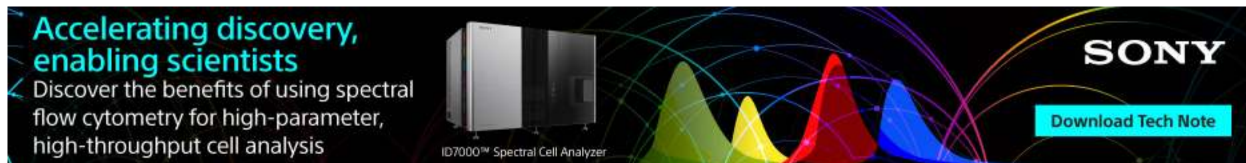

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IDO-Independent Suppression of T Cell Effector Function by IFN-γ–Licensed Human Mesenchymal Stromal Cells

Raghavan Chinnadurai,* Ian B. Copland,* Seema R. Patel,* and Jacques Galipeau*,†

Human bone marrow–derived mesenchymal stromal cells (MSCs) inhibit proliferation of activated T cells, and IFN-γ plays an important role in this process. This IFN-γ–licensed veto property is IDO-dependent. To further decipher the mechanistic underpinnings of MSC veto function on T cells, we investigated the effect of MSCs and IFN-γ–licensed MSCs on T cell effector function as assayed by cytokine secretion of T cells. Although MSCs and IFN-γ–licensed MSCs inhibit T cell proliferation, only IFN-γ–licensed MSCs significantly inhibit Th1 cytokine (IFN-γ, TNF-α, and IL-2) production by T cells. Additionally, IFN-γ–licensed MSCs inhibit T cell degranulation as well as single, double, and triple cytokine–producing T cells. Although IFN-γ–licensed MSCs upregulate their IDO activity, we found that MSC IDO catalytic function is dispensable with regard to MSC-driven inhibition of T cell effector function. Novel flow cytometry based functional screening of MSC-expressed, IFN-γ–licensed inhibitory molecules identified B7H1 and B7DC/PD1 pathways as essential effectors in blocking T cell function. Small interfering RNA–mediated blocking of B7H1 and B7DC reverses the inhibitory potential of IFN-γ–licensed MSCs on T cell effector function. Mechanistic analysis revealed that clustering of MHC and coinhibitory molecules are indispensable for the inhibitory effect of IFN-γ MSCs. Although exogenous IL-2 reverses B7H1-Ig–mediated inhibition of T cell proliferation, it does not affect the veto function of IFN-γ MSCs on both T cell proliferation and effector function. Our results reveal a new immunosuppressive property of IFN-γ–licensed MSCs that inhibits T cell effector function independent of IDO but through the ligands for PD1. ***The Journal of Immunology*****, 2014, 192: 1491–1501.**

Marrow mesenchymal stromal cells (MSCs) play a role in maintaining the hematopoietic niche, regulate marrow immune homeostasis, and can differentiate into bone, cartilage, and adipocytes as a mesenchymal progenitor pool (1, 2). Although endogenous clonogenic marrow MSCs represent a small minority of all nucleated cells in marrow space, typically 1:100,000, these same cells possess a robust mitogenic potential when cultured ex vivo (3, 4). This proliferative potential allows for manufacture of a cellular pharmaceutical product of sufficient number to treat one or a few subjects with product derived from a single small-volume marrow harvest. The ensuing cellular product typically possesses potent veto function on T cell proliferation and other immune cell types that may be exploited for treatment of immune disorders (5–8).

The therapeutic potential of transfused MSCs has been demonstrated in animal models of experimental autoimmune encephalitis, diabetes, rheumatoid arthritis, myocardial infarction, acute lung injury, retinal degeneration, acute renal failure, and transplant rejection (9, 10). In human phase II clinical trials, MSCs were shown to be effective in treating graft-versus-host disease (11), although study of an industrial MSC product in a phase III trial was negative (12–14). Nevertheless, >200 clinical trials are registered to test the use of MSCs to treat targeted immune-related disorders (multiple sclerosis, graft-versus-host disease, Crohn's disease) and cardiovascular conditions (myocardial infarction, ischemia) (15). Although host factors, source, and functionality of MSCs may contribute to inconsistent outcomes of clinical trials, a better understanding of cellular immunology of MSCs would help to optimize and possibly enhance the pharmaceutical utility of such cells (13, 16). It has been shown that the immunosuppressive properties of endogenous MSCs are dependent on the presence of IFN-γ in the microenvironment, because neutralizing Abs to IFN-γ receptor abolish the antiproliferative properties of MSCs on T cells (17). Indeed, the in vitro veto function of culture-expanded MSCs is substantially enhanced by prelicensing with IFN-γ and provides some insight in the physiological responsiveness of MSCs to inflammatory cues (18–20). IFN-γ not only modulates the immune properties of MSCs but also their differentiation potential. Croitoru-Lamoury et al. (21) demonstrated that addition of IFN-γ greatly inhibits the osteogenic and adipogenic markers and subsequently inhibits the differentiation of MSCs. Therefore, an analysis of MSC immune plasticity in response to exogenous IFN-γ stimulation (6, 22) and how this serves as an immune checkpoint to T cell activity are of physiological and translational interest.

Once naive T cells are exposed to an activation signal they proliferate, and a subset becomes effector T cells as manifested by cytokine secretion and degranulation, based on their memory status (23, 24). It has been reported that unlicensed MSCs inhibit T cell proliferation but not effector function (25). Indeed, there is no systematic analysis of cytokine-licensed MSC immunosuppressive effect on T cell function. To address this issue, we interrogated the cellular immunology of resting and IFN-γ–licensed MSCs on T cell

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R.C. designed the research plan, performed most experiments, analyzed results, and wrote the manuscript; I.B.C. prepared mesenchymal stromal cells from multiple donors and provided platelet lysates; S.R.P. performed Western blot for IDO; and J.G. designed the research plan, analyzed results, and wrote the manuscript.

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Abbreviations used in this article: MSC, mesenchymal stromal cell; 1MT, 1-methyl-DL-tryptophan; SEB, staphylococcal enterotoxin B; siRNA, small interfering RNA.

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proliferation and effector function and found that MSC B7H1/B7DC upregulation is a key component of T cell suppression that occurs parallel to and independently of similarly upregulated MSC IDO catalytic activity. These data provide insight into the intrinsic veto properties of MSCs in response to inflammatory cues and also provide mechanistically definable parameters that may inform the development of optimized MSC transfusional products for treatment of immune ailments.

Materials and Methods

MSC isolation and culture

Human MSCs were isolated from bone marrow aspirates collected from the iliac crest of consenting subjects. Bone marrow aspirates were diluted 1:2 with PBS and layered onto a Ficoll density gradient to isolate mononuclear cells. The cells were centrifuged at $400 \times g$ for 20 min and thereafter plated in complete human MSC medium (α-MEM, 10% human platelet lysate, 100 U/ml penicillin/streptomycin) at 200,000 cells/cm^2. Nonadherent hematopoietic cells were removed by changing the medium after 3 d of culture, and MSCs were allowed to expand for 7 d. Thereafter, the cells were passaged weekly and reseeded at 1000 cells/cm^2. After the third passage, the MSC cultures were assayed by flow cytometric analysis for the absence of $CD45^+$ and $CD31^+$ contaminating cells and expression of CD44, CD73, CD90, and CD105 (BD Biosciences, San Jose, CA). All assays were performed using MSCs between passages 4 and 7.

Manufacturing of human platelet lysate

Outdated platelet pheresis products were obtained from the Emory University Blood Bank following American Red Cross consent and an Emory Institutional Review Board waiver. Platelets were fractured using a double freeze-thaw procedure to release their contents. Pools of five fractured platelet units were then filtered through a 40-μm Pall blood transfusion filter (Pall Biomedical, Fajardo, Puerto Rico) and then aliquoted and spun for 20 min at $4000 \times g$ at room temperature, filtered (40 μm), and then recalcified to 20 mM $CaCl_2$. Following centrifugation, platelet lysate was filtered to 0.2 μm and then stored at −80°C until use.

IFN-γ licensing and MSC phenotype

MSCs were seeded in a six-well plate at 5000 cells/cm^2 on day 0. On day 1, the medium was removed and recombinant human IFN-γ (Invitrogen, Carlsbad, CA) was added at the concentration of 50 ng/ml in the MSC culture medium. On day 4, cells were trypsinized and subjected to flow cytometry analysis of expression of HLA-ABC, HLA-DR, CD80, CD86, B7H1, B7DC, CD95L, and appropriate isotype controls (BD Biosciences). For the IDO mRNA expression analysis, the cells were subjected to total RNA extraction using an RNeasy Plus Mini kit (Qiagen, Valencia, CA). Subsequently, total cDNA was prepared using a QuantiTect reverse transcription kit (Qiagen), and SYBR Green (PerfeCTa SYBR Green FastMix; Quanta Biosciences, Gaithersburg, MD) real-time PCR was performed with IDO primers (5′-GCCCTTCAAGTGTTTCACCAA-3′, 5′-CCAGCCAGACAAATATATGCGA-3′) and GAPDH primers (5′-CTCTCTGCTCCTCCTGTTCGAC-3′, 5′-TGAGCGATGTGGCTCGGCT-3′) with an Applied Biosystems 7500 fast real-time PCR system thermal cycler. The fold change in mRNA expression was calculated by the $\Delta\Delta C_t$ method. For Western blot analysis, proteins were detected using primary rabbit anti-human IDO1 (1:1000; EMD Millipore, Billerica, MA) or rabbit anti-human β-actin (1:1000; Cell Signaling Technology, Danvers, MA) and secondary HRP-coupled goat anti-rabbit IgG (H+L) (1:10,000; Bethyl Laboratories, Montgomery, TX) and revealed using an ECL system (Amersham Pharmacia Biotech, Piscataway, NJ).

MSC and T cell coculture

MSCs were seeded onto 96-well plates with the density of 5000/cm^2. The cells were licensed for 48 h without or with IFN-γ at a concentration of 50 ng/ml in MSC culture medium. PBMCs prepared from healthy individuals by Ficoll density gradient were resuspended in RPMI 1640 complete medium (10% heat-inactivated serum, 100 U/ml penicillin/streptomycin, L-glutamine, 10 mM HEPES). One hundred thousand PBMCs were added to each well and were stimulated with 500 ng/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich, St. Louis, MO) or 0.5-μl Dynabeads (Life Technologies, Oslo, Norway) or anti-CD3 (OKT3) (BioLegend, San Diego, CA) and anti-CD28 (BD Biosciences). For intracellular cytokine staining, cells were incubated with brefeldin A at the concentration of 10 μg/ml (Sigma-Aldrich) for 12–14 h and subsequently subjected to intracellular flow cytometry staining procedures of BD Cytofix/Cytoperm according to the manufacturer's instructions and with the Abs allophycocyanin–Cy7–anti-CD3, FITC–TNF-α, PE–IL-2, and allophycocyanin–IFN-γ (BD Biosciences). Degranulation assay was performed with CD107 Ab (BD Biosciences) staining during stimulation. Phenotyping of IFN-γ–secreting T cells was performed with Abs Tim3, CD253, CD85J, CD272, CD244, CD160, CD134, CD95, PD1 (BioLegend), CD200, CCR7, and CD45RA (BD Biosciences). For Ki67 proliferation assay, cells were incubated for 4 d in the absence of brefeldin A and were subjected to intracellular Ki67 staining according to the manufacturer's instructions (BD Biosciences).

T cell assays with tryptophan catabolites and cytokines

1-Methyl-DL-tryptophan (1MT; 1 mM concentration; Sigma-Aldrich) or the cytokines IFN-γ (Invitrogen), IL-2 (Roche), and TNF-α (R&D Systems, Minneapolis, MN) were added to the PBMCs cocultured with and without IFN-γ–licensed MSCs as described above and published earlier (22). L-tryptophan and the tryptophan catabolites L-kynurenine, quinolinic acid (2,3-pyridinedicarboxylic acid), anthranilic acid, and kynurenic acid (Sigma-Aldrich) were added at the indicated concentrations to the PBMCs stimulated with 500 ng/ml SEB. The cells were subjected to either intracellular cytokine staining assay or proliferation assay as described above.

B7H1-Ig inhibition assay

Ninety-six–well plates were coated with 1 μg/ml anti-CD3 (OKT3) (BioLegend) alone or in combination with B7H1-Ig fusion protein (R&D Systems) overnight. On the next day, the unbound proteins were washed away. The PBMCs were added to the plate in the presence and absence of cytokines, and subsequently proliferation and cytokine staining assays were performed as described above. For the non–crosslinking assay, B7H1-Ig was coated onto the plate at the indicated concentrations and anti-CD3 was added onto the PBMCs at 1 μg/ml.

Small interfering RNA transfection of human MSCs

MSCs were seeded in 96-well plates at a concentration of 5000 cells/well 1 d prior to transfection with nontargeting control small interfering RNA (siRNA) or B7H1 and B7DC SMARTpool siRNA (Dharmacon, Lafayette, CO). During transfection, the cells were conditioned with serum-free 10 mM HEPES containing α-MEM for 30 min. Two microliters 100 μM siRNA solution or 3 μl DharmaFECT 1 reagent was added to 250 μl α-MEM containing 10 mM HEPES. The siRNA solution and the DharmaFECT reagent were mixed and incubated at room temperature for 30 min. Fifty microliters of the siRNA transfection mixture was added to each well. The cells were then incubated for 5 h and then the transfection medium was replaced with MSC culture medium. After 12 h the cells were stimulated with and without IFN-γ at the indicated concentrations for 36–48 h and subjected to coculture with PBMCs for intracellular cytokine staining assay.

Statistical analysis

Data were analyzed with the GraphPad Prism 5.0 software. For the comparison of two groups, a paired t test was applied. A two-sided p value <0.05 was considered statistically significant.

Results

IFN-γ–licensed human MSCs inhibit effector function of T cells

Flow cytometry analysis of resting MSCs demonstrates that MHC class I but not MHC class II is expressed by MSCs. Upregulation of both of these molecules was observed on MSCs licensed with IFN-γ, but not TNF-α. The costimulatory molecules B7-1 and B7-2 were not upregulated by IFN-γ, TNF-α, or by both in combination (Fig. 1A). We analyzed the veto effect of MSC IFN-γ licensing on T cell function. Our results demonstrate that both MSCs and IFN-γ–licensed MSCs inhibit $Ki67^+$ T cell proliferation efficiently. However, intracellular cytokine staining assays demonstrate that only IFN-γ–licensed MSCs significantly inhibit the secretion of IFN-γ by T cells when compared with nonlicensed MSCs (Fig. 1B). We further validated these observations by testing the inhibitory effect of MSCs (and IFN-γ–licensed MSCs) isolated from the bone marrow of four independent donors. We observed that as an aggregate ($n = 4$), IFN-γ–licensed MSCs gain the ability to

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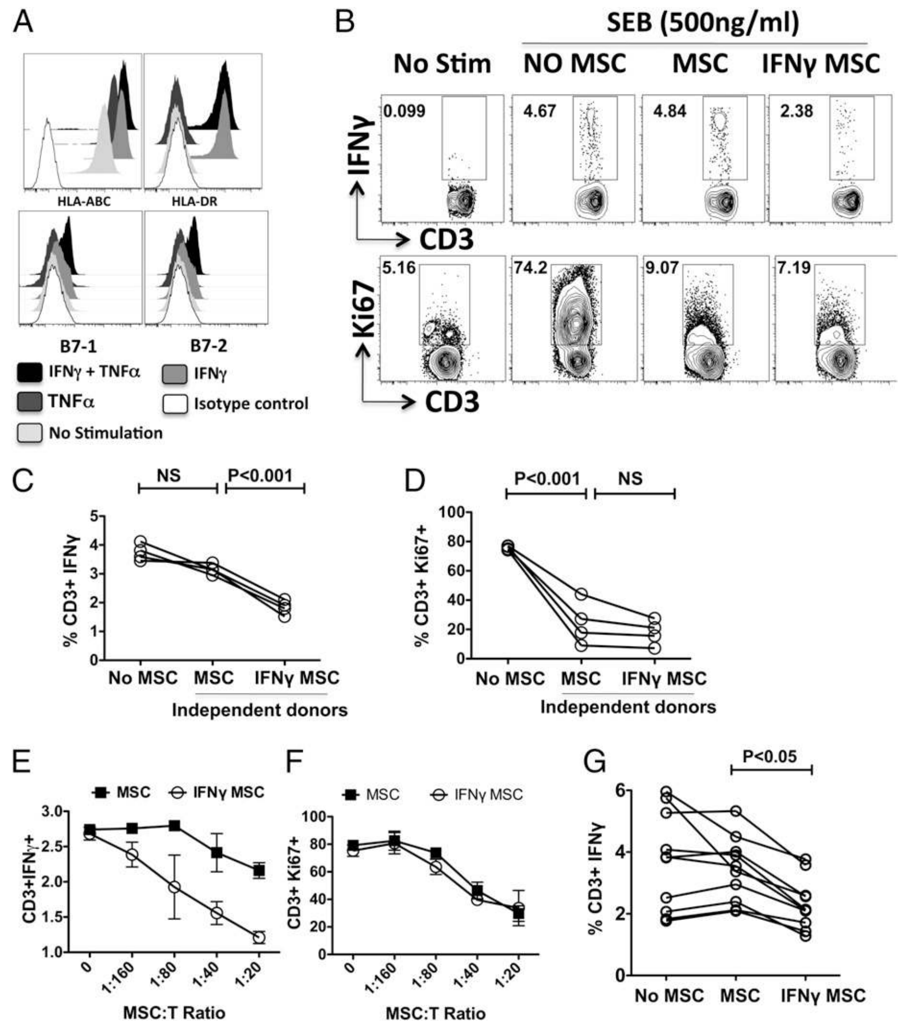

FIGURE 1. IFN-γ–licensed human MSCs inhibit IFN-γ secretion on T cells. (**A**) Human MSCs were stimulated by either IFN-γ, TNF-α alone, or in combination with the concentration of 50 ng/ml for 48 h. The cells were stained with the Abs to the surface markers for HLA-ABC, HLA-DR, B7-1, and B7-2 and subjected to flow cytometry. (**B**) PBMCs were cocultured in the presence and absence of either MSCs or IFN-γ–licensed MSCs and were stimulated with SEB. For intracellular IFN-γ cytokine staining assay, the cells were incubated with brefeldin A for 12–14 h and subsequently stained with Abs to CD3 and IFN-γ for flow cytometry. For Ki67 proliferation assay, the cells were incubated for 4 d in the absence of brefeldin A and were subjected to the Ab staining to CD3 and Ki67. (**C**) Cumulative intracellular IFN-γ cytokine staining assay or (**D**) Ki67 proliferation assay was performed with MSCs isolated from the bone marrow of four independent donors. Paired *t* test analysis was performed through Prism software to get the *p* values. (**E**) Intracellular IFN-γ cytokine staining assay or (**F**) Ki67 proliferation assay was performed with the indicated ratios between MSCs and PBMCs. (**G**) Intracellular IFN-γ cytokine staining assay was performed with PBMCs isolated from the blood of independent donors. Paired *t* test analysis was performed through Prism software to get the *p* values. Representative data are shown from three independent experiments

significantly block cytokine production by T cells, a feature not deployed by resting MSCs (Fig. 1C). Both resting and licensed MSCs are equivalent in their ability to block T cell proliferation (Fig. 1D), and these MSC-mediated effects on T cells occur in a dose-dependent manner (Fig. 1E, 1F). This suppressive effect of IFN-γ MSCs on T cell effector function is IFN-γ dose-dependent (Supplemental Fig. 1). To test whether T cell response differs among distinct normal subjects, we analyzed the veto effect of MSCs and IFN-γ–licensed MSCs from a single donor on PBMCs collected from nine independent normal volunteers. Our results demonstrate that T cell susceptibility to MSC veto effect varies between normal responder subjects, but the cumulative analysis remains significant (Fig. 1G).

IFN-γ–licensed human MSCs inhibit T cell degranulation and cytokine production

Following in vitro activation, a subset of $CD3^+$ T cells produces cytokines de novo as detected by intracellular cytokine staining. Among these, proinflammatory cytokines produced by $CD3^+$ T cells such as IFN-γ, IL-2, and TNF-α play an important role in a T cell–driven immune response, and we found that IFN-γ–licensed MSCs block production of all three cytokines when assessed independently (Fig. 2A). A more complex picture emerges when analyzing the contemporaneous production of IFN-γ, IL-2, and TNF-α by activated T cells. More than 93.8% of activated $CD3^+$ T cells are triple-negative, and we observed that the cytokine-secreting effector T cell pool (6.2%) contains a spectrum of single, double, or multiple cytokine-producing cells (Fig. 2B). Boolean gating analysis identified seven independent populations of cytokine-producing $CD3^+$ T cells (Fig. 2B), and we tested the effect of MSCs on these subsets. Cumulative data from experiments with multiple donors demonstrate that IFN-γ–licensed MSCs significantly inhibit triple-positive (IFN-γ^+IL-2^+TNF-α^+) (Fig. 2C), TNF-α^+ double-positive (IFN-γ^+IL-2^-TNF-α^+ and IFN-γ^-IL-2^+TNF-α^+) (Fig. 2D), and single (IFN-γ^-IL-2^-TNF-α^+ and IFN-γ^+IL-2^-TNF-α^-) (Fig. 2E) cytokine-producing T cells. The frequency of IFN-γ^-IL-2^+TNF-α^- and IFN-γ^+IL-2^+TNF-α^- populations was too low to consider for analysis. To test the effect of MSCs on T cell degranulation, we used CD107 expression as a surrogate of T cell perforin release following SEB activation. Our results demonstrate that IFN-γ–licensed MSCs significantly inhibit the degranulation $CD3^+CD8^+$IFN-γ^+ T cells (Fig. 2F), and this finding was replicated using MSCs from three independent donors (Fig. 2G).

IDO catalytic activity is dispensable for the inhibition of T cell function by IFN-γ–licensed human MSCs

We and others had shown that human MSC suppression of T cell proliferation is dependent on IDO induction and catalytic activity (8, 22). In this study, we tested whether suppression of responder T cell cytokine production is IDO-dependent as well. We show that stimulation of MSCs with IFN-γ upregulates IDO expression at both RNA and protein levels (Fig. 3A–C). Blocking of IDO catalytic activity with 1MT negates the suppressive effect of MSCs on T cell proliferation (Fig. 3D). However, addition of 1MT

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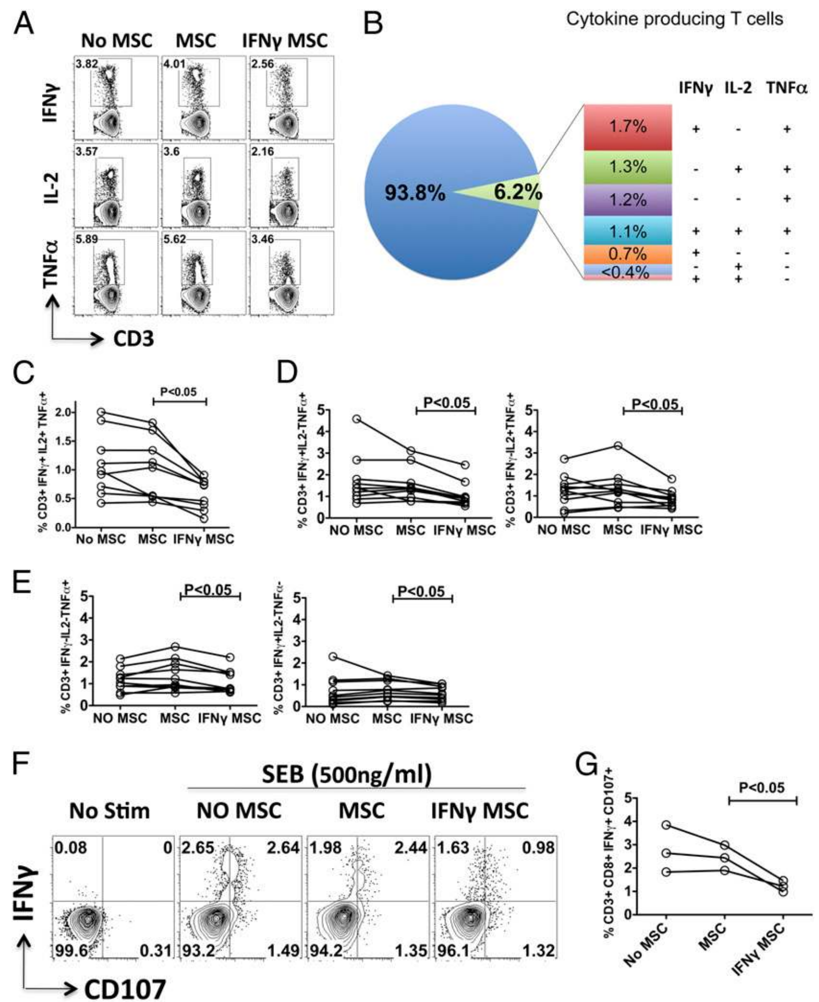

FIGURE 2. IFN-γ–licensed human MSCs inhibit degranulating, triple, double, and single cytokine-producing T cells. (**A**) PBMCs were cocultured in the presence and absence of either MSCs or IFN-γ–licensed MSCs with SEB and brefeldin A for 12–14 h. Cells were subsequently stained with Abs to CD3 and IFN-γ, IL-2, and TNF-α for flow cytometry. Percentages of $CD3^{+}IFN\text{-}\gamma^{+}$, $CD3^{+}IL\text{-}2^{+}$, and $CD3^{+}TNF\text{-}\alpha^{+}$ are shown. (**B**) Diagram depicts the relative distribution of the single, double, and triple cytokine-producing T cells in an average of eight donors. The frequencies of single, double, and triple cytokine-producing T cells were derived from the Boolean gating analysis in FlowJo software. Cumulative (**C**) triple ($CD3^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{+}TNF\text{-}\alpha^{+}$), (**D**) double ($CD3^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}TNF\text{-}\alpha^{+}$, $CD3^{+}IFN\text{-}\gamma^{-}IL\text{-}2^{+}TNF\text{-}\alpha^{+}$), and (**E**) single ($CD3^{+}IFN\text{-}\gamma^{-}IL\text{-}2^{-}TNF\text{-}\alpha^{+}$, $CD3^{+}IFN\text{-}\gamma^{+}IL\text{-}2^{-}TNF\text{-}\alpha^{-}$) cytokine-producing T cells in the presence and absence of either MSCs or IFN-γ–licensed MSCs. Paired *t* test analysis was performed through Prism software to get the *p* values. (**F**) Representative degranulation assay. PBMCs were cocultured in the presence and absence of either MSCs or IFN-γ–licensed MSCs with SEB, brefelding A, and Ab to CD107 for 12–14 h. Cells were subsequently stained with Abs to CD3 and IFN-γ for flow cytometry. (**G**) Cumulative of $CD3^{+}CD107^{+}IFN\text{-}\gamma^{+}$ T cells in the presence and absence of either MSCs or IFN-γ–licensed MSCs from three independent donors.

does not impede the ability of IFN-γ–licensed MSCs to suppress IFN-γ secretion by T cells (Fig. 3D), suggesting that an IDO-independent mechanism is at play. We further investigated the direct effect of tryptophan catabolites (akin to those molecules arising from IDO-mediated tryptophan catabolism) on T cell proliferation and function. Our results demonstrate that only kynurenine blocks T cell proliferation whereas other catabolites such as L-tryptophan, kynurenic acid, quinolinic acid, and anthranilic acid have no effect on T cell proliferation (Fig. 3E). In support of the IDO blocking assay results, we also observed that none of the catabolites in the tryptophan pathway inhibit IFN-γ secretion by T cells (Fig. 3F). Collectively, our results demonstrate that although IDO activity by IFN-γ–licensed MSCs inhibits T cell proliferation via conversion of tryptophan to kynurenine, this mechanistic pathway does not impact MSC suppression of effector T cell function.

Functional screening of IDO-independent inhibitory pathways in IFN-γ–licensed MSCs identifies B7H1/DC-PD1 mechanism

To identify contact-dependent MSC inhibitory pathways, we employed a flow cytometry–based functional screening approach interrogating candidate cell surface effectors based on published literature (26). In this screening approach, we performed coculture of PBMCs with MSCs and IFN-γ–licensed MSCs and used intracellular CD3 T cell $IFN\text{-}\gamma^{+}$ cytokine expression as a measure of response as a means to delineate candidate pathways. We anticipated three possible scenarios of reduction in T cell $IFN\text{-}\gamma^{+}$ cytokine expression: 1) both inhibitory $receptor^{+}$ or inhibitory $receptor^{-}$ T cell populations show reduction in $IFN\text{-}\gamma^{+}$ frequencies (and hence no association to augmented MSC activity), 2) only the inhibitory $receptor^{-}$ T cell population show reduction in $IFN\text{-}\gamma^{+}$ frequency (negative correlation with licensed MSCs), or 3) only the inhibitory $receptor^{+}$ T cell population show reduction in $IFN\text{-}\gamma^{+}$ frequency (this scenario suggests a causal link between MSC licensing and T cell–specific response). The identification of inhibitory receptors that recapitulate scenario 3 are identified as potential inhibitory pathways involved in IFN-γ–licensed MSC suppressor function (Fig. 4A). Based on this screening strategy, we have identified two inhibitory receptors, PD1 and CD95, on T cells that display the scenario 3 pattern (Fig. 4B, Supplemental Fig. 2). B7H1 and B7DC are the ligands for the inhibitory receptor PD1 on T cells. Similarly, CD95L is the ligand for CD95 on T cells. To confirm whether these ligands are expressed by licensed MSCs, we performed flow cytometric analysis for the surface expression of B7H1, B7DC, and CD95L on MSCs and IFN-γ–licensed MSCs. We have found that CD95L is not expressed on resting MSCs nor is it upregulated by IFN-γ licensing (Fig. 4C). However, both B7H1 and B7DC are expressed on MSCs and they are upregulated by IFN-γ stimulation (Fig. 4C). Moreover, coculturing of MSCs and activated PBMCs in transwells upregulates B7H1 and B7DC expression in 16 h. The addition of brefeldin A in the 16-h intracellular cytokine staining assay accumulated IFN-γ intracellularly and blocked its secretion, and it did not induce the upregulation of B7H1 and B7DC on MSCs (Supplemental Fig. 3). As a result, coinhibitory molecules were not upregulated in the 16-h intracellular cytokine staining assay and provided the de novo immunosuppressive effect of MSCs prelicensed with IFN-γ. To test

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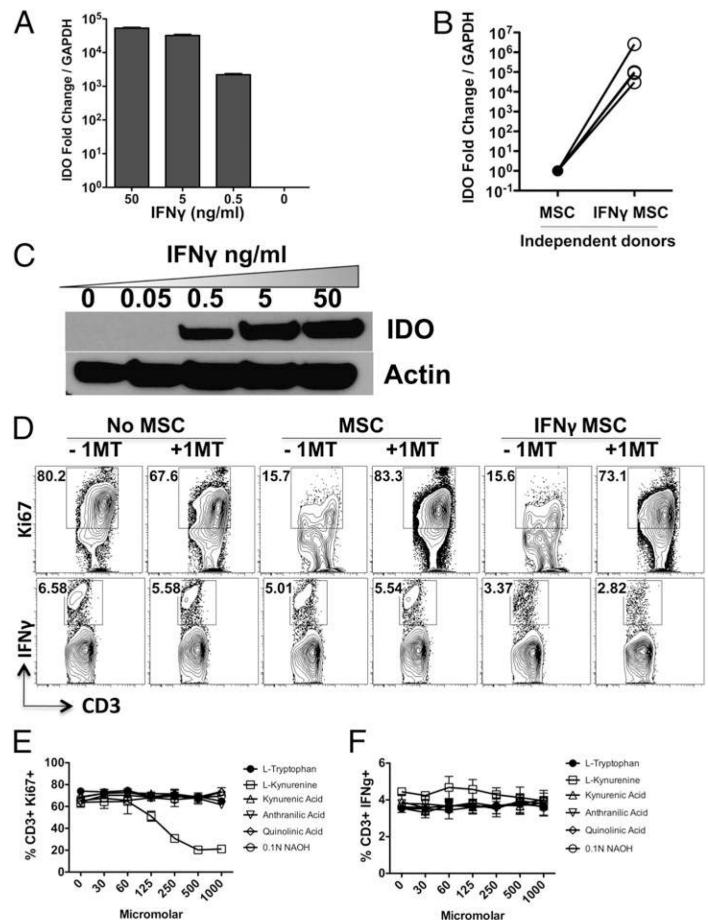

FIGURE 3. IDO is dispensable for the inhibition of T cell function by IFN-γ–licensed human MSCs. (**A**) Human MSCs were stimulated with indicated concentrations of IFN-γ for 48 h. The RNA was extracted from the cells and the expression levels of IDO mRNA were quantitated by quantitative SYBR Green real-time PCR. GAPDH mRNA levels were used as internal controls. The $\Delta\Delta C_t$ method was applied to calculate the fold change. (**B**) Cumulative fold change of IDO expression among MSCs from four independent donors is shown. (**C**) Western blot analysis to show the IDO expression at protein levels. Actin was used as an internal control. (**D**) 1MT was added at the concentration of 1 mM to PBMCs cocultured in the presence and absence of IFN-γ–licensed MSCs. For 12–14 h intracellular cytokine staining assay, intracellular IFN-γ was measured on T cells by flow cytometry. For Ki67 proliferation assay, intracellular Ki67 was measured after 4 d. Tryptophan catabolites were cocultured with PBMCs at the indicated concentrations and (**E**) intracellular IFN-γ or (**F**) Ki67 was measured 12–14 h or 4 d afterward, respectively. Representative data are shown from three independent experiments.

the functional relevance of B7H1 in attenuating cytokine production by activated T cells, we demonstrated that B7H1-Ig inhibits IFN-γ production only on $PD1^+$ T cells (Fig. 4D). Collectively, our screening identified MSC-expressed B7H1/B7DC as a candidate pathway involved in the inhibition of cytokine production by $PD1^+$ T cells.

Clustering of MHC and coinhibitory molecules are indispensable for the suppression of T cell function by IFN-γ–licensed MSCs

The activation of T cells contained within PBMC preparations can be accomplished either with SEB or CD3 and CD28-targeted Abs, and both of these stimulants activate T cells in a distinct manner. SEB links MHCs and TCR with high affinity, whereas anti-CD3 and anti-CD28 bind to the CD3/CD28 TCR complex independent of MHC and thereby activate T cells (27). To understand the orchestrated mechanism of action of coinhibitory molecules in conjunction with MHC, we compared the inhibitory effect of IFN-γ–licensed MSCs on T cells stimulated with SEB or anti-CD3/CD28 beads. Our results demonstrate that IFN-γ–licensed MSCs inhibit T cell function efficiently only with SEB but not with beads (Fig. 5A, 5C). Additionally, in contrast to SEB, bead-stimulated $PD1^+$ T cells are not susceptible to inhibition by IFN-γ–licensed MSCs (Fig. 5B, 5C). Addition of anti-CD28 in the SEB stimulation culture did not interfere with the inhibitory potential of IFN-γ–licensed MSC inhibitory potential (data not shown). This suggests that CD28 costimulation signal provided by beads does not override the inhibitory effect of IFN-γ–licensed MSCs. Phenotypic analysis demonstrated that bead-stimulated IFN-γ^+ T cells contain a higher proportion of central memory T cells ($CCR7^+$ $CD45RA^-$) than with SEB stimulation (Supplemental Fig. 4). However, no difference was observed in the proportion of effector memory T cells ($CCR7^-CD45RA^-$) between SEB and bead-stimulated IFN-γ^+ T cells (Supplemental Fig. 4). Importantly, only SEB-stimulated effector and central memory populations were inhibited by IFN-γ–licensed MSCs (Fig. 5D). These results suggest that differential inhibition susceptibility of SEB versus bead-stimulated T cells to IFN-γ–licensed MSCs is modulated by factors distinct to the responder T cell phenotype. To address this issue, we compared the inhibitory potential of B7H1-Ig protein crosslinked or noncrosslinked with anti-CD3. Our results demonstrate that B7H1-Ig protein crosslinked with anti-CD3 inhibits T cell function efficiently compared with the non–crosslinked form (Fig. 5E). These results suggest that clustering of MHC and coinhibitory molecules is necessary for the inhibition of T cell function by IFN-γ–licensed MSCs.

Inflammatory cytokine milieu does not alter the inhibitory potential of IFN-γ–licensed MSCs

Although the clinical outcome of MSC therapy is influenced by many host factors and donor MSC functionality (13), current understanding about the anti-inflammatory properties of MSCs and IFN-γ–licensed MSCs in the inflammatory cytokine milieu is lacking. Previous studies have shown that addition of IL-2 overcomes B7H1-mediated inhibition of T cell proliferation (28). We have observed that exogenous IL-2 but not IFN-γ or TNF-α overcomes the B7H1-Ig–mediated inhibition of T cell proliferation (Fig. 6A). However, none of these inflammatory cytokines modulates B7H1-Ig–mediated inhibition of IFN-γ cytokine secretion by T cells (Fig. 6B). Importantly, T cell proliferation and cytokine secretion inhibition by IFN-γ–licensed MSCs was not affected by the exogenous addition of inflammatory cytokines (Fig. 6C,

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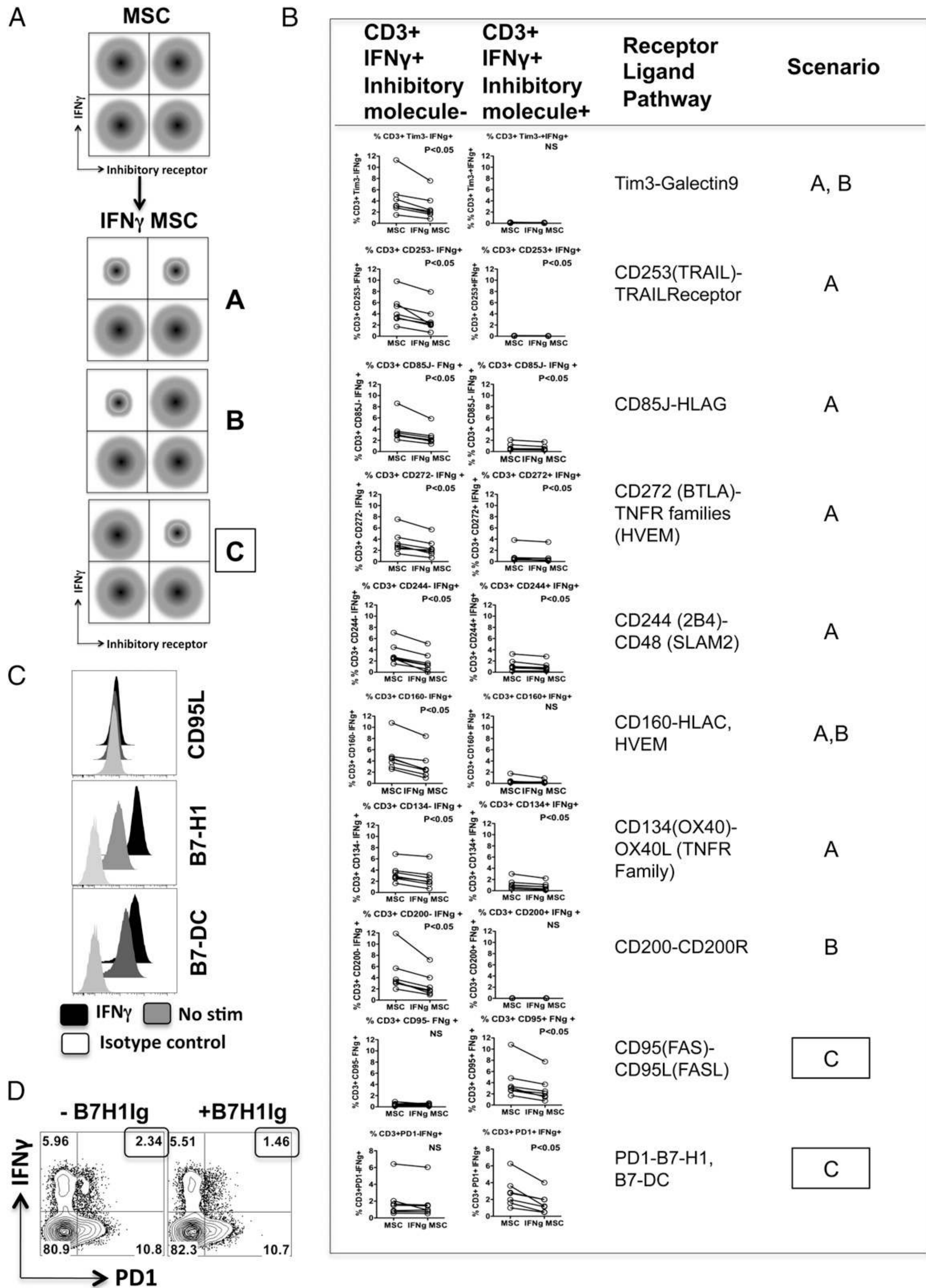

FIGURE 4. B7H1 and B7DC-PD1 is the potential coinhibitory pathway identified on IFN-γ–licensed MSCs through flow cytometry–based screening. (**A**) Schematic flow diagram depicts the possible outcomes of the frequencies of IFN-γ^+inhibitory receptor$^+$ and IFN-γ^+inhibitory receptor$^-$ populations. Boxed possible outcome represents the potential inhibitory pathway involved in the inhibitory mechanism of IFN-γ–licensed MSCs. (**B**) Entire screening scheme with the inhibitory receptor pathways and possible outcome is been shown. PBMCs cocultured with MSCs or IFN-γ–licensed MSCs were stained for CD3, IFN-γ, and the respective inhibitory receptors. Cumulative multiple donor frequencies of IFN-γ^+inhibitory receptor$^+$ and IFN-γ^+inhibitory receptor$^-$ populations are shown. Paired *t* test analysis was performed through Prism software to get the *p* values. (**C**) B7H1, B7DC, and CD95L expression is shown on the unstimulated (dark gray) and IFN-γ–stimulated MSCs for 48 h (black). Appropriate isotype controls (light gray) are also shown. (**D**) PBMCs were stimulated on the plates coated with anti-CD3 in the presence and absence of B7H1-Ig. Flow cytometry analysis of $CD3^+PD1^+IFN\text{-}\gamma^+$ and $CD3^+PD1^-IFN\text{-}\gamma^+$ frequencies are shown. Representative data are shown from three independent experiments

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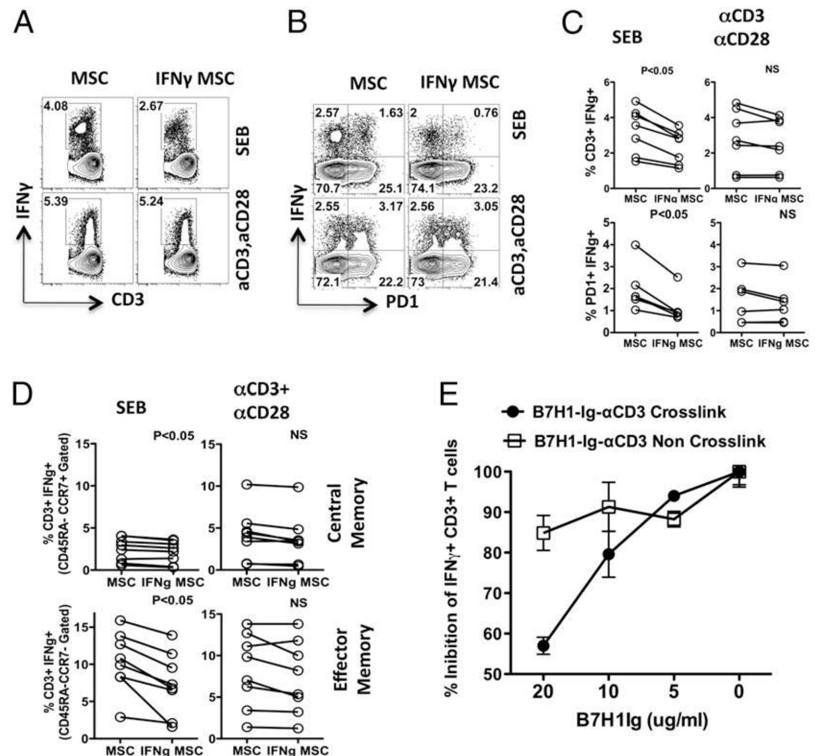

FIGURE 5. Clustering of MHC and coinhibitory molecules is indispensable for the inhibition of T cell function by IFN-γ–licensed MSCs. Representative (**A**) $CD3^+IFN\text{-}\gamma^+$ frequencies, (**B**) $CD3^+PD1^+IFN\text{-}\gamma^+$ frequencies, and (**C**) cumulative data of PBMCs from independent donors stimulated either with SEB or anti-CD3 and anti-CD28 beads are shown. MSCs or IFN-γ–licensed MSCs were cocultured with PBMCs and stimulated either with SEB or anti-CD3 and anti-CD28 beads for 12–14 h with brefeldin A, and subsequently the cells were stained for the appropriate markers and subjected to flow cytometry analysis. (**D**) Cumulative data show the susceptibility of $CCR7^-CD45RA^-$ (effector memory) T cells to the inhibition by MSCs and IFN-γ–licensed MSCs stimulated with SEB or anti-CD3 and anti-CD28 beads. (**E**) Percentage inhibition of $CD3^+IFN\text{-}\gamma^+$ T cells cocultured with crosslinked or non–crosslinked anti-CD3 and B7H1-Ig. For crosslinking, B7H1-Ig protein was coated with anti-CD3 overnight. For non-crosslinking, B7H1-Ig protein was coated alone, and anti-CD3 was added in solution with PBMCs. Percentage inhibition was calculated by considering $CD3^+IFN\text{-}\gamma^+$ T cells as 100% in the absence of B7H1-Ig protein. Representative data are shown from three independent experiments

6D). These results demonstrate that an IL-2–rich microenvironment can modulate B7H1-mediated inhibition of T cell proliferation but not function. However, MSCs and IFN-γ–licensed MSCs inhibit T cell proliferation and function effectively in the presence of inflammatory cytokines.

B7H1 and B7DC knockdown reverses the inhibitory potential of IFN-γ–licensed MSCs on T cell function

To investigate the functional role of B7H1 and B7DC on the inhibitory effects of MSCs and IFN-γ–licensed MSCs on T cell function, we used an siRNA knockdown approach (Fig. 7A). Our results demonstrate that MSCs transfected with B7H1 and B7DC siRNA and subsequently stimulated with IFN-γ show reduction in the upregulation of B7H1 and B7DC but not MHC class I and IDO (Fig. 7B, 7C). These results demonstrate that the siRNA approach is specific to the knockdown of B7H1 and B7DC. We then investigated the inhibitory potential of B7H1 and B7DC siRNA-transfected IFN-$\gamma^{+/-}$–licensed MSCs on T cell function. Cumulative data of paired analysis within independent donors demonstrate that compared with control siRNA, B7H1 and B7DC-transfected IFN-γ–licensed MSCs significantly decrease their inhibitory effects (Fig. 7D). Collectively, our results demonstrate that B7H1 and B7DC expression by IFN-γ–licensed MSCs directly inhibits T cell function.

Discussion

Previous studies have found that unlicensed MSCs solely block mitogen-driven T cell proliferation and do not inhibit the effector functions of T cells (25, 29). We have confirmed previous observations that IFN-γ stimulation of MSCs upregulates MHC class I, MHC class II, B7H1, B7DC, and IDO but does not induce expression of CD80 and CD86 (21, 30). In this study, we have further demonstrated that IFN-γ–licensed MSCs inhibit not only T cell proliferation but effector function as well, and that this effect is independent of IDO catalytic activity and functionally rests on the de novo expression of B7H1/B7DC inhibitory molecules. Based on the two-signal hypothesis for the activation of T cells, professional APCs such as dendritic cells provide both TCR and costimulatory engagements (31). The absence of costimulatory molecules, upregulation of coinhibitory molecules, and IDO by IFN-γ are all consistent with MSC-driven T cell suppressor functionality.

There are now numerous suppressor pathways suggested to be operative in human MSCs distinct to IFN-γ–augmented IDO catalytic activity cells (32, 33), and we tested which among these were augmented by IFN-γ licensing. We specifically examined the correlation between MSC licensing and T cell cytokine suppression with MSC expression of galectin-9 (34), TRAIL (35), HLAG (36), TNFR (37), SLAM2 (38), HLAC (39), OX40L (40), CD200R (41), FASL (42), and B7H1/B7DC (26). Among these, we identified and validated B7H1 and B7DC as the sole inhibitory contact factors deployed by IFN-γ licensing (26). We did not detect the surface expression of CD95L by MSCs. Considering the published report on the role of MSCs in inducing T cell apoptosis by CD95L, the regulation of its surface expression on MSCs needs to be studied in the future (42). It is well known that B7H1 and B7DC inhibit the proliferation of $PD1^+$ T cells (43), and IFN-γ–licensed mouse MSCs have been shown previously to inhibit T cell proliferation through B7H1 (44, 45). Our results further define a role of MSC-expressed B7H1 and B7DC in suppressing the T cell effector function independent of proliferation as well.

These data add to a growing body of knowledge examining the immune plasticity of MSCs and their veto function (46). Interestingly, resting MSCs express immune-augmenting leukines CCL2 and IL-6. Although counterintuitive, the CCL2 expressed by MSCs is subjected to partial N-terminal cleavage converting CCL2 to an antagonist with profound inhibitory effects on Th17 cells and immunoblasts as well (47, 48). Additionally, MSC-derived IL-6 will promote regeneration of radiation-injured gut epithelium and has been suggested to play an important role in converting monocytes to suppressor macrophages (49, 50). It has also been previously shown that IFN-γ–licensed MSCs will upregulate expression of TLR2 and TLR3 in a manner seen with IFN-α–licensed MSCs as well. Dis-

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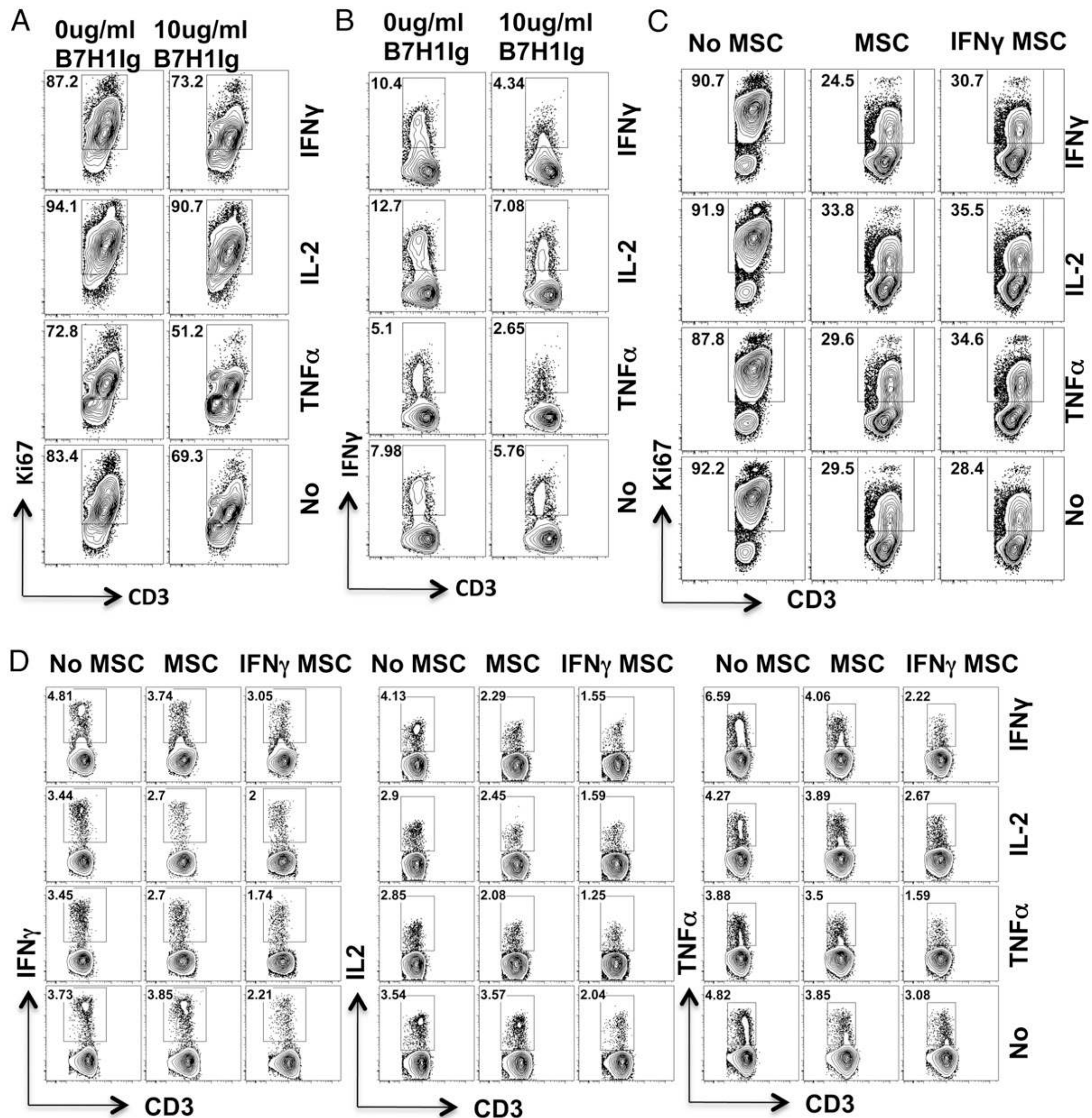

FIGURE 6. Inflammatory cytokines do not reverse the inhibitory potential of IFN-γ–licensed MSCs. PBMCs were stimulated (**A**, **B**) on the plates coated with anti-CD3 alone or in combination with B7H1-Ig, or (**C**, **D**) in the presence and absence of MSCs and IFN-γ–licensed MSCs with SEB. Indicated cytokines were added to the culture at a final concentration of 500 ng/ml. (B and D) Intracellular cytokine staining was performed 12–14 h after initiation of culture with brefeldin A with Abs to CD3 and IFN-γ, IL-2, and TNF-α. (A and C) Ki67 proliferation assay was performed after 4 d with the Abs to CD3 and Ki67. Representative data are shown from two independent experiments.

tinctly, TNF-α licensing of MSCs does not upregulate IDO or B7H1, but will markedly upregulate TLR2 and TLR7 expression. Importantly, although IFN-γ and TNF-α lead to distinct responses in MSCs, when used in combination there is a synergistic effect on augmentation of IDO and COX2 expression by human MSCs as well as inhibition of Th17 cells (51–53). Although TGF-β from MSCs has been invoked in genesis of regulatory T cells, its expression is not increased by IFN-γ. IL-10 is never expressed by MSCs and it is not augmented by cytokine licensing (22, 54). Thus, our findings in combination with others confirm the independent and overlapping inhibitory mechanisms that IFN-γ–licensed MSCs possess to inhibit T cell responses at different stages.

We have used the superantigen SEB to stimulate T cells as a surrogate system that mimics the physiological activation of Ag-specific T cells by MHC and TCR engagement in an effort to gain novel insights complementary to in vitro observations made with responder T cells pharmacologically activated with anti-CD3 or PMA/ionomycin stimulation (27). SEB specifically activates T cells expressing Vβ receptor by linking it to HLA-DR on APCs (27). The strong affinity of SEB to HLA-DR and TCR provides the stimulus for T cell activation independently of their antigenic affinity and of their secondary receptors such as CD4 or CD8, which can bind to MHC class II or MHC class I, respectively. Our mechanistic studies with SEB and anti-CD3 have demonstrated that clustering of MHC and coinhibitory molecules are indispensable for the inhibition of T cell function by IFN-γ–licensed MSCs. Moreover, IFN-γ–licensed MSCs inhibit T cell cytokine production only through a contact-dependent mechanism (data not shown). In agreement with our results, Yokosuka et al. (55) described that PD1 engagement with its ligand, PDL1, causes the clustering of PD1 and

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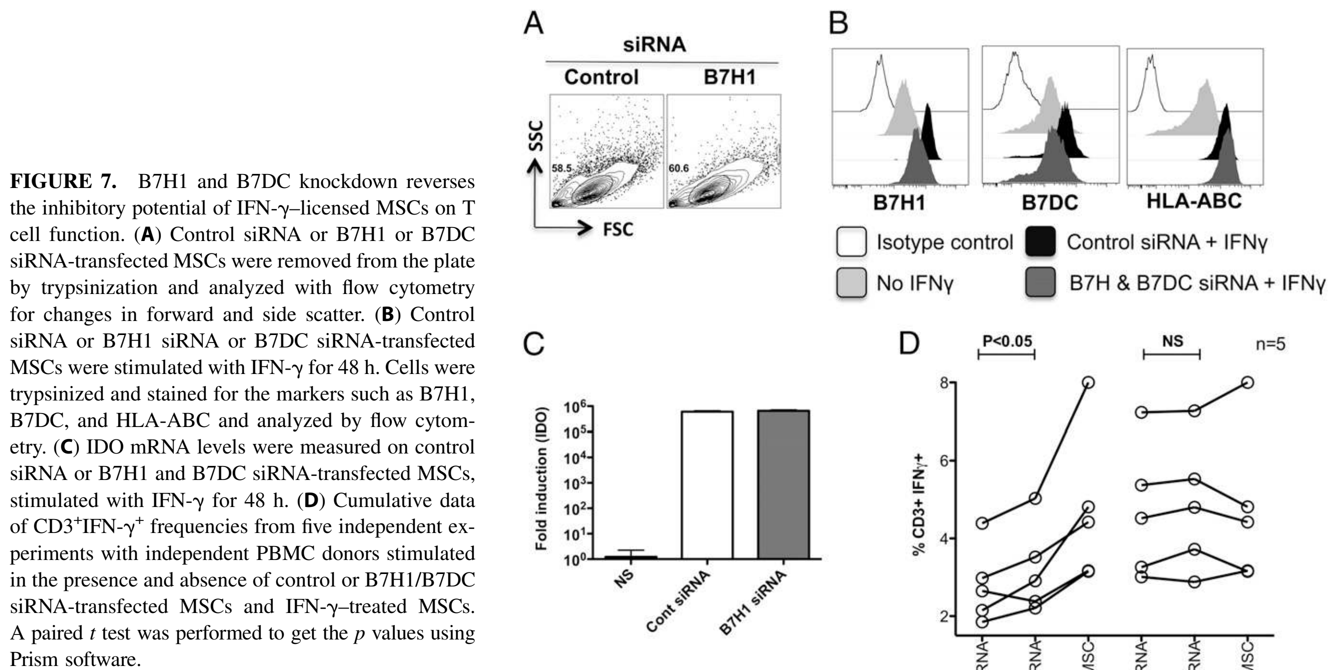

FIGURE 7. B7H1 and B7DC knockdown reverses the inhibitory potential of IFN-γ–licensed MSCs on T cell function. (**A**) Control siRNA or B7H1 or B7DC siRNA-transfected MSCs were removed from the plate by trypsinization and analyzed with flow cytometry for changes in forward and side scatter. (**B**) Control siRNA or B7H1 siRNA or B7DC siRNA-transfected MSCs were stimulated with IFN-γ for 48 h. Cells were trypsinized and stained for the markers such as B7H1, B7DC, and HLA-ABC and analyzed by flow cytometry. (**C**) IDO mRNA levels were measured on control siRNA or B7H1 and B7DC siRNA-transfected MSCs, stimulated with IFN-γ for 48 h. (**D**) Cumulative data of $CD3^{+}IFN\text{-}\gamma^{+}$ frequencies from five independent experiments with independent PBMC donors stimulated in the presence and absence of control or B7H1/B7DC siRNA-transfected MSCs and IFN-γ–treated MSCs. A paired *t* test was performed to get the *p* values using Prism software.

TCR, which subsequently mediates the suppression of T cells. PD1 clustering and PD1-TCR colocalization in the microcluster is necessary for the suppression mediated by PD1.

T cell anergy can be reversed by exogenous IL-2, and we tested whether environmental IL-2 will mitigate the inhibitory effect of MSCs on T cells (56). Interestingly, we observed that whereas IL-2 can override PD1-driven blockade of T cell proliferation, exogenous IL-2 does not interfere with IFN-γ–licensed ability of MSCs to block T cell effector function and proliferation. These data suggest that MSCs deploy suppressor pathways additive to B7H1/B7DC, which complement its effect and are incurious to IL-2 effect.

Culture-expanded bone marrow–derived MSCs possess a unique transcriptome that distinguishes them from fibroblasts and hematopoietic stem cells, and these cells retain cell physiological properties that likely reflect the innate biological functions of the clonal marrow-nested progenitors from which they arose (57). It is now recognized that MSCs can home to sites of injury and malignant proliferation and participate in the remodeling and tissue structure where they likely play a regenerative and immune-attenuating role (58). Our in vitro analyses of culture-expanded MSCs and their immune plasticity in response to IFN-γ offer a surrogate insight into the likely in vivo cell biology of native MSCs in response to inflammatory cues. Because most of the IFN-γ to which MSCs would be exposed in vivo likely originates from activated T cells and NK cells, understanding the mechanisms at play between IFN-γ–licensed MSCs and activated T cells provides valuable insights into the physiological as well as the maladapted interactions between MSCs and effector cells (19). The important insight in the present study is that licensed MSCs deploy complementary and nonoverlapping T cell veto mechanisms, namely, IDO and B7H1/B7DC upregulation. These observations may inform a strategy targeting tumor microenvironment as part of a cancer immunotherapy strategy where contemporaneous PD1 and IDO blockade may be required for optimally overcoming immune escape mechanisms by tumor cloaked in host-derived stromal cells (59, 60). Furthermore, these data inform the translational use of culture-expanded MSCs as a transfusion product to treat autoimmune and alloimmune ailments (16). By considering the combinatorial IFN-γ–licensed direct effect of MSCs on T cell proliferation, effector function, and the indirect effect on T cell proliferation through soluble factors, we predict that IFN-γ–prelicensed autologous MSCs would generate a more potent and primed cell therapy product for suppression of immune ailments (61). The inhibitory potential of IFN-γ–licensed MSCs on the highly inflammatory $TNF\text{-}\alpha^{+}$–producing T cells we observed is of significant interest because this T cell effector subset may be associated with the pathogenesis of autoimmune disorders amenable to MSC cellular therapies (62).

In conclusion, in the present study we define in part the immune plasticity of the IFN-γ–licensed MSC immunosuppressive effect on T cell proliferation and effector function and we provide the rationale for testing IFN-γ–licensed MSCs as an augmented MSC therapy to treat immune disorders.

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Disclosures

The authors have no financial conflicts of interest.

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