Soluble molecules are key in maintaining the immunomodulatory activity of murine mesenchymal stromal cells

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Summary

Mesenchymal stromal cells (MSCs) possess both immuno-privileged and immuno-inhibitory properties that contribute to their therapeutic effects. Ex vivo expansion is required to obtain sufficient cells for therapy, but might also alter their immunological properties. To date there has been no systematic study of MSC immunobiology during extended culture. Here, we demonstrate that both immuno-privilege and immunosuppressive properties of MSCs change with increasing passage. We demonstrate that although MSCs exhibit powerful immunosuppressive effects through secretion of transforming growth factor-β (TGF-β) and induction of interleukin-10, these effects are diminished by a concomitant increase in MSC immunogenicity. Interferon-γ treatment for 3 days induced extendedly cultured MSCs to express significantly higher levels of major histocompatibility complex class I. In vivo, this results in cells that induce significant delayed-type hypersensitivity reactions in allogeneic recipients. Importantly, these effects are alleviated by isolation of the transplanted MSCs using a semi-permeable barrier. Under these conditions, even MSCs cultured through as many as 14 passages still exhibit immuno-inhibitory effects in vivo. Furthermore, the levels of anti-inflammatory molecule TGF-β secreted by MSCs were maintained in the extended culture. These data shed light on the variable results of allogeneic MSCs in transplantation and suggest alternative strategies for prolonging the effect of allogeneic MSCs in cell-based therapy.

Key words: Mesenchymal stromal cells, Allogeneic, Immunogenecity, Immunosuppression, Stem cell transplantation

Introduction

Mesenchymal stromal cells (MSCs) can potentially be used to treat various congenital, malignant and metabolic diseases. Until a valid patient-customized cell source becomes readily available, allogeneic MSCs could provide a useful substitute for autologous MSCs. However, immune reactivity to allogeneic MSCs presents a significant challenge to their clinical applications. Previous studies have shown allogeneic MSCs to be both immuno-privileged and immunosuppressive (Le Blanc, 2006; Brooke et al., 2007; Granero-Molto et al., 2008), suggesting that these cells could be transplanted to unmatched recipients with a reduced requirement for immune suppression. Various studies over the last decade have attested to the potential of allogeneic MSCs for treating diseases of the heart (Quevedo et al., 2009; Hare et al., 2009), kidney (Herrera et al., 2004; Tögel et al., 2009), liver (Abdel Aziz et al., 2007; di Bonzo et al., 2008; Liska et al., 2009) and brain (Qu et al, 2008); for alleviating graft-versus-host disease (GvHD) (Ringdén et al., 2006; Le Blanc et al., 2008; Zhou et al., 2010); for eradicating tumors (Zischek et al., 2009) and for reconstituting the hematopoietic system (Koç et al., 1999; Majumdar et al., 2000). Nevertheless, the rejection of transplanted allogeneic MSCs raises questions about their immuno-privileged status (Eliopoulos et al., 2005; Badillo et al., 2007) and about some of the failures of allogeneic MSC therapy (Cilloni et al., 2000; Djouad et al., 2003; Duffield et al., 2005; Coyne et al., 2006; Sudres et al., 2006; Carr et al., 2008; Rose et al., 2008; Carvalho et al., 2008). Even when shown to work, tissue regeneration by MSCs is generally suboptimal. Relatively small numbers of transplanted donor MSCs are retained in damaged tissues (Koç et al., 2002; Krampera et al., 2003; di Bonzo et al., 2008) and the percentage of integrated MSCs can be as low as 0.4% at 60 days after infusion (Coyne et al., 2006). Detection of transplanted MSCs in vivo using various tracking methods suggests that transplanted MSCs can be completely eliminated from the host tissue within 14 days (Carr et al., 2008), although other reports have shown that MSCs are detectable within host tissue 1 year post-transplantation (Le Blanc et al., 2008). Krampera et al (Krampera et al., 2003) reported that cell–cell interaction is necessary for the immuno-inhibitory properties displayed by MSCs, and others (Tse et al., 2003; Di Nicola et al., 2002) found that soluble factors mediated immune inhibition by MSCs.

Variations in the immunological results of MSCs in transplantation have been ascribed to many factors, including batch or lot number (Neuhuber et al., 2005). It remains to be proven whether interbatch variation is inherent to MSCs or an

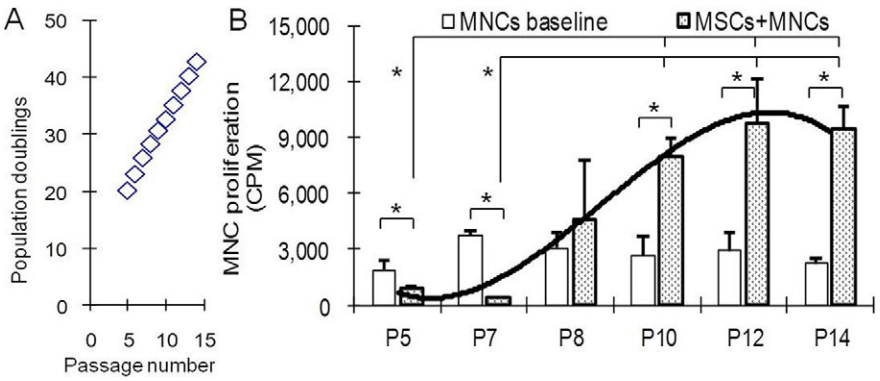

Fig. 1. Immunogenicity of MSCs is elevated with increasing passage number in vitro. (**A**) Cell counts of MSCs increased with increasing passage number. (**B**) The proliferation of BALB/c spleen MNCs induced by inactivated C57BL/6 MSCs was enhanced with increasing passage number. All bars represent mean ± s.d. of triplicates of two independent experiments (*$P<0.05$). The solid curve represents the trendline of MNC numbers elicited by MSCs at increasing passage number.

experimental artifact. Given that long-term in vitro culture alters the biology and differentiation potential of MSCs (Izadpanah et al., 2008; Kretlow et al., 2008), it is possible that the immune properties of MSCs might also be altered during extended in vitro culture (Crisostomo et al., 2006) and that variations in their therapeutic efficacy are related to these changes. To date, there has not been a systematic study of changes in MSC immunobiology during extended culture.

Results

Alteration of immune properties of MSCs at different passage numbers in vitro

We investigated the immunological properties of MSCs during prolonged in vitro culture. MSCs from mouse bone marrow were isolated by rapid adhesion to plastic and expanded with the same batch of MSC medium. With serial passage, MSCs proliferated (Fig. 1A) and displayed changes in alloreactivity (Fig. 1B). Before passage 8 (P8) (approximately 10 divisions after P5), MSCs from C57BL/6 mice cultured with mononuclear cells (MNCs) from BALB/c did not induce an allogeneic response significantly above background, and inhibited the spontaneous proliferation of naïve BALB/c MNCs (Fig. 1B). From P10, MSCs induced the first significant allogeneic response, which increased in parallel with increasing number of passages (Fig. 1B).

The immunosuppressive properties of MSCs were further investigated using two standard in vitro immunological assays. In the first assay, MSCs from C57BL/6 mice were added to a one-way mixed lymphocyte culture (MLC) in which the stimulator cells were the mitomycin C-treated C57BL/6 MNCs and the responder cells were BALB/c MNCs (Fig. 2A). P7 MSCs were able to inhibit the MLC by up to 90% but this declined with increasing passage number to 10% at P14 (approximately 22 divisions after P5) (Fig. 2A). Similarly, MSCs also inhibited

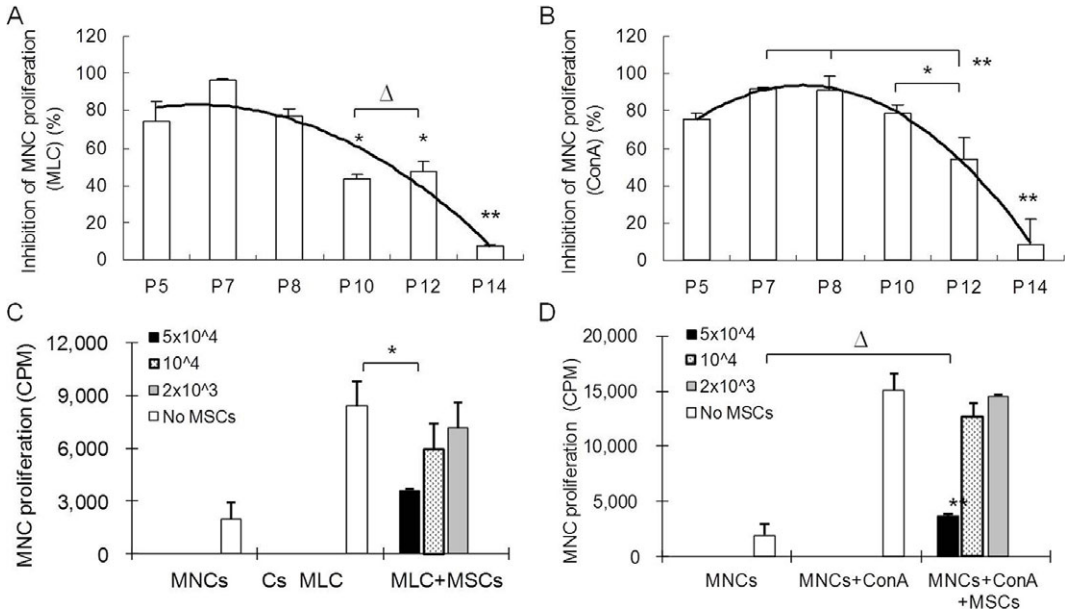

Fig. 2. Immuno-inhibitory effect of MSCs decreases with increasing passage number in vitro. (**A**) In MLC, the inhibition on BALB/c MNC proliferation by C57BL/6 MSCs decreased with increasing passage number. (**B**) Despite ConA stimulation, BALB/c MNC proliferation was inhibited by C57BL/6 MSCs. The immuno-inhibitory effect of MSCs decreased with increasing passage number. (**C**) In MLC, the inhibition on BALB/c MNC proliferation was significant when the number of P14 MSCs increased up to 5×10^4 cells. (**D**) Despite ConA stimulation, BALB/c MNC proliferation was inhibited by 5×10^4 of P14 MSCs. Bars in A and B represent mean ± s.d. of triplicates of two independent experiments. Bars in C and D represent mean ± s.d. of triplicates in one of three independent experiments. *$P<0.05$; **$P<0.01$; Δ, no statistical difference. The solid curves in A and B represent the inhibition trendlines of MNC proliferation elicited by MSCs at increasing passage number.

proliferation of concanavalin A (ConA)-stimulated BALB/c MNCs (Fig. 2B). This too decreased with increasing passage number. To determine whether the weak inhibitory effects of P14 MSCs on lymphocyte proliferation could be enhanced by increasing the ratio of MSCs to responder lymphocytes, different numbers of MSCs were added to the MLC (Fig. 2C) or to the ConA-stimulated MNCs (Fig. 2D). Both inhibitory effects were significant when the MSC number was increased to 5×10^4 cells/well.

Cytokine-mediated immune inhibition of MSCs at late passage numbers in vitro

The mechanisms underlying the immunogenic and immuno-inhibitory effects of P14 MSCs were investigated further. With the ratio of MSCs to MNCs fixed at 1:10, P14 MSCs inhibited the proliferation of MNCs stimulated by allogeneic MNCs with and without direct contact (Fig. 3A). The inhibition was greater when the MSCs were separated from the MNCs using a transwell system (Fig. 3B). Interestingly, transwells containing P8 and P14 MSCs exerted similar inhibitory effects (Fig. 3C).

Effects of TGF-β and interleukin-10 on the immuno-inhibition of MSCs in vitro

Because soluble molecules appear to play an important role in the inhibition of lymphocyte proliferation by MSCs up to P14, and because we have previously shown that transforming growth factor-β (TGF-β) and interleukin-10 (IL-10) were involved in immuno-inhibition by MSCs in rabbits (Liu et al., 2006), the effects of these molecules were further investigated in this study. Cultured alone, P14 MSCs secreted TGF-β (Fig. 4A), but not IL-10 (Fig. 4B). When MSCs were added to MLC, the concentration of TGF-β in the supernatant increased significantly, regardless of whether MSCs were separated from or in direct contact with MNCs (Fig. 4A). By contrast, IL-10 secretion significantly increased when MSCs were separated from MNCs by transwell inserts (Fig. 4B).

Further assay of culture supernatant showed that the addition of MSCs significantly decreased the amounts of pro-inflammatory factors, including interferon-γ (IFN-γ) and IL-13 (Fig. 4C,D), in MLC. Blocking TGF-β alone or with IL-10 increased the levels of IFN-γ and IL-13 significantly (Fig. 4C–E). There was no detectable IL-3, IL-4 or IL-17 in co-cultures of MNCs and MSCs (data not shown).

In vivo immune properties of MSCs at different passage numbers

We next determined whether the immunogenic and immunomodulatory properties of MSCs observed in vitro could be demonstrated in vivo. P8 and P14 MSCs were chosen as representative populations. BALB/c mice were primed by injection of C57BL/6 MSCs subcutaneously into the abdominal wall and challenged via the footpads with C57BL/6 MNCs 10 days later. Footpad swelling was determined 24 hours later. Footpad swelling induced by both P8 and P14 MSCs was significantly reduced compared with MNCs of the same strain (Fig. 5A). When MSCs at P8 or P14 were injected into the abdominal wall subcutaneously on the same day of challenge, the footpad swelling of BALB/c primed with MSCs at P8, but not P14, was further inhibited (Fig. 5B). Hematoxylin and eosin (H&E) staining of the footpads showed reduced cell infiltration in the footpads of mice primed with MSCs compared with those primed with allogeneic MNCs (Fig. 5C). There was no significant difference between the footpad swelling in mice primed with P8 or P14 MSCs. Major histocompatibility complex-I (MHC-I) expression on MSCs 48 hours post-implantation showed positive staining for both P8 and P14 MSC grafts (Fig. 5E).

MHC-I expression on MSCs under IFN-γ stimulation in vitro

MHC-I is the molecule most responsible for allo-immune reaction. We found stronger allo-immune reactions and weaker immuno-inhibitory effects of MSCs after P10 (Fig. 1B and Fig. 2A,B). It is unclear whether MSCs after P10 express an increased level of MHC-I on their surface. Flow cytometric results revealed that MSCs at all passage numbers expressed similar low levels of MHC-I in vitro (Fig. 5E).

In the 5-day MLC, there was a high level of IFN-γ secretion (Fig. 4C). It is unknown whether the sensitivity of MHC-I expression to IFN-γ is different among MSCs at different passage numbers. Consistently, MSCs at all passage numbers displayed increasing MHC-I expression with the extension of IFN-γ treatment (Fig. 5D,E). However, after 3 days of IFN-γ

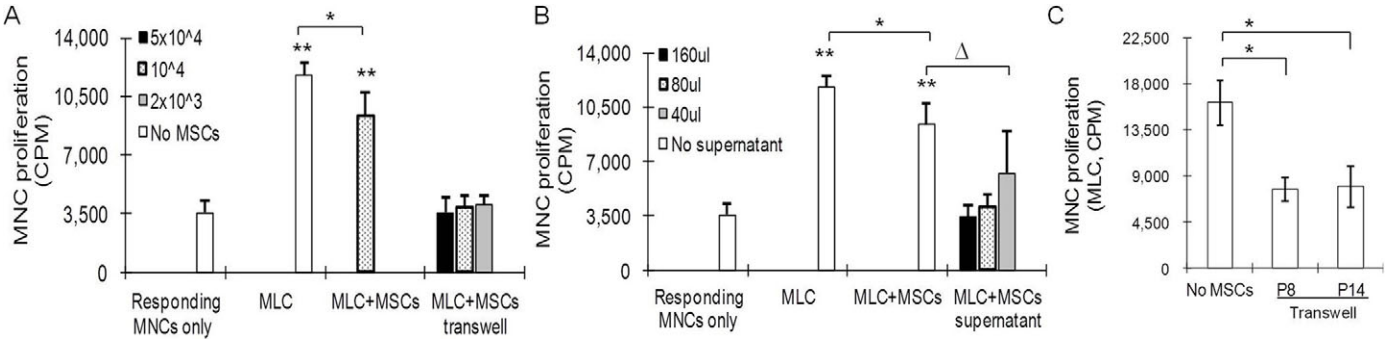

Fig. 3. Immuno-inhibitory effect of P14 MSCs was mediated by soluble molecules rather than by direct cell–cell contact. (**A**) Transwell-contained P14 MSCs exerted a significantly stronger inhibitory effect on MNC proliferation than that by direct MNC–MSC contact. (**B**) Addition of 80 μl or a larger volume of P14 MSC supernatant exerted a significantly stronger inhibitory effect on MNC proliferation than that by direct MNC–MSC contact. (**C**) Transwell-contained P8 and P14 MSCs had a similar inhibitory effect on MNC proliferation. Bars represent mean ± s.d. of triplicates in two independent experiments. $*P<0.05$; $**P<0.01$; Δ, no statistical difference.

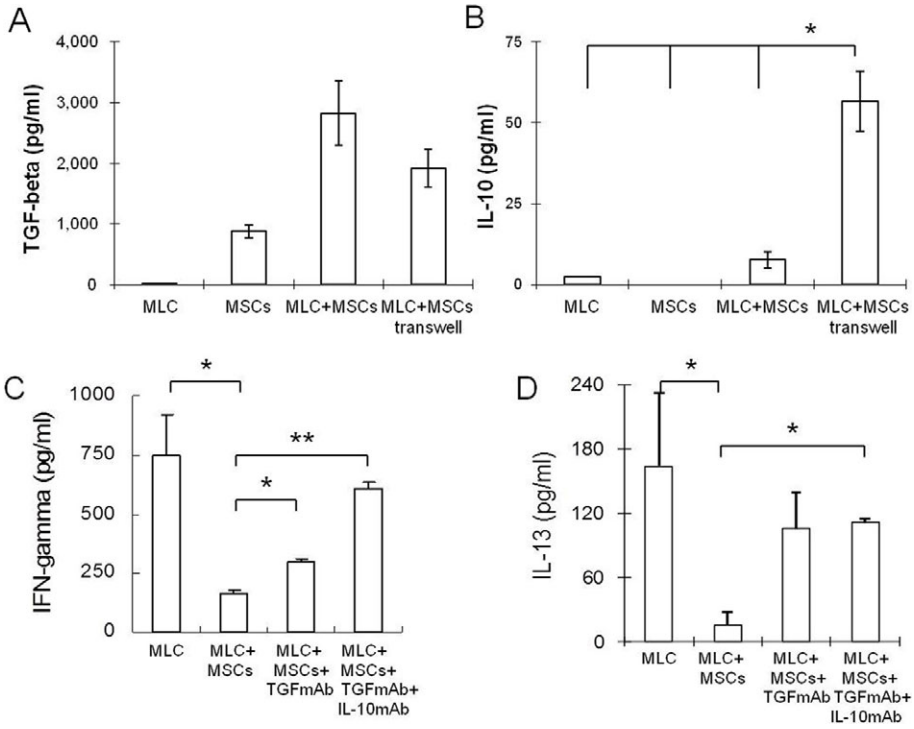

Fig. 4. Effect of TGF-β and IL-10 on the immuno-inhibition of MSCs in vitro. (**A**) TGF-β concentration was analyzed in the supernatant of MSCs under various culture conditions. (**B**) IL-10 concentration was analyzed in the supernatant of MSCs under various culture conditions. (**C**) IFN-γ concentration was analyzed in the supernatant of MSC and MLC co-culture with or without the blocking action of TGF-β and IL-10 mAbs. (**D**) IL-13 concentration was analyzed in the supernatant of MSC and MLC co-culture with or without the blocking action of TGF-β and IL-10 mAbs. Bars represent mean ± s.d. of duplicates of two independent experiments. *$P<0.05$; **$P<0.01$.

treatment, the increase in MHC-I expression on MSCs at passage numbers after P10 was significantly higher than the increases before P10 (Fig. 5E).

No significant change in TGF-β secretion by MSCs with extended in vitro culture

As mentioned earlier, the expression of MHC-I on MSCs was more easily induced at later passage numbers under the same dosage of IFN-γ stimulation, which partly accounts for the increase of immungenicity. We further investigated whether the decrease in immuno-inhibition was due to reduced TGF-β production by MSCs at later passage numbers. However, the bioassay showed that there was a similar inhibitory effect on Mv1Lu cell proliferation by MSCs at P8 and P14 (Fig. 5F). This suggests that there were similar levels of active TGF-β in their supernatants. When P8 and P14 MSCs were co-cultured with MLC, their supernatants exerted similar inhibitory effects on Mv1Lu cell proliferation. Furthermore, both had stronger inhibitory effects than the sole MSC supernatant (Fig. 5F). These results suggest that P8 and P14 MSCs secreted similar amounts of active TGF-β in response to an immune response, and this pro-inflammatory environment induces MSCs to produce more TGF-β.

Cytokine-mediated immuno-inhibitory effects of MSCs in vivo

To further verify the immuno-inhibitory effect of cytokines secreted by MSCs, P14 MSCs were placed in diffusion chambers and then implanted subcutaneously at challenge. Although the footpad swelling primed by P14 MSCs could not be inhibited by directly injecting the same cells at challenge, it was significantly inhibited by these chamber-encapsulated MSCs at 24 hours after challenge (Fig. 6A).

To determine whether TGF-β and IL-10 contributed to the immuno-inhibitory effect of the supernatant of P14 MSCs in vivo, blocking monoclonal antibodies (mAbs) were added to the chamber-encapsulated P14 MSCs. Successful blocking by TGF-β mAb was confirmed through the detection of much lower TGF-β levels in the sera of the BALB/c mice at 48 hours after mAb blocking compared to control animals minus mAb blocking (data not shown). However, no IL-10 was detected in the sera of the groups tested. Other pro-inflammatory cytokines, such as IL-3 and IL-4, were also undetectable in the sera of all groups. Delayed-type hypersensitivity (DTH) results at 24 hours after challenge showed no significant increase of footpad swelling rescued by MSC supernatant, regardless of whether cells were exposed to either TGF-β mAb alone or both mAbs (Fig. 6B), and there was no significant difference in the footpad swelling between the single mAb blocking and double mAb blocking.

Discussion

This study evaluates the relationship between MSC passage number and the immunostimulatory and immunosuppressive properties of MSCs. We demonstrate that with increasing passage number, MSCs become increasingly immunogenic and evoke greater allogeneic lymphocyte proliferation and diminishing immunosuppressive activity in vitro, which became statistically significant from P10 onwards. In vivo, the magnitude of footpad swelling caused by MSCs at P8 and P14 was not significantly different, regardless of their in vitro immunogenic status. In vivo, isolation of MSCs using a permeable chamber reduced the induction in MSC immunogenicity and permitted the cells to mediate immunosuppressive effects at greater passage numbers.

Increased immunogenicity with increasing passage number might be due to the induction of MHC-I molecules on MSCs.

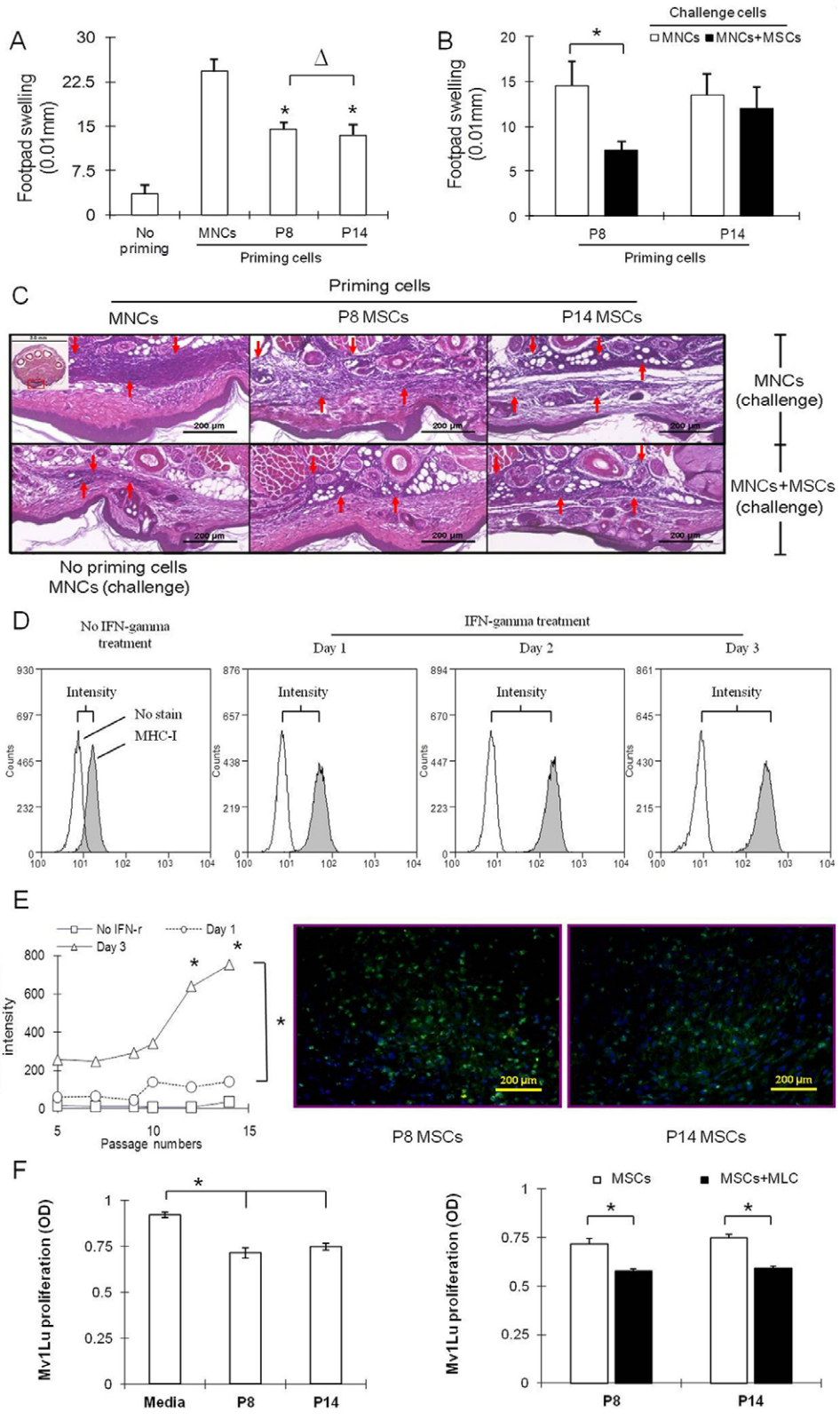

Fig. 5. Immune properties of MSCs in vivo, and MHC-I expression. (**A**) Footpad swelling of BALB/c mice was triggered by C57BL/6 MNCs after priming with C57BL/6 MSCs at either P8 or P14. (**B**) Footpad swelling of BALB/c mice was significantly alleviated by the co-implantation of P8 MSCs but not P14 MSCs at the challenge time point. (**C**) Footpads of all groups were harvested 48 hours after challenge and were stained by H&E. The co-implantation of P8 MSCs with the challenge of MNCs reduces lymphocyte infiltration. The inset image shows an example of the cross-sectional view of a mouse footpad at 1.25× objective magnification. Square box in the inset image shows the area photographed in the other images. Red arrows indicate the areas of lymphocyte infiltration. (**D**) After stimulation with IFN-γ in vitro, MHC-I expression on MSCs increased over time. (**E**) After 3 days of IFN-γ treatment in vitro, the MHC-I expression on MSCs at passage numbers greater than P10 was significantly stronger than on those before P10. Both P8 and P14 MSC grafts harvested 48 hours after challenge were positively stained with FITC-conjugated MHC-I. (**F**) In the TGF-β bioassay, the supernatant from MSCs at both P8 and P14 exerted similar inhibitory effects on Mv1Lu proliferation. Similar results were also observed in the supernatant derived from the co-culture of MSCs and MLC. However, the supernatant from MSC–MLC co-culture exerted significantly stronger inhibition than the supernatant from sole MSC culture. In A and B, bars represent mean ± s.d. of the thickness of all mouse footpads in the respective groups (n=3–6). In A and F, bars represent mean ± s.d. of triplicates of two independent experiments. *P<0.05; Δ, no statistical difference.

MHC expression by MSCs can be induced upon exposure to pro-inflammatory molecules IFN-γ (Le Blanc et al., 2003; English et al., 2007). Although only low levels of MHC-I expression were observed on all MSCs in normal culture conditions, significant expression was observed on both P8 and P14 MSCs after IFN-γ treatment and in vivo. MSCs after P10 expressed significantly stronger MHC-I after 3 days of low dose IFN-γ treatment than MSCs before P10.

Although MSCs at P14 displayed weak immuno-inhibitory effects in vitro, the supernatant of P14 MSCs exhibited strong

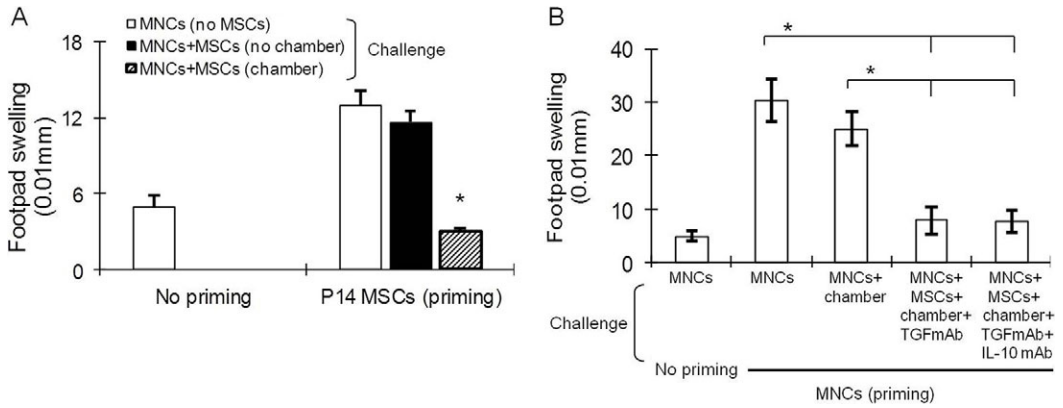

Fig. 6. Immuno-inhibitory effect of MSCs and their supernatant in vivo. (**A**) Footpad swelling of BALB/c mice was significantly relieved by co-implantation of chamber-loaded P14 MSCs with MNC challenge. (**B**) Application of TGF-β and IL-10 mAbs could not significantly reverse the inhibition on footpad swelling of BALB/c by the co-implantation of chamber-loaded P14 MSCs with MNC challenge. Data were based on the measurement of footpads 24 hours after challenge. Bars represent mean ± s.d. of the thickness of all mouse footpads in the respective groups (*$P<0.05$ compared with positive control).

immuno-inhibitory effects in co-cultures of MNCs and MSCs. This suggests a combination of immunostimulatory properties that require cell–cell contact and soluble immuno-inhibitory effects that do not require such contact. Similarly, a significantly enhanced inhibitory effect on allogeneic MNC proliferation and footpad swelling could be achieved by separating MSCs at P14 from the graft environment. Meanwhile, the TGF-β secretion levels were unchanged throughout extended culture of MSCs, as measured in the collected medium. These data imply that soluble molecules play a dominant role in the immuno-inhibitory properties of MSCs and that, over an extended period of time, these secreted molecules can partly counteract the immunostimulatory properties of MSCs. The decreasing immuno-inhibitory activity of MSCs could be caused by the increasing expression of MHC-I but not by the decreasing levels of anti-inflammatory cytokines. It was previously reported that some pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1α and IL-1β (Krampera et al., 2006; English et al., 2007; Ren et al., 2008), can activate the anti-inflammatory properties of MSCs by inducing production of soluble anti-inflammatory factors. Additionally, the inhibitory effect on footpad swelling exerted by encapsulated P14 MSCs injected into the abdominal wall indicates that soluble molecules are able to exert an inhibitory activity that is distal to the target site.

Our previous work with rabbit MSCs demonstrated that both TGF-β and IL-10 contribute to MSC immuno-inhibition (Liu et al. 2006). We therefore determined whether these molecules were responsible for the immuno-inhibitory properties of mouse MSCs. It was shown that TGF-β was at a high level in both MSC–MNC contact and separation conditions, whereas IL-10 was at a high level only when MSCs were separated from MNCs. This suggests that MSCs secretions are able to induce MNCs to produce IL-10, and that direct physical contact between MSCs and MNCs inhibits this. We also observed that blocking TGF-β and IL-10 increased the levels of pro-inflammatory cytokines IFN-γ and IL-13 in the co-culture of MSCs and MNCs. The results suggest that these two factors play an important role in the allogeneic immuno-inhibition of MSCs. However, the blockade of TGF-β and IL-10 in vivo could not reverse the immuno-inhibition of MSCs to the level of the group without MSCs. This implies that the immuno-inhibition of MSCs is the final result of multiple anti-inflammatory factors. TGF-β and IL-10 are not dominant factors that can completely account for this immuno-inhibition of murine MSCs. As reported, other mediators could include indolamine 2,3-dioxygenase, prostaglandin E2, HLA-G5 and nitric oxide (Le Blanc, 2006; Ren et al., 2008; Selmani et al., 2008).

In vivo, allogeneic MSCs are reported to prime weaker DTH responses than allogeneic MNCs (Barrington-Leigh, 1984; Cher and Mosmann, 1987; Luo and Dorf, 2007), suggesting that the capacity of MSCs to induce an immune response is limited. It is probable that MSCs attract lymphocytes to the graft area (Ren et al., 2008) or prevent donor T lymphocytes from migrating to target tissue by trapping these cells in secondary lymphoid organs (including lymph nodes) (Li et al., 2008). The infiltration of lymphocytes at the MSC graft sites was indeed observed and was reported in our previously published study (Liu et al., 2009). These data therefore suggest that MSCs affect lymphocyte migration.

In summary, the gradual loss of immuno-privileged and immuno-suppressive properties with increasing passage number is an innate feature of MSCs that partly explains the onset of immunogenicity. TGF-β and IL-10 mediate some of the immuno-inhibitory activities of MSCs, which might not require the presence of MSCs at the target site. These data provide a theoretical basis for prolonging the effect of MSCs in immunological therapy by encapsulation of these cells. These data further highlight the importance of reporting the passage number or the number of cell divisions undergone by MSCs in immunological studies, an aspect that is frequently overlooked.

Materials and Methods

Animals

All experimental protocols involving mice were approved by the Institutional Animal Care and Use Committee of the National University of Singapore. Female C57BL/6 and BALB/c mice, 6–8 weeks old, were purchased from the Comparative Medicine Centre and housed in the satellite Animal Holding Unit of the National University of Singapore.

Isolation and expansion of MSCs

Bone marrow from the femurs and tibiae of C57BL/6 mice were cultured in MesenCult basal medium (05501, Lot# 07B21265, Stem Cell Technologies, Canada), supplemented with 20% (v/v) MSC stimulatory supplements (05502, Lot# 06M20759, Stem Cell Technologies) and 1% (v/v) penicillin–streptomycin

solution (PS; Sigma) at 37℃ for 3 days. Adherent cells were selected for subsequent serial passages. To remove contaminating hematopoietic cells, P2 cells were incubated with silica microspheres (Bangs Laboratories, Fishers, IN) in RPMI-1640 basic media (Sigma) at 37℃ for 1 hour and subjected to Ficoll-Hypaque (GE Healthcare, UK) fractionation. The interlayer cells were cultured to confluency and negatively selected with a Lineage Cell Depletion Kit (Miltenyi Biotec, Cologne, Germany) following the manufacturer's instructions. Negatively selected cells were seeded at 1×10^4 cells/cm^2 up to P5, and at 5×10^3 cells/cm^2 for subsequent passages. At P5, MSCs with negative expression of lineage markers, CD11b, CD45, CD117 and MHC-II, and positive expression of CD34, Sca1, CD73, and MHC-I (at low levels) were characterized by flow cytometry (Liu et al., 2009). These cells were successfully induced to osteogenic, chondrogenic and adipogenic lineages in vitro (Liu et al., 2009), and were continuously expanded. All media in this experiment were tested as mycoplasma-free by the supplier. Cell proliferation was shown by the population doublings, which were calculated as $\log2(N/N_0)$, where N is the final number of cells and N_0 is the initial number of cells.

Alloimmune responses of MSCs in vitro

Alloreactivity of MSCs

BALB/c spleen MNCs (1×10^5 cells/well) were co-cultured with mitomycin C-inactivated MSCs (1×10^4 cells/well) at varying serial passage numbers in 200 µl of media [RPMI-1640 containing 5% FBS (Biowest, Nuaillé, France), 50 µM 2-mercaptoethanol (Sigma) and 1% PS] within 96-well U-bottom plates for 5 days. The same number of BALB/c MNCs in culture without MSCs was utilized as negative control. At 16 hours before harvesting, 0.5 µCi/well of [^{3}H]thymidine (BioRad Laboratories) was incorporated into proliferating cells and cell numbers recorded as counts per minute (CPM) using a scintillation counter (TopCount NXT, PerkinElmer). The readings of all mitomycin C-treated C57BL/6 MNCs were less than 550 CPM.

One-way mixed lymphocyte culture

MSCs (1×10^4 cells/well) at various serial passage numbers were added into the co-culture of BALB/c MNCs (1×10^5 cells/well) and inactivated C57BL/6 MNCs (1×10^5 cells/well) in triplicates and incubated for 5 days. The co-culture of BALB/c and C57BL/6 MNCs in wells without MSCs was utilized as positive control. The inhibition of BALB/c MNC proliferation (%) was calculated as $(CPM_{positive\ control}-CPM_{experimental\ group})\times100/CPM_{positive\ control}$.

Mitogen-induced MNC proliferation assay

MSCs (1×10^4 cells/well) at various serial passage numbers were co-cultured with BALB/c MNCs (1×10^5 cells/well) and 3 µg/ml of ConA (Sigma) in 96-well U-bottom plates for 48 hours. Tritium-labeled thymidine was added 8 hours before harvesting, and the cell numbers were recorded as CPM. The co-culture of BALB/c MNCs and ConA in wells without MSCs was utilized as positive control. The inhibition of BALB/c MNC proliferation was calculated as described above.

Transwell and supernatant assays

Different numbers of MSCs at P14 (2×10^3, 1×10^4 and 5×10^4, respectively) were seeded into eight-well strip transwell inserts (0.2 µm pore size; Nunc, Roskilde, Denmark) on 96-well plates that contained BALB/c MNCs (1×10^5 cells/well) and inactivated C57BL/6 MNCs (1×10^5 cells/well). After 5 days, the proliferating cell numbers were recorded as previously described. To double-check the secretion effect, supernatants were collected from 5-day cultures of MSCs at P14. Then, 160, 80 and 40 µl of MSC supernatants were transferred into 96-well flat bottom plates that contained BALB/c MNCs (1×10^5 cells/well) and inactivated C57BL/6 MNCs (1×10^5 cells/well). The volume of each well in this supernatant assay was topped up to 200 µl with culture media. After 5 days, the proliferating cell numbers were recorded. The co-culture of BALB/c MNCs and inactivated C57BL/6 MNCs in wells without MSCs served as positive controls. For comparison with the immuno-inhibitory effect of MSCs by direct contact, the co-culture of BALB/c MNCs, inactivated C57BL/6 MNCs and MSCs was also tested on the same plate. To measure the supernatant effect of MSCs at P14 on lymphocyte proliferation, MSCs at P8 (1×10^4 cells) were seeded into the transwell inserts on the 96-well plates containing MLC, and the cell numbers were recorded as previously described.

Supernatant collection for multiplex assay and TGF-β bioassay

MSCs at P14 (1×10^5 cells/well) were seeded into 24-well plates directly or within transwell inserts (0.4 µm pore size; BD Falcon) on 24-well plates. Each well of these 24-well plates contained 1×10^6 of BALB/c MNCs and 1×10^6 of inactivated C57BL/6 MNCs in 1 ml of media. To test the effect of TGF-β and IL-10 on the immuno-inhibitory properties of MSCs, TGF-β (Clone 1D11, R&D Systems) and IL-10 (Clone JESS-2A5, eBioscience) blocking mAbs were added into the above wells. Both blocking antibodies were utilized at a concentration of 10 µg/ml. After 5 days, the supernatants were kept for TGF-β bioassay or stored at −80℃ for multiplex testing. Additionally, supernatants from MSC monocultures were collected as controls.

MHC-I expression on MSCs after IFN-γ treatment

MSCs at all tested passage numbers (1×10^4 cells/cm^2) were incubated with recombinant murine IFN-γ (100 U/ml, Cat# 315-05, PeproTech, Rocky Hill, NJ) in T25 flasks for up to 3 days. Cells before and 24 or 72 hours after IFN-γ treatment were stained with MHC-I antibody for flow cytometry analysis. The fluorescence intensity of MHC-I on the cell surface was calculated by the deduction of the median fluorescence of cells without MHC-I antibody staining from that of the stained cells within the same sample.

Alloreactivity of MSCs in vivo

Delayed-type hypersensitivity (DTH) test

After anesthesia, BALB/c mice were injected subcutaneously with 1×10^6 of MSCs in 200 µl of PBS on both sides of the abdominal wall (priming). At 10 days post-implantation, 5×10^6 of C57BL/6 MNCs in 25 µl of PBS were injected into one hind footpad of BALB/c subcutaneously (challenge). Another 25 µl of PBS were injected into the other hind footpad of BALB/c subcutaneously as the internal negative control. The footpad thickness was measured 24 hours after immunological challenge with a micrometer (Mitutoyo, Japan) and the footpad swelling was calculated as described previously (Luo and Dorf, 2007; Yamashita et al., 2009). To establish experimental controls, footpads challenged with C57BL/6 MNCs without priming served as negative controls. Footpads primed with C57BL/6 MNCs and challenged with C57BL/6 MNCs served as positive controls. There were 4–6 mice per group.

To study the inhibitory effect of MSCs on footpad swelling, two methods of infusion were applied at the time of challenge. One method was to subcutaneously inject MSCs (5×10^6 cells/ml, 180 µl) into BALB/c abdominal wall. The other method was to include MSCs (5×10^6 cells/ml, 180 µl) in diffusion chambers (Millipore) that were inserted into the BALB/c abdominal wall subcutaneously. To study the effects of TGF-β1 and IL-10 on MSC immuno-inhibition, TGF-β1 blocking mAb (100 µg/ml) and IL-10 blocking mAb (100 µg/ml) were premixed with MSCs. To control the release of blocking antibodies locally, 0.5% of hydrogel (PuraMatrix, 3DM, Cambridge, MA) was used to embed MSCs and antibodies in the diffusion chambers.

Harvesting MSC grafts for fluorescence staining

At 48 hours post-challenge, the whole skin around the MSC priming area was cut, fixed and embedded in OCT compound media (Jung, Leica Microsystems, Wetzlar, Germany). The successful injection showed protrusion of skin. Specimens were sectioned at 6 µm in thickness. The slides were blocked and stained with FITC-conjugated MHC-I mAb (1:12.5; K^b, clone 2G5, eBioscience) at room temperature for 1 hour. Slides were mounted in Vectashield mounting medium (H-1200, Vector Laboratories) and observed under fluorescence microscopy.

MSC, MNC and serum harvest for flow cytometry and multiplex assay

After IFN-γ treatment, MSCs were harvested at 24 and 72 hours by trypsinization. At 48 hours post-challenge, BALB/c spleen and blood of all groups were harvested. MNCs were derived from the fractionation of spleen and blood cells on Ficoll-Hypaque for subsequent flow cytometry analysis. Serum was stored at −80℃ for multiplex analysis.

Harvesting footpads for H&E staining

At 48 hours post-challenge, BALB/c footpads of all groups were harvested and fixed in 4% paraformaldehyde. After decalcification in 10% acetic acid for 2 weeks, footpads were cross-sectioned at the root of toes before paraffin embedding. Sections of 5 µm thickness were made and stained with H&E. Images were captured under an inverted microscope (Olympus IX70, Japan). All pictures were taken at 10× objective magnification.

Multiplex assay

Harvested supernatant and serum was tested with Procarta mouse cytokine assay kits (Panomics, Fremont, CA) following the manufacturer's instructions. The kits were designed for examining IL-3, IL-4, IL-10, IL-13, IL-17 and IFN-γ levels in a single sample, as well as the total TGF-β1 level. Briefly, premixed antibody beads were added into a pre-wet filter plate and washed. Then, 50 µl of samples, 25 µl of premixed detection antibodies and 50 µl of streptavidin-PE were added and incubated in sequence. Each incubation step took 30 minutes. Lastly, 120 µl/well of reading buffer was added and results read using a Luminex 100 plate reader (Qiagen). For TGF-β1 assay, cell culture supernatant and serum were activated with 1 N HCl and neutralized with 1.2 N NaOH in 0.5 M HEPES as instructed.

TGF-β bioassay

To confirm the effect of the active form of TGF-β in the MSC supernatant, the inhibition of Mv1Lu cell proliferation by active TGF-β was examined (Mazzieri et al., 2000). Freshly harvested supernatant (100 µl) was added to 4×10^4 Mv1Lu cells (CCL-64, ATCC) per well in 96-well plates and cultured at 37℃ for 24 hours. Subsequently, 20 µl of MTS was added into each well of the co-culture and incubated for further 3 hours, prior to measurement of optical density at 490 nm with a microplate reader.

Statistical analysis

Quantitative results were analyzed by *t*-tests or ANOVA (SPSS 13.0 for Windows). A value of $P<0.05$ and $P<0.01$ was considered significantly different.

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