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Mesenchymal stem cells suppress B-cell terminal differentiation

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Objective. **Mesenchymal stem cells (MSCs) have been shown to possess immunomodulatory properties on a diverse array of immune cell lineages. However, their effect on B lymphocytes remains unclear. We investigated the effect of MSCs on B-cell modulation with a special emphasis on gene regulation mediated by MSC humoral factors.**

Materials and Methods. **MSCs were isolated from C57BL/6 bone marrow and expanded in culture. Splenic B cells were purified using anti-CD43 antibody and immunomagnetic beads. B cells and MSCs were cocultured in separate compartments in a transwell system. For B-cell stimulation, lipopolysaccharide was used in vitro and T-dependent and T-independent antigens were used in vivo.**

Results. **In MSC cocultures, lipopolysaccharide-stimulated B-cell proliferation was suppressed, $CD138^+$ cell percentage decreased, and the number of apoptotic $CD138^+$ cells decreased. In the B/MSC coculture, the IgM^+ cell percentage was higher and the IgM amount released in the medium was lower than in the control. The B-lymphocyte–induced maturation protein–1 messenger RNA expression in the coculture was suppressed throughout the 3-day culture period. Conditioned media derived from MSC cultures prevented terminal differentiation of B cells in vitro and significantly suppressed the antigen-specific immunoglobulin M and immunoglobulin G1 secretion in mice immunized with T-cell–independent as well as T-cell–dependent antigens in vivo.**

Conclusion. **Results indicate that humoral factor(s) released by MSCs exert a suppressive effect on the B-cell terminal differentiation. Suppression may be mediated through inhibition of B-lymphocyte–induced maturation protein–1 expression, but the nature of the factor(s) is yet to be determined.**

Bone marrow (BM) is a complex tissue containing diverse lineages of hematopoietic and stromal cells that support hematopoiesis [1]. Marrow stroma contains a small subpopulation of undifferentiated cells referred to mesenchymal stem cells (MSCs). MSCs are capable of rapidly proliferating ex vivo and differentiating into various mesenchymal lineages [2,3], offering a tool for clinical applications [4,5]. MSCs have also shown immune regulatory properties [6–8]. We have shown in rats that MSCs facilitate induction of mixed hematopoietic chimerism and islet allograft tolerance [9]. MSCs exert suppressive effects on T cells [10–12], natural killer cells [13], as well as dendritic cells [14]. With respect to mature B cells, human MSCs have been shown to inhibit B-cell proliferation, differentiation, and chemotaxis in vitro [15], although the mechanism involving in B-cell modulation is largely unknown.

Exposure of mature B cells to lipopolysaccharides (LPS) induces expression of B-lymphocyte–induced maturation protein–1 (Blimp-1), leading to the terminal differentiation of B cells into plasma cells [16]. Blimp-1 is postulated to be the master transcriptional regulator required for B-cell terminal differentiation by directly repressing transcription factors that, in turn, regulate several important gene programs [17]. Ectopic expression of Blimp-1 has been shown to be sufficient for inducing B-cell terminal differentiation in *bcl-1* lymphoma, a model used for testing differentiation of mature B cells into plasma cells [18,19]. In the

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Blimp-1–conditional knockout mouse, Blimp-1 is required for differentiation of plasma cells, preplasma memory B cells [20], and maintenance of plasma-cell longevity in the BM [21].

We investigated the potential of MSCs to modulate mature B cells using mice, focusing on gene regulation and MSC-released humoral factors. Our results show that MSCs reduce the plasma cell generation in cocultured B cells in vitro. Humoral factor(s) from MSCs released in culture suppress antigen (Ag)-specific immunoglobulin (Ig) M and IgG1 secretion in vivo in animals immunized with T-cell–independent (T-ID) as well as T-cell–dependent (T-D) antigens. B-cell suppression is mediated by MSC-released humoral factor(s), does not require cell–cell contact, and is associated with reduced *Blimp-1* messenger RNA (mRNA) expression. Monocyte chemotactic protein–1 (MCP-1), interleukin (IL)-10, transforming growth factor–β (TGF-β), and indolamine 2,3-dioxygenase (IDO) are not involved in the B-cell suppression, and the nature of humoral factor(s) remains to be elucidated.

Materials and methods

Animals and immunization

Female C57BL/6 and BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the City of Hope Animal Resources Center. BALB/c mice were immunized with intraperitoneal (IP) injections of either a T-D Ag (50 μg NP_{12}-Ficoll) or a T-ID Ag (50 μg alum-precipitated NP_{19}-KLH, both from Biosearch Technologies, Novato, CA, USA) in 250 mL phosphate-buffered saline (PBS). The animal protocol used in this study was approved by the City of Hope Research Animal Care Committee.

Isolation and expansion of MSCs and preparation of splenic B cells

BM cells from C57BL/6 mice were cultured in 25 cm^2 tissue culture flasks (8×10^5 cells/cm^2) using Murine MesenCult Basal Medium containing 20% MSC Stimulatory Supplements (StemCell Technologies, Vancouver, BC, Canada) at 37°C in air plus 5% CO_2. After 72 hours, nonadherent cells were decanted and thereafter the medium was changed every 3 to 4 days. When adherent cells reached 70% to 80% confluence, they were trypsinized and passaged. MSCs were differentiated to adipocytes or osteocytes using the culture method described by Peister et al. [22]. Adipocytes were detected by Oil Red O (Sigma-Aldrich, St Louis, MO, USA) staining and osteocytes by alkaline phosphatase staining. B cells were prepared from the spleen by depleting non–B cells using a phycoerythrin (PE)–anti-CD43 antibody (Ab) (BD Biosciences, San Jose, CA, USA) and magnetic beads coated with anti-PE Ab (Miltenyi Biotec, Gladbach, Germany). The Ab-labeled cells were then separated by a magnetic-activated cell sorting system (Miltenyi Biotec). The resulting B-cell fraction contained >95% $CD19^+$ B cells.

Monoclonal antibodies and fluorescein-activated cell sorting (FACS) analysis

Fc receptors were blocked by incubating cells with 5 μg/mL anti-CD16/32 Ab (BD Biosciences). Antibodies used for labeling included: monoclonal Abs conjugated to allophycocyanin–anti-CD19; biotin: –anti-H-$2K^b$, –anti-I-A^b, –anti-FAS-L, –anti-CD40L, –anti-IgM^b, –anti-IgD^b, –anti-IgG3; fluorescein isothiocyanate: –anti-Sca-1, –anti-CD34, –anti-CD40; –anti IgG3; and PE: –anti-c-kit, –anti-CD11b, –anti-CD45, –anti-CD80, –anti-CD86, –anti-CD138 (all from BD Biosciences). Cells labeled with biotinylated Abs were visualized by incubating with allophycocyanin-conjugated streptavidin. For cell-proliferation assays, B cells were labeled with carboxyfluorescein diacetate, succinimidyl ester (CFSE; Molecular Probes/Invitrogen, Carlsbad, CA, USA) as described elsewhere [21] and analyzed using FACSCalibur (Becton Dickinson, San Jose, CA, USA).

Transwell cultures

Transwell cultures were set up in six-well culture plates. Each well contained an insert with a 0.4-μm pore size membrane (Corning, Corning, NY, USA) to separately culture B cells from MSCs. MSCs (10^5 cells/well) were seeded in wells 6 to 8 hours before placing 10^6 B cells in the insert. Complete culture medium (CCM) was added in a volume of 4 mL/well. This medium was RPMI-1640 supplemented with 3 μg/mL LPS, 10% fetal bovine serum, 50 μM 2-mercaptoethanol, and antibiotics. Cells were cultured for 3 days in a tissue culture incubator.

ELISA for antibody titration

The titer of IgM and IgG3 in B-cell cultures, or NP-specific IgM, IgG1, IgG2a, IgG2b, or IgG3 in serum samples from the immunized mice was measured by enzyme-linked immunosorbent assay (ELISA). ELISA plates were prepared by coating 96-well plates with either goat anti-mouse IgG plus IgM (Caltag Laboratories, Burlingame, CA, USA) or NP_{20}-BSA (Biosearch Technology, Novato, CA, USA). After incubation, wells were washed then incubated with biotinylated goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 Ab (Caltag Laboratories), followed by incubation with avidin-peroxidase (Sigma-Aldrich) using *o*-phenylenediamine dihydrochloride (Sigma-Aldrich) in citrate buffer as substrate. The reaction was read at 450 nm on a multiscan 96-well plate reader (GENios, TECAN US Inc., NC, USA).

Cell proliferation assay by 3H-thymidine uptake

B cells (10^6 cells/well) were cultured 48 hours in a 96-well plate in the presence of LPS either with or without MSCs (1 or 2×10^5/well). Cells were pulsed with 1 μCi ^{3}H-thymidine for the last 8 hours and ^{3}H-thymidine uptake was measured by a liquid scintillation counter (Microbeta Trilux1450, Wallac, Waltham, MA, USA).

Apoptosis analysis

An Annexin-V kit (BD Biosciences) was used to detect apoptotic cells in the $CD138^+$ and $CFSE^-$ cell fractions by FACS analysis.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from the cultured B cells using TRI-REAGENT RNA isolation reagent (Molecular Research Center, Cincinnati, OH, USA). RNA was reverse-transcribed using Superscript III and Oligo (dT) (Invitrogen, Carlsbad, CA, USA) in a final volume of 50 mL. Semi-quantitative PCR using 1 mL complementary DNA (cDNA) was performed as follows. An initial 2-minute incubation at 92–95°C for denaturation was followed by annealing at 30 or 35 cycles of PCR at 50°C for *μS* (secretion form of IgM

heavy chain mRNA) and at 52°C for *μM* (membrane form of IgM heavy chain mRNA), and for 45 cycles at 55°C for hypoxanthine phosphoribosyltransferase (*HPRT*). Polymerization was done at 72°C for 1 minute. The following PCR primers were used for the cDNA amplification: the *μM* primers, 5′-GGCTTTGAGAACCTG TGGA-3′ and 5′-TTACAGCTCAGCTGTCTGT-3′; the *μS* primers, 5′-TCTGCCTTCACCACAGAAG-3′ and 5′-TAGCATGGTCAAT AGCAGG-3′; and the *HPRT* primers, 5′-GCTGGTGAAAAGGAC CTCT-3′ and 5′-CACAGGACTAGAACACCTGC-3′. The following primers and probes were purchased from Applied Biosystems (Foster City, CA, USA) for cDNA amplification to perform quantitative real-time PCR: *Blimp-1* (Mm01187285ml); X-box binding protein 1 (*XBP-1*) (Mm01187751ml); interferon regulatory factor-4 (IRF-4) (Mm00516431ml); B-cell lymphoma 6 (*Bcl-6*) (Mm01342169ml); paired box gene 5 (*PAX-5*) (Mm01345231ml); *β-actin* (401846). TaqMan Universal PCR master mix (Applied Biosystems) was used for qRT-PCR with 2 mL cDNA in five replicates. The average threshold cycles of the replicates were used to calculate the fold-change between endogenous gene expression in the day 0 sample and the specific gene expression in the day 1, 2, and 3 samples. Cycle for *β-actin* was used to normalize results. Relative quantification was calculated using the comparative Ct method.

Determination of cytokines and chemokines released in the culture medium

Cytokines and chemokines released into the culture medium were detected using RayBio Mouse Cytokine Array I (RayBiotech, Norcross, GA, USA). Membrane-bound cytokines/chemokines were revealed by horseradish peroxidase (HRP)–conjugated streptavidin.

Preparation and tests of conditioned media

Five types of conditioned medium (CM) (Table 1) were prepared by culturing cells with CCM (4 mL/well) in the transwell system. CM1 was CCM containing LPS (3 μg/mL, CCM-LPS) with no cells; CM2 was produced by culturing MSCs (10^5/well) in CCM without LPS; CM3, 4, and 5 used CCM-LPS to culture MSCs, B cells (10^6/well), and MSCs and B cells, respectively. At the end of 3-day culture, the supernatant was collected, aliquoted, and stored at –80°C until use. For in vitro experiments, purified B cells (10^6/well) were cultured in a 24-well plate with 1 mL/well desired CM and generation of $CD138^+$ cells was analyzed on day 3 by FACS. For in vivo experiments, BALB/c mice immunized with NP_{12}-Ficoll were injected IP with 300 mL desired CM or PBS (control) on days 0 (day of first immunization), 2, and 4. Mice immunized with alum-precipitated NP_{19}-KLH were treated on days 2, 4, and 6. Serum samples were collected for Ab assays on weeks 1, 2, and 3. Control serum was from BALB/c mice immunized with alum-precipitated NP_{19}-KLH (50 mg) without CM treatment.

Table 1. Mesenchymal stem cell–conditioned media

	CCM	CM1	CM2	CM3	CM4	CM5
LPS	−	+	−	+	+	+
MSCs	−	−	+	+	−	+
B cells	−	−	−	−	+	+

CCM= complete culture medium; CM = conditioned medium; LPS = lipopolysaccharide; MSC = mesenchymal stem cell

Statistical analysis

Statistical analysis was performed using unpaired Student's *t*-test. *p* Values <0.05 were considered to be significant.

Results

Characteristics of mouse MSCs

Cells isolated from C57BL/6 BM and passaged more than five times in culture exhibited a spindle-shaped morphology (Fig. 1A-a) and differentiated into adipocytes (Fig. 1A-b) and osteocytes (Fig. 1A-c). Cells were negative for hematopoietic markers (c-kit, CD34, CD45 and CD11b) and immunophenotypic makers ($H\text{-}2K^b$, $I\text{-}A^b$, CD86, CD40, CD40L and FAS-L), but expressed Sca-1 and CD80 Ags (Fig. 1B). Thus, the characteristics of our cells were comparable to those of murine MSCs reported by others [3,10,22–24]. However, we did not test if these cells met specified stem cell criteria, including long-term self-renewing, our MSCs would have been more appropriate to be expressed as multipotent mesenchymal stromal cells as suggested by the International Society for Cellular Therapy [25].

MSCs prevent the terminal differentiation of LPS-stimulated B cells into plasma cells

In control transwell cultures without MSCs, the $CD138^+$ cell percentage increased by LPS stimulation from 0.5% preculture to 7.8% on day 3 and remained at the similar level on day 4. In contrast, in the B/MSC (at a 10:1 ratio) cocultures, the $CD138^+$ cell percentage increased to 2.0% on day 3 with no further increase on day 4 (Fig. 2A). Thus, presence of MSCs reduced the plasma cell number to approximately one-fourth of the control level. To determine if MSCs prevent B-cell terminal differentiation, CFSE-labeled B cells were stimulated with LPS and cultured with or without MSCs for 3 days to measure the expression of CFSE and several differentiation markers by FACS. B cells in both groups divided up to seven cell divisions during this period (Fig. 2B-a), indicating that MSCs do not accelerate cell-cycle progression. Expression of IgM and IgD decreased gradually as cell division progressed. The reduction of IgM on the B cells cocultured with MSCs was slightly slower than control B cells (Fig. 2B-b). In contrast, IgD expression was still high on divisions 3 and 4 of B cells cocultured with MSCs, and then slowly decreased. CD138 expression was detected on some of the control B cells after five cell divisions, but the less detectable with B cells cocultured with MSCs. The slow reduction of surface IgD and the slow induction of CD138 expression on the B cells cocultured with MSCs might indicate that MSCs decelerate the terminal differentiation of B cells stimulated with LPS.

MSCs selectively suppress LPS-stimulated B-cell differentiation into IgM-forming cells

To determine whether MSCs suppress immunoglobulin production, B-cell expression of *μM* and *μS* mRNA on

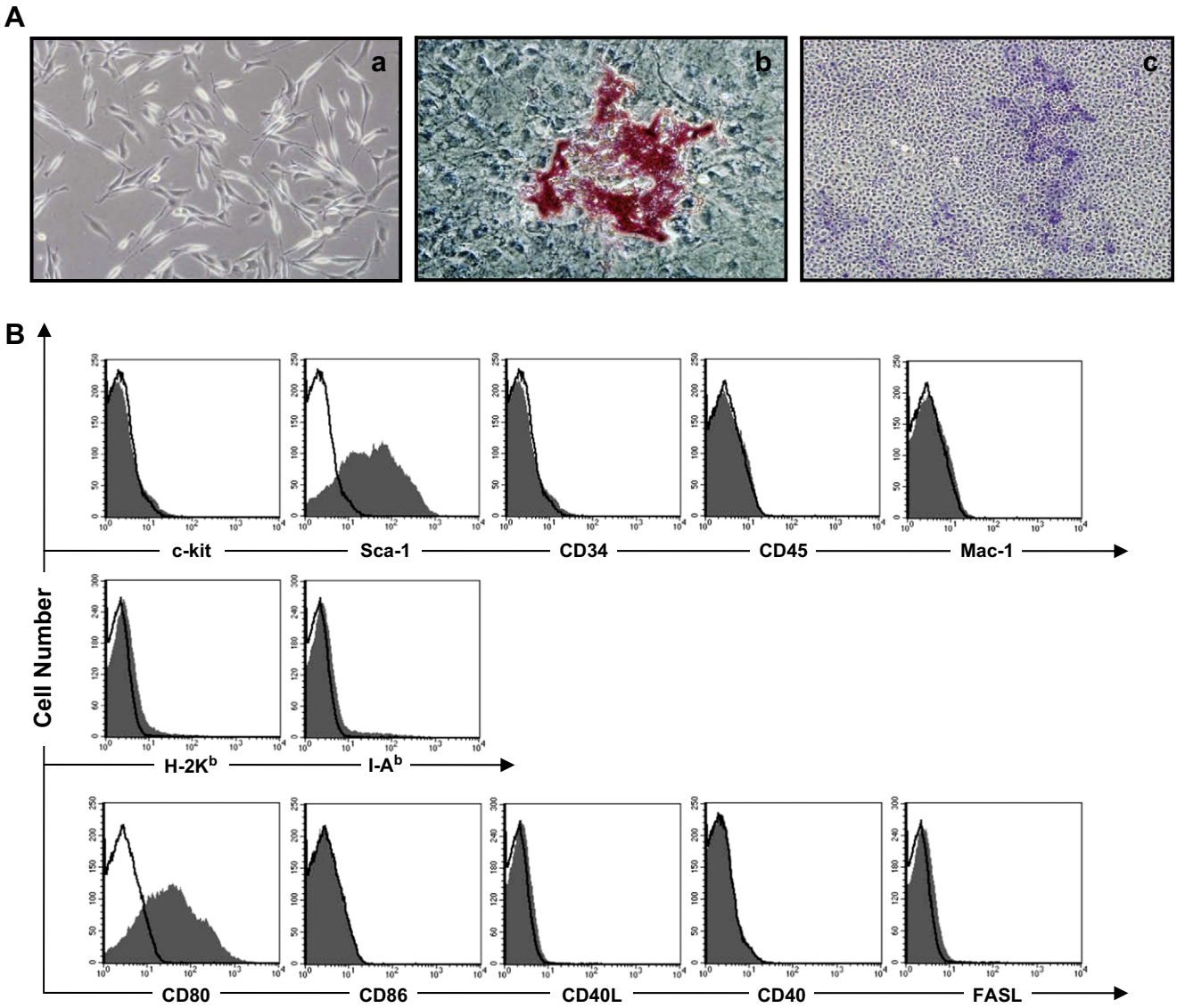


Figure 1. Characteristics of mesenchymal stem cells (MSCs) isolated from mouse bone marrow and expanded in culture: (**A**) MSCs were spindle-shape in monolayer culture (**A-a**, 100×), differentiated into adipocytes (**A-b**, Oil Red O staining, 200×) and osteocytes (**A-c**, alkaline phosphatase staining, 40×); (**B**) fluorescein-activated cell sorting analysis of MSCs using hematopoietic and immunophenotypic markers. Shaded histograms represent cells stained with a specific antibody (Ab) and open histograms represent unstained control cells.

day 3 was examined using RT-PCR, but no clear difference was observed (Fig. 3A). IgM antibody production was significantly lower in the medium taken from the B/MSC cocultures than in the control medium (29.0 ± 1.6 ng/mL vs 69.9 ± 23.2 ng/mL, n = 3; $p < 0.05$) and the IgG3 titer was twofold higher in the coculture medium (5.1 ± 0.7 ng/mL vs 2.9 ± 0.2 ng/mL, n = 3; $p < 0.01$) (Fig. 3B). Furthermore, the $IgG3^+$ B-cell percentage was significantly higher in the cocultures than in the controls (2.1% ± 1.6% vs 0.9% ± 0.2%, n = 5; $p < 0.01$) (Fig. 3C-a, C-b). CFSE labeling revealed the presence of a higher number of $IgG3^+CFSE^-$ B cells in the cocultures than the control cultures (Fig. 3D). These results indicate that MSCs augment IgG3 expression of B cells and influence the Ig class switch recombination from IgM to IgG3.

MSCs also suppress B-cell proliferation, but do not induce plasma cell apoptosis

^{3}H-thymidine incorporation performed on day 2 demonstrated the suppression of LPS-stimulated B-cell proliferation by MSCs (Fig. 4A). At a 5:1 of B and MSC ratio, MSCs suppressed the ^{3}H-thymidine uptake of B cells to approximately half of the control levels (1.6 ± 0.8 × 10^4 cpm vs 2.9 ± 0.1 × 10^4 cpm, n = 3; $p < 0.05$). In contrast, no suppression was observed at a 10:1 ratio. MSCs also suppressed B-cell division as tested on day 3 using

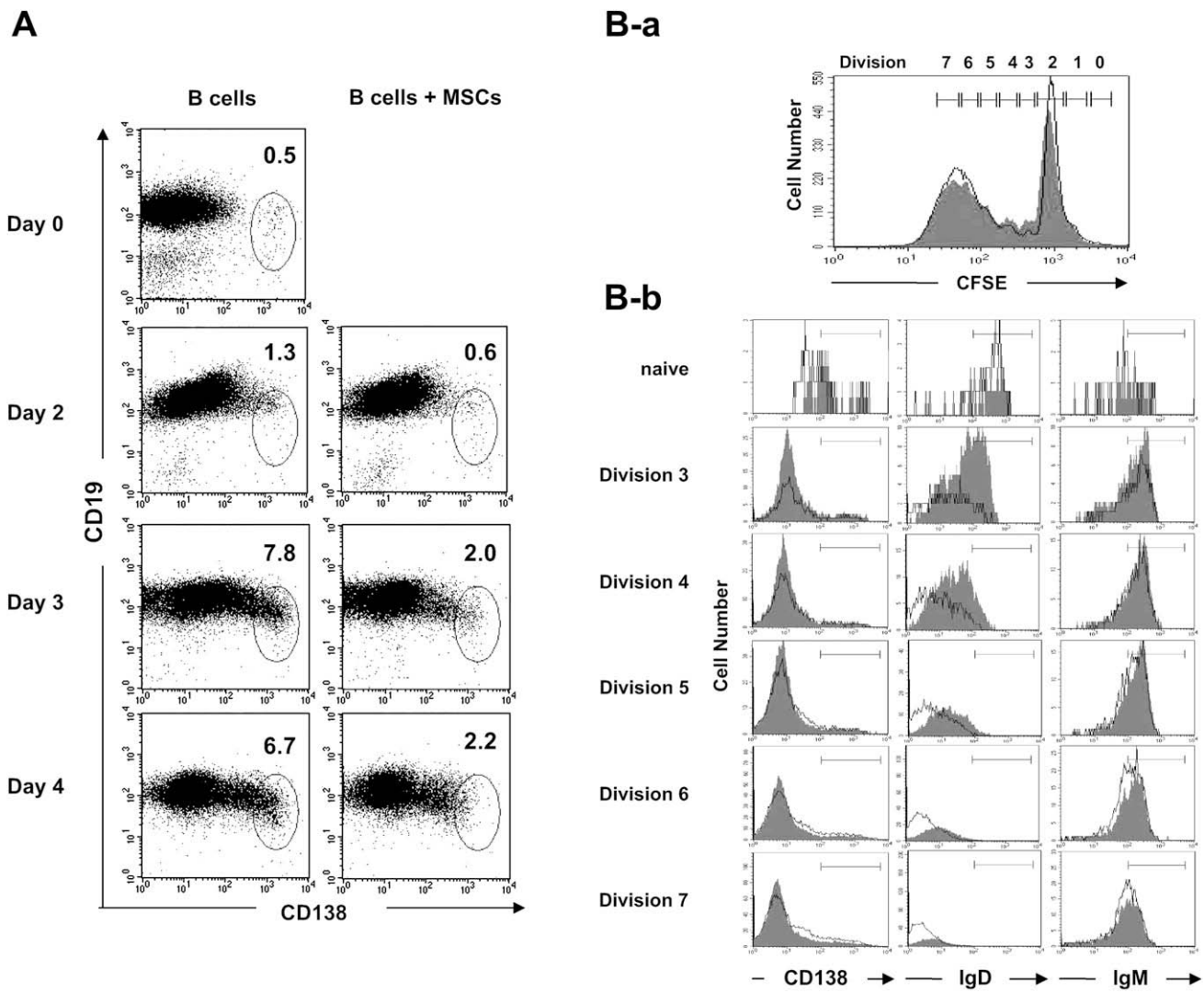


Figure 2. Mesenchymal stem cells (MSCs) inhibit B-cell terminal differentiation: (**A**) CD138 expression was analyzed daily for 4 days by fluorescein-activated cell sorting (FACS) on BALB/c B cells cocultured with C57BL/6 MSCs (1:10 ratio) in separate compartments in the transwell system; (**B-a**) Cell division of the carboxyfluorescein diacetate, succinimidyl ester (CFSE)–labeled B cells cultured with or without MSCs was analyzed by FACS on day 3; (**B-b**) Expression of IgM, IgD, and CD138 on the cocultured (filled histograms) and control (open histograms) B cells was analyzed by FACS (representative of three independent experiments).

CFSE-labeled B cells (Fig. 4B). The number of $CFSE^{low}$ dividing B cells was significantly lower in the B/MSC cocultures at all ratios tested (10:1, 5:1, 2:1) as compared to that of B-cell alone cultures (Fig. 4B). These results show that the suppression of LPS-mediated B-cell proliferation requires higher number of MSCs than that required for the suppression of B-cell differentiation. The number of dividing ($CFSE^{-}$) cells expressing CD138 was significantly lower in the B/MSC cocultures than in the control cultures (2.6% vs 5.4%) (Fig. 4C-a). To determine whether the low $CD138^{+}$ cell number in the cocultures was a result of cell apoptosis, B cells were stained on day 3 for CD138 and Annexin-V. The percentage of Annexin-$V^{+}PI^{-}CD138^{+}$ cells was significantly lower in the B/MSCs than that in B cells alone (10.8% ± 0.5% vs 17.2% ± 0.8%, n = 4; $p <$ 0.01) (Fig. 4C-b and C-c), suggesting that the decreased plasma cell numbers was not due to apoptosis caused by the presence of MSCs.

MSCs downregulate expression of Blimp-1 mRNA during the B-cell terminal differentiation

To examine genes involved in suppression of B-cell terminal differentiation by MSCs, expression of *Blimp-1*, *XBP-1*, *IRF-4*, *PAX*-5, and *Bcl-6* mRNA by B cells was examined using RT-PCR. To compare expression levels, the endogenous mRNA level on day 0 was defined as 1.0. *Blimp-1* expression continuously increased in control B cells during the 3-day culture period (Fig. 5). *Blimp-1* expression was significantly lower in B cells cocultured with MSCs and the difference was highest on day 2

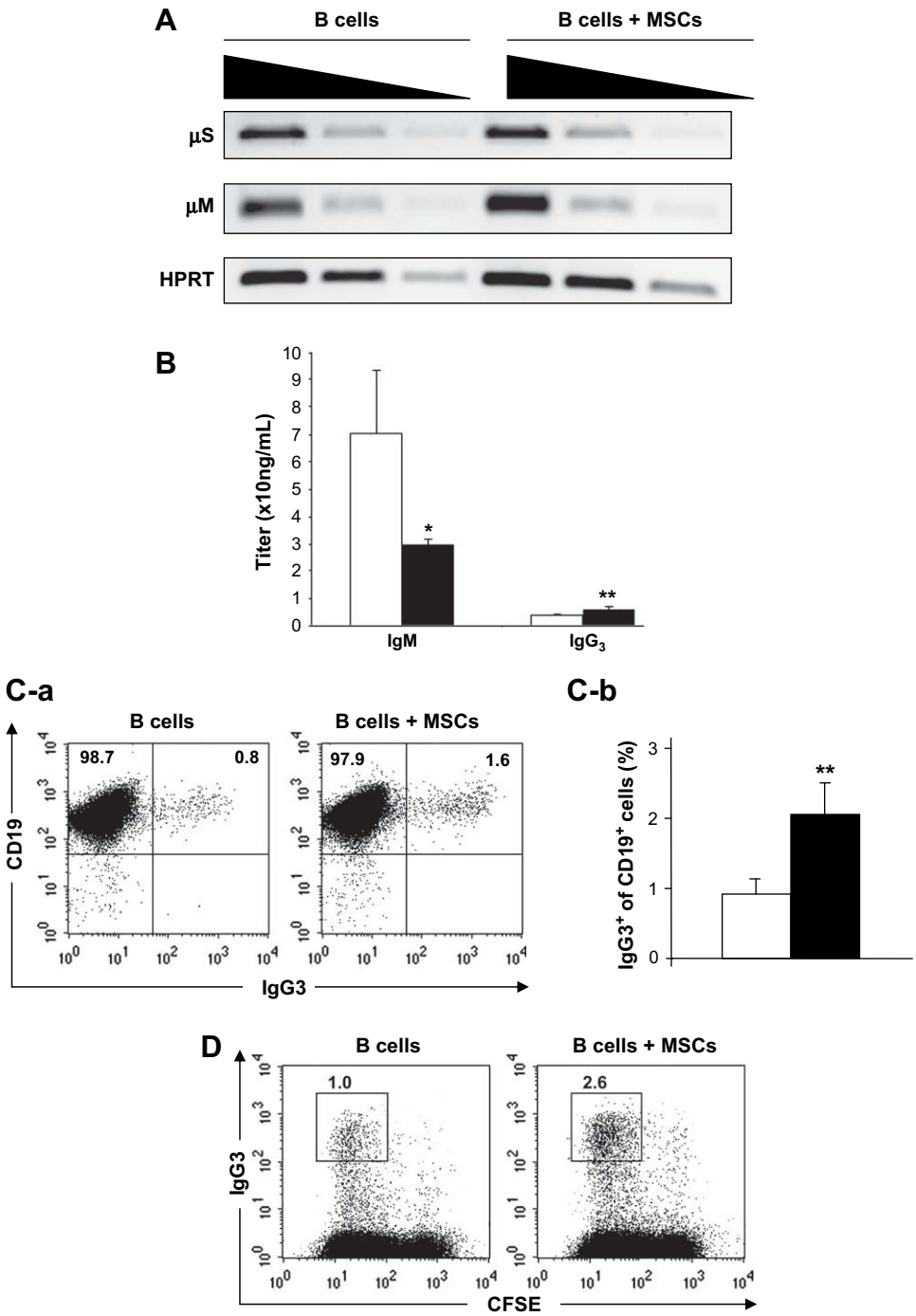


Figure 3. Mesenchymal stem cells (MSCs) selectively suppress lipopolysaccharide (LPS)-stimulated B-cell differentiation into IgM-forming cells and augment IgG3 expression: (**A**) messenger RNA (mRNA) was extracted on day 3 from the B cells cultured with or without MSCs. Expression of *μM* and *μS* mRNA was analyzed by semi-quantitative real-time polymerase chain reaction (RT-PCR). Fourfold dilution series of the complementary DNA were used as input material for the PCR with primers specific for *μM*, *μS*, or for hypoxanthine phosphoribosyltransferase (*HPRT*) as a reference and *HPRT* mRNA as an internal control for the amount of mRNAs (representative of n = 2); (**B**) titers of IgM and IgG3 were measured by enzyme-linked immunosorbent assay in the supernatant of B cells cultured alone (open bar) or cocultured with MSCs (solid bar) for 3 days (titers are shown as mean ± standard deviation, n = 3; $*p < 0.05$; $**p < 0.01$); (**C**) MSCs and carboxyfluorescein diacetate, succinimidyl ester (CFSE)–labeled B cells (1:10 ratio) were cocultured in the presence of LPS. (**C-a**) The numbers indicate $IgG3^-CD19^+$ and $IgG3^+CD19^+$ fractions on day 3; (**C-b**) The percentage of surface $IgG3^+$ cells in $CD19^+$ cells in B cells cultured alone (open bar) or with MSCs (solid bar) (n = 5; $**p < 0.01$); (**D**) The percentages of $CFSE^-$ $CD138^+$ cells were determined on day 3 by fluorescein-activated cell sorting and shown in each square (representative of n = 5).

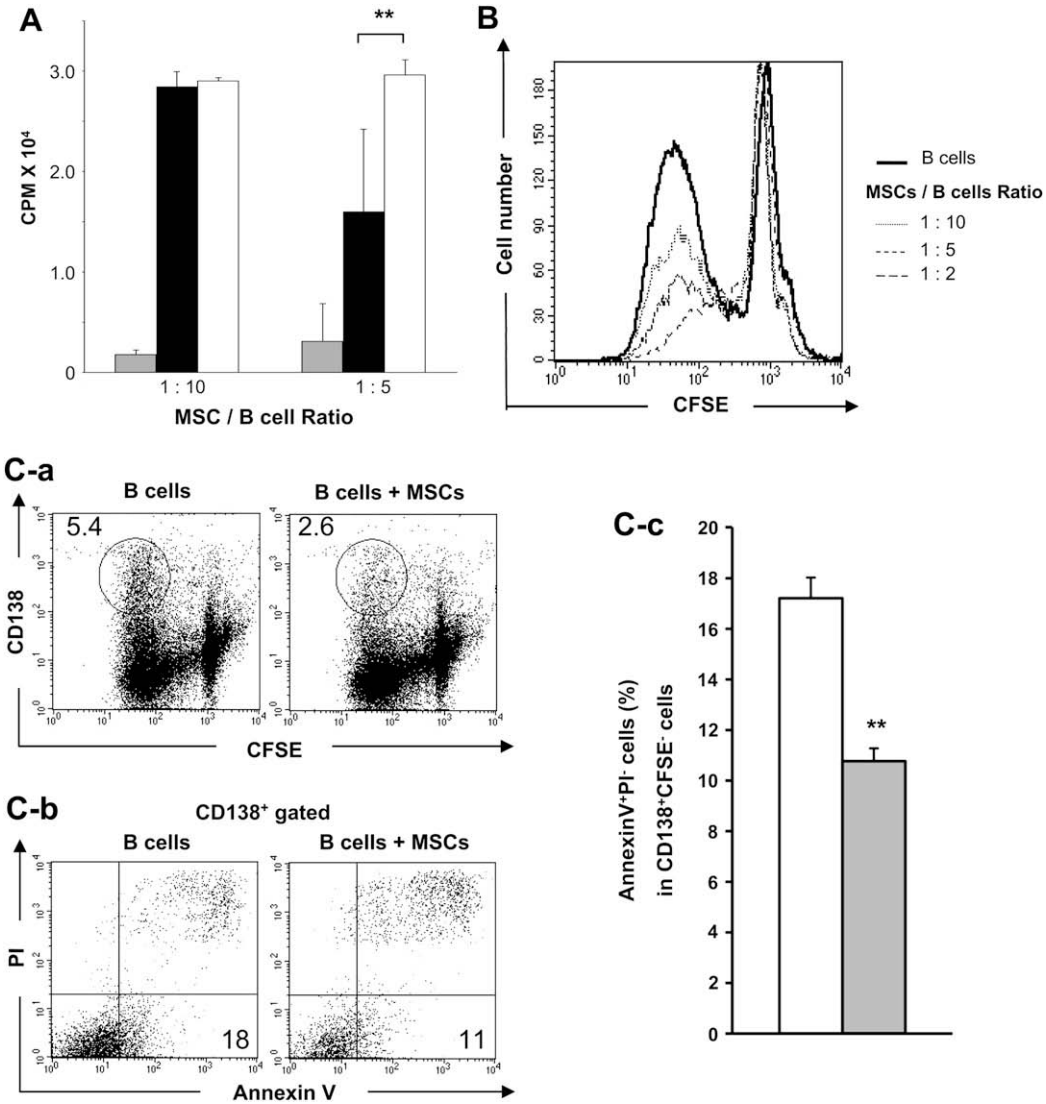


Figure 4. Mesenchymal stem cells (MSCs) suppress B-cell proliferation, but do not induce plasma cell apoptosis: (**A**) ^{3}H-thymidine uptake on day 2. B cells with MSCs at 1:10 or 1:5 ratio (solid bar), B cells cultured alone (open bar), and MSC alone (shaded bar) were cultured in the presence of lipopolysaccharide (LPS) (cpm; mean ± standard deviation, n = 3); (**B**) MSCs and carboxyfluorescein diacetate, succinimidyl ester (CFSE)–labeled B cells were cultured at three different ratios (1:10, 1:5, and 1:2) in transwells in the presence of LPS using B cells alone as controls. Histograms show the B-cell division on day 3; (**C**) MSCs and CFSE-labeled B cells were cocultured (1:10) in the presence of LPS. (**C-a**) The number in each oval indicates the percentage of $CFSE^{-}CD138^{+}$ cells on day 3; (**C-b**) The numbers at low-right corners indicate the percentage of Annexin-$V^{+}PI^{-}$cells in the $CD138^{+}$ population in B/MSC and B alone cultures; (**C-c**) The shaded bar shows the percentage of Annexin-$V^{+}PI^{-}$ cells in $CD138^{+}CFSE^{-}$ cells in B/MSC cultures (10:1) and the open bar shows that in the B-cell alone cultures (mean ± standard deviation, triplicate tests of n = 4; $^{**}p < 0.01$).

(cocultured vs control B cells: 1.5 ± 0.3 vs 6.1 ± 1.5, n = 5; $p < 0.01$). *Blimp-1* expression in the cocultured B cells was also lower on day 3 (10.2 ± 1.7 vs 15.2 ± 4.0, n = 5; $p < 0.05$). There was no significant difference in the *XBP-1* expression in B cells in both groups. The *IRF-4* expression in the cocultured B cells was significantly suppressed only on day 2 (2.0 ± 0.5, vs 2.8 ± 0.4, n = 5; $p < 0.05$) and the *PAX-5* expression was significantly increased as compared to the control B cells (coculture vs control: 0.8 ± 0.2 vs 0.3 ± 0.1 on day 1 and 0.6 ± 0.1 vs 0.3 ± 0.1 on day 3, n = 5; $p < 0.01$). During culture, the *Bcl-6* expression gradually decreased as the *Blimp-1* expression increased in control B cells, while it was expressed significantly higher in cocultured B cells, but only on day 2 (0.7 ± 0.1 vs 0.4 ± 0.1, n = 5; $p < 0.05$). In summary, the expression of *Blimp-1* mRNA was suppressed throughout the culture period in B cells cocultured with MSCs. Conversely, *PAX-5* expression increased. These results demonstrate that MSCs prevent terminal differentiation of B cells by downregulation of *Blimp-1*.

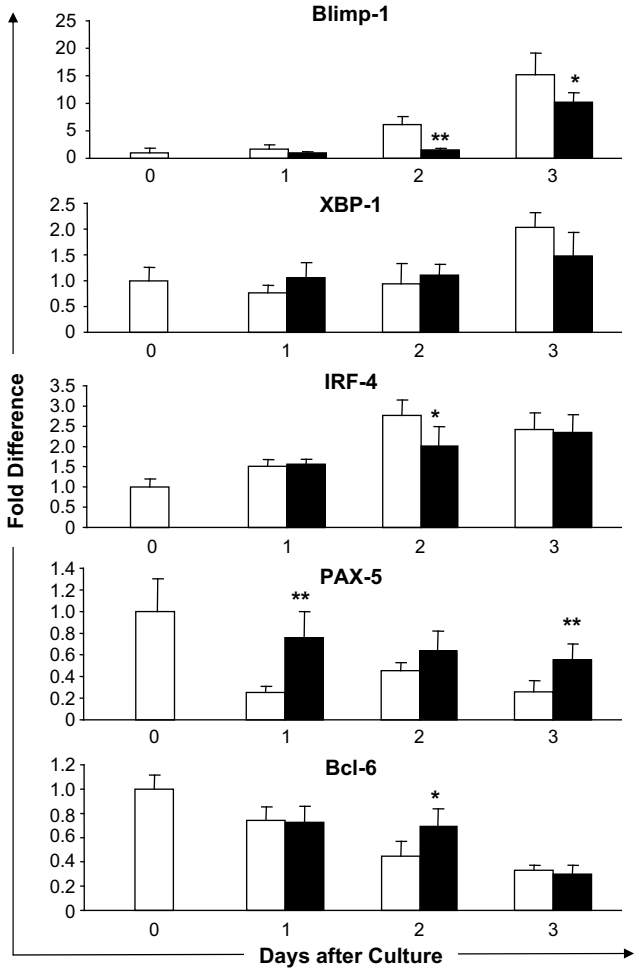


Figure 5. B-lymphocyte–induced maturation protein–1 (*Blimp-1*) expression is suppressed in B cells cocultured with mesenchymal stem cells (MSCs): messenger RNA (mRNA) was extracted from B cells cultured alone or with MSCs in the presence of lipopolysaccharide (LPS) in transwells for 3 days. Expression levels of *Blimp-1*, X-box binding protein–1 (*XBP-1*), interferon regulatory factor–4 (IRF-4), paired box gene–5 (*PAX-5*), and B-cell lymphoma 6 (*Bcl-6*) mRNA were analyzed by real-time polymerase chain reaction. Results were calculated by the comparative threshold cycle (Ct) method, with the Ct for the β-actin used to normalize the results. Expression of each gene was calculated with the endogenous level of the corresponding gene in untreated B cells defined as 1. Each of X-axis indicates the days of culture. Solid and open bars show results of B/MSC cell cocultures and B cell alone, respectively (mean ± standard deviation of 5 mRNA samples from two independent experiments; $*p < 0.05$; $**p < 0.01$).

Inhibition of B-cell differentiation is mediated by MSC-released humoral factor(s)

The culture system used in this study did not allow direct cell–cell contact between MSCs and B cells and, therefore, suppression of B-cell differentiation did not require cell–cell contact and must have been mediated through humoral factor(s) secreted by MSCs. To further confirm this, purified BALB/c B cells were cultured for 3 days in 24-well plates using three different concentrations (100%, 50%, and 25%) of three different conditioned media, CM3, CM4, and CM5 (Table 1). CCM containing 3 μg/mL LPS was used for control cultures, as well as to dilute the test CMs. The CM3 and CM5 cultures containing MSC humoral factor(s) suppressed generation of $CD138^+$ cells, while the CCM and CM4 cultures did not (Fig. 6A).

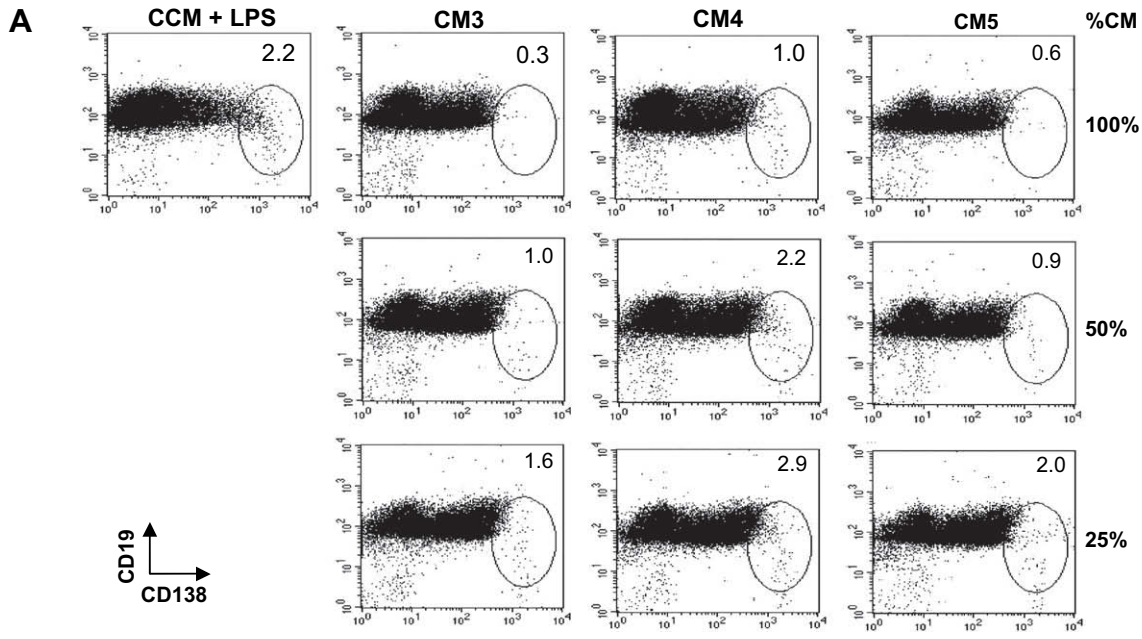
Possible humoral factors involved in B-cell suppression could be cytokines. Therefore, cytokines and chemokines present in CMs were assayed using the Cytokine Antibody Array I. As shown in Figure 6B, only MCP-1 was detected in CM3, MCP-1, and IL-6 were positive in CM5, and no cytokine or chemokine was detected in CM4 derived from the B-cell alone culture. We then tested a possible involvement of MCP-1 in the $CD138^+$ cell suppression by adding anti–MCP-1 monoclonal Ab at various concentrations to B cells cultured in CM3 or CM5. Three days later, only a few $CD138^+$ cells were recovered from all of these cultures, suggesting that MCP-1 was not responsible for suppression of B-cell differentiation (data not shown).

Administration of MSC-derived CMs reduces antigen-specific IgM and IgG1 production in mice immunized with T-independent or T-dependent antigen

To test the suppression of B-cell function by MSC-humoral factor(s) in vivo, test CMs were injected IP to mice immunized with NP_{12}-Ficoll from day 0, or alum-precipitated NP_{19}-KLH from day 2. Serum samples were collected after 2 and 3 weeks for measurement of NP-specific Ab titers. Because some of the injected CMs contained LPS, which might influence the recipient Ab response, the NP-specific Ig titers were compared between the groups injected with CM not containing LPS (PBS vs CM2) and between the groups injected with LPS-containing CM (CM1 vs CM3). In addition, CM2 and CM3 contained MSC products. In mice treated with CM2 or CM3, the titers of all isotypes of NP-specific Igs were lower than those measured in mice administered LPS-free PBS or CM1 (Fig. 7A and B). The NP-specific IgM-titer was significantly lower, with ranges of 51% to 84% in mice immunized with T-D as well as T-ID antigens (Table 2). Similarly, NP-specific IgG1 titer in mice immunized with T-D Ag was reduced to 64% to 69% of the controls. These results clearly show that humoral factor(s) from MSCs released in culture medium are capable of suppressing B-cell function in vivo.

Discussion

Culture-expanded MSCs consistently suppressed the terminal differentiation of B cells into plasma cells. Previous investigations measured MSC effects by ^{3}H-thymidine uptake of B cells stimulated by various other antigens [11,15,26] and all of these studies with one exception [15], showed the MSC's inhibitory effect on B-cell proliferation as we did. However, the recent studies have reported two opposite effects on B-cell differentiation. Two studies



B

Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
Pos	Pos	Neg	Neg	GCSF	GM-CSF	IL-2	IL-3
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13
IL-4	IL-5	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70	IL-13
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
IL-17	IFN-γ	MCP-1	MCP-5	RANTES	SCF	sTNFRI	TNF-α
Tp	VEGF	Blank	Blank	Blank	Blank	Blank	Pos
Tp	VEGF	Blank	Blank	Blank	Blank	Blank	Pos

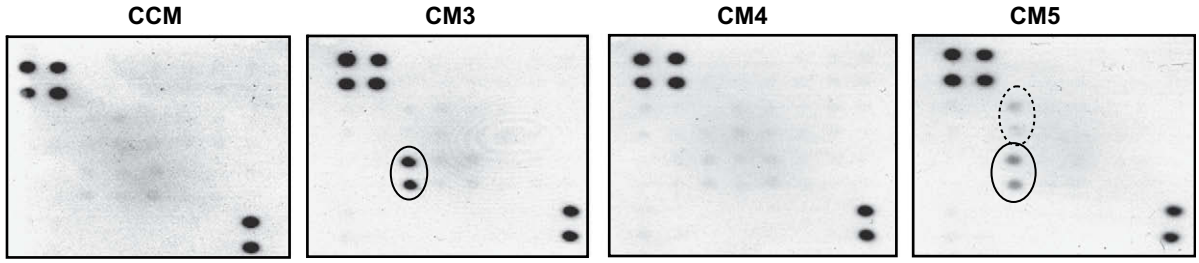


Figure 6. Mesenchymal stem cells (MSCs) prevent B-cell differentiation into plasma cells by releasing humoral factor(s): (**A**) 10^6 B cells were cultured with three different concentrations (100%, 50%, or 25%) of conditioned medium (CM) 3, CM4, or CM5 in 24-well plates for 3 days. CMs containing lipopolysaccharide (LPS) were diluted with LPS-containing complete CM (CCM), and those without LPS were diluted with CCM. B cells cultured with CCM containing LPS were used as controls. The numbers in each oval indicate the percentages of $CD138^+$ $CD19^{+/-}$ cell fractions (n = 3); (**B**) The presence of cytokines/chemokines in CM3, CM4, and CM5 was screened using the RayBio Mouse Cytokine Array I. The layout of the array is shown on the top. The protein array was incubated separately with test CM and CCM as a control. Results are shown at the bottom. Solid dots indicate the signal for monocyte chemotactic protein–1 (MCP-1), and the circled dots indicates the signal for interleukin-6 (IL-6). (Pos = positive control; Neg = negative control; Tp = thrombopoietin). Spot intensity relative to the positive and negative control offers an indication of the relative amount of chemokines/cytokines present in the CM.

showed "suppression of B cell differentiation" [15,27] and the other two showed "augmentation" [28,29]. However, even in the latter studies, Rasmusson et al. found the suppression of LPS-stimulated B-cell differentiation by MSC-secreted humoral factors [28]. These discrepancies may be due to various factors and conditions, including different signaling pathways initiated by the stimuli through the BCR, TLR, or CD40 molecules, via cell–cell contact, or humoral factors, the strength of the stimuli, the species of the MSC origin, the purity of B cells, and/or MSCs. We have shown that suppression of LPS-stimulated B-cell proliferation in vitro requires a higher MSC to B-cell ratio than that required for suppressing B-cell differentiation. Moreover, our study has suggested that the decreased numbers of

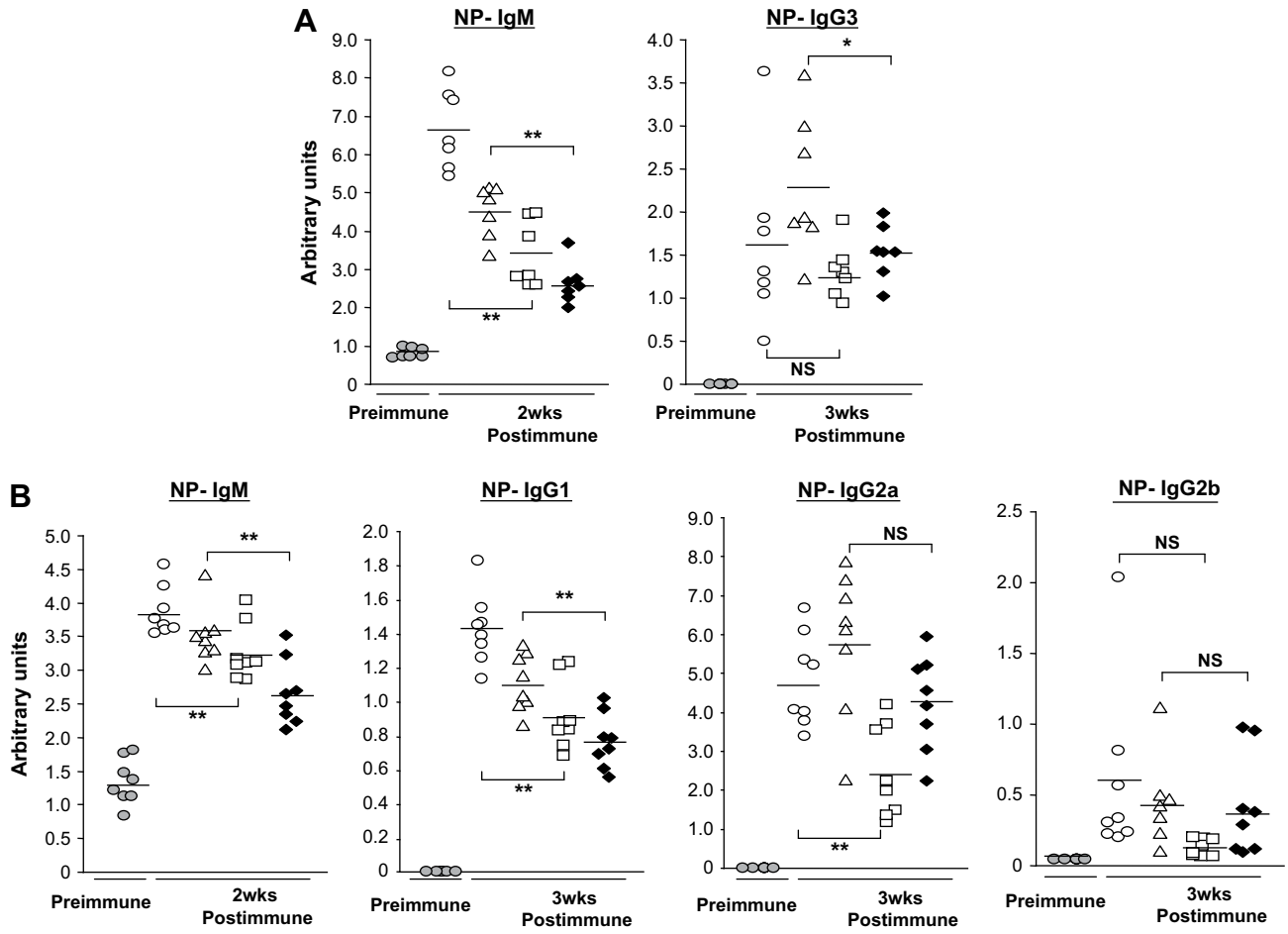


Figure 7. Antigen-specific Ig secretion is suppressed in the immunized mice treated with conditioned medium (CM), a culture supernatant obtained from C57BL/6 MSCs cultures: (**A**) CM or control phosphate-buffered saline (PBS) was injected intraperitoneally (IP) on days 0, 2, and 4 into BALB/c mice immunized on day 0 with 50 μg NP12-Ficoll (T-dependent Ag). Serum samples were analyzed for NP-specific IgM on days 0 and 14, and IgG3 on days 0 and 21; (**B**) CM or PBS was injected IP on days 2, 4, and 6 into BALB/c mice immunized with 50 μg alum-precipitated NP19-KLH (T-independent Ag). Serum samples were analyzed for NP-specific IgM on days 0 and 14, and IgG1, IgG2a, and IgG2b antibodies (Abs) on days 0 and 21. Ab titers were shown as arbitrary units by enzyme-linked immunosorbent assay. Shaded circles, open circles, triangles, squares, or black diamonds represent preimmune, PBS, CM1, CM2, or CM3, respectively (n = 7 for Fig. 7A; n = 8 for Fig. 7B; NS, not significant; $*p < 0.05$; $**p < 0.01$).

differentiated plasma cells in the B/MSC cocultures are not mediated by apoptosis.

Transcription factors Blimp-1 [19], XBP-1 [30], and IRF-4 [31] have been postulated to be the master regulators of B-cell terminal differentiation [16]. Blimp-1 represses the expression of both PAX-5 [32,33] and Bcl-6 [34–36], which are required for preservation of B-cell phenotypes and germinal center reactions. Generation of plasma cells also requires repression of PAX-5 and Bcl-6 expression [16]. Among these genes, only the expression of *Blimp-1* mRNA was continuously suppressed during the 3-day culture period in the B/MSC cocultures, although decreased *Blimp-1* expression may possibly be due to decreased plasma cell numbers. Expression of *PAX-5* mRNA increased relative to *Blimp-1* suppression. TLR-4, bound to LPS on B cells, sends signals to initiate the transcription factors nuclear factor–κB and activator protein–1 [37], which subsequently induce *Blimp-1* expression [38,39]. The *Blimp-1* promoter is directly regulated by Bcl-6 [40] or activator protein–1 [38,39]. Thus, the humoral factor(s) released by MSCs may influence this signaling pathway, leading to suppression of *Blimp-1*. The simultaneous suppression of the *Blimp-1*and *IRF-4* mRNAs

Table 2. Reduction of NP-specific Ig titers by injections of mesenchymal stem cell culture supernatants

Antigen	T -independent		T-dependent			
Antibody isotype	IgM	IgG3	IgM	IgG1	IgG2a	IgG2b
CM2/PBS (no LPS)	51**	81^{NS}	84**	64**	51**	21^{NS}
CM3/CM1 (LPS)	58**	66*	76**	69**	73^{NS}	91^{NS}

Percentage of reductive antigen NP-specific Ig titers was calculated from the average of each group injected with conditioned medium (CM) or phosphate-buffered saline (PBS) (n = 8 for T-independent antigen and n = 7 for T-dependent).
LPS = lipopolysaccharide; NS = not significant.
$*p < 0.05$.
$**p < 0.01$.

on day 2 may further enhance inhibition of B-cell terminal differentiation.

Using the transwell culture system, we have shown that cell–cell contact is not necessary for MSCs to suppress B-cell function. The involvement of the humoral factor(s) was further demonstrated by culturing B cells in MSC culture supernatants (CM3 and CM5). The presence of MCP-1 in both CM3 and CM5 detected by the Cytokine Antibody Array I is consistent with previous finding that MSCs are capable of secreting MCP-1 [41]. However, the addition of anti–MCP-1 monoclonal Ab to the CM3 and CM5 did not inhibit suppression of B-cell differentiation, indicating no direct involvement of this cytokine. MSCs are also shown to secret IL-10, TGF-β [42], and IDO [43] in response to interferon-γ stimulation. IL-10 and TGF-β are representative of suppressive cytokines [44], and IDO is shown to be involved in suppression of T-cell activation by catalyzing tryptophan conversion to kynurenine [45]. However, the Cytokine Antibody Array analysis did not detect IL-10 in MSC culture supernatants. Moreover, neither TGF-β nor IDO appeared to be involved in B-cell suppression as indicated by our neutralizing experiments using anti–TGF-β mAb or 1-methyl-D-tryptophan (data not shown).

Immunomodulatory properties of MSCs on mature B cells have never been investigated in vivo. To determine whether the humoral factors from MSCs can also effectively suppress B-cell function in vivo, Ig titers were measured in serum samples taken from mice immunized with T-ID or T-D Ag and treated with a specific CM. The class switch recombination from IgM to IgG-subtypes in B cells is influenced by various cytokines secreted by $CD4^+$ T cells and antigen-presenting cells. In order to exclude the effects of LPS on T cells and antigen-presenting cells, the results of CMs with or without LPS were compared to those obtained with appropriate controls. These in vivo results have clearly shown a significant reduction of IgM and IgG1 titers specific to NP by mice treated with MSC culture supernatant. We speculate that MSCs and MSC-derived CM would also suppress memory B-cell differentiation into plasma cells, leading to suppression of antibody production, although the timing of CM administration may be critical.

In summary, we have demonstrated that MSCs exert a suppressive effect on the terminal differentiation of B cells both in vitro and in vivo by releasing humoral factor(s). Suppression of B-cell differentiation may be mediated by downregulation of *Blimp-1* expression by MSC-humoral factor(s).

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