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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) possess immunosuppressive properties, yet when treated with IFN-γ they acquire APC functions. To gain insight into MSC immune plasticity, we explored signaling pathways induced by IFN-γ required for MHC class II (MHC II)-dependent Ag presentation. IFN-γ-induced MHC II expression in mouse MSC was enhanced by high cell density or serum deprivation and suppressed by TGF-β. This process was regulated by the activity of the type IV CIITA promoter independently of STAT1 activation and the induction of the IFN regulatory factor 1-dependent B7HL/PD-L1 encoding gene. The absence of direct correlation with the cell cycle suggested that cellular connectivity modulates IFN-γ responsiveness for MHC II expression in mouse MSC. TGF-β signaling in mouse MSC involved ALK5 and ALK1 TGF-βRI, leading to the phosphorylation of Smad2/Smad3 and Smad1/Smad5/Smad8. An opposite effect was observed in human MSC where IFN-γ-induced MHC II expression occurred at the highest levels in low-density cultures; however, TGF-β reduced IFN-γ-induced MHC II expression and its signaling was similar as in mouse MSC. This suggests that the IFN-γ-induced APC features of MSC can be modulated by TGF-β, 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 (Pen/Strep; Wisent Technologies) 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
days. Human primary MSC were provided to us by D. J. Prockop (Tulane University, New Orleans, LA) and maintained in α-MEM, 2 mM l-glutamine (Wisent Technologies), 16.5% FBS (Atlanta Biologicals), 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. Prockop 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. Prockop, personal communication).

Human MSC were expanded for one to six passages by plating every 7 days at either 100 or 2000 cells/cm², 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-α were purchased from BioSource International and rhTGF-β1 was obtained from R&D Systems.

Cell lines

C57BL/6 mouse DC2.4 dendritic cells (DC) and chicken egg white albumin (OVA)-specific I-A²-restricted MF2.2D9 CD4⁺ T helper cells 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. Prockop 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. Prockop, personal communication).

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expression response compared with high-density cell cultures (Fig. 2A). In both, however, the 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 in cells plated at high or low density, and expression patterns were similar to those described above (data not shown). Thus, MSC from high-density cell cultures display increased responsiveness to 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 MSC 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-A^b-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-γ-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 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).
FIGURE 4. TGF-β signaling in mouse MSC. A, TGF-β growth effect. C57BL/6 MSC were plated in 96-well plates at the indicated densities in complete medium for 2 days. Cells were left untreated or treated with 40 pM rhTGF-β1 for 3 days and subjected to a MTT viability assay. Shown are the means of triplicates ± SDs of one of three representative experiments. A two-way ANOVA with a Bonferroni post test were used to compare replicate means (\( * \), \( p < 0.05 \); \( ** \), \( p < 0.01 \); \( *** \), \( p < 0.001 \)). B, ALK1 and ALK5 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. bEnd.3 endothelial cells were harvested at 80% confluence. Total RNA was prepared from adherent live cells and processed for RT-PCR analysis of mRNA expression 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, \( \alpha \)-tubulin expression as a control (Fig. 3D). By contrast, none of the CIITA isoforms was expressed in unstimulated MSC and only the type IV was present in IFN-γ-activated cells (Fig. 3D). Therefore, CIITA up-regulation occurs mostly via the activation of promoter IV in MSC stimulated with IFN-γ. In addition, CIITA expression is regulated by cell density and is likely a key regulator of the regulation of MHC II expression in MSC.

TGF-β signaling pathways and effects on IFN-γ induction of MHC II expression in murine 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., astrogliaoma cells or DC (21–25). We examined the response of our cell line 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 semi-quantitative RT-PCR assays. ALK5 mRNA was expressed in all
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-βR 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-β 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-β-induced, dose-dependent Smad1/5/8 phosphorylation, while low-cell density cultures displayed basal phosphorylation levels (Fig. 4D). Lastly, we tested whether TGF-β 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-β 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-β (Fig. 5B). Quantitative RT-PCR assays demonstrated that TGF-β pretreatment resulted in a ∼3-fold down-regulation of IFN-γ-induced CIITA mRNA levels (Fig. 5C). TGF-β-mediated inhibition of CIITA and MHC II induction by IFN-γ was independent of STAT1 because its phosphorylation was not inhibited by TGF-β (Fig. 5D). Thus, TGF-β 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.

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

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.
while SR-MSC became large flat cells and acquired a mature phenotype refractory to osteogenic differentiation compared with cells plated at 100 cells/cm² (Fig. 6B and data not shown). As we described above in mouse MSC, TGF-β induced a partial growth inhibition in human MSC unless cells were overconfluent (cell density ≥20,000 cells/cm², Fig. 6C) and its signaling involved Smad1/5/8 and Smad2/3 (Fig. 6D). We next investigated the effects of TGF-β and cell density on MHC II levels in human MSC. First, we tested whether IFN-γ up-regulates the total expression of MHC II or the trafficking to the cell surface of a preexisting pool of MHC II molecules, as previously suggested (29). MSC from two donors were exposed to IFN-γ for 24 h and WCE were subjected to immunoblot analysis of total MHC II levels. Results showed that MHC II expression was not constitutive and depended on activating stimulus, e.g., IFN-γ (Fig. 7A). In addition, TGF-β inhibited the up-regulation of IFN-γ-induced MHC II (Fig. 7B). However, with difference to mouse MSC, human MSC cultures plated at ≥2000 cells/cm², which contained less FSC<sub>low</sub>SSC<sub>low</sub>...
small cells (R2 population, Fig. 7C), displayed decreased levels of IFN-γ-induced MHC II expression compared with cells plated at 100–200 cells/cm², as observed by flow cytometry (Fig. 7C) and immunoblot (Fig. 7D) analyses. Thus, cell proliferation, differentiation, and IFN-γ-induced MHC II levels are regulated by TGF-β 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-meditated Ag presentation when activated with IFN-γ (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 on human or mouse MSC was alternatively observed to be constitutive (11, 30), up-regulated (10, 12, 29, 31) or not (32) by IFN-γ or inhibited by IFN-γ (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-γ 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-γ-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/cm² were in log phase of growth at the time of harvesting 4 days later, whereas cultures plated at 12,000 cells/cm² reached the stationary phase and confluence. Despite these differences, IFN-γ-induced MHC II expression and Ag processing. We observed that MHC II expression and mediated Ag processing induced by IFN-γ 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-γ-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.

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MHC II Ag presentation pathways are specific to the so-called professional APC of hematological origin, i.e., DC, macrophages, and B lymphocytes. We know very little about mechanisms involved in the turnover at the cell surface of MHC II molecules. Immature DC display fully efficient MHC II Ag-presenting machinery; however, the recycling of surface MHC II molecules is significantly faster compared with mature DC (39). Le Blanc et al. (29) observed that nonactivated MSC express cyttoplasmic MHC II molecules and that IFN-γ triggers their transport to the cell surface. In our hands, intracellular MHC II expression by resting human MSC was absent. We observed that MHC II expression is inducible and depends on activating stimuli, e.g., IFN-γ. Nevertheless, our previous flow cytometry analysis of MHC II expression on MSC clonal populations demonstrated that a small percentage displayed a constitutive surface expression of MHC II (10), suggesting that MSC preparations may vary in the proportion of such cells. The majority of cell types activated with IFN-γ up regulate MHC II via the activation of CIITA promoter IV (40). Yet, very few of them are capable of MHC II-mediated Ag presentation. Some intestinal, skin, and thymic cortical epithelial cells can mediate MHC II Ag presentation to immunoregulatory gut T cells. The human T82 intestinal epithelial cell line acquires MHC II Ag processing after IFN-γ exposure. In these cells, the transcription of genes encoding MHC II as well as the MHC II chaperones HLA-DM and invariant chain relies on STAT1-mediated IFN-γ transduction and induction of CIITA (41). In this study, we observed that IFN-γ-induced MHC II expression and Ag processing was switched on by CIITA expression subsequently to CIITA promoter IV activation in MSC.

CIITA promoter IV is regulated by STAT1, IRF-1, and upstream transcription factor I transcription factors (20). IL-1β and TGF-β were found to down-regulate its activity, yet they do not interfere with STAT1 and/or IRF-1 activation (21, 22, 24, 25). Similarly, we observed that cell density and TGF-β signaling critically regulated CIITA expression and MHC II Ag processing in MSC, whereas they had no effect on IFN-γ-induced STAT1 phosphorylation nor on the expression of B7-H1. B7-H1 expression was described dependent on the STAT1-induced IRF-1 transcription factor (42). No clear model has been suggested to account for the antagonistic regulation of transcriptional responses induced by IFN-γ and TGF-β. We observed that TGF-β signaling in human and mouse MSC may involve ALK5 and Smad2/3 as well as ALK1 and Smad1/5/8. A study suggested that the activation of ALK1 up-regulates the expression of STAT1 and thus interferes with IFN-γ signaling in HUVEC (43). Other authors attributed the inhibition of CIITA promoter IV by TGF-β to the activation of ALK5 and Smad3 transcription factor; however, no putative Smad3 binding site has been found (24). One hypothesis is that STAT1 and Smad3 compete for interaction with limiting amounts of cotranscriptional factors such as p300/CBP (44). Further characterizations will be necessary to elucidate molecular factors involved in the cross-talk between IFN-γ and TGF-β signaling in mouse and human MSC. In summary, the molecular mechanisms governing the immune plasticity of MSC described in this study could be of relevance for their use in clinical settings and may help explain the disparity between outcome in murine models of disease and what has been observed in vitro and in vivo in humans. In the only mouse study so far describing the effect of MSC on GVHD arising from allogeneic bone marrow transplantation (alloBMT), donor MSC were cultured at high-cell density (6000 cells/cm²) (14). Based on our observations, these cells would be intrinsically primed to be responsive to inflammatory stimulus and adopt MHC II-mediated APC-like features. Indeed, it is well described that conditioning regimens and alloBMT increase plasma levels of inflammatory 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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