

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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Online Supplementary Appendix

Design and Methods

Cells and drug treatment

Peripheral blood mononuclear cells (PBMC) were obtained from venous blood samples of healthy donors after Ficoll-Hypaque density gradient separation.¹ 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).¹ 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.² BMSC expressed SH3/CD73, SH4, SH2/CD105, CD44, β 1-integrin/CD29, ICAM1/CD54, HLA-I, prolyl-4-hydroxylase, alkaline phosphatase, collagen, vimentin, bone sialoprotein, and osteopontin but not CD45, CD31, CD34, CD33, CD3, CD2, CD16, CD14, ICAM2, ICAM3, CD80, CD86, CD83, and HLA-DR (*data not shown*). BMSC were treated for different time periods (24-36-48-72 h) with each statin (10-1.0-0.1 μ M). On the basis of preliminary experiments, fluvastatin was chosen as a prototype of these drugs. Pre-incubation with 10 μ M for 24-48 h 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 10 μ M and DMSO (solvent of all these inhibitors) at 1:1000 in culture medium. Among the three inhibitors, manumycin A was chosen as FTI-276 and GGTI-286 were less potent at the 10 μ M concentration and they did not exert constant and reproducible inhibition of RhoA GTP-binding protein. The cyclo-oxygenase 2 inhibitors indomethacin ester-4-methoxy-phenyl and NS-398 were used at 10-1-0.1 μ M. The 10 μ M concentration was used in experiments aimed to determine the role of PGE₂ in inducing inhibition of T-cell proliferation as reported.^{3,4} Indeed, at this concentration the production of PGE₂ during BMSC-PBMC culture was almost abolished (*data 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 labeling with PI as described.⁵ 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 labeling. In some experiments, apoptotic cells were identified after permeabilization and PI staining as cells with a less than 2n DNA content as described.⁵ Irradiated BMSC (5000rads) were used to compare the effects of each drug with an effective apoptotic signal.⁶

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 isothiocyanate (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 *versus* number of cells or as mean fluorescence intensity (MFI, a.u.).

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

2×10^4 /sample BMSC were incubated for 48 h at 37°C in 5% CO₂ atmosphere with one or another combination of drugs, washed and then co-cultured with 10^5 PBMC at 1:5 ratio and proliferation of PBMC to either PHA (1 μ g/mL) or anti-CD3 mAb (JT3A, IgG2a, 20ng/mL)⁷ 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 labeling PBMC with carboxy fluorescein succinimidyl ester (CFSE, Invitrogen s.r.l., Molecular Probes, Carlsbad, CA, USA) according to the manufacturer's instructions, by cytofluorimetric evaluation of progressive loss of this fluorochrome along time, proportional to cell division⁸ 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 radiolabeled thymidine. To determine the effect of

soluble factors on T-cell proliferation, PBMC were seeded on Millicell transwell (TW) with 0.3 μm 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 96 well plates for 24 h 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 24 h, culture supernatants (SN) were harvested. To evaluate constitutive production of cytokines, BMSC were cultured alone for 24 h and SN were harvested. The SN were analyzed for the content of PGE₂ 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- β 1 content was analyzed after treatment for 1 h of each SN with 1N HCl followed by 1N NaOH with a TGF β 1 specific kit (Bender MedSystem). Culture SN collected for these experiments were also used to evaluate kynurenine concentration by spectrophotometric analysis as described.⁴

Evaluation of RhoA activation

To analyze the effect of fluvastatin and farnesyl transferase inhibitors on RhoA activation, BMSC were incubated for 24 h in medium alone or in medium containing fluvastatin 10 μM or manumycin A 10 μM 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.

Cytoskeleton analysis by confocal microscopy

2.5×10^4 BMSC seeded on 0.2mm thin round glass slides were incubated for 24 h 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 30 min 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 an IX81 motorized microscope (Olympus). Samples were observed with PlanApo 40x NA1.00 or 60x NA1.40 oil objectives and data analyzed with the 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.¹ The resulting cell populations was always greater than 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 ratio to detect the pro-survival effect of BMSC on B cells. Samples were analyzed on days 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) as 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.⁹ 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 μm pores (Millipore) put in 24w plates with BMSC seeded on the bottom to avoid BMSC-B cell contact.

Results

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) β 1 and hepatocyte growth factor (HGF), present in BMSC-lymphocyte co-cultures, may inhibit lymphocyte proliferation.^{10,11} First, we tested whether fluvastatin could regulate this inhibiting effect. Then, we analyzed whether PGE₂, IL10, TGF β 1 and HGF were detectable in conditioned medium obtained from BMSC-lymphocyte co-cultures. Conditioned medium from 24 h co-cultures of BMSC and PBMC was able to reduce by 50% T-lymphocyte proliferation to anti-CD3 mAb (*Online Supplementary Figure S3A*). The SN derived from co-cultures of PBMC and BMSC pre-incubated with fluvastatin or manumycin A exerted a similar inhibiting effect (*Online Supplementary Figure S3A*). 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 the *Online Supplementary Figure S3B*, 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 ($r=0.4771$) (*Online Supplementary Figure S3C*). 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 (*data not shown*). As shown in the *Online Supplementary Table S1*, 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) (Online Supplementary Figure S3D); significant reduction of T-cell proliferation was also exerted by exogenously added PGE₂ from 1000 to 10ng (80% to 50% of maximal cell proliferation) (Online Supplementary Figure S3F). 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 24 h of stimulation (Online Supplementary Figure S3E). 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, 50 μM 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 (*data 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 (*data 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 different inhibiting soluble factors are present in conditioned medium from PBMC-BMSC co-cultures 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.

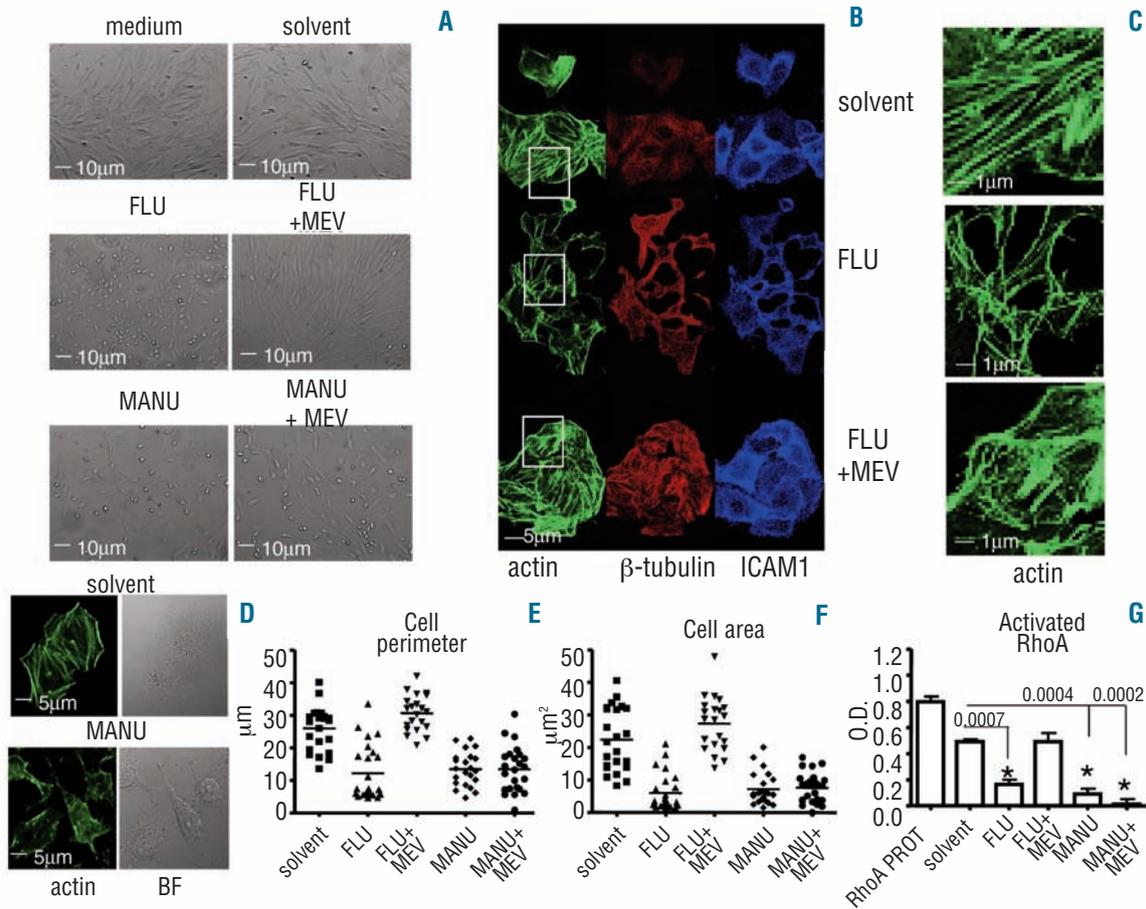
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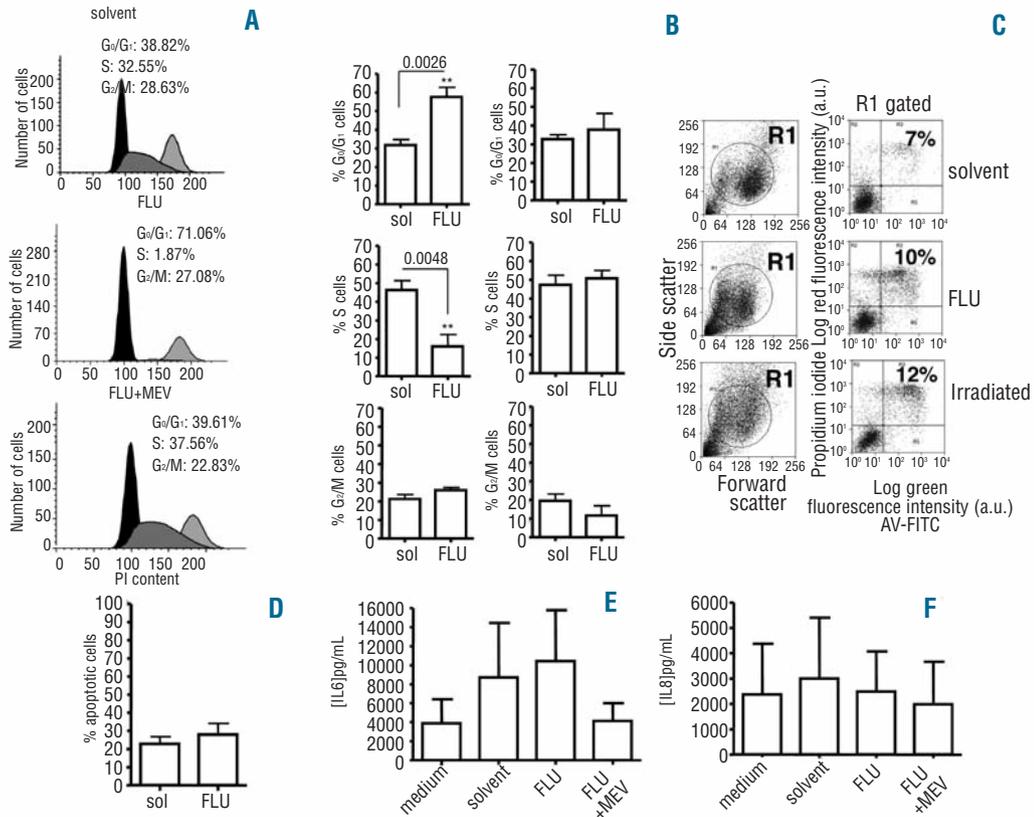
Online Supplementary Table S1. Supernatants harvested from BMSC-PBMC co-cultures contain IL10 and inhibit T-lymphocyte proliferation.

| Exp. n. | BMSC ¹ PBMC IL10 pg/mL | BMSC ¹ PBMC % T-cell prol. | BMSC ² PBMC FLU IL10 pg/mL | BMSC ² PBMC FLU % T-cell prol. | BMSC ³ PBMC MANU IL10 pg/mL | BMSC ³ PBMC MANU % T-cell prol. |
|-------------|---|---|---|---|---|---|
| 1 | 1848 | 35 | 874 | 65 | 667 | 69 |
| 2 | 1822 | 56 | 1925 | 52 | 931 | 61 |
| 3 | 1733 | 53 | 1341 | 58 | 2166 | 39 |
| 4 | 1458 | 56 | 1095 | 70 | 750 | 64 |
| 5 | 30 | 78 | 15 | 90 | 14 | 88 |
| 6 | 13 | 85 | 30 | 83 | 12 | 82 |
| Mean | 1150 | 60 | 880 | 69 | 756 | 67 |
| ±SD | ±885 | ±18 | ±751 | ±14 | ±791 | ±17 |

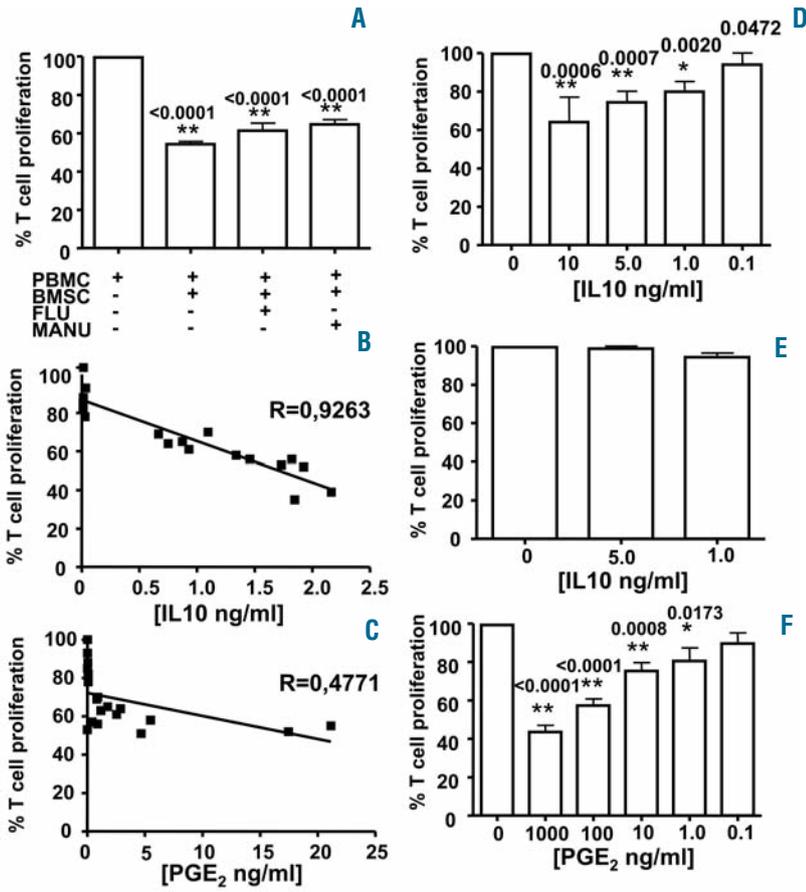
PBMC were cultured for 24 h with BMSC (°) or with BMSC pre-treated for 48 h with 10μM of fluvastatin (°, FLU) or with 10μM of manumycin A (°, MANU). Then supernatants were harvested and analyzed for IL10 content by specific ELISA (results are reported as pg/mL). SN were tested for the ability of inhibiting 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.



Online Supplementary Figure S1. 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 10 μM fluvastatin alone (FLU) or with 1mM L-mevalonate (FLU+MEV) or with 10 μM manumycin A alone (MANU) or with L-mevalonate (MANU+MEV) for 48 h 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 (labeled with phalloxin Alexa488, green) and β -tubulin (labeled with anti- β -tubulin mAb followed by anti-isotype GAM alexafluor535, red) and ICAM1 (labeled 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 μm^2 (F) using the Analysis FIVE computer program. Each symbol indicates the mean value of 5 single cells, for each condition at least 100 cells were analyzed. Horizontal bars indicate the mean of each culture condition. Results are representative of 6 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 \pm SD of 4 independent experiments. Statistical significance of results is indicated.



Online Supplementary Figure S2. 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 μ M) or fluvastatin and L-mevalonate (1mM) for 48 h and the G₀/G₁ (black) or S (dark gray) or G₂/M (light gray) 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 h, washed and cultured in medium for additional 24 h and then analyzed for PI content (right subpanels). Results are expressed as percentage of BMSC cells in G₀/G₁ or S or G₂/M phase and are the mean \pm SD of 6 independent experiments. Results were analyzed by one-tail Student's t-test at 95% confidence. The *P* values are shown when statistically significant. **(C)** BMSC cultured for 24 h 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 h. Results are shown as mean \pm SD from 6 independent experiments. **(E)** and **(F)** Basal production of IL6 (**E**) or IL8 (**F**) by BMSC pre-treated with fluvastatin. BMSC were cultured as indicated for 24 h 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 \pm SD of 6 independent experiments.



Online Supplementary Figure S3. 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 24 h (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 6 independent experiments in panel A, D, E and F. The P value of the indicated culture conditions versus 100% of T-cell proliferation is shown when statistically significant.