# 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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## 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. Here 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, such 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 cell 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 morphological, 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 cell-induced T-cell suppression and B-lymphocyte survival.

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

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## 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 hematologic 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 a striking improvement in 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 isoprenoid 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]

Here, 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, USA). 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, Italy) and D. Vercelli (Scientific Institute San Raffaele, Milan, Italy), respectively. The anti-β1integrin (CD29) mAb (3E1, IgG1) was a kind gift of Dr L. Zardi (IST-Genoa, Italy). 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-276, 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).

Information on cells and drug treatment, analysis of cell cycle and apoptotic assay, immunofluorescence and cytofluorimetric analysis, BMSC-PBMC co-cultures and evaluation of T-cell proliferation, cytokine detection assays, evaluation of RhoA activation, cytoskeleton analysis by confocal microscopy and analysis of pro-survival effect of BMSC on B cells is available in the *Online Supplementary Appendix*.

### Statistical analysis

Results were analyzed by applying the one tail Student's t-test at 95% confidence level. This test was chosen as drug treatment led to modifications of the mean of experimental values in one way analysis. The statistical significance is shown in each figure. Pearson's 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 (*Online Supplementary Figure S1A*) 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 pyrophoshates and produces 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 (*Online Supplementary Figure S1A*). 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 center of the cell (*Online Supplementary Figure S1B* and *C*). This distribution was completely lost in BMSC pre-incubated with fluvastatin (10 μM for 24 h) whereas the distribution of tubulin microtubules was not affected (*Online Supplementary Figure S1B*). A similar effect on actin distribution was detected in BMSC treated with manumycin A (*Online Supplementary Figure S1D*). The addition of L-mevalonate to fluvastatin, at the onset of cell culture, completely restored the actin distribution in BMSC (*Online Supplementary Figure S1B*). The observed morphological alterations were also confirmed by measuring the perimeter and the area of BMSC cultured in the presence of fluvastatin or maunmycin A (*Online Supplementary Figure S1E* and *F*). 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 (*Online Supplementary Figure S1G*). Manumycin A strongly reduced the amount of RhoA activated protein present in BMSC and L-mevalonate did not prevent the effect of manumycin A (*Online Supplementary Figure S1G*).

### 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 Figure 1A, 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 (Figure 1B). 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 (Figure 1A and B). 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 (Figure 1A and B). Also, pre-incubation of BMSC with manumycin A reduced the expression of the above indicated surface molecules (Figure 1C and D). However, upon treatment with manumycin A only the decrease in HLA-I expression was statistically significant (Figure 1D). The addition of L-mevalonate did not revert the effect of manumycin A (*data not shown*).

### Fluvastatin impairs the S-phase of the cell cycle in BMSC but it does not induce apoptosis nor affect 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, such 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 $G_0/G_1$ phase but not $G_2$/M phase after 48 h of treatment (*Online Supplementary Figure S2A* and *B*). These results were indeed statistically significant for $G_0/G_1$ and S phases (*Online Supplementary Figure S2B*). 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 $G_0/G_1$ phase to S phase of the cell cycle was observed by 24 h (*Online Supplementary Figure S2B*). 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 10 μM fluvastatin for 24-48-72 h and apoptosis analyzed after annexin V and PI staining. We found that the percentages of dying cells in fluvastatin-treated BMSC cultures after 24 h were comparable to those detected in control cultures (Figure 2C). As shown in Figure 2D, the amount of dying cells in fluvastatin cultures was not statistically significant at 48 h of incubation compared to solvent-treated cells. Although not shown, at 72 h of treatment about 50-60% of BMSC were dying. Importantly, when fluvastatin was washed out after 48 h treatment, the apoptotic BMSC detected after additional 24 h of culture were similar to those detected in control cultures (*data not shown*). Thus, almost all BMSC were living cells after 48 h 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 48 h and 72 h after irradiation more than 50% and 85% of BMSC were dying, respectively (*data 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 (*Online Supplementary Figure S2E* and *F*). Alltogether, these finding indicate that treatment of BMSC for 48 h does not impair the survival of BMSC or 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 48 h with fluvastatin, washed and used for co-culture experiments with CFSE-labeled 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 2A,

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 pre-treated BMSC, lymphocyte cell clumps were evident again and this effect was abrogated when BMSC were incubated with fluvastatin and L-mevalonate (Figure 2A). As shown in Figure 2B, 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 48 h pre-incubation of BMSC with fluvastatin 10 μM (Figure 2B, D and E). This inhibiting effect was not detected when BMSC were pre-incubated with 1 μM fluvastatin for 48 h (*data 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) (Figure 2B). Ten micromolar concentration of other HMG-CoA reductase inhibitors as mevastatin, atorvastatin, simvastatin exerted superimposable effects to those of fluvastatin on BMSC (*data not shown*). Also treatment with manumycin A strongly affects the BMSC-mediated inhibition of T-cell proliferation (Figure 2C, 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) (Figure 2F) 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%) (Figure 2F *vs.* 2D and E). Interestingly, BMSC treated with fluvastatin for 48 h did not affect the inhibition of T-cell proliferation (Figure 2F) detected in PBMC-BMSC TW cultures. This suggests that fluvastatin does not alter the efficiency of putative inhibiting factors produced when BMSC and PBMC were not in contact.

Results concerning conditioned medium from co-cultures of fluvastatin-treated BMSC and PBMC inhibition of T-cell proliferation are presented in the *Online Supplementary Appendix*.

### 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

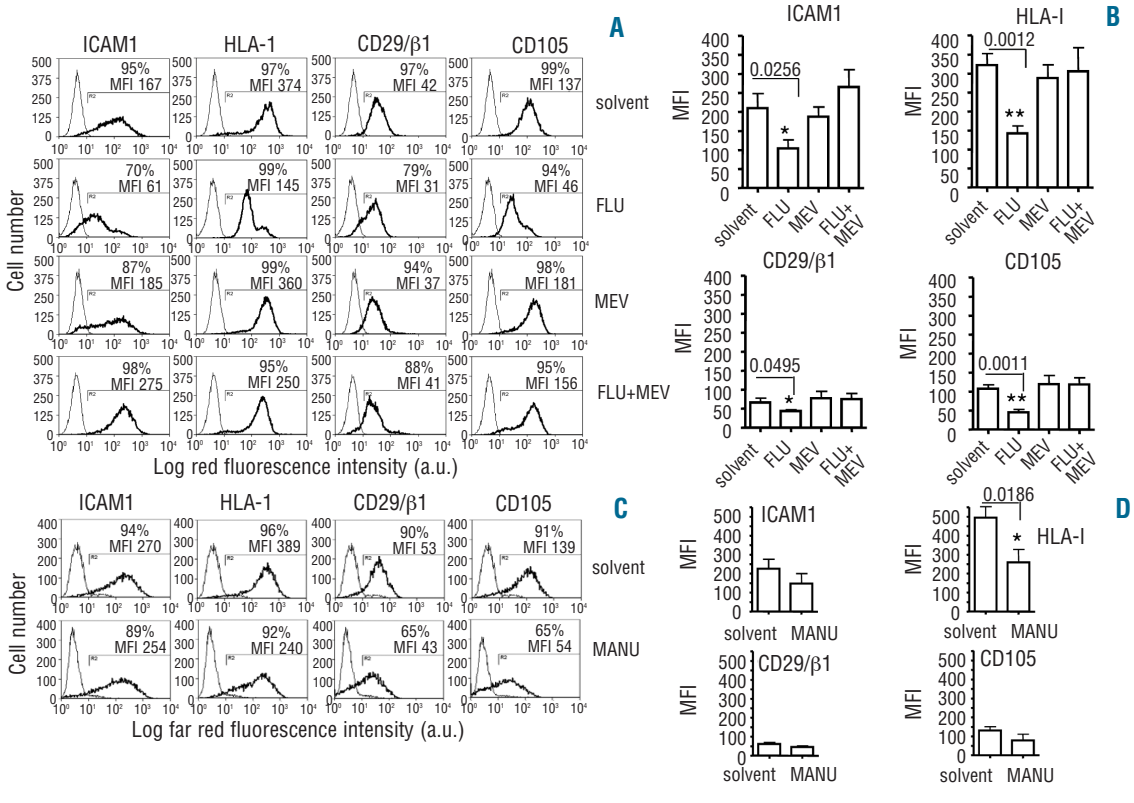


**Figure 1.** Effect of fluvastatin and manumycin A on the expression of surface molecules of BMSC. (A) BMSC were cultured for 48 h with DMSO (solvent, diluted 1:1000 in culture medium) or 10μM fluvastatin or 1mM L-mevalonate or with fluvastatin and L-mevalonate. Then cells were stained with antibodies 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 6 independent experiments. Results are shown as MFI and they were analyzed by one-tail Student's 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 6 independent experiments. (D) Results of each molecule analyzed in panel C are depicted as the mean±SD of 6 independent experiments. Results are shown as MFI in a.u. and they were analyzed by one-tail Student's t-test at 95% confidence. The *P* values are shown when statistically significant.

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 days 3, 5 and 7. As shown in Figure 3A (left, and Figure 3C) 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 (Figure 3A, left and Figure 3C). 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 (Figure 3B, left and Figure 3C). 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%) (Figure 3A, right and Figure 3D). Importantly, BMSC rescued B cells from corticosteroid-induced apoptosis, indeed only 30% of B cells were dying in BMSC-B cell co-cultures. 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 (Figure 3B, right and Figure 3D). In parallel experiments, we analyzed whether BMSC could spare B cells from apoptosis also

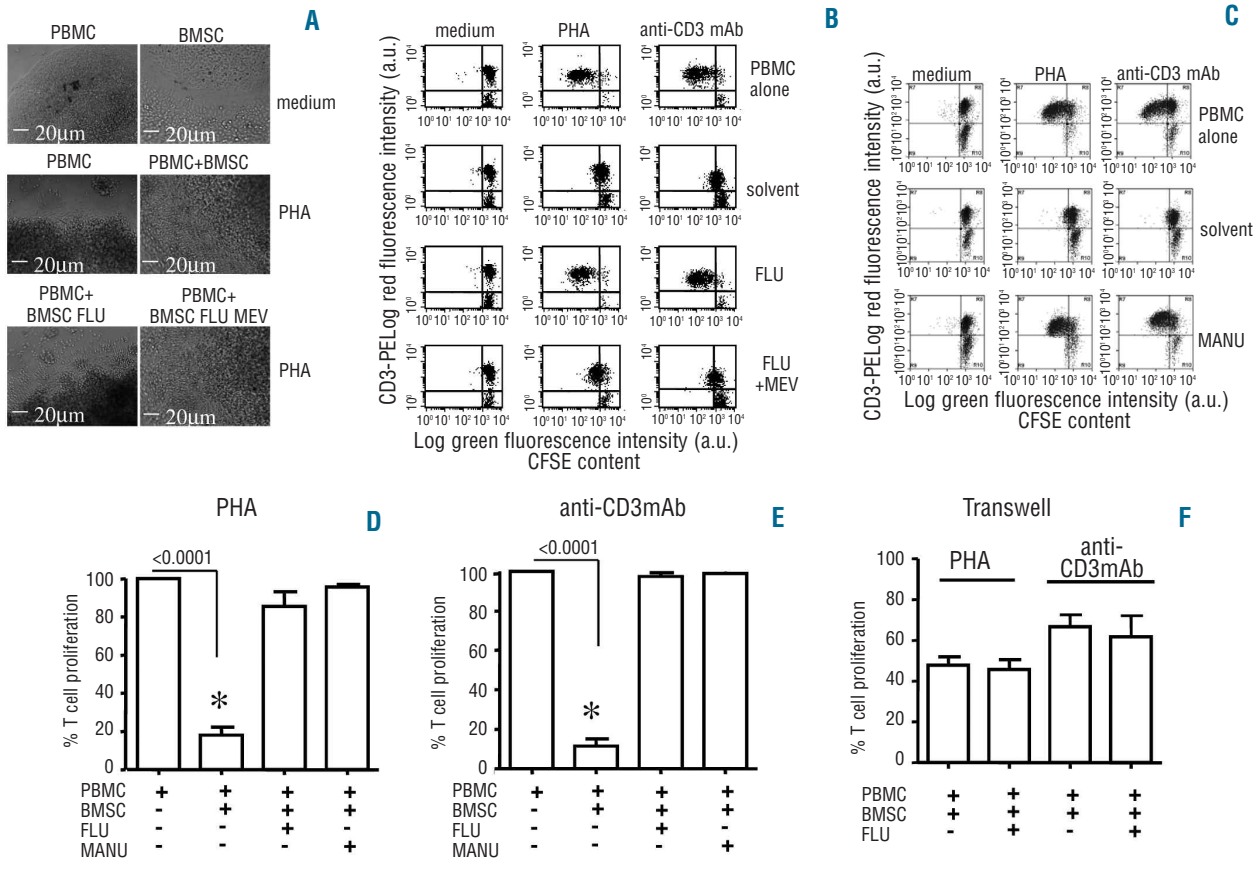


**Figure 2.** Fluvastatin inhibits BMSC-mediated immunosuppression of T-lymphocyte proliferation. (A) Peripheral blood mononuclear cells (PBMC) were cultured in U-bottomed plates with medium (upper left) or with PHA or PHA and BMSC (middle) or with PHA and BMSC pre-treated with fluvastatin alone (lower 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 labeled 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 48 h 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 plot 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 6 independent experiments. (C) CFSE labeled 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-CD3mAb 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 6 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's t-test at 95% confidence. The *P* values are shown when statistically significant.

when B cells and BMSC were separated by a transwell and whether fluvastatin could influence this effect. As shown in Figure 3F, the BMSC-mediated anti-apoptotic effect was strongly reduced when B cells and BMSC were not in contact (compare Figure 3C second column with Figure 3E second column). Dexamethasone completely abolished the slight anti-apoptotic effect observed in the transwell system (Figure 3E). Fluvastatin did not significantly affect the anti-apoptotic effect on B cells either in absence or presence of dexamethasome (Figure 3E). Finally, we should note that BMSC do not give a pro-survival signal to T cells along a period of 7-10 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 (*data not shown*). This suggests that peripheral T cells are less prone to spontaneous apoptosis than peripheral B cells.

## Discussion

Here, 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 treatment with fluvastatin (48 h) did not induce a significant apoptotic effect in

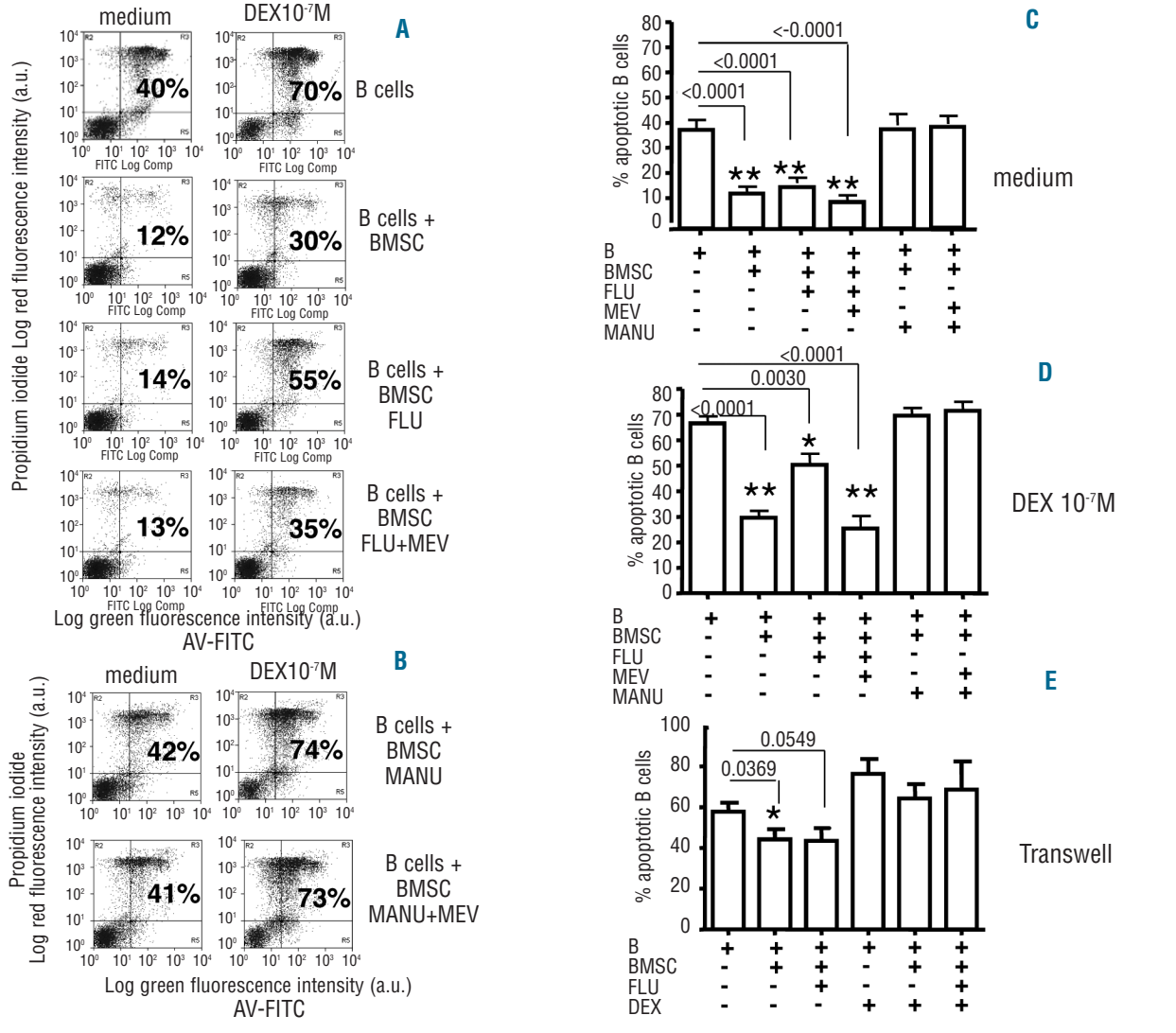


Figure 3. Effect of fluvastatin on BMSC-mediated pro-survival effect on peripheral blood B cells. (A) Peripheral blood B lymphocytes were cultured with medium (left panels) or with $10^{-7}$M dexamethasone (DEX $10^{-7}$M) for five days either alone (first row) or with BMSC either untreated (second row) or pre-treated with 10 μM fluvastatin for 48 h (third row, FLU) or with fluvastatin and 1mM L-mevalonate (fourth row, FLU+MEV). Dot plots are divided as described for Figure S2 panel C. (B) B cells were cultured as in panel A with BMSC pre-treated with 10μM manumycin A (upper row, MANU) or with manumycin A and L-mevalonate (lower row, MANU+MEV) for 48 h. 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 6 independent experiments. Results are analyzed by one-tail Students' t-test at 95% confidence. The *P* values are shown when statistically significant.

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.[30,33-35] 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 downregulation 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 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 (*Online Suplementary Table S1*) and $PGE_2$ (*data not shown*) are released in co-cultures of BMSC and PBMC also when BMSC were pre-treated 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 $PGE_2$ 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 $PGE_2$. 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 $PGE_2$ in BMSC- and/or fibroblast-like-mediated immunosuppression.[9,14,36]

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 (Figure 3E) compared to that detected when B cells were in contact with BMSC (Figure 3C). 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.[14,37-39] 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 favoring T cell-mediated effector functions.

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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