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TGF-β Promotes Immune Responses in the Presence of Mesenchymal Stem Cells

Chunliang Xu,* Pengfei Yu,* Xiaoyan Han,* Liming Du,* Jianhe Gan,† Ying Wang,* and Yufang Shi*,‡

Mesenchymal stem cells (MSCs) possess potent immunosuppression capacity and could exert strong therapeutic effects in many diseases, especially inflammatory disorders, in animal models and clinical settings. Although inflammatory cytokines are critical in inducing the immune modulatory properties of MSCs, detailed molecular mechanisms are yet to be fully understood. TGF-β is a well-known anti-inflammatory cytokine and exists in various inflammatory processes; therefore, we investigated whether it could synergize with MSCs in suppressing immune responses. To our surprise, we found that TGF-β actually reversed the immunosuppressive effect of MSCs on anti-CD3 activated splenocytes. Using TGF-β unresponsive MSCs, we demonstrated that the TGF-β directly acted on MSCs. Furthermore, we showed that the effect of TGF-β is exerted through inhibiting inflammatory cytokines induced inducible NO synthase (iNOS) expression in a SMAD3-dependent manner. Interestingly, we found that TGF-β produced by MSCs could act in an autocrine manner to reduce inflammatory cytokine-induced inducible NO synthase expression by MSCs themselves. Therefore, our study revealed a previously unrecognized property of TGF-β in promoting immune responses in the presence of MSCs. ***The Journal of Immunology*****, 2014, 192: 103–109.**

Mesenchymal stem cells (MSCs) are tissue stem cells that were first described in bone marrow, and then in many other tissues, including adipose tissues, dental tissues, umbilical cord, and tumors (1–3). Thus far, no specific marker has been identified on the surface of MSCs. The current identification of MSCs relies largely on the following properties: adherence to tissue culture plastic; differentiation into adipocytes, osteoblasts, and chondroblasts; positive expression of CD105, CD73, CD29, and CD90; and negative for CD31, CD45, and MHC class II molecules (4). Although MSCs are believed to be critical for keeping cellular homeostasis of metazoan species, most information about these cells is derived from cell populations that are expanded in vitro (5, 6). The in situ physiologic roles of MSCs are still elusive, and detailed analysis is still waiting for the identification of specific surface markers. Nevertheless, because of their potential application in treating inflammatory diseases, many studies have been focused on the immune modulatory property of MSCs expanded in vitro (7).

MSCs have great potential in regenerative medicine, and they also have been shown to provide a therapeutic effect in various diseases, such as osteogenesis imperfecta, graft versus host disease, systemic lupus erythematosus, inflammatory bowel disease, and other inflammation-related diseases in both animal models and clinical settings (8–10). Interestingly, recent studies revealed that some of the therapeutic effects of MSCs are attributed to their potent immunosuppressive capacities (11–13). The MSCs can inhibit the proliferation and function of T cells and B cells, hinder the maturation and function of dendritic cells, and impair the function of NK cells. Although MSCs are being evaluated vigorously in clinical trials to treat various inflammation-related diseases, the mechanism of their immunosuppression is still not completely understood (12). Various molecules have been reported to execute the immunosuppression function of MSCs, including inducible NO synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), TGF-β, PGE_2, and galectins (11). Recently, we found that MSCs are not innately immunosuppressive, but acquire this capability after activation with inflammatory cytokines, IFN-γ in combination with TNF-α, IL-1β, or IL-1α (14). Further study revealed that mouse MSCs use iNOS, whereas human MSCs use IDO to perform their immunosuppressive functions (15).

TGF-β family molecules are pleiotropic cytokines with important roles in cancer, immunoregulation, and wound healing (16). Three TGF-β isoforms have been described in mammalian species, including TGF-β1, TGF-β2, and TGF-β3. TGF-β1, the prototype of this family, is a well-known immunosuppressive molecule (17). Interestingly, TGF-β1 was reported to be responsible for MSC-mediated inhibition of T cells (18). To study the role of TGF-β in immunosuppression by MSCs, we used the

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The online version of this article contains supplemental material.

Abbreviations used in this article: DN, dominant negative; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible NO synthase; MSC, mesenchymal stem cell; NC, negative control; siRNA, small interfering RNA.

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coculture system with MSCs and activated T cells. Surprisingly, we found that in contrast to its well-established immunosuppressive role, TGF-β reversed the suppressive effect of MSCs on T cell proliferation. Further study showed that TGF-β1 inhibited inflammatory cytokine-induced iNOS expression in a SMAD3-dependent manner. These results revealed a previously unrecognized immune-promoting effect of TGF-β1.

Materials and Methods

Cells

MSCs were generated from bone marrow from tibias and femurs of 6-10 wk old mice as previously reported (19). The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Cells were used before 20th passage. Human umbilical cord–derived MSCs were used for the 4th to 10th passages. Splenocytes were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol.

Plasmids

The mouse iNOS promoter sequence was amplified from C57BL/6 genomic DNA with primers listed in Table I and inserted between the Mlu I and Bgl II restriction sites of pGL2-basic vector to obtain plasmid pGL2-iNOS-promoter. The sequence of dominant negative (DN) form of human TGF-β receptor 2 was amplified from the genomic DNA of TGF-β receptor 2 DN transgenic mice and inserted between the *Eco*R I and *Bam*H I sites of pLVX-IRES-zsGreen vector (20). NF-κB reporter plasmid was purchased from Beyotime (Haimen, China). All other primers used in this study are listed in Table I.

Reagents

Recombinant mouse IFN-γ, TNF-α, TGF-β, anti-mouse CD3, anti-mouse CD28, and polyclonal anti-mouse TGF-β–RII-PE were purchased from R&D Systems (Minneapolis, MN); CFSE and Griess reagent were obtained from Sigma-Aldrich (St. Louis, MO); SMAD3 and negative control (NC) small interfering RNA (siRNA) were from Thermo Scientific (Lafayette, CO); Lipofectamine 2000 and TRIzol reagent were obtained from Invitrogen (Carlsbad, CA); Luciferase assay kit was obtained from Promega (Madison,WI); Taqman reverse transcription kit was obtained from ABI (Carlsbad, CA); SYBR Green reagent was obtained from Takara (Dalian, China); mouse iNOS and GAPDH monoclonal Abs were obtained from Cell Signaling Technology (Danvers, MA).

Real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), and reverse-transcribed into cDNA with the Taqman reverse transcription kit (ABI, Carlsbad, CA). cDNA was used as template in real-time PCR with SYBR Green reagent to determine specific gene expression. Primers used are listed in Table I.

Nitrate quantification by Griess test

Culture supernatant (50 μl) and standards were transferred to a flat-bottom, 96-well plate, and then 50 μl Griess reagent was added. After incubation for 5 min in the dark at room temperature, the relative amount of nitrate in each well of the plate was determined colorimetrically by absorbance at 540 nm.

Transfection

MSCs were transfected as previously reported (21). For siRNA transfection, MSCs were seeded the day before transfection in antibiotic-free medium. SMAD3 and NC siRNA were transfected with Lipofectamine 2000 following the product instructions (at 100 nM final concentration) the following day and then incubated for additional 48 h. For plasmid transfection, 2 μg plasmid was introduced into 2×10^6 MSCs with Amaxa Nucleofector using program U-023 and Nucleofector Kit V (Amaxa, Cologne, Germany).

Luciferase reporter assay

Plasmid containing iNOS promoter or NF-κB promoter linked to luciferase reporter was introduced into MSCs with the Amaxa Nucleofector device and seeded into 96-well plates. After 24 h, different cytokines were added to the corresponding wells, cells were collected at different time points, and luciferase activities of these cells were determined with the Luciferase assay kit.

Proliferation assay

For proliferation assay using [^{3}H]-methyl-thymidine incorporation, MSCs were plated in 96-well plates at different numbers per well. The next day, supernatant was discarded and 2×10^5 splenocytes in complete medium were added per well with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28. After 48 h, 1 μCi [^{3}H]-methyl-thymidine (Tdr, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, Shanghai, China) was added to each well. After 4 to 6 h, cells were frozen, thawed, and harvested. The radioactivity of incorporated [^{3}H]-methyl-thymidine was measured by scintillation. For CFSE-based proliferation assay of T cells, MSCs were plated at 1×10^4 per well in 96-well plates. Supernatant was discarded the next day, and 2×10^5 CFSE-labeled splenocytes in complete medium were added to each well with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28. After 72 h, cell proliferation was assessed by the CFSE signal changes on flow cytometry using a FACSCalibur.

Flow cytometry

To determine whether TGF-β alters the expression level of TGF-βRII on MSCs, we treated MSCs with different cytokines as indicated in the legend for Supplemental Fig. 2 for 24 h. Next, we stained these cells with polyclonal anti-mouse TGF-β–RII-PE to analyze the expression of both membrane-bound and intracellular TGF-β–RII on flow cytometry using a FACSCalibur.

Statistical analysis

Data are presented as mean ± SD. Statistical significance was assessed by unpaired two-tailed Student *t* test: $*p < 0.05$; $***p < 0.001$.

Results

TGF-β reverses the immunosuppression capacity of MSCs

MSCs have been shown to possess potent immunosuppression capacity in vitro and in vivo. To investigate factors that regulate the immunosuppression capacity of MSCs, we adopted the MSCs and splenocytes coculture system, in which the combination of anti-CD3 and anit-CD28 can strongly induce T cell proliferation. Using this system, we have demonstrated that MSCs directly inhibit T cell proliferation via concerted actions of chemokines and NO (14). Because TGF-β is a well-known immunosuppressive factor (16), we hypothesized that TGF-β could synergize with MSCs in inducing more potent immunosuppression. Unexpectedly, we found that TGF-β1 (10 ng/ml) did not promote; rather, it reversed the immunosuppressive effect of MSCs on T cells at an MSC: splenocyte ratio of 1:20, as assessed by [^{3}H]-methyl-thymidine incorporation (Fig. 1A). To verify this observation, we used different MSC:splenocyte ratios and conducted dose-response experiments with different concentrations of TGF-β1 and another isoform—TGF-β2. We found that both TGF-β1 and TGF-β2 effectively reversed immunosuppression by MSCs when used even at low concentrations (1 ng/ml; Fig. 1B). To verify our observations further, we adopted another proliferation assay based on CFSE. We labeled splenocytes with CFSE, cocultured them with MSCs, and examined the CFSE signal changes in T cells using flow cytometry. Again, we found that TGF-β1 effectively reversed the immunosuppressive effect of MSCs on T cell proliferation (Fig. 1C). Overall, in both assays, although TGF-β alone slightly inhibited T cell proliferation, when it was added into the splenocytes and MSCs coculture, the immunosuppressive effect of MSCs was greatly abolished. This result contradicted our hypothesis.

TGF-β reverses immunosuppression by acting directly on MSCs

In our coculture system, TGF-β1 could have acted on MSCs or some cell types of the splenocyte population. To determine the target cells of TGF-β in our experimental system, we generated TGF-β–unresponsive MSCs. MSCs were transfected with plasmids encoding a dominant negative form TGF-β receptor II (TGF-βRIIdn), which is known to abolish TGF-β signaling. Cells transfected with control

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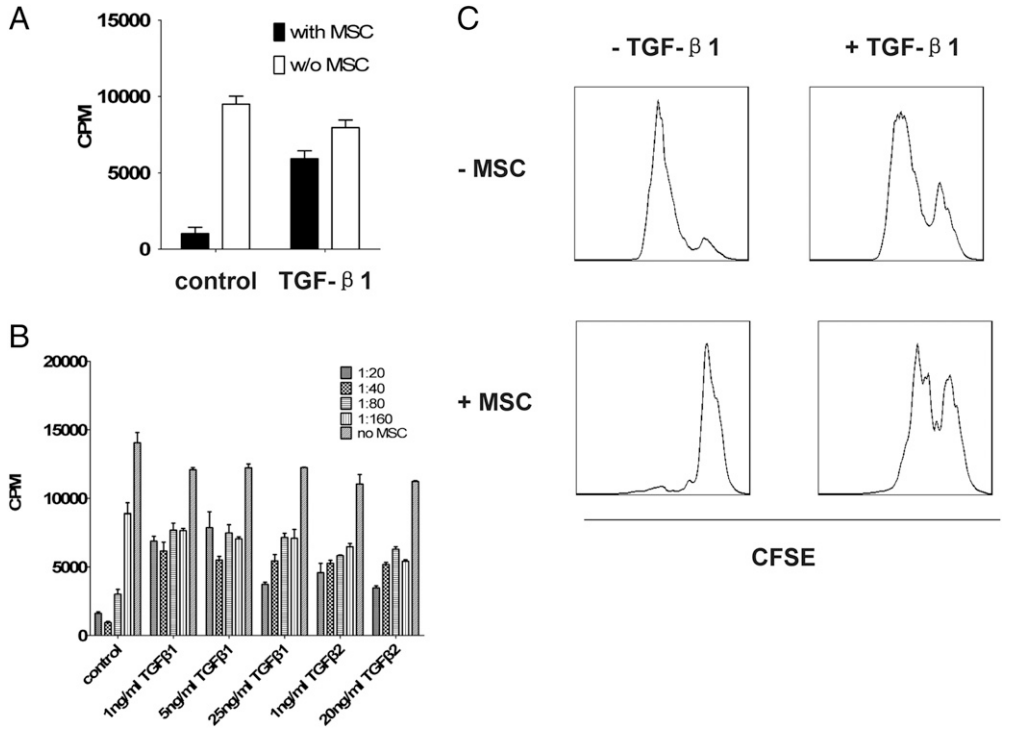

FIGURE 1. TGF-β abolished immunosuppression by MSCs. (**A**) MSCs were seeded at 1×10^4 per well in a 96-well plate. The next day, 2×10^5 splenocytes with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 Abs in RPMI 1640 complete medium were added into each well, and 10 ng/ml TGF-β1 was added at 0 and 24 h to the indicated wells. After 48 h, 1 μCi of [^{3}H]-methyl-thymidine was added to each well. After an additional 4 to 6 h, cells were frozen, thawed, and harvested. The radioactivity of incorporated [^{3}H]-methyl-thymidine was measured with scintillation. (**B**) Different numbers of MSCs (1×10^4, 0.5×10^4, 0.25×10^4, 0.125×10^4 per well) were seeded in 96-well plates. The next day, 2×10^5 splenocytes with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 Abs in RPMI 1640 complete medium were added into each well, and different concentrations of TGF-β1 and TGF-β2 were added at 0 and 24 h to the corresponding wells. Cell proliferation was measured as in (A). (**C**) MSCs were plated at 1×10^4 per well in 96-well plates. The next day, supernatant was discarded, and 2×10^5 CFSE-labeled splenocytes were added to each well with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28. TGF-β1 (10 ng/ml) was added at 0, 24, and 48 h. After 72 h, cell proliferation was assessed by the CFSE signal examined by flow cytometry using a FACSCalibur.

plasmid and TGF-βRIIdn plasmid were designated as MSC-GFP and MSC–TGF-βRIIdn, respectively. MSCs were effectively transfected as shown by GFP expression detected by flow cytometry (Fig. 2A). We next performed immunosuppression assay with these transfected cells. Consistent with the result presented in Fig. 1, TGF-β reversed the immunosuppressive effect of control plasmid transfected MSC-GFP cells. However, this reversal effect was completely abolished in MSC–TGF-βRIIdn cells (Fig. 2B). This result clearly demonstrated that TGF-β acted directly on MSCs to reverse their immunosuppressive effect.

TGF-β inhibited iNOS expression in MSCs

Our recent investigations showed that the induction of iNOS by inflammatory cytokines is essential for mouse MSC–mediated immunosuppression (14). It is important to point out that we have shown that MSCs express only iNOS, but not neuronal NOS or endothelial NOS; therefore, supernatant nitrate is a good estimate of iNOS activity in MSCs. To investigate the mechanisms through which TGF-β reverses the immunosuppressive effect of MSCs, we examined the supernatant for nitrate concentration in the coculture system. Indeed, we found that TGF-β1 reduced nitrate in the su-

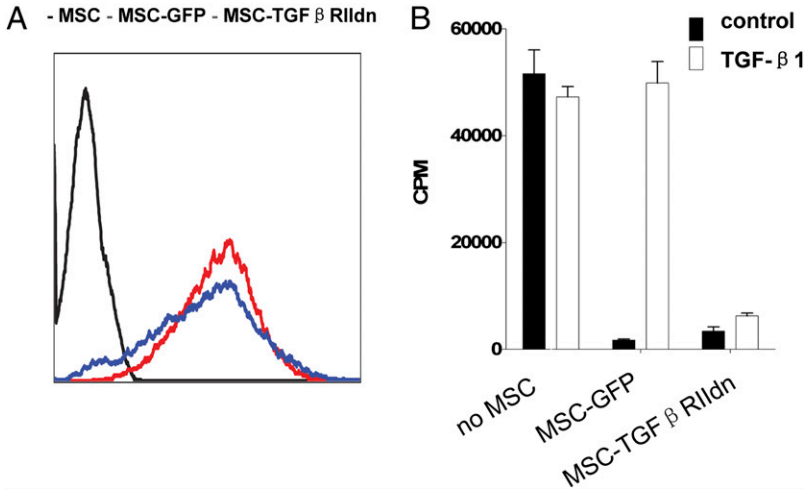

FIGURE 2. TGF-β reverses immunosuppression by acting directly on MSCs. (**A**) MSCs were transfected with control vector (MSC-GFP) or TGF-βRIIdn plasmid (MSC–TGF-βR2dn), and the transfection efficiency was examined by GFP expression by flow cytometry. (**B**) MSC-GFP and MSC–TGF-βRIIdn were plated at 1×10^4 per well in 96-well plates. The next day, 2×10^5 splenocytes with 1 μg/ml anti-CD3 and 1 μg/ml anti-CD28 Abs in RPMI 1640 complete medium were added into each well, and 10 ng/ml TGF-β1 was added at 0 and 24 h to the indicated wells. After 48 h, 1 μCi of [^{3}H]-methyl-thymidine was added to each well. After an additional 4 to 6 h, cells were frozen, thawed, and harvested. The radioactivity of incorporated [^{3}H]-methyl-thymidine was measured with scintillation.

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pernatant of the splenocytes and MSCs coculture system (Fig. 3A). As we have shown before, the expression of iNOS in MSCs is induced by inflammatory cytokines (14). To determine the effect of TGF-β on iNOS expression, we treated MSCs directly with inflammatory cytokines in the presence or absence of TGF-β. As shown in Fig. 3B, TGF-β1 significantly reduced the inflammatory cytokine-induced iNOS mRNA in MSCs with primers listed in Table I. The same inhibitory effect was also observed in iNOS protein and supernatant nitrate level examined by Griess test and Western blot analysis, respectively (Fig. 3C, 3D). Furthermore, we performed dose-response experiments and found that the inhibition of TGF-β1, TGF-β2, and TGF-β3 was potent, and they could block iNOS expression when used at 1 ng/ml (Fig. 3D and Supplemental Fig. 1). An interesting observation is that when a high dose of TGF-β2 and TGF-β3 (10 ng/ml and 100 ng/ml, respectively) was used, the inhibitory effect on iNOS expression was largely abolished (Fig. 3D and Supplemental Fig. 1). To investigate the underlying mechanism, we analyzed the TGF-βRII expression of MSCs and found that a high dose of TGF-β dramatically decreased the extracellular membrane-bound TGF-βRII expression (Supplemental Fig. 2A), but it had no effect on the intracellular TGF-βRII expression (Supplemental Fig. 2B). These data clearly showed that TGF-β reversed the immunosuppressive effect of MSCs by inhibiting iNOS expression in MSCs.

TGF-β inhibited iNOS transcription in a SMAD3-dependent manner

The effect of TGF-β on iNOS expression has not been reported in MSCs. To discern how TGF-β inhibited iNOS, we examined the effect of TGF-β1 on iNOS transcription, mRNA degradation, and protein degradation. As shown in Fig. 4A, inflammatory cytokines dramatically increased the iNOS promoter activity, which is consistent with the critical role of inflammatory cytokines in iNOS expression in MSCs. Interestingly, TGF-β1 alone had no effect on the iNOS promoter activity, whereas it completely inhibited the iNOS promoter activity induced by inflammatory cytokines; the same is true for TGF-β2 (Fig. 4A and Supplemental Fig. 3A). On the other hand, TGF-β1 had no effect on iNOS mRNA degradation (Supplemental Fig. 3B) or iNOS protein degradation (Supplemental Fig. 3C). TGF-β is known to exert its biological functions through SMAD-dependent or -independent pathways, or both (22). Next, we studied which pathway was involved in the inhibition of iNOS expression by TGF-β in MSCs using siRNA-mediated knockdown of SMAD3, which is an important molecule in the SMAD-dependent pathway (23). Knockdown of SMAD3 by siRNA was efficient (Fig. 4B). Interestingly, the inhibition of iNOS expression by TGF-β1 was almost eliminated by knockdown of SMAD3 (Fig. 4C), suggesting that TGF-β inhibited iNOS expression through a SMAD-dependent pathway. Furthermore, the NF-κB pathway has been shown to be an important pathway for the inflammatory cytokines induced iNOS expression (24). Therefore, we tested whether the NF-κB pathway is inhibited by TGF-β1. With the NF-κB reporter assay, we found that TGF-β1 had no effect on the activation of NF-κB pathway induced by inflammatory cytokines, indicating that the inhibition of iNOS transcription by TGF-β is not through the NF-κB pathway (Fig. 4D). Therefore, TGF-β1 inhibited iNOS expression in MSCs transcriptionally in an SMAD3-dependent manner.

MSC-derived TGF-β inhibited iNOS expression in an autocrine manner

As discussed above, TGF-β is effective in modulating immunosuppression by MSCs. Therefore, we examined the amount of TGF-β released by MSCs and its role in the immunosuppression capacity of MSCs. We first quantified TGF-β expression in two lines of MSCs with real-time PCR and ELISA. We found that MSCs expressed a high level of TGF-β1 mRNA and released >300 pg/ml of TGF-β when cultured at 0.5×10^6 cells/ml for 48 h (Fig. 5A, 5B). Therefore, we expected that the TGF-β released from MSCs could participate in regulating the potency of MSC-mediated immunosuppression. The effect of MSC-derived TGF-β on the inflammatory cytokine-induced iNOS expression was examined by blocking TGF-β signaling in MSCs with three methods, including transfection with SMAD3 siRNA or TGF-βRIIdn and the use of SB431542, which is a specific chemical inhibitor of the TGF-β receptor. We found that when the TGF-β receptor or signaling process was inhibited in MSCs, the expression of iNOS induced by inflammatory cytokines was much higher, as shown by the increased supernatant nitrate concentrations (Fig. 5C–E). Therefore, TGF-β produced by MSCs plays an important role in

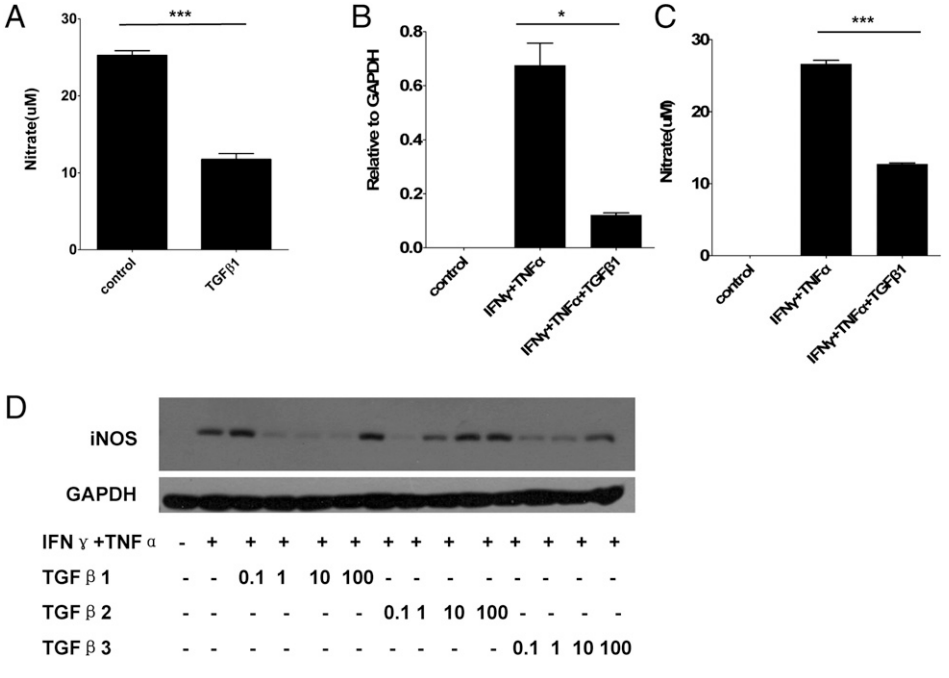

FIGURE 3. TGF-β inhibited inflammatory cytokine-induced iNOS expression in MSCs. (**A**) Supernatant nitrate concentrations in the coculture of splenocytes and MSCs with or without TGF-β1 as described in Fig. 2 were determined by Griess test. (**B** and **C**) MSCs were cultured at 5×10^5 cells/ml and stimulated with 10 ng/ml IFN-γ and 10 ng/ml TNF-α with or without 10 ng/ml TGF-β1. The iNOS mRNA level and nitrate in the culture supernatant were determined with real-time PCR and Griess test 24 h later, respectively. (**D**) MSCs were activated by 10 ng/ml IFN-γ and 10 ng/ml TNF-α with different concentrations of TGF-β1, TGF-β2, and TGF-β3 (0, 0.1, 1, 10, and 100 ng/ml) for 24 h, and iNOS protein was determined with Western blotting analysis using an mAb specific for iNOS. $*p < 0.05$, $***p < 0.001$.

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Table I. Primers for real-time PCR and Plasmids

Primers	Forward	Reverse
Real-time PCR		
Human		
IDO	5′-GCCCTTCAAGTGTTTCACCAA-3′	5′-CCAGCCAGACAAATATATGCGA-3′
β-Actin	5′-TTGCCGACAGGATGCAGAAGGA-3′	5′-AGGTGGACAGCGAGGCCAGGAT-3′
Mouse		
iNOS	5′-CAGCTGGGCTGTACAAACCTT-3′	5′-CATTGGAAGTGAAGCGTTTCG-3′
SMAD3	5′-ACCACAGCATGGACGCAGGTTC-3′	5′-GCACCAAGAAGGCCGGCTCAC-3′
TGF-β1	5′-TCACTGGAGTTGTACGGCAGTG-3′	5′-TCGAAAGCCCTGTATTCCGTC-3′
GAPDH	5′-CACATTGGGGGTAGGAACAC-3′	5′-ACCCAGAAGACTGTGGATGG-3′
β-Actin	5′-TTCCAGCCTTCCTTCTTGGG-3′	5′-TGTTGGCATAGAGGTCTTTACGG-3′
Plasmids		
iNOS promoter	5′-GCGCACGCGTGATATGCTGAAATCCA-3′	5′-GCGCAGATCTTCCGTGGAGTGAACAA-3′
TGF-βRdn	5′-ACGGAATTCGCCACCATGGGTCGGGGGCTGCTC-3′	5′-ACGGGATCCCTAAACGCGGTAGCAGTAGAAG-3′

modulating inflammatory cytokine-induced iNOS expression in MSCs. Finally, we examined whether TGF-β has similar roles in human MSCs. We have previously reported that IDO is necessary for the immunosuppressive effect of human MSCs. We found that three isoforms of TGF-β all have the ability to inhibit the inflammatory cytokine-induced IDO expression in human MSCs (Supplemental Fig. 4A). Thus, our data clearly show that in the presence of TGF-β, the immunosuppression of MSCs is turned off.

Discussion

MSCs exist in almost all tissues and are responsible for tissue repair and regeneration. Although the physiologic roles of MSCs are still elusive, MSCs that are expanded in vitro have been evaluated extensively for their application in alleviating various immune disorders and tissue and organ injuries (9). It is clear that the potent therapeutic effects of MSCs are achieved through their potent immunosuppressive capacity and regenerative potentials. It is generally believed that when MSCs are recruited into the injury site, they can either resolve the inflammation and accelerate tissue repair when acute inflammation occurs or maintain the inflammation status, resulting in chronic inflammation (12). In this study, we demonstrate that the well-known immunosuppressive cytokine family members, TGF-β, could in fact abolish MSC-mediated immunosuppression. The mechanism of the immunosuppression of MSCs has been pursued extensively (11); however, discrepant conclusions were made by different groups. We found that MSCs are not immunosuppressive in the resting state, but acquired this capacity after activation by inflammatory cytokines (14). When comparing MSCs from different species, we found that there is

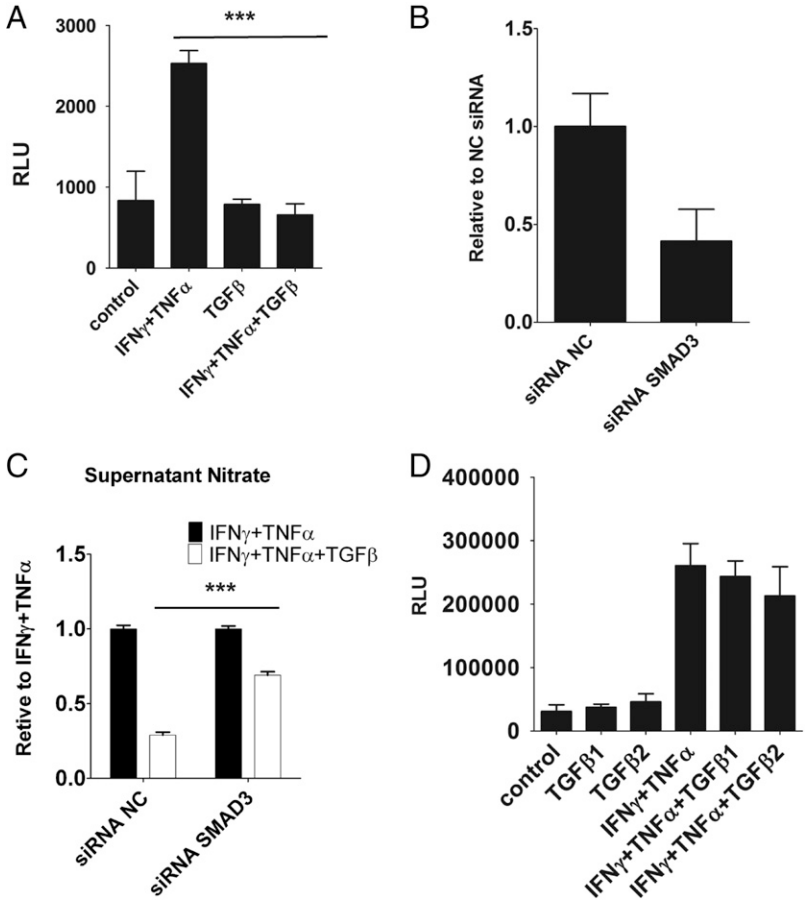

FIGURE 4. TGF-β inhibited iNOS transcription in an SMAD3-dependent manner. (**A**) MSCs (2×10^6) were transfected by iNOS promoter reporter plasmid, pGL2–iNOS promoter, using Amaxa Nucleofector and seeded into 96-well plate at 1×10^5 cells/well. Twenty-four hours later, 10 ng/ml IFN-γ, 10 ng/ml TNF-α, and 10 ng/ml TGF-β1 were added to the indicated wells. Forty-eight hours later, the cells were collected and the reporter activity was measured with the Luciferase assay kit. (**B** and **C**) MSCs were seeded into six-well plates at 1×10^5 cells/well the day before transfection in antibiotic-free medium. The next day, SMAD3 and NC siRNA were transfected with Lipofectamine 2000 following the product instructions (100 nM at final concentration). (B) Knockdown efficiency was determined 48 h later with real-time PCR. Transfected cells were activated with 10 ng/ml IFN-γ and 10 ng/ml TNF-α with or without 10 ng/ml TGF-β1. (C) The supernatant nitrate concentrations were determined 24 h later by Griess test. (**D**) MSCs (2×10^6) were transfected by NF-κB reporter plasmid using Amaxa Nucleofector and seeded onto a 96-well plate at 1×10^5 cells/well. Twenty-four hours later, 10 ng/ml IFN-γ, 10 ng/ml TNF-α, and 10 ng/ml TGF-β1 or TGF-β2 were added to the indicated wells. Four hours later, the cells were collected and the reporter activity was measured with the Luciferase assay kit. ***$p < 0.001$.

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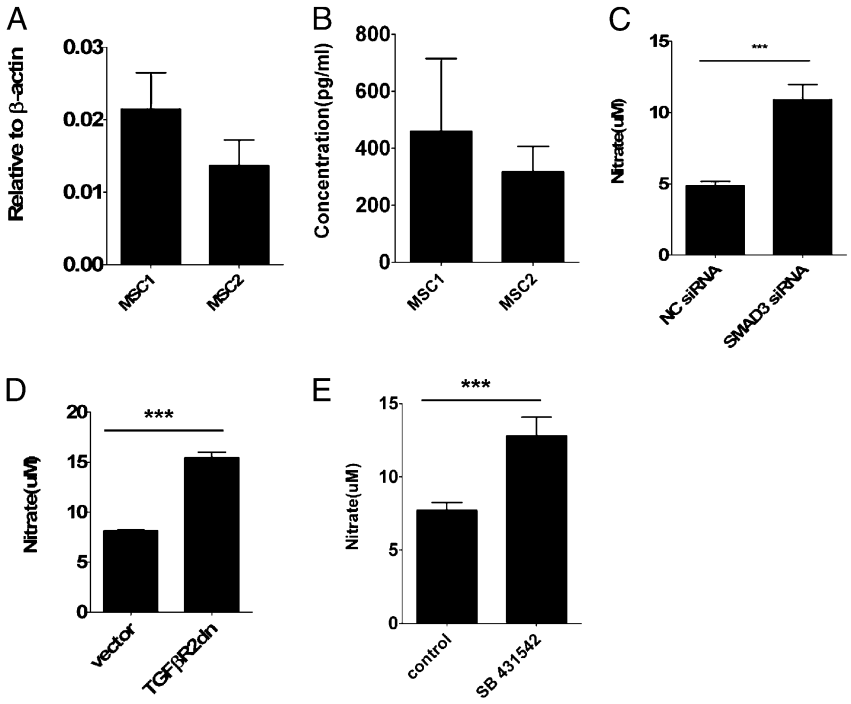

FIGURE 5. MSC-derived TGF-β inhibited iNOS expression in an autocrine manner. (**A** and **B**) TGF-β1 mRNA and the concentration of TGF-β1 in the supernatant of two MSC lines were determined with real-time PCR and ELISA, respectively. (**C**) MSCs were seeded onto a six-well plate at 4×10^5 cells/well the day before transfection in antibiotic-free medium. The next day, SMAD3 and NC siRNA were transfected with Lipofectamine 2000 following the product instructions (100 nM at final concentration). Transfected cells were activated with 10 ng/ml IFN-γ and 10 ng/ml TNF-α 48 h later, and nitrate concentration in the supernatant was determined with a Griess test 24 h after the addition of cytokines. (**D**) MSCs transfected with control vector and TGF-βRIIdn vector were activated with 10 ng/ml IFN-γ and 10 ng/ml TNF-α, and nitrate concentrations in the supernatant were determined with a Griess test 24 h after the addition of cytokines. (**E**) MSCs were pretreated with TGF-β receptor inhibitor SB431542 for 24 h before activation of 10 ng/ml IFN-γ and 10 ng/ml TNF-α. Nitrate concentrations in the supernatant were determined with a Griess test 24 h later. ***$p < 0.001$.

a species variation in the mechanism of immunosuppression: mouse MSCs use iNOS, whereas human and monkey MSCs use IDO to conduct their immunosuppressive activities (15). We believe that our findings partly explain the discrepant conclusions of the molecular mechanisms of MSC-mediated immunosuppression among different research groups.

TGF-βs are multifunctional cytokines with three isoforms: TGF-β1, TGF-β2, and TGF-β3. They have different expression patterns and functional variations in vivo, but have relatively similar functions in in vitro assays. TGF-β1 is the prototype of TGF-βs and is the predominant isoform expressed by cells of the immune system (16). TGF-β1 can suppress immune response either directly by inhibiting the function of immune cells, such as T cells, B cells, NK cells, and macrophages, or indirectly by inducing regulatory T cells (17). Interestingly, TGF-β1 was reported to be an important factor in the immunoregulatory function of MSCs (18). Based on these observations, we originally reasoned that the addition of TGF-β to the coculture of MSCs with activated splenocytes would suppress immune responses more potently than when used alone. Unexpectedly, we found that TGF-β in fact abolished the immunosuppressive capacity of MSCs by inhibiting inflammatory cytokine-induced iNOS expression in mouse MSCs and IDO in human MSCs. Although TGF-β was previously reported to inhibit iNOS expression in macrophages induced by inflammatory cytokines (25, 26), the mechanisms of iNOS induction are different in MSCs and macrophages. TGF-β inhibited iNOS transcription in MSCs as demonstrated in our study. On the other hand, TGF-β accelerated iNOS protein degradations in macrophages. We also found that the effect of TGF-β–induced inhibition of iNOS expression in MSCs was SAMD3-dependent (Supplemental Fig. 4B). An interesting observation is that TGF-β acts differently on the iNOS expression along with increasing concentrations. TGF-β had no effect on iNOS expression at a low concentration (0.1 ng/ml), and maximal inhibition was achieved at ~1 ng/ml. However, the inhibition ability of TGF-β1 on iNOS expression remained constant, whereas the effects of TGF-β2 and TGF-β3 became weaker at higher concentrations (>10 ng/ml). We showed that a high-dose of TGF-β can reduce the membrane expression of TGF-βRII while having no effect on its intracellular expression level; this might partly explain the dose response of TGF-β.

TGF-β is known to be present in various tissues. It has been reported that its expression increases in various tissues with damage, especially when accompanied by inflammation (16). TGF-β is abundantly present in the tumor microenvironment and plays important roles in the evasion of tumor immune recognition (27, 28). Interestingly, we recently reported that MSCs promote tumor growth by modulating immune responses in tumors (2). We isolated tumor-associated MSCs from mouse spontaneous lymphoma (lymphoma-associated MSCs) and found that L-MSCs can more potently promote lymphoma growth than that of their bone marrow counterparts. Further study revealed that L-MSCs release larger amount of CCR2 ligand, through which monocytes and macrophages are recruited into tumors and promote tumor growth (2). Intriguingly, bone marrow MSCs acquire the tumor-promotion effect of L-MSCs when treated with inflammatory cytokine TNF-α. It is important to determine the relation between TGF-β and MSCs regarding the effects on immune responses in the tumor microenvironment. Overall, because the immune promoting effect of TGF-β is a new concept, we believe the investigations on the role of TGF-β in inflammatory sites, such as the tumor microenvironment, would provide novel information for the understanding of the pathogenesis of various diseases.

In conclusion, we found that TGF-β reversed the immunosuppressive effect of MSCs on anti-CD3 activated splenocytes, and this effect is exerted through inhibiting inflammatory cytokine-induced iNOS expression in an SMAD3-dependent manner. Our study revealed a previously unrecognized property of TGF-β in promoting immune responses in the presence of MSCs.

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Disclosures

The authors have no financial conflicts of interest.

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