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ORIGINAL ARTICLE

Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation

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Conflicts of interest
There is no conflict of interest for any of the authors of the manuscript.

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Summary

Bone marrow-derived mesenchymal stromal cells (MSC) have emerged as useful cell population for immunomodulation therapy in transplantation. Moving this concept towards clinical application, however, should be critically assessed by a tailor-made step-wise approach. Here, we report results of the second step of the multistep MSC-based clinical protocol in kidney transplantation. We examined in two living-related kidney transplant recipients whether: (i) pre-transplant (DAY-1) infusion of autologous MSC protected from the development of acute graft dysfunction previously reported in patients given MSC post-transplant, (ii) avoiding basiliximab in the induction regimen improved the MSC-induced Treg expansion previously reported with therapy including this anti-CD25-antibody. In patient 3, MSC treatment was uneventful and graft function remained normal during 1 year follow-up. In patient 4, acute cellular rejection occurred 2 weeks post-transplant. Both patients had excellent graft function at the last observation. Circulating memory $CD8^+$ T cells and donor-specific $CD8^+$ T-cell cytolytic response were reduced in MSC-treated patients, not in transplant controls not given MSC. $CD4^+FoxP3^+$Treg expansion was comparable in MSC-treated patients with or without basiliximab induction. Thus, pre-transplant MSC no longer negatively affect kidney graft at least to the point of impairing graft function, and maintained MSC-immunomodulatory properties. Induction therapy without basiliximab does not offer any advantage on $CD4^+FoxP3^+$Treg expansion (ClinicalTrials.gov number: NCT 00752479).

Introduction

Notwithstanding promising preclinical [1–11] and early clinical [12] results with bone marrow-derived mesenchymal stromal cells (MSC), moving the concept of MSC-based therapy forward towards clinical application in solid organ transplantation should be critically assessed. There are few protocols of MSC-based therapy in organ transplantation [13–18]. Our clinical MSC protocol in renal transplant recipients was conceived as a tailor-made step-wise approach every few patients to look for the unexpected and ultimately identify a definite protocol that allows to create favourable conditions for tolerance while avoiding unwanted effects. This strategy has been adopted because MSC-therapy in organ transplantation is a very innovative potentially useful approach to transplant tolerance, but still

in its infancy. Indeed, while initial results appear promising, there remain many open questions as to how these cells have to be administered and how they may function to modulate host immune response *in vivo* in clinical transplant setting. Along this line, our protocol was focussed to characterize the safety and tolerability of peri-transplant MSC infusion and define the biological/mechanistic effects of this cell therapy. Similar to the approach to the pathophysiology of a rare condition in few patients that may contribute to the understanding of other more common disorders, intensively studying few transplant patients given MSC would possibly enlighten the path to elucidate safety issues and mechanistic immunomodulatory pathways rather than jumping altogether on large trials before fundamental questions have been addressed. Admittedly, this reflects the opinion of the authors. Still we believe that playing with cells and potent biological agents for which there is uncertainty about safety and efficacy and that may have unexpected side effects justify cautiousness. Thus, we initially started with two living-related donor kidney recipients who were given *ex vivo* expanded, autologous, bone marrow-derived MSC at day 7 post-transplant, after induction therapy with basiliximab/low-dose thymoglobulin [15]. MSC infusion did promote on long-term a pro-tolerogenic environment characterized by lower memory/effector $CD8^+$ T cells, expansion of $CD4^+$ Tregs and reduction in donor-specific $CD8^+$ T-cell cytotoxicity, compared with control kidney transplant recipients given the same induction therapy but not MSC. However, few days after cell infusion, both MSC-treated patients developed acute renal insufficiency. Histological and immunohistochemical analysis of graft infiltrating cells did exclude an acute cellular or humoral rejection, but intragraft recruitment of neutrophils together with MSC, as well as complement-C3 deposition were observed [15]. It was hypothesized that the subclinical inflammatory environment of the graft in the few days of postsurgery could have favoured the prevalent intragraft recruitment and activation of the infused MSC promoting a pro-inflammatory milieu with eventual acute renal dysfunction (engraftment syndrome), as reported by others with combined kidney and bone marrow transplantation [19]. Therefore, to gain insight into the clinical observation in these two patients given MSC post-transplantation, we moved back to a clinically relevant murine kidney transplant model, and found that a single administration of cells before (DAY-1) but not after renal transplantation avoided the acute deterioration of graft function, while maintaining the immunomodulatory effects associated with MSC treatment**,** including a marked Treg expansion [8].

These experimental findings did represent a gain of knowledge to further implement our clinical protocol with the aim to create favourable conditions for MSC-promoting immunomodulation and Treg expansion, avoiding the unwanted effect of acute deterioration of graft function associated with the prevalent intragraft localization of MSC when given at day 7 post-transplantation.

Moreover, our first two MSC-treated transplant recipients were given induction therapy which included the anti-IL-2-receptor (CD25) monoclonal antibody basiliximab [15]. Recent evidence in kidney transplant patients showed that basiliximab may cause a transient loss of $CD25^+FoxP3^+$Treg cells in the circulation [20]. Together these findings led us to eliminate basiliximab from the induction regimen used in previous step 1 with the aim to possibly maximize the expansion of $CD25^+FoxP3^+$Treg cells.

Therefore, in this study (step 2) in two additional living-related kidney transplant recipients, we sought to: (i) look for unwanted and unexpected events when autologous bone marrow-derived MSC are administered at DAY-1 pretransplantation, (ii) evaluate the induction therapy that would maximize the MSC-induced Treg expansion and immunomodulation in the setting of pretransplant cell infusion, (iii) get insights on the mechanisms underlying the promotion *in vivo* of a pro-tolerogenic environment, if any, by MSC-based therapy.

Patients and methods

Patients

A 37-year-old man (patient 3) on peritoneal dialysis caused by end-stage renal disease (ESRD) secondary to IgA nephropathy received a renal transplant from his father, mismatched for two HLA haplotypes (one mismatch on HLA-A and one on HLA-DR) (Table 1).

Table 1. Baseline patients' characteristics.

	Patient 3	Patient 4	Control group 1* RATG alone	Control group 2* Bas/RATG
Age	37	34	56 ± 9	42 ± 15
Gender (M/F)	M	M	5/1	4/2
HLA mismatches median (range)	2	3	4 (3–5)	2 (0–4)
Cross-match	Negative	Negative	Negative	Negative
Anti-donor HLA Abs	Negative	Negative	Negative	Negative

*Control group 1: kidney transplant recipients given induction therapy with RATG alone and not given MSC ($n = 6$). Control group 2: kidney transplant recipients given Bas/RATG induction therapy but not MSC ($n = 6$).

Data are mean ± SD. HLA mismatches range was 0–3 for living-donors ($n = 3$) and 3–4 for deceased donor ($n = 3$).

RATG, rabbit anti-thymocyte globulin; Bas, basiliximab.

A consecutive 34-year-old man (patient 4) on ESRD secondary to medullary sponge disease received a preemptive renal transplant from his mother, mismatched for three HLA haplotypes (one on HLA-A, HLA-B and HLA-DR, respectively) (Table 1). Although negative for anti-donor HLA-antibodies, he was positive for nondonor-specific anti-HLA DR4 antibodies.

Four to six months before transplantation both of them underwent right posterior superior iliac crest aspiration under local anaesthesia. MSC were isolated and *ex vivo* expanded according to Good-Manufacturing-Practice procedures (Cell Therapy Laboratory "G Lanzani", Ospedali Riuniti di Bergamo, authorization no. aM-189/2008 Agenzia Italiana del Farmaco, AIFA [21,22]). The day before kidney transplantation (DAY-1), autologous MSC were administered intravenously (2.0×10^6 cells/kg body weight) after premedication with chlorphenamine and hydrocortisone. Immunophenotyping of peripheral blood T-cell subsets and monitoring of T-lymphocyte function were performed before and up to day 360 and 180 post-transplant, in patients 3 and 4, respectively. Written informed consent was obtained from recipients and living donors. All treatment protocols were approved by the Istituto Superiore di Sanità (ISS, Rome, Italy, authorization no. 45253(06)-PRE.21-882) and by the Institutional Review Board of the Ospedali Riuniti di Bergamo (authorization no. 352, March 18, 2008).

The patients received induction therapy with low-dose rabbit anti-thymocyte globulin (RATG) infusion (thymoglobulin, 0.5 mg/kg daily starting immediately pretransplantation up to day 6 post-transplant). Maintenance immunosuppression was with cyclosporine A (CsA, target trough blood levels of 300–400 ng/ml up to day 7 postsurgery, and 100–150 ng/ml at month 5 post-transplantation), mycophenolate mofetil (plasma trough mycophenolic acid levels [23] of 0.5–1.5 μg/ml), and steroids. Five hundred milligrams of methylprednisolone were administered before the first RATG infusion and continued for 2 more days post-transplantation (250 and 125 mg, respectively). Thereafter, oral prednisone (75 mg) was administered, which was progressively tapered and discontinued after day 7 postsurgery. As controls, historical kidney transplant recipients with a deceased donor ($n = 6$, Table 1) given induction therapy with low-dose RATG and the same maintenance immunosuppression were also considered. From these patients, PBMC samples taken before and at days 180 and 360 post-transplant were available. Donor cells for functional studies were, however, available only from one patient. Thus, as additional controls, six patients receiving a living-related ($n = 3$) or deceased kidney ($n = 3$) with comparable HLA mismatches (Table 1), but not MSC from whom donor cells were available were studied. They were given basiliximab and low-dose RATG and the same maintenance immunosuppressive regimen of MSC-treated patients.

This induction therapy has been introduced in our clinical practice in kidney transplantation since 2005 to minimize side effects associated with the standard dose of RATG [24]. Moreover, to avoid the risk of insufficient anti-rejection activity, we integrated this regimen of very low dose of RATG (approximately half the currently recommended doses for induction and one-third to one-fourth of doses administered in the large majority of previous reports [25–27]) with basiliximab (20 mg day i.v. at day 0 and day 4 post-transplantation) with the rationale of inhibiting those lymphocytes eventually surviving low-dose RATG exposure. The dual induction regimen allows to achieve rapid and effective lymphocyte depletion while simultaneously allowing safe minimization of maintenance immunosuppression, especially when low dose RATG is started before patient referral to the surgical room [28]. With this perioperative minimal induction therapy, the rate of acute graft rejection was very low (4%) [28]. This is in line with recent findings with a similar regimen of dual antibody induction therapy with ATG and daclizumab [29].

MSC isolation and expansion

Mesenchymal stromal cells were processed and cultured as previously reported [21,22]. Cells were classified as MSC based on their ability to differentiate into bone, fat and cartilage, and by flow cytometric analysis (positive for CD44, CD29, CD73, HLA-ABC, CD90, and CD105, but negative for CD14, CD34, CD45 and HLA-DR) responding to defined criteria for MSC stated by the International Society of Cell Therapy [30]. The final product was characterized with respect to viability, purity and therapeutic potential. Detailed methods for MSC isolation and expansion and characterization are given in Data S1.

Immunophenotyping of peripheral blood lymphocytes

Blood mononuclear cells were stained with fluorochrome conjugated monoclonal antibodies against CD3, CD4, CD8, CD45RO, CD45RA, CD25 (clone MA251, which binds to a CD25 epitope different from that recognized by basiliximab), CD127 and FoxP3. Multicolour flow cytometry was used to identify T-cell subsets with standard techniques and equipment (FACSAria – BD Bioscience) [31].

Ex vivo functional immunological assays

Transplant patients were monitored before and every 6 months post-transplantation for alloimmune response

against donor and third-party antigens by cell-mediated lympholysis [15].

Histology and immunohistochemistry

Detailed methods for histological and immunohistochemical analysis are given in Data S1.

Statistical analyses

Variations in peripheral blood $CD4^+$ and $CD8^+$ T-cell counts, percentage and counts of T-cell subpopulations and $CD8^+$ T-cell-mediated lysis from control kidney transplant recipients not given MSC as well as variation in peripheral T-cell counts and percentages between the two control groups were assessed by ANOVA. The statistical significance level was defined as $P < 0.05$.

Results

Clinical course

In patient 3, pretransplant infusion of MSC was uneventful. After kidney transplantation, renal function rapidly improved and normalized within 3 days (Fig. 1a). Thereafter, the graft function remained stable up to day 360 post-transplantation. At this time point, a 'protocol' biopsy showed no signs of acute rejection (Fig. 1b). The patient is in good health with stable graft function at the last available evaluation (day 540 post-Tx: serum creatinine 1.0 mg/dl; proteinuria 0.14 g/24 h).

Patient 4 also received pretransplant MSC infusion with no side effects. Renal function rapidly improved and normalized on day 3 post-transplant (serum creatinine 1.3 mg/dl). From day 14 onwards, a rapid progressive increase in serum creatinine was observed up to 2.3 mg/dl (Fig. 1c). Renal ultrasound showed normal structure and resistivity index (IR: 0.58). CsA trough levels were in the therapeutic range. A moderate increase in temperature (38 °C) was documented. Thoracic X-ray, viral blood tests and urine culture were negative. On day 17 post-transplant, a kidney biopsy was performed which showed moderate–severe acute cellular rejection (Fig. 1d). Intravenous pulses of methylprednisolone were started. After tapering, the corticosteroid was maintained at the daily dose of 8 mg. Renal function progressively improved and serum creatinine returned to normal value within 10 days (serum creatinine 1.3 mg/dl). At 12 months post-transplant, the patient was in good health with stable graft function (serum creatinine 1.25 mg/dl; proteinuria 0.11 g/24 h).

In the control groups not receiving MSC, at 180 post-transplantation serum creatinine levels were 1.58 ± 0.28 and 1.47 ± 0.40 mg/dl and proteinuria values were 0.27 ± 0.12 and 0.26 ± 0.16 g/24 h in patients given low-dose RATG or the combination of Bas/low-dose RATG, respectively. At 360 post-transplantation, serum creatinine levels were 1.45 ± 0.31 and 1.54 ± 0.34 mg/dl and proteinuria values were 0.26 ± 0.17 and 0.18 ± 0.19 g/24 h, respectively. No acute rejection episodes occurred in these control patients during the 1-year follow-up.

Histology and immunohistochemistry

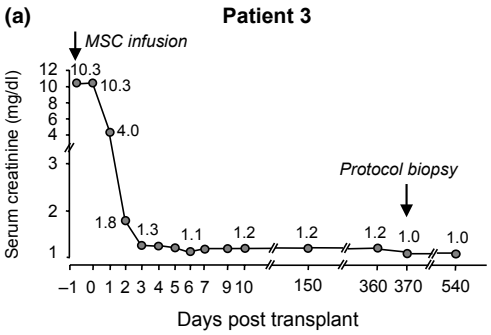
The 1-year protocol biopsy from patient 3 showed very mild signs of CsA chronic nephrotoxicity including interstitial fibrosis, thickness of vessel wall as well as focal atrophic lesions of the tubular epithelium (Fig. 1b). Graft infiltrating CD44/CD105-double positive cells, considered as *bona-fide* MSC [15] were negligible (Fig. 1e). Early post-transplant biopsy of patient 4 showed moderate-interstitial fibroedema and severe lymphocyte infiltrate of perivascular interstitium and tubular epithelium, consistent with acute cellular rejection (Fig. 1d). Staining for C4d was negative. Intragraft $CD4^+$, $CD8^+$ T cells and $CD20^+$ B cells were lower than in a control group of patients with acute cellular rejection, but higher than in transplant recipients without graft rejection (Fig. 1f). The number of granulocytes in the graft was negligible and comparable to control graft biopsies with acute cellular rejection (Fig. 1f). MSC were negligible in the graft of patient 4 (Fig. 1e).

Immunophenotyping of peripheral blood lymphocytes

In patients 3 and 4, and in control kidney transplant recipients, RATG induced profound $CD4^+$ and $CD8^+$ T-cell depletion in the peripheral blood (Fig. 2).

In patient 3, percentage and counts of memory/effector $CD8^+CD45RO^+RA^-$ T cells markedly decreased within day 7 post-transplant and remained lower than pretransplant values thereafter (Fig. 3a and b). In patient 4, the percentage of $CD8^+CD45RO^+RA^-$ T cells was reduced at day 7 post-transplant as compared with pretransplant values, remained stable thereafter up to day 180 with the exception of a transient increase at day 14 and further decreased at day 360 (Fig. 3a). $CD8^+CD45RO^+RA^-$ T-cell counts during the whole post-transplant period were lower than pretransplant values (Fig. 3b). Conversely, in control patients given induction therapy with low-dose RATG alone, percentages and counts of $CD8^+CD45RO^+RA^-$ T cells significantly increased at days 180 and 360 post-transplant as compared with pretransplant values (Fig. 3a and b). In control patients given Bas/low-dose RATG, the percentages and counts of $CD8^+CD45RO^+RA^-$ T cells up to day 360 post-transplant was comparable with pretransplant values (Fig. 3a and b).

The percentage of $CD4^+CD25^{high}FoxP3^+CD127^-$ regulatory T cells (Treg) was mildly reduced in patient 3,



(e)	CD105⁺CD44⁺ MSCs (cells/3 mm²)
Pt 3	**4.4**
Ctr: Per-protocol biopsy	1.36
Ctr: Normal kidneys (n = 4)	1.5 ± 2.6
Pt 4	**5.5**
Pt. with acute rejection	0

(f)	CD4⁺ T cells	CD8⁺ T cells	CD20⁺ B cells	CD68⁺ macrophages	Granulocytes	C3 (semiquantitative score)
	(cells/field)					
Pt 3	**14.6**	**16.7**	**6.2**	**30.6**	**7.7**	
Ctrs: per-protocol biopsies (n = 3)	9.5 ± 5.6	14 ± 7	3 ± 2.4	25 ± 8	5.8 ± 4.2	
Pt 4	**45.0**	**28.2**	**8.6**	**37.3**	**8.6**	**0.6**
Pts with acute rejections (n = 3)	80 ± 35	103 ± 35	20 ± 16	58 ± 2	5.5 ± 1.2	0.2 ± 0.3

Figure 1 Post-transplant course of graft function and histologic and immunohistochemic analysis of kidney graft biopsies from patients given pretransplant MSC infusion. (a) Profile of serum creatinine levels during the 1 year follow-up and (b) a representative image of Gomori's trichrome staining on protocol kidney graft biopsy (original magnification 200×) of patient 3 are shown. (c) Profile of serum creatinine levels during the 6 months follow-up and (d) a representative image of H&E staining on kidney graft biopsy taken at day 17 post-transplant (original magnification 200×) of patient 4 are shown. Measured GFR was 62.5 ml/min/1.73 m^2 at day 540 post-transplant in patient 3 and 51.06 ml/min/1.73 m^2 at 6 months post-transplant in patient 4. Panel (e) reports intragraft CD105 and CD44 double positive cell counts in patients 3 and 4 and in sections of normal renal tissue from patients undergoing nephrectomy for renal carcinoma. The total number of double-positive cells counted in 3 mm^2 (corresponding to the area of about 30 high-power fields) is reported. Panel (f) reports counts of intragraft cell infiltrate and score of C3 complement deposition in patients 3 and 4. As controls, renal biopsies from patients with acute graft rejection (n = 3) within 15–100 days postoperatively, and patients (n = 3) undergoing per-protocol biopsy at 1 year post-transplant were analyzed in parallel (means ± SD). For both immunofluorescence and immunoperoxidase analyses the number of positive cells were counted in at least 20–30 high power fields. Complement deposition was scored for intensity (absent, faint, moderate, intense: 0–3) in at least 20–30 high power fields. MSC, mesenchymal stromal cells.

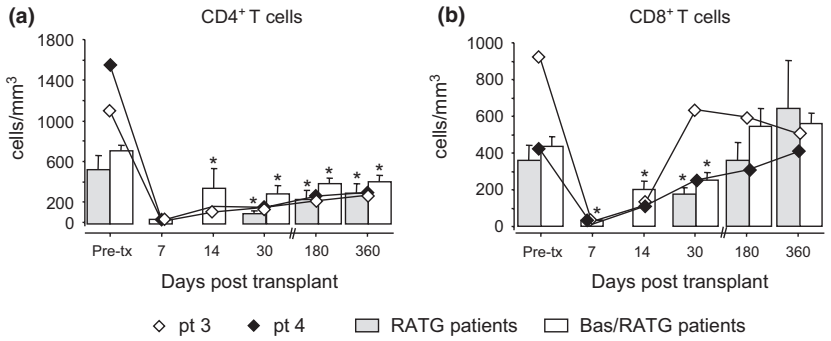


Figure 2 Profile of repopulating CD4+ and CD8+ T-cell counts. Absolute number of CD4+ (a) and CD8+ (b) T cells in peripheral blood of patient 3 (open diamonds), patient 3 (black diamonds) and control patients given RATG alone (grey histograms) or Bas/RATG (white histograms) not given MSC from baseline to 360 days post-transplant. Data are mean ± SE. *$P < 0.05$ versus pretransplant. At days 180 and 360 post-transplant, CD4+ T-cell counts remained lower than post-transplant values, whereas CD8+ T cells approached pretransplant values both in patients given MSC and in the control groups not receiving the cell therapy.

remained unchanged in patient 4 till 180 days post-transplant and decrease at day 360 as compared with pretransplant value during the respective observation period (Fig. 3c). In both patients, Treg counts in the post-transplant period were lower than pretransplant except a marked increase in patient 4 at day 14 (Fig. 3d). In control patients given low-dose RATG alone, the percentage of Tregs at days 180 and 360 post-transplantation was comparable with pre-transplant value (Fig. 3c), whereas Treg cell numbers were significantly reduced (Fig. 3d). In the additional control group given Bas/low-dose RATG, a transient decrease in the percentage and counts of Treg up to day 30 post-transplant was documented, with complete recovery to pretransplant value thereafter (Fig. 3c and d). Thus, the ratio of percentage of Treg/memory-effector $CD8^+$ T cells was higher in patient 3, but not in patient 4 given MSC than in control recipients (Fig. 3e), whereas higher ratio of cell number of Treg/memory-effector $CD8^+$ T cells was found in both patients (Fig. 3f).

Effect of current induction regimen without basiliximab on FoxP3+ T-cell profile

We compared the peripheral blood profile of $CD4^+CD25^{high}FoxP3^+CD127^-$ Treg in patients 3 and 4 given MSC and the induction therapy that avoids basiliximab with that in our previous MSC-treated patients 1 and 2 who received both basiliximab and low-dose RATG as induction regimen [15]. Patient 3 but not 4 showed an initial decline in $CD4^+CD25^{high}FoxP3^+CD127^-$ Treg, less marked then that found in patients 1 and 2 (Fig. 4a). However, in all patients the Treg count recovered to pretransplant values between days 30 and 180 after transplantation and remained unchanged thereafter.

As basiliximab has been shown to down-regulate the expression of CD25 on Treg *in vivo* in renal transplant patients [32], we also evaluated percentages of total FoxP3-expressing $CD4^+$ T cells in MSC-treated patients. Total FoxP3 expressing $CD4^+$ T cells underwent a significant expansion during the first 30 days post-transplant in all MSC patients but one (patient 1). At days 180 and 360, the level of $CD4^+Foxp3^+$ Treg was similar among MSC-treated and control patient group (Fig. 4b).

Ex vivo immunologic functional assay

In patient 3, the cytolytic function of $CD8^+$ T cells was completely abrogated in response to donor antigens and reduced against third-party antigens (Fig. 5). In patient 4, the $CD8^+$ T-cell-mediated lympholysis against donor and third-party cells was completely abrogated at day 180 post-transplant. At day 360, the anti-donor $CD8^+$ T-cell-mediated lympholysis still remained undetectable whereas anti-third party response recovered to pretransplant values. In the patient given induction therapy with low-dose RATG alone, the anti-donor and anti-third party cytolytic response at days 180 and 360 post-transplant were similar to pretransplant levels, with a marked increase in the anti-third party response at day 180 (Fig. 5). In the control group of patients given Bas/low-dose RATG, the $CD8^+$ T-cell cytolytic response toward donor antigens was transiently reduced at day 180 post-transplant as compared to pretransplant values and did not significantly change in response to third-party antigens (Fig. 5).

Discussion

The main purposes of the study were to: (i) establish whether DAY-1 pretransplant infusion of autologous bone marrow-derived MSC as compared to our previous protocol of MSC treatment at day 7 post-transplant in the context of kidney transplantation protects from cell-induced

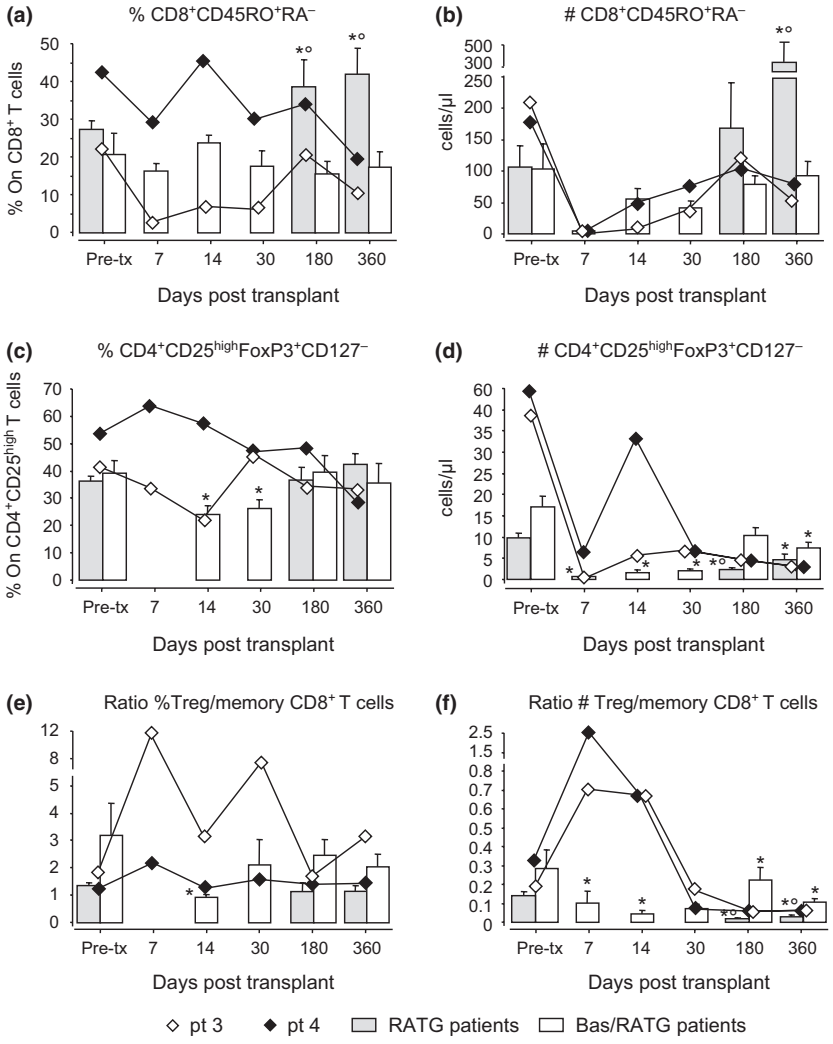


Figure 3 Profile of memory and regulatory T cells in the peripheral blood. Percentages (within total $CD8^+$ T cells) (a) and cell numbers (b) of memory $CD45RO^+RA^-$ T cells and percentages (within $CD4^+CD25^{high}$ T cells) (c) and cell number (d) of regulatory $FoxP3^+CD127^-$ cells from patient 3 (open diamonds) and 4 (black diamonds) and from control patients given RATG alone (grey histograms) or Bas/RATG (white histograms) not given MSC from baseline to 360 days post-transplant. Panels (e) and (f) represent ratios of either percentages or cell number of $CD4^+CD25^{high}$ $FoxP3^+CD127^-$ T cells/memory $CD45RO^+RA^-CD8^+$T cells from patient 3 (open diamonds) and 4 (black diamonds) and from control patients given RATG alone (grey histograms) or Bas/RATG (white histograms) not given MSC from baseline to 360 days post-transplant. Data are means ± SEM. $*P < 0.05$ versus pre-tx; $°P < 0.05$ versus Bas/RATG patients.

impairment of graft function and (ii) evaluate the effect on circulating Treg of the induction regimen without basiliximab as compared with our previous induction therapy including this anti-CD25 antibody in MSC-treated patients.

Pre-transplant MSC infusion protects from post-transplant cell-induced graft dysfunction

None of the two patients developed cell-mediated impairment of graft function after pretransplant MSC infusion. In the first patient (3) the cell treatment was uneventful and graft function remained normal during the 1 year follow-up post-transplantation. These findings translated to clinics a recent observation in a murine model of kidney transplantation that the time of MSC infusion in respect to the allograft dictates the possibility to develop early graft dysfunction as a consequence of preferential intragraft localization of infused cells [8]. Thus, in mice given MSC the day before kidney transplantation, the cells mainly localized into the spleen. None of the animals developed kidney graft dysfunction. At variance, post-transplant MSC infusion resulted in preferential homing of cells into the grafts, associated with graft dysfunction. This observation is consistent with previously published data of preferential MSC homing to the site of

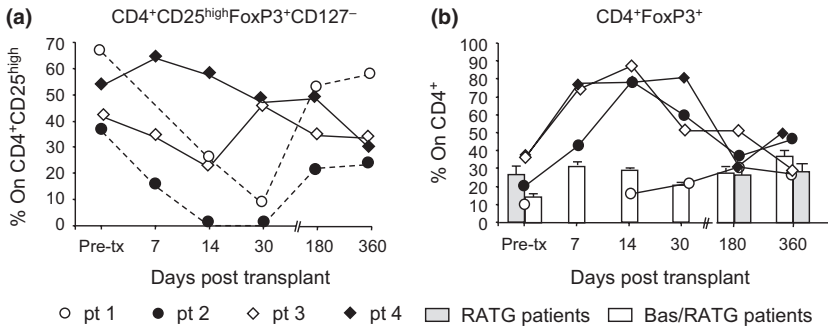


Figure 4 Profile of $CD4^+CD25^{high}Foxp3^+CD127^-$ Tregs and of total FoxP3-expressing $CD4^+$ T cells in the peripheral blood of MSC-treated patients. Percentage of regulatory $FoxP3^+CD127^-$ cells within $CD4^+CD25^{high}$ T cells (a) and of $FoxP3^+$ cells within $CD4^+$ T cells (b) from patient 3 (open diamonds) and patient 3 (black diamonds) given MSC and induction therapy with low-RATG alone compared with that of our previous MSC-treated kidney transplant patients 1 and 2 who received basiliximab/low-RATG as induction therapy. Grey and white histograms are percentages of $CD4^+Foxp3^+$ T cells from control patients given RATG alone or combined Bas/RATG, respectively. Follow-up is from baseline (pre-tx) to day 360 post-transplantation.

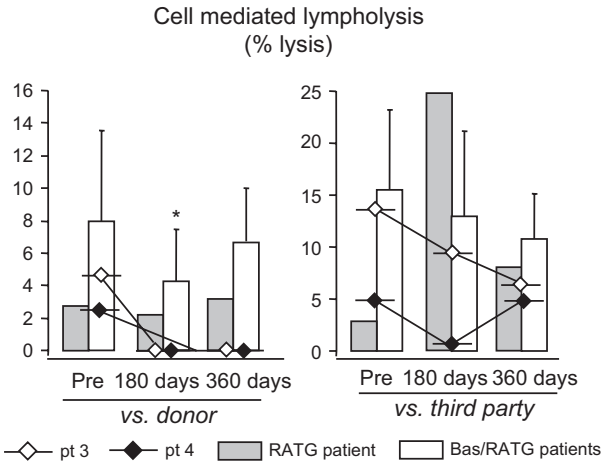


Figure 5 $CD8^+$ T-cell function by T-cell-mediated lympholysis assay. Cell-mediated lympholysis as percentage of specific lysis at 50:1 effector-target ratio against donor and third party antigens in patient 3 (open diamonds) and 4 (black diamonds) in a control patient given RATG alone (grey histograms) or patients given combined Bas/RATG (white histograms) on PBMC taken pre-transplant (pre) and at days 180 and 360 post-transplantation. Data are means ± SEM. $*P < 0.05$ versus pre.

tissue damage in experimental models of stroked brains [33], tumours [34], ischemic myocardium [35], and acute renal failure [36]. Experimental evidence in rodent models of acute renal injury has shown increased production in the kidney of hyaluronic acid (HA), the ligand for CD44 molecule expressed on MSC cell surface [36,37]. Therefore, we would like to suggest that in patients 3 and 4, the infusion of MSC pretransplantation in an environment not yet hosting a kidney graft with subclinical inflammatory tissue injury, as it occurs few days post-surgery, might lead to preferential cell recruitment into lymphoid organs, because of lack of the intragraft HA chemotactic signal.

The second patient (4), given MSC the day before kidney transplantation, had acute renal dysfunction 14–17 days postsurgery, and the graft biopsy showed evidence of acute cellular rejection. Higher HLA haplotype mismatches in patient 4 than in 3 can possibly explain the occurrence of rejection in the former. Although based on findings in a single MSC-treated patient, there is also the possibility that autologous MSC may have low capacity to control host immune response in the context of high alloreactive environment. Of note, recent evidence in a large-cohort of living-related kidney transplants has shown that the use of autologous MSC alone compared with anti-IL-2-receptor antibody induction therapy resulted in lower incidence of acute rejection at 6 month post-transplant [17]. Similarly to our patient 3, but at variance to patient 4, in this large-cohort of transplant recipients given MSC alone, HLA mismatching was on average lower than 3 [17]. Thus, with high HLA haplotype mismatches, adequate induction therapy including basiliximab could be of value to help the development of immunomodulatory function of MSC in the early post-transplant period, limiting the risk of acute graft rejection.

Impact on Treg profile of basiliximab-free induction therapy

The α-chain of the IL-2 receptor, known as CD25, is not solely expressed on activated/effector T cells, but also on Treg constitutively expressing the $CD4^+CD25^{high}$ phenotype [38]. Specific cell markers for Tregs also include the transcription factor forkhead-box-P3 (FoxP3) [39] and more recently the down-regulation of the IL-7 receptor (CD127) [39–41]. Thus, the question was raised whether avoiding the anti-CD25 antibody basiliximab in the current induction regimen would better favour the emergence of

Treg in the circulation after cell-therapy than with induction therapy including basiliximab as we adopted in our previous two MSC-treated kidney transplant patients [15]. Here we found no major difference in the profile of circulating $CD4^+CD25^{high}FoxP3^+CD127^-$ or $CD4^+FoxP3^+$ T cells in the present two patients given pretransplant MSC under the induction therapy that avoids basiliximab compared to the previous two patients receiving post-transplant MSC in the setting of combined basiliximab/low-RATG induction regimen [15]. This is in line with recent observation in liver transplant recipients that *in vivo* CD25 blockade with basiliximab did not lead to Treg changes as the proportion of $FoxP3^+$ cells among $CD4^+$ T cells and the level of FoxP3 expression were unaffected [42]. Moreover, others have shown *in vitro* that in the presence of basiliximab, $CD4^+CD25^{high}FoxP3^+$ cells were reduced because of the down-regulation of CD25 expression but the suppressive function of $CD4^+CD25^-FoxP3^+$ T cells was maintained [43]. Together these findings indicate that CD25 molecule is not essential for *in vivo* maintenance of human Treg in the peripheral blood, and that basiliximab is unlikely to negatively influence strategies involving Treg to promote tolerance after organ transplantation as the MSC-based therapy.

In this study, we also wanted to gain insight into the *in vivo* effect of pretransplant MSC infusion on T-cell subsets and function after peritransplant T-cell depletion with low-dose RATG induction therapy that avoids basiliximab. We found that in both patient 3 and (albeit less markedly) in patient 4, but not in transplant recipients given low-RATG alone or combined with basiliximab and not MSC, the percentage of memory/effector $CD8^+$ T cells within the overall $CD8^+$ T-cell population in peripheral blood decreased post-transplantation. At the 1 year follow-up of the two patients, memory/effector $CD8^+$ T cells remained lower than pretransplant values. These findings are reminiscent of changes in memory/effector $CD8^+$ T-cell profile in the initial two kidney transplant recipients given MSC at day 7 post-transplantation in the context of step 1 protocol [15]. The expansion of memory T cells that escape deletion after lymphoablation represents a major barrier to transplant tolerance [44]. With the limitation of few patients studied, overall our findings indicate that, at variance with current T-cell depleting induction therapy with RATG or Alemtuzumab, autologous MSC enable to control memory/effector $CD8^+$ T-cell proliferation long-lasting independently of whether a pre- or post-transplant cell infusion protocol is adopted. The mechanism(s) responsible for the MSC-mediated suppression of memory $CD8^+$ T-cell proliferation remains ill defined. A possible role of MSC-produced TGF-β [45,46], which antagonizes the effect of IL-15 – a cytokine relevant to memory $CD8^+$ T-cell expansion [47] – is proposed.

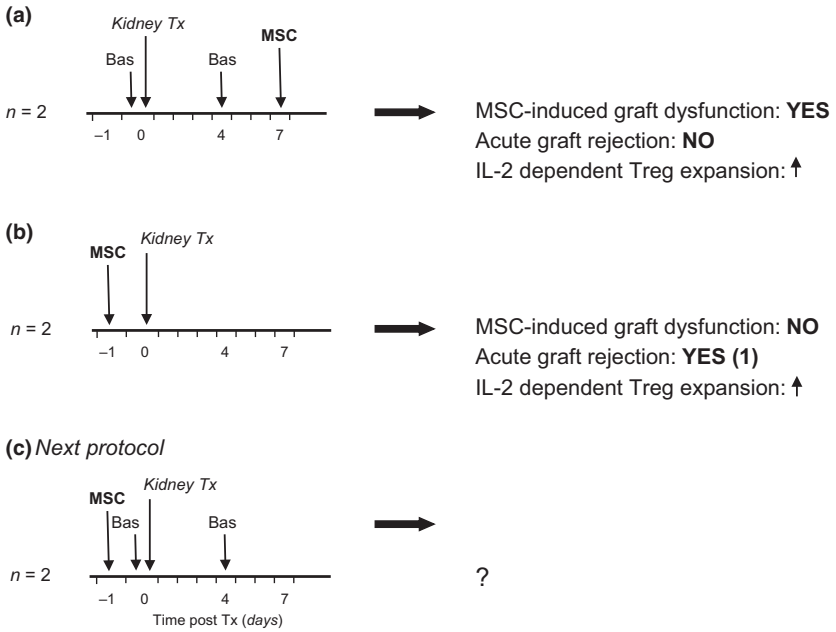


Figure 6 Clinical protocol of MSC-treated kidney transplant recipients implemented at our centre. The clinical MSC-based protocol on living-related kidney transplant recipients was conceived as a tailored-made step-wise gain of knowledge every two patients to eventually identify a definite protocol that allows to create favourable conditions for tolerance avoiding unwanted effects. Here are depicted the initial protocol (a) of post-transplant MSC infusion in patients 1 and 2 with induction therapy of basiliximab and low-RATG, the protocol (b) adopted thereafter in patient CM and DA of pretransplant MSC infusion with induction therapy of low-RATG alone. The study protocols are shown with major clinical and immunologic outcomes. The next up-dated protocol (c) to be tested in additional two patients is also reported. Bas, basiliximab.

Evidence from experimental models of solid organ transplantation suggests that the mechanisms of MSC-induced tolerance also include Tregs [4,6,8,9]. Here we found that the number of $CD4^+CD25^{high}Foxp3^+CD127^-$ Treg in the peripheral blood of MSC-treated patients slowly expanded after a marked reduction because of the depleting action of the induction therapy, although the effect was only marginally higher than in control groups. Given the inconsistent effect on Treg count in the two patients receiving MSC, and the very small difference in the Treg profile as compared to controls, we advice caution to conclude for a robust impact of MSC treatment on Treg expansion, at least in peripheral blood of kidney transplant recipients. Nevertheless, MSC therapy did result in a clear increase in the ratio of Treg/memory $CD8^+$ T-cell count, suggesting a unique skewing toward regulation of host immune response. Indeed, as previously documented in patients undergoing post-transplant MSC infusion [15], the change in the memory/effector $CD8^+$ T-cell profile was associated with a profound and persistent reduction in donor-specific $CD8^+$ T-cell cytolytic activity. These effects were not seen in kidney transplant recipients given low-RATG alone or combined with basiliximab induction therapy without MSC. Thus, MSC may have an additional effect beyond classical immunosuppressants of promoting inhibition of memory $CD8^+$ T-cell function that persists with time.

We acknowledge the many limitations of this preliminary work in few patients, that however has helped to get more insights on some of the open questions dealing with therapeutic administration of MSC on kidney transplant patients. Our findings also highlights that the time is probably not yet ripe for large clinical trials with MSC on organ transplantation.

In summary, in the second step of the multi-step clinical protocol under consideration here we documented that: (i) pretransplant (DAY-1) infusion of MSC provides a safety advantage over the protocol of post-transplant (day 7) cell administration, in that no longer associates with cell-induced impaired graft function, while maintains MSC immunomodulatory properties; (ii) induction therapy without basiliximab does not further expand $CD4^+FoxP3^+$ Treg pool as compared to the induction therapy with basiliximab, while exposing patients to the possibility of acute rejection early post-transplant [48]. Therefore, as further implementation of knowledge, we plan as next step a clinical protocol of pretransplant infusion of autologous bone marrow-derived MSC with basiliximab/low-RATG exactly as in step 1 (Fig. 6) where no patients had acute rejection.

Authorship

NP, FC and GR: participated in all stages of the study, made interpretation of the study findings, prepared the first draft of the report and the final manuscript. FC, MT, RAC and MC: performed immunophenotyping and functional immunological assays. MN: participated in research design and data interpretation. MI and CC: performed MSC isolation and characterization. EG, AR and GR: were in charge of patient care and monitoring. PC and PR: performed immunohistochemic analysis. GR: supervised all the study.

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Supporting information

Additional Supporting Information may be found in the online version of this article:
Data S1. Materials and methods.

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