ARTICLE

Cell–cell contact with proinflammatory macrophages enhances the immunotherapeutic effect of mesenchymal stem cells in two abortion models

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Mesenchymal stem cells (MSCs), which are pluripotent cells with immunomodulatory properties, have been considered good candidates for the therapy of several immune disorders, such as inflammatory bowel diseases, concanavalin A-induced liver injury, and graft-versus-host disease. The embryo is a natural allograft to the maternal immune system. A successful pregnancy depends on the timely extinction of the inflammatory response induced by embryo implantation, followed by the switch to a tolerant immune microenvironment in both the uterus and the system. Excessive infiltration of immune cells and serious inflammatory responses are triggers for embryo rejection, which results in miscarriage. Here, we demonstrated that adoptive transfer of MSCs could prevent fetal loss in a lipopolysaccharide (LPS)-induced abortion model and immune response-mediated spontaneous abortion model. The immunosuppressive MSCs alleviated excessive inflammation by inhibiting $CD4^+$ T cell proliferation and promoting the decidual macrophage switch to M2 in a tumor necrosis factor-stimulated gene-6 (TSG-6)-dependent manner. Cell-to-cell contact with proinflammatory macrophages increased the TSG-6 production by the MSCs, thereby enhancing the suppressive regulation of T cells and macrophages. Moreover, proinflammatory macrophages in contact with the MSCs upregulated the expression of CD200 on the stem cells and facilitated the reprogramming of macrophages towards an anti-inflammatory skew through the interaction of CD200 with CD200R on proinflammatory macrophages. Therefore, the results demonstrate that a TSG-6-mediated paracrine effect, reinforced by cell-to-cell contact between MSCs and proinflammatory macrophages, is involved in the mechanism of MSC-mediated abortion relief through the induction of immune tolerance. Our study also indicates the potential application of MSCs in clinical recurrent miscarriages.

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INTRODUCTION

Emerging evidence has demonstrated that mesenchymal stem cells (MSCs) are a promising tool for immunotherapy, as a result of their differentiation potential and immunoregulatory properties. Good therapeutic results have been obtained with MSC treatment in clinical and basic studies, such as for graft-versus-host diseases, autoimmune disorders, and inflammatory diseases.[1–3] MSCs display their immunotherapeutic effect through the regulation of both adaptive and innate responses, depending on soluble factors or cellular contact for their function. T-cell proliferation was suppressed by MSCs via the secretion of soluble factors, such as transforming growth factor-beta (TGF-β), prostaglandin E2 (PGE2), indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), and hepatocyte growth factor (HGF).[4–7] MSCs also possess the ability to induce the differentiation of $CD4^+$ T cells into $CD25^+Foxp3^+$ regulatory T cells (induced Tregs) through the expression of TGF-β.[8] With regard to innate immunity, MSCs have been reported to promote the formation of M2 macrophages in vivo and in vitro, which is the critical mechanism in anti-inflammation or the induction of immune tolerance.[9,10]

Miscarriage, which accounts for 15% of clinically recognized pregnancies in humans, induces substantial suffering and frustration to the expectant mother and her family.[11] Although embryo implantation is an inflammatory process, a successful pregnancy depends on the timely extinction of the inflammatory response induced by embryo implantation and switches to an immune tolerant microenvironment in both the uterus and system.[12,13] Once the specific immune tolerance during pregnancy is disturbed, adverse pregnancy outcomes, such as miscarriage, preterm delivery, or fetal growth restriction, may occur.[14–17] Infection is one of the most common causes of miscarriage. LPS, derived from gram-negative bacteria, is a potent initiator of inflammation and is widely used in experimental models. The administration of LPS to pregnant mice will switch the immune

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tolerance to inflammatory responses, resulting in fetal loss.[18,19] In addition to infection-induced miscarriage, immune response-mediated pregnancy loss accounts for more than 60% of unexplained recurrent spontaneous abortion. Most immune abortions are associated with a damaged maternal immune tolerance accompanied by systemic and local increased proinflammatory cytokines and activated immune responses. As MSCs are known to induce tolerance via their immunosuppressive ability in clinical and experimental studies,[20,21] the possibility of whether miscarriages could be alleviated by MSCs deserves investigation. To the best of our knowledge, associated research in this regard has not been carried out to date.

Herein, we investigated the treatment effects of MSCs on LPS-induced and spontaneous abortion, as well as the mechanisms involved. We found that the transplantation of C57BL/6 mouse-derived MSCs significantly reduced fetal loss in these mice. MSC treatment decreased the systemic and local (decidual) T-cell-mediated inflammatory responses. Proinflammatory macrophages in the decidua switched to an anti-inflammatory phenotype after MSC administration. The MSCs caused immunosuppression to alleviate abortion relying on a soluble factor, TSG-6. Notably, the cell-to-cell interaction between the proinflammatory macrophages and MSCs promoted the production of MSC-derived TSG-6, as well as the expression of CD200 on MSCs. Furthermore, the binding of the MSC surface marker CD200 with its receptor (CD200R) on macrophages was indispensable for the reprogramming of proinflammatory macrophages by MSCs, which suggests that direct contact with proinflammatory macrophages increases the immunosuppressive ability of MSCs in abortion models.

RESULTS

MSCs alleviate LPS-induced abortion by suppressing systemic and decidual inflammation

To first investigate whether MSCs have a therapeutic effect on inflammation-induced abortion, we injected mouse bone marrow-derived MSCs (Supplementary Figure 1) into LPS-challenged pregnant C57BL/6 mice. All pregnant mice were administered LPS (0.25 mg/kg) at Gd 7.5, followed by intraperitoneal injections with 2×10^6 MSCs or PBS at both 2 and 24 h, respectively (Fig. 1a). As a result, the resorption rate at GD 13.5 was significantly lower in the MSC-treated mice than that in the PBS-treated mice (Fig. 1b). The levels of a series of inflammatory cytokines (such as TNF-α, IFN-γ, IL-1β, IL-27, and IL-6) were markedly increased in the peripheral blood of the mice treated with PBS, while they were significantly reduced in that of the MSC-treated mice (Fig. 1c). Flow cytometric (FCM) analysis of peripheral blood mononuclear cells (PBMCs) indicated that the $CD4^+$ T cells from the MSC-treated mice produced significantly less inflammatory cytokines (including TNF-α, IFN-γ, and IL-1β) than did those from the PBS-treated mice (Fig. 1d). Similar results were obtained in the decidua (Fig. 1e–g). The transcript levels of CD4 and the proinflammatory cytokines TNF-α, IFN-γ, and IL-1β in the decidua were significantly lower in the MSC-treated group than those in the PBS-treated group (Fig. 1e). These data were confirmed by immunostaining for CD4 in decidua, where the MSC-treated group showed fewer $CD4^+$ infiltrates (Fig. 1f). Consistent results were also noted in the FCM determination of inflammatory cytokine production by decidual $CD4^+$ T cells (Fig. 1g). These data suggest that the MSC treatment efficiently ameliorates LPS-induced fetal loss by attenuating systemic inflammation, as well as local inflammation of the decidua. Furthermore, MSCs characterized with a defined marker profile were detected in the uterus of the pregnant C57BL/6 mouse. We also found that the percentage of this cell population was gradually increased with pregnancy progression (Supplementary Figure 2). Collectively, MSCs play a role in the maintenance of normal pregnancy via their immunosuppressive capacity (Table 1).

A shift of macrophages to an anti-inflammatory phenotype occurs in the decidua of LPS-challenged mice after MSC treatment

MSCs have been demonstrated to have multiple immunoregulatory abilities and are capable of influencing both the innate and adaptive immune responses.[22–26] We further investigated the immune cell populations in the decidua, the most important interface at which pregnancy immunity occurs. As shown in Fig. 2a, the mRNAs of F4/80 and CD4 were substantially increased after LPS stimulation, whereas those of the other immune cell markers remained unchanged. MSCs administration significantly reduced the abundance of CD4 mRNA but not that of F4/80 mRNA. Immunostaining for F4/80 in the decidua confirmed that the increased number of macrophages after LPS injection could not be reverted by MSCs (Fig. 2b). However, the mRNA expression of iNOS (an M1 marker) in the decidua was significantly decreased after the MSC administration, whereas the mRNA expression of arginase 1 (Arg1, an M2 marker) was significantly increased (Fig. 2c). Similar results were noted in the double immunostaining for F4/80 and iNOS or Arg1 in the decidua (Fig. 2d and Supplementary Figure 3). These results suggest that MSC treatment may contribute to switching the LPS-induced M1 to the M2 phenotype. FCM analysis of the $F4/80^+$ populations in decidual immune cells (Fig. 2e) showed that MSC treatment significantly increased the M2 marker (CD206 and Arg1) expression but decreased the M1 marker (CD11c and iNOS) expression. Moreover, the LPS-induced decreased production of the anti-inflammatory cytokine IL-10 by $F4/80^+$ decidual macrophages (dMφ) was remarkably reverted after MSC treatment. Moreover, the proinflammatory cytokines IL-12, TNF-α, and IFN-γ produced by $F4/80^+$ dMφ in LPS-challenged mice were significantly inhibited by the MSCs (Fig. 2f). Thus, the promotion of the macrophage transition from the M1 to M2 phenotype is involved in the MSC mechanism against LPS-induced fetal loss.

MSC-derived TSG-6 contributes to the immunosuppression in LPS-challenged pregnant mice

Previous studies have indicated that MSCs exerted their immunoregulatory effect through the secretion of paracrine factors. We therefore examined whether the factors secreted by MSCs might play a role in anti-inflammation in LPS-challenged pregnant mice. Real-time RT-PCR examination of the gene expression levels of the paracrine factors in the decidua showed that the TSG-6 mRNA expression was significantly higher in the MSC-treated group than that in the control group (Fig. 3a). Similar results were obtained at the protein level (Fig. 3b). These data suggest that MSCs might modulate T cells and macrophages in LPS-challenged mice via TSG-6. To verify this concept, MSCs transfected with TSG-6-siRNA were cocultured with macrophages or $CD4^+$ T cells in vitro. RAW264.7 cells (a murine macrophage cell line) treated with LPS and IFN-γ were induced to the M1 phenotype, which mimicked the inflammatory macrophages in the LPS-challenged uterus. MSCs transfected with scrambled (SCR) siRNA remarkably reduced the expression of M1 markers (i.e., CD11c and iNOS), as well as the production of the inflammatory cytokine TNF-α. In comparison, activated RAW264.7 cells cocultured with TSG-6-silenced MSCs displayed significantly higher expression levels of CD11c, iNOS, and TNF-α (Fig. 3c). These data indicated that MSC-derived TSG-6 was involved in suppressing inflammatory macrophages. In addition, $CD4^+$ T cells isolated from the blood of normal pregnant mice were cocultured with TSG-6-silenced MSCs or SCR siRNA-transfected MSCs. Flow cytometry was subsequently employed to analyze the proliferation of CFSE-labeled T cells activated with anti-CD3/CD28 antibody. The SCR siRNA-transfected MSCs significantly inhibited $CD4^+$ T-cell proliferation, whereas the TSG-6-silenced MSCs showed no effect in this regard (Fig. 3d). Accordingly, the T-cell suppressive ability of MSCs is TSG-6-dependent. These ex vivo experiments suggest that MSC-derived TSG-6 is capable of attenuating proinflammatory macrophages

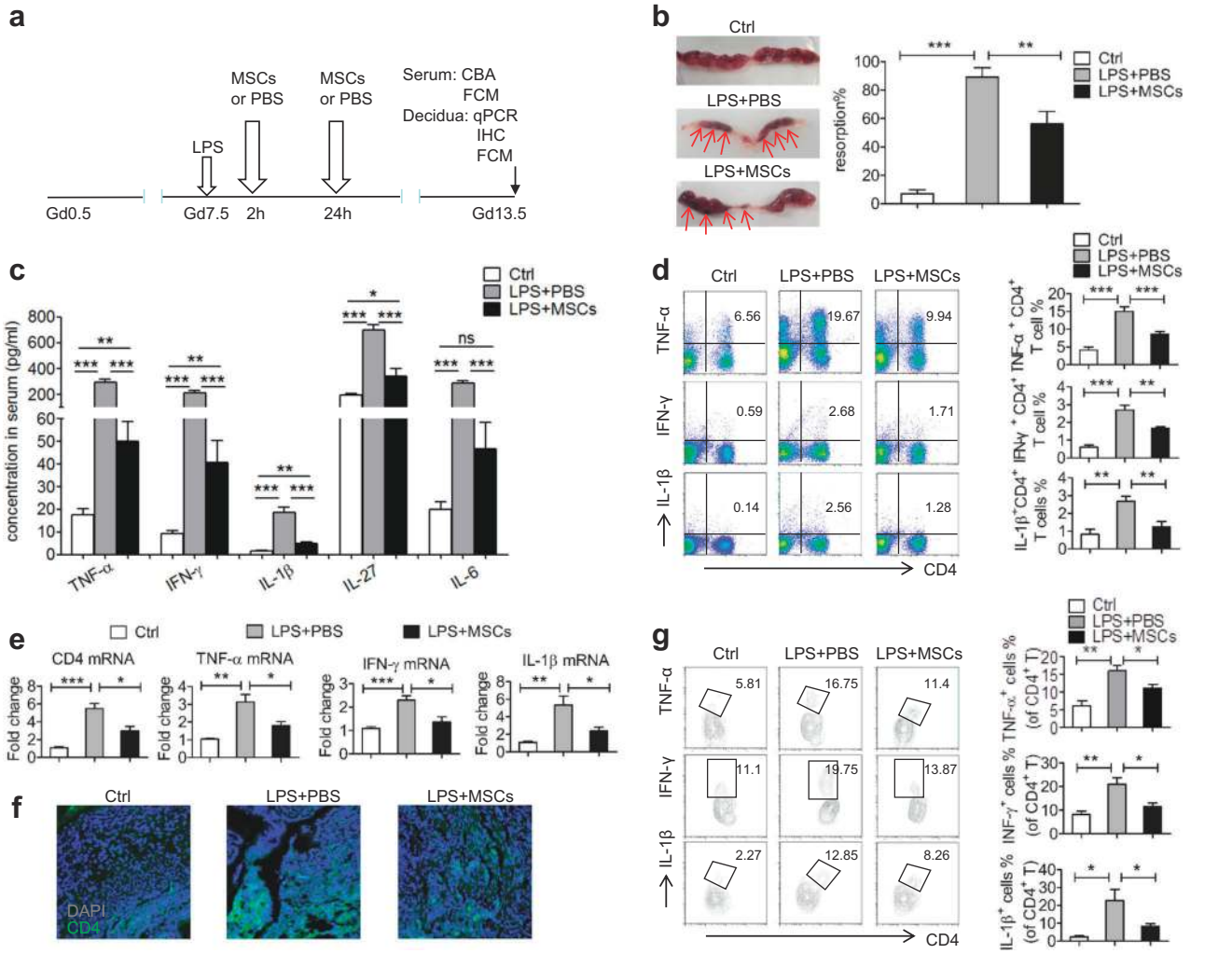

Fig. 1 MSCs ameliorate LPS-induced fetal loss. **a** Schema of MSC treatment in a model of LPS-induced abortion. **b** Representative images of embryos in the uterus from normal mice and LPS-challenged mice treated with PBS or MSCs. Data depict the embryo resorption rate in pregnant mice treated as indicated. **c** Serum concentrations of proinflammatory cytokines measured with the LEGENDplex assay (BioLegend). **d** Representative and quantitative flow cytometry results for CD4, TNF-α, IFN-γ, and IL-1β expression in peripheral blood mononuclear cells (PBMCs). Data depict the percentages of TNF-α^+CD4$^+$ cells, IFN-γ^+CD4$^+$ cells, and IL-1β^+CD4$^+$ cells among the total PBMCs. **e** Real-time RT-PCR assays of decidual mRNAs. Data indicate the mRNA levels as the relative ratio to the levels in control mice. **f** Immunostaining for CD4 in the decidua (original magnification of 100×). **g** Representative and quantitative flow cytometry results for CD4, TNF-α, IFN-γ, and IL-1β expression in decidual immune cells. Data depict the percentages of TNF-α^+, IFN-γ^+, and IL-1β^+ cells among decidual CD4$^+$ T cells. Data are the means ± SEM of six to ten mice per group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. CBA, cytometric bead array

and T-cell proliferation. To further demonstrate the role of TSG-6 in the therapeutic effect of MSCs against LPS-induced abortion, we intraperitoneally injected 2×10^6 si-TSG-6 MSCs, 2×10^6 si-SCR MSCs, or the control (PBS) into the mice at 2 and 24 h after LPS administration. Compared with the mice treated with si-SCR-MSCs, the mice treated with siTSG-6-MSCs exhibited a significantly higher resorption rate (Fig. 3e) and increased levels of proinflammatory cytokines in the serum (Fig. 3f). Similarly, the levels of CD4, TNF-α, IFN-γ, IL-1β, and iNOS mRNAs were substantially increased in the decidua of the mice treated with TSG-6-silenced MSCs, whereas the Arg1 mRNA was markedly reduced in this group (Fig. 3g). Consistent results were noted in the immunostaining of CD4 or F4/80 in the decidua of these three groups. After TSG-6-silenced MSC treatment, more obvious CD4$^+$ cell infiltration in the decidua was observed. There was no significant difference in the number of F4/80$^+$ dMφ between the two MSC-treated groups (Fig. 3h and Supplementary Figure 4). More importantly, CD4$^+$ T cells that infiltrated the decidua exhibited a greater inflammatory status with significantly higher expression of TNF-α, IFN-γ, and IL-1β in the mice that had received TSG-6-silenced MSCs (Fig. 3i). The dMφ in the mice treated with TSG-6-silenced MSCs displayed significantly lower expression of CD206 and Arg1 and significantly higher expression of CD11c and iNOS than did those in the mice treated with si-SCR-MSCs (Fig. 3j). These findings suggest that TSG-6 contributes to MSC-mediated macrophage switching and T-cell suppression activities, leading to the rescue of LPS-induced abortion.

Contact with proinflammatory macrophages promotes TSG-6 production and the immunosuppressive ability of MSCs

The results in Fig. 3c, d demonstrate the role of MSC-derived TSG-6 in suppressing proinflammatory macrophages and T cells. Despite these results, a high expression of TSG-6 was not observed in MSCs cultured alone. Notably, a remarkably increased expression of TSG-6 was detected in MSCs cocultured with LPS/IFN-γ-activated RAW264.7 cells (Fig. 4a), which suggests that

Table 1. Primers used for real-time PCR

Primer name	Sequence (5′-3′)
GAPDH	TGTTGAAGTCGCAGGAGACAACCT
	AACCTGCCAAGTATGATGACATCA
TSG-6	GGCTGGCAGATACAAGCTCA
	TCAAATTCACATACGGCCTTGG
IDO	TGGCGTATGTGTGGAACCG
	CTCGCAGTAGGGAACAGCAA
IL13	CAGCCTCCCCGATACCAAAAT
	GCGAAACAGTTGCTTTGTGTAG
COX-2	TGCTGGAAAAGGTTCTTCTACGG
	GAACCCAGGTCCTCGCTTATG
IL1Ra	GACCCTGCAAGATGCAAGCC
	GAGCGGATGAAGGTAAAGCG
TGF-β	TTGCCCTCTACAACCAACACAA
	GGCTTGCGACCCACGTAGTA
HGF	CCTGTCAGCGTTGGGATTC
	CTCGGATGTTTGGGTCAGTGG
Foxp3	CTGGTATATGCTCCCGGCAA
	GTTCTTGTCAGAGGCAGGCT
F4/80	TGCATCTAGCAATGGACAGC
	GCCTTCTGGATCCATTTGAA
iNOS	CAGATCGAGCCCTGGAAGAC
	CTGGTCCATGCAGACAACCT
Arg1	CAGAAGAATGGAAGAGTCAG
	CAGATATGCAGGGAGTCACC
CD4	AGCATGTCAGGGGCAAATGA
	TGGCTTGGATGTGTGTTGGT
CD8	CCTGACATGCAGCCTTACCA
	AGGCTATCAGTGTTGTGGGC
CD49b	TACTGGTTGGTTCACCGTGG
	CCCAGGCTCATGTTGGTCTT
CD54	GGCAGCAAGTAGGCAAGGAC
	TGGCGGCTCAGTATCTCCTC
CD90	ACTTCACCACCAAGGATGAG
	CAGCATCCAGGATGTGTTCT
CD106	GCTGCGAGTCACCATTGTTC
	CTGACAGGCTCCATGGTCAG
CD200	TGTAATCCAGCCTGCCTACA
	AGGCAAGCTGTTCCTGAGAC
PD-L1	TGGAGTATGGCAGCAACGTC
	GTCCTCCTCTCCTGCCACAA

macrophages, in turn, have an impact on the functions of MSCs. To further explore the mechanisms of macrophage-mediated regulation of MSCs, M1 (induced by LPS/IFN-γ-treated RAW264.7 cells) or M2 (induced by IL-4-treated RAW264.7 cells)[27,28] macrophages were cocultured with MSCs in a cell-to-cell contact system (CC) or a transwell chamber (TW). As shown in Fig. 4b, M1 cocultured with MSCs induced significantly high TSG-6 expression in both the CC and TW systems, whereas M2 had no effect on the TSG-6 production by MSCs in either system. Interestingly, M1 in the CC system promoted stronger MSC-derived TSG-6 expression than it did in the TW system, which suggests that both soluble factors and direct cell-to-cell contact may contribute to the enhanced TSG-6 expression. Accordingly, MSCs that were cultured directly with M1 or in the TW system significantly suppressed the expression of CD11c, iNOS, and TNF-α by these RAW264.7 cells. This modulation by MSCs was more effective in the CC system than in the TW system. Furthermore, the expression levels of CD11c, iNOS, and TNF-α were not influenced before and after M2 coculture with MSCs in either system (Fig. 4c). After coculture with M1 or M2 in the CC or TW system for 48 h, the MSCs were separated to assess whether their T-cell suppressive capacity was influenced. The FCM analysis showed that the inhibition of T-cell proliferation by the MSCs was significantly enhanced after the M1 +MSCs direct coculture, whereas it was abolished by the coculture in the TW system. Moreover, the suppressive ability of the MSCs cocultured with M2 in the CC or TW system was comparable to that of the MSCs cultured alone (Fig. 4d). These data suggest that the direct contact of MSCs with M1 results in increased expression of TSG-6, which improves the immunosuppressive property of stem cells.

CD200 on MSCs is indispensable for suppressing proinflammatory macrophages

We have shown that the regulation of macrophages by MSCs depends on direct cell-to-cell interaction in addition to soluble factors. Whether and what membrane molecule(s) are involved in the switch of proinflammatory macrophages by MSCs in LPS-induced abortion are unclear. To further explore the potential mechanism, we cocultured M1 with siTSG-6-MSCs or siSCR-MSCs in the CC or TW system. We found that the inhibited expression of M1 markers (CD11c and iNOS) and the inflammatory cytokine TNF-α by siSCR-MSCs was attenuated in the CC coculture system of TSG-6-silenced MSCs and M1 (Fig. 4e). Similar results were obtained in the TW coculture system. However, it was noted that significant differences existed between the TSG-6-silenced MSC groups of the two coculture systems. Specifically, TSG-6-silenced MSCs significantly reduced the expression of CD11c, iNOS, and TNF-α by M1 in the CC coculture system, but not in the TW system (Fig. 4e). Thus, the mechanism of MSC suppression of proinflammatory macrophages involves cell-to-cell contact in addition to the role of TSG-6. The expression of membrane-bound inhibitory molecules or adhesion molecules on MSCs was evaluated by real-time PCR after 48 h coculture with M1 or M2. The mRNA expression of CD200 was significantly increased on the MSCs cocultured with M1 in both the CC and TW systems, which suggests that M1 promoted CD200 expression via soluble factors (Fig. 4f). The mRNA levels of CD54, CD90, CD106, PD-L1, and Ceacam-1 on MSCs showed no significant differences between these indicated coculture systems (Supplementary Figure 5). Consistent results were obtained in the protein expression of CD200 on MSCs by FCM (Fig. 4g). The receptor of CD200 (CD200R) was also detected on M1, although its expression was not affected before and after the coculture with MSCs (Fig. 4h). These data suggest that the CD200–CD200R interaction may account for the cell-to-cell contact required for enhancing the MSCs' suppressive modulation of proinflammatory macrophages. We subsequently attempted to verify our hypothesis by blocking the interaction of CD200 and CD200R in the M1+MSC coculture system. Pretreatment with anti-CD200R antibody partially abrogated the suppressed expression of CD11c on M1 by siSCR-MSCs in the CC coculture system; however, it failed to exert this effect in the TW coculture system (Fig. 4i). Furthermore, the inhibition of CD11c on M1 was attenuated by TSG-6-silenced MSCs, which was further weakened as a result of the use of anti-CD200R antibody in the CC coculture system but not in the TW coculture system. Similar results were observed in the FCM analyses for the expression of iNOS and TNF-α produced by M1 (Fig. 4i). These data demonstrate that the M1-promoted expression of CD200 on MSCs, which interacts with M1-CD200R, in turn increases the immunosuppressive property of MSCs to shift the proinflammatory macrophage phenotype.

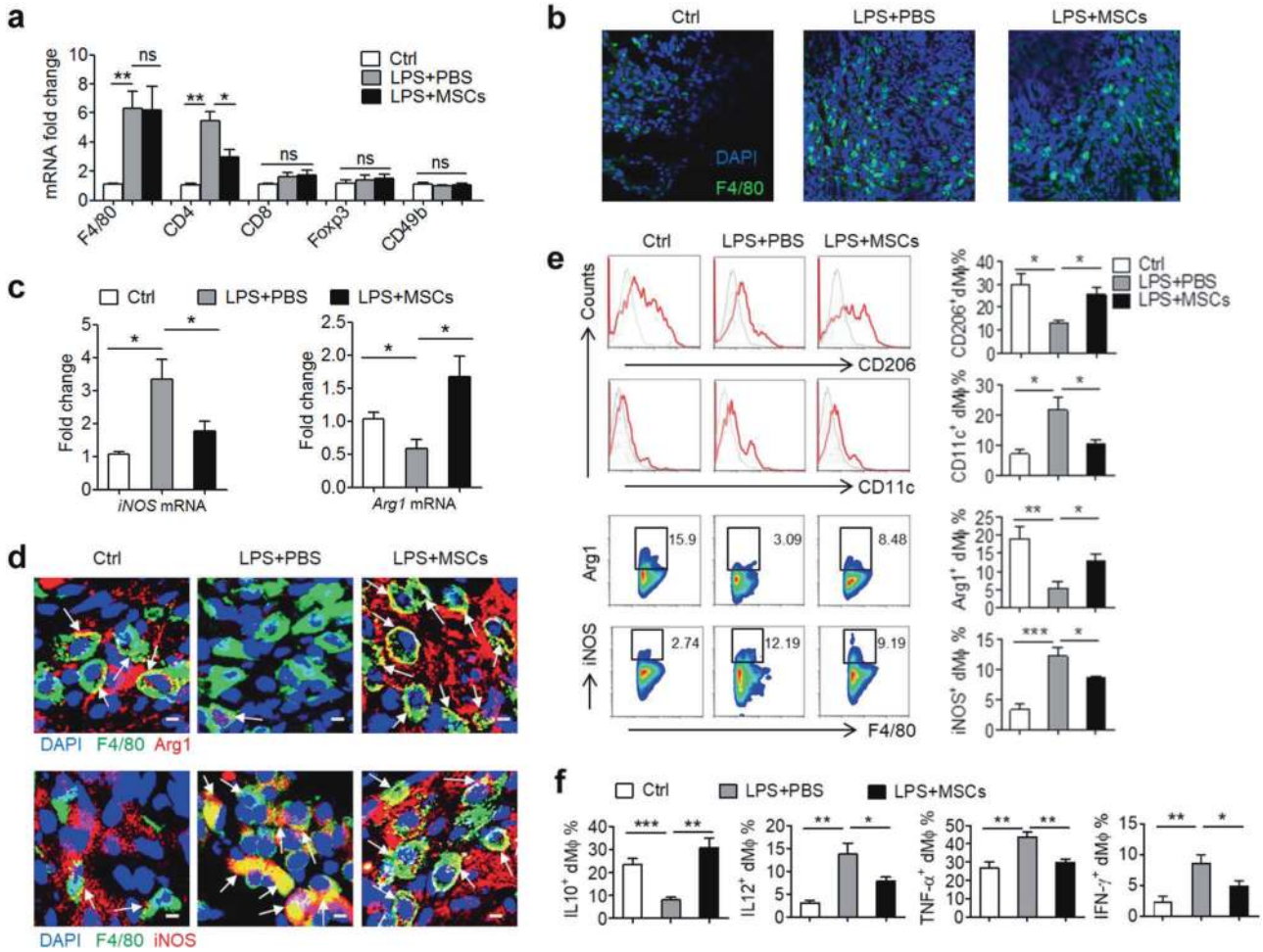

Fig. 2 MSCs induce M2 macrophage switching in the decidua. **a** mRNA levels of markers for decidual immune cells from normal mice and LPS-challenged mice treated with PBS or MSCs. Data indicate the mRNA levels as the relative ratio to the levels in control mice. **b** Immunostaining for F4/80 in the decidua (original magnification of 100×). **c** Real-time RT-PCR analysis of the relative expression of iNOS and Arg1 mRNA in the decidua. Data indicate the mRNA levels as the relative ratio to the levels in control mice. **d** Immunostaining for iNOS or Arg1 in F4/80$^+$ decidual macrophages (Scale bar represents 2.5 μm). **e** Representative and quantitative flow cytometry results for CD206 (an M2 marker), CD11c (an M1 marker), Arg1 (an M2 marker), and iNOS (an M1 marker) expression in F4/80$^+$ decidual macrophages. **f** Flow cytometric analysis of anti-inflammatory (IL-10) and proinflammatory (IL-12, TNF-α, and IFN-γ) cytokines in F4/80$^+$ decidual macrophages. Data are the means ± SEM of six to ten mice per group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; ns, not significant

Direct contact with proinflammatory macrophages is required for MSCs to alleviate LPS-induced fetal loss

We have proven in vitro that cell-to-cell contact with proinflammatory macrophages is essential for the immunosuppressive effect of MSCs. Whether MSCs rely on this mechanism to alleviate LPS-induced abortion was ultimately tested. To determine whether MSCs were present in the pregnant uterus, pregnant (Gd7.5) or nonpregnant mice were administered an intraperitoneal injection (i.p.) of 2×10^6 Zs-Green$^+$ MSCs (enhanced green fluorescent protein labeled MSCs), and another injection was administered after 24 h. Imaging of the Zs-Green$^+$ cells was performed after 6 days. The in vivo optical images showed that following adoptive transfer, Zs-Green$^+$ MSCs were predominantly enriched in the uterus of the pregnant mouse relative to those in the virgin mouse (Fig. 5a). In addition, Zs-Green$^+$ MSCs were detected in the decidua of pregnant mice, particularly in the LPS-challenged mice, by confocal microscopy (Fig. 5b). These findings suggest that the pregnant uterus may be more likely to attract the homing of MSCs, which substantially increases the possibility of having direct contact with decidual immune cells, including macrophages. Moreover, we found that the mRNA expression of TSG-6 was significantly increased after MSC transplantation, in both normal pregnant and LPS-challenged pregnant mice. More importantly, the increased TSG-6 mRNA expression by MSCs was substantially higher in the LPS-treated pregnant mice than in the untreated pregnant animals (Fig. 5c). Consistent results were obtained in the immunoblot assay for the TSG-6 protein (Fig. 5d). This promoted expression of TSG-6 in decidual MSCs from LPS-treated mice may be induced by the proinflammatory macrophages via contact with the transplanted stem cells in the uterus. Furthermore, the role of the CD200–CD200R-mediated interaction between MSCs and macrophages in LPS-challenged mice was also investigated. We intraperitoneally injected 2×10^6 sh-CD200 MSCs, 2×10^6 sh-SCR MSCs, or the control (PBS) into the LPS-induced abortion mice at 2 and 24 h after LPS administration. The mice treated with CD200-silenced MSCs exhibited a significantly higher resorption rate (Fig. 5e, f) and increased levels of proinflammatory cytokines (IFN-γ and IL-27) in the serum (Fig. 5g). Similarly, the TNF-α, IFN-γ, and iNOS mRNA levels were substantially increased in the decidua of the mice treated with CD200-silenced MSCs, whereas the Arg1 mRNA expression was markedly reduced (Fig. 5h). The mRNA expression levels of F4/80 and CD4 in the decidua were comparable between the mice that received CD200-silenced MSCs and those that received sh-SCR MSCs (Fig. 5h). In

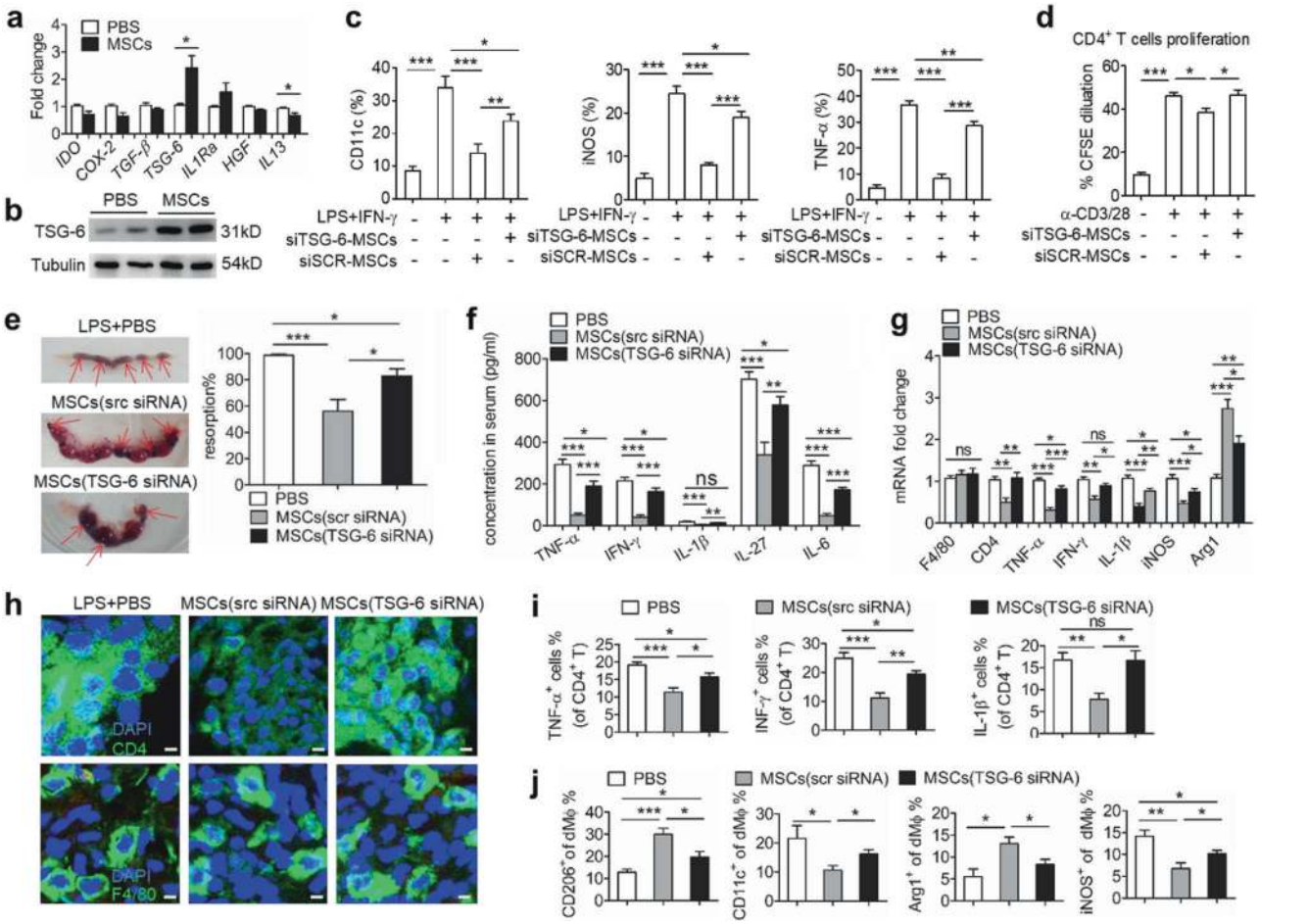

Fig. 3 The immunosuppressive activity of MSCs in LPS-induced abortion partially depends on TSG-6. **a** Relative fold changes in the mRNA levels of indicated molecules in the deciduas of LPS-challenged mice treated with PBS or MSCs, as determined by real-time RT-PCR. Data indicate the mRNA levels as the relative ratio to the levels in PBS-treated mice. **b** Immunoblots showing TSG-6 expression levels in the deciduas of PBS- and MSC-treated groups after LPS challenge. The results are representative of two separate experiments. **c** RAW264.7 cells were polarized for 24 h into proinflammatory M1 using IFN-γ (20 ng/mL) and LPS (1 μg/mL). After three washes, the induced M1 macrophages were cocultured with TSG-6-knocked down MSCs or scrambled (SCR) siRNA-transfected MSCs for 48 h. Flow cytometry was performed to determine the expression of CD11c and iNOS (M1 markers) and the proinflammatory cytokine TNF-α by RAW264.7 cells. **d** Flow cytometry results for CFSE dilution in $CD4^+$ cells purified from blood of naïve mice following coculture with TSG-6-knocked down MSCs or SCR siRNA-transfected MSCs. Data in **c** and **d** are the means ± SEM of three experiments. **e** Representative images of embryos in the uterus from LPS-challenged mice injected with PBS, TSG-6 siRNA-silenced MSCs, or SCR siRNA-transfected MSCs. The graphical data depict the embryo resorption rate in pregnant mice treated as indicated. **f** Serum concentrations of proinflammatory cytokines measured with the LEGENDplex assay (BioLegend). **g** Real-time RT-PCR analysis of the relative expression of the indicated molecules in the deciduas from the LPS-challenged mice treated as described in **e**. Data indicate the mRNA levels as the relative ratio to the levels in PBS-treated mice. **h** Immunostaining for CD4 or F4/80 in the deciduas (Scale bar represents 2.5 μm). **I** Flow cytometric analysis of proinflammatory cytokines (TNF-α, IFN-γ, and IL-1β) produced by decidual $CD4^+$ T cells from mice treated as described in **e**. **j** Flow cytometric analysis of M2 (CD206 and Arg1) and M1 (CD11c and iNOS) markers in $F4/80^+$ decidual macrophages from mice treated as described in **e**. Data are the means ± SEM of five to eight mice per group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; ns, not significant

addition, the FCM analysis showed that the inhibition of proinflammatory cytokines (TNF-α, IFN-γ, and IL-1β) in decidual $CD4^+$ T cells by SCR shRNA-transfected MSCs was not affected in the mice treated with CD200-silenced MSCs (Fig. 5i). However, the dMφ in the mice treated with CD200-silenced MSCs displayed significantly lower expression of CD206 and Arg1 and significantly higher expression of CD11c and iNOS than those in the mice treated with sh-SCR MSCs (Fig. 5j). These data confirm the critical role of CD200 in the MSC-mediated switching of the proinflammatory macrophage phenotype in LPS-challenged pregnant mice. The results in Fig. 5 indicate that MSCs (incorporated by adoptive transfer) home into the uterus and make contact with decidual macrophages in LPS-challenged pregnant mice, which leads to the increased expression of MSC-derived TSG-6, as well as the MSC-enhanced immunosuppression of proinflammatory macrophages in a CD200-dependent manner.

MSCs reduce the resorption of the abortion-prone mouse model via TSG-6 and direct contact with M1

In parallel experiments, we investigated the effects of MSCs in the spontaneous abortion (SA) model, a well-established mouse model for studying immune abortion. It is characterized by increased inflammation in the maternal immune system with activated NK cells and macrophage infiltration and an enhanced Th1 response. Recent findings show that MSCs effectively reduce the high resorption of the SA model via modulating the Th1/Th2 responses. However, the specific molecular mechanism has not previously been disclosed. We have found that MSCs suppress

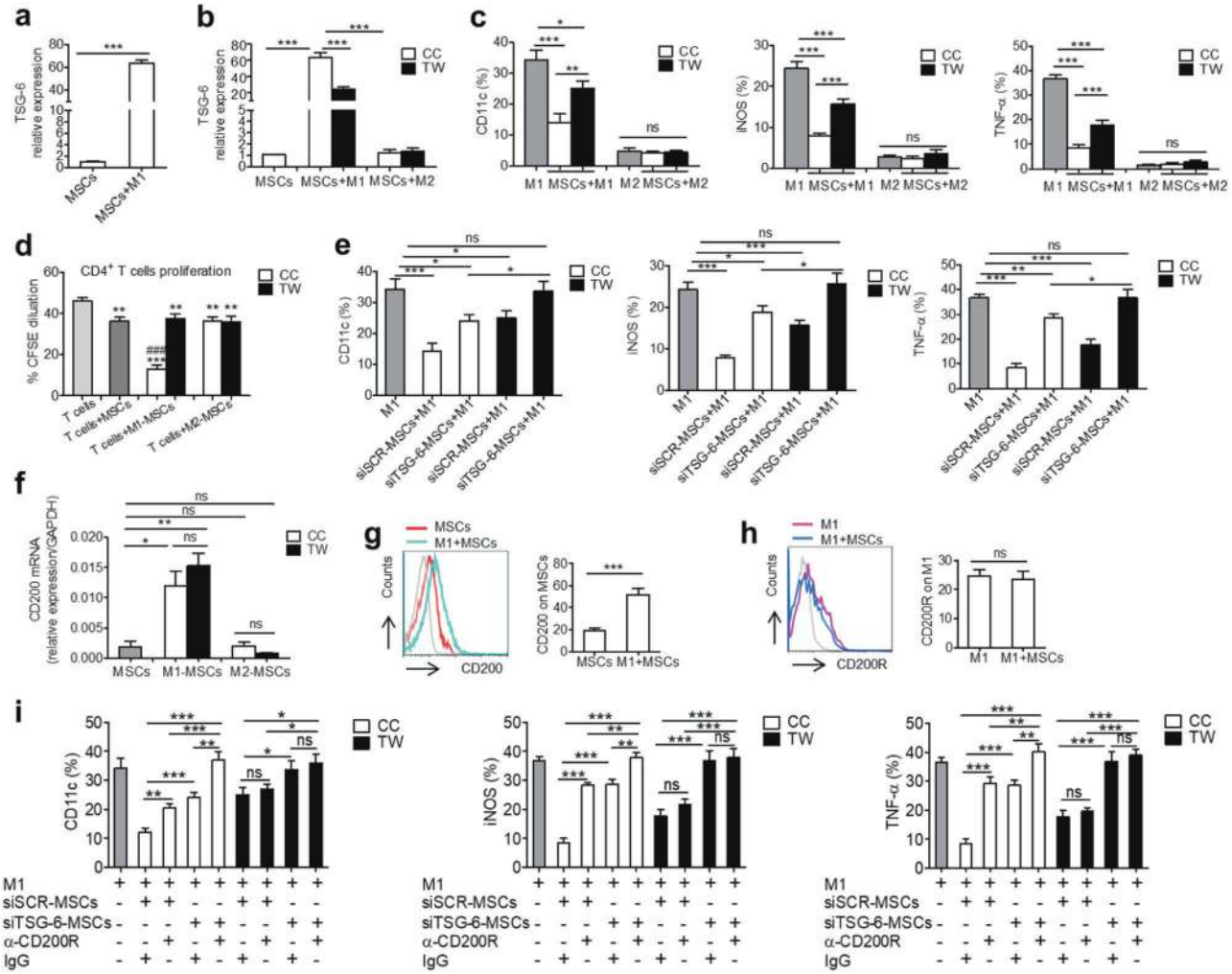

Fig. 4 The immunosuppressive activity of MSCs is enhanced after cell-to-cell interaction with M1 macrophages in vitro. **a** Real-time RT-PCR for TSG-6 expression in MSCs cultured alone or together with M1 (RAW264.7 cells treated with IFN-γ and LPS). **b** Real-time RT-PCR analysis of TSG-6 expression by sorted MSCs. **c** Flow cytometric determination of the percentages of RAW264.7 cells that were positive for CD11c, iNOS, and TNF-α. For **b** and **c**, the MSCs were cultured with M1 (RAW264.7 cells treated with IFN-γ and LPS) or M2 (RAW264.7 cells treated with IL-4) in direct cell-to-cell contact (CC) or using a transwell chamber (TW) system for 48 h. **d** MSCs were cultured with M1 or M2 as described in **b** and **c**, and the sorted MSCs were then cultivated with $CD4^+$ cells purified from the blood of naïve mice. The immunosuppressive activity of the MSCs was assayed as described in Fig. 3d. **e** M1 was cocultured with TSG-6-knocked down MSCs or scrambled (SCR) siRNA-transfected MSCs in a CC or TW system for 48 h. Flow cytometry was performed to determine the expression of CD11c, iNOS, and TNF-α by RAW264.7 cells. **f** Real-time RT-PCR for CD200 expression in MSCs cocultured with M1 or M2 in a CC or TW system for 48 h. **g**, **h** Representative and quantitative flow cytometry results for CD200 on MSCs (**g**) and CD200R (the receptor of CD200) on M1 (**h**). **i** Anti-CD200R antibody or control antibody (IgG) was added to the cocultured system described as **e**. Flow cytometry was performed to determine the expression of CD11c, iNOS, and TNF-α by RAW264.7 cells. Data are the means ± SEM of three to four independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; ns, not significant

inflammation through TSG-6 and the contact interaction with M1 to rescue LPS-induced abortion, although whether the mechanism also works in the SA model remains to be verified. Thus, PBS, 2×10^6 MSCs, 2×10^6 TSG-6 siRNA-transfected MSCs, 2×10^6 SCR siRNA-transfected MSCs, 2×10^6 CD200 shRNA-transfected MSCs, or 2×10^6 SCR shRNA-transfected MSCs were injected into the SA model at GD 7.5 and GD 8.5. Consistent with the results in the LPS-induced abortion model treated with MSCs, the relatively higher resorption of the SA model was remarkably reduced after MSC injection. However, a compromised therapeutic effect was observed in the SA model treated with TSG-6 siRNA-transfected MSCs or CD200 shRNA-transfected MSCs (Fig. 6a). Furthermore, significantly more infiltrated $dCD4^+$T cells with higher proinflammatory cytokine production in the SA model were efficiently reduced by MSC treatment, while TSG-6 siRNA-transfected MSCs or CD200 shRNA-transfected MSCs exerted clearly weakened effects (Fig. 6b, c). In addition, we found that the increased number of dMφ in the SA model was not affected by the MSC injection, although the phenotype was significantly transformed from M1 to M2 (Fig. 6d, e). Similar but weakened effects were obtained in the SA model treated with TSG-6 siRNA-transfected MSCs or CD200 shRNA-transfected MSCs (Fig. 6e). The results in Fig. 6 demonstrated that MSCs also have a therapeutic effect in the immune abortion model and the underlying mechanism involves the secretion of TSG-6 and CD200-mediated signaling to reshape the activated macrophages and suppress the pro-inflammatory T cells.

DISCUSSION

The embryo is a natural allograft to the maternal immune system. During embryo implantation, a localized inflammatory reaction

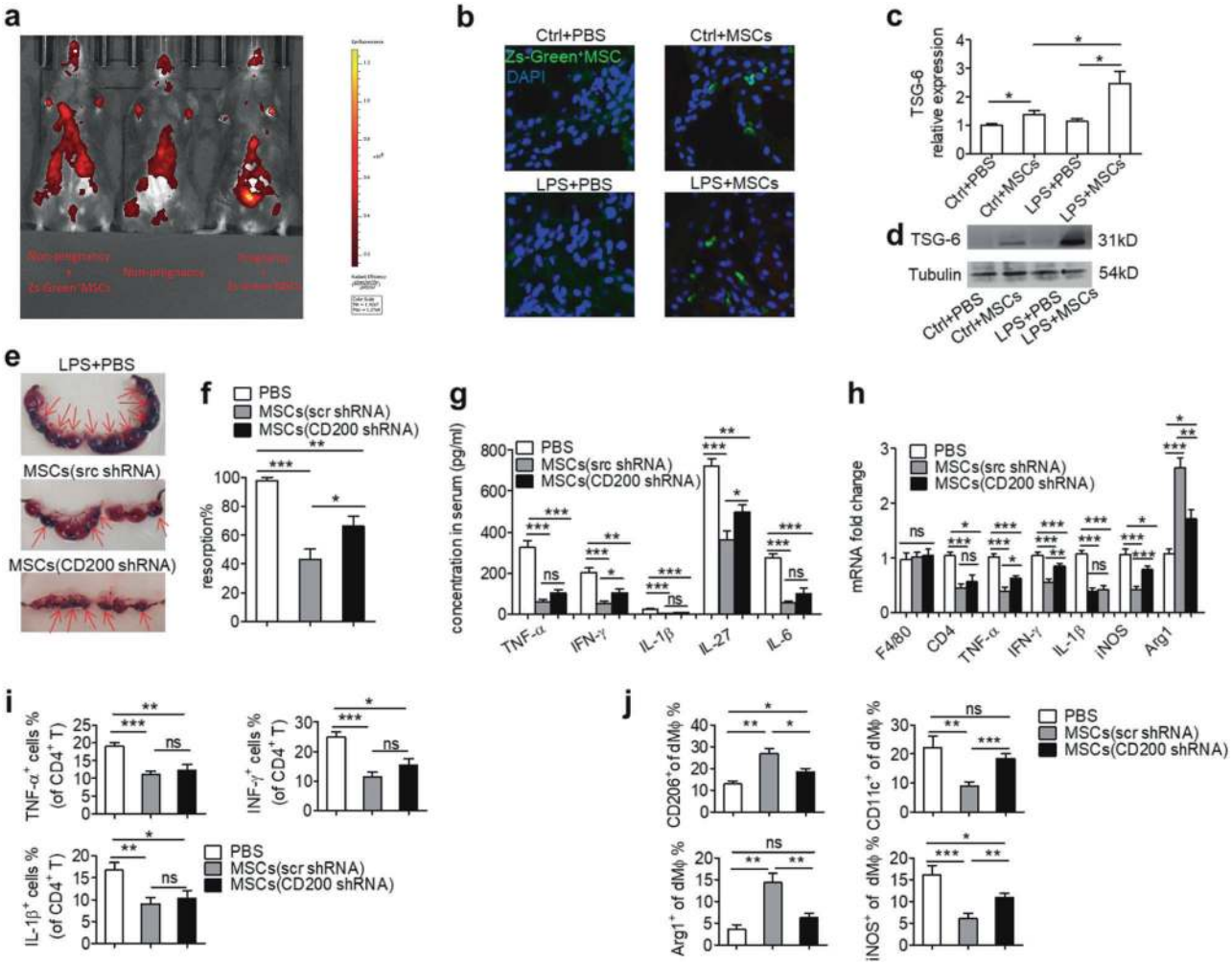

Fig. 5 Cell-to-cell interaction with M1 contributes to the therapeutic effect of MSCs against LPS-induced abortion. **a** In vivo optical image of the Zs-Green$^+$ MSCs in virgin and pregnant mice. **b** Images of decidual frozen sections from normal mice and LPS-challenged mice treated with PBS or Zs-Green$^+$ MSCs. **c**, **d** The deciduas from normal pregnant mice and LPS-challenged pregnant mice treated with PBS or MSCs were obtained to determine the expression of TSG-6 mRNA by real-time RT-PCR (**c**) and TSG-6 protein by immunoblot assay (**d**). The results are representative of three separate experiments. **e**, **f** Representative images of embryos in the uterus from LPS-challenged mice injected with PBS, CD200 shRNA-silenced MSCs, or scrambled (SCR) shRNA-transfected MSCs (**e**). Data depict the embryo resorption rate in pregnant mice treated as indicated (**f**). **g** Serum concentrations of proinflammatory cytokines measured with the LEGENDplex assay (BioLegend). **h** Real-time RT-PCR analysis of the relative expression of the indicated molecules in the deciduas from the LPS-challenged mice treated as described in **e** and **f**. Data indicate the mRNA levels as the relative ratio to the levels in the PBS-treated group. **i**, **j** Flow cytometric analysis of proinflammatory cytokines (TNF-α, IFN-γ, and IL-1β) produced by decidual CD4$^+$ T cells (**i**). Flow cytometric determination of M2 (CD206 and Arg1) and M1 (CD11c and iNOS) markers in F4/80$^+$ decidual macrophages (**j**). For **i** and **j**, decidual immune cells were separated from the mice treated as described in **e** and **f**. Data are the means ± SEM of five to eight mice per group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; ns, not significant

occurs, which is critical for pregnancy establishment.[12,29] However, successful pregnancy maintenance depends on the timely extinction of the inflammatory response, followed by a switch to a tolerant status in the maternal immune system. It is commonly accepted that the immune tolerance during normal pregnancy plays a critical role in preventing rejection of the paternal antigen-carrying fetus.[30] Broken immune tolerance due to uncontrolled inflammation or dampened immunosuppression leads to an abnormal pregnancy, such as miscarriage.[11] Immune disorder-mediated miscarriage has accounted for approximately 60% of unexplained recurrent miscarriages; however, the effective treatment options are uncertain to date. Immunotherapies for miscarriage, including transfusion of paternal leukocytes, passive immunization with intravenous immunoglobulin (IVIG) or third-party donor white cells or trophoblast membrane transfusions, are disputed due to the conflict efficacy in different studies and the potential side effects.[31] Therefore, reliable methods to relieve the high rate of miscarriage are urgently needed. MSCs have been used therapeutically in clinical trials and subsequently in practice to treat immune-related disorders, including graft-versus-host disease, rheumatoid arthritis, and Crohn's disease.[25,26,32] MSC therapy has been proven to be effective and safe, and it is supported by scientists worldwide.[33–35] With respect to reproductive system diseases, emerging studies have reported MSC therapy for premature ovarian failure.[36,37] Several patients who received MSCs recovered their normal menstrual cycles and gave birth to babies. Furthermore, transplanted MSCs were found to recruit to the endometrium in response to injury, which implies the potential for uterine regeneration and repair.[38,39] However, MSCs applied in pregnancy-associated diseases have rarely been reported. In this study, the high embryo resorption rate induced by LPS was significantly reduced by MSC treatment. The LPS-

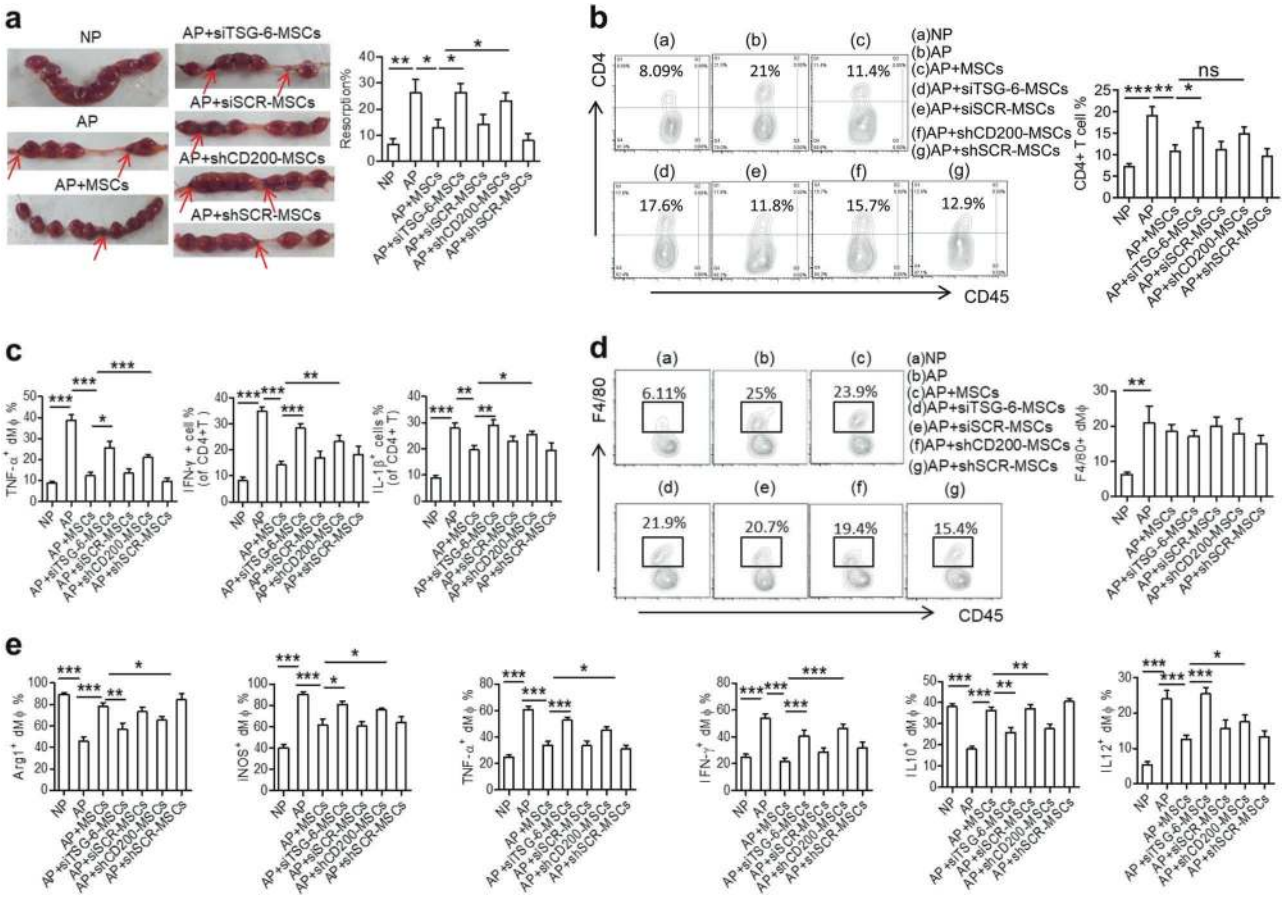

Fig. 6 MSCs prevent fetal loss via TSG-6 and the contact with M1 in the abortion-prone model. **a** Representative images of embryos in the uterus from the normal pregnancy model (established by female CBA/J mice mated with male Balb/c mice) and the spontaneous abortion model (established by female CBA/J mice mated with male DBA/2 mice) injected with PBS, MSCs, TSG-6 siRNA-silenced MSCs, SCR siRNA-transfected MSCs, CD200 shRNA-silenced MSCs, or SCR shRNA-transfected MSCs. Data depict the embryo resorption rate in pregnant mice treated as indicated. **b** Representative and quantitative flow cytometry results for $CD4^+$ T cells infiltrating the decidua. **c** Flow cytometric analysis of proinflammatory cytokines (TNF-α, IFN-γ, and IL-1β) produced by decidual $CD4^+$ T cells. **d** Representative and quantitative flow cytometry results for F4/80+ macrophages in the decidua. **e** Flow cytometric determination of M2 (Arg1) and M1 (iNOS) markers, as well as the cytokine production in $F4/80^+$ decidual macrophages. For **b**–**e**, decidual immune cells were separated from the mice treated as described in **a**. Data are the means ± SEM of five to eight mice per group. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$; ns, not significant

induced activation of $CD4^+$ cells in the peripheral blood and the high levels of proinflammatory cytokines in the serum were significantly inhibited by MSC treatment. In the decidua, the increased infiltration of activated $CD4^+$ T cells due to LPS stimulation was dampened by MSCs, and the proinflammatory phenotype of macrophages was also suppressed. Similar results were obtained in the spontaneous abortion model treated with allo-transplantation of MSCs. These results demonstrate the therapeutic effect of MSCs in preventing pregnancy loss, which suggests the promising clinical application of MSCs in miscarriage.

Mouse bone marrow-derived MSCs (BM-MSCs), obtained from the femurs of C57/BL6 mice, were transplanted into the two abortion models in our study. In addition to bone marrow, mouse MSCs can also be isolated from adipose tissues. AD-MSCs (adipose-derived MSCs) with a comparable therapeutic efficacy and larger quantities have been suggested as an alternative for BM-MSCs.[40] Nevertheless, BM-MSCs are the most commonly used in clinical trials to date, including the reproductive system disease and premature ovarian failure.[36] Moreover, evidence indicates that BM-MSCs have a lower immunogenicity than that of AD-MSCs. Accordingly, BM-MSCs cultured at passages 3–7 were transplanted into abortion models and remarkably ameliorated pregnancy loss.

According to published research, MSCs are applied to immunotherapy mainly via secreted soluble factors, such as IDO, PGE2, IL-10, TGF-β, and TSG-6.[1,41–45] In our study, we found that MSC-derived TSG-6 contributed to the inhibition of T-cell proliferation and the transition of macrophages from the M1 to M2 phenotype in LPS-induced or spontaneous abortion mice. TSG-6 expression knockdown in MSCs remarkably compromised the reduction of fetal loss in these two abortion models and weakened the ability to suppress the proliferation and activation of $CD4^+$ T cells, as well as reprograming macrophages towards the M2 phenotype. These data demonstrate the role of TSG-6 in the immunosuppression by MSCs of both adaptive and innate immunity to ameliorate abortion. Notably, MSC-secreted TSG-6 is typically reported to regulate innate immune cells,[46] particularly the polarization and TNF-α production of macrophages.[47–50] Our findings indicate that TSG-6 derived from MSCs is also competent in suppressing T-cell proliferation and their proinflammatory responses, similar to the function of hMSC-derived TSG-6 in inhibiting Th1 differentiation in autoimmune diabetes.[51] Therefore, both T-cell suppression and M2 switching are TSG-6-dependent in the infection abortion and immune abortion mice treated with MSCs.

Although a majority of reports suggest that the immunoregulatory property of MSCs is dependent on their secretion of soluble factors, the direct cell-to-cell contact also plays a substantial role in their function.[21] Upregulated CD54 on hMSCs in contact with M1 macrophages increased the stem cells' inhibitory effect on T-cell proliferation.[52] Murine MSCs inhibited the differentiation of myeloid progenitors (MPs) in the inflammatory environment via the interaction of the MSC surface-expressed CD200 with MP-expressed CD200R1.[53] Our results suggest that the direct interaction between MSCs and M1 macrophages is indispensable in the MSC treatment of LPS-induced abortion. The in vitro experiments showed that the expression of TSG-6 was low by MSCs themselves. Moreover, coculture with M1, but not M2, substantially increased the expression of TSG-6 by MSCs and was significantly higher in the CC system than in the TW chamber, which suggests that M1 promotes the expression of TSG-6 via soluble factors, as well as direct contact. It is known that TNF-α is the effective inducer of TSG-6,[54] and the M1 macrophage is characterized by a high production of TNF-α. Thus, TNF-α was speculated to mediate the upregulation of TSG-6 by M1 in MSCs. With regard to the direct contact with M1 being responsible for increasing the MSC-derived TSG-6 expression, the specific molecular mechanisms have not been determined and are worth further investigation. Consistently, our in vivo investigation showed that the intraperitoneally injected MSCs preferred to be recruited to the pregnant uterus, where they have the opportunity to be in contact with the decidual immune cells, including macrophages. More importantly, the expression of TSG-6 in the decidua from LPS-challenged pregnant mice after MSC injection was significantly higher than that in normal pregnant mice that received MSCs. The macrophages in the LPS-challenged mice displayed a more proinflammatory phenotype than those in normal pregnant mice, which may account for the notably increased TSG-6 expression in the decidua after MSC treatment. As the therapeutic effect of MSCs against LPS-induced abortion depended on TSG-6, we wondered whether the immunosuppressive modulation on innate and adaptive immune cells by MSCs could be influenced after their coculture with M1, which is capable of promoting the expression of TSG-6 in MSCs. As expected, MSCs cocultured directly with M1 exhibited a more potent regulation of proinflammatory macrophage suppression than those cocultured indirectly with M1. Similarly, the inhibitory effect of MSCs on T-cell proliferation was significantly enhanced after the direct or indirect coculture with M1, and the reinforcement was more significant in the M1+MSC direct coculture system. In contrast, the suppressed proliferation of T cells by MSCs was not affected by their coculture with M2.

However, the more potent enhancement of the immunosuppressive ability of MSCs in the CC coculture system was not entirely due to the different abilities to promote TSG-6 expression. We noted that MSCs transfected with TSG-6 siRNA completely lost the capacity to switch the proinflammatory phenotype of M1 in the TW coculture system, whereas MSCs treated in the same way simply displayed a weakened rather than a deprived capacity to reprogram M1 in the direct CC coculture system. This finding suggests that in addition to the role of the soluble factor TSG-6, the direct interaction between M1 and MSCs is involved in the immunoregulation of M1 by MSCs. A further study was performed to elucidate the underlying molecular mechanism. We found that the interaction between the MSC surface-expressed CD200 and its M1-expressed receptor CD200R contributed to the MSC-mediated switch of proinflammatory macrophages. CD200 (OX-2 antigen) is a type-1 membrane glycoprotein, and its interaction with CD200R results in the inhibition and/or downregulation of myeloid cell activity.[55,56] Here, we showed that the expression of CD200 on MSCs was significantly upregulated by M1, but not M2, in both the direct and indirect coculture systems, which suggests that it may be the characteristic cytokine profile of M1 that stimulates the increased CD200 expression on MSCs. Blocking the CD200 and CD200R interaction with the anti-mouse CD200R antibody significantly attenuated the MSC immunosuppressive effect on M1. These in vitro data indicated that M1 upregulates the expression of CD200 on MSCs, which, in turn, promotes the MSC-mediated M1 switching towards the M2-like phenotype. Moreover, MSCs transfected with CD200 shRNA remarkably dampened the switch of proinflammatory macrophages towards the anti-inflammatory phenotype, as well as the restricted inflammation in the system and decidua, thereby compromising the therapeutic effect against abortion.

In conclusion, we have demonstrated the mechanism through which MSCs reduce LPS-induced and spontaneous abortion. A mechanism of cross-talk among MSCs and immune cells in abortion mice was identified, in which cell-to-cell contact is highlighted. Direct contact with proinflammatory M1 not only contributed to the increased production of TSG-6 by MSCs but also upregulated the CD200 expression on MSCs. The increased TSG-6 further suppressed the proliferation of T cells and facilitated the switching of proinflammatory macrophages to the anti-inflammatory phenotype, while the upregulated CD200 mediated the direct interaction between MSCs and proinflammatory macrophages, aiding in the transition of the proinflammatory macrophages (Fig. 7). The cross-talk confirmed that MSCs are immune regulators, as well as "immune sensors", sensing the dynamic microenvironment caused by soluble factors and the cell contact-mediated signal. These results will be useful for understanding the complicated mechanism of MSC-induced immune tolerance and providing the theoretical basis for their application in miscarriage. However, there are limitations when translating our results to a human clinical therapy due to the differences between humans and mice. The transplanted time, dosing and route in humans should be carefully adjusted and optimized in future clinical trials. Furthermore, due to the incomplete silencing by the application of siRNAs or shRNAs in this study, the significant difference between the therapeutic effect of MSCs and that of TSG-6 or CD200 silenced MSCs was not highly remarkable. More

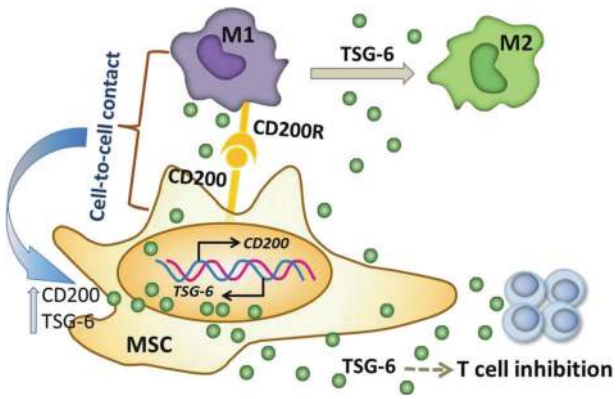

Fig. 7 Schema of how MSCs exhibit immunosuppressive modulation to induce immune tolerance against abortion. MSCs displayed immunosuppressive modulation in abortion models to reduce the fetal loss in a paracrine manner and a cell contact-dependent manner. MSC-derived TSG-6 shifted the macrophages from a pro-inflammatory phenotype (M1) to an anti-inflammatory phenotype (M2), as well as inhibited the proliferation and inflammatory response of $CD4^+$ T cells. This paracrine effect was reinforced by the MSC-M1 contact, which promoted the production of TSG-6. In addition, CD200 on MSCs interacting with CD200R on M1 played an indispensable role in the transition of M1 to M2 by MSCs. Notably, M1 exerted a promotion effect on the CD200 expression of MSCs. Therefore, soluble factor, TSG-6 and the contact with M1 are involved in the immunosuppression by MSCs against abortion

effective experimental means are required to further confirm the mechanism of MSC therapy.

MATERIALS AND METHODS

Isolation, expansion, and characterization of MSCs

Mouse bone marrow-derived MSCs were obtained from the femur bone of euthanized C57BL/6 mice as previously described.[57] In summary, cells were cultured in Mesenchymal Stem Cell Medium (ScienCell, San Diego, California, USA) at 37 °C in a 5% CO_2 humidified incubator. After 24 h, the non-adherent cells were removed, and fresh medium was added. Adherent cells were detached with 0.25% Trypsin-EDTA and passaged when they reached 90% confluence to maintain their proliferative phenotype. MSCs at passages 3–7 were used for the following experiments to avoid the long-term culture associated senescence and attenuated properties. To confirm the mesenchymal nature of the cells, the phenotype was analyzed by flow cytometric analysis, and the differentiation potential was assessed by adipogenic and osteogenic assays. Adipogenesis was induced by adipogenic induction medium (Cyagen Biosciences, Santa Clara, California, USA) for 12–20 days and confirmed by Oil red O staining to show the red stained lipid droplets. Osteogenesis was induced by osteogenic induction medium (Cyagen biosciences, Santa Clara, California, USA) for 2–4 weeks, and calcium deposition was shown by Alizarin red staining. No spontaneous induction was observed in uninduced cultures.

Mice and LPS-induced abortion model

Male and female C57BL/6 mice (8–10 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. All experimental procedures that involved animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (China), and permission was provided by the Human Research Ethics Committee of Obstetrics and Gynecology Hospital of Fudan University. Female mice were mated in natural cycling with the males. Detection of a vaginal plug was chosen to indicate day 0.5 of gestation. Pregnant females were intraperitoneally injected with 0.25 mg/kg LPS (Sigma-Aldrich, St. Louis, MO, USA) at GD 7.5 to induce abortion. The LPS-treated mice were then randomly divided into two groups. Through intraperitoneal injection, one group was administered MSCs (2×10^6 cells in 200 μL of PBS) and the other group was administered 200 μL of PBS, at both 2 and 24 h, respectively. All mice were sacrificed at GD 13.5 to examine and calculate the embryo resorption rate.

TSG-6 siRNA transfection

MSCs were transfected with TSG-6 siRNA (Santa Cruz Biotechnology, Dallas, Texas 75220, USA) or scrambled (scr) siRNA (Santa Cruz Biotechnology, Dallas, Texas 75220 USA) diluted in transfection medium. The knockdown efficiency of TSG-6 in MSCs was 89% as assessed by real-time RT-PCR.

CD200 shRNA transfection

MSCs were transfected with CD200-specific or nonspecific control short hairpin A (shRNA) (Santa Cruz Biotechnology, Dallas, Texas 75220, USA) using transfection reagent in shRNA transfection media according to the manufacturer's protocol. The knockdown efficiency of CD200 was 76% as validated by real-time RT-PCR 48 h after transfection.

Mice and spontaneous abortion model

Male DBA/2 and female CBA/J mice (8–10 weeks old) were purchased from Shanghai SLAC Laboratory Animal Co. The spontaneous abortion model was established by the female CBA/J mice mated in natural cycling with the male DBA/2. The normal pregnant model was established by the female CBA/J mice mated with the male BALB/C mice as the control group. Detection of a vaginal plug was chosen to indicate day 0.5 of gestation. The spontaneous abortion mice were randomly divided into six groups. Each group was treated with PBS, MSCs, siTSG-6 transfected MSCs, scramble siRNA transfected MSCs, shCD200 transfected MSCs or scramble shRNA transfected MSCs (2×10^6 cells in 200 μL of PBS), respectively, at GD 7.5 and GD 8.5. The normal pregnant mice were administered PBS 200 μL. All mice were sacrificed at GD 13.5 to examine and calculate the embryo resorption rate.

Cytometric bead array

Serum inflammatory cytokines were measured with the LEGENDplex Mouse Inflammation Panel (BioLegend, 9727 Pacific Heights Blvd San Diego, CA 92121, USA) according to the manufacturer's instructions.

Flow cytometry and cell sorting

Cells were washed and incubated with appropriate fluorochrome-conjugated antibodies for staining. Flow cytometric analysis was performed on a CyAn ADP analyzer (Beckman Coulter, Inc. Kraemer Boulevard Brea, CA, USA), and the data were analyzed with FlowJo version 6.1 software (Tree Star, Asland, OR, USA). For flow cytometric sorting, cells were stained with specific antibodies and isolated on a BD FACSAria cell sorter (BD Biosciences, Becton Drive Franklin Lakes, USA).

Western blotting analysis

Whole-cell protein extracts were prepared by lysing cells in radioimmunoprecipitation assay buffer supplemented with proteinase inhibitors (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). The protein yield was quantified using the bicinchoninic acid protein assay. After denaturation, equal amounts of protein were separated by SDS–polyacrylamide gel electrophoresis before wet-transfer onto polyvinylidene difluoride membranes. Nonspecific binding sites were blocked by incubating the membranes with 5% bovine plasma albumin (BSA) in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBS-T) for 1 h, which were rinsed and incubated with primary antibody solutions (1:500 for TSG-6 (R&D systems) and 1:5000 for tubulin (Abcam, Kendall Square, Suite B2304 Cambridge, MA 02139-1517, USA)) diluted in blocking buffer (5% BSA and 1× TBS-T) overnight at 4 °C. Primary antibodies were removed by washing the membranes four times in TBS-T, and the membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:3000) (Abcam, Kendall Square, Suite B2304 Cambridge, MA 02139-1517, USA). After three washes with TBS-T, immunopositive bands on the blots were visualized with the enhanced chemiluminescence detection system (Merck Millipore, Darmstadt, Germany).

Quantitative real-time PCR

Total RNA was extracted with TRIzol Reagent (Invitrogen, 168 Third Avenue Waltham, MA USA) and then reverse-transcribed into first-strand complementary DNA (cDNA) (Qiagen, Str.140724 Hilden, Germany) according to the manufacturer's instructions. The synthesized cDNA was amplified with specific primers and SYBR Green (Qiagen, Str.140724 Hilden, Germany) with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Waltham, Massachusetts, USA). Triplicate samples were examined in each condition. A comparative threshold cycle (CT) value was normalized for each sample by using the $2-DDCT$ method.

Statistical analysis

The statistical software Prism 6 (GraphPad) was used for data analyses. Statistical significance was determined by Student's *t*-test. Multiple means were compared by ANOVA. Error bars in figures indicate the SEM. Statistical significance was set at $P < 0.05$. Statistically significant results are expressed using asterisks, where $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

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AUTHOR CONTRIBUTIONS

L.Y.H., experimental design, performance of experiments and data analysis, and manuscript writing; Z.D. and X.L., performance of experiments, analysis of data, and generation of figures; Z.J., L.Y.K. and H.J.F., generation of figures and literature search; Z.Y.Y., Z.X.X. and L.D.J., study design and data interpretation; D.M.R., study design, conception of experiments and data interpretation.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41423-019-0204-6) contains supplementary material.

Competing interests: The authors declare that they have no conflict of interest.

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