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ORIGINAL ARTICLE

A critical role of IFNγ in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1

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Bone-marrow-derived mesenchymal stem cells (MSCs) have been shown to possess immunosuppressive properties, e.g., by inhibiting T cell proliferation. Activated T cells can also enhance the immunosuppression ability of MSCs. The precise mechanisms underlying MSC-mediated immunosuppression remain largely undefined, although both cell-cell contact and soluble factors have been implicated; nor is it clear how the immunosuppressive property of MSCs is modulated by T cells. Using MSCs isolated from mouse bone marrow, we show here that interferon gamma (IFNγ), a well-known proinflammatory cytokine produced by activated T cells, plays an important role in priming the immunosuppressive property of MSCs. Mechanistically, IFNγ acts directly on MSCs and leads to up-regulation of B7-H1, an inhibitory surface molecule in these stem cells. MSCs primed by activated T cells derived from IFNγ–/– mouse exhibited dramatically reduced ability to suppress T cell proliferation, a defect that can be rescued by supplying exogenous IFNγ. Moreover, siRNA-mediated knockdown of B7-H1 in MSCs abolished immunosuppression by these cells. Taken together, our results suggest that IFNγ plays a critical role in triggering the immunosuppresion by MSCs through up-regulating B7-H1 in these cells, and provide evidence supporting the cell-cell contact mechanism in MSC-mediated immunosuppression.

***Keywords*:** mesenchymal stem cells (MSCs), immunosuppression, IFNγ, B7-H1, siRNA, proliferation inhibition
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Introduction

Mesenchymal stem cells (MSCs) are rare cells of non-hematopoietic stromal origin, residing in the bone marrow (BM) [1] and a great variety of other tissues [2], which are able to differentiate into cells of the mesodermal lineage, including osteoblasts, adipocytes and chondrocytes [3]. This makes them an attractive cell source for the development of novel cell-based therapies that are usually referred to as cell transplantation and regenerative medicine [4, 5]. Besides their potential use in tissue repair and regeneration, MSCs have recently been discovered to possess immunomodulatory properties whereby they exert a profound inhibitory impact on T cell proliferation *in vitro* and *in vivo*. *In vitro*, studies carried out using human or mouse MSCs indicate that these cells act in a dose-dependent fashion to strongly suppress T lymphocyte proliferation triggered by cellular stimuli, non-specific mitogenic stimuli or antigen [6-8]. *In vivo*, MSCs may improve the result of allogeneic transplantation by promoting hematopoietic engraftment and limiting graft-versus-host diseases (GVHD), as well as ameliorating autoimmune diseases such as experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) [7, 9-12]. Although the immunosuppressive properties of MSCs have been well established [13, 14], the mechanisms are still largely obscure. Soluble factors such as hepatocyte growth factor (HGF), transforming growth factor (TGF-β) [15, 16], indoleamine 2,3-dioxygenase (IDO) [17], interleukin-10 (IL-10) [18] and unidentified factors [15, 16, 19] as well as contact-dependent mechanisms [20, 21] have been implicated.

Interferon gamma (IFNγ) is a major proinflammatory cytokine secreted by activated T and NK cells [22]. IFNγ

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can strongly up-regulate immune-related genes including major histocompatibility complex (MHC) [19, 23] and co-stimulatory molecules CD80 and CD86 [22, 24]. Recently, English *et al*. [25] reported that IFNγ produced by activated T cells is required for murine MSCs to exert their immunosuppression function, and the effect of mMSCs appears to be mediated by a soluble factor IDO secreted from these stem cells. B7-H1, also called PD-L1 (programmed death legend 1), is found in various tissues and acts as an inhibitory co-stimulatory molecule during immune responses [26]. A recent study by Augello *et al*. [27] showed that Ab-mediated neutralization of B7-H1 on MSCs led to diminished ability of immunosuppression. However, the potential inter-relationship between IFNγ and B7-H1 in the T-MSC interplay during MSC-mediated immunosuppression has not been examined.

In this study, we found that higher concentrations of IFNγ were present in the supernatant of the MSC/activated T co-culture system. This was accompanied by up-regulation of B7-H1 on the surface of MSCs and significant suppression of T lymphocyte proliferation. Co-culturing of MSCs with T lymphocytes derived from IFNγ–/– mice dramatically reduced their ability to suppress T lymphocyte proliferation, and, interestingly, did not lead to B7-H1 up-regulation. Supplement of exogenous IFNγ restored B7-H1 up-regulation and inhibition of lymphocyte proliferation. Finally, we found that knockdown of B7-H1 in MSCs abolished their ability to inhibit T cell proliferation. Our results suggest that the cytokine IFNγ triggers the immunosuppression function of MSCs via up-regulation of B7-H1 expression. These findings may have implications in designing immune intervention strategies for amelioration of autoimmune disease symptoms [28, 29] and for eliciting anti-rejection response in transplantation [30] and regenerative medicine [19].

Results

Characterization of murine bone-marrow-derived MSCs: multipotent differentiation and immunosuppressive effect

MSCs were derived from C57BL/6J mouse BM according to an established protocol [31]. These cells exhibited spindle-shaped morphology and continuous proliferation. MSCs from the 8th passage were analyzed for expression of cell surface molecules by flow cytometry. As reported in other studies, these cells were found to express c-kit, Sca-1 and SSEA-1, and they were negative for FLK-1, Thy1.1 and MHC-II (Figure 1A). These cells did express MHC class I (K^b and D^b) but were negative for CD11b, CD31 and CD34 (data not shown). To further characterize the derived MSCs, we examined their capacity of multilineage differentiation into adipocytes, osteoblasts and chondrocytes, and our results showed that they could differentiate into all three cell types under appropriate conditions (Figure 1B). These results indicated the successful culture of MSCs, and MSCs at the 8th passage were used for all subsequent functional assays in this study.

To examine their inhibitory effect on T lymphocyte proliferation, MSCs were added at various ratios to freshly isolated lymphocytes (responser, R) stimulated with irradiated allogenic cells (stimulator, S) (called mixed lymphocyte cultures, MLR), (Figure 1C-1) or freshly isolated lymphocytes (responser, R) activated with 3 μg/ml mitogen ConA (Figure 1C-2) or freshly isolated lymphocytes (responser, R) stimulated with 100 ng/ml of anti-CD3 plus 200 ng/ml anti-CD28 (TCR stimulation pathway, labeled with sti) (Figure 1C-3). T cell proliferation was measured using [^{3}H]-thymidine incorporation. The results showed that proliferation was strongly inhibited by MSCs in all of the above assays in an MSCs cell-number-dependent manner (data not shown), which is consistent with previous reports.

High level of IFNγ in the supernatant of stimulated lymphocytes co-cultured with MSCs

Because some previous reports have shown that IL-10 and TGF-β are involved in the immunosuppression response mediated by MSCs, we first examined the cytokine profile in the supernatant collected from co-cultured lymphocytes and MSCs using ELISA. The results showed that IL-10 or TGF-β was not detectable (Figure 2A), suggesting that under our co-culture conditions IL-10 and TGF-β are not involved in the proliferation inhibition mediated by MSCs. We also examined the concentrations of IL-12 and TNF-α, two cytokines known to enhance immune responses. The results showed that TNF-α but not IL-12 was detected. IFNγ has been implicated by a previous study to be important for MSC-mediated immunosuppression [22, 25]; we thus examined its level in our co-culture system which consisted of stimulated splenocytes (SP) in the presence of MSCs, and found that IFNγ was present at a high level in the culture supernatant (more than 750 pg/ml) (Figure 2A). The concentration of IFNγ was not affected by the presence (MSC + SP) or absence of MSCs (Figure 2A), suggesting that it was produced by the stimulated lymphocytes. Intracellular staining showed that both the CD4+ T and CD8+ T cells produced IFNγ from the beginning of their stimulation (Figure 2B). This suggested that IFNγ was produced by SP and there was more IFNγ production from CD8+ T cells (Figure 2B-lower) than from CD4+ T cells (Figure 2B-upper). This indicated that CD8+ T cells were the dominant IFNγ-producing population and this result is consistent with Krampera *et al*. [22].

IFNγ up-regulates B7-H1 expression on MSCs

A previous study showed that IFNγ induced B7-H1 ex-

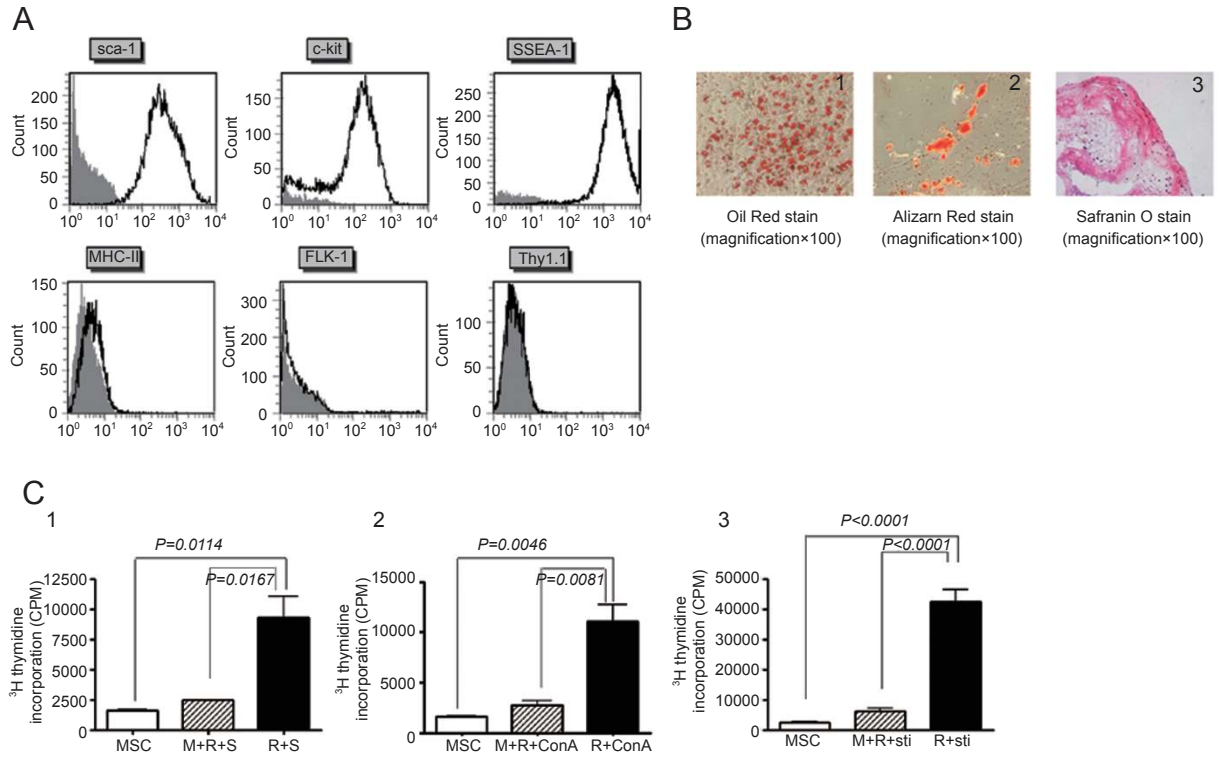

Figure 1 Phenotype, multipotent differentiation and immunosuppression of bone marrow-derived MSCs. Mononuclear cells obtained from C57BL/6J mouse bone marrow were cultured and their multilineage differentiation capacity was confirmed *in vitro*. **(A)** Phenotype profile of 8th passage MSCs; cells were harvested and stained for the indicated surface markers as described in Materials and Methods. From left to right are: sca-1, c-kit, SSEA-1 (upper row); and MHC-II, FLK-1, Thy1.1 (lower row). **(B)** Multilineage differentiation capacity of 8th passage MSCs. 1. MSC differentiation into adipocytes identified with oil red staining *in vitro* (× 100). 2. MSC differentiation into osteocytes identified with Alizarin Red-Tris-HCL staining *in vitro* (× 100). 3. MSC differentiation into chondrocytes identified with Safranin O staining *in vitro* (× 100). **(C)** MSCs inhibit lymphocyte proliferation stimulated by heterogenic cells (mixed lymphocyte reaction, MLR), mitogen (ConA) and anti-CD3 plus anti-CD28 *in vitro*. 1. MSCs inhibit MLR: Open bar indicates MSCs alone in culture. Hatched bar indicates that MSCs (M) were added to the mixed lymphocyte (R + S) culture. Black bar indicates a mixed lymphocyte reaction (R + S) without MSCs. 2. MSCs inhibit lymphocyte proliferation stimulated by ConA. Open bar indicates MSCs alone in culture. Hatched bar indicates that MSCs (M) were added to the lymphocytes incubated with 3.0 μg/ml ConA (R + ConA). Black bar indicates lymphocytes stimulated by 3.0 μg/ml ConA (R + ConA) without MSCs. 3. MSCs inhibit lymphocyte proliferation stimulated by anti-CD3 and anti-CD28. Open bar indicates MSCs alone in culture. Hatched bar indicates that MSCs (M) were added to the lymphocytes (R) incubated with 100 ng/ml anti-CD3 and 200 ng/ml anti-CD28 (sti). Black bar indicates lymphocytes (R) stimulated by anti-CD3 and anti-CD28 (sti). Values represent means ± SD. Similar results were obtained from more than five independent experiments.

pression in murine MSCs but the up-regulation of B7-H1 was not involved in immunosuppresion [25]. Conversely, Augello *et al*. showed that Ab-mediated neutralization of B7-H1 on murine MSCs diminished immunosuppression. To clarify the potential role of B7-H1, we were interested in investigating whether B7-H1 expression was induced by IFNγ in our co-culture system, and, if so, whether the expression of B7-H1 could mediate the immunosuppressive effect of MSCs. The expression of B7-H1 was examined using real-time PCR and flow cytometry. The results showed that naïve MSCs did not express significant levels of B7-H1; however, when MSCs were co-cultured with stimulated lymphocytes, both mRNA and protein levels of B7-H1 increased significantly ($P = 0.0158$ and $P = 0.0001$, respectively) (Figure 3A-1, 2). This result indicated that activated lymphocytes up-regulated B7-H1 expression on MSCs. The supernatant collected from the co-culture had a similar ability to stimulate B7-H1 expression in MSCs (Figure 3B), suggesting that cytokine(s) in the supernatant might be responsible for inducing B7-H1 expression. To probe which cytokine was responsible for B7-H1 up-regulation, we performed Ab-mediated neutralization experiments, and the results showed that only the specific Ab against IFNγ was able to abolish the induction of B7-H1 expression

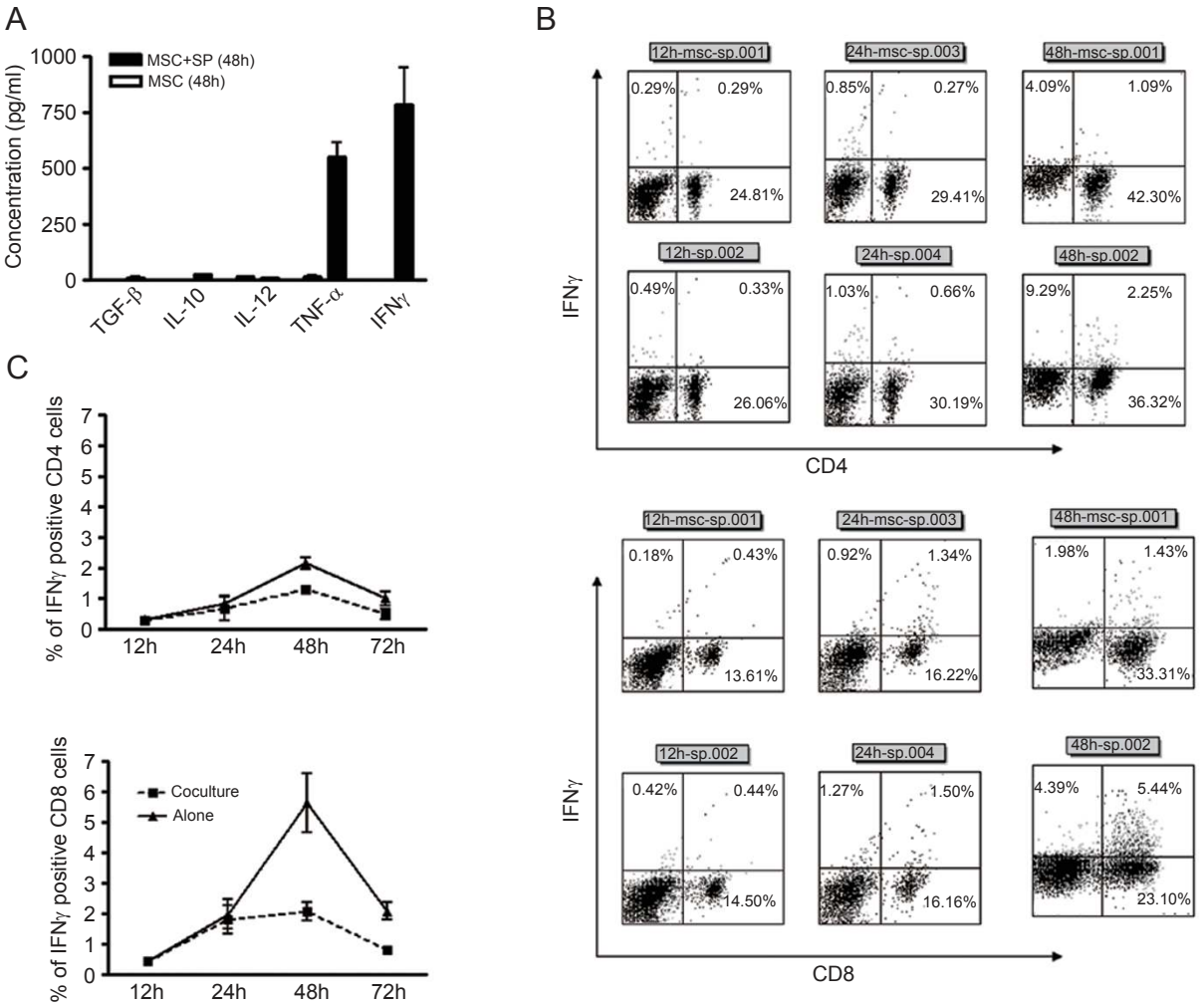

Figure 2 High level of IFNγ detected in lymphocytes and the supernatant of co-culturing system. Levels of IFNγ, TGF-β, IL-10, IL-12 and TNF-α in supernatants were detected by ELISA. **(A)** Concentration of these cytokines in the supernatants of co-culture. The cytokines examined are indicated under the *X*-axes; *Y*-axis denotes concentration. **(B)** Flow cytometry detected the intracellular staining of IFNγ in CD4+ and CD8+ subset levels in the presence or absence of MSCs. (The upper row represents the presence of MSCs, and lower row represents absence of MSCs in CD4+ and CD8+ subsets, respectively.) **(C)** The time points detected are indicated under the *X*-axis; the *Y*-axis denotes % of IFNγ -positive cells. (Upper figure represents CD4+ subset, and lower figure represents CD8+ subset.)

(Figure 3B). To further confirm the role of IFNγ, individual cytokines were added to naive MSCs; as expected, only IFNγ could stimulate B7-H1 expression (Figure 3C). We also examined the expression of other members of the B7 family, B7-H2, B7-H3 and B7-H4, and found that they were not up-regulated by IFNγ (Figure 3D).

To further explore the time course of B7-H1 expression induced by IFNγ, we added exogenous IFNγ into the culture of naïve MSCs and analyzed B7-H1 expression pattern. The results showed that within the dose range from 0.05 to 5 ng/ml IFNγ could induce B7-H1 expression in a dose-dependent manner (Figure 3E-1). Moreover, B7-H1 expression increased to a maximum within 24 h after IFNγ treatment, and the level lasted for at least 120 h (Figure 3E-2). Thus, the expression of B7-H1 was rapidly induced in MSCs in response to IFNγ.

Activated lymphocytes derived from IFNγ –/– mouse did not induce B7-H1 expression and failed to trigger immunosuppression function of MSCs

To investigate whether IFNγ signaling plays an important role in triggering the immunosuppression function of MSCs, and the potential role of B7-H1 induction by IFNγ, we used lymphocytes derived from IFNγ knock-

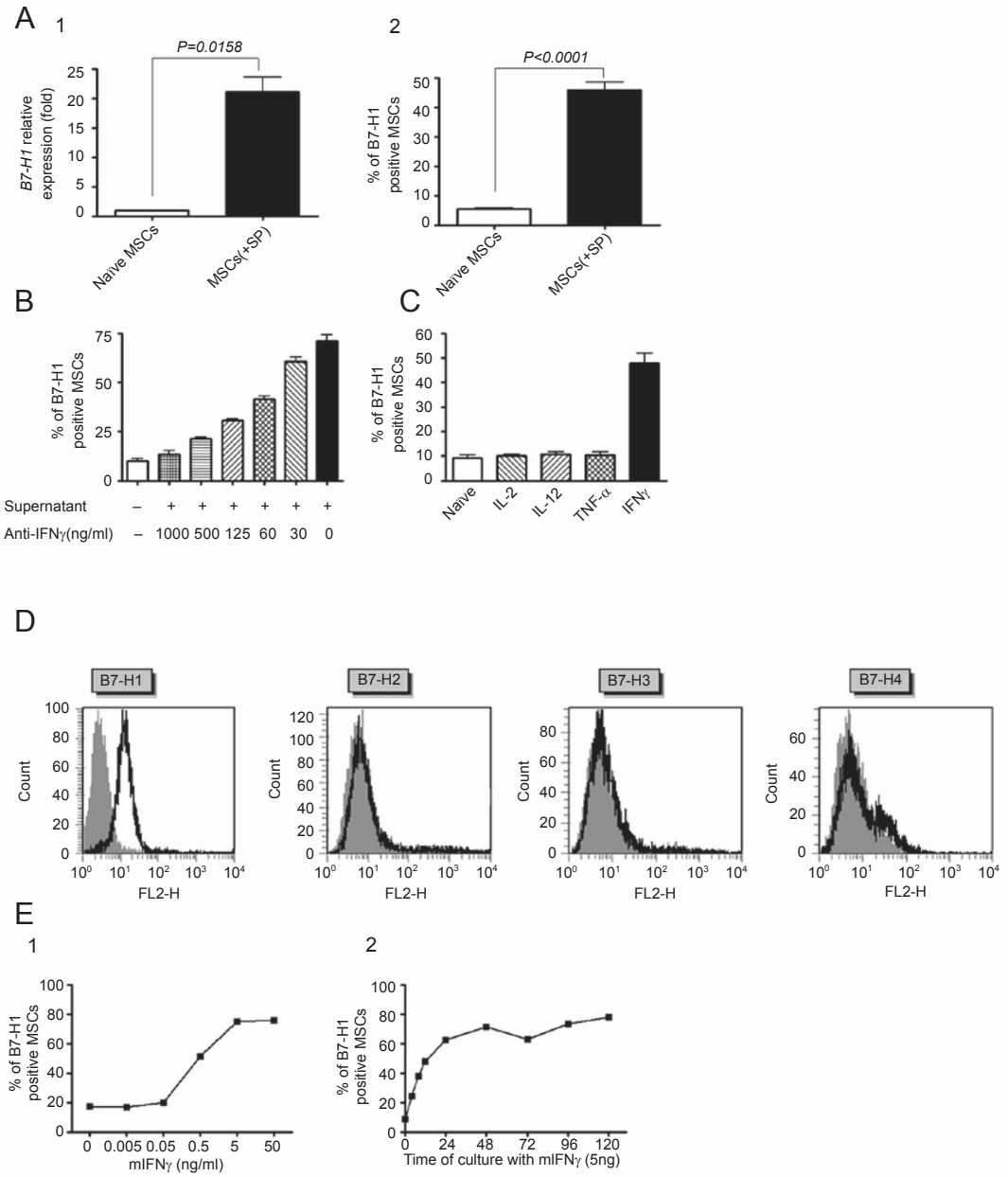

Figure 3 Exogenous IFNγ initially increased B7-H1 expression on MSCs. **(A)** Detection of the B7-H1 molecule from the gene and protein levels. 1. B7-H1 transcription in MSCs examined by real-time PCR. The open bar represents B7-H1 transcription in MSCs cultured without stimulated lymphocytes, black bar represents B7-H1 transcription in MSCs co-cultured with stimulated lymphocytes. 2. Percent of B7-H1 expression on MSC surface as detected by flow cytometry with specific anti-mouse B7-H1 antibody labeling. The open bar represents B7-H1 expression on MSCs cultured without stimulated lymphocytes, and black bar represents B7-H1 expression on MSCs co-cultured with stimulated lymphocytes. **(B)** B7-H1 molecule expression profiles on MSCs after incubation with supernatant collected from lymphocytes culture medium stimulated by anti-CD3 and anti-CD28 for 24 h. In blocking assay, incubation of the supernatant with anti-mouse IFNγ Ab was carried out in 1 000, 500, 125, 60 and 30 ng for 1 h, respectively, prior to culturing MSCs. After culturing for 24 hr, the MSCs were collected and B7-H1 expression profile detected by FACS. **(C)** Expression profile of B7-H1 expression on MSCs co-cultured with different inflammatory cytokines. Open bar represents B7-H1 expression on naïve MSCs, left hatched bar represents co-culturing with IL-2, right hatched bar represents co-culturing with IL-12, grid bar represents co-culturing with TNF-α and black bar represents co-culturing with IFNγ. **(D)** Expression profiles of B7-Hs family molecules on MSCs after incubation with IFNγ for 72 h. Gray areas represent B7-Hs molecule expression without IFNγ stimulation. The black line represents B7-Hs molecule expression with exogenous IFNγ added. **(E)** B7-H1 expression profile induced by exogenous IFNγ. 1. B7-H1 expression profile upon stimulation with increasing concentrations of exogenous IFNγ. 2. Kinetic curve of B7-H1 expression on MSCs incubated with 5 ng/ml exogenous IFNγ.

out mice (IFNγ–/–) in the co-culture with wild-type (wt) MSCs. Interestingly, co-culture with activated IFNγ–/– T lymphocytes failed to up-regulate B7-H1 expression in MSCs (Figure 4A). Meanwhile, proliferation of stimulated lymphocytes derived from IFNγ–/– mice increased significantly compared with that of wt lymphocytes under similar co-culture conditions (Figure 4B) ($P < 0.0001$). Thus, IFNγ–/– lymphocytes failed to induce B7-H1 expression in MSCs and were unable to efficiently trigger the immunosuppression function of these cells.

To confirm the critical role of IFNγ in inducing B7-H1 expression and in triggering MSC-mediated immunosuppression, exogenous IFNγ was added into the co-culture of MSCs with IFNγ–/– lymphocytes; B7-H1 expression and immunosuppresion capacity of MSCs were then examined. The results showed that exogenous IFNγ could rescue B7-H1 expression (data not shown) and restore the ability of the MSCs to inhibit lymphocyte proliferation (Figure 4C). Taken together, our results indicate that IFNγ plays a critical role in triggering MSC-mediated immunosuppression,

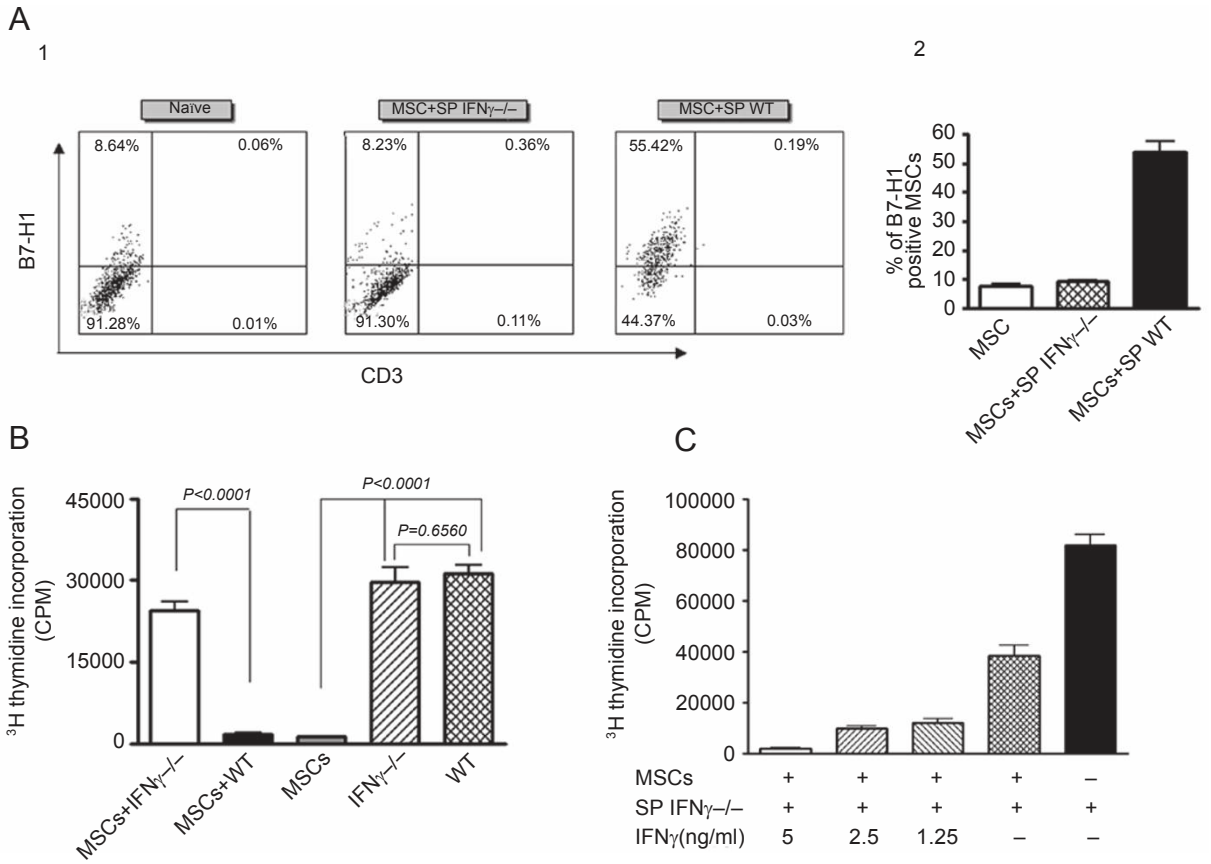

Figure 4 IFNγ–/– mouse-derived lymphocytes upon the stimuli neither triggered the B7-H1 expression on MSCs, nor mediated immunosuppression. **(A)** Wild-type B6-derived MSCs expressing B7-H1 co-culture in the wt- or kc-derived lymphocytes. 1. In naïve state, co-culture with IFNγ–/– mouse-derived lymphocytes upon stimulation and co-culture with B6 wild-type mouse-derived lymphocytes upon stimulation. The *Y*-axis represents B7-H1, *X*-axis represents CD3. (Dot-plot from left to right, data shown are representative of at least three experiments.) 2. Percent of B7-H1 molecule expression on MSCs co-culturing with kc mouse-derived or wt-derived lymphocytes upon the same stimuli. **(B)** MSCs immunosuppression on IFNγ–/– mouse-derived and wt mouse-derived lymphocytes upon stimulation, respectively. The open bar represents the proliferation of co-cultured MSCs and stimulated IFNγ–/– lymphocytes, black bar represents proliferation of co-cultured MSCs and stimulated wt lymphocytes, gray bar represents MSCs basic culture proliferation level, hatched bar represents the proliferation of stimulated IFNγ–/– lymphocytes and grid bar represents the proliferation of stimulated wt lymphocytes. Abbreviations: IFNγ–/– represents stimulated IFNγ–/– lymphocytes by anti-CD3 and anti-CD28 (100 and 200 ng/ml, respectively), wt represents stimulated wt lymphocytes as the counterpart above. **(C)** Exogenous IFNγ restores the proliferation of lymphocytes derived from IFNγ–/– mouse inhibited by fMSCs. The different bars represent the proliferation under different doses of exogenous IFNγ labeled under the X-axis. Abbreviations: IFNγ–/– represents stimulated IFNγ–/– lymphocytes, wt represents stimulated wt lymphocytes.

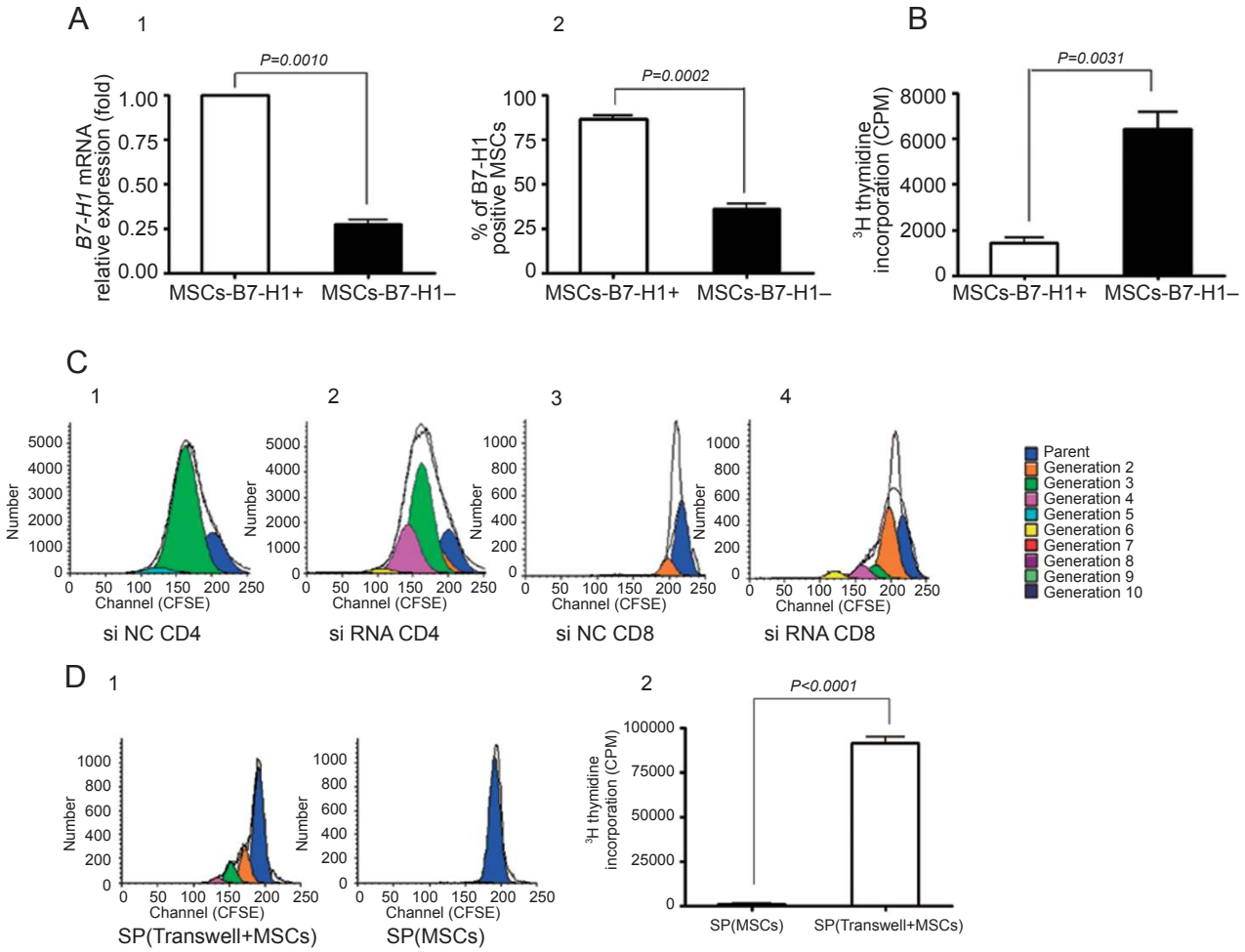

Figure 5 Immunosuppressive function of MSCs on different lymphocyte subsets mediated via the PD-1/B7-H1 pathway, and cell-cell contact needed. **(A)** B7-H1 special siRNA knocks down the gene and relative protein expression. 1. B7-H1 transcription in MSCs after B7-H1 RNAi administration. Open bar represents the B7-H1 mRNA level in MSCs with negative control RNAi treatment (MSCs-B7-H1+). Black bar represents the B7-H1 mRNA level in MSC-B7-H1–. 2. B7-H1 protein expression on MSCs after B7-H1 RNAi administration: open bar represents B7-H1 expression on MSCs with negative control RNAi treatment (MSCs-B7-H1+), black bar represents B7-H1 expression level on MSC-B7-H1–. **(B)** Lymphocyte proliferation inhibition profile after incubation with MSC-B7-H1–. Open bar represents lymphocyte proliferation upon co-culture with MSCs-B7-H1+. Black bar represents lymphocyte proliferation upon co-culture with MSCs-B7-H1–. **(C)** Generations in T cell subset inhibition assay with CFSE labeling. siNC CD4 represents the generation of CD4+ T cells incubated with MSCs-B7-H1+. siRNA CD4 represents CD4+ T incubated with MSCs-B7-H1–. siNC CD8 represents the generation of CD8+ T cells incubated with MSCs-B7-H1+. siRNA CD8 represents CD8+ T incubated with MSCs-B7-H1– (as shown from 1 to 4). **(E)** In the transwell experiments MSCs play the immunosuppression via the cell-cell contact fashion. 1. Generation assay of lymphocytes labeled with CFSE combined with APC-conjugated-CD3 mouse antibody label. The left figure represents lymphocytes incubated with the transwell system. Right figure represents lymphocytes incubated without the transwell system. 2. Proliferation assay of lymphocytes collected from the co-culture with transwell membrane (open bar) or the co-culture system without membrane (black bar), respectively, by [^{3}H]-thymidine incorporation.

likely via up-regulation of B7-H1 in MSCs.

Knockdown of B7-H1 in MSCs abolished their immunosuppression function

To obtain direct evidence that B7-H1 induction by IFNγ is critical in MSC-mediated inhibition of T cell proliferation, we used B7-H1 specific siRNA to knockdown its expression. Among the four synthesized siRNAs, one was found to effectively reduce B7-H1 expression (termed MSCs-B7-H1–) while the negative control (termed MSCs-

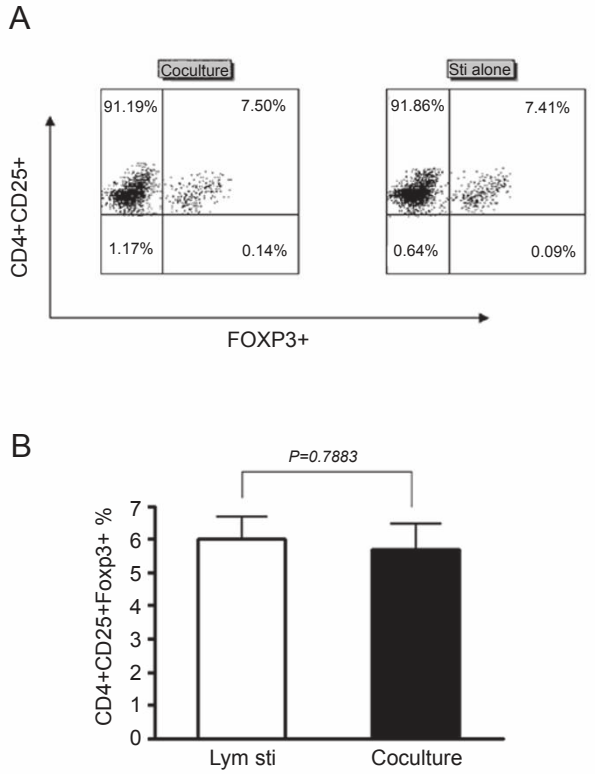

Figure 6 Treg subset was not involved in the immunosuppression function mediated by MSCs. **(A)** Flow-cytometry-detected CD4+CD25+Foxp3+ subset in the lymphocytes stimulated upon the anti-CD3/CD28 without MSCs (left) and with MSCs (right); data shown are representative of at least three experiments. **(B)** Bar graph of the CD4+CD25+Foxp3+ subset presence in lymphocytes stimulated upon the anti-CD3/CD28 without MSCs (open bar) and with MSCs (black bar). Abbreviations: lym sti represents lymphocytes stimulated upon the anti-CD3/CD28 without MSCs; co-culture represents lymphocytes stimulated upon the anti-CD3/CD28 with MSCs.

B7-H1+) had no effect on the B7-H1 expression in MSCs as shown by real-time PCR ($P = 0.001$) (Figure 5A-1). This effect was confirmed at protein level by flow cytometry analysis ($P = 0.0002$) (Figure 5A-2). In the co-culture with stimulated lymphocytes, the B7-H1 knockdown MSCs exhibited a significantly reduced ability to inhibit lymphocyte proliferation ($P = 0.0102$) (Figure 5B). Cell division assay showed that both CD4+ T cells and CD8+ T cells were inhibited using CSFE tracer (Figure 5C). These results suggested that B7-H1 expression is important for MSC-mediated immunosuppression.

As B7-H1 is one of the inhibitory co-stimulators expressed on cell surface which functions via interaction with its receptor PD-1 on T or B cells [26], cell-cell contact is necessary for B7-H1-mediated immunosuppression. Interestingly, PD-1 expression was increased in activated T lymphocytes (data not shown), regardless of whether MSCs were present or not. To determine whether cell-cell contact is needed in the inhibition mediated by MSCs, trans-membranes were used to separate stimulated lymphocytes (added in the upper chamber) and MSCs (cultured in the lower chamber). The results showed that the proliferation of lymphocytes separated from MSCs by the transmembrane was significantly higher than that of co-cultured cells without the transmembrane ($P = 0.0001$) (Figure 5D-1). Cell division assay further suggested that the transmembrane blocked MSC-mediated inhition (Figure 5D-2). Thus, cell-cell contact is required for MSC-mediated inhibition of lymphocyte proliferation under our experimental conditions.

Immunosuppression by MSCs does not involve Tregs

There have been conflicting reports on the potential involvement of regulatory T cells (Tregs) in MSC-mediated immunosuppresion [32-34]. To clarify whether Tregs play any role in the inhibition of lymphocyte proliferation in our co-culture system, we examined the Treg population and found that the percent of CD4+CD25+FoxP3+ cells was maintained at a similar level in lymphocytes co-cultured with or without MSCs (Figure 6A and 6B). In addition, cytokines secreted by Tregs (such as IL-10 and TGF-β) are not detected in our system (Figure 2A). Thus, our result suggests that immunosuppression by MSCs observed in our experiments is not mediated by Tregs.

Discussion

IFNγ is a major proinflammatory cytokine secreted by activated T and NK cells [22], and can strongly up-regulate many immune-related genes including MHC [19, 23] and co-stimulatory molecules [22, 24]. Recently, IFNγ was shown to be a double-edged sword in immune response because it can induce Tregs that further inhibit immune response by release of inhibitory cytokines (such as IL-10 and TGF-β) or by cell-cell contact [35]. More recently, English *et al*. reported that IFNγ produced by activated T cells is required for murine MSCs to exert their immune-suppression function, and the effect of mMSCs appears to be mediated by a soluble factor IDO secreted from these stem cells [25].

B7-H1 was initially reported mainly as a co-inhibitor molecule by Chen's group and now has been identified on resting T cells, B cells, DCs and macrophages, where it is further up-regulated upon activation [26, 36]. Other groups also reported that B7-H1 is present on endothelial cells and cells from other non-lymphoid organs [36-39]. The exact immune functions of B7-H1 are not fully understood. With respect to immunosuppression mediated by MSCs, Augello

et al. first reported that murine MSCs inhibit lymphocyte proliferation by activation of the B7-H1-mediated signaling pathway. They found that MSCs inhibited the activation and proliferation of murine lymphocytes via engagement of the inhibitory molecule B7-H1 to its cognate receptor on target immune cells, and MSC-mediated inhibition could be blocked by anti-B7-H1 antibody [27]. Intriguingly, Stagg *et al*. [23] found that IFNγ induced B7-H1 expression in human MSCs but B7-H1 up-regulation was not involved in immunosuppression by human MSCs. Thus, the inter-relationship between IFNγ and B7-H1 in the MSC-lymphocyte interplay merits further investigation.

In this report, we analyzed the pattern of cytokines in the supernatant of stimulated lymphocytes co-cultured with MSCs, and found that IL-10, IL-12 and TGF-β were not detectable but both IFNγ and TNFα were present at high levels. Co-culture with stimulated lymphocytes significantly up-regulated B7-H1 expression on MSCs, an effect which can be mimicked by supernatants from the co-culture. Further analyses indicated that IFNγ present in the co-culture supernatant was primarily responsible for the induction of B7-H1 expression on MSCs. To investigate whether IFNγ signaling plays an important role in triggering the immunosuppression function of MSCs, and to probe the potential role of B7-H1 induction by IFNγ, we performed similar co-culture experiments using lymphocytes derived from IFNγ–/– knockout mice. Our results indicated that IFNγ produced by lymphocytes plays a critical role in triggering immunosuppression by MSCs, likely through induction of B7-H1 expression. Consistent with this notion, supplement of exogenous IFNγ into the co-culture of activated IFNγ–/– lymphocytes with wt MSCs restored both B7-H1 induction and MSC-mediated immunosuppression. In support of an important role of B7-H1, knockdown of B7-H1 expression on MSCs by RNAi significantly attenuated the immunosuppression function of MSCs.

Both cell-cell contact and soluble factors have been implicated in MSC-mediated immunosuppression, and different experimental settings often led to different results [33, 40]. In our study, we found that priming of MSCs by lymphocyte-derived IFNγ led to up-regulation of B7-H1 on MSCs, which appeared to be critical for mediating the immunosuppression function of these cells. B7-H1 is known to function by interaction with its cognate receptor on target cells, a process mediated by cell-cell contact. Consistent with this notion, our co-culture experiments using transmembranes to separate lymphocytes and MSCs showed that inhibition of lymphocyte proliferation was largely abolished without direct contact with MSCs.

Recent reports showed that IFNγ is a key factor to induce CD4+CD25+ Tregs [41, 42]. While some reports showed that induced Tregs were involved in the proliferation inhibition mediated by MSCs [32, 34], other studies showed the opposite results [33]. Immunosuppression by MSCs observed in our experiments does not appear to be mediated by Tregs.

MSCs, due to their immunomodulation property, may have wide applications in clinical settings, such as amelioration of autoimmune diseases, reducing rejection response in transplantation and in regenerative medicine. In this study, we revealed a new interplay between MSCs and T cells, whereby activated T cells prime MSCs for immunosuppression through IFNγ signaling and subsequent up-regulation of B7-H1 in MSCs, while primed MSCs in turn suppress T cell proliferation via B7-H1-mediated inhibitory signaling. Whether MSCs mediate immunosuppression *in vivo* via B7-H1 up-regulation by IFNγ is under investigation.

Materials and Methods

Isolation and culture of murine MSCs

MSCs were generated from C57BL/6J, 6-8-week old, female mice (Shanghai Laboratory Animal Center, Chinese Academy of Sciences). Mice were sacrificed by cervical dislocation; femurs and tibiae were removed and cleaned of all connective tissue. BM cells were collected by flushing femurs and tibiae with 1 × phosphate-buffered saline (PBS) buffer using a 26-gauge needle. Material was filtered and washed twice with centrifugation at 1 500 rpm for 5 min in 1 × PBS/4% FCS medium. To initiate MSC culture, cells were plated in 75-cm^2 dishes (BD Biosciences) at a concentration of 5 × 10^5/ml/cm^2 nucleated cells in Dulbecco modified Eagle medium (DMEM) with low glucose (HyClone) supplemented with 10% FCS (HyClone), 2 mM L-glutamine (Invitrogen Life Technologies), 50 μM β-ME, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies). Cultures were incubated at 37 ºC in a 5% CO_2 atmosphere. After 48 h, non-adherent cells were removed by washing with 1 × PBS, and fresh medium was added. Medium was changed every 3-4 days. When the cells reached 80% confluence, the monolayer was washed twice with 1 × PBS, then lifted by incubation for 2-3 min at 37 ºC with a 0.25% trypsin solution containing 0.01% EDTA (Invitrogen Life Technologies). Trypsin was neutralized by the addition of fresh complete medium. The resulting suspension was then expanded by plating at 6 000-10 000 cells/cm^2 in 75-cm^2 dishes. Identical conditions were used for subsequent passages [31].

Differentiation of murine MSCs

Before further expansion and experimental use, as an additional quality control, we tested MSCs cultures for their ability to undergo differentiation into chondrocytes, osteocytes and adipocytes. Cells were trypsinized, harvested and plated at 5 × 10^4 cells per well in 24-well plates in complete medium. Medium was replaced 24 h after plating. Chondrogenic differentiation was induced in some of the pelleted cells of the initial culture by ascorbic acid (50 μg/ml) and TGFβ-1 (1 ng/ml) in complete medium. After 2 weeks, plates were washed with 1 × PBS, fixed with 4% paraformaldehyde, and stained with 0.05% alcian blue. Osteogenic differentiation was induced in other cells of the initial culture by adding ascorbic acid (50 μg/ml), sodium β-glycerophosphate (10 mM) and dexamethasone (10^{-8} M)

in complete medium. After 2 weeks, plates were washed with 1 × PBS, fixed with 4% paraformaldehyde and stained with 1% alizarin red. Adipogenic differentiation was induced in a third aliquot of cells of the initial culture by the addition of dexamethasone (10^{-7} M) and insulin (6 ng/ml), in complete medium. After 2 weeks, plates were washed with PBS, fixed with 4% paraformaldehyde and stained with Oil red O [31].

Flow cytometric analysis

Flow cytometric analysis was performed in PBS with 1% FBS with the following mAbs: R-phycoerythrin (PE)-conjugated anti-mouse CD45 (clone 30-F11, Biolegend), H-2K^b (clone AF6-88.5, Biolegend), I-A^b (clone AF6-120.1, Biolegend), CD25 (PE, clone PC61, eBioscience), B7-DC (PE, clone TY25, eBioscience), B7-H1 (PE, clone MIH5, eBioscience), B7-H3 (PE, clone M3.2D7, eBioscience), B7-H4 (PE, clone 188, eBioscience), c-kit (PE-Cy5, clone ack2, eBioscience), FLK-1 (PE, clone Avas12a1, eBioscience), SSEA-1 (FITC, Santa Cruz), sca-1 (PE, clone D7, eBioscience) and Thy1.1 (FITC, clone HIS51, eBioscience). Flow cytometry was performed using a FACS Calibur cytometer (BD Biosciences) and analyzed using Cellquest software and Modfit software (BD Biosciences). For intracellular staining, cells were fixed and permeabilized (BD cytofix/cytoperm^{TM}, San Diego, CA) before incubation with PE-conjugated anti-IFNγ (BD Pharmingen), and Golgiplug (BD Biosciences, San Diego, CA) was added for the final 6 h before staining. Isotypic control analyses were performed in parallel.

Proliferation and blocking assays

In proliferation assays, mouse lymphocytes (1×10^5 per well) were cultured in triplicate in complete DMEM (DMEM with 10% fetal calf serum, HEPES, β-mercaptoethanol, L-glutamine, sodium pyruvate and penicillin/streptomycin) in 96-well flat-bottomed plates. Cells were cultured in the presence or absence of the pre-adherent MSCs and stimulated by mouse anti-CD3 (clone 145-2C11, eBioscience) and anti-CD28 (clone 37.51, eBioscience) (100 and 200 ng/ml, respectively) at 37 ºC in 5% CO_2 for 72 h. Cells were pulsed with 1 μCi [^{3}H]-thymidine (Shanghai Institute of Atomic Nucleus, Chinese Academy of Sciences) during the last 16-18 h of culture prior to harvest. [^{3}H]-thymidine incorporation was measured as cpm using a β-plate counter. IFNγ–/– knocknout mouse were provided by Dr Xiaodong Wu (Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences, Shanghai, China), who did the same experiments as the protocol of wt mice. Percentage of inhibition on the proliferation of lymphocytes derived from IFNγ–/– knockout or wt mouse was calculated as [1– (CPM of stimulated lymphocytes co-cultured with MSCs/the sum of CPM of lymphocytes upon the stimuli of anti-CD3/anti-CD28 alone)] × 100%. In blocking assays, first collecting the supernatant of lymphocytes upon the stimuli of anti-CD3 and anti-CD28, then incubate it with anti- mouse IFNγ antibody (clone: R4-6A2, eBioscience) for 1 h prior to culturing the MSCs. After culturing for 24hr, MSCs are trypsinized and detected the B7-H1 expression profile by FACS.

Real-time PCR

Total RNA from MSCs or lymphocytes, the latter co-cultured with MSCs or activated by anti-CD3/CD28, was extracted using TRIzol reagent (Invitrogen Life Technologies), then reverse transcribed using a First Strand cDNA Synthesis kit (Fermentas Life Science); cDNA was analyzed by real-time PCR using SYBR Green Master Mix (Applied Biosystems). Thermocycler conditions comprised an initial holding at 50 ºC for 2 min and a subsequent holding at 95 ºC for 10 min, which was followed by a two-step PCR program at 95 ºC for 15 s and 60 ºC for 60 s for 40 cycles. In the end, a dissociation stage was added using 95 ºC for 15 s, 60 ºC for 15 s and 95 ºC for 15 s. For β-actin, B7-H1, IDO and PGE2, two sets of primers were used: β-actin amplicon forward, TGT CCA CCT TCC AGC AGA TGT, and reverse, AGC TCA GTA ACA GTC CGC CTA GA; B7-H1 amplicon forward, CTA CGG TGG TGC GGA CTA CA, and reverse, CAT GCT CAG AAG TGG CTG GAT; IDO forward, GCC CAC CGG AAC TTC CTT T, and reverse, CAC TCG TTA TAA GCT TTC GTC AAG TC; PGE2 forward, TGC CAT GTA CCT CAT CAG CAA, and reverse CGG CTG TCA CCC ACT TGT T. Data were collected and quantitatively analyzed on an ABI Prism 7900 Sequence Detection System [41] (Applied Biosystems).

siRNA oligo transfection and CFSE cell tracer-labeling

MSCs were precoated on 12-well plates (Costar, Corning Incorporated). The siRNA oligo and the GenePORTER reagent (Gene Therapy Systems) were diluted with serum-free medium, then mixed and incubated at room temperature for 20 min. Culture medium was aspirated from the cells, the mixture added to the cells and the culture incubated at 37 ºC for 3-5 h. Post-transfection, 1 volume of medium containing 20% FCS was added and the culture incubated overnight under 5-10% CO_2 at 37 ºC. At 24 h post-transfection, addition of cells or stimulation by IFNγ was undertaken. Carboxyfluorescein diacetate, succinimidyl ester (CFSE) labeling of lymphocytes was performed as described previously. Briefly, cells were extensively washed and resuspended at a final concentration of 3×10^6 cells/ml in 0.1% BSA PBS. CFSE was added at a final concentration of 5 μM and incubated for 10 min at room temperature. Staining was quenched by addition of 5 volumes of ice-cold culture medium. This was followed by washing with 10% heat-inactivated fetal calf serum- containing DMEM-LG. Cells then received the stimulus of anti-CD3/CD28 in the presence or absence of MSCs for 3-4 days.

Statistical analysis

Differences in cell surface molecular expression and cpm of proliferation tests were analyzed by the Mann-Whitney *U* test. A Student's *t*-test was used to analyze the differences between the groups. One-way ANOVA was initially performed to determine whether an overall statistically significant change existed before using the two-tailed paired or unpaired Student's *t*-test. A *P*-value of < 0.05 was considered statistically significant.

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