Reduction in murine acute GVHD severity by human gingival tissue-derived mesenchymal stem cells via the CD39 pathways

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Abstract

Human gingival tissue-derived mesenchymal stem cells (GMSCs) present an accessible source of mesenchymal stem cells (MSCs) for treating autoimmune diseases. Here we show that human GMSCs can prevent and treat acute graft-versus-host disease (GVHD) in two different mouse models. Our results indicate that besides exhibiting suppressive function in vitro and in vivo, GMSCs may also regulate the conversion of Tregs to Th1 and/or Th17-like cells, as well as stabilize Foxp3 expression. Furthermore, GMSC-mediated prevention of acute GVHD was dependent on CD39 signaling that play an important role in the function and stability of Tregs. Finally, we also observed stronger protective ability of GMSCs with greater expansion ability compared with BMSCs or ASCs. These results indicate that human GMSCs have the potential to be used to treat GVHD.

Introduction

Administration of mesenchymal stem cells (MSCs) represents a promising treatment for patients suffering from autoimmune disorders. Exogenous mesenchymal stem cells have been shown to inhibit T-cell proliferation1, as well as improve outcomes in preclinical murine models of GVHD2 and clinical steroid refractory GVHD in children3. Use of gingival-derived MSCs (GMSCs)—a population of stem cells that exists in the human gingival tissue—has several advantages over that of bone marrow stromal cells (BMSCs): easier isolation, better population homogeneity, and more rapid proliferation4.

Acute GVHD is a severe complication of allogeneic hematopoietic stem cells and solid organ transplantation that is associated with significant morbidity and mortality. Current strategies to treat acute GVHD do not produce long-lasting responses and vary greatly between different individuals5. Thus, developing effective GVHD prevention and treatment strategies is key to improve the state of transplantation medicine.

CD39 is an ectoenzyme that hydrolyzes ATP and adenosine diphosphate (ADP) into adenosine monophosphate (AMP). Located on the surface of endothelial cells and circulating platelets, CD39 plays a role in the suppressive function of human and mouse regulatory T cells (Tregs)6. Previous data from our laboratory demonstrated that CD39 signaling is involved in mediating the protective effect of GMSCs7. Here, we investigated the potential therapeutic effects of GMSCs and the role that CD39
plays in this GMSC-mediated GVHD attenuation. Our data show that human GMSCs have therapeutic potential in ameliorating lethal acute GVHD through adenosine receptors.

**Materials and methods**

**Animals**

BALB/c (H-2d), C57BL/6 (H-2b; termed B6), DBA/2 (H-2d), and B6D2F1 (H-2b/d) mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 Foxp3-GFP-knock-in mice were generously provided by Dr. Talil Chattila (UCLA) and bred in our animal facility. Mice were used at age of 8–12 weeks. All murine experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees at University of Nanjing Medical University.

**GMSCs, BMSCs, and adipose stromal/stem cell (ASC) preparation**

Human gingiva samples were collected following routine dental procedures at Nanjing Medical University, with approval by the Institutional Review Board. Human GMSCs were obtained as previously described14.

Human BMSCs were isolated by differential adhesion from a 30 ml BM aspirate obtained from the iliac crest of two human donors (Lonza, Hopkinton, MA) at the First Affiliated Hospital of Nanjing Medical University in China with approval by the ethics committee of Jiangsu People’s Hospital. Mononuclear cells (MNC) were enriched from the BM by using ACK Lysis Buffer (Lonza, Walkersville, MD) and long-term culture. The cells were cultured in MSC growth medium consisting of Minimum Essential Medium Alpha supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY), 1% Penicillin-Streptomycin (Sigma Aldrich, St. Louis, MO), 2.5 μg/L FGF (R&D Systems, Minneapolis, MN), 2 ml/L Gentamicin (Sigma Aldrich, St. Louis, MO), and 2.2 g/L NaHCO3 (Sigma Aldrich, St. Louis, MO) at 37 °C with 5% carbon dioxide. On day 5, non-adherent cells were removed, and the growth media was fully replaced. Adherent cells were then expanded for another two weeks. Cells were washed with phosphate-buffered saline (PBS) (Thermo Fisher Scientific Waltham, MA), and the media was replaced on day 14.

**Adipose stromal/stem cell (ASC) preparation**

Following ethics approval by Jiangsu People’s Hospital, human ASCs were isolated from donated subcutaneous lip aspirates and tissue from abdominoplasties of two donors using previously described methods8,9. Briefly, liposuction tissues were washed with PBS, digested for 1 h in PBS supplemented with 1% bovine serum albumin, 0.1% collagenase type 1 and 2 mM CaCl2. The stromal vascular fraction (SVF) was found in the pellet after centrifugation at 300 g at room temperature. The SVF cells were then expanded in DMEM/F12 Ham’s medium supplemented with 10% fetal bovine serum and 1% antibiotic/antifungal agents until >80% confluent. Adherent ASCs were dislodged from tissue culture flasks using trypsin digestion. The cells were characterized by cell surface immunophenotyping, as well as in vitro (data not shown).

**Induction of CD4+ Tregs in vitro**

Naïve CD4+CD25-CD62L+ T cells were purified from the spleens of Foxp3-GFP C57BL/6 mice via magnetic isolation (Miltenyi Biotec). GMSCs or fibroblast cells were co-cultured with naïve CD4+CD25+CD62L+ T cells (1:5), and stimulated with beads coated with anti-CD3 and anti-CD28 mAb (1:5) in the presence of IL-2 (100 IU/ml) and TGF-β (5 ng/ml) to induce Tregs. GMSCs and fibroblast cells were allowed to adhere to the plate overnight before the co-culture. In some experiments, rmIL-6 (10 ng/mL) and/or rmIL-1β (10 ng/mL) were also added. After 3 days, cells were harvested and analyzed by flow cytometry for CD25, Foxp3, and CD39 expression.

**Treg immunosuppression assays**

WT naïve CD4+ T cells were labeled with CFSE and cultured in a 96-well bottom plate with anti-CD3/CD28-conjugated beads or anti-CD3-conjugated beads and APCs at a cell to bead ratio of 1:1. Serially diluted Treg cells were co-cultured for 72 h and cellular proliferation by CFSE was measured by flow cytometry.

**Acute GVHD models**

Model I: BALB/c hosts were given total body irradiation (TBI; 800 cGy) from a 200-KV x-ray source. Within 24 h, BALB/c mice were intravenously injected with 5 × 10^6 T cell-depleted BM cells and 2 × 10^6 T cells from C57BL/6 mice10,11. Mice were given medicated water (25 μg/ml neomycin/0.3 U/ml polymyxin B; Sigma-Aldrich)12–16. The survival of mice was monitored daily, and body weight was measured weekly.

Model II: Acute GVHD was induced by intravenous injection of 50 × 10^6 splenocytes isolated from C57BL/6 mice into non-irradiated B6D2F1 mice as previously reported17. Mice were sacrificed after 2 weeks, and splenocytes were stained for the expression of H2Kb, H2Kd, and immune cell lineage markers using flow cytometry18,19.

**Chimerism assay**

In Model II, mice were sacrificed on day 14, and the spleen cells were examined by flow cytometry. The proportion of donor to host cell was determined by staining H2-Kb and H2-Kd (donor cells are H2-Kb^+/d−; host cells are H2-Kb^b+/d+).
In vivo cytotoxic T cell activity

In vivo cytotoxic activity was determined using carboxyfluorescein succinimidyl ester (CFSE, Thermo Fisher Scientific Waltham, MA) labeled target cells as described by manufacturer’s protocol. Briefly, spleen cells from DBA/2 mice were stained with 0.5 μM CFSE (CFSElow) and spleen cells from C57BL/6 mice were stained with 5 μM CFSE (CFSEhi). F1 mice were injected intravenously with a 1:1 mixture (1 × 10⁷ cells each) from both donors as target cells. CFSE staining density allowed distinction between injected DBA/2 and C57BL/6 cells. Five hours after adoptive cell transfer, mice were sacrificed. Their splenocytes were analyzed by flow cytometry to determine the percentage of CFSElow and CFSEhi cell populations. The absolute number of each target cell population was calculated based on the total spleen cell counts from individual mice. This number was multiplied by the percentage of CFSElow and CFSEhi cells as determined by flow cytometry for each respective mouse. The percent specific lysis was determined using frequency of donor cells: % lysis = (%CFSElow in normal F1 – % CFSElow in experiments/%CFSElow in normal F1).

Flow cytometry

Spleen was prepared and stained with CD3 (Clone 17A), CD4 (Clone GK1.5), CD8 (Clone 53–67), CD19 (Clone 1D3), CD25 (Clone PC61), CD39 (Clone TU66), IFN-γ (Clone XMG1.2), TNF-α (Clone MP6-XT22), IL-17A (Clone N49-653), IL-4 (Clone 11B11), IL-10 (Clone JES5-16E3), IL-2 (Clone JES6-5H4), Helios (Clone 22F6), Fas (Clone Jo2), H2Kb (Clone AF6-88.5), H2Kd (Clone SF1-1.1.1) and analyzed on a FACScalibour and a FACSLSR. Intracellular staining was performed with a Foxp3/Transcription Factor Staining Buffer Set (eBioscience).

Statistical analyses

All data are represented as the mean ± SEM. Multiple regression and Student’s t-tests were used to determine statistical significance of non-survival data. Survival difference was determined by the Kaplan-Meier log-rank test. P < 0.05 was considered statistically significant.

Results

GMSC-induced iTregs exhibit suppressive function in vitro

Previous studies demonstrated that GMSCs can generate induced regulatory T cells (iTregs) in vivo without altering the natural regulatory T cell (nTreg) population. iTFregs have also been shown to inhibit murine acute GVHD. Therefore, we tested the effect of GMSCs on iTreg generation in vitro. CD4⁺CD25⁺ CD62L⁻ naïve T cells were purified from C57BL/6 Foxp3-GFP-knock-in mice and co-cultured with GMSCs or fibroblasts (1*10⁶:2*10⁵) (5:1) in the presence of anti-mouse CD3/CD28 mAb beads and IL-2 with or without TGF-β. GMSCs did not affect Foxp3 expression in CD4⁺CD25⁺ T cells cultured for 3 days independently of the presence of TGF-β in vitro. However, GMSCs increased the proportion of Foxp3-GFP⁺ cells co-expressing the CD39 ectonucleotidase when TGF-β was also present in the culture.

We next assessed the stability of iTregs in response to IL-1β and IL-6, two pro-inflammatory cytokines known to increase the degradation of Foxp3 and convert Tregs in effector T cells. After 3 days, iTregs were purified from the coculture containing GMSCs or fibroblasts using anti-CD4 mAb-conjugated beads. The cells were then cultured with DMSO or IL-1β and IL-6 for additional 3 days in the presence of IL-2. As predicted, Foxp3 expression was reduced in the fibroblast-containing iTreg group upon exposure to IL-1β and IL-6 (Fig. 1c), while the GMSC-containing iTreg group resisted Foxp3 downregulation. Also, the frequencies of IL-17A and IFN-γ in the fibroblast-containing iTreg group was higher than in the GMSC-containing iTreg group (Fig. 1c). The effect of IL-1β and IL-6 on iTreg function was also examined. iTregs purified as described above were co-cultured with CFSE-labeled CD4⁺ T cells (2*10⁵:8*10⁵) (1:4) for 3 days, and then analyzed by flow cytometry. Whereas iTregs previously cocultured with GMSCs and fibroblasts both showed similar ability to suppress T-cell expansion under normal conditions, only the fibroblast-induced iTregs lost their suppressive function following exposure to IL-1 and IL-6 (Fig. 1d).

Administration of GMSCs improves survival and reduces pro-inflammatory cytokine production in acute GVHD in vivo

Next, we examined whether GMSCs can effectively eliminate acute GVHD in vivo. To accomplish this, two different acute GVHD models (C57BL/6-to-BALB/c and C57BL/6-to-B6D2F1) were used. In the C57BL/6-to-BALB/c model, mice were lethally irradiated and then rescued with an allogeneic donor BM. 2 × 10⁶ GMSCs or fibroblast cells were injected the same day as acute GVHD was induced. Mortality and weight loss were used to quantify progression of acute GVHD as previously described. Recipients of GMSCs showed significantly improved survival (Fig. 2a, 87% vs. 0%, P < 0.0001) and weight loss (Fig. 2b; P < 0.031) compared to the non-treated or fibroblast-treated control groups. When GMSCs were injected 7 days after BM transplantation, a strong immunosuppressive environment ensued, with 87% long-term survival compared to 0% in the non-treated or fibroblast cell treated controls. These results indicated that exogenous GMSCs were effective in treating acute GVHD (Fig. 2b, 87% vs. 0%, P < 0.0025; Fig. 2d, P = 0.03). Because GMSCs enhanced CD39 expression in
Fig. 1 (See legend on next page.)
iTregs in vitro (Fig. 1) and Tregs can suppress acute GVHD24, we examined the frequency of CD39\(^+\) iTregs in this model. Compared with the fibroblast-treated group, the proportion of CD39\(^+\) Foxp3\(^+\) cells was significantly increased in the GMSC-treated group on day 15 (Fig. 2e). Most of these Foxp3\(^+\) cells were Helios\(^-\) iTregs, and no significant difference in the frequency of Helios\(^+\) nTregs was observed between the treatment groups on day 3 and day 15 (Fig. 2f). We investigated why severity of acute GVHD was reduced in our murine models of the disease. Previous studies have suggested that cytokine production by donor cells contributed to the progression of acute GVHD25,26. To determine whether GMSCs could suppress acute GVHD through controlling cytokine production by staining with IFN-\(\gamma\), IL-17, IL-4, TNF-\(\alpha\), IL-2, and IL-10, we detected the production of cytokines after injecting 15 days. Injection of GMSCs into mice with acute GVHD significantly reduced the percentages of cells secreting pro-inflammatory cytokines such as IFN-\(\gamma\), IL-17, IL-4, and TNF-\(\alpha\). However, the IL-2 production was significantly increased in GMSCs group than Fibroblast group, which suggested that GMSCs can increase the differentiation and function of iTreg through enhancing the secretory of IL-2. Other Th2 cytokines such as IL-5 and IL-13 were undetectable in this model, and iTreg treatment did not alter their levels (data not shown) (Fig. 2g).

In Model II of parent-into-F1 hybrid strain combination [C57BL/6-to-B6D2F1], 50 \times 10^6 C57BL/6 spleen cells were intravenously injected into non-irradiated immunocompetent D2B6F1 mice. After 14 days, mice were sacrificed, and the spleen cells were examined by flow cytometry. Donor and host cells can be distinguished by staining H2K\(^d\) and H2K\(^b\) since donor cells are H2K\(^b\)/H2K\(^d\) and host cells are H2K\(^b\)/H2K\(^d\). Nearly 50% of the administered donor cells were found in acute GVHD 2 weeks after adoptive cell transfer. Co-transfer of GMSCs markedly reduced donor engraftment and cell number (Fig. 3a). GMSCs suppressed the expansion of both donor CD4\(^+\) and CD8\(^+\) T cells. Host B cells, which are highly sensitive to depletion during active GVHD, were abundant in GMSC-treated mice compared to untreated GVHD controls and fibroblast-treated recipients (Fig. 3b). We attributed this observation to decreased FasL expression on donor CD8\(^+\) T cells and decreased Fas expression on host B cells (Fig. 3b) in GMSC-treated mice27.

To determine whether GMSCs suppressed the cytotoxic capacity of donor T cells, CFSE\(^hi\)-labeled C57BL/6 and CFSE\(^low\)-labeled DBA/2 splenocytes were mixed at a 1:1 ratio and administered in vivo with or without GMSCs20,28. After five hours, an approximately equal ratio of CFSE\(^hi\)-labeled B6 and CFSE\(^low\)-labeled DBA/2 splenocytes was recovered in the GMSC-treated group of non-transplanted F1 mice (Fig. 3c). In contrast, only 40% of DBA/2 cells were found in the untreated GVHD group. These results suggested selective killing of DBA/2 cells, while administration of GMSCs prevented the CTL priming response of F1 recipients against donor splenocytes.

iTregs play a role in GMSC-mediated protection against acute GVHD in vivo

To better understand the role of iTregs in GMSC-mediated protection against acute GVHD, we blocked Treg function by treating recipients in our GVHD Model I with an anti-CD25 antibody (clone PC61)29,30. PC61 was injected (IP) on the same day as BM and T cell transfer (IV) into the C57BL/6-to-BALB/c model. PC61 abrogated the protective effects of GMSCs during acute GVHD (Fig. 4a, 73% \pm 26%, P = 0.03; Fig. 4b, P = 0.025).

Because GMSCs enhanced CD4\(^+\) iTreg suppressive function without affecting Foxp3 expression in vitro (Fig. 1a), we studied the function of GMSC- and fibroblast-primed iTregs in vivo. GMSCs or fibroblasts were co-cultured with CD4\(^+\) CD25\(^-\) CD62L\(^-\) Naive T cells in the presence of TGF-\(\beta\) and IL-2 for 3 days to generate iTregs. Then, 2 \times 10^6 pretreated iTregs were purified by depleting the GMSCs or fibroblasts using anti-CD4-beads. These cells were injected into acute GVHD Model I. We observed that only GMSC-primed iTregs conferred protection against acute GVHD (Fig. 4c, d).
Fig. 2 (See legend on next page.)
Discussion

GMSCs have been shown to be efficacious in different disease models, including collagen-induced arthritis21, colitis31–33, wound healing34, and Xeno-Graft-versus–Host Disease35. However, no group has yet
Fig. 3 Adoptive transfer of GMSCs markedly suppressed the engraftment of donor cells in model two of acute-GVHD. Acute-GVHD (C57BL/6-to-F1) was induced as described in Methods. After two weeks, mice were sacrificed; donor and host splenic lymphocyte subsets were analyzed by flow cytometry. Donor and host cells can be distinguished by staining H2-Kd and H2-Kb—donor cells are H2-Kb+/d− and host cells are H2-Kb+/d+.

**a** H2-Kb vs. H2-Kd for donor and host cells after 14 days. Relative engraftment and absolute numbers of donor cells are shown for each group. Each symbol represents one mouse and two independent experiments was performed. ****P < 0.0001, unpaired t test.

**b** Absolute number of donor CD8+ cells; host CD8+ cells, donor CD4+ cells; host CD4+ cells; donor CD19+ cells; host CD19+ cells; donor Fas+ in CD8+ cells; host Fas+ in CD8+ cells. Each symbol represents one mouse and two independent experiments was performed. ****P < 0.0001, ns, no significance, unpaired t test.

**c** CSFE (CFSEhi and CFSELow) was determined by Flow cytometry, and cells number from DBA targets and B6 targets was counted. Each symbol represents one independent experiments. *P < 0.0001, ****P < 0.0001, unpaired t test.
reported that GMSCs can be used to treat acute GVHD. To answer this question, GMSCs were derived from human gingival tissues. Two distinct acute GVHD models were used to monitor GMSCs treatment. Our results clearly demonstrated that the superior effect of GMSCs in reducing the severity of acute GVHD generation and in treating mice with ongoing GVHD.

Several factors may explain the mechanism by which GMSCs suppress acute GVHD. First, GMSCs reduced levels of several pro-inflammatory cytokines, particularly IL-17, IL-4, and IFN-γ, while increased levels of IL-2 which play a important role in the differentiation and function of iTreg. Second, GMSCs inhibited the proliferation of donor CD8 cells and reduced the cytotoxicity of donor CD8 cells to host CD19 cells. Furthermore, GMSCs reduced the expression of Fas on B cells and FasL on CD8 cells, which prevented killing of target B cells. Therefore, it appears that GMSCs protected the donor cells from apoptosis. Third, we detected higher expression of Foxp3 in GMSC-primed group compared to fibroblast controls in our acute GVHD model. Additionally, although GMSCs did not enhance Foxp3 expression in vitro, GMSCs increased the stability of Foxp3 expression and sustained the suppressive function of CD4 iTregs under inflammatory conditions.

Multiple studies have shown that the immunoregulatory function of GMSCs is associated with higher Treg frequency in vivo. A population of Foxp3CD39+ iTregs is highly expressed in TGF-β-generated CD4+ iTregs as a result of adding GMSCs to cell cultures in vitro; indeed, GMSCs were identified as having stronger regulatory function and stability. We also detected higher Foxp3 expression in the GMSC-primed group compared to the Fibroblast-primed group, and most of the Foxp3+ cells lacked Helios expression. In addition, our experiments showed that, although GMSCs had no effect on Foxp3 expression, GMSCs increased the stability of Foxp3 expression and sustained the suppressive function of CD4+ iTregs during the inflammatory response.

Recently, a population of CD4+CD39+Foxp3+ T cells was identified as having a regulatory function in the autoimmune disease model. Our results suggest that Helios− Foxp3+ T cells are a unique cell population that may be induced by GMSCs in acute GVHD. Interestingly, while the number of Treg cells as defined by Foxp3 expression did not increase, the survival of GMSC-treated
Fig. 5 (See legend on next page.)
group was much longer. As MSCs may not be able to infiltrate organs of interest, it is possible that soluble factors secreted by GMSCs may regulate Treg cell function.

In our previous study, we found that GMSCs treated collagen-induced arthritis through the CD39 pathway. We examined whether CD39 is also an important pathway for GMSC treatment in acute GVHD. Transferred GMSCs not only express CD39, but also increase the frequency of CD39+ T cells in spleen. The correlation between the frequency of CD39+/CD39− Treg and the score of aGVHD was calculated by Linear regression-compare slopes. Each symbol represents one mouse and three independent experiments were performed. Log-rank (Mantel-Cox) Test for survival curve and unpaired t test for weight loss curve. ****P < 0.0001.

The percentage of Foxp3+CD39+, IL-17A+ and IFN-γ+ in CD4+ T cells in spleen were measured by Flow Cytometry. The correlation of Foxp3+CD39−/CD39+ Treg and the score of aGVHD was calculated by Linear regression-compare slopes. Each symbol represents one mouse and three independent experiments were performed. Log-rank (Mantel-Cox) Test for survival curve and unpaired t test. *P < 0.05; **P < 0.01; ***P < 0.001.

Together, our data indicate that GMSCs have a greater potential therapeutic effect against acute GVHD than BMSCs or ASCs. Adoptive transfer of GMSCs provided the clinical outcome in our acute GVHD model using older passaged cells compared to these other mesenchymal stromal cell types. Efficiency of using older passaged cells means that more cells can be made available for treatment.

These experiments suggest that human GMSCs selectively promote the Foxp3+/CD39+ subset of iTregs. The ability of GMSCs to increase survival and reduce severity of acute GVHD appears to be associated with up-regulation expansion, differentiation of Tregs in vivo through the CD39 pathways. Human GMSCs present a novel therapeutic strategy for preventing and treating acute GVHD, as well as autoimmune diseases. Given the
promising results outlined above, future studies in the clinic will be needed to assess the true prophylactic and therapeutic potential of this readily accessible and unique source of MSCs.

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Conflict of interest
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