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ORIGINAL ARTICLE

The effect of rabbit antithymocyte globulin on human mesenchymal stem cells

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Summary

Mesenchymal stem cells (MSCs) possess immunomodulatory properties which are of key interest for their application in autoimmunity and transplantation. In transplantation, administration of MSCs has shown promising results in preclinical models and has recently moved to clinical trials. Therefore, it is important to study the interactions between MSCs and immunosuppressive drugs currently used in transplantation. We aimed to analyze the effect of rabbit antithymocyte globulin (rATG) MSCs. MSCs were obtained from perirenal fat of kidney donors and exposed to ranging doses of rATG (Thymoglobulin®, Genzyme; 0.5–100 μg/ml). Binding of rATG, effects on viability and susceptibility to be killed by cytotoxic lymphocytes as well as effects on their immunosuppressive potential of MSCs were tested. rATG binds dose-dependently to MSCs. This binding was associated with slightly impaired viability after 48 and 72 h when compared with nonexposed MSCs. In contrast to nontreated MSCs, rATG preexposed MSCs were susceptible to be lysed by cytokine-activated $CD8^+$ cytotoxic cells and NKT cells. The capacity of MSCs to suppress the proliferation of anti-CD3/CD28 activated CD4 and CD8 T cells were reduced by the presence of rATG in the culture. rATG reduces the viability and antiproliferative capacity of MSCs in a dose-dependent manner and converts them into targets for CD8 T cells and NKT cell lysis.

Introduction

Mesenchymal stem cells (MSCs) have immunomodulatory properties that make them promising candidates as immunosuppressive agents in the transplantation setting. Many animal models have been used to describe their therapeutic properties in heart, skin, islets, bone marrow, liver, and kidney transplantation without concomitant immunosuppression [1–5]. In the context of multitarget therapy commonly used in clinical transplantation, MSCs are unlikely to be used as single agent [6–8]. The study of potential interactions with conventional immunosuppressants currently used in organ transplantation may be of interest in order to design immunosuppressive regimens with a strong mechanistic rationale.

The interaction between MSC and immunosuppressive drugs used in transplantation has been studied *in vitro* [9,10] and *in vivo* [11,12]. From those studies, it is known that calcineurin inhibitors (Cyclosporin and Tacrolimus) and mTOR inhibitors (Rapamycin) are not an optimal combination with MSC therapy as they reduce the immunosuppressive ability of MSCs while the cell cycle inhibitor mycophenolic acid (MPA) acts synergistically with the immunosuppressive effect of MSCs.

Rabbit antithymocyte globulin (rATG) is a purified, pasteurized, gamma immune globulin, obtained by immunization of rabbits with human thymocytes. This immunosuppressive product contains an incompletely known composition of cytotoxic antibodies directed against antigens expressed on human lymphocytes. Analysis of the active fraction of Thymoglobulin rATG revealed that 7% of the rabbit IgG is specific for human peripheral blood lymphocytes and 93% are nonspecific rabbit IgG [13]. It is used as induction therapy during the first days after organ

transplantation to decrease the incidence of acute rejection or as a T cell-depleting agent before bone marrow transplantation, as well as in the treatment for acute rejection episodes after transplantation. rATG binds to the surface of lymphocytes and monocytes and temporally depletes these cells from the circulation, thereby preventing acute rejection in the graft [14]. Moreover, rATG has been shown to induce $CD4^+$ $CD25^{bright}$ $FoxP3^+$ Treg *in vitro* [15].

Therefore, it is likely that MSC therapy will be used after organ transplantation simultaneously with circulating rATG antibodies, as in fact it has been done [6].

In this study, we aimed to analyze the effect of rATG on MSC properties and their interactions with circulating immune cells.

Materials and methods

Isolation and culture of perirenal adipose tissue-derived MSCs

Perirenal adipose tissue that became available as a waste product during the kidney donation procedure was collected after obtaining written informed consent as approved by the Medical Ethical Committee of the Erasmus Medical Centre Rotterdam (protocol no. MEC-2006-190). The tissue was collected in minimum essential medium-α (MEM- α) (Gibco-BRL, Paisley, UK) supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml) (1% P/S; Gibco-BRL) and 4 mM L-glutamine and stored at 4 °C for 3–16 h. MSCs were isolated as described previously [16]. Cultures were kept at 37 °C, 5% CO_2, and 95% humidity and refreshed twice weekly with MEM-α with 1% P/S, and 15% fetal bovine serum (FBS; Biowhittaker, Verviers, Belgium). At 90% confluence, adherent cells were removed from culture flasks by incubation in 0.05% trypsin–EDTA (Gibco-BRL) at 37 °C and cells were used for experiments described below or frozen at −150 °C until further use. MSCs were used for experiments between passages 2 and 5 and tested for their phenotypic markers and osteogenic and adipogenic potential as described before [17]. At least three different MSCs were used in each experiment.

Immunosuppressive drugs

Rabbit antithymocyte globulin (Genzyme, Cambridge, MA, USA) was dissolved in sterile water at 5 mg/ml. Rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA) was used as antibody control.

Rabbit IgG binding assay

Mesenchymal stem cells were trypsinized and washed and then divided into 100 000 cells/tube in polypropylene tubes. Graded doses of rATG were added to tubes (0, 0.5, 1, 5, 10, 50, and 100 μg/ml) or rabbit IgG (0, 0.5, 1, 5, 10, 50, and 100 μg/ml) and incubated for 30 min. MSCs were then washed and incubated with antibodies: sheep anti-rabbit IgG-PE (Serotec, Oxford, UK), CD13-PE-Cy7 (BD Biosciences, San Jose, CA, USA), CD105-FITC (BD Biosciences), CD90 APC (BD Biosciences), HLA-ABC-APC (BD Biosciences) and HLA-DR-FITC (BD Biosciences) for 30 min in the dark. Cells were then washed and analyzed by flow cytometry in a FACS Canto II (BD Biosciences). Some cells were preincubated for 24 h with 50 ng/ml IFNγ (U-Cytech, Utrecht, the Netherlands) and 20 ng/ml TNFα (PeproTech, London, UK) for 24 h and continued the same procedure described above. Three different MSC donors were used for this experiment.

Viability assessment by MTT assay

Mesenchymal stem cell were seeded in a 96-well plate at 5000 cell/well and let them adhere overnight. rATG (0.5, 1, 5, 10, 50, and 100 μg/ml) was added to the wells in standard MSC culture medium (MEM-α + 15% non-heat inactivated FBS) for 24, 48, and 72 h. The MTT assay was used to determine viable cell numbers. Briefly, medium was replaced with 100 μl of fresh medium before the assay, and 20 μl of 5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) in PBS was added and incubated for 4 h at 37 °C. Dimethyl sulfoxide (DMSO) (100 μl) was then added and absorbance read at 595 nm using a Victor^2 1420 multilabel counter (Perkin Elmer, Waltham, MA, USA). Six different MSC donors were used for this experiment.

Isolation of peripheral blood mononuclear cells

Peripheral blood samples were collected from healthy volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, using Ficoll Isopaque (δ = 1.077; Amersham, Uppsala, Sweden), and frozen at −150 °C until use.

Flow cytometric analysis of proliferation

Peripheral blood mononuclear cells from healthy donors were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; 1 μM). 1.25×10^5 PBMCs-CFSE labeled were stimulated with anti-CD3/anti-CD28 antibodies (1 μl/10^6 PBMCs; BD Biosciences) in a 24-well plate containing 0.25×10^5 MSC per well in RPMI medium with 1% P/S and 10% heat-inactivated human serum. MSCs were seeded into wells and let to adhere for 4–5 h in RPMI medium with 1% P/S and 10% heat-inactivated human serum. After MSC adhesion rATG (0.5, 1, 5, 10, 50, and 100 μg/ml) or

rabbit IgG (10 μg/ml) was added to wells and immediately after PBMC in a ratio of 5:1 (PBMC:MSC). PBMCs were recovered after 96 h and stained for CD3-Amcyan, CD4-APC, CD8-PE-Cy7, CD20-PerCP, and CD25-PE and proliferation was analyzed by dilution of the CFSE staining on the different subsets on a FACS Canto II flow cytometer (BD Biosciences). Three different MSC donors were used for this experiment.

Cell purification by fluorescence-activated cell sorting of effector cells for cell-mediated lysis (CML)

Peripheral blood mononuclear cells were cultured with IL-2 (200 IU/ml) and IL-15 (10 ng/ml) (PeproTech, Rocky Hill, NJ, USA) for 7 days. They were then washed twice with phosphate-buffered saline (PBS) with 1% heat-inactivated FBS and stained with antibodies against CD3-AmCyan, CD16-phycoerythrin (CD16-PE), CD56-allophycocyanin (CD56-APC), CD4-fluorescein isothiocyanate (CD4-FITC), CD8-PE-cyanine (CD8-PE-Cy7), and 7-amino-actinomycin D (Via-Probe) (all from BD Biosciences) at room temperature and protected from light for 30 min. Cells were sorted with FACS Aria-II with FACSDiva software (BD Biosciences). Viable lymphocytes were gated for CD3. The $CD3^-$ cells were gated for $CD16^+$ $CD56^+$ to obtain NK cells, and $CD3^+$ cells were gated for $CD16^+$ $CD56^+$ to obtain NKT cells and for $CD8^+$ $CD16^-$ $CD56^-$ to obtain CD8 cells. Purified NK, NKT and CD8 cells were cultured for another 24 h with IL-2 (200 IU/ml) and IL-15 (10 ng/ml) and then used in the lysis assay as effector cells.

CML assay

Cytotoxicity-mediated lysis of MSCs was determined by europium release assay as described previously [18]. In brief, target cells (MSCs preincubated for 24 h with 50 μg/ml of rATG) were labeled with europium–diethylenetriaminepentaacetate (DTPA) (Sigma-Aldrich). Effector cells were incubated with 5000 target cells at effector-to-target (*E*:*T*) ratios of 40:1 to 0.3:1 in round-bottom 96-well plates (Nunc, Roskilde, Denmark) for 4 h at 37 °C in RPMI medium with 1% P/S and 10% heat-inactivated human serum. In this experiment, we used heat-inactivated serum to avoid the effect of complement on the cytolitic cells. The plates were then centrifuged and 20 μl of the supernatant was transferred to 96-well plates with low background fluorescence [fluoroimmunoplates (FluoroNunc plates); Nunc]. Subsequently, 100 μl of enhancement solution (PerkinElmer, Groningen, the Netherlands) was added to each well. Released europium fluorescence was measured using a fluorometer Victor^2 1420 multilabel counter (Wallac).

Maximal release of europium by target cells was measured by incubation of 5000 labeled target cells with 1% Triton (Sigma-Aldrich, Zwijndrecht, the Netherlands) for 4 h. Spontaneous release of europium was measured by incubation of labeled target cells without effector cells for 4 h. Percentage leakage was then calculated as: (spontaneous release/maximal release) × 100%. The average europium leakage of MSCs was 19.1 ± 2.0%. When MSCs were exposed to rATG, the spontaneous leakage was 21.2 ± 2.0%. The percentage cytotoxicity-mediated lysis was calculated as follows: percent lysis = (measured lysis − spontaneous release)/(maximal release − spontaneous release) × 100%. For the NK, NKT and CD8 T-CML experiments, we used a combination of three different PBMCs and six different MSC donors. For the CD4 T-CML, a combination of two different PBMCs and four different MSC donors was used.

Statistical analysis

Data were analyzed using two-way ANOVA for repeated measures using Graphpad Prism software (Graphpad software, La Jolla, CA, USA). Parametric data are expressed as mean ± standard error of the mean (SEM). Statistical significance was defined as $P < 0.05$ (two-tailed).

Results

rATG binds to MSCs in a dose-dependent manner

Mesenchymal stem cells were incubated with 0, 0.5, 1, 5, 10, 50, and 100 μg/ml of rATG for 30 min and binding of rabbit polyclonal antibodies on the MSC surface was assessed by flow cytometry. Rabbit IgG was used as a negative control. rATG dose-dependently bound to MSCs (Fig. 1a and b) while rabbit IgG control did not bind to MSC at any concentration (in Fig. 1a depicted representative 10 μg/ml and Fig. 1b shows the range 0.5–100 μg/ml). Under inflammatory conditions (e.g. exposure to IFNγ and TNFα), the immunosuppressive potential of MSCs is increased [19] and the surface molecule phenotype is changed, so is important to consider a different rATG binding in this condition. Percentage of positive cells and the MFI of rabbit Ig binding were not significantly increased by the preincubation of the MSCs with IFNγ and TNFα (Fig. 1b). We also observed that there was no unspecific binding of rabbit IgG at any of the concentrations tested (0.5–100 μg/ml) (Fig. 1b). The incubation time (30 min or 24 h) had no influence on the binding of rATG on MSCs (data not shown). Typical MSC markers (Fig. 1c) and HLA class I and II (Fig. 1d) were not affected by the incubation with rATG.

Effect of rATG on the viability of MSC

Viability of MSCs exposed for 24, 48, and 72 h to increasing doses of rATG or to 10 μg/ml of rabbit IgG control was

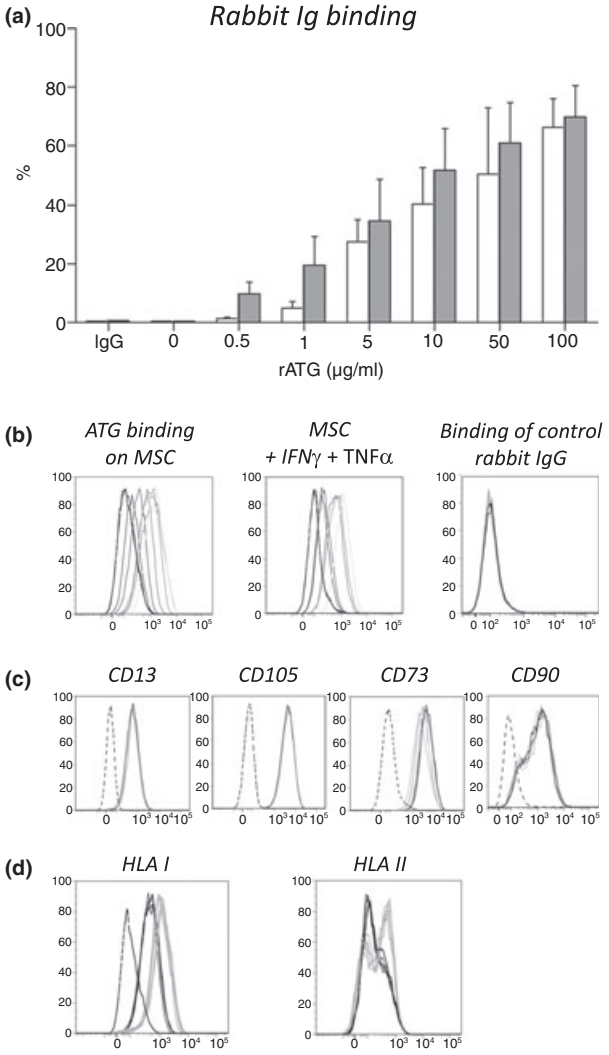

Figure 1 Rabbit antithymocyte globulin (rATG) binds to in a dose-dependent manner. (a) Mesenchymal stem cells (MSCs) were incubated with graded doses of rATG for 30 min, washed and then incubated with PE-labeled sheep antirabbit IgG. The percentage of rATG positive cells increased dose-dependently (white bars) and was not significantly increased by the pretreatment of MSCs with Inflammatory cytokines IFNγ and TNFα (gray bars) $n = 3$. (b) Histograms show this effect and the absence of unspecific of rabbit IgG control at any concentration tested (0.5–100 μg/ml). (c) Typical MSC markers were not affected by rATG binding (black to gray line represents lower to higher rATG concentration). Dashed line is unstained control. (d) The expression of HLA I and II is up-regulated on MSCs upon incubation with inflammatory cytokines (gray lines). However, incubation with different concentrations of rATG did not affect the expression. Dashed line represents unstained control.

tested by MTT assay. In this assay, the culture wells contain only MSC with medium supplemented with nondecomplemented serum and rATG or IgG control. Incubation for 24 h with rATG had no effect on the viability of MSCs.

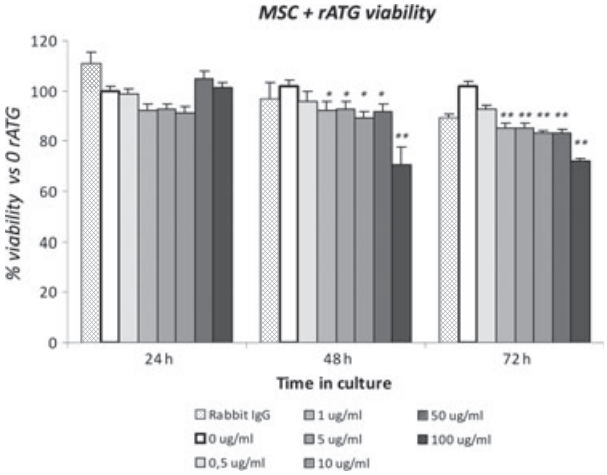

Figure 2 Rabbit antithymocyte globulin (rATG) reduces viability of Mesenchymal stem cells (MSCs). At 48 and 72 h after incubation of MSCs with rATG the viability was reduced as measured by MTT assay. 10 μg/ml of rabbit IgG was used as a control. Data is presented as mean values with SEM. $*P \leq 0.05$, $**P \leq 0.01$ vs. 0 μg/ml ATG ($n = 6$).

After 48 h, MSCs cultured with rATG doses from 1 to 100 μg/ml showed reduced viability that was even lower after 72 h of incubation. At this latest time-point, viability was significantly reduced even at very low doses of rATG (0.5 μg/ml) (Fig. 2).

Lower antiproliferative efficacy of MSCs in the presence of rATG

To test the effect of rATG on the antiproliferative effect of MSC, PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies and cultured with MSCs (ratio MSC: PBMC 1:5) in the presence of ranging doses of rATG for 96 h. MSCs inhibited the proliferation (as measured by CFSE dilution) of stimulated PBMCs by 77.9 ± 13% (mean ± SEM). rATG reduced the immunosuppressive effect of MSCs on both CD4 and CD8 populations in a dose-dependent manner (Fig. 3a and b). The presence of rATG in the co-culture promoted CD25 expression especially within the CD4 population (Fig. 3c and d).

rATG increases the NKT cell and $CD8^+$ CML of MSCs

It has been previously demonstrated that activated NK cells are capable to lyse allogeneic MSCs [20]. We analyzed the effect of rATG binding to MSCs on the CML by cell sorted $CD3^-$ $CD16^+$ $CD56^+$ (NK cells). There was no difference on the lysis performed by activated NK cells whether MSCs were preincubated with rATG or not (Fig. 4a). However, within the nonNK cells sorted fraction, we observed a rATG-dependent lysis of MSCs (Fig. 4b). To further

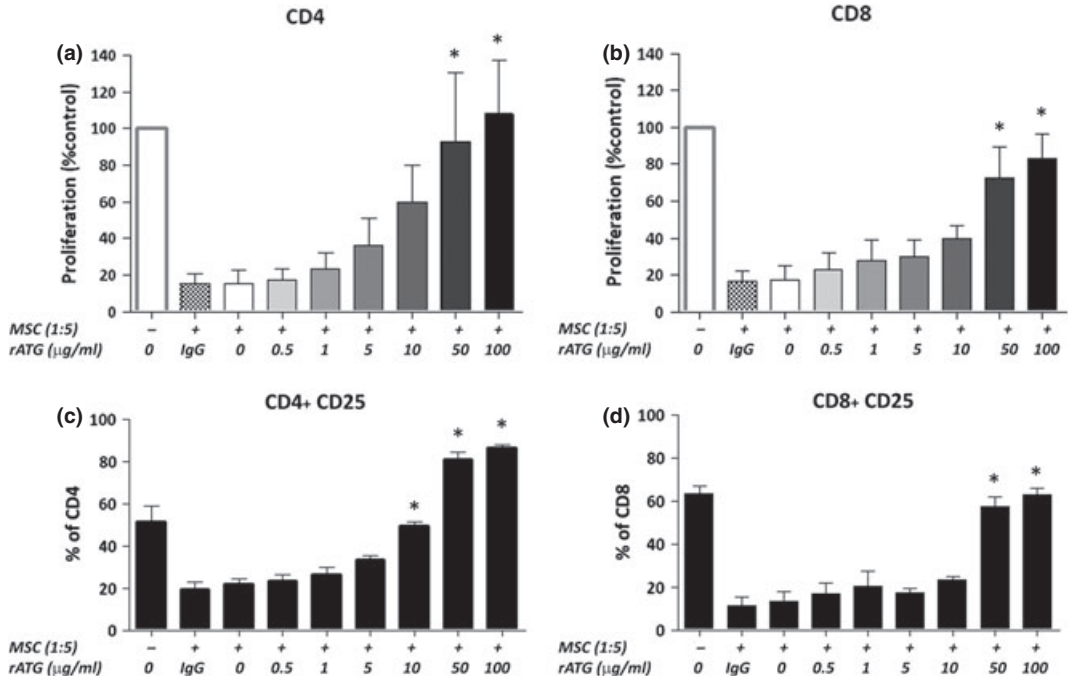

Figure 3 Mesenchymal stem cells (MSCs) have lower immunossupressive capacity in the presence of rabbit antithymocyte globulin (rATG). MSC were incubated with anti-CD3/anti-CD28 activated peripheral blood mononuclear cells at a ratio of 1:5 in the presence of graded doses of rATG and proliferation checked by CFSE dilution. 10 μg/ml of rabbit IgG was used as a control. (a) At this ratio, MSCs can suppress the proliferation of CD4 T cells and (b) CD8 T cells up to 80% versus no MSCs and 0 μg/ml rATG. When those cells were co-incubated with rATG, this suppressive effect was gradually lost. (c) The percentage of $CD4^+$ $CD25^+$ cells and (d) $CD8^+$ $CD25^+$ cells was increased by the presence of ranging doses of rATG. Proliferation was checked at 96 h of co-culture. Results are mean percentages of three experiments by duplicate with SEM. $*P < 0.05$ vs. 0 μg/ml ATG, $**P < 0.01$ vs. 0 μg/ml ATG, $***P < 0.001$ vs. 0 μg/ml ATG.

analyze which population was preferentially lysing rATG treated MSCs, we sorted the NK depleted fraction into $CD3^+$ $CD16^+$ $CD56^+$ (NKT cells), $CD3^+$ $CD4^+$ (CD4 T cells) and $CD3^+$ $CD16^-$ $CD56^-$ $CD8^+$ (CD8 T cells). Preincubation of MSCs with rATG increased their susceptibility to be lysed by NKT cells and CD8 T cells while CD4 T cells showed negligible lysis capacity (Fig. 4c).

Discussion

Our data reveal a dose-dependent binding of rATG on MSCs that is translated in a reduction of their viability and their conversion into targets for NKT and CD8 cell-mediated lysis. The presence of rATG reduces the antiproliferative ability of MSCs on activated CD4 and CD8 cells.

The deleterious effects of rATG on MSCs are relevant for potential application in clinical transplantation (solid organ and bone marrow). MSCs have shown very potent and long-lasting immunosuppressive effects in preclinical models of transplantation [21,22], however, their translation in the clinical setting will be accompanied by pharmacological co-medication for ethical/safety reasons. The effect of calcineurin inhibitors (Tacrolimus, Cyclosporine), mTOR inhibitors (Rapamycin), and cell cycle inhibitors MPA on MSCs has been studied acknowledging a synergistic effect of MPA and MSC and an antagonistic effect of Cyclosporine, Tacrolimus, and Rapamycin on MSC immunosuppressive capacity [9,11]. However, nothing was known of the effect of rATG on MSC abilities.

In the first pilot study of MSC treatment on two kidney transplant patients, [6] no short term beneficial effect was observed. Those patients received basiliximab and low-dose rATG induction therapy and MSC injection 7 days after transplantation. After the results were obtained in our study, we speculate that this might be at least partially because of the deleterious effect of rATG on MSCs. New trials suggest that MSC could substitute the use of induction therapy in kidney transplantation, as MSC proved as efficient as Basiliximab in reducing acute rejection rates in these patients [8]. This experience together with our results would suggest that the use of MSC without rATG brings positive results in the outcome of kidney transplantation.

We must take into account that these trials were performed using autologous bone marrow MSC and our experiments are done with allogeneic adipose tissue MSC. However, adipose tissue derived MSC have proven to

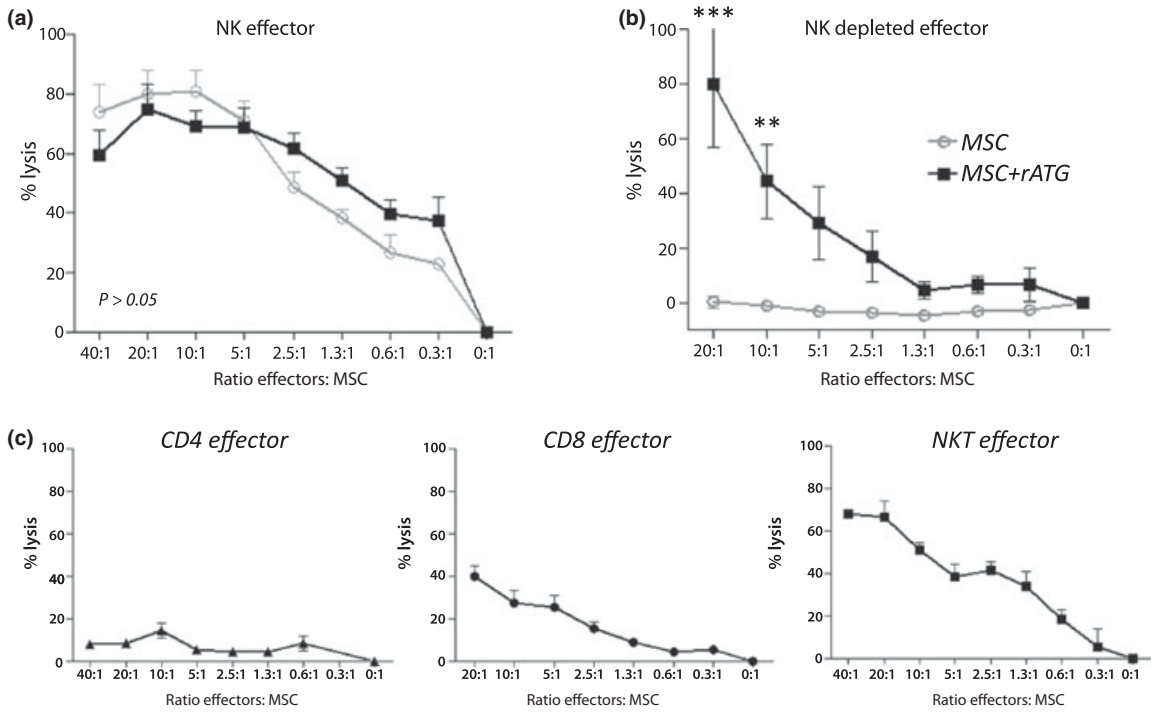

Figure 4 Rabbit antithymocyte globulin (rATG) increases NKT and CD8 T-cell-mediated lysis (CML) of mesenchymal stem cells (MSCs). (a) Sorted NK cells ($CD3^-$ $CD16^+$ $CD56^+$) were incubated for 4 h with europium-labeled MSCs. NK cells are able to lyse MSCs (gray line) and preincubation of MSCs with rATG (50 μg/ml) had no effect on NK CML (black line), $n = 6$. (b) The same experiment was performed with the NK cell depleted fraction and demonstrated that this mixed cell fraction (peripheral blood mononuclear cells depleted from $CD3^-$ $CD16^+$ $CD56^+$) was able to specifically lyse rATG preincubated MSCs (black line). (c) Further separation of the NK depleted fraction in NKT ($CD3^+$ $CD16^+$ $CD56^+$, $n = 6$), CD8 cells ($CD3^+$ $CD8^+$ $CD16^-$ $CD56^-$, $n = 4$) and CD4 ($CD3^+$ $CD4^+$) fractions revealed that NKT cells and CD8 cells were responsible for the lysis of rATG preincubated MSCs. $^{**}P < 0.01$ versus no ATG, $^{***}P < 0.001$ versus no ATG in the NK depleted fraction.

possess similar or even more potent immunomodulatory capacity *in vitro* [23] and *in vivo* [24,25] than bone marrow derived MSCs [26].

The half-life of rATG is 3–8 days [27], with an average maximal total Thymoglobulin level ranging from 171 μg/ml in patients receiving 14 doses (1.5 mg/kg/dose) to 66 μg/ml for patients receiving 6 doses (1.5 mg/kg/dose). A gradual decline in Thymoglobulin was observed following discontinuation of treatment but by day 90, 81% of the patients have still detectable levels of Thymoglobulin [13]. Therefore, care should be taken in the therapeutic use of rATG and MSCs in transplantation.

Mesenchymal stem cells have proven antiproliferative properties on activated lymphocytes [17]. This effect is one of the main attractive properties for the use of MSC as a therapeutic tool in transplantation. In our setting, MSCs show antiproliferative properties as well as inhibition of activation of CD4 and CD8 T cells. The presence of rATG reduces the immunosuppressive effect of MSC dose-dependently. This effect can be caused by the impairment of the viability of the MSCs or by the effect that rATG enhances proliferation of $CD25^+$ T cells [28].

One important point to take into account is the additive effect of rATG and MSCs to increase the proportion of $CD25^+$ T cells. The potential of rATG to generate induced regulatory T cells has been thoroughly studied [15,29] and recently the same effect has been described for MSCs [30]. However, when both therapeutic agents are present there is an induction of activated T cells rather than regulatory T cells, as observed by the reduced immunosuppressive capacity of MSC. Moreover, i*n vivo*, the increased numbers of regulatory T cells observed after rATG treatment have been shown to be because of thymic emigration and homeostatic proliferation in lymphodepleted patients [31].

The viability of MSCs after rATG incubation is an important issue as well. rATG has a direct effect on MSCs viability and converts them in targets for CD8 and NKT cells. The expansion of cytokine-induced-killer cells by rATG has been reported [32], and based on our results, rATG appears to label the cells thereby converting them in targets for cytotoxic T cells.

In conclusion, our results suggest a deleterious effect of rATG on the survival and the immunosuppressive ability of MSCs. This effect should be taken into account when

designing new clinical trials with MSC therapy in transplantation.

Authorship

MF: wrote the manuscript, participated in the research design and in the performance of the research cited in this manuscript. CCB: participated in the data analysis and the writing of the manuscript and in its critical appraisal. SSK: participated in the research cited in this manuscript. AUE: participated in the data analysis of the manuscript. MRvR: participated in the data analysis of the manuscript. WW: participated in the writing of the manuscript and in its critical appraisal. MGH: participated in the writing of the manuscript and in its critical appraisal. JMG: participated in the research design, the writing of the manuscript and in its critical appraisal. MJH: participated in the data analysis, the writing of the manuscript and in its critical appraisal.

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