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Haematologica 2010 [Epub ahead of print]

Citation: Musso A, Zocchi MR, and Poggi A. Relevance of the mevalonate biosynthetic pathway in the regulation of bone marrow mesenchymal stromal cell-mediated effects on T cell proliferation and B cell survival. Haematologica. 2010; 95:xxx

doi:10.3324/haematol.2010.031633

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Relevance of the mevalonate biosynthetic pathway in the regulation of bone marrow mesenchymal stromal cell-mediated effects on T cell proliferation and B cell survival

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Running title: Mevalonate pathway and BMSC function.

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Key words: statin, bone marrow mesenchymal stromal cells, RhoA, T cell proliferation, immunosuppression, B cell survival, cholesterol.

Funding
This work was supported by grants from Compagnia del San Paolo n.2007.2065 to AP, from Final. Min. Sal. 2006 (Targeting tumor-related immunosuppression for new combined approaches of immunotherapy), Final.Min.Sal.2008 (Innovative therapeutic models for the treatment of hematological malignancies) and AIRC2009 to MRZ (IG8727) and AP (IG8761).
Abstract

Background. Bone marrow mesenchymal stromal cells can suppress T lymphocyte proliferation, but promote survival of normal and malignant B cells, thus representing a possible target for new therapeutic schemes. Herein, we defined the effects of cholesterol synthesis inhibitors on the interaction between these mesenchymal stromal cells and T or B lymphocytes.

Design and Methods. We exposed mesenchymal stromal cells to inhibitors, as fluvastatin, of the 3-hydroxy-3-methylglutaryl-coenzymeA reductase, responsible for the synthesis of mevalonate, the precursor of cholesterol. Also, these cells were treated with manumycin A, a farnesyl transferase inhibitor which blocks the mevalonate-dependent isoprenylation of small guanosin triphosphate binding proteins. First, mesenchymal stromal cells morphology, cytoskeleton assembly, cell cycle, survival and cytokine production were evaluated. Then, these cells were co-cultured with either T or B lymphocytes and we analyzed: 1) the inhibition of T cell proliferation to mitogenic stimuli; 2) B cell survival.

Results. Fluvastatin altered the assembly of actin microfilaments, inactivated RhoA guanosin triphosphate binding protein, inhibited the S-phase of the cell cycle, induced apoptosis in a small fraction of cells but preserved cytokine production. Preincubation of mesenchymal stromal cells with fluvastatin, or manumycin A, down-regulated the expression of adhesion molecules, reduced cell-to-cell interactions and prevented the inhibition exerted by these stromal cells on CD3/T cell receptor-induced lymphocyte proliferation. Mevalonic acid could revert morphologic, phenotypic and functional effects of fluvastatin. Finally, fluvastatin significantly reduced the mesenchymal stromal cells-mediated rescue of B cells in the presence of dexamethasone, although it did not function in the absence of corticosteroids.

Conclusions. Fluvastatin-mediated effects on bone marrow mesenchymal stromal cells were conceivably due to the inhibition of isoprenylation of small guanosin triphosphate binding proteins, occurring for the lack of mevalonate. Altogether these findings suggest that drugs acting on the mevalonate biosynthetic pathway can regulate mesenchymal stromal cells-induced T cell suppression and B lymphocyte survival.
Introduction

Bone marrow mesenchymal stromal cells (BMSC) play a key role in driving proliferation and maturation of hematologic cell precursors in the bone marrow niche (1-4). In addition, the interaction between BMSC and effector lymphocytes can lead to the suppression of T and natural killer cell proliferation and effector function (5-16). Thus, BMSC-mediated immunosuppression has been exploited to favor engraftment after bone marrow transplantation and limit graft-versus-host-disease (GVHD) (17,18). In turn, BMSC can promote survival, proliferation and differentiation of B lymphocytes (19); also, BMSC-mediated immunosuppression has been reported to favor tumor growth in animal models (20). Recently, BMSC have been considered a target for the therapy of hematological malignancies as it is becoming evident that these cells can help neoplastic stem cells to proliferate and evade the immune-mediated control (15,16, 20-22). Indeed, the use of thalidomide or lenalidomide, hitting the mesenchymal stromal component besides myeloma cells, has been introduced in the treatment of multiple myeloma, with striking amelioration of prognosis of this disease (20-22). More recently, it has been proposed to use lenalidomide together with statins (22-24): these drugs are inhibitors of the enzyme 3-hydroxy-3-methylglutaryl-CoenzymeA (HMG-CoA) reductase involved in cholesterol biosynthesis and are commonly used for the treatment of hypercholesterolemia (25-27). Statins are also able to affect proliferation of smooth muscle cells and inhibit lymphocyte function both in vitro and in vivo (25-27). Many effects of statins depend on the disruption or depletion of membrane rafts: lipid rafts are cholesterol rich membrane micro-domains that play a key role in signal transduction mechanisms (28,29). In addition, statins reduced the production of isoprenoids intermediates responsible for the activation of small guanosin triphosphate (GTP) binding proteins of Rho and Ras family involved in the regulation of actin cytoskeleton assembly and proliferation, respectively (30,31).

Herein, we have analyzed the effects of the HMG-CoA reductase inhibitor fluvastatin on the morphology, phenotype and function of BMSC. We found that BMSC–mediated inhibition of T cell proliferation is fully prevented by fluvastatin pre-treatment of BMSC, mainly due to the lack of
isoprenylation of small GTP binding proteins; this effect was reverted by mevalonic acid, the metabolic product of HMG-CoA reductase. Of note, in the presence of dexamethasone, fluvastatin treatment reduced the BMSC-mediated rescue of B cells, although it did not inhibit the pro-survival signals delivered by BMSC to B lymphocytes in the absence of corticosteroids.

**Design and Methods**

**Monoclonal antibodies (mAbs) and reagents**

The anti-CD45 mAbs (TA218/12, IgM; T205/23; IgM), the anti-CD31 mAb (89D3), the anti-CD16 mAbs (NK1, IgG1; NK54, IgG2a), the anti-CD18 mAb (70H12, IgG2a), the anti-CD54 mAb (ICAM1, clone SM89, IgM), the anti-CD44 mAbs (T61/12, IgG1, TA153/G8, IgG2a) were obtained in our laboratory as described (32). The anti-CD3 mAb (UCHT-1, IgG1) was from Ancell (Bayport, MN55003, USA). The anti-HLA class-I W6/32 (IgG2a), the anti-SH2 (CD105, IgG1), the anti-SH3 (CD73, IgG2b), the anti-SH4 (IgG1), the anti-CD34 (clone IgG1) the anti-CD11a (LFA1α, TS1.22, IgG1), the anti-CD18 (LFA1β, TS1.18, IgG1) producing hybridomas were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Anti-HLA class I mAb (clone 3A3, IgM) and anti-CD14 mAb (63D3, IgG1) were kindly provided by E. Ciccone (Institute of Anatomy, University of Genoa) and D. Vercelli (Scientific Institute San Raffaele, Milan) respectively. The anti-β1 integrin (CD29) mAb (3E1, IgG1) was a kind gift of Dr L. Zardi (IST-Genoa). The anti-ICAM2 and anti-ICAM3 mAbs were from Bender MedSystem (CA 94010, USA). The anti-prolyl-4-hydroxylase mAb (clone 5B5, IgG1) was purchased from Dako (Denmark). Phytohemagglutinin (PHA) was from Sigma Chemicals Co. (St. Louis, MO, USA) and recombinant (r) IL10 was from PeproTech EC (PeproTech, Rocky Hill, NJ, USA). Complete medium was composed of RPMI1640 (Biochrom, Berlin, Germany) with 10% of fetal calf serum (FCS, Biochrom) supplemented with 1% antibiotics (penicillin and streptomycin) and 1% L-glutamine (Biochrom). The farnesyl and geranylgeranyl transferase inhibitors FTI-I, GGTI-286 and manumycin A, the HMG-CoA reductase inhibitors mevastatin, atorvastatin, simvastatin, fluvastatin,
the cyclo-oxigenase 2 inhibitors indomethacin ester-4-methoxy-phenyl and NS-398 were purchased from Calbiochem (EMD Biosciences Inc. San Diego, CA, USA), mevalonic acid, anti-vimentin, anti-tubulin-α mAbs, DMSO and dexamethasone were purchased from Sigma (Sigma-Aldrich, 20151, Milan, Italy). Alexafluor488-conjugated phalloidin was from Invitrogen (Invitrogen S.r.l., Molecular Probes Brand, San Giuliano Milanese, Italy).

**Cells and drug treatment**

Peripheral blood mononuclear cells (PBMC) were obtained from venous blood samples of healthy donors after Ficoll-Hypaque density gradient separation (33). In some experiments B cells from PBMC were obtained using RosetteSep negative separation kit (StemCell Technologies SARL, 3800 Grenoble, France). The purity of B cell populations was always more than 95% (n=10) (33). BMSC were obtained by culturing bone marrow cell suspensions from healthy donors (Ethics Committee n. 0026910/07 renewed on 03/2009) for bone marrow transplantation (11). BMSC expressed SH3/CD73, SH4, SH2/CD105, CD44, β1-integrin/CD29, ICAM1/CD54, HLA-I, prolyl-4-hydroxylase, alkaline phoshatase, collagen, vimentin, bone sialoprotein, and osteopontin but not CD45, CD31, CD34, CD33, CD3, CD2, CD16, CD14, ICAM2, ICAM3, CD80, CD86, CD83, and HLA-DR (not shown). BMSC were treated for different time periods (24-36-48-72h) with each statin (10-1.0-0.1µM). On the basis of preliminary experiments, fluvastatin was chosen as a prototype of these drugs. Pre-incubation with 10µM for 24-48h of fluvastatin was determined as the optimal concentration to obtain phenotypic and functional effects on BMSC without inducing remarkable apoptotic effects. The inhibitors of farnesyl and/or geranylgeranyl transferase were used at 10µM and DMSO (solvent of all these inhibitors) at 1:1000 in culture medium. Among the three inhibitors, manumycin A was chosen as FTI-I and GGTI-286 were less potent at the 10µM concentration and they did not exert constant and reproducible inhibition of RhoA GTP-binding protein. The cyclo-oxigenase 2 inhibitors indomethacin ester-4-methoxy-phenyl and NS-398 were
used at 10-1-0.1μM. The 10μM concentration was used in experiments aimed to determine the role of PGE$_2$ in inducing inhibition of T cell proliferation as reported (9, 16). Indeed, at this concentration the production of PGE$_2$ during BMSC-PBMC culture was almost abolished (not shown). After extensive washes, BMSC were analyzed for morphology, surface phenotype and cytokine production or used in co-culture experiments with lymphocytes. In some experiments, 1mM of L-mevalonate (solubilized in ethanol 1:1000), the metabolic product of HMG-CoA reductase, was added at the onset of the treatment with statins.

**Analysis of cell cycle and apoptotic assay**

Analysis of cell cycle was performed on BMSC cultured in the different conditions described and harvested at the indicated time points. Cell cycle was analyzed after permeabilization and labelling with PI as described (34). Samples were run on a FACScalibur cytofluorimeter (Becton Dickinson, Paolo Alto, USA) and analyzed with the Modfit program for cell cycle analysis. Apoptotic cells were evaluated using FITC-annexin V and PI labelling. In some experiments, apoptotic cells were identified after permeabilization and PI staining as cells with a <2n DNA content as described (34). Irradiated BMSC (5000rads) were used to compare the effects of each drug with an effective apoptotic signal (35).

**Immunofluorescence and cytofluorimetric analysis**

Immunofluorescence on either BMSC or lymphocytes was performed with the indicated mAbs followed by the addition of anti-isotype specific goat anti-mouse (GAM) antisera (Southern Biotechnology, CA, USA) conjugated with phycoerythrin (PE), with fluorescein isothyocianate (FITC) or with AlexaFluor647 (Invitrogen) as indicated. Control samples were stained with isotype-matched irrelevant mAb (Becton Dickinson) followed by anti-isotype specific GAM-PE or GAM-FITC or GAM-AlexaFluor647. Samples treated with manumycin A were stained with antibody and GAM-AlexaFluor647 as manumycin A-treated cells did not show auto-fluorescence in far-red field.
Samples were run on a FACScalibur (Becton Dickinson). Data were analyzed using CellQuest computer program and are expressed as Log fluorescence intensity vs. number of cells or as mean fluorescence intensity (MFI, a.u.).

**BMSC-PBMC co-cultures and evaluation of T cell proliferation**

2x10⁴/sample BMSC were incubated for 48h at 37°C in 5% CO₂ atmosphere with one or another combination of drugs, washed and then co-cultured with 10⁵ PBMC at 1:5 ratio and proliferation of PBMC to either PHA (1µg/ml) or anti-CD3 mAb (JT3A, IgG2a, 20ng/ml) (32) was evaluated after four days of culture: indeed, in preliminary experiments this was the time by which maximal proliferation of PBMC alone to these stimuli was achieved. Cell proliferation was assessed after labelling PBMC with carboxy fluorescein succinimidyel ester (CFSE, Invitrogen s.r.l., Molecular Probes, Carlsbad, CA) according to manufacturer’s instructions, by cytofluorimetric evaluation of progressive loss of this fluorochrome along time, proportional to cell division (36) on gated T cells labeled with anti-CD3 mAb (UCHT-1, IgG1). In some experiments cell proliferation was also evaluated using the ATPlite kit (Perkin Elmer Italia, Milan, Italy). In preliminary experiments, these two assays gave comparable results to assays based on uptake of radiolabelled thymidine. To determine the effect of soluble factors on T cell proliferation, PBMC were seeded on Millicell transwell (TW) with 0.3µm pores (Millipore Corporation, Billerica, MA) put in 24w plates with BMSC seeded on the bottom to avoid BMSC-PBMC contact. Moreover, supernatants (SN) obtained from PBMC, BMSC or BMSC, either treated or not with different drugs, washed and co-cultured with PBMC, were added, 1:2 diluted, at the onset of PBMC cultures stimulated with either PHA or anti-CD3 and proliferation was assessed as described above.
Cytokine detection assays

$10^5$/sample PBMC (responder, R) were cultured in 24 well plates for 24h alone or with BMSC, treated as described with one or another combination of drugs, at 1:4 BMSC:R ratio in RPMI1640 complete medium at 37°C. After 24h, culture supernatants (SN) were harvested. To evaluate constitutive production of cytokines, BMSC were cultured alone for 48h and SN were harvested. The SN were analyzed for the content of PGE$_2$ by the ELISA kit (Cayman Chemical, Ann Harbor, Michigan 48108, USA) following the manufacture’s instructions. In some experiments, SN were analyzed with Multiplex Cytokine Kit (Bender MedSystem GmbH, Vienna, Austria) for detection of interleukin (IL)6, IL8 and IL10. TGF-β1 content was analyzed after treatment for 1h of each SN with 1N HCl followed by 1N NaOH with a TGFβ1 specific kit (Bender MedSystem). Culture SN collected for these experiments were also used to evaluate kynurenine concentration by spectrophotometric analysis as described (16).

Evaluation of RhoA activation

To analyze the effect of fluvastatin and farnesyl transferase inhibitors on RhoA activation, BMSC were incubated for 24h in medium alone or in medium containing fluvastatin 10µM or manumycin A 10µM with or without L-mevalonate 1mM. Thus, BMSC were harvested and lysed. Cell extracts were equalized to obtain identical protein concentrations and then were used to perform G-LISA test following the manufacturer’s instruction (Cytoskeleton Inc., Denver, CO 80223, USA). The concentration of activated RhoA in each sample was evaluated comparing sample absorbance at 490nm with a standard curve with known amounts of active RhoA protein.
Cytoskelektton analysis by confocal microscopy

2.5x10^4 BMSC seeded on 0.2mm thin round glass slides were incubated for 24h with one or another combination of drugs. Then, cells were fixed with formaldehyde 1% and permeabilized with 0,1% Triton-X-100. Each slide was stained with AlexaFluor488-conjugated phalloidin, anti-tubulin α mAb and anti-ICAM1 mAb for 30min at 4°C. After extensive washes, slides were incubated with anti-isotype GAM-AlexaFluor535 for anti-β-tubulin and with GAM-AlexaFluor647 for anti-ICAM1. Samples were then analyzed by FV500 (Fluoview confocal Laser Scanning Microscope System, Olympus Europe GMBH, Hamburg, Germany) equipped with an Argon laser to excite AlexaFluor488, a He-Neon Green laser at 513nm to excite AlexaFluor535 and a He-Neon red laser at 633nm to excite AlexaFluor647 dye associated to a IX81 motorized microscope (Olympus). Samples were observed with PlanApo 40x NA1.00 or 60x NA1.40 oil objectives and data analyzed with FluoView 4.3b computer program (Olympus). Each image has been taken in sequence mode to avoid cross-contribution of each fluorochrome. Results are shown in pseudocolor.

Analysis of pro-survival effect of BMSC on B cells

B cells were isolated from healthy donors PBMC using the Rosettesep B cell enrichment kit (Stemcell Technologies) as previously reported (33). The resulting cell populations was always >95% CD20^+ (range of 6 experiments 95-100%). B cells were seeded with BMSC at the ratio of 10:1. This ratio was the optimal to detect the pro-survival effect of BMSC on B cells. Samples were analyzed on day 3, 5 and 7 for apoptotic B cells after FITC-annexin-V and PI staining. Day 5 was chosen as in preliminary experiments (n=10) at this day more than 40% of B cells were dying in the absence of BMSC and less than 20% of B cells were dying in the presence of BMSC. Also, BMSC were treated with one or another drug as indicated above, washed and used in co-culture experiments. The corticosteroid dexamethasone was added at 10^{-6}-10^{-7}-10^{-8}M and results obtained with 10^{-7}M are shown (37). In some experiments, to determine the role of soluble factors produced
by BMSC on B cell survival, B cells were seeded in transwell (TW) with 0.3\(\mu\)m pores (Millipore) put in 24w plates with BMSC seeded on the bottom to avoid BMSC-B cell contact.

**Statistical analysis**

Results were analyzed by applying the one tail student t test at 95% confidence. This test was chosen as drug treatment led to modifications of the mean of experimental values in one way. The statistical significance is shown in each figure. The Pearson coefficient of correlation \(r\) was determined between the amount of IL10 or PGE\(_2\) present in the SN from BMSC-PBMC co-culture and the degree of percent of maximal T cell proliferation.

**Results**

**Fluvastatin inhibits the activation of RhoA and alters actin microfilament distribution in BMSC**

Inhibition of HMG-CoA reductase blocks the production of mevalonate which is involved in the synthesis of geranyl and farnesyl pyrophosphates. In turn, these molecules are responsible for cholesterol biosynthesis and isoprenylation of small GTP-binding protein (25-27, 29-31). Isoprenylation is essential for activation of the cytoskeletal associated protein RhoA; the correct function of RhoA determines the redistribution of actin microfilaments during adhesion to substrate or in cell-to-cell interactions (29-31). Thus, we analyzed the morphology of BMSC and the distribution of actin-filaments in BMSC exposed to the competitive HMG-CoA reductase inhibitor fluvastatin. Bright field analysis (fig.1A) revealed that BMSC displayed a fibroblasts-like morphology which was lost when cells were incubated with fluvastatin. Indeed, upon fluvastatin treatment, BMSC appeared as smaller cells than their untreated counterpart. L-mevalonate, the
metabolic product of HMG-CoA reductase, blocked the fluvastatin effect. Also manumycin A, a potent inhibitor of farnesyl transferase which catalyses the transfer of farnesyl pyrophosphates and produce isoprenoid derivatives responsible for the ADP-ribosylation and activation of RhoA (29,30), altered the morphology of BMSC. In this case, the addition of L-mevalonate did not revert the effect of manumycin A (fig.1A). Confocal microscopy analysis showed that cultured BMSC adhered to the substrate displaying a flattened shape and actin microfilaments were organized in linear subcellular structures both at the periphery and centre of the cell (fig. 1B and at higher magnification fig.1C). This distribution was completely lost in BMSC pre-incubated with fluvastatin (10μM for 24h) whereas the distribution of tubulin microtubules was not affected (fig. 1B). A similar effect on actin distribution was detected in BMSC treated with manumycin A (fig.1D). The addition of L-mevalonate to fluvastatin, at the onset of cell culture, completely restored the actin distribution in BMSC (figure 1B). The observed morphologic alterations were also confirmed by measuring the perimeter and the area of BMSC cultured in the presence of fluvastatin or manumycin A (fig. 1E and 1F). The evaluation of the amount of activated RhoA present in BMSC revealed that this GTP-binding protein is strongly activated in BMSC but not in fluvastatin pre-incubated cells; L-mevalonate did block the effect of fluvastatin (fig.1G). Manumycin A strongly reduced the amount of RhoA activated protein present in BMSC and L-mevalonate did not prevent the effect of manumycin A (fig.1G).

**BMSC treated with fluvastatin show reduced expression of molecules involved in cell-to-cell interaction**

Interaction between myeloma cells and BMSC has been shown to contribute to multidrug resistance; this phenomenon, termed cell adhesion-mediated drug resistance, can be partially overcome by statins in vitro, due to their effects on GTP binding proteins (23). Thus, we analyzed the effect of fluvastatin on the surface phenotype of BMSC. As shown in fig.2A, we found that the expression of some surface molecules involved in BMSC-lymphocyte interaction, was reduced,
although still detectable by pre-incubation of BMSC with fluvastatin. This effect was markedly evident on HLA-I (p<0.0012) and CD105 (p<0.001) with a reduction of about 50% of mean fluorescence intensity (fig.2B). The expression of ICAM1 (p<0.0256), and CD29/β1 integrin, (p<0.0495) was also affected by fluvastatin treatment but to a lesser extent (fig.2A and 2B). Although not shown, also LFA3 and CD44 molecules were expressed at a lower level in fluvastatin-treated BMSC. No effect was detectable in solvent treated cells. When BMSC were pre-incubated with fluvastatin and L-mevalonate, surface expression of all these molecules was rescued (fig.2A and 2B). Also, pre-incubation of BMSC with manumycin A reduced the expression of the above indicated surface molecules (fig.2C and 2D). However, upon treatment with manumycin A only the decrement of HLA-I expression was statistically significant (fig.2D). The addition of L-mevalonate did not revert the effect of manumycin A (not shown).

Fluvastatin impairs the S-phase of the cell cycle in BMSC but it does not induce apoptosis nor affects secretion of constitutive cytokines

Due to its action on cytoskeleton and RhoA GTP-binding proteins, we analyzed the effect of fluvastatin on cell cycle progression of BMSC, on BMSC survival and on constitutive release of cytokines by BMSC. Indeed, other GTP-binding protein, as Ras and Raf, involved in the regulation of cell proliferation can be affected by fluvastatin as previously reported (25,27); in addition, secretion of cytokines may occur via granule exocytosis depending on cytoskeleton activity (25,27,31). We found that fluvastatin strongly reduced or even abrogated the S phase of the cell cycle, partially affected G0/G1 phase but not G2/M phase after 48h of treatment (fig.3A and 3B). These results were indeed statistically significant for G0/G1 and S phases (fig.3B). It is of note that L-mevalonate completely restored the S phase of the cell cycle. Upon removal of fluvastatin from culture medium a strong progression of BMSC from G0/G1 phase to S phase of the cell cycle was observed by 24h (fig.3B). This suggests that the effects of fluvastatin treatment on BMSC proliferation are fully reversible. In parallel experiments, we analyzed whether fluvastatin can affect
BMSC survival. To this aim, BMSC were incubated with 10µM fluvastatin for 24-48-72h and apoptosis analyzed after annexin V and PI staining. We found that the percentages of dying cells in fluvastatin-treated BMSC cultures, after 24h, were comparable to those detected in control cultures (fig.4C). As shown in fig. 4D, the amount of dying cells in fluvastatin cultures was not statistically significant at 48h of incubation compared to solvent-treated cells. Although not shown, at 72h of treatment about 50-60% of BMSC were dying. Importantly, when fluvastatin was washed out after 48h treatment, the apoptotic BMSC detected after additional 24h of culture were similar to those detected in control cultures (not shown). Thus, almost all BMSC were living cells after 48h fluvastatin treatment and washing out this drug. In parallel experiments, we analyzed the effect of irradiation on BMSC. These experiments have been performed to determine BMSC sensitivity to an effective apoptotic stimulus. We found that after irradiation the number of dying cells progressively increased along time. Indeed, at 48hr and 72hr after irradiation more than 50% and 85% of BMSC were dying respectively (not shown). Finally, we analyzed the possible effects of fluvastatin on the release of IL6 and IL8 that are known to be constitutively produced by BMSC (9,14). We observed that fluvastatin apparently did not inhibit the constitutive secretion of IL6 and IL8 (fig.3E and F). Alltogether, these finding indicate that treatment of BMSC for 48h does not impair the survival of BMSC and their ability to produce the cytokines analyzed.

**Fluvastatin inhibits BMSC immunosuppressive effect on T cell proliferation**

BMSC can inhibit T cell proliferation thus exerting a potent immunosuppressive effect (5-15). Thus, we analyzed whether this effect was affected by fluvastatin. To this aim, BMSC were treated for 48h with fluvastatin, washed and used for co-culture experiments with CFSE-labelled PBMC at the 1:5 BMSC:PBMC ratio and proliferation of T cells was analyzed by staining cell cultures with anti-CD3 mAb to gate T cells: reduction of CFSE content along time was proportional to cell proliferation. As shown in figure 4A, evident clumps of PBMC were detectable in the presence of PHA. These clumps were not evident in PBMC cultured with PHA and BMSC. In PBMC co-
cultures with fluvastatin-pretreated BMSC, lymphocyte cell clumps were evident again and this effect was abrogated when BMSC were incubated with fluvastatin and L-mevalonate (fig.4A). As shown in figure 4B, BMSC strongly inhibited T cell proliferation induced by the polyclonal mitogen PHA or through the engagement of the CD3/TCR complex. This effect was almost completely abolished by 48h pre-incubation of BMSC with fluvastatin 10µM (fig.4B, D and E). This inhibiting effect was not detected when BMSC were pre-incubated with 1µM fluvastatin for 48h (not shown). BMSC-mediated inhibition was completely restored when BMSC were pre-incubated with fluvastatin and L-mevalonate (the metabolic product of HMG-CoA reductase) (fig. 4B). Ten micromolar concentration of other HMG-CoA reductase inhibitors as mevastatin, atorvastatin, simvastatin exerted superimposable effects to those of fluvastatin on BMSC (not shown). Also treatment with manumycin A strongly affects the BMSC-mediated inhibition of T cell proliferation (fig.4C, D and E). In parallel experiments, we analyzed whether BMSC could exert any inhibition on T cell proliferation when PBMC were separated from BMSC by a transwell (TW) (fig.4F) and whether fluvastatin could affect also this inhibiting effect. In this culture system, BMSC were less effective in inhibiting T cell proliferation (that decreases from 100 to 65% for CD3-mediated stimulation) compared to PBMC-BMSC cultured in contact (from 100 to 10%) (fig. 4F vs 4D and 4E). Interestingly, BMSC treated with fluvastatin for 48h did not affect the inhibition of T cell proliferation (fig.4F) detected in PBMC-BMSC TW cultures. This suggests that fluvastatin do not alter the efficiency of putative inhibiting factors produced when BMSC and PBMC were not in contact.

**Conditioned medium from co-cultures of fluvastatin-treated BMSC and PBMC inhibits T cell proliferation**

It has been reported that several soluble factors such as prostaglandin E2 (PGE2), kynurenine, interleukin (IL)10, transforming growth factor (TGF)β1 and hepatocyte growth factor (HGF), present in BMSC-lymphocyte co-cultures, may inhibit lymphocyte proliferation (13,14). First, we
tested whether fluvastatin could regulate this inhibiting effect. Then, we analyzed whether PGE2, IL10, TGFβ1 and HGF were detectable in conditioned medium obtained from BMSC-lymphocyte co-cultures. Conditioned medium from 24h co-cultures of BMSC and PBMC was able to reduce by 50% T lymphocyte proliferation to anti-CD3 mAb (suppl.fig.1A). The SN derived from co-cultures of PBMC and BMSC pre-incubated with fluvastatin or manumycin A exerted a similar inhibiting effect (suppl.fig.1A). Although not shown, the conditioned medium harvested from BMSC or PBMC alone or from PBMC-BMSC co-cultured in transwells, to avoid PBMC-BMSC contact, did not exert any effect. Then, we analyzed a set of SN (n=14) from PBMC-BMSC co-cultures for the presence of IL10 and PGE2 and we evaluated a possible correlation with T cell proliferation. As shown in suppl.fig.1B, we found a strong inverse correlation between the amount of IL10 (r=0.9263) found in a given SN and the degree of CD3/TCR-mediated T cell proliferation. A lower correlation was found for PGE2 (r=0.4771) (suppl.fig.1C). No statistically significant correlation was found between IL10 and PGE2 levels in these SN (r=0.4841). The conditioned medium harvested from BMSC or PBMC alone contained very low amounts of IL10 (10-50pg/ml) or PGE2 (30-70pg/ml) and similar amounts of these factors were detected in PBMC-BMSC co-cultures separated by a transwell (not shown). As shown in suppl. table 1, the amounts of IL10 found in co-cultures of fluvastatin- or manumycin-treated BMSC and PBMC were superimposable to those detected in untreated BMSC-PBMC co-cultures. A strong correlation between the level of IL10 and the inhibiting effect on T cell proliferation was detected for SN from co-cultures of BMSC treated with fluvastatin (R=0.9574) or manumycin (R=0.9844). Furthermore, we analyzed whether and at what concentration IL10 or PGE2 can inhibit T cell proliferation. A statistically significant effect of exogenous recombinant IL10 added to cultures was observed from 10 to 1ng (60% to 80% of proliferation) (suppl.fig.1D); significant reduction of T cell proliferation was also exerted by exogenously added PGE2 from 1000 to 10ng (80% to 50% of maximal cell proliferation) (suppl.fig.1F). It is of note that recombinant IL10 could affect T cell proliferation only when it was added at the onset of cell culture and not after 24h of stimulation (suppl.fig.1E). Although not
shown, the amount of kynurenine found in SN (6-10 μM) was always 10-100 fold lower than that useful for inducing inhibition of lymphocyte proliferation. Indeed, 50μM of purified kynurenine slightly inhibited proliferation (80% of proliferation to anti-CD3 mAb) while 1mM of kynurenine strongly affected lymphocyte proliferation (20% of proliferation to anti-CD3 mAb). We did not detect HGF in BMSC-PBMC co-culture SN (not shown), while the amount of TGFβ1 found in these SN was similar (500-1300pg/ml) to that found in SN from BMSC cultured alone (350-1300pg/ml). As the SN from BMSC cultured alone did not inhibit T cell proliferation to PHA or anti-CD3 mAb stimuli (not shown), it is unlikely that TGFβ1 is the main factor responsible for the inhibiting activity found in BMSC-PBMC co-culture SN. Taken together, these findings suggest that in conditioned medium from PBMC-BMSC co-cultures are present different inhibiting soluble factors but only IL10 and to a lesser extent PGE2 may be involved in the observed inhibition of T cell proliferation. Thus, we performed a series of experiments by adding to PBMC-BMSC co-cultures a blocking anti-IL10 polyclonal antiserum or inhibitors of PGE2 synthesis (indomethacin and/or NS398). Although not shown, both anti-IL10 antiserum and/or PGE2 synthesis blockers did not affect the BMSC-mediated inhibiting effect on T cell proliferation in PBMC-BMSC co-cultures.

**Effect of fluvastatin on pro-survival signals delivered by BMSC to B cells**

It has been reported that BMSC can deliver survival signals to B cells (19). Thus, we analyzed whether fluvastatin-treated BMSC can spare B cells from spontaneous apoptosis in *in vitro* culture. To this aim, highly purified B cells from peripheral blood were cultured with BMSC and the percentage of apoptotic cells was analyzed on day 3, 5 and 7. As shown in figure 5A (left, and fig.5C) about 40% of B cell were dying by apoptosis on day 5 of culture in complete medium in the absence of any survival factor added. Importantly, BMSC exerted a strong survival effect on B cells as on day 5 less than 15% of B cells were apoptotic. Fluvastatin-treated BMSC were still able to spare B cells from spontaneous apoptosis; the incubation of BMSC with fluvastatin and L-mevalonate did not affect the pro-survival effect on B cells (fig. 5A, left and fig.5C). On the other
hand, pre-treatment of BMSC with manumycin A almost abolished the BMSC-mediated anti-apoptotic effect on B cells and the addition of L-mevalonate did not restore this effect (fig. 5B, left and fig.5C). Furthermore, we analyzed whether BMSC can counteract the pro-apoptotic signal delivered by corticosteroid on B cells. Indeed, we found that in the presence of $10^{-7}$M of dexamethasone the percentage of dying B cells was increased at day 5 compared to B cells cultured in medium alone (from 40 to 70%) (fig.5A, right and fig.5D). Importantly, BMSC rescued B cells from corticosteroid-induced apoptosis, indeed only 30% of B cells were dying in BMSC-B cell cocultures. Fluvastatin pre-treatment of BMSC strongly reduced the anti-apoptotic signal delivered to B cells in the presence of corticosteroid (55% vs 30% of dying cells). In this case, pre-treatment of BMSC with fluvastatin and L-mevalonate did restore the BMSC-mediated pro-survival signal to B cells. On the other hand, manumycin A completely blocked the BMSC pro-survival signal to B cells and L-mevalonate did not influence this effect (fig.5B right and fig.5D). In parallel experiments, we analyzed whether BMSC could spare B cells from apoptosis also when B cells and BMSC were separated by a transwell and whether fluvastatin could influence this effect. As shown in fig.5F, the BMSC-mediated anti-apoptotic effect was strongly reduced when B cells and BMSC were not in contact (compare fig.5C second column with fig.5E second column). Dexamethasone completely abolished the slight anti-apoptotic effect observed in the transwell system (fig.5E). Fluvastatin did not significantly affect the anti-apoptotic effect on B cells either in absence or presence of dexamethasome (fig.5E). Finally, we should note that BMSC do not give a pro-survival signal to T cells along a period of seven-ten days; indeed, the large majority of T cells were living cells either in the presence or in the absence of BMSC for this period of time (not shown). This suggests that peripheral T cells are less prone to spontaneous apoptosis than peripheral B cells.
Discussion

Herein, we show that the HMG-CoA reductase inhibitor fluvastatin is able to prevent the immunosuppressive effect exerted by BMSC on T lymphocyte proliferation. Moreover, BMSC deliver to B cells a strong pro-survival signal that is affected by fluvastatin only in the presence of corticosteroids. In addition, in our experiments fluvastatin inhibited the activation of the small GTP-binding protein RhoA, leading to strong alterations of actin microfilament distribution in BMSC and consequent detaching of BMSC from substrate. Furthermore, fluvastatin reduced the expression of some surface molecules involved in cell-to-cell interactions and it affected cell cycle progression of BMSC. On the other hand, a short time treatment with fluvastatin (48h) did not induce a significant apoptotic effect in BMSC and did not affect the constitutive production of IL6 and IL8 by BMSC. It is known that inhibition of HMG-CoA reductase by statins, reduces the availability of L-mevalonate and impairs cholesterol synthesis (25-27). The lack of L-mevalonate decreases also the synthesis of isoprenoid molecules, such as farnesylpyrophosphate and geranylpyrophosphate. The isoprenylation of small GTP-binding proteins as Ras, Raf and Rho is a post-translational modification necessary to the conversion of these proteins to the active forms that bind GTP. Members of Rho and Ras family are known to have a key role in regulating cell shape and motility or secretion and proliferation respectively (30, 39-41). Thus, the effects of fluvastatin reported here may be dependent on the decrease of cell membrane cholesterol content and/or on the inhibition of small GTP-binding proteins. Our present data suggest that fluvastatin treatment inhibits the immunosuppressive effect of BMSC by interfering with the isoprenylation and activation of GTP-binding proteins. Indeed, manumycin A, a potent inhibitor of isoprenylation, which does not alter cholesterol content of cellular membranes (25) can exert the same effect of fluvastatin on BMSC. These data are also supported by the observation that addition of L-mevalonate, the metabolic product of HMG-CoA reductase, which is essential for cholesterol synthesis, could rescue BMSC-induced immunosuppression when BMSC were incubated with fluvastatin but not with manumycin A. On the other hand, the fluvastatin-mediated effect on BMSC phenotype is mainly dependent on
the blocking of cholesterol synthesis, rather than on the inhibition of isoprenylation, as manumycin A reduced the surface expression of adhesion molecules to a lesser extent than fluvastatin. Indeed, fluvastatin significantly reduces the expression of several surface molecules, as CD29/β1-integrin, ICAM1, HLA-I and CD105, involved in cell-to-cell and cell to substrate adhesion. Of note, primary drug resistance in multiple myeloma has been related to the overexpression of adhesion molecules that follows persistent interaction of myeloma cells with BMSC in the bone marrow microenvironment; this phenomenon, termed cell adhesion-mediated drug resistance, can be partially overcome by statins in vitro (23). In our experiments, fluvastatin-induced down-regulation of adhesion molecules on BMSC might also limit the interactions between lymphocytes and BMSC. This would reduce the inhibiting signals delivered by BMSC to T cells upon cell-to-cell contact, and allow in turn, T lymphocytes to respond to proliferating signals. This hypothesis is supported by two observations: first, when cultured on fluvastatin-treated BMSC, T lymphocytes not only recover the ability to respond to mitogenic stimuli, but also detach from BMSC and adhere one to each other, forming those cell clumps that were lost in the presence of untreated BMSC; second, BMSC-mediated inhibition on T cell proliferation was stronger when BMSC and lymphocyte contact was allowed than when lymphocytes and BMSC were separated by a transwell. In the latter case, fluvastatin did not alter the inhibiting effect of BMSC on T cell proliferation, suggesting that the drug is effective only on BMSC-mediated inhibition consequent to cell-to-cell contact and not on that mediated by soluble factors. This would imply that fluvastatin does not inhibit the production and/or secretion of cytokines. This interpretation is in line with: a) the finding that fluvastatin-treated BMSC constitutively produce the same amounts of IL6 and IL8 secreted by untreated BMSC and b) the immunoregulatory cytokine IL10 (supplemental table 1) and PGE2 (not shown) are released in co-cultures of BMSC and PBMC also when BMSC were pretreated with fluvastatin.

Our present data do not clarify which soluble factor is involved in the observed inhibition of T cell proliferation, as IL10 blocking antiserum and/or PGE2 synthesis inhibitors do not prevent the
immunosuppressive effect detected in lymphocyte-BMSC co-cultures. Nevertheless, we found a strong and statistically significant correlation between the content of IL10 in SN from BMSC-PBMC co-cultures and the degree of inhibition on T lymphocyte proliferation. A less strong correlation was found for PGE2. These data, would support the idea that IL10 gives a relevant contribution to the inhibiting activity present in BMSC-PBMC co-cultures, besides the reported role of PGE2 in BMSC- and/or fibroblast-like-mediated immunosuppression (9,14,42).

It has been recently reported that BMSC can support B cell survival by delivering anti-apoptotic and pro-differentiating signals (19). We found that fluvastatin can significantly inhibit the pro-survival signal delivered by BMSC to B cells in the presence of dexamethasone, although this signal is marginally inhibited in the absence of corticosteroids. This finding supports the hypothesis that fluvastatin can enhance the pro-apoptotic effect of corticosteroid by influencing the BMSC-B cell interaction. Also in this case, fluvastatin-mediated effects can be reverted by L-mevalonate, that allows the synthesis of isoprenoid molecules, and reproduced by manumycin A, that inhibits isoprenylation blocking the activation of small GTP binding protein. The finding that mevalonate could not rescue B cells in the presence of manumycin A would suggest that isoprenylation of GTP-binding proteins is essential also for BMSC-mediated pro-survival effect on B cells. Reduction in BMSC-B cell interaction, due to the effects of fluvastatin on actin cytoskeleton and adhesion molecules, might contribute to the inhibition of the survival signal delivered to B cells, although the need of corticosteroids to evidence this phenomenon is still to be fully clarified. Our experiments also show that the anti-apoptotic signal delivered to B cells by BMSC is mainly dependent on B-BMSC contact, as in transwells this signal was very low (fig.5E) compared to that detected when B cells were in contact with BMSC (fig.5C). In the transwell culture system corticosteroids are effective in inducing the apoptosis of most B cells. This would imply that corticosteroids can markedly inhibit the production, release or membrane expression of B cell survival factors. The identification of these factors would be relevant to define combinations of fluvastatin or isoprenylation blockers and corticosteroids potentially useful in the therapy of B cell
lymphoproliferative diseases. In conclusion, our present data indicate that the mevalonate biosynthetic pathway can regulate some BMSC-mediated functions, suggesting that interference with cholesterol synthesis may down-regulate the immunosuppressive effect of BMSC on T cells and, in the presence of corticosteroids, also impair the survival signals to B cells. This would further reinforce the idea that fluvastatin may be used for the treatment of diseases where stromal cells present in the microenvironment play a key role in promoting neoplastic growth (14, 43-45). In this context, it has been suggested to use statins together with thalidomide or lenalidomide for the treatment of multiple myeloma (22, 23). We propose that statins may also potentiate the effect of corticosteroids on tumor cells of the B lineage and at the same time relieve T cells from suppressive effects, thus favouring T cell-mediated effector functions.

**Authorship and Disclosures**

AM performed the research and analyzed data, MRZ and AP design the research, coordinate the research, analyzed data and wrote the paper. AP is the principal investigator and takes primary responsibility for the paper.

The Authors reported no potential conflict of interest.
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Legend to figures

Figure 1. Effect of fluvastatin on BMSC morphology, actin distribution and RhoA GTP-binding protein activation.

(A). BMSC were cultured in medium (medium) or with DMSO (solvent) or with 10µM fluvastatin alone (FLU) or with 1mM L-mevalonate (FLU+MEV) or with 10µM manumycin A alone (MANU) or with L-mevalonate (MANU+MEV) for 48hr and observed under the microscope in bright field with Olympus IX71 microscope (200x magnification).

(B). BMSC incubated with solvent or fluvastatin or fluvastatin and L-mevalonate as in panel A were analyzed by confocal microscopy (400x magnification, Olympus IX81 microscope, objective 40xoil, NA1.40) for the expression and distribution of actin (labelled with phallotoxin Alexa488, green) and β-tubulin (labelled with anti-β-tubulin mAb followed by anti-isotype GAM alexafluor535, red) and ICAM1 (labelled with anti-ICAM1 mAb followed by anti-isotype GAM alexafluor647, blue). (C). Digital zoom (5x) of white squares in panel B. (D). BMSC incubated with solvent or manumycin A and analyzed by confocal microscopy, as in panel B, for the distribution of actin (left) or in bright field (right) (400x magnification).

(E) and (F). Perimeter (E) and area (F) of BMSC incubated as in panel A; data are expressed as µm (E) or µm² (F) using the Analysis FIVE computer program. Each symbol indicate the mean value of
5 single cells, for each condition at least 100 cells were analyzed. Orizontal bars indicate the mean of each culture condition. Results are representative of six independent experiments for panels A,B,C,D,E and F. (G). Activation of RhoA protein was analyzed on BMSC incubated as indicated in panel A and compared to that of 1ng RhoA (RhoA PROT). Results are shown as OD read at 490nm of normalized amount of RhoA for each cell lysate and are the mean±SD of 4 independent experiments. Statistical significance of results is indicated.

**Figure 2.** Effect of fluvastatin and manumycin A on the expression of surface molecules of BMSC. (A). BMSC were cultured for 48h with DMSO (solvent, diluted 1:1000 in culture medium) or 10µM fluvastatin or 1mM L-mevalonate or with fluvastatin and L-mevalonate. Then cells were stained with antibodies to the indicated surface molecules (ICAM1, HLA-I, CD29/β1 integrin and CD105) followed by PE-conjugated anti-isotype specific GAM. Results are expressed as Log red fluorescence intensity (arbitrary units, a.u.) and are representative of 6 independent experiments. In each subpanel are shown the fluorescence intensity of an isotype matched control antibody (thin line) and that of the indicated molecule analyzed (bold line). The percentage of positive cells and the mean fluorescence intensity (MFI) of the molecule analyzed are also shown. (B) Results of each molecule analyzed in panel A are depicted as the mean±SD of six independent experiments. Results are shown as MFI and they were analyzed by one tail student t test at 95% confidence. The p values are shown when statistically significant. (C). BMSC were cultured with DMSO as in panel A or with manumycin A and analyzed for the expression of the indicated surface molecules with specific mAbs followed by Alexafluor647-conjugate anti-isotype specific GAM. Results are expressed as Log far red fluorescence intensity in a.u. and are representative of six independent experiments. (D). Results of each molecule analyzed in panel C are depicted as the mean±SD of six independent experiments. Results are shown as MFI in a.u. and they were analyzed by one tail student t test at 95% confidence. The p values are shown when statistically significant.
**Figure 3.** Effect of BMSC treatment with fluvastatin on cell cycle progression, apoptosis and secretion of constitutive cytokines.

(A). BMSC were cultured with DMSO (solvent of fluvastatin 1:1000 diluted) or with fluvastatin (10 µM) or fluvastatin and L-mevalonate (1 mM) for 48 hr and the G0/G1 (black) or S (dark grey) or G2/M (light grey) phases of cell cycle were analyzed after PI staining. Results are expressed as log red fluorescence intensity (a.u.) and are representative of 6 independent experiments. (B) Analysis of BMSC cell cycle after 48 h treatment with fluvastatin as in (A) (left subpanels). In some experiments BMSC were cultured with fluvastatin for 48 hr, washed and cultured in medium for additional 24 hr and then analyzed for PI content (right subpanels). Results are expressed as % of BMSC cells in G0/G1 or S or G2/M phase. and are the mean±SD of six independent experiments. Results were analyzed by one tail student t test at 95% confidence. The p values are shown when statistically significant. (C). BMSC cultured for 24 hr as indicated (with solvent of fluvastatin, fluvastatin or in medium after irradiation) were analyzed for their forward scatter (FSC) and side scatter (SSC) (left). Reactivity of cells with FITC-annexin V and PI gated on viable and dying cells excluding cellular debris (R1 gated) is shown on the right. Quadrants of each dot plot are divided into four regions of cells: upper left (AV−PI+), upper right (AV−PI+), lower left (AV−PI−) and lower right (AV+PI−). In the upper right quadrant are indicated the percentages of AV+PI+ cells considered as apoptotic. (D) Percentage of apoptotic cells among BMSC cultured in solvent (sol) or with fluvastatin (FLU) and analyzed after 48 hr. Results are shown as mean±SD from six independent experiments. (E) and (F). Basal production of IL6 (E) or IL8 (F) by BMSC pretreated with fluvastatin. BMSC were cultured as indicated for 24 hr and culture supernatants were harvested and analyzed by specific ELISA for the presence of IL6 or IL8. Results are expressed as pg/ml and are the mean±SD of six independent experiments.

**Figure 4.** Fluvastatin inhibits BMSC-mediated immunosuppression of T lymphocyte proliferation.
(A). Peripheral blood mononuclear cells (PBMC) were cultured in U-bottomed plates with medium (upper left) or with PHA or PHA and BMSC (middle) or with PHA and BMSC pretreated with fluvastatin alone (lower left) or fluvastatin and L-mevalonate (lower right), BMSC cultured in medium alone are also shown (upper right). Bright field images of cell cultures were taken on day 3 of culture (100x magnification, IX71 Olympus microscope). (B). PBMC labelled with CFSE were cultured alone (first row) or with BMSC (second, third and fourth rows) in medium (left) or with PHA (middle) or anti-CD3 mAb (right). Some experiments were performed with BMSC pre-incubated for 48hr with fluvastatin (third row) or fluvastatin and L-mevalonate (fourth row). On day 4 cells were harvested and stained with anti-CD3 mAb (to identify T cells) followed by anti-isotype PE-conjugated GAM. Each dot plots was divided into four regions: upper left: proliferating T cells, upper right: parental (non proliferating) T cells, lower left: proliferating non-T cells, lower right: parental non-T cells. Results are expressed as Log green fluorescence intensity (a.u.) vs Log red fluorescence intensity (a.u.) and are representative of at least six independent experiments. (C). CFSE labelled PBMC were cultured in the indicated conditions (PBMC alone, PBMC-BMSC co-cultures in solvent alone or with maumycin A, MANU) and proliferating T cells analyzed as in panel B. (D) and (E). Percent of T cell proliferation upon stimulation with PHA (D) or anti-CD3 mAb (E) detected in the presence of BMSC either untreated (PBMC-BMSC) or treated with fluvastatin (FLU) or with manumycin A (MANU). (F) Proliferation of T cells stimulated with PHA or anti-CD3 mAb analyzed when PBMC was separated from BMSC by transwell in the indicated culture conditions. Results are expressed as percent of proliferation evaluated by ATP-lite proliferation kit and are the mean±SD of six independent experiments. To normalize results obtained in different experiments, T cell proliferation to PHA (D, F) or anti-CD3 mAb (E, F) was considered as 100%. Results are analyzed by one tail student t test at 95% confidence. The p values are shown when statistically significant.
Figure 5. Effect of fluvastatin on BMSC-mediated pro-survival effect on peripheral blood B cells.

(A). Peripheral blood B lymphocytes were cultured with medium (left panels) or with $10^{-7}$M dexamethasone (DEX $10^{-7}$M) for five days either alone (first row) or with BMSC either untreated (second row) or pretreated with $10\mu$M fluvastatin for 48h (third row, FLU) or with fluvastatin and 1mM L-mevalonate (fourth row, FLU+MEV). Dot plots are divided as described for figure 3 panel C. (B). B cells were cultured as in panel A with BMSC pretreated with $10\mu$M manumycin A (upper row, MANU) or with manumycin A and L-mevalonate (lower row, MANU+MEV) for 48h. Cells were stained with FITC-AV and PI and the percentage of AV$^+$PI$^+$ apoptotic cells are indicated in the upper right quadrant of each dot plot. (C), (D) and (E). B cells were cultured in medium (C) or with $10^{-7}$M dexamethasone (DEX $10^{-7}$M) (D) or in a transwell separated from BMSC seeded in the lower chamber. The percentage of apoptotic B cells were detected in the indicated culture conditions. Data are expressed as percentage of AV$^+$PI$^+$ apoptotic B cells and are the mean±SD of six independent experiments. Results are analyzed by one tail student t test at 95% confidence. The p values are shown when statistically significant.
Figure 1
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**Supplementary Table 1.** Supernatants harvested from BMSC-PBMC co-cultures contain IL10 and inhibit T lymphocyte proliferation.

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PBMC were cultured for 24h with BMSC<sup>1</sup> or with BMSC pretreated for 48hr with 10µM of fluvastatin<sup>2</sup>, FLU or with 10µM of manumycin A<sup>3</sup>, MANU). Then supernatants were harvested and analyzed for IL10 content by specific ELISA (results are reported as pg/ml). SN were tested for the ability of inhibiting T cell proliferation to anti-CD3 mAb with an ATP-lite proliferation kit. To normalize results obtained in different experiments, T cell proliferation to anti-CD3 mAb obtained in the absence of BMSC was considered as 100 and results are expressed as percentage of this value. Mean±SD is also shown.
Supplementary Figure 1. Effect of supernatants from PBMC-BMSC co-cultures, compared to exogenous IL10 or PGE₂ on T lymphocyte proliferation triggered by anti-CD3 mAb. To normalize results obtained in different experiments, T cell proliferation to anti-CD3 mAb in the absence of SN was considered as 100%. (A). Effect of SN harvested from PBMC or PBMC-BMSC co-cultures either untreated or treated with fluvastatin (FLU) or manumycin A (MANU). (B) and (C). Correlation curve and r Pearson's coefficient between percent of T cell proliferation and IL10 (B) or PGE₂ (C) content in 14 SN harvested from PBMC-BMSC co-cultures. (D) and (E). Effect of the indicated doses of exogenous IL10 on T cell proliferation added at the onset (D) or after 24h (E) of cell culture. (F). Effect of the addition of different doses of exogenous PGE₂, added at the onset of cell culture, on T cell proliferation. Results are expressed as percent of T cell proliferation evaluated by ATP-lite assay kit and are the mean±SD of six independent experiments in panel A, D, E and F. The p value of the indicated culture conditions vs. 100% of T cell proliferation is shown when statistically significant.