Interferon-γ–Dependent Inhibition of B Cell Activation by Bone Marrow–Derived Mesenchymal Stem Cells in a Murine Model of Systemic Lupus Erythematosus

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Objective. Bone marrow–derived mesenchymal stem cells (BM-MSCs) are multipotent cells characterized by immunomodulatory properties and are therefore considered a promising tool for the treatment of immune-mediated diseases. This study was undertaken to assess the influence of murine BM-MSCs on the activation of B cells in (NZB × NZW)F1 mice as an animal model of systemic lupus erythematosus (SLE).

Methods. We evaluated the in vitro effects of BM-MSCs on the proliferation and differentiation to plasma cells of splenic mature B cell subsets, namely follicular and marginal zone B cells isolated from (NZB × NZW)F1 mice. Lupus mice were also treated with BM-MSCs, and serum autoantibodies, proteinuria, histologic changes in the kidney, and survival rates were monitored.

Results. BM-MSCs inhibited antigen-dependent proliferation and differentiation to plasma cells of follicular and marginal zone B cells in vitro. This inhibitory effect was dependent on interferon-γ (IFNγ) and was mediated by cell-to-cell contact, involving the programmed death 1 (PD-1)/PD ligand pathway. In vivo treatment with BM-MSCs did not affect the levels of anti–double-stranded DNA antibodies or proteinuria. However, a reduction in glomerular immune complex deposition, lymphocytic infiltration, and glomerular proliferation was observed.

Conclusion. Our findings indicate that BM-MSCs affect B cell receptor–dependent activation of both follicular and marginal zone B cells from lupus mice. This inhibitory effect is IFNγ-dependent and cell contact–dependent. MSCs in vivo do not affect the production of autoantibodies, the level of proteinuria, or the mortality rates. Nonetheless, the significant improvement in histologic findings in the kidney supports the potential role of MSCs in the prevention of glomerular damage.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the presence of serum autoantibodies directed against nucleic acids. It is presumed that, analogous to other experimental models as well as other autoimmune diseases, a defect in T cell tolerance and/or activation might be at the origin of the disease (1,2). Nonetheless, several lines of evidence support a crucial role of B lymphocytes in the pathogenesis of SLE, both in mice and in humans. Autoantibodies directed to DNA, RNA, or other nuclear and cytoplasmic antigens have been detected in serum from humans with SLE as well as from strains of lupus-prone mice, and serum autoantibodies have been correlated with disease activity in humans with SLE, particularly in
those with lupus nephritis (3). Moreover, results from clinical studies suggest that B cell depletion with rituximab improves the clinical manifestations of SLE (4).

Bone marrow–derived mesenchymal stem cells (BM-MSCs) are a heterogeneous population of self-renewing and multipotent cells. BM-MSCs can be rapidly expanded in vitro and can differentiate into bone cells, fat cells, and cartilage cells (5). Moreover, they have been hypothesized to possess immunomodulatory properties because they affected both the phenotype and the function of a number of cells belonging to the innate or adaptive immune system (6). This led to the idea that these cells could be beneficial in the treatment of autoimmune diseases (7).

It is presumed that the effect of the interaction between MSCs and cells of the immune system vary depending on the microenvironment in which this interaction takes place (8,9). Indeed, we have previously shown that human BM-MSCs could support and enhance the in vitro polyclonal expansion of circulating human B cell subsets from both normal donors and SLE patients (10). However, it has been reported that BM-MSCs can also inhibit B cell proliferation and effector function both in vitro (11) and in vivo (12).

In the present study, we found that murine BM-MSCs did not affect polyclonal B cell activation mediated by Toll-like receptor 9 (TLR-9) agonist, while they inhibited the B cell receptor (BCR)–dependent proliferation and differentiation to plasma cells of splenic B cells derived from (NZB × NZW)F₁ mice. This suppressive effect was cell-dose dependent and was significantly enhanced by exogenous interferon-γ (IFNγ). The capacity of IFNγ to induce the suppressive activity of BM-MSCs was not dependent on the enzyme indoleamine 2,3-dioxygenase (IDO), but was dependent on cell-to-cell contact mediated by the interaction between programmed death 1 (PD-1) and PD ligand 1 (PDL-1). Injection of BM-MSCs into (NZB × NZW)F₁ mice did not affect the generation and maintenance of specific serum autoantibodies, but significantly ameliorated the kidney histopathology scores, indicating that administration of BM-MSCs might be beneficial in patients with SLE-associated glomerulonephritis.

**MATERIALS AND METHODS**

**Media and reagents.** For B cell culture, we used RPMI 1640 medium (BioWhittaker) supplemented with 10% fetal bovine serum (FBS; defined) (HyClone), 2 mM Glutamax (Gibco), 10 mg/liter of nonessential amino acids (Gibco), 1 mM pyruvate (Gibco), 50 units/ml of penicillin (Gibco), 50 units/ml of streptomycin (Gibco), and 5 × 10⁻²M 2-mercaptoethanol (Sigma).

**Generation of BM-MSCs.** Briefly, bone marrow was flushed out of the tibias and femurs of 6–8-week-old C57BL/6J mice. After 2 washings by centrifugation at 1,500 revolutions per minute (352g) for 5 minutes in phosphate buffered saline (PBS), cells were plated in 75-cm² tissue culture flasks at a concentration of 0.3–0.4 × 10⁶ cells/cm² using murine MesenCult as medium (Stem Cell Technologies). Cells were maintained at 37°C in a humidified incubator with an atmosphere of 5% CO₂, and the medium was refreshed every 3–4 days for about 4–5 weeks. After this time, adherent cells were collected following a 10-minute incubation at 37°C with 0.05% trypsin/Annexin V (Sigma–Aldrich). The identity of MSCs was confirmed according to their immunophenotype, based on the expression of positive markers CD9 and Sca-1 antigen and negative CD45 (all from BD Pharmingen). Cells between passages 20 and 25 were used in the experiments.

**Isolation of follicular and marginal zone B cells.** To avoid inadvertent activation of B cells, B cells were isolated by negative selection (CD43 depletion) with the use of CD43 microbeads (Miltenyi Biotec) according to the manufacturer’s instructions. Following staining for B220 (BD Biosciences), CD24, CD21, CD23 (eBioscience), follicular, and marginal zone B cells were sorted with a FACSAria (Becton Dickinson). The purity of the sorted cells was >95%.

**Proliferation assay.** B cell subsets were labeled with 0.5 μM 5,6-carboxyfluorescein diacetate N-succinimidyl ester (Molecular Probes) for 8 minutes at room temperature. Purified B cells subsets were cultured with mouse BM-MSCs irradiated at 10,000 rads at different ratios (1:1, 3:1, and 9:1 ratio of B cells to BM-MSCs) in a 96-well flat-bottomed plate with 2.5 μg/ml of CpG-containing oligodeoxynucleotide (CpG ODN) 1826 (5’TCC-ATG-ACG-TTC-CTG-ACG-CTT-3’TIB Molbiol), 25 ng/ml of soluble CD40L (R&D Systems), 2.5 μg/ml of F(ab’)² anti–mouse IgM (Jackson ImmunoResearch), and 1,000 units/ml of interleukin-2 (IL-2) (Proleukin; Prometheus). The proliferation profile of propidium iodide–negative viable B220-positive cells was analyzed on day 3 of culture. IFNγ was added at a concentration of 5 ng/ml, and the IDO inhibitor 1-methyl-1H-tryptophan was added at a concentration of 100 μM. MSCs were incubated with anti–PID-1 and anti–PDL-1 (eBioscience) at 2.5 μg/ml as well as with 2 irrelevant isotype antibodies, anti–CD4 (BioLegend) and anti–CD3 (R&D Systems), which served as controls.

**Evaluation of apoptosis.** Apoptotic cells were detected at 20 hours by intracellular staining for annexin V and 7-aminoactinomycin D using an Annexin V–PE Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer’s instructions.

**Differentiation analysis by enzyme-linked immunoassay (ELISpot) assay and enzyme-linked immunosorbent assay (ELISA).** Plates (96-well flat-bottomed; Greiner) were coated with isotype-specific goat anti-mouse IgG or IgM antibodies (SouthernBiotech). Plates were washed and blocked with PBS–10% FBS for 2 hours at room temperature. After washing, serial dilutions of culture supernatants were added, and incubation was continued for 2 hours at room temperature. Plates were washed again, alkaline phosphatase–conjugated goat anti-mouse IgG or IgM was added, and plates were incubated for 2 hours at room temperature. The reaction
was developed with Sigma 104 substrate. Plasma cells secreting IgG or IgM were detected using an ELISpot assay. Briefly, 96-well plates (Millipore catalog no. MAIPS4510) were coated with 10 μg/ml of purified goat anti-mouse IgG or IgM (SouthernBiotech). After washing and blocking with PBS–1% bovine serum albumin (BSA) for 30 minutes, serial dilutions of cultured B cells were added, and plates were incubated overnight at 37°C. Plates were then washed and incubated with isotype-specific secondary antibodies, followed by streptavidin–horseradish peroxidase (Sigma). The assay was developed with aminoethylcarbazole (Sigma) as a chromogenic substrate.

Transwell experiments. Transwell chambers with 0.2-μM pore membranes (Nunc) were used to physically separate the stimulated B cells from the MSCs. Follicular and marginal zone B cells (3 × 10^5 cells/well) were cocultured in the lower Transwell chamber, and the C57BL/6J MSCs were placed in the upper chamber at the same ratios as described above (1:1, 3:1, and 9:1 ratio of B cells to BM-MSCs).

Protein kinase signaling pathways. The analysis of phosphoproteins was performed after 16 hours and 24 hours of culture for marginal zone and follicular B cells, respectively. Cells were stained for surface marker B220 and then permeabilized and fixed using a Cytotox/PermePert kit (BD Biosciences) according to the manufacturer’s instructions. Intracellular staining was performed with primary antibodies against phospho-ERK, phospho-p38, or phospho-Akt (Cell Signaling Technology), as well as a secondary biotinylated IgG antibody (Jackson ImmunoResearch). Streptavidin–phycoerythrin (PE) (BD PharMingen) was used for signal detection.

In vivo treatment of mice. Female (NZB × NZW)F1 mice were purchased from Harlan Italy. Mice were used for isolation of lymphoid organs between the ages of 9 weeks and 33 weeks. Mice were treated intravenously with 1.25 × 10^6 BM-MSCs at weeks 27, 28, and 29. Mice were bred and maintained at the animal facility of the Advanced Biotechnology Center in Genoa. The care and use of the animals were in compliance with the laws of the Italian Ministry of Health and with the guidelines of the European Community.

Detection of antinuclear antibodies (ANAs) and anti-double-stranded DNA (anti-dsDNA) antibodies by immunofluorescence and ELISA. Serum levels of circulating ANAs were detected using permeabilized HEp-2 cells (Biomed Instruments), and anti-dsDNA antibodies were determined by immunofluorescence using Crithidia luciliae (Biomed Instruments) and by ELISA. IgG ANAs and anti-dsDNA were detected with fluorescein isothiocyanate–conjugated goat anti-mouse IgG (SouthernBiotech) and were scored by an observer (FS) who was blinded to the experimental group. For anti-dsDNA ELISAs, polystyrene plates were coated with poly-L-lysine and calf thymus DNA (both from Sigma). Plates were postcoated for 45 minutes with 50 μg/ml of polyglutamic acid, blocked for 45 minutes with PBS–3% BSA, and then serial
EFFECT OF BM-MSCs ON B CELL ACTIVATION IN A MURINE MODEL OF SLE

Figure 2. A, Proliferation of follicular (FO) and marginal zone (MZ) B cells incubated either alone or in combination with bone marrow–derived mesenchymal stem cells (BM-MSCs) at different ratios. Cultures were stimulated with interleukin-2, CpG-containing oligodeoxynucleotide 1826, anti-Ig, and CD40L, either alone or in the presence of interferon-γ (IFNγ), and proliferation was measured on day 3 of culture as the absolute number of B220-positive, propidium iodide–negative cells which have diluted 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (numbers at the top of the histograms). Shown is a representative experiment of 11 experiments performed. B, Percentage of B220-positive apoptotic cells (annexin V+/7-aminoactinomycin D– [7-AAD–] and annexin V+/7-AAD+) in follicular and marginal zone B cells cultured as described in A, in the presence or absence of IFNγ. Values are the mean and SEM of 3 experiments. C, IgM and IgG secretion in culture supernatants (top), as determined by enzyme-linked immunosorbent assay (top), and frequency of immunoglobulin-secreting cells (ISCs) (bottom), as determined by enzyme-linked immunosorbent assay (bottom). Cells were cultured as described in A, in the presence (solid bars) or absence (open bars) of IFNγ, and assays were performed on day 4 of culture. Values in B and C are the mean and SEM of 6 experiments. Significant comparisons were as follows: for IgM (μg/ml) in follicular cells, **P = 0.0013 and *P = 0.0177; for IgG (μg/ml) in follicular cells, * at a 1:1 ratio = P = 0.0165 and * at a 3:1 ratio = P = 0.0190; for IgM (μg/ml) in marginal zone cells, **P = 0.0082 and *P = 0.0349; for IgG (μg/ml) in marginal zone cells, ***P = 0.0005 and *P = 0.0192; for IgM (ISC/well) in follicular cells, **P = 0.0019 and *P = 0.0377; for IgG (ISC/well) in follicular cells, ***P < 0.0001 and **P = 0.0023; and for IgM (ISC/well) in marginal zone cells, ***P = 0.0005 and *P = 0.0038; and for IgG (ISC/well) in marginal zone cells, *** at a 1:1 ratio = P < 0.0001, *** at a 3:1 ratio = P = 0.0007, and *P = 0.0138.

dilutions of serum (from 1:100 to 1:3,200) were incubated overnight. Specific antibodies were detected with alkaline phosphatase–conjugated goat anti-mouse IgG (SouthernBiotech).

Histopathologic assessment of kidneys. Mice were killed, and their kidneys were isolated, preserved in 10% formalin, and embedded in paraffin. Histopathologic assessments were performed on 4-μm formalin-fixed, paraffin-embedded sections that had been stained with periodic acid–Schiff. A total of 50 sequential glomeruli from the superior, middle, and inferior cortices of each kidney were scored for the presence of proliferating glomeruli and were then expressed as the percentage of proliferating glomeruli of the total number of glomeruli analyzed. Lymphocytic infiltration of 50 sequential glomeruli from each section was evaluated and scored using a scale of 0–3+, where 0 = normal, 1 = mild, 2 = moderate, and 3 = severe abnormalities.

Kidney cryosections (4 μm) were fixed in ice-cold acetone, washed with PBS, blocked with normal goat serum, and then incubated with fluorescein-conjugated goat IgG, goat anti-mouse IgG, and goat anti-mouse IgM (SouthernBiotech). The relative fluorescence intensity for IgM and for IgG was scored separately using a scale of 0–3+, where 0 = no apparent staining as compared with the isotype control, 1 = detectable staining, 2 = moderate staining intensity, and 3 = maximum staining intensity. Two experienced evaluators (AG and AM) who were blinded with regard to the experimental group evaluated the samples.

Assessment of proteinuria. Proteinuria was evaluated colorimetrically using Albustix (Bayer) at weeks 25, 26, 27, 28, 29, and 32.

Statistical analysis. Proteinuria data were analyzed by Fisher’s exact test. All the other parameters were analyzed by Mann-Whitney U test and Student’s t-test. Statistical significance was defined as P < 0.05. The time to the occurrence of death was assessed by Kaplan-Meier cumulative survival functions.
Results

Requirement of IFN-γ priming for BM-MSC–mediated inhibition of B cell proliferation and differentiation to plasma cells. The pool of mature murine splenic B cells is mainly composed of 2 anatomically and functionally distinct subsets: follicular and marginal zone B cells. These subsets can be identified by the differential expression of surface markers (13). We isolated B cells from the spleens of (NZB × NZW)F1 mice by negative enrichment with the surface marker CD43 to avoid the inadvertent activation of B cells. CD43–negative cells were then sorted with antibodies specific for CD21, CD24, and CD23 to purify the follicular (CD23+CD24–CD21+) and marginal zone (CD23–CD24+CD21+) B cells (14).

We have previously shown that human BM-MSCs promote the proliferation and differentiation into immunoglobulin-secreting cells (ISCs) of human transitional, naive, and memory B cells stimulated with CpG ODN 1826 (used as an agonist of TLR-9) in the absence of BCR triggering (10). Thus, in the present study, we stimulated follicular and marginal zone B cells with CpG ODN 1826 and IL-2, either alone or in combination with BM-MSCs. BM-MSCs did not inhibit the proliferative response of either follicular or marginal zone B cells to TLR-9 stimulation, even at a 1:1 ratio (Figure 1A). Thus, the CpG responsiveness of murine splenic B cells to CpG ODN 1826 was not influenced by BM-MSCs.

Next, we sought to determine whether BM-MSCs could negatively influence splenic B cell responsiveness. Since IFN-γ has been previously shown to be crucial for the immunomodulatory activity of BM-MSCs (8,9), we added IFN-γ to cocultures of B cells and MSCs. The addition of IFN-γ to the cocultures did not induce inhibition of the proliferation of either follicular or marginal zone B cells (Figure 1A). In addition, the differentiation to plasma cells, which was measured as immunoglobulin secretion and as the ISC frequency, was
not affected by BM-MSCs, whether they had been left untreated or had been treated with IFNγ (Figure 1B).

The production of autoantibodies in systemic autoimmune diseases is often related to the synergistic engagement of the BCR and TLR-9 in response to DNA-containing antigens (15). Thus, we stimulated follicular and marginal zone B cells with CpG, anti-Ig, CD40L, and IL-2 in the presence of BM-MSCs. BM-MSCs inhibited follicular and marginal zone B cell proliferation at a 1:1 ratio, while this effect was lost at a 9:1 ratio of B cells to MSCs. Proliferation of both follicular and marginal zone B cells was strongly impaired in the presence of IFNγ, and this effect was observed at all ratios of B cells to MSCs that were tested (Figure 2A). Remarkably, MSCs did not induce apoptosis in either follicular or marginal zone B lymphocytes (Figure 2B). Furthermore, BM-MSCs did not significantly inhibit IgM or IgG secretion by follicular or marginal zone cells and did not influence the frequency of ISCs of either isotype. The addition of IFNγ induced a significant inhibition of plasma cell differentiation at a 1:1 and a 3:1 ratio of B cells to MSCs (Figure 2C). Moreover, preincubation of BM-MSCs with IFNγ displayed comparable inhibition of follicular and marginal zone B cell proliferation, thus suggesting a direct involvement of IFNγ in the modulation of BM-MSC signals (data not shown).

**IDO-independent, but cell-to-cell contact-dependent, inhibition of B cell proliferation and differentiation to plasma cells mediated by BM-MSCs.** BM-MSC-mediated inhibition of T cell proliferation and differentiation to cytokine-secreting cells has been reported to be dependent on, among other factors, the
expression of IDO, which catalyzes the conversion of tryptophan to kynurenine. IDO expression in BM-MSCs has been shown to be regulated by IFN-H9253 (8). To determine whether IDO could also be involved in the inhibition of B cell proliferation and differentiation, we tested in our assay system a competitive inhibitor of the IDO pathway, 1-methyl-DL-tryptophan (16). The proliferation and differentiation to plasma cells of both follicular and marginal zone B cells were not restored in the presence of IDO inhibitor (Figures 3A and B).

We next evaluated the effect of BM-MSCs on 3 of the major signaling axes involved in BCR-mediated activation of follicular and marginal zone B lymphocytes: the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin pathway, the MEK-1/ERK-1/2 pathway, and the p38 MAPK pathway. We analyzed specific intracellular phosphoproteins by flow cytometry in stimulated follicular and marginal zone B cells. As shown in Figure 5A, BCR stimulation of follicular and marginal zone B cells led to an increased level of p38, ERK, and Akt phosphorylation at 24 and 16 hours of stimulation. Coculture with MSCs resulted in inhibition of p38, ERK, and Akt phosphorylation in follicular and marginal zone B cells. These findings suggest that the inhibition of...
BCR signaling by BM-MSCs affected all 3 signaling pathways, perhaps acting upstream of BCR signaling, splitting into the 3 axes.

**Requirement of PD-1/PDL-1 interaction for BM-MSC–mediated inhibition of B cell activation.** One of the molecular mechanisms suggested to be crucially involved in the inhibition of T cell and B cell activation by BM-MSCs is the interaction of the inhibitory molecule PD-1 with its ligand PDL-1 (17). PD-1 was expressed on resting follicular and marginal zone B cells at lower levels than PDL-1 (Figure 5C), whereas murine BM-MSCs expressed PD-1 and PDL-1 at similar levels, as previously reported (18) (Figure 5C). We sought to determine whether IFNγ treatment could modulate the expression of PD-1 and PDL-1 on BM-MSCs and on follicular and marginal zone B lymphocytes. BM-MSCs displayed a significant increase in PDL-1 expression after IFNγ treatment (Figure 5C), whereas no effect on PD-1 expression was observed (data not shown). Analogously, IFNγ affected PDL-1 expression on both follicular and marginal zone B lymphocytes while PD-1 expression was unaltered.

In order to verify whether the inhibitory molecular pathway induced by PD-1/PDL-1 interactions could be involved in the inhibition of BCR stimulation of murine B lymphocytes by BM-MSCs, we used specific monoclonal antibodies against PD-1 and PDL-1 to block PD-1/PDL-1 interactions. Treatment with monoclonal antibodies against anti–PD-1 and anti–PDL-1 completely restored p38, ERK, and Akt phosphorylation in both follicular and marginal zone B cells upon stimulation with anti-Ig and TLR-9 agonist and partially rescued B cell proliferation at a 3:1 ratio of B cells to MSCs (Figures 5B and D).

These results suggest that PD-1/PDL-1 interactions between MSCs and B lymphocytes inhibit the signal transduction pathways triggered by productive B cell stimulation by antigen and affect B cell expansion.
Lack of effect of in vivo cell therapy with BM-MSCs on dsDNA autoantibodies and proteinuria, but significant improvement in histopathologic changes in the kidney. We next tested whether BM-MSCs might have beneficial in vivo effects on B cell activation and SLE pathogenesis in (NZB × NZW)F₁ mice. Beginning at age 27 weeks, when IgG anti-dsDNA were detectable in the serum of (NZB × NZW)F₁ mice, we initiated a therapeutic protocol consisting of an intravenous infusion of 1.25 × 10⁶ BM-MSCs, which was administered weekly for 3 weeks. The following parameters were evaluated: serum levels of IgG autoantibodies, degree of proteinuria, histopathologic changes in the kidney, and percentages of mice surviving over time.

Infusion of BM-MSCs did not significantly affect serum concentrations of IgG anti-dsDNA or IgG ANAs, as measured by immunofluorescence (Figure 6B). Moreover, serum levels of IgG₁, IgG₂a, IgG₂b, and IgG₃ anti-dsDNA were unaffected by treatment with BM-MSCs (Figure 6B). We also measured the frequencies of total and anti-dsDNA–specific ISCs and found that they were not modified by treatment with BM-MSCs (data not shown). In contrast, despite the lack of a significant impact of BM-MSC infusions on proteinuria and percentage of survival in these mice (Figures 6C and D), histopathologic analysis of the kidney revealed a significant improvement in glomerular proliferation, lymphocytic infiltration, and IgG immune complex deposition in mice treated with BM-MSCs (Figure 6A). These results indicate that BM-MSCs could have a beneficial effect on kidney function in experimental SLE, independently of their lack of effect on serologic anti-dsDNA and ANA immunoglobulins.

DISCUSSION

SLE is a systemic autoimmune disease characterized by the continuous generation of autoantibody-producing cells (i.e., autoreactive plasma cells) through mechanisms that are not yet fully understood. Nevertheless, it is well established that both B lymphocytes and T lymphocytes are critical in the pathogenesis of the disease through autoantibody-dependent and -independent mechanisms (19). Steroids and immunosuppressants are currently used to treat many patients with SLE (20). The efficacy of these agents lies in their ability to suppress inflammation and to block or partially reduce abnormal T cell and B cell activation (21). Therefore, targeting both the B lymphocytes and the T lymphocytes as well as their interaction could represent an alternative approach to the currently available pharmacologic methods.

MSCs can interact with cells of both the innate and adaptive immune system, leading to the modulation of several effector functions. However, the mechanisms involved in their function are still a matter of debate and may be diverse or only partially overlapping (8). With regard to the effect of MSCs on human B cell activation, previous studies have yielded contradictory data (11,12,22,23), despite some evidence of an in vivo effect on antibody production (13,24). Thus, in order to verify whether BM-derived MSCs could affect systemic autoimmune responses, we investigated their impact on the proliferation and differentiation of B cells isolated from (NZB × NZW)F₁ mice.

We found that BM-MSCs did not influence B cell activation of either follicular or marginal zone B cells upon TLR-9 stimulation. In contrast, they inhibited the proliferation of both follicular and marginal zone B cells after stimulation with both BCR and TLR-9 agonists if MSCs were used at high doses, and this inhibitory effect was increased when exogenous IFNγ was added to the cultures. This observation is consistent with previous studies by Krampera et al (8) and Spaggiari et al (25), in which they showed that CD4, CD8, and natural killer cells are inhibited by MSCs through an IFNγ-dependent mechanism.

Experiments were performed in an attempt to clarify the mechanisms involved in the suppressive activity of MSCs. First, we established that IFNγ-dependent inhibition of B cell proliferation and differentiation by MSCs was dependent on cell-to-cell contact but not on soluble factors. Second, we found that MSCs were able to inhibit the activation of both follicular and marginal zone B cells when BCR was engaged, but not when TLR-9 alone was triggered. These 2 observations led us to hypothesize that inhibitory surface molecules could trigger the IFNγ-dependent suppressive activity of BM-MSCs. In this context, a good candidate was the costimulatory molecule PD-1 and its ligand PDL-1, which control an inhibitory pathway of T cell activation (18). It is noteworthy that in a previous study, PD-1 was shown to inhibit BCR signaling in B lymphocytes by recruiting SH2 domain–containing phosphatase 2 to its phosphotyrosine and by dephosphorylating key signal transducers of BCR signaling (26). Furthermore, Augello et al (17) reported a possible involvement of the PD-1/PDL-1 pathway in the suppressive effect of BM-MSCs on T lymphocytes.

Two lines of evidence from the present study support our hypothesis. First, IFNγ treatment increased the expression of PDL-1 on BM-MSCs and follicular and marginal zone B lymphocytes. Second, blocking
antibodies specific for PD-1 and PDL-1 completely restored p38, ERK, and Akt phosphorylation and partially restored the proliferation of both follicular and marginal zone B cells. Thus, our data suggest that IFN-γ is a crucial soluble factor in the induction of the suppressive activity of MSCs on mouse B lymphocytes, and the interaction of PD-1 with PDL-1 may be one of the pathways involved in their immunomodulatory activity. This conclusion is also confirmed by the findings reported by Ren et al (27) in which MSCs generated from IFN-γ-deficient or IFN-γ receptor–deficient mice did not display immunosuppressive activity. Furthermore, IFN-γ alone or in combination with tumor necrosis factor α (TNFα) and IL-1β stimulated the production of T cell–attracting chemokines as well as inducible nitric oxide synthase, which inhibits T cell activation through the production of nitric oxide by mouse MSCs (27). These observations support the notion of the importance of the microenvironment in driving the final outcome of the immunomodulatory activity of MSCs on cells of the innate or adaptive immune system.

In order to elucidate these microenvironment-driven effects, we also studied the in vivo impact of MSCs on the activation of B lymphocytes in the (NZB × NZW)F1 mouse model of SLE. These mice display hyperresponsiveness of the T cell effector/memory compartment, with a significant increase in the frequency of IFN-γ- and TNFα-secreting cells in healthy laboratory strains (Schena F, et al: unpublished observations). In this proinflammatory microenvironment, in vitro inhibition of B cell activation by MSCs does not translate to reduced in vivo production of autoantibodies. This lack of effect can be related to 2 different mechanisms. First, it has been proposed that in murine lupus, the production of anti-dsDNA autoantibodies is controlled by IFN-γ and TNFα signaling (28). This is consistent with our finding of a lack of effect of MSCs on in vitro stimulation of both follicular and marginal zone B lymphocytes with TLR-9. The second consideration concerns the cellular mechanisms that sustain autoantibodies over time. Hoyer et al (29) demonstrated that in (NZB × NZW)F1 mice, the number of splenic antibody-secreting cells increases during ages 1–5 months and becomes stable thereafter. Thus, serum autoantibodies in our animal model of lupus could be maintained by a pool of long-lived plasma cells generated in young mice and not affected by the presence of MSCs.

Treatment of (NZB × NZW)F1 mice with allogeneic BM-MSCs did not have an effect on proteinuria. Two recent studies showed significant amelioration of immunopathology and a decrease in serum autoantibody levels in MRL/pr mouse after treatment with human MSCs (30,31). This discrepancy with our results could be related to the xenogeneic source of the MSCs used for the treatments, as well as to the different experimental models of SLE that were used. Unlike (NZB × NZW)F1 mice, MRL/lpr mice are homozygous for the Fas mutation and show systemic autoimmunity related to a massive lymphadenopathy due to uncontrolled T cell proliferation and associated with autoantibody production, glomerulonephritis, and arthritis. Fas is a member of the TNF receptor superfamily. It is a crucial mediator of apoptotic cell death and is involved in maintaining T lymphocyte homeostasis. It is reasonable that in our model, the effects of MSCs were mainly related to inhibition of the exaggerated proliferation of T cells, which in turn, provide help for polyclonal B cell activation and autoantibody production.

Despite the lack of effect on the production of anti-dsDNA autoantibodies and proteinuria, treatment with MSCs had a dramatic effect on 3 different histopathologic parameters of the kidney: glomerular proliferation, lymphocytic infiltration, and immune complex deposition. These effects could be related to a direct interaction of MSCs with renal cells. Consistent with these observations, a direct effect of MSCs on the kidney has been documented in a mouse model of acute kidney injury, in which MSCs exerted their beneficial effects on tubular cell repair by producing the mitogenic and prosurvival factor insulin-like growth factor 1 (32). In a rat model of acute renal failure, MSCs have also been shown to exert a protective effect by a paracrine mechanism, tuning down proinflammatory cytokines such as IL-1β, TNFα, and IFN-γ (33).

In conclusion, we have shown that BM-MSCs inhibited antigen-dependent proliferation and differentiation to plasma cells of both follicular B cells and marginal zone B cells in vitro. This inhibitory effect was dependent on IFN-γ and was mediated by cell-to-cell contact, which involved interactions between PD-1 and PDL-1. Although BM-MSCs inhibited B cell antigen–dependent proliferation and differentiation in vitro, the in vivo treatment with BM-MSCs did not affect levels of anti-dsDNA antibody or proteinuria. However, we observed a reduction in glomerular IgG immune complex deposition, lymphocyte infiltration, and in particular, glomerular proliferation, suggesting a potential role of mesenchymal stem cells in preventing glomerular damage, an issue that needs further investigation.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Traggiai had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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