Supporting Information

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SI Materials and Methods

Mice. C57BL/6 (H-2^b) and BALB/c (H-2^d) were purchased from Harlan Laboratories, Inc. Ovalbumen (OVA) peptide$^{323-339}$-specific, MHC class II-restricted, TCR transgenic DO11.10 (H-2^d) and MHC class I-restricted, TCR transgenic OT-I mice were provided by F. Grassi (Institute for Research in Biomedicine, Bellinzona Switzerland). Experiments were conducted in accordance with the institutional guidelines for animal care and use of the Institutional Animal Care and Use Committee of the Advanced Biotechnology Center (ABC, Genoa, Italy).

Bone Marrow-Derived Mesenchymal Stem Cells and Dendritic Cells. Mesenchymal stem cells (MSC) were isolated and expanded as previously described (1). MSC were obtained from C57BLl/6 (H-2^b) and BALB/c (H-2^d) mice, respectively. MSC were CD9$^+$, Sca-1$^+$, CD44$^+$, CD11b$^-$, CD34$^-$, CD45$^-$, and MHC cl.II$^-$. Bone marrow (BM)-derived dendritic cells (DC) were generated as previously described (2, 3). To assess the effect of MSC on DC activation, at day 7 BM-derived DC (1×10^6/mL) were stimulated for 24 h with 10 μg/mL of LPS (Sigma-Aldrich) in the presence or absence of MSC at a 1:3 ratio. Phenotype analysis and cytokine secretion were performed on DC isolated from adherent MSC by gentle aspiration. Isolated DC were CD11c$^+$ (>70%) and Sca-1$^-$ (<1%).

FACS Analysis. Cells were stained with antibodies specific for surface and intracytoplasmic antigens, including APM components, acquired on FACS Calibur and analyzed by CellQuest software (Becton Dickinson Company). Cells were stained with the following antibodies: anti-CD45 cytocrome C (CyC), anti-stem cell antigen-1 (Sca-1) phycoerythrin (PE), unconjugated anti-CD9, anti-CD3e PE, anti-CD4 peridin chlorophyll-α protein (PerCP), anti-CD4 fluorescein isothiocyanate (FITC), anti-CD8 PerCP, anti-MHC cl.I PE, anti-MHC cl.II PE, anti-CD44 (FITC and PE), anti-CD62L allophycocyanin (APC), anti-B220 PE, anti-CD138 PE, and anti-CD11c APC and anti-CD69 PE (all from BD Pharmingen). Anti-CD40 FITC, anti-CD80 FITC, anti-CD86 (FITC and PE), anti-CD11b FITC, anti-CD34 PE, biotin-conjugated anti–CCR-7 and anti-CD49dβ1 mAbs were purchased from AbD Serotec. Anti-DX5 APC, anti-CD83 FITC were purchased from eBioscience. Clonotype (DO11.10)-specific KJ1-26 mAb was from Caltag. Rat anti-mouse IgG1/2a FITC (BD Pharmingen) and Streptavidin-PE (AbD Serotec) were used as secondary reagents for indirect staining of CD9$^+$ MSC and CCR7$^+$ DC, respectively. For intracellular IL-12 staining, APC-conjugated anti–IL-12 antibody (BD Pharmingen) was used after fixation and permeabilization (BD Pharmingen).

In Vitro CD4 and CD8 Cell Cultures. After LPS activation, mature ctr-DC and MSC-conditioned DC were pulsed with increased doses of pOVA$^{323-339}$ (0.02, 0.2, 2 μM kindly provided by Dr. E. Millo, CEBR University of Genoa, Italy) or with OVA antigen (10μg/mL) for 3 h. Naive CD4$^+$ and CD8$^+$ T cells were isolated by positive selection with beads (Miltenyi Biotec); CD4$^+$CD44$^-$CD25$^-$CD62L$^+$ naive T cells were sorted with a FACSAria (Becton Dickinson). Naive CD4$^+$ pOVA$^{323-339}$ specific T cells and CD8$^+$ OVA-specific T cells were labeled with 2.5 mM of 5-(and 6)-carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes). Naive CD4$^+$ T cells and CD8$^+$ T cells were cultured with activated-OVA pulsed DC. T cell activation and CFSE dilution of CD8$^+$ OVA specific T cells were evaluated at 48 h and 72 h, respectively. CFSE dilution of naive CD4$^+$ OVA specific T cells was analyzed at day 3. To quantify cell recoveries, FACS acquisitions were standardized by fixed numbers of calibration beads (BD Pharmingen, Allschwil, Switzerland).

Western Blot Analysis. Following LPS stimulation, (0, 30, and 60 min) DC cultured with or without MSC were lysed in ice-cold lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Nonidet P-40, 20 mM Tris pH 7.0, 1 μM protease inhibitor mixture, 1 mM sodium orthovanadate and 1 mM phenyl methylsulphonyl fluoride). Resuspended samples were run on an SDS/PAGE under reducing conditions and subsequently blotted to nitrocellulose membranes (Amersham Biosciences) using the indicated rabbit mAbs: antiphospho-Erk1/ErK2 (1:1,000), antiphospho-p38 (1:500), and antiphospho-Akt (1:1,000) (Cell Signaling Technology). A mouse anti–β-tubulin antibody was used as charge control (Calbiochem). After washing with TBS-T, filters were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000) for 30 min and developed with Amersham ECL-plus detection system (GE Healthcare).

Antigen-Processing Machinery Staining. Mouse anti-human mAbs specific for antigen processing machinery (APM) components were kindly provided by Soldano Ferrone (Department of Immunology Cancer Cell Center, Buffalo, NY). After LPS activation, control DC and MSC-conditioned DC were harvested and then fixed with 2% paraformaldehyde, treated in the microwave, and permeabilized with saponin. Cells were incubated with an appropriated amount of anti-APM mAbs: δ-specific mAb SY-5, MB1-specific mAb SJJ-3, and LMP-10–specific mAb TO-7 (4).

RT-PCR. Total RNA were isolated from MSC (with or without LPS) or from LPS activated BM-derived DC (with or without MSC) using TRIzol Reagent (Invitrogen). To exclude the possibility that contaminating MSC expressing low levels of Sca-1 could affect PCR experiments on DCs recovered from cocultures, we evaluated Sca-1 expression on MSC after LPS stimulation at different doses (1, 5, 10, and 50 μg/mL). The expression of Sca-1, on electronically gated CD9$^+$CD44$^+$CD34$^-$CD45$^-$ MSC, was not affected by LPS treatment, considering both the percentage of positive cells as well as the mean fluorescence intensity. (Fig. S5).

First-strand cDNAs were generated using 2 μg of RNA using SuperArray's RT^2 First Strand Kit (SABiosciences; http://www.sabiosciences.com). Real-time PCR was performed with a Roche Light Cycler 480 (Roche Diagnostic; http://www.roche-applied-science.com). To analyze genes related to TLR4, signaling we used the RT^2 Profiler PCR Array mouse Toll-Like Pathway (SABiosciences). To evaluate the gene expression of cytokines, indoleamine 2,3-dioxygenase (IDO) and Toll-like receptors (TLRs), we performed quantitative RT-PCR . cDNA was obtained from 1 μg of RNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostic). The expression levels of TNF-α, IL-10, IL-12, IDO, and TLR1-9 genes were quantified in 96-well optical reaction and real-time PCR reactions were performed in triplicate in a final volume of 20 μL containing 20 ng cDNA, 10 μL of Lyght-Cycler 480 SYBR Green I Master (Roche Diagnostic) and 1 μM of each primer pair (TIB MolBiol). GAPDH was used as housekeeping gene to normalize the expression data.

The thermal protocol included an enzymatic activation step at 95 °C (5 min) and 45 cycles of 95 °C (10 s), 60 °C (10 s), and 72 °C (10 s). The melting curve of the PCR products was also recorded to check the reaction specificity. The relative gene expression of tar-

get genes in comparison of the GAPDH reference gene was conducted following the comparative CT threshold method (5) and the normalized expression was then expressed as relative quantity of mRNA (fold-induction) with respect to the control sample.

The specific primers used were as follows: IL-12 (Right 5′-GGAGTCCAGTCCACCTCTACA-3′and Left 5′-ATCGTTTTGCTGGTGTCTCC-3′); TNF-α (Right 5′-GGTCTGGGCCATAGAACTGA-3′ and Left 5′-TCTTCTCATTCCTGCTTGTGG-3′); IL-10 (5′-TGTCCAGCTGGTCCTTTGTT-3′ and Left 5′-CAGAGCCACATGCTCCTAGA-3′); IDO (Right 5′-AAGGACCCAGGGGCTGTAT-3′ and Left 5′-GGGCTTTGCTCTACCACATC-3′) (TIB Molbiol). For TLR expression, probes with the following Applied Biosystems assay identification numbers were used:

TLR1: Mm00446095_m1
TLR2: Mm00442346_m1
TLR3: Mm00446577_g1
TLR4: Mm00445274_m1
TLR5: Mm00546288_s1
TLR6: Mm02529782_m1
TLR7: Mm00446590_m1
TLR8: Mm01157262_m1
TLR9: Mm00446193_m1

ELISA. IL-10, TNF-α, and IL-12p70 in supernatants obtained from control DC and MSC-conditioned DC after 24 h LPS activation were analyzed by ELISA (using the Quantikine ELISA kit, R&D Systems, for TNF-α and IL-12p70, and the ELISA MAX Deluxe set. Biolegend, for IL-10) following the manufacturer's instructions. To address the possibility that LPS may affect the behavior of TLR4-bearing MSC during the coculture with DCs, we repeated the experiments exposing DCs to the supernatants from MSC cultured with or without LPS. MSC grown to confluence were cultured alone with or without LPS (10 μg/mL) for 48 h and the cell-supernatants were harvested. DC were cultured for 24 h with LPS (10 μg/mL) in the absence or presence of MSC and MSC supernatants. Phopsho p38 mitogen activated protein kinase (MAPK) was performed using the Cell-Based p38 MAPK (Thr180/Tyr182) ELISA kit (RayBiotec, Inc.) according to the manufacturer's instructions.

DC Migration Study. DC radioactive labeling was performed with ^{99m}Tc-exametazime (HMPAO, Ceretec; GE Healthcare) according to a procedure described previously (6, 7). Labeling efficiency, ranged 50% to 60% in all experiments, and stability was checked in three experiments by measuring the persistence of radioactivity within cells over 4 h and was > 95%. Before immunization, mice were anesthesized with trichloroacetaldeide and positioned over the head of a large field γ-camera (GE Millennium) equipped with a high-resolution collimator, and settled with a window energy of 5% centered over the 140-KeV photopeak of ^{99m}Tc. Image size was 256 × 256 × 32 and zoom factor was optimized to encompass the investigated mouse. DC injection was performed soon after the start of a dynamic acquisition (300 × 1 s; 30 × 10 s, 20 × 30 s, 40 × 60 s frames), for a total of 60 min. Time activity curves in the injection site and in the draining lymph node were plotted expressing the counting rate in these regions divided by that of the whole body. Injection site and draining lymph nodes counts were thus expressed as number of DC retained into the two regions at each time according to the following calculation (ROI, region of interest):

$$\text{Number of DC} = \frac{\text{Counting rate}_{(\text{ROI})} \times \text{Number of injected DC}}{\text{Counting rate}_{(\text{whole body})}}$$

Statistical Analysis. Statistical differences were determined using the Student's t test for independent samples from GraphPad Prism (GraphPad Softwares Inc. La Jolla, CA). The significance levels are indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.005$, $^{***}P < 0.0005$.

1. Zappia E, et al. (2005) Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 106:1755–1761.
2. Inaba K, et al. (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176:1693–1702.
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4. Whiteside TL, Stanson J, Shurin MR, Ferrone S (2004) Antigen-processing machinery in human dendritic cells: Up-regulation by maturation and down-regulation by tumor cells. *J Immunol* 173:1526–1534.
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7. Blocklet D, et al. (2003) 111In-oxine and 99mTc-HMPAO labelling of antigen-loaded dendritic cells: In vivo imaging and influence on motility and actin content. *Eur J Nucl Med Mol Imaging* 30:440–447.

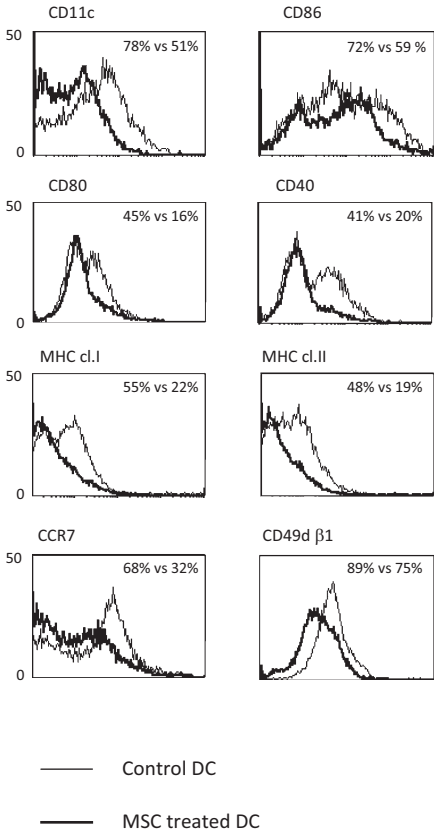

Fig. S1. MSC impaired LPS-induced maturation of DCs. Surface expression of CD11c, CD86, CD40, CD80, MHC class I and class II, CCR7, and CD49dβ1, analyzed by flow cytometry, is shown on DC activated by LPS in the absence (thin line) or in the presence of MSC (thick line). Histograms are representative of one of five independent experiments. Percentage of surface markers of control DC vs. MSC-conditioned DC is shown at the top of the histograms.

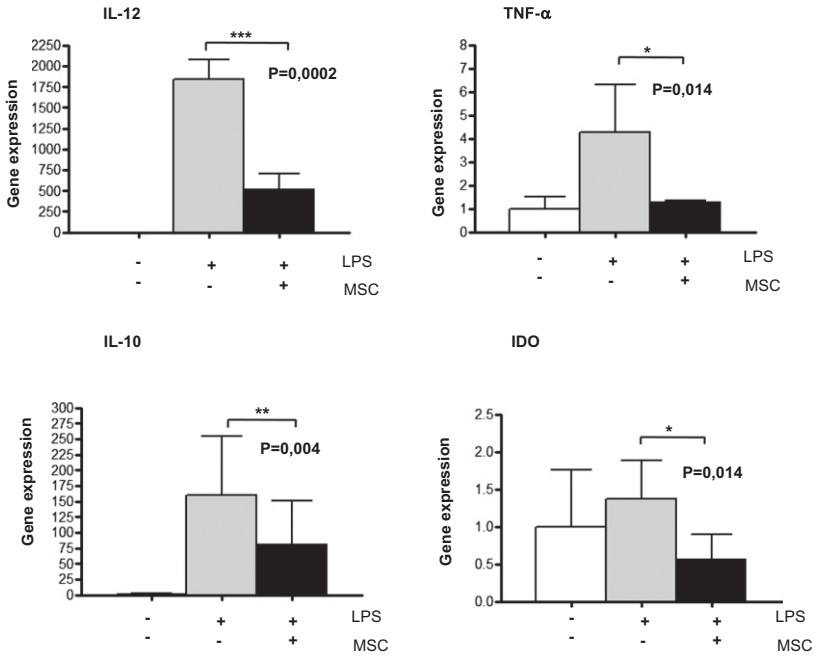

Fig. S2. MSC inhibit gene expression of cytokines and IDO of LPS-stimulated DC. mRNA was obtained from control DC and MSC-conditioned DC following LPS stimulation. Gene expression of IL-12, TNF-α, IL-10, and IDO was evaluated by real-time PCR on control DC (gray bar) and MSC-conditioned DC (black bar) after 10 μg/mL of LPS stimulation (***$P = 0.0002$, *$P = 0.014$, **$P = 0.004$, respectively). Control DC in the absence of LPS stimulation (white bar) was used as internal control. Mean ± SD from ctr-DC ($n = 6$) and MSC-conditioned DC ($n = 6$).

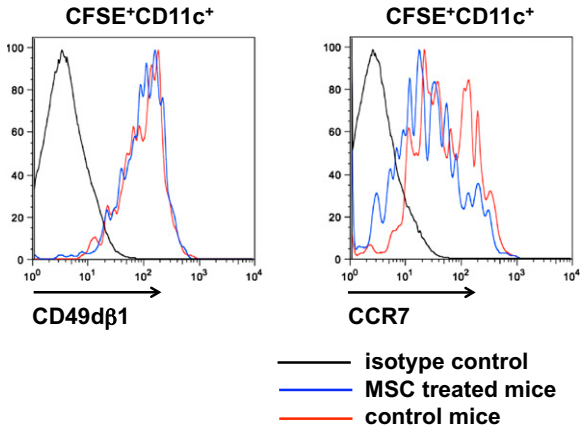

Fig. S3. CCR7^{high}CFSE^{+}CD11c^{+} DC are decreased in the draining lymph node of MSC-injected mice. The surface expression of CCR7 and CD49dβ1 analyzed by flow cytometry is shown on electronically gated CFSE^{+}CD11c^{+} DC recovered from the draining lymph node of MSC-treated mice (blue line) compared with PBS-injected control animals (red line); the isotype-matched control is depicted (black line). Histograms are representative of one of three independent experiments.

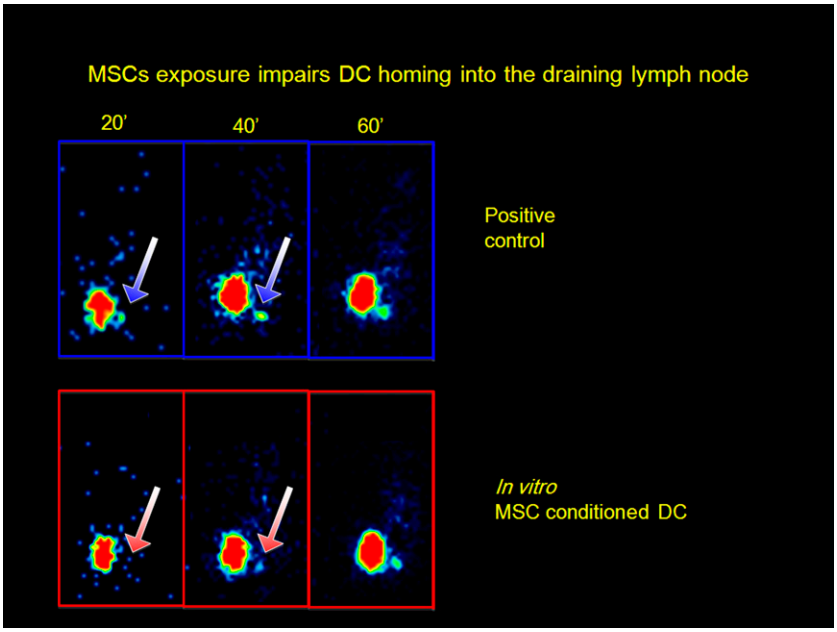

Fig. S4. MSC halt homing of DC to the draining lymph nodes. A representative in vivo scintigrafic image (one of four independent experiments) displays a significant reduction of the early uptake of ^{99m}Tc-HMPAO–labeled DC in the draining lymph node of mice treated with in vitro MSC-conditioned OVA-pulsed 99mTc-HMPAO–labeled DC (*Lower*) compared with control mice treated with OVA-pulsed, 99mTc-HMPAO–labeled DC (*Upper*).

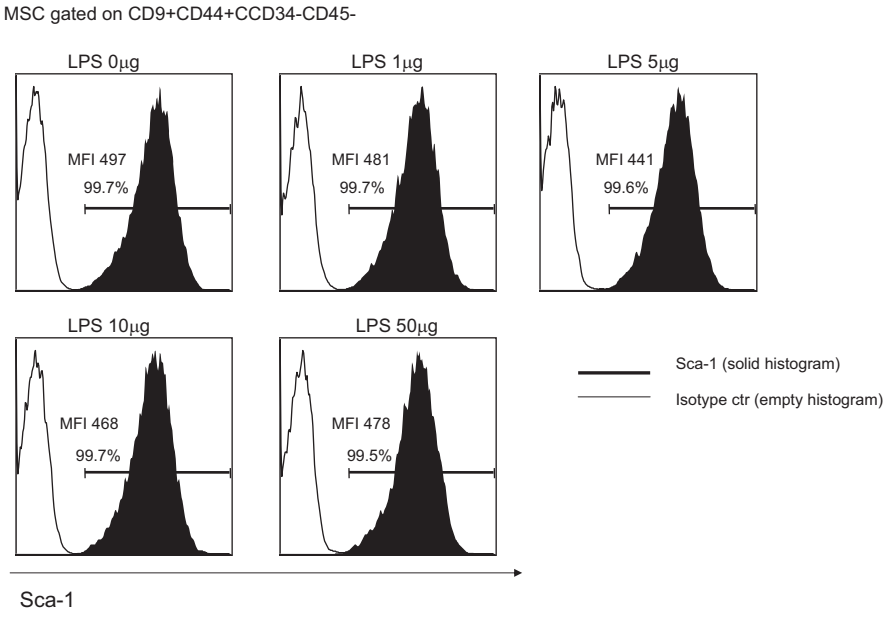

Fig. S5. Sca-1 expression on MSC after LPS stimulation. Surface expression, analyzed by flow cytometry, of Sca-1 (solid histogram) and isotype control (empty histogram) is shown on MSC activated with LPS at different doses (0, 1, 5, 10, and 50 μg/mL). MSC were gated on $CD9^{+}CD44^{+}CD34^{-}CD45^{-}$. Histograms are representative of one of three independent experiments. Percentage of surface markers as well as the mean fluorescence intensity is shown at the top of the histograms.

Table S1. Genes down-modulated in MSC conditioned DC vs. ctr-DC after LPS activation

Gene symbol	Description	Ctr- DC (*)	MSC-conditioned DC (*)	Fold down-Regulation
CxCl 10	Chemokine (C-X-C) ligand	0.02	0.002	−11.7
MAP2K3	Mitogen-activated protein kinase kinase 3	0.07	0.003	−21.38
MAP2K4	Mitogen-activated protein kinase kinase 4	0.04	0.02	−2.21
MyD88	Myeloid differentiation primary response gene (88)	0.008	0.002	−4.67
NF-κB1	Nuclear factor of κ light polipeptide gene enhancer in B-cells 1	0.55	0.14	−4.01

The table depicts the relative expression levels, using the RT^2 Profiler PCR Array mouse Toll-Like Pathway (SABiosciences), of genes of TLR signaling pathway that were down-regulated over four folds among 84 genes related to the TLR-mediated signal transduction following LPS stimulation in MSC-conditioned DC compared with control DC. The values are represented as the difference in Ct values normalized to housekeeping gene expression. Comparison in gene expression were done using the $2^{-}\Delta Ct$ (*) method (5).