Generation of Donor-specific T Regulatory Type 1 Cells From Patients on Dialysis for Cell Therapy After Kidney Transplantation

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Background. T regulatory type 1 (Tr1) cell–mediated induction of tolerance in preclinical models of transplantation is remarkably effective. The clinical application of such a therapy in patients on dialysis undergoing kidney transplantation should take into account the possible alterations of the immune system observed in these patients. Herein, we aimed at testing the ability to generate donor-specific Tr1 cell–enriched lymphocytes from patients on dialysis on the waiting list for kidney transplantation. **Methods.** The Tr1 cell–enriched lymphocytes were generated by coculturing interleukin-10–producing dendritic cells obtained from healthy donors with peripheral blood mononuclear cells (PBMCs) of patients on dialysis, following the same protocol used in a previous cell therapy clinical trial to prevent graft-versus-host disease. Alternatively, purified $CD4^+$ T cells were used instead of total PBMCs. The ability to generate clinical-grade Tr1 cell–enriched products was defined by testing the reduced response to restimulation with mature dendritic cells generated from the original donor (i.e., anergy assay). **Results.** The Tr1 cell–enriched medicinal products generated from PBMCs of patients on dialysis showed a low anergic phenotype, incompatible with their eventual clinical application. This was irrespective of HLA matching with the donor or the intrinsically reduced ability to proliferate in response to alloantigens. On the contrary, the use of purified $CD4^+$ T cells isolated from patients on dialysis led to the generation of a highly anergic donor-specific medicinal product containing an average of 10% Tr1 cells. **Conclusions.** The Tr1 cell–enriched medicinal products can be efficiently generated from patients on dialysis by carefully tailoring the protocol on the patients' immunological characteristics.

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Kidney transplantation is the only curative treatment for patients suffering from end-stage renal disease on dialysis.[1,2] At present, combined immunosuppressive treatments decrease the incidence of acute rejection, achieving 1-year graft survival rates above 90% in many transplant centers.[3] Unfortunately, the efficacy of immunosuppressive drug treatment is counter-balanced by undesired side-effects such as nephrotoxicity,[4] metabolic disorders,[5] cardiovascular diseases,[6] infections,[7] and malignancies.[8] Therefore, drug minimization and induction of donor-specific tolerance is a key clinical goal.[9]

T regulatory cells (Tregs) have been shown to induce tolerance after transplantation in several preclinical models.[10,11] The Tregs are categorized into 3 major subgroups based on their ontogeny[12,13]: thymus-derived Tregs, which develop in the thymus and are present in healthy individuals from birth; peripheral Tregs (pTreg), which are generated in the periphery under various tolerogenic conditions; and in vitro–induced Tregs. T regulatory type 1 (Tr1) cells are a subset of pTreg characterized by elevated production of interleukin (IL)-10 selectively in response to the antigen (Ag) they have been primed with.[14] Tr1 cells are induced by Ag stimulation via an IL-10–dependent process in vitro and in vivo.[14] Our group performed the first-in-man clinical trial infusing IL-10–anergized donor T cells containing Tr1 cells specific for the host Ags to prevent graft-versus-host disease (GvHD) after hematopoietic stem cell transplantation (ALT-TEN trial).[15] The medicinal product containing host-specific Tr1 cells and memory T cells able to respond to pathogens (named mixed lymphocyte reaction (MLR)/10) was infused in patients with hematological cancers after hematopoietic stem cell transplantation. The long-term follow-up showed no safety concerns relating to the cell therapy, complete

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TABLE 1.

Characteristics of subjects used as responders for the generation of the MLR/DC-10 cell products in Figure 2A. Data are median (interquartile range)

	Patients (n = 8)	Symbol	Controls (n = 8)
Age, y	50.5 (31-59)		45 (36-56)
Sex (M)	5/8		4/8
Time of dialysis, y	3.1 (1.3-4.7)		N/A
Cause of CKD	Hypertension (n = 1)	●	N/A
	IgA nephropathy (n = 1)	■	
	PKD (n = 1)	▲	
	T1D (n = 1)	▼	
	CAKUTs (n = 1)	◆	
	Unknown (3)	● ◒ ⊗	
Dialysis modality	HD: n = 7		N/A
	PD: n = 1	⊗	

CKD indicates chronic kidney disease; PKD, polycystic kidney disease; T1D, type 1 diabetes; CAKUT, congenital anomalies of the kidney and urinary tract; HD, hemodialysis; PD, peritoneal dialysis.

immune reconstitution in 5 of 12 patients, disease remission, and reduced severity of acute GvHD in 4 patients.[15] Ag-specific Tr1 cells have been also used in patients with Crohn disease.[16]

The ONE Study (www.onestudy.org) is a cooperative international multicenter clinical trial aiming at developing and testing the safety of different immunoregulatory cell products in living donor kidney transplant recipients.[17] Our group participates to this study to test the tolerogenic ability of a donor-specific Tr1 cell–enriched medicinal product. The fundamental prerequisite for the clinical use of Tr1 cells in kidney transplanted patients is the development of an effective method for their ex vivo generation from patients on dialysis. The immune system of these patients is characterized by several immunological alterations, such as (i) lymphopenia,[18] (ii) presence of activated proinflammatory monocytes,[19,20] (iii) reduced frequency of highly immunogenic circulating dendritic cells (DC),[21-23] (iv) defective humoral immunity,[24] (v) hyporesponsiveness to T-cell priming,[25] and (vi) high levels of plasmatic proinflammatory molecules.[25-27] Importantly, thymus-derived Tregs expanded in vitro from patients on dialysis are less suppressive and more plastic (i.e., more prone to produce IL-17) as compared to those generated from control healthy subjects.[28] Conversely, few data are available on pTreg cells generated from patients on dialysis.[29] Berglund et al[30] reported donor-specific IL-10 production from a cell population generated by coculturing T cells from patients on dialysis with immature DCs (iDCs) from healthy subjects. Our group previously characterized a subset of IL-10–producing human dendritic cells, termed DC-10, which can be induced in vitro from circulating monocytes in the presence of IL-10.[31] Coculture of in vitro–generated DC-10 with allogeneic peripheral blood mononuclear cells (PBMCs) for 10 days in the presence of exogenous IL-10 (MLR/DC-10) was shown to be an effective way of generating Tr1 cell–enriched products.[32] Accordingly, we expect to use donor-derived DC-10 for the generation of Tr1 cell–enriched medicinal product in The ONE Study. However, no data on the functional characterization of the MLR/DC-10 from patients on dialysis has been reported. Herein, we aimed at testing whether the protocol used for the generation of MLR/DC-10 protocol previously developed by our group[32] is suitable for generating donor-specific Tr1 cell–enriched product to be used in The ONE Study. Our findings indicate that the MLR/DC-10 cell product generated by using total PBMCs shows low anergic phenotype when obtained from patients on dialysis. Therefore, we optimized the protocol by depleting non-CD4$^+$ cells and obtained a cell product endowed with the safety features required for clinical application.

MATERIALS AND METHODS

Healthy Donors and Patients

Peripheral whole blood was collected from patients with end-stage renal disease on dialysis waiting for kidney transplantation at the San Raffaele Hospital (n = 13) and from family-related kidney donors (n = 5) or from healthy volunteer blood donors (n = 11). Buffy coats from healthy volunteers (n = 25) were also obtained. Kidney living donors and volunteer blood donors were both considered healthy control subjects in this study and named *controls*. Our goal was to test the protocol for Tr1 cell generation using a representative patient population likely to be enrolled in The ONE Study clinical trial at our clinical center. Therefore, patients were not stratified for dialysis modality, pathogenesis of kidney failure, or uremic versus dialysis but rather all patients on waiting list for kidney transplantation at our institute were included in this study. Characteristics of patients and healthy controls are listed in Table 1 (includes donors used for the generation of MLR/DC-10 cell products) and Table 2 (includes donors used for the generation of CD4$^+$/DC-10 cell products). All subjects enrolled in this study provided written informed consent before blood withdrawal, in accordance with the local ethics committee's approval (protocol PERIBLOOD) and with the Declaration of Helsinki.

DC Generation

The IL-10–producing DC (named DC-10) and mature DC (mDC) were generated from control subjects as previously described.[31,32] Briefly, PBMCs were isolated from peripheral blood or buffy coats collected from controls by density-gradient centrifugation on Lymphoprep (Axis-Shield, Oslo, Norway). CD14$^+$ monocytes were isolated by autoMACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and cultured in RPMI 1640 (BioWhittaker, Verviers, Belgium) supplemented with 10% FCS (BioWhittaker) and 100 U/mL

TABLE 2.

Characteristics of patients used as responders for the generation of CD4/DC-10 cell products shown in Figure 5A. Data are median (interquartile range)

	Patients (n = 5)	Symbol
Age, y	45 (26-48)	
Sex (M)	3/5	
Time of dialysis, y	2.5 (4)	
Cause of CKD	Hypertension (n = 1)	●
	SLE (n = 1)	■
	PKD (n = 1)	▲
	T1D (n = 1)	⊗
	Unknown (1)	▼
Dialysis modality	HD: n = 4	
	PD: n = 1	⊗

M indicates male; CKD, chronic kidney disease; SLE, systemic lupus eritematosus; PKD, polycystic kidney disease; T1D, type 1 diabetes; HD, hemodialysis; PD, peritoneal dialysis.

penicillin-streptomycin (BioWhittaker) with 10 ng/mL rhIL-4 (R&D Systems, Minneapolis, MN) and 100 ng/mL rhGM-CSF (R&D Systems, Minneapolis, MN, USA) for 7 days in the presence (DC-10) or absence (mDC) of 10 ng/mL rhIL-10 (CellGenix GmbH, Freiburg, Germany). Mature DCs were matured on day 5 with lipopolysaccharide from *Escherichia. coli* (1 μg/mL, Sigma Chemicals, St Louis, MO).

MLR/DC-10 and CD4/DC-10 Generation

Irradiated DC-10 generated from controls were used as stimulators, whereas total PBMCs (for MLR/DC-10 generation) or immunomagnetic positively selected $CD4^+$ T cells (for CD4/DC-10 cell product generation) from controls or patients on dialysis were used as responders with a responder-to-stimulator ratio of 10:1. The PBMCs or $CD4^+$ T cells were cocultured with DC-10 in X-VIVO 15 medium (BioWhittaker) supplemented with 5% human AB serum (BioWhittaker) and 100 U/ml penicillin-streptomycin for 10 days with addition of exogenous IL-10 as previously described.[32] As reference, in each experiment, mDC from control subjects were generated in parallel to DC-10 and upon irradiation cocultured with PBMCs (for MLR/mDC generation) or $CD4^+$ T cells (for CD4/mDC generation) for a total of 10 days without IL-10 supplementation.

Anergy Assay

At the end of the 10-day coculture, MLR/DC-10 and MLR/mDC or $CD4^+$/DC-10 and CD4/mDC cell products were collected, washed, and plated in a secondary MLR with donor-derived mDC at 10:1 ratio (responder:stimulator) to test their antidonor responsiveness. ^{3}H-thymidine (Sigma-Aldrich) was added 48 hours after culture for the last 12 hours. Anergy is defined as donor-specific hyporesponsiveness, and it is calculated as follows: [MLR/mDC – MLR/DC-10:MLR/mDC] × 100 (or [CD4/mDC – CD4/DC-10:CD4/mDC] × 100). In the ALT-TEN trial, the anergy cutoff value for MLR/10-below which the cell product was not considered safe to be infused into patients-was 67%. The anergy cutoff value for CD4/DC-10 cell product instead, was set in this study at 60%. Those values were defined based on the average anergy -2 times standard deviation values obtained from experiments performed in more than 30 control subjects.[15] The anergy assay was the release criterion for the MLR/10 medicinal product used in the ALT-TEN trial.[15]

Flow Cytometry

The phenotype of in vitro generated mDC and DC-10 was evaluated by flow cytometry. The expression of the following surface markers was tested after culture: CD1a (anti-CD1a Alexa488), CD14 (anti-CD14 APC-H7), CD16 (anti-CD16 APC-H7), and CD86 (anti-CD86 PE). All monoclonal antibodies were obtained from BD Pharmingen (San Jose, CA). Cells were washed 2 times with (PBS, 0.5-1%, 10% fetal bovine serum, 0.1% NaN3 sodium azide) and incubated at room temperature for 30 minutes. The Tr1 cell enrichment in the CD4/mDC and CD4/DC-10 cell products was evaluated with the following monoclonal antibodies: CD3 PerCp-Cy5.5 (Biolegend), CD4 Pe-Cy7 (BD Bioscience, San Jose, CA), CD45RO PacificBlue (Biolegend), LAG-3 PE (R&D System), CD49b Alexa488 (Biolegend). Cells were incubated at 37 °C for 30 minutes instead of room temperature. Samples were acquired using the BD FACSCanto II (Becton Dickinson, San Jose, CA) and data were analyzed with FlowJo software.

Enzyme-Linked Immunoassorbent Assay

Supernatants were collected from the MLR/DC-10 and CD4/DC-10 coculture 96 hours after plating (primary MLR) or 48 hours after restimulation with donor-derived mDC (secondary MLR) to test interferon (IFN)-γ levels. Levels of IFN-γ were determined by capture enzyme-linked immunosorbent assay according to the manufacturer's instructions (BD Biosciences).[33] The detection limit of IFN-γ was 60 pg/mL.

Statistical Analysis

Comparisons between groups were performed using the Mann-Whitney *U* test. For all analyses, a 2-tailed *P* value of 0.05 or less was considered significant. Data are shown as medians. Statistical analyses were performed using the statistical software GraphPad Prism 6.0 (San Diego, CA).

RESULTS

The MLR/DC-10 Cell Product Generated from Patients on Dialysis Displays Low Anergic Phenotype

We first tested the feasibility of generating Tr1 cell–enriched products from the peripheral blood (PB) of patients on dialysis using the MLR/DC-10 protocol.[32] The DC-10 and mDCs were generated in vitro from $CD14^+$ monocytes purified from PBMC of controls. After 7 days of culture in the presence of polarizing cytokines, DC-10 and mDC were collected and analyzed by flow cytometry. Both cell subsets had the expected phenotype.[31] Namely, DC-10 were $CD1a^-CD14^+CD16^+CD86^+$, whereas mDCs were $CD1a^+CD14^-CD16^-CD86^{high}$ (Figure 1).

The MLR/DC-10 cell product was obtained by a 10-day coculture of DC-10 generated from PB or buffy coats of controls (stimulators) with PBMC obtained from patients on dialysis or control subjects (responders) in the presence of exogenous IL-10. In parallel, as control, the MLR/mDC cell product was generated. Characteristics of the subjects enrolled in the study and used to generated data shown in Figures 1–4 are listed in Table 1. Controls and patients on dialysis were matched for age and sex. Patients on dialysis were on renal replacement treatment (n = 7/8 on hemodialysis and n = 1/8 on peritoneal dialysis) for a median of 3.1 years, and the pathogenesis of chronic kidney disease was variable (including autoimmune diseases, hypertension, genetic and congenital diseases, or unknown diseases). To test the anergy of the MLR/DC-10 cell product, cells were restimulated with donor-derived mDC, and their proliferative capacity was compared to that of the MLR/mDC product restimulated with the same donor-derived mDC. We first tested the anergic phenotype of MLR/DC-10 obtained from coculturing PBMC from patients on dialysis and DC generated from the PB of family-related control subjects (Figure 2A, samples 1-5). As previously defined by our group, the cutoff anergy value for MLR/DC-10 cell products was set at 67%.[15] Such anergic value was not achieved in 3 of the 5 generated MLR/DC-10 cell products. To exclude that the reduced anergy observed in the MLR/DC-10 products was due to the partial HLA-matching between patients on dialysis and the family-related control donors, MLR/DC-10 cell products were generated with PBMC of patients on dialysis and DC-10 generated from the PB of unrelated healthy control donors who were assumed to be fully HLA mismatched. A limited anergic value was also

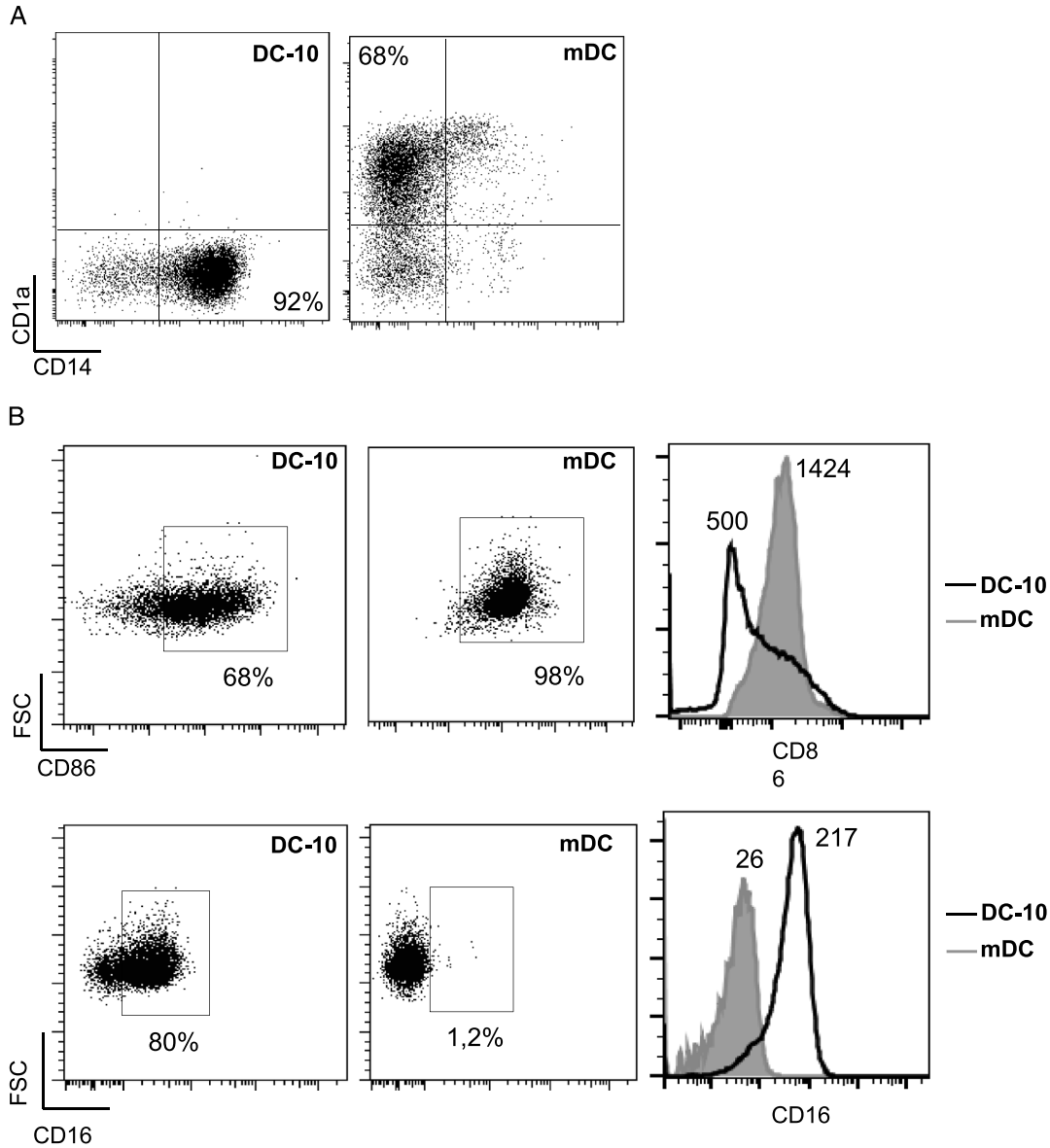

FIGURE 1. Phenotypic characterization of DC-10 and mDC generated from controls. A, Magnetically isolated $CD14^+$ cells were cultured for 7 days in presence of rhIL-10 obtaining a population of DC-10 that retains $CD14^+$ expression but lack the expression of CD1a (one representative dot plot is shown out of 5). Mature DCs, cultured in absence of rhIL-10 and matured with LPS at day 5. They lose CD14 and acquire CD1a expression (one representative dot plot is shown out of 5). B, Further characterization shows that CD86 expression is higher in terms of frequency (dot plots) and MFI (histograms) on mDC than DC-10 (1 representative dot plot and histogram is shown out of 5) and that DC-10 express high levels of CD16 whereas mDC are negative for this marker (1 representative dot plot and histogram is shown out of 5). Histograms show direct comparison of CD86 and CD16 on DC-10 (empty) and mDC (filled). Numbers indicate MFI of the tested marker. LPS indicates lipopolysaccharide; MFI, median fluorescence intensity.

detected in MLR/DC-10 cell products generated with fully mismatched pairs (Figure 2A, samples 6-8), suggesting that the level of HLA-match does not impact anergy induction. On the other hand, highly anergic MLR/DC-10 cell products were generated from all except 1 control subject, confirming our previous findings (Figure 2B).[32] Overall, MLR/DC-10 cell products generated from patients on dialysis showed reduced anergic phenotype as compared to those generated from control subjects (Figure 2C).

The MLR/mDC Culture Is a Proper Reference to Test Donor-Specific Anergy

It is known that T cells isolated from patients on dialysis display reduced cell proliferation in vitro upon culture with allogeneic PBMCs (i.e., in primary MLR assays) ([25] and data not shown). Given that the proliferation of MLR/mDC cell product is our reference value in the anergy assay, a possible explanation for the low anergic phenotype observed in the MLR/DC-10 cell products generated from patients on dialysis is that the MLR/mDC culture is an improper cell culture reference. Interestingly, both IFN-γ levels measured in the culture supernatants (Figure 3A) and cell proliferation (Figure 3B) were similar in MLR/mDC cell products generated from patients on dialysis and from control subjects. These data suggest that the MLR/mDC is a proper reference cell product to test the MLR/DC-10 cell product responsiveness.

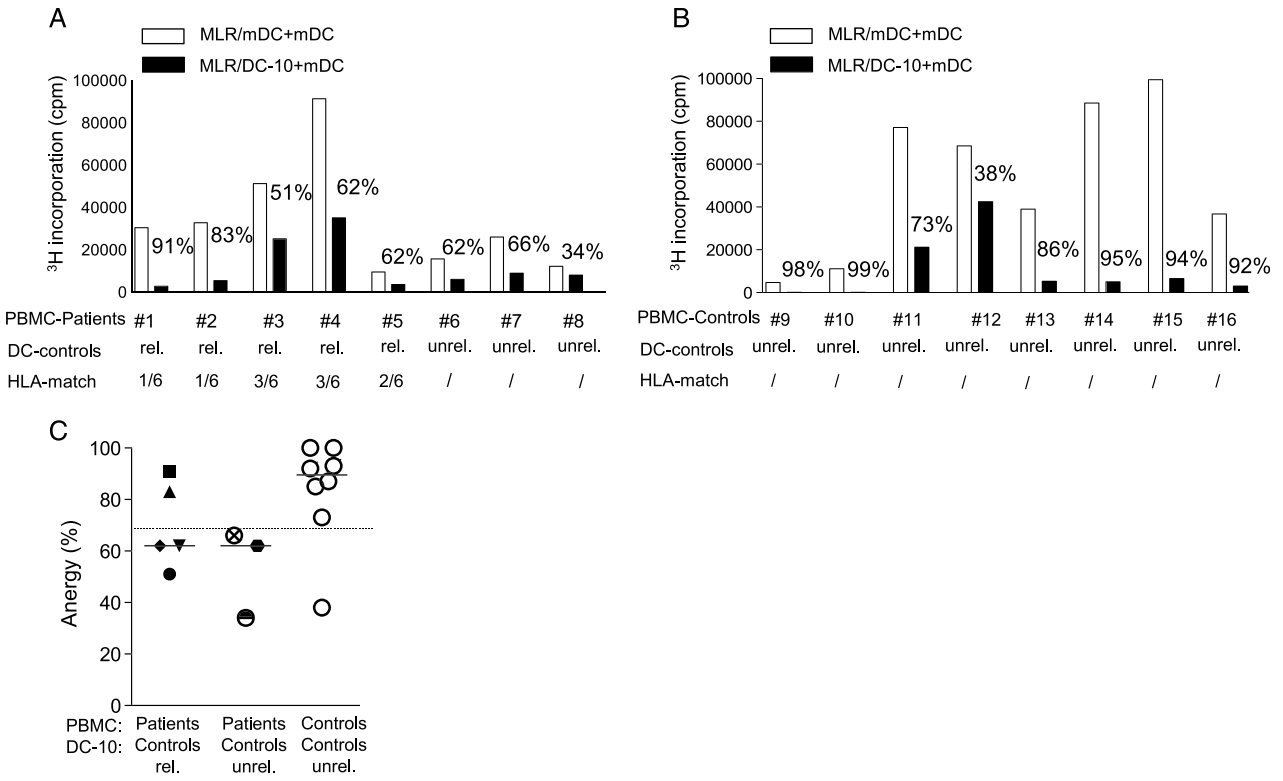

FIGURE 2. Anergic phenotype of MLR/DC-10 cell product generated from dialysis patients and controls. A, MLR/DC-10 cell product obtained by co-culturing PBMC from patients on dialysis with DC-10 derived from PB of controls (n = 5 family related and n = 3 unrelated donors) was restimulated with donor-derived mDC and cell proliferation was assessed by thymidine (^{3}H) incorporation. MLR/mDC cell product proliferation upon restimulation with donor-derived mDC was used as a reference value. The anergy value is shown as percentage on top of the bar graphs. Patients and controls characteristics are listed in Table 1. B, MLR/DC-10 and MLR/mDC cell products were generated using DCs generated from buffy coats of controls and PBMCs of unrelated healthy subjects. The anergy value is shown as a percentage on top of the bar graphs. Donor characteristics are not available. C, Anergy values of MLR/DC-10 cell products obtained from patients on dialysis and controls are shown. The dotted line is set at 67%, which is the cutoff value to consider the cell product safe for infusion into patients. Each symbol represents one patient. Controls were tested in parallel. Lines represent median value of each data-set (ns, Mann-Whitney *U* test). C.p.m indicates counts per minute; Ctrl: healthy controls; rel, relative; unrel, unrelated donor.

The Low Anergic Phenotype of MLR/DC-10 Cell Products From Patients on Dialysis Is Not Due to the Enrichment of IFN-γ–Producing Cells

It is known that PBMC from patients on dialysis produce more proinflammatory cytokines, such as IL-2[25,34] and IL-12.[35] Thus, we tested whether the reduced anergic phenotype detected in MLR/DC-10 cell products generated from patients on dialysis was due to enrichment in IFN-γ–producing cells during the 10-day cocultures. The IFN-γ levels measured in the culture supernatants were similar in patients on dialysis and control subjects (Figure 4), thus suggesting that the reduced anergy observed was likely not due to enrichment in IFN-γ–producing cells.

Development of a Novel Protocol for Tr1 Cell–Enriched Medicinal Product Tailored on Patients on Dialysis

An alternative explanation for the low MLR/DC-10 anergic phenotype observed when cells from patients on dialysis were used is the presence, in the starting PBMC, of cells that could hamper the in vitro differentiation of CD4$^+$ T cells into Tr1 cells. To test this, we set up a new protocol for the generation of Tr1 cell-enriched cultures (named CD4/DC-10) in which purified CD4$^+$ T cells (rather then total PBMC) from PB of patients or buffy coats from controls were cultured with DC-10 generated from buffy coats of controls in the presence of IL-10. In parallel, the control culture was obtained by coculturing CD4$^+$ T cells with mDC generated from buffy coats of control subjects (named CD4/mDC). Characteristics of this group of patients on dialysis used to generate data shown in Figure 5 are listed in Table 2.

The setup of the CD4/DC-10 protocol performed with PB of controls confirmed that 1:10 DC-10:CD4$^+$ cell ratio was optimal to obtain an anergic cell product and reproducible results (data not shown). In addition, the anergy cutoff value of the CD4/DC-10 cell product optimized on controls was set at 60% (data not shown). The CD4/DC-10 cell product was then tested for final cell composition, and it was confirmed to be purely composed of CD4$^+$ T cells (data not shown). The anergy assay was performed by restimulating CD4/DC-10 cell product with donor-derived mDC and by comparing their proliferative capacity to that of CD4/mDC cell product restimulated with the mDC of the same donors. This modified protocol led to the generation of a CD4/DC-10 cell product from patients on dialysis equally anergic to that obtained from controls (Figure 5A–B).

The Tr1 cell content in the CD4/DC-10 cell products was tested by measuring the frequency of CD4$^+$CD45RA$^-$LAG-3$^+$CD49b$^+$ T cells, according to the surface markers recently identified.[36] An average frequency of 10% of Tr1 cells in the CD4/DC-10 cell products was observed—while approximately only 1% of Tr1 cells in the CD4/mDC cell products—irrespective of whether obtained from patients

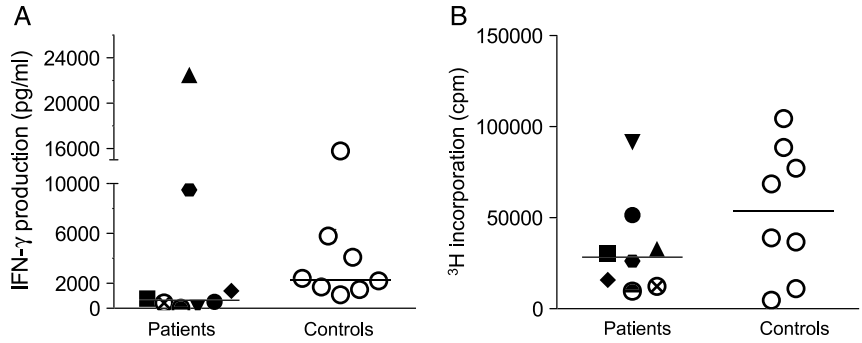

FIGURE 3. Characterization of the MLR/mDC cell products used as reference. MLR/mDC cell products obtained from patients on dialysis and controls were restimulated with donor-derived mDC. Levels of IFN-γ in the supernatant (A) and cell proliferation (B) were assessed 28 hours after activation. Each symbol represents 1 donor (listed in Table 1). Controls were tested in parallel. Lines represent median value of each data set (*ns*, Mann–Whitney-U test).

on dialysis or controls (Figure 5C for 1 representative plot). These data indicate that culturing CD4$^+$ T cells from patients on dialysis with DC-10 generated from healthy control subjects in the presence of exogenous IL-10 is an efficient method for the generation of Tr1 cell–enriched medicinal product to be used in cell therapy trials.

DISCUSSION

Our results show that anergic donor-specific Tr1 cell–enriched medicinal products for cell therapy application in the context of The ONE Study cannot be generated from dialysis patients using the MLR/DC-10 protocol previously developed by our group.[32] Thus, we set up a novel protocol by using purified CD4$^+$ T cells instead of total PBMC. This change led to the generation of a cell product suitable for cell therapy in patients with kidney failure.

The immune system of patients on dialysis has been extensively characterized, and it is currently recognized to be altered. Patients on renal replacement therapy are characterized by a proinflammatory peripheral status.[21,25,37] Nonetheless, T-cell responsiveness to in vitro alloantigen stimulation is impaired.[23] Given the well-known immunological alterations occurring in patients on dialysis, as a prerequisite for the initiation of cell therapy arm of The ONE Study clinical trial,[17] we tested whether the generation of anergic donor-specific Tr1 cell–enriched lymphocytes was feasible in these patients.

To date, only 1 group reported the generation of Tr1 cell–enriched products from patients on dialysis.[29] However, in this study, the cell product was generated by using iDC rather than DC-10 as donor-derived cells.[29] It is now well accepted that DC-10 are more effective than iDC for Tr1 cell induction.[31] In addition, despite the fact that IL-10 production was shown to be alloantigen-specific, no assessment of the cell product anergic phenotype (fundamental requirement for safety concerns) or Tr1 cell frequency was reported. Thus, there was a strong need for the development of a clinical grade protocol for the generation of donor-specific Tr1 cells from patients on dialysis.

The protocol previously developed by our group,[32] which envisaged the use of total PBMC as starting cells, did not lead to the generation of a clinical grade product. A Tr1 cell–enriched product endowed with a low anergic phenotype, indeed, represents a possible risk for the recipient because of the potential proliferation in vivo on encountering donor antigens after kidney transplantation.

The reduced ability to generate anergic Tr1 cell–enriched products from patients on dialysis using the MLR/DC-10 protocol might be due to the use of an improper reference for anergy assessment (i.e., MLR/mDC cell product proliferation). Indeed, the well-known T-cell hyporesponsiveness of dialysis patients toward alloantigens in a primary MLR[25] might also affect their responsiveness in a secondary MLR. However, our data indicate that both cell proliferation of and IFN-γ production by MLR/mDC cell products restimulated with mDC in a secondary MLR were not different from those in healthy donors, making the MLR/mDC cell product proliferation a reliable reference value for defining MLR/DC-10 cell product anergy. In addition, given the alteration of the immune system of patients on dialysis featured by the increase of proinflammatory cytokines, such as IL-2 and IL-12,[25,34,35] we excluded that the generation of highly anergic Tr1 cells was hampered by an increased IFN-γ production in the MLR/DC-10 cell product. Thus, we hypothesized that a proinflammatory cell fraction residing in the bulk PBMC population of patients on dialysis would hamper Tr1 cell–enriched product generation. Dendritic cells from patients on dialysis have for instance been described to induce high proliferation of T cells from healthy subjects.[23]

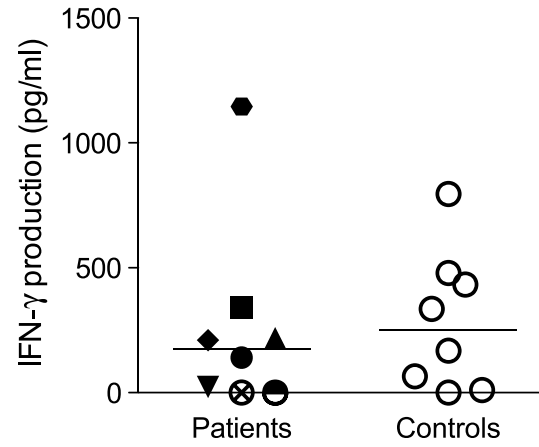

FIGURE 4. IFN-γ release from MLR/DC-10 cell products. After the 10-day coculture, MLR/DC-10 cell products were restimulated with mDC of the original donors, and IFN-γ levels were tested in the supernatant obtained from patients on dialysis, and controls. Each symbol represents 1 donor (listed in Table 1). The MLR/DC-10 cell products obtained from controls were tested in parallel. Lines represent median value of each data set (ns, Mann-Whitney *U* test).

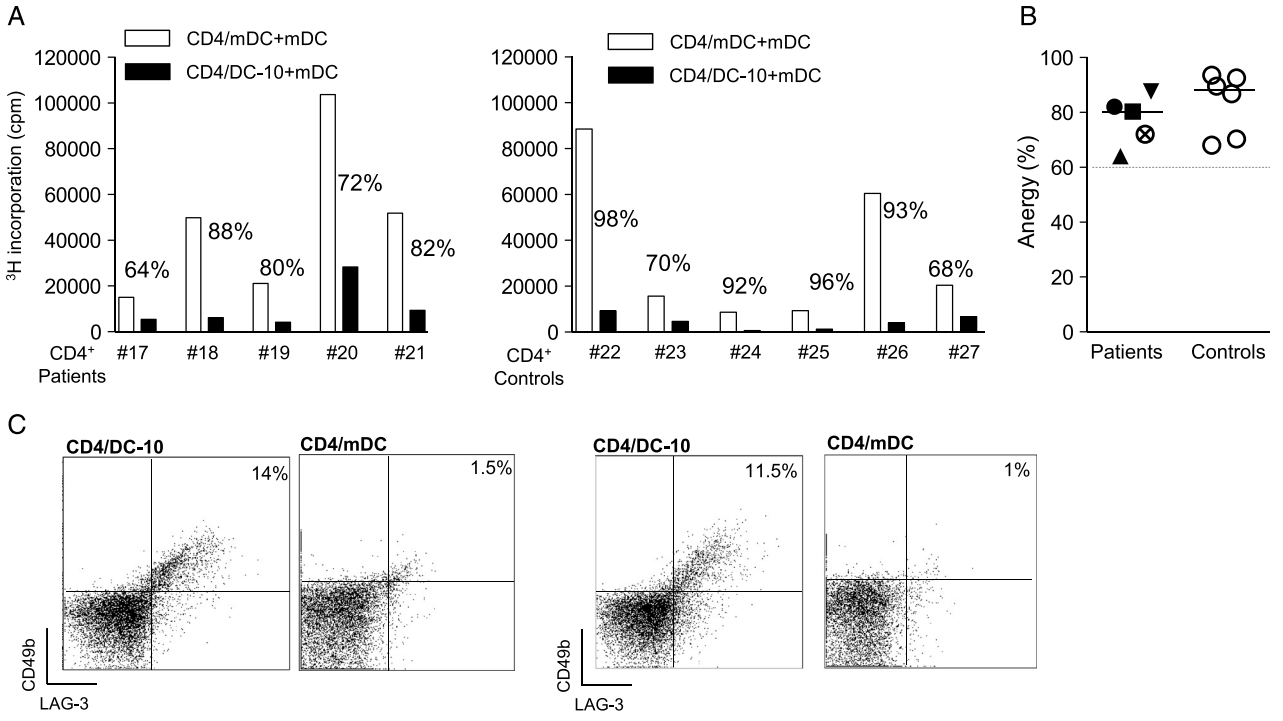

FIGURE 5. Characterization of the CD4/DC-10 cell products generated from patients on dialysis and controls. A, CD4/DC-10 obtained by co-culturing CD4$^+$ T cells from patients on dialysis (left) or controls (right) with control–derived DC-10 were restimulated with mDC derived from the original donors and cell proliferation was assessed by ^{3}H incorporation. The CD4/mDC cell product proliferation upon restimulation with mDC was used as reference value. The anergy value is shown as a percentage on top of the bar graphs. B, Anergy values of CD4/DC-10 cell products obtained from patients on dialysis and controls are shown (ns, Mann-Whitney *U* test). The dotted line is set at 60%, cutoff value to consider the CD4/DC-10 cell product safe for infusion into patients. C, Tr1 cell enrichment in the CD4/DC10 cell products generated from patients on dialysis (left panels) and controls (right panels) is shown as LAG-3$^+$CD49b$^+$ cells on CD4$^+$CD45RA$^-$ cells in both CD4/DC-10 and CD4/mDC cell products. One representative dot plot out of 3 is shown.

Moreover, monocytes produce high levels of proinflammatory cytokines on stimulation.[20] Thus, CD4$^+$ T cells of patients on dialysis were purified and cocultured with donor-derived DC-10 using the CD4/DC-10 protocol. Thanks to this novel approach, we were able to obtain a medicinal product with the following characteristics: (i) highly anergic toward donor-derived mDC; (ii) enriched in Tr1 cells (around 10%); and (iii) purely composed of CD4$^+$ T cells (whereas the MLR/DC-10 cell product was contaminated by a small fraction of CD8$^+$ T cells and natural killer cells, data not shown). Overall, the CD4/DC-10 cell product is endowed with the characteristics of safety that are required for clinical application.

Given the overwhelming uremia, continuous exposure to the dialysis filter membrane and nutritional deficiencies, the immune system of patients on dialysis lays in a proinflammatory status. This might affect the in vitro generation of Tr1 cell–enriched lymphocytes, rendering the MLR/DC-10 cell product not suitable for patients on dialysis, contrary to what was observed in patients with hematological cancer disease.[15] One explanation for the different ability to generate clinical grade Tr1 cell–enriched product between patients on dialysis and those with hematological cancer[15] may reside in the fact that, in the ALT-TEN study, T cells were collected from healthy donors, and DCs were generated from patients with the aim to prevent GvHD.[15] It might be possible that the generation of DC is less "patient-dependent" than is that of Tr1 cells for patients on dialysis.

The major limitation of our study is the inclusion of patients with a variety of primary and secondary kidney diseases that eventually all lead to renal replacement therapy. The limited number of patients enrolled in our study did not allow a direct correlation between disease etiology and lack of anergy in the MLR/DC10 cell products. Further studies enlarging the patient population to stratify for disease etiology are required to get hints on the mechanisms underlying the failure of Tr1 cell generation but using total PBMCs.

A relevant point of discussion is the in vivo effect of immunosuppressive drugs used in The ONE Study (i.e., mycophenolate mofetil and FK506) on Tr1 cells. Although data are too preliminary to draw definitive conclusions, immune monitoring performed so far on patients enrolled in the reference group trial of The ONE Study shows no negative effect of the abovementioned immunosuppressive drugs on Tr1 cells (data not shown).

An additional important issue is the number of Tr1 cells needed to be transferred to generate donor-specific tolerance. Data in preclinical models of islet transplantation suggest that 2×10^6 of CD4$^+$ T cells, containing an average 10% of Tr1 cells, induce graft tolerance in immunocompetent mice in the absence of active immunosuppression.[10] Direct translation from mouse to human is hard but one could anticipate that 10% of Tr1 cells in the final medicinal product should be enough to induce tolerance. Only data from clinical trials will hopefully elucidate this important matter.

To conclude, we want to emphasize the necessity of carefully screening the immune system of patients before

enrolling in cell therapy–based clinical trials. We believe in the relevance of setting up disease-tailored protocols aiming at minimizing possible negative outcomes and finally safeguarding patient health.

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REFERENCES

1. Sayegh MH, Carpenter CB. Transplantation 50 years later—progress, challenges, and promises. *N Engl J Med*. 2004;351(26):2761–2766.
2. Oniscu GC, Brown H, Forsythe JL. Impact of cadaveric renal transplantation on survival in patients listed for transplantation. *J Am Soc Nephrol*. 2005;16(6):1859–1865.
3. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. *N Engl J Med*. 2010;363(15):1451–1462.
4. Naesens M, Lerut E, Sarwal M, et al. Balancing efficacy and toxicity of kidney transplant immunosuppression. *Transplant Proc*. 2009;41(8):3393–3395.
5. Mathis AS, Dave N, Knipp GT, et al. Drug-related dyslipidemia after renal transplantation. *Am J Health Syst Pharm*. 2004;61(6):565–585.
6. Kasiske BL, Guijarro C, Massy ZA, et al. Cardiovascular disease after renal transplantation. *J Am Soc Nephrol*. 1996;7(1):158–165.
7. Morelon E, Stern M, Kreis H. Interstitial pneumonitis associated with sirolimus therapy in renal-transplant recipients. *N Engl J Med*. 2000;343(3):225–226.
8. London NJ, Farmery SM, Will EJ, et al. Risk of neoplasia in renal transplant patients. *Lancet*. 1995;346(8972):403–406.
9. Battaglia M, Roncarolo MG. Immune intervention with T regulatory cells: past lessons and future perspectives for type 1 diabetes. *Semin Immunol*. 2011;23(3):182–194.
10. Gagliani N, Jofra T, Stabilini A, et al. Antigen-specific dependence of Tr1-cell therapy in preclinical models of islet transplant. *Diabetes*. 2010;59(2):433–439.
11. Blazar BR, Taylor PA, Panoskaltsis-Mortari A, et al. Interleukin-10 dose-dependent regulation of CD4+ and CD8+ T cell-mediated graft-versus-host disease. *Transplantation*. 1998;66(9):1220–1229.
12. Abbas AK, Benoist C, Bluestone JA, et al. Regulatory T cells: recommendations to simplify the nomenclature. *Nat Immunol*. 2013;14(4):307–308.
13. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. *Immunity*. 2013;38(3):414–423.
14. Roncarolo MG, Gregori S, Battaglia M, et al. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*. 2006;212:28–50.
15. Bacchetta R, Lucarelli B, Sartirana C, et al. Immunological outcome in haploidentical-HSC transplanted patients treated with IL-10-anergized donor T cells. *Front Immunol*. 2014;5:16.
16. Desreumaux P, Foussat A, Allez M, et al. Safety and efficacy of antigen-specific regulatory T-cell therapy for patients with refractory Crohn's disease. *Gastroenterology*. 2012;143(5):1207–1217 e1-2.
17. Geissler EK. The ONE Study compares cell therapy products in organ transplantation: introduction to a review series on suppressive monocyte-derived cells. *Transplant Res*. 2012;1(1):11.
18. Yoon JW, Gollapudi S, Pahl MV, et al. Naive and central memory T-cell lymphopenia in end-stage renal disease. *Kidney Int*. 2006;70(2):371–376.
19. Kim HW, Woo YS, Yang HN, et al. Primed monocytes: putative culprits of chronic low-grade inflammation and impaired innate immune responses in patients on hemodialysis. *Clin Exp Nephrol*. 2011;15(2):258–263.
20. Malaponte G, Bevelacqua V, Fatuzzo P, et al. IL-1beta, TNF-alpha and IL-6 release from monocytes in haemodialysis patients in relation to dialytic age. *Nephrol Dial Transplant*. 2002;17(11):1964–1970.
21. Agrawal S, Gollapudi P, Elahimehr R, et al. Effects of end-stage renal disease and haemodialysis on dendritic cell subsets and basal and LPS-stimulated cytokine production. *Nephrol Dial Transplant*. 2010;25(3):737–746.
22. Hesselink DA, Betjes MG, Verkade MA, et al. The effects of chronic kidney disease and renal replacement therapy on circulating dendritic cells. *Nephrol Dial Transplant*. 2005;20(9):1868–1873.
23. Choi HM, Woo YS, Kim MG, et al. Altered monocyte-derived dendritic cell function in patients on hemodialysis: a culprit for underlying impaired immune responses. *Clin Exp Nephrol*. 2011;15(4):546–553.
24. Litjens NH, Huisman M, van den Dorpel M, et al. Impaired immune responses and antigen-specific memory CD4+ T cells in hemodialysis patients. *J Am Soc Nephrol*. 2008;19(8):1483–1490.
25. Hendrikx TK, van Gurp EA, Mol WM, et al. End-stage renal failure and regulatory activities of CD4 + CD25bright + FoxP3+ T-cells. *Nephrol Dial Transplant*. 2009;24(6):1969–1978.
26. Tarakcioglu M, Erbagci AB, Usalan C, et al. Acute effect of hemodialysis on serum levels of the proinflammatory cytokines. *Mediators Inflamm*. 2003;12(1):15–19.
27. Weichhart T, Kopecky C, Kubicek M, et al. Serum amyloid A in uremic HDL promotes inflammation. *J Am Soc Nephrol*. 2012;23(5):934–947.
28. Afzali B, Edozie FC, Fazekasova H, et al. Comparison of regulatory T cells in hemodialysis patients and healthy controls: implications for cell therapy in transplantation. *Clin J Am Soc Nephrol*. 2013;8(8):1396–1405.
29. Berglund D, Korsgren O, Lorant T, et al. Isolation, expansion and functional assessment of CD4 + CD25 + FoxP3+ regulatory T cells and Tr1 cells from uremic patients awaiting kidney transplantation. *Transpl Immunol*. 2012;26(1):27–33.
30. Berglund D, Karlsson M, Biglarnia AR, et al. Obtaining regulatory T cells from uraemic patients awaiting kidney transplantation for use in clinical trials. *Clin Exp Immunol*. 2013;173(2):310–322.
31. Gregori S, Tomasoni D, Pacciani V, et al. Differentiation of type 1 T regulatory cells (Tr1) by tolerogenic DC-10 requires the IL-10-dependent ILT4/HLA-G pathway. *Blood*. 2010;116(6):935–944.
32. Bacchetta R, Gregori S, Serafini G, et al. Molecular and functional characterization of alloantigen-specific anergic T cells suitable for cell therapy. *Haematologica*. 2010;95(12):2134–2143.
33. Levings MK, Gregori S, Tresoldi E, et al. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25 + CD4+ Tr cells. *Blood*. 2005;105(3):1162–1169.
34. Rostaing L, Peres C, Tkaczuk J, et al. Ex vivo flow cytometry determination of intracytoplasmic expression of IL-2, IL-6, IFN-gamma, and TNF-alpha in monocytes and T lymphocytes, in chronic hemodialysis patients. *Am J Nephrol*. 2000;20(1):18–26.
35. Libetta C, Rampino T, Dal Canton A. Polarization of T-helper lymphocytes toward the Th2 phenotype in uremic patients. *Am J Kidney Dis*. 2001;38(2):286–295.
36. Gagliani N, Magnani CF, Huber S, et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med*. 2013;19(6):739–746.
37. Costa E, Lima M, Alves JM, et al. Inflammation, T-cell phenotype, and inflammatory cytokines in chronic kidney disease patients under hemodialysis and its relationship to resistance to recombinant human erythropoietin therapy. *J Clin Immunol*. 2008;28(3):268–275.