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Mesenchymal Stem Cells Inhibit Human Th17 Cell Differentiation and Function and Induce a T Regulatory Cell Phenotype

Soufiane Ghannam,* Jérôme Pène,* Gabriel Torcy-Moquet,† Christian Jorgensen,* and Hans Yssel*

Mesenchymal stem cells (MSCs) exert immunomodulatory properties via the inhibition of T cell activation and proliferation. Because of the deleterious role of Th17 cells in the pathogenesis of inflammatory disease, we investigated whether proinflammatory cytokines could modify the expression of adhesion molecules on human MSCs, thereby contributing to increased Th17 cell adhesion to MSCs and, as a consequence, modulating the function of the latter cells. IFN-γ and TNF-α synergistically enhanced the expression of CD54 by MSCs, enabling the CCR6 chemokine ligand CCL20 to induce in vitro adhesion of Th17 cells to MSCs. MSCs prevented the in vitro differentiation of naive $CD4^+$ T cells into Th17 cells and inhibited the production of IL-17, IL-22, IFN-γ, and TNF-α by fully differentiated Th17 cells; this was mediated, in part, via PGE_2, the production of which was enhanced in cocultures with Th17 cells. Moreover, MSCs induced the production of IL-10 and trimethylation of histone H3K4me3 at the promoter of the *FOXP3* gene locus, whereas it suppressed trimethylation of the corresponding region in the RORC gene in Th17 cells. These epigenetic changes were associated with the induction of fork head box p3 and the acquisition by Th17 cells of the capacity to inhibit in vitro proliferative responses of activated $CD4^+$ T cells, which was enhanced when MSCs were preincubated with IFN-γ and TNF-α. These results showed that, under inflammatory conditions, MSCs mediate the adhesion of Th17 cells via CCR6 and exert anti-inflammatory effects through the induction of a T cell regulatory phenotype in these cells. ***The Journal of Immunology*****, 2010, 185: 302–312.**

Mesenchymal stem cells (MSCs), also referred to as bone marrow-derived stromal cells, represent a heterogenous population of fibroblast-like cells that can be isolated from bone marrow, as well as from many adult tissues, and that are precursors of three main cell types of the mesodermal lineage, including osteocytes, chondrocytes, and adipocytes (reviewed in Ref. 1). MSCs are involved in different developmental pathways and in a large number of diverse biological processes. In particular, bone marrow-derived MSCs exert broad immunomodulatory effects, because they inhibit the proliferation of T cells (2, 3) and the activation, cytotoxicity, and cytokine production of NK cells in vitro (4). Furthermore, MSCs interfere with the in vitro differentiation of $CD34^+$ hematopoietic precursor cells and monocytes into dendritic cells (5–8), and they decrease the expression of MHC class II and several costimulatory molecules, such as CD80 and CD86, as well as the production of IL-12 by mature dendritic cells, thereby impairing their capacity to present Ag. Moreover, MSCs have anti-inflammatory effects, as shown by their capacity to decrease the production of TNF-α by myeloid dendritic cells and to increase the production of IL-10 by plasmacytoid dendritic cells (9). Human MSCs were also found to inhibit the in vitro proliferation of B cells and their differentiation into plasma cells (10), although their effect is certainly more complex, because it was recently reported that bone marrow-derived MSCs are able to induce polyclonal expansion and differentiation of B cells (11).

Several molecular mechanisms that might underlie the immunosuppressive effects of MSCs have been reported, involving cell surface molecules and soluble factors. Inhibition of murine T lymphocyte proliferation by bone marrow MSCs was shown to be dependent on cellular interactions requiring activation of the programmed death-1 pathway (12), although no information is available with respect to human cells. Among potentially immunosuppressive soluble factors, IFN-γ was found to play a pivotal role in the inhibition of human T cell and NK cell function, as a result of its capacity to induce the production of IDO by MSCs (13, 14), resulting in the depletion of tryptophan, which is an essential amino acid for lymphocyte proliferation. However, in a study using genetically deficient mouse strains, it was reported that IFN-γ alone was insufficient and that the immunosuppressive function of MSCs also required the concomitant presence of TNF-α, IL-1α, or IL-1β, which, together, induced the expression of high levels of several chemokines and inducible NO synthase (iNOS) (15), responsible for the cytokine-induced immunosuppression by MSCs. These results confirmed previously published findings identifying iNOS as a single mediator capable of mediating immunosuppression by MSCs (16), and they ruled out several other soluble factors proposed as mediators of immunosuppression by MSCs, including IL-10, TGF-β, PGE_2, and IDO (15). It needs to be determined whether this difference between mouse and human

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; FOXP3, fork head box p3; iNOS, inducible NO synthase; MFI, mean fluorescence intensity; MSC, mesenchymal stem cell; OSM, oncostatin M; ROR, retinoid-related orphan receptor; Treg, T regulatory.

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MSCs is species dependent. Finally, it was shown recently that human MSCs secrete a soluble isoform of the nonclassic HLA class I molecule HLA-G5 in an IL-10–dependent manner and that HLA-G5 might contribute to the expansion of $CD4^+CD25^{high}$ $FOXP3^+$ T regulatory (Treg) cells (17). However, results from the latter study also showed that HLA-G5 produced by MSCs inhibits the secretion of IFN-γ, thereby impairing the production of IDO, which contrasts with earlier reports (13, 14). Together, these results indicate that multiple mechanisms may underlie the immunomodulatory effects of MSCs on innate and adaptive immune responses, and they point to certain discrepancies reported in the literature.

Recently, a novel subset of Th cells was identified and denominated Th17 cells, on the basis of their production of high levels of IL-17 (18). Th17 cells protect the host against extracellular pathogens encountered at mucosal surfaces, and they play a detrimental role in experimental murine models of inflammatory disease, such as multiple sclerosis and rheumatoid arthritis, as well as in human inflammatory bowel disease and psoriasis (19–22). In addition to IL-17A and IL-17F, fully differentiated Th17 cells produce high levels of TNF-α, IL-22, IL-26, and CCL20; furthermore, they express the chemokine receptor CCR6 at their cell surface (23–26). Although the precise mechanism is controversial, human Th17 cell differentiation from naive cells is induced by the combined activity of TGF-β, IL-1β, and IL-23; IL-21 reportedly is involved in this process as well, through its effects on naive T cells (27–29). Together, these cytokines modulate IL-17 production by inducing the expression of a key lineage-specific transcription factor, the retinoid-related orphan receptor (ROR)C, which is the homolog of RORγt in the mouse (30–32). It was shown in the mouse that in an inflammatory environment, characterized by the presence of IL-6, endogenous TGF-β is able to differentiate Treg cells into IL-17–producing cells (33), suggesting a strong interlineage relationship between both types of cells. However, whether an opposite mechanism is operational, in that Th17 cells might be converted into T cells with immunoregulatory properties, remains to be determined.

Because of the immunomodulatory activity of bone marrow-derived MSCs, we investigated their effect on the differentiation of the Th17 lineage, as well as on the function of fully differentiated Th17 cells, in particular in the context of an inflammatory environment. Moreover, because MSC-induced immunosuppressive effects require cell–cell contact, we determined whether the interaction between T cells and MSCs is governed by molecular mechanisms similar to those involved in the arrest of T cells to endothelial cells under flow conditions.

In this article, we show that Th17 cells adhere to MSCs through the joined involvement of CCR6 and CD11a/CD18 and their respective ligands and that these interactions lead to the induction of a regulatory phenotype in fully polarized tissue-infiltrating human Th17 cells.

Materials and Methods

T cells

$CD4^+$ peripheral blood human T cells or $CD4^+CD45^+$ naive cord blood T cells (purity 99%) were isolated from freshly collected, heparinized peripheral (Etablissement Français du Sang, Montpellier, France) or cord (Department of Obstetrics, Centre Universitaire Hospitalier Arnaud de Villeneuve, Montpellier, France) blood, respectively, using the RosetteSep method (StemCell Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's specifications. In vitro differentiated Th17 cells were obtained by stimulating 2×10^5 $CD4^+$ cord blood T cells with magnetic beads (T cell/bead ratio 4:1), coated with anti-CD3 and anti-CD28 mAbs (Expander beads, Invitrogen), and subsequent culture with rIL-1β, rIL-6, rTGF-β (R&D Systems, Minneapolis, MN; final concentration 10 ng/ml), and rIL-23 (a kind gift of Dr. Hugues Gascan, Inserm U564, Angers, France; final concentration 100 ng/ml) in 24-well culture plates (BD Biosciences, San Jose, CA). Cells were used after 7 d of culture. Fully in vivo differentiated human Th17 cell clones were generated from tissue-infiltrating T cells, as described previously (24). In the current study, these T cell clones were isolated from biopsies taken from cutaneous lesions of patients with psoriasis vulgaris or intestinal lesions of patients with active Crohn's disease (kindly provided by Dr. Nadia Raison-Peyron, Service de Dermatologie and Dr. Michel Veyrac, Service de Chirurgie Digestive, Centre Universitaire Hospitalier St. Eloi). Biopsies were obtained after informed consent forms were signed, according to the protocol established by the Ethics Committee of the University Hospitals of Montpellier. T cell clones were cultured in the presence of an irradiated feeder cell mixture and PHA (Murex, Norcross, GA), as described (34), propagated with rIL-2 (2 ng/ml; R&D Systems), and used in experiments 10–14 d after the onset of propagation. All cultures and experimental procedures were carried out in Yssel's medium (35), supplemented with 1% human AB^+ serum (Etablissement Français du Sang, Lyon, France).

Mesenchymal stem cells

Human bone marrow MSCs were obtained from Biopredic International (Rennes, France) from healthy volunteers undergoing orthopedic surgery. MSCs were cultured in Yssel's medium, supplemented with 10% FCS (Biowest, Nuaillé, France), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 ng/ml bovine fibroblast growth factor (R&D Systems). In this study, MSCs obtained from three donors were used at the third or fourth passage. For flow cytometry or experiments measuring cell arrest under flow conditions (see below), MSCs were cultured for 48 h in the presence of IL-1β, IFN-γ, TNF-α, or oncostatin M (OSM), either alone or in combination (each cytokine at a concentration of 10 ng/ml; R&D Systems).

Flow cytometry analysis

Flow cytometry analysis of T cells and MSCs was carried out as described by Scheffold et al. (36), using PE-conjugated anti-CCR6 (R&D Systems), anti-CD54, CD62L and CD62P, integrin β7, CD29, CD58, CD49d, and CD11a mAbs and isotype control (BD Biosciences). Cells were analyzed using a FACSCalibur flow cytometer, and data were collected for 10,000 events/sample using CellQuest Pro software (both from BD Biosciences).

Detection of cytokine production and expression of transcription factors by intracellular and intranuclear staining, respectively

Intracellular staining was performed on T cells after 6 h of activation with PMA (1 ng/ml) and ionomycin (1 μg/ml; both from Calbiochem, San Diego, CA), in the presence of brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, MO) for the final 3 h of culture. Then, the cells were fixed overnight at 4°C with paraformaldehyde 3% and subsequently stained with fluorochrome-conjugated mAbs. Flow cytometry analysis of cytokine production was carried out using IL-17–allophycocyanin-specific (R&D Systems, San Diego, CA), IL-17–PE-specific, IL-10–Alexa Fluor 647-specific (eBioscience, San Diego, CA), and IL-22–allophycocyanin-specific (a kind gift of Dr. Wen-Jun Ouyang, Genentech, South San Francisco, CA) mAbs. The expression of transcription factors was assessed using PE-conjugated mAbs specific for fork head box p3 (FOXP3) (Miltenyi Biotec, Paris, France) and RORC (eBioscience). All mAbs were used at concentrations recommended by the manufacturer. Immunostaining and cell fluorescence were carried out as mentioned above (36).

Measurement of cell adherence under flow conditions

Adherence of T cells to MSCs under flow conditions was measured according to the technique described by Alon et al. (37). MSCs were cultured on fibronectin-coated glass coverslips until a confluent monolayer was obtained, which was then placed in a flow chamber (Flow Chamber System, Provitro, Berlin, Germany). MSCs were preincubated with IFN-γ and TNF-α (10 ng/ml) for 48 h prior to their use in the assay. T cells were labeled with CFSE (10 μM for 10^6 T cells; Molecular Probes, Eugene, OR), resuspended at a concentration of 1×10^6 cells/ml in Yssel's medium, supplemented with 1% human serum, and injected into the flow chamber, under conditions of shear stress, using a precision peristaltic pump IPC4N (Ismatec, Glattbrugg, Switzerland). The effect of CCL20 (20 ng/ml; R&D Systems) was tested by adding this CCR6 ligand to the T cells, during the 3 min prior to when the cells reached the flow chamber. All flow experiments were performed at 37°C, using a heated microscope plate chamber and prewarmed medium. At first, the cells were drawn through the chamber

Table I. *Primers used to amplify the relevant regions of the* FOXP3 *and* RORC *genes*

Amplicon Name		5′-Primer Sequence-3′
RORC −14 kb	Forward	GGCGCCTGTCATCCTACTCACTGCA
	Reverse	CCTTGGCTCCCTGTCCTTCTCAGCA
RORC +800 kb	Forward	TGCCCAATCCAGGGACATGAGGAC
	Reverse	TGCCCAATCCAGGGACATGAGGAC
RORC +1926 kb	Forward	GGGTCAGTGCCACTGGTCCTTCTCA
	Reverse	TGTCACAGTCCCAAGCCCCATGTG
Foxp3 −8 kb	Forward	TTGGACAAGGACCCGATGCCCA
	Reverse	GAAGATCTCGGCCCTGGAAGGTTCC
Foxp3 +1017 kb	Forward	GAGAGCCCAGCCATGATCAGCCTCA
	Reverse	GGGACTTGGGGGTTCTGTGAAGCCA
Foxp3 +2132 kb	Forward	AATCTGCACTCCTGCTTTGCCCTGC
	Reverse	GGACATGTGGGGTTGCTGCTAAGGG

as a bolus at 1 dyne/cm^2 until the first cells entering the chamber was observed under the microscope. The shear flow was then reduced to 0.2 dyne/cm^2 for 1 min to allow T cell accumulation; it was subsequently increased to 0.75 dyne/cm^2 for a 5-min period during which lymphocyte arrest was recorded on a single field in the center of the chamber, using an Axiovert 25 B/W epifluorescence inverted microscope, coupled to an Axiocam high-resolution numeric camera (both from Zeiss, Oberkochen, Germany). Finally, after washing the flow chamber with medium, 10 adjacent fields around the center of the chamber were also recorded.

Cocultures of MSCs and T cells

MSCs were cocultured at 5 × 10^4 cells/ml with naive CD4$^+$CD45RA$^+$ T cells during their in vitro differentiation into Th17 cells or with fully differentiated Th17 clones in 24-well plates in a final volume of 1 ml. After 3 d, the T cell cultures were separated from the MSC monolayer, transferred to a new culture plate, and propagated with 2 ng/ml rIL-2. Seven days later, the T cells were collected and analyzed for function and phenotype.

Measurement of suppressive activity

The suppressive activity of T cells was determined by their capacity to inhibit anti-CD3/CD28 mAb-induced allogenic T cell proliferation. One million purified CD4$^+$ T cells were labeled with CFSE and stimulated with anti-CD3/CD28 mAb-coated Expander beads (T cell/bead ratio 4:1) in the presence or absence of an equal amount of fully differentiated Th17 cells that had been stimulated for 4 d in the presence or absence of MSCs, as described above. Cultures were carried out in 24-well plates. The effect of proinflammatory cytokines on the MSC-mediated immunomodulatory properties was determined by adding 10 ng/ml TNF-α and IFN-γ to the cultures. After 3 d of culture, cell division of the proliferating CD4$^+$ T cells was analyzed by flow cytometry.

Measurement of cytokine and PGE$_2$ production by ELISA

For the measurement of cytokine production, 10^6 T cells, cultured in the presence or absence of MSCs, were activated with plate-bound anti-CD3 mAb (mAb SPV-T3b; 10 μg/ml in PBS; Beckman Coulter, Villepinte, France) and the anti-CD28 mAb L293 (1 μg/ml; BD Biosciences, Le Pont-De-Claix, France) in a final volume of 1 ml. After 48 h of culture, the culture supernatants were collected, and the production of IL-4 and IFN-γ was analyzed as described (24); the production of IL-5, IL-10, and IL-13 was analyzed using Eli-pair kits (Diaclone, Besançon, France); and the production of IL-17, IL-22, OSM, and CCL20 was analyzed using DuoSet kits (R&D Systems). PGE$_2$ production was measured in the culture supernatants of MSCs, cultured in the presence or absence of Th17 cells for 3 d, using an ELISA kit (R&D Systems) according to the manufacturer's instructions.

Quantitative RT-PCR

Total RNA was extracted from cell lysates obtained with TRIzol reagent (Invitrogen). For mRNA analysis, 500 ng total RNA was reverse transcribed using Multiscribe reverse transcriptase (Applied Biosystems, Foster City, CA). The following primers, designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer38.cgi), were used: *FOXP3*: sense, 5′-CCAGCCATGATCAGCCTCAC-3′, antisense, 5′-CCGAAAGGGTGCTGTCCTTC-3′; *IL10*: sense, 5′-CAAATGAAGGATCAGCTGGACAA-3′, antisense, 5′-GCATCACCTCCTCCAGGTAAAAC-3′; *IL-17*: sense, 5-CCCGGACTGTGATGGTCAAC-3′, antisense, 5′-GCACTTTGCCTCCCAGATCA-3′; *RORC*: sense, 5′-GCATGTCCCGAGATGCTGTC-3′, antisense, 5′-CTGGGAGCCCCAAGGTGTAG-3′; and ribosomal protein S9: sense 5′-AAGGCCGCCCGGGAACTGCTGAC-3′, antisense, 5′-ACCACCTGCTTGCGGACCCTGATA-3′. Quantitative PCR was performed using the FastStart DNA Master SYBR Green I kit and a LightCycler 480 Detection system (both from Roche, Meylan, France), as specified by the manufacturer. For quantification, values express the relative mRNA level of specific gene expression as obtained using the $2^{-\Delta Ct}$ method and *Rsp9* housekeeping gene for normalization.

Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously (38). For each ChIP experiment, chromatin was isolated from 2 × 10^7 T cells. Abs against histone H3K4me3, as well as an anti-GAPDH mAb, as a negative control, were used (all from Upstate Biotechnology, Lake Placid, NY). The data were normalized with inputs taken from samples before the immunoprecipitation and treated under the same conditions. Primers used to amplify the relevant regions of the *FOXP3* and *RORC* genes are listed in Table I.

Results

IFN-γ and TNF-α synergistically enhance the expression of CD54 by MSCs

The interaction of various selectins and integrins with their ligands is crucial for the arrest of T lymphocytes to inflamed endothelial cells under flow conditions (39). To determine whether the

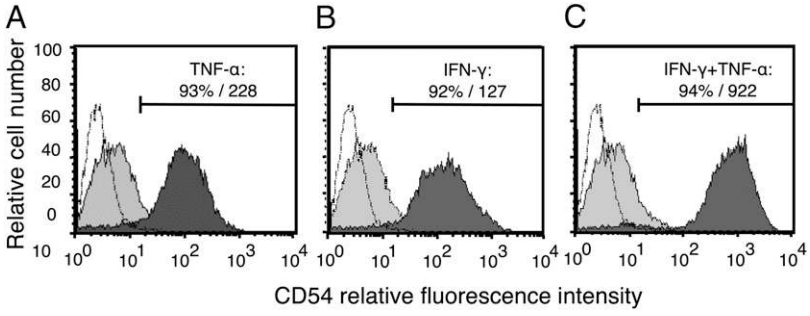

FIGURE 1. IFN-γ and TNF-α synergistically enhance the expression of CD54 by MSCs. One million MSCs were cultured in the absence (light gray graph) or presence (dark gray graph) of TNF-α (*A*), IFN-γ (*B*), or the combination of both cytokines (each at 10 ng/ml) (*C*) for 48 h, after which the expression of CD54 was determined by flow cytometry. The *x*- and *y*-axes represent fluorescence (four decade log scale) and relative cell number, respectively. Cells stained with control isotype-matched mAb are represented by the dotted lines. Values represent the percentage of stained cells and MFI. MFI, mean fluorescence intensity.

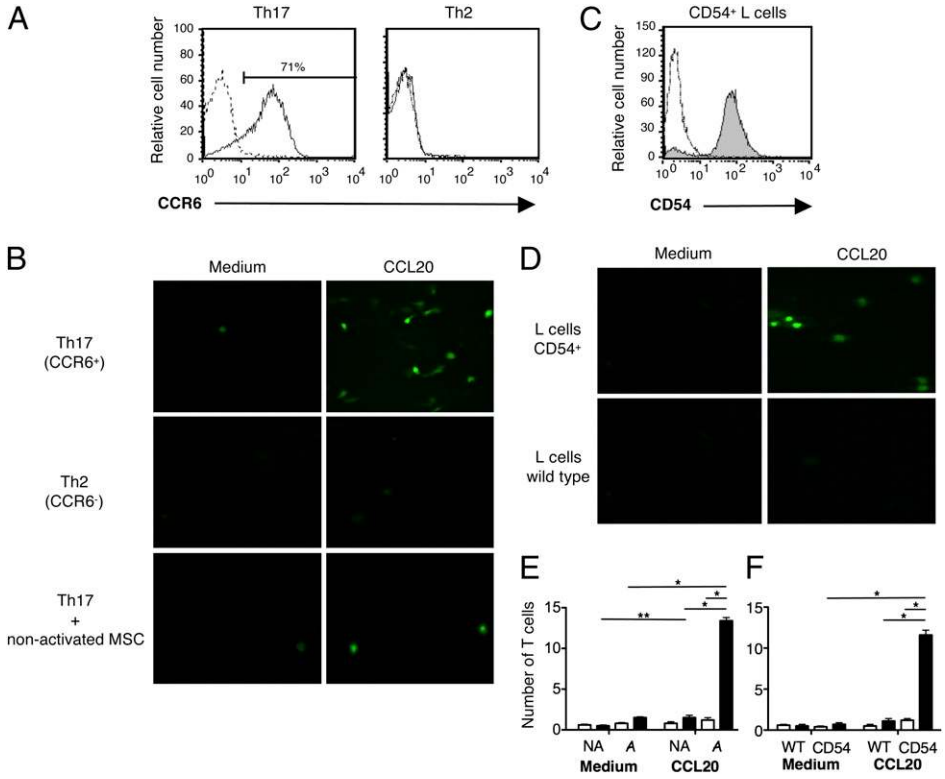

FIGURE 2. CCL20 induces in vitro adhesion of human Th17 cells to MSCs. *A*, CCR6 expression by fully differentiated human Th17 and Th2 lymphocyte clones, as determined by flow cytometry. The *x*- and *y*-axes represent fluorescence (four decade log scale) and relative cell number, respectively. Cells stained with control isotype-matched mAb are represented by dashed lines. *B*, Th17 and Th2 lymphocyte clones were loaded with CFSE and injected into a flow chamber containing a confluent monolayer of MSCs preincubated or not for 48 h with TNF-α and IFN-γ at a concentration of 10 ng/ml, in the presence or absence of CCL20 (20 ng/ml). Magnification ×12.5. *C*, CD54 expression by L cells, transfected with CD54 cDNA, as determined by flow cytometry. Cells stained with control isotype-matched mAb are represented by the open graph. *D*, Adhesion of Th17 cells to L cell transfectants expressing CD54 or to control wild-type L cells in the absence or presence of CCL20. Magnification ×12.5. *E* and *F*, The number of T cells adhered, in the presence or absence of CCL20, to MSCs (*E*) preincubated in medium alone (NA) or with TNF-α and IFN-γ (A), or to L cells (*F*), expressing CD54 (CD54) or not (WT), used as control cells. Values represent the mean ± SD of the number of T cells counted in 10 adjacent fields around the center of the chamber per experiment, for a total of three experiments. Statistical analysis was carried out by applying the Student *t* test. $*p < 0.0005$; $**p < 0.05$.

adherence of T cells to MSCs involves similar molecular interactions, we first determined the expression levels of the selectins CD62L and CD62P; the integrins β7, CD29, CD58, CD49d, and CD11a; and CD54, the ligand of CD11a/CD18, on MSCs. Furthermore, we tested the capacity of proinflammatory cytokines to induce or enhance the expression of the latter surface molecules on these cells. All MSCs constitutively expressed CD29, CD49d, and CD58 at their cell surface, which was not modulated by the addition of TNF-α, IFN-γ, IL-1β, and OSM, either alone or in combination (data not shown). The integrins β7 and CD11a, as well as the selectins CD62L and CD62P, were neither expressed by MSCs nor induced by the above-mentioned cytokines. In contrast, although CD54 was expressed at low levels by ~10% of the MSCs, its expression was strongly enhanced or induced by TNF-α and IFN-γ in a synergistic manner on all cells (Fig. 1).

Table II. *CCL20-mediated adhesion of Th17 cells to MSCs is dependent on the expression of CD54*

	Medium		CCL20	
Cell Type	Th2	Th17	Th2	Th17
MSC[a]				
Activated	0.8 ± 0.2	1.5 ± 0.3	1.2 ± 0.3	13.4 ± 0.4
Nonactivated	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	3.5 ± 0.1
L cell[b]				
$CD54^+$	0.5 ± 0.2	1.1 ± 0.3	1.2 ± 0.2	11.6 ± 0.6
Wild-type	0.6 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.7 ± 0.2

Data represent the mean ± SEM of the number of T cells recorded in 10 adjacent fields around the center of the chamber.

[a]CCL20-induced adhesion of fully differentiated, CFSE-loaded, human Th17 and Th2 lymphocyte clones to monolayers of MSCs, preincubated for 48 h in medium or with TNF-α and IFN-γ, was measured using a flow chamber. Experimental conditions are as described in the legend for Fig. 2.

[b]Wild-type L cells and L cells expressing the human CD54 molecule were used as controls.

CCL20 induces in vitro adhesion of human Th17 cells to MSCs

Engagement of CCR6 by its ligand CCL20 was shown to induce a conformational change of the CD11a/CD18 structure, thereby allowing the binding of this molecule to its counterstructure CD54 (40). To determine whether MSCs are able to induce the adherence of Th17 cells in a CCR6-dependent way, CFSE-labeled Th17 cells were put in contact in the flow chamber with TNF-α– and IFN-γ–incubated MSCs, in the absence or presence of CCL20. CFSE-labeled Th2 cells that do not express CCR6 (Fig. 2*A*, Table II) and MSCs that had not been treated with proinflammatory cytokines were used as a negative control. No T cell adhesion to untreated MSCs or to TNF-α– and IFN-γ–treated MSCs was observed in the absence of CCL20 (Fig. 2*B*, 2*E*). CCL20 induced a small, but consistent, adherence of Th17 cells to untreated MSCs, which was strongly enhanced when TNF-α– and IFN-γ–treated MSCs were used (Fig. 2*B*, 2*E*). In contrast, Th2 cells failed to adhere to TNF-α– and IFN-γ–treated MSCs in the absence or in the presence of this CCR6 ligand (Fig 2*B*, 2*E*). Finally, to determine

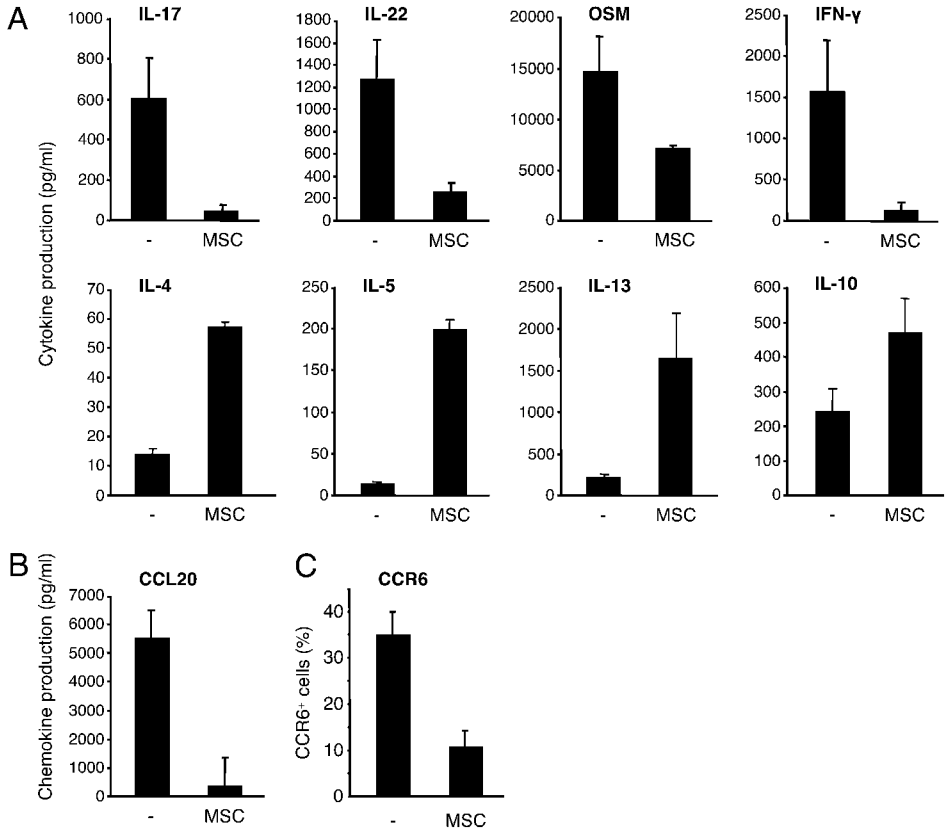

FIGURE 3. MSCs inhibit the differentiation of naive human T cells into Th17 cells. Naive $CD4^+$ cord blood-derived T cells were stimulated with Expander beads in the presence of rIL-1β, rIL-6, rTGF-β, and rIL-23, as well as in the presence (MSC) or absence (−) of MSCs, as described in *Materials and Methods*. After 7 d of culture, the cells were analyzed directly or stimulated with immobilized anti-CD3 and soluble anti-C28 mAbs prior to analysis. The presence of cytokines (*A*) and CCL20 (*B*) was determined by ELISA in the culture supernatants of cells stimulated for 48 h. *C*, Cell surface CCR6 expression was determined by flow cytometry.

whether the arrest of Th17 cells to MSCs was mediated through the interaction between CD11a/CD18 and CD54, as reported for endothelial cells, the capacity of CD54-expressing L cell transfectants (Fig. 2*C*) to mediate cellular adhesion was analyzed. As shown in Fig. 2*D* and 2*F* and Table II, Th17 cells firmly adhered to CD54-transfected, but not to wild-type, L cells in the presence of CCL20.

MSCs inhibit the differentiation of naive human T cells into Th17 cells

The effect of MSCs on the differentiation of the Th17 lineage was analyzed using purified, cord blood-derived, naive $CD4^+$ T cells, differentiated in vitro following stimulation via CD3 and CD28 in the presence of rIL-1β, IL-6, IL-23, and TGF-β, in the presence or absence of bone marrow-derived MSCs. Consistent with reports in the literature (27, 28), this combination of cytokines induced the generation of a population of IL-17–, IL-22–, IFN-γ–, TNF-α–, and CCL20-producing cells (Fig. 3*A*, 3*B*). In addition, T cells differentiated under Th17-polarizing conditions in vitro produced high levels of OSM and low levels of IL-4, IL-5, IL-13, and IL-10 (Fig. 3*A*). Moreover, ~35% of the differentiated cells expressed CCR6 at their surface (Fig. 3*C*), confirming the Th17 phenotype of these cells. The presence of MSCs during the Th17 lineage-differentiation process strongly inhibited the production of all inflammatory cytokines, as well as the production of CCL20,

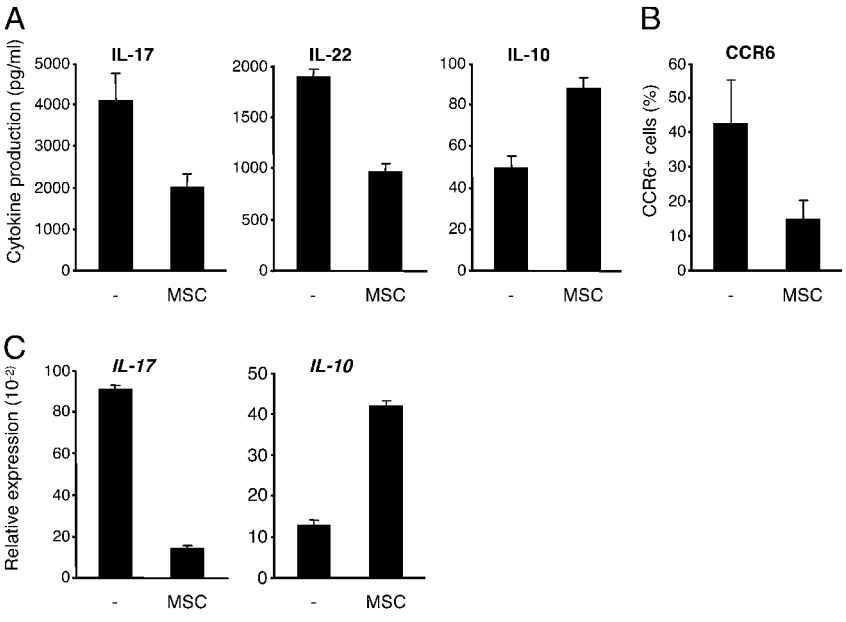

FIGURE 4. MSCs induce a Treg cell phenotype in fully differentiated Th17 cells. Fully differentiated Th17 T cell clones were stimulated with Expander beads in the presence (MSC) or absence (−) of MSCs and were treated as described in the legend for Fig. 3. *A*, The presence of cytokines was determined by ELISA in the culture supernatants of cells stimulated for 48 h. *B*, Cell surface CCR6 expression was determined by flow cytometry. *C*, Expression of IL-17 and IL-10 transcripts was measured by quantitative RT-PCR.

whereas it slightly induced, albeit in a statistically significant manner, the production of IL-4, IL-5, IL-13, and IL-10. The presence of MSCs also prevented the induction of CCR6 expression on differentiating T cells.

MSCs inhibit the production of inflammatory cytokines by fully differentiated Th17 cells

We next analyzed the capacity of MSCs to modulate the cytokine production profile of fully differentiated Th17 lymphocyte clones by stimulating these cells with anti-CD3 and anti-CD28 mAbs in the presence or absence of MSCs. The presence of MSCs in these cultures during the activation of the T cells resulted in the inhibition of the production of IL-17 and IL-22 and an increase in the production of IL-10 (Fig. 4*A*), as well as in a decrease in the percentage of cells expressing CCR6 (Fig. 4*B*). Moreover, results from intracellular staining of Th17 cells, cultured in the presence or absence of MSCs, showed that MSCs diminished the frequency of IL-17– and IL-22–producing T cells, which was accompanied by the induction of a population of IL-10–producing cells (Fig. 5*A*), whereas the production of IL-10 was also induced in T cells that continued to produce IL-17 (Fig. 5*B*). The expression of IL-17 mRNA was downregulated in Th17 cells cocultured with MSCs, with a concomitant upregulation of the expression of IL-10 transcripts, indicating that the MSCs modulate the cytokine gene-expression profile at the transcriptional level (Fig. 4*C*).

Cell–cell contact and Th17 cell-induced PGE_2 are involved in the inhibitory effects of MSCs

Soluble factors secreted by MSCs were reported to mediate the inhibitory properties of these cells (1). Therefore, to determine whether the effects of MSCs are due to cellular cell contact or to secreted factors, T cells and MSCs were cocultured in a transwell system. As shown in Fig. 6*A*, the effects of MSCs on the production of IL-17, IL-22, and IL-10 by the Th17 cells were only partially maintained in a transwell cell-culture system, indicating that cellular cross-talk and soluble factors are involved in their inhibitory activity. Although MSCs constitutively produced PGE_2, the production of this inflammatory mediator was strongly enhanced in the presence of Th17 cells (Fig. 6*B*). Finally, the addition of the PGE_2 inhibitor indomethacin partially abolished the suppressive effects of MSCs (Fig. 6*C*). Human MSCs were found to secrete IDO, as measured following the addition of L-tryptophan to the culture supernatants; this production, in contrast to that of PGE_2, was not modified in the presence of Th17 cells. However, the addition of 1methyl-D-tryptophan, a specific inhibitor of IDO, to the cocultures of MSCs and Th17 cells, did not modify the cytokine production profile of the latter (data not shown). Finally, MSCs did not produce iNOS, which was also not induced in the presence of Th17 cells (data not shown).

MSCs reciprocally modulate the expression of RORC and FOXP3 in fully differentiated Th17 cells

Because of the differential effects of MSCs on the respective production of IL-17, IL-22, and IL-10, the expression of the Th lineage-specific transcription factors RORC and FOXP3 was investigated. Naive T cells differentiated along the Th17 lineage pathway in the presence of MSC expressed decreased levels of RORC transcripts; in contrast, expression levels of FOXP3 mRNA were strongly enhanced (Fig. 7*A*). Similarly, coculture of differentiated Th17 cells with MSCs resulted in a decrease in RORC mRNA expression, with a concomitant induction of the expression of FOXP3 transcripts when the Th17 cells were subsequently activated via CD3 and CD28 (Fig. 7*B*). In contrast, induction of

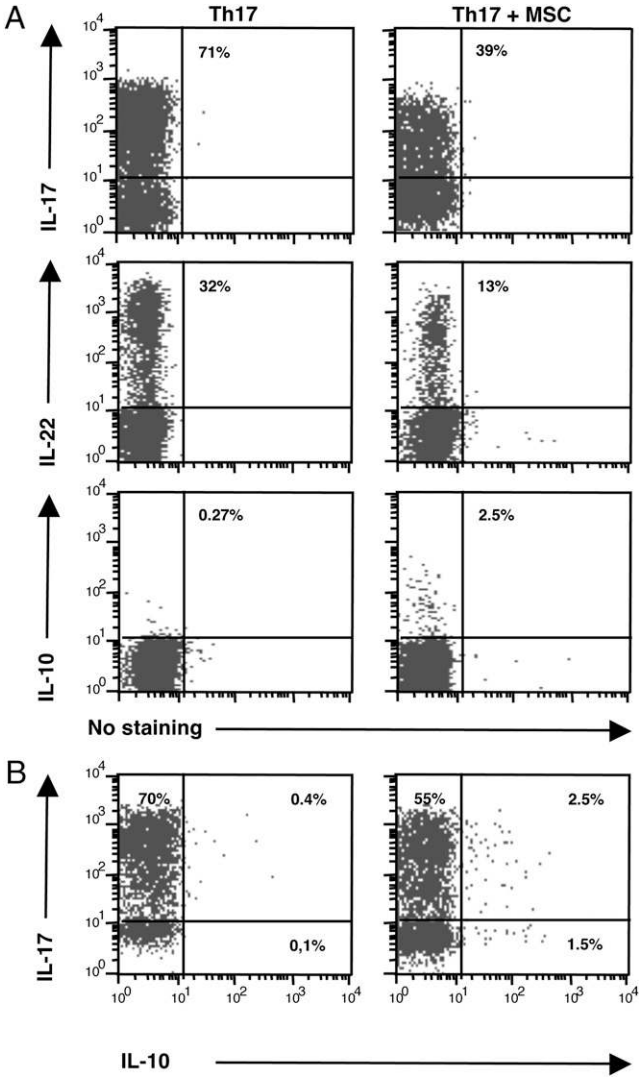

FIGURE 5. MSCs inhibit the frequency of IL-17– and IL-22–producing Th17 cells and induce an IL-10–producing Treg cell phenotype in fully differentiated T cells. The cytokine production by Th17-differentiated cell clones, activated for 6 h with PMA and ionomycin, was analyzed by intracellular flow cytometry, as indicated in *Materials and Methods*. Dot plots of the gated living cells are shown. *A*, Numbers represent the percentages of each cytokine-producing cell population. *B*, Numbers represent the percentages of cells producing IL-17, IL-10, or both cytokines.

FOXP3 expression was not observed in the Th17 cells that had not been in contact with MSCs prior to their activation (Fig. 7*A*, 7*B*). This reciprocal effect of MSCs was confirmed on the expression of the RORC and FOXP3 proteins by intranuclear-staining experiments (Fig. 7*C*, 7*D*). Consistent with its enhancing effect on the production of IL-10, PGE_2 produced by MSCs increased the frequency of FOXP3-expressing Th17 cells (Fig. 7*D*).

MSCs induce trimethylation of H3K4me3 at the FOXP3 *gene loci and expression of FOXP3 in fully differentiated Th17 cells*

Epigenetic modifications of histones in the nucleosome, in particular of histone 3 in the promoter region of the RORC and FOXP3 genes, have been associated with induction or repression of the DNA accessibility for the gene-transcription machinery (41). Therefore, to investigate whether MSCs modulate the accessibility of FOXP3 and RORC gene loci, ChIP analysis was used to determine, in the genomic region of FOXP3 and RORC loci, the

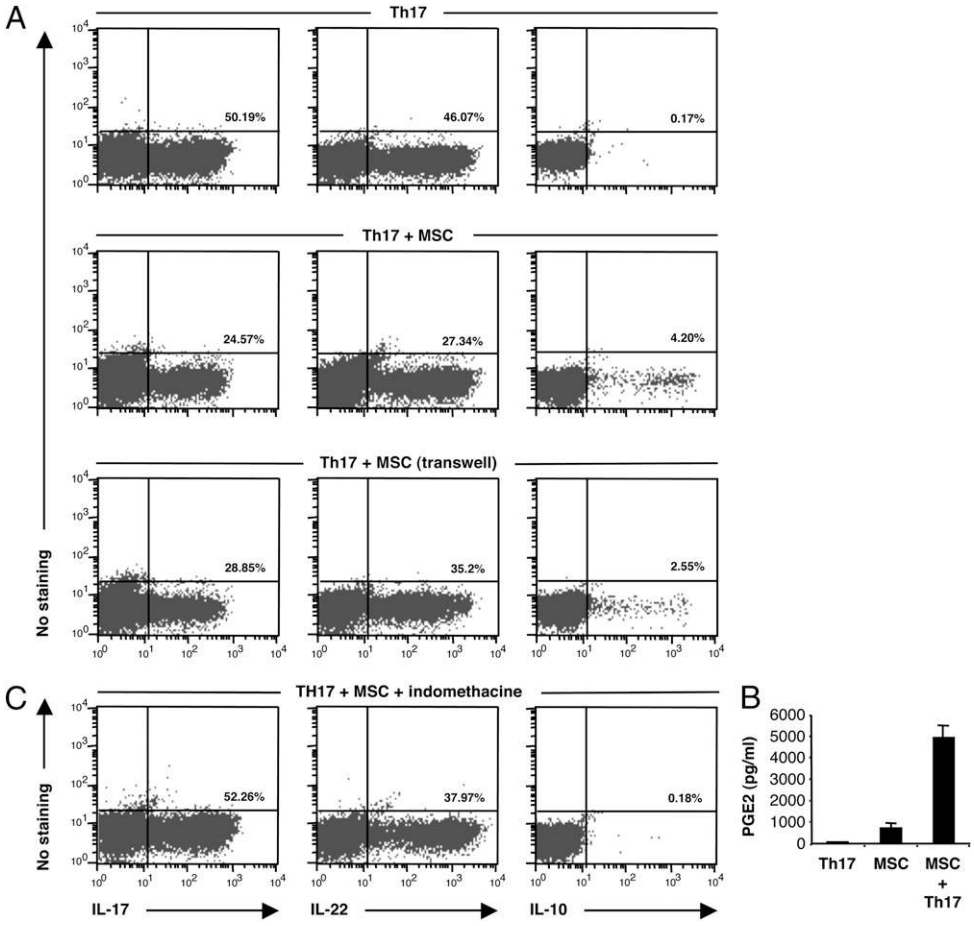

FIGURE 6. Cell contact and Th17 cell-induced PGE_2 are involved in the inhibitory effects of MSCs. *A*, Th17-differentiated cells were stimulated with Expander beads, in the absence (Th17) or presence of MSCs, added together and without (Th17 + MSCs) or with indomethacin (Th17 + MSCs + indomethacin) or in transwells (Th17 + MSCs [transwell]). Then, T cells were collected and treated as described in the legend for Fig. 5. Numbers represent the percentage of IL-17–, IL-22–, or IL-10–producing T cells. *B*, Production of PGE_2, as determined by ELISA in their respective culture supernatants, by Th17-differentiated cells stimulated with Expander beads, MSCs alone, or cocultures of both cell types. Values are the mean ± SD of the values obtained in three experiments.

trimethylation of histone H3 on lysine 4 (H3K4me3), which is an epigenetic mark of active genes (Fig. 8*A*). By comparing the relative levels of immunoprecipitated fragments, we found minimal levels of H3K4me3 at the FOXP3 gene in Th17 cells cultured in the absence of MSCs, whereas the levels of this modification were elevated in Th17 cells that had been cocultured with MSCs. In contrast, the levels of H3K4me3 were decreased at the RORC gene on Th17 cells cocultured in the presence of MSCs (Fig. 8*B*), compared with those cells cultured in the absence of MSCs.

MSCs induce a functional Treg cell phenotype in fully differentiated Th17 cells

To determine whether the induction of FOXP3 mRNA expression by MSCs was associated with the acquisition of immunosuppressive activity, we analyzed whether a cloned Th17 cell line, cocultured with MSCs, acquired the ability to inhibit the proliferation of allogenic $CD4^+$ T cells. Th17 cells that had not been cultured in the presence of MSCs did not affect the anti-CD3/anti-CD28–driven proliferation of $CD4^+$ T cells (Fig. 9*A*, 9*B*). Compared with $CD4^+$ T cells stimulated in the presence of Th17 cells that had not been in contact with MSC (Fig. 9*B*), those stimulated in the presence of Th17 cells cocultured for 48 h with MSCs inhibited proliferative $CD4^+$ T cells responses (Fig. 9*C*). Moreover, the presence of TNF-α and IFN-γ during the coculture of the Th17 cells and MSCs enhanced the antiproliferative activity of the Th17 cells (Fig. 9*D*), indicating that the presence of a local inflammatory environment contributes to the immunosuppressive effects mediated by the MSCs.

Discussion

In the current study, we investigated the capacity of MSCs to interfere with the functional activity of Th17 cells, a population of $CD4^+$ T cells that plays an important role in the pathogenesis of several autoimmune and inflammatory diseases. Using naive, cord blood-derived $CD4^+$ T cell lines cultured under conditions of in vitro Th17 lineage differentiation, primary, short-term–cultured MSCs specifically inhibited the production of proinflammatory cytokines, including IL-17, IL-22, IFN-γ, TNF-α, and OSM, as well as that of the CCR6 ligand CCL20, whereas they enhanced the production of IL-10 by the differentiating Th17 cells. The same modulatory action on the cytokine-production profile was observed on fully differentiated Th17 lymphocyte clones that had been derived from lesional tissues of patients with inflammatory disease; this effect was accompanied by the induction of the transcription factor FOXP3, as well as immunosuppressive activity in the latter cells, suggestive of a Treg cell phenotype.

MSC-mediated immunomodulatory effects on T cells involve cellular interactions; however, little information is available about the nature of the cell surface molecules that play a role in the latter process. Using an in vitro model of cellular adhesion under flow conditions that was adapted to study MSC–T lymphocyte interactions,

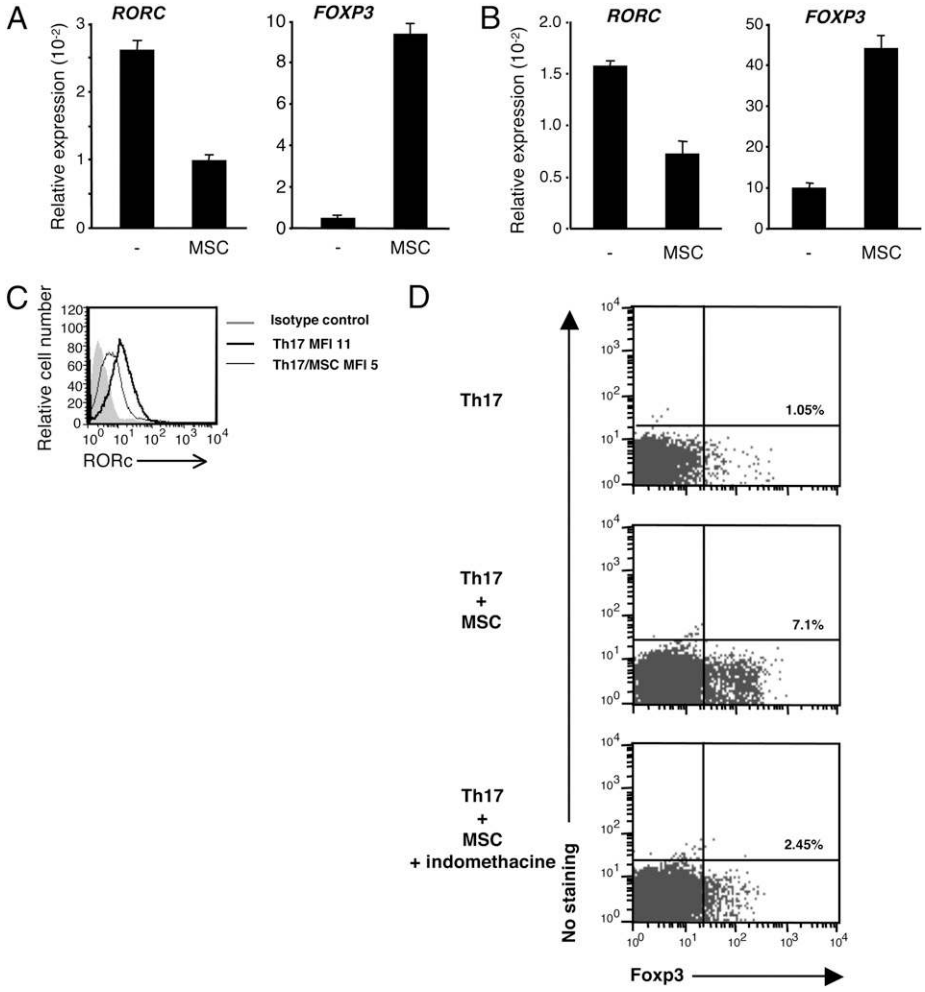

FIGURE 7. MSCs induce the conversion of fully differentiated Th17 cells into functional Treg cells. Naive $CD4^+$ cord blood-derived T cells (*A*) or fully differentiated Th17 T cell clones (*B–D*) were stimulated with Expander beads for 7 d in the presence of rIL-1β, rIL-6, rTGF-β, and rIL-23, or 4 d, respectively, as well as in the presence or absence of MSCs, as described in *Materials and Methods*. Then, T cells were separated from MSCs and treated as follows. *A* and *B*, RORC and FOXP3 mRNA expression by T cells, cocultured in the presence (MSC) or absence (−) of MSCs, and activated for 6 h with plate-bound anti-CD3 and anti-CD28 mAbs, as determined by real-time quantitative PCR. Data are mean ± SD of the values obtained in three independent experiments. *C*, Intranuclear expression of RORC by T cells, cocultured with (Th17/MSCs) or without (Th17) MSCs, and activated for 6 h with PMA and ionomycin, as described in the legend for Fig. 5. Shaded graph represents the control staining with isotype-matched mAb. *D*, Intranuclear expression of FOXP3 by T cells, cocultured with (Th17 + MSC) or without (Th17) MSCs or in the presence of indomethacin (Th17 + MSC + indomethacin) and activated for 6 h with PMA and ionomycin, as described in the legend for Fig. 5. Numbers represent the percentage of cells expressing FOXP3.

we emphasize the importance of C11a/CD18, its ligand CD54, and the chemokine receptor CCR6 in mediating the adherence of Th17 cells to MSCs. Although primary MSCs express low levels of CD54 at their cell surface, the presence of inflammatory cytokines strongly enhanced its expression, with a synergistic effect of TNF-α and IFN-γ, whereas IL-1β had little or no effect. The high

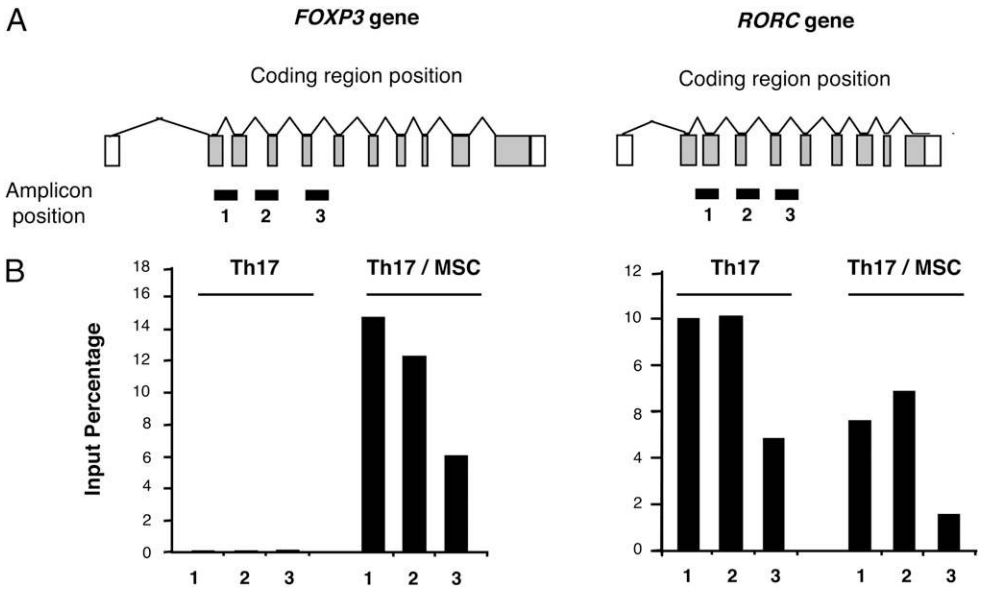

FIGURE 8. MSCs induce epigenic modification of H3K4me3 in fully differentiated Th17 cells. *A*, Genomic region analyzed in this study, showing the structure of the *FOXP3* and *RORC* gene. Numbers correspond to the amplicons studied at each gene locus, as described in Table I. *B*, H3K4me3 modification at the *FOXP3* and *RORC* genes in Th17 cells cocultured with (Th17 / MSC) or without (Th17) MSCs. Chromatin fragments of T cells were analyzed by ChIP as described in *Materials and Methods*. Immunoprecipitated chromatin DNA was amplified by quantitative PCR, with primers derived from different sites of *FOXP3* and *RORC* genes.

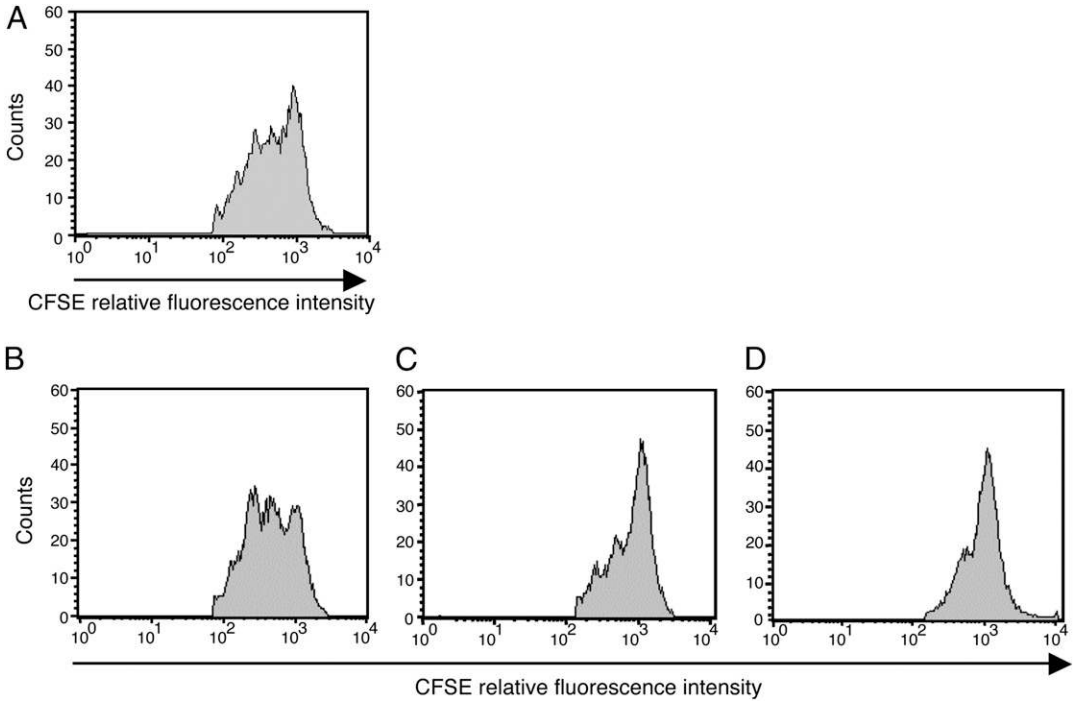

FIGURE 9. MSCs induce a functional Treg cell phenotype in fully differentiated Th17 cells. The proliferation of $CD4^+$ T cells stimulated with anti-CD3/CD28 mAb-coated Expander beads (T cell/bead ratio 4:1) was determined by following the number of cell divisions of the CFSE-labeled T cells. Graphs show the proliferation of $CD4^+$ cells alone (*A*) or $CD4^+$ cells cultured in the presence of fully differentiated Th17 T cell clones that had been stimulated for 4 d in the absence (*B*) or presence (*C*) of nonactivated MSCs or activated MSCs cultured for 48 h with TNF-α and IFN-γ (*D*), as described in *Materials and Methods*.

expression levels of CD54 on the MSCs, together with conformational changes in T cell-expressed C11a/CD18 brought about by the engagement of a chemokine receptor with its ligand (reviewed in Ref. 39), resulted in the adherence of Th17 cells to inflamed MSCs, thereby corroborating previously reported results showing that CCL20 is able to induce the adherence of peripheral blood T lymphocytes to immobilized CD54 under flow conditions (40). Th17 cells, but not Th1 or Th2 cells, secrete CCL20 following TCR-mediated activation (24, 27), providing the initial events leading to their chemokine- and integrin-mediated adhesion to MSCs. Although these results indicate that MSCs are able to induce a low degree of T cell adhesion under noninflammatory conditions, because of their constitutive low expression of CD54, they also show that conditions of inflammation strongly increase the probability of MSCs to recruit inflammatory Th17 cells and, as a consequence, to modulate their function toward a regulatory phenotype.

The immunomodulatory activity of human MSCs on T cells, in particular their capacity to inhibit in vitro T cell proliferation and cytokine production, was reported previously (5, 6), although the molecular mechanisms underlying this activity are still subject to controversy. In the current study, MSCs inhibited the production of IL-17, IL-22, IFN-γ, and OSM and enhanced that of IL-4, IL-5, IL-13, and IL-10 by differentiating Th17 cells. These results corroborate an earlier report in the literature showing that human MSCs decrease IFN-γ production in vitro by T cells subjected to Th1 cell-polarizing conditions, whereas they increase IL-4 production under conditions of Th2 cell differentiation (9). The inhibition of the production of proinflammatory cytokines by MSCs was not due to a specific effect on the proliferative response of the cells, because cell survival in cultures of naive cells in the presence or absence of MSCs was not significantly different (data not shown). Importantly, we showed that MSCs are able to modulate the cytokine-production profile of fully in vivo differentiated Th17 cells isolated from human inflamed lesional tissue by inhibiting the expression of the Th17-specific transcription factor RORC, as well as the production of the Th17 signature cytokines IL-17 and IL-22. Concomitantly, MSCs induced the expression of IL-10 mRNA and protein by Th17 cells. Because the latter cells represent a clonal population, these results indicate that MSCs are able to convert strongly polarized proinflammatory T cells into an anti-inflammatory and IL-10–producing T cell population. In contrast to the adhesion of Th17 cells to MSCs, which required cell–cell interactions, the immunomodulatory effects of the latter cells on the inflammatory cytokine-production profile were dependent, in part, on their secretion of soluble, proinflammatory factors, with an important role for PGE_2, thereby confirming and extending a previous report in the literature (9). Moreover, in a reciprocal manner, the production of PGE_2 by MSCs was strongly increased by soluble factor(s) produced by Th17 cells. A likely candidate is TNF-α, because this cytokine is produced at high levels by the Th17 clones used in this study (24), which is in line with results reported previously in the literature, showing that TNF-α induced the production of PGE_2 by human and mouse stromal cells (42, 43). Two other factors, IDO and iNOS, were reported to mediate the immunosuppressive activity of MSCs. Although human MSCs secrete IDO, this mediator does not seem to be involved in their anti-inflammatory effects on Th17 cells through the modification of their cytokine-production profile. Finally, the results presented in this study showed that Th17 cells do not induce the production of iNOS, thereby eliminating the contribution of this factor to the modulatory effects of MSCs in humans, contrary to what has been shown in experimental mouse models.

In cultures of differentiating Th17 cells, MSCs induced the expression of FOXP3, raising the possibility that they are able to generate Treg cells among the non-Th17 population and/or to induce the expression of this transcription factor in already committed Th17 cells. The former possibility is supported by the results from a recently published study showing that MSCs promoted the

proliferation and expansion of $CD4^+CD25^{hi}FOXP3^+$ Treg cells in cultures of peripheral blood T lymphocytes (17). However, the results from the current study showed that MSCs are also able to induce FOXP3 expression in fully differentiated clonal Th17 cells, thereby endowing them with an immunoregulatory function. Although Th17 and Treg cells display discrete and opposite functions, their programs of differentiation are strongly linked, with TGF-β as a common denominator that is essential for the differentiation of both populations. The strong interrelationship between these cells is underscored by the observation, initially based on studies in the mouse, that endogenous TGF-β–producing Treg cells can be converted into IL-17–producing T cells in a proinflammatory cytokine environment, which is controlled by IL-6 (33, 44) or by dendritic cells activated via dectin-1 (45). The plasticity of Treg cells was also confirmed in humans, showing that the latter cells could be differentiated into RORC- and CCR6-expressing, IL-17–producing T cells following stimulation with allogenic monocytes in the presence of recombinant human IL-2 and recombinant human IL-15, which was found to be independent from the presence of IL-6 (46). Furthermore, in a recent study, the presence of a $CD4^+FOXP3^+RORC^+CCR6^+$ IL-17–producing T cell population with immunosuppressive activity was reported in human peripheral blood and lymphoid tissues (47). Although human peripheral blood $CD4^+FOXP3^+CCR6^-$ Treg cells could be differentiated in vitro into $RORC^+$ IL-17–producing T cells in the presence of IL-1β, IL-2, IL-21, and IL-23 (47), the current study provides evidence for the reverse process in that differentiated Th17 cells can be converted in T cells with immunosuppressive activity. FOXP3 expression is induced in T cells following activation (48). However, the expression of this transcription factor was only observed in Th17 cells that had been cultured with MSCs (Fig. 7); it was undetectable in Th17 cells cultured in the absence of MSCs, indicating that MSCs specifically induced the expression of FOXP3 transcripts. This induction was associated with a concomitant decrease in RORC expression, leading to a T cell phenotype characterized by the coexpression of both transcription factors, reminiscent of the $CD4^+FOXP3^+RORC^+CCR6^+$ peripheral blood T cells described by Voo et al. (47).

Although the activity of transcription factors is important in the regulation of T cell subset differentiation, epigenetic mechanisms also play a critical role, because they determine the chromatin accessibility of their respective promoters, leading to the engagement of the transcription machinery. In particular, post-translational modifications of lysines 4 and 27 of histone H3 are implicated in the stability of FOXP3, RORC, GATA3, and Tbx21 gene expression, and the degree of trimethylation of each of these four gene loci constitutes the functional signature of the different Th cell subpopulations that they encode (41). It was shown in the current study that MSCs induced the trimethylation of H3K4me3 at the *FOXP3* gene locus, whereas it suppressed, but not abolished, the trimethylation of the corresponding region in the RORC promoter. As reported previously, these epigenetic changes are indicative of the acquisition of an induced Treg cell profile (41), which was induced, as shown in the current study, in T cells that were initially characterized as bona fide Th17 cells.

Furthermore, the induction of FOXP3 in differentiated Th17 cells by MSCs was associated with the acquisition of immunosuppressive activity in vitro. Importantly, the presence of TNF-α and IFN-γ, thus mimicking an inflammatory environment, strongly enhanced the capacity of MSCs to induce Treg cell function in the Th17 cells. These results are reminiscent of those obtained with genetically modified mice showing that the immunosuppressive capacity of MSCs is not intrinsic but is induced by the proinflammatory cytokines, with IFN-γ playing a major role in combination with TNF-α, IL-1α, or IL-1β (16). Moreover, as shown in the current study, the combination of these cytokines also induces the expression on MSCs of adhesion molecules, such as CD54, which are important in the recruitment of T cells. Although MSCs do not seem to secrete CCL20, the chemokine that initiates the series of events leading to recruitment and possible sequestering of Th17 cells, this CCR6 ligand is produced by activated Th17 cells themselves (25, 31). Because the close proximity between T cells and MSCs is required for the latter to exert optimal immunosuppressive activity, these results suggest that in the absence of active inflammation and tissue damage, MSCs would neither mobilize the inflammation-inducing T cells nor exert their immunosuppressive effects. The production of proinflammatory cytokines, including TNF-α and IFN-γ (27, 25, 31), which are able to induce the secretion of immunosuppressive mediators (e.g., PGE_2), as well as the expression of CCR6 and the production of its ligand by Th17 cells, contributes to the initiation and perpetuation of a particular inflammatory environment in which Th17 cells favor their own migration and sequestration, thus rendering them susceptible to the acquisition of immunoregulatory capacities, and instruct MSCs to become immunosuppressive. Finally, the implication that follows from the results obtained in the current study is that fully differentiated Th17 cells are flexible; their cytokine production profile and function can be modulated in the context of an inflammatory environment.

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Disclosures

The authors have no financial conflicts of interest.

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Corrections

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The third author's last name was published incorrectly. The correct name is Gabriel Moquet-Torcy.

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