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Inhibition of Immune Synapse by Altered Dendritic Cell Actin Distribution: A New Pathway of Mesenchymal Stem Cell Immune Regulation

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Immune synapse formation between dendritic cells (DCs) and T cells is one of the key events in immune reaction. In immunogenic synapses, the presence of fully mature DCs is mandatory; consequently, the modulation of DC maturation may promote tolerance and represents a valuable therapeutic approach in autoimmune diseases. In the field of cell therapy, bone marrow mesenchymal stem cells (MSCs) have been extensively studied for their immunoregulatory properties, such as inhibiting DC immunogenicity during in vitro differentiation and ameliorating in vivo models of autoimmune diseases (e.g., experimental allergic encephalomyelitis). MSCs seem to play different roles with regard to DCs, depending on cell concentration, mechanism of stimulation, and accompanying immune cells. The aim of this work was to elucidate the immunogenic effects of MSC/DC interactions during DC activation (LPS stimulation or Ag loading). Human monocyte-derived DCs, bone marrow-derived MSCs, and circulating lymphocytes obtained from healthy donors, as well as the laboratory-generated influenza virus hemagglutinin-derived peptide, aa 306–318 peptide-specific T cell line were used for this study. We demonstrate that MSCs mediate inhibition of DC function only upon cell–cell contact. Despite no modification observed in cell phenotype or cytokine production, MSC-treated DCs were unable to form active immune synapses; they retained endocytic activity and podosome-like structures, typical of immature DCs. The transcriptional program induced by MSC–DC direct interaction supports at the molecular pathway level the phenotypical features observed, indicating the genes involved into contact-induced rearrangement of DC cytoskeleton. The Journal of Immunology, 2010, 185: 5102–5110.

B one marrow mesenchymal stem cells (MSCs) are multipotent cells able to differentiate in vitro and in vivo into tissues of mesenchymal and nonmesenchymal origin. MSCs have been extensively studied for their immunoregulatory properties, and there is wide agreement that they inhibit in vitro T cell proliferation and dendritic cell (DC) immunogenicity and can ameliorate in vivo autoimmune diseases, such as experimental allergic encephalomyelitis (1, 2). MSCs have been approved as treatment for severe graft-versus-host disease (3). A Notch-Jagged1 cross-talk between MSCs and T cells may be responsible for T cell immunosuppression, and LPS stimulation may inhibit this suppression by downregulating Jagged1 expression via TLR4 (4). However, even if MSC stimulation by LPS is followed by upregulation of TLR4 expression, it does not affect the ability of MSCs to induce the differentiation of nonimmunogenic DCs (5). Conflicting results were reported for MSC/B cell interaction. Corcione et al. (6) showed MSC-mediated inhibition of the proliferation and differentiation of human peripheral B cells into Ig-secreting cells; in contrast, a more recent study concluded that B cells can efficiently respond to the TLR9 agonist CpG 2006 in the absence of BCR triggering when cocultured with MSCs and that MSCs stimulate the proliferation and differentiation of B into Ig-secreting cells isolated from patients affected by systemic lupus erythematosus (7). The extent to which the effects of MSCs on immune-system cells are mediated by soluble factors and/or cell–cell contact has yet to be fully understood. Different properties of MSCs may depend on the mechanism of stimulation, cell concentration, and type of coexisting immune cells.

Focusing our research on MSC/DC interactions during DC activation, we show that upon direct physical interaction with MSCs, DCs become unable to form active immune synapses with lymphocytes, despite their expression of a mature phenotype and normal IL-12/IL-10–production profile. In contrast, MSC-treated DCs retain endocytic activity and podosome-like structures, typical of “immature” DCs. We also show that the behavior of MSC-treated DCs is accompanied by rearrangement of the cytoskeleton and reprogramming of mRNA expression, as well as that cell–cell contact, assessed by electron microscopy, is mandatory for MSC-mediated inhibition of DC immunogenicity.

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Materials and Methods

Cells

DCs were generated from human monocytes of healthy donors, as previously described (8). Briefly, anti-CD14+ monocytes were positively sorted by magnetic microbeads (Miltenyi Biotec, Bologna, Italy). Monocytes were cultured for 6 d in medium supplemented with GM-CSF (1000 U/ml) and IL-4 (1000 U/ml; both from Labogen, Milano, Italy). Immature DCs were activated for 24 h of incubation with LPS (1 μg/ml; Sigma-Aldrich, St. Louis, MO) or 3 h of Ag loading (10 μg/ml; influenza virus hemagglutinin-derived peptide, aa 306–318; FluHA 306–318). DCs were negatively selected from PBMCs using the T cell isolation kit II from Miltenyi Biotec. The FluHA 306–318–specific T cell line was generated in our laboratory through semicloning methodology (9). Ag specificity was tested periodically. To analyze immune synapse formation between Ag-specific T cells and MSCs, MSCs were obtained as previously reported (5). Briefly, whole bone marrow was collected from the iliac crest in tubes containing acid citrate dextrose and centrifuged for 10 min at 700 × g; the interface between plasma and red cell pellet (buffy coat) was recovered and diluted 1:10 in HBSS without calcium and magnesium (Euroclone, Milano, Italy) to be counted. Cells were then plated in 75-cm² flasks (1.6 × 10⁶ cells/cm²) in DMEM with low glucose (Life Technologies, Invitrogen, Milano, Italy); 10% FBS (HyClone, Milano, Italy) was added, and the cells were incubated at 37˚C in humidified atmosphere containing 95% air and 5% CO₂. At confluence, the adherent cells were harvested with 0.05% trypsin and 0.02% EDTA (Eurobio, Toulouse, France) and resuspended in complete medium (primary culture). Cells were plated again at 10² cells/cm² in 100-mm dishes, and expansion of the cells was obtained with successive cycles of trypsinization and reseeding. Cells were used at different passages. Two DC/MSC ratios, 10:1 and 1000:1, were used in all experiments. Before testing their stimulatory function, DCs cocultured with MSCs were negatively selected from coculture by labeling with PE-conjugated CD105 Abs, followed by incubation with anti-PE magnetic microbeads (Miltenyi Biotec).

All human participants gave written informed consent, and the study was authorized by the local ethical committee (protocol number 2999 18.264-04).

Immunocytochemistry

To analyze immune synapse formation between Ag-specific T cells and Ag-loaded DCs, DCs were loaded with FluHA 306–318 peptide for 3 h, with or without the presence of MSCs. In one experimental condition, MSCs were pretreated with purified azide-free mouse monoclonal Abs against VCAM-1 (clone P3C4; Santa Cruz Biotechnology, Santa Cruz, CA; used at 30 μg/ml following the manufacturer’s instructions) and N-cadherin (clone GC-4; Sigma-Aldrich; used at 80 μg/ml following the manufacturer’s instructions); after overnight incubation at 37°C with blocking Abs, MSCs were washed twice in fresh medium and cocultured with DCs. In agreement with results reported by Ren et al. (10), this experimental condition functionally reduces the MSC/DC cell–cell adhesion by 30%. This is the mean value from two experiments in which adhesion was evaluated as the number of DCs in contact with MSCs in six microscope fields randomly selected by a 20× objective (field size was 0.9 × 0.9 mm). After Ag loading and negative separation from MSCs, DCs were reseeded on coverslips (3 × 10⁵ cells) and left to adhere for 30 min at 37°C, then an equal number of T cells was added. After 45 min at 37°C, T cell/DC conjugates were washed with PBS, fixed with 4% phosphate buffered parafomaldehyde for 10 min, permeabilized with 0.05% saponin (ICN Biomedicals, Zurich, Switzerland), and 2% BSA (Sigma-Aldrich) in PBS, and labeled with monoclonal mouse anti-human CD43 Ab (10 μg/ml; BD Pharmingen, San Diego, CA), followed by rabbit anti-mouse IgG Alexa Fluor 546 (1:200; Molecular Probes, Eugene, OR) or were diluted in PBS, 2% BSA, and 0.05% saponin and then incubated for 2 h at room temperature, respectively.

To analyze actin/Rac1/PAK1 distribution, DCs preincubated with or without the presence of MSCs were seeded on glass coverslips (5 × 10⁶ cells) after 24 h of LPS stimulation for actin labeling and after 5 min of LPS stimulation for Rac1 and PAK1 labeling. Cells were fixed and permeabilized, as described above, for immune synapse analysis. Actin was labeled with FITC-conjugated phalloidin (0.1 μg/ml; Sigma-Aldrich) for 30 min at room temperature. Primary and secondary Abs were diluted in PBS with 2% BSA and 0.05% saponin and incubated for 2 h at room temperature. The following Abs were used: monoclonal mouse anti-human CD11c, PE conjugated (1:50; BD Pharmingen); monoclonal rabbit anti-human PAK1 (2 μg/ml; USBiological, Milano, Italy), followed by tetramethylrhodamine isothiocyanate-conjugated monomolecular mouse anti-rabbit (1:200; Sigma-Aldrich); or monoclonal mouse anti-human Rac1 (2 μg/ml; USBiological), followed by Alexa Fluor 488-conjugated rabbit anti-mouse IgG (1:200; Molecular Probes).

Confocal images were acquired using a Leica TCS SP5 microscope (Leica Microsystems, Mannheim, Germany) equipped with a He/Ne/Ar laser source, using a Leica Plan Apo ×63/1.40 NA oil-immersion objective. A series of optical sections (1024 × 1024 pixels each: pixel size, 200 × 200 nm) was taken at intervals of 0.35 μm. Confocal images were deconvolved with Imagel 3D deconvolution software (National Institutes of Health [NIH], Bethesda, MD). Densitometric analysis was performed with ImageJ software (NIH), measuring the average fluorescence intensity on regions of interest in single focal-plane images.

T cell-proliferation tests

MLR was performed in 96-well U-bottom plates (Nunc, Roskilde, Denmark). A total of 1×10⁵ CD4+ T cells were cultured for 5 d in RPMI 1640 with 10% FCS, together with 1×10⁶ allogeneic DCs/well activated with LPS, with or without MSCs. Experiments were conducted in quadruplicate. At day 5, the proliferative response was measured by [3H]thymidine (1 μCi/ml; Amersham Bioscience, Piscataway, NJ) incorporation test. [3H]thymidine was added for the last 8 h of culture. Plates were harvested (Tomtec Mach III, Wallac, Turku, Finland) on glass fiber filters (PerkinElmer, Wellesley, MA), and the [3H]thymidine uptake was measured by liquid scintillation in a Microbeta 1450 Trilux counter (Wallac). The FluHA 306–318–specific T cell line (1×10⁵ cells/well) was tested for the proliferative response to autologous DCs (1×10⁵ cells/well) loaded with peptide Ag for 3 h, with or without the presence of MSCs; where indicated, MSCs were pretreated with anti-VCAM-1 or anti-N-cadherin blocking Abs, as previously described. After 48 h, the proliferative response was evaluated as described above. Transwell experiments were performed in Transwell permeable supports (0.4-μm polyester membrane; Costar, Cambridge, MA).

T cells were recovered after 5 d of culture in an MLR performed with DCs activated with or without the presence of MSCs, as described above. After 6 h of resting at 4°C, T cells (1×10⁵ cells/well) were stimulated with IL-2 (50 U/ml) and tested for subsequent proliferative responses.

Electron microscopy and immunoelectron microscopy

DCs were activated with LPS for 24 h in the presence of MSCs, and the cocultures were fixed, while attached to cover-slips, with 2% (w/v) formaldehyde and 2.5% glutaraldehyde in 0.1 mol L⁻¹ cacodylate buffer (pH 7.4) for 30 min for conventional microscopy and for 10 min for immunoelectron microscopy, at room temperature. Then they were removed with a scraper and pelleted. For immunoelectron microscopy, endogenous peroxidases were blocked with 3% H₂O₂ in PBS, and 30 min. DCs were fixed with mouse monoclonal Abs (BD Pharmingen), followed by peroxidase-labeled polyclonal goat anti-mouse Abs (1:100, Sigma-Aldrich), and visualized with diaminobenzidine. In all cases, cells were osmicated and embedded in epoxy resin. Sections were observed using a Jeol 1010 electron microscope (Tokyo, Japan) at 80 kV. Immunolabeled cells were observed unstained; otherwise, the sections were stained with 1% aqueous solution of 0.05% uranyl acetate/0.02% Phillips lead citrate.

Densitometry. Labeled cell membranes in experiments with LPS were subjected to densitometry, by outlining thin (2–5-mm wide) strips on the digitalized photomicrographs and analyzing them with the macro for gel analysis by Image 1.63 software (NIH). The gray level of cytoplasm was subtracted from that of all peaks, and the total area of each peak corresponding to a plasma membrane was assumed as the intensity of labeling of that membrane, in arbitrary units.

Statistics for densitometry. Because the intensity of labeling was widely distributed and not all of the membranes appeared labeled, a two-step statistical analysis was applied. The data for each experimental condition and cell type (i.e., MSCs versus DCs, independent of the cell side) were first analyzed by the χ² test, to verify whether the number of labeled cells depended on the cell type. Upon visual inspection of data showing skewness, the data relative to the labeled membranes were analyzed in the log transformed data. This procedure was repeated for the log transformed data of different cell types and the different surfaces of DCs (i.e., whether or not they were facing another cell) for each tagged membrane molecule. When this analysis gave significant results, the Student t test for unpaired values was used to check which values diverged from one other. All tests applied were two tailed, and p < 0.01 was considered significant (the relatively low value of p needed for significance was selected because of the large number of comparisons). Because compound immunofluorescence and densitometry of the intensities of labeling, the corresponding results are given as median ± SD of the median, and the SE is asymmetrically distributed around the median (11).
DC surface markers

DC surface markers were evaluated by flow cytometry with a four-color Epic XL cyometer (Beckman Coulter, Fullerton, CA), equipped with Expo 32 software. The Abs used (Beckman Coulter) were directed against the following Abs (the tags are given in parentheses): CD80 (FITC), CD86 (PE), HLA-DR (ECD), and CD83 (PCC). Cell vitality was tested with propidium iodide (Molecular Probes). The cells were labeled in PBS with 1% FCS for 25 min at 4°C, washed twice, and analyzed immediately.

Cytokine determination

DCs were activated with LPS (1 μg/ml) for 24 h, with or without MSCs; the supernatants were collected and stored at −80°C for cytokine determination by ELISA (QuantiKine kit, R&D Systems, Minneapolis, MN).

Dextran uptake

Endocytosis by resting or LPS-activated DCs was examined as described by Sallusto et al. (12). Briefly, 1 × 10⁵ cells were resuspended in RPMI 1640 with 10% FCS, equilibrated at 37°C or 0°C for 10 min, and pulsed with FITC-conjugated dextran (40,000 Da; Molecular Probes), 1 mg/ml for 45 min at 37°C or 0°C. Cells were washed four times with cold PBS and analyzed by flow cytometry; propidium iodide was used to exclude dead cells. The labeling of cells pulsed at 0°C was subtracted from that of cells pulsed at 37°C to obtain a measure of the endocytosed dextran.

Transcriptional analysis

A total of 1 × 10⁶ DCs were cocultured with MSCs at a ratio of 10:1 in the presence of LPS; alternatively, DCs or MSCs were cultivated with LPS. After 4 h, cells were collected. RNA preparation was performed with TRIzol reagent. Labeling, hybridization on a HT12 WholeGenome BeadArray (Illumina, San Diego, CA), and scanning were performed according to the Illumina reference protocols. The analysis was performed on three donors.

Array preprocessing

Bead-summary data saved from Illumina BeadStudio were preprocessed in several steps. The background signal was assessed and corrected using the intensity signal from the control probes present on the array and then quantile normalization was performed. In addition to background correction, Illumina probe identifiers were converted to nucleotide universal identifiers (13) specific for the nucleotide sequence of each probe. The computation was performed using the lumi package (14) written in the R programming language.

Data were submitted to Array Express under accession number E-MTAB-286 (http://www.ebi.ac.uk/microarray-as/aef).

Differential expression, annotation, and visualization

Differential expression analysis was carried out using the Rank Product algorithm (15), taking into account the differences among donors. The p values estimating differential expression were corrected for multiple testing, and genes with a corrected p value ≤0.05 were selected. Differentially expressed gene (DEG) lists from the various data sets were uploaded in the Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov) to perform functional annotation. The upregulated and downregulated gene lists were annotated separately. Gene Ontology enrichment over Biological Process was performed using the Biological Network Gene Ontology plug-in for the Cytoscape Desktop. To perform sample and gene clustering, a matrix with the absolute expression of the differentially expressed genes was extracted from the normalized data and then clustered using the Pearson correlation, bootstrapping genes, and samples over 1000 iterations. Visualization of the genes on a subset of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using the KEGG web service, with the aid of in-house programs.

Identification of predicted relationships

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) Web site (http://string.embl.de) was used to infer knowledge about the known interactions of the DEGs annotated in KEGG pathways. For each pathway, lists of gene symbols representing DEGs in that pathway were uploaded into STRING, and the minimum confidence score was set to medium (0.4).

Pathway analysis

Pathway analysis was performed using the procedure of Beltrame et al. (16). Briefly, the Fisher Exact Test was run over paired case-control ratios (cocultures versus single cells) calculated from the normalized data, using the public pathway set. Then, p values were transformed into pathway signatures and signed binary enrichment factors (sBEFs). sBEFs were clustered, using bootstrapping with support trees (17). This computation was carried out with the TIGR MultiExperiment Viewer (TmeV, Dana-Farber Cancer Institute, Boston, MA) 4.4.

Results

MSCs inhibit the ability of DCs to perform active immune synapses after short-term coculture

Standard MLR was used to assess the ability of DCs cocultured with MSCs during LPS activation to stimulate CD4⁺ T cells (Fig. 1A). Upon coculture with MSCs, DCs were no longer able to induce T cell-proliferative responses. To address whether this result depended on soluble factors or cell–cell contact, we cocultured MSCs and DCs in contact with each other or in a Transwell system and stimulated them with LPS for 24 h. The three cell groups (control without MSCs, Transwell coculture, and contact coculture) were tested by a classical MLR assay (Fig. 1B): DCs activated in contact with MSCs were virtually unable to stimulate the lymphocytes, whereas the stimulation provided by DCs activated in a Transwell was similar to that of controls. After 5 d of MLR, as described in Fig. 1B, the T cells were recovered and tested for proliferative response to IL-2 stimulation. The T cells previously allostimulated by DCs activated in contact with MSCs did not proliferate, even upon IL-2 challenge (Fig. 1C). The ability to reverse MSC inhibition by blocking adhesion molecules V-CAM-1 and N-cadherin was tested during Ag-specific T cell line stimulation. We compared T cell proliferation after stimulation with autologous DCs loaded with Ag in the presence or absence of MSCs pretreated with anti–V-CAM-1 and anti–N-cadherin Abs (Fig. 1D) (see Materials and Methods). Our data show that the tolerogenic effect of MSCs on DCs was significantly reversed in the presence of Abs blocking adhesion molecules (one-sample t test; p = 0.003).

The ability of mature DCs to engage in active immune synapses with T cells is instrumental to inducing T cell activation. To address whether DCs loaded with Ag in the presence of MSCs are able to establish immune synapses during lymphocyte stimulation, we analyzed CD43 distribution at the contact surface between DCs and a FluHA 306–318-specific T cell line by confocal microscopy. When FluHA 306–318 cells were challenged with peptide-loaded DCs, we observed the formation of active immune synapses, where CD43 underwent relocation away from contact areas in almost all of the cell conjugates analyzed (Fig. 2A, upper left panels). On the contrary, DCs cocultured with MSCs contacted lymphocytes on areas resembling immune synapses but did not undergo relocation of CD43 (Fig 2A, upper right panels), as confirmed by densitometry analysis (Fig. 2A, lower panels). To evaluate the direct mechanistic effect of adhesion in this phenomenon, we performed DC Ag loading in the presence of MSCs previously treated with blocking Abs against V-CAM-1 and N-cadherin. We counted the number of active immune synapses per microscope field, using an ×40 objective, in six randomly selected fields (Fig. 2B); field size was ∼387 × 387 μm. The mean number of active synapses per field (± SD) was 5.6 ± 1.2 in the control sample, which decreased to 3.2 ± 0.6 (p = 0.049) when peptide loading was done in the presence of MSCs. When the MSCs were preincubated with anti–V-CAM-1 or anti-N-cadherin blocking Abs, the mean number of active synapses was 6 ± 0.9 and 4.2 ± 0.6, respectively. Pretreatment with anti–V-CAM-1 Abs significantly reversed (one-sample t test, p = 0.032) the inhibitory effect of MSCs on DC Ag-presentation capability.

MSCs and DCs: two ways to be in contact

Because DCs and MSCs appeared to adhere to each other during coculture (Fig. 3A), electron microscopy and immunoelectron
microscopy were used to analyze the contacts between these two cell types. Using electron microscopy, contact zones between MSCs and DCs were mainly suggestive of adherens junctions (Fig. 3B). Rarely, we found contact zones suggestive of gap junctions (Fig. 3C). The expression of CD54, on average, was significantly (p, 0.01) less intense on the surfaces of DCs in contact with MSCs than on the rest of the cell surface, whereas CD43 was equally distributed on the surfaces of DCs, suggesting that upon contact between MSCs and DCs, the latter cells undergo only partial redistribution of the CD54 molecule alone (Fig. 4).

**MSC/DC short-term interaction interferes with DC endocytosis but does not influence DC phenotype and cytokine production**

We investigated what influence, if any, coculture with MSCs during DC activation had on DC phenotype, cytokine production, and endocytosis. The cell phenotype, expressed as the percentage of cells positive for CD80, CD83, CD86, and HLA DR, did not change when activation took place in the presence of MSCs (Fig. 5A). Endocytosis, as evaluated by dextran-FITC uptake, decreased upon activation in the absence of MSCs, whereas this decrease was not observed upon DC activation in the presence of MSCs, whereas this decrease was not observed upon DC activation in the presence of MSCs (Fig. 5B). The production of IL-10 and IL-12, two key cytokines produced by DCs, did not vary significantly upon activation in the presence of MSCs (Fig. 5C, 5D).

**DCs do not undergo RAC-1 and actin redistribution upon MSC/DC short-term interaction**

Actin distribution is regulated by small GTPases of the Rho family, such as Rac1, which has PAK1 as an immediate target and is responsible for polarization-related morphological changes. Rac1 is upregulated by LPS exposure, and activation of Rac1 is associated with its rapid recruitment into lipid rafts and podosome disassembly. Because actin redistribution is involved in endocytosis, we addressed whether coculture with MSCs during DC activation by LPS influences the behavior of this protein. As shown by confocal microscopy, DCs activated by LPS without MSCs completely lost their podosomes, which are typical of immature DCs, and showed uniform distribution of actin along the cell surface (Fig. 6A, a–d). On the contrary, MSC-treated DCs retained structures that were suggestive of podosomes, as shown by colocalization of actin and CD11c (Fig. 6A, e–h).

DCs activated without MSCs expressed and concentrated Rac1 together with PAK1 along the cell surface (Fig. 6B, i–l), whereas Rac1 was minimally expressed, and PAK1 remained distributed in the cytoplasm of MSC-treated cells (Fig. 6B, m–p).

**Transcriptional sensing of DCs upon contact with MSCs**

To gain insight into the molecular mechanisms subverting DC reprogramming by MSCs, we investigated the transcriptional...
response of DCs to cell contact with MSCs in the presence of LPS (see Materials and Methods). We performed microarray analysis by comparing DCs cocultured with MSCs with DCs or MSCs cultured alone, in triplicate. In this way, we obtained a statistically robust list of DEGs; in particular, 422 genes resulted differentially expressed in the cocultures with respect to DCs alone and 403 with respect to MSCs alone (Supplemental Table I). To investigate the regulation of pathways and cellular networks in our samples, we performed a pathway analysis (16) and clustered the results as sBEFs (Supplemental Fig. 1). The transcriptional changes induced by the contact of MSCs with DCs were dissected out by focusing on the comparison between the coculture condition and the DCs cultured alone by means of the Pearson correlation. As previously described, we identified clusters of genes whose expression was differentially modulated in the presence or absence of MSCs. Among them, several genes were mapped on KEGG pathways, which resulted significantly affected according to a false discovery rate-corrected Fisher exact test ($p < 0.05$), in particular the regulation of actin cytoskeleton, adherens junctions, gap junctions, and tight junctions (Fig. 7A, Supplemental Fig. 2). Among the members of the actin cytoskeleton-regulation pathway, we observed an increased expression of the genes coding for integrins ITGA11 (4.21-fold change) and ITGB5 (6.04-fold change), as well as fibronectin ($\text{FN1}$; 3.09-fold change). In addition, we observed an upregulation of genes coding for focal adhesion kinase (FAK) ($\text{PTK2}$; 2.38-fold change), the noncatalytic region of tyrosine kinase adaptor protein-associated protein Nap125 ($\text{NCKAP1}$; 2.95-fold change), myosin L chain kinase (MLCK) ($\text{MLCK}$; 2.60-fold change), and myosin regulatory L chain 9 (MYL9) ($\text{MLC}$; 2.62-fold change). The upregulation of MYLK and MYL9 is in agreement with an upregulation of myosin II ($\text{MYH10}$; 3.61-fold change), part of the same chain of reactions. Among genes mapped on adherens, gap, and tight-junction pathways, we found increased expression of nectin 3 ($\text{PVRL3}$; 4.09-fold change), connexin 43 ($\text{Cx43}$; 23.36-fold change), and coractin ($\text{CTTN}$; 2.99-fold change), respectively.

In contrast, the LPS pathway was not influenced by the presence of MSCs, as evidenced by the lack of over- or downregulation of
involved genes in the coculture condition, with respect to DCs alone.

Known interactions between our DEGs and other genes strongly suggest that nectins and nectin-like molecules could interact with FAK (PTK2), which, in turn, cooperates with integrins in actin cytoskeleton organization (Fig. 7B).

**Discussion**

The extent to which effects of MSCs on immune system cells are mediated by soluble factors and/or cell–cell contact is still being debated. Elucidating this fundamental issue is crucial to understanding how different properties of the DC interaction with MSCs depends on cell concentration and localization.

Our results are extremely innovative in this field of research. In the present work, we show how fully differentiated DCs activated in contact with MSCs induce stable not responsiveness of viable CD4+ T cells. Further, we demonstrate that this depends on the inability of DCs to establish active immune synapses with lymphocytes. This inability is associated with other features of immaturity retained by DCs: altered Rac1 redistribution and alteration in the gene networks responsible for actin cytoskeleton organization.

We studied the immunomodulatory effects of MSCs cocultured with DCs during LPS activation or Ag loading, and we showed by Transwell experiments that the tolerogenic effect of MSCs on DCs is dependent on cell–cell contact interactions, not on soluble factors (i.e., IL-6 and PGE2), as reported in other experimental conditions (18–23). It was suggested that a shift from soluble factors to cell–cell contact mechanisms is associated with MSC dilution (22), which was high (MSC/DC cell ratio 1:10 and 1:1000) in our experimental setting. Electron microscopy images showed that MSCs and DCs came in intimate contact with each other stabilized by adherens junctions, accompanied by the uneven distribution of CD54, but not of CD43, on DCs, indicating that these contacts cannot be assimilated to immune synapses (24). These findings, together with the unaltered costimulatory molecule expression and cytokine production by DCs, suggest that the inability of MSC-treated DCs to engage in active synapses with lymphocytes is an active process rather than the expression of an exhausted or damaged cell phenotype (25).

Data from other studies showed that, in humans and mice, TNF-α– or LPS-stimulated maturation of DCs in the presence of MSCs modifies the expression of costimulatory molecules needed for the activation of lymphocytes (4, 23, 25–27). Our data showed that MSC-treated DCs maintain endocytic activity typical of the immature phenotype, indicating that LPS activates membrane molecule and cytokine expression, whereas MSC contact inhibits full cell maturation. The process by which DCs move from the immature phenotype suitable for Ag acquisition to the mature one suitable for Ag presentation is a broader change that involves more than mere upregulation of MHC class II and CD86 membrane molecules; it also includes reorganization of the cytoskeleton (28). A recent model proposed DC activation as the integra-
tion of many stimuli not in a linear system (29). In our experiments, MSC-treated DCs showed colocalization of actin with CD11c, indicating the presence of podosome-like structures typical of immature, not immunogenic, DCs (30). On the contrary, cells activated without MSCs lose these structures and have actin distributed all around the cell, as expected for mature DCs (31).

**FIGURE 5.** MSC/DC short-term interaction interferes with DC endocytosis but does not influence DC immunophenotype or cytokine production. A, Immunophenotype. B, Flow-cytometry measure of endocytosis: labeling without LPS activation (upper panels) and labeling after LPS activation (lower panels). Filled curve, negative control. ELISA determination of IL-10 (C) and IL-12 (p40+p70) (D); each circle represents a sample.

**FIGURE 6.** Coculture with MSCs during activation affects the organization of DC cytoskeleton. Confocal laser-scanning microscopy of DCs activated with LPS but without MSCs (controls; a-d, i-l) or in the presence of MSCs (e-h, m-p). A, Green, F-actin; red, CD11c. B, Green, Rac1; red, PAK1. Colocalization as determined by xz view is depicted in d, h, l, and p. Original magnification ×60.
Actin distribution is regulated by small Rho GTPases (32), and it was reported that Rac1 is activated shortly after LPS stimulation (33). We showed that 5 min of LPS stimulation, in the absence of MSCs, led to the activation of Rac1 and PAK1 and that this process was inhibited in MSC-treated DCs. These findings suggest that the induction of nonimmunogenic DCs depends on interference with rearrangement of the cytoskeleton during or upon activation. Together with the description of a new DC-regulation mechanism through adhesion, we attempted to further understand the possible molecules involved. For this purpose, we performed a study of mRNA transcription, focusing on expression pathways specific for adhesion and activation. In each condition, we exposed the cells to LPS to understand the role of MSCs in controlling DC immune response and to consider the effect of LPS on the transcriptional program. The signaling pathways described in the Results are consistent with our imaging and functional data and support the idea that MSC immunomodulation acts through several molecules involved in adhesion, as confirmed by our adhesion-blocking experiments. Indeed, the importance of adhesion and adhesion molecules in the immunomodulatory action of MSCs on T cells was demonstrated recently by Ren et al. (10). They showed that ICAM-1 and VCAM-1 are required for lymphocyte–MSC adhesion and play an important role in MSC-mediated immunosuppression. We found upregulation of connexin 43 (Cnx43) gene, a major gap junction protein, and the adhesion receptor nectin 3, which is primarily involved in the regulation of cell–cell adhesion and contact-dependent inhibition of cell movement and proliferation (34). Moreover, we found upregulation of the gene for FAK which, in addition to mediating the response to adhesion to the extracellular matrix (35), is involved in the formation of intercellular adherens junctions and in the activation of adhesion-dependent signal pathways, including actin network rearrangement (36, 37). Known interactions between the DEGs found in this study and other genes strongly suggest that nectins and nectin-like molecules could interact with FAK (PTK2) to drive actin cytoskeleton organization. In this scenario, DCs and MSCs seem to use the nectin-based cell–cell adhesion to subsequently reorganize actin cytoskeleton.

The observation that the expression of the genetic networks induced by the LPS pathway is not affected by the presence of MSCs indicates that the MSC messages are primarily mediated by molecules that are part of the cytoskeleton and cell–cell junctions, and, therefore, are influenced solely by contact rather than by other soluble signals. Strikingly, this gene-expression result is fully in agreement with the other cell-based assays, indicating the validity of our observation.

It is known that actin reorganization requires Rac signaling, a pathway that is thought to be mediated by the protein Scar/WAVE (Wiskott-Aldrich syndrome protein-family verprolin homologous protein) (38, 39). The absence of Rac1 expression, in association with the upregulation of Nap125 (NCKAP1 gene), whose function is to inhibit the WAVE1/HSPC300 complex, could explain the lack of proper cytoskeleton reassembling that we observed. Moreover, the absence of PAK expression took into account the expression of MYLK and MYLK-associated genes (MLC) on myosin expression.
In conclusion, we propose that, at low concentrations, MSCs modulate DC function by interfering with the activation process through an alteration of the cytoskeleton organization; this depends on cell–cell contact mechanisms and has long-lasting effects. As a consequence, DCs retain features of immaturity (podosome-like processes, active endocytosis) while undergoing some sort of maturation (expression of cell membrane molecules and cytokines), thus becoming tolerogenic and unable to efficiently engage in immune synapses with T cells. Whole-gene-expression analysis indicates that molecular pathways of actin cytoskeleton, adherens junctions, gap junctions, and tight junctions are the core molecular determinants of this differential immune response. The master regulators of this interaction are under investigation.

Disclosures

The authors have no financial conflicts of interest.

References