

REGULAR ARTICLE

Mesenchymal stromal cells up-regulate CD39 and increase adenosine production to suppress activated T-lymphocytes

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Abstract Mesenchymal stromal cells (MSCs) suppress T cell responses through mechanisms not completely understood. Adenosine is a strong immunosuppressant that acts mainly through its receptor A_{2a} (ADORA2A). Extracellular adenosine levels are a net result of its production (mediated by CD39 and CD73), and of its conversion into inosine by Adenosine Deaminase (ADA). Here we investigated the involvement of ADO in the immunomodulation promoted by MSCs. Human T lymphocytes were activated and cultured with or without MSCs. Compared to lymphocytes cultured without MSCs, co-cultured lymphocytes were suppressed and expressed higher levels of ADORA2A and lower levels of ADA. In co-cultures, the percentage of MSCs expressing CD39, and of T lymphocytes expressing CD73, increased significantly and adenosine levels were higher. Incubation of MSCs with media conditioned by activated T lymphocytes induced the production of adenosine to levels similar to those observed in co-cultures, indicating that adenosine production was mainly derived from MSCs. Finally, blocking ADORA2A signaling raised lymphocyte proliferation significantly. Our results suggest that some of the immunomodulatory properties of MSCs may, in part, be mediated through the modulation of components related to adenosine signaling. These findings may open new avenues for the development of new treatments for GVHD and other inflammatory diseases.

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Introduction

Mesenchymal stromal cells (MSCs) are multipotential cells with the ability to differentiate into various components of the

marrow microenvironment, such as bone, adipose, and stromal tissues (Friedenstein et al., 1966; Pittenger et al., 1999). Another important propriety of MSCs is the immunosuppressive effect they have on several immune cells, including B, T, and Natural Killer (Corcione et al., 2006; Di Nicola et al., 2002; Krampera et al., 2003; Rasmusson et al., 2005; Spaggiari et al., 2006; Tse et al., 2003). One of the mechanisms by which MSCs modulate immune responses is through generation of regulatory T-cells (Tregs) (Di et al., 2008; Maccario et al., 2005; Prevosto et al., 2007). Recently, it was shown that Tregs

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produce adenosine, an endogenous purine nucleoside that plays an important role in Tregs-mediated immunosuppression (Borsellino et al., 2007; Deaglio et al., 2007; Kobie et al., 2006; Mandapathil et al., 2010).

Adenosine is constitutively present at low levels in the extracellular space, but its concentration increases in metabolic stress (Sitkovsky and Lukashev, 2005). Adenosine has various immunoregulatory activities that are mediated through four adenosine receptors: A_1 , A_{2a} , A_{2b} , and A_3 (Thiel et al., 2003). It has been suggested that A_{2a} adenosine receptors (ADORA2A) act as "sensors" for extracellular adenosine levels (Sitkovsky and Ohta, 2005). In immune cells, ADORA2A signaled by adenosine induce an elevation of cytoplasmic cAMP, a strong immunosuppressive agent (Sitkovsky et al., 2004; Ohta and Sitkovsky, 2001; Gessi et al., 2007; Fredholm et al., 2003) that suppress T cell and cytokine secretion and ultimately prevents inflammation (Lappas et al., 2010; Koshiba et al., 1997).

During inflammation, adenosine production is increased and mediated by two sequential enzymes, CD39 and CD73. CD39, a member of the ecto-nucleoside triphosphate diphosphohydrolase family, converts ATP and ADP into 5'-AMP whereas CD73, an ecto-5'-nucleotidase, converts 5'-AMP into adenosine (Resta et al., 1998; Eltzschig et al., 2003; Behdad et al., 2009). Adenosine levels are controlled by Adenosine Deaminase (ADA) that converts adenosine into inosine (Franco et al., 1998). ADA can bind to CD26, which reduces the local concentration of adenosine on the cell surface and prevents the binding between adenosine with its receptors, mainly ADORA2A. The lack of this signal allows T cells to escape from the adenosine-mediated inhibition mechanism, leading to inflammation (Morimoto and Schlossman, 1998; Kameoka et al., 1993; Dong et al., 1996).

Although generation of adenosine by Tregs has been reported (Borsellino et al., 2007; Deaglio et al., 2007; Kobie et al., 2006; Mandapathil et al., 2010), the role of MSC in the modulation of adenosine levels, in inflammatory situations, such as those observed following T-lymphocyte activation, has not been established. Here we investigated the involvement of components related to adenosine signaling in the immunomodulation promoted by MSCs. Our results show that under inflammatory stimuli, such as that provided by activated T-lymphocytes, an increased percentage of MSCs express CD39, resulting in an increased production of adenosine. Specific blocking of adenosine signaling through ADORA2A receptors resulted in increased T-lymphocyte proliferation, confirming the immunosuppressive role of MSC-derived adenosine. We also show that, in the presence of MSC, T-lymphocytes (including Tregs) express CD73. These findings have important implications in the understanding of the molecular mechanisms underlying the immunomodulatory properties associated with MSCs. Finally, this knowledge may help in the development of new protocols for the treatment of GVHD and other inflammatory diseases.

Results

MSC characterization

Cultured MSCs derived from human bone marrow showed capacity for differentiation into adipocytes, chondrocytes, and osteocytes and a typical MSC immunophenotype was

observed in the cultured samples. MSCs were positive for CD73, CD90, CD29, CD13, CD44, CD49e and HLA-class I markers and negative for hematopoietic cells markers (CD34, CD14, CD45, and glycophorin A), endothelial cells (CD31 and KDR), and HLA-class II (Supplemental Fig. 1).

Suppression of T cell proliferation by MSCs

Proliferation of activated T cells was inhibited by MSCs. We observed that only half of the cells co-cultured with MSCs incorporated BrdU, as compared to cells cultured without MSCs. For instance, while the percentage of proliferating cells was 37.93% for T cells cultured without MSCs, only 18.43% of the cells cultured with MSC actively incorporated BrdU (Fig. 1). This experiment was repeated three times and similar results were found (data not shown).

ADA and ADORA2A mRNA levels in $CD4^+$ T cells cultivated with or without MSCs

T cells cultured with MSCs had lower levels of ADA transcripts ($p=0.0460$) (Fig. 2A) and higher levels of ADORA2A transcripts ($p<0.0001$) (Fig. 2B), when compared to cultured cells without MSCs

ADA activity quantification

Although not statistically significant, a higher ADA activity was observed in the culture media obtained from T cells cultivated with MSCs, as compared to that observed in cultures without MSCs (Supplemental Fig. 2).

Expression of CD26, CD39 and CD73 in $CD4^+$ T cells, in $CD4^+CD25^{hi}$ Tregs, and in MSCs

Disregarding the presence of MSC in the culture, the percentage of cells expressing CD26 was higher in Tregs than in $CD4^+$ T cells (Fig. 3A). Of notice, the percentage of $CD4^+$ T cells expressing CD26 was significantly lower when co-cultured with MSCs than without ($p=0.0016$), while the percentage of Tregs expressing CD26 was not changed.

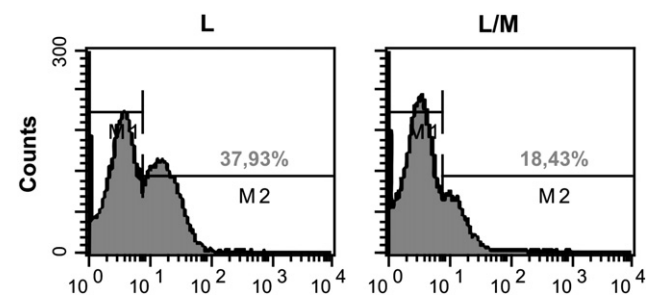


Figure 1 $CD3^+$ lymphocyte proliferation assay. Representative flow cytometry of BrdU incorporation by $CD3^+$ lymphocytes. $CD3^+$ lymphocytes were activated by anti-CD2/CD3/CD28 beads and cultured without (L) or with (L/M) MSCs. Evaluation was performed on the fifth day following activation.

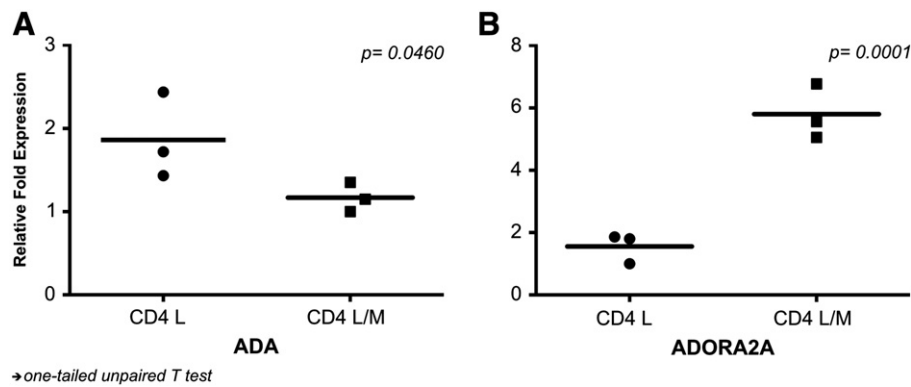


Figure 2 ADA and ADORA2A mRNA levels. CD3⁺ T cells from 3 healthy volunteers were activated with anti-CD2/CD3/CD28 beads and cultured without or with MSCs. On the fifth day following activation, CD4⁺ T cells were immunomagnetically purified from lymphocyte only cells (CD4 L) and from lymphocyte plus MSCs (CD4 L/M), and real time PCR was performed. (A) ADA mRNA levels; (B) ADORA2A mRNA levels. The relative fold values were obtained by the $2^{-\Delta\Delta Ct}$ method using the median Ct value of the samples from cells cultivated without MSCs, as a reference.

We observed that the percentage of cells expressing CD39 was significantly higher in Tregs than CD4⁺ T cells. There was no difference between activated T cells cultured with or without MSCs (Fig. 3B).

A very small percentage (~1.5%) of T cells (both, CD4⁺ T cells or Tregs) expressed CD73 in cultures carried in the absence of MSC; nevertheless, when cultured in the presence of MSC, a higher percentage of these cells expressed CD73. Moreover, although not statistically significant, in MSC co-cultures, the percentage of Tregs expressing CD73 was 31% higher than the observed for CD4⁺ T cells (Fig. 3C). Of notice, the percentage of double positive CD39⁺CD73⁺ Tregs raised dramatically upon co-culture with MSCs (Fig. 3D).

Finally, the evaluation of CD39 and CD73 in MSC, cultured alone or in the presence of activated T cells, revealed that the percentage of MSCs expressing CD39 (Fig. 3E) was significantly increased ($p=0.0094$) from 15% to approximately 35%. Almost all MSC express CD73 when cultured alone and, in the presence of activated T cells, this percentage remained above 90% (Fig. 3F). As a result, the percentage of double positive CD39⁺CD73⁺ MSC was three times higher, when co-cultured with activated lymphocytes, as compared to MSC cultured alone (Fig. 3G).

Adenosine quantification

Higher levels of adenosine were found in the supernatant of MSC co-cultures than in the cultures without MSCs ($p=0.0009$). In addition, we observed that in the presence of supernatant of activated T cells, MSCs produced two times more adenosine than the MSCs cultured without inflammatory stimulus (Fig. 4).

Adenosine signaling through ADORA2A is involved in T-lymphocyte suppression by MSCs

As expected, proliferation of lymphocytes pre-treated with ZM 241385 (a highly selective ADORA2A antagonist) and co-cultured with MSCs, was significantly higher than similarly

co-cultured untreated T-lymphocytes with a mean of 29% more proliferating cells, $p=0.013$ (Fig. 5).

Discussion

Our study shows that in an inflammatory environment, such as that provided by activated T-lymphocytes, MSCs elevate CD39 expression and increase the production of adenosine, suppressing T cell proliferation. In addition, suppressed T cells present elevated levels of ADORA2A, that when blocked, partially restores T-lymphocyte proliferation.

These results are in line with the known strong immunosuppressive effects of extracellular adenosine accumulation, mediated mainly through ADORA2A adenosine receptors (Sitkovsky and Ohta, 2005), and with previous reports showing the role of adenosine production by Tregs in immune response suppression (Deaglio et al., 2007; Kobie et al., 2006; Mandapathil et al., 2010; Schmitt et al., 2009). Moreover the cellular expression of ADORA2A in T cells is directly proportional to the intensity of T cell response to adenosine (Apasov et al., 2000; Armstrong et al., 2001), inhibiting T cell activation and expansion (Huang et al., 1997). Concordantly, our results show that in co-culture, the presence of MSCs induced a higher expression of ADORA2A on CD4⁺ T cells. Also, adenosine concentration and CD39 expression were significantly higher on MSCs cultured with activated T cells.

Adenosine levels in co-cultures (mean of 72 ng/mL) are partially resultant from the production mediated by Tregs, nevertheless, at least more than half of the adenosine found in co-cultures can be attributed to MSCs, as adenosine concentration in the medium of MSCs cultured alone reached up to 43 ng/mL. Moreover, when the medium of MSCs cultured alone was substituted by that of activated lymphocytes cultured alone (in the 3rd day of culture), the concentration rose up to 91 ng/mL. Although it can be speculated that 5'-AMP generated from ATP by CD39⁺ Tregs could serve as substrate for the production of adenosine by CD73⁺ MSCs, our results indicate that MSC plays a major role in the production of adenosine in co-cultures.

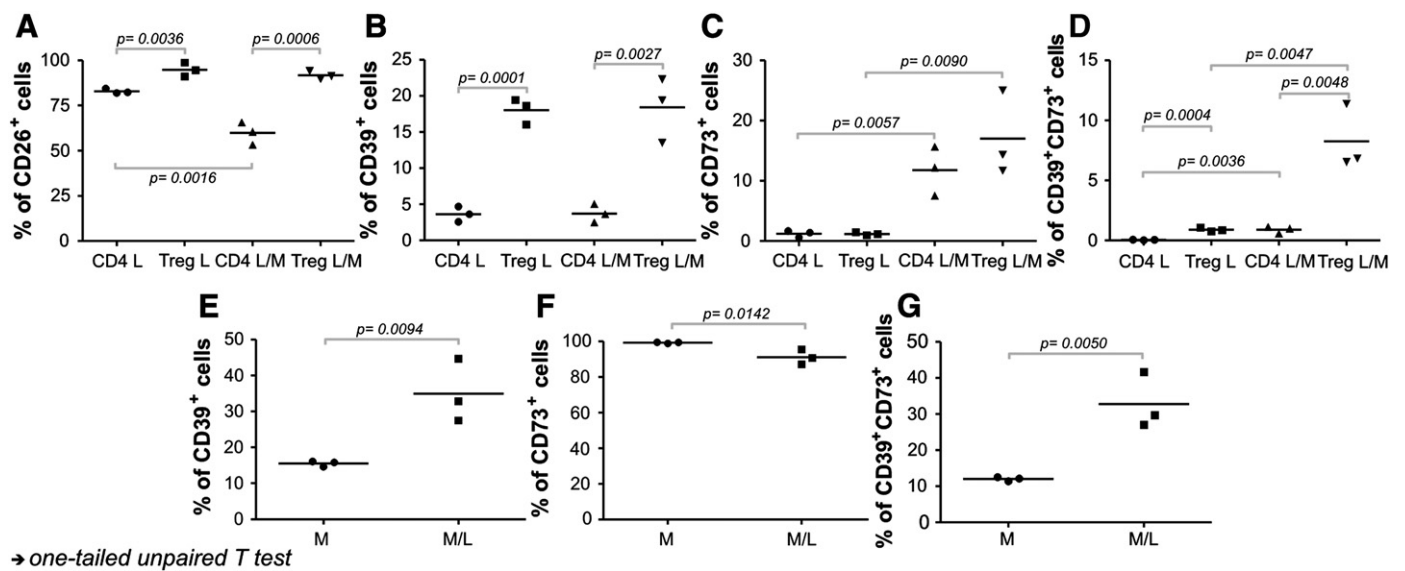


Figure 3 CD26, CD39 and CD73 expression. CD3⁺ T cells from 3 healthy volunteers were activated with anti-CD2/CD3/CD28 beads and cultured with or without MSCs. CD26, CD39 and CD73 expression was analyzed in CD4⁺ T cells and CD4⁺CD25^{hi} cells (Tregs) were obtained from cultures without MSCs (CD4 L and Treg L) or with MSCs (CD4 L/M and Treg L/M). CD39 and CD73 were also analyzed in MSC without (M) or with (M/L) activated lymphocytes. (A) CD26 expression in CD4⁺ T cells and Tregs cultured without or with MSCs. In co-culture, CD26 expression in Tregs was $91.7 \pm 1.3\%$, whereas CD4⁺ T cells expressed $59.8 \pm 3.5\%$ ($p=0.0006$). T cells cultivated without MSCs showed $94.7 \pm 2.2\%$ and $82.9 \pm 0.7\%$ of CD26 expression in Tregs and CD4⁺ T cells, respectively ($p=0.0036$). In addition, expression of CD26 was lower in CD4⁺ T cells from co-culture than from culture without MSCs ($p=0.0016$); (B) CD39 expression in CD4⁺ T cells and Tregs cultured without or with MSCs. The expression of CD39 was significantly higher in Tregs than CD4⁺ T cells, either in activated T cells cultured with ($p=0.0027$) and without ($p=0.0001$) MSCs; (C) CD73 expression in CD4⁺ T cells and Tregs cultured without or with MSCs. CD4⁺ T cells from both cultures showed a higher expression of CD73 ($11.8 \pm 2.3\%$) than CD4⁺ T cells cultivated without MSCs ($1.2 \pm 0.3\%$) ($p=0.0057$). In Tregs from co-culture, the CD73 expression was significantly higher than the corresponding cells from culture without MSCs ($17.0 \pm 4.0\%$ against $1.2 \pm 0.1\%$) ($p=0.0090$); (D) percentage of CD39 and CD73 double positive CD4⁺ T cells and Tregs cultured without or with MSCs; (E) CD39 expression in MSC without or with activated lymphocytes. MSCs presented a higher expression of CD39 ($34.9 \pm 5.0\%$ against $15.5 \pm 0.4\%$) compared to MSCs cultured without T cells ($p=0.0094$); (F) CD73 expression in MSC without or with activated lymphocytes. MSCs presented a lower expression of CD73 ($91.1 \pm 2.4\%$ against $99.3 \pm 0.2\%$) compared to MSCs cultured without T cells ($p=0.0142$). (G) Percentage of CD39 and CD73 double positive MSC cultured without or with activated lymphocytes.

Our study also shows that MSCs induce lower expressions of ADA on CD4⁺ T cells. Although not statistically significant, ADA activity was higher in MSC co-cultures. It is possible that a larger number of samples would reveal a significant difference in ADA activity between cultures. The increased ADA activity observed may be explained, at least in part, by the lower percentage of CD4⁺ T cells expressing CD26, when in the presence of MSCs. With lower levels of CD26 more ADA molecules would be expected to be found free in the supernatant.

Interestingly, even with higher ADA activity, adenosine levels were higher in co-culture. Mandapathil et al. (2010) revealed that low CD26 expression is a characteristic of naturally-occurring Tregs, whereas CD4⁺ T cells have elevated expression of this protein. Differently, we found that the expression of CD26 was higher in Tregs than in CD4⁺ T cells, in both culture systems. This elevated CD26 expression in Tregs may constitute a protective mechanism that increases ADA surface levels, preventing Tregs to be affected by adenosine signaling (Dong et al., 1996). Inversely, in CD4⁺ T cells co-cultured with MSC, the reduced expression of CD26, and the consequent low levels of ADA on the cell surface, would allow adenosine mediated suppression to occur.

Recent data have shown that CD39 surface molecule is expressed predominantly in CD4⁺CD25^{hi} Tregs (Mandapathil et al., 2010; Schmitt et al., 2009), suggesting that this ecto-enzyme may be useful to define human Tregs subsets (Borsellino et al., 2007; Dwyer et al., 2007; Schmitt et al., 2009). Differently than CD4⁺CD25^{hi} Tregs, this CD4⁺CD25^{hi} CD39⁺ subset is able to suppress pathogenic Th17 cells (Fletcher et al., 2009). Consistent with other authors, in our study, the CD39 was predominantly expressed in CD4⁺CD25^{hi} Tregs, both generated in the absence or in the presence of MSC.

CD73 can be detected in different cell types and it is possible that its physiological role differs according to the cell context (Zimmermann, 1992). Besides the enzymatic activity of CD73, its different functions include signal activation in leukocytes (Robinson, 1991) and T cell adhesion into endothelium (Airas et al., 1993). Recent data show that the expression of CD73 is very low in T cells, and that this protein is mainly localized in the cytosol of human naturally-occurring Tregs (Alam et al., 2009). Differently, murines present elevated CD73 expression on the surface of CD4⁺ T cells and CD4⁺CD25^{hi} Tregs (Alam et al., 2009; Kobie et al., 2006). In our study, CD4⁺ T cells and CD4⁺CD25^{hi} Tregs generated by T cell activation presented low CD73

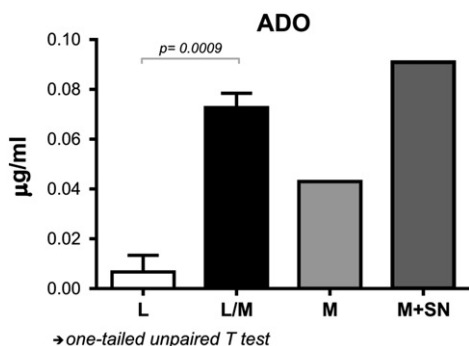


Figure 4 Adenosine quantification. CD3⁺ T cells from 3 healthy volunteers were activated with anti-CD2/CD3/CD28 beads and cultured without (L) or with (L/M) MSCs. T cell culture supernatant presented higher levels of adenosine (ADO) in the co-culture than in the culture without MSCs (0.072 ± 0.005 µg/mL against 0.006 ± 0.006 µg/mL; $p=0.0009$). In addition, MSC culture produced 0.043 µg/mL of adenosine, whereas in the presence of supernatant of activated T cells, the adenosine production was 0.091 µg/mL.

expression on the surface and in the cell cytoplasm (data not shown). However, CD4⁺ T cells and CD4⁺CD25^{hi} Tregs induced by MSCs showed increased levels of CD73 expression on the cell surface. These apparently conflicting results may be explained by methodological differences used in each study and the fact that CD73 stability on human cells surface is low (Thomson et al., 1990; Airas et al., 1993).

During the revision of our manuscript, a paper coincidentally reported that adenosine produced by murine MSCs coexpressing CD39/CD73, mediates the inhibition of T cell proliferation (Sattler et al., 2010). As shown by us, in their report, they showed that MSCs coexpress the ectoenzymes CD73 and CD39 and that they can generate adenosine, which inhibits the proliferation of activated T lymphocytes, through signaling mediated by the adenosine receptor ADORA2A. Our work with human cells corroborates with their independent findings (with mice cells). More impor-

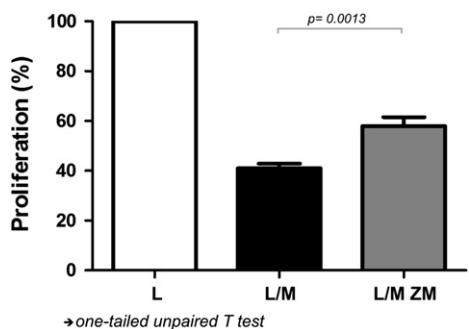


Figure 5 Inhibition of ADORA2A signaling. CD3⁺ T-cells were activated with anti-CD2/CD3/CD28 beads and cultured alone (L) or with MSCs (L/M). Alternatively, T-cells were pre-treated at 0.3 µM of the ADORA2A antagonist ZM 241385 (L/M ZM 241385). T-lymphocyte treatment with ZM 241385 induced an increase in T-cell proliferation by 29% compared to untreated T-lymphocytes ($p=0.013$). Proliferation is shown relative to activated T-cells cultured in the absence of MSCs. Results are the mean (\pm SD) of two independent experiments.

tantly, our work adds further insights into the cross talk between activated lymphocytes and MSCs (mediated by inflammatory and immunomodulatory signals) leading to increased percentages of double positive CD39⁺CD73⁺, both, in MSCs and Treg lymphocytes.

Interestingly, activation of ADORA2A limits graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (Lappas et al., 2005). Our results indicate that the modulation of components related to adenosine signaling by MSCs, may be one of the mechanisms underlying the claimed potential of this cells in the treatment of GVHD (Le Blanc et al., 2008; Le Blanc et al., 2004). Our findings may open new avenues for the development of new treatments for GVHD and other inflammatory diseases.

Materials and methods

This study was approved by the Institutional Ethics Committee and all samples were obtained after individual informed consent was provided.

Isolation and characterization of MSCs

Approximately 10 mL of human bone marrow aspirates was collected from healthy donors. Mononuclear cells were separated by Histopaque®-1077 (Sigma, St. Louis, MO, USA). MSCs isolated by plastic adherence as previously described (Silva et al., 2003), and cultured at a concentration of $2-4 \times 10^5$ cells/mL in 25 cm² flasks with alpha-MEM (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 15% fetal bovine serum (FBS – HyClone, Logan, UT, USA), 2 mmol/l L-glutamine, and 100 U penicillin/streptomycin (Sigma, St. Louis, MO, USA). Non-adherent T cells were removed after 72 h and the formation of small colonies was observed 3 to 4 days later. The plastic-adherent MSCs from the fourth passage were used in the experiments.

Cultured MSCs were immunophenotypically characterized using the following monoclonal antibodies: CD-33-FITC, CD45-FITC, CD31-FITC, HLA-DR-FITC, Cadherin-5-FITC, Glycophorin-FITC, CD73-PE, CD146-PE, CD90-PE, CD29-PE, CD44-PE, CD13-PE, CD49e-PE, HLA-ABC-PE, CD34-PE, CD14-PE, CD54-PE, CD166, and AC-133-PE (Pharmlingen, San Jose, CA, USA). Adipogenic, osteogenic, and chondrogenic MSC differentiation potential were evaluated as previously described (Silva et al., 2003). Basically, after incubation with the specific differentiation medium, cells were fixed and stained by the von Kossa method (calcium deposition, for osteocyte differentiation), with Sudan II and Scarlet stains (lipid accumulation, for adipocyte differentiation), or immunostained with anti-type II collagen (for chondrocyte differentiation). The cells were analyzed with Axioskop 2.0 Zeiss microscope equipped with an AxioCam camera (Carl Zeiss, Oberkochen, Germany).

Isolation of peripheral blood mononuclear cells and immunomagnetic selection of T-cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers and separated using Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden). Next, they were washed three times with PBS 1×. We used the Pan T

cell isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations for the immunomagnetic selection of CD3⁺ cells. Briefly, non-CD3⁺ T cells were depleted from PBMCs using a mixture of antibodies and secondary antibodies linked to magnetic microbeads, using magnetic columns and VarioMACS magnet (Miltenyi Biotec). Selected CD3⁺ T cells with purity above 95%, determined by flow cytometry, were used in further experiments.

T-cell activation

Selected CD3⁺ T cells were activated using the T Cell Activation/Expansion Kit, according to the manufacturer's instructions (Miltenyi Biotec). In short, purified T cells, resuspended in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS (HyClone), were briefly incubated with anti-biotin magnetic particles, pre-loaded with biotinylated antibodies against human CD2, CD3, and CD28 (in a ratio of 1 particle for 2 cells), mimicking activation by antigen-presenting cells.

Immediately following activation, 2.5×10^5 activated T cells were cultured with or without 5×10^4 MSCs, previously adhered to the bottom of a 24 well plate in a total volume of 1 mL of medium (RPMI, 10% FBS). After 3 days, 20 U/mL of human recombinant IL-2 (Peprotech, Rocky Hill, NJ, USA) was added to the cultures and evaluations were carried on the 5th day.

Proliferation assay

To evaluate the capacity of MSCs to modulate lymphocyte proliferation, bromodeoxyuridine (BrdU) incorporation by lymphocytes cultured in the presence or absence of MSCs was analyzed, according to the manufacturer's instructions (BrdU Flow Kit, BD Biosciences). In short, following activation, 5×10^5 CD3⁺ T-cells were cultured in the presence or absence of 1×10^5 MSC, previously adhered to wells of a 12 well plate. Following a period of 5 days, BrdU was added to wells, cultured for 1 h and stained with PerCP-anti-CD3 and APC-anti-BrdU monoclonal antibodies. FACsort flow cytometer and CellQuest software (Becton-Dickinson, Mountain View, CA, USA) were used to analyze fluorescent T cells.

Adenosine Deaminase activity

The relative ADA activity, in medium obtained from T cells cultured either in the presence or absence of MSCs, was measured using the Adenosine Deaminase Quimiada Kit (Ebram, São Paulo, SP, Brazil), according to the manufacturer's instructions. This assay measures the enzymatic deamination of adenosine into inosine (carried by ADA), indirectly, by quantifying the variation in the absorbance derived from the formation of colored quinone, resulting from a sequence of reactions carried by enzymes present in the reaction buffer. In brief, 50 μ L of medium from cultures was incubated with 110 μ L of Buffer A (Tris-HCl 50 mM pH 8, 4-AAP 0.2 mM, PNP 0.1 U/mL, XOD 0.2 UI/mL, peroxidase 0.6 UI/mL, BSA 5%, and sodium azide 0.01%) for 5 min and after the first absorbance measurement, 55 μ L of Buffer B was added (Tris-HCl 50 mM pH 4, adenosine 10 mM and N-Ethyl-N-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, hydrate 2 mM) and a final absorbance measurement was done 8 min later. All samples

were processed in parallel in a microplate spectrophotometer Versamax system (Molecular Devices, Sunnyvale, CA, USA) and the difference between the final and the first measured absorbance was used as a relative measure of activity.

Real-time PCR

For gene expression analysis, CD4⁺ T cells were positively selected using anti-CD4 linked magnetic beads (Miltenyi Biotec) from CD3⁺ cells obtained from the fifth day of culture, following activation with anti-CD2/CD3/CD28 beads.

To analyze the expression of ADA and ADORA2A, CD3⁺ T cell from 3 healthy volunteers were activated with anti-CD2/CD3/CD28 beads and following a 5 day period, CD4⁺ T cells were immunomagnetically purified (purity above 95%) and real-time PCR was performed. Total RNA from CD4⁺ cells was obtained using TRIZOL reagent (Invitrogen) and reverse transcribed to cDNA using the High Capacity cDNA Archive Kit (Applied BioSystems, Foster City, CA), following the manufacturer's instructions. Real-time PCR for ADA (Hs01110945_m1) and ADORA2A (Hs00169123_m1) were done (in duplicate) with TaqMan probes and MasterMix (Applied BioSystems). To normalize sample loading, the differences of threshold cycles (Δ Ct) were derived by subtracting the Ct value for the internal reference (mean of GAPDH and β -actin) from the Ct values of the evaluated genes. The relative fold value was obtained by the $2^{-\Delta\Delta$ Ct} method (Pfaffl et al., 2001) using the median Ct value of the samples from cells cultivated without MSCs, as a reference. The $\Delta\Delta$ Ct was calculated by subtracting the reference Δ Ct from the Δ Ct values of the samples. Expression of all samples was measured in a single plate for each gene evaluated.

Flow cytometry

Magnetic isolated CD3⁺ T cells from three independent samples were activated and cultured with or without MSCs (as described above), and evaluated on the fifth day after activation by flow cytometry, using monoclonal antibodies and corresponding isotope controls (Becton-Dickinson), as described below.

T cells were evaluated for the expression of CD26, CD39 and CD73 in CD4⁺CD25^{hi} regulatory T cells (Treg) and in CD4⁺CD25^{lo} T cells. For this, two tubes were labeled independently with anti-CD4-PC5, anti-CD25-APC and anti-CD26-FITC; or with anti-CD4-PC5, anti-CD25-FITC, CD39-APC and anti-CD73-PE. Additionally, T cells were characterized according to size and complexity.

The percentage of cells expressing CD39 and CD73 among MSCs (as defined by a gate on CD45 negative cells), was obtained through the use of anti-CD45-FITC, anti-CD39-APC and anti-CD73-PE.

Determination of adenosine levels by high pressure liquid chromatography (HPLC)

Adenosine levels were quantified by HPLC-UV, as previously described (Valerio et al., 2009). The analysis was performed on HPLC system consisting of a Shimadzu Model (Kyoto, Japan) LC 10 AD pump, Shimadzu Model SPD-10A ultraviolet detector and chromatopac C-R6A integrator (Shimadzu). Chromatographic separation was achieved at room temperature on a LiChrospher 100 RP-18 column [size 125 \times 4 mm, 5 mm

particle size (Merck, Darmstadt, Germany)]. The mobile phase consisted of 0.5% of acetonitrile, 5% of methanol, and 94.5% of sodium acetate buffer 0.25 M, pH 6.3. HPLC-grade water from Milli-Q system (Millipore, Bedford, MA, USA) was used. The flow rate was 1.0 mL min⁻¹, and the ultraviolet detector set at 254 nm. An adenosine standard curve (0.32–4 mg·mL⁻¹) was constructed in pure culture medium. For this purpose, the stock solution of adenosine at 4 mg·mL⁻¹ was prepared in methanol and stored at –20 °C, and further diluted in culture medium to yield the concentrations described above.

A total of 2.5 × 10⁵ activated T cells were cultivated with or without 5 × 10⁴ MSCs for 5 days. Also, 5 × 10⁴ MSCs were cultivated in the same conditions, without lymphocytes. On the third day of culture, the supernatant of activated T cells (inflammatory situation) cultured without MSCs was added to wells containing MSCs. After 5 days, 500 µL of each culture supernatant was added to tubes containing 1 mL of acetonitrile, and immediately vortexed by 1 min and centrifuged, to extract proteins and to stabilize adenosine. Next, collected supernatant was dried under air flow and the pellet was resuspended in 200 µL of mobile phase (0.5% of acetonitrile, 5% of methanol and 94.5% of sodium acetate buffer 0.25 M, pH 6.3). Finally, resuspended samples were vortexed for 20 s and 100 µL of each sample was used for chromatographic analysis. Adenosine standards were used to confirm the retention time and the required run-time. Adenosine was quantified comparing the retention time with the standards. Under the conditions used, the limit of detection was 10 ng/mL and the limit of quantification was 20 ng/mL.

Specific inhibition of ADORA2A signaling

To confirm the immunosuppressive role of adenosine signaling, through ADORA2A receptors, on lymphocytes co-cultured with MSCs, a potent and highly selective ADORA2A antagonist (ZM 241385, TOCRIS Bioscience) was used.

T-cells were labeled by incubating them in a PBS solution containing 0.1% human albumin and CFSE at a concentration of 2.5 µM (Molecular Probes/Invitrogen) for 10 min at 37 °C, when labeling was stopped by adding cold RPMI containing 10% fetal calf serum. Labeled lymphocytes were then activated and cultured with or without MSCs for 5 days (as described). In parallel, T-lymphocytes were pre-treated with 0.3 µM of ZM 241385 for 30 min, washed with PBS and resuspended in medium before being co-cultured with MSCs, as described elsewhere (Mandapathil et al., 2010). Results were obtained from two independent experiments.

Statistical analysis

Where appropriate statistical significance was determined using one-tailed unpaired *T*-test, considering *p* ≤ 0.05.

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