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Regulation of MHC Class II Expression and Antigen Processing in Murine and Human Mesenchymal Stromal Cells by IFN-γ, TGF-β, and Cell Density

Raphaëlle Romieu-Mourez, Moïra François, Marie-Noëlle Boivin, John Stagg, and Jacques Galipeau

Mesenchymal stromal cells (MSC) are bone marrow-derived mesenchymal progenitors thought to give rise to cells that constitute the hematopoietic microenvironment. MSC are classically expanded in vitro from the plastic-adherent cell population from bone marrow aspirates. MSC can serve as precursors for the generation of a variety of nonhemopoietic tissues, including bone, cartilage, muscle, and neural elements. As a result, they have been extensively studied as potential tools to decipher this enigma, we investigated the molecular mechanisms underlying IFN-γ-induced MHC II-mediated APC functions in MSC. Our observations made in mouse and human MSC suggest that cell culture parameters, such as cell density, serum factors, and cell density in vitro, although not in the same way in mouse and human MSC, via their convergent effects on CIITA expression. The Journal of Immunology, 2007, 179: 1549–1558.

Materials and Methods

MSC culture and treatment

Mouse primary MSC were obtained from bone aspirates of 4- to 6-wk-old BALB/c or C57BL/6 mice like previously shown (12). Both femurs and tibias from each leg were isolated and cleaned from any remaining flesh. Bones were then flushed with complete medium (high glucose DMEM, 10% FBS, and 100 U/ml penicillin and streptomycin) to extract the bone marrow, which was plated for 5 days. Fresh complete medium was added every 3–4 days until the culture reached 80% confluence. In routine maintenance culture, mouse MSC were seeded at 500 cells/cm² every 4 days since they reached confluence after 7...
cytometer and data were analyzed using CellQuest software (BD cytometry analysis was performed on 20,000 events using a FACSCalibur Systems. T hybridoma cells (10^5) were added in a total volume of 200 µl. MSC populations were tested for the absence of CD31/H11001. 4–20% Tris-glycine gel (Invitrogen Life Technologies) and transferred for 24 h. Afterward, cells were lifted by incubation for 2 min (OVA)-specific I-Ab-restricted MF2.2D9 CD4^+ T hybridoma cell line were provided by K. L. Rock (University of Massachusetts, Worcester, MA) and cultured in RPMI 1640, 10% FBS, and 100 U/ml Pen/Strep. Donors 240L, 5066R and 5068L were a 24 year-old male, 22 year-old female, and, 24 year-old male, respectively. Karyotypes of MSC from donor 240L has been established by D. J. Prokop and colleagues. For this task, cells were cultured and harvested using standard cytogenetic procedures. Twenty metaphases were examined and normal karyotypes were observed, in the absence of consistent numerical or structural chromosome anomalies (D. J. Prokop, personal communication). Human MSC were expanded for one to six passages by plating cells every 7 days at either 100 or 2000 cells/cm^2, as indicated. Both human and mouse MSC populations were tested for the absence of CD31^+ or CD45^+ cells, expression of CD34, CD44, CD73, CD90, and CD105, and ability to differentiate into adipocytes and osteocytes as described elsewhere (12). Recombinant human (rh) and mouse (rm) IFN-γ were purchased from BioSource International and rhTGF-β was obtained from R&D Systems. Cell lines C57BL/6 mouse DC2.4 dendritic cells (DC) and chicken egg white albumin (OVA)-specific I-A^b-restricted MF2.2D9 CD4^+ T hybridoma cell line were provided by K. L. Rock (University of Massachusetts, Worcester, MA) and cultured in RPMI 1640, 10% FBS, and 100 U/ml Pen/Strep and 50 µM 2-ME. Mouse Btk CL.4 (ATCC TIB-81) fibroblastic cells and B9.d.5 (ATCC CRL-2999) endothelial cells were purchased from American Type Culture Collection. Primary HUVEC were obtained from Cambrex. Flow cytometry analysis The following Abs were used for flow cytometry analysis: PE-conjugated anti-mouse CD45 (clone 30-F11), CD105 (clone M37/18; eBioscience), CD90 (clone 16-10A1), D^3 or K^2 (MHC II, clone K9H5 or ApF6-88.5), I-A^b (MHC II, clone Af6-120.1), or B7-H1 (clone MIIH5; eBioscience); FITC-conjugated anti-human CD105 (clone 8E11; Chemicon International); biotin-coupled anti-human CD90 (clone SE10) or CD45 (clone H30); PE-conjugated anti-human CD31 (clone WM-59) or CD73 (clone AD2); allophycocyanin-conjugated anti-human CD34 (clone 581) or CD44 (clone G44-26); PerCP-conjugated anti-human HLA-DR Ab (MHC II, clone L243), and isotypic controls. Except where indicated, Abs were purchased from BD Biosciences. For cell cycle analysis of mouse MSC, surface staining of I-A^b was first performed and cells were fixed with 1% paraformaldehyde (PFA), washed with PBS and with the BD Biosciences Perm/Wash solution, and stained overnight with 7-aminoactinomycin D (BD Biosciences). The absence of genomic DNA contamination was demonstrated using the relative quantification (based on the relative expression of target genes vs Gapdh) was used for PCR amplification in a 20-µl reaction volume containing the Thermus aquaticus Taq polymerase (Upstate Biotechnology). Type-specific primers for mouse C20c to type I, type II, and type IV were designed by Pai et al. (16). GAPDH primers were as described previously (17). Other mouse primers were: Accvii (ALK1, NM_006912.1) forward 1613-ACA CCCACATCCTCAAC-1631 and reverse 1667-ACGCCACCTCTCT CATCATCG-1688 (ampliplex 76 bp); Tggbr (ALK5, NM_005707.2) forward for 1236- AAATTCCTGACAGCTGTTT-1255 and reverse 1280-GTGA CGAGTTCTCATAA/AGGCAACTG-1305 (ampliplex 70 bp); and Actb (β-actin, NM_007393.1) forward 1010-TGACAGGATGCGAGGA-1029 and reverse 1067-CGTCAGAGGAGAAGATG-1084 (ampliplex 75 bp). These primers were designed using the Roche Applied Science Universal Probe Library web site and had an annealing temperature of 58°C. Real-time PCR Quantitative PCR assays for CIITA and β-actin mRNAs were performed in duplicate using the Universal Probe Library system (Roche Applied Science). Primers were used for determination of total mouse CIITA mRNA levels (AF100709.1 in the region shared by all isoforms) were: forward 2939-GATGTTGAGAAGCTGTATC-2958 and reverse 2982-TGCTATC TCTGAGGTTTTC-3001 and were used with the Universal Probe Library 110. Primers specific for mouse Actb (β-actin) were as described above and used with Universal Probe Library 106. Quantitative PCR assays specific for mouse Gapdh were performed using the SYBR Green I reagent (Roche Applied Science) as previously detailed (17). PCR efficiency corrections were determined for all primers by establishing standard PCR curves performed on a mixture of cDNA pooled from IFN-γ-macrophages, splenocytes, and MSC. Data were collected using the LightCycler 2.0 Real-Time PCR System (Roche Applied Science) and analyzed using the relative quantitation (based on the relative expression of target genes vs β-actin or GAPDH as reference genes) with a normalized calibration with an efficiency correction method on the LightCycler analysis software. The absence of genomic DNA contamination was demonstrated routinely by analysis of PCR performed with total RNA and with each of the primer sets. Growth response to TGF-β MSC were seeded in 96-well plates and treated or not with 4–40 pM (0.1–1 ng/ml) rhTGF-β for 3 days before performing a MTT assay using the Cell Titer96 reagent (Promega) to measure cell viability. Conversion of tetrazolium compound to a 490-nm absorbing formazan product was measured on an ELX800 microplate reader (Bio-Tek Instruments). Immunoblot analysis Cell pellets were resuspended in Cell Lysis Buffer (Sigma-Aldrich) complemented with protease inhibitors (Roche). Protein concentration of whole cell extracts (WCE) was determined using a Bradford protein assay (Bio-Rad). Equal amounts of WCE (20–40 µg) were run on a 4–20% Tris-glycine gel (Invitrogen Life Technologies) and transferred onto a 0.45-µm polyvinylidene fluoride membrane (Millipore). Primary Abs were specific for: phospho-Stat1 (Tyr401; Cell Signaling), phospho-Smad2/Smad3 (Ser465/467; Cell Signaling), phospho-Smad1/Smad5/Smad8 (Ser463/465; Cell Signaling), HLA-DR (B2A2; Santa Cruz Biotechnology) or α-Tubulin (Tu-02; Santa Cruz Biotechnology). Secondary Abs were either HRP-conjugated sheep anti-rabbit IgG or rabbit anti-mouse IgG (GE Healthcare) and were revealed using the ECL Advance solution (GE Healthcare). Immunoblots were exposed to films (Kodak BioMax MR films) for 5 to 10 min. Figures show scans of films with exposures below saturating levels of the chemoluminescent signal.

**RT-PCR** DNA-free total RNA was prepared using the RNeasy kit with DNase digestion (Qiagen). RNA (2 µg) was reverse transcribed with the avian myeloblastosis virus reverse transcriptase (Roche Applied Science) using the random primer p(dN)6. An aliquot of one-fifth of the resulting cDNA was used for PCR amplification in a 20-µl reaction volume containing the Thermus aquaticus Taq polymerase (Upstate Biotechnology). Type-specific primers for mouse C20c to type I, type II, and type IV were designed by Pai et al. (16). GAPDH primers were as described previously (17). Other mouse primers were: Accvii (ALK1, NM_006912.1) forward 1613-ACA CCCACATCCTCAAC-1631 and reverse 1667-ACGCCACCTCTCT CATCATCG-1688 (ampliplex 76 bp); Tggbr (ALK5, NM_005707.2) forward for 1236- AAATTCCTGACAGCTGTTT-1255 and reverse 1280-GTGA CGAGTTCTCATAA/AGGCAACTG-1305 (ampliplex 70 bp); and Actb (β-actin, NM_007393.1) forward 1010-TGACAGGATGCGAGGA-1029 and reverse 1067-CGTCAGAGGAGAAGATG-1084 (ampliplex 75 bp). These primers were designed using the Roche Applied Science Universal Probe Library web site and had an annealing temperature of 58°C.
(data not shown) occurred mostly in high cell density MSC cultures. By contrast, there was no difference in MHC I or B7-H1/PD-L1 expression levels up-regulated by IFN-γ in low- and high-cell density cultures; in addition, expression of endoglin (CD105) was unchanged by IFN-γ or cell density. Two other C57BL/6 as well as one BALB/c MSC preparations were tested for IFN-γ-induced MHC II and B7-H1 expression in cells plated at high or low density, and expression patterns were similar to those described above (data not shown).

Next, MHC II-mediated Ag processing was tested. C57BL/6 mouse MSC cultures at different cell densities were incubated for 24 h with IFN-γ and OVA as a source of soluble exogenous Ag. Positive controls included the C57BL/6 DC2.4 immortalized DC line, which displays constitutive APC functions. APC were fixed with PFA and exposed to the CD4+ MF2.2D9 T hybridoma cells that are specific for an OVA C-terminal epitope (OVA 323–339, ISQAVHAAHAEINAGR) presented in the context of I-Aβ molecules. T hybridoma cells are not dependent on costimulation signals for activation and respond to the presence of specific Ag-MHC complexes on APC by the production of IL-2. Levels of IL-2 were measured supernatants from APC and T hybridoma cells cocultures by ELISA. It revealed that high-density MSC cultures were more easily primed for MHC II-mediated processing of OVA when activated with IFN-γ compared with low-density cultures (Fig. 1B). As expected, low- and high-density cultures of IFN-γ-activated BLK CL.4 fibroblasts did not process OVA, although they expressed MHC II (data not shown). Thus, MSC from high-density cell cultures display increased responsiveness to IFN-γ for MHC II expression and Ag processing.

IFN-γ-induced MHC II expression in mouse MSC is not correlated with the cell cycle and is increased by serum starvation or cell connectivity

To test whether MHC II up-regulation by IFN-γ was dependent on the cell cycle status of MSC, IFN-γ-activated MSC from low- and high-cell density cultures were subjected to analysis of MHC II expression and DNA content levels by flow cytometry. High-density cultures accumulated cells mostly in the G0-G1 and G2-M phases (Fig. 2A, left panel). Low-density MSC cultures contained asynchronous cells and the higher proportion of cells in the S phase, which is most likely linked to optimal cell culture conditions since cells in the S phase were less numerous after serum deprivation in low-density MSC cultures. As described above, low-density MSC cultures have a blunted IFN-γ-induced MHC II expression response compared with high-density cell cultures (Fig. 2A, right panel). In both, however, the proportion of cells in the G0-G1, S, or G2-M phases expressing MHC II was similar. Surprisingly, IFN-γ-induced MHC II expression was strongly up-regulated by the removal of serum factors, and this effect occurred regardless of the cell cycle status. Accordingly, serum-starved low-density MSC cultures exhibited increased ability to process soluble OVA and present I-Aβ-restricted OVA epitopes to CD4+ T hybridomas (Fig. 2B). Thus, the ability of mouse MSC to express MHC II in response to IFN-γ is not directly related to the cell cycle but can be up-regulated by high cell density, possibly through increased cell connectivity, or the removal of serum factors.

Signaling pathways involved in IFN-γ-induced MHC II expression in murine MSC

To study the regulation of the IFN-γ response, a major downstream event to the activation of the IFN-γ receptor, namely, STAT1 activation, was assessed. The phosphorylation of STAT1
was detected as early as 10 min after IFN-γ stimulation in high- and low-confluent MSC cultures, as seen in immunoblots on WCE using a phospho-specific Ab (Fig. 3A). STAT1 phosphorylation was monitored in cells exposed to decreasing concentrations of IFN-γ and an identical response was observed in high- and low-confluent MSC cultures (Fig. 3B). Consistent with this finding, the IFN regulatory factor 1 (IRF-1)-dependent B7-H1 encoding gene was also unaffected by cell density (Fig. 1A), and these results suggest that IFN-γR-mediated activation of STAT1 and IRF-1 are not impaired in low-density mouse MSC cultures.

The regulation of MHC II expression and Ag processing occurs predominantly at the transcriptional level and the CIITA protein is a potent activator for MHC II gene transcription (20). CIITA mRNA expression was assessed in MSC by quantitative RT-PCR analysis of CIITA forms I, III, and IV and β-actin as a control. Twenty-five, 30, 35, and 40 cycles were conducted for each amplification and PCR products were analyzed by electrophoresis on 3% agarose gels. Photographs show ethidium bromide fluorescence of PCR products before reaching saturation levels for CIITA form IV and β-actin and at 40 cycles for CIITA forms I and III. Negative controls to test genomic DNA contamination included PCR performed with RNA samples not subjected (−) to reverse transcription (RT).

FIGURE 2. Absence of correlation between cell cycle and IFN-γ-induced MHC II expression in high- or low-density mouse MSC cultures. A, Flow cytometry analyses of high- vs low-cell density or serum-starved mouse MSC cultures. C57BL/6 MSC were plated at 12,000 or 550 cells/cm² in complete medium. Where indicated, medium was replaced with serum-free medium 2 days later. Twenty-four hours later, cells were left untreated or treated with 30 U/ml rmIFN-γ for an additional day. Cells were harvested by trypsinization and incubated with PE-conjugated anti-I-Aβ Ab or PE-conjugated mouse IgG2a isotype (negative) control Ab. Cells were then fixed, permeabilized, counterstained with 7-aminoactinomycin D (7AAD) for DNA content, and analyzed by flow cytometry. Cell cycle phase is denoted as G0-G1, S, and G2-M. B, APC assay on serum-starved mouse MSC cultures. C57BL/6 MSC or BLK CL.4 cells were plated at 550 cells/cm². Where indicated, medium was replaced with serum-free medium 2 days later. Twenty-four hours later, all cell cultures were treated with 30 U/ml rmIFN-γ in the presence or absence of 2 mg/ml OVA for an additional day and processed as described in Fig. 1B.

FIGURE 3. IFN-γ-induced STAT1 phosphorylation and expression of CIITA in high- or low-density mouse MSC cultures. A, STAT1 phosphorylation. C57BL/6 MSC were plated at 12,000 or 550 cells/cm² in complete medium for 3 days. Cells were left untreated or treated with 30 U/ml rmIFN-γ for 10 or 20 min. Samples of WCE were run on a SDS-PAGE gel and subsequently subjected to immunoblot analysis of STAT1 phosphorylation at residue Tyr701 or α-tubulin expression, as a control. B, Same as A, except that cells were left untreated or treated with different doses of rmIFN-γ (0.15–10 U/ml) for 20 min. C, CIITA expression. C57BL/6 MSC or BLK CL.4 cells were plated at 12,000 or 550 cells/cm² in complete medium for 3 days. Splenocytes and peritoneal macrophages were harvested from C57BL/6 mice. Cells were left untreated or treated with 30 U/ml rmIFN-γ for 18 h. Total DNase-treated RNA was prepared and processed for quantitative RT-PCR analysis of CIITA mRNA levels with primers and probe common for all isoforms. Levels of CIITA were obtained by normalizing to GAPDH levels. Similar results were obtained when using β-actin as a reference gene for normalization (data not shown). D, Same as C, except that RNA were processed for RT-PCR analysis of mRNA expression of CIITA forms I, III, and IV and β-actin as a control. Twenty-five, 30, 35, and 40 cycles were conducted for each amplification and PCR products were analyzed by electrophoresis on 3% agarose gels. Photographs show ethidium bromide fluorescence of PCR products before reaching saturation levels for CIITA form IV and β-actin and at 40 cycles for CIITA forms I and III. Negative controls to test genomic DNA contamination included PCR performed with RNA samples not subjected (−) to reverse transcription (RT).
cytes in complete medium for 2 days. Cells were left untreated or treated with rhTGF-β1 for 3 days and subjected to a MTT cell viability assay measured by tetrazolium reduction to a 490-nm absorbance live cells and processed for RT-PCR analysis of mRNA expression.

**FIGURE 4.**

**TGF-β signaling pathways and effects on IFN-γ induction of MHC II expression in mouse MSC.**

Various factors such as IL-1β, IL-4, TGF-β, and IL-10 have been shown to block the up-regulation of MHC II expression induced by IFN-γ in various cell types, e.g., astroglia cells or DC (21–25). We examined the response of MSC to TGF-β with respect to cell viability, signaling, and regulation of MHC II expression. MSC were moderately sensitive to either growth increase or inhibition by TGF-β at high- or low-cell density, respectively (Fig. 4A). Mammals express one TGF-β type II receptor (TGF-βRII) and two TGF-βRI, i.e., ALK5 and ALK1/TSR-I. Although ALK5 is ubiquitously expressed, ALK1 was observed predominantly present in blood vessels, mesenchyme of the lung, submucosal layer of the stomach and intestines, and at sites of epithelial and mesenchymal interactions during mouse embryo development (26) and in vitro in human endothelial cell lines (27). In this study, expression of ALK5 and ALK1 mRNA was assessed in primary MSC, splenocytes, and peritoneal macrophages as well as in the bEnd.3 endothelial and BLK CL.4 fibroblast cell lines by semiquantitative RT-PCR assays. ALK5 mRNA was expressed in all of ALK5, ALK1, and gapdh as a control. Twenty-five, 30, 35, and 40 cycles were conducted for each amplification and PCR products were analyzed by electrophoresis on 3% agarose gels. Photographs show ethidium bromide fluorescence of PCR products obtained before reaching saturation levels. Amplicon sizes of ALK5, ALK1, and gapdh were 70, 76, and 550 bp, respectively. Primers specific for ALK5 formed dimers (indicated by *) that migrated on a 3% agarose gel slightly below specific PCR product and that were visible in most samples, including in PCR performed in the absence of cDNA (ddH2O lane, top panel). C, TGF-β signaling. C57BL/6 MSC were plated at 12,000 or 550 cells/cm² in complete medium for 3 days. bEnd.3 cells were grown at 80% confluence. Cells were serum-starved for 2 h and left untreated or treated with rhTGF-β1 (0.3–30 pM) for 45 min. Samples of WCE were run on a SDS-PAGE gel and subsequently subjected to immunoblot analysis of Smad2 dually phosphorylated at residues Ser465 and Ser467 (top panel), Smad1/5/8 dually phosphorylated at residues Ser400 and Ser423 (middle panel), and α-tubulin expression as a control (bottom panel). D, Same as C, except that BLK CL.4 cells were analyzed.
tested cell types (Fig. 4B). ALK1 mRNA levels appeared, as expected, increased in bEnd.3 cells compared to other cell types. In addition, observed results suggested that levels of ALK1 transcripts were increased by low- or high-density culture conditions in MSC or BLK CL.4 cells, respectively. These results prompted us to probe downstream events to TGF-β binding. ALK5 signals through receptor-regulated Smad2/Smad3 (Smad2/3) while ALK1 activates receptor-regulated Smad1/Smad5/Smad8 (Smad1/5/8) (28). WCE from TGF-β1-activated MSC, BLK CL.4, and bEnd.3 cells were subjected to immunoblot analysis of receptor-regulated Smad activation using phospho-specific Abs. Low-density MSC cultures, compared with high-density cultures, demonstrated increased sensitivity to TGF-β1 pertaining to the phosphorylation of both Smad2/3 and Smad1/5/8 (Fig. 4C). A different response was detected in BLK CL.4 cells, in which only high-cell density cultures displayed a TGF-β1-induced, dose-dependent Smad1/5/8 phosphorylation, while low-cell density cultures displayed basal phosphorylation levels (Fig. 4D). Lastly, we tested whether TGF-β1 regulates MHC II expression and Ag processing in mouse MSC. Flow cytometry data showed that IFN-γ-induced expression of surface MHC II was inhibited by pretreatment with TGF-β1 in MSC, while levels of MHC I, B7-H1, and CD80 were unchanged (Fig. 5A). Accordingly, MHC II-restricted processing of soluble OVA and presentation to T hybridoma cells was reduced by TGF-β1 (Fig. 5B). Quantitative RT-PCR assays demonstrated that TGF-β1 pretreatment resulted in a ~3-fold down-regulation of IFN-γ-induced CIITA mRNA levels (Fig. 5C). TGF-β1-mediated inhibition of CIITA and MHC II induction by IFN-γ was independent of STAT1 because its phosphorylation was not inhibited by TGF-β1 (Fig. 5D). Thus, TGF-β1 signaling, which involves ALK5, ALK1, Smad2/3, and Smad1/5/8, does not affect IFN-γ-R activity and STAT1 phosphorylation. Nonetheless, it inhibits IFN-γ-induced MHC II-restricted Ag presentation in MSC and this effect correlates with the decrease in CIITA and MHC II levels.

**Figure 5.** TGF-β-mediated modulation of IFN-γ-induced signaling and MHC II processing in mouse MSC. A, Flow cytometry analyses. C57BL/6 MSC were plated at 12,000 cells/cm² for 2 days and pretreated or untreated with 40 pM rhTGF-β1 for 24 h before 30 U/ml rmIFN-γ was added, where indicated, for another 24 h. Cells were then analyzed by flow cytometry for K b, I-A b, B7-H1, and CD80 expression. B, APC assay. C57BL/6 MSC were plated at the indicated densities for 2 days and treated or untreated with 40 pM rhTGF-β1. The next day, cells were harvested by trypsinization and cocultured in 96-well plates with OVA-specific I-A b-restricted CD4 T hybridoma cells (MF2.2D9; 10⁶ cells) in the presence of 30 U/ml rmIFN-γ and OVA (0–2.5 mg/ml). After 20 h, supernatants were collected and tested for IL-2 release by CD4 T hybridoma cells using ELISA. Means of triplicates ± SDs of one two representative experiments are shown. C, CIITA expression. C57BL/6 MSC were plated at 12,000 or 550 cells/cm² for 2 days and treated or untreated with 40 pM rhTGF-β1. The next day, cells were left untreated or treated with 30 U/ml rmIFN-γ for 16 h and processed for determination of CIITA mRNA levels as described in Fig. 3C. D, STAT1 phosphorylation. C57BL/6 MSC were plated at 12,000 cells/cm² for 2 days, pretreated or untreated with 40 pM rhTGF-β1 for 24 h, and stimulated or not with rmIFN-γ (0.15–10 U/ml) for 20 min. Samples of WCE were analyzed for STAT1 phosphorylation as described in Fig. 3A.

**Regulation of growth, differentiation and IFN-γ-induced MHC II expression in human MSC**

Human MSC were established from three normal donors. Flow cytometry analyses showed that cells were negative for CD31, CD45, and CD34 and expressed CD44, CD73, CD90, and CD105 (Fig. 6A). In addition, when exposed to specific differentiation-inducing agents, human MSC acquired an adipogenic (data not shown) or osteogenic phenotype (see Fig. 6B). Mouse MSC can grow well in culture for extended periods, get confluent, and still behave like stem/progenitor cells. In contrast, human MSC become senescent after several in vitro passages. At early passages, single-derived human MSC clones become heterogeneous as they expand. They contain small, round, or spindle-shaped (fibroblast-like) rapidly self-renewing MSC (RS-MSC) and larger, slowly renewing MSC (SR-MSC) (18). RS-MSC have the greatest potential for multilineage differentiation (18) and in vivo engraftment (19). In agreement with these reports, we observed that early passage human MSC plated at high-cell density (2000 cells/cm²) did not reach confluence after a 7-day culture period but lost RS-MSC
while SR-MSC became large flat cells and acquired a mature phenotype refractory to osteogenic differentiation compared with cells plated at 100 cells/cm² for 7 days and analyzed for the expression of CD31, CD45, CD34, CD44, CD73, CD90, and CD105 by flow cytometry. Plots show isotype control IgG-staining profiles (dotted line) vs specific Ab-staining profiles (thick line). B. Initial cell density affects MSC differentiation. Cells were cultured for two passages at 100 or 2000 cells/cm² and equal numbers of cells (10⁵) were seeded in 6-well plates and subjected to a bone differentiation assay that was revealed by Alizarin Red staining. C. Growth response to TGF-β. Passage two MSC (plated in routine at 100 cells/cm²) were seeded at the indicated cell density in 96-well plates for 3 days. Cells were then left untreated or treated with rhTGF-β1 (4 or 40 pM) for 3 days and subjected to a MTT cell viability assay as described in Fig. 4A. D, TGF-β signaling. MSC cultured from passages one to five at either 100 or 2000 cells/cm², as indicated, or HUVEC were serum-starved for 2 h and left untreated or treated with rhTGF-β1 (1–30 pM) for 45 min. Samples of WCE were run on a SDS-PAGE gel and subsequently subjected to immunoblot analysis of Smad2 dually phosphorylated at residues Ser⁴⁶⁵ and Ser⁴⁶⁷ (top panel), Smad1 dually phosphorylated at residues Ser⁴⁶⁵ and Ser⁴⁶³, as well as Smad5 and Smad8 phosphorylated at equivalent sites (middle panel) or α-tubulin expression as a control (bottom panel).

FIGURE 6. Proliferation, differentiation, and TGF-β signaling pathways in human MSC (donor 240L). A, Expression of MSC markers. Cells (passage 1) were cultured at 100 cells/cm² for 7 days and analyzed for the expression of CD31, CD45, CD34, CD44, CD73, CD90, and CD105 by flow cytometry. B, Initial cell density affects MSC differentiation. Cells were cultured for two passages at 100 or 2000 cells/cm² and equal numbers of cells (10⁵) were seeded in 6-well plates and subjected to a bone differentiation assay that was revealed by Alizarin Red staining. C, Growth response to TGF-β. Passage two MSC (plated in routine at 100 cells/cm²) were seeded at the indicated cell density in 96-well plates for 3 days. Cells were then left untreated or treated with rhTGF-β1 (4 or 40 pM) for 3 days and subjected to a MTT cell viability assay as described in Fig. 4A. D, TGF-β signaling. MSC cultured from passages one to five at either 100 or 2000 cells/cm², as indicated, or HUVEC were serum-starved for 2 h and left untreated or treated with rhTGF-β1 (1–30 pM) for 45 min. Samples of WCE were run on a SDS-PAGE gel and subsequently subjected to immunoblot analysis of Smad2 dually phosphorylated at residues Ser⁴⁶⁵ and Ser⁴⁶⁷ (top panel), Smad1 dually phosphorylated at residues Ser⁴⁶⁵ and Ser⁴⁶³, as well as Smad5 and Smad8 phosphorylated at equivalent sites (middle panel) or α-tubulin expression as a control (bottom panel).
small cells (R2 population, Fig. 7C), displayed decreased levels of IFN-\(\gamma\)-induced MHC II expression compared with cells plated at 100–200 cells/cm\(^2\), as observed by flow cytometry (Fig. 7C) and immunoblot (Fig. 7D) analyses. Thus, cell proliferation, differentiation, and IFN-\(\gamma\)-induced MHC II levels are regulated by TGF-\(\beta\) and cell density in human MSC, although not in the same way as in mouse MSC.

**Discussion**

Divergent observations have been reported on the use of MSC as immunomodulatory cells. Suppression of in vitro and in vivo immune reactions by MSC has been substantially documented, as detailed in a recent review (9). Nonetheless, we observed that MSC are able to induce in vivo allogeneic immune reactions (12) or to acquire MHC II-mediated Ag presentation when activated with IFN-\(\gamma\) (10). Nauta et al. (13) reported that allogeneic mouse bone marrow implantation was favored by coinjection of host MSC but inhibited by donor MSC (13). In line with these divergent observations, expression of MHC II expression in human or mouse MSC was alternatively observed to be constitutive (11, 30), up-regulated (10, 12, 29, 31) or not (32) by IFN-\(\gamma\) or inhibited by IFN-\(\gamma\) (11). Some of these discrepancies regarding the immune plasticity of MSC may be related to underrecognized variable: cell culture conditions, including the cell density and resultant connectivity of MSC. The behavior of human MSC, and to a much lesser extent of rodent MSC, seems sharply dependent on cell density. Indeed, MSC late passages or cultures grown for extended times at high density lose RS-MSC, and SR-MSC become large flat cells as they acquire a mature phenotype unfavorable to multilineage differentiation, replication, and in vivo engraftment (18, 19, 33). We therefore studied the effect of MSC density on MHC II expression and Ag processing. We observed that MHC II expression and-mediated Ag processing induced by IFN-\(\gamma\) were down-regulated by low-cell density in mouse C57BL/6 (Fig. 1) or BALB/c (data not shown) MSC. This plasticity appears rapidly regulated because differences were observed after plating cells at either low or high density for only 4 days. Surprisingly, an opposite effect was observed in human MSC where IFN-\(\gamma\)-induced MHC II expression occurred at the highest levels in low-density cultures (Fig. 7, C and D). In both cases, however, this suggests that MHC II-related immunostimulatory functions in MSC are critically regulated by culture conditions such as cell density.

Using mouse MSC, we investigated possible mechanisms involved in the cell density-dependent regulation of MHC II expression. C57BL/6 MSC cultures plated at 550 cells/cm\(^2\) were in log phase of growth at the time of harvesting 4 days later, whereas cultures plated at 12,000 cells/cm\(^2\) reached the stationary phase and confluence. Despite these differences, IFN-\(\gamma\)-induced MHC II expression occurred regardless of the cell cycle in mouse MSC (Fig. 2A). Transfer of conditioned medium from confluent cells to dividing cells or vice versa did not change the regulation of MHC II expression, suggesting that secreted factors do not play a role in this response (data not shown). This raises the possibility that contact-dependent receptors modulate APC functions in mouse MSC. Candidate receptors known to be expressed by MSC and deemed to play a role in Ag presentation include: CD44, a glycosaminoglycan present in extracellular matrices and in association with cell surfaces that acts as a receptor for hyaluronate (34); cadherins mediating \(\text{Ca}^{2+}\)-dependent cell-cell adhesion, or connexins in intercellular gap junctions. MSC have been shown to express several connexins, including connexin 43, and form functional gap junction intercellular communications when maintained at high confluence (35, 36), a feature shared by professional APC and proposed to be involved in immune activation (37, 38).
cytokines, such as IFN-γ, IL-1, and TNF-α (45). Therefore, i.v. infusion of MSC primed for APC response in an inflammatory allogeneic environment may not display suppressive features, such as attenuating GVHD and may indeed elicit a MHC II-dependent immune response. In contrast, some research groups that have dealt with human MSC in Phase II clinical trials to treat or prevent GVHD used cells that were cultured at high densities (4000–6000 cells/cm²) (4, 5). Because we show that human MSC do not appear to be primed for a MHC II-mediated APC phenotype by high-cell culture densities, a distinguishing feature relative to mouse MSC, it is conceivable that human MSC may indeed retain a suppressive phenotype in the setting of alloBMT. Our investigations also lead us to speculate that tight control of in vitro culture conditions or TGF-β pretreatment may skew the MSC immune phenotype even more so toward a gain of immunosuppression and thus influence the outcome of their infusion in vivo. This would improve their use as an anti-inflammatory and suppressive regulatory cell in the setting of life-threatening GVHD and morbidity autoimmune ailments as well.

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Disclosures

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References


