

Soluble mediators from mesenchymal stem cells suppress T cell proliferation by inducing IL-10

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Abbreviations: B6, C57BL/6; CS, culture supernatant; DCs, dendritic cells; IDO, indoleamine 2,3-dioxygenase; MLR, mixed lymphocyte reaction; MSC, mesenchymal stem cell; 1-MT, 1-methyl-DL-tryptophan; sp, splenocyte

Abstract

Mesenchymal stem cells (MSCs) can inhibit T cell proliferation; however, the underlying mechanisms are not clear. In this study, we investigated the mechanisms of the immunoregulatory activity of MSCs on T cells. Irradiated MSCs co-cultured with either naïve or pre-activated T cells in a mixed lymphocyte reaction (MLR) significantly suppressed T cell proliferation in a dose-dependent manner, irrespective of allogeneic disparity between responders and MSCs. Transwell assays revealed that the suppressive effect was primarily mediated by soluble factors that induced apoptosis. Splenocytes stimulated with alloantigen in the presence of the MSC culture supernatant (CS) produced a significant amount of IL-10, which was attributed to an increase in the number of IL-10 secreting cells, confirmed by an ELISPOT assay. The blockade of IL-10 and IL-10 receptor interaction by anti-IL-10 or anti-IL-10-receptor antibodies abrogated the suppressive capacity of MSC CS, indicating that IL-10 plays a major role in the suppression of T cell proliferation. The addition of 1-methyl-DL-tryptophan

(1-MT), an indoleamine 2,3-dioxygenase (IDO) inhibitor, also restored the proliferative capacity of T cells. In conclusion, we demonstrated that soluble mediators from culture supernatant of MSCs could suppress the proliferation of both naïve and pre-activated T cells in which IL-10 and IDO play important roles.

Keywords: apoptosis; immunosuppression; indoleamine-pyrrole 2,3-dioxygenase; interleukin-10; mesenchymal stem cells; T-lymphocytes

Introduction

Mesenchymal stem cells (MSCs) are multipotential non-hematopoietic cells (Wakitani *et al.*, 1995; Pittenger *et al.*, 1999) that can not only expand *ex vivo* without any particular changes in phenotype and function (Reyes *et al.*, 2001) but also differentiate *in vitro* into several mesenchymal cell lineages such as adipocytes, chondrocytes, osteocytes, myocytes, astrocytes and neurons (Krampera *et al.*, 2003; Kato *et al.*, 2004; Glennie *et al.*, 2005; Zappia *et al.*, 2005; Nauta *et al.*, 2006; Stagg *et al.*, 2006). Another important characteristic of MSCs is their immunosuppressive effect on several immune cells, including dendritic cells (DCs) (Jiang *et al.*, 2005), B lymphocytes (Corcione *et al.*, 2006) and T lymphocytes (Di Nicola *et al.*, 2002; Krampera *et al.*, 2003; Tse *et al.*, 2003; Groh *et al.*, 2005; Rasmusson *et al.*, 2005). This makes MSCs attractive candidates for applications in regenerative medicine. Several recent studies have suggested that allotransplantation of MSCs may be feasible in clinical application such as metachromatic leukodystrophy and graft-versus-host disease (Frumento *et al.*, 2002). Aggarwal and Pittenger (2005) showed that MSCs altered the phenotype of specific immune cells, providing evidence that transplantation of allogeneic MSCs could be clinically applicable. Despite all of this evidence for the therapeutic potential of MSCs, in order for MSCs to be accepted broadly as a therapeutic modality, the exact molecular mechanisms underlying their suppressive ability should be more fully understood. At present, these mechanisms remain unclear. Therefore, it is critical to identify the precise mechanisms by which MSCs exert their suppressive capabilities. Evidence from *in vitro* stimulation tests has demonstrated that both cell-cell contact and

soluble factors contribute to the immunomodulatory function of MSCs. There are confounding data, however, with regard to the mechanisms involved. As for the soluble factors, indoleamine 2,3-dioxygenase (IDO) (Meisel *et al.*, 2004), prostaglandin E2 (PGE₂) (Aggarwal and Pittenger, 2005), TGF- β and nitric oxide (Sato *et al.*, 2007; Ren *et al.*, 2008) have all been reported to be involved. Furthermore, MSCs induce cell division arrest and apoptosis of activated T cells (Glennie *et al.*, 2005; Plumas *et al.*, 2005; Zappia *et al.*, 2005), but the mechanisms behind the cell division arrest and apoptosis are not clearly defined.

To understand the precise mechanisms by which the MSCs exert their immunomodulatory activity, we investigated the effect of MSCs on T cell activation, including proliferation, expansion and cytokine secretion, and tried to define the extent of the contribution of each factor involved by using blocking studies. We observed that MSCs effectively suppressed the proliferation of alloreactive T cells in mixed lymphocyte reaction (MLR). Transwell experiments revealed that the suppressive capacity of MSCs could be attributed to soluble mediators, which was confirmed by showing that MSC culture supernatant (CS) had the same suppressive activity as the MSCs themselves. Splenocytes activated in the presence of MSC CS produced a significantly higher amount of IL-10; additionally, a blockade of IL-10/IL-10R interaction by antibodies or of IDO activity by 1-MT abrogated the suppressive capacity of MSC CS.

Results

MSCs effectively suppressed not only naïve but also pre-activated T cell proliferation irrespective of the allogeneic disparity between T cells and MSCs

To investigate the ability of MSCs to suppress T cell proliferation, different doses of MSCs were added to MLR with a ratio of splenocyte to MSCs of 2,000:1, 200:1 or 20:1. Both BALB/c and C57BL/6 MSCs effectively inhibited the proliferation of both allogeneic and syngeneic T cells to a similar degree at 72 h after stimulation. Maximum inhibition was achieved with the 20:1 ratio; at 24 h after stimulation, however, the suppressive effect was not evident for the 200:1 and 2,000:1 ratios (Figure 1A). MSCs could suppress pre-activated T cells since the addition of MSCs at 36 h after the initiation of the MLR showed a comparable inhibitory effect to the addition of MSCs at the start of the MLR (Figure 1B). This indicated that MSCs exerted their inhibitory effects not only on naïve T cells but also on pre-activated T cells.

Soluble factors in culture supernatant of MSCs suppressed T cell proliferation in MLR

To determine whether the suppression of T cell proliferation required direct cellular contact by MSCs, splenocytes were separated from MSCs in a transwell chamber and activated by MLR. While MLR was occurring in the lower chamber, BALB/c MSCs were cultured in the upper chamber. In this system, soluble elements produced from MSCs in the upper chamber were free to pass the transmembrane while the cells were not allowed to contact each other. As shown in Figure 2A, BALB/c MSC suppressed T cell proliferation indicating that MSC suppression of T cell proliferation was not dependent on cell-to-cell contact but was caused by soluble factors. In order to confirm the ability of soluble factors from MSCs to suppress T cell proliferation, 72 h culture supernatant was added to the MLR at a 1:1 ratio. MLR CS and BALB/c fibroblast CS were used as controls. As shown in Figure 2B, significant suppression of T cell proliferation was observed only in the group with MSC CS, suggesting that soluble factors contained in the culture supernatant of MSCs could suppress T cell proliferation.

MSC culture supernatant caused apoptosis of proliferated splenocytes rather than cell cycle arrest

To determine whether the suppression of T cell proliferation by MSCs or MSC CS was due to the induction of cell death or cell cycle arrest, we examined whether MSC CS could delay the cell cycle or trigger apoptosis of splenocytes by estimating values of each cell-cycle phase. After 72 h incubation of MSC CS and T cells in splenocytes stimulated with anti-CD3 and anti-CD28 antibodies, the apoptotic population of splenocytes was increased more than twice, and MSC CS could not interrupt or delay the entry of them into the cell cycle. In contrast, MSC treatment instead of MSC CS caused almost all T cells to remain in the G0/G1 phase and also induced apoptosis of T cells (Figure 3A). In further experiments, MSCs were added to proliferating T cells among the splenocytes stimulated with anti-CD3 and anti-CD28 antibodies, and the level of apoptosis was analyzed by flow cytometry with annexin V and SYTOX green staining to confirm MSC-mediated apoptosis. Annexin V binds phosphatidylserine, which is released from the outer cell membrane early in the process of apoptosis (Koopman *et al.*, 1994), and SYTOX green dye cannot permeate live cells but stains apoptotic cells with intense green fluorescence by binding to cellular nucleic acids. Annexin V single positive and SYTOX green

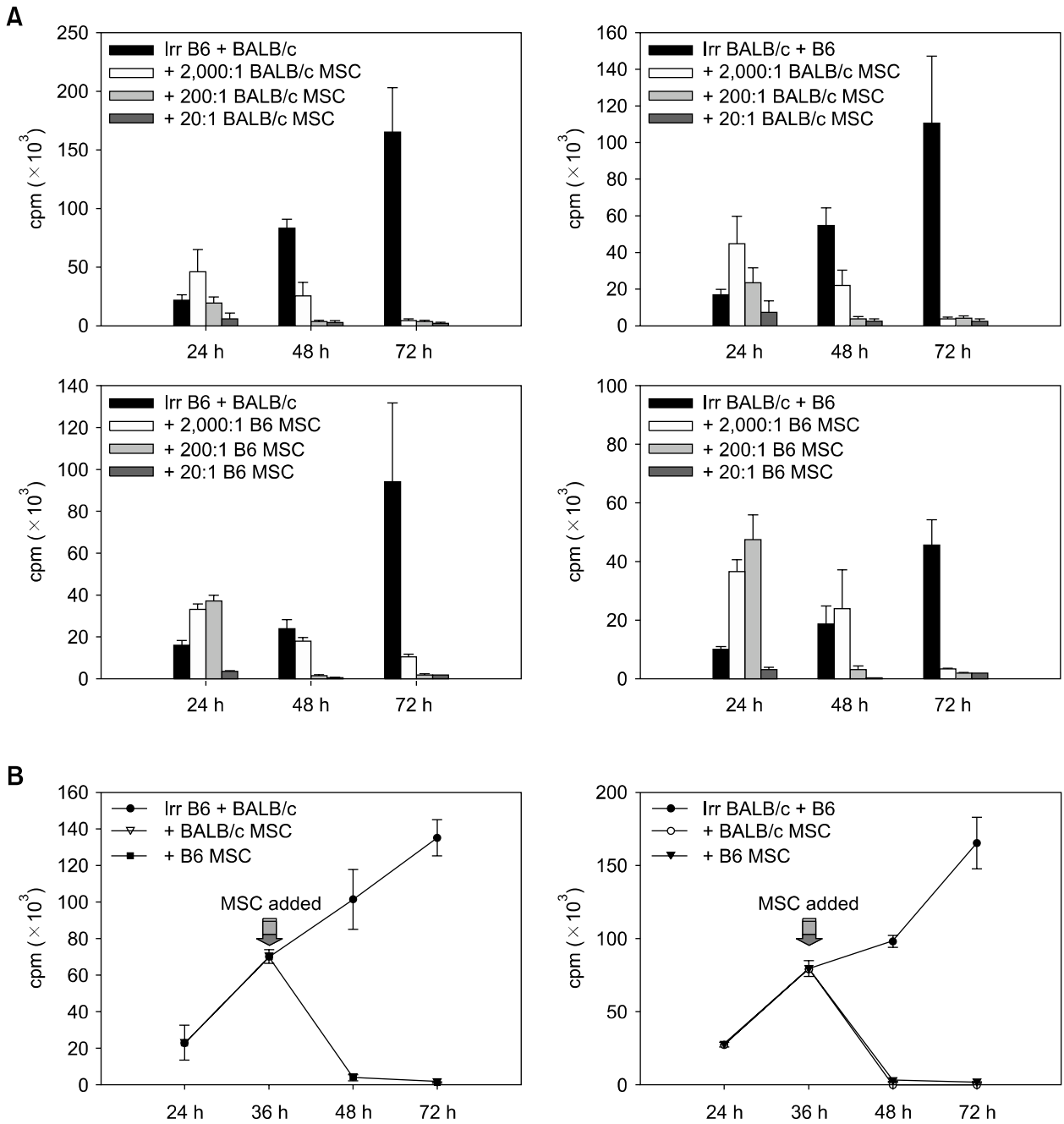


Figure 1. MSCs suppressed both naïve and pre-activated T cell proliferation in a time and dose-dependent manner. 5×10^5 BALB/c splenocytes and 5×10^5 C57BL/6 splenocytes were used as either stimulators or responders in MLR. (A) BALB/c or C57BL/6 MSCs were added to MLR at the start of the reaction, and the ratios of splenocytes to MSCs were 2,000:1, 200:1 or 20:1. The culture periods were 24 h, 48 h and 72 h. (B) BALB/c or C57BL/6 MSCs were added to the MLR at 36 h after the start of the reaction. The ratio of splenocytes to MSCs was 20:1. The culture continued for 24 h, 36 h, 48 h or 72 h. The results are expressed as the mean counts per minute (cpm) \pm SD for three independent experiments.

and annexin V double positive cells indicated early and accumulated apoptosis, respectively. Almost 94 percent of the splenocytes (early apoptosis: 15.7%, accumulated apoptosis: 78.2%) underwent apoptosis when they were co-cultured with MSCs

for 72 h. In contrast, cells cultured without MSCs (early apoptosis: 24.7%, accumulated apoptosis: 19.3%) showed a much lower percentage of cells undergoing apoptosis (activation-induced cell death was present even without any stimulation)

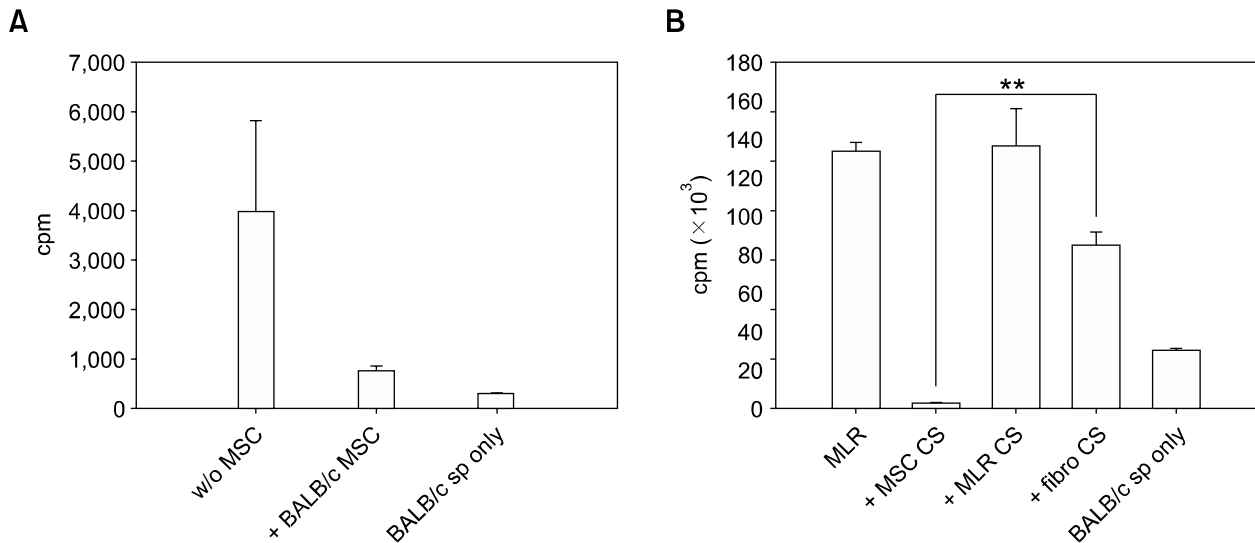


Figure 2. Suppression of T cells by MSCs was not dependent on cell-cell contact but was evoked by soluble factors. 5×10^5 BALB/c splenocytes were stimulated with 5×10^5 γ -irradiated C57BL/6 splenocytes in MLR for 72 h. (A) The MLR took place on the bottom of a transwell, and MSCs were added to the indicated upper wells (splenocytes/MSCs ratio was 20:1). (B) Supernatant from day 3 cultures of BALB/c MSCs was added to the MLR at 0 h. MLR culture supernatant and BALB/c fibroblast culture supernatant were used as controls. MLR culture supernatant was obtained from 2×10^6 BALB/c splenocytes co-cultured with 2×10^6 γ -irradiated C57BL/6 splenocytes in 25 cm² tissue culture flasks for 14 days. BALB/c fibroblast culture supernatant was collected from primary BALB/c fibroblast cells on a 100 mm plate fully cultured for 3 days. The volume of each culture supernatant added was 50% that of MLR. Results are expressed as mean values (\pm SD) of cpm obtained in triplicate cultures. ** $P < 0.001$.

(Figure 3B).

MSC culture supernatant stimulated splenocytes to secrete a significant amount of IL-10

To evaluate the effect of MSC CS on IL-10 secretion in splenocytes, the profile of IL-10 secretion in splenocytes stimulated in the presence of MSC CS was analyzed by an ELISPOT assay. BALB/c splenocytes stimulated with irradiated C57BL/6 splenocytes were cultivated on the ELISPOT plate with BALB/c MSC CS for 48 h (Figure 4A). For the controls, BALB/c fibroblast CS was used. The number of IL-10 secreting cells was only changed by the MSC CS treatment. In addition, no IL-10 was detected in MSC CS, whereas little IL-10 was present in MLR CS (Figure 4B). Because IL-10 is a well known inhibitor of T cell proliferation and activation, we next investigated whether T cell proliferation recovered when MSC CS was used in MLR with blocking antibodies for either IL-10 or IL-10 receptor. As shown in Figure 4C, T cell proliferation was significantly restored in MSC CS with each antibody, but this phenomenon was not observed in treatment with fibroblasts. These findings suggested that MSC CS drove splenocytes to produce IL-10. Moreover, MSC CS-mediated IL-10 production allowed the proliferation of T cells to be suppressed by a paracrine effect.

IDO relation to MSC culture supernatant to suppress T cell

IDO cleaves tryptophan, an essential amino acid for T cell growth, and converts it to kynurenine (Mellor and Munn, 1999). 1-methyl-tryptophan (1-MT) interferes with IDO activity as a competitive inhibitor (Bodaghi *et al.*, 1999). To examine whether indoleamine-2,3-dioxygenase (IDO) was related to T cell suppression by MSC CS, 1-MT was added to MLR with MSC CS. The proliferation of T cells in the group treated with MSC CS and 1-MT was almost completely recovered (Figure 5). According to this result, we can state that IDO is sufficient to suppress T cell activity mediated by MSC CS.

Discussion

Recently, MSCs have been actively studied with a view toward tissue regeneration and organ transplantation due to their ability to differentiate into several tissues and their potential to regulate many immune cells (Aggarwal and Pittenger, 2005; Glennie *et al.*, 2005; Groh *et al.*, 2005; Jiang *et al.*, 2005; Plumas *et al.*, 2005; Poggi *et al.*, 2005; Rasmusson *et al.*, 2005; Sordi *et al.*, 2005; Zappia *et al.*, 2005; Corcione *et al.*, 2006; Nauta *et al.*, 2006; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2006; Stagg *et al.*, 2006; Taylor *et al.*, 2006). It has

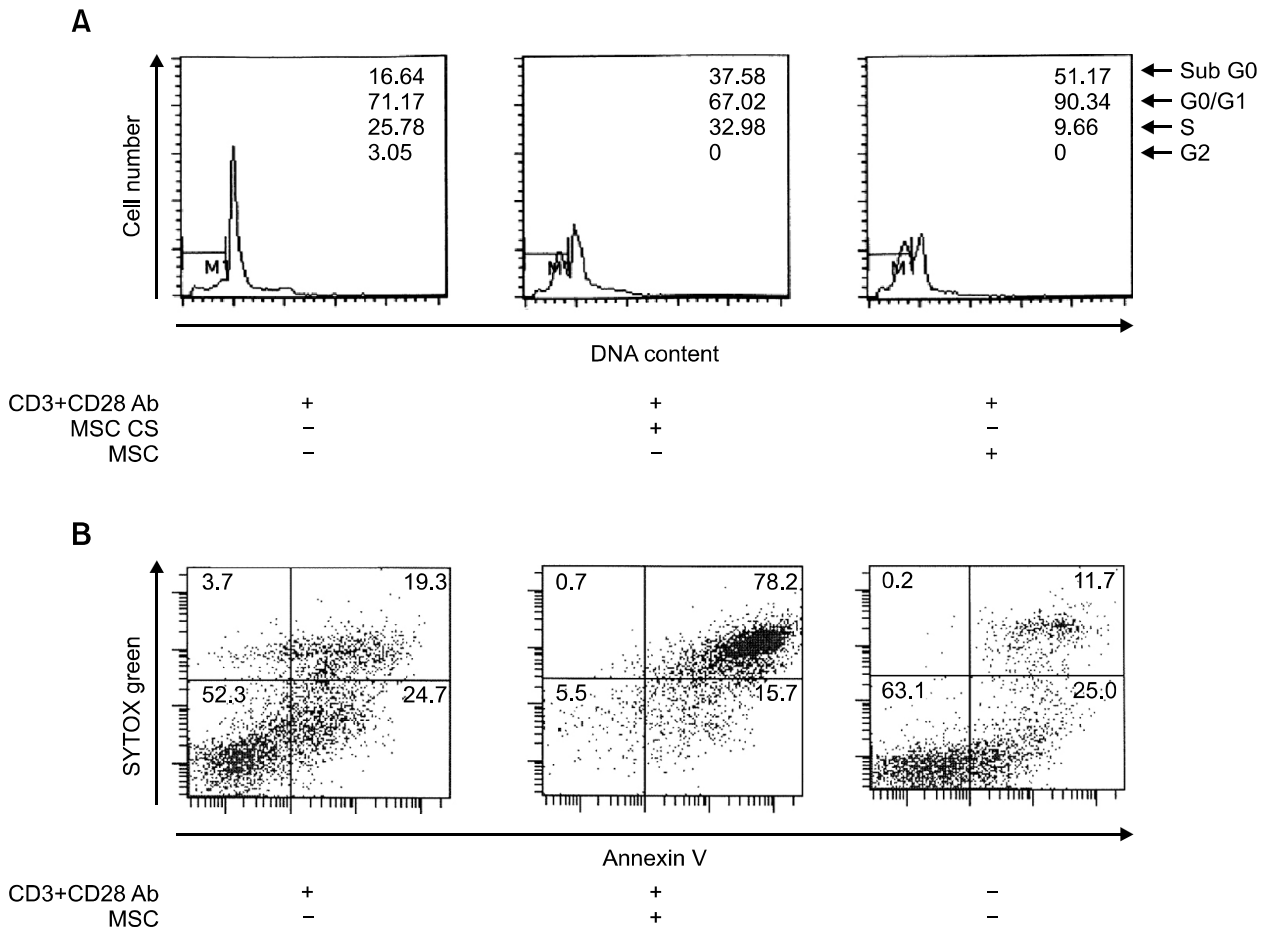


Figure 3. MSC culture supernatant caused apoptosis of splenocytes rather than cell cycle arrest. 5×10^5 BALB/c splenocytes were stimulated with anti-CD3 (3 μ g/ml) and CD28 (1 μ g/ml) antibodies for 72 h. (A) 100 μ l MSC culture supernatant or 2.5×10^4 MSCs were treated in the indicated groups. DNA content was assessed using PI staining. (B) 2.5×10^4 MSCs were added to the proper group. Apoptotic cells were stained with annexin V (PE) and SYTOX[®] green. These assays were analyzed using flow cytometry, and MSCs were excluded from the analysis by positive gating of CD45. One representative of three separate experiments is shown.

been well known that MSCs inhibit T cell proliferation (Di Nicola *et al.*, 2002; Krampera *et al.*, 2003; Potian *et al.*, 2003; Tse *et al.*, 2003; Meisel *et al.*, 2004; Aggarwal and Pittenger, 2005; Groh *et al.*, 2005; Rasmusson *et al.*, 2005), in addition to modulating DC functions, altering antigen-presenting cell maturation and interacting with NK cells (Beyth *et al.*, 2005; Jiang *et al.*, 2005; Poggi *et al.*, 2005; Sotiropoulou *et al.*, 2006; Spaggiari *et al.*, 2006). Several studies have revealed that immunomodulatory effects of MSCs are mediated by cell-cell contact and soluble factors such as prostaglandin E2, IDO and TGF- β (Di Nicola *et al.*, 2002; Krampera *et al.*, 2003; Potian *et al.*, 2003; Tse *et al.*, 2003; Meisel *et al.*, 2004; Aggarwal and Pittenger, 2005; Groh *et al.*, 2005; Rasmusson *et al.*, 2005). All of these mechanisms act directly on T cells, which we assume would not be enough to explain the bystander effect of MSCs. It is still

unclear where these soluble factors come from and what causes them to be secreted.

In this report, we showed the following. (1) Murine MSCs inhibited the proliferation of either syngeneic or allogeneic T cells, irrespective of their activation status, i.e., unprimed and already activated effector T cells. (2) These effects were caused by not only MSCs but also MSC CS. (3) There was no requirement for direct MSC-T cell contact. (4) MSC CS induced apoptosis of splenocytes including T cells rather than cell-cycle arrest, and (5) it caused splenocytes to secrete cytokines, especially IL-10. (6) Neutralization of IL-10 and IL-10 receptor moderately restored the proliferation of T cells. (7) MSC culture supernatant also induced IDO in the MLR, which caused suppression of T cells. The IDO inhibitor 1-MT was able to almost completely reverse this suppression of T cells.

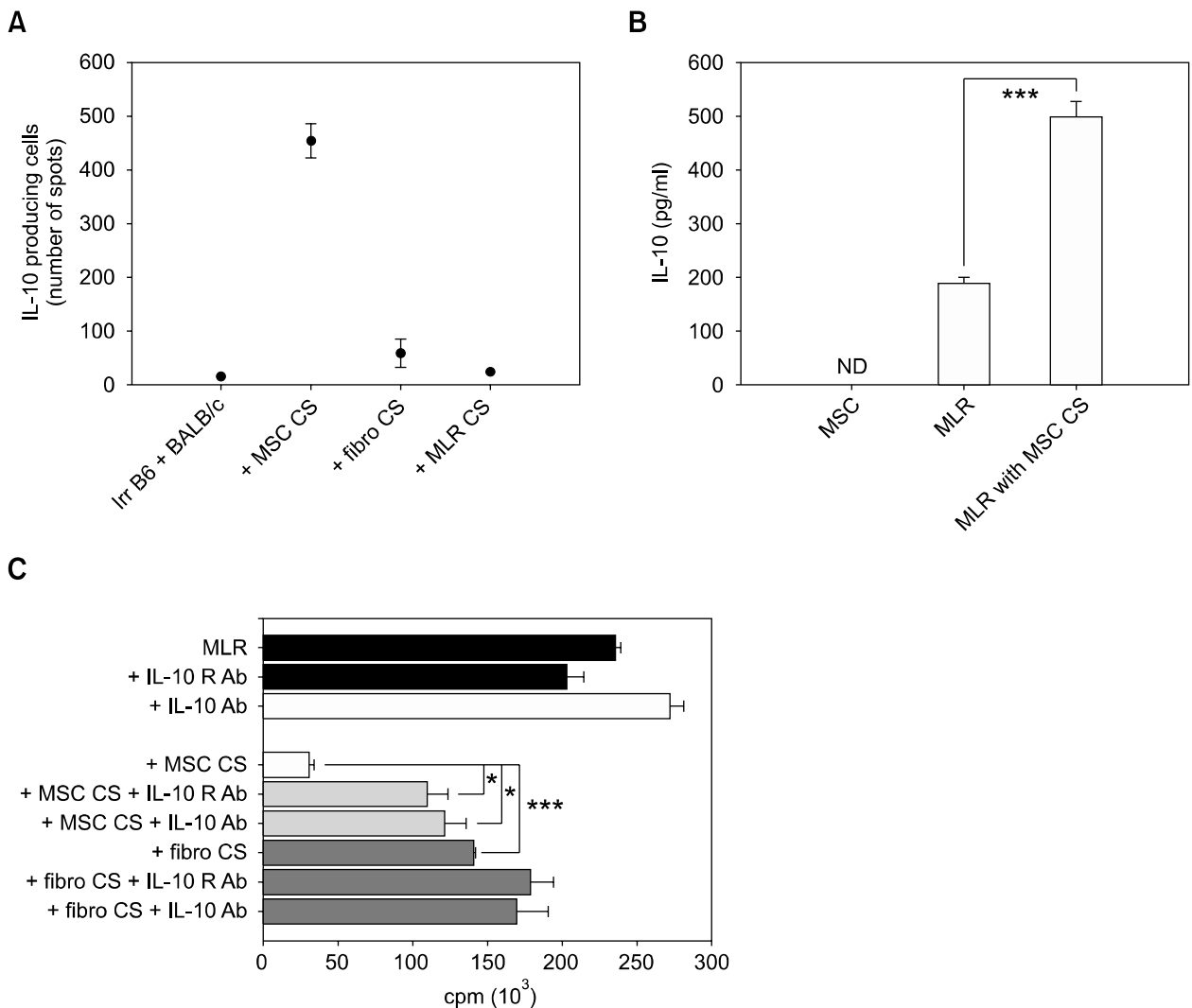


Figure 4. Soluble factors from MSCs affected IL-10 secretion of splenocytes. (A) 5×10^5 BALB/c and 5×10^5 γ -irradiated C57BL/6 splenocytes were used as responder and stimulator cells, respectively, in MLR for 72 h. (A) ELISPOT assays were performed with or without culture supernatant from BALB/c MSCs. BALB/c fibroblast culture supernatant and MLR culture supernatant were used as controls. 100 μ l of each supernatant was added per well. (B) The concentration of IL-10 in MLR culture supernatant, MSC culture supernatant or MLR with MSC CS was measured by ELISA. $***P < 0.0001$. ('ND' means not detected) (C) Blocking of IL-10 and IL-10 receptor interaction was performed in the absence or presence of MSC culture supernatant, BALB/c fibroblast culture supernatant or MLR culture supernatant. A neutralizing IL-10 or IL-10 receptor Ab (5 μ g/ml, respectively) was used in the indicated groups. The results shown are expressed as mean \pm SD from three independent experiments. $*P < 0.05$, $***P < 0.0001$.

In Figure 1A, MSCs effectively suppressed T cell activation when different doses of MSCs (2000:1, 200:1 and 20:1) were used in the MLR at different times, and even when the T cells were allogeneic with the MSCs. The immunomodulatory effect of MSCs on T cells, however, was not evident except for the 20:1 ratio. It seems that few MSCs activated T cell proliferation rather than suppression. MSCs themselves were γ -irradiated with 60cGy to blunt MSC proliferation, and the cpm value of MSC culture alone was similar to that of non-stimulated responder cell culture. Moreover, the phenomenon of early activation of T cells by few MSCs did not

occur when MSC CS was used in the MLR instead of MSCs at 24 h and 48 h (data not shown). Therefore, it appears that MSCs interacted with T cells at early time points, causing activation of the T cells. The results of experiments without direct cell contact, however, emphasize that T cell contact with MSCs is not necessary to inhibit T cell proliferation. It should be considered, though, whether contact between T cells and MSCs is fully dispensable.

Although MSCs have been known to induce apoptosis (Plumas *et al.*, 2005), whether apoptosis is induced by MSC CS or not remains unclear. In

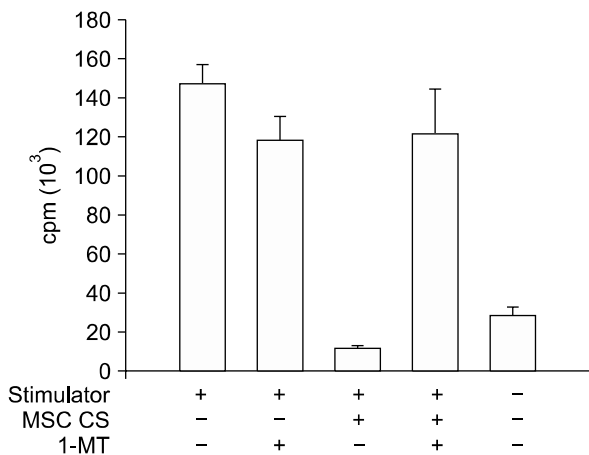


Figure 5. IDO is related to the immunomodulatory effect of MSC culture supernatant on T cells. MLR was done with 5×10^5 splenocytes of BALB/c or C57BL/6 as responder or γ -irradiated stimulator, respectively, for 72 h. In the indicated groups, 1 mM 1-MT was used with or without 100 μ l MSC CS in appropriate wells. T cell proliferation was measured by thymidine uptake by T cells. The results shown are expressed as mean \pm SD from three independent experiments.

this study, we showed that MSC CS induced cell cycle arrest independent of cellular apoptosis, while MSCs themselves induced splenocyte apoptosis, some of which was cell-cycle arrest dependent. Therefore, we can say that T cell cycle arrest requires cell-cell contact, while induction of cellular apoptosis demands not only cell contact but also soluble factors.

In an ELISPOT assay, IL-10 was secreted from splenocytes and not from MSCs, indicating that T cells might be influenced by paracrine effect. Moreover, downregulation of T cell proliferation by MSC CS was limited when IL-10 or IL-10 receptor was intercepted by neutralizing antibodies. It is well known that IL-10 is a suppressive cytokine for proliferation and cytokine production of T cells, and it plays a key role in inducing T cell anergy. Antigen-specific T cell suppression by IL-10 is essential in peripheral tolerance to allergens, autoantigens, transplantation antigens and tumor antigens (Taylor *et al.*, 2006). Previous studies showed that IL-10 led to T cell apoptosis (Georgescu *et al.*, 1997; Ayala *et al.*, 2001; Oshima *et al.*, 2002), and it appears that IL-10 from T cells influenced by MSC CS caused T cell apoptosis. It needs to be further investigated whether the increase of IL-10 is necessary and sufficient to suppress the T cell proliferation and induce apoptosis.

IDO is known to induce T cell suppression by MSCs (Meisel *et al.*, 2004; Ryan *et al.*, 2007). However, the involvement of IDO in MSC CS mediated T cells suppression has not been reported.

Our results showed almost complete restoration of T cell proliferation by adding 1-MT; therefore, IDO seems to be one of the factors related to the immunomodulatory effects of MSC CS. T cell proliferation is thought to be suppressed by a reduced amount of tryptophan, while apoptosis is induced by an increased level of kynurenine, a metabolite formed from tryptophan (Frumento *et al.*, 2002; Terness *et al.*, 2002). Tryptophan is an essential factor for T cell proliferation, while kynurenine blocks Ag-driven specific T cell proliferation, possibly leading to T cell death (Meisel *et al.*, 2004). It has been reported that IL-10 secreted by regulatory T cells induced IDO secretion from APCs in a T cell proliferation niche by MLR (Finger and Bluestone, 2002). In our study, we observed IL-10 and IDO in MSC CS play an important role in suppression of T cell proliferation independently as shown in Figure 4 and 5. However, the addition of both antiIL-10 Ab and 1-MT at the same time did not show the additive effect in restoration of suppressive effect by MSC CS (data not shown) suggesting that IL-10 secreted by stimulated splenocytes might induce the production of IDO from APCs contained in the splenocytes. This should be further confirmed by showing dose-effect response between IL-10 and IDO in splenocytes. The results described thus far show that soluble factors from MSCs induced IL-10 production by splenocytes, which in turn suppressed the proliferation of the T cells themselves. We postulate that this underlies the bystander immunomodulatory effect exerted by MSCs *in vivo*.

Methods

Isolation and characterization of murine MSCs

C57BL/6 (H2-K^b) mice were purchased from Charles River Laboratories. MSCs were collected from bone marrow. Femurs and tibias were flushed with PBS containing 150 μ g/ml gentamycin. The cells obtained were cultured in 100 mm cell culture plates with high-glucose DMEM (Gibco Laboratories, Grand Island, NY), which was supplemented with heat-inactivated 10% FBS, 5% horse serum, 50 μ g/ml gentamycin, 2 mM L-glutamine, 100 μ M non-essential amino acids, 10 mM HEPES and 55 μ M 2-mercaptoethanol. Cells were incubated at a concentration of 10^6 /ml at 37°C in a 5% CO₂ atmosphere. After 72 h, non-adherent cells were removed. When the plate was ~80% confluent, adherent cells were trypsinized and split. The MSCs of BALB/c mouse origin were obtained from Dr. Elizabeth Simpson (Imperial College Faculty of Medicine, London, UK). The cells were cultivated in complete DMEM-10 (DMEM containing heat-inactivated 10% FBS, 50 μ g/ml gentamycin, 2 mM L-glutamine, 100 μ M non-essential amino acids, 10 mM HEPES and 55 μ M 2-mercapto-

ethanol). Surface antigen expression of the MSCs was confirmed by flow cytometry. 1×10^6 MSCs were incubated at 4°C for 1 h in the following monoclonal antibodies: FITC conjugated anti-CD106, CD11c, H2K^d, I-A^b (BD Pharmingen, San Diego, CA) and CD34 (eBioscience, San Diego, CA); phycoerythrin (PE) conjugated anti-H2K^b and I-A^d (BD Pharmingen, San Diego, CA); biotin conjugated anti-CD14, CD31, and CD45 (eBioscience, San Diego, CA). Cells were washed with PBS containing 1% BSA and 0.1% sodium azide. Biotinylated cells were incubated with PerCP-streptavidin (BD Pharmingen, San Diego, CA) at 4°C for 30 min. The cell suspensions were washed with PBS containing 0.5% BSA and 0.1% sodium azide and then analyzed by FACScan (Becton Dickinson, Heidelberg, Germany) using Cell Quest software. MSCs from both mouse strains were positive for CD106 and negative for CD45, CD14, CD34, CD31, and CD11c. MSCs from the BALB/c strain expressed neither MHC class I nor class II molecules, whereas those from the C57BL/6 strain expressed a modest level of MHC class I molecules with no MHC class II molecules. Osteogenic differentiation was induced by 10 mM β -glycero-phosphate, 50 μ g/ml ascorbic acid 2-phosphate and 10^{-9} M dexamethasone in complete DMEM-10. Cultures for osteogenesis were maintained for 3-4 weeks, and differentiation medium was re-supplied twice a week. Cell differentiation for osteogenesis was identified by culture staining with 2% Alizarin Red S for 20 min at room temperature after fixation with 10% formalin for 20 min. To induce adipogenic differentiation, MSCs were cultured with 10^{-9} M dexamethasone and 5 μ g/ml insulin in complete DMEM-10 for 3-4 weeks, changing the media twice a week. Cultured cells were fixed with 10% formalin for 20 min and incubated with Oil Red O for 1 h. Photographs of the cells were taken with a digital camera (Nikon Infinity) attached to a light microscope (Nikon ECLIPSE TS 100). MSCs from both strains differentiated into adipocytes and osteoblasts.

Preparation of MSC culture supernatants

1×10^5 cells of BALB/c MSCs were plated in a 100 mm plate and cultured for 72 h in complete DMEM-10. When the plate was confluent with about 90% cells, culture supernatant was harvested and centrifuged at $1,200 \times g$ at room temperature for 10 min. The upper aqueous solution was aliquoted in 1.5 ml microtubes and kept in a -70°C deep freezer.

Mixed lymphocyte reactions (MLRs)

BALB/c and C57BL/6 splenocytes were used as either stimulator or responder cells. Splenocytes were isolated by mechanical dissociation using fully frosted slides followed by red blood cell lysis with Red Blood Cell Lysing Buffer (Sigma-Aldrich, St-Louis, MO). Prior to the experiment, stimulator cells were irradiated with 20 Gy of γ -ray to prevent them from reacting against responder cells. Both BALB/c and C57BL/6 MSCs were irradiated with 60 cGy of γ -ray. A co-culture was then made by adding 5×10^5 stimulator and 5×10^5 responder cells per well (1:1) in 96-well round-bottom plates in 0.2 ml complete DMEM-10. 2.5×10^2 , 2.5×10^3 or 2.5×10^4 MSCs were added to

the co-culture at different time intervals, $t=0$ or $t=36$ h. Control cultures did not contain MSCs. In selected MLR experiments, MSCs were substituted with culture supernatant from MSCs. Along with the stimulator/responder population in the MLR, the prepared MSC supernatant mixed with 50% fresh media was added at different time points, $t=0$ and $t=36$ h of the reaction. Proliferation was assessed by 1 μ Ci of [³H]-thymidine (Amersham, Buckinghamshire, UK) incorporation. [³H]-thymidine was added to MLRs at 24 h, 36 h, 48 h or 72 h, and the cells were harvested onto glass fiber filters using a 96-well cell harvester (Inotech, Bertold, Japan) after 18 h of extended culture. All of the T cell proliferation assays were done in triplicate. [³H]-thymidine incorporation was measured by a microbeta TriLux luminescence counter (Perkin Elmer, Wellesley, MA). The results were represented as counts per minute (cpm).

Transwell cultures

A transwell system (1.0 μ m pore size membrane, Corning, Acton, MA) was used to prevent MSCs from contacting the splenocytes. BALB/c splenocytes were used as stimulators, and C57BL/6 splenocytes were used as responders. 5×10^5 stimulator and 5×10^5 responder cells were loaded into the lower chamber of the well, and 2.5×10^4 MSCs were added to the upper chamber at $t=0$ and $t=36$ h of the reaction. After 72 h, the reaction was pulsed with [³H]-thymidine for a further 18 h.

Analysis of cell-cycle arrest and apoptosis

In the cell-cycle arrest assay, the stimulated cells were harvested and stained with an allophycocyanin (APC) conjugated anti-CD45 Ab (BD Pharmingen, San Diego, CA), then fixed with a solution containing a ratio of PBS with 50% FBS to 70% ethanol of 3:7 at 4°C overnight. The next day, the cells were centrifuged and stained with 300 μ l propidium iodide stock solution (50 μ g/ml) for 15 min. After staining, the DNA content of the cells was analyzed by FACSCanto II (Becton Dickinson, Heidelberg, Germany) using FACSDiva software. To assess apoptosis, the Vybrant apoptosis Assay Kit #8 (R-phycoerythrin annexin V/SYTOX green) (Invitrogen, Carlsbad, CA) was used. Briefly, cells were harvested and washed with annexin-binding buffer, then stained with R-phycoerythrin annexin V and SYTOX Green for 30 min on ice. After staining, the cells were analyzed by FACSCanto II using FACSDiva software.

ELISPOT and ELISA for the measurement of IL-10

96-well ELISPOT plates (Millipore, Bedford MA) were coated with anti-IL-10 capture mAb (diluted in sterile PBS) in a volume of 100 μ l with 1:60 dilution and incubated overnight at 4°C. On the day of the experiment, the plates were washed five times with PBS-containing 0.05% Tween-20 (PBST) and blocked for 30 min with complete DMEM-10 at 37°C. Both 5×10^5 C57BL/6 splenocytes (γ -irradiated stimulator cells) and 5×10^5 BALB/c splenocytes (responder cells) in 100 μ l of complete DMEM-10

were cultured with 100 μ l of complete DMEM-10, BALB/c MSC CS or fibroblast CS for 48 h. After the incubation, cells were discarded and the plate was washed five times with PBST. A 1:60 dilution of detection antibody in PBS was added to the plate, which was then incubated for 1 h at 37°C. After washing five times with PBST, streptavidin-alkaline phosphatase (Promega, Madison, WI) was added to each well and incubated for 1 h at 37°C. The plate was then washed four times with PBST and two times with PBS. The developing solution composed of 20 μ l/ml of NPT/BCIP stock solution (Roche, Indianapolis, IN) in alkaline phosphatase substrate buffer (100 mM Tris-Cl pH 9.5, 100 mM NaCl and 5 mM MgCl₂ in distilled water) was added. The resulting spots were counted and analyzed on a computer-assisted AID EliSpot Reader System (AID, Straßberg, Germany). The amount of IL-10 in the MSC or MLR CS was quantified by ELISA (Duoset ELISA development system, R&D, MN) according to the manufacturer's instruction.

Blocking IL-10 and IL-10 receptor interaction and IDO inhibition

BALB/c splenocytes were co-cultured with irradiated C57BL/6 splenocytes in the absence or presence of MSC CS or fibroblast CS, with or without IL-10 or IL-10 receptor neutralizing antibodies (5 μ g/ml) for 72 h. For IDO inhibition, 1 mM 1-methyl-DL-tryptophan (1-MT, Sigma-Aldrich, St-Louis, MO) was added instead of neutralizing antibodies. [³H]-thymidine was pulsed for 18 h.

Statistical analysis

Values for all measurements are presented as mean \pm SD. Comparisons for all pairs were performed by Student's *t*-test. Significance levels were set at a *P* value of 0.05.

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