Mesenchymal Stem Cells (MSCs) Attenuate Cutaneous Sclerodermatous Graft-Versus-Host Disease (Scl-GVHD) through Inhibition of Immune Cell Infiltration in a Mouse Model

Ji-Young Lim1,4, Da-Bin Ryu1,4, Sung-Eun Lee1, Gyeongsin Park2,3 and Chang-Ki Min1,3

Human chronic graft-versus-host disease (GVHD) shares clinical characteristics with a murine sclerodermatous GVHD model that is characterized by skin thickening and lung fibrosis. A B10.D2 → BALB/c transplant model of sclerodermatous GVHD was used to address the therapeutic effect of mesenchymal stem cells (MSCs) on the development of chronic GVHD. The clinical and pathological severity of cutaneous sclerodermatous GVHD was significantly attenuated in MSC-treated recipients relative to sclerodermatous GVHD control subjects. After MSC treatment, skin collagen production was significantly reduced, with consistent down-regulation of Tgfb expression. Effects of MSCs on molecular markers implicated in persistent transforming growth factor-β signaling and fibrosis, such as PTEN, phosphorylated Smad-2/3, and matrix metalloproteinase-1, were observed in skin tissue. MSCs neither migrate to the skin nor affect the in vivo expansion of immune effector cells, but they inhibited the infiltration of immune effector cells into skin via down-regulation of CCR4 and CCR8 expression on CD4+ T cells and CCR1 on CD11b+ monocyte/macrophages. MSCs diminished expression of chemokines such as CCL1, CCL3, CCL8, CCL17, and CCL22 in skin. MSCs were also dependent on stimulated splenocytes to suppress fibroblast proliferation. Our findings indicate that MSCs attenuate the cutaneous sclerodermatous GVHD by selectively blocking immune cell migration and down-regulating chemokines and chemokine receptors.


INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an effective treatment for leukemia and genetic disorders. The incidence of chronic graft-versus-host disease (CGVHD) is believed to be increasing, in part due to the use of peripheral blood progenitor cells, nonmyeloablative conditioning, early withdrawal of immunosuppressants, and longer survival due to better supportive care (Flowers et al., 2002; Zecca et al., 2002), but currently available therapies using long-term immunosuppressive treatments have shown limited efficacy.

Sclerodermatous GVHD (Scl-GVHD) in skin is the most common pathological change in human CGVHD and affects almost all organs and tissues, manifested by a marked increase in collagen deposition. The main clinical manifestations of Scl-GVHD include lichenoid lesions, sclerodermatous lesions, disorders of pigmentation (e.g., areas of hypopigmentation and hyperpigmentation), and leopard-skin eruptions (widespread, well-delimited, hyperpigmented macules) (Cho et al., 2009; Filipovich et al., 2005; Peñas et al., 2002). There are very few experimental models that have been developed to examine CGVHD. Among them, the B10.D2 (H-2d) → BALB/c (H-2b), major histocompatibility complex-matched and minor histocompatibility antigen-mismatched model replicates human Scl-GVHD and systemic sclerosis. Skin thickening and pulmonary fibrosis with increased collagen and excessive extracellular matrix deposition develop predominantly, and fibrosis also may affect the liver, kidneys, gastrointestinal tract, and parotid glands in Scl-GVHD mice (Anderson et al., 2003; McCormick et al., 1999; Zhang et al., 2002). Although proinflammatory processes and vasculopathy dominate the early stages of Scl-GVHD, progressive tissue fibrosis is the key feature of late-stage disease in each organ (Varga and Abraham, 2007).
Mesenchymal stem cells (MSCs) generated from bone marrow (BM) aspirate have self-renewal and differentiation capacities with ex vivo expansion and in vitro differentiation into the osteogenic, chondrogenic, or adipogenic lineages (Dominici et al., 2006). Reproducible immunomodulatory properties of MSCs and clinically relevant characteristics, including ease of expansion and safe infusion profile, are well known. Furthermore, because allogeneic MSCs do not also stimulate the immune reaction, presumably because of minimum surface markers such as major histocompatibility complex class II molecules (Abumaree et al., 2012), MSCs have attracted great interest for use as a cell-based immunotherapy for steroid-refractory GVHD (Le Blanc et al., 2008). However, the biologic mechanisms underlying MSC effects in vivo remain unclear (Auletta et al., 2010; Horwitz et al., 2011), with mixed clinical results and tempered enthusiasm for the use of MSCs after allo-HSCT (Galipeau, 2013). Several clinical trials assessing the efficacy of MSCs in immune-mediated diseases are currently underway (Kaplan et al., 2011; Wang et al., 2011).

We used an established allo-HSCT model to elucidate the in vivo immunomodulatory mechanisms of MSCs that mediate anti-inflammatory and anti-fibrotic effects in Scl-GVHD recipients. Given the complex inflammatory milieu associated with GVHD, it is possible that anti-inflammatory therapy using third-party MSCs would be beneficial. The murine allo-HSCT model has helped define in vivo MSC-mediated immunosuppressive mechanisms that contribute to attenuation of Scl-GVHD severity.

RESULTS

Characteristics of murine MSCs

MSCs are characterized by expression of several surface markers and display multipotent differentiation along mesenchymal lineages (Dominici et al., 2006). Phenotypic characterization of M210B4 cells and primary MSCs by flow cytometric analysis using standard markers showed that they were more than 95% positive for Sca-1 and CD44 and were negative for CD34, CD45, Flk-1, CD117, and CD11b (see Supplementary Figure S1 online).

Early injection of M210B4 or primary MSCs attenuated the severity of murine Scl-GVHD in skin

M210B4 MSCs were intravenously administered to allogeneic recipients on days 3, 5, and 7 after allo-HSCT. When compared with the allogeneic control group, early treatment with M210B4 MSCs significantly inhibited the severity of Scl-GVHD, as evidenced by decreased average skin score (Figure 1a). However, mice receiving M210B4 MSCs did not show a marked recovery in body weight (data not shown). As shown in Figure 1b, the alleviated severity of skin disease was also verified by histopathologic findings, including histopathologic scores and dermal thicknesses. Moreover, fibrosis areas and collagen amounts in the skin in the MSC-treated group were significantly lower than those in the allogeneic control group (Figure 1c). In parallel, the mRNA expression levels of collagen type 1α1 (COL1A1), 1α2 (COL1A2), and 3α1 (COL3A1) were significantly reduced in skin tissue after MSC treatment, compared with those among allogeneic Scl-GVHD controls (Figure 1d). On the other hand, those parameters in the lungs did not differ between the two groups (see Supplementary Figure S2a—d online), nor did MSC treatment influence collagen parameters in the liver (data not shown). To confirm the Scl-GVHD protection effect of primary MSCs, BM-derived C57BL/6 MSCs were intravenously administered at $3 \times 10^5$ cells to allogeneic recipients on days 3, 5, and 7. Similarly, when compared with the allogeneic control group, early treatment with primary MSCs also significantly inhibited the severity of Scl-GVHD with recovery in body weight (Figure 1e). To see the therapeutic effects of MSCs after Scl-GVHD development, M210B4 MSCs or primary BM MSCs were injected early (days 14, 21, and 28) or late (days 28, 30, and 32) after onset of clinical Scl-GVHD. Both types of MSCs did not have therapeutic effects (see Supplementary Figure S3a, S3b online). Together, these data indicate that MSCs early after allo-HSCT play a role in suppressing cutaneous Scl-GVHD, but not visceral Scl-GVHD, by more effectively exerting an antifibrotic effect in skin.

MSCs reduced mRNA expression of various cytokines in the skin

Cytokines have been reported to play an important role in the pathogenesis of Scl-GVHD (Zhou et al., 2007). In skin, as shown in Figure 2a, expression of mRNAs for the cytokines transforming growth factor (TGF)-β, INF-γ, IL-10, IL-1β, and IL-6 were up-regulated in animals with Scl-GVHD, compared with non-GVHD controls not receiving allogeneic splenocytes, 14 days after allo-HSCT. However, expression levels of these cytokine mRNAs were decreased after MSC treatment. In the lung, on the other hand, mRNA expression levels of TGF-β, INF-γ, and IL-10 were increased in allogeneic recipients, and among them, TGF-β and IFN-γ were reduced after injection of MSCs (see Supplementary Figure S4a online). Furthermore, MSC treatment increased the mRNA levels of IFN-γ and IL-10 in the liver (see Supplementary Figure S5a online).

Twenty-eight days after allo-HSCT, mRNA expression of TGF-β alone was consistently elevated in the skin of mice with Scl-GVHD compared with non-GVHD controls, and MSC treatment persistently reduced cutaneous TGF-β levels for a prolonged period after allo-HSCT (Figure 2b). These data show that the infusion of MSCs early after allo-HSCT altered the expression of various proinflammatory cytokines in skin at an early time point and that the GVHD-protective effect of MSCs on TGF-β expression is sustainable in this model.

MSCs restored PTEN expression and normalized phosphorylated Smad-2/3 and matrix metalloproteinase-1 (MMP-1) levels in skin

TGF-β plays a central role in pathological tissue fibrosis, and overexpression of PTEN abrogates TGF-β-induced Smad-2/3 phosphorylation (Bu et al., 2008). PTEN expression was decreased and phosphorylated Smad-2/3 was increased in this murine Scl-GVHD model (Huu et al., 2013). MSC treatment restored PTEN expression and reduced the level of Smad-2/3 phosphorylation in the skin at days 14 and 28 (Figure 3a and 3b, respectively). In the lung, however, the levels of PTEN and Smad-2/3 phosphorylation did not differ between MSC-treated allogeneic recipients and Scl-GVHD controls (see Supplementary Figure S4b). Furthermore, neither did MSC treatment influence levels of PTEN or Smad-2/3 phosphorylation in the liver (see Supplementary Figure S5b).

In injured human skin, MMP-1 is induced as wound-edge keratinocytes bind type I collagen in the dermis,
and the ability of this proteinase to cleave collagen is key to facilitating keratinocyte movement (Pilcher et al., 1997). MMP-1 expression in the skin, but not in the lungs, was reduced in MSC-treated allogeneic recipients compared with Scl-GVHD controls (Figure 3, and see Supplementary Figure S4b). In the liver, MMP-1 levels were not altered by treatment with MSCs (see Supplementary Figure S5b).

Figure 1. MSCs attenuated the severity of skin scleroderma graft-versus-host disease (Scl-GVHD) and improved skin fibrosis. (a) BALB/c mice transplanted with T-cell-depleted bone marrow (TCD-BM) and spleen cells from B10.D2 mice (Scl-GVHD) have chronic dermatitis and an increased average skin score. However, mice receiving M210B4 cells (Scl-GVHD + MSCs) have markedly decreased chronic dermatitis and skin scores. BALB/c mice receiving transplantations with cells from B10.D2 TCD-BM (non-GVHD control) do not show dermatitis or hair loss. (b–c) Slides were scored, and soluble collagen was measured as described in the Materials and Methods section. Representative photomicrographs of histopathological changes from the non-GVHD controls, Scl-GVHD, and Scl-GVHD + MSCs groups on day 14 (upper panel) and day 28 (lower panel) after transplantation. Sections were stained with hematoxylin and eosin or Masson’s trichrome. Skin fibrosis was compared by determining dermal thickness, trichrome area/total area, and soluble collagen production. (d) mRNA expression of collagen 1α1, 1α2, and 3α1 on day 14 (upper panel) and day 28 (lower panel) after transplantation. (e) Recipient mice receiving primary MSCs (Scl-GVHD + primary MSCs) also exhibited less severe skin Scl-GVHD. Original magnification ×100. Scale bar = 100 μm. Each value indicates mean ± standard error of the mean of n = 4–7 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001; Scl-GVHD versus Scl-GVHD + MSCs or Scl-GVHD + primary MSCs. BMT, bone marrow transplantation; D, day; GVHD, graft-versus-host disease; MSC, mesenchymal stem cell; Scl, scleroderma.

In vivo detection of injected MSCs
To examine the lifespan of primary MSCs in GVHD target organs and secondary lymphoid organs, allogeneic
recipients receiving MSCs were sacrificed at 14, 21, 28, and 35 days after allo-HSCT. MSCs were frequently observed in lung, liver, spleen, and peripheral lymph nodes (LNs), but not in skin (Figure 4).

**Infiltration of immune effector cells into each organ and in vitro assays**

It has been hypothesized that CD11b⁺ monocyte/macrophage activation by host-reactive CD4⁺ T cells is an initiating event in Scl-GVHD (McCormick et al., 1999). Secondary lymphoid organs like the spleen and LNs are important sites of donor T-cell activation and expansion during allo-HSCT (Beilhack et al., 2005). Flow cytometric analyses 14 days after allo-HSCT, performed on the skin, spleen, and peripheral LNs, showed that the frequency and number of splenic CD4⁺ T cells (Figure 5a) and CD11b⁺ monocyte/macrophages (Figure 5b) were significantly higher in Scl-GVHD controls than in non-GVHD controls. After MSC treatment, the frequency and number of CD4⁺ T cells and CD11b⁺ monocyte/macrophages in the skin were markedly decreased compared with those in Scl-GVHD mice, but the frequency and number of those cells in spleen, LNs, lung, and liver were similar between the two groups (Figure 5a, 5b, and see Supplementary Figures S4c, S5c).

Figure 2. Decreased mRNA expression of skin cytokines in the MSC-treated group after allo-HSCT. BALB/c mice underwent transplantation with T-cell–depleted bone marrow (TCD-BM) and spleen cells from B10.D2 mice and were injected with M210B4 MSCs or not (Scl-GVHD + MSCs and Scl-GVHD, respectively), as in Figure 1a. Non-GVHD controls received TCD-BM alone. Expression of TGF-β, INF-γ, IL-10, IL-1β, IL-6, and TNF-α mRNA in skin (a) 14 days and (b) 28 days after allo-HSCT. Each value indicates mean ± standard error of the mean of n = 4–7 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001; Scl-GVHD versus Scl-GVHD + MSCs. allo-HSCT, allogeneic hematopoietic stem cell transplantation; D, day; GVHD, graft-versus-host disease; MSC, mesenchymal stem cell; Scl, sclerodermatous; TNF, tumor necrosis factor; TGF, transforming growth factor.

Figure 3. MSCs restored PTEN expression and normalized Smad-2/3 phosphorylation status and matrix metalloproteinase-expression in skin tissue. BALB/c mice underwent transplantation with T-cell–depleted bone marrow (TCD-BM) and spleen cells from B10.D2 mice and were injected with M210B4 MSCs or not (Scl-GVHD + MSCs and Scl-GVHD, respectively), as in Figure 1a. Non-GVHD controls received TCD-BM alone. Immunohistochemical staining (left) was quantified (right) at (a) 14 and (b) 28 days after transplantation. Original magnification ×400. Scale bar = 12.5 μm. Each value indicates mean ± standard error of the mean of n = 4–7 mice per group. **P < 0.01, ***P < 0.001; Scl-GVHD versus Scl-GVHD + MSCs. D, day; GVHD, graft-versus-host disease; MSC, mesenchymal stem cell; Scl, sclerodermatous.

Figure 2. Decreased mRNA expression of skin cytokines in the MSC-treated group after allo-HSCT. BALB/c mice underwent transplantation with T-cell–depleted bone marrow (TCD-BM) and spleen cells from B10.D2 mice and were injected with M210B4 MSCs or not (Scl-GVHD + MSCs and Scl-GVHD, respectively), as in Figure 1a. Non-GVHD controls received TCD-BM alone. Expression of TGF-β, INF-γ, IL-10, IL-1β, IL-6, and TNF-α mRNA in skin (a) 14 days and (b) 28 days after allo-HSCT. Each value indicates mean ± standard error of the mean of n = 4–7 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001; Scl-GVHD versus Scl-GVHD + MSCs. allo-HSCT, allogeneic hematopoietic stem cell transplantation; D, day; GVHD, graft-versus-host disease; MSC, mesenchymal stem cell; Scl, sclerodermatous; TNF, tumor necrosis factor; TGF, transforming growth factor.

Figure 3. MSCs restored PTEN expression and normalized Smad-2/3 phosphorylation status and matrix metalloproteinase-expression in skin tissue. BALB/c mice underwent transplantation with T-cell–depleted bone marrow (TCD-BM) and spleen cells from B10.D2 mice and were injected with M210B4 MSCs or not (Scl-GVHD + MSCs and Scl-GVHD, respectively), as in Figure 1a. Non-GVHD controls received TCD-BM alone. Immunohistochemical staining (left) was quantified (right) at (a) 14 and (b) 28 days after transplantation. Original magnification ×400. Scale bar = 12.5 μm. Each value indicates mean ± standard error of the mean of n = 4–7 mice per group. **P < 0.01, ***P < 0.001; Scl-GVHD versus Scl-GVHD + MSCs. D, day; GVHD, graft-versus-host disease; MSC, mesenchymal stem cell; Scl, sclerodermatous.
Next, we tested the capacity to suppress immune effector cells and the antifibrotic effect of MSCs. They significantly inhibited in vitro proliferation of CD4\(^+\) T and CD11b\(^+\) cells in stimulated splenocytes (Figure 5c) via suppression of pro-inflammatory cytokines (Figure 5d). Human dermal fibroblasts (HDFs) were co-cultured with primary MSCs in the presence or absence of stimulated splenocytes. MSCs with stimulated splenocytes significantly inhibited proliferation of HDFs, confirming that the antifibrotic effect of MSCs is immune cell dependent (Figure 5e). Together, these data indicate that MSCs did not affect the in vivo expansion of immune cells in the spleen or LNs but did affect their migration into skin without direct antifibrotic effect in this Scl-GVHD model.

**MSCs reduced expression of skin chemokines and their related chemokine receptors**

Organ-specific homing of immune cells is regulated by chemokines and their receptors, and up-regulation of cutaneous chemokines/cytokines has been reported in Scl-GVHD (Shi et al., 2007; Zhang et al., 2002). At day 14, mRNA for the chemokines CCL1, CCL3 (MIP-1), CCL8, CCL17, and CCL22 were up-regulated in mice with Scl-GVHD compared with non-GVHD controls (Figure 6a [skin], and see Supplementary Figure S4d [lung]). As shown in Figure 6a and Supplementary Figures S4d and S5d, at day 14, their mRNA expressions in the skin were significantly reduced by MSCs but not in the lung and liver. At day 28, mRNA expressions of CCL1, CCL3, CCL8, and CCL17 were reduced after MSC treatment in the skin alone. The suppressive effect of MSCs on those chemokines at the protein level was likewise observed in the skin, but not in the lung or liver, on days 14 and 28 (Figure 6b, and see Supplementary Figures S4e, S5e). Accordingly, chemokine receptors involved in skin migration, including CCR4 and CCR8 on CD4\(^+\) T cells, as well as CCR1 on CD11b\(^+\) monocyte/macrophages, were significantly down-regulated after MSC treatment (Figure 6c). Elevated CXCR4 expression on immune cells, which is associated with SDF-1/CXCL12, has been shown to play a crucial role in the migration of immune cells, consequently resulting in GVHD target tissue damage (Le Huu et al., 2014), but MSC treatment did not affect the SDF-1 and CXCR4 axis in this study (see Supplementary Figure S6a, S6b online). CCL2 and CCR2 are also not changed after MSC treatment (see Supplementary Figure S6c).

**DISCUSSION**

The use of human MSCs to prevent or treat CGVHD with sclerodermatous changes has been limited by an incomplete understanding of the mechanism of action of these cells (Galipeau, 2013; Horwitz et al., 2011). In this study, we showed that administration of BM-derived third-party MSCs early after allo-HSCT clearly reduced the severity of alopecia and fibrosis of the Scl-GVHD mice. Potent antifibrotic activity was observed only in the skin, as determined by both skin manifestation and histopathology, and not in the lung or liver. Early MSC treatment down-regulated TGF-\(\beta\) expression, mainly in skin tissues, continuously for 28 days, and accordingly, PTEN expression was restored, and phosphorylated Smad-2/3 and MMP-1 levels were reduced, only in the skin. Although MSCs were not recruited into the skin, MSCs specifically decreased the infiltration of CD4\(^+\) T and CD11b\(^+\) effector cells into the skin, exclusively, but not into secondary lymphoid organs or other GVHD targets, such as the lung or liver, via the complex interaction of chemokines and chemokine receptors. These results suggest that MSC...
MSCs Attenuate Scl-GVHD by Inhibiting Immune Cell Recruitment

Figure 5. Reduced infiltration of immune cells into skin after MSC treatment and in vitro anti-inflammatory and antifibrotic function of MSCs. BALB/c mice underwent transplantation with T-cell–depleted bone marrow (TCD-BM) and spleen cells from B10.D2 mice and were injected with M210B4 MSCs or not (Scl-GVHD + MSCs and Scl-GVHD, respectively), as in Figure 1a. Non-GVHD controls received TCD-BM alone. Flow cytometric analyses were performed using skin, spleen, and peripheral LNs. The frequency and number of (a) CD4+ T cells and (b) CD11b+ monocyte/macrophages are shown 14 days after transplantation. Each value indicates mean ± standard error of the mean of n = 4–7 mice per group. *P < 0.05, **P < 0.01, ***P < 0.001; Scl-GVHD versus...
fibrosis of the skin and inner organs and infiltration by cutaneous mononuclear cells (predominantly CD4 T cells and CD11b+ monocyte/macrophages) starting 14 days after allo-HSCT (McCormick et al., 1999; Yamamoto, 2010). The pathophysiologic processes implicated in the high fibrosis of Scl-GVHD are largely unknown. It has been shown that inflammatory cytokines and/or chemokines released from immune effector cells, endothelial cells, fibroblasts, and other various cell types in the GVHD target organs are involved in the initiation and progression of excessive fibrotic process (Distler and Distler, 2008). Extracellular matrix components, mainly type I and III collagen, are extraordinarily accumulated in Scl-GVHD. As far as we know, the anti-fibrotic effect of MSCs has been examined only in a model of bleomycin-induced lung fibrosis (Kumamoto et al., 2009; Moodley et al., 2009; Ortiz et al., 2007; Zhao et al., 2008). Despite the available in vitro and in vivo data suggesting that MSCs may have a beneficial effect on sclerodermatous lesions, to the best of our knowledge there have been no studies that have found how MSCs are involved in regulating the severity of Scl-GVHD in this model.

Few data are clinically available regarding MSC-based therapy in patients with scleroderma or CGVHD. Scl-GVHD represents 10–15% of cases of human CGVHD (Vogelsang et al., 2003). Some patients with severe refractory systemic sclerosis have received intravenous infusions of allogeneic MSCs (Christopeit et al., 2008; Keyszer et al., 2011). No conclusions about the efficacy of MSC transplantation can be drawn from these clinical cases, although skin improvement was noted in some patients, albeit in the absence of detailed immunomonitoring. MSCs seemed to be more promising and effective in CGVHD of the oral mucosa, gastrointestinal tract, liver, and skin in one published report (Ringdén et al., 2006). Some patients with severe scleroderma showed a partial response to MSC treatment, whereas MSCs might be ineffective in patients with bronchiolitis obliterans. Similarly, this study also found that MSCs had different therapeutic effects on two target organs, the skin and lungs.

This activation of MSCs in response to stimulation by TGF-β, which has a major role in the pathogenesis of Scl-GVHD, might limit the clinical use of allogeneic MSCs, because activation of the TGF-β signaling pathway could lead to an increase in the synthesis of target genes, including the gene encoding collagen type 1 (Vanneaux et al., 2013). In this study, up-regulated TGF-β mRNA expression was sustained in allogeneic recipients after the induction of CGVHD, and MSC treatment early after allo-HSCT persistently suppressed

**Figure 6.** MSCs reduced mRNA expression of skin chemokines and their related chemokine receptors. BALB/c mice underwent transplantation with T-cell–depleted bone marrow (TCD-BM) and spleen cells from B10.D2 mice and were injected with M210B4 MSCs or not (Scl-GVHD + MSCs and Scl-GVHD, respectively), as in Figure 1a. Non-GVHD controls received TCD-BM alone. Levels of (a) mRNA and (b) protein for CCL1, CCL3, CCL8, CCL17, and CCL22 in the skin 14 days (left) and 28 days (right) after allo-HSCT. (c) Levels of mRNA for CCR4 and CCR8 in splenic CD4+ T cells and CCR1 and CCR5 in splenic CD11b+ monocyte/macrophages. Each value represents the mean ± standard error of the mean of n = 4–7 mice per group. *P < 0.05; **P < 0.01, ***P < 0.001, Scl-GVHD versus Scl-GVHD + MSCs. allo-HSCT, allogeneic hematopoietic stem cell transplantation; D, day; GVHD, graft-versus-host disease; MSC, mesenchymal stem cell; Scl, sclerodermatous.
TGF-β mRNA expression in the skin but not in the lung. PTEN deficiency has been reported to be associated with fibrosis (Nho et al., 2006), and TGF-β has been shown to activate phosphorylation of the Smad3 signaling pathway in fibroblasts (Gu et al., 2007). Our results are consistent with studies reporting that molecular markers implicated in TGF-β signaling, such as PTEN, Smad-2/3 phosphorylation, and MMP-1, are involved in the regulation of fibrosing activity (Huu et al., 2013).

To study other possible mechanisms, we assayed the migration of immune cells to assess how MSCs might participate in the amelioration of CGVHD. The inhibition of lymphocyte migration attenuates ScI-GVHD (Huu et al., 2013). Chemokines are crucial mediators of leukocyte trafficking into sclerotic skin (Hasegawa and Sato, 2008). In this model, elevated expression of skin profibrotic chemokines, such as CCL11, CCL3, CCL8, CCL17, and CCL22, were downregulated by administration of MSCs, consequently resulting in decreased infiltration of immune cells into skin tissues. CCL2, CCL3, and CCL5 have been identified as the chemokines most critical to tissue fibrosis and inflammation in systemic sclerosis, and their expression was found to precede the progression of skin and pulmonary fibrosis in this ScI-GVHD model (Zhang et al., 2002). Increased levels of the T helper type 2 chemokines CCL17, CCL22 (Fujii et al., 2004), and CCL27 (Hayakawa et al., 2005) in the serum of patients with systemic sclerosis have also been linked to the extent of skin sclerosis. CCR4 and its ligands (CCL17 and CCL22) are important for the recruitment of memory T cells into the skin in various cutaneous immune diseases (Kusumoto et al., 2007). After MSC infusion, the expression of CCL17 and CCL22 proteins was also significantly reduced in the skin of ScI-GVHD mice but not in the lungs or liver. Our study showed that MSCs affected the associated chemokine receptors expressed on CD4+ and CD11b+ immune cells. Absence of MSCs in the skin (Figure 4) and dependency of an in vitro antifibrotic effect of MSCs on the stimulated splenocytes (Figure 5e) also suggest that blockade of immune cell migration contributes to reduced ScI-GVHD severity (Baggiolini, 1998; Huu et al., 2013). However, it would be important to further investigate the molecular mechanisms that underlie the differences in effects on dermal and pulmonary fibrosis, because these differences in the immunomodulatory properties of MSCs may have functional relevance to the therapeutic applications of MSCs.

In conclusion, we have shown that administration of MSCs early after transplantation effectively reduces the severity of skin fibrosis in murine ScI-GVHD, suggesting that MSC treatment is a potential candidate for use in treating patients with ScI-GVHD and scleroderma. MSCs might have a role in antifibrotic effects with influence on the immune effector cells and, in particular, by reducing their infiltration into skin.

MATERIALS AND METHODS

Experimental allo-HSCT and MSCs

Female B10.D2 (H-2b) and BALB/c (H-2d) mice (8–12 weeks old) were purchased from Shizuoka Institute for Laboratory Animals (Japan SLC, Shizuoka, Japan). Briefly, recipient (BALB/c) mice were lethally irradiated with 650 cGy using a 137Cs source (Gammacell 3000 Elan, MDS Nordion, Ottawa, Ontario, Canada). Approximately 6 hours later they were injected intravenously via the tail vein with donor (B10.D2) T-cell–depleted BM (3 × 10^6 cells/mouse) and spleen cells (5 × 10^6 cells/mouse) (referred to as ScI-GVHD mice). A control group of BALB/c recipient mice received either B10.D2 donor BM without T cells (non-GVHD controls) or BALB/c BM with T cells (syngeneic controls). The M210B4 cell line (CRL-1972, ATCC, Manassas, VA) is a clone derived from BM stromal cells from a C57BL/6J × C3H/HeJ F1 mouse, and primary MSCs were obtained from C57BL/6 mice. M210B4 cells (ScI-GVHD + MSCs) or primary MSCs (ScI-GVHD + primary MSCs) were administered after allo-HSCT at a dose of 3 × 10^5 cells/mouse. All animal experiments were approved by the institutional animal care and use committees of the Catholic University of Korea.

GVHD skin score

The clinical skin GVHD score was modified as previously described (Anderson et al., 2003). The minimum score was 0, and the maximum score was 8.

Histopathological analysis

Formalin-fixed, paraffin-embedded tissue sections were subjected to hematoxylin-eosin staining for microscopic examination and Masson’s trichrome staining for fibrosis. Slides were scored by a pathologist (blinded to experimental groups). Dermal thickening from the bottom of epidermis to fat was evaluated for each animal as previously described (Lim et al., 2014). Collagen deposition was quantified on trichrome-stained sections as the ratio of blue-stained area to total stained area using digital analyzer software ImageJ (National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov).

Protein extracts and measurement of soluble collagen

Tissue samples were homogenized in 2 ml of buffer solution and centrifuged at 3,000 r.p.m. for 20 minutes, after which supernatants were harvested. Total protein concentrations in supernatant were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Total soluble collagen was quantified using the Sircol Soluble Collagen Assay (Bio-color, Belfast, Ireland) as previously described (Oh et al., 2011).

Immunohistochemical staining and in vivo tracking

More detailed information of immunohistochemical methods for PTEN, phosphorylated Smad, and MMP-1 are described in the Supplementary Materials online. Immunohistochemical stains were evaluated for the presence of positively staining cells in the dermis as previously described (Le Huu et al., 2014). Information regarding fluorescent detection for in vivo tracking primary MSCs is described in the Supplementary Materials.

Stimulation of splenocytes and HDFs and their co-culture with primary MSCs

Splenocytes (1 × 10^5) form B10.D2 mice were stimulated with 2 μg/ml anti-CD3/CD28 (eBioScience, San Diego, CA) and 5 μg/ml lipopolysaccharide (Sigma-Aldrich, St. Louis, MO) for 72 hours. Human dermal fibroblasts were labeled with carboxyfluorescein succinimidyl ester (Invitrogen, Carlsbad, CA), and then were stimulated with 5 μg/ml concanavalin A (Sigma-Aldrich). Stimulated splenocytes were co-cultured with HDFs (1 × 10^5) in the presence of MSCs (1 × 10^5). Suspended cells were obtained for FACS analysis and supernatants for cytokine ELISA. Stimulated human dermal fibroblasts and primary MSCs were co-cultured with or without stimulated splenocytes. To verify the proliferation of human dermal...
fibroblasts, H-2b negative cells were analyzed for CFSE using LSRII (BD Pharmingen, San Diego, CA).

**Measurements of cytokines and chemokines by ELISA**
Concentrations of TGF-β, IFN-γ, IL-10, IL-1 β, IL-6, and TNF-α (R&D Systems, Minneapolis, MN) in supernatants of co-cultures, and CCL1, CCL2, CCL3, CCL8, CCL17, and CCL22 in supernatants of homogenized tissues were measured by ELISA.

**Statistical analysis**
All values are expressed as mean ± standard error of the mean. Statistical comparisons between groups were performed using a parametric independent sample t test if there were five or more animals per group or using the Mann-Whitney test if there were fewer than five animals per group.

**CONFLICT OF INTEREST**
The authors state no conflict of interest.

**ACKNOWLEDGMENTS**
This research was supported by a grant (14172MFD5974) from the Ministry of Food and Drug Safety in 2016.

**SUPPLEMENTARY MATERIAL**
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2017.02.986.

**REFERENCES**


Horwitz EM, Maziarz RT, Kebrïaei P. MSCs in hematopoietic cell transplantation. Biol Blood Marrow Transplant 2011;17(1 Suppl.):S21–9.


www.jidonline.org 1903

J-Y Lim et al.
MSCs Attenuate Scl-GVHD by Inhibiting Immune Cell Recruitment


