Mesenchymal Stem Cells Effectively Modulate Pathogenic Immune Response in Experimental Autoimmune Encephalomyelitis

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Objective: To evaluate the ability of mesenchymal stem cells (MSCs), a subset of adult stem cells from bone marrow, to cure experimental autoimmune encephalomyelitis.

Methods: The outcome of the injection of MSCs, in mice immunized with the peptide 139-151 of the proteolipid protein (PLP), was studied analyzing clinical and histological scores of treated mice. The fate of MSCs labeled with the green fluorescent protein was tracked in vivo by a photon emission imaging system and postmortem by immunofluorescence. The modulation of the immune response against PLP was studied through the analysis of in vivo T- and B-cell responses and by the adoptive transfer of MSC-treated encephalitogenic cells.

Results: MSC-treated mice showed a significantly milder disease and fewer relapses compared with control mice, with decreased number of inflammatory infiltrates, reduced demyelination, and axonal loss. In contrast, no evidence of green fluorescent protein–labeled neural cells was detected inside the brain parenchyma, thus not supporting the hypothesis of MSCs transdifferentiation. In vivo, PLP-specific T-cell response and antibody titers were significantly lower in MSC-treated mice. When adoptively transferred, encephalitogenic T cells activated against $PLP_{139-151}$ in the presence of MSCs induced a milder disease compared with that induced by untreated encephalitogenic T cells. These cells showed decreased production of interferon-γ and tumor necrosis factor-α and did not proliferate on antigen recall, and thus were considered anergic.

Interpretation: Overall, these findings suggest that the beneficial effect of MSCs in experimental autoimmune encephalomyelitis is mainly the result of an interference with the pathogenic autoimmune response.

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The bone marrow is composed of a stromal component that tightly interacts with osteoblasts and endothelial cells, contributing to the creation of the proper microenvironment supporting the survival of hematopoietic stem cells in a quiescent state and fostering hematopoiesis.[1] Such stromal scaffold is formed of cells of mesenchymal origin,[2] named mesenchymal multipotent progenitor cells or mesenchymal stem cells (MSCs) because of their capacity of differentiating into multiple tissues of the mesenchymal lineage.[3] These cells have been reported also to (trans)differentiate into almost any cell type from the three lineages, increasing hopes for their utilization in regenerative medicine.[4]

More recently, MSCs have been demonstrated to have a number of unique immunological properties.[5] In fact, MSCs can inhibit proliferation of T,[6] B,[7] and dendritic cells[8] through the induction of cell division arrest. MSCs can also inhibit proliferation of natural killer cells[9] and impair dendritic cell maturation, as well as antigen presentation.[10] MSCs have shown also some kind of immune privilege because of their capacity of escaping, in some experimental conditions, allogeneic immune responses.[11,12] These features provide the conceptual support for their in vivo utilization in immune-mediated diseases such as organ transplantation[13] and graft-versus-host disease.[14] More recently, we demonstrated that MSCs can ameliorate also experimental autoimmune encephalomyelitis (EAE), an ex-

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perimental model for human multiple sclerosis.[15] In that study, the intravenous (IV) injection of MSCs in C57BL/6J mice immunized with the peptide 35-55 of myelin oligodendrocyte glycoprotein (MOG) significantly improved clinical severity of EAE, decreasing central nervous system (CNS) inflammation and demyelination. Here, we further validate the rationale for the treatment of multiple sclerosis with MSCs by demonstrating that these cells can *ameliorate* relapsing-remitting EAE induced in SJL mice with the peptide 139-151 of the proteolipid protein ($PLP_{139-151}$), through an effect impairing both the cellular and humoral arm of the encephalitogenic immune response without any evidence of local transdifferentiation into neural cells.

Materials and Methods

Isolation and Characterization of Mesenchymal Stem Cells

Bone-marrow MSCs were isolated from 6- to 8-week-old C57BL/6J mice (Harlan, S. Pietro al Natisone, Italy), as described elsewhere.[15] In brief, marrow cells, flushed out of tibias and femurs, were plated in $75cm^2$ tissue culture flasks at the concentration of 0.3 to 0.4×10^6 cells/cm^2 using Murine Mesencult as medium (Stem Cell Technologies, Vancouver, British Columbia, Canada). Cells were cultured in plastic plates as adherent cells and kept in a humidified 5% CO_2 incubator at 37°C, refreshing medium every 3 days for about 6 weeks when cells reached 80% confluence. On treatment with 0.05% trypsin solution containing 0.02% ethylenediaminetetraacetic acid (Sigma-Aldrich, St. Louis, MO), marrow cells were plated in $25cm^2$ flasks at 1.2 to 2.0×10^4 cells/cm^2 for the subsequent 4 or 5 passages. Thereafter, cells were routinely seeded at 4 to 10×10^3 cells/cm^2, reaching 80% confluence after 4 to 5 days. Mature MSCs, obtained after four to five passages in culture, were defined by their capacity of differentiating, under appropriate conditions in vitro, into adipocytes, chondrocytes, and osteocytes and by the expression of CD9, Sca-1, CD73, and CD44 and the lack on their surface of the hematopoietic markers CD45, CD34, and CD11b.[5] The phenotype was analyzed using the following monoclonal antibodies directed against mouse surface markers: PE-Cy5–conjugated rat anti–mouse CD45 (#30-F11), R-PE–conjugated rat anti–mouse Sca-1 (#Ly6A\E), purified rat anti–mouse CD9 (#KMC8), R-PE–conjugated rat anti–mouse CD73 (#Ty/23) (all purchased from BD PharMingen, San Diego, CA). Fluorescein isothiocyanate rat anti–mouse CD11b (#M1/70.15) and R-PE rat anti–mouse CD34 (#MEC14.7) were purchased from Serotec (Oxford, United Kingdom). Fluorescein isothiocyanate anti–rat $IgG_{1/2a}$ (BD PharMingen, San Diego, CA) was used as secondary reagents for an indirect staining of CD9-positive cells. All the analyses were performed using a fluorescence-activated cell sorting Calibur flow cytometer (BD PharMingen). The CELL Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used for data analysis. A total of 20,000 events were acquired for each sample.

Evaluation of MSC differentiation was achieved as follows. For the induction of adipogenesis, MSCs were cultured for 2 weeks in the presence of 10ng/ml insulin (Sigma) and 1×10^{-8}M dexamethasone (Sigma) and stained by Oil Red O. Osteogenic induction was achieved on culture of MSCs with 50μg/ml L-ascorbic acid-2 phosphate (Sigma), 10mM glycerol 2-phosphate disodium salt (Sigma), and 1×10^{-8}M dexamethasone (Sigma) for 3 weeks. Osteogenic differentiation was detected by Alazirin red staining. Chondrogenic induction was obtained culturing MSCs with 0.1mM L-ascorbic acid-2 phosphate (Sigma), 10ng/ml human transforming growth factor-β_1 (Pharmingen-BD), and 1×10^{-8}M dexamethasone (Sigma) for 2 weeks and detected by Alcian blue staining.

To track marrow cells in vivo, we transfected MSCs with a second-generation lentiviral vector pRRLsin.PPT-hCMV engineered with the enhanced green fluorescent protein (eGFP) gene, as described elsewhere.[15]

Experimental Autoimmune Encephalomyelitis Induction and Treatment Protocols

Female SJL/J mice, 6 to 8 weeks old, were purchased from Harlan Italy. All animals were housed in pathogen-free conditions and treated according to the guidelines of the Animal Ethical Committee of the Advanced Biotechnology Center (Genoa) and that of the Besta National Neurological Institute. Mice were immunized with 0.3ml of an emulsion composed of 0.15ml incomplete Freund's adjuvant (Difco, Detroit, MI) supplemented with 4mg/ml *Mycobacterium tuberculosis* (strain H37Ra; complete Freund's adjuvant; Difco) and 200μg $PLP_{139-151}$ (Espikem Florence, Italy) diluted in an equal volume of phosphate-buffered saline (PBS) and injected subcutaneously in the flanks at two sites. Immunization with $PLP_{139-151}$ was followed by IV administration of 300ng pertussis toxoid (LIST Biological Laboratories, Hornby, ONT, Canada) on day 0 and after 48 hours. EAE–affected mice were injected IV on day 12 after immunization with 10^6 MSCs diluted in PBS (100μl) without Ca^{2+} and Mg^{2+} (Sigma) or with an equal volume of PBS in control mice. In one experiment, mice were injected with 10^6 of the NIH/3T3 fibroblast cell line (ATCC; American Type Culture Collection, Manassas, VA). For the induction of EAE by adoptive transfer, lymph nodes were obtained from donor SJL/J female mice (age, 8 weeks) 10 days after immunization with $PLP_{139-151}$ (0.1mg) in complete Freund's adjuvant. Lymph nodes cells (LNCs) were stimulated in vitro for 72 hours with 20μg/ml $PLP_{139-151}$ in presence or absence of MSCs at a 1:8 ratio (MSCs/LNCs) and injected IV into strain-, age-, and sex-matched recipient mice. Mice received also an IV injection of pertussis toxoid (200ng in 0.2ml PBS) at day 0 and 48 hours later. Weight and clinical score, assigned as described previously,[16] were recorded daily. Mice were followed, unless moribund, for at least 60 days after injury. Disease incidence, onset, and maximum score were recorded for each mouse and expressed as mean ± standard deviation. The cumulative disease score is the mean of the sum of the neurological score recorded daily for each mouse during the whole period of observation, and it was used for statistical analysis by Mann–Whitney *U* test.

Cells Preparation and Proliferation Assays

Mononuclear cells were isolated from the spleen and lymph nodes of $PLP_{139-151}$-immunized mice, either treated with MSCs or vehicle alone, and used at the concentration of 2.5×10^6/ml in a final volume of 200μl/well into 96-well flat-bottomed microtiter plates (Sarstedt, Newton, NC) in the presence of 20μg/ml $PLP_{139-151}$ peptide (Espikem, Florence, Italy) or 1μg/ml anti-CD3 antibody (R&D Systems, Minneapolis, MN). In experiments addressing direct inhibition of T-cell proliferation, MSCs were added at a 1:8 ratio (MSCs/LNCs). In some proliferation experiments, NIH/3T3 fibroblasts were used as controls at the same cell ratio. T-cell proliferation was measured by standard ^{3}H-thymidine incorporation assay after 72 hours of incubation in complete RPMI medium.

Cytokine Production

The levels of interferon-γ (IFN-γ) and tumor necrosis factor-α of mononuclear cells obtained from the spleen and lymph nodes of EAE–affected mice treated with MSCs or vehicle alone were measured on supernatants derived from 72-hour-long cultures of cells stimulated by anti-CD3 (R&D Systems) or $PLP_{139-151}$ utilizing a commercially available kit of enzyme-linked immunosorbent assay (R&D Systems). Similarly, we measured cytokine levels before passive transfer experiments on supernatants derived from cultures of encephalitogenic LNCs from EAE–affected mice, stimulated by anti-CD3 (R&D Systems) or $PLP_{139-151}$ in the presence or absence of MSCs.

Antibody Response

Blood was collected from the tail of mice, and total specific IgG, IgG1, IgG2a, IgG2b, and IgG3 antibodies were measured by enzyme-linked immunosorbent assay, as described elsewhere.[16] In brief, 96-well microtiter plates (Immunol; Thermo Lab Systems, Franklin, MA) were coated overnight at 4°C with 0.1ml $PLP_{139-151}$, diluted in 0.1M $NaHCO_3$ buffer (pH 8) at a concentration of 0.01mg/ml. The plates were blocked with PBS 10% fetal calf serum (blocking buffer) for 2 hours. Samples were diluted in blocking buffer at different concentrations (1:100 for IgG, IgG1, and IgG2a; 1:250 for IgG3; and 1:5,000 for IgG2b), and antibody binding was tested by the addition of peroxidase-conjugated monoclonal goat anti–mouse IgG, IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology Associates, Birmingham, AL) each at 1:5,000 dilution in blocking buffer. Enzyme substrate was added and plates were read at 450nm on a microplate reader (Termo Lab Systems Multiscan, Ascent).

Histology

After death, mice were transcardially perfused with 4% paraformaldehyde, and spinal cords collected in the same fixative and then embedded in paraffin. Five-micrometer sections were analyzed for histological examination using hematoxylin and eosin for detection of inflammatory infiltrates, Luxol fast blue for myelin, and Bielschowsky silver impregnation for detection of axonal structures under an Olympus Provis AX70 (Olympus Italia, Segrate, MI) optical microscope. Spinal cord demyelination was identified on individual images, traced manually on the composite images and their surface areas measured using Image Pro-PLUS 4 software (Media Cybernetics, Silver Spring, MD), and expressed as a percentage of the total surface area.[17] Axonal density was counted on an average of six adjacent spinal cord sections per mouse (at ×40 magnification). To quantify inflammatory activity in spinal cords, we performed immunohistochemistry with a purified rat anti–mouse CD3 monoclonal antibody (#145-2C11; R&D Systems), purified rat anti–mouse Mac3 monoclonal antibody (#M3/84; BD PharMingen), and a purified rat anti–mouse B220 monoclonal antibody (#RA3-6B2; BD PharMingen), which identify T cells, macrophages, and B cells, respectively. We used a mouse anti–glial fibrillary acidic protein (anti-GFAP) monoclonal antibody (#G-A-5; Chemicon, Temecula, CA) for the detection of astrocytes, a mouse anti–neuron-specific nuclear protein (NeuN; #A60; Chemicon) for neurons, and a mouse anti-oligodendrocyte marker O4 monoclonal antibody (#81; Chemicon) for mature oligodendrocytes. All primary antibodies were demonstrated with a biotin- or rhodamine-labeled secondary anti–rat antibody (Serotec) followed by diaminobenzidine tetrahydrochloride detection according to standard protocol. Negative control samples were prepared omitting the primary antibody. Quantification was performed on six sections per animal, from approximately three segments apart. Images were captured at 40× magnification with an Olympus DP70 digital camera using the acquisition software Image-Pro Plus by counting the number of pixels above a set threshold. The captured images were coded and quantified in a blinded manner. CNS pathological score was expressed as mean ± standard deviation on determination of the total number of positive elements exclusively in the white matter and reported as number of positive elements per square millimeter. Histology scores were analyzed by Mann–Whitney *U* test.

Monitoring of Enhanced Green Fluorescent Protein–Labeled Mesenchymal Stem Cells

IV-injected eGFP-labeled MSCs were tracked along time using an in vivo biophotonic imaging system, which allows detection of photon-emitting cells in animal tissues. In brief, mice were anesthetized by a constant flow of 1.5% isoflurane from the IVIS manifold and imaged using an IVIS Image System 100 Series (Xenogen Corporation, Alameda, CA). The biofluorescence signals (photons/sec) emitted from the mice were captured by a high-sensitivity charge-coupled device camera and analyzed using Living Image software (Xenogen Corporation).

To overcome poor sensitivity inside the skull and allow identification of cells inside the CNS, we tracked eGFP-positive cells in ornithine carbamyl transferase–embedded neural tissues by means of immunofluorescence examination as described elsewhere,[15] using a rabbit anti-GFP polyclonal antibody (Cat. N. A11122; Molecular Probes, Eugene, OR), followed by detection with an anti–rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes). We used 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO) for cell nucleus detection, whereas NeuN-, GFAP-, and O4-positive cells were detected as described earlier and shown with an anti–rat antibody conjugated with Alexa 594 (Molecular Probes).

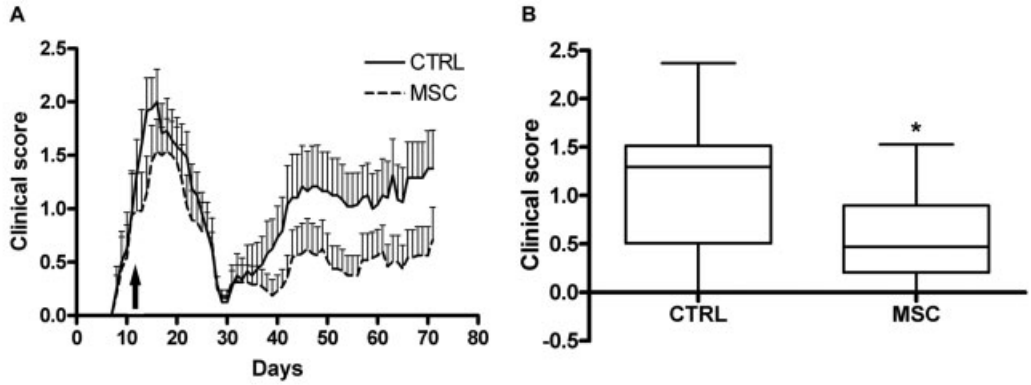

Fig 1. Intravenous injection of mesenchymal stem cells (MSCs) ameliorates $PLP_{139-151}$-induced experimental autoimmune encephalomyelitis (EAE). (A) MSCs (dashed line) *injected at disease onset* (black arrow) *reduce the severity of EAE compared with control (CTRL;* solid line*) mice. (B) Distribution of the average scores for the whole observation period for each mouse of MSC-treated and control mice (*$p < 0.05$, *Mann–Whitney* U *test). PLP = proteolipid protein.*

Results

Mesenchymal Stem Cells Ameliorate Relapsing-Remitting Experimental Autoimmune Encephalomyelitis

We previously demonstrated that C57BL6/J-derived MSCs injected IV into MOG_{35-55}-immunized mice before or after disease onset ameliorated chronic EAE.[15] Thus, before exploring the therapeutic potential of C57BL/6J MSCs in allogeneic SJL recipients, we sought to verify their capacity of inhibiting proliferation of LNCs from SJL mice. As expected, MSCs inhibited proliferation of LNCs stimulated with anti-CD3 up to 90% compared with control animals (2.758cpm for MSC-treated LNCs compared with 35.740 for control animals, mean of 5 experiments; $p < 0.01$, Mann–Whitney U test), confirming that inhibition of T-cell proliferation works across major histocompatibility complex barrier.[11] No significant inhibition of proliferation was observed when NIH/3T3 fibroblasts were added to the culture (data not shown). Next, we IV injected 10^6 MSCs into SJL mice immunized with $PLP_{139-151}$ at the onset of the first symptoms of EAE around day 12 after immunization. MSC-treated mice developed a considerably milder disease compared with PBS-treated control mice, as demonstrated by a significant difference in disease course (Fig 1A) and in the distribution of mean clinical score for each mouse (see Fig 1B). A significant difference was observed also when cumulative disease score and, more important, relapse rate was considered (Table). In contrast, no amelioration of EAE was observed when 10^6 NIH/3T3 were IV injected (data not shown). These results suggest that MSCs have a potent effect on the clinical expression of relapsing-remitting EAE.

The injection of MSCs resulted in a statistically significant reduction of all pathology scores in MSC-treated compared with control mice (Fig 2; see the Table). In particular, MSCs significantly reduced demyelination ($p < 0.01$) and inflammation as shown by a decreased number of CD3-positive T cells ($p < 0.01$), B220-positive B cells ($p < 0.01$), and Mac3-positive macrophages ($p < 0.01$). In addition, in MSC-treated mice, we detected less evidence of axonal sufferance compared with control mice as demonstrated by a significant difference in density of axons detected by silver staining ($p < 0.05$).

Mesenchymal Stem Cells Enter the Central Nervous System but Do Not Transdifferentiate into Neural Cells

eGFP-transfected MSCs were injected into $PLP_{139-151}$-immunized SJL mice to confirm that they can colonize lymphoid organs and reach the CNS. IVIS Detection System detected a distinct positive signal in the spleen and cervical lymph nodes 2 days after injection (not shown) and still clearly presents 1 month after immu-

Table. Clinical Pathological Features of Experimental Autoimmune Encephalomyelitis–Affected Mice

Treatment	Treatment Schedule (dpi)	Mice, N	Disease Onset (dpi)	Maximum Clinical Score	Cumulative Disease Score	Relapse Rate	CD3-Positive Infiltrates (cells/mm^2)	Mac3-Positive Infiltrates (cells/mm^2)	B220-Positive Infiltrates (cells/mm^2)	Demyelination (%)	Axonal Density (axon/mm^2)
Control	12	20	12.22 ± 1.07	2.67 ± 0.23[a]	61.68 ± 8.62[b]	1.40 ± 0.22[b]	127.20 ± 6.34[b]	114.30 ± 4.95[b]	81.60 ± 3.83[b]	31.50 ± 4.12[b]	114.98 ± 8.80[b]
MSC	12	20	12.06 ± 0.65	2.00 ± 0.25[a]	34.13 ± 6.29[b]	0.55 ± 0.15[b]	62.92 ± 8.58[b]	40.22 ± 11.29[b]	40.08 ± 7.48[b]	6.34 ± 0.97[b]	324.69 ± 30.15[b]

[a] $p < 0.05$; [b] $p < 0.01$.

dpi = days post-immunization; MSC = mesenchymal stem cells.

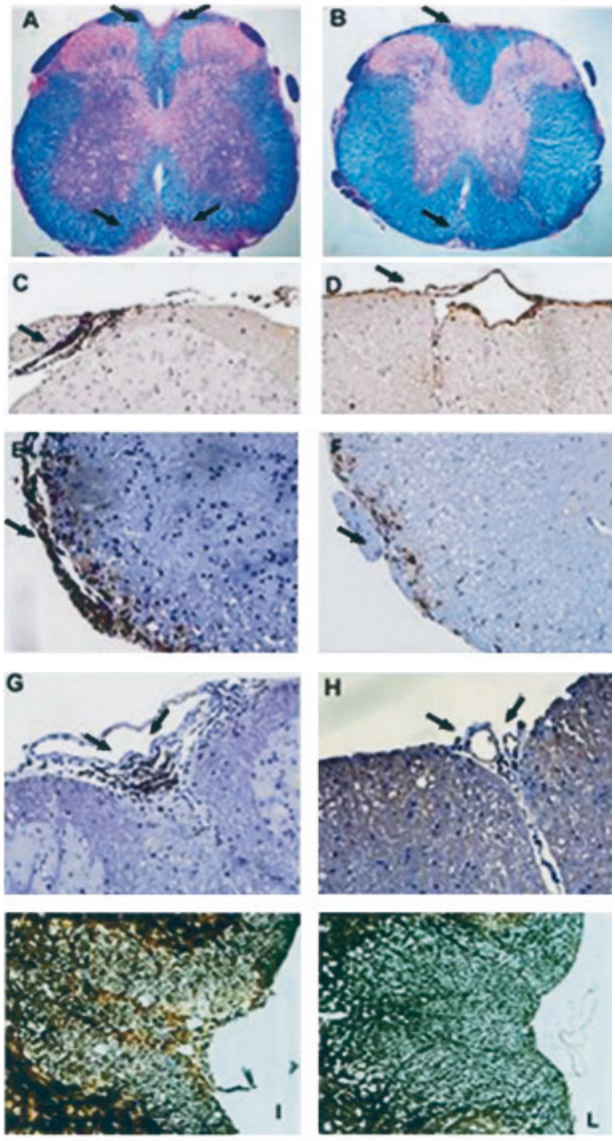

Fig 2. Pathological findings in the central nervous system (CNS) of mice with $PLP_{139-151}$-induced experimental autoimmune encephalomyelitis (EAE). Control mice are shown in A, C, E, G, and I, whereas mesenchymal stem cell (MSC)–treated animals are depicted in B, D, F, H and L. Luxol fast blue staining of the spinal cord shows demyelination in control (A) and in MSC-treated mice (B) (×4; black arrows*). Histology for (C, D) $CD3^+$ T cells (×40;* black arrows*), (E, F) $MAC3^+$ macrophages (×40;* black arrows*), (G, H) $B220^+$ B cells (×40;* black arrows*), and (I, L) Bielschowsky silver impregnation (×40). PLP = proteolipid protein.*

nization (Figs 3A, B). Next, we confirmed that photon emission detected by the IVIS System arose from the presence of labeled MSCs inside the tissues as demonstrated by the detection of eGFP-positive elements inside the spleen (see Fig 3C) and lymph nodes (see Fig 3D). Immunofluorescence was used also to track MSCs inside the CNS due to the scattered biosignal detected with the IVIS Detection System beyond the skull bones. eGFP-positive cells were not observed inside the CNS 2 days after injection, but were clearly detected 1 month after administration, mostly close to inflammatory infiltrates (Figs 4A, B). To verify whether MSCs inside the CNS could transdifferentiate into neural cells and therefore contribute to tissue repair, we analyzed 48 sections from each MSC-treated animal searching for colocalization of markers of astrocytes (GFAP; see Fig 4C), neurons (NeuN; see Fig 4D), and oligodendrocytes (O4; see Fig 4E) with eGFP. Regardless of the presence of several eGFP-positive elements inside the parenchyma, we could not detect any clear evidence of colocalization of neural markers and eGFP signal, suggesting that, on injection in SJL mice, MSCs do not noticeably differentiate into neural cells.

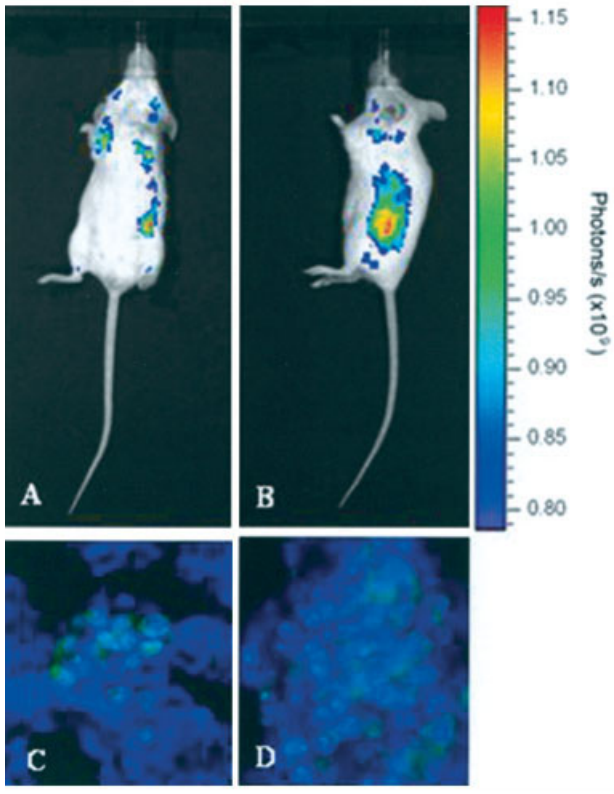

Fig 3. Green fluorescent protein (GFP)–labeled cells are detected in the spleen of SJL mice on intravenous injection 1 month after immunization. Mouse images show the bioluminescent signal from the spleen and the cervical lymph nodes detected in one representative animal (from the top in A and from the right side in B) 1 month after immunization. The bioluminescent signal is given as photons per second. The presence of GFP-positive cells in the spleen (C) and in a lymph node (D) of the same mouse is confirmed by immunofluorescence (original magnification, 40×).

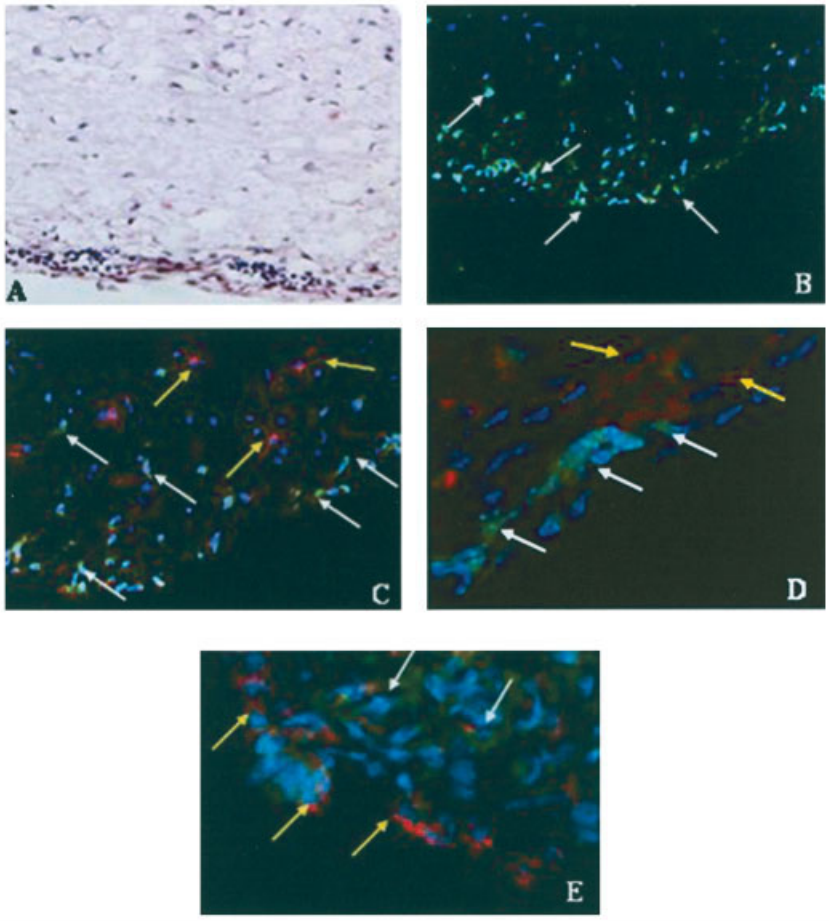

Fig 4. Enhanced green fluorescent protein (eGFP)–positive cells are detected inside the inflamed central nervous system of mesenchymal stem cell (MSC)–treated experimental autoimmune encephalomyelitis (EAE)–affected mice. (A) Hematoxylin and eosin staining of the spinal cord showing a subpial inflammatory infiltrate (40×). (B) In the same section, several eGFP-positive cells are detected (original magnification, 40×; white arrows*). (C) Glial fibrillary acidic protein (GFAP; original magnification, 40×;* yellow arrows*), (D) neuron-specific nuclear protein (NeuN; 100×,* yellow arrows, *D), and (E) O4 (100×,* yellow arrows*) immunoreactivities are depicted together with eGFP-labeled cells.*

Mesenchymal Stem Cells Halt Effector Cells on In Vivo Injection

Next, we asked whether MSCs can affect the pathogenic cellular response against $PLP_{139-151}$ on IV administration into EAE–affected mice. In Figure 5, we show that proliferation of T cells from the lymph nodes and the spleen of MSC-treated mice is significantly impaired compared with control mice. Interestingly, such inhibition occurs both on stimulation with anti-CD3 (see Fig 5A) and $PLP_{139-151}$ (see Fig 5B), and it is paralleled by a significant decrease in the production of tumor necrosis factor-α and IFN-γ of spleen cells and LNCs of MSC-treated mice compared with control mice (see Figs 5A, B). To verify whether MSCs could also affect the pathogenic B-cell response, we measured the production of $PLP_{139-151}$-specific immunoglobulins from the blood of EAE–affected mice receiving MSC administration and from control mice. MSC injection significantly inhibited total antigen-specific IgG production, as well as that of each IgG subclass, compared with control mice (see Fig 5C). These results suggest that, on in vivo administration, MSCs can block the pathogenic T- and B-cell response against $PLP_{139-151}$.

Mesenchymal Stem Cells Inhibit Proliferation and Inflammatory Cytokines Production of Encephalitogenic T Cells and Improve Adoptively Transferred Experimental Autoimmune Encephalomyelitis

If MSCs can truly block pathogenic cells, then they should affect the encephalitogenic potential of $PLP_{139-151}$-specific T cells. Thus, we first cultured T cells obtained from the lymph nodes of mice immunized with $PLP_{139-151}$ in the presence of $PLP_{139-151}$ alone or together with MSCs at 8:1 ratio (LNC/MSC), and after 72 hours, we measured T-cell proliferation and cytokine production. Encephalitogenic LNCs cultured in

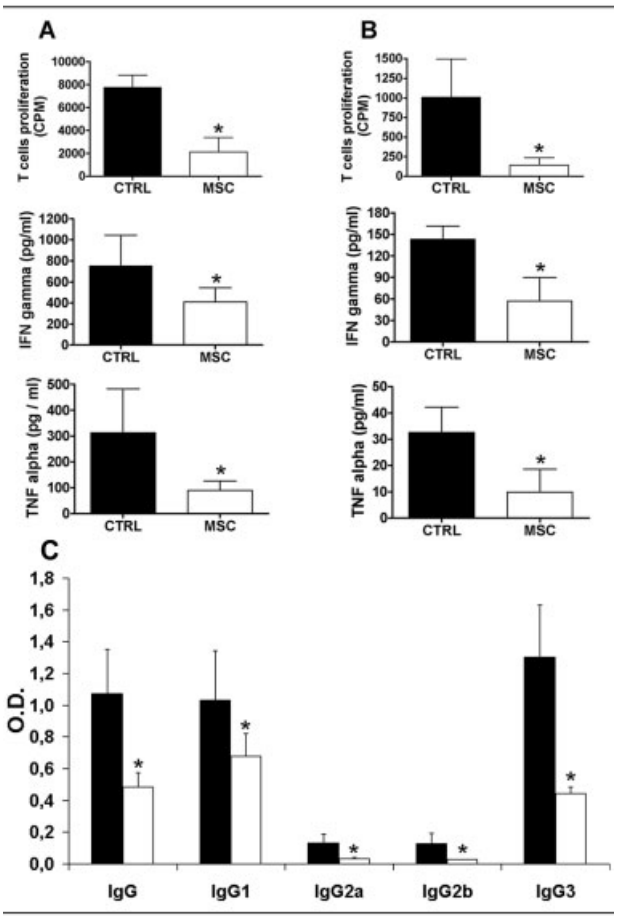

Fig 5. Mesenchymal stem cells (MSCs) suppress in vivo T- and B-cell responses. Proliferation (expressed as mean counts per minute [CPM] ± standard deviation [SD] of three independent experiments) and cytokines production (expressed as mean pg/ml ± SD from three separate experiments) of mononuclear cells (MNCs) from the spleen of MSC-treated (white bars) *and control mice (CTRL;* black bars*) stimulated with anti-CD3 (A) or $PLP_{139-151}$ (B). (C) the IgG production from MSCs-treated and control mice is depicted (expressed as mean OD ± SD from three separate experiments).* *p < *0.05, Mann–Whitney* U *test. IFN = interferon; PLP = proteolipid protein; TNF = tumor necrosis factor.*

the presence of MSCs proliferated significantly less and produced lower amounts of tumor necrosis factor-α and IFN-γ than control cells (Figs 6A, B). Interleukin-2 (IL-2), IL-4, and IL-10 production was not affected by MSC treatment (data not shown). Next, to test the ability of MSC-treated encephalitogenic T cells to induce EAE, we removed LNCs in suspension from adherent MSCs and verified T-cell purity/MSCs contamination by flow cytometry before adoptive transfer experiments. MSC contamination was always negligible, being less than 2%. Thus, we injected either 10^7 encephalitogenic control cells or MSC-treated LNCs into naive SJL mice. MSC-treated encephalitogenic LNCs induced a significantly milder disease compared with control encephalitogenic LNCs (see Fig 6C); this was confirmed by a decreased cumulative score (11.27 ± 2.38 in mice receiving MSC-treated LNCs vs 27.33 ± 3.41 in mice receiving control LNCs; $p < 0.01$, Mann–Whitney U test) and a lower maximal clinical score (1.73 ± 0.32 vs 2.87 ± 0.26, respectively; $p < 0.05$, Mann–Whitney U test). Then, we sought measuring whether MSC pretreatment of encephalitogenic LNC can impact also the generation of an in vivo T-cell response and IgG production against $PLP_{139-151}$ in the adoptively transferred animals. We observed that mice injected with MSC-treated LNCs produced a lower amount of PLP-specific IgG of all subclasses (see Fig 6D), showed a

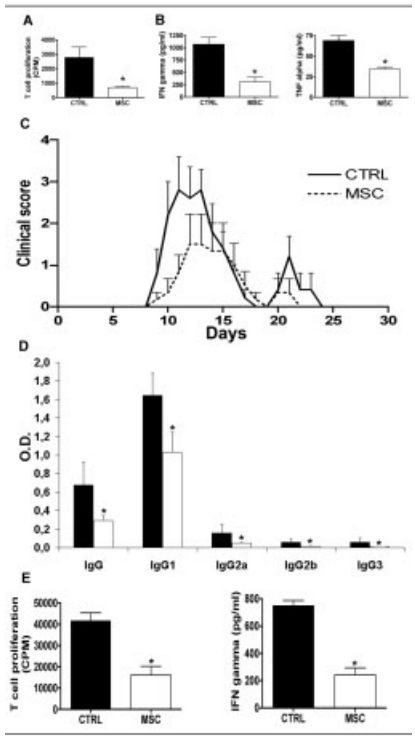

Fig 6. Mesenchymal stem cells (MSCs) ameliorate adoptively transferred experimental autoimmune encephalomyelitis (EAE). T-cell proliferation (A) and cytokine production (B) of encephalitogenic lymph nodes cells (LNCs) cultured in the absence (black bars) *or presence* (white bars) *of MSCs. (C) Clinical course of EAE in mice adoptively transferred with LNCs cocultured in the presence* (dotted line) *or absence of MSCs* (thick line). *(D) IgG production in mice receiving LNCs treated in the presence* (white bars) *or absence of MSCs* (black bars) *(expressed as mean optic density (OD) ± standard deviation [SD] of five to seven individual mice per group). A representative experiment of two independent experiments is depicted. (E) In vivo $PLP_{139-151}$-induced proliferation (expressed as mean counts per minute [CPM] ± SD) and cytokine production (expressed as mean pg/ml ± SD) of LNCs obtained from mice adoptively transferred with encephalitogenic LNCs cultured in the presence* (white bars) *or absence of MSCs (**p < *0.05, Mann–Whitney* U *test). CTRL = control; PLP = proteolipid protein.*

lower proliferative response to $PLP_{139-151}$, and produced less IFN-γ on antigen stimulation compared with mice receiving encephalitogenic control LNCs (see Fig 6E). These results suggest that MSCs can truly alter the capacity of encephalitogenic LNCs to transfer full-blown EAE to healthy recipient mice.

Discussion

Because of their capacity of modulating several effector functions of immune cells,[5] bone-marrow–derived MSCs have been postulated to be an appealing therapeutic approach for immune-mediated diseases. Such a concept has already translated into a clinical approach as MSCs have been used for the treatment of severe graft-versus-host disease refractory to conventional therapies.[14] In addition, MSCs could be regarded also as a promising tool for the treatment of autoimmunity. To this end, we recently demonstrated for the first time that murine MSCs improve the chronic progressive course of EAE induced in C57B/6J mice on immunization with MOG_{35-55}. This therapeutic effect was caused by the induction of peripheral T-cell tolerance.[15] In this study, we further dissect such therapeutic potential by addressing the capacity of IV-injected MSCs in SJL mice to treat relapsing-remitting EAE induced by $PLP_{139-151}$, a myelin antigen capable of inducing a potent T- and B-cell response. Similar to that observed in the chronic model of EAE induced by MOG_{35-55}, MSCs significantly improved EAE also in this relapsing-remitting model induced by $PLP_{139-151}$. Of interest, we observed a clear reduction of the relapse rate in mice treated with MSCs, suggesting that these cells are effective on relapsing-remitting disease, a clinical course frequently associated with human autoimmune diseases. We also evaluated the in vivo effect of MSCs on the B-cell response against myelin in mice with EAE. Indeed, MSCs have been shown to inhibit B-cell proliferation and differentiation to antibody secreting cells in vitro,[7] but no data are currently available on the effect of MSCs on an in vivo antigen-specific B-cell response. Here, we demonstrate for the first time that MSCs significantly inhibited B-cell infiltration of the CNS and, more important, decreased the in vivo production of pathogenic antibodies involved in the autoimmune response against myelin.

On IV administration in EAE–affected mice, MSCs labeled with eGFP rapidly reached the spleen and lymph nodes where they could be detected for a long time after immunization, supporting their capacity of induction of long-term tolerance. eGFP-positive cells were detected inside the inflamed brain at day 30 after immunization. At that time, eGFP-positive cells were detected inside the spinal cord of EAE–affected mice. However, we could not observe any noticeable colocalization between eGFP and NeuN or O4, markers of neurons and mature oligodendrocytes, respectively, suggesting that MSC transdifferentiation into neural cells does not occur in our experimental system. This is in contrast with Zhang and colleagues' study,[18] which observed that in the CNS of EAE–affected mice treated with MSCs, less than 5% of injected cells expressed NG2, a marker of oligodendrocyte progenitors, suggesting some degree of neural differentiation. Although we cannot exclude that eGFP-positive MSCs may eventually transdifferentiate at later stages of the disease, we consider such a biological event negligible for therapeutic effect. In fact, the clinical effect of MSCs is maximal in the early stages of disease and certainly before they massively enter the CNS, as demonstrated in this study and in Zappia and colleagues' article.[15] These results support the concept that, in EAE, the therapeutic effect of MSCs is not due to tissue repair sustained by regeneration of damaged neurons and oligodendrocytes, but instead to the modulation of the autoimmune attack to myelin. Confirming this hypothesis, in this article, we demonstrated that MSCs significantly affect the capacity of encephalitogenic $PLP_{139-151}$-specific LNCs to adoptively transfer diseases into naive recipients as a consequence of a decreased reactivity to $PLP_{139-151}$ of LNC-treated cells. Similar to that observed in the actively induced model of EAE, T and B cells from passively transferred mice resulted irresponsive to PLP. Although it is not known whether a single injection of MSCs may affect for a long time the pathogenic immune response, it is noticeable that such cell therapy is capable of consistently decreasing the number of relapses and the late motor disability in this animal model.

Taken together, these findings suggest that MSCs can effectively ameliorate relapsing-remitting EAE through the inhibition of pathogenic T- and B-cell responses directed against the immunizing antigen, although they do not provide clear evidence for the use of MSCs as a reparative strategy in CNS autoimmunity. As a consequence, MSCs may be beneficial for the treatment of human multiple sclerosis especially in the early stages of the disease, where the immune response against myelin plays a key role in the development of disease.[19] A phase I study is warranted to address the safety profile of human MSCs in a chronic, often severely disabling, but not life-threatening disease such as multiple sclerosis.

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