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MSCs inhibit bone marrow-derived DC maturation and function through the release of TSG-6

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ABSTRACT

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that are characterized by the ability to take up and process antigens and prime T cell responses. Mesenchymal stem cells (MSCs) are multipotent cells that have been shown to have immunomodulatory abilities, including inhibition of DC maturation and function *in vivo* and *in vitro*; however, the underlying mechanism is far from clear. In this study we found that MSCs can inhibit the maturation and function of bone marrow-derived DCs by releasing TSG-6. In the presence of MSCs, lower expression of mature DC surface phenotype (CD80, CD86, MHC-II, and CD11c) was observed. In addition, typical DC functions, such as the production of IL-12 and the ability to prime T cells, were decreased when co-cultured with MSCs. In contrast, knockdown of TSG-6 reduced the inhibitory effect of MSCs on DC. Moreover, we found that TSG-6 can suppress the activation of MAPKs, and NF-κB signaling pathways within DCs during Lipopolysaccharides (LPS) stimulation. In conclusion, we suggest that TSG-6 plays an important role in MSCs-mediated immunosuppressive effect on DC.

1. Introduction

Mesenchymal stem cells (MSCs) possess the immunomodulatory activity, which has attracted considerable attention in recent years. MSCs regulate a wide range of immune cells [1]. For example, MSCs inhibit proliferation of T and B lymphocytes [2,3], prevent differentiation of monocytes into dendritic cells (DCs), and inhibit DC maturation [4,5]. Due to the immunosuppressive properties, MSC-based therapy has been successfully applied in various immune-related diseases, such as graft versus host disease (GvHD), systemic lupus erythematosus (SLE), autoimmune encephalomyelitis, and multiple sclerosis (MS) [6,7,1,8–10].

DCs are the most potent antigen-presenting cells, and play a key role in the initiation of primary immune responses and the induction of tolerance. DCs have the ability to take up and process antigens, up-regulating several immune-related molecules, such as co-stimulatory, adhesion, and integrin molecules to prime naive T cells [11]. Once activated by DCs, these T cells can complete the immune response by interacting with other cells, such as B cells for antibody formation, macrophages for cytokine release, and targets for lysis [12]. In addition to the capacity to stimulate naive T cells, DCs can also interact directly with B [13] and natural killer cells [14]. Given the critical role in the initiation of primary immune responses, DCs are becoming a vital target for immunosuppression to prevent allograft rejection, autoimmune diseases, and other immune-related diseases.

Recent studies have focused on the influence of MSCs on DCs. Many reports have demonstrated that MSCs exert a potent suppressive effect on the maturation and function of myeloid or monocyte DCs. For example, Djouad et al. [15] observed that MSCs can produce IL-6, which may be involved in reversing the maturation of DCs into a less mature phenotype, and in the partial inhibition of bone marrow progenitor differentiation into DCs. Spaggiari et al. [16] demonstrated that PGE2 secreted by MSCs plays a major role in MSC-mediated inhibitory effects on DCs during the progression from monocytes to immature DCs (iDCs). In brief, the soluble factors released by MSCs may be involved in the mechanism by which MSCs exert their inhibitory effect on DC maturation.

TNFa-stimulating gene (TSG)-6, an anti-inflammatory protein produced by MSCs in response to inflammatory cytokines [17], has been revealed to produce immune modulating effects in several animal models [18–20]. Research has demonstrated that TSG-6 secreted by MSCs endowed the ability of suppressing

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NF-κB signaling in resident macrophages induced by zymosan in the peritoneum [21]. Because of the importance of NF-κB signal transduction pathways in regulating expression of functionally important immune molecules expressed by DCs [22], therefore, we sought to determine whether TSG-6 produced by MSCs has an inhibitory effect on DCs maturation and function.

In the current study we observed that MSCs can affect DC maturation and function by secreting TSG-6. Knockdown of TSG-6 reduce the inhibitory effect of MSCs on DCs. Furthermore, our data showed that TSG-6 suppressed MAPKs and NF-κB signaling activation during the progression from iDCs to mDCs induced by LPS. Therefore, we demonstrated that TSG-6 play an important role in MSCs-mediated inhibition of DCs maturation and function.

2. Materials and methods

2.1. Ethics statements

Six-to-eight-week-old female C57BL/6 and naive BALB/c mice were purchased from the Laboratory Animal Center of Southern Medical University (Guangzhou, China). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80-23, 1996 revision). All of the experimental procedures were approved by the Southern Medical University Ethics Committee.

2.2. MSC isolation and culture

MSCs were prepared from mouse bone marrow (BM) cells, as previously described [23] with minor modifications. MSCs were isolated from the BM of male C57BL/6 mice tibias and femurs. MSCs were assessed by flow cytometry for the expression of the typical markers (CD9, CD44, and Sca-1), and negative for CD11b, CD34, and CD45. MSCs were used in the experiments only from passages 3–8.

2.3. Generation of mouse BM-derived DCs

BM-derived DCs were generated as previously described with minor modifications [24]. BM cells were extracted from marrow cavities of femurs and tibias. The erythrocytes were lysed using erythrocyte lysis buffer (R&D Systems, Minneapolis, MN, USA), then washed three times in serum-free RPMI-1640 medium and cultured in 6-well plates (Costar, Cambridge, MA, USA) at 1×10^6 cells/well containing 10% FBS, 20 ng/mL recombinant murine granulocyte–macrophage colony-stimulating factor (GM-CSF; Peprotech, Rocky Hill, NJ, USA), and 20 ng/mL recombinant murine interleukin-4 (IL-4; Peprotech) at 37 °C in a humidified 5% CO_2 humidified atmosphere. DC maturation was induced at day 5 with 200 ng/mL of LPS (Sigma–Aldrich, St. Louis, MO, USA) stimulation for another 2 days. Cytofluorimetric analysis was performed to evaluate the DC maturation phenotype (CD80, CD86, MHC-II, and CD11c).

2.4. Transfection of MSCs with TSG-6 siRNA

A total of 2×10^5 MSCs were plated in 6-well dishes and cultured for 24 h; then, the cells were transfected with TSG-6 siRNA (sc-39820; Santa Cruz Biotechnology Inc, Paso Robles, USA) using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). To confirm the silencing effect of the TSG-6 siRNA, after 48 h, RNA was extracted from aliquots of the cells and analyzed for TSG-6 expression using real-time RT-PCR.

2.5. DC and MSC co-cultures

A 6-well transwell system (0.3-mm pore size membrane; Corning, Cambridge, MA, USA) was used to assess the effect of MSCs on DCs without cell-to-cell contact. At day 5, 10^6 iDCs (cultured alone with GM-CSF and IL-4) were placed in the lower chamber and stimulated for another 48 h with 200 ng/mL of LPS in the presence or absence of MSCs or TSG-6-siRNA MSCs in the upper chamber at a 10:1 ratio.

2.6. Flow cytometry

For phenotypic analysis of the cell surface marker expression, cells were harvested, resuspended in PBS, and incubated for 20 min with phycoerythrin- or FITC-conjugated monoclonal antibodies (mAbs) on ice. For MSCs, cells were stained with antibodies against CD9, CD44, Sca-1, CD11b, CD34, and CD45. For DCs, cells were stained with antibodies against CD11c, CD80, CD86, and MHC-II (eBioscience, San Diego, CA, USA). Mouse IgG1 isotype-control antibodies were used in parallel as negative controls. Stained cells were then washed twice and resuspended in cold buffer and analyzed with flow cytometry (FACS Calibur; BD Biosciences), and the results were processed using FlowJo software (Tree Star, Inc). The results are expressed as the percentage of positively stained cells relative to the total cell number.

2.7. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Levels of IL-12 mRNA in DCs were quantified by real-time RT-PCR. Total RNA was isolated from DCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the total RNA of each sample was reversely transcribed with oligo dT and SuperScript III RT (Invitrogen). PCR was carried out using an ABI 7500HT fast real-time PCR System (Applied Biosystems). GAPDH was used as the endogenous control. The primers which were used are listed in Supplementary Table 1.

2.8. Cytokine analysis

The concentration of IL-12p70 was determined by an enzyme-linked immunosorbent assay (ELISA) using commercially-available kits (R&D Systems) on supernatants derived from 48 h DC cultures and transwell co-cultures with MSCs, according to the manufacturer's protocol. Interferon-gamma (IFN-γ) secreted from T cells were assayed by ELISA. Quantitative analysis of TSG-6 was performed by ELISA on supernatants derived from MSC cultures for 12, 24, and 48 h in the presence or absence of DCs stimulated by LPS according to the manufacturer's instructions. TSG-6 concentrations were determined with a standard curve constructed by titrating standard TSG-6.

2.9. T-cell proliferation assay

Purified $CD3^+$ T cells (responder cells) from naive BALB/c mice spleens were added to round-bottom 96-well plates (Costar) at 1×10^5 cells/well and incubated at 37 °C in 5% CO_2. The iDCs, mDCs, and DCs conditioned by MSCs or TSG-6-siRNA MSCs were incubated with mitomycin C (50 μg/mL; Sigma–Aldrich) for 1 h at 37 °C to inhibit cell proliferation. These cells were washed 3 times with PBS as stimulator cells and co-cultured with T cells at various ratios (1:40, 1:20, 1:10, and 1:5). T-cell proliferation was detected using a cell counting kit (CCK)-8, according to the manufacturer's instructions (Dojindo Laboratories, Tokyo, Japan) after co-culture with DCs for 5 days.

2.10. Western blot analysis

To define the MAPKs and AKT activation, the phosphorylation of JNK, p38, ERK, and AKT were measured by Western Blot. For NF-κB assay, the phosphorylation of NF-κB and the nuclear translocation of p65 were measured by Western Blot. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with primary antibodies against JNK, p38, ERK, AKT, NF-κB p65 and phospho-JNK, phospho-p38, phospho-ERK, phospho-AKT phospho-NF-κB p65 (Cell Signaling Technology, Danvers, MA, USA) at a 1:1000 dilution at 4 °C overnight.

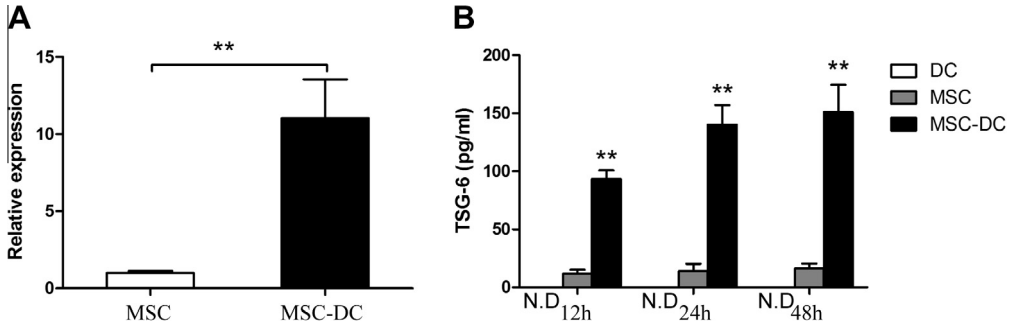

Fig. 1. TSG-6 expression and production by MSCs is up-regulated in MSC-DC cocultures. (A) Real-time polymerase chain reaction analysis showed that the expression of TSG-6 in MSCs. (B) TSG-6 levels were measured by ELISA assay in culture supernatant of MSCs, DCs or MSCs-DCs group at 12, 24, and 48 h. Data was expressed as means ± SD. $^{**}p < 0.01$, (MSC-DC group versus MSC group, N.D none detectable).

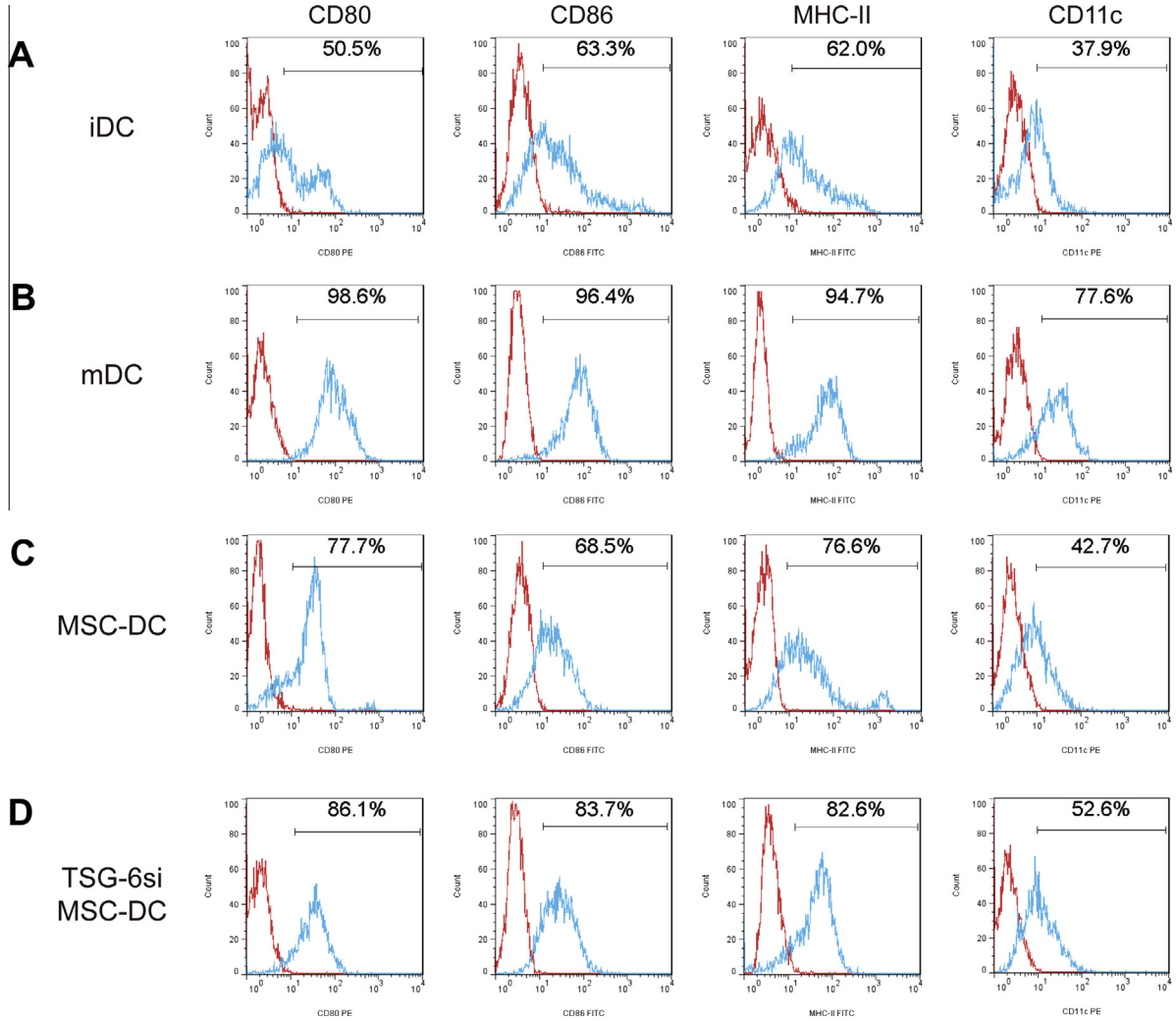

Fig. 2. Phenotype analysis of DCs under different conditions. Surface expression of CD80, CD86, MHC class II and CD11c was analyzed by flow cytometry. (A) iDCs were positive for CD80 (50.5%), CD86 (63.3%), MHC-II (62.0%), and CD11c (37.9%). (B) Mature DCs expressed very high levels of CD80 (98.6%), CD86 (96.4%), MHC-II (94.7%) and CD11c (77.6%). (C) However, MSCs reduce the expression of the four markers compared with mDCs (CD80 77.7%, CD86 68.5%, MHC-II 76.6%, and CD11c 42.7%). (D) A partial restoration of the four mature surface markers (CD80 86.1%, CD86 83.7%, MHC-II 82.6%, and CD11c 52.6%; Fig. 2D) expression were observed in TSG-6si MSC-DCs group.

Secondary antibody incubation occurred for 1 h at room temperature. Immunoblots were visualized using enhanced chemiluminescence (ECL; Thermo Scientific). The expression levels from whole cell extract were normalized against GAPDH (Cell Signaling Technology). The Protein levels of nuclear extracts were normalized against Histone H3 (Cell Signaling Technology).

2.11. Statistical analysis

All experiments were completed at least three times and data are expressed as the mean ± SD. Statistical analyses were performed using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) One-way analysis of variance (ANOVA) or two-tail Student's *t*-test was used to determine the significance between groups. Significance was determined at a $p < 0.05$ level.

3. Results

3.1. Characterization of MSCs

MSCs at passage 3 were analyzed for the expression of cell surface molecules by flow cytometry. Flow cytometry analysis confirmed that the cells were positive for CD9 (99.2%), CD44 (94.9%), and Sca-1 (95.7%), and had low expression of CD11b (0.156%), CD34 (0.280%), and CD45 (1.25%); (Supplement Fig. 1).

3.2. MSCs increased expression and secretion of TSG-6 in Co-culture

We next tested the hypothesis that MSCs could increase the expression and secretion of TSG-6 co-culture with DCs during LPS stimulation. TSG-6 expression was increased approximately 11-fold in MSCs cocultured with DCs compared with MSCs cultured alone (Fig. 1A). TSG-6 produced by MSCs, DCs cultured alone and co-culture in the presence of LPS was also measured via ELISA at 12 h, 24 h, and 48 h. MSCs produced low level of TSG-6, whereas, this production was significantly increased on co-culture with DCs. Notably, DCs cultured alone did not produce any detectable TSG-6 (Fig. 1B).

3.3. MSC inhibition of DC maturation stimulated by LPS depends on the expression of TSG-6

After 5 days, DC differentiation was assessed by analyzing the expression of CD80 (50.5%), CD86 (63.3%), MHC-II (62.0%), and CD11c (37.9%; Fig. 2A) Then, the iDCs were stimulated by LPS for 48 h to acquire the mature phenotype. As shown in Fig. 2B, mDCs showed high levels of expression of the following surface markers: CD80 (98.6%); CD86 (96.4%); MHC-II (94.7%); and CD11c (77.6%). However, MSC-conditioned DCs showed decreased levels of expression of the following surface markers, suggesting a significant impairment of DCs maturation (Fig. 2C): CD80 (77.7%), CD86 (68.5%);, MHC-II (76.6%), and CD11c (42.7%). When we utilized MSC transfected with siRNA for TSG-6 (Supplement Fig. 2) in the co-culture with DC, we detected a partial restoration of the four surface markers (CD80 86.1%, CD86 83.7%, MHC-II 82.6%, and CD11c 52.6%; Fig. 2D) expression compared to DC in the presence of wild-type MSCs.

3.4. DCs co-cultured with MSCs reduce IL-12 expression and secretion

The significant inhibition of IL-12 expression by DCs activated by LPS in the presence of MSCs was confirmed by QRT-PCR. As shown in Fig. 3A, co-culturing DCs with MSCs reduced the expression of IL-12 mRNA within DCs post-stimulation ($p < 0.01$). To further examine whether or not MSCs could affect IL-12 secretion in DCs, the supernatants of DCs in the presence or absence of MSCs were assayed for IL-12 p70 by ELISA. The production of IL-12 p70 was enhanced in mDCs. MSC exposure reduced the levels of IL-12 p70 in the supernatants of LPS-activated DCs compared with DCs cultured alone ($p < 0.01$; Fig. 3B). However, silencing of

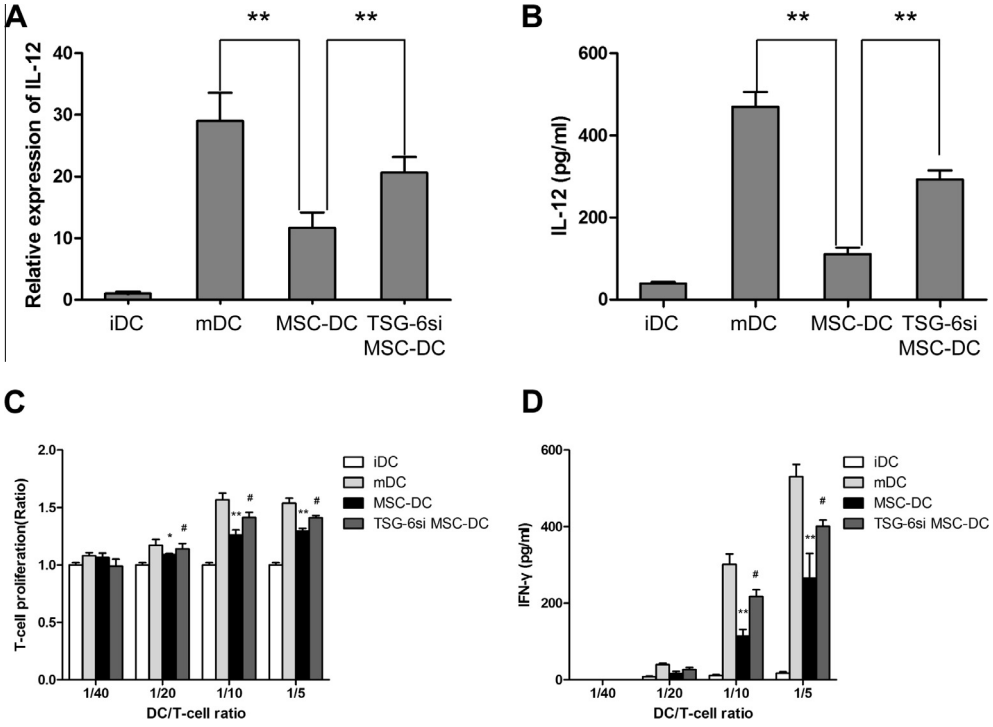

Fig. 3. MSCs inhibit the DCs functions depend on TSG-6. (A) The expression of IL-12 mRNA in DCs was confirmed by QRT-PCR 24 h. (B) IL-12p70 production was measured in culture supernatant of DCs cultured alone or with MSCs after 48 h stimulation with LPS. (C) In MLR experiments, T-cell proliferation was determined by using CCK-8 assay. (D) The production of IFN-γ by T cells was analyzed by ELISA. Data were expressed as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ (mDCs group versus MSC-DC group), $^{\#}p < 0.05$ (TSG-6si MSC-DC group versus MSC-DC group).

TSG-6 within MSCs attenuate the inhibitory effect of MSCs on reducing LPS-induced DCs activation and resulted in a striking increased production of IL-12 compared with wild type MSCs ($p < 0.01$; Fig. 3A and B).

3.5. DCs co-cultured with MSCs reduce T-cell activation

The inhibitory activity exerted by MSCs on DC maturation and function was further analyzed in mixed lymphocyte reactions (MLRs). We designated DCs without LPS treatment as the control group. As shown in Fig. 3C, DCs cultured in the presence of MSCs exhibited a clearly impaired capability of stimulating T-cell proliferation at DC-to-T-cell ratios of 1:20, ($p < 0.05$) 1:10 ($p < 0.01$), and 1:5 ($p < 0.01$) compared with the mDC group. There was no significant difference at a DC-to-T-cell ratio of 1:40. In addition, T cells stimulated by DCs co-cultured with MSCs secreted lower levels of IFN-γ compared with mDCs at DC-to-T-cell ratios of 1:10 ($p < 0.01$) and 1:5 ($p < 0.01$). There was no significant difference at a DC-to-T-cell ratio of 1:20. It should be noted that at a DC-to-T-cell ratio of 1:40, T-cells did not produce detectable IFN-γ (Fig. 3D). In accordance with the above results, MSCs transfected with TSG-6 siRNA had less impact on inhibiting DCs induced T-cell proliferation and IFN-γ secretion (Fig. 3C and D).

3.6. TSG-6 inhibit MAPKs and NF-κB signaling during LPS-induced DC maturation

To determine whether or not TSG-6 affect DC maturation by affecting MAPKs intracellular signaling pathways, phosphorylation of JNK, p38 and ERK in DCs were determined by Western blot analysis. The results reported in Fig. 4 show that LPS-induced phosphorylation of JNK (Fig. 4A), p38 (Fig. 4B) and ERK p38 (Fig. 4C) was significantly reduced in the TSG-6-DC group ($p < 0.01$).

DC maturation is induced by LPS through activation of NF-κB signaling [22]. To determine whether or not TSG-6 affect DC maturation through inactivation of NF-κB signaling, the phosphorylation of NF-κB and the translocation of p65 to the nucleus in DCs were measured. As shown in Fig. 4E and F, TSG-6 reduced phosphorylation of NF-κB (Fig. 4E) and nuclear translocation of p65 (Fig. 4F).

LPS stimulation increased phospho-Akt levels in DCs (Fig. 4D). However, difference in LPS-induced phosphorylation of Akt in

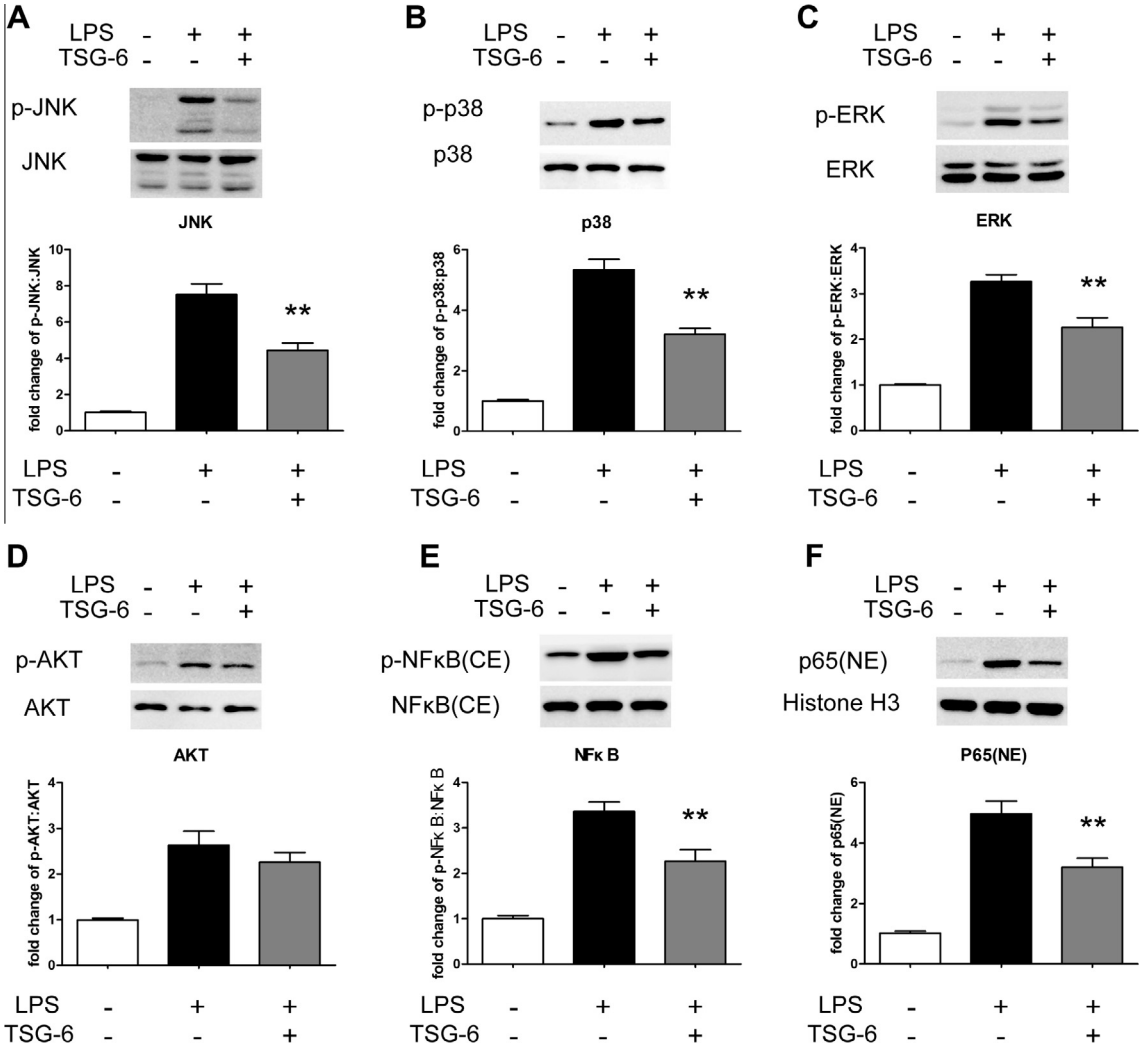

Fig. 4. TSG-6 inhibit MAPKs and NF-κB signaling in DCs. For MAPKs, AKT and NF-κB assays activation assays, expression of phosphorylated and total JNK (A), p38 (B), ERK (C), AKT (D) NF-κB (E) and the nucleus translocation of p65(F), within DCs were determined by Western blotting. Each result above was from one representative experiment out of three experiments. Bar graphs in (A–F): A densitometric analysis was performed. Histograms showed the fold change of the ratio between the activated phosphorylated JNK, p38, ERK, AKT, NF-κB and total JNK, p38, ERK, AKT, NF-κB in DCs; The P65 level in nucleus was normalized to histone H3. The data were shown as means ± SD, the expression was normalized to controls. $^{**}p < 0.01$ (LPS + DCs group versus TSG-6 + LPS + DCs group).

DCs between TSG-6 treated and untreated was less remarkable when compared with those of MAPK and NF-κB.

4. Discussion

In the present study we investigated the mechanisms responsible for MSC-mediated inhibition of DC maturation and function *in vitro*. MSCs mediate a potent inhibition on DC maturation and function such as decreasing expression of DC maturation surface markers and reducing IL-12 production and its capability of stimulating T-cell responses in MLRs. What's more, the inhibitory effect is possibly through TSG-6.

TSG-6 is an anti-inflammation protein with multiple immune-modulating effects and the expression of TSG-6 in MSCs is increased when induced by proinflammatory cytokines such as TNF-α and IL-1 [17]. In this study, we sought to determine whether TSG-6 is involved in MSC-mediated immunosuppressive effects on DCs and the possible underlying mechanisms.

First, we found that the production of TSG-6 by MSCs co-cultured with DC stimulated by LPS was significantly increased compared with monocultures of MSCs. Then we found that the expression of mature phenotypes (CD80, CD86, MHC-II, and CD11c) on DCs was significantly decreased when DCs were co-cultured with MSCs compared with DCs cultured alone. Because DC maturation was evaluated not only on the basis of the surface expression of the maturation phenotype, but also according to the acquisition of typical DC functions, such as the production of IL-12 and the ability to trigger T cell responses in MLRs [16]. Therefore, we further analyzed the level of expression of IL-12 mRNA in DCs by QRT-PCR and IL-12 p70 in the supernatants by ELISA and T-cell activation in MLRs. Our results showed that MSCs can affect DCs functions, leading to a decreased production of IL-12 and T cell activation. In contrast, knock down of TSG-6 by siRNA attenuate the inhibitory effect of MSCs on DCs maturation compared with wide-type MSCs. The production of IL-12 by DCs and T-cell activation in MLR were obviously higher in TSG-6-siRNA MSCs treated group than those in MSCs treated group. The data obtained in experiments using TSG-6 siRNA demonstrate that TSG-6 might play an important role in inhibiting DC maturation.

To elucidate the molecular mechanism underlying TSG-6-mediated inhibition of DC maturation, we next examined the activation of JNK, p38, ERK of MAPKs, AKT and NF-κB signal transduction pathways in DCs. The MAPKs signal pathway plays a crucial role in regulating various cellular responses, including cell differentiation, proliferation, and survival, and previous studies have demonstrated that the three members of the MAPK superfamily JNK, p38, and ERK play an important role in DC maturation and IL-12 secretion [25]. The NF-κB pathway is an important transcription factor that regulates many genes with key roles in DC maturation and immune response induced by LPS [22], inhibition of these pathways are likely to suppress DC maturation and function. Our results revealed a significant reduction in the levels of phosphorylation of JNK, p38, ERK, NF-κB and the translocation of p65 to the nucleus in DCs in the presence of MSCs. AKT pathways also known to be involved in DCs maturation, however, our data showed that TSG-6 has no obvious influence on AKT pathway. In short, our results suggest that the inhibitory effects of TSG-6 may be partly explained by the inactivation of the MAPKs, and NF-κB intracellular signaling pathways within DCs, leading to reduced transcriptional expression of IL-12 and other functionally important immune molecules, and thus suppressing DC maturation and function.

DCs play a central role in controlling immunity and the induction of T cell-mediated immune diseases, such as GVHD, allergies, and autoimmune diseases [26]. Immature DCs also serve to promote tolerance induction [27]. It is very important to apply MSCs to reduce the incidence and degree of GVHD and other immune-related diseases due to the unique immunosuppressive properties of MSCs.

The first limitation of our study was that many soluble factors have been shown to be related to the inhibitory effect of MSCs, such as PGE2, IDO, IL-10, and TGF-β, and the results presented here do not rule out the effects of other immunomodulatory factors. Which cytokines are the most important to induce this inhibitory mechanism of MSCs on DCs deserves further studies. In addition, our study was only carried out *in vitro*. Further research needs to be performed *in vivo* to verify our results.

In summary, we have investigated the inhibitory mechanism of MSC activity on DCs. The results of this study offer new insight into the mechanisms responsible for MSC-mediated inhibition of DC maturation and function. These findings may promote the future clinical application of MSCs in immune-related diseases, although there are many issues to be solved. The clinical application of MSCs for the treatment of immune-related diseases deserves further research.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.001.

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