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Original Article

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Human mesenchymal stem cells inhibit antibody production induced *in vitro* by allostimulation

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Abstract

Background. Antibodies directed against alloantigens are implicated in the pathogenesis of several immune reactions complicating transplantation, including humoral rejection after solid organ transplantation. Mesenchymal stem cells (MSCs) have immunomodulatory capacity, since *in vivo* they may prolong skin graft survival in the animal model and can rescue patients with life-threatening graft-*versus*-host disease.

Methods. To investigate whether MSCs exert an inhibitory effect on antibody production during allostimulation, we stimulated peripheral blood mononuclear cells, obtained from healthy controls or sensitized patients undergoing dialysis for end-stage renal failure, in mixed lymphocyte culture (MLC), and evaluated immunoglobulin production either in the absence or in the presence of third-party allogeneic MSCs. We also evaluated the effect of MSCs on B-cell allostimulation performed adding to MLC a polyclonal stimulus delivered by an agonist anti-CD40 monoclonal antibody.

Results. We found that the addition of MSCs at the beginning of MLC considerably inhibited immunoglobulin production in standard MLC, irrespective of the MSC dose employed. Conversely, immunoglobulin secretion induced by direct CD40-CD40L binding was not significantly inhibited. Furthermore, we demonstrated, in one sensitized patient, that secretion of donor-specific anti-HLA class I antibodies detected both in baseline serum and in the supernatant of control MLC was inhibited by the addition of MSCs. Mechanistically, the addition of MSCs induced a striking decrease of IL-5 production in the cultures.

Conclusions. Our findings suggest that third-party MSC are able to suppress allo-specific antibody production *in vitro*, and may therefore help overcome a positive cross-match in sensitized transplant recipients.

Keywords: alloantigen-specific Ig; humoral immune response; kidney transplantation; mesenchymal stem cells

Introduction

Antibodies directed against alloantigens are implicated in the pathogenesis of several immune reactions complicating transplantation. In particular, in solid organ transplant (SOT) recipients, the role of humoral immunity in acute and chronic rejection has been highlighted by the recent histological characterization of antibody-mediated rejection and by the detection of donor-reactive antibodies with sensitive techniques [1,2]. In this latter setting, in an effort to expand the donor pool, trials of allograft transplantation across the HLA barrier have employed desensitization strategies, including the use of plasmapheresis, intravenous immunoglobulins, anti-B-cell monoclonal antibodies and splenectomy, associated with high-intensity immunosuppressive regimens [3–5]. These measures have proven only partially successful in preventing or treating humoral rejection in high-risk patients, while causing a significant increase in the risk of severe infectious complications occurring after transplantation. Thus, the development of new therapeutic tools able to blunt alloantibody production would be a welcome complementation to existing protocols.

Mesenchymal stem cells (MSCs) play a central role in the development and differentiation of the lymphohematopoietic system [6,7]. Infusion of *ex vivo* expanded human MSCs demonstrated that both recipient- and donor-derived human MSCs can facilitate haematopoietic recovery in the setting of both autologous and allogeneic HSCT [8]. MSCs not only have a favourable effect on engraftment of haematopoietic progenitors, but also display immunoregulatory activities. Several *in vitro* studies have demonstrated

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the ability of MSCs to modulate T-cell-mediated immune response. We and others have shown that MSCs can inhibit T-cell proliferation and cytotoxicity directed towards alloantigens, as well as alloantigen-induced dendritic cell (DC) differentiation [9–12]. Moreover, treatment with MSCs may resolve steroid-resistant graft-*vs*-host disease in humans [13]. It has been recently suggested that MSCs may promote the emergence of CD4+/CD25+ regulatory T cells *in vitro* [12], and, in the context of solid organ transplant, MSCs were shown to prolong skin graft survival in a primate model [13]. Further rationale for the use of MSCs in SOT trials could derive from the demonstration that MSCs are capable of modulating B-cell alloresponses.

Few studies have analysed the effects of MSCs on B-cell function [14–18], and data regarding the modulatory effects of MSCs on alloantigen-specific humoral response in humans are lacking. To verify whether MSCs exert an inhibitory effect on antibody production during allostimulation, we devised an *in vitro* system for immunoglobulin (Ig) production during mixed lymphocyte culture (MLC), and evaluated the impact of third-party MSC addition on the resulting antibody response.

Materials and methods

Cell harvesting

Peripheral blood samples were collected from five highly sensitized children with end-stage renal failure receiving dialysis whilst awaiting kidney transplantation, and 10 healthy adult controls. Patients' parents and healthy controls provided their informed, written consent to be included in the study. Human MSCs were obtained from bone marrow (BM) samples of healthy subjects donating to a sibling for allogeneic HSCT, after obtaining written informed consent from donors or their legal guardians. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient from heparinized samples.

The Institutional Review Board of the Pediatric Hematology/Oncology Unit, Fondazione IRCCS Policlinico San Matteo, Pavia approved the protocol.

Ex vivo culture of human MSCs

MSCs were expanded from BM mononuclear cells using a previously reported method [12]. Briefly, mononuclear cells were seeded at a density of 10^6 cells/ml in 75 cm^2 flasks (Corning-Costar, Celbio, Milano, Italy) in MesenCult medium with MSC-supplements (Stem Cell Technologies, Vancouver, Canada) and incubated at 37°C in 5% humidified CO_2 atmosphere. After 24 h, non-adherent cells were discarded, fresh medium was added and half the medium 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 and re-plated at a concentration of 5×10^5 cells per flask for the next passage. Cultured MSCs, isolated after either the second or the third passage, included a single-phenotype population, as defined by flow cytometry, positive for CD29, CD44, CD73, CD90, CD105, CD166, CD13 and HLA-class I molecules and negative for CD45, CD31, CD34, CD14, HLA-DR, CD80 and CD86 antigens. The adipogenic and osteogenic differentiation capacity of MSCs were determined as previously described [12]. Briefly, to detect the osteogenic differentiation, cells were stained for alkaline phosphatase (AP) activity using Fast Blue (Sigma-Aldrich) and for calcium depositions with Alzarin Red (Sigma-Aldrich). Adipogenic differentiation was evaluated through the morphological appearance of fat droplets with Oil Red O (Sigma-Aldrich).

In vitro Ig production

Spontaneous and allo-stimulated *in vitro* Ig production was evaluated according to the following method. B-cell enrichment of responder PBMC was obtained, after removal of monocytes by cell adherence, through *partial* depletion of CD4+ and *depletion of* CD8+ T cells by selection with magnetic microspheres coated with anti-CD4 and anti-CD8 mAbs (MACS, Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany), according to manufacturer's instructions. The responder cell population obtained included a mean 46.5 (95% CI 30–62) CD3+ cells, of which 43% (95% CI 32–53) were CD4+ and 1% (95% CI 0.2–1.2) CD8+, 43.5% CD3-/CD56+ NK cells, 0.7% (95% CI 0.1–2) CD14+ cells, and 9.9% (95% CI 4.6–15) CD19+/CD20+ B cells.

Responder cells (1×10^5) were stimulated in MLC with 1×10^5 irradiated (3000 cGy) stimulator PBMC (either a pool of allogeneic PBMC derived from healthy subjects HLA-disparate with the controls, or a pool of stimulator PBMC bearing HLA antigens recognized by patient's alloantibodies found to be positive at the cross-match test) to reach a final volume of 200 μl/well in 96-well plates. A total of 5×10^4 irradiated autologous feeder PBMC, derived from the discarded fractions after magnetic microsphere separation, were also added to the cultures. We also investigated a condition in which a polyclonal stimulus through the use of agonist anti-CD40 mAb and recombinant human (rh)IL-10 was added to B-cell allostimulation in MLC [19]. MLC were plated in the absence (ctrl-MLC) or presence (MSC–MLC) of third party MSCs at the two responder:MSC ratios of 4:1 and 20:1. In some experiments, we added the condition where MSC were not added directly to the cultures, but plated on transwell permeable supports (Corning Costar, Corning, NY, USA), in order to allow soluble factor transit in the absence of direct contact between MSC and other cellular subsets. The third party MSCs employed were obtained from three different donors. In each experiment, MSCs from a single donor, according to availability, were employed.

Control cultures included wells with responders alone, stimulators and feeders alone, and irradiated responder cells in the presence of irradiated stimulators and autologous feeders. All cultures were performed in Iscove's medium, supplemented with 5 μg/ml bovine insulin, 0.5% bovine serum albumin, 2 μg/ml ethanolamine, 20 mM HEPES buffer (all from Sigma-Aldrich, St. Louis, MO, USA), 50 μg/ml gentamycin, 2 mM L-glutamine and 5% heat-inactivated fetal-bovine serum (all from Gibco, Life

Technologies Ltd, Paisley, UK). All experiments were run in 10 replicates. Supernatants were harvested after 10-day incubation at 37°C in humidified 5% CO_2 atmosphere.

Supernatant Ig and cytokine quantification

Human IgG (hIgG), hIgM and hIgA concentration in the supernatants was determined by an enzyme-linked immunosorbent assay (ELISA) technique, as previously described [19]. In brief, microtiter plates (Greiner Bio-One, Frickenhausen, Germany) were coated with rabbit anti-hIgG, anti-hIgM and anti-hIgA antibodies (Dako, Glostrup, Denmark) by incubating for 3 h at 37°C and then overnight at 4°C. The following day, diluted supernatants were plated, and incubated for 2 h at 37°C. Subsequently, rabbit anti-hIgG, or anti-hIgM, or anti-hIgA antibodies conjugated to horseradish peroxidase (Dako) were added. The concentration of each isotype was extrapolated from the standard curve included in each plate. Ig concentration was expressed as μg/ml. Intra- and inter-assay variability, calculated as coefficient of variation, was always <10%.

IL-4, IL-5, IL-6, IL-10, IL-12 and transforming-growth factor β (TGFβ) concentrations in the MLC supernatant were quantified by ELISA using monoclonal antibody pairs (Pierce Endogen, Rockford, IL, USA). Plates were coated with purified antibodies at the appropriate concentrations. Standard curves were prepared with recombinant human cytokine (Pierce Endogen). Biotin-labeled antibodies (Pierce Endogen) were added and HRP-conjugated streptavidine (Pierce Endogen) was used to develop the reactions. Plates were read at 450 nm (Titertek Plus MS 212M).

Antibody-dependent cellular cytotoxicity (ADCC) assay

Specific alloantibody content was evaluated through an ADCC assay, performed according to a previously published method [20]. Plasma or MLC supernatants from sensitized patients or plasma from randomly selected healthy donors were tested. Target cells consisted of PHA blasts bearing HLA class I antigens recognized by patient's alloantibodies found reactive at the cross-match test. PBMC from randomly selected healthy donors were employed as effectors. In detail, 5×10^5 target cells were labeled with ^{51}Cr by overnight incubation. Cells were washed three times, re-suspended in RPMI medium, and 50 μl cell suspension/well was plated in U-bottom 96-well plates. Fifty microlitres plasma or supernatant were added to each well and left for 30 min at 37°C. Final plasma concentrations for the assay were 1:4, 1:10, 1:100, 1:1000 and 1:10 000. Supernatant was used undiluted, reaching a final concentration of 1:4. After extensive washing, effector cells were added to the test at an effector:target ratio of 100:1 and 30:1 in 100 μl RPMI for sample count, while 100 μl RPMI alone or 100 μl triton solution were added for spontaneous release and maximum release counts, respectively. All conditions were plated in triplicate. Cytotoxic activity was measured as previously described [12]. Spontaneous release from the target cells was consistently less than 25%. Results were expressed as % specific lysis.

Statistical analysis

Data were expressed as mean ± SD or median and range, as appropriate. The Wilcoxon test for matched samples was employed to compare groups. A P-value <0.05 was considered to be statistically significant. Statistical analysis was performed using the SAS System (SAS, Cary, NC, USA).

Results

Effects of MSCs on Ig production

Previously published reports suggested that, in human *in vitro* models, MSC-mediated inhibition of both T lymphocyte proliferation and cytotoxicity induced by allogeneic stimulus as well as B lymphocyte proliferation and Ig production in response to polyclonal stimulation is dose-dependent, and maximal at a 1:1 responder (R):MSC ratio [12,17]. In particular, at R:MSC ratios lower than 2:1, the inhibitory effect of MSC on purified B-cell function resulted undetectable [17,18]. While these data were obtained with purified B cells, we decided to evaluate the effects of MSCs on alloantigen-induced Ig production through a more physiological experimental system where T-helper effect on B-cell function is maintained. Thus, we chose to employ an *in vitro* system of non-purified PBMC responders at a responder (R):MSC ratio of 4:1. We focused on B-cell differentiation to Ig-producing cells rather than proliferation, since in a mixed T/B cell system, the effective role of MSCs on specific subset proliferation may result difficult to un-ambiguously dissect.

In a first set of experiments, we determined whether we could detect Ig production following stimulation of B cell enriched PBMC (mean % CD19+ B cells at baseline: 4.2 ± 2.6; mean% CD19+ B cells after enrichment: 9.9 ± 6.2), obtained from ten healthy controls and five sensitized patients with end-stage renal failure, in a one-way MLC. We found that MLC stimulation was able to induce production of IgG, IgA and IgM in all ten healthy controls and in four out of the five patients (mean values of controls: IgG 1583 ng/ml, range 140–4100; IgA 1902 ng/ml, range 70–4500; IgM 1167 ng/ml, range 65–2403; mean values of patients: IgG 866 ng/ml, range 126–1400 ng/ml; IgA 135 ng/ml, range 85–215 and IgM 240 ng/ml, range 187–294) (Figure 1). In one patient, the pooled allogeneic stimulator PBMC selected were not able to induce antibody production (IgG 5.9; IgA 5.1; IgM 10.7). Responders alone, irradiated stimulators and feeders alone, or irradiated responders in the presence of irradiated stimulators and autologous feeders showed minimal Ig production (Figure 1). Addition of MSC at a 4:1 R:MSC ratio induced a marked inhibition of Ig production, with reduced IgG secretion (median fold-decrease: controls, 7.2, range 1.2–43; patients, 5, range 1.8–10) ($P < 0.001$), IgA secretion (median fold-decrease: controls, 3.8, range 1.2–15.8; patients, 3.6, range 1.8–5.6) ($P < 0.01$), and IgM secretion (median fold-decrease: controls, 17, range 0.5–64; patients, 3.6, range 1.7–4.9) ($P < 0.05$) (Figure 1). In order to assess reproducibility of results, we repeated three experiments with the same responder and stimulator pair, in the presence of allogeneic MSCs derived from (a) the same third party individual (interassay

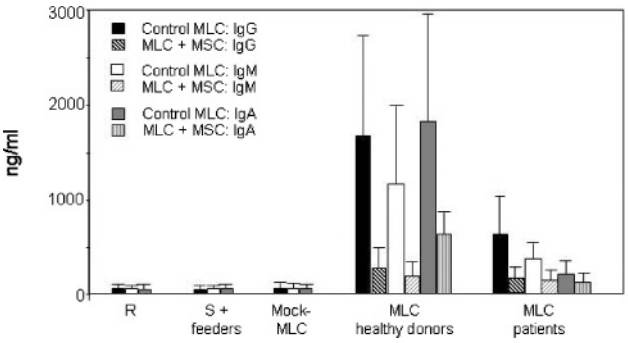

Fig. 1. Third party MSCs significantly inhibit antibody production *in vitro* in the presence of T cells. PBMC were stimulated in MLC with or without third party MSCs (R:MSC ratio 4:1). Control cultures included responders alone (R), irradiated stimulators + feeders alone (S + feeders), and irradiated responders + stimulators + feeders (mock MLC). B cell differentiation was evaluated by quantitation of IgG, IgM and IgA in culture supernatants by ELISA. Mean Ig concentrations (ng/ml) and standard deviation for 10 healthy controls and five patients are shown. Intra-assay variability, calculated as coefficient of variation, was always <10%.

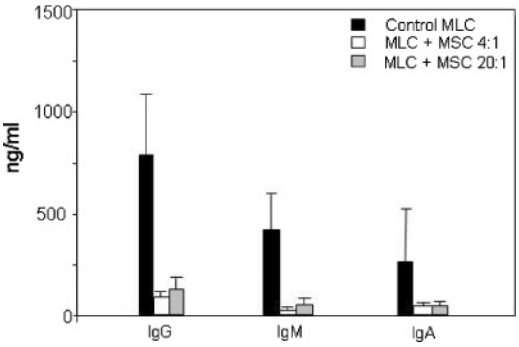

Fig. 2. Allogeneic MSCs inhibit antibody production *in vitro* both at low and high R:MSC ratio. B-cell enriched PBMC from four of the 10 healthy controls were stimulated in MLC with or without allogeneic MSCs (R:MSC ratio 4:1 and 20:1). B-cell differentiation was evaluated by quantitation of IgG, IgM and IgA in culture supernatants by ELISA. Mean Ig concentrations (ng/ml) and standard deviation for the four healthy controls are shown.

variability); (b) the three different MSC donors. In all cases, we could observe comparable Ig production inhibition (variability of Ig G, IgM and IgA production fold-decrease, expressed as coefficient of variation, <25%).

In a second set of experiments conducted on a small number of healthy subjects ($n = 4$), we tested the effect of a lower R:MSC ratio (20:1), that has been reported to have opposing effects on alloreactive T-cell activity inhibition or stimulation in different subjects, and on Ig production in MLC [14]. Ig secretion of control MLC was compared to antibody levels detected in MLC plated in the presence of MSC at the 4:1 and 20:1 ratios. The degree of inhibition of Ig production in the cultures with MSC added at the 20:1 ratio was not statistically different from the inhibition induced by MSC at the 4:1 ratio (Figure 2).

MSCs may inhibit production of specific alloantibodies in sensitized patients

To evaluate whether, in sensitized patients, production of specific alloantibodies was inhibited, we elected to employ a functional assay rather than flow cytometry, since the

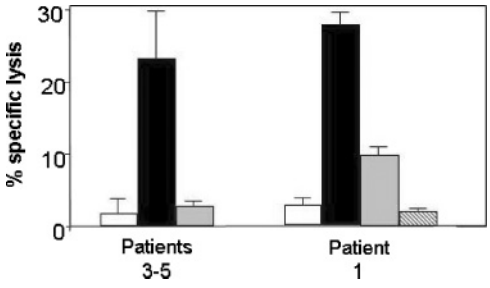

Fig. 3. Third party MSCs inhibit production of specific alloantibodies *in vitro*. The presence of specific alloantibodies in sensitized patients' sera or MLC supernatants was evaluated by means of an antibody-dependent cellular cytotoxicity assay. PBMC from third party healthy volunteers were tested in a chromium release assay against PHA blasts bearing HLA class I antigens recognized by alloantibodies detected in the patients' sera, after incubation of the target cells with patient serum (*black bars*) or with the corresponding supernatants from control MLC (*gray bars*) or MLC plated in the presence of MSCs (*striped bars*). Control cytotoxicity on targets not previously incubated with serum or supernatants is also reported (*white bars*). Results of each experiment represent mean cytotoxicity at a 100:1 E:T ratio. In the left panel, each bar represents the mean cytotoxicity of the three subjects ± standard deviation.

amount of Ig measured in the supernatants by ELISA was at least three log lower than that usually found in sera, and therefore hardly detectable by the standard flow cytometry bead technique employed to measure anti-HLA antibodies.

The ability of sensitized patients' sera to induce killing in an ADCC assay was first analysed. We found that alloantibodies present in patients' sera, collected at the time of MLC experiments, mediated lysis of target cells bearing the corresponding HLA antigens. We then evaluated whether the same alloantibodies were also present in control MLC supernatants at a concentration sufficient to mediate ADCC killing of the same targets. In three of the four evaluable patients, the supernatant from control MLC was not able to mediate sizeable cytotoxicity, likely due to the low concentration of alloantibodies. In one patient, anti-HLA A68 antibodies could be detected in serum at the time of evaluation. Patient lymphocytes were stimulated in MLC with HLA-A68+ PBMC, and the supernatant collected from control MLC was able to induce killing of HLA-A68+ targets. Addition of MSC to the cultures abrogated specific antibody production, as testified to by the observation that supernatant recovered from MSC-MLC did not mediate lysis of HLA-A68+ targets (Figure 3).

MSC-dependent inhibition of Ig production is mainly exerted through suppression of T-cell help, and is facilitated by contact between MSC and other cellular subsets.

Since our finding of significant Ig production inhibition by MSCs added to the cultures at low R:MSC ratios was apparently in contrast with previous reports [17,18], we proceeded to verify whether this difference could be due to the presence of T lymphocytes in our culture system. We, therefore, repeated the previous set of MLC stimulation experiments, including a condition in which MLC was performed in the presence of agonist anti-CD40 mAb and rIL-10, a system described to by-pass T-cell help and induce direct stimulation of memory B-cell pool expansion and Ig production [21]. We observed that, while alloantigen-induced Ig production in a conventional MLC was significantly suppressed by MSCs added at a 4:1 R:MSC ratio, secretion of

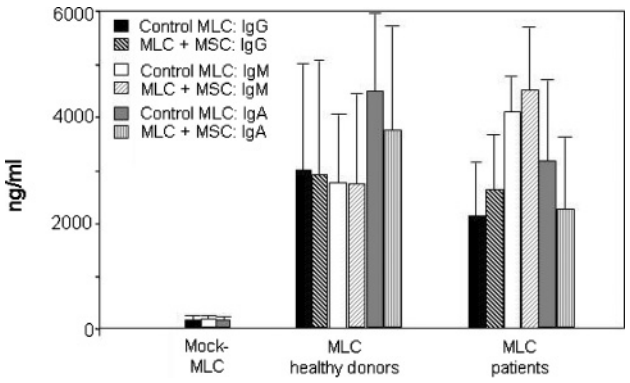

Fig. 4. MSC have little or no inhibitory effect on Ig production when it is facilitated by CD40 agonist engagement. PBMC were stimulated in MLC in the presence of agonist anti-CD40 mAb and rIL-10 with or without allogeneic MSC (R:MSC ratio 4:1). Control cultures included irradiated responders + stimulators + feeders (mock MLC). B-cell differentiation was evaluated by quantitation of IgG, IgM and IgA in culture supernatants by ELISA. Mean Ig concentrations (ng/ml) and standard deviation for the 10 healthy controls and five patients are shown.

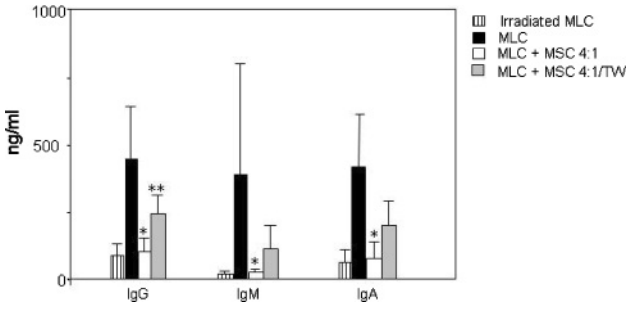

Fig. 5. Inhibition of *in vitro* antibody production by third party MSCs is less pronounced when MSCs are not in direct contact with other cellular subsets of MLC. PBMC from four of the 10 healthy controls were stimulated in MLC with (*white bars*) or without (*black bars*) allogeneic MSC (R:MSC ratio 4:1), added directly to the cultures or plated on transwell supports (*gray bars*). B-cell differentiation was evaluated by quantitation of IgG, IgM and IgA in culture supernatants by ELISA. Controls consisted of irradiated MLC (*striped bars*). Mean Ig concentrations (ng/ml) and standard deviation for the four healthy controls are shown. * indicates a $P < 0.05$, $^{**}P = 0.08$.

IgG, IgA and IgM induced via direct CD40 binding was not significantly inhibited (Figure 4). These results suggest that suppression of Ig production mediated by MSCs takes place at a step that precedes T-cell help through CD154-CD40 binding between T and B cells.

In order to gain mechanistic insight into the phenomenon, we determined whether MSC-mediated inhibition of Ig production was maintained if MSCs were separated from the other cellular components of MLC by means of a permeable support that allowed diffusion of soluble factors in the absence of direct contact. We found that Ig secretion was impaired also in the transwell culture condition, although inhibition, in contrast with what is observed if MSCs were added directly to the MLC, was not statistically significant (Figure 5). We then measured the levels of cytokines possibly influencing Ig secretion in the supernatants collected at the end of culture. After 10-day MSC culture, TGFβ, and IL-6 but no IL-4, IL-5, IL-10 or IL-12 were detected in the supernatant. During control MLC, TGFβ, IL-5, IL-6 and IL-10 were variably secreted, while neither IL-4 nor IL-12 was measurable. In the presence of MSC, we observed a marked increase in the levels of IL-6 and TGFβ, and a slight increase in the levels of IL-10 (Figure 6). The effect of MSCs on production of TGFβ was almost abrogated in the transwell culture system, while no significant difference was observed for IL-6 or IL-10 secretion, indicating that MSC need cell contact in order to influence TGFβ production in MLC. Interestingly, MSC, either added directly or plated in transwells, completely abrogated IL-5 production in the MLC supernatant (Figure 6).

Discussion

In this paper, we have examined the effect of human MSC on Ig production stimulated by alloantigens in an *in vitro* model of PBMC cultivated in MLC. We show that, in this model, MSC added at both R:MSC ratios of 1:4 and 1:20 exert a potent inhibitory effect on IgG, IgA and IgM secretion. This effect is largely dependent on a suppressive activity involving a step that precedes expansion of memory B-cell pool induced by CD154-CD40 binding. Moreover, notwithstanding the low antibody concentrations in MLC supernatants compared with sera, we were able to show that, at least in one sensitized patient, secretion of donor-specific anti-HLA antibodies detected in baseline serum and in the supernatant of control MLC was inhibited by addition of MSC.

Our data are consistent with the findings of B-cell inhibition reported in animal models [15,16], and may also be reconciled with previous observations in humans. In detail, a paper demonstrated MSC-mediated inhibition of human B-cell proliferation and antibody production *in vitro* after polyclonal stimulation [17], while another report showed no MSC effect on proliferation of purified B-lymphocytes after exposure to a combination of allogeneic antigen-presenting cells and CpG [18]. Differently from our *in vitro* PBMC model, those studies employed purified B cells, and could therefore only evaluate the direct effect of MSCs on B-cell function, which was mainly evident at the highest R:MSC ratio of 1:1. At lower concentrations, MSCs do not exhibit direct activity, and suppression of B-cell function may be visible only in the presence of T cells, as in our study, or upon addition of exogenous IFNγ to the purified B-cell culture, as in the report by Krampera *et al*. [18]. In a recent study that utilized a model of bacterial/viral antigen stimulation of PBMC, Rasmussen *et al*. demonstrated that MSC-dependent inhibition of antibody secretion in human B cells is directly correlated to the strength of the baseline stimulus [22]. These data are in agreement with our finding, giving the fact that allostimulation in our model was a strong inducer of Ig production. *However*, from the analysis of available data, it can be hypothesized that MSC-mediated inhibition of B lymphocyte function is predominantly due to the effect of T-cell help suppression rather than to a direct effect on B lymphocytes, given the observation that Ig production is not inhibited by MSC when CD40 engagement is obtained by agonist anti-CD40L antibody rather than by the direct help of T cells.

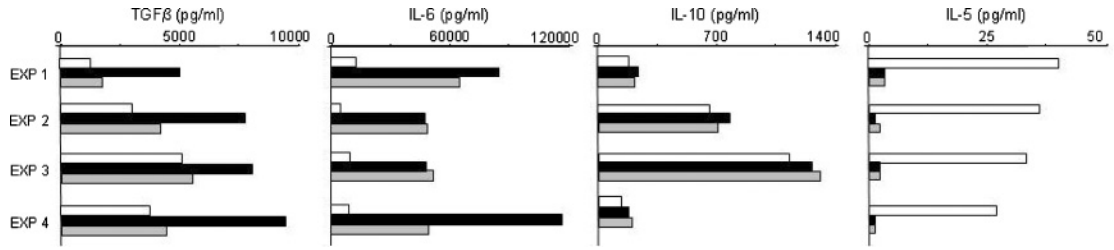

Fig. 6. Effect of MSC co-culture on cytokine secretion in a MLC. PBMC from four of the 10 healthy controls were stimulated in MLC with (*black bars*) or without (*white bars*) third party MSCs (R:MSC ratio 4:1), added directly to the cultures or plated on transwell supports (*gray bars*). Supernatants were collected at the end of the culture period and analysed for the various secreted factors by ELISA. Bars represent cytokine levels (pg/ml) from the four independent experiments reported also in Figure 4. Since measurable levels of TGFβ could be dosed in the culture media, TGFβ values reported were subtracted of the culture media background.

In our culture system, inhibition of Ig secretion is maintained if MSCs are separated from the other components of MLC by a permeable membrane support, this indicating that secreted factors play a relevant role in the immunomodulatory activity. However, the inhibitory effect is greater when MSCs are directly in contact with immune cells, suggesting that cell–cell contact facilitates immunomodulation exerted by MSCs. It has been proposed that MSCs may interact with T cells by inducing a Th1 to Th2 shift, with increased secretion of anti-inflammatory cytokines, among which IL-4 [11]. Given the expected increased levels of IL-4, together with the marked increase of IL-6, in T-cell-MSC co-cultures, one would predict induction of activated B-cells differentiation into Ig-secreting cells. Thus, the observed blunted Ig secretion in the presence of MSCs must be due to an alternative effect exerted on B cells. In this regard, the increased TGFβ levels observed in MLC after addition of MSCs might have counteracted the activity of IL-6. Moreover, it was recently demonstrated that the immunomodulatory effects of MSCs may be partly mediated by secretion of prostaglandin E2 (PGE_2) [11]. PGE_2 was shown to inhibit antigen-specific human T-cell responses, and in particular, together with a reduced secretion of IL-2 and IFNγ, the production of IL-4 and IL-5 [23]. Indeed, in our *in vitro* MLC system, we observed that the addition of MSC induced a striking decrease of IL-5 invariably found in the control MLC cultures. Thus, in our *in vitro* MLC model of Ig production, the inhibitory effect of PGE_2 on IL-5, and possibly IL-4 (undetectable at the end of our control MLC cultures), may have been prevalent on the increased IL-4 production observed when MSCs were interacting with T cells in a Th2-inducing condition culture system [11]. In addition to the mechanism proposed, MSCs may also modulate T-cell dependent B-cell responses through inhibition of antigen-presenting cell function [11,12,24], induction of regulatory CD25+/CD4+ T cells [11,12], or induction of apoptosis in activated T cells through the conversion of tryptophan into kynurenine by indoleamine 2,3-dioxygenase (IDO) expressed on MSC [25].

Following allostimulation, Ig were secreted in large amounts in our *in vitro* system, with healthy controls showing higher production compared with end-stage renal failure patients undergoing dialysis. This observation was unexpected, since in the case of healthy controls the only previous encounter with alloantigens might have occurred as a result of pregnancy or transfusion. It has been described that alloimmunization by pregnancy or transfusion results in production of multiple antibodies rather than a monospecific response towards disparities, and that some of these responses are cross-reactive [26]. Therefore, it is conceivable that the robust antibody secretion induced *in vitro* by allostimulation includes Ig with multiple specificities, and among those, in addition to alloantibodies, also antibodies directed against nominal antigens. Notwithstanding the state of general immunosuppression described for dialyzed patients [27], likely responsible for the lower Ig production after stimulation in MLC, differences between patients and controls were markedly reduced when Ig production was induced via direct CD40 binding. These data suggest that, in dialyzed patients, T-cell or DC compartments may be more compromised than T-independent B-cell function.

The results of this study, if confirmed through further *in vitro* studies and in animal models, could support the use of MSCs, possibly in association with other strategies, to modulate B-cell alloreactivity.

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References

1. Moll S, Pascual M. Humoral rejection of organ allografts. *Am J Transplant* 2005; 5: 2611–2618
2. Mc Kenna RM, Takemoto SH, Terasaki PI. Anti-HLA antibodies after solid organ transplantation. *Transplantation* 2000; 69: 319–326
3. Snanoudj R, Beaudreuil S, Arzouk N *et al*. Immunological strategies targeting B cells in organ grafting. *Transplantation* 2005; 79: S33–36
4. Gloor JM, DeGoey SM, Pineda AA *et al*. Overcoming a positive crossmatch in living-donor kidney transplantation. *Am J Transplant* 2003; 3: 1017–1023
5. Takemoto SK, Zeevi A, Feng S *et al*. National conference to assess antibody-mediated rejection in solid organ transplantation. *Am J Transplant* 2004; 4: 1033–1041
6. Pittenger MF, Mackay AM, Beck SC *et al*. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; 284: 143–147

7. Noort WA, Kruisselbrink AB, in't Anker PS *et al*. Mesenchymal stem cells promote engraftment of human umbilical cord blood-derived CD34+ cells in NOD/SCID mice. *Exp Hematol* 2002; 30:870–878
8. Lazarus HM, Koc ON, Devine SM *et al*. Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant* 2005; 11: 389–398
9. Di Nicola M, Carlo-Stella C, Magni M *et al*. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or non specific mitogenic stimuli. *Blood* 002; 99: 3838–3843
10. Krampera M, Glennie S, Dyson J *et al*. Bone marrow mesenchymal stem cells inhibit the response of naïve and memory antigen-specific T cells to their cognate peptide. *Blood* 2003; 101: 3722–3729
11. Aggarwal S, Pittinger MF. Human mesenchymal stem cells modulate alloantigen immune cell responses. *Blood* 2005; 105: 1815–1822
12. Maccario R, Podestà M, Moretta A *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: 516–525
13. Le Blanc K, Rasmusson I, Sundberg B *et al*. Treatment of severe acute graftversus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; 363: 1439–1441
14. Bartholomew A, Sturgeon C, Siatskas M *et al*. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002; 30: 42–48
15. Glennie S, Soeiro I, Dyson PJ *et al*. Bone marrow mesenchymal stem cells induce division arrest energy of activated T cells. *Blood* 2005; 105: 2821–2827
16. Augello A, Tasso R, Negrini SM *et al*. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005; 35: 1482–1490
17. Corcione A, Benvenuto F, Ferretti E *et al*. Human mesenchymal stem cells modulate B cell functions. *Blood* 2006; 107: 367–372
18. Krampera M, Cosmi L, Angeli R *et al*. Role for IFN-g in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006; 24: 386–398
19. Avanzini MA, Locatelli F, Dos Santos C *et al*. B lymphocyte reconstitution after hematopoietic stem cell transplantation: functional immaturity and slow recovery of memory CD27+ B cells. *Exp Hematol* 2005; 33: 480–486
20. Yamada T, Watanabe N, Nakamura T, Iwamoto A. Antibody-dependent cellular cytotoxicity via humoral immune epitope of Nef protein expressed on cell surface. *J Immunol* 2004; 172: 2401–2406
21. Marconi M, Plebani A, Avanzini MA *et al*. IL-10 and IL-4 co-operate to normalize in vitro IgA production in IgA-deficient (IgAD) patients. *Clin Exp Immunol* 1998; 112: 528–532
22. Rasmusson I, Le Blanc K, Sundberg B, Ringden O. Mesenchymal stem cells stimulate antibody secretion in human B cells. *Scand J Immunol* 2007; 65: 336–343
23. He X, Stuart JM. Prostaglandin E2 selectively inhibits human CD4+ T cells secreting low amounts of both IL-2 and IL-4. *J Immunol* 1999; 163: 6173–6179
24. Beyth S, Borovsky Z, Mevorach D *et al*. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T cell unresponsiveness. *Blood* 2005; 105: 2214–2219
25. Meisel R, Zibert A, Laryea M *et al*. Human bone marrow stomal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004; 103: 4619–4621
26. Mizutani K, Terasaki P, Rosen A *et al*. Serial ten-year follow-up of HLA and MICA antibody production prior to kidney graft failure. *Am J Transplant* 2005; 5: 2265–2272
27. Vogtlander NPJ, Brown A, Valentijn RM *et al*. Impaired response rates, but satisfying protection rates to influenza vaccination in dialysis patients. *Vaccine* 2004; 22: 2199–2201

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