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The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes

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ABSTRACT

Background
Mesenchymal stem cells are multilineage non-hematopoietic progenitor cells that play a key role in supporting the lymphohematopoietic system. Their distribution in bone marrow and secondary lymphoid organs allows an intimate interaction with T- and B-lymphocytes. While their effect on T-lymphocytes has been extensively analyzed, data on the effect of mesenchymal stem cells on B cells are more limited. We analyzed the effects of mesenchymal stem cells on B-lymphocytes and the pathways involved in these effects.

Design and Methods
The effect of MSC on the proliferation and viability of B cells was evaluated using MTT assays, annexin/7-amino-actinomycin D and propidium iodide staining. The B-cell maturation pattern was established using flow cytometry based on the expression of different markers related to the differentiation of B cells, such as CD38, CD138, CD19 and CCR7, and to the expression of surface and intracellular immunoglobulins. Finally, western blot assays were used to identify the pathways involved in the effects of mesenchymal stem cells on B-lymphocytes.

Results
Mesenchymal stem cells increased viability and blocked the cell cycle of B-lymphocytes in the G_0/G_1 phase. *In vitro* exposure of B cells to plasmacytoid dendritic cells induced B-cell differentiation as shown by an increased number of $CD38^{++}/CD138^{++}$ cells, which also displayed higher levels of cytoplasmic immunoglobulin and lower levels of CD19, CCR7 and surface immunoglobulin. Interestingly, this maturation pattern was inhibited by adding mesenchymal stem cells to the culture. Finally, mesenchymal stem cells modified the phosphorylation pattern of the extracellular response kinase 1/2 and p38 pathways which are both involved in B-cell viability, proliferation and activation.

Conclusions
Mesenchymal stem cells increase B-cell viability while inhibiting proliferation, arresting B-lymphocytes in the G_0/G_1 phase of the cell cycle. The presence of mesenchymal stem cells blocked B-cell differentiation as assessed by flow cytometry. Finally, mesenchymal stem cells modified the activation pattern of the extracellular response kinase and the p38 mitogen-activated protein kinase pathways in B-lymphocytes.

Key words: mesenchymal stem cells, B lymphocytes, cell survival, B-cell differentiation.

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Introduction

Mesenchymal stem cells (MSC) are multipotent non-hematopoietic progenitor cells capable of differentiating into various lineages including osteoblasts, chondrocytes and adipocytes.[1,2] In addition, MSC provide cytokines and growth factors supporting the development and differentiation of the lymphohematopoietic system. MSC can, however, also display an important immunoregulatory activity. With regards to this, previous studies have clearly demonstrated that MSC inhibit T-cell responses induced by mitogens and alloantigens.[3] This effect has been related to inhibition of cyclin D2 and upregulation of $p27^{kip1}$ induced by MSC, which arrest T-lymphocytes in the G_1 phase.[4] MSC also cause T helper 1 cells (T_H1) to decrease their production of interferon γ.[5] Furthermore, MSC modulate the immune response through the induction of regulatory antigen-presenting cells with T-cell suppressing properties.[6] Accordingly, MSC reduce the expression of co-stimulatory molecules and down-regulate interleukin-12 secretion among mature antigen-presenting cells.[7] These properties have supported the use of MSC in the treatment of graft-versus-host disease in the setting of allogeneic transplantation.[8,9]

Contrary to their well-known effect on T cells and antigen-presenting cells, the effect of MSC on B cells remains unclear. MSC have been implicated in B-cell development, in bone marrow, spleen and lymphoid follicles,[10] exerting a negative control on B-cell lymphopoiesis through the expression of activin A, a pleiotropic molecule which is a member of the transforming growth factor-β superfamily. In addition, Corcione *et al.* reported that B-cell proliferation is inhibited by MSC,[11] but this has not been confirmed by other authors.[12] With regards to B-cell differentiation, MSC have been reported to induce both stimulation and impairment of immunoglobulin production by B-lymphocytes.[11,13] The distribution of MSC in bone marrow and secondary lymphoid organs allows an intimate interaction between both cell subsets, which contributes to normal lymph node development[14] as well as to the support of tumor B cells[15] in follicular lymphomas. Finally, the interaction between B cells and MSC cannot be considered as a simple bi-directional cross-talk; other cell subsets, such as dendritic cells are involved. With regard to this, plasmacytoid dendritic cells (pDC) have been reported to induce plasma cell differentiation of B-lymphocytes[16] and, in secondary lymphoid organs, all these different cell subsets interact in order to generate an appropriate immune response.

In this study we analyzed the effect of MSC and pDC on B-lymphocytes and the pathways involved in these effects. We found that, while pDC induced B-lymphocyte differentiation, MSC blocked proliferation and differentiation but also increased B-cell viability. These effects were mediated, at least in part, through the extracellular response kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways. The current study may allow us to establish a model defining the normal interactions between MSC, B-lymphocytes and dendritic cells; such a model could serve to elucidate the potential role of these interactions in the pathogenesis of diseases such as lymphoproliferative disorders and immune-mediated disorders.[15,17,18]

Design and Methods

Cell isolation

MSC were isolated and expanded, as previously reported,[18,19] from healthy donors after informed consent had been obtained. The informed consent form was approved by the Local Ethical Committee. Low density mononuclear cells from bone marrow were separated with a Ficoll-Paque (Seromed® Biochrom KG) gradient and plated for adherence on a plastic surface (3-5 days) in culture medium with DMEM (Gibco), L-glutamine (4 mM), D-glucose (5.56 mM), penicillin (100 U/mL) and streptomycin (10 mg/mL) plus 10% fetal bovine serum (Gibco). Cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2. Twice a week, adherent cells were fed by complete replacement of the medium. When the layer was confluent, cells were detached using trypsin and then subcultured at a concentration of 10,000 cells/cm^2 until confluence. This process was repeated at least three times in order to obtain a sufficient number of cells for analysis. Adipogenic, osteogenic and chondrogenic differentiation was induced as previously described[19] and as recommended by the ISCT consensus.[20] The cells were phenotyped using the following conjugated monoclonal antibody combinations: fluorescein isothiocyanate (FITC)/phycoerythrin (PE)/peridin chlorophyll protein-cyanine-5 (PerCP-Cy5)/allo-phycocyanin (APC): CD90/CD73/CD45/CD34; CD105/CD166/ HLA-DR/CD34; CD14/CD106/CD19/CD45. Data were acquired using a FACScalibur flow cytometer (Becton Dickinson Biosciences – BDB, San Josè, CA, USA) and analyzed with the Paint-A-Gate program (BDB).

B- and T-lymphocytes were obtained from the buffy coats of healthy donors using magnetic activated cell sorting (MACS) CD19- and CD3-conjugated microbeads, following the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA). The cells' purity was analyzed using the following monoclonal antibody combination: CD45-FITC/CD20-PE/CD3- PerC-Cy5/ CD14-APC. Positively selected cells contained > 95% B or T cells, as assessed by flow cytometry.

pDC were obtained from the buffy coats of healthy donors also by MACS using the depletion program, according to the manufacturer's instructions (Miltenyi Biotech). The cells were purified using the following monoclonal antibody combinations: FITC/PE/PerC-Cy5/APC: CD3-CD19-CD56-CD14/CD123/HLA-DR/ BDCA2, CD3-CD19-CD56-CD14/CD123/CD11c/ BDCA2, CD3-CD19-CD56-CD14/CD123/BDCA4/ HLA-DR. Anti-CD3-CD19-CD56-CD14 (obtained from Cytognos, Salamanca, Spain). Selected cells contained >85% pDC, as assessed by flow cytometry.

Cell viability assays

Two hundred thousand MSC were seeded in the upper chamber of a 0.4 μm pore transwell insert (Corning) and 10^6 positively selected B-lymphocytes were seeded in the

lower chamber of the transwell on a 24-well culture plate. Cells were cultured for 4 to 7 days in 1 mL of RPMI 1640 medium supplemented with 10% human AB serum (Sigma) plus plate-bound-anti-Ig 10 mg/mL (Jackson Immune Reseach Laboratories, Dianova, West Grove, PA, USA) in combination with 10 μg/mL CpG-C ODN 2395 (Coley Pharmaceutical Group, Massachusetts, MA, USA). In some experiments, 2 μg/mL anti-CD40L (rCD40L, Immunotech, Marseille, France) and 100 U/μL interleukin-4 (R&D Systems, Minneapolis, MN, USA) were also added to the culture medium.

The effect of MSC on B-cell growth (viability and/or proliferation) was initially assessed by measuring the 3-(4.5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) dye absorbance of the cells. For this purpose, 10^5 B cells/100 μL were plated in triplicate into 96-well tissue culture dishes in culture medium with or without 10^4 MSC. On the day of the assay, MTT (0.5 mg/mL) was added to the medium in each well, and the plates were returned to the incubator for 3 hours. Then, 10 μL of SDS 0.1 N HCl were added to the culture for each condition. The plate was shaked in the dark for 2 hours to dissolve the MTT formazan crystals. The absorbance of the samples was then recorded at 570 nm with a 630 nm reference filter. The MTT absorbance was assessed on the fourth day of culture. Three wells were analyzed for each condition, and the results are presented as the mean ± SD of triplicates of a representative experiment that was repeated at least three times.

For the detection of apoptosis, the annexinV-PE/7 amino-actinomycin (7-AAD) apoptosis detection kit from BDB was used as previously described.[20] Briefly, at least 10^6 lymphocytes were washed and resuspended in binding buffer (1:10 diluted in phosphate-buffered saline) maintaining a cell concentration of 1×10^6/mL. T-lymphocytes were added to keep a proportion of 3:1 between B- and T-lymphocytes. The annexin V-PE (5 μL) and 7-AAD (5 μL) were added for 15 min. In order to identify the B- and T-lymphocytes, anti-CD20-FITC and anti-CD3-APC were also added. For every condition, 50,000 events were collected and analyzed. The samples were acquired using Trucount™ tubes (BD), which contain a calibrated number of fluorescent microbeads. The absolute count of annexin V-PE plus 7-AAD negative cells was calculated using the following equation: (the number of events in the region containing annexin V-PE plus 7-AAD negative cells/number of events in the absolute count in the bead region) x (number of beads per test / test volume). Paint-A-Gate Pro software (BD) was used for analyses.

Proliferation assays

B-lymphocyte cell cycle and DNA content studies were also performed. For this purpose 5×10^5 lymphocytes were cultured using either the culture medium plus anti-Ig in combination with CpG-C, as previously described, or with medium plus phorbol ester (12-o-tetradodecanoyl-phorbol-13-acetate) (TPA; Sigma, St Louis, MO, USA) for 4 days and were stained with FITC-conjugated monoclonal antibodies CD2, CD3, CD5 and CD7 for 15 min. Afterwards, 500 μL of solution B containing 0.5 g/L of RNAse (Sigma) were added for 10 min in the dark. Finally, 500 μL of solution C containing 0.42 g/L of propidium iodide (Sigma) were added to each tube and cells were incubated in the dark for 15 min. After this period the cells' DNA content was measured using a FACScan flow cytometer (BDB). At least 20,000 events were acquired. The distribution of cells in the phases of the cell cycle was analyzed using the model included in the ModFit LT™ (Verity Software House, Inc, Maine, MI, USA) software program, after excluding cell debris and cell doublets in a FSC/FL2 area and a FL2 width/FL2 area dot plot, respectively.

Differentiation assays and immunophenotypic characterization

One hundred thousand MSC were seeded in the upper chamber of a 24-transwell plate (Corning) and 5×10^5 positively selected B-lymphocytes were seeded in the lower chamber of the transwells. Furthermore, pDC were added to either the upper or the lower chamber to keep a proportion of 5:1 with B cells. The cells were cultured for 7 days in 1 mL of RPMI 1640 medium supplemented with 10% human AB serum plus 10 mg/mL anti-Ig and 10 μg/mL CpG-C ODN 2395.

B-cell maturation and differentiation were analyzed using the following monoclonal antibody combinations: FITC/PE/PerC-Cy5/APC: CD138/CD20/CD19/CD38, CD138/CD27/CD19/CD38, CD45RA/CCR7/CD19/CD38 as previously reported by our group for the identification of plasma cells.[21-23] Furthermore, the expression of immunoglobulin both at the membrane and intracellular levels was analyzed using the following monoclonal antibody combinations: λ-FITC/-PE/CD19-PerC-Cy5/CD38-APC. Intracellular immunoglobulin was detected, after staining for surface proteins, using a direct immunofluorescence technique.The IntraStain kit (Dako Cytomation, Denmark) was used for this purpose, strictly following the manufacturer's recommendations. After staining for intracytoplasmic antigens, cells were washed and resuspended in 0.5 mL of phosphate-buffered saline until their presence was shown using the flow cytometer.

Data were acquired on a FACSCalibur flow cytometer (BDB) using the Cell Quest software program (BDB) and the mean fluorescence intensity (MFI, expressed in arbitrary linear units scaled from 0 to 10^4) was analyzed using the Paint-A-Gate Pro (BDB) software for each antigen, gated on the live population. An analysis of surface antigens was performed on the gated population using the Cell-Quest software for both intracellular cytokines and the percentage of positive cells.

Western blot analysis

In order to perform western blot assays, B cells were cultured in the lower chamber of a 0.4 μm pore transwell insert and MSC (with or without pDC) were cultured separately in the upper chamber. Accordingly, purified B cells were obtained for the analyses. The B cells were pelleted, washed twice with phosphate-buffered saline (Gibco) and 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 pepstatin, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM sodium orthovanadate and 1 mM phenylmethylsulphonyl fluoride). Samples were centrifuged at 13,000 rpm at 4°C for 10 min and the supernatants were

collected into new tubes. Cell extracts were resuspended in sample buffer, boiled and separated using sodium dodecyl sulphate gel electrophoresis. After transfer to PVDF membranes (Millipore), filters were blocked for 1 hour in 10 mM Tris pH 7, 150 mM NaCl, 0, 1% Tween 20 (TBST) plus; 1% bovine serum albumin (BSA) and then incubated with the corresponding antibody. Membranes were incubated with mouse anti-pErk 1-2 (1:5000), rabbit anti-Erk 1-2 (1:2000) (both from Santa Cruz Biotechnology, Inc.), rabbit anti-p-p38 (1:1000), mouse anti-p38 (1:1000), anti-pAkt (1:1000) and anti-PARP (1:1000) (from Cell Signaling Technology). After washing with TBST, filters were incubated with horseradish peroxidase-conjugated secondary antibodies for 30 min and bands were visualized in a luminol-based detection system with p-iodophenol enhancement.

Statistical analysis

Mean values and their SD as well as ranges and median values were calculated for each variable using the SPSS software program (SPSS 11.0, Chicago, IL, USA). Paired-sample T-tests were performed to compare the

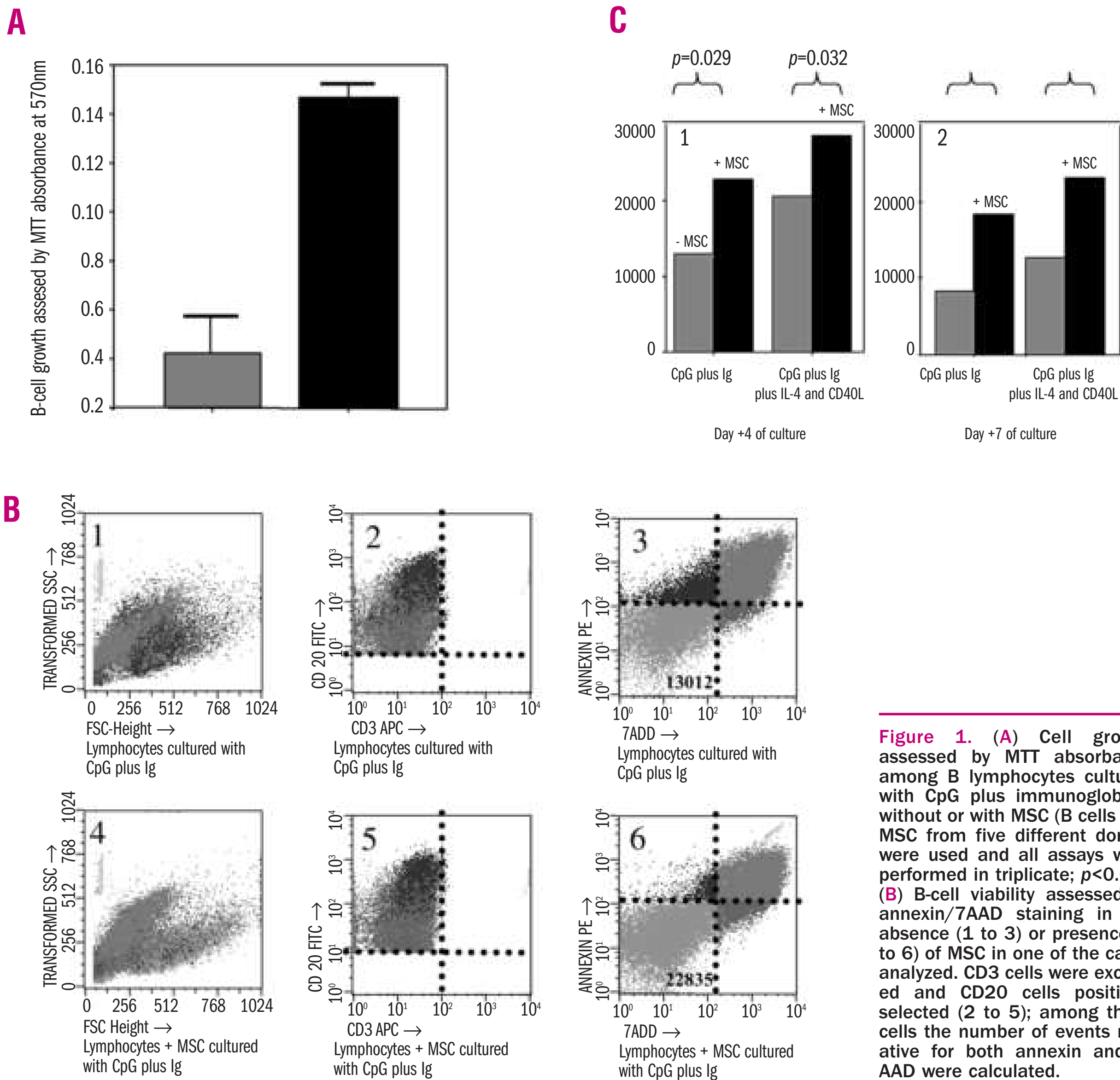

Figure 1. (A) Cell growth assessed by MTT absorbance among B lymphocytes cultured with CpG plus immunoglobulin without or with MSC (B cells and MSC from five different donors were used and all assays were performed in triplicate; $p<0.01$). (B) B-cell viability assessed by annexin/7AAD staining in the absence (1 to 3) or presence (4 to 6) of MSC in one of the cases analyzed. CD3 cells were excluded and CD20 cells positively selected (2 to 5); among these cells the number of events negative for both annexin and 7-AAD were calculated.

(C) (1) B-cell viability assessed by annexin/7AAD staining after 4 days of culture significantly increased in the presence of MSC: mean values (standard deviation) of annexin/7AAD negative events (viable B cells) for samples cultured with CpG plus immunoglobulin (Ig) in the absence or presence of MSC were: 13012 (SD: 5662) *vs.* 22835 (SD: 8553) events, respectively; p=0.029 Similar data were obtained for samples cultured with CpG plus Ig plus interleukin-4 (IL-4) and CD40L, mean values being 20581 (SD: 6962) *vs.* 28543 (SD: 3954) for samples cultured without or with MSC, respectively; p=0.032. (2) B-cell viability after 7 days of culture: mean values (standard deviation) of annexin/7AAD negative events for samples cultured with CpG plus Ig in the absence or presence of MSC were: 8146 (SD: 2446) *vs.* 18227 (SD: 6147) events, respectively; p=0.022. Similar data were obtained for samples cultured with CpG plus Ig plus IL-4 and CD40L, mean values being 12574 (SD: 7353) *vs.* 23181 (SD: 7936) for samples cultured without or with MSC, respectively; p=0.021. (B cells and MSC from seven different donors were used)

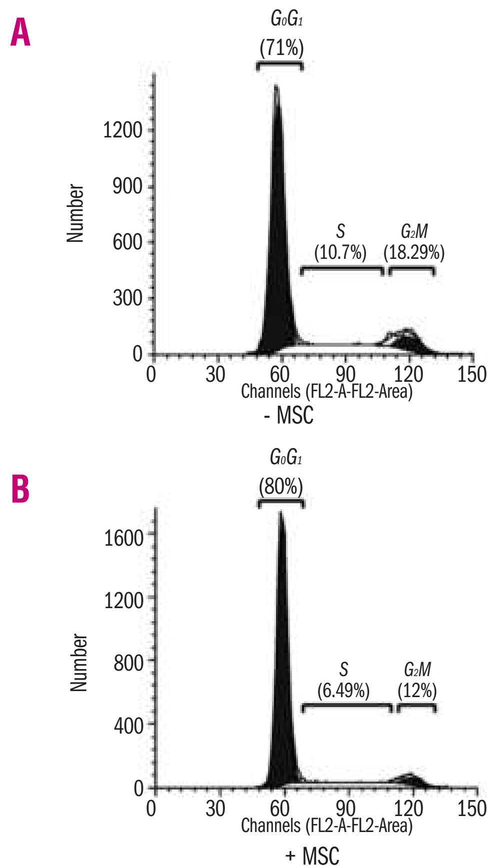

Figure 2. Mean percentage of B cells cultured in the presence of TPA without (A) or with (B) MSC in the G_0G_1 phase (71.43% *vs.* 80.97%, respectively; *p*=0.014), the G_2M phase (18.29% *vs.* 12.53%, respectively; *p*=0.008) and the S phase (10, 27% *vs.* 6.49%, respectively; *p*=0.035) of the cell cycle. (the figure shows data from a representative case of the seven cases analyzed).

effect of MSC on the viability, proliferation and differentiation of B-lymphocytes within the different types of culture. Non-parametric testing was also performed for the MTT assays. The number of cases for each experiment are specified in the corresponding figures. *p* values less than 0.05 were considered statistically significant.

Results

The effect of mesenchymal stem cells on the proliferation and viability of B-lymphocytes

MTT uptake in B-lymphocytes increased in the presence of MSC upon culture with plate-bound-anti-Ig plus CpG-C (Figure 1A), suggesting increased B-cell survival and/or proliferation. Accordingly, we decided to evaluate the effect of MSC on B-lymphocyte viability and the cell cycle more specifically. As shown in Figures 1B and 1C, the presence of MSC increased B-cell viability (doubling the number of viable B cells from 13012 to 22835 in annexinV-PE/7-AAD-negative events within B-lymphocytes in the absence versus presence of MSC, respectively), thus explaining the increased MTT uptake for those cases cultured with anti-Ig and CpG-C in the presence of MSC. Similar results were obtained after culture in the presence of anti-Ig plus CpG-C, plus anti-CD40L and interleukin-4 (Figure 1B and 1C) after 4 and 7 days of culture.

Next, we analyzed the effect of MSC on B-cell proliferation. The combination of anti-Ig plus CpG-C with or without anti-CD40L and interleukin-4 induced a low B-cell proliferation with less than 5% of cells being in the S or G_2M phases of the cell cycle. In order to obtain better evidence of the effect of MSC on B-cell cycling, TPA was used to induce a more intense stimulation of B-cell proliferation and, under this culture condition, MSC significantly decreased the percentage of B cells in the S and G_2M phases, as shown in Figure 2. Contrarily, within cultures grown with CpG plus anti-Ig with or without anti-CD40L and interleukin-4, the effect of MSC on B-cell cycling could not be observed since most cells were in the G_0/G_1 phases.

Thus, MSC promote B-lymphocyte survival while, under highly proliferative conditions, they arrest the B-cell cycle.

The effect of mesenchymal stem cells on B-lymphocyte differentiation

Once we had confirmed the effect of MSC on B-cell survival and proliferation, we examined their effect on B-cell differentiation, since several studies have confirmed a link between proliferation and differentiation among B-lymphocytes.[14,24] For this purpose, we used ten samples of bone marrow from different healthy donors and a similar number of buffy coats in order to obtain B cells. The different culture conditions were analyzed a minimum of five times. We examined the expression of various surface antigens related to B-cell differentiation. We did not observe a clear maturation pattern of B cells upon culture with either anti-Ig plus CpG-C or TPA or with or without anti-CD40L and interleukin-4 (*data not shown*). Accordingly, we decided to examine the effect of pDC on B-cell maturation since pDC have been reported to induce B-cell differentiation.[16] As shown in Figures 3A and 3B, we confirmed that the presence of pDC increased both the percentage of $CD38^{++}/CD138^{++}$ cells as well as the mean fluorescence intensity (MFI) of both markers for the whole B-cell population. This effect was observed both allowing direct contact between lymphocytes and pDC and when B cells and pDC were cultured in transwell plates. As shown in Figures 3A and 3B, the presence of MSC in the culture significantly decreased the percentage of $CD38^{++}/CD138^{++}$ cells as well as the MFI for both markers.

Accordingly, different B-cell subpopulations could be identified depending on the expression of CD38 and CD138, which represented a continuum in B-cell maturation. To ensure that these subpopulations represented different stages of maturation we analyzed the expression of cytoplasmic immunoglobulin as a surrogate marker of differentiation and, as shown in Figure 3C, the levels of cytoplasmic immunoglobulin were significantly higher among the $CD38^{++}$ cells, whereas the expression of surface immunoglobulin decreased

expected, an inverse correlation was observed between the expression of these markers and that of CD38 (Figure 3C).

Thus, pDC encouraged B-lymphocyte differentiation, while this effect was inhibited in the presence of MSC.

Pathways involved in the effect of mesenchymal stem cells on B-lymphocytes

In order to define the pathways involved in the effects of MSC on B-lymphocytes, we performed western-blot analysis focusing on the pathways involved in cell survival and proliferation. We evaluated each pathway in three different experiments. For this purpose, we used B-lymphocytes cultured for 48 hours in the presence of

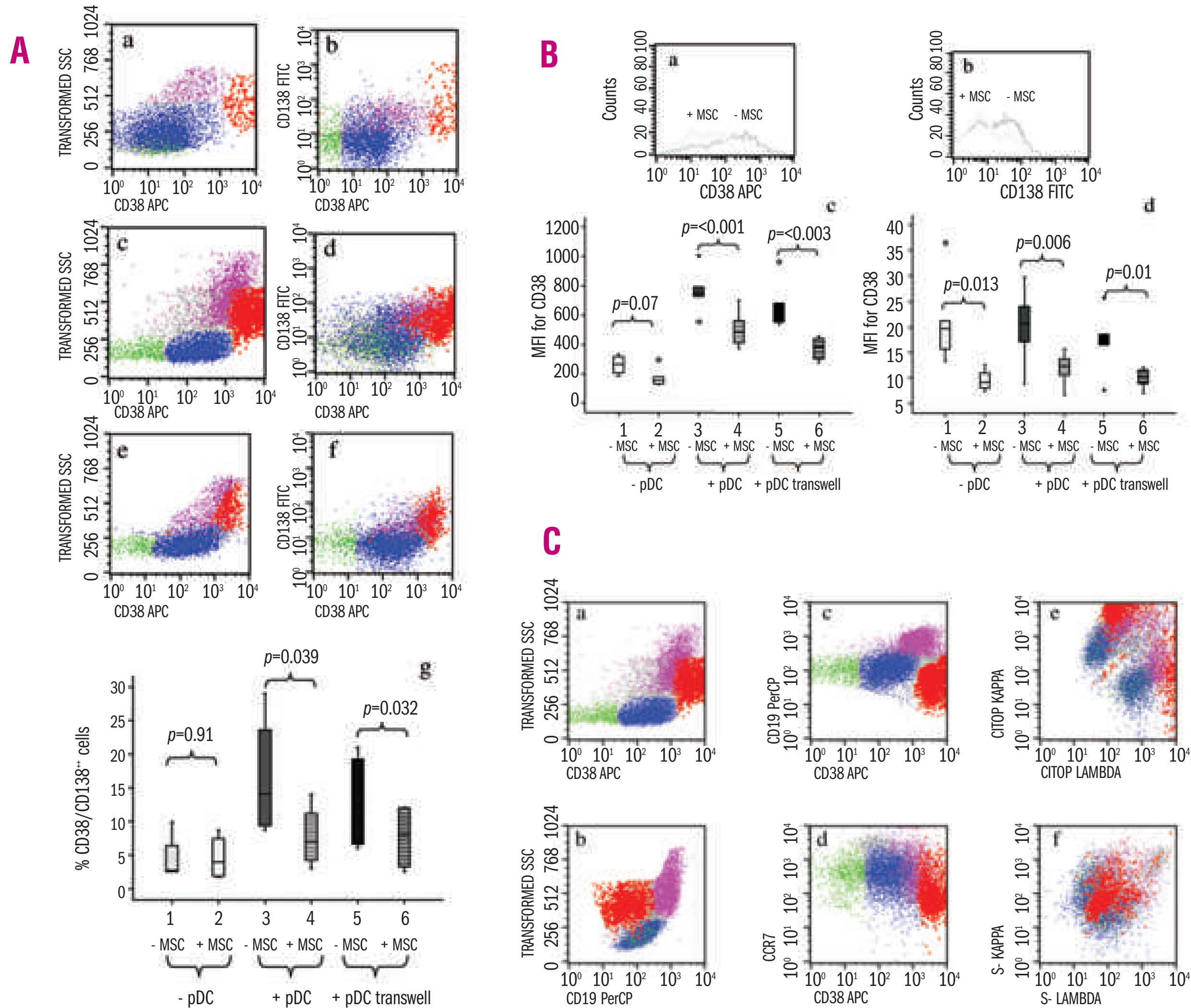

Figure 3. (A) Percentage of CD38^{++}/CD138^{++} (red events) cells after 7 days of culture of B-lymphocytes with: (a, b) CpG and Ig; (c, d) CpG and Ig plus pDC (e, f) CpG and Ig plus pDC and MSC. A representative experiment out of the ten cases analyzed is shown. (g) Percentage of CD38^{++}/CD138^{++} cells after culture of B lymphocytes with: (1) anti-Ig and CpG alone (mean percentage: 4.47%) (2) the same as (1) plus MSC (mean percentage: 4.6%) (3) anti-Ig and CpG with pDC (mean percentage: 16.47%) (4) the same as (3) plus MSC (mean percentage: 7.67%) (5) anti-Ig and CpG with pDC in transwell (mean percentage: 12.8%) (6) the same as (5) plus MSC (mean percentage: 7.6%) (B) (a) MFI for CD38 after culturing B lymphocytes with anti-Ig plus CpG plus pDC (shown in green) or with the same condition plus MSC (shown in yellow); (b) MFI for the CD138 after culture in the same conditions; (c) MFI for the CD38 after culturing B-lymphocytes (1) with anti-Ig plus CpG (MFI: 260.9) (2) the same as (1) plus MSC (MFI: 173.6) (3) with pDC (MFI: 762.7) (4) the same as (3) plus MSC (MFI: 501.5) (5) with pDC in transwells (MFI: 663.03) (6) plus MSC (MFI: 373.1); (d) MFI for CD138 of B-lymphocytes cultured (1) with anti-Ig plus CpG (MFI: 21.01) (2) the same as (1) plus MSC (MFI: 9.56) (3) with pDC (MFI: 20.16) (4) the same as (3) plus MSC (MFI: 11.88) (5) with pDC in transwells (MFI: 17.08) (6) plus MSC (MFI: 10.04). (C) (a) Maturation pattern of B cells from CD38^{-} (immature B cells, in green), CD38$^{+/-}$ (mature B cells, in blue), CD38^{+} (lymphoplasmocytes, in violet) and CD38^{++} (plasma cells, in red); (b) the same populations identified based upon CD19 expression: red events displayed lower MFI for CD19 as compared to the rest: 590 (SD:330) *vs.* 113 (SD: 60), p=0.01; (c and d) the expression of CD38 inversely correlated with both MFI for CD19 and CCR7; (e) Cytoplasmic kappa light expression was significantly higher for the CD38^{++} cells than for the rest: MFI = 2341 (SD= 1394) among CD38^{++} cells (shown in red) *vs.* 934 (SD=655) among CD38^{+} cells (shown in violet) (p<0.001). Respective values for cytoplasmic lambda light chains were 1461 (SD=694) *vs.* 720 (SD=428) for CD38^{++} *vs.* CD38^{+} cells, respectively (p<0.001); (f) MFI for surface kappa light chain was 306 (SD= 96) among CD38^{++} cells *vs.* 221 (SD=129) among CD38$^{+/-}$ cells (p=0.1) Values for surface lambda light chains were 217 (SD=90) *vs.* 156 (SD=95) for CD38^{+}, and CD38$^{+/-}$ cells, respectively (p=0.15). A representative experiment out of the ten cases analyzed is shown.

immunoglobulin plus CpG or TPA with and without MSC. Regarding the pathways involved in cell proliferation and viability, we observed that Akt phosphorylation and PARP cleavage occur upon stimulation with immunoglobulin plus CpG but not with TPA. The addition of MSC did not modify these results. In contrast, the presence of MSC clearly influenced the ERK 1/2 and p38 pathways, but these effects depended on the culture conditions. Thus, MSC induced phosphorylation of ERK 1/2 MAPK and inhibited phosphorylation of p-p38 in B-cells cultured with immunoglobulin plus CpG (low proliferative conditions) while the contrary occurred in B cells cultured with TPA (highly proliferative conditions) (Figure 4).

To evaluate the effect of MSC in the pathways involved in B-cell differentiation, we performed a western-blot analysis after culturing B cells in the presence of pDC, since that was the condition which allowed optimal B-cell differentiation. As shown in Figure 4, phosphorylation of both ERK 1/2 and p38 was stimulated in the presence of MSC after 48 hours of culture with pDC.

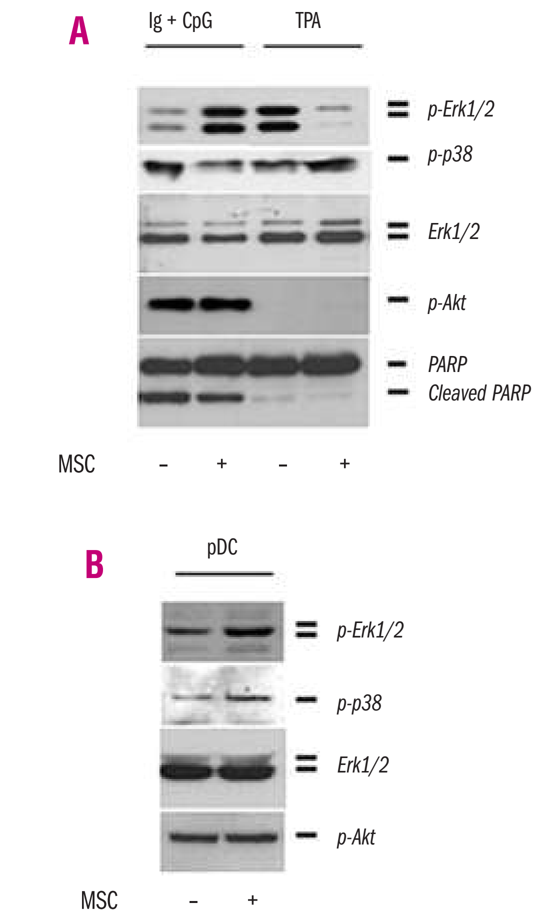

Figure 4. Western blot assays in B cells cultured for 48 hours in the presence of Ig plus CpG or TPA (A) or in presence of pDC (B).

Discussion

Several studies have been devoted to evaluating the immunosuppressive effect of MSC. Interestingly, MSC can inhibit monocyte-derived dendritic cell maturation as well as T-lymphocyte proliferation.[3-7] Furthermore, MSC reside in bone marrow and secondary lymphoid organs, where they play a crucial role in organogenesis,[14] and can migrate to injured tissues. These properties have already been exploited in the clinical setting for the treatment of graft-versus-host disease.[8,9] Thus, MSC could provide chemokines which regulate lymphocyte proliferation and survival. Benvenuto *et al.*[24] have recently reported that MSC increase the survival of unstimulated T cells and inhibit the proliferation of activated T cells. This is in accordance with previous studies indicating that MSC arrest T cells in the G_1 phase of the cell cycle[4] and that this effect is mediated by the inhibition of cyclin D2 and the upregulation of $p27^{kip1}$. In this study we observed, using a different approach, that MSC both inhibited B-cell proliferation and promoted B-cell survival. We also noted that the magnitude of these effects varied depending on the culture conditions, so, as expected, the former effects were more evident on proliferating B cells while the latter effects were more evident on resting B cells. In addition, our results add further evidence to that from a previous study by Ame-Thomas *et al.*[25,26] which showed that MSC support malignant B-cell survival in follicular lymphomas. Interestingly, it has been demonstrated that a cross-talk between follicular lymphoma cells and their microenvironment, including stromal cells, delivers the growth factors required for lymphoma genesis.[25,26]

Tangye *et al.*[25,26] elegantly described how the B-cell differentiation machinery is linked to cell division, showing that class switching proceeds in concert with clonal expansion. As the antigen is required to continue driving proliferation, a feedback can operate and should a successful class of antibody be produced then the antigen is cleared and further cell division stops. On the other hand, persistence of the antigen can continue to stimulate cells to divide and later to switch isotypes. Thus, B cells generate immunoglobulin-secreting cells through a division-linked mechanism. Accordingly, inhibition of B-cell cycling also blocks plasma cell maturation.[25,26] With this concept in mind and considering the inhibitory effect of MSC on B-cell division, we analyzed the effect of MSC on B-cell differentiation. As reported by Poeck *et al.*,[16] we found that co-culture of B cells with pDC induced plasma cell differentiation. More specifically, we analyzed the expression pattern of different antigens related to B-cell maturation and, in all cases, the presence of pDC increased the expression of maturation markers such as CD38 and CD138. We further evaluated the maturation pattern by confirming that the higher the expression of CD38 and CD138 in B cells the more intense the MFI for cytoplasmic immunoglobulin. All these markers enable the identification of plasma cells.[21-23] It is, however, worth noting that the higher the expression of the above mentioned markers, the lower the expression of surface immunoglobulin, CD19 and CCR7 which also characterizes earlier stages of B-cell differentiation, as previously reported.[27]

Regarding the effect of MSC on the plasma cell differ-

entiation of B cells, previous studies have provided contradictory data. Thus, in accordance with our study, Corcione *et al*.[11] reported that MSC inhibit B-cell proliferation and differentiation; this conclusion was reached as a result of the detection of immunoglobulin production in the presence of CpG, CD40L, anti-immunoglobulin, and interleukins 2, 4 and 10. In contrast, Traggiai *et al*.[28] recently showed that MSC may induce both the expansion and differentiation of B cells stimulated with an agonist of Toll-like receptor 9 in the absence of B-cell receptor triggering. In addition, Rasmusson *et al*.[13] showed that MSC increase antibody secretion by human B cells stimulated with lipopolysaccharide, cytomegalovirus or varicella-zoster virus although this effect varies depending on the stimulus used to trigger B lymphocytes so that, in cases in which lipopolysaccharide, cytomegalovirus or varicella-zoster virus induce a weak response, MSC can stimulate IgG secretion: the contrary occurs when a strong primary stimulus is used. These results should not be considered as contradictory and, in fact, are in accordance with our data since, as previously shown, the predominant effect of MSC on B-cell viability and proliferation varies depending on the proliferative status of the B cells, so that the increased viability induced by MSC in resting B cells could explain the higher production of IgG upon weak stimulation. We used pDC to stimulate B cells because we found that this approach induced the most clear differentiation pattern and, additionally, allowed reproduction, *in vitro*, of the interactions between the different cell subsets involved in the immune response which takes place *in vivo* in both hematopoietic and lymphoid organs. Assays were performed using transwells, confirming that the effect of MSC on B-lymphocytes is mostly dependent on soluble factors and also showed that, interestingly, the effect of pDC on plasmacytic differentiation is observed irrespectively of whether the culture involves direct or transmembrane contact with pDC.

Regarding the pathways involved in these effects, we focused our analysis on those which are involved in cell survival and proliferation. Under conditions which induced a low proliferative rate of B cells, we observed that MSC increased ERK1/2 and inhibited p38 MAPK phosphorylation, which may explain the increased viability of B-lymphocytes in the presence of MSC. Remarkably, the opposite effect was observed under culture conditions which favored B-cell proliferation, so that MSC inhibited ERK1/2 phosphorylation and induced activation of p38 MAPK in B cells cultured with TPA. Interestingly, both pathways have emerged as central regulators of cell proliferation through the control of cell growth and cycle progression. Thus, ERK1/2 signaling promotes G_1 progression[29] whereas p38 signaling negatively regulates cyclin-D1 transcription which, in turn, contributes to cell cycle arrest in the G_1 phase,[30,31] thus explaining the effect of MSC on B-cell proliferation. Interestingly, Suzuki *et al*.[32] showed that a stromal cell-derived factor (SDF, CXCL12) favors T-cell survival by a mechanism mediated through the activation of phosphatidylinositol 3-kinase and MAPK pathways. Similar findings were reported by Palmesino *et al*.,[33] who showed that the stimulation of B cells with CXCL12 results in the activation of a MAPK cascade and internalization of CXCR4, although this effect of SDF may depend on the stage of maturation.[34]

With regards to the effect of MSC on signaling pathways involved in B-cell maturation, western-blot assays, performed on B-lymphocytes cultured in the presence of pDC, showed that MSC induced ERK 1/2 phosphorylation. It is worth mentioning in relation to this that, in B cells, ERK 1/2 as well as p38 becomes activated after the binding of unmethylated viral and bacterial CpG DNA to Toll-like receptor 9. However, mammalian DNA contains rare hypomethylated CpG regions which are capable of co-stimulating autoreactive lymphocytes and can, in turn, promote systemic humoral autoimmunity.[35] Nevertheless, as shown by Rui *et al*.,[36] the continuous self-signaling to ERK through Toll-like receptor 9 inhibits CpG-induced plasma cell differentiation, thus avoiding autoimmunity. According to our results, MSC induce a persistent activation of ERK in B cells cultured with pDC, which is similar to that observed among anergic B cells constantly exposed to self-antigens[36] and would suggest a role for MSC in the regulation of immune response homeostasis. Moreover, according to Rui *et al*.[36] B-cell receptors do not become desensitized by the activation of the ERK pathway and, considering the effect of MSC on B-cell survival, could set B-lymphocytes to respond to immunologic challenges. Finally, as reported in the present study concerning B cells, it is worth mentioning that the ERK signaling pathway has been shown to promote different outcomes in a PC12 cell line, including differentiation and proliferation, thus providing an example of the wide spectrum of cell fate decisions induced by this pathway, depending on the intensity and the duration of the activation signal.[37]

Our study also shows that other pathways involved in cell survival, such as PI3K-Akt, and markers of caspase activation, such as PARP, are not modified by MSC, at least not under the conditions used in the current study.

In conclusion, MSC promote survival and inhibit proliferation and maturation of B cells. These effects are mediated by activation of MAPK pathways such as pERK 1/2 and p38. This study contributes to establishing an *in vitro* model defining the normal cross-talk between the different cell subsets involved, *in vivo*, in the control of the immune response and lymphoid proliferation and differentiation.

Authorship and Disclosures

ST performed the cell cultures and western blot assays; JAPS was the main researcher of the project; MD-C cultured and expanded the MSC and determined their; LIS-A performed the flow cytometry assays and separated the dendritic cells; BB was responsible for the proliferative assays; AL supervised the B-lymphocyte flow cytometry assays; AB contributed to cell culture assays; EO supervised the western blot assays; FMS-G was responsible for MSC differentiation; CdC critically reviewed the manuscript; JSM critically reviewed the research project and the manuscript.

The authors reported no potential conflicts of interest.

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