response to LPS-treated lungs, suggesting that injured lung cells produce soluble factors driving BMDMSC chemotaxis. Next, it was determined if injured lung cells could respond to factors produced by BMDMSCs. For these experiments lung cells obtained from LPS-treated mice were separated from BMDMSCs by a transwell insert that either prevented or permitted cell-contact. Under both conditions, lung cells produced significantly lower amounts of MIP-1α, IL-1β, IL-12, IL-6, and Regulated upon Activation, Normal T-cell Expressed, and Secreted (RANTES). A greater suppression in levels of MIP-1α, and RANTES was observed when contact with BMDMSC was permitted. What emerged from these findings was the identification that injured lung cells induced BMDMSC migration, and that MSC regulate inflammatory signaling processes in injured lung cells.

Gupta *et al.* recently reported attenuation of lung injury, and improved survival via intrapulmonary delivery of BMDMSCs in an endotoxin model.[18] Infusion of BMDMSCs 4 h after intratracheal LPS, decreased lung edema at 24 h and 48 h. Protein infiltration into the lung, a measure of leakiness of the alveolar capillary barrier, was decreased at 48 h, but not at 24 h. The authors also demonstrate a decrease in MIP-2 levels in the lung lining fluid by 24 h followed by a decrease in TNF-α, at 48 h. An acute increase (8 h) in IL-10 was noted in the plasma and lining fluid with BMDMSC infusion. Significant histological improvement in lung injury was observed despite low levels of donor BMDMSC engraftment in the lung. The differences in mediator production observed by Gupta *et al. versus* the authors likely relate to differences both in the dose and mode of administration of endotoxin and BMDMSCs. Nevertheless, it is evident that both intravenous and intratracheal infusion of BMDMSC curbs the severe acute inflammatory response systemically and in the lung, and significantly attenuates lung injury.[19]

Using an innovative cell-based and gene-based approach, Mei *et al.* report that the protective effect of BMDMSCs in the LPS-injured mouse lung is greatly potentiated by infusion of BMDMSC overexpressing angiopoietin 1(ANGPT1), a vasculoprotective gene.[20] BMDMSC infusion, 30 minutes after i.v. LPS, significantly decreased airspace neutrophil count 3 d after LPS administration. In mice given BMDMSCs expressing ANGPT1 (BMDMSC-pANGPT1), the level of inflammation was further reduced and could not be distinguished from control animals. Infusion of BMDMSC-pANGPT1 resulted in a significantly greater suppression of IFN-γ and IL-1β compared to BMDMSCs alone. Indeed TNF-α in the lining fluid was significantly reduced only with BMDMSC-pANGPT1 infusion. In concert with our observations and those by Gupta *et al.*, the protective effect seen with BMDMSC therapy did not require high level of engraftment in the lung. These results demonstrate that protection conferred by BMDMSCs can be augmented by gene therapy approaches, where a synergy of the anti-inflammatory effect of BMDMSC with improved preservation of endothelial function by overexpressing ANGPT1, can improve outcomes. Taken together, these findings demonstrate a role of BMDMSCs in mitigating the inflammatory response to LPS (Table I) and as a consequence, attenuating lung injury. These results indicate the potential for BMDMSC as a therapy in ALI, a disease with a staggering mortality rate and limited treatment options.

TABLE I.—*Effect of BMDMSC infusion on endotoxin-induced cytokine levels* in vivo.

Stimulus	Dose	Recipient	MSC	Control	Cytokines	Ref.
E. coli LPS O11 B6, i.p	1 mg/kg	Mice, C57 BL6J female	Bone marrow derived 5×10^5, i.v immediately post-LPS syngeneic	LPS only	↓ Plasma IL-1β, IFNγ, IL-6, MIP-1α, KC	Xu *et al.* 2007
E. coli LPS O55 B5, i.p	1 mg/kg	Mice, C57 BL6J male	Bone marrow derived 7.5×10^5, i.t 4 h post-LPS syngeneic	LPS/Saline LPS/apoptotic MSC LPS/3T3 fibroblasts	↓ BAL TNF-α, MIP-2 ↑ Plasma, BAL IL-10	Gupta *et al.* 2007
E. coli LPS O55 B5, i.t	40 mg/kg	Mice, C57 BL6J female	Bone marrow derived 7.5×10^5, i.v 0.5 h post-LPS syngeneic	LPS/Saline	↓ BAL IL-1β ↓ Long homogenate TNF-α, IL-6, KC	Mei *et al.* 2007

E. coli: Escherichia Coli; i.p.: intraperitoneal; i.t. intratracheal; i.v.: intravenous.

MSC and chronic obstructive pulmonary disease

Chronic obstructive pulmonary diseases (COPD) of the lung are frequently progressive syndromes resulting in destruction of alveolar septa leading to air-space enlargement and a consequent decrease in alveolar surface area.[21] Emphysema, defined as airspace enlargement of the adult lung, frequently develops as a component of COPD. COPD was ranked sixth among the causes of death globally in 1990 but is projected to be the third most common cause of death by 2020.[22] In the United States, COPD is currently the fourth leading cause of death.

Cigarette smoking and air pollution are the major risk factors associated with emphysematous changes in the adult lung. While numerous aspects of the pathogenesis of emphysema remain to be understood, a salient aspect of the disease is an up regulation of inflammatory processes leading to apoptosis of epithelial cells, proteolysis of the terminal air-spaces and lung extracellular matrix components. BMDMSCs are being considered as a therapy in COPD both for their ability to regenerate type I and type II cell in the air-spaces and due to their immunomodulatory effects. Currently, a Phase II clinical trial is establishing the safety and efficacy of multiple administrations of allogeneic BMDMSCs in patients with moderate to severe COPD (Clinical trails identifier - NCT00683722).

There is one study in literature that has investigated the effects of BMDMSC administration in a rat model of emphysema.[23] Rats were exposed to Co60 irradiation and intra-tracheal papain treatment after which BMDMSCs were infused intravenously. Lungs were harvested after 28 days and histological changes in the lung were compared between the different treatment groups. As expected, emphysematous changes in the lung, as quantified by mean linear intercept, increased in irradiated, papain treated rats. Infusion of BMDMSCs significantly protected against air-space enlargement. Furthermore, the percentage of apoptotic cells was also significantly decreased with BMDMSC treatment. Immunohisto-chemical analysis of lung sections revealed co-staining of engrafted BMDMSCs with the type II-epithelial cell marker, surfactant protein-C, suggesting that BMDMSCs may have differentiated into lung-cell types. These data suggest that BMDMSCs can protect against progression of emphysema by mechanisms relating to epithelial cell regeneration and also potentially due to paracrine effects resulting in decreased alveolar apoptosis.

MSC and pulmonary hypertension

Pulmonary hypertension (PH) is a rapidly progressive and often fatal disease characterized by increased pulmonary arterial pressure, right-heart dysfunction, and lung vascular remodeling leading to loss of the distal pulmonary vasculature.[24] Strategies aimed at promoting neovascularization and regeneration of the lost vasculature are of immense therapeutic interest in patients with PH. Because BMDMSCs produce growth factors such as vascular endothelial growth factor (VEGF) that promote neovascularization, there is interest in BMDMSC-based therapies in PH.

Furthermore, BMDMSCs are mobilized from the bone marrow to the peripheral blood during chronic hypoxia.[25] This raises the possibility that exogenous infusion of BMDMSCs may bolster endogenous reparative mechanisms.

Studies by Baber *et al.* demonstrate that administration of BMDMSCs attenuates monocrotaline-induced PH in rats.[26] BMDMSCs were infused intratracheally, 14 days after intravenous challenge with monocrotaline. Results showed that BMDMSC infusion attenuated monocrotaline-induced increase in pulmonary arterial pressure and improved pulmonary vascular resistance. Immunohistochemistry analysis of lung sections revealed that immunolabeled BMDMSCs were detected in the lung parenchyma surrounding the airways, but not in the pulmonary vessel walls. Thus, the benefits were attributed to paracrine effects of BMDMSCs in the lung parenchyma which resulted in improved vascular endothelial function. This finding is corroborated by studies Horimoto *et al.* who found that intravenous infusion of BMDMSCs 7-days after subcutaneous monocrotaline improved right ventricular (RV) hypertrophy.[27] Interestingly, infusion of BMDMSCs over-expressing endothelial nitric oxide synthase (eNOS) further improved RV. These results suggest that protection conferred by BMDMSCs can be augmented by gene therapy approaches, where a synergy of the paracrine effects of BMDMSCs with improved preservation of vascular function by overexpressing eNOS, can improve outcomes.

Together with the observation that the peripheral BMDMSC population is significantly augmented during hypoxia,[25] these *in vivo* data suggest that BMDMSCs home to the lung and produce growth factors during hypoxia. Indeed, *ex vivo* studies demonstrate that treating pulmonary artery rings to conditioned medium from hypoxia-stressed BMDMSCs prior to subjecting arteries to hypoxia, attenuates hypoxia-induced vasoconstriction.[28] This raises the possibility that hypoxia-stressed BMDMSCs secrete soluble factors that improve hypoxia-induced alterations in pulmonary vasoreactivity. Further research into identifying BMDMSC-derived factors will aid not only in identifying therapeutic targets but will also lead to a better mechanistic understanding of the disease process. However, it should be noted that mesenchymal precursors from the monocyte/macrophage lineage termed fibrocytes are also recruited to the vasculature during hypoxic vascular remodeling.[29] It has also been shown that BMDMSCs cultured in the presence of a demethylating agent increase expression of fibrocyte cell surface marker.[30] Fibrocytes play a role in fibrotic tissue remodeling by increasing expression of matrix components such as collagen and fibronectin. Because BMDMSCs and fibrocytes are both adherent populations care must be taken to distinguish these cell types by the presence of markers such as CD45 prior to *in vivo* infusion.

MSC and asthma and allergy

Another area in pulmonary medicine where the immune regulatory potential of BMDMSCs is being actively investigated is Asthma. Asthma is one of the most common chronic inflammatory diseases affecting an estimated 300 million worldwide.[31] Its pathogenesis stems from the complex interplay between the allergic response, inflammatory/immune cells, and airway hyperresponsiveness, which leads to bronchoconstriction and eventually airway remodeling. To date, multiple studies have proven that BMDMSCs have immune regulatory properties and can reduce acute inflammation. Now, investigators are looking at bone marrow–derived BMDMSCs in decreasing the inflammatory response of an ovalbumin (OVA)-induced asthma mouse model.[32] Weiss *et al.* found that administration of BMDMSCs significantly attenuated OVA-induced increases in airway hyperresponsiveness as well as the number of eosinophils in bronchoalveolar lavage fluid after OVA challenge. In addition, they saw a significant decrease of T_H2 cytokines in the lungs of BMDMSC-treated mice. The anti-inflammatory effects were seen with both syngeneic and allogeneic BMDMSC administration, consistent with the concept of BMDMSC immunoprivilege. These findings though preliminary, open yet another door for potential therapy of asthma.

Ongoing studies are also investigating the mechanisms of BMDMSCs affecting dendritic cell activation and antigen presentation, and its effects on T-cell lineage commitment and T-cell effector function.[32] The above mechanisms involve the acute phase of asthma, and if left unchecked, the resulting uncontrolled inflammation eventually leads to airway remodeling and fibrosis characteristic of severe uncontrolled asthma. The pathogenesis of chronic airway remodeling involves effector molecules such as transforming

growth factor-b and vascular endothelial growth factor, along with other cytokines and inflammatory cells.[33] The authors speculate that BMDMSCs through the regulation of these molecules, not only can control the inflammatory acute phase, but potentially can also affect the process of remodeling. However, studies providing proof of this concept are needed.

Another disease closely related to asthma is allergic rhinitis (AR). Not only because they have similar pathogenic processes occurring on different segments of the airway, but recent investigations are now strengthening the relationship that presence of AR significantly predisposes to the development of asthma later in life.[34] When both diseases coexist, it is known that treating AR can also lead to improvement in asthma symptoms and severity.[35] These relationships are significant because it further contributes to asthma morbidity, but it also opens up another potential target for cellular therapy.

In a recent study, Cho *et al.* demonstrated that in a mouse model of AR; adipose tissue-derived stem cells (ASC) inhibit the allergic response.[36] They were able to simulate experimental allergic rhinitis by sensitizing BALBc mice to OVA. The treatment involved intravenous infusion of cultured ASC pooled from allogenic mice prior to OVA sensitization. Following infusion and sensitization, blood was collected for analysis of IgE, IgG1 and IgG2a in the serum. They also harvested splenocytes for cytokine determination and nasal mucosa for immunohistochemistry. The results showed significantly lower levels of IgE, IgG1, IgG2a, and IgG1/IgG2a, along with decreased IL-4, IL-5 and elevated Interferon-γ levels in the OVA-sensitized mice treated with ASC compared to untreated mice. Histologic analysis of nasal mucosal sections of ASC treated OVA mice showed migration of ASC into the tissues along with markedly less inflammatory cell and eosinophil infiltration of the mucosal layers. Interestingly, the investigators also observed significantly less symptoms of sneezing and nose rubbing in the OVA-ASC treated mice. These animal experiments are examples that serve as the groundwork for expanding the growing number of applications for stem cells to include not only the inflammatory, but also allergic pulmonary disease.

MSC and fibrotic lung disorders

While BMDMSC have shown to consistently attenuate inflammation in numerous experimental models of injury, it is important to recognize that time window is a critical factor in optimizing the protective effect of BMDMSC transplantation. Recent data by Yan *et al.* indicate that infusion of BMDMSC at a later stage of lung injury can in fact be deleterious.[37] GFP+ BMDMSC were infused at 4 h, 60 d or 120 d after lung irradiation. Cells infused early (4 h) engrafted to the lung at low levels and were distributed around alveolar and bronchial epithelium. In contrast, cells injected at a later stage (60 d and 120 d) were detected in the interstitium as myofibroblasts indicating that differentiation of BMDMSC occurred in response to mediators produced in the injured tissue. These data point to the conclusion that infusion of BMDMSC during an ongoing fibrotic response may augment fibrosis. Thus, time window is a critical factor in BMDMSC infusion and must be given due consideration to optimize the protective effects of BMDMSC in lung injury. Because infusion of BMDMSCs during an ongoing fibrotic response can augments fibrosis, BMDMSC-based therapy may not be ideal in patients with fibrotic disorders.

MSC and oxidative stress

Oxidative stress is a hallmark of inflammation,[38, 39] and studies in patients with various inflammatory lung diseases have shown that increased oxidant burden is associated with the progression and severity of the disease process.[40-42] (11, 43, 47). Because oxidative stress is intimately related to inflammation and tissue injury, the role of BMDMSCs in modulating the redox environment is a rapidly emerging area of interest. The potential redox modulatory effects of BMDMSCs are especially relevant to ALI, a disease characterized by dramatic perturbations in the systemic redox environment.

Oxidative stress in ALI

The idea that highly reactive oxygen metabolites, produced by activated leukocytes, caused tissue injury [42] was advanced before the clinical description of ALI in 1967.[44] The acute pulmonary injury caused by these reactive oxygen species (ROS) was believed to occur in a pathway that was parallel to the ongoing inflammatory response. We now know that ROS and redox signaling pathways, that are not strictly ROS-mediated, converge with cellular and humoral com-

ponents of the immune system and this interaction is a key pathway in the pathogenesis of ALI and ARDS.

Over the years, studies in humans have consistently shown two things. Firstly, patients with ALI and ARDS show increased levels of oxidative stress compared to healthy controls and second that higher levels of oxidative stress in ARDS patients correlate with poorer outcomes. For instance, elevation in plasma hypoxanthine, a substrate for superoxide and hydrogen peroxide, is associated with increased mortality in ARDS patients.[45] In this study, hypoxanthine was highly negatively correlated with loss of protein thiol groups in the plasma, indicating oxidative modification of extracellular protein thiols. The redox state of thiol residues on extracellular proteins is regulated by two, low-molecular weight thiol/disulfide control systems, cysteine (Cys) and its disulfide cystine (CySS) and glutathione (GSH) and glutathione disulfide (GSSG).[46]

Cys and GSH are critical determinants of cytokine expression during activation of the immune system, and alteration in Cys and GSH homeostasis is a central feature of inflammation and tissue injury.[47] Therefore, understanding the regulation of Cys and GSH redox systems during inflammation by BMDMSCs is important not only to fully delineate the systemic effects of BMDMSCs but also to identify potential therapeutic targets. In this section, the authors identify known redox regulatory and antioxidant defense systems in BMDMSCs. Several studies have investigated the redox-dependent effects on BMDMSCs in response to extracellular signals and these are addressed. Finally, the authors discuss emerging evidence from their laboratory that suggests that BMDMSCs can attenuate oxidation of Cys and GSH redox systems *in vivo*. Together these discoveries are contributing to the novel concept of BMDMSCs as a therapeutic modality in attenuating oxidative stress in inflammatory lung diseases.

Antioxidant defense systems in MSC

The inflammatory state is characterized by elevated levels of reactive oxygen and reactive nitrogen species, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and nitric oxide (NO).[1] These reactive species serve multiple functions including killing phagocytosed microorganisms, removal of cell and tissue debris, and induction of signaling events related to inflammation and repair. However, ROS can also cause tissue necrosis and inflammation which contribute to increased tissue injury and destruction. Indeed, elevated oxidant burden is purported pathogenic component in numerous inflammatory lung diseases including ALI, IPF, and COPD.

Because BMDMSCs are mobilized during tissue injury, and participate both in the attenuation of inflammation and in tissue repair and regeneration, one may speculate that BMDMSCs possess enhanced antioxidant defense systems to survive and function in a hostile environment. Indeed, studies in endothelial progenitor cells (EPC) demonstrate lower basal ROS production in EPCs compared to umbilical vein endothelial cells or microvascular endothelial cells.[48] Furthermore, EPCs also express higher mRNA and protein levels of antioxidant enzymes such as manganese-superoxide dismutase (Mn-SOD), catalase, and glutathione peroxides (GPx) which could confer a higher resistance to oxidant stress.

Existing evidence suggests that BMDMSCs may also be enriched for the presence of antioxidant protective genes. Comparison of the human bone marrow-derived fibroblast cell-line V54/2 to the peripheral blood-derived fibroblast cell-line L87/4, revealed that V54/2 cells expressed higher levels of glutathione-S-transferase (GST), an enzyme system involved in the detoxification by electrophiles by conjugation to GSH.[49] Detailed comparisons of antioxidant defense systems between BMDMSCs and other stromal cells such as lung fibroblasts, as well as cell types of epidermal and endodermal origin will lead to a better understanding of the antioxidant capacity of BMDMSCs.

A number of studies have also investigated the effects of various stressors on ROS production and antioxidant levels in BMDMSCs (Figure 2). Takahata *et al.* reported that stimulation of the mesenchymal stem cell-line C3H10T1/2 with adrenaline led to an increase in cellular GSH levels via a process involving nuclear factor E2 p45-related factor-2 (Nrf-2)-mediated activation of the CySS/ glutamate antiporter, xCT.[50] This suggests β_2 adrenergic stimulation of BMDMSCs can lead to increase influx of CySS from the extracellular compartment to within the cells for the purpose of GSH synthesis. In addition to hormones, temperature has also been studied as a stressor. Stolzing *et al.* investigated whether culturing BMDMSCs under reduced temperature conditions impacts ROS production and antioxidant defense systems.[51] MSCs were derived from the bone marrow

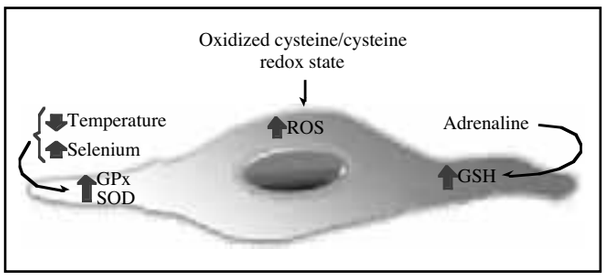

Figure 2.—Extracellular signals induce redox dependent changes in BMDMSCs. *In vitro* studies demonstrate that MSCs respond to exogenous signals ranging from stress hormones to nutrient availability. Adrenaline nduces CySS import which results in increased GSH synthesis. Exposing cells to an oxidized extracellular cysteine/cystine redox state induces upregulation of ROS production by a mechanism likely involving oxidation of redox sensitive Cys residues on the membrane. Selenium regulates expression of antioxidant enzymes such as GPx and SOD, as does culture temperature conditions.

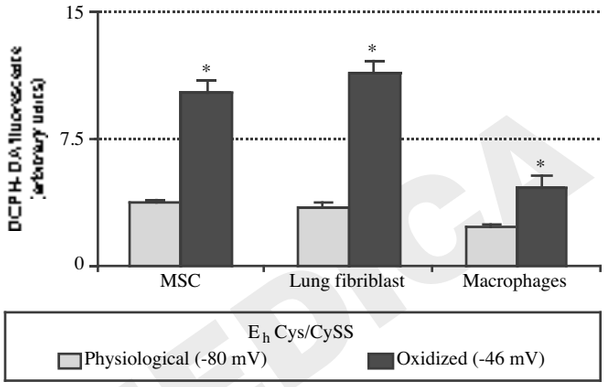

Figure 3.—Oxidized extracellular E_h Cys/CySS induces ROS production in BMDMSCs. BMDMSCs, primary lung fibroblasts, and the macrophage cell-line, RAW 264.7 were pre-incubated with an ROS-sensitive dye, DCFH-DA (100 μM) for 30 min, before treating with -80 mV and -46 mV redox media for 5 min at 37 °C. Oxidation of DCFH-DA to fluorescent DCF was measured on a microplate reader. MSCs exposed to oxidized Cys/CySS redox (-46 mV) show a 2-fold increase in ROS production compared to cells exposed to a physiological redox potential of -80 mV (*P<0.001). Similar responses are observed in lung fibroblasts and macrophages suggesting that responses to oxidized Cys/CySS redox state may be conserved between cell-types. Data are mean + SE of 4 replicates of a representative experiment.

and were cultured either at 32 °C or 37 °C. BMDMSCs cultured at 32 °C expressed higher levels of GPx, lower levels of ROS and demonstrated decreased NO levels and a decrease in markers of oxidative stress such as, malondialdehyde and protein carbonyl content. Interestingly, the decrease in oxidant stress was associated with a decrease in apoptosis. Similarly studies by Ebert *et al.* have shown that supplementing telomerase-immortalized human MSCs with selenium decreases ROS production and increases GPx activity, while cells cultured under selenium deficient conditions demonstrated increased DNA damage as evidenced by formation of micronuclei.[52]

In addition to the role of exogenous insults and nutrient deficiency on antioxidant capacity of BMDMSCs, *in vitro* studies demonstrate that MSCs may have the capacity to modulate the redox environment. For instance, adipose tissue derived MSC conditioned medium (ADCM) demonstrated antioxidant capacity comparable to 100 μM ascorbic acid. Furthermore, culturing tert-butyl hydroperoxide-treated dermal fibroblasts with ADCM improved cell viability. This study suggests that MSCs actively secrete antioxidant factors which may confer protection in the setting of inflammatory lung diseases.[53] However, studies in matrigel angiogenesis assay have demonstrated that direct contact of MSCs with endothelial cells (EC: MSC ratio; 1:1 to 1:3) led to increased ROS production resulting in endothelial cell apoptosis and ultimately to capillary degeneration. A drop in cytotoxicity was observed when MSC numbers were decreased by an order of magnitude. These studies indicate that *in vivo* effects of MSCs may vary depending on the MSC number and on the interacting cell population.[54]

MSC and thiol/disulfide redox state

Studies emerging from our laboratory suggest a reciprocal interaction between BMDMSCs and the extracellular thiol/disulfide redox state. In unpublished observations we have found that BMDMSCs exposed to an oxidized extracellular Cys/CySS redox state *in vitro* demonstrate a greater than two-fold upregulation in cellular ROS production (Figure 3). Because Cys/CySS redox state is oxidized in the setting of endotoxin-induced lung injury, understanding the effects of Cys/CySS redox on BMDMSC function and anti-inflammatory effects is paramount. Indeed preliminary observations from our laboratory suggest that production of interleukin-1 antagonist is decreased in endotoxin-stimulated cells that are exposed to an oxidized Cys/CySS redox state compared to physiological redox state. These findings suggest that therapies to preserve oxidation of Cys/CySS during ALI may improve the anti-inflammatory effects of BMDMSCs *in vivo*.

While redox state changes can impact critical signaling events in BMDMSCs, studies in a mouse model of endotoxin-induced lung injury are revealing that BMDMSC infusion improves Cys and GSH homeostasis. What these data show is that in addition to modulating the systemic inflammatory environment, infusion of BMDMSCs alters the systemic redox environment to a less oxidizing value. While the mechanistic basis for these changes is unclear it is likely that processes related to increased recycling and transport of thiols and disulfides may be involved.

BMDMSCs represent not only an emerging therapeutic modality, but also a paradigm for the resolution of inflammation. Therefore, identifying the mechanisms by which BMDMSCs modulate thiol/disulfide redox status will aid in understanding the regulation of these systems during the resolution of inflammation, and may unveil potential therapeutic targets. Furthermore, determining whether the anti-inflammatory effects of BMDMSCs can be augmented by dietary or pharmacological interventions to preserve Cys/CySS and GSH/GSSG redox state represents a therapeutic strategy that can be readily translated to the clinic.

MSC and the alcoholic lung

Alcohol abuse and the lung

Alcohol is one of the most commonly used and abused beverages throughout history. Numerous epidemiologic and experimental studies have described alcohol's health-related benefits, such as cardio- and neuro-protection when consumed in moderation.[55] However, long term alcohol abuse leads to dependency followed by multiple complications affecting different organ systems. One devastating consequence of alcohol abuse is immune and bone marrow suppression.[56] To date, a multitude of studies have shown that alcohol can alter the immune system by quantitatively and qualitatively disrupting both cytokine signaling and the immune regulatory cells.[57-59]

The link between alcohol and its detrimental effects towards the lung date back a century ago to observations made by William Osler, who noted that alcoholics had increased risk of having lung infections such as pneumonia and tuberculosis. This predisposition was initially attributed to alterations of immune function along with disruption of upper airway defenses resulting in unopposed entry of pathogens into the lungs.[60] It was not until the study by Moss,[60] showing alcoholism as an independent risk factor for developing acute respiratory distress syndrome (ARDS), that the concept of alcohol serving as a priming agent for inflammatory lung injury was born. This intriguing relationship later spawned investigations ultimately leading to the concept of the "alcoholic lung".[62]

Multiple mechanisms are involved in the pathogenesis of the alcoholic lung (Figure 4). These pathologic processes involve interactions between the different cellular components and the inflammatory mediators including: 1) perturbations in GM-CSF signaling by alveolar macrophages [63, 64] leading to epithelial barrier dysfunction and impaired innate immunity; 2) depletion of glutathione,[65-67] increased angiotensin II [68] followed by increased nitric oxide,[69] superoxide and NADPH oxidase [70] in the epithelial lining resulting in increased oxidative stress and cellular apoptosis; 3) increased TGF-β1 [71] and fibronectin [72] worsening pro-inflammatory cytokine production; 4) upregulated soluble endothelial selectin;[73] and, 5) changes in epithelial tight junctions (or claudins) [74] disrupting the endothelial-alveolar capillary barrier integrity leaking to "leaky" alveoli. Other pathways include impairment of the immune cell proliferation and functions leading to alterations in TNF-α, interleukins, interferon-γ, NF-κB, immunoglobulins, etc., further worsening the inflammatory response and weakening the host defense to infections. These are discussed in further detail in the following reviews.[57-60, 75]

MSC and the alcoholic lung

The effects alcohol on stem cells has not been fully characterized. Orthopedic literature has looked into effects on alcohol on BMDMSC differentiation and found that exposure to alcohol enhances adipogenic differentiation [76] and inhibits osteogenesis. So far, there have been no studies on the effects of alcohol on BMDMSC immune modulation, nor are there investigations on BMDMSC regulating alcohol-mediated inflammatory injury.

Preliminary *in vitro* experiments in our institution have shown that chronic alcohol exposure by stem cells increases their expression of TGF-β1 and fibronectin, suggesting a shift towards a pro-inflammatory and possibly, a pro-fibrotic state. Acute exposure on the other hand, decreases TNF-α secretion consistent with an anti-inflammatory effect. The

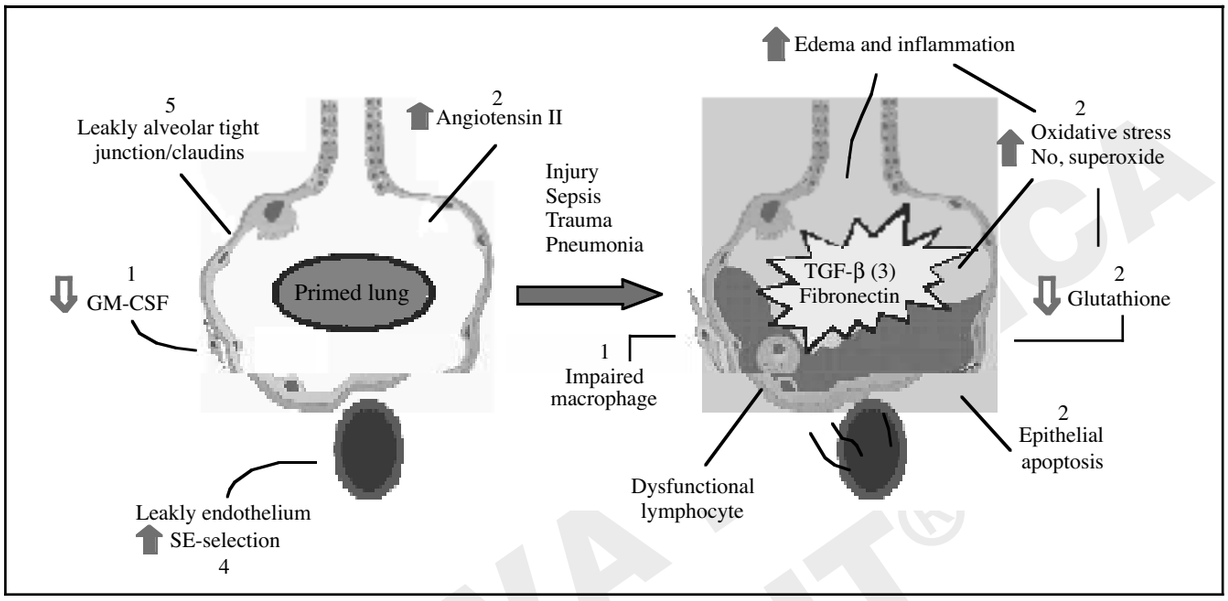

Figure 4.—Pathogenesis of the alcoholic lung. Multiple mechanisms are involved in the pathogenesis of the Alcoholic lung . These pathologic processes involve- 1) Perturbations in GM-CSF signaling by alveolar macrophages; 2) depletion of glutathione; 3) increased TGF-β1 and fibronectin expression; 4) upregulation of soluble endothelial selectin and; 5) changes in epithelial tight junction proteins.

authors are speculating that part of alcohol's detrimental effects on the immune system could be related to the bone marrow suppression of mesenchymal stem cell proliferation. Given the significant influence of BMDMSC on immune regulation, their depletion could exacerbate the dysfunctional inflammatory response observed in alcoholics. Could replacement of these cells modulate the alcohol-induced pro-inflammatory state? Can cellular therapy reverse the effects of chronic alcoholism on the immune system? Can BMDMSCs attenuate ARDS in the alcoholic lung? At this time, these questions remain unanswered. However, as discussed and illustrated above, the mechanisms whereby alcohol exerts its immune dysregulation are also pathways influenced by BMDMSCs. Further studies are needed to expand and correlate these findings with the goal of translating it towards clinical application.

Conclusions

Since the description of the immunomodulatory effects of BMDMSCs *in vitro* to their current applications in inflammatory lung diseases; BMDMSCs truly represent a bench to bedside paradigm. Indeed, considerable opportunity exists to extend the *in vivo* findings to the clinic to test whether MSC-based interventions are beneficial in patients with ALI, PH, and asthma.

At the same time, however, studies are needed to fill gaps in our understanding of the mechanistic role and the potential efficacy of BMDMSCs in inflammatory lung diseases. It must be noted that in most of the *in vivo* studies of ALI, BMDMSCs have been administered either before or immediately after the inflammatory challenge. Care should be taken to design experiments to test the efficacy of BMDMSCs when administered during the progression of lung injury/inflammation so that clinical outcomes can be readily envisioned.

Similarly, differences in outcomes between intra-pulmonary *versus* intra-venous routes must be addressed. Because ALI is a heterogenous clinical entity that can result from pulmonary insults such as aspiration of gastric contents and extra-pulmonary insults such as sepsis and pancreatitis, the efficacy of BMDMSCs must be tested in various models of ALI.

It is possible that the effects of BMDMSCs may vary depending on the underlying cause of ALI, and also on the route of administration. Finally, studies investigating the effects of BMDMSCs in models of live bacteria-induced ALI are paramount, to ensure that the anti-inflammatory effects of BMDMSCs does not compromise bacterial clearance. The way forward likely lies in identifying points of control in the inflammatory response that are modulated by BMDMSCs and apply that knowledge to design complementary therapies in ALI. If the immense therapeutic potential of BMDMSCs can be realized in the clinic, it will represent a breakthrough in the treatment of inflammatory lung diseases.

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