Aging Mesenchymal Stem Cells Fail to Protect Because of Impaired Migration and Antiinflammatory Response

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Abstract

Rationale: Aging is characterized by functional impairment and reduced capacity to respond appropriately to environmental stimuli and injury. With age, there is an increase in the incidence and severity of chronic and acute lung diseases. However, the relationship between age and the lung's reduced ability to repair is far from established and necessitates further research in the field.

Objectives: Little is currently known about age-related phenomena in mesenchymal stem cells (MSCs). On account of their ability to protect the endothelium and the alveolar epithelium through multiple paracrine mechanisms, we looked for adverse effects that aging might cause in MSC biology. Such age-related changes might partly account for the increased susceptibility of the aging lung to injury.

Measurements and Main Results: We demonstrated that old mice have more inflammation in response to acute lung injury. To investigate the causes, we compared the global gene expression of aged and young bone marrow–derived MSCs (B-MSCs). Our results revealed that the expression levels of inflammatory response genes depended on the age of the B-MSCs. We demonstrated that the age-dependent decrease in expression of several cytokine and chemokine receptors is important for the migration and activation of B-MSCs. Finally, we showed by adoptive transfer of aged B-MSCs to young endotoxemic mice that aged cells lacked the antiinflammatory protective effect of their young counterparts.

Conclusions: Taken together, the decreased expression of cytokine and chemokine receptors in aged B-MSCs compromises their protective role by perturbing the potential of B-MSCs to become activated and mobilize to the site of injury.

Keywords: aging; lung injury; ARDS; lung repair; mesenchymal stem cells

At a Glance Commentary

Scientific Knowledge on the Subject: Although our understanding of the biology of aging has advanced remarkably, the mechanisms linking aging to lung diseases remain unclear. Furthermore, little is currently known about age-related phenomena in mesenchymal stem cells (MSCs), and more specifically how aging affects their biological activity during repair processes in response to injury.

What This Study Adds to the Field: We demonstrate that aging of bone marrow–derived MSCs has a direct consequence on their activation, migration, and expression of soluble factors, all aspects that are required for an appropriate response during lung injury.

(Received in original form June 8, 2013; accepted in final form January 30, 2014)

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Supported by NIH grants R01 HL083019 and K01 HL084683-05 and by a grant from the American Federation of Aging.

Author Contributions: Conception, hypothesis delineation, and design, M.L.B., M.G.K., B.J.M., and M.R.; acquisition of data, analysis, and interpretation, M.L.B., L.H., M.G.K., C.L.L.-C., L.M., B.M.E., T.J.R., N.K., N.C., A.L.M., and M.R.; writing of the article, M.L.B., M.G.K., B.J.M., N.C., A.L.M., and M.R.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 189, Iss 7, pp 787–798, Apr 1, 2014

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Originally Published in Press as DOI: 10.1164/rccm.201306-1043OC on February 21, 2014

Internet address: www.atsjournals.org

Aging, as a natural physiological phenomenon, is characterized by a decline of homeostasis, functional impairment, and reduced capacity to respond appropriately to injury (1, 2). With age, different layers of defects affect vital functions and translate not only as increasing difficulty in adapting to environmental stimuli but also as increased vulnerability to diseases. In an aged lung, pulmonary function progressively deteriorates, inflammation increases, and the incidence of acute and chronic lung diseases rises (1, 3–5). The aptitude of stem cells and in particular bone marrow–derived mesenchymal stem cells (B-MSCs) in conferring lung protection in various preclinical models of acute and chronic injury has been previously described (6–10). Under normal conditions, quiescent MSCs are activated by inflammatory cytokines (11–14), inducing mobilization and secretion of a repertoire of soluble factors, several with demonstrated cytoprotective and antiinflammatory properties (15–18). The process of cell migration involves the activation of various chemokine receptors such as CCR2, CCR7, CXCR4, CXCR5, and CX3CR1 (12, 13, 19–22) and is facilitated by the production of matrix metalloproteinases that can degrade components of the extracellular matrix (23, 24).

Little is currently known about age-related phenomena of MSCs and how they affect their response to injury (25–28). Given that aging adversely affects the ability of B-MSCs to respond to injury (3), we hypothesized that aged MSCs undergo changes that impact their beneficial function during lung injury. Indeed, the current study demonstrates that B-MSCs from old animals do not protect as efficiently as those from young animals when tested in young mice injured by intraperitoneal injection of LPS, a well-established model of lung injury (29–38). To investigate the underlying mechanisms leading to such B-MSC dysfunction, we set out to compare the global gene expression profiles of aged and young B-MSCs. Our results reveal that a large number of genes involved in inflammatory response have lower expression levels in aged B-MSCs. It is interesting that this occurs without any increase in the expression of markers of cell senescence. In concordance with our previous work establishing the important role of B-MSC activation for their protective function (39), we find that the *in vitro* activation of aged B-MSCs results in

reduced migration and expression of soluble factors compared with young B-MSCs. Furthermore, we establish that gene expression of several cytokine and chemokine receptors such as tumor necrosis factor receptor (TNFR), IFN- γ receptor (IFNGR), and CCR7 decreases in aged B-MSCs. Our *in vivo* experiments corroborate our *in vitro* observations showing that the adoptive transfer of aged B-MSCs (murine and human) to young injured mice lacked the antiinflammatory protective effect of young B-MSCs and that aged B-MSCs have lower migration rates in a classical parabiosis model independently of the injured animal's age. In both cases, aged B-MSCs were associated with increased severity of lung injury.

In summary, our group has identified that the failure of aged B-MSCs to become activated and respond to lung injury is in consequence to the down-regulation of multiple cytokine and chemokine receptors.

Methods

Mouse Model of LPS-induced Lung Injury

Young (3-mo-old) and old (24-mo-old) female wild-type C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used in the various experiments. To induce lung injury mice were injected intraperitoneally with *Escherichia coli* LPS (1 mg/kg body weight; Sigma, St. Louis, MO). Mouse or human B-MSCs (5×10^5) were administered intravenously to young mice treated with LPS.

Results

Lung Inflammation Increases with Age in a Murine Model of LPS-induced Lung Injury

To compare the severity of lung injury between young and old mice, 3- and 24-month-old mice were injected intraperitoneally with LPS to generate lung injury (40). All the animals survived and were killed for analysis 48 hours after LPS injection. Several parameters were used to determine the degree of injury. Lung wet-to-dry weight ratios were measured, revealing only a mild increase in fluid content in the lungs of old mice after LPS injury (Figure 1A). Bronchoalveolar lavage (BAL) was performed at the time of sacrifice and supernatant was used for

cytokine quantification, which showed that the levels of IL-1 β , TNF- α , IL-6, and IL-10 were increased in old mice (Figure 1B). Moreover, old mice suffered a significant increase in whole and differential cell counts in their BAL (Figures 1C and 1D). Conversely, blind analysis of histological sections demonstrated that young mice had less lung vascular congestion and lower inflammatory cell infiltration (Figure 1E, panels 1 and 2), resulting in a lower histological lung injury score (Figure 1F). Our results confirm observations in other models of lung injury, in which old mice suffer more severe injury due to abnormal lung repair (2, 4, 5, 41, 42).

Aged B-MSCs Have Decreased Expression of Genes Involved in Activation, Antiinflammatory Response, and Migration without Increasing Markers of Cell Senescence

Gene expression profiles of young and aged B-MSCs, freshly sorted by fluorescence-activated cell sorting (FACS), were composed and compared with each other. Because of the low number of B-MSCs in bone marrow, three animals were pooled together for each sample, resulting finally in three pools of young B-MSCs and three pools of aged B-MSCs. Total RNA from young and aged B-MSCs was hybridized to Agilent mouse gene expression microarrays (Agilent Technologies, Santa Clara, CA) and genes that were differentially expressed between sorted B-MSCs from young and old animals were identified (Gene Expression Omnibus [GEO] link GSE44403). The transcript levels of several of the differentially expressed genes were later confirmed by real-time PCR (RT-PCR), where higher ΔC_T values correspond to a lower number of transcripts (see Table E1 in the online supplement). Our results show that aging induced a significant change in the gene expression profile of 927 genes (Figure 2A); it is interesting that the vast majority (840) of these genes had lower expression in aged B-MSCs as demonstrated by higher ΔC_T values. In agreement with the microarray results, RT-PCR data from 15 cytokines or cytokine receptors (Figure 2B), 3 chemokine receptors (Figure 2C), and 3 other transcripts (*Timp2*, *Marcks*, and *Mmp9*) that are involved in migration (Figure 2D) revealed decreased expression in aged

Figure 1. Lung inflammation increases with age in a murine model of LPS-induced lung injury. Several parameters of lung inflammation were assessed 48 hours after LPS-induced acute respiratory distress syndrome (ARDS) in young and old mice. (A) Wet-to-dry ratio indicates significantly increased water content in the lungs of old mice after injury. (B) Cytokine levels are increased in the bronchoalveolar lavage (BAL) of old mice ($n_o = 4$, $n_y = 6$, analyzed in triplicate by Luminex, Austin, TX). (C) Total counts of inflammatory cells are higher in the BAL of old mice ($n_o = 4$, $n_y = 6$). (D) Macrophages, lymphocytes, and neutrophils are elevated in the BAL of old mice ($n_o = 4$, $n_y = 6$). (B and D) Open bars, young mice; solid bars, old mice. (E) Representative histological sections of lung obtained from young mice (panel 1) and old mice (panel 2) after endotoxin treatment. (F) Lung inflammation score based on multiple histological sections from all animals. Data are represented as means $\pm$ SEM. Lym = lymphocytes; MAC = macrophages; n_o = number of old mice analyzed per experiment; n_y = number of young mice analyzed per experiment; Neu = neutrophils; TNF = tumor necrosis factor. Scale bars: 100 μ m. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

B-MSCs. Although *Ifngr1* and *Il17rc* were not in the differentially expressed gene list from our microarray analysis, they were included in the RT-PCR assay and proved to be significantly decreased in aged B-MSCs. It is interesting that we did not find differences in the expression of genes involved in cell senescence (Figure 2E).

Gene expression also shows that several metalloproteinases were down-regulated in aged B-MSCs (see Tables E2 and E3), suggesting that release of B-MSCs could be affected by aging. Furthermore, genes that are involved in osteogenic differentiation were down-regulated in aged B-MSCs (see

Table E4). Functional analysis of the differentially expressed genes revealed that many of them are implicated in biological functions such as cell migration, chemotaxis, inflammatory response, and activation (see Table E5). Furthermore, IL-10, IL-6, TNFR1 and TNFR2, and CXCR4 are among the canonical pathways associated with the down-regulated genes (see Table E6).

Aged B-MSCs Exhibit Reduced Production of Multiple Soluble Factors after *In Vitro* Stimulation

To understand how reduction in the expression of cytokine receptors in aged

B-MSCs could influence activation of the cells in an inflammatory environment, we obtained serum from young mice injected with LPS (LPS-serum), which was confirmed to contain multiple cytokines (see Figure E2A). Activation of B-MSCs with 0.5% LPS-serum resulted in dramatic differences in the gene expression of *Il1rn*, *Kgf*, *inos*, *Tgfb2*, and *Tgfb3* between young and aged B-MSCs, suggesting that aged B-MSCs have impaired activation (Figure 3A). To provide direct evidence that the decrease in B-MSC activation by the TNF- α and IFN- γ cytokines is a consequence of the age-related

Figure 2. Differential gene expression in aged versus young bone marrow-derived mesenchymal stem cells (B-MSCs). (A) Microarray map of sorted B-MSCs from young and old mice, showing 927 genes that are differentially expressed. (B–E) Real-time PCR detection of genes that are differentially expressed in microarrays including (B) cytokine receptors, (C) chemokines, (D) *Marcks*, *Mmp9*, and *Timp2*, and (E) markers of cell senescence. Open bars, young mice; solid bars, old mice. Values are expressed as means $\pm$ SEM; $n = 3$ samples per group, done in triplicate. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

down-regulation of the cytokine receptors, we blocked the receptors TNFR2 (with an antagonist) and IFNGR1 (with a neutralizing antibody) in young B-MSCs. The successful blocking of TNFR was confirmed by measuring the TNF- α -mediated phosphorylated/activated form of c-Jun N-terminal kinase-1 (JNK1) (43). Western blotting against phosphorylated JNK1 revealed impaired activation of the TNF- α intracellular signaling cascade in the cells where TNFR was blocked, similarly to aged B-MSCs (Figures 3B and 3C). No change was observed in the expression levels of total JNK. Blocking of TNFR2 in young B-MSCs resulted in decreased expression of *Il1rn*, *Nos2*, *Tgfb3*, and *Mmp9* as determined by RT-PCR (higher ΔC_T values correspond to lower number of transcripts) and a lower secretion level of IL-10, as determined by

ELISA (Figures 3D–3H). The observed levels were comparable to those of aged cells. Additional blocking of IFNGR1 had a synergistic effect on the expression levels of all the previously mentioned genes.

Antiinflammatory Capacity of Aged B-MSCs Is Decreased *In Vivo*

To evaluate differences in the antiinflammatory capacity of young and aged B-MSCs *in vivo*, 5×10^5 aged or young B-MSCs were administered intravenously to young mice that had been injected intraperitoneally with LPS to induce lung injury. Animals were killed 48 hours after injury and the antiinflammatory capacity of aged and young B-MSCs was assessed indirectly by measuring lung inflammation parameters.

We found that the injured mice that received aged B-MSCs not only had moderate wet-to-dry ratios (Figure 4A) but they also had elevated BAL levels of the cytokines IL-1 β , TNF- α , and IL-6 (Figure 4B). The severity of lung inflammation in mice treated with aged B-MSCs, as opposed to mice treated with young B-MSCs, was further confirmed by the increased number of inflammatory cells in BAL (Figures 4C and 4D) and the increase in lung vascular congestion and inflammatory cell infiltration (Figure 4E, panels 1–3), resulting in higher lung injury scores (Figure 4F).

These data are contrary to the effect observed when young B-MSCs were administered to 24-month-old mice with LPS-induced ARDS. As we demonstrate in Figure 4G, total cell counts in the BAL of mice that received young B-MSCs is lower than in mice that received LPS alone. This

Figure 3. Down-regulation of cytokine receptors in aged bone marrow-derived mesenchymal stem cells (B-MSCs) affects their activation. (A) The expression (RT-PCR) of antiinflammatory factors is significantly lower in aged B-MSCs after 0.5% LPS-serum cell activation. Open bars, young mice; solid bars, old mice. (B) Western blot showing that phosphorylated c-Jun N-terminal kinase (p-JNK) is lower in aged activated B-MSCs (column O) compared with young activated B-MSCs (column Y). Blocking of TNFR2 alone (column YT) or together with IFNGR1 (column YTI) in young activated B-MSCs inhibits the phosphorylation of JNK. β -Actin was used as a loading control. (C) Bar graph depicting the results of two TNFR2 blocking experiments. (D–H) Real-time PCR detection of (D) *Il1rn*, (E) *Nos2*, (F) *Tgfb3*, (G) *Mmp9*, and (H) *Il10* after blocking of TNFR2 and/or IFNGR1 in young activated B-MSCs. The values are expressed as means $\pm$ SEM; $n = 3$ samples per group, done in triplicate. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. IFNGR1 = IFN- γ receptor-1; inh = inhibitor; TNFR2 = tumor necrosis factor receptor-2.

observation clearly correlates with the histological sections stained with hematoxylin and eosin, where we observed lower numbers of inflammatory cells recruited into the injured lung (Figure 4H).

Decreased Expression of Chemokine Receptors in Aged B-MSCs Is Associated with Reduced Migration Capacity after *In Vitro* Stimulation

According to our microarray data, B-MSCs from old animals suffered a decrease in the expression of multiple genes that could be implicated in cell migration. RNA from B-MSCs (passage 0) was tested by RT-PCR for the expression of chemokine receptors *Ccr7*,

Cx3cr1, *Cxcr5*, as well as *Timp2*, *Marcks*, and *Mmp9* (higher ΔC_T values in aged B-MSCs correspond to lower number of transcripts) (Figures 2C and 2D). To understand how the down-regulation of chemokine receptors in aged B-MSCs could influence cell migration we incubated them in LPS-serum. After confirming that the mouse LPS-serum contained the chemokines macrophage inflammatory protein-3 β (ligand of CCR7) and fractalkine (ligand of CX3CR1) (see Figure E2C), it was used to stimulate aged and young B-MSCs (an optimal LPS-serum concentration for activating B-MSCs was defined as 0.5% of the culture medium; Figure E3B). The migration of cells was

dramatically different between activated young and aged B-MSCs. In contrast to young B-MSCs (Figure 5A, panels 3 and 4), aged B-MSCs (Figure 5A, panels 1 and 2) were not able to close the wound after 48 hours of stimulation. To provide direct evidence that the down-regulation of chemokine receptors affects the migration of B-MSCs, we blocked the receptor CCR7 in young B-MSCs, using a neutralizing antibody. The successful blocking of CCR7 was corroborated by Western blot against the phosphorylated form of JNK (Figures 5B and 5C) (44–46). The migration of young B-MSCs was assessed at 48 hours in a Transwell chamber with LPS-serum in the

Figure 4. Aged bone marrow–derived mesenchymal stem cells (B-MSCs) are not as protective as their young counterparts in LPS-induced lung injury. Several parameters of lung inflammation were assessed 48 hours after LPS-induced acute respiratory distress syndrome (ARDS) in young mice and concomitant administration of either young or aged B-MSCs (5×10^5). (A) Wet-to-dry ratio indicates increased water content in the lungs of mice that received aged B-MSCs ($n_o = 6$, $n_y = 7$). (B) Cytokine levels are increased in the bronchoalveolar lavage (BAL) of mice that received aged B-MSCs ($n_o = 6$, $n_y = 6$, analyzed in triplicate by multiplexed bead-based immunoassays). (C) Total counts of inflammatory cells are higher in the BAL of mice that received aged B-MSCs ($n_o = 6$, $n_y = 6$). (D) Macrophages, lymphocytes, and neutrophils are elevated in the BAL of mice that received aged B-MSCs ($n_o = 6$, $n_y = 6$). (E) Representative histological sections of lung obtained from LPS-treated mice without B-MSC treatment (panel 1), mice treated with young B-MSCs (panel 2), and mice treated with aged B-MSCs (panel 3). (F) Lung inflammation score based on multiple histological sections of all animals. To evaluate the protective effect of young B-MSCs in old mice, 5×10^5 young B-MSCs were administered intravenously to old (24-mo-old) mice. After 48 hours mice were killed and (G) BAL cell counts and (H) histological sections stained with hematoxylin and eosin were analyzed. Data are presented as means $\pm$ SEM. Lym = lymphocytes; Mac = macrophages; n_o = old mice analyzed per experiment; n_y = young mice analyzed per experiment; Neu = neutrophils; TNF- α = tumor necrosis factor- α . Scale bars: 100 μ m. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

lower well. Blocking of CCR7 reduced the migration rates to levels comparable to those achieved by aged B-MSCs (Figure 5D, panels 1 and 3; and Figure 5E).

Aging Adversely Affects B-MSC Migration *In Vivo*

Previous studies had demonstrated the homing of infused B-MSCs into the injured lung. The biological relevance of this observation has been obscure because of the inability to demonstrate the migration and recruitment of MSCs to the lung from

distal organs. An option is the development of chimeric mice. However, the resistance of B-MSCs to radiation limits their use in studies of B-MSC biology. To overcome this restriction we used a parabiosis model, which is a unique model to evaluate *in vivo* cell migration (47–52). This model results from the surgical attachment of two mice that develop a common circulation. The parabiotic configuration allowed tracking of green fluorescent protein (GFP)-labeled B-MSCs migrating from a donor GFP⁺ mouse into the lungs of an injured recipient

wild-type mouse. Cells can be tracked and quantified by FACS and indirect immunofluorescence assay. In the present assay GFP⁺ cells that were recruited into the injured lungs were detected and counted by FACS (Figure 6A).

In agreement with the *in vitro* experiments, the number of GFP-labeled B-MSCs from old donors was significantly lower in the young recipient lung (Figure 6B) and blood (Figure 6C) samples. Our results suggest that B-MSCs experience a defect in activation rather than lung recruitment

Figure 5. Aged bone marrow-derived mesenchymal stem cells (B-MSCs) migrate less than young MSCs. (A) Wound-healing assays of young and aged B-MSCs incubated with serum from endotoxemic mice (LPS-serum): aged B-MSCs at (panel 1) time 0 and (panel 2) 48 hours, and young B-MSCs at (panel 3) time 0 and (panel 4) 48 hours. (B) Western blot showing that phosphorylated JNK (p-JNK) is lower in aged activated B-MSCs (Old) compared with young activated B-MSCs (Young). Antibody blocking of CCR7 in young activated B-MSCs inhibits the phosphorylation of JNK (Young + CCR7 inhib). β -Actin was used as a loading control. (C) Bar graph depicting the results of CCR7-blocking experiments ($n = 2$). (D) Migration of B-MSCs in Transwell chambers after 48 hours of stimulation with LPS-serum: (panel 1) aged B-MSCs, (panel 2) young B-MSCs, and (panel 3) young B-MSCs subjected to CCR7 blocking. (E) Bar graph depicting the number of migrating cells ($n = 3$). Solid bars, old B-MSCs; open bars, young B-MSCs. Values represent means $\pm$ SEM; $n =$ number of experiments performed. Scale bars: 100 μ m. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$.

or retention. Importantly, recipient wild-type mice in parabiosis with old GFP⁺ donors had elevated levels of inflammatory cytokines in their serum (Figure 6D), increased lung vascular permeability (Figure 6E), and higher counts of inflammatory cells in their BAL (Figures 6F and 6G). Furthermore, they experienced more lung vascular congestion and inflammatory cell infiltration (Figure 6H, panels 1 and 2), having overall more severe lung injury scores (Figure 6I). Similar results were obtained with parabiotic pairs using old recipient mice (our unpublished data).

Human B-MSCs Derived from Old Donors Have Decreased Protective Capacity

To investigate whether our observations in murine B-MSCs apply to human B-MSCs

(hB-MSCs), we obtained hB-MSCs from cadaveric vertebral bones of young and old donors (referred to as young and aged hB-MSCs, respectively). The gene expression levels of the *TNFR1*, *IL6R*, and *IFNGR1* genes that were identified as differentially expressed in young and aged murine B-MSCs were assayed by RT-PCR and found to be down-regulated also in aged hB-MSCs (Figure 7A). To understand how the down-regulation of cytokine receptors in aged hB-MSCs could influence the activation of cells in an inflammatory environment we stimulated aged and young hB-MSCs with various concentrations of ARDS-serum obtained from patients with ARDS. Activation of hB-MSCs, documented as an increase in the secretion of IL-10, was optimal when using 0.5%

human ARDS-serum in the culture medium (see Figure E3D) and the IL-10 concentration was higher in young hB-MSCs compared with aged hB-MSCs.

The migration of aged and young hB-MSCs was quantified as described previously, using a Transwell chamber with ARDS-serum in the lower well. In agreement with our observations on murine B-MSCs, the migration of aged hB-MSCs after 48 hours was lower than the migration of young hB-MSCs (Figure 7B). Moreover, aged and young hB-MSCs were compared in wound-healing assays on incubation with human ARDS-serum. In contrast to young hB-MSCs (Figure 7C, panels 3 and 4), aged hB-MSCs were unable to close the wound after 48 hours of stimulation (Figure 7C, panels 1 and 2).

Figure 6. Migration of aged bone marrow–derived mesenchymal stem cells (B-MSCs) in parabiotic mice. Young wild-type acute respiratory distress syndrome (ARDS) mice were coupled with either young or old green fluorescent protein–positive (GFP⁺) mice to create parabiotic pairs. (A) The migration of GFP⁺ B-MSCs after 48 hours was tracked by flow cytometric analysis of total lung cell suspension in the wild-type mice. (B and C) The frequency of GFP⁺ B-MSCs derived from either young or old donors in (B) the lung and (C) the blood of wild-type mice. (D) Bronchoalveolar lavage (BAL) cytokine levels, (E) water content in the lung as measured by wet-to-dry ratio, (F) total count of cells in BAL, and (G) BAL macrophage, lymphocyte, and neutrophil ratios were elevated in the parabionts coupled with old GFP⁺ mice. (H) Histology of lungs from wild-type parabionts with (panel 1) young and (panel 2) old GFP⁺ donors. (I) Lung inflammation score based on histological sections. Open bars, young mice; solid bars, old mice. Values represent means $\pm$ SEM; n = 6 mice per group. Scale bars: 100 μ m. * P < 0.05, ** P < 0.005, *** P < 0.0005. Lym = lymphocytes; Mac = macrophages; Neu = neutrophils; TNF- α = tumor necrosis factor- α .

To evaluate *in vivo* the differences in the protective capacity of young and aged hB-MSCs, we administered intravenously 5×10^5 aged or young hB-MSCs to mice injected intraperitoneally with LPS and compared their recovery with that of animals that received saline only. All animals were killed 48 hours after injury. BAL analysis showed that despite the protective effect of both young and aged hB-MSCs, the injured

animals that had received aged hB-MSCs had significantly higher BAL levels of proinflammatory IL-1 β and IL-6 compared with the animals that received young hB-MSCs (Figure 7D), suggesting an age-related decrease in the protective capacity of the cells. In accordance with this, lung vascular permeability was significantly reduced only in the mice treated with young hB-MSCs (Figure 7E). Although the administration of

both young and aged hB-MSCs reduced the cell count in BAL, mice treated with aged hB-MSCs had significantly higher numbers of BAL lymphocytes and neutrophils (Figures 7F and 7G). Also in agreement, lung vascular congestion and inflammatory cell infiltration (Figure 7H, panels 1–3) as well as the lung injury score (Figure 7I) were higher in mice treated with aged hB-MSCs compared with mice treated with young hB-MSCs.

Figure 7. Aged human bone marrow-derived mesenchymal stem cells (hB-MSCs) are not as protective as their young counterparts in lung injury. (A) Real-time PCR of cytokine receptors expressed in aged and young hB-MSCs ($n = 3$, in triplicate). (B) Migration of hB-MSCs in Transwell chambers after 48 hours of stimulation with serum from patients with acute respiratory distress syndrome (ARDS) ($n = 4$). (C) Wound-healing assays of hB-MSCs incubated with serum from patients with ARDS: aged hB-MSCs at (panel 1) time 0 and (panel 2) 48 hours; young hB-MSCs at (panel 3) time 0 and (panel 4) 48 hours. Several parameters of lung inflammation were assessed 48 hours after LPS-induced ARDS in young mice and concomitant administration of saline or either young or aged hB-MSCs (5×10^5): (D) cytokine levels, (E) wet-to-dry ratio, (F) total counts of inflammatory cells, and (G) macrophage, lymphocyte, and neutrophil content in mice treated with saline (gray bars), young hB-MSCs (open bars), or aged hB-MSCs (black bars) ($n = 6$). (H) Representative histological sections of lung obtained from injured mice treated with saline (panel 1), mice treated with young hB-MSCs (panel 2), and mice treated with aged hB-MSCs (panel 3). (I) Lung inflammation score based on histological sections ($n = 6$). Values are expressed as means $\pm$ SEM. Scale bars: 100 μ m. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$. BAL = bronchoalveolar lavage; Lym = lymphocytes; Mac = macrophages; Neu = neutrophils; TNF- α = tumor necrosis factor- α .

Discussion

On the basis of extensive research done by us, and others, demonstrating the protective effect of young-B-MSCs in animal models of LPS injury (9, 16, 31, 35, 53–66) and our finding that old mice develop more lung inflammation in such models, we

hypothesized that the observed susceptibility to injury could be due to an age-related decrease in B-MSC function.

We compared global gene expression profiles between B-MSCs from old and young mice. To make sure that the transcriptomes reflected faithfully the natural state of B-MSCs, we performed all

our experiments with either freshly FACS-sorted cells or cultured FACS-sorted cells at passage 0. Our microarray analysis revealed that B-MSCs from old animals had significantly different expression levels of genes that were involved mainly in cell activation, migration, and differentiation but not different expression levels of genes

involved in cell senescence. It is interesting that the vast majority of these genes (90%) were decreased in old animals, implicating this as an underlying cause of the attenuated potential of aged B-MSCs, which is consistent with previous publications related to B-MSC aging and exhaustion (67).

An interesting finding was that aged B-MSCs had suffered a transcriptional decrease in receptors that play an important role in B-MSC biology. TNFR1 and TNFR2 mediate the majority of TNF- α responses and are also necessary for the activation of B-MSCs (11, 68). IL-6R promotes migration and cell survival of B-MSCs (69). IL-18 receptor accessory protein (IL-18RAP) is essential for IL-18-mediated signaling (70). IL-1R2 is a decoy receptor that sequesters IL-1 β (71). Not only do B-MSCs from old animals have lower expression of cytokine receptors but they also produce lower amounts of soluble factors and show reduced motility after *in vitro* activation. IL-1RN is an antiinflammatory factor that competes for binding to IL-1R1 (72), keratinocyte growth factor (KGF) has cytoprotective effects in endothelial and epithelial cells (65, 73), IL-10 is an antiinflammatory cytokine (16), and inducible nitric oxide synthase (iNOS) produces NO that inhibits the proliferation of T cells (17, 74). Further proof that the reduction in cytokine receptors plays an important role in the activation of B-MSCs, or rather the lack of it, was obtained by monitoring the expression of IL-10, IL-1RN, iNOS, transforming growth factor (TGF)- β_3 , and matrix metalloproteinase-9 (MMP9) after blocking TNFR2 and IFNGR1 in B-MSCs from young animals. Although B-MSCs from young mice show a dramatic increase in the expression of all five genes subsequent to activation, TNFR2 and IFNGR1 blocking causes a drop in expression to levels comparable to those in B-MSCs

from old mice. Our findings are in accordance with existing literature showing that TNF- α stimulation triggers MMP9 production in B-MSCs, which in turn promotes chemotactic migration through the extracellular matrix (75).

B-MSCs from old animals also have significant down-regulation of multiple chemokine receptors and other genes involved in B-MSC migration. In particular, chemokine receptors CCR7, CX3CR1, and CXCR5 are involved in B-MSC chemotaxis (19, 20). It has been demonstrated that CCR7 expression can depend, in T cells and dendritic cells, on TNF- α activation. The metalloproteinase MMP9 degrades extracellular matrix and basement membranes to allow cell migration across the blood vessels (76), and MARCKS (myristoylated alanine-rich C-kinase substrate) is involved in directed chemotaxis of B-MSCs (77). When we tested B-MSCs from old animals for their ability to migrate *in vitro* under inflammatory conditions we found that aging adversely affects their migration capacity and wound-healing ability. This is further confirmed by showing that the migration of young B-MSCs can be reduced to levels comparable to aged B-MSCs by blocking the CCR7 receptor.

The *in vivo* lung injury experiments are an important part of our study as they clearly demonstrate that aged endogenous B-MSCs not only fail to migrate efficiently to the injury site but once there they also fail to produce enough of the antiinflammatory agents that characterize their younger forms. Even when a large number of exogenous B-MSCs are administered intravenously, aged B-MSCs are significantly less efficient in resolving inflammation compared with young B-MSCs. Although animal models offer

invaluable insight concerning cellular mechanisms, it is critical to translate our observations into the human ARDS physiopathology. By using B-MSCs obtained from young and aged individuals we were able to demonstrate that aged hB-MSCs, similar to what occurs on murine MSCs, suffer a down-regulation of cytokine receptors that is accompanied by a decrease in activation, a decrease in migration, and consequently a decrease in their ability to regulate inflammation caused by LPS injury. Our observations are in agreement with data from Lavasani and colleagues demonstrating the rejuvenating effect of young but not aged wild-type MSCs in progeric mice (*Ercc1*^{-/-}) (78).

Our study is based on an unbiased evaluation of global gene expression changes that occur as B-MSCs age. We determined that differences between freshly isolated B-MSCs from young and old animals depend mainly on genes that are involved in cell activation, cell migration, and production of antiinflammatory molecules. Our *in vitro* and *in vivo* results converge to support the notion that underexpression of these genes can cause a profound decrease in the ability of B-MSCs to migrate to the site of an injury and resolve inflammation. Our data are relevant for understanding the mechanism of aging in B-MSCs and how it impacts health, and also for achieving effective stem cell therapeutic interventions. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the University of Pittsburgh Cancer Institute (UPCI) Luminex Core Facility for assistance with Luminex assays, and Theresa A. Heinrich for helping in the review of the manuscript, and Albert and Vera Donnenberg for generously providing the human B-MSCs.

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