Original Articles

Generation of CD4$^+$ or CD8$^+$ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction

Claudia Prevosto, Marta Zancolli, Paolo Canevali, Maria Raffaella Zocchi, Alessandro Poggi

From the Laboratory of Experimental Oncology D, National Cancer Research Institute, 16132-Genoa, Italy (CP, PC, AP); Laboratory of Tumor Immunology, San Raffaele Scientific Institute, 20132, Milan, Italy (MZ, MRZ).

Funding: supported by grants from the Italian Association for Cancer Research (AIRC2005-7 to AP and AIRC2006 to MRZ) and from the Ministero della Salute 2005-2008. CP is a fellow supported by FIRC (Liliana Di Somma fellowship).

Manuscript received January 9, 2007.
Manuscript accepted May 2, 2007.

Correspondence:
Alessandro Poggi, PhD, MD, National Cancer Research Institute (IST) Genoa, Laboratory of Experimental Oncology D, Department of Translational Oncogenesis, Largo R. Benzi 10, 16132 Genoa, Italy.
E-mail: alessandro.poggi@istge.it

ABSTRACT

Background and Objectives

Mesenchymal stem cells (MSC) have been proposed as a way to treat graft-versus-host disease based on their immunosuppressive effect. We analyzed whether regulatory T cells can be generated in co-cultures of peripheral blood mononuclear cells (PBMC) and MSC.

Design and Methods

MSC were obtained from the bone marrow of four healthy donors and nine patients with acute leukemia in complete remission following chemotherapy. Short-term (4 days) co-cultures of MSC and autologous or allogeneic PBMC were set up, the lymphocytes harvested and their regulatory activity assessed.

Results

Lymphocytes harvested from MSC-PBMC co-cultures strongly inhibit (up to 95%) mixed lymphocyte reaction (MLR), recall to alloantigen, and CD3- or phytohemagglutinin-induced lymphocyte proliferation. These lymphocytes, termed regulatory cells (Reg_c), were all CD45$^+$CD2$^+$ with variable proportions of CD25$^+$ cells (range 40-75% n=10) and a minor fraction expressed CTLA4 (2-4%, n=10) or glucocorticoid-induced tumor necrosis factcor receptor-related gene (0.5-4% n=10). Both CD4$^+$ and CD8$^+$ Reg_c purified from MSC-PBMC co-cultures strongly inhibited lymphocyte proliferation at a 1:100 Reg_c:responder cell ratio. CD4$^+$ Reg_c expressed high levels of forkhead box P3 (Foxp3) mRNA while CD8$^+$ Reg_c did not. The effectiveness of Reg_c, whether CD4$^+$ or CD8$^+$, was 100-fold higher than that of CD4$^+$CD25^{+high} regulatory T cells. Reg_c were also generated from highly purified CD25$^-$ PBMC or CD4$^+$ or CD8$^+$ T cell subsets. Soluble factors, such as interleukin-10, transforming growth factor-β and prostaglandin E_2 did not appear to be involved in the generation of Reg_c or in the Reg_c-mediated immunosuppressive effect. Furthermore, cyclosporine A did not affect Reg_c generation or the immunosuppression induced by Reg_c.

Interpretation and Conclusions

These findings indicate that powerful regulatory CD4$^+$ or CD8$^+$ lymphocytes are generated in co-cultures of PBMC with MSC. This strongly suggests that these regulatory cells may amplify the reported MSC-mediated immunosuppressive effect.

Key words: mesenchymal stem cells, regulatory CD4$^+$ T cells, regulatory CD8$^+$ T cells, lymphocyte triggering.

Haematologica 2007; 92:881-888

©2007 Ferrata Storti Foundation

Bone marrow mesenchymal stem cells (MSC) are able to give rise, under appropriate culture conditions, to osteoblasts, chondrocytes, adipocytes and even neurons.[1-6] Thus, it has been proposed to use MSC to repair injured tissues, or after appropriate manipulation, to treat inherited disorders.[1-6] In addition, several studies have shown that MSC can also exert a tolerogenic effect.[7-16] Indeed, MSC inhibit lymphocyte proliferation in mixed lymphocyte reaction (MLR) when added as a third party as well as T lymphocyte proliferation in response to polyclonal mitogenic stimuli.[7-16] The administration of MSC was found to abolish graft-versus-host disease (GVHD) in a young patient who underwent bone marrow transplantation, strongly suggesting that these cells could be used *in vivo* as regulators of the immune response.[17] The MSC-mediated immunosuppressive effect is thought to be dependent on soluble factors released by these cells, such as transforming growth factor (TGF)-β[8], hepatocyte growth factor (HGF)[8], prostaglandin E_2 (PGE_2)[15] or metabolites of tryptophan generated by activation of indoleamine-dioxigenase (IDO) present in MSC.[14]

It is commonly accepted that immunosuppression can be accomplished by lymphocyte populations termed regulatory T cells.[18-33] The regulatory T-cell population resides mainly within the $CD4^+$ T-cell subset namely: $CD4^+CD25^{+high}$ forkhead box P3$^+$ (Foxp3$^+$) (T_{reg}) cells, $CD4^+IL10^+Foxp3^-$ (Tr1) cells, and $CD4^+TGF\text{-}\beta^+$(Th3) cells.[27,30-33] In addition, also $CD8^+$ T-cell subsets such as $CD8^+CD25^+$, $CD8^+CD28^-$ and $CD8^+IL\text{-}10^+$ can down-regulate lymphocyte activation and proliferation.[27,30-33] The suppressive mechanism can involve the direct interaction of a given regulatory subset and responder lymphocytes and/or the release, by the regulatory cells of immune regulating cytokines including TGF-β or IL10[30-33] which inhibit lymphocyte response.

A role of regulatory cells in MSC-mediated immunosuppression has been suggested by the increment of $CD4^+CD25^+CTLA4^+$ cells found in co-cultures of allogeneic MSC when added to MLR as a third party.[34] Thus, besides soluble mediators released by MSC, the MSC-mediated immunosuppressive effect could be amplified by the action of T_{reg}. However, it is not clear whether $CD4^+CD25^+CTLA4^+$ cells obtained from co-culture of MSC in MLR may function as T_{reg} and, if so, their effectiveness, and whether these putative T_{reg} are generated only when MSC are added as a third party to MLR. In this study, we investigated whether MSC induce by themselves, in co-cultures with lymphocytes, the generation of highly effective regulatory cells expressing either CD4 or CD8. Regulatory activity, compared to that of conventional $CD4^+$ T_{reg} cells was analyzed in MLR, recall to alloantigen as well as CD3- or phytohemagglutinin(PHA)-driven T-cell proliferation.

Results

Inhibition of lymphocyte proliferation by lymphocytes harvested from MSC-PBMC co-cultures.

We first analyzed whether, in MSC-PBMC co-cultures, MSC could induce the generation of cells with regulatory/immunosuppressive activity. To this aim, MSC were cultured with PBMC for 4 days then cells were harvested and added to primary MLR and cell proliferation was analyzed by ^{3}H thymidine uptake on day 7 (Figure 1A). We found that the addition of cells from MSC-PBMC co-cultures to MLR strongly inhibited cell proliferation (range of inhibition 65-95%, n=5). On this basis, these cells were thereafter termed regulatory cells (Reg_c). This effect was evident not only at 1:1 and 1:10 but also at 1:100 Reg_c: responding (R) PBMC ratios (60-80% range of inhibition, n=5) and it was still detectable at 1:250 to 1:500 Reg_c:R ratios (45-55% of inhibition, n=3). Comparable results were also obtained when analyzing proliferation after staining of responding cells with CFSE (*not shown*). Reg_c were generated from PBMC-MSC co-cultures using autologous (Figure 1) or allogeneic MSC (*not shown*).

Given the down-regulating effect of Reg_c on MLR, we next explored whether these cells could also affect lymphocyte proliferation to recall of alloantigen. To this aim, Reg_c were added to secondary MLR and proliferation was analyzed on day 3 after the second challenge with stimulating PBMC. We found that, Reg_c down-regulated cell proliferation to recall of alloantigen (Figure 1B) as well: indeed, at a 1:100 Reg_c:responder lymphocyte ratio, there was a 69-95% inhibition (n=4) of the second response to alloantigen. Furthermore, Reg_c could down-regulate CD3- (Figure 1C) or PHA-mediated triggering (Figure 1D). The close interaction between Reg_c and responding cells was necessary to achieve the inhibition of either MLR or recall to alloantigen or CD3-driven activation, as Reg_c had no effect on lymphocyte proliferation when they were separated from responder PBMC by a porous filter in Millicell chambers (Figure 1A-D). Furthermore, the addition to MLR or secondary MLR or CD3-stimulated PBMC of either unstimulated PBMC or lymphocytes activated for 4 days with either PHA or anti-CD3 monoclonal antibody (mAb), at the same ratios used above, did not affect lymphocyte proliferation (*not shown*). It is of note that the generation of Reg_c in co-culture of lymphocytes and MSC was achieved only when there was a close interaction between these two cell populations. Indeed, no Reg_c were harvested from PBMC-MSC co-cultures when these cells were separated from each other by a transmembrane porous filter in a Millicell chamber (*not shown*).

Reg_c are represented by $CD4^+$ and $CD8^+$ lymphocytes

On the basis of the ability of Reg_c generated in MSC-PBMC co-cultures to down-regulate lymphocyte proliferation, we explored whether these cells can express markers typical of $CD4^+$ T_{reg}.[30-33] We found that Reg_c were all

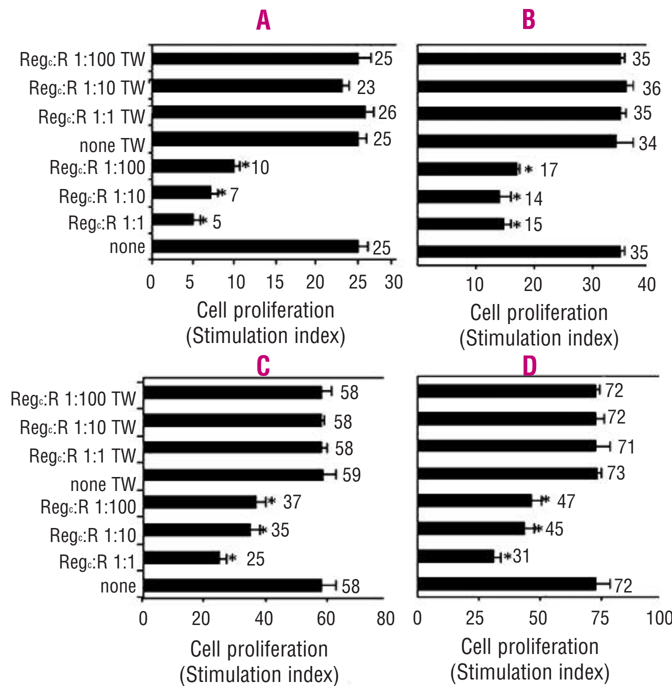

Figure 1. Cells generated from MSC-PBMC co-cultures (Reg$_c$) inhibit lymphocyte proliferation. Cells (Reg$_c$) generated from 4-day co-cultures of MSC with PBMC were added at the onset of culture at the indicated Reg$_c$:PBMC (as responder cells, R) ratios to MLR (A) or recall to alloantigen (B) or to PBMC stimulated with anti-CD3 mAb (C) or to PBMC stimulated with PHA (1 μg/mL) (D). In some experiments, the Reg$_c$ were separated from R by the membrane of a Millicell chamber (TW) in order to avoid physical contact between Reg$_c$ and R. In these experiments Reg$_c$ were generated by co-culturing PBMC with autologous MSC, and Reg$_c$ were autologous to responding PBMC. Cell proliferation was analyzed by ^{3}H-thymidine uptake during the last 18 h of cultures (7 days for MLR, 3 days for recall to alloantigen and 3 days for CD3$^-$ and PHA-mediated triggering). Results are expressed as stimulation index, that is the ratio between the ^{3}H-thymidine uptake of a given culture condition and the uptake of R alone. None: cell proliferation of PBMC in MLR (A), recall to alloantigen (B) or upon stimulation with either anti-CD3 mAb (C) or PHA (D) in the presence of PBMC from which Reg$_c$ were derived; although not shown, this cell proliferation was similar to that obtained in the absence of any cell population added to cultures. Numbers in each panel indicate the stimulation index in each culture condition. Columns represent the mean of five independent experiments and the bars indicate the standard deviation of these experiments. Asterisks: $p<0.01$.

Table 1. Phenotype of Reg$_c$ obtained from the indicated cell populations after co-culture with MSC.

	PBMC %	*CD4$^+$* %	*CD8$^+$* %	*CD25$^-$PBMC* %
CD3$^+$ T cells	70-85	95-98	90-95	70-85
CD2$^+$	80-95	98-100	98-100	90-95
CD4$^+$	57-75	98-100	1-2	65-70
CD8$^+$	35-45	3-8	98-100	30-45
CD25$^+$	40-75	45-80	2-5	1-3
CD28$^+$	65-85	80-95	80-95	65-85
CD69$^+$	4-15	20-35	5-10	1-2
CD16$^+$CD56$^+$ NK cells	3-10	1-2	0.5-2	4-8
CD19$^+$sIgM$^+$ B cells	5-10	0.5-2	0.3-3	5-10
GITR$^+$	2-4	2-5	0.5-1	0.5-1
CTLA4$^+$	1-3	1-4	0.5-2	0.5-2

Unfractionated PBMC or CD25$^-$ PBMC or highly purified CD4$^+$ or CD8$^+$ cells were cultured for 4 days with MSC, after this period cells were harvested and analyzed for their regulatory activity as Reg$_c$ (see the text) and were stained with anti-CD3 or anti-CD2 or anti-CD4 or anti-CD8 or anti-CD25 or anti-CD28 or anti-CD69 or anti-GITR or anti-CTLA4 (the expression of CTLA4 was analyzed in the cytoplasm and at the cell surface) mAb followed by anti-isotype specific goat anti-mouse PE-conjugated to determine the phenotype of Reg$_c$. In some experiments cells were double stained with anti-CD16-FITC and CD56-PE to identify natural killer cells or anti-CD19-PerCP and anti-surface IgM-FITC to identify B cells within Reg$_c$ populations. Data are expressed as the range of positive cells in six independent experiments.

CD45$^+$ and that the large majority of them were T cells as they expressed CD3 and CD2 antigens (95-98%, Table 1). Furthermore, a large proportion of Reg$_c$ were CD4$^+$ (57-75%), a fraction expressed CD8 (35-45%) while only a minor fraction expressed markers of CD4$^+$ T$_{reg}$, such as CTLA-4 (analyzed both at the cell surface and in the cytoplasm after cell permeabilization) and GITR (Table 1).

To determine the ability of MSC to generate Reg$_c$ from different lymphocyte subsets, MSC were co-cultured with highly purified CD25$^-$ PBMC or CD4$^+$ or CD8$^+$ T lymphocytes. The Reg$_c$ obtained from these cultures were analyzed for their inhibiting effect on lymphocyte proliferation. Reg$_c$ generated from CD25$^-$PBMC, CD4$^+$ or CD8$^+$ T cells inhibited lymphocyte proliferation in MLR at similar Reg$_c$:PBMC ratios (Figure 2A). It is of note that briefly activated (4 days) PHA blasts or long-term (30 days) activated CD4$^+$ or CD8$^+$ T cells did not affect lymphocyte proliferation induced by MLR (Figure 2B) or anti-CD3 mAb or PHA (*not shown*) when added to cell cultures at the same ratios used for Reg$_c$. Both CD8$^+$ and CD4$^+$ cells, obtained from MSC-PBMC co-cultures and purged of possible contaminating MSC by immunodepletion with anti-CD105 mAb, exerted a strong inhibition (80-95%, n=5) on lymphocyte proliferation regardless of the stimulus used (anti-CD3 mAb or PHA, Figure 2C; or MLR, *not shown*). Finally, we analyzed whether the expression of the Foxp3 transcription factor, typical of T$_{reg}$, was increased in MSC-PBMC co-cultures and in which lymphocyte population. A two to three fold increase in Foxp3 mRNA expression was found in MSC-PBMC co-cultures compared to PBMC alone (Figure 2D). This increase was not detected when CD25$^-$ PBMC were co-cultured with MSC (Figure 2D), although Reg$_c$ derived from CD25$^-$ PBMC-MSC co-cultures can inhibit cell proliferation (Figure 2A). Furthermore, the finding that the degree of regulatory activity was independent of the expression of Foxp3 mRNA was confirmed in different Reg$_c$ populations. Indeed, CD8$^+$ Reg$_c$ expressed a lower level of Foxp3 than PHA-activated lymphocytes (Figure 2D) but, unlike PHA blasts, they strongly down-regulated lymphocyte proliferation (Figure 2A and 2B). On the other hand, CD4$^+$ Reg$_c$ expressed higher levels of Foxp3 than CD8$^+$ Reg$_c$

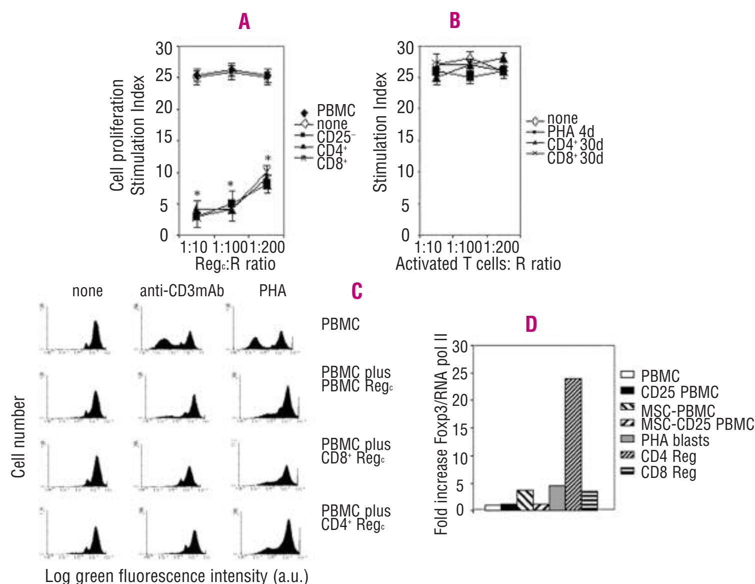

Figure 2. Different subsets of Reg$_c$ inhibit lymphocyte proliferation. A. Highly purified CD25⁻ or CD4⁺ or CD8⁺ lymphocytes were cultured for 4 days with MSC and then added as Reg$_c$ to the onset of MLR at the indicated Reg$_c$:PBMC (R) ratios (1:10, 1:100, 1:200), asterisks: $p<0.01$ (n=6). B. 4 day-activated PHA blasts or long-term cultured CD4⁺ or CD8⁺ lymphocytes (3 days) as activated T cells were added to the onset of MLR at the indicated activated T cells:R ratios (1:10, 1:100, 1:200). Results are expressed as stimulation index (SI), that is, the ratio between the ^{3}H-thymidine uptake of a given culture condition and the uptake of R alone. C. Unfractionated PBMC or CD8⁺ or CD4⁺ Reg$_c$ derived from PBMC co-cultured with MSC for 4 days were added at the onset of the proliferation assay to autologous PBMC labelled with CFSE stimulated with anti-CD3 mAb or PHA at the Reg$_c$:R ratio of 1:100. None: unstimulated cells. D. The expression of forkhead box p3 (Foxp3) mRNA was analyzed in PBMC or CD25⁻PBMC alone or co-cultured with MSC (MSC-PBMC, MSC-CD25⁻PBMC) or PHA blasts or CD4⁺Reg$_c$ or CD8⁺Reg$_c$ lymphocyte populations and expressed as change in fold increase, relative to the level of RNA polymerase II subunit A, using quantitative real-time PCR. PBMC were taken to have the reference value of 1. Data are representative of two independent experiments.

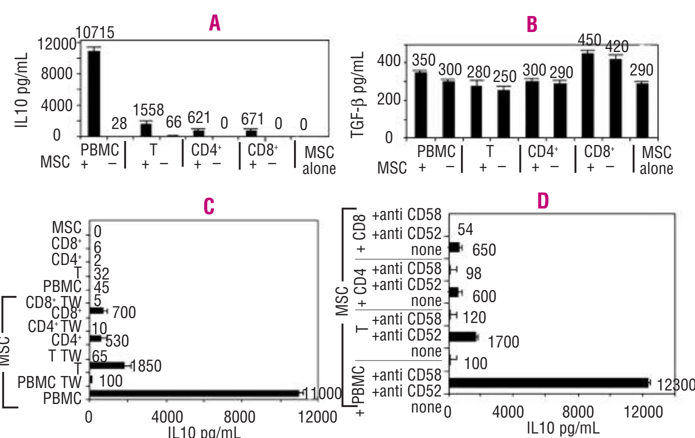

Figure 3. Production of IL10 and TGF-β in co-cultures of lymphocytes and MSC. The presence of IL10 (A) and TGF-β (B) cytokines was analyzed in culture supernatant (SN) harvested after 24 h of culture of PBMC or T or CD4⁺ or CD8⁺ cells with (⁺) or without (⁻) autologous MSC, or of MSC alone. The production of IL10 was evaluated also when lymphocytes were separated from MSC by a transwell (TW) (C) or in the presence of a combination of anti-CD58 and anti-CD2 mAb (5 μg/mL). (D). Results are expressed as pg/mL of the indicated cytokine evaluated with a fluorescence cytokine kit (Bender System) for IL10 or by ELISA for TGF-β. Numbers in panels indicate the amount of each cytokine. Less than 30 pg/mL of TGF-β were detected in the complete medium (*data not shown*).

(Figure 2D) but the regulatory activity of these two different cell populations was comparable (Figure 2C).

MSC stimulate PBMC and T-cell subsets to produce IL10 and TGFβ, immune-regulating cytokines.

The generation of Reg$_c$ in co-cultures of MSC with lymphocytes prompted us to determine whether immune-regulating cytokines, such as IL10 or TGF-β, were responsible for this effect. First, we analyzed whether these two cytokines were released in MSC-lymphocyte co-cultures. Secretion of IL10 and TGF-β in culture supernatants harvested from co-cultures of PBMC with MSC was analyzed after 24 hours of incubation. High levels of the immunosuppressive cytokine IL10 were found in MSC-PBMC co-culture supernatants (Figure 3A) while the amount of TGF-β was similar to that produced by MSC alone (Figure 3B). Less than 30 pg/mL of TGF-β were detected in the complete medium (*not shown*). In the presence of MSC, IL10 was secreted at 50 to 200-fold higher amounts than upon anti-CD3 mAb stimulation or MLR (*not shown*). To determine whether MSC can trigger a subset of PBMC to produce IL10, we performed co-cultures of MSC with purified T, CD4⁺ or CD8⁺ T lymphocytes. Highly purified *ex vivo* isolated T (99-100%, n=4), CD4⁺ T (96-100%, n=4) and CD8⁺ T (95-100%, n=4) cells were obtained by negative selection. We found that both unfractionated T cells, CD4⁺ or CD8⁺ T lymphocyte subsets co-cultured with MSC can produce IL10 although at a lower level compared to unfractionated PBMC. Next, we determined whether contact between either PBMC or lymphocyte subsets and MSC was necessary to induce lymphocyte activation. To this aim, PBMC or T-cell subsets were seeded in Millicell filter chambers in culture plates separated from MSC adherent at the bottom of culture wells. Parallel experiments with lymphocytes and MSC in direct contact were performed as a control. The presence of IL10 in culture supernatants was thus analyzed after 24 hours of incubation (Figure 3). Secretion of

this cytokine was strongly reduced (>90% inhibition) when responder cells (PBMC, or T or CD4+ or CD8+ cells) were separated from MSC by the porous filter of Millicell chambers (Figure 3C). Furthermore, we analyzed the surface molecular structures responsible for the delivery of the triggering signal to PBMC or T-cell subsets.

To this aim, a series of co-culture experiments was performed in the presence of mAb directed to surface receptor/ligand pairs including CD2/LFA2-CD58/LFA3 and CD11a/LFA1-CD54/ICAM1, possibly involved in cell to cell interaction.[22] As shown in Figure 3D, we determined that the interaction of CD2/LFA2 and CD58/LFA3 is responsible for the delivery of the activation signal that leads to cytokine production. Indeed, the addition of anti-CD2 and/or anti-CD58 mAb strongly inhibited (65-80%, n=4) the production of IL10 (Figure 3D) by T cells as well as CD4+ and CD8+ T-cell subsets. On the other hand, the addition of anti-CD11a/LFA1 mAb, alone or in combination with anti-CD54/ICAM1 mAb, had a lower inhibitory effect (range 25-35% in six independent experiments, *not shown*). The amount of TGF-β detected in culture supernatants did not change if lymphocytes and MSC were separated by a transwell or when the co-cultures were performed in the presence of anti-LFA3/CD58 mAb (*data not shown*). The addition of mAb specific for CD2 or CD58 or CD11a or CD54 (alone or in combination) neither inhibited nor induced the generation of Reg_c (*not shown*).

Comparison of CD4+ or CD8+ Reg_c and CD4+CD25+T_{reg} effectiveness

We addressed the question of whether the effectiveness of the inhibition exerted by Reg_c was comparable to that of naturally occurring CD4+CD25+ T_{reg}. As shown in Figure 4A-B, purified CD4+CD25+ T_{reg} inhibited by 50-70% PBMC proliferation to alloantigen or by 30-50% upon triggering with PHA (n=3) at a 1:1 CD4+CD25+ $Treg$:responder (R) lymphocyte ratio. This inhibiting effect was reduced by 50-70% and was abolished at ratios of 1:10 and 1:100 CD4+CD25+ T_{reg}:R, respectively. On the other hand, Reg_c (either CD4+ or CD8+) derived from the same donors of purified CD4+CD25+ T_{reg} cells, almost blocked lymphocyte proliferation at 1:1 and 1:10 Reg_c:R ratios while Reg_c inhibited proliferation by 80% or 60% at 1:100 and 1:200 Reg_c:R ratios, respectively.

Role of soluble factors in inducing the generation of Reg_c and Reg_c-mediated immunosuppression

We next analyzed whether IL10, TGF-β and PGE_2 were involved in the generation of Reg_c as well as in the Reg_c-mediated regulation of lymphocyte proliferation. Co-cultures of lymphocytes and MSC were performed in the presence of anti-IL10 and/or anti-TGF-β blocking antibodies and/or NS398, an inhibitor of PGE_2 synthesis, to evaluate the effect of these soluble factors on Reg_c precursors (pCD4+ and pCD8+ of Figure 4C). As shown in Figure 4C, the anti-IL10 and the anti-TGF-β antibodies, alone or

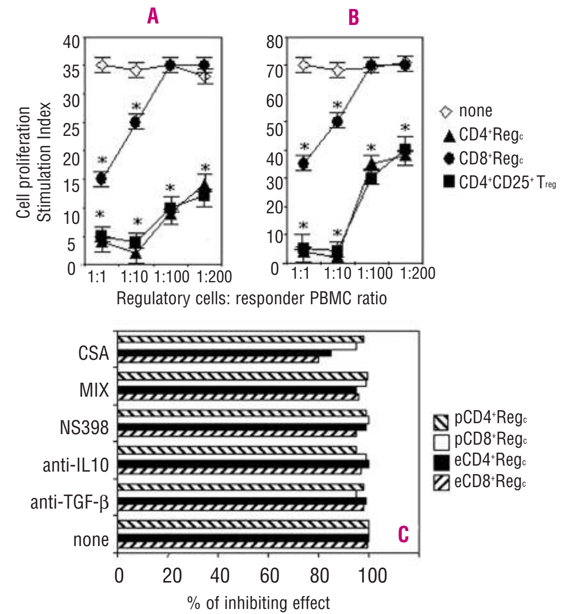

Figure 4. Comparison between the effectiveness of Reg_c and CD4+CD25+ Treg and role of soluble factors and CSA in the generation of Reg_c and in Reg_c-mediated immunosuppression. CD4+Reg_c or CD8+Reg_c or CD4+CD25+ T_{reg} cell populations were added to autologous PBMC triggered with anti-CD3 mAb (A) or PHA (B) at the indicated regulatory cell-responder PBMC ratios (1:1, 1:10, 1:100, 1:200) and proliferation analyzed on day 3 by ^{3}H-thymidine uptake. Results are expressed as stimulation index (SI), that is, the ratio between the ^{3}H-thymidine uptake of a given culture condition and the uptake of responder PBMC alone. None: indicates the SI in the presence of PBMC from which Reg_c or T_{reg} were derived; this SI was similar to that observed in the absence of any cell population added (*data not shown*). C. CSA (500 ng/mL) or anti-IL10 mAb (5 μg/mL) or anti-TGF-β mAb (5 μg/mL) or NS398 (COX-2 inhibitor, 15 μM) or a combination of anti-IL10 and anti-TGF-β mAb and NS398 (MIX) were added to lymphocyte-MSC co-cultures of precursors of Reg_c (pCD4+Reg_c, pCD8+Reg_c) or to effector Reg_c (eCD4+Reg_c or eCD8+Reg_c) during the regulatory assay. Asterisks: p<0.01.

in combination with the NS398 inhibitor (MIX), did not affect the generation of Reg_c (Figure 4C). We then determined whether IL10, TGF-β and PGE_2 could be involved in the immunosuppressive mechanism mediated by Reg_c on lymphocyte proliferation.

Thus, the anti-IL10 and anti-TGF-β antibodies, alone or together with NS398, were added at the onset of the regulatory assay, in order to analyze the effect of each soluble factor on the function of Reg_c effector cells (eCD4+ and eCD8+ of Figure 4C). Again, the blockade of IL10, TGF-β and PGE_2 did not affect the Reg_c-mediated immunosuppressive effect on the stimulation by anti-CD3 (Figure 4C) or alloantigen or PHA (*data not shown*).

Effect of CSA on the generation of Reg_c and on Reg_c-mediated immunosuppression

Given the inhibiting effect of Reg_c on lymphocyte proliferation, we next explored whether the immunosuppressive drug CSA could affect Reg_c generation or Reg_c-mediated immunosuppression. Indeed, CSA is administered to down-regulate lymphocyte response to prevent

and treat graft-versus-host disease (GVHD). As MSC have been used for the treatment of GVHD, it is of relevance to determine whether CSA could reduce MSC-induced generation of Reg_c or immunosuppression exerted by Reg_c. When generated in the presence of CSA (500 ng/mL), CD4$^+$ or CD8$^+$ Reg_c still exerted a strong inhibiting effect on PHA-induced lymphocyte proliferation (up to 95% at a 1:100 Reg_c:responder cell ratio) comparable to that exerted by Reg_c generated without CSA (Figure 4C).

However, the inhibition of lymphocyte proliferation to anti-CD3 mAb or alloantigen exerted by Reg_c obtained in the presence of CSA (range of inhibition 50-85% n=4) was less effective than that displayed by Reg_c generated without CSA (range of inhibition 70-95%, n=4) (*data not shown*). Finally, we analyzed whether CSA could impair the immunosuppressive function of CD4$^+$ or CD8$^+$ Reg_c, pretreated with the drug, washed and added to the regulatory assay. We found that CSA treatment of Reg_c did not affect the Reg_c-mediated immunosuppression (Figure 4C).

Discussion

In this study, we have shown that MSC by themselves trigger the generation of highly effective CD4$^+$ or CD8$^+$ regulatory T cells (Reg_c). Although CD4$^+$ Reg_c do not express markers typical of naturally occurring CD4$^+$ CD25$^+$ T_{reg}, such as CTLA4 and GITR, they display a stronger immunoregulatory activity than CD4$^+$CD25$^+$$T_{reg}$. On the other hand, we found that CD8$^+$ Reg_c can express CD28, at variance with typical CD8$^+$CD28$^-$ regulatory T cells.[33]

Several reports have claimed that MSC can exert a pleiotropic immunosuppressive effect down-regulating (i) lymphocyte proliferation in MLR, when MSC are added as a third party[7-16], and (ii) cytokine production by both antigen-presenting cells and T cells.[15] This effect was mainly ascribed to the production by MSC of cytokines (TGF-β[8] HGF[8]), metabolites (PGE_2[15]) or enzymes (IDO).[14] The reported production of soluble factors and the consequent strong inhibition of MLR was observed after adding MSC, as a third party, to MLR at a MSC:PBMC ratio of 1:1 or 1:2.[7-16] This raises a question: are these MSC:PBMC ratios achieved *in vivo*? Indeed, according to the immunosuppression observed *in vivo*,[17] relatively high numbers of MSC should be injected to obtain this effect. This may be of great relevance in planning the dose of MSC to administer. Here, we provide functional evidence that immunosuppression can also be produced by Reg_c generated upon interaction with MSC. This finding might explain the therapeutic effect observed *in vivo* following the injection of relatively low numbers of MSC compared to the number of lymphocytes present in a given patient.[17]

The role of naturally occurring CD4$^+$CD25$^+$ T_{reg} in MSC-mediated immunosuppressive effect is still controversial.[34,39] Indeed, Krampera *et al*.[39] reported that MSC induce a strong anti-proliferative effect not associated with any effect on enhancement of T regulatory activity. On the other hand, Maccario *et al*.[34] showed that lymphocyte populations containing variable proportions of putative CD4$^+$CD25$^+$ T_{reg} were obtained when MSC were added to MLR, as a third party, at ratios of 1:1 and 1:2 MSC:PBMC. However, the regulatory function of these *bona fide* CD4$^+$CD25$^+$ T_{reg} has not been analyzed. In this study, we have shown that effector CD4$^+$, but also CD8$^+$, regulatory lymphocytes (Reg_c) can be derived from PBMC-MSC co-cultures; indeed, both CD4$^+$ and CD8$^+$ Reg_c can inhibit MLR, recall to alloantigen, and PHA- and CD3-driven stimulation. Furthermore, we found that not only CD4$^+$ but also CD8$^+$ cells, sorted before the co-culture with MSC, can give rise to functional Reg_c. Our results do not exclude the presence of naturally occurring T_{reg} within the CD4$^+$ Reg_c; however, the ratio at which CD4$^+$ Reg_c exerted their inhibiting effect is very low (1:100-1:250 Reg_c:PBMC ratio) compared to that reported in the literature and in this study for Tre_g (1:1-1:10 Tre_g:PBMC ratio).[18,22,29,32,33] Taken together these results indicate that MSC can induce the generation of highly effective Reg_c from different lymphocyte subsets (CD25$^-$, CD8$^+$ or CD4$^+$) and that Reg_c are present in different lymphocyte subsets co-cultured with MSC (CD8$^+$ or CD4$^+$). It is difficult to determine whether our CD4$^+$$Reg_c$ or CD8$^+$$Reg_c$ are novel populations of regulatory cells. Indeed, the findings that in our experimental system CD4$^+$$Reg_c$ do not express CTLA4 and GITR, while CD8$^+$$Reg_c$ express CD28 would suggest that Reg_c are different from some of the typical regulatory T cells described.[30-33,40]

The possibility that residual contaminating MSC could be present within Reg_c populations, and thus they may have a role in inhibiting lymphocyte proliferation, is very unlikely as the whole population of Reg_c expressed the leukocyte marker CD45. Moreover, cells which do not express markers of MSC, such as CD105 or SH3 and SH4, can down-regulate lymphocyte proliferation. Finally, the number of MSC possibly present in a given Reg_c populations should be less than 1% (as this is the threshold of immunofluorescence), thus the ratio between MSC and responding lymphocytes should be 1:100.000, when Reg_c function at a Reg_c:responder cell ratio of 1:100. This number of MSC is not compatible with any inhibition of cell proliferation also according to the literature.[1-16] The molecular mechanism through which Reg_c can exert their potent regulatory effect remains to be elucidated. It has been proposed that Tre_g may down-regulate immune responses through direct interaction of CTLA4 on suppressor T cells and CD80 or CD86 on effector cells;[32,33] this interaction may also occur between suppressor T cells and antigen-presenting cells leading to activation of IDO, reduction of extracellular free tryptophan and consequent inhibition of T-cell proliferation.[32,33] Reg_c generated in our MSC-PBMC cocultures did not express CTLA4, making it unlikely that the above mentioned mechanism is primarily involved

in mediating the immunosuppressive effect in this experimental system. Furthermore, Reg_c can exert their inhibiting effect independently of Foxp3 transcription factor expression, unlike what is observed for $Treg$.[30-33] In our experimental system, the soluble factors IL10, TGF-β and PGE_2 did not appear to be involved in Reg_c generation and Reg_c-mediated regulatory function. One could hypothesize that soluble factors other than TGF-β, IL10 and PGE2 are responsible for Reg_c-mediated effect. Indeed, we found that during MSC-lymphocytes co-cultures, high amounts of IFN-γ and TNF-α can be released; these cytokines may be involved in the regulation of cell proliferation (*not shown*). However, similar amounts of IFN-γ and TNF-α are released during alloantigen stimulation or triggering of lymphocytes with PHA or anti-CD3 mAb, suggesting that, if these cytokines are involved, they are not sufficient to explain the Reg_c-mediated inhibiting effect. In addition, the regulation of lymphocyte proliferation may occur by induction of apoptosis. Indeed, we have found an increase of the number of apoptotic cells in lymphocyte cultures triggered with anti-CD3 mAb in the presence of Reg_c; experiments are in progress to determine the mechanism of this apoptotic event. Finally, a role for other mediators, such as adenosine, could be an alternative explanation for the potent IL10/TGF-β independent Reg_c-mediated regulation. It has been recently reported that adenosine can play a critical role in T-cell mediated regulation of inflammation in colitis by inhibiting the production of inflammatory cytokines without affecting IL10 and TGF-β secretion.[44]

The frequency of cells with inhibiting activity within the populations of Reg_c is still to be determined. It is conceivable that a large fraction, and not a small subset, of lymphocytes derived from co-cultures with MSC display regulatory functions, as Reg_c can exert their regulating activity at very low Reg_c:responding cell ratios. It is of note that neither surface nor cytoplasmic markers expressed by conventional described subsets of regulatory cells[19,30-32] are selectively present on Reg_c; this hampers the determination of the frequency of regulatory cells within Reg_c populations.

In conclusion, we could hypothesize that two different mechanisms of MSC-mediated immunosuppression might occur, depending on the ratio between MSC and responding cells: the first one mainly mediated by soluble factors (TGF-β and PGE_2) during the interaction between MSC and lymphocytes at 1:1 and 1:10 MSC-lymphocyte ratios and the second one dependent on Reg_c generation evident at lower ratios, such as 1:2000. Thus, MSC may exert their immunosuppressive function directly through the release of factors such as TGF-β, HGF, IDO and PGE_2 able to inhibit responder T-cell proliferation; on the other hand this immunosuppressive effect could be amplified by the generation of Reg_c. Importantly, the generation of Reg_c and the regulatory effect mediated by the different subpopulations of Reg_c appear to be independent of CSA treatment. This finding is of great relevance as CSA is commonly used in the prevention and treatment of GVHD, suggesting that CSA can co-operate with Reg_c in inducing immunosuppression when MSC are administered.

Finally, it has been recently reported that NK cells can regulate MSC survival.[37,41-43] Thus, one can hypothesize that NK cells would affect the generation of Reg_c by eliminating MSC and consequently regulating the MSC-mediated immunosuppressive effect. This should be considered when it is planned to administer MSC to patients in whom latent viral infections can be reactivated by immunosuppressive treatment.

Authors' Contributions

CP and MZ contributed equally to this work; PC second author, MRZ third author, AP last author; CP and MZ designed and performed experiments. PC performed experiments; MRZ and AP performed and designed some experiments, analyzed and interpreted the data, drafted and revised the article. All the authors approved the final version of this manuscript. AP takes primary responsibility for the paper and created all figures and Table 1.

Conflict of Interest

The authors reported no potential conflicts of interest.

References

1. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.
2. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximaze the yelds of early progenitors and evaluate their quality. Stem Cells 2002;20:530-41.
3. Kawada H, Fujita J, Kinjo K, Matsuzaki Y, Tsuma M, Miyatake H, et al. Non-hematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 2004;104:3581-7.
4. Lazarus HM, Haynesworth SE, Gerson SL, Rosenthal NS, Caplan AI. Ex vivo expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. Bone marrow Transplant 1995; 16: 557-64.
5. Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY et al.Isolated allogeneic bone-marrow derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. Proc Natl Sci USA Acad 2002;99:8932-7.
6. Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res 2000;61:364-70.
7. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. Transplantation 2003;75:389-97.
8. Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Stucchi C, Cleris L. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 2002; 99: 3838-43.
9. Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy 2003; 5:

485-9.
10. Le Blanc K, Tammik L, Sundberg B, Haynesworth SE, Ringden O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. Scand J Immunol 2003; 57:11-20.
11. Potian JA, Aviv H, Ponzio NM, Harrison JS, Rameshwar P. Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. J Immunol 2003; 171:3426-34.
12. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J et al. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 2003; 102: 3837-44.
13. Beyth S, Borovsky Z, Mevorach D, Liebergall M, Gazit Z, Aslan H et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T cell unresponsiveness. Blood 2005; 105:2214-9.
14. Meisel R, Zibert A, Laryea M, Gobel U, Daubener U, Dilloo D. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619-21.
15. Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815-22.
16. Augello A, Tasso R, Negrini SM, Amateis A, Indiveri F, Cancedda R at al. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed cell death 1 pathway. Eur J Immunol 2005;35:1482-90.
17. Le Blanc K, Rasmusson I, Sundberg B, Gotherstrom C, Hussan M, Uzunel M et al. Treatment of severe acute graft-versus host disease with third party haploidentical mesenchymal stem cells. Lancet 2004; 363:1439-41.
18. Mahic M, Yaqub S, Johansson CC, Tasken K, Aandahl EM. Foxp3+ CD4+CD25+ adaptive regulatory T cells express cyclooxygenase-2 and suppress effector T cells by a prostaglandin E2-dependent mechanism. J Immunol 2006; 177:246-54.
19. Sakaguchi S. Regulatory T cells: key controllers of immunologic self-tolerance Cell 2000; 101: 455-8.
20. Yamagiwa S, Gray JD, Hashimoto S, Horwitz DA. A role for TGF-, in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. J Immunol 166:7282-9.
21. Strom TB, Carpenter CB. Prostaglandin as an effective antirejection therapy in rat renal allograft recipient. Transplantation 1983; 35:279-81.
22. Baratelli F, Lin Y, Zhu L, Yang SC, Henze-Vourc'h N, Zeng G et al. Prostaglandin E2 induces Foxp3 gene expression and T regulatory cell function in human CD4+ T cells. J. Immunol 2005; 175:1483-90.
23. Rifa'I M, Kawamoto Y, Nakashima I, Suzuki H. Essential role of CD8+ CD122+ regulatory T cells in the maintenance of T cell homeostasis. J Exp Med 2004; 200:1123-34.
24. Xystrakis E, Dejean AS, Bernard I, Druet P, Liblau R, Gonzales-Dunia D, et al. Identification of a novel natural regulatory CD8 T-cell subset and analysis of its mechanism of regulation. Blood 2004; 104:3294-301.
25. Zheng SG, Wang JH, Koss MN, Quismorio F Jr, Gray JD, Horwitz DA. CD4+ and CD8+ regulatory T cells generated ex-vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus host disease with lupus-like syndrome. J Immunol 2004; 172:1531-9.
26. Wei S, Kryczek I, Zou L, Daniel B, Cheng P, Mottram P et al. Plasmocitoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma. Cancer Res 2005; 65:5020-26.
27. Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. Immunol Rev 2006; 212: 28-50.
28. Groux H. Type 1 T-regulatory cells: their role in the control of immune responses. Transplantation 2003;15: 8S-12S.
29. Annunziato F, Cosmi L, Liotta F, Lazzeri E, Manetti R, Vanini V, et al. Phenotype, localization and mechanism of suppression of CD4+ CD25+ regulatory thymocytes. J Exp Med 2002;196:379-87.
30. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. Nat Immunol 2005;6:1142-51.
31. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. Nat Rev Immunol 2002; 2:389–400.
32. Sakaguchi S. Naturally arising CD4+ regulatory T cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004; 22: 531–62.
33. Von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat Immunol 2005; 6:338-43.
34. Maccario R, Podestà M, Moretta A, Cometa A, Comoli P, Montagna D et al. Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica 2005; 90:516-25.
35. Moretta A, Poggi A, Pende D, Tripodi G, Orengo AM, Pella N et al. CD69-mediated pathway of lymphocyte activation: anti-CD69 monoclonal antibodies trigger the cytolytic activity of different lymphoid effector cells with the exception of cytolytic T lymphocytes expressing T cell receptor α. J Exp Med 1991;174:1393-8.
36. Poggi A, Tomasello E, Revello V, Nanni L, Costa P, Moretta L. p40 regulates NK cell activation mediated by NK receptors for HLA class I antigens and TCR-mediated triggering of T lymphocytes. Int Immunol 1997;9:1271-9.
37. Poggi A, Prevosto C, Massaro AM, Negrini S, Urbani S, Pierri I, et al. Interaction between human natural killer cells and bone marrow stromal cells induces NK cell triggering. Role of NKp30 and NKG2D receptors. J Immunol 2005;175:6352-60.
38. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-DDCT) method. Methods 2001; 25:402-8.
39. Krampera M, Cosmi L, Angeli R, Pasini A, Lotta F, Andreini A et al. Role of IFN-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells 2006;24:386-98.
40. Zou W. Regulatory T cells, tumour immunity and immunotherapy. Nat Rev 2006;6:295-307.
41. Poggi A, Massaro AM, Negrini S, Pierri I, Balocco M, Michelis G, et al. Evidence for killing of mesenchymal stem cells (MSC) by autologous natural killer lymphocytes. Blood (ASH Annual Meeting Abstracts), Nov 2004; 104:1290.
42. Spaggiari GM, Capobianco A, Becchetti S, Mingari MC, Moretta L. Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood 2006;107:1484-90.
43. Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells 2006; 24:74-85.
44. Naganuma M, Wiznerowicz EB, Lappas CM, Linden J, Worthington MT, Ernst PB. Cutting edge: critical role for A2A adenosine receptors in the T cell-mediated regulation of colitis. J Immunol 2006;177:2765-9.