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Cutting Edge: Immunological Consequences and Trafficking of Human Regulatory Macrophages Administered to Renal Transplant Recipients

James A. Hutchinson,*† Paloma Riquelme,*† Birgit Sawitzki,‡ Stefan Tomiuk,†§ Patrick Miqueu,‡ Maaz Zuhayra,‡ Hans H. Oberg,‡ Andreas Pascher,** Ulf Lützen,‖ Uwe Janßen,‡ Christiane Broichhausen,* Lutz Renders,†‡ Friedrich Thaiss,†§ Ernst Scheuermann,§§ Eberhard Henze,‖ Hans-Dieter Volk,‡† Lucienne Chatenoud,†‡ Robert I. Lechler,†‖ Kathryn J. Wood,‡‡ Dieter Kabelitz,§ Hans J. Schlitt,* Edward K. Geissler,*† and Fred Fändrich†***

Regulatory macrophages (Mregs) were administered to two living-donor renal transplant recipients. Both patients were minimized to low-dose tacrolimus monotherapy within 24 wk of transplantation and subsequently maintained excellent graft function. After central venous administration, most Mregs remained viable and were seen to traffic from the pulmonary vasculature via the blood to liver, spleen, and bone marrow. By 1 y posttransplantation, both patients displayed patterns of peripheral blood gene expression converging upon the IOT-RISET signature. Furthermore, both patients maintained levels of peripheral blood FOXP3 and TOAG-1 mRNA expression within the range consistent with nonrejection. It is concluded that Mregs warrant further study as a potential immune-conditioning therapy for use in solid-organ transplantation. The results of this work are being used to inform the design of The ONE Study, a multinational clinical trial of immunomodulatory cell therapy in renal transplantation. The Journal of Immunology, 2011, 187: 2072–2078.

Conditioning the response of organ transplant recipients to donor alloantigen using cell-based therapies is now becoming a clinically feasible strategy, and, as the potential risks are better understood and minimized, such approaches are gaining credibility. Compared to longer established techniques, such as donor-specific blood transfusion and bone marrow transplantation, newer methods involving the ex vivo induction, expansion, and purification of tolerance-promoting cells offer the substantial advantages that the quality and dose of cell products can be tightly controlled. Moreover, by expansion or induction of cells in culture and by specifically purifying tolerogenic cell types, the number of viable suppressor cells administered to the patient can be maximized, and the dangers of sensitization and graft-versus-host reactions may be reduced.

Efforts in our laboratory to develop a cell product for promoting transplant tolerance in the clinical setting have focused on a type of suppressor macrophage, the human regulatory macrophage (Mreg) (1–7). Mregs exhibit a number of properties that might make them particularly suitable for clinical purposes, in particular, the cells are fully differenti- ated and potently T cell suppressive (8). Mregs derive from CD14+ peripheral blood monocytes in the absence of other cell types when monocytes are cultured for 6 d in medium supplemented with human AB serum before stimulation with IFN-γ for a further 24 h. Cell populations generated in this manner are homogeneously CD14low/HLA-DRCD80CD86CD16TCDLR2TLR4low and CD163low (8).

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The sequences presented in this article have been submitted to the National Center for Biotechnology Information/Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE24172.

Address correspondence and reprint requests to Dr. James A. Hutchinson, Department of Surgery, University Hospital Regensburg, Franz-Josef-Strauß-Allee-11, Regensburg 93053, Germany. E-mail address: james.hutchinson@klinik.uni-regensburg.de

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Abbreviations used in this article: 1-D/L-MT, 1-methyl-D/L-tryptophan; IOT, Indices of Tolerance; Mreg, regulatory macrophage; RISET, Reprogramming the Immune System for Establishment of Tolerance; SPECT, single photon emission computed tomography.

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This report describes the treatment of two living-donor renal transplant recipients with preoperative infusions of donor-derived Mregs. Both patients were successfully weaned to low-dose tacrolimus monotherapy within 24 wk of transplantation and remain in excellent clinical condition. Throughout their follow-up, the patients were intensively monitored using cutting-edge immunomonitoring assays provided by the European Union Framework Program 6 Reprogramming the Immune System for the Establishment of Tolerance (RISET) consortium, including flow cytometry, serial gene expression profiling, and analysis of TCR Vβ usage by peripheral blood T cells. The two patients are now 3 y posttransplantation with stable graft function and receive minimal maintenance immunosuppression.

Materials and Methods

Ethics

In a modification of the TAIIC-II trial protocol (http://www.clinicaltrials.gov, NCT00223067), two patients were treated with Mreg therapy at the discretion of two senior consultants as "individueller heilversuch" (individual healing attempts). The patients and their donors gave full, informed, written consent to the procedure and follow-up investigations. Human leukocytes for experimental use were obtained with approval of the local ethics committee (ethics vote 09/100) and informed consent of the donors.

In vitro characterization of human Mregs

Mregs were prepared according to published methods (1). For flow cytometry, harvested Mregs were resuspended in ice-cold staining buffer (Dulbecco’s modified PBS, 1% BSA, and 0.1% NaN3) and blocked for 30 min with 10% FCS block (Miltenyi Biotec) before staining with fluorochrome-conjugated primary Abs for 1 h. 7-aminocoumarin D exclusion was used for live/dead discrimination. To assess the T cell-suppressive capacity of Mregs, CFSE-labeled CD3+ T cells and Mregs were set in direct coculture for 5 d. Subsequently, T cell proliferation and absolute numbers were assessed by flow cytometry, as described elsewhere (1). The mechanism of Mreg-mediated T cell suppression was investigated in direct 1:1 Mreg/T cell cocultures. 1-methyl-DL-tryptophan (1-D/L-MT; Sigma-Aldrich), an inhibitor of IDO, was completely dissolved in 5N HCl with gentle heating and agitation, before adjusting the solution to pH 7.2.

Production of Mregs for infusion into patients

Mregs for administration to patients were prepared under strict GMP conditions according to an adaptation of a previously published method (1). Briefly, donor PBMC were obtained by leukapheresis and Ficoll density gradient separation. Plastic-adherent PBMC were plated at a density of 35 × 10^6 monocytes/175 cm^2 culture flask (Cell+ T175 flask; Sarstedt) in 30 ml RPMI 1640-based medium without phenol red (Lonza) supplemented with 10% human AB serum (Lonza), 2 mM l-glutamine (Lonza), 100 U/ml penicillin, 10 μg/ml streptomycin (Lonza), and recombinant human M-CSF (R&D Systems) at a final concentration of 5 ng/ml carried on 0.1% human serum albumin (Aventis). The cells were cultured for 6 d with complete medium exchanges on days 1, 2, and 4. On day 6, cultures were stimulated with 25 ng/ml recombinant human IFN-γ (Miltenyi; Boehringer Ingelheim). On day 7, the adherent cell fraction was recovered by trypsin–EDTA treatment (TrypsLE Express without Phenol Red; Invitrogen) followed by gentle scraping. Mregs from all flasks were pooled and resuspended in complete medium.

Clinical management of patients CA and MM

Mregs were administered to patients CA and MM in the context of an immunosuppressive protocol comprising tacrolimus, steroids, and azathioprine. To reduce the risk of preoperative infusion of Mregs sensitizing recipients, infusions were given under cover of 2 mg/kg/d azathioprine, commencing 3 d prior to administration of Mregs and continuing for 8 wk postoperatively (9). Mregs were administered 6 (MM) or 7 (CA) d prior to transplantation by central venous infusion. From the day of transplantation onwards, the patients were immunosuppressed with tacrolimus and steroids; steroids were weaned over the first 10 wk postoperatively, leaving the patients with maintenance tacrolimus monotherapy, aiming for trough serum levels between 4 and 8 ng/ml.

Radiological investigations

Radiological investigations were performed by the Department of Nuclear Medicine at Universitätsklinikum Schleswig-Holstein according to standard clinical protocols. For short-term cell tracking studies, patient MM received a total of 5 × 10^6 Mregs labeled with 45 MBq [111In]-oxine (Covidien). Single photon emission computed tomography (SPECT) images were interpreted by an expert radiologist.

Gene expression profiling

Gene expression profiling data have been deposited in the National Center for Biotechnology Information/Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with accession numbers GSE14655 and GSE24172.

Statistics

Reported values are mean ± SD in all cases. All n-values signify the number of independent biological replicates using cells from separate donors. Error bars shown in Fig. 1 represent SEM. A paired t test was used for all significance testing. Statistical treatment of microarray data is described in the text and figure legends.

Results

Production and quality control of clinical Mreg preparations

A basic scientific understanding of the development, function, and possible immunological role of naturally occurring counterparts of the human Mreg has lead to an optimized method of cell production for clinical purposes. The quality of Mreg preparations produced by this method can be tightly specified in terms of cell surface phenotype, cell morphology, and potency in suppressing mitogen-driven T cell responses. Specifically, Mregs are consistently CD14^−/low-HLA-DR^CD80^/−/low-HLA-DR^CD86^/−/low-HLA-DR^CD16^/−/low-HLA-DR^CD163^/low (Fig. 1A).

Mregs suppress T cell responses in vitro

To assess the T cell-suppressive capacity of Mregs, a flow cytometric assay was used to quantify mitogen-driven CD4^+ and CD8^+ T cell proliferation and, in parallel, to make absolute counts of T cells in direct coculture with Mregs. T cells cultured alone did not proliferate and, by the fifth day of culture, 17 ± 6.5% of CD4^+ and 22.1 ± 11.1% of CD8^+ T cells had died spontaneously (Fig. 1B, 1C). Direct coculture of Mregs with allogeneic T cells did not stimulate an MLR reaction, nor did Mregs rescue cocultured lymphocytes. The strong proliferation of both CD4^+ and CD8^+ T cells observed after PHA stimulation was profoundly suppressed by coculture with allogeneic Mregs (Fig. 1D). Strikingly, fewer CD4^+ and CD8^+ T cells remained in coculture with Mregs when stimulated with PHA (p < 0.001 and p = 0.009, respectively), which points to an Mreg-mediated elimination of activated T cells (Fig. 1C). This disappearance of T cells during Mreg cocultures is most likely due to phagocytosis of dying T cells by Mregs because examination of Mregs by transmission electron microscopy after coculture with T cells revealed the presence of numerous phagolysosomes (Supplemental Fig. 1). Suppression of T cell proliferation by Mregs was observed at Mreg/T cell ratios >1:8 (Fig. 1D). Inhibition of IDO activity with 1 mM 1-D/L-MT restored the ability of T cells cocultured in the presence of allogeneic Mregs to proliferate in response to PHA (Fig. 1E).

Administration of Mregs to patient MM

Patient MM, a 23-y-old female with renal failure owing to IgA nephropathy, received a kidney transplant from her 58-y-old mother, with whom she had single HLA-B and HLA-DR
mismatches. CMV and EBV serology was negative in donor and recipient. Six days prior to transplantation, MM was given $4.3 \times 10^8$ viable donor-derived Mregs (an equivalent of $8.0 \times 10^6$ cells/kg) by central venous infusion under cover of 1 mg/kg/d azathioprine (Fig. 2A, Supplemental Fig. 2).

Transplantation was without complications and initial graft function was satisfactory, in that serum creatinine levels had fallen to 1.4 mg/dl within 1 wk. Azathioprine was discontinued from week 8 postoperatively without adverse effect, and steroid therapy was discontinued by week 14, after which MM was maintained on tacrolimus monotherapy. Protocol biopsies at 8 and 24 wk posttransplantation showed no signs of rejection. Currently, MM is 3 y posttransplantation and in a stable clinical condition on tacrolimus 2 mg twice daily with trough levels of 4 to 5 ng/ml.

Profiling of peripheral blood TCR Vβ usage showed no mono- or oligoclonal T cell expansion, which is indicative of T cell nonreactivity to the transplanted kidney (Supplemental Fig. 3A) (10). Peripheral blood TOAG-1 mRNA expression levels are usually reduced during acute rejection of kidney allografts, but MM consistently maintained levels of TOAG-1 expression similar to healthy controls (Supplemental Fig. 3B) (11). No anti-HLA Abs, which were assayed on a monthly basis, were detected following the administration of Mregs.

Knowing the fate of Mregs after administration to a patient is central to any understanding of their potential immunomodulatory effects in vivo. Therefore, $5 \times 10^7$ (12%) of the $4.3 \times 10^8$ Mregs given to patient MM were labeled with 45 MBq $^{111}$In oxine and administered at the same time as the unlabeled Mregs. Immediately after the infusion, a whole-body SPECT study was performed and again at four later time points (Fig. 2B). Initially, the Mregs were seen to be...
trapped in the lungs, but after 2.5 h, cells were detected in circulation and had begun to accumulate in the liver and spleen. Twenty-two hours after M reg administration, the signal from the lung fields had much diminished, the cells having accumulated in the liver, spleen, and hematopoietically active bone marrow. The absence of signal from the patient’s urinary tract throughout the investigation indicates that the majority of infused M regs remained alive (12).

**Administration of M regs to patient CA**

Patient CA was an athletic 47- y-old male patient with severe chronic renal failure owing to nephrosclerosis as a consequence of long-standing arterial hypertension. CA was transplanted with a kidney from an unrelated 40- y-old living donor (Fig. 3A). Donor and recipient were fully HLA mismatched (donor: HLA-A3, 2; HLA-B41, 55; HLA-DR11, 13; recipient: HLA-A2, 2; HLA-B7, 35; HLA-DR4, 14), and both were CMV positive. One week prior to transplantation, a total of $7.5 \times 10^8$ viable donor-derived M regs (equivalent to $7.1 \times 10^6$ cells/kg) were administered to CA by slow central venous infusion (Supplemental Fig. 2). No evidence of impaired pulmonary perfusion caused by M reg administration was found (Fig. 3B). Seven days later, the patient underwent transplantation without complications. Serum creatinine levels gradually declined over the subsequent 10 wk to <2 mg/dl. Azathioprine treatment was stopped after the 8th week postoperatively, and steroids were discontinued by the 10th week, leaving the patient with tacrolimus as his sole maintenance therapy. Protocol biopsies taken at weeks 8, 24, and 52 showed no signs of acute cellular rejection, although occasional clusters of CD20+ B cells of unknown pathological significance were observed (data not shown). At 36 mo posttransplantation, CA had stable renal function with a creatinine of 1.43 mg/dl. Patient CA is being maintained on once-daily 5 mg sustained-release tacrolimus (Advagraf; Astellas Pharma) monotherapy; at 36 mo, his trough tacrolimus level was 2.7 ng/ml.

After administration of M regs, but prior to transplantation, the expression of TOAG-1 mRNA in the peripheral blood of CA increased 5.5-fold (Supplemental Fig. 3B). A similar increase in TOAG-1 was observed in patient MM after M reg treatment. As observed in MM, patient CA’s expression of FOXP3 mRNA began to rise from the fourth week postoperatively (Supplemental Fig. 3B), which corresponded

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**FIGURE 2.** Administration of M regs to patient MM. A. The management and clinical course of patient MM. B. Anterior-posterior scintigrams reconstructed from SPECT imaging data show the distribution of $^{111}$In-labeled M regs at various intervals after administration by central venous infusion: 1, M regs were initially trapped in the pulmonary vasculature; 2, by 2.5 h postinfusion, M regs were detected in circulation; 3 and 4, within 4.5 h, M regs had begun to accumulate in the liver and spleen; 5, on the day after M reg administration, the majority of M regs had exited the lungs and were no longer detectable in the blood; 6 and 7, M regs also accumulated in hematopoietically active bone marrow.

**FIGURE 3.** Administration of M regs to patient CA. A. The management and clinical course of patient CA. B. Total of $7.5 \times 10^8$ viable donor-derived M regs were administered to CA by slow central venous infusion using a giving set with a 200- µm cell filter. During and after infusion, the patient displayed no symptoms or signs of pulmonary embolism. To directly assess the degree to which M reg infusion might have impaired pulmonary perfusion, CA was investigated by ventilation and perfusion scintigraphy, both before and after M reg administration. Prior to cell administration, there was no evidence of a ventilation or perfusion defect: the perfusion fraction to the right lung was 59% and 41% to the left (L). After cell infusion, no localized or generalized perfusion defect was noted, and the perfusion fraction for each lung was unaltered.
Serial analysis of Indices of Tolerance marker gene expression in the peripheral blood of patients MM and CA

A pattern of gene expression in peripheral blood that correlates with a drug-free, tolerant state in kidney transplant recipients has been defined by the Indices of Tolerance (IOT) research network (13). Using the RISE T 2.0 Agilent custom microarray platform (Agilent Technologies), serial analyses of gene expression in the peripheral blood of patients MM and CA were performed (Fig. 4). The dataset from MM and CA was then compared with the dataset obtained from the cohort of kidney transplant recipients studied by IOT. Expression profiles of the 10 most discriminatory biomarkers of tolerance identified by the IOT study were extracted from the complete quantile normalized, log2-transformed dataset. From this data, pairwise correlation coefficients were calculated comparing the medians of the IOT-tolerant patient group with MM and CA at each separate time point, and heatmaps of reporter-wise median-centered log2 data were generated.

Throughout her postoperative course, the profile of gene expression displayed by patient MM was very similar to that of the tolerant patient group. Patient CA followed a different course, upregulating SH2D1B, H535T1, TCL1A, FCRL1, FCRL2, and CD79b and downregulating SLC8A1 and TLR5 only later in the follow-up period. In consequence, CA initially most resembled the group of chronically rejecting and stably immunosuppressed patients, but by week 32 closely resembled the clusters of patients classified as tolerant or healthy controls.

Discussion

Several alternative cell types are now approaching the point of preclinical development that might allow them to be properly investigated as adjunct immunosuppressive therapies in early-stage clinical trials, including certain tolerogenic DC subsets, regulatory T cells, mesenchymal stem cells, and M regs. Yet clinical studies using cellular therapies in transplantation remain controversial because many in the field doubt the clinical practicality of such therapies and their safety. It is certainly true that administration of cell preparations to patients is not without potential complications, but these risks can be minimized by refining cell preparations and clinical monitoring of the recipient: in both these respects, we have learned much from the cases of MM and CA.

Should we be surprised by the clinical outcomes of MM and CA? Although a high proportion of renal transplant recipients with stable graft function several years after transplantation may be weaned to tacrolimus monotherapy, quite a different situation pertains to our patients. Both MM and CA underwent an early, rapid reduction in immunosuppression, such that both received tacrolimus monotherapy by 14 and 10 wk, respectively, and were further minimized to 4–6 ng/ml trough tacrolimus levels by 24 wk. At 3 y posttransplantation, patient CA is clinically stable with once-daily 5 mg sustained-release tacrolimus, and patient MM is being stably maintained with tacrolimus 2 mg twice daily. Despite their early minimization of immunosuppressive therapy, neither patient MM nor CA underwent an acute rejection episode during the 3-y follow-up period. Shapiro et al.’s study (14) remains a benchmark trial of minimized tacrolimus monotherapy in renal transplant recipients. In this study, 150 patients were treated with 5 mg/kg antithymocyte globulin with bolus prednisone as induction and were subsequently treated with tacrolimus monotherapy. Under this regimen, 37% of patients underwent acute rejection by 4 mo. After the fourth month, 113 patients underwent a stepwise minimization of tacrolimus dosing; these patients were followed up for a mean of 11 ± 5.4 mo, during which time 23% of patients underwent acute rejection. Other studies with the aim of establishing renal transplant patients on tacrolimus monotherapy after antithymocyte globulin induction (15) or alemtuzumab induction (16) achieved similar outcomes in terms of acute rejection rates and the proportion of patients tolerating monotherapy. Despite receiving no conventional induction therapy, neither MM nor CA underwent acute rejection or showed any signs of subclinical rejection at their last protocol biopsy. From clinical experience, for the patients to have tolerated such an abrupt weaning of immunosuppressive therapy is not without potential complications, but these risks can be minimized by refining cell preparations and clinical monitoring of the recipient: in both these respects, we have learned much from the cases of MM and CA.

FIGURE 4. Using the RISE T 2.0 Agilent custom microarray platform, serial analyses of gene expression in the peripheral blood of patients MM and CA were performed. The resulting dataset was then compared with that obtained from the IOT-RISET patients. Expression profiles of the 10 most discriminatory biomarkers of tolerance identified by the IOT-RISET study are presented. The color coding of the heatmaps represents reporter-wise median-centered log2 ratios of the combined IOT, CA, and MM datasets: red, relative upregulation; green, relative downregulation; black, no differential regulation. The convergence over time of patient MM and CA’s marker gene expression upon the tolerance-associated IOT-RISET gene signature is best appreciated by considering pairwise Pearson correlation coefficients comparing the medians of the IOT-RISET drug-free, tolerant patient group with MM and CA at each sampling time point (bottom right panel).
immunosuppression is a surprising result and suggestive of attenuated antidonor reactivity.

Serial analyses of the immunological status of the two patients, especially by gene expression profiling, support the contention that the reactivity of MM and CA against their grafts was attenuated. As time progressed, the expression pattern of the IOT-RISET gene set in MM and CA tended toward that of a group of tolerant kidney transplant recipients or healthy untransplanted individuals (13). The frequency of individuals displaying the IOT-RISET gene signature among the cohort of stably immunosuppressed patients on calcineurin inhibitor monotherapy studied by IOT-RISET was only 14.7%. Considering that this group of patients were all 6 to 9 years after transplantation and had been purposefully selected for their stable clinical state, we can reasonably expect the frequency of patients displaying the IOT-RISET gene signature within the general pool of renal transplant recipients to be lower.

Could selection of MM and CA have biased our interpretation of their clinical outcomes? Patient CA was fully mismatched against his unrelated donor. Patient MM, who received a kidney from her 58-y-old mother, was at risk for being presensitized against noninherited maternal Ags (17). Therefore, neither donor-recipient pair could be said to be especially more likely to have a better outcome than the general pool of living-donor transplant recipients.

After systemic administration, human M regs could influence recipient responses against donor alloantigen by multiple mechanisms: M regs might have direct effects on alloreactive and regulatory T cells or may act indirectly through a systemic release of cytokines or alloantigen. At the cellular level, though, it has been shown that M regs profoundly suppress mitogen-stimulated T cell proliferation in vitro and that this activity is partly mediated by the tryptophan-catabolizing enzyme IDO. In mice, IDO is indispensable for maintaining maternal tolerance of allogeneic conceptuses (18) and participates in the establishment of allograft tolerance by adoptive transfer of T reg cells (19). Therefore, it is at least plausible that human M regs might influence responses to allografts through the action of IDO.

Central to any understanding of the possible mechanisms by which M regs might act is the question of whether they transiently or stably engraft in the recipient or die shortly after administration. Tracking studies with In-labeled cells in patient MM revealed that donor-derived M regs migrated to specific sites and remained viable for at least the first 30 h posttransfusion, although their fate beyond this time is not known. However, given that M regs are believed to be fully differentiated, postmitotic cells, it must be assumed that they cannot establish a permanent state of chimerism.

In conclusion, these pilot case studies demonstrate the feasibility of treating renal transplant recipients with M regs. Reassuringly, no unexpected adverse reactions were observed in either patient. Tracking M regs has provided us with a preliminary description of their distribution after infusion, which is essential information in the assessment of any new therapeutic agent. Taken together, the clinical outcomes of the patients and their peripheral blood gene expression profiles must be seen as promising results, albeit of a preliminary nature. We are still years away from proving any clinical efficacy of M reg treatment as an adjunct immunosuppressive therapy, which could only be properly demonstrated in a large-scale clinical trial. However, it is our opinion that further clinical trials of M reg treatment as an adjunct therapy aimed at establishing renal transplant recipients on minimal, calcineurin inhibitor-based maintenance immunosuppression are warranted. This concept is now being carried forward within The ONE Study, a European Union-funded multinational clinical trial.

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Disclosures

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