Induction of Long-Lasting Regulatory B Lymphocytes by Modified Immune Cells in Kidney Transplant Recipients

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ABSTRACT

Background We recently demonstrated that donor-derived modified immune cells (MICs)—PBMCs that acquire immunosuppressive properties after a brief treatment—induced specific immunosuppression against the allogeneic donor when administered before kidney transplantation. We found up to a 68-fold increase in $CD19^{+}CD24^{hi}CD38^{hi}$ transitional B lymphocytes compared with transplanted controls.

Methods Ten patients from a phase 1 clinical trial who had received MIC infusions before kidney transplantation were followed to post-transplant day 1080.

Results Patients treated with MICs had a favorable clinical course, showing no donor-specific human leukocyte antigen antibodies or acute rejections. The four patients who had received the highest dose of MICs 7 days before surgery and were on reduced immunosuppressive therapy showed an absence of *in vitro* lymphocyte reactivity against stimulatory donor blood cells, whereas reactivity against third party cells was preserved. In these patients, numbers of transitional B lymphocytes were 75-fold and seven-fold higher than in 12 long-term survivors on minimal immunosuppression and four operationally tolerant patients, respectively ($P<0.001$ for both). In addition, we found significantly higher numbers of other regulatory B lymphocyte subsets and a gene expression signature suggestive of operational tolerance in three of four patients. In MIC-treated patients, *in vitro* lymphocyte reactivity against donor blood cells was restored after B lymphocyte depletion, suggesting a direct pathophysiologic role of regulatory B lymphocytes in donor-specific unresponsiveness.

Conclusions These results indicate that donor-specific immunosuppression after MIC infusion is long-lasting and associated with a striking increase in regulatory B lymphocytes. Donor-derived MICs appear to be an immunoregulatory cell population that when administered to recipients before transplantation, may exert a beneficial effect on kidney transplants.

Clinical Trial registry name and registration number: MIC Cell Therapy for Individualized Immunosuppression in Living Donor Kidney Transplant Recipients (TOL-1), NCT02560220

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Cellular therapies represent a novel approach to immunosuppression after solid organ transplantation. Monocenter and multicenter studies have investigated the hypothesis of whether cellular therapies can help to reduce immunosuppression and associated side effects, such as infection and malignancy, while maintaining or even improving efficacy in terms of prevention of rejection.[1,2] The One

Study was designed to test a total of seven different cell therapies, including polyclonal or donor-specific regulatory T lymphocytes (Tregs), regulatory macrophages, and regulatory dendritic cells, along with reduced immunosuppressive medication, compared with a reference group of patients receiving standard immunosuppressive therapy.[3] This study provided some important insights: (*1*) cell therapy in combination with reduced immunosuppression appears to be feasible without excessively increased rejection rates, (*2*) the sparing of immunosuppression may ultimately lead to lower infection rates, and (*3*) a change in regulatory B lymphocyte subsets in treated patients may be detectable, namely a discrete increase in immunosuppressive marginal zone B lymphocytes in patients treated with polyclonal Tregs.[3–5]

Recently, we published the results of the TOL-1 phase 1 study using modified immune cells (MICs) to prevent rejection after living donor kidney transplantation.[6] MICs are donor-derived PBMCs that acquire immunosuppressive properties after brief incubation with an alkylating agent.[7–11] MIC production is rapid and easily reproducible, allowing cell infusion into the recipient on the day of PBMC collection from the donor. In a rat heart transplantation model, 10^8 donor-derived MICs, when administered before transplantation, resulted in permanent graft acceptance in more than 50% of animals without additional immunosuppressive therapy.[10] This effect was not seen when monocyte-depleted or third party MICs were administered, suggesting a donor-specific effect mediated by monocytes within the PBMC pool. From August 2015 to February 2017, MICs were prepared from ten living kidney donors by unstimulated leukapheresis and administered at a dose of 1.5×10^6 to 1.5×10^8 MICs/kg body weight to ten prospective transplant recipients (groups A–C) following good manufacturing practice (GMP).[6] Two or seven days later, a kidney graft from the same respective donor was transplanted. Group C patients, who had received the highest cell dose 7 days before transplantation and low post-transplantation immunosuppression with reduced cyclosporin A (CyA) and enteric-coated mycophenolate sodium (EC-MPS) without corticosteroids, showed low antidonor T lymphocyte reactivity *in vitro*. Although MICs were immediately removed from systemic circulation and no permanent chimerism was established, the frequency of a specific regulatory B lymphocyte subset, $CD19^+CD24^{hi}CD38^{hi}$ transitional B lymphocytes (TrBs), increased up to 68-fold in these patients compared with the frequency in transplanted controls without MIC infusions. This led us to the hypothesis that long-term donor-specific immunosuppression is maintained by TrBs. An increase of TrBs in the context of transplantation was first described in operationally tolerant patients and thought to play a role in the maintenance of kidney function in these patients.[12,13] Meanwhile, the ten MIC-treated patients in our series have completed a follow-up period of 3 years. The clinical and immunologic results of these ten patients are presented herein, highlighting the four patients in group C who received the highest MIC dose 7 days before surgery. We now show evidence that the immunosuppressive effect of MICs in transplant recipients persists after 3 years of follow-up and is maintained by IL-10–producing TrBs.

Significance Statement

In previous work, the authors demonstrated that kidney transplant recipients developed donor-specific unresponsiveness when they were given a pretransplant infusion of modified donor-derived PBMCs. In this study, they provide evidence that the immunosuppressive properties of these cells persist and the donor-specific unresponsiveness is long-lasting. In the four patients who received the highest dose of the modified immune cells, administration of these cells was associated with a striking increase in IL-10–producing regulatory B lymphocytes and evidence of the consensus gene expression signature of operational tolerance. *In vitro*, donor-specific unresponsiveness was abolished after B lymphocyte depletion, suggesting a direct pathophysiologic role for regulatory B lymphocytes. These findings support the notion that modified donor-derived PBMCs may be useful in kidney transplantation, but this approach requires further validation and rigorous controlled randomized studies.

METHODS

TOL-1 Study and 3-Year Follow-Up

From August 2015 to February 2017, 14 donor and recipient pairs were screened for enrollment in the TOL-1 study and ten patients eventually received the MICs intravenously (R1–R7, R11, R12, and R14) on the day of donor leukapheresis as a single administration as described previously (Supplemental Figure 1 and Supplemental Table 1).[6] Patients were treated in a stepwise manner, increasing the dose from group A (1.5×10^6 MICs/kg body weight) to group B (1.5×10^8 MICs/kg body weight) to account for potential adverse effects associated with the MIC product. Because a very low MIC count was thought to carry an increased risk of recipient sensitization (patients in group A received only 1% of the MICs compared with patients in groups B and C), MICs were administered only 2 days before transplantation during the dose escalation phase. From group B to C, the administration time

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point was shifted from day −2 to day −7 before transplantation. Based on preclinical experiments, day −7 was considered the optimal time point for administration. Administration of MICs at the time or after transplantation did not have the same protective effect.[9,14–16] The presence of HLA antibodies in MIC-treated patients was excluded in all patients by complement-dependent cytotoxicity crossmatch, ELISA crossmatch, and Luminex Single Antigen test on day −1 before transplantation and after MIC infusion. Immunosuppressive maintenance therapy and post-transplant care were provided according to center practice, with CyA, EC-MPS, and methylprednisolone administered from the day of surgery, without induction with an IL-2 receptor antagonist (Supplemental Figure 2). After the study end point on day 30, patients were followed to day 1080 after kidney transplantation with regular outpatient visits according to the center's practice. Routine screening for BK virus (at each outpatient visit) and donor-specific HLA antibodies (DSA) (at days 7, 30, 180, and every 6 months thereafter, plus additional screening for DSA in these TOL-1 patients at days 60, 90, 135, and 270) was performed. Biomaterials were collected during follow-up and retrospectively evaluated.

Control Groups

Supplemental Tables 2 and 3 provide details on controls. They were chosen because on these for the study selected controls there was sufficient biomaterial available.

Modified Immune Cell Product Manufacturing

The MIC product was based on donor PBMCs obtained by unstimulated leukapheresis. The leukapheresis products were processed under GMP conditions according to the manufacturing authorization of the regional (DE_BW_01_-MIA_2015_0032/DE_BW_01_Uniklinik HD_Med Klinik V GMP-Facility, Regierungspräsidium, Tübingen, Germany) and national (submission number 2252/01, Paul-Ehrlich-Institut, Langen, Germany, for the TOL-1 study) regulatory authorities. Cells were incubated with mitomycin C and then washed three times. The final product (MICs) consisted of human cells from peripheral blood, resuspended in a buffer solution to a maximum total volume of 100 ml. The product was released for infusion into humans after the appropriate quality controls.[6]

HLA Antibody Detection and Crossmatch Techniques

HLA antibodies were detected using the complement-dependent cytotoxicity panel-reactive antibody assay, ELISA, and Luminex Single Antigen methodologies as previously described.[6] For the evaluation of pre- and post-transplant HLA antibodies with the Luminex Single Antigen test, the positivity cutoff was set at 1000 mean fluorescence intensity.

Lymphocyte Proliferation Assay

Lymphocytes were separated from heparinized whole blood using lymphocyte gradient centrifugation (Lymphodex; inno-train, Kronberg, Germany). Lymphocytes were washed and adjusted to a cell number of 10^6/ml. A cell suspension of 100 μl (10^5 lymphocytes) was added to each well of a 96-well cell culture plate. Cells were stimulated polyclonally for 3 days with 100 μl of the following mitogens: pokeweed mitogen (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), phytohemagglutinin (Remel; Thermo Fisher Scientific, Dartford, UK), concanavalin A (Sigma-Aldrich), or CD3 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ). In addition, recipient cells were stimulated for 5 days in a mixed lymphocyte culture, antigen specifically with 10^5 cells of the original transplant donor per well, or unspecifically with 10^5 pooled third party cells per well. The pool consisted of cells from four MHC class 2–incompatible donors. Stimulator cells were irradiated to prevent their proliferation. At the beginning of the cell culture, all cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (CellTrace, Invitrogen; Fisher Scientific, Schwerte, Germany). After 3 and 5 days of cell culture, respectively, green fluorescence intensity of cells was analyzed in the blast region of a CFSE/Fw-Sc dot plot using a FACSCalibur flow cytometer (BD Biosciences). Proportion of blasts with low CFSE fluorescence (CFSElow blasts) was determined, and background proliferation was subtracted, determined in a cell culture without stimulus run in parallel. All assays were carried out in triplicate.

Lymphocyte Proliferation Assay with Depleted Responder Cells

Lymphocytes were depleted of B lymphocytes using dynabeads coated with CD19 monoclonal antibody (Dynabeads CD19; Thermo Fisher Scientific). Lymphocytes and B cell–depleted lymphocytes were stimulated with original donor cells. After 5 days of cell culture, proliferation of lymphocytes was determined using CFSE (CellTrace, Invitrogen) and flow cytometry (FACSCalibur, BD Biosciences). All assays were carried out in triplicate.

Lymphocytes were depleted of $CD19^+CD24^{hi}CD38^{hi}$ TrBs using a FACSAria cell sorter (BD Biosciences). Lymphocytes and depleted lymphocytes were stimulated with irradiated pooled third party or original donor cells. Cell cultures were run in parallel with the addition of a monoclonal anti–IL-10 antibody (anti–IL-10 mab, no azide, rat anti-human and viral IL-10; BD Biosciences) to block IL-10–mediated inhibition of T cell proliferation. After 7 days of cell culture, proliferation of patient lymphocytes was determined using CFSE (CellTrace, Invitrogen) and flow cytometry (FACSCalibur, BD Biosciences). All assays were carried out in triplicate.

Determination of Lymphocyte Subsets in Peripheral Blood

Relative and absolute numbers of lymphocyte subsets were determined in heparinized whole blood using Trucount tubes,

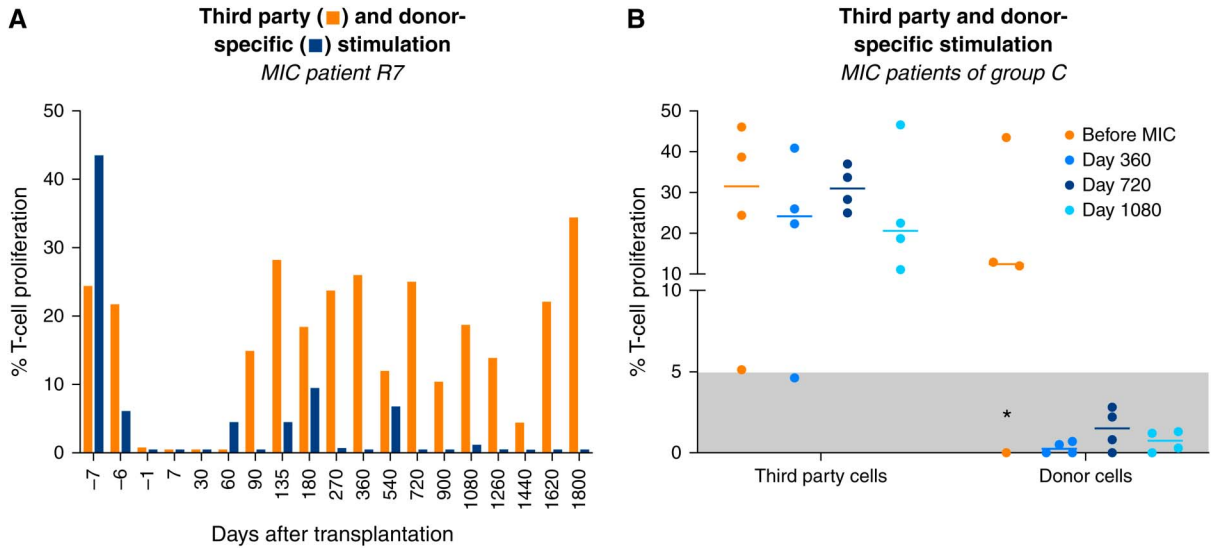

Figure 1. Third party and donor-specific lymphocyte response of MIC-treated patients *in vitro*. (A) Third party (orange bars) and donor-specific (dark blue bars) stimulation of blood lymphocytes from individual patient R7 is shown exemplarily for before (days −7, −6, −1) and after (days 7–1800) transplantation, as well as before (day −7) and after (from day −6) MIC infusion. (B) Third party and donor-specific stimulation of blood lymphocytes from MIC-treated patients of group C on day 360 (blue ●), day 720 (dark blue ●), and day 1080 (light blue ●) versus before MIC treatment on day −7 (orange ●). Individual measurements and median are shown. Values outside the normal range for healthy individuals are highlighted in gray. The findings indicate preserved immunologic responsiveness of recipient T lymphocytes against irradiated third party cells with reduced responsiveness to donor cells after transplantation compared with before transplantation and MIC infusion. Stimulatory cells consisted of irradiated allogeneic peripheral blood mononuclear cells. T lymphocyte proliferation was assessed by CFSE staining. *Patient R14 who had 0 HLA-A, -B, -DR mismatches with the donor.

monoclonal antibodies, and a FACSCalibur flow cytometer (all from BD Biosciences).

CD45 PerCP, CD3 FITC, CD4 APC, CD8 PE, CD19 APC, CD56+CD16 PE, CD20 APC, CD25 PE, and HLA-DR PE monoclonal antibodies were incubated with heparinized whole blood for 15 minutes at room temperature in the dark. Red cells were lysed for 15 minutes using NH_4Cl lysis solution (BD Biosciences). Thereafter, cells were ready for flow cytometric analysis.

When Tregs were determined, CD4 PerCP, CD25 APC, and CD127 FITC monoclonal antibodies (BD Biosciences) were incubated with heparinized whole blood for 30 minutes at room temperature in the dark. Red cells were lysed for 10 minutes using NH_4Cl lysis solution (BD Biosciences). Thereafter, cells were washed with PBS (Gibco, Grandisland, NY), permeabilized (Permeabilizing Solution 2, BD Biosciences) for 10 minutes at room temperature in the dark and washed again. FoxP3 PE monoclonal antibody was added for 30 minutes. Cells were washed, incubated in PBS for 30 minutes and washed again. Additional Treg subsets were determined using fluorochrome-labeled CD152, Helios, IL-10, and TGF-β1 monoclonal antibodies (BD Biosciences). Absolute and relative numbers of Tregs were analyzed using a FACSCalibur or a FACSCanto flow cytometer (BD Biosciences).

When regulatory B lymphocytes were determined, CD19 APC, CD24 PE, CD38 PerCP, and CD27 FITC monoclonal antibodies were incubated with freshly obtained heparinized whole blood for 30 minutes at room temperature in the dark. Red cells were lysed using NH_4Cl lysis solution (BD Biosciences) and washed in PBS (Gibco). Additional regulatory B lymphocyte subsets were determined using fluorochrome-labeled CD1d, CD25, CD71, CD73, and CD147 monoclonal antibodies. When IL-10–positive regulatory B lymphocytes were determined, cells were incubated with different monoclonal antibodies for 30 minutes, washed with PBS, permeabilized, washed, incubated with IL-10 FITC monoclonal antibody, and washed again. Absolute and relative numbers of regulatory B lymphocytes were analyzed using a FACSCalibur or a FACSCanto flow cytometer (BD Biosciences).

Immunohistochemical Staining for CD20, CD3, and FoxP3

Immunohistochemistry was performed on an automated immunostainer (Ventana BenchMark Ultra, Ventana Medical Systems, Tucson, AZ). Sections were cut, deparaffinized, rehydrated, and pretreated with an antigen retrieval buffer (Tris/Borat/EDTA, pH 8.4). After blocking of endogenous peroxidase, the slides were incubated with monoclonal antibodies directed against CD3 (clone 2GV6; Roche, Rotkreuz, Switzerland), CD20 (clone L26; Roche) at the provided dilutions of the ready-to-use kits and FoxP3 (clone D2W8E; Cell Signaling Technology, Danvers, MA) at a dilution of 1:25, followed by

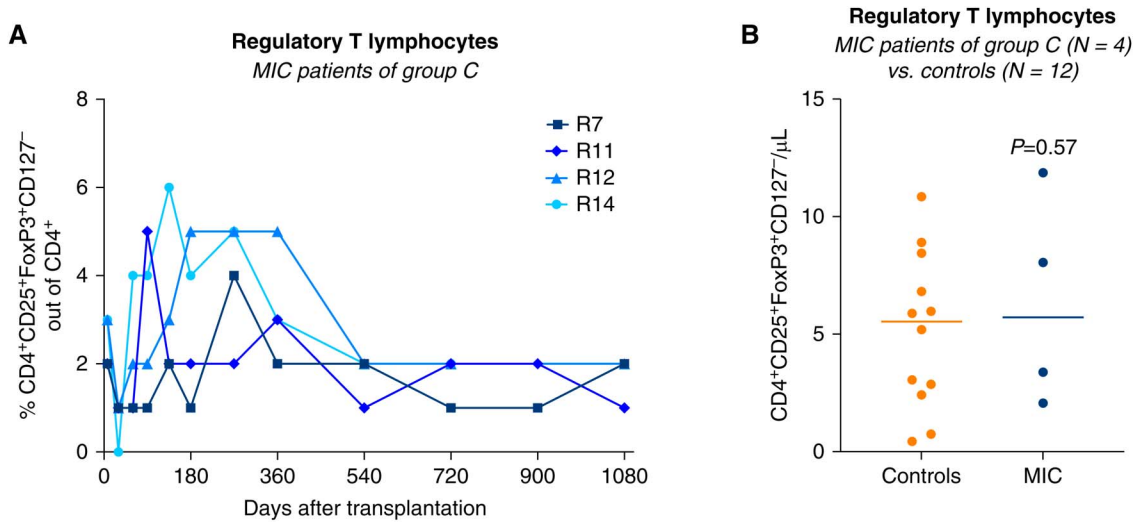

Figure 2. Regulatory T lymphocytes in MIC-treated patients compared with regulatory T lymphocytes in controls. (A) Individual measurements for percentage of $CD4^+CD25^+FoxP3^+CD127^-$ Tregs in patients R7 (■), R11 (◆), R12 (▲), and R14 (●) from day 0 to day 1080 are shown. (B) On day 1080, median Treg numbers in MIC-treated patients (dark blue ●) were not significantly different from numbers in 12 transplanted controls (orange ●) on triple drug immunosuppressive therapy matched for time after transplantation. Individual measurements and median are shown.

incubation with OptiView Amplifier for FoxP3. Visualization was achieved using DAB as chromogen. Before mounting, slides were counterstained with hematoxylin. After digitalization, slides were analyzed by selecting the high-power field (30,483 μm^2) with the highest leukocyte infiltration and counting the numbers of CD20-positive, CD3-positive, and FoxP3-positive lymphocytes in this area.

Determination of Cytokine and Chemokine Levels in Plasma

Plasma levels of cytokines and chemokines were determined according to the instructions of the manufacturer using the Luminex Performance Assays: Human High Sensitivity Cytokine Base Kit A, Human Cytokine Base Kit A, Magnetic Luminex Performance Assay Base Kit, TGF-β (R&D Systems, Wiesbaden, Germany). In total 350 μl plasma was incubated with antibody-coated beads for 3 hours, followed by biotin-labeled antibody for 1 hour and fluorochrome-conjugated streptavidin for 30 minutes. Assays were analyzed using the Luminex LX100/200 system (Luminex B.V., MV 's-Hertogenbosch, The Netherlands).

Gene Expression Analysis

PBMCs were collected by Ficoll-Paque density gradient centrifugation and stored in RNAprotect Cell Reagent (Qiagen, Hilden, Germany) at −80°C. Phenol-free total RNA was extracted with RNeasy Plus Mini Kits (Qiagen), and reverse transcription was carried out with a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. For quantitative real-time PCR, the RNA was quantified and normalized using an ultraviolet/visible photometer (Eppendorf, Hamburg, Germany). Gene expression of a consensus gene expression signature of operational tolerance in kidney transplantation[17] was measured by TaqMan gene expression assays (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific) according to the manufacturer's instructions (Supplemental Table 4). Relative gene expression values were calculated by the comparative $\Delta\Delta$Ct method using geometric means of four housekeeping genes (*ACTB, B2M, GAPDH*, and *HPRT1*) as reference. Recently published multivariable linear regression models were used for drug adjustment and calculation of predicted probabilities.[17]

Statistical Analyses

Because of the small number of patients and the exploratory approach of the trial, mostly descriptive statistical methods were applied. For comparison with different control groups, the two-group Mann–Whitney *U* test and the one-way ANOVA with Tukey's multiple comparisons test were used as deemed appropriate. In the text and tables, continuous data are summarized as the median and range and categorical data as absolute and relative frequencies. Longitudinal data are displayed over time, stratified by group. Unless otherwise indicated, the results in figures are presented as individual measurements (and the median) or the median and interquartile range.

Study Approval

The TOL-1 study was reviewed and approved by the ethics committee of the University of Heidelberg, Heidelberg, Ger-

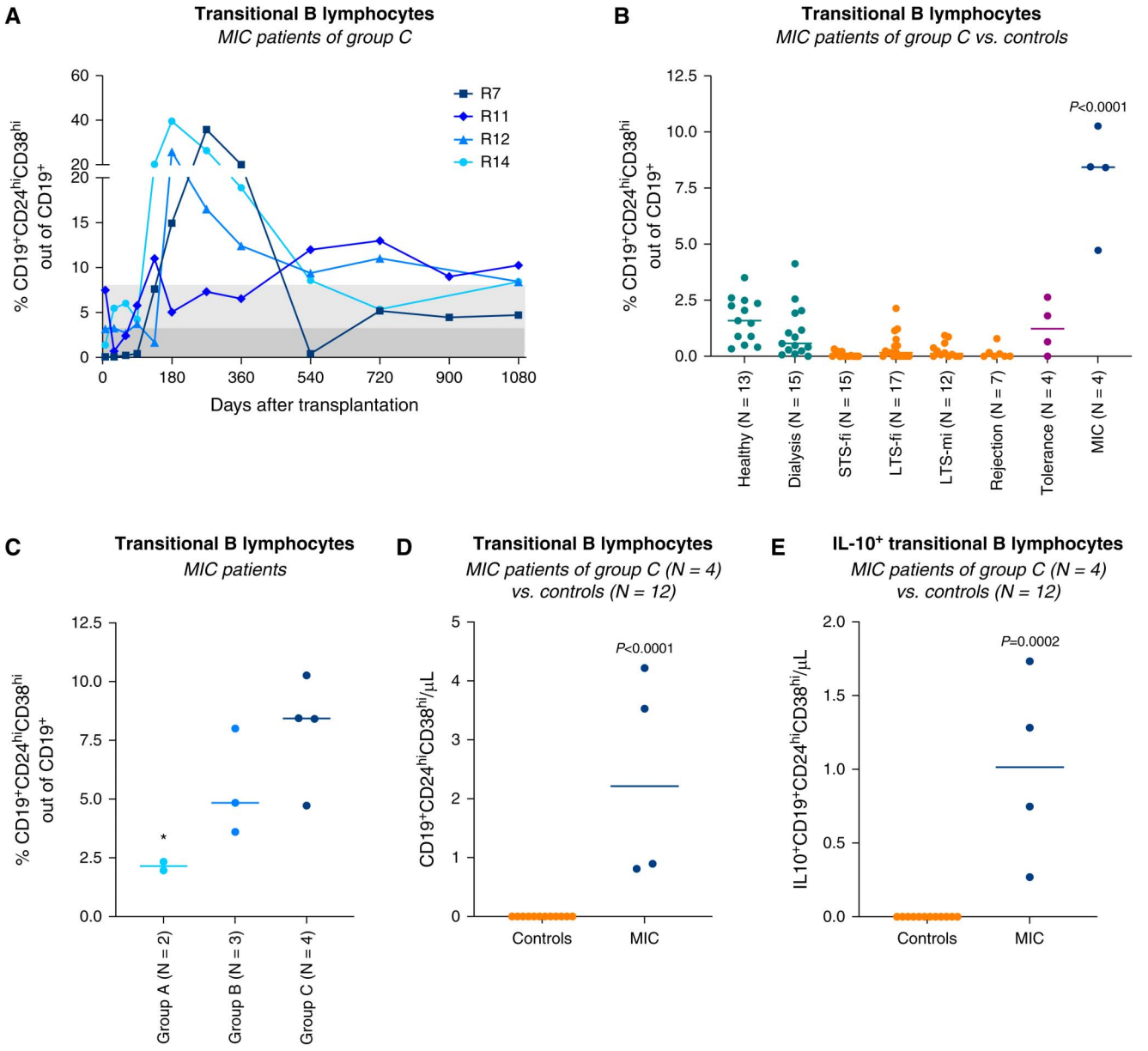

Figure 3. Regulatory B lymphocytes in MIC-treated patients compared with regulatory B lymphocytes in controls. (A) Individual measurements for percentage of $CD19^+CD24^{hi}CD38^{hi}$ TrBs in MIC-treated patients R7 (■), R11 (◆), R12 (▲), R14 (●) from day 0 to day 1080 are shown. TrBs were highest from day 135 to day 360 after kidney transplantation and remained above baseline values during follow-up. Values usually found in transplanted patients on triple drug immunosuppressive therapy are highlighted in dark gray, values usually found in operationally tolerant patients in light gray. (B) Percentage of TrBs in MIC-treated patients of group C (dark blue ●) were compared with measurements in healthy controls or patients on dialysis (green ●), transplanted controls (orange ●), and four operationally tolerant patients without MIC infusions (magenta ●). Individual measurements and median are shown. On day 1080, TrBs in MIC-treated patients of group C were 75 and seven times higher compared with TrBs in transplanted long-term survivors on minimal immunosuppression (LTS-mi) or operational tolerant patients, respectively ($P<0.001$). (C) There was a stepwise increase of TrB percentages from group A to C patients. (D) $CD19^+CD24^{hi}CD38^{hi}$ and (E) $IL\text{-}10^+CD19^+CD24^{hi}CD38^{hi}$ TrB numbers in MIC-treated patients of group C (dark blue ●) were compared with numbers in a second independent cohort of 12 transplanted controls (orange ●). Individual measurements and median are shown. On day 1080, TrBs and IL-10–producing TrB numbers in MIC-treated patients of group C were >100 times higher compared with TrB numbers in transplanted controls ($P<0.001$). LTS, transplanted long-term survivor; STS, transplanted short-term survivor; fi, full immunosuppression; mi, minimal immunosuppression. *Not enough biomaterial was available for measurement in patient R1.

many, and the Paul-Ehrlich-Institut, Langen, Germany (ethics number: AFmo-549/2014; Paul-Ehrlich-Institut, Vorlagen-Nr. 2252/01; EudraCT number: 2014-002086-30; Clinicaltrials.gov identifier: NCT02560220). The study was performed in compliance with the provisions of the Declaration of Helsinki and Good Clinical Practice guidelines. Written informed consent was obtained from participants before inclusion in the study. During follow-up, patient data and biomaterials were collected and analyzed according to protocols approved by the ethics committee of the University of Heidelberg (ethics numbers: 082/2005, 083/2005, S-395/2011, and S-225/2014).

RESULTS

Clinical Outcomes out to Year 3 after Transplantation

This is the 3-year follow-up of patients who originally participated in a 30-day, single-arm, phase 1 clinical trial to determine the safety and feasibility of intravenous administration of donor-derived MICs for individualized immunosuppression in living donor kidney transplant recipients (TOL-1 study, Supplemental Figure 1 and Supplemental Table 1).[6] Patients received a prescribed dose of either 1.5×10^6 MICs/kg body weight on day −2 (n=3, group A) or 1.5×10^8 MICs/kg body weight on day −2 (n=3, group B) or day −7 (n=4, group C, patients R7, R11, R12, R14) before living donor kidney transplantation, in addition to post-transplant immunosuppression with CyA, EC-MPS, and methylprednisolone. Immunosuppressive therapy was modified in group C patients to lower CyA and lower EC-MPS doses without corticosteroids during follow-up beyond day 30 to minimize the risk of infectious complications of combined cell-based and chemical immunosuppressive therapy (Supplemental Figure 2). During follow-up to day 1080, no *de novo* DSA and no acute rejection episodes were detected (Table 1 and Supplemental Table 5). Kidney graft function was stable with a median serum creatinine of 1.40 mg/dl (range 1.04–2.10 mg/dl), a median eGFR according to the CKD Epidemiology Collaboration (CKD-EPI) of 61 ml/min per 1.73 m^2 (range 41–93 ml/min per 1.73 m^2), and a median urinary protein excretion of 14 g/mol creatinine (range 5–90 g/mol creatinine, Supplemental Figure 3). No opportunistic infections and no cytomegalovirus or BK virus replications were diagnosed during intensive post-transplant screening. A total of 12 nonopportunistic infectious episodes were noted in six of the ten patients, with only two episodes occurring during years 2 and 3. No malignancy or post-transplant lymphoproliferative disorders were detected (Table 1).

Reduced Antidonor T Lymphocyte Responses

Group C patients had received the highest MIC dose 7 days before transplantation. Extensive immunologic testing was performed in these patients because, on the basis of preclinical studies and the 1-year results of this phase 1 study, it was anticipated that these patients would show the strongest donor-specific immunosuppression.[6–11,14–16] For example, measurement in patient R7 showed decreased responsiveness to cells from the original donor already 1 day after MIC infusion, with preserved responsiveness to third party cells (Figure 1A). This effect was maintained throughout follow-up to day 1800 (year 5), the latest follow-up for this patient. When all four patients from group C were analyzed, they showed preserved lymphocyte proliferation in response to allogeneic stimulation with third party cells before and after transplantation as an indication of an intact general immune response (Figure 1B). In contrast, the T cell response against the specific donor was absent on days 360, 720, and 1080, indicating persistent donor-specific unresponsiveness.

Lymphocyte Subpopulations and Tregs

Because no donor chimerism was detectable in MIC-treated patients,[6] it is reasonable to assume that regulatory cells of the recipient must have been responsible for the donor-specific immunosuppression after MIC infusion. Supplemental Figure 4 shows the evolution of different lymphocyte subpopulations and Figure 2A gives the evolution of $CD4^+CD25^+FoxP3^+CD127^-$ Tregs as percentage of the total $CD4^+$ lymphocyte pool for the four patients of group C to day 1080 after transplantation. Treg percentages (as well as numbers of different Treg subsets, Supplemental Figure 5) showed a transient rise to a median of 4.5% (range 2%–5%) on day 270, but remained stable thereafter at a median of 3% (range 2%–5%), 2% (range 1%–2%), and 2% (range 1%–2%) on days 360, 720, and 1080, respectively, compared with before MIC infusion (median 2.5%, range 2%–4%, Figure 2A). On day 1080, Treg numbers in the four MIC-treated group C patients (median 5.7/μl, range 2.1–11.9/μl) were not significantly different from Treg numbers in 12 matched transplanted controls on standard immunosuppressive therapy (median 5.5/μl, range 0.4–10.8/μl, P=0.68, Figure 2B). Likewise, numbers of FoxP3-positive cells in graft biopsies appeared not to be different between group A, B, and C patients (Supplemental Figure 6).

Strongly Increased Regulatory B Lymphocyte Frequencies

In contrast to Tregs, analysis of regulatory B lymphocytes revealed impressive changes during the course after transplantation. $CD19^+CD24^{hi}CD38^{hi}$ TrBs as the percentage of the total $CD19^+$ lymphocyte pool increased from a median of 6% (range 0%–11%) before MIC infusion and transplantation to 20% (5%–40%) on day 180. TrBs subsequently dropped to a median value of 8% (range 5%–13%) by day 720 and remained in this range to day 1080 (median 8%, range 5%–10%). These values are still markedly higher than baseline TrB percentages and also higher than percentages reported in the literature for stable immunosuppressed (0%–5%, indicated in Figure 3A in dark gray)[18–20] or operationally tolerant patients (3%–8%, indicated in Figure 3A in light gray).[12,13,21–26] TrB percentages of

Table 1. Outcomes and complications in MIC-treated patients out to day 1080

Parameter	Total Treated (*n*=10)	Group A (*n*=3)	Group B (*n*=3)	Group C (*n*=4)
Patients with biopsy-proven rejection, *n* (%)	1 (10)	1 (33)	0 (0)	0 (0)
Rejection episodes, *n*	1	1	0	0
Acute TCMR (≥Banff IA)	0	0	0	0
Chronic active TCMR	1	1[a]	0	0
Acute ABMR	0	0	0	0
Chronic active ABMR	0	0	0	0
Patients with *de novo* DSA, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)
Patients with opportunistic infections, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)
Infectious episodes, *n*	0	0	0	0
Pneumonia	0	0	0	0
CMV reactivation >1000 copies/ml	0	0	0	0
BKV replication >10,000 copies/ml	0	0	0	0
BKV-associated nephropathy	0	0	0	0
Other infection	0	0	0	0
Patients with nonopportunistic infections, *n* (%)	6 (60)	2 (67)	1 (33)	3 (75)
Infectious episodes, *n*	12	2	1	9
CV-associated infection	1	1	0	0
Urinary tract infection	7	0	1[a]	6
Postoperative wound infection	1	0	0	1
Pneumonia	1	0	0	1
Other infection	2	1[a]	0	1
Patients with PTLD or malignancy, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)

TCMR, T cell–mediated rejection; ABMR, antibody-mediated rejection; DSA, donor-specific HLA-A, -B, -DR, -DQ antibodies; CMV, cytomegalovirus; BKV, BK virus; CV, central venous catheter; PTLD, post-transplant lymphoproliferative disease.
[a]Episodes during years 2 and 3.

group C patients were further compared with TrB percentages of controls without MIC infusion (Figure 3B). On day 1080 after transplantation, TrB percentages in MIC-treated patients of group C were five, 15, >100, 52, and >100 times higher than TrB percentages in healthy controls (n=13), dialysis patients (n=15), short-term survivors on full immunosuppression (STS-fi, n=15), long-term survivors on full immunosuppression (LTS-fi, n=17), and chronic rejectors (n=7), respectively (P<0.001 for all comparisons). Strikingly, TrB percentages on day 1080 were 75-fold higher than TrB percentages in 12 transplanted control patients with well-functioning grafts and low immunosuppressive therapy (long-term survivors with minimal immunosuppression, LTS-mi) and still seven-fold higher than TrB percentages in four stable, operationally tolerant patients without immunosuppressive therapy (P<0.001 for both comparisons, Figure 3B). When comparing MIC-treated group C patients with a second independent cohort of 12 transplanted control patients who received regular immunosuppressive therapy and were matched for the post-transplant period, the TrB numbers were >100-fold higher (P<0.001, Figure 3D). Importantly, the IL-10–producing TrB numbers were also >100-fold higher than in transplanted controls (P<0.001, Figure 3E and Supplemental Figure 7) suggesting that TrBs in MIC-treated group C patients are capable of producing the immunosuppressive cytokine IL-10. This is also reflected by the persistently detectable levels of IL-10 in the serum of patients during post-transplant follow-up (Supplemental Figure 8C). Although there was no appreciable increase in TrB percentages in patients of groups A and B during the first year after transplantation,[6] it now appeared that there was a dose-dependent increase in TrB percentages from patients of group A (median 2%, range 2%–2%) to those of group B (median 5%, range 4%–8%, Figure 3C) at year 3 after surgery. As expected from the preclinical experiments, the greatest increase was observed in group C patients (median 8%, range 5%–10%, Figure 3, B and C). In addition, significantly higher numbers were found for other regulatory B lymphocyte subsets in different stages of B cell development including $CD19^{+}CD24^{hi}CD27^{+}$ memory B lymphocytes (P=0.029, Supplemental Figure 9).

Three of Four MIC-Treated Group C Patients Have the *COMBINED-g7* Consensus Gene Expression Signature of Operational Tolerance

Because MIC-treated patients of group C had immunologic characteristics similar to those of patients with operational tolerance, we were further interested in whether these patients also had the recently published consensus gene expression signature of operational tolerance (*COMBINED-g7*).[17] The mean changes in unadjusted and drug-adjusted gene expression of the seven studied genes are shown in Figure 4, A and B, respectively. Strikingly, using the drug-adjusted *COMBINED-g7* consensus gene expression signature, three of four (75%) MIC-treated patients in group C were identified as operationally tolerant at 3 years with a high probability of >0.85 (cutoff 0.32, Figure 4C). MIC-treated patients of group A or B (with

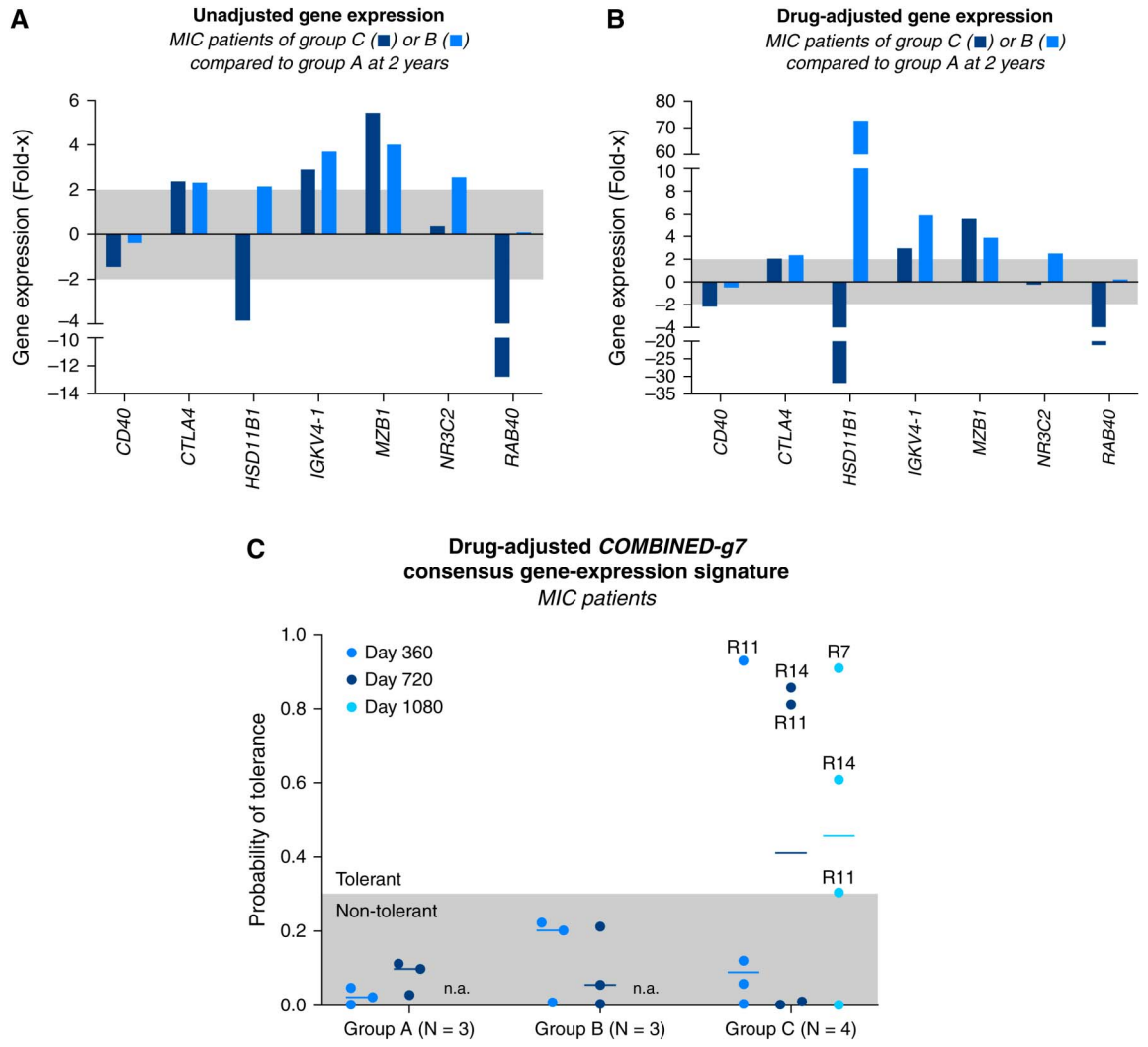

Figure 4. ***COMBINED-g7* consensus gene expression signature of operational tolerance.** (A and B) Mean changes in unadjusted (A) and drug-adjusted gene expression (B) of the seven studied genes are shown. In group C (dark blue bars) or B patients (blue bars), a three- and 5.9-fold increase in drug-adjusted *IGKV4-1* gene expression was observed compared with group A patients (B). (C) Using the drug-adjusted *COMBINED-g7* consensus gene expression signature, three of four (75%) MIC-treated patients in group C were identified as operationally tolerant at 3 years with a high probability of >0.85 (cutoff 0.32). Individual measurements and median are shown.

only 2 years of follow-up) and patient R12 from group C (with up to 4 years of follow-up, 4-year data not shown) were not classified as operationally tolerant. Interestingly, patient R12 differed from the other group C patients in that he appeared to have a preactivated immune system from the very beginning (and also before MIC administration) as indicated by higher activated $CD8^+$ cells (Supplemental Figure 10). Despite the preactivation, this patient never developed DSA or rejection during rigorous screening.

Immunosuppressive Properties Are Located in the B Cell Compartment

MIC-treated patients showed a marked increase in (IL-10–producing) regulatory B lymphocytes. If these lymphocytes indeed are responsible for the immunosuppressive effect of MIC therapy, B cell depletion should lead to a reversal of the effect. To test this hypothesis, we compared the *in vitro* antidonor T cell response of the four MIC-treated group C patients with the T cell response of four transplanted controls without MIC infusions. The response against the respective specific donor was absent in MIC-treated patients, whereas transplanted controls showed preserved T lymphocyte reactivity to cells derived from their donors (Figure 5A). When the same *in vitro* experiment was repeated after B cell depletion of the recipient lymphocytes, no change in antidonor T cell reactivity was observed in the transplanted controls (Figure 5B). In contrast, in MIC-treated group C patients, T cell reactivity against stimulatory donor blood

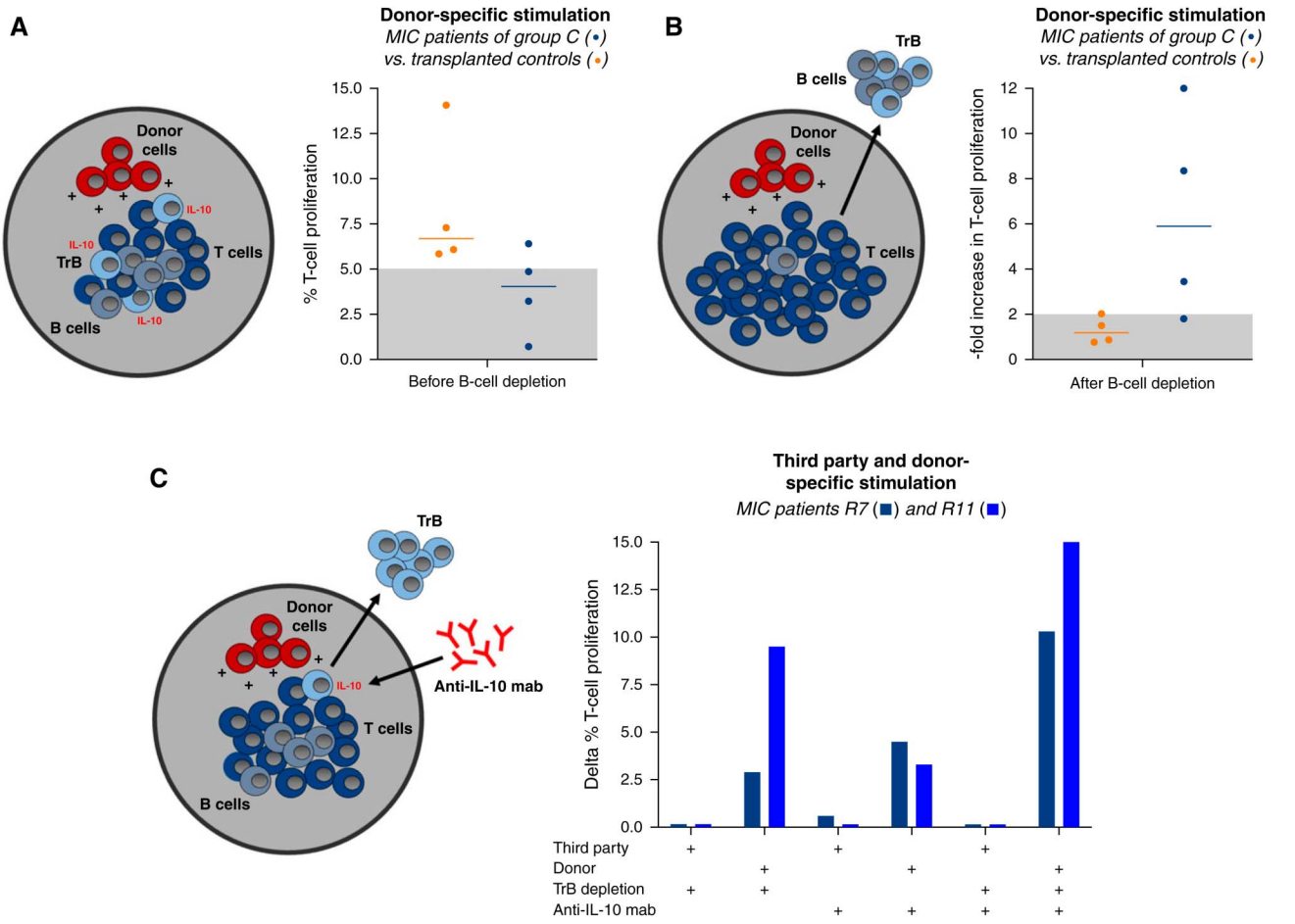

Figure 5. Third party and donor-specific lymphocyte response of MIC-treated patients after B lymphocyte depletion and IL-10 neutralization *in vitro*. (A and B) Donor-specific stimulation of blood lymphocytes from MIC-treated patients of group C (dark blue ●) were compared with measurements in transplanted controls (orange ●). Individual measurements and median are shown. Values outside the normal range for healthy individuals are highlighted in gray. (A) Compared with MIC-treated patients, transplanted controls without MIC treatment showed higher antidonor T cell response. (B) When the same analysis was performed after B lymphocyte depletion of recipient cells, T cell reactivity against stimulatory donor blood cells was restored in MIC-treated compared with transplanted control patients. (C) Antidonor T cell response of blood lymphocytes from MIC-treated patients R7 (dark blue bars) and R11 (blue bars) increased after depletion of $CD19^{+}CD24^{hi}CD38^{hi}$ TrBs, and/or addition of a monoclonal anti–IL-10 antibody (anti–IL-10 mab) indicating a functional relevance of this regulatory cell type. Individual increase in T cell proliferation after compared with before intervention (TrB depletion, anti–IL-10 mab) is shown. Stimulatory cells consisted of irradiated allogeneic peripheral blood mononuclear cells. T lymphocyte proliferation was assessed by CFSE staining.

cells was restored after B cell depletion, suggesting a direct pathophysiologic role of regulatory B lymphocytes in donor-specific unresponsiveness.

We now wanted to know whether TrBs were responsible for these observations and whether the effects of TrBs were mediated *via* the immunosuppressive cytokine IL-10. Therefore, we repeated the above experiment with depletion of TrBs instead of total B lymphocytes and in the absence or presence of an anti–IL-10 mab in two group C patients (R7 and R11) in whom sufficient biomaterial was available (Figure 5C). In this experiment, the antidonor T cell response increased by a median of 6% when TrB-depleted patient lymphocytes were stimulated with donor cells, and by a median of 13% after simultaneous treatment with the anti–IL-10 mab. In contrast, no such increase was observed during stimulation with third party cells. This can be interpreted as an indication of a functional relevance of IL-10–producing TrBs for donor-specific unresponsiveness.

DISCUSSION

The present analysis is a 3-year follow-up of ten patients from a phase 1 clinical trial who originally received MICs for the prevention of kidney transplant rejection.[6] The four patients of group C, who had received the highest MIC

number 7 days before surgery, showed persistent donor-specific immunosuppression as indicated by an absence of post-transplant cellular stimulation reactivity when tested against their respective donors. This was accompanied by a consistent increase of IL-10–producing $CD19^{+}CD24^{hi}CD38^{hi}$ TrBs and other regulatory B lymphocyte subpopulations at different stages of maturation. Most strikingly, donor-specific immunosuppression of the antidonor T cell response was abolished *in vitro* after B lymphocyte depletion, suggesting a direct pathophysiologic role of regulatory B lymphocytes in donor-specific unresponsiveness. These findings together with a gene expression signature consistent with operational tolerance in three of four (75%) MIC-treated group C patients provide compelling evidence that we were able to actively induce an operationally tolerant phenotype by high-dose MIC treatment 7 days before surgery.

B lymphocytes were first identified as a potential regulatory cell type when a randomized controlled trial found an excessively increased risk of T cell–mediated rejections after anti-CD20 compared with IL-2 receptor antibody induction therapy in kidney transplant recipients.[27] This finding led to the hypothesis that a particular cell type within the B lymphocyte compartment must have regulatory rather than alloreactive properties. Shortly thereafter, two independent research groups identified specific B lymphocyte gene signatures in the blood of operationally tolerant as compared with stable immunosuppressed patients.[12,13] Flow cytometric analysis revealed that TrBs in particular were markedly elevated in these patients, a finding later confirmed by other groups.[21–26] Two studies of tolerance induction by infusion of polyclonal Tregs within the framework of The One Study also showed increased regulatory B lymphocyte percentages as a significant flow cytometric result[3–5] (although describing a slightly different phenotype), and an increase in regulatory B lymphocytes has recently been reported after chimerism induction with combined hematopoietic stem cell and kidney transplantation.[28] Higher regulatory B lymphocyte numbers were not only observed in operationally tolerant patients but also associated with good kidney graft function in stable immunosuppressed patients.[18–20] In a prospective study of 73 kidney transplant recipients, Shabir *et al.* reported that TrBs were linked to rejection-free survival.[18] In that study, patients with >3% TrBs showed no rejection episodes. Comparable results were reported in the study of Tebbe *et al.*,[19] where higher TrB percentages were associated with rejection-free survival and better kidney graft function. We found an increase of TrBs from a median of 6% before MIC infusion and transplantation to a median of 20% on day 180 after transplantation, which was higher than frequencies reported in the literature and frequencies in transplanted control patients in our study. During follow-up to day 1080, TrB percentages remained above the 3% threshold reported by Shabir *et al.*[18] with median percentages of 4%–40%. These percentages are still considerably higher than percentages reported for stable immunosuppressed and operationally tolerant patients.[12,13,18–26] There has been speculation as to whether the high TrB levels are merely the result of reduced immunosuppression and not related to operational tolerance. This has now been ruled out in several studies. In a cross-sectional analysis of 117 transplant recipients, Bottomley *et al.* found no significant association between steroids and TrBs.[29] Rebollo-Mesa *et al.,* in the Genetic Analysis of Molecular Biomarkers of Immunologic Tolerance (GAMBIT) study, observed a slightly higher proportion of TrBs in patients receiving reduced or no corticosteroids, but this result was not fully explained by reduction of immunosuppression.[30] We have recently shown that higher TrB percentages were not associated with lower immunosuppression,[31] and already in our first analysis, we found that on day 135 after transplantation, before steroids were stopped in three of four group C patients, the median TrB percentage was already 9% (range 2%–20%), which was considerably higher than in transplanted controls.[6] In the current analysis, TrB percentages in MIC-treated patients were 75-fold higher than TrB percentages in patients with minimal immunosuppression (LTS-mi) and seven-fold higher than TrB percentages in operationally tolerant patients without MIC treatment, clearly showing that the increase in TrBs cannot be explained by immunosuppression reduction alone. Significantly higher TrB numbers, including higher numbers of IL-10–producing TrBs, were also found in comparison with a second independent cohort of kidney transplant recipients matched for time after surgery. In addition, there was an increase in TrB percentages from group A (infusion of a low MIC number 2 days before surgery) to group C (infusion of a high MIC number 7 days before surgery) patients. Most importantly, we were able to classify three of four (75%) MIC-treated group C patients as operationally tolerant on the basis of the recently published *COMBINED-g7* consensus gene expression signature, which adjusts gene expression for immunosuppressive therapy.[17] Using the cutoff of 0.32, Christakoudi *et al.* "misclassified" only five of 186 (3%) stable kidney transplant recipients on immunosuppressive therapy and one of 34 (3%) chronic rejectors as operationally tolerant when validating this signature.[17]

One might argue that regulatory B lymphocytes may only be a marker or a pathophysiologic driver of stable graft function and operational tolerance. Data from animal models, however, suggest that regulatory B lymphocytes directly promote allograft tolerance (*e.g.*, by upregulation of IL-10, TGF-β, or the induction of Tregs).[32–34] Compared with transplanted controls, we found significantly increased frequencies of IL-10–producing TrBs in MIC-treated patients which was reflected in detectable serum levels of IL-10 (and TGF-β1).[6] An increase of the immunosuppressive cytokines was detected as early as 2–7 days after MIC infusions, before transplantation, as indicated by proteome analysis.[6] Further support for the assumption that regulatory B lymphocytes are pathophysiologically relevant in the promotion of tolerance come from our *in vitro* experiments. MIC-treated patients showed a

suppressed T lymphocyte response to donor antigen compared with immunosuppressed control patients. When recipient B lymphocytes were removed from culture, there was a restoration of recipient lymphocyte reactivity to donor antigen, whereas no such change was observed in immunosuppressed patients without MIC treatment. This can be interpreted as an indication for an immunosuppressive activity of these B lymphocytes with the active component being the IL-10–producing TrBs.

At this stage we can only speculate on the exact mechanisms of regulatory B lymphocyte induction. Data from clinical studies show that prevention of costimulatory signaling (*e.g.*, by belatacept) led to improved kidney graft function that was associated with increased frequencies of IL-10–producing regulatory B lymphocytes in graft biopsies.[35] In preclinical studies, Khiew *et al.* recently demonstrated allograft tolerance in a mouse heart transplantation model after treatment with a costimulatory blocker together with donor spleen cells, an approach resembling MIC treatment.[36] In this model, tolerance was mediated by tolerant B cells that were able to suppress naive B cell production of IgG in an antigen-specific manner. MICs were infused into the patient immediately after GMP production, when they were still viable but no longer able to divide. At that stage, we saw low expression of stimulatory molecules (*e.g.*, costimulatory molecules CD80 and CD86, and HLA-DR) and high expression of immunosuppressive molecules (*e.g.*, adrenomedullin).[7,9,37] While in the patient's body, they entered an early apoptotic phase and were immediately phagocytosed by the patient's antigen-presenting cells without eliciting a proinflammatory response.[7,9]

In this study, we found a transient increase in Treg frequencies (and various Treg subset numbers) out to day 360, similar to the changes observed for TrBs, but no significant differences at 3 years compared with 12 transplanted controls. We also found no clear evidence of increased FoxP3-positive Treg numbers in the available graft biopsies. Nevertheless, we cannot exclude the possibility that Tregs play a critical role in the induction and persistence of donor-specific unresponsiveness. It is known that IL-10 produced by TrBs promotes differentiation of $CD4^+$ T cells toward a Treg phenotype,[38,39] and only recently Louis *et al.* found a concomitant sharp decrease in Treg and TrB numbers in DSA-positive patients who later developed antibody-mediated rejection, as compared with those who did not, again highlighting the possible importance of both regulatory (T and B) cell populations in maintaining stable graft function.[40]

This study has several limitations. We show the 3-year follow-up of ten patients from a 30-day, single-arm, phase 1 clinical trial, in which only four patients received the highest MIC number 7 days before surgery, which was found most effective. Control groups were selected retrospectively, and MIC-treated patients were still on (albeit reduced) immunosuppressive therapy during follow-up. In addition, HLA matching happened to be marginally better in group C compared with group A and B patients. We are well aware that these results are still preliminary and require further validation. Nevertheless, the results of high regulatory B lymphocyte numbers after MIC infusions together with our *in vitro* findings of abolished donor-specific unresponsiveness after B lymphocyte depletion, and more specifically after removal of TrBs, lead us to hypothesize that donor-specific immunosuppression is mediated by regulatory B lymphocytes.

In summary, MIC infusions before living donor kidney transplantation led to a dose-dependent increase in regulatory B lymphocyte populations and long-lasting donor-specific immunosuppression. We feel that the observation of long-lasting MIC-induced donor-specific unresponsiveness, although preliminary because the main finding is based on only four patients, is striking and highly promising for the development of improved treatment of transplant patients. A phase 2 study is underway to test the hypothesis that a larger series of MIC-treated patients, compared with patients treated with standard of care but without MIC infusions, will develop an operationally tolerant-like phenotype (EudraCT number: 2021-000561-33, ClinicalTrials.gov Identifier: NCT05365672).

DISCLOSURES

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AUTHOR CONTRIBUTIONS

D. Czock, C. Kleist, C. Morath, G. Opelz, M. Schaier, A. Schmitt, M. Schmitt, C. Sommerer, P. Terness, and M. Zeier conceptualized the study; D. Czock, V. Daniel, C. Morath, G. Opelz, M. Schaier, A. Schmitt, M. Schmitt, P. Terness, and M. Zeier were responsible for methodology; V. Daniel, C. Morath, M. Schaier, M. Schmitt, and M. Zeier were responsible for resources; C. Kleist, C. Morath, M. Schaier, A. Schmitt, and M. Schmitt were responsible for funding acquisition; C. Morath and M. Schaier were responsible for project administration, software, validation, and visualization; C. Morath, M. Schaier, P. Terness, and M. Zeier were responsible for supervision; L. Benning, L. da Silva, U. Merle, C. Morath, C. Nusshag, G. Ponath, M. Schaier, A. Schmitt, C. Sommerer, C. Speer, and M. Zeier were responsible for investigation; V. Daniel, C. Morath, and G. Opelz wrote the original draft; C.M. Alvarez, M. Aly, L. Benning, G.A. Böhmig, L. da Silva, V. Daniel, A. Hückelhoven-Krauss, E. Ibrahim, F. Kälble, C. Kleist, A. Mehrabi, U. Merle, C. Morath, C. Müller-Tidow, C. Nusshag, J. Reiser, M. Schaier, S. Scherer, C. Schwab, A. Schmitt, M. Schmitt, P. Schnitzler, C. Sommerer, C. Speer, C. Süsal, P. Terness, T. Tran, R. Waldherr, L. Wang, and M. Zeier were responsible for data curation and formal analysis and reviewed and edited the manuscript; and D. Czock and G. Opelz reviewed and edited the manuscript.

DATA SHARING STATEMENT

All data used in this study are available in this article.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://links.lww.com/JSN/B632.

Supplemental Table 1. Baseline patient characteristics.

Supplemental Table 2. Baseline characteristics of selected controls for Figures 2B, 3D, 3E, 5A, and 5B and Supplemental Figure 9.

Supplemental Table 3. Baseline characteristics of selected controls for Figure 3B.

Supplemental Table 4. Gene symbols and TaqMan assay IDs.

Supplemental Table 5. Biopsy results in MIC-treated patients out to day 1080.

Supplemental Figure 1. TOL-1 study design and follow-up to day 1080.

Supplemental Figure 2. Immunosuppressive therapy.

Supplemental Figure 3. Kidney function and integrity.

Supplemental Figure 4. Lymphocyte subpopulations.

Supplemental Figure 5. Regulatory T lymphocyte subsets.

Supplemental Figure 6. Immunohistochemistry in biopsy specimens.

Supplemental Figure 7. Gating strategy for IL-10^{+}CD19^{+}CD24^{hi}CD38^{hi} TrBs.

Supplemental Figure 8. Cytokines.

Supplemental Figure 9. Regulatory B lymphocyte subsets.

Supplemental Figure 10. Course of lymphocytes in individual patients.

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