Comparison of Regulatory T Cells in Hemodialysis Patients and Healthy Controls: Implications for Cell Therapy in Transplantation


Summary

Background and objectives Cell-based therapy with natural (CD4+CD25hiCD127lo) regulatory T cells to induce transplant tolerance is now technically feasible. However, regulatory T cells from hemodialysis patients awaiting transplantation may be functionally/numerically defective. Human regulatory T cells are also heterogeneous, and some are able to convert to proinflammatory Th17 cells. This study addresses the suitability of regulatory T cells from hemodialysis patients for cell-based therapy in preparation for the first clinical trials in renal transplant recipients (the ONE Study).

Design, setting, participants, & measurements Healthy controls and age- and sex-matched hemodialysis patients without recent illness/autoimmune disease on established, complication-free hemodialysis for a minimum of 6 months were recruited. Circulating regulatory T cells were studied by flow cytometry to compare the regulatory T cell subpopulations. Regulatory T cells from members of each group were compared for suppressive function and plasticity (IL-17–producing capacity) before and after in vitro expansion with and without Rapamycin, using standard assays.

Results Both groups had similar total regulatory T cells and subpopulations I and III. In each subpopulation, regulatory T cells expressed similar levels of the function-associated markers CD27, CD39, HLA-DR, and FOXP3. Hemodialysis regulatory T cells were less suppressive, expanded poorly compared with healthy control regulatory T cells, and produced IL-17 in the absence of Rapamycin. However, Rapamycin efficiently expanded hemodialysis regulatory T cells to a functional and stable cell product.

Conclusions Rapamycin-based expansion protocols should enable clinical trials of cell-based immunotherapy for the induction of tolerance to renal allografts using hemodialysis regulatory T cells.


Introduction

Although solid-organ transplantation is the treatment of choice for end stage kidney disease (ESKD), the use of broad-spectrum immunosuppressive drugs results in accelerated mortality (1), is toxic to transplants, and does not prevent chronic rejection (2). Thus, the establishment of clinical tolerance to engrafted tissues to minimize or eliminate immunosuppression is a key research goal.

Natural CD4+CD25hiCD127loFOXP3+ regulatory T cells (Tregs) that physiologically prevent autoimmune diseases by inhibiting target cells, including responder (CD4+CD25−) T cells (Tresps) and antigen-presenting cells (3), can prevent allograft rejection in animal models (4,5). Experimental induction of immunologic transplant tolerance in animal models is associated with increased Treg numbers in both the transplant and regional lymphoid tissue (6,7). This finding mirrors recent human data correlating Treg infiltration of renal transplants and outcome in borderline rejection (8).

Functional Tregs from healthy individuals can now be selected and expanded polyclonally in vitro under good manufacturing practice (GMP) conditions (4,9,10). Thus, clinical cell therapy with Tregs is a realistic possibility. Indeed, case series and phase I studies have shown beneficial outcomes in type 1 diabetes mellitus (11) and the prevention or treatment of post-bone marrow transplantation graft versus host disease in humans (9,12,13).

Human Tregs are heterogeneous, being divided into three functionally distinct populations based on differential expression of the naïve cell marker CD45RA, CD25, and the transcription factor FOXP3 (14): populations I (CD4+CD25hiCD127loCD45RA+), II (CD4+CD25brightCD127loCD45RA−), and III (CD4+CD25hiCD127loCD45RA−). Population I is the most abundant in umbilical cord blood and matures

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to populations III and II on activation. The latter are effector Tregs that are highly suppressive and short-lived, whereas the former are circulating memory-type Tregs (14). Other surface markers have also been used to delineate functional Treg subsets, such as costimulatory molecules (CD27) (15), ectoenzymes (CD39) (16), HLA-DR (17), and the memory T cell marker CD45RO (14,18). Treg phenotypes can be plastic (19), and some Treg subpopulations (especially those subpopulations in population III) (14) have the capacity to express proinflammatory cytokines and transcription factors more typically seen in Th1 and Th17 lineages (14,20,21). Consequently, successful programs of cell therapy will be critically dependent on the selection of the most appropriate Treg populations for infusion into humans.

ESKD patients awaiting transplantation are immunologically unique in showing features of both immunodeficiency (22–24) and chronic inflammation/immune dysregulation (25–27). ESKD patients have reduced peripheral blood Tregs (28) and naïve T cells (29), implying a deficiency of naïve Tregs, the population most resistant to Th17 conversion in vitro (14). Here, we sought to characterize the phenotype and function of Tregs from ESKD patients on hemodialysis (HD) compared with healthy controls (HCs) and determine if Tregs from these patients could be expanded ex vivo in GMP-compatible conditions.

Materials and Methods

Participant Selection

Fourteen patients with ESKD established on HD for at least 6 months without HD-associated complications and fourteen age- and sex-matched HCs were recruited after informed consent (Institutional Review Board approval 09/H0707/86). Exclusion criteria included recent illness (within the previous 2 months), significant anemia, autoimmune disease, current/previous immunosuppressive medicines, and previous transplantation. HCs were clinically well for at least 2 months. Participant characteristics are summarized in Table 1.

CD4+ T Cell Enrichment

PBMCs isolated from fresh blood as previously described (30) were enriched for CD4+ T cells using the MACS CD4+ T Cell Isolation Kit II (Miltenyi Biotech, Germany) according to the manufacturer's instructions. Enrichment was typically to >90% CD4+ lymphocytes.

Flow Cytometry

Flow cytometry using CD4+ T cells stained with CD4-Qdot 605, CD39-FITC, CD127-PerCP-Cy5.5, HLA-DR-APC-EF450 (eBioscience, United Kingdom), CD25-PE (MA251), CD27-APC-H7, CD45RO-PECy7 (all Becton-Dickinson, San Jose, CA), and CD45RA-AF700 (Biolegend, United Kingdom) was carried out on an FACSCalibur or LSR II flow cytometer (Becton-Dickinson). Appropriate isotype controls and Fluorescence Minus One controls were used to assign gates. Gating strategies used to identify Treg subpopulations are shown in Supplemental Figure 1 (14).

Intracellular staining for FOXP3 used the kit and anti–FOXP3-FITC from eBioscience according to the manufacturer's instructions. For double-double staining with IL-17-PE (eBioscience), PMA (50 ng/ml; Sigma), ionomycin (1 mM; Sigma), and Brefeldin A (3 μg/ml; eBioscience) were added to cell cultures 4.5 hours before intracellular staining.

Ex Vivo Treg Expansion

PBMCs were depleted for CD8+ followed by enrichment for CD25+ using magnetic beads (Miltenyi). Enriched cells were typically >95% CD25hiFOXP3+ (data not shown). Cells were plated at 10^6/ml in X-VIVO 15 Medium (Lonza, MD) containing 5% human AB serum (Biosera, United Kingdom) + 100 nM Rapamycin (31,32) (Rapamune, Wyeth, United Kingdom) and activated with anti-CD3/CD28–coated microbeads (2:1 bead:cell ratio; Dynabeads; Invitrogen, United Kingdom). HuRecombinant IL-2 (500 IU/ml; Proleukin; Novartis, United Kingdom) was added at day 4 and replenished every second day. Every 10–12 days, beads were removed by magnetic adherence, and

<table>
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<td>Sex (men:women)</td>
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N/A, not applicable; ESKD, end stage kidney disease

aThis patient initially had ESKD of unknown etiology, which was later assumed to be caused by IgA nephropathy. He has been retained in the analysis.

bBoth assumed to be secondary to hypertensive nephropathy.
cells were restimulated as before with fresh Rapamycin and IL-2.

**Suppression Assay**
Suppression of Tresp proliferation by Tregs was assessed using the standard carboxyfluorescein succinimidyl ester dilution method (33) comparing allogeneic Tresp from normal volunteers cultured alone with Tresp cocultured 1:1 with Tregs at Treg:Tresp ratios of 1:1, 1:5, and 1:10 (constant Tresp numbers). For comparison of suppression of Tresp by Tregs, the Tregs:Tresp ratio needed to cause 50% suppression (IC50; the higher the IC50, the less suppressive the Tregs) and maximum suppression (Smax) were calculated for each population as per the methods described in refs. 34 and 35.

**IL-17 Production**
To assess IL-17 production, Tregs were cultured in the presence of IL-2 (10 IU/ml), IL-1β (10 ng/ml), IL-6 (4 ng/ml), and TGF-β (5 ng/ml; R&D Systems) as described in ref. 32 before harvest of supernatants at 5 days.

ELISA
ELISA for human IL-17 was carried out using the DuoSet ELISA Kit from R&D (Abingdon, United Kingdom) according to manufacturer’s instructions and expressed as IL-17 concentration per million live cells in the assay.

**Data Analysis**
Data analysis used GraphPad Prism (LaJolla, CA). Data are presented as mean ± SD or median (interquartile range) for parametric and nonparametric data, respectively, and compared using paired t or Wilcoxon signed rank tests as appropriate. A P value <0.05 was considered statistically significant.

**Results**

**Participant Demographics**
Fourteen HD patients and fourteen age- and sex-matched HCs participated in this study based on the inclusion and exclusion criteria listed in Materials and Methods. Demographic data are summarized in Table 1.

**HD Patients Have Treg Populations Corresponding to HCs**
To compare circulating Tregs between HD patients and HCs, enriched CD4+ T cells were phenotyped by flow cytometry. The percentage of Tregs in the total CD4+ population did not differ between the two cohorts (5.2% ± 2.0% compared with 4.6% ± 1.7% for HCs and HD patients, respectively, P = 0.41) (Figure 1 and Table 2). Because previous reports had suggested that patients on HD should have fewer naïve Tregs relative to HCs (29), we next compared the three populations described in the work by Miyara et al. (14) in HCs and patients on HD. Population III was the largest subpopulation of Tregs in both groups (Figure 1 and Table 2). Although the proportions of Treg populations I and III were similar between both groups, a paucity of cells in population II was seen in HD patients (Figure 1).

Naïve CD45RA+ Tregs decrease with age, with a reciprocal increase in the proportion of CD45RO+ Tregs (36), which may influence the feasibility of isolating certain Treg populations for therapeutic use. To determine whether significant age-dependent changes in Treg subsets occurred in HD patients, the effect of age on Treg populations I and III was examined by linear regression. Neither group showed a significant reduction in population I Tregs (HC: R^2 = 0.02, P = 0.71; HD: R^2 = 0.004, P = 0.86) nor an appreciable increase in population III Tregs (HC: R^2 = 0.06, P = 0.49; HD: R^2 = 0.004, P = 0.86) with age.

To see whether differences existed in Treg activation and maturation between HCs and HD patients, the expression of Treg activation/maturation markers was compared between Treg populations in both groups (Figure 2) (14–18). As expected, in both cohorts, stepwise increases in the percentage of cells expressing CD39, HLA-DR, and FOXP3 between populations I, III, and II were seen, correlating with maturation status (Figure 2, B and C). Expression of these markers on Treg subpopulations did not, however, differ between patients on HD and HCs, indicating that Treg maturation and activation within individual subsets is similar between HD patients and HCs.

**Tregs from Dialysis Patients Can Be Expanded Using Existing GMP-Compliant Protocols While Retaining Suppressive Function**
Having established that HD patients have the same numbers of Tregs as HCs with normal expression of maturation and activation markers within subsets, albeit with slight reduction in population II, we next sought to determine whether they could be expanded in vitro under the GMP-compatible conditions (13,32) as required for a program of cell therapy.

Freshly isolated Tregs from five HD patients and five HCs were expanded in vitro as described in Materials and Methods in the presence or absence of Rapamycin. Baseline CD25 and FOXP3 expression was similar in Tregs isolated from both cohorts (Supplemental Figure 2, A and B). Tregs from both groups expanded rapidly in the presence or absence of Rapamycin (Figure 3A). Although Tregs from HD patients proliferated slower than the Tregs from HCs in the absence of Rapamycin, no significant difference was observed between HC and HD Tregs in the presence of Rapamycin (Figure 3A). Expression of FOXP3 and CD25 was comparable at the end of expansion (Supplemental Figure 2, C and D).

Carboxyfluorescein succinimidyl ester dilution assays were performed to assess the ability of freshly isolated and ex vivo expanded Tregs from HCs and HD patients to suppress proliferation of allogeneic Tresp (Figure 3B). To compare suppressive capacity of HC Tregs against those of Tregs from HD patients at baseline, the IC50 (the higher the IC50, the less suppressive the Tregs) and Smax were calculated from nonlinear regression plots (Figure 3C) as previously described (35).

Freshly isolated HD Tregs had impaired ability to suppress Tresp proliferation relative to the HCs, which was evidenced by a higher median IC50 and a lower Smax than their counterparts (Figure 3, C and D), consistent with previous reports (28). In vitro expansion in the presence of Rapamycin resulted in highly suppressive Tregs from HD patients, comparable in suppressive ability with freshly
isolated Tregs from HCs (Figure 3, E–I). In vitro expansion in the absence of Rapamycin resulted in poorly suppressive Tregs from both HC and HD groups. Indeed, only three HC Tregs and one HD Treg expanded without Rapamycin had maximal suppressive capability of 50% or more at 1:1 ratio. Consequently, although freshly isolated Tregs from HD patients are functionally impaired, GMP-compatible ex vivo expansion in the presence of

Figure 1. Comparison of regulatory T cell (Treg) numbers between healthy controls (HCs) and hemodialysis (HD) patients. (A and B) Representative examples from 14 participants of Treg populations I–III in (A) HCs and (B) patients on HD. Please note that the different appearance of the dot plots is because of data acquisition on different FACS machines on separate days. (C) Pooled data from 14 participants in each cohort showing mean percentage (± SD) of total Tregs (CD4⁺CD25hiCD127lo) in the CD4⁺ population and the percentage of Treg subpopulations I–III within that population. *P<0.05.

Table 2. Treg subpopulations in HCs and HD patients

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean Percent (± SD) of Cells in HCs (n=14)</th>
<th>Mean Percent (± SD) of Cells in HD Patients (n=14)</th>
<th>P</th>
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<tr>
<td>CD4⁺CD25hiCD127lo</td>
<td>5.2±2.0</td>
<td>4.6±1.7</td>
<td>0.41</td>
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<tr>
<td>Population I</td>
<td>34.3±18.8</td>
<td>42.3±18.1</td>
<td>0.27</td>
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<tr>
<td>Population II</td>
<td>4.7±4.7ab</td>
<td>1.8±1.5b</td>
<td>&lt;0.05</td>
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<tr>
<td>Population III</td>
<td>60.3±16.8a</td>
<td>54.5±17.1</td>
<td>0.37</td>
</tr>
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</table>

n=14 for both groups.
*P<0.05 compared with population I.
bP<0.05 compared with population III.
Rapamycin efficiently expands these Tregs and produces potently suppressive cells.

Rapamycin Effectively Prevents IL-17 Production by Expanded HD Tregs

Because Treg plasticity to a Th17 phenotype after infusion in humans may confer an undesirable proinflammatory phenotype, we next evaluated the ability of these cells to produce IL-17 in a proinflammatory milieu. Freshly isolated Tregs from HD patients stained marginally more positively for IL-17 and after activation in IL-17-inducing cytokines (IL-2, IL-1β, IL-6, and TGF-β [32]), produced more IL-17 than those Tregs from HCs, but these differences did not reach statistical significance (Figure 4, A–C). After expansion ex vivo, low-level staining for IL-17 was observed in Tregs from both HCs and HD patients, with HD Tregs, as before, being slightly more positive for IL-17 (Figure 4, D and E). Rapamycin did not significantly alter IL-17 staining, although there was a tendency for slightly higher IL-17 expression in Tregs expanded in the presence of the drug (Figure 4, D and E) ($P$>$0.05$ for all comparisons). However, when IL-17 production was analyzed in 5-day supernatants by ELISA, Tregs from patients on HD expanded without Rapamycin produced substantial quantities of IL-17 (Figure 4F), with Rapamycin clearly inhibiting this production (Figure 4F).

Taken together, these findings suggest that expansion of Tregs from HD patients with Rapamycin decreases their capacity to produce IL-17 under inflammatory conditions.

Discussion

Tregs are ideal candidates for cell-based therapy to induce solid organ transplant tolerance. Such strategies have been successful in animal models (4,5). Experimental induction of transplant tolerance is characterized by increased Treg frequency within both the transplanted organ and the regional lymphoid tissue (6,7). The likelihood of positive outcomes using Treg-based therapies in humans is suggested by data from human renal transplantation, where Treg infiltration of transplants correlates with better outcomes from borderline rejection (8), and demonstrations that GMP-grade functional Tregs can be expanded from PBMCs of healthy donors (4,9,10).

For Treg cellular therapy to be viable, three technical challenges need to be overcome: Tregs need to be present in peripheral blood in sufficient numbers to be isolated, they need to be expandable in vitro, and they need to be functionally stable. Although these requirements have been shown for Tregs from healthy donors (4,9,10), they have not been shown for patients with ESKD on HD awaiting a renal transplant. Indeed, these individuals show features
Figure 3. | In vitro expansion of Tregs from HD patients under good manufacturing practice (GMP)-compatible conditions and suppressive function before and after expansion. (A) Fold expansion of Tregs per restimulation (restim) in the presence and absence of Rapamycin. *P<0.05 relative to no Rapamycin. (B–D) Baseline suppression of carboxyfluorescein succinimidyl ester (CFSE)-labeled autologous responder T cells (Tresps). (B) A representative example of suppression of CFSE dilution in the absence (0:1) and presence (1:1) of Tregs at 1:1 ratio. (C) Representative regression plot of percent suppression against Treg:Tresp ratio for the calculation of the Treg:Tresp ratio sufficient for 50% suppression (IC50); straight lines show IC50 for HCs, and dotted lines show IC50 for HD patients. (D) Cumulative IC50 and maximum suppression.
of chronic inflammation/immune dysregulation (25–27). Although some of the chronic inflammation is attributable to excessive immune system activation (37), abnormalities in Tregs have also been described, particularly a defect in Treg numbers and function (28,38); some of this defect is ascribed to high apoptosis in the presence of uremic factors, particularly oxidized LDL (38). However, these studies did not include strict age and sex matching of patients to controls (one of them did not include HCs at all) (28), included patients with immunologic and inflammatory diseases (28), and did not address important phenotypic characteristics required of Tregs for cell-based therapy.

In the present study, a comprehensive analysis of Treg phenotypes in patients with ESKD compared with age- and sex-matched controls was performed. We accept that the study is limited by the expansion of only five Treg lines from each group, although this number is in keeping with the majority of previous literature in the field. We showed that patients on HD have equivalent Treg subsets I and III to their matched controls and express almost identical Treg surface markers on these subsets (CD27, CD39, HLA-DR, and FOXP3). Population II Tregs are effector Tregs, with potent suppressive function, that die quickly on activation (14), and therefore, the deficiency of population II Tregs from five HCs and five patients on HD. Please note that only three IC_{50} values could be calculated for HD Tregs, because the other two were poorly suppressive. (E–I) Suppressive capacity of Tregs after expansion in vitro in the absence (−Rapa) and presence (+Rapa) of Rapamycin. (E and F) Representative examples of suppression of CFSE dilution in the absence (0:1) and presence (1:1) of Tregs at 1:1 ratio for (E) HC and (F) HD Tregs. (G) Representative regression plot of percent suppression against Treg:Tresp ratio; straight lines show IC_{50} for HCs, and dotted line show IC_{50} for paired HD patient. The example is taken from a Rapamycin-treated pair of Tregs. (H and I) IC_{50} and S_{max} for Tregs expanded with and without Rapamycin compared with freshly isolated Tregs. Please note that missing IC_{50} values are because of poor suppressive ability. *P<0.05.

Figure 4. | IL-17 production by ex vivo-expanded HD Tregs. (A and B) IL-17 and FOXP3 staining in freshly isolated Tregs from HCs and HD patients showing (A) representative and (B) cumulative data from five independent experiments. (C) IL-17 production from freshly isolated HC and HD Tregs is activated in the presence of inflammatory cytokines; cumulative data from five independent experiments are shown. (D and E) IL-17 staining in HC and HD Tregs expanded ex vivo in the presence or absence of Rapamycin, showing (D) representative and (E) cumulative data from five independent experiments. (F) IL-17 production from HC and HD Tregs expanded ex vivo in the presence or absence of Rapamycin, showing cumulative data from five independent experiments. *P<0.05.
observed in HD patients may be the result of increased apoptosis (38) or impaired generation of effector Tregs in the context of chronic inflammation. In either case, population II does not expand ex vivo and therefore, is not amenable to use for cell therapy. We did not find paucity in naïve (population I) Tregs as suggested previously (29). Furthermore, an additional analysis of naïve and memory subsets of Tregs did not support the difference reported in the previous publication (29) (data not shown). Importantly, because population I Tregs are the subset most resistant to Th17 conversion in vitro (14), Tregs expanded from this population may minimize the potential for immune-mediated graft loss during cell therapy (18).

Although FOXP3 expression was similar in freshly isolated Tregs from patients and controls, impaired Treg-mediated suppression of autologous Tresps was seen in HD patients. Because this finding was apparent in the absence of uremic serum, we believe that the majority of the functional defect is cell-intrinsic (i.e., the Tregs are defective) rather than cell-extrinsic (uremic toxins, oxidized LDL, and hypercytokinemia). However, HD Tregs were easy to expand in vitro, particularly in the presence of Rapamycin, to a number sufficient for programs of cell therapy, and Rapamycin rescued the suppressive function of HD Tregs, making them suitable for cell-based therapy. Using the Rapamycin-based protocol in this paper, we predict being able to achieve sufficient Treg expansion to administer between 3×10^6 and 5×10^6 cells/kg on a single occasion (as predicated by the ONE Study). The number that may actually be required for tolerance induction will depend on their in vivo survival, their migratory ability, and the degree of infectious tolerance imparted by the infusion of the Tregs (39).

Finally, we observed that Rapamycin effectively diminished the capacity of Tregs from HD patients to produce or secrete (40,41) IL-17 in the presence of an inflammatory stimulus. Rapamycin promotes the expansion of phenotypically and functionally stable Tregs in GMP-compatible systems while impairing the proliferation of CD4+CD25+ T effectors (13,32,42). Thus, in the absence of Rapamycin treatment, the elevated IL-17 production by HD Tregs may be attributed to increased contaminating Th17 cells or a greater propensity for these cells to differentiate into Th17 cells (consistent with the chronic inflammation seen in HD and/or with vitamin D insufficiency/deficiency in ESKD, because this vitamin prevents Th17 differentiation) (43). Nevertheless, this finding is the clearest indication yet that Treg-based cell therapy for renal allograft transplantation should use a Rapamycin-based protocol. Rapamycin is an ideal choice for Treg-based therapy in solid organ transplantation, because it preferentially expands the most stable Treg subpopulation (population I) and inhibits IL-17 production from other subpopulations (32,44). Rapamycin interferes with early T cell signaling events (Akt/mammalian target of rapamycin), which are critical in T cell fate decisions (45). In addition to preferentially expanding population I Tregs, Rapamycin also induces expression of CD62L and CD27 on Tregs (44), which are permissive for homing to sites of antigen encounter after transplantation and also markers for the most suppressive of Tregs in vivo and in vitro (46–49).

Together, these data are a significant advance on prior publications, including the work by of Berglund et al. (50), which did not analyze Treg subpopulations in HD, baseline suppressive function, GMP-based clinical grade expansion, or Treg plasticity. We have shown, for the first time, that Tregs from patients on HD have similar subpopulations to age- and sex-matched healthy donors and can be expanded ex vivo under GMP-compatible conditions in the presence of Rapamycin to a functional and stable cell product that can be used in clinical trials of Treg-based immunotherapy for the induction of tolerance to renal allografts. The results support the rationale behind the use of Treg-based immunotherapy among patients receiving kidney transplants as planned in the ONE Study.

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Disclosures

None.

References


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This article contains supplemental material online at http://cjasn.asnjournals.org/lookup/suppl/doi:10.2215/CJN.12931212/-/DCSupplemental.
Supplemental Figure 1. Gating strategy for Treg sub-populations adapted from Miyara et al. 14.

A representative example of the strategy employed for HCs and HD patients to identify Treg populations I, II and III. Lymphocytes were identified by their forward scatter (FSC) vs side scatter (SSC) profile (A) and gated on the CD4 marker (B). The CD25hiCD127lo cells were identified (C) and three populations were gated according to expression of CD45RA and CD25 (D). As Miyara et al. are not specific about how they distinguish populations I, II and III, we adopted the following criteria based on the figures published in their paper: Population I - CD45RA+ cells from the vertical apex to the point where population II begins; Population III - from the x-axis to the lower boundary of Population I, the width being equal to that of Population I; Population II - any CD45RA- cells with greater CD25 staining than population III.
Supplemental Figure 2. Purity of isolated Tregs before and after *in vitro* expansion.

Tregs from five healthy controls (HCs) and five patients on dialysis (HD patients) were isolated under GMP-compatible conditions and expanded using standard protocols *in vitro* in the presence (+Rapa) and absence (-Rapa) of Rapamycin. Purities were compared at separation and following expansion by flow cytometry. A and C show representative dot plots for CD25 and FOXP3 at baseline and after expansion respectively; B and D show cumulative percentage positive for FOXP3 at baseline and after expansion respectively.