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Down-Regulation of MHC-II in Mesenchymal Stem Cells at High IFN-γ Can Be Partly Explained by Cytoplasmic Retention of CIITA

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Mesenchymal stem cells (MSCs) are located in postnatal bone marrow, show plasticity, are linked to various bone marrow disorders, exhibit phagocytosis, exert Ag-presenting properties (APC), and are immune suppressive. Unlike professional APCs, MSCs respond bimodally to IFN-γ in MHC-II expression, with expression at 10 U/ml and baseline, and down-regulation at 100 U/ml. The effects at high IFN-γ could not be explained by down-regulation of its receptor, IFN-γR1. In this study, we report on the mechanisms by which IFN-γ regulates MHC-II expression in MSCs. Gel shift assay and Western blot analyses showed dose-dependent increases in activated STAT-1, indicating responsiveness by IFN-γR1. Western blots showed decreased intracellular MHC-II, which could not be explained by decreased transcription of the master regulator CIITA, based on RT-PCR and in situ immunofluorescence. Reporter gene assays with PIII and PIV CIITA promoters indicate constitutive expression of PIII in MSCs and a switch to PIV by IFN-γ, indicating the presence of factors for effect promoter responses. We explained decreased MHC-II at the level of transcription because CIITA protein was observed in the cytosol and not in nuclei at high IFN-γ level. The proline/serine/threonine region of CIITA showed significant decrease in phosphorylation at high IFN-γ levels. An understanding of the bimodal effects could provide insights on bone marrow homeostasis, which could be extrapolated to MSC dysfunction in hematological disorders. The Journal of Immunology, 2008, 180: 1826–1833.

The postnatal bone marrow is home to two major stem cells, hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (1–3). The anatomy of human bone marrow is organized such that HSCs are found in proximity to the endosteum, whereas MSCs surround the trabeculae and blood vessels (1, 2). HSCs and MSCs are mesodermal, and each can generate specialized cells through multiple lineages. Although HSCs give rise to blood and immune cells, MSCs can form cells such as adipocytes, chondrocytes, and stromal cells (3). In addition, MSCs have shown evidence of plasticity by trans-differentiating into cells of other germ layers, including ectodermal neurons and endodermal hepatocytes (4–6).

The plastic nature of MSCs, combined with the ease of expansion from adult bone marrow aspirates and other sources, supports their candidacy for translation to cell therapy for various types of diseases. Although only a subset of MSCs expresses MHC-II, there are concerns of rejection in settings of allogeneic transplantations. This fear has been partly alleviated by the cells’ immune-suppressive properties (7–9). The mechanisms by which MSCs mediate immune suppression are complex and could be partly explained by veto function, the blunting of maturation process by APCs, and the production of anti-inflammatory cytokines (7, 9, 10). Despite these immune-suppressive properties, MSCs also exhibit proinflammatory functions. MSCs have been shown to act as phagocytes and APCs, and allow for T-cytotoxic responses to viral infection, although with reduced efficiency (7–9).

In classical APCs such as macrophages, IFN-γ induces the expression of MHC-II in a dose-dependent manner, partly via activation of STAT-1 (11, 12). Although MSCs have been shown to exhibit APC and phagocytic functions, their responses to IFN-γ differ from macrophages (7). At low IFN-γ levels, MHC-II is expressed in MSCs, thereby providing them with APC capability (7, 10). In contrast, at high IFN-γ levels, MHC-II expression is down-regulated, making them incapable of APC functions (7, 13). A study of IFN-γ regulation of MHC-II expression in MSCs is therefore relevant to the general immunology of these stem cells and to understand related hematological disorders that have been linked to aberrant MSC physiology. For instance, MSCs from patients with myelodysplastic syndrome and severe aplastic anemia show impaired immunomodulatory functions in which they fail to properly elicit T cell suppression (14, 15). Multiple myeloma and systemic lupus erythematosus have also been associated with deficiencies in cytokine production in MSCs (16, 17).

Also, the mechanisms by which APC functions are regulated in MSCs would be relevant to hematopoiesis. The strategic location of
MSCs, because they are in contact with bone marrow vasculature in the abruminal region where they can monitor cells and other molecules entering and exiting the bone marrow, suggests gatekeeper functions and a protective role (1). In fact, the observation of MSCs with capability to reduce the response of invading foreign insults and reduce inflammation at the interface between the periphery and bone marrow cavity during a limited time frame supports a protective function (7). Thus, the response of MSCs to IFN-γ could be relevant to hematopoiesis because this cytokine, as well as other inflammatory mediators, could lead to hematopoietic suppression (18–20). The down-regulation of MHC-II in MSCs at high IFN-γ levels may therefore represent a novel regulatory mechanism to prevent hematopoietic dysfunction. In this study, we report on a reversible effect of high IFN-γ levels on MHC-II expression on MSCs, partly through the control of the master transcription regulator, CIITA.

Materials and Methods

Reagents

Ficoll-Hypaque, actinoynycin D, leptomycin B, DMEM with high glucose, and RPMI 1640 were purchased from Invitrogen Life Technologies. Texas Red phalloidin (F-actin) was obtained from Molecular Probes.

Abs and cytokines

The following Abs were purchased from BD Biosciences: PE HLA-DR mAb; FITC and PE anti-murine IgG isotype control; and FITC anti-CD45, anti-CD14, and anti-CD29. Anti-CD105 (Src homology 2) and IFN-γ were obtained from Genome Therapeutics; anti-CD105 from T Cell Diagnostics; rabbit anti-STAT-1 from Cell Signaling Technology; rabbit anti-acetyl-histone H3 from Upstate Biotechnology; rabbit anti-phospho-STAT-1 from Cell Signaling Technology; β-actin mAb from Sigma-Aldrich; and IL-10 from R&D Systems. Secondary Ab, anti-goat IgG FITC was purchased from Open Biosystems. Murine phospholated proline/serine/threonine (P/S/T) mAb was purchased from PerkinElmer.

Human subjects

Bone marrow aspirates were obtained from the posterior iliac crest of healthy donors between 18 and 30 years. Use of human subjects adhered to guidelines approved by the Institutional Review Board of the University of Medicine and Dentistry of New Jersey.

Culture of MSCs

MSCs were cultured from bone marrow aspirates, as described (9). Unfractionated aspirates (2 ml) were added to DMEM containing 10% FCS (D10 medium), and then transferred to tissue culture Falcon 3003 petri dishes (Fisher Scientific). Plates were incubated for 3 days, (D10 medium), and then transferred to plasma-treated, tissue culture Falcon 3003 petri dishes (Fisher Scientific). Plates were incubated for 3 days, and then fixed with 2% paraformaldehyde for 20 min. Next, the cells were permeabilized and blocked with 0.1% Triton X-100, 1% BSA in PBS for 45 min. Cells were stained with primary Ab, CIITA, overnight at 4°C at a dilution of 1/500. This was followed by incubation with secondary anti-goat IgG FITC at 1/1000 final dilution. Cytoskeletal staining for cell visualization was developed with Texas Red phalloidin (F-actin) at 6.0 μM final dilution.

Polymerase chain reaction

Total RNA was isolated from 2 × 10^6 MSC with RNaseasy kit (Qiagen), and 1.5 μg was reverse transcribed with Superscript II RT kit (Invitrogen Life Technologies). The cDNA was subjected to semiquantitative PCR with primers specific for the upstream and downstream regions of CIITA cDNA, accession number NM_000246.1. The sequence-spanning upstream regions of CIITA cDNA (P/S/T) were 5′-GAA ACC CCT CCC AGA GG-3′ (sense) and 5′-GAG ACC GAA ACC CCT CCC AGA GG-3′ (antisense). The sequence-spanning downstream regions of CIITA cDNA (leucine-rich repeat (LRR)) were 5′-GGG AAA GCT TGT GCA GAC TC-3′ (sense) and 5′-GGG AAA GCT TGT GCA GAC TC-3′ (antisense).

EMSA

EMSA was performed with double-stranded 22- bp sequence that has a STAT-1 binding site corresponding to the IFN-γ-activated sequence (GAS) element in the promoter region of the human IFN regulatory factor-1 gene, as described (21). MSCs were grown to 80% confluence and stimulated with IFN-γ for 15 min at 37°C. Cells were washed with ice-cold PBS and then trypsinized. Nuclear extracts were prepared from the harvested cells for EMSA, as described (21). Supershift assay was performed, as previously described (21). Briefly, the supershift was performed with rabbit anti-STAT-1 Ab at 1/10 final dilution. The reactions were incubated at room temperature for 20 min, followed by electrophoresis with 4 μl of the reaction mixture.

Immunoprecipitation/Western blot

Immunoprecipitation of CIITA was performed with cytoplasmatic and nuclear extracts, and phosphorylated STAT-1 (p-STAT-1) with whole cell extracts. CIITA was immunoprecipitated by incubating with anti-CIITA at 1/1000 final dilution. After this, the reactions were incubated with protein G Sepharose at 4°C for 1 h. The reactions were centrifuged at 4°C, 10,000 × g for 30 min, and the pellets were washed once with 1× PBS, resuspended in sample buffer, and then electrophoresed on 12% SDS-PAGE (Bio-Rad). Proteins were transferred to polyvinylidene difluoride membranes (PerkinElmer), which were incubated with anti-CIITA at 1/1000 dilution, 4°C overnight. After this, membranes were washed and incubated with HRP-conjugated mouse anti-goat IgG (1/2,000) for 2 h at 4°C. HRP was developed with chemiluminescence detection reagents (PerkinElmer). The m.w. were determined by comparison with Kalediume scope- prestained standards (Bio-Rad).

Western blot analyses were performed as above, except that the membranes were incubated with the following primary Abs: murine anti-human HLA-DR, anti-acyetyl-histone H3, anti-p-STAT-1, or anti-β-actin. The primary Abs were developed with the respective HRP-conjugated IgG.

Phosphorylated P/S/T was studied by first immunoprecipititating CIITA with cytoplasmatic extracts. This was followed by analyses for phospho-P/S/T with Western blots.

Immunochemistry

Cells were stimulated with IFN-γ at 10 or 100 U/ml for various time periods, with or without the addition of actinoynycin D and leptomycin B, and then fixed with 2% paraformaldehyde for 20 min. Next, the cells were permeabilized and blocked with 0.1% Triton X-100, 1% BSA in PBS for 45 min. Cells were stained with primary Ab, CIITA, overnight at 4°C at a dilution of 1/500. This was followed by incubation with secondary anti-goat IgG FITC at 1/1000 final dilution. Cytoskeletal staining for cell visualization was developed with Texas Red phalloidin (F-actin) at 6.0 μM final dilution.

Reporter gene assays

MSCs were added to six-well plates at 10^4 well. Cells were transiently transfected with effectene (Qiagen), as described (7). Each transfection was performed in triplicates with 2 μg of pGL3-CIITA-PII, pGL3-CIITA-PIV, or pGL3. The CIITA reporter gene vectors were provided by P. van den Elsen (Leiden University Medical Center, Leiden, The Netherlands). After 24 h, transfectants were unstimulated or stimulated with 10 or 100 U/ml IFN-γ. After 16 h, cells were collected in lysis buffer (Promega) and then
subjected to freeze-thaw cycles in a dry ice/ethanol bath. Cell-free lysates were quantified for luciferase activity/µg total protein. The activities subtracted the luciferase background obtained with vector alone, which were <5 relative light units. Total protein was determined with a kit purchased from Bio-Rad DC protein assay method.

Band density analysis

Band densities were analyzed using UN-SCAN-IT gel software (Silk Scientific) using the bands for housekeeping genes for normalizations.

Statistical analysis

Data were analyzed using ANOVA and Tukey-Kramer multiple comparisons test. A p value of <0.05 was considered significant.

Results

STAT-1 activation by high and low IFN-γ concentrations

In immune cells, IFN-γ mediates the activation of STAT-1 (24). We have previously reported the down-regulation of MHC-II expression by 100 U/ml IFN-γ with no significant change in its receptor, IFN-γRI (7). We now ask whether high IFN-γ levels blunted IFN-γRI activation by EMSA and Western blot analysis. MSCs from two different bone marrow donors were stimulated for 10 min with 0.1, 10, and 1000 U/ml IFN-γ. Negative controls were stimulated with 10 ng/ml IL-10. Nuclear extracts were incubated with a 32P-labeled GAS dsDNA probe, and STAT-1 binding was determined by EMSA. A representative gel (Fig. 1A) shows STAT-1 binding at all concentrations for MSC from both donors. The band intensities for STAT-1 were proportional to IFN-γ concentration (Fig. 1C). Together, this section shows activation of IFN-γRI at low and high IFN-γ levels.

Endogenous MHC-II in MSCs

The inverse relationship between IFN-γ levels and MHC-II expression could not be explained by deficiency in IFN-γRI signaling (Fig. 1). We therefore asked whether undetectable membrane MHC-II could be due to retention in the cytosol. This question was addressed with MSCs stimulated with IFN-γ at 10 and 100 U/ml, based on their reported differences in affecting MHC-II expression (7). MSCs from three different donors were stimulated with IFN-γ. After 1, 2, and 4 h, cytoplasmic and nuclear extracts were analyzed by Western blot for HLA-DR. Unstimulated cultures showed light bands up to 4 h (Fig. 2A). Consistent with the EMSA results, representative Western blots showed bright bands of p-STAT-1 at each IFN-γ concentration (Fig. 1C). Together, this section shows activation of IFN-γRI at low and high IFN-γ levels.

FIGURE 1. Activation of IFN-γRI by IFN-γ. A, MSCs from two different donors were incubated with various concentrations of IFN-γ. After 15 min, nuclear extracts were studied in gel shift assays for STAT-1 with 32P-GAS DNA sequences. IL-10 served as negative control. B, MSC nuclear extracts were studied in supershift assay for binding specificity of STAT-1 with 32P-GAS DNA sequences. +, Indicates addition of STAT-1 Ab to the sample; −, indicates no Ab in the sample. C, MSCs were stimulated for 15 min with various concentrations of IFN-γ. Whole cell extracts were studied for p-STAT-1 by Western blot.

FIGURE 2. MHC-II expression in IFN-γ-stimulated MSCs. A, Triplicate cultures were stimulated in parallel with 10 or 100 U/ml IFN-γ for the three following time periods: 1, 2, and 4 h. The cytoplasmic (C) and nuclear (N) fractions were analyzed by Western blot for MHC-II. Parallel analyses were done for unstimulated MSCs. Membranes were stripped and reprobed with anti-β-actin. Blot represents three different experiments. A, Shows a representative blot from three experiments, each performed with a different donor. The MHC-II bands were observed at 34 kDa. B, Figure displays the normalized band densities of the Western blots shown in A.
marked reduction in cytoplasmic MHC-II up to 2 h in MSCs stimulated with the higher concentration (100 U/ml) of IFN-γ. In contrast, MHC-II showed constant expression at 10 U/ml IFN-γ or unstimulated. RT-PCR was performed for CIITA with primers specific for types III and IV. Normalization was done with GAPDH-specific primers. The figure represents three experiments, each performed with MSC from a different donor. C, Normalized band densities of the PCR experiments shown in B. *, p < 0.05 vs unstimulated MSCs in CIITA-PIII transfectants. **, p < 0.05 vs unstimulated and 10 U/ml IFN-γ in CIITA-PIV transfectants.

**FIGURE 3.** Reporter gene activities for CIITA-PIII and CIITA-PIV and endogenous expressions. A, MSCs from five different donors were transfected with pGL3-CIITA-PIII or pGL3-CIITA-PIV or with pGL3 alone. After 16 h, transfectants were stimulated with 10 or 100 U/ml IFN-γ. After an additional 16 h, cell extracts were quantified for luciferase activities (mean ± SD, n = 5). B, MSCs were stimulated with 10 or 100 U/ml IFN-γ or unstimulated. RT-PCR was performed for CIITA with primers specific for types III and IV. Normalization was done with GAPDH-specific primers. The figure represents five different experiments. C, Figure shows the normalized band densities for the PCR bands displayed in B.

Activity of CIITA-PIV and CIITA-PIII in MSCs

CIITA has been described as the master regulator of MHC-II transcription (25). It is considered a critical component of MHC-II transcription because its absence directly results in lack of class II complexes (26). We therefore asked whether decreased MHC-II protein in MSCs treated with 100 U/ml IFN-γ (Fig. 2) might be explained by aberrant CIITA transcription. To address this question, we used a reporter gene assay with pGL3 containing CIITA-PIII and CIITA-PIV promoters. The former is associated with constitutive expression of the CIITA gene, whereas CIITA-PIV is responsive to IFN-γ (27). MSCs were transfected with pGL3-CIITA-PIII or pGL3-CIITA-PIV, and after 16 h, transfectants were stimulated with 10 or 100 U/ml IFN-γ. After additional 16-h incubation, luciferase activities were quantified with whole cell extracts. MSCs transfected with pGL3-CIITA-PIII showed significantly (p < 0.05) reduced luciferase activities at both concentrations of IFN-γ (Fig. 3A, left group). In contrast, transfectants with pGL3-CIITA-PIV showed no significant (p > 0.05) change in luciferase activity at 10 U/ml, but significant (p < 0.05) increase at 100 U/ml IFN-γ (Fig. 3A, right group).

We next determined whether the reporter gene activities correlate with endogenous CIITA mRNA. We therefore performed RT-PCR with total RNA from MSCs, unstimulated and stimulated with IFN-γ at 10 and 100 U/ml. Normalizations were done by RT-PCR with GAPDH-specific primers. We observed inverse relationship in band densities between CIITA-PIII and CIITA-PIV,
indicating that the reporter gene activities mimic endogenous mRNA levels (Fig. 3B). Quantitation of band intensities revealed a similar inverse relationship as the reporter gene assays (Fig. 3C). Because CIITA is necessary for the stability of the MHC-II enhanceosome, the data indicate that reporter gene activity for CIITA-IV promoter and endogenous mRNA cannot explain decreased MHC-II in MSC stimulated with 100 U/ml IFN-\( \gamma \)/H9253.

Expression of CIITA in IFN-\( \gamma \)-stimulated MSCs

Reporter gene activity with CIITA promoter is an indication that the MSCs are capable of activating CIITA (Fig. 3). However, these studies were done with ectopic insertions of the two different CIITA promoters. In addition, previous studies have reported decreased MHC-II expression in MSC stimulated with 100 U/ml IFN-\( \gamma \).

Expression of CIITA in IFN-\( \gamma \)-stimulated MSCs

FIGURE 5. Cytoplasmic and nuclear CIITA in IFN-\( \gamma \)-stimulated MSCs. A, Cells were stimulated with 10 or 100 U/ml IFN-\( \gamma \) for 0.5, 1, 2, or 4 h, and at each time point, nuclear and cytoplasmic extracts were isolated and then analyzed by Western blots for CIITA. B, Nuclear extracts from 10 U/ml IFN-\( \gamma \) stimulations and cytoplasmic extracts from 100 U/ml IFN-\( \gamma \) stimulations were studied for ribosomal protein (16 kDa) and for acetyl-histone H3 protein (17 kDa). The figure represents three experiments, each performed with a different donor. The m.w. for bands was observed at \(-128\) kDa. C–E, Representative of three experiments for CIITA by in situ immunofluorescence (FITC, green) in a background of staining for F-actin (red) (C), in the presence of 10 ng/ml actinomycin D (D), or in the presence of 10 ng/ml actinomycin D and 10 ng/ml leptomycin B (E). Figure represents three experiments, each with a different donor (C).
(598 bp) indicate no change in CIITA mRNA for IFN-γ stimulations at 10 and 100 U/ml as compared with unstimulated MSCs. The results are also presented as normalized band densities (Fig. 4C). Because the results for a specific section of the mRNA contrast the changes in PIII and PIV mRNA (Fig. 3B), we propose that these results might be due to the activation of CIITA-PI in MSCs (28).

CIITA protein in nuclear and cytoplasmic extracts

Luciferase activity and RT-PCR for CIITA could not offer a mechanistic insight to the observed reduction of MHC-II protein in MSCs stimulated with 100 U/ml IFN-γ (Figs. 3 and 4). We therefore asked whether higher levels of IFN-γ could cause retention of CIITA in the cytosol because this would affect MHC-II transcription. Nuclear and cytoplasmic extracts from MSCs, stimulated with 10 and 100 U/ml IFN-γ for 0.5, 1, 2, and 4 h, were analyzed by immunoprecipitation/Western blots for CIITA. The results indicate nuclear CIITA at 10 U/ml IFN-γ, which was undetectable in the cytoplasmic extracts (Fig. 5A, upper row). At 100 U/ml, bands for CIITA were observed only in cytoplasmic CIITA (Fig. 5A, second row).

Because Fig. 5A (left panel) showed CIITA protein only in the cytoplasm, we next validated the purity of these extracts. The goal is to ascertain that nuclear proteins at 100 U/ml IFN-γ did not contaminate the cytoplasmic extracts. We therefore performed Western blots for ribosomal protein (cytoplasmic) and acetyl-histone H3 (nuclear). Representative studies (Fig. 5B, right panel) showed bright bands for ribosomal proteins and undetectable bands for acetyl histone H3, indicating no contamination with nuclear extracts. Similarly, nuclear extracts from 10 U/ml IFN-γ showed no evidence of cytoplasmic contamination (Fig. 5B, left panel).

To confirm the results by Western blots (Fig. 5B), we analyzed the MSCs for intracellular localizations of CIITA by immunofluorescence. MSCs were stimulated with 10 or 100 U/ml IFN-γ for 0.5, 1, 2, and 4 h and then labeled with anti-CIITA and Texas Red phalloidin (F-actin) for clearer visualization. The results show nuclear localization of CIITA in MSCs stimulated with 10 U/ml IFN-γ at all time points (Fig. 5C, top panel). Conversely, cells stimulated with 100 U/ml IFN-γ show cytoplasmic localization (Fig. 5C, bottom panel). In fact, intense cytoplasmic staining was visible for MSCs stimulated with 100 U/ml IFN-γ for 1 h. These results are consistent with our Western analyses, and the experimental evidence indicates retention of cytoplasmic CIITA protein in MSC stimulated with 100 U/ml IFN-γ, but not for 10 U/ml.

Because both Western blots and immunofluorescence studies suggest retention of CIITA in the cytoplasm (Fig. 5, A–C), we next designed studies to further confirm these observations. We first treated the MSCs as for Fig. 5C in the presence of 10 ng/ml transcriptional inhibitor, actinomycin D. Despite blocking of transcription, we did not observe nonspecific effects on translation (Fig. 5D). The concentration of actinomycin D was based on optimization studies in which we observe a gradual decrease in CIITA message by 16 h by RT-PCR. After this, we repeated the experiment in the presence of both actinomycin D and the inhibitor of nuclear export, leptomycin B (10 ng/ml). We did not observe nuclear accumulation at high IFN-γ. This indicates that the mechanism could not be explained by increased nuclear export of CIITA (Fig. 5E). This is consistent with retention of CIITA in the cytosol at high IFN-γ.

Phosphorylation of P/S/T domains of CIITA

The P/S/T region of CIITA (Fig. 4A), which is a P/S/T site (proline aa 163–195, serine aa 209–237, threonine aa 260–322) is generally involved in protein-protein interactions following phosphorylation of the P/S/T region that correlate to increases in MHC-II expression (29, 30). Thus, we studied whether the retention of CIITA in the cytosol could be explained by altered phosphorylation of the P/S/T region with CIITA. To address this question, we stimulated MSCs with IFN-γ at low and high levels and then immunoprecipitated the cytosolic proteins with anti-CIITA, followed by Western analyses for phospho-P/S/T (Fig. 6). The results showed a faint band at high levels (100 U/ml) of IFN-γ, despite a bright band for total CIITA (Fig. 6). In summary, the analyses indicate reduced phosphorylation of the P/S/T region of CIITA at high levels of IFN-γ.

Discussion

We have previously reported an atypical response of MSCs to high levels of IFN-γ with regard to membrane expression of MHC-II (7). At high levels of exogenous IFN-γ, MHC-II was decreased, but was maintained at baseline and low IFN-γ levels (7). We now report on a novel mechanism by which the master regulator of MHC-II, CIITA, is regulated in MSCs at different concentrations of IFN-γ. In addition, we have shown evidence for a possible switch of one active CIITA promoter to the IFN-γ-responsive type IV promoter. These studies were done with reporter gene assays that showed high activity by CIITA-PIII promoter and reduced activity following IFN-γ stimulation, which corresponded with mRNA expressions (Fig. 3, A and B). Baseline CIITA-IV promoter in unstimulated MSCs was activated following IFN-γ stimulation (Fig. 3A). The switch in promoter responsiveness is similar to parallel studies in B cells (27). The overall findings by which MSCs responded differently to changes in IFN-γ levels are consistent with the immune-enhancing and immune-suppressive roles of MSCs within different microenvironmental settings (10). This report is significant to several clinical problems, including immune responses of MSCs at cell therapy, in particular, transplantation across allogeneic barriers. A significant finding is the induced expression by RT-PCR as determined with primers specific to two different regions of the mRNA (Fig. 4). This contrasts the changes observed in the RT-PCR for specific CIITA (Fig. 3B). We attributed this difference to the activation of the PI promoter of CIITA, although further investigations are required to support this premise. Because the biology of CIITA has not been studied in MSCs, the concept that CIITA could lack the NLS is an important question. This question is mainly based on other reports that show a balance of CIITA between the cytosol and nuclei of immune cells (31). Because Fig. 4 shows no evidence of its loss, future studies will begin to focus on the coprotein(s) that has (have) been associated with NLS (32).

The ability of MSCs to decrease MHC-II expression at high IFN-γ is especially significant to bone marrow biology. Of relevance is the suppressive effects of high IFN-γ to hemopoiesis (33). The fact that CIITA is a key factor in preventing MSCs from...
expressing MHC-II indicates that the stem cells might have an innate ability to regulate exacerbated APC functions. By reducing APC properties, the cells not only prevent increases in IFN-γ production, but also cause decreased production of other inflammatory mediators, which could suppress hematopoiesis (34). Functionally, the decrease in MHC-II expression at high IFN-γ levels correlated with the loss of IFN-γ-treated MSCs to elicit allogeneic responses. It is, however, unclear whether high levels of IFN-γ also cause MSCs to exhibit profound immune suppressor functions. Perhaps this change in MHC-II expression in MSCs at high IFN-γ might begin to explain the mechanism by which MSCs exert veto properties as third party cells (7, 9).

The immune biology of MSCs and the mechanisms by which these stem cells revert to immune suppressor cells could provide insights into the understanding of several immune-mediated dysfunctions, including hematological disorders linked to autoimmune processes (14, 16, 17). Dysregulated MSC functions have been linked to the pathophysiology of aplastic anemia, which is considered an immune-mediated disorder with increased suppressor T cells, and high levels of IFN-γ (14). MSCs have also been reported to be dysfunctional in patients with myelodysplastic disorders (15). MSCs have been suggested as a partial replacement of immune-suppressive therapy in bone marrow transplantation to decrease graft-vs-host disease (34). An understanding by which MSCs transition to immune suppressor functions when MHC-II expression is decreased might be important in improving the effectiveness in delivering stem cell therapy for graft-vs-host disease.

Down-regulated membrane expression of MHC-II cannot be explained by the cells’ inability to activate STAT-1 (Fig. 1). Rather, the decreased expression of MHC-II is controlled by CIITA. The key control point does not appear to be at the level of transcription, as indicated by reporter gene assay and by RT-PCR for endogenous CIITA (Figs. 3 and 4). The lack of nuclear CIITA at high IFN-γ levels indicates a defect in its transport (Fig. 5). This defect is rapid and is also reversible, which is indicated by reduced cytoplasmic MHC-II up to 2-h incubation with high IFN-γ, followed by an increase at 4 h (Fig. 2). The findings are intriguing and insightful because they will begin to lead into future studies to determine how MHC-II transcription and translation are regulated in MSCs, and also produce avenues to examine how a microenvironment might affect MSC functions. An examination of the complex method by which CIITA recruits cofactors makes it logical to appreciate how the loss of nuclear CIITA could rapidly prevent MHC-II transcription (Fig. 7).

The LRR motif within the C-terminal of CIITA protein (Fig. 4A) is a conserved domain for molecules involved in multiple functions such as RNase inhibition, activation of GTPases, protein-protein interactions, signal transduction and transcription, and nuclear localization (35, 36). The CIITA protein has been reported to form self-associations as a result of the horseshoe-like structure of LRR, which are necessary for MHC-II transcription (36). Although we have proposed a defect in nuclear translocation of CIITA at high IFN-γ level, we have not observed evidence of transcripts in which the LRR region is omitted because this might explain the hindrance to nuclear transport of CIITA.

The P/S/T region of CIITA (Fig. 4A) is generally involved in protein-protein interactions (29, 30). However, we showed reduced phosphorylation of this site, suggesting that the mechanism of cytotoxic response (Fig. 5) might be explained by the lack of efficient phosphorylation. These are intriguing findings that are the subject of further research in the laboratory.

The consensus is that MSCs can escape recognition by alloreactive T cells and are therefore beneficial in a transplantation setting of MHC discrepancy. However, there is still lack of agreement on the mechanism by which MSCs mediate immune suppression. Studies have reported up-regulation of MHC-II by high level of IFN-γ at 48 h in MSCs with failed T cell response (34). Our results show reduced MHC-II at 2 h, and this might explain a different MSC subset (Fig. 2). IFN-γ has been reported to stimulate osteoclast formation during T cell activation (37). This report is highly significant to our findings because MSCs are precursors of osteoblasts (2). These are exciting findings that could be relevant for diseases linked to bone loss. In conclusion, this study is relevant to future clinical application with MSCs and also for hematological disorders linked to MSC dysfunctions.

The strategic position of MSCs surrounding bone marrow blood vessels that monitor cells entering and exiting bone marrow may implicate a protective role for MSCs that hinders ablation of bone marrow during insults such as inflammatory responses following infection of bone marrow. The conversion of MSCs to immune suppressor cells with concomitant down-regulated expression of MHC-II may also elucidate several hematological conditions such as aplastic anemia in which lymphocytes autoattack HSC. An understanding by which MSCs mediate immune functions may be relevant for therapeutic intervention for patients with immune-linked hematological disorders.

The cartoon shown in Fig. 7 summarizes the salient findings of this study. The responses of low (a) and high (b) concentrations of IFN-γ are similar up to the translation of CIITA. However, the mechanism diverges as CIITA is transcribed and is required to re-enter the nuclei. Future studies are therefore required to determine the mechanisms at which CIITA transport is affected because such an understanding will be a key to future applications in which

![Figure 7. Molecular effects of IFN-γ on CIITA gene transcription.](image-url)
it would be necessary to suppress MHC-II re-expression for cellular therapy. This study is currently extended to future investigation in our laboratory on in-depth analyses of the CIITA-PIV promoter. In addition to IFN-γ, the regulation of MHC-II expression also involves other factors such as TGF-β1 (38).

Disclosures
The authors have no financial conflict of interest.

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