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

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Background and Objectives. Experimental evidence and preliminary clinical studies have demonstrated that human mesenchymal stem cells (MSC) have an important immune modulatory function in the setting of allogeneic hematopoietic stem cell (HSC) transplantation. We extended the evaluation of mechanisms responsible for the immune regulatory effect derived from the interaction of human MSC with cells involved in alloantigen-specific immune response in mixed lymphocyte culture (MLC).

Design and Methods. Dendritic cell (DC) differentiation, T- and natural killer (NK)-lymphocyte expansion, alloantigen-specific cytotoxic activity and differentiation of CD4+ T-cell subsets co-expressing CD25 and/or CTLA4 molecules were assessed, comparing the effect observed using third-party MSC with that obtained employing MSC autologous to the MLC responder.

Results. We found that human MSC strongly inhibit alloantigen-induced DC1 differentiation, down-regulate alloantigen-induced lymphocyte expansion, especially that of CD8+ T cells and of NK lymphocytes, decrease alloantigen-specific cytotoxic capacity mediated by either cytotoxic T lymphocytes or NK cells and favor the differentiation of CD4+ T-cell subsets co-expressing CD25 and/or CTLA4. More effective suppressive activity on MLC-induced T-cell activation was observed when MSC were third-party, rather than autologous, with respect to MLC-responder cells.

Interpretation and Conclusions. Our results strongly suggest that MSC-mediated inhibition of alloantigen-induced DC1 differentiation and preferential activation of CD4+CD25+ T-cell subsets with presumed regulatory activity represent important mechanisms contributing to the immunosuppressive activity of MSC. Collectively, these data provide immunological support for the use of MSC to prevent immune complications related to both HSC and solid organ transplantation and to the theory that MSC are *universal* suppressors of immune reactivity.

Key words: mesenchymal stem cells (MSC), mixed lymphocyte culture (MLC), dendritic cells (DC), regulatory T-cells, tolerance.

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Mesenchymal stem cells (MSC) from bone marrow (BM) include precursors able to differentiate along multiple mesenchymal lineages and can be extensively expanded *in vitro*, while maintaining their differentiation ability.[1,2] *Ex vivo* expanded MSC can be identified by assessing both their differentiation capacity *in vitro*, and their peculiar surface phenotype.[1,3] MSC play a crucial role in the development and differentiation of the lymphohematopoietic system by promoting cell-to-cell interactions and by secreting a number of growth factors and regulatory cytokines. In this regard, experiments in NOD/SCID mice demonstrated that MSC promote better engraftment of human cord blood-derived CD34+ cells.[4,5] The clinical application of *ex vivo* expanded human MSC demonstrated that the infusion of either recipient- or donor-derived human MSC can improve hematopoietic recovery in the setting of both autologous and allogeneic hematopoietic stem cell (HSC) transplantation.[2,6-8] MSC do not only have a favorable effect on engraftment of hematopoietic progenitors, but also display an important immunoregulatory activity. In fact, a preliminary clinical study suggested that the co-infusion of non-irradiated donor-derived human MSC with HSC reduces the incidence and severity of graft-versus-host disease (GVHD) in recipients of an allograft from an HLA-identical sibling.[9] Moreover, a recent case report demonstrated a striking effect of haploidentical MSC infusion in promoting resolution of severe, treatment-refractory, acute GVHD.[10] Several *in vitro* studies have addressed the issue of the capacity of MSC to modulate T-cell-mediated immune responses.[3,11-23] Experiments in animal mod-

els showed that mouse BM-MSC inhibit the activation of both naive and memory antigen-specific T cells in response to their cognate peptide.[15] This immunosuppressive activity of mouse MSC was not dependent on the secretion of inhibitory soluble factors and did not require the presence of $CD4^+CD25^+$ regulatory cells.[15] However, recently published data demonstrated that mouse MSC reduce T-cell responsiveness mainly by inducing division arrest anergy of activated T cells.[23] Irradiated human MSC co-cultivated with alloantigen-stimulated peripheral blood mononuclear cells (PBMC) in mixed lymphocyte culture (MLC) induce dose-dependent inhibition of T-lymphocyte proliferation and of alloantigen-specific cytotoxic activity.[3,13,15,17,19,20] Even though MSC express HLA-class I and LFA-3 molecules constitutively, and HLA class II molecules and ICAM-1 upon γ-interferon treatment, they are unable to induce proliferation of allogeneic lymphocytes.[14,16] Most human MSC-mediated immunosuppressive function on alloantigen-activated T-lymphocyte proliferation was related to the secretion of anti-proliferative soluble factors.[13,14,19,20] On the other hand, previously published data do not exclude that a part of the immunosuppressive effect of human MSC on alloantigen-induced T-cell activation is dependent on cell-to-cell contact mechanisms.[13] Effector cells recovered from primary MLC, performed in the presence of either third-party MSC or MSC autologous to the MLC responder cells (autologous), were able to proliferate efficiently in secondary MLC, thus suggesting that the presence of MSC during the primary MLC caused neither clonal deletion of alloreactive T-cells nor generation of dominant T-cell suppressor activity.[13]

The aim of the present study was to extend the analysis of the mechanisms responsible for the important immunoregulatory effect displayed by *ex vivo* expanded human MSC in the setting of allogeneic HSC transplantation.[9,10] To this purpose, we evaluated the *in vitro* interaction between *ex vivo* expanded human MSC and the alloantigen-specific immune response elicited in primary and in secondary MLC. In contrastto the strategy in most previously reported *in vitro* studies, we decided to employ non-irradiated MSC for MLC experiments, reasoning that irradiation may be expected to impair the cell-survival and differentiation ability of MSC, as well as their proliferative capacity thus altering their interaction pattern with lymphocyte subsets. It has been demonstrated that MSC infused into non-human primates are distributed into a wide variety of tissues, where they may persist for several months, proliferate and participate in cell turnover and replacement of engrafted organs.[24] Therefore, the addition of non-irradiated rather than irradiated MSC to primary MLC could be expected to mimic better *in vitro* interaction patterns between human MSC and alloantigen-induced immune responses elicited *in vivo* by co-infusion of MSC and HSC in the setting of allogeneic transplantation.[8-10]

Dendritic cell (DC) differentiation, T- and NK-lymphocyte expansion, alloantigen-specific cytotoxic activity and differentiation of $CD4^+$ T-cell subsets displaying a regulatory/suppressive phenotype, such as co-expression of CD25 and/or CTLA4 antigens, were assessed in MLC, comparing the effects observed using third-party MSC with those obtained employing autologous MSC.

Design and Methods

Cell harvesting

Human MSC were obtained from BM samples from healthy subjects donating BM to a sibling for allogeneic HSC transplantation, after obtaining written informed consent. Heparinized peripheral blood samples were obtained from the same healthy family donors employed for MSC expansion, and from other unrelated healthy volunteers. The Institutional Review Boards of Pediatric Hematology/Oncology, IRCCS Policlinico San Matteo, Pavia and Centro Cellule Staminali, Department of Hematology/Oncology, Ospedale San Martino, Genova approved the study.

Flow cytometry analysis

FITC, PE, PerCP, or PerCPCy5.5-conjugated monoclonal antibodies (MoAb) specific for the following antigens were employed: (i) CD45, CD14, CD34, CD13, CD80, CD86, HLA A-B-C, HLA-DR (BD Biosciences, Mountain View, CA; BD PharMingen, San Diego, CA, USA), CD29, CD105, CD166 (Serotec, Kidlington, Oxford, UK), CD44 (Immunotech, Marseille, France), and CD106 (Southern Biotechnology, Birmingham, AL, USA), for the assessment of MSC surface phenotype; (ii) HLA DR, CD11c, CD123, CD34, CD14, CD3, CD19, CD20, CD16, CD56, and anti-IgM (BD Biosciences) for the identification of DC; and (iii) CD3, CD4, CD8, CD56, CD25, and CD152 (BD Biosciences, BD PharMingen) for the evaluation of lymphocyte subsets. Appropriate isotype-matched controls (BD Bioscience) were included. Intracellular staining for CTLA-4 (CD152) was performed with the Cytofix/Cytoperm kit (BD PharMingen). In brief, cells were stained with MoAb to surface antigens (CD4 and CD25), washed, fixed, permeabilized and stained for intracellular CTLA-4 with anti-CD152 MoAb. Two-color or three-color cytometry, through direct immune fluorescence and FACScalibur flow cytometry (BD Biosciences), was performed using a previously described method.[25]

Ex vivo culture of human MSC

MSC were expanded from BM mononuclear cells

using a previously reported method.[3] Briefly, mononuclear cells were seeded at a density of 10^6 cells/mL in 75 cm^2 flask (Corning-Costar, Celbio, Milan, Italy) in MesenCult medium and MSC-supplements (StemCell Technologies, Vancouver, Canada) and incubated at 37°C in a 5% humidified CO_2 atmosphere. After 24 hours, non-adherent cells were discarded, fresh medium was added and then half the medium was replaced twice a week. When cultures reached more than 90% confluence, adherent cells were detached with 0.05% trypsin (Euroclone, Wetherby, West York, UK), washed twice with complete medium, counted with a nuclear stain (0.1% methylviolet in 0.1M citric acid), and replated at a concentration of 5×10^5 cells per flask for the next passage. The capacity of MSC to differentiate along adipogenic, osteogenic and chondrogenic lineages was demonstrated, in preliminary experiments, according to previously described methods.[1,3]

Mixed lymphocyte cultures

PBMC were obtained by Ficoll-Hypaque density gradient from heparinized peripheral blood samples of the healthy family BM donors employed for studying MSC expansion, and from other unrelated healthy volunteers. The cells were employed on the same day of collection or cryopreserved for later use. The primary MLC was performed by incubating, at 37°C in a humidified 5% CO_2 atmosphere, 5×10^4 responder (R) PBMC and 5×10^4 irradiated (3,000 cGy) stimulator (S) PBMC/micro-well, in 96-well round-bottom or flat-bottom (in the case of evaluation of DC differentiation) tissue-culture plates, in a final volume of 200 μL of RPMI 1640 supplemented with 2 mM L-glutamine, 50 μg/mL gentamycin (Gibco-BRL, Life Technologies, Paisley, UK) and 5% human pooled serum (complete medium RPMI-HS), in the absence (ctrl-MLC) or presence (MSC-MLC) of different numbers (5×10^4 = 1:1 R-PBMC:MSC ratio; 5×10^3 = 10:1 R-PBMC:MSC ratio) of MSC. The MSC were added to primary MLC to either autologous R-PBMC (autologous setting) or R-PBMC of an unrelated subject (third-party setting), together with S-PBMC, that were allogeneic both to R-PBMC and MSC. The secondary MLC was performed by adding 50 μL of fresh medium, containing 5×10^4 irradiated S-PBMC, directly to the same micro-wells of primary-MLC effectors, after a 10-day primary MLC. A range of 25-50 replicate micro-wells was plated for each experimental condition and pooled to perform immunity assays. DC were identified as positive for HLA-DR and negative for the lineage markers CD3, CD19, CD20, surface IgM, CD56, CD16, CD14, and CD34; anti-CD11c and IL-3Rα (CD123) were used for identification of DC1 and DC2 subsets, respectively.[26] T and NK-lymphocyte subset expansion was evaluated by counting numbers of $CD3^+/CD4^+$ or $CD3^+CD8^+$ T cells and of $CD3^-/CD56^+$ NK cells per mL of culture, recovered after both 7-day primary and secondary MLC and comparing these with the initial numbers (day 0). For the evaluation of alloantigen-specific cytotoxic activity, recombinant interleukin-2 (rIL-2, 5 U/mL; Proleukin, Chiron Emeryville, CA, USA) was added to each well, on day +3 and +6 for primary MLC and on day +3 for secondary MLC. In the case of secondary MLC, supernatant obtained from R-PBMC stimulated for 3 days with PHA (4 μg/mL, Roche Mannheim, Germany) was also added to the cultures on day 0, at a concentration of 1/5 v/v.[27] Primary and secondary MLC were incubated for 10 and 8 days, respectively, and then tested in the cytotoxicity assay.

Cytotoxicity assay

Alloantigen-specific cytotoxic activity was tested in a 5-hour ^{51}Cr-release assay as previously described.[27] Result were expressed as lytic units/10^6 effector cells, 1 lytic unit (LU) being defined as the number of effector cells required to induce 30% lysis of ^{51}Cr-labeled target cells (LU_{30}). ^{51}Cr-labeled target cells included PHA-activated S-PBMC (S-PHA) and MSC lines employed for the primary MLC. When indicated, the NK-sensitive tumor cell line K562 (*cold* NK-sensitive target cells) was added in 20-fold excess of ^{51}Cr-labeled targets in microtiter wells. The presence of a large excess of *cold* NK-sensitive target cells decreases the cytotoxic activity of NK and/or NK-like cells towards allogeneic S-PHA ^{51}Cr-labeled targets.[28]

Statistical analysis

Statistical analysis was performed using the SAS System (SAS Inc., Cary, NC, USA) and the SPSS computer program (SPSS Inc., Chicago, IL, USA). The non-parametric Mann-Whitney rank-sum test for continuous variables was used for comparing differences between the median values obtained in the different experimental conditions.

Results

Surface phenotype of in vitro expanded MSC

In keeping with data previously reported by Le Blanc *et al.*,[3] *in vitro* expanded MSC obtained from 18 separate experiments, isolated after either the second or the third passage, included more than 97% of a single-phenotype population defined by flow cytometry as positive for CD29, CD44, CD105, CD106, CD166, CD13 and HLA-class I molecules and negative for CD45, CD34, CD14, HLA-DR, CD80 and CD86 antigens.

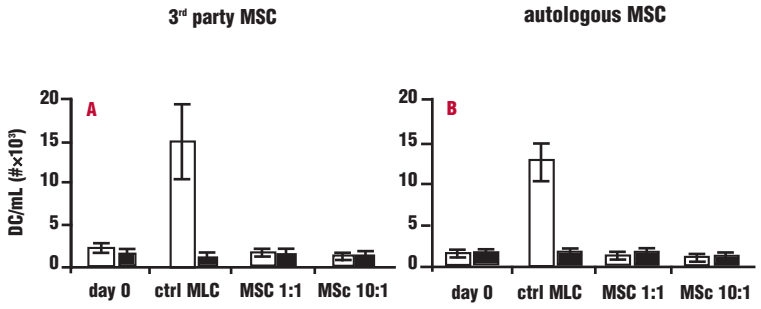

Figure 1. Effect of human MSC on DC differentiation induced by allogeneic stimulus. DC differentiation was assessed in primary MLC, performed in the absence (ctrl-MLC) or in the presence of either third-party (A) or autologous (B) MSC at a R-PBMC/MSC ratio of 1:1 (MSC 1:1) or of 10:1 (MSC 10:1). The number of DC1 (white columns) and DC2 (black columns) per mL of culture, before (day 0) and after 5 days of MLC, are reported; results are expressed as mean (columns) and range (bars) values of three separate experiments.

Effect of MSC on DC differentiation induced by allogeneic stimulus

It was observed in preliminary experiments that PBMC activation induced in primary MLC causes the differentiation of DC, mainly of DC1 phenotype, and that DC peak values are reached between day 3 and day 5 of culture and decline thereafter.

Evaluation of DC differentiation in 3 separate experiments of primary MLC showed that both autologous and third-party MSC strongly inhibited DC1 differentiation (Figure 1). More than 90% of DC differentiating in ctrl-MLC expressed CD11c (DC1) and only a few DC (<5%) alternatively expressed CD123 (DC2). Conversely, more than 80% of the few DC differentiated in MSC-containing MLC (MSC-MLC) co-expressed CD11c and CD123 molecules. R-PBMC alone or R-PBMC plus either third-party or autologous MSC displayed a sharp decrease in the number of DC/mL of culture, less than 30% of the initial number (day 0) being recovered after 3 to 5 days of culture.

Effect of MSC on lymphocyte expansion induced by allogeneic stimulus

In agreement with data reported by previous studies,[3,13,14,19,20] the results of experiments reported in Figure 2 confirmed that addition of MSC to primary MLC caused a dose-dependent inhibition of alloantigen-induced expanding capacity of T-cell subsets. In primary MLC, the suppressive effect was evident on both $CD4^+$ and $CD8^+$ positive T-cell subsets. In detail, the numbers of both $CD4^+$ and $CD8^+$ T-cells measur-

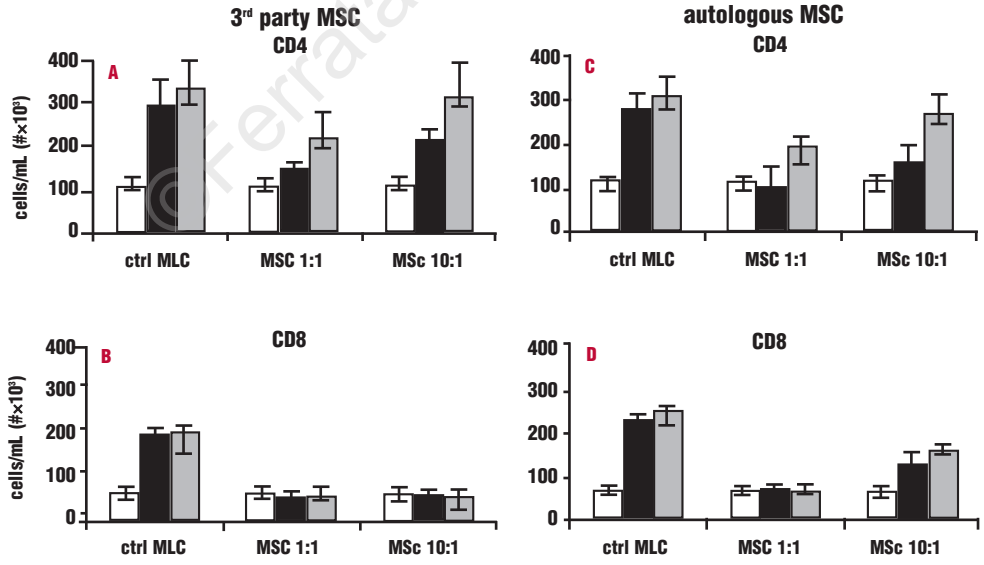

Figure 2. Effect of human MSC on T-lymphocyte expansion induced by allogeneic stimulus. Recovery of $CD4^+$ (A and C) and $CD8^+$ (B and D) T-lymphocytes, in respect to the initial cell number (white columns), was assessed after 7-day primary (black columns) and 7-day secondary (gray columns) MLC, performed in the absence (ctrl-MLC) or presence of either third-party (A and B) or autologous (C and D) MSC. The MSC were added at a R-PBMC/MSC ratio of 1:1 (MSC 1:1) or of 10:1 (MSC 10:1) Results are expressed as number of cells/mL of culture; median (columns) and range (bars) values of 8 experiments with third-party (A and B) and of 5 experiments with autologous MSC (C and D) are reported.

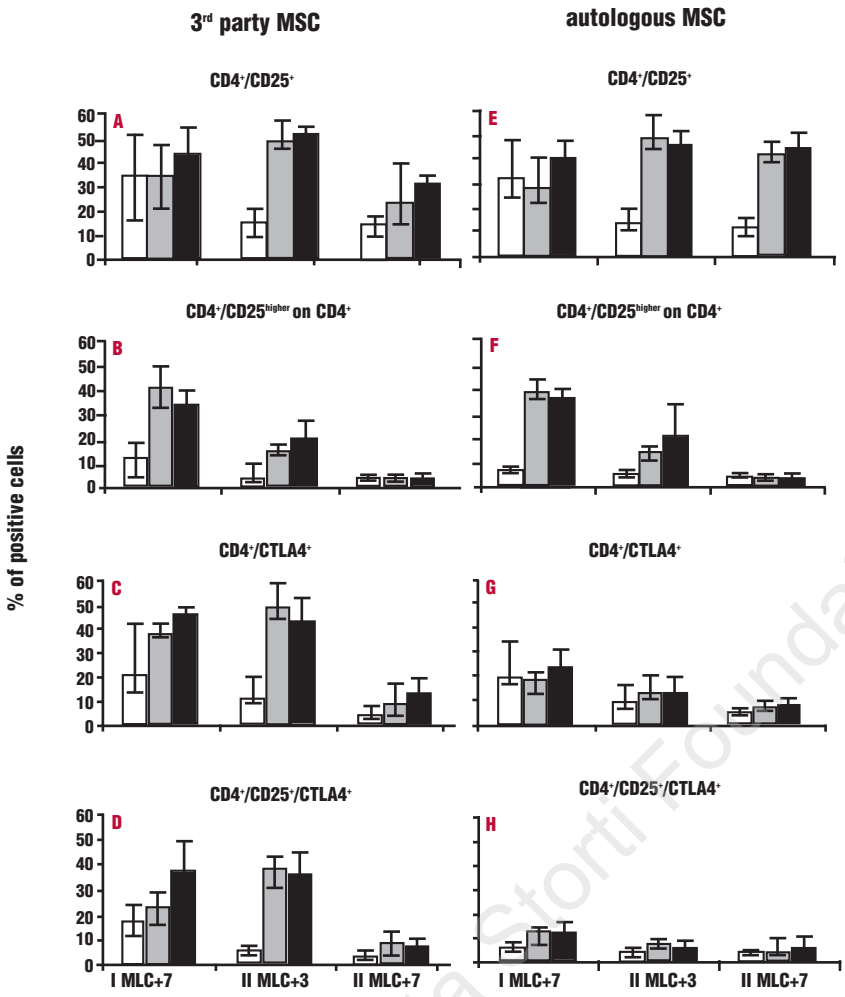

Figure 3. Effect of human MSC on differentiation of $CD4^+CD25^+$ T lymphocyte subsets induced by allogeneic stimulus. Percentages of various $CD4^+$ T lymphocyte subsets were calculated on cells recovered after 7-day primary MLC (I MLC +7), 3- and 7-day secondary MLC (II MLC +3, II MLC +7), performed in the absence (ctrl-MLC: white columns) or presence of third-party (A-D) or autologous (E-H) MSC. The MSC were added at a R-PBMC/MSC ratio of 1:1 (gray columns) or of 10:1 (black columns). Percentages of total $CD4^+CD25^+$ (A and E), $CD4^+CD25^{bright}$, (calculated on gated $CD4^+$ cells, B and F), $CD4^+CTLA4^+$ (C and G) and $CD4^+CD25^+CTLA4^+$ PBMC (D and H) are reported. Results are expressed as median (columns) and range (bars) values of 4 experiments with third-party (A-D) and of 4 experiments with autologous MSC (E-H).

able in the presence of autologous MSC were significantly lower ($p<0.01$) than those recovered in primary ctrl-MLC; this difference was even more evident in the presence of third-party MSC ($p<0.001$, see also Figure 2). R-PBMC cultured with MSC at both R-PBMC:MSC ratios, in the absence of S-PBMC did not display any modification in the number of either $CD4^+$ or $CD8^+$T-lymphocytes after 7-10 days primary MLC in comparison to day 0.

Following stimulation in secondary MLC, we observed partial (at a R-PBMC:MSC ratio of 1:1) or complete (at a R-PBMC:MSC ratio of 10:1) recovery of $CD4^+$ T-lymphocyte expansion (Figure 2A and 2C), while partial recovery of $CD8^+$ T cells was recorded only in cultures with autologous MSC added at a R-PBMC:MSC ratio of 10:1 (Figure 2D). The capacity of $CD8^+$ T-lymphocyte expansion did not recover when third-party MSC were employed (Figure 2B) or when autologous MSC were added at a R-PBMC:MSC ratio of 1:1 (Figure 2D). In detail, the numbers of $CD4^+$ T cells were significantly lower in the presence of MSC ($p<0.001$ for third-party MSC; $p<0.01$ for autologous MSC, as compared to secondary ctrl-MLC) only when a R-PBMC:MSC ratio of 1:1 was employed, while there was no effect on cell recovery when we used a R-PBMC:MSC ratio of 10:1. As compared to secondary ctrl-MLC, the number of $CD8^+$ T cells recovered was lower in the presence of both third-party ($p<0.001$) and autologous ($p<0.01$) MSC at any R-PBMC:MSC ratio employed.

The effect of MSC on NK-lymphocyte expansion induced in primary and secondary MLC was similar to that observed for $CD4^+$ T cells (*data not shown*).

Effect of MSC on differentiation of $CD4^+CD25^+$ T-cell subsets induced by allogeneic stimulus

The preferential recovery of $CD4^+$ T cells observed in secondary MSC-MLC prompted us to investigate whether $CD4^+$ T-lymphocytes recovered from the cultures co-expressed CD25, in particular high levels of this molecule ($CD25^{bright}$), and/or CTLA-4 (Figure 3), this phenotype having been associated with regulation/suppression of immune responses.[29-32] The percentage of total $CD4^+CD25^+$ cells was strikingly high-

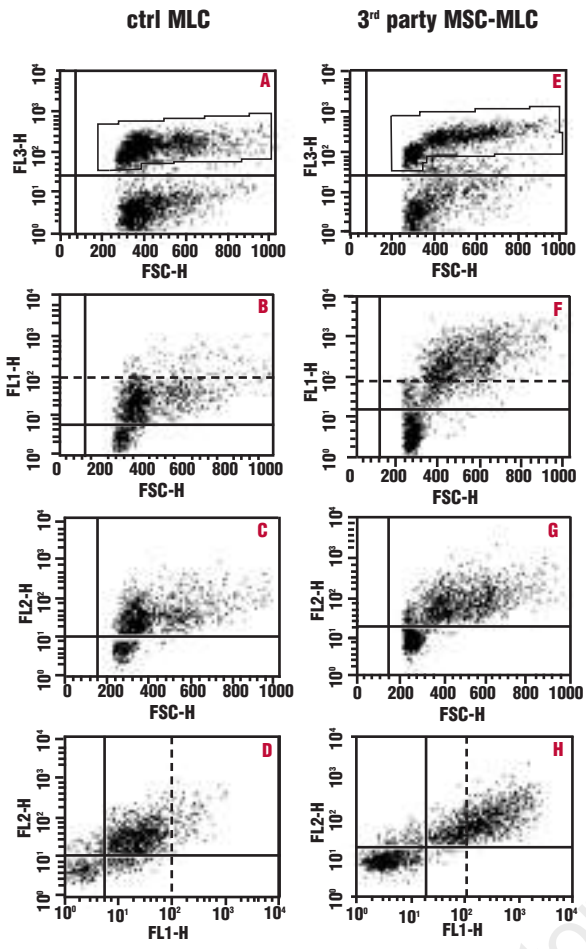

Figure 4. Co-expression of CTLA4 in the CD4^{+}CD25^{bright} T lymphocyte subset induced by allogeneic stimulus. Percentages of CD25^{bright} (B, F), CTLA4^{+} (C, G), and CD25^{bright}CTLA4^{+} (D, H) cells were evaluated on gated CD4^{+} T lymphocytes (A,E). Representative results of one of the experiments illustrated in Figure 3, concerning 7-day primary MLC performed in the absence (ctrl-MLC, A-D) or presence of MSC at a R-PBMC/MSC ratio of 1:1(E-H), are reported. Solid line: threshold for isotype controls, dotted line: threshold for CD25^{bright} expression.

er in the presence than in the absence of MSC only after secondary MLC (Figure 3A and 3E). However, the proportion of CD4^{+}CD25^{bright} cells, after both primary and 3-day secondary MLC (Figure 3B and 3F), was higher in MSC-MLC than in ctrl-MLC, the highest values being found in primary MLC at the R-PBMC:MSC ratio of 1:1. Similarly, after both primary and secondary MLC, percentages of CD4^{+}CTLA4^{+} cells (Figure 3C and 3G) and CD4^{+}CD25^{+}CTLA4^{+} cells (Figure 3D and 3H) were increased in the presence of MSC. In detail, percentage values of CD4^{+}CTLA4^{+} and CD4^{+}CD25^{+}CTLA4^{+} were remarkably higher in the presence of third-party MSC, though these subsets were also slightly increased in the presence of autologous MSC if compared with ctrl-MLC (Figure 3C and 3D and Figure 3G and 3H, respectively). In all culture conditions, CD4^{+}CD25^{bright} cells co-expressed CTLA4; moreover, in the presence of third-party MSC, CD4^{+}CD25^{dim} and CD4^{+}CD25^{neg} cells could also co-express CTLA4 (Figures 3 and 4).

When R-PBMC were cultured with autologous or third-party MSC in the absence of S-PBMC, less than 10% of CD4^{+} T lymphocytes expressed CD25 and/or CTLA4 molecules; at day 0, CD4^{+}CD25^{+} and CD4^{+}CTLA4^{+} R-PBMC were <10% and <4%, respectively.

Effect of MSC on cell-mediated cytotoxic activity induced by allogeneic stimulus

Both alloantigen-specific cytotoxic T lymphocytes (CTL) and alloreactive NK or NK-like cells are able to mediate alloantigen-induced cell-mediated cytotoxic activity, with alloreactive NK cells contributing to cytolytic function when effector and target cells are KIR-ligand incompatible.[33-35] In order to evaluate the effect of human MSC on both CTL-mediated and NK-mediated alloantigen-specific cytotoxic activity, experiments were performed using R-PBMC and S-

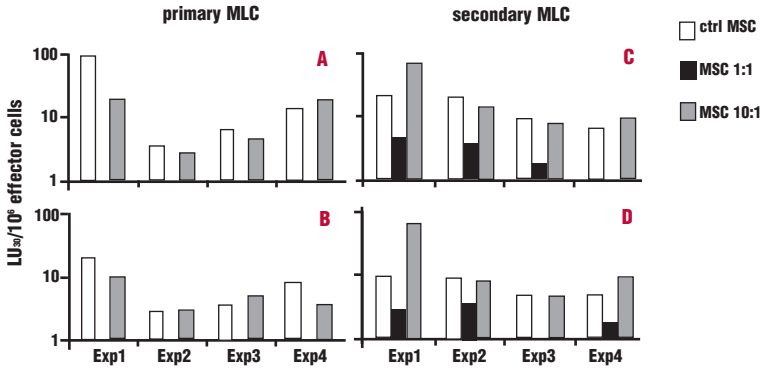

Figure 5. Effect of third-party human MSC on cell-mediated cytotoxic activity induced by allogeneic stimulus. Specific lysis of allogeneic S-PHA target cells, detected by using various effector to target (E:T) ratios (from 50:1 to 3:1), were assessed after primary CTL-MLC (A and B) and secondary CTL-MLC (C and D), performed in the absence (white columns) or presence of third-party MSC added at a R-PBMC/MSC ratio of 1:1 (black column) or 10:1 (gray column). Cytotoxic activity was evaluated in the absence (A and C) or presence (B and D) of *cold* NK-sensitive target cells (K562). Results are expressed as LU$_{30}$/10^{6} effector cells; data from 4 separate experiments are reported.

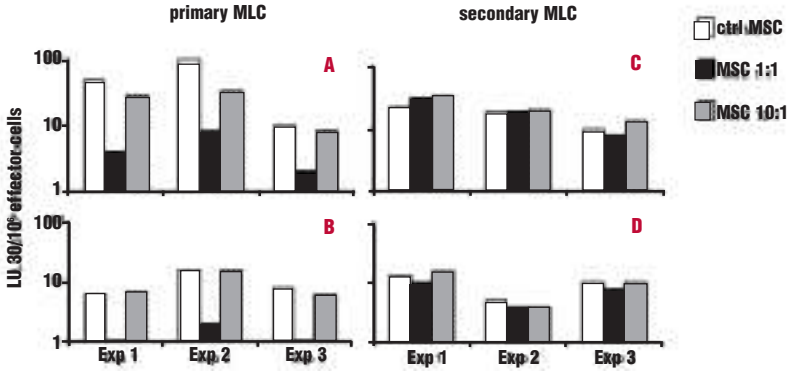

Figure 6. Effect of autologous human MSC on cell-mediated cytotoxic activity induced by allogeneic stimulus. Specific lysis of allogeneic S-PHA target cells, detected by using various effector to target (E:T) ratios (from 50:1 to 3:1), were assessed after primary CTL-MLC (A and B) and secondary CTL-MLC (C and D), performed in the absence (white columns) or presence of autologous MSC added at a R-PBMC/MSC ratio of 1:1 (black column) or of 10:1 (striped column). Cytotoxic activity was evaluated in the absence (A and C) or presence (B and D) of *cold* NK-sensitive target cells (K562). Results are expressed as $LU_{30}/10^6$ effector cells; data from 3 separate experiments are reported.

Table 1. Distribution of T and NK lymphocyte subsets among the effector cell populations employed for cytotoxicity assays reported in Figures 5 and 6. Range values of the percentage of cells expressing T- and NK-surface markers are reported.

Cell Population	*Primary MLC*			*Secondary MLC*		
	Ctrl	*MSC 1:1*	*MSC 10:1*	*Ctrl*	*MSC 1:1*	*MSC 10:1*
Third-party MSC						
$CD3^+$	57-70	89-95	90-93	88-95	91-97	90-95
$CD3^+$ $CD4^+$	33-40	79-83	65-70	48-55	78-86	70-75
$CD3^+$ $CD8^+$	20-28	10-12	17-25	34-41	11-15	17-25
$CD3^{neg}$ $CD56^+$	21-36	2-7	4-7	3-9	1-7	2-8
Autologous MSC						
$CD3^+$	41-67	73-88	65-80	85-92	79-90	78-91
$CD3^+$ $CD4^+$	18-35	47-64	34-60	57-61	67-77	46-66
$CD3^+$ $CD8^+$	21-30	15-20	8-18	27-35	11-16	22-29
$CD3^{neg}$ $CD56^+$	23-51	8-15	16-27	4-11	3-14	7-15

PBMC obtained from KIR-ligand incompatible subjects, identified by HLA-B and HLA-C high-resolution molecular typing.

Results obtained in primary MLC, in the absence of *cold* NK-sensitive target cells, showed that both third-party (Figure 5A) and autologous (Figure 6A) MSC were able to display a dose-dependent inhibitory effect on alloantigen-specific cytotoxic activity, which was particularly striking at a R-PBMC/MSC ratio of 1:1, and when third-party MSC were employed. In secondary MLC, dose-dependent inhibition of alloantigen-specific cytotoxic activity was only detected when third-party MSC were employed (Figure 5C), while autologous MSC were unable to down-regulate cytolytic function (Figure 6C).

In keeping with previously reported data,[17] cytotoxic activity towards human MSC was absent in all culture conditions, and specific lysis of ^{51}Cr-labeled allogeneic target cells (S-PHA) was not affected by addition of MSC, at an effector:MSC ratio of 10:1, during the 5-hour cytotoxicity assay (*data not shown*). In all experiments, addition of *cold* NK-sensitive target cells decreased cytotoxic activity in both primary and secondary ctrl-MLC, thus confirming a contribution of NK or NK-like effectors, beside alloantigen-specific CTL, to the cytolytic function elicited in MLC (Figure 5B and Figure 6B). Addition of *cold* NK-sensitive target cells also decreased cytotoxic activity in MSC-MLC. Collectively, these data suggest that MSC employed at a 1:1 R-PBMC:MSC ratio are able to inhibit both CTL and NK-cell cytotoxic functions strongly, but when used at a 10:1 R-PBMC:MSC ratio, they display a quite variable effect which may vary from an inhibitory to an enhancing role.

Flow cytometric evaluation of the distribution of T- and NK-lymphocyte subsets among effector-cell populations employed for cytotoxicity assays confirmed that the interaction of MSC with alloantigen-stimulated PBMC particularly down-regulated the expansion of $CD8^+$ T-lymphocytes and of NK cells. In line with this pattern of immune regulatory function, percentages of $CD4^+$ T lymphocytes were higher in all culture conditions of MSC-MLC than in ctrl-MLC (Table 1).

Discussion

Preliminary evidence suggests that the use of human MSC could improve HSC engraftment and prevent or cure severe GVHD after allogeneic HSC transplantation.[6,9,10] The effectiveness of human MSC in controlling severe GVHD seems to be related to the immuno-regulatory role these cells play in suppressing alloantigen-specific T-cell activation.[11-21] The results of the present study provide further insights into some of the MSC-mediated immune-regulatory mechanisms acting *in vitro* on T-cell activation, by demonstrating that human MSC: (i) strongly inhibit alloantigen-induced DC1 differentiation; (ii) down-regulate alloantigen-induced lymphocyte expansion, especially that of $CD8^+$ T cells

and of NK lymphocytes; (iii) favor the differentiation of $CD4^+$ T-cell subsets co-expressing CD25 and/or CTLA4; (iv) decrease the alloantigen-specific cytotoxic capacity mediated by either CTL or NK cells; and (v) exert more effective suppressive activity on MLC-induced T-cell activation when they are allogeneic rather than autologous with respect to responder cells. The inhibitory effect of human MSC on alloantigen-induced DC differentiation is in agreement with recent studies showing the capacity of MSC to alter DC1 function[21] and antigen-presenting cell maturation,[22] and could be related to their capacity of producing anti-inflammatory cytokines, such as tumor growth factor-β,[13] known to inhibit *in vitro* activation and maturation of DC.[36] Moreover, in agreement with recently published data,[37] it can be hypothesized that the preferential differentiation of $CD4^+CD25^+$ cells, a lymphocyte subset with presumed regulatory/suppressive function, obtained in the presence of MSC, also contributes to restraining DC differentiation. DC are known to be the most powerful stimulators of alloreactive T-cell response;[38] therefore, MSC-induced down-regulation of DC1 differentiation may play an important role in the inhibition of alloantigen-induced T- and NK-cell expansion and cytotoxic activity. The remarkable inhibition of alloantigen-induced $CD8^+$ T lymphocyte expansion in both primary and secondary MLC, and of NK cells mainly in primary MLC, could also be related to the MSC-induced preferential differentiation of $CD4^+CD25^+$ regulatory T-cell subsets, which are known to suppress proliferation of effector lymphocytes.[29,32] Conversely, preferential differentiation of $CD4^+CD25^+$ T-lymphocyte subsets, and in particular of $CD4^+CD25^{bright}$ cells co-expressing CTLA4, was not apparently strictly related to the inhibition of alloreactive cytotoxic capacity. Indeed, down-regulation of alloreactive-cytolysis was MSC dose-dependent, while preferential differentiation of $CD4^+CD25^+$ cell subsets was the same, independently of the R-PBMC:MSC ratios employed. In agreement with data reported by Rasmusson *et al.*,[17] we found that the dose-dependent inhibitory effect of MSC on cell-mediated cytotoxic activity was mostly due to suppression of the proliferation of cytotoxic lymphocytes, rather than to a direct inhibition of cytolytic capacity. $CD4^+CD25^+$ T cells, and in particular $CD4^+CD25^{bright}$ cells co-expressing CTLA-4, have been demonstrated to be involved in both induction of tolerance against self-antigens and maintenance of homeostasis of the immune response directed towards nominal antigens.[29-32] Differentiation of $CD4^+CD25^+$ lymphocytes is inducible by stimulation with immature DC;[30,39] their activation requires antigen-dependent stimulation via T-cell receptors, while their suppressive effector function is antigen non-specific.[29] Recent evidence suggests the co-existence of different subsets of $CD4^+CD25^+$ cells displaying their regulatory activity by cell-cell contact or by secretion of anti-inflammatory cytokines, such as interleukin-10.[40] In particular, it has been demonstrated that $CD4^+CD25^+$ T cells confer suppressive properties to other T cell subsets, a process termed *infectious tolerance*.[41] Although several experimental data demonstrated the ability of $CD4^+CD25$ T-regulatory cells to suppress alloreactivity *in vitro* and either to prevent or to attenuate GVHD in animal models,[29,42-46] two recent studies suggest that differentiation of these cells may not be sufficient for preventing occurrence of acute or chronic GVHD in patients given allogeneic HSCT.[47,48] In view of these data, the MSC-dependent, preferential differentiation of alloantigen-reactive $CD4^+CD25^+$ T-cell subsets displaying a regulatory/suppressive phenotype here described, although in keeping with down-regulation of MLC-induced lymphocyte proliferation and cytolytic activity, may not be the principal mechanism accounting for the recently described striking effect of MSC infusion in promoting resolution of severe acute GVHD.[10] Indeed, it is worth considering that several pieces of evidence demonstrated that human MSC exert a relevant proportion of their inhibitory effect on lymphocyte activation through the release of soluble factors and by indoleamine 2,3-dioxygenase-mediated degradation of tryptophan, an amino acid essential for lymphocyte proliferation.[13,14,19,20] Therefore, it is conceivable that the preferential differentiation of $CD4^+CD25^+$ T-cell subsets is favored by other mechanisms of MSC-mediated inhibition of alloantigen-induced effector cell activation and expansion, and, in turn, these cells contribute to propagate and extend suppressor activity.

Our results clearly demonstrate that third-party human MSC are more effective than autologous MSC in suppressing both alloantigen-induced lymphocyte proliferation and alloantigen-specific cytotoxic activity. This finding could, at least in part, be explained by considering that higher percentages of $CD4^+CTLA^+$ and $CD4^+CD25^+CTLA4^+$ T cells were detected when third-party, rather than autologous, MSC were added to MLC. CTLA4 is a T-lymphocyte glycoprotein expressed following activation and is known to deliver an inhibitory signal to T cells and to mediate apoptosis of previously activated T lymphocytes.[49] Our data strongly suggest that interactions between human MSC and alloantigen-activated lymphocytes promote expression of CTLA-4 on $CD4^+$ T cells. The evidence that third-party, rather than autologous, MSC are more efficient in mediating this function may be interpreted by hypothesizing that T-cell recognition of alloantigens expressed by MSC may further facilitate the preferential generation and expansion of activated $CD4^+$ T cells expressing CTLA4. Although the use of third-party MSC needs to be further tested in a clinical context, this experimental finding encourages the use of third-party MSC for the prevention of immune complications

related to either HSC or solid organ transplantation and provides support to the theory that MSC are *universal* suppressors of immune reactivity.

RM, MP, AM, PC, DM, AI, FF and FL contributed to the conception and design of the study, analysis and interpretation of data and critical review the manuscript; AC and LD contributed to carrying out, analyzing and interpretating the immunology assays; GP and SP contributed to ex-vivo expansion of MSC. The authors declare that they have no potential conflicts of interest.

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