Regulatory T Cells and T Cell Depletion: Role of Immunosuppressive Drugs

Marina Noris,*† Federica Casiraghi,*† Marta Todeschini,*† Paolo Cravedi,*† Daniela Cugini,*† Giuseppe Monteferrante,*† Sistiana Aiello,*† Linda Cassis,*† Eliana Gotti,* Flavio Gaspari,* Dario Cattaneo,*† Norberto Perico,* and Giuseppe Remuzzi*†

**Department of Immunology and Organ Transplantation, Ospedali Riuniti–Mario Negri Institute for Pharmacological Research, and †Center for Research on Organ Transplantation, Chiara Cucchi De Alessandri & Gilberto Crespi, Bergamo, Italy*

Allogeneic immune responses are modulated by a subset of host T cells with regulatory function (Treg) contained within the $CD4^+CD25^{high}$ subset. Evidence exists that Treg expand after peritransplantation lymphopenia, inhibit graft rejection, and induce and maintain tolerance. Little, however, is known about the role of Treg in the clinical setting. IL-2 and activation by T cell receptor engagement are instrumental to generate and maintain Treg, but the influence of immunosuppressants on Treg homeostasis in humans *in vivo* has not been investigated. This study monitored Treg phenotype and function during immune reconstitution in renal transplant recipients who underwent profound T cell depletion with Campath-1H and received sirolimus or cyclosporine (CsA) as part of their maintenance immunosuppressive therapy. $CD4^+CD25^{high}$ cells that expressed FOXP3 underwent homeostatic peripheral expansion during immune reconstitution, more intense in patients who received sirolimus than in those who were given CsA. T cells that were isolated from peripheral blood long term after transplantation were hyporesponsive to alloantigens in both groups. In sirolimus- but not CsA-treated patients, hyporesponsiveness was reversed by Treg depletion. T cells from CsA-treated patients were anergic. Thus, lymphopenia and calcineurin-dependent signaling seem to be primary mediators of $CD4^+CD25^{high}$ Treg expansion in renal transplant patients. These findings will be instrumental in developing "tolerance permissive" immunosuppressive regimens in the clinical setting.

J Am Soc Nephrol 18: 1007–1018, 2007. doi: 10.1681/ASN.2006101143

Renal allotransplantation is the treatment of choice for most ESRD (1). However, the substantial benefit that is obtained from transplantation is offset by the adverse effects of immunosuppression (2). The field of transplant medicine now needs to focus on drug efficiency/safety, with tolerance being the ultimate goal. Immune activation in graft rejection involves multiple effector cell populations, but with few exceptions, $CD4^+$ and/or $CD8^+$ lymphocytes have a requisite role (3). In experimental models of organ transplantation, allogeneic immune responses are modulated by a subset of host T cells with regulatory function (Treg), contained within the $CD4^+CD25^{high}$ subset (4), that can inhibit graft rejection and maintain tolerance to alloantigens. Little, however, is known about the role of Treg in the clinical setting.

Experimental evidence exists that Treg expand after lymphopenia that is induced by T cell–depleting agents (5–7). Recently, the humanized anti-CD52 mAb Campath-1H (Alemtuzumab; Schering Plough, Milan, Italy) was used to reduce markedly both circulating and bone marrow lymphocytes for several months in kidney transplant recipients (8). Thus far, in the kidney transplant setting, Campath-1H induction has been followed by diverse immunosuppressive regimens (8–11), in accordance with the principle of minimum use of posttransplantation immunosuppression. Among the agents that are associated with Campath-1H, sirolimus does not interfere with calcineurin-dependent IL-2 synthesis and signaling (12,13) and promotes *in vitro* Treg expansion upon T cell receptor (TCR) stimulation (14) or the *de novo* induction of Treg from naïve $CD4^+$ T cells (15), events that would favor tolerance induction *in vivo*. However, it is theoretically possible that calcineurin inhibitors, including cyclosporine (CsA) and tacrolimus (16,17), represent a barrier to immune tolerance in organ transplantation given the following: (*1*) Treg have a crucial role in maintaining immune tolerance (4); (*2*) IL-2 has a well-defined physiologic role in generating and maintaining Treg, because IL-2 and IL-2 receptor β knockout mice die of autoimmunity, which is prevented by Treg administration (18–20); and (*3*) calcineurin inhibitors block IL-2 formation in T lymphocytes (17). This, however, has never been tested *in vivo* in humans. To clarify the role of lymphopenia and of calcineurin-dependent signaling in Treg homeostasis in clinical transplantation, we treated kidney transplant recipients with Campath-1H to deplete lymphocytes profoundly and examined the impact of low-dosage sirolimus- or low-dosage CsA-based maintenance immunosuppression on the emergence and activity of Treg.

Received October 23, 2006. Accepted December 20, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

M.N. and F.C. contributed equally to this study.

Address correspondence to: Dr. Marina Noris, Mario Negri Institute for Pharmacological Research, Via Gavazzeni 11, 24125, Bergamo, Italy. Phone: +39-035-319888; Fax: +39-035-319331; E-mail: noris@marionegri.it

ISSN: 1046-6673/1803-1007

Materials and Methods

Patients

Twenty-one white primary kidney transplant recipients were enrolled under a protocol that was approved by the Ethics Committee of the Renal Transplant Center of Bergamo. Recipients were selected according to age 18 to 70 yr, current panel reactive antibodies <10%, non-HLA identical to the donor and a negative cross-match test, and random allocation on a 1:1 basis to either low-dosage sirolimus or low-dosage CsA added to low-dosage mycophenolate mofetil (MMF) and induction therapy with Campath-1H. Baseline characteristics are shown in Table 1. Schematic representation of the study design is outlined in Figure 1. Informed written consent was obtained from all participating patients according to the Declaration of Helsinki.

Immunosuppressive Therapy

At surgery, patients were given methylprednisolone (500 mg intravenously), followed by a 30-mg infusion of Campath-1H. Methylprednisolone was also infused on days 1 (250 mg) and 2 (125 mg) after transplantation. Low-dosage sirolimus (Rapamune, Wyeth-Lederle, Rome, Italy) was started on day 1 (4 mg/d orally, then adjusted to target trough levels of 5 to 10 ng/ml, by HPLC [21]). CsA was given intravenously (1 to 2 mg/kg per d) on day 1, then shifted to oral CsA (2 mg/kg twice daily) and adjusted to trough blood concentrations of 120 to 220 ng/ml (by HPLC [22]) in the first month and of 70 to 120 ng/ml thereafter (Table 2). In both groups, patients were also given MMF (Cell Cept; Roche, Milan, Italy) at the oral dosage of 500 mg twice daily from day 1.

Follow-Up and Patient Outcome

Patients were followed up for 24 mo. Mean serum creatinine concentration progressively decreased during the first 2 mo and remained stable in the two study groups up to study end. GFR, measured by plasma clearance of iohexol (23), showed a stable graft function at 6, 12, and 24 mo (Figure 2) in both groups. In sirolimus-treated patients, however, GFR was numerically higher than in the CsA group at all time points. The adverse events were comparable in the two study groups (Table 3). Three patients, one on sirolimus and two on CsA, had an acute rejection episode at 14, 9, and 210 d after transplantation, respectively, and fully recovered with intravenous methylprednisolone. One patient who was on sirolimus died from sepsis 18 mo after transplantation.

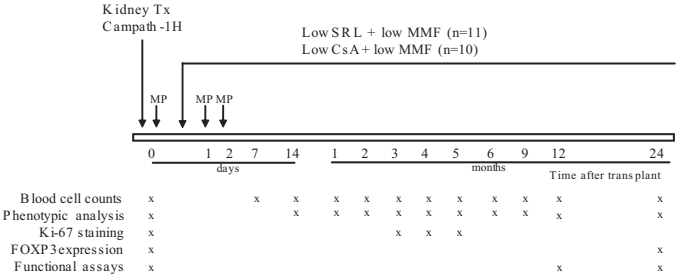

Figure 1. Schematic representation of study design. Twenty-one white primary kidney transplant recipients were randomly allocated on a 1:1 basis to either low-dosage sirolimus (SRL group; $n = 11$) or low-dosage cyclosporin A (CsA group; $n = 10$) added on to low-dosage mycophenolate mofetil (MMF) and induction therapy with Campath-1H (C1H). Immunosuppressive treatments are indicated by arrows. At surgery, patients were given 500 mg of methylprednisolone intravenously, followed by 30 mg of Campath-1H intravenously. Methylprednisolone was also infused on days 1 (250 mg) and 2 (125 mg). Sirolimus (low SRL) was started on day 1 (4 mg/d orally, then adjusted to expected target trough levels). Intravenous CsA (low CsA) was started soon after surgery (1 to 2 mg/kg per d); on day 1, CsA was shifted to oral CsA (2 mg/kg twice daily) and adjusted according to trough blood concentrations. In both groups, patients were also given MMF at the oral dosage of 500 mg twice daily starting on day 1 (low MMF). Phenotypic and functional assessments are outlined on the left. Timing of each evaluation is marked with a cross.

Peripheral Leukocyte Count and Lymphocyte Phenotype

Peripheral blood mononuclear cells (PBMC) were monitored by flow cytometry using TruCount tubes and analyzed by FACSCalibur cytom-

Table 1. Distribution of patient demographics[a]

Demographic	Overall ($n = 21$)	SRL ($n = 11$)	CsA ($n = 10$)
Age (yr)	49 (24 to 70)	51 (31 to 69)	47 (24 to 70)
Gender (M/F)	13/8	6/5	7/3
Type of donor			
cadaveric	19	11	8
living	2	0	2
Mismatches	4.0 ± 1.3	4.0 ± 1.4	4.0 ± 1.2
Cause of renal failure			
IgA nephropathy	6	3	3
membranous nephropathy	1	1	0
other glomerulonephritis	1	1	0
polycystic kidney disease	3	1	2
interstitial inflammation	1	1	0
pyelonephritis	3	3	0
unknown	6	1	5

[a]Data are median (range) or mean ± SD. CsA, cyclosporin A; SRL, sirolimus.

Table 2. SRL and CsA trough levels during the 24-mo follow-up after transplantation

Months after Transplantation	Mean SRL Trough (ng/ml)	Mean CsA C0 (ng/ml)
1	7.6 ± 2.9	143 ± 89
3	8.6 ± 3.1	124 ± 50
6	9.3 ± 3.6	113 ± 27
12	9.3 ± 2.1	99 ± 36
18	10.3 ± 5.0	87±22
24	10.4 ± 3.7	75 ± 32

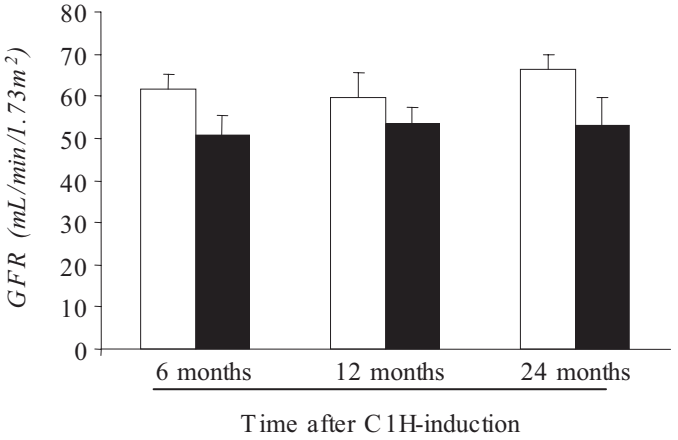

Figure 2. Kidney graft function. GFR at 6, 12, and 24 mo after C1H induction in SRL-treated ($n = 11$, $n = 10$ at 24 mo; □) and CsA-treated ($n = 10$ ■) groups. Data are means ± SEM.

eter (BD Bioscience, San Jose, CA). For T lymphocyte subset immunophenotyping, fresh blood samples and PBMC were incubated with fluorochrome-conjugated mAb against human CD3, CD4, CD8, CD25, CD69, CD45RO, and Ki-67 antigens (BD Bioscience). The samples were analyzed by FACSort or by FACSAria cytometers (BD Bioscience). For each marker, blank samples with isotype-matched control antibodies were analyzed.

FOXP3 Expression in CD4$^+$ Subset

After gating for CD3, CD4$^-$, CD4$^+$, CD4$^+$CD25$^-$, and CD4$^+$CD25^{low}, CD4$^+$CD25^{high} T cells were isolated by cell sorting (FACSAria; BD Bioscience), and purity was >99%. Total RNA was reverse-transcribed to cDNA, and quantitative real-time PCR was performed on a TaqMan ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green PCR Core reagents and the following primers: FOXP3, forward (300 nM) 5′-AGC CAT GGA AAC AGC ACA TTC-3′ and reverse (300 nM) 5′-GAG CGT GGC GTA GGT GAA A-3′. β2-Microglobulin served as housekeeping gene. The ΔΔCt equation was used to compare the FOXP3 gene expression in each sample with the expression in a pool of CD3$^+$CD4$^+$ cells from healthy subjects (calibrator). Results were expressed as arbitrary units taking the expression in the calibrator as 1.

Measurement of Lymphocyte Alloreactivity

Mixed Lymphocyte Reaction. Recipient PBMC (1×10^5) were cultured in RPMI/20% human serum type AB, with irradiated (4000 RAD) stimulator cells from donor and third-party subjects (either PBMC from living donors or CD2-depleted spleen cells from cadaveric donors). Third-party controls were chosen, to the extent possible, such that the HLA mismatches were the same as those between the donor and the recipient. Cells were incubated for 6 d and pulsed with 1 μCi [^{3}H]thymidine during the last 16 h. The stimulation index was calculated by the ratio of cp/min allogeneic combinations (donor or third-party) to the cp/min self combinations.

Table 3. Patients with adverse events that required treatment or hospitalization[a]

Adverse Events	Overall	SRL	CsA
Infectious			
CMV reactivation	4	—	4
pneumonia	2	1	1
urinary tract infection	4	3	1
herpes zoster reactivation	2	2	—
sepsis	2	1	1
Surgical			
perirenal hematoma	2	—	2
hemoperitoneum	1	—	1
lymphocele	1	—	1
pleural effusion	1	—	1
Medical			
fever of unknown origin	10	3	7
leukopenia	3	1	2
diarrhea	2	—	2
myocardial ischemia	2	1	1
thrombocytopenia	1	—	1
gout	1	—	1
thrombophlebitis	1	—	1
diabetes	3	1	2

[a]CMV, cytomegalovirus.

Enzyme-Linked Immunosorbent Spot Assays. Enzyme-linked immunosorbent spot (ELISPOT) assays were performed using BD ELISPOT Human IFN-γ reagents. Responder PBMC (300,000/well) were plated in ELISPOT plates (Millipore, Billerica, MA) in the presence of irradiated donor or third-party stimulators (300,000). Plates were incubated overnight, and the resulting spots were counted by Immunospot image analyzer (Aelvis Elispot Scanner system). Aliquots of responder PBMC were also incubated with medium alone (negative controls) or in the presence of 10 μg/ml phytohemagglutinin (PHA) (positive controls). Results are the mean value of IFN-γ spots/300,000 recipient PBMC after subtracting IFN-γ spots in negative controls (usually two or fewer).

Statistical Analyses

Results are mean ± SEM. Baseline characteristics of patients were compared by χ^2 test or by t test as appropriate. Variations in peripheral blood count, phenotype, and functional data were assessed by ANOVA. The statistical significance level was defined as $P < 0.05$. The Bonferroni correction for multiple comparison was applied.

Results

Phenotype of Peripheral Blood Leukocytes in Renal Transplant Patients after Campath-1H

Campath-1H profoundly depleted total B cells, NK cells, monocytes, and T cells and CD4$^+$ and CD8$^+$ T cell subsets

(Figure 3, A and B). Confirming previous reports (24), the depleted $CD4^+$ T cell pool, however, was enriched by memory $CD45RO^+$ cells (Figure 3C). Campath-1H–induced cell depletion was not influenced by the type of maintenance immunosuppression therapy (Figure 3, D through I). $CD3^+CD4^+$ T cells recovered very slowly: At month 24 after transplantation, their count was approximately one third of baseline values in both sirolimus- and CsA-treated patients (Figure 3H). In the siroli-

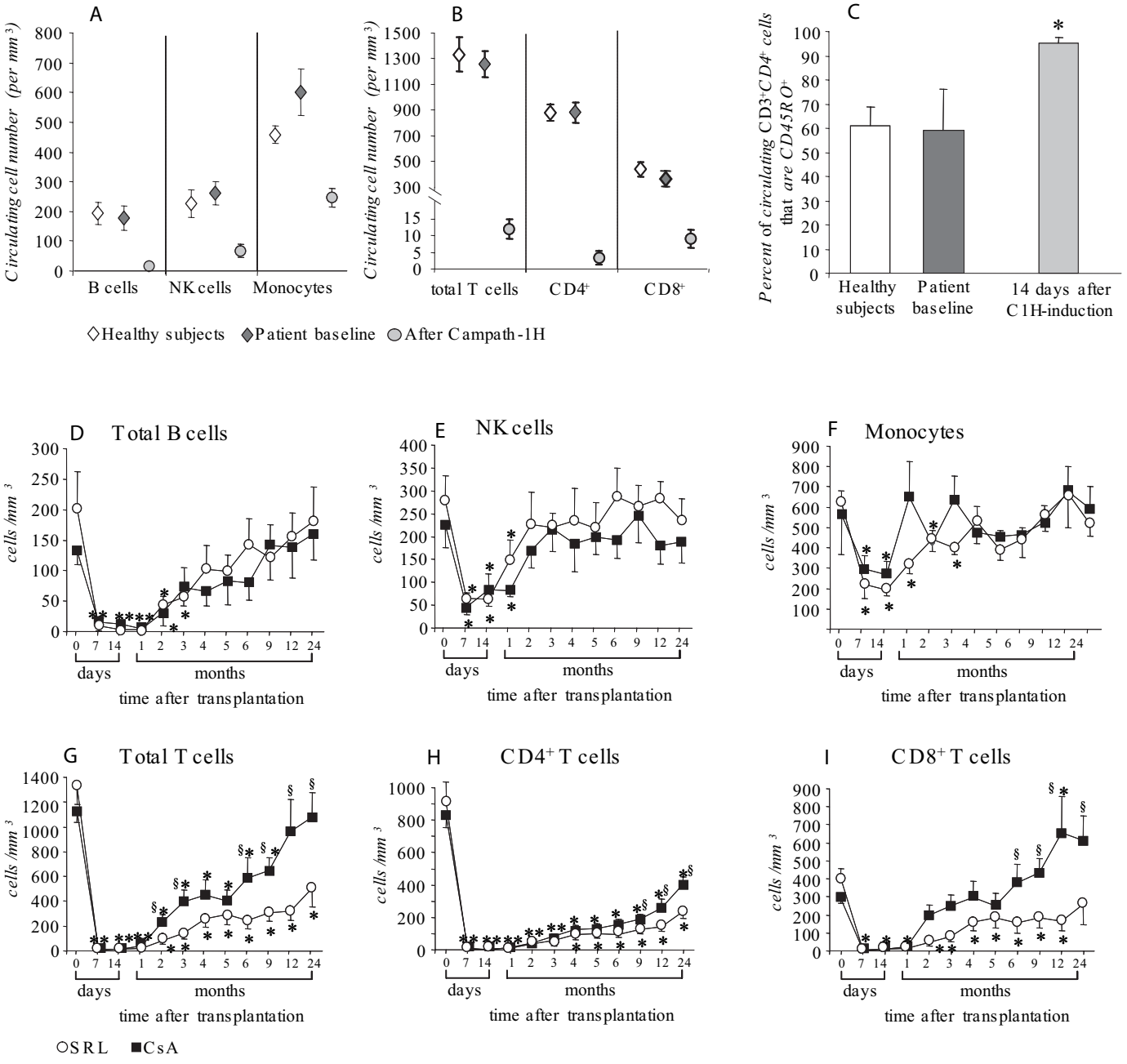

Figure 3. Effect of C1H on circulating leukocyte subsets in renal transplant patients. Using flow cytometry analysis, absolute numbers of total $CD3^-CD19^+$ B cells and $CD3^-CD16^+CD56^+$ NK cells (A) and of total $CD3^+$ T cells and of $CD3^+CD4^+$ ($CD4^+$) and $CD3^+CD8^+$ ($CD8^+$) subsets (B) were calculated for healthy subjects ($n = 11$) and patients at baseline (before transplantation, $n = 21$) and at 14 d after transplantation and C1H induction ($n = 21$; data from SRL- and CsA-treated patients were cumulated because no difference was recorded between the two groups at this time). Absolute numbers of monocytes were obtained from a complete blood count that was done on the same day (A). (C) Percentage of memory $CD45RO^+$ cells within $CD3^+CD4^+$ from healthy subjects and from patients at baseline and at 14 d after transplantation and C1H induction (cumulative data from the SRL and the CsA group). (D through I) Kinetics of absolute numbers of repopulating total $CD3^-CD19^+$ B cells, $CD3^-CD16^+CD56^+$ NK cells, monocytes, $CD3^+$ T cells, and $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets in the peripheral blood of C1H-treated renal transplant patients from baseline (time 0) to 24 mo after transplantation. ○, SRL recipients ($n = 11$, $n = 10$ at 24 mo); ■, CsA recipients ($n = 10$). Data are means ± SEM. *$P < 0.05$ *versus* time 0; §$P < 0.05$ *versus* SRL-treated group at the same time point.

mus group, $CD3^+CD8^+$ T cell counts remained lower than before transplantation until month 24 after surgery, whereas in the CsA group, they returned to preoperative values within 4 to 5 mo (Figure 3I). B cells, NK cells, and monocytes recovered faster than $CD4^+$ and $CD8^+$ T cells, reaching pretransplantation values within 6 (B cells) and 3 mo (NK cells and monocytes) after transplantation in both groups (Figure 3, D through F).

Sirolimus- but Not CsA-Based Maintenance Immunosuppression Is Associated with In Vivo *Expansion of $CD4^+CD25^{high}$ Cells after Campath-1H*

The number and the proportion of circulating $CD4^+$ cells that comprised $CD25^{high}$ cells were comparable in participants, analyzed at baseline, and in healthy individuals ($n = 11$; mean age 36 yr; range 32 to 55; Figure 4, A and B). Campath-1H induced a profound and unselective depletion of $CD4^+CD25^-$, $CD4^+CD25^{low}$ (effector T cells) (25) and $CD4^+CD25^{high}$ subsets (Figure 4, A through C) in transplant patients.

From month 1 after transplantation, the percentage of $CD25^{high}$ cell subset in total $CD3^+CD4^+$ T cells progressively increased over baseline in sirolimus-treated patients (Figure 4, D through F), reaching values that were significantly higher than before transplantation from months 4 to 24 postoperatively. In the CsA group, the trend of $CD4^+CD25^{high}$ cell percentage to increase was milder: Values that were significantly higher than before transplantation were recorded only at month 6 (Figure 4, D through F). As a consequence, the Treg/effector T cells ratios were significantly higher ($P < 0.05$) in the sirolimus than in the CsA group from months 2 through 24 (data not shown).

For assessment of whether enrichment in $CD4^+CD25^{high}$ T cells was associated with cell proliferation, staining for Ki-67 (a nuclear cell proliferation–associated antigen that is expressed in all active stages of cell cycle) (26) was performed in cells that were taken from patients at baseline and at the time of maximal $CD4^+CD25^{high}$ cell expansion (3 to 5 mo after transplantation). Higher cycling was observed in the three $CD4^+CD25$ cell subsets from all patients who were studied 3 to 5 mo after transplantation ($P < 0.05$) than in cells from patients at baseline and from healthy individuals (Figure 5C), which is consistent with $CD4^+$ T cell recovery after Campath-1H–induced lymphopenia (Figure 3H). Of note, the proportion of $CD4^+CD25^{high}Ki\text{-}67^+$ cells was significantly ($P < 0.05$) higher in the sirolimus- than in the CsA-treated group, whereas no difference in the percentages of both $CD4^+CD25^{low}Ki\text{-}67^+$ and $CD4^+CD25^-Ki\text{-}67^+$ cells was found between the two groups (Figure 5C).

Expanding $CD4^+CD25^{high}$ Cells Express the Treg Hallmark FOXP3

The previous results document that Campath-1H–induced T cell depletion favors the emergence of $CD4^+CD25^{high}$ T cells in patients who receive sirolimus maintenance therapy. However, CD25 cannot be regarded as a specific hallmark of Treg, because also effector/memory T cells express this molecule on their surface (27). To ascertain whether the high levels of CD25 expression in regenerating $CD4^+$ cells upon Campath-1H induction reflected a regulatory phenotype, we evaluated the mRNA expression level of *FOXP3*, a gene that encodes a transcription factor that is required for Treg development and function (28,29). Using electronically sorted $CD3^+CD4^+$ subpopulations, we saw the highest levels of FOXP3 expression in $CD25^{high}$ subset both in patients and in healthy individuals, with intermediate and low levels of FOXP3 expression in the $CD25^{low}$ and $CD25^-$ subsets, respectively (Figure 5A). FACS analysis showed that the large majority (99.0%; range 98.5 to 99.5%) of $CD4^+CD25^{high}$ cells from all groups were $CD69^-$, excluding that they were activated cells (30). There was a significantly ($P < 0.05$) increased FOXP3 expression in $CD4^+CD25^{high}$ cells that were isolated 24 mo after transplantation from sirolimus- compared with CsA-treated patients or from participants at baseline and healthy individuals (Figure 5A).

To quantify Treg in the total $CD3^+CD4^+$ cell population, we further evaluated FOXP3 expression in positively selected $CD3^+CD4^+$ T cells. Cells that were isolated 24 mo after transplantation from sirolimus-treated patients had higher ($P < 0.05$) FOXP3 expression compared with $CD3^+CD4^+$ cells from the same patients at baseline or from healthy individuals (Figure 5B), which is consistent with both $CD4^+CD25^{high}$ T cell expansion that was observed through flow cytometry and increased FOXP3 expression in this cell subset. Notably, in CsA-treated patients, FOXP3 expression in $CD3^+CD4^+$ T cells was lower ($P < 0.05$) than in the sirolimus group (Figure 5B). Altogether, these results indicate that, after lymphocyte depletion by Campath-1H induction, sirolimus but not CsA increased the pool of FOXP3-expressing $CD4^+CD25^{high}$ cells. We saw no evidence in any group of significant FOXP3 expression in $CD4^-$ cells (FOXP3 expression <0.01 arbitrary units).

$CD4^+CD25^{high}$ T Cells from Sirolimus-Treated Patients Suppress T Cell Alloreactivity Ex Vivo

Treg are anergic T cells that respond poorly to allogeneic stimuli and are also capable of inhibiting the alloreactive response of effector T cells (31). Therefore, we performed functional assays to address whether the emergence of Treg was associated with host T cell hyporesponsiveness against donor antigens. Both the proliferative response of T cells to alloantigens in MLR and the frequency of previously activated/memory T cells by ELISPOT for IFN-γ after overnight exposure to alloantigens were analyzed (32). Samples were taken before transplantation and at two different time intervals after T cells had repopulated the peripheral blood at an adequate amount (at months 12 and 24 after transplantation). In sirolimus-treated patients, the anti-donor T cell proliferative response and the frequencies of IFN-γ–producing donor-reactive cells were significantly ($P < 0.05$) reduced at both posttransplantation points as compared with before transplantation (Figure 6, A and B). Posttransplantation anti–third-party alloreactivity was significantly lower than pretransplantation as well (Figure 6, A and B). These results could not be attributed to incomplete recovery of T cell count or to a state of general immunosuppression caused by maintenance therapy, because T cells that were isolated at the same time points responded normally to a polyclonal T cell stimulus with PHA (Figure 6B). In CsA-treated

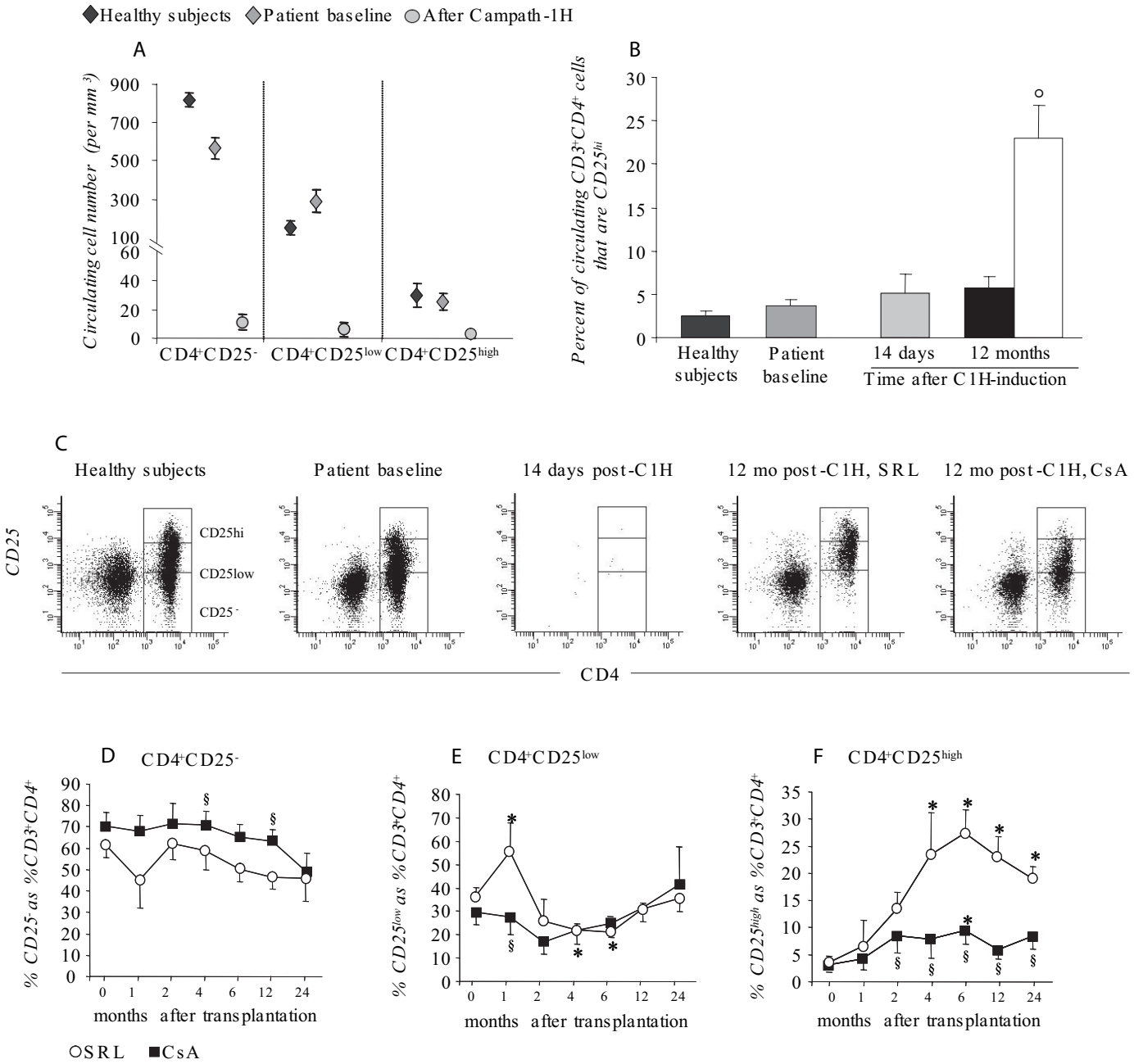

Figure 4. SRL maintenance therapy favors expansion of $CD4^+CD25^{high}$ subset after C1H induction. (A) Using flow-based frequency enumeration and absolute $CD3^+CD4^+$ cell counts that were obtained on the same day, absolute numbers of $CD3^+CD4^+CD25^-$, $CD3^+CD4^+CD25^{low}$, and $CD3^+CD4^+CD25^{high}$ cells were calculated in healthy subjects ($n = 11$) and in patients at baseline ($n = 21$) and at 14 d after transplant (after C1H, $n = 21$). (B) Percentage of $CD25^{high}$ within the $CD3^+CD4^+$ subset from healthy subjects ($n = 11$) and from patients at baseline ($n = 21$), at 14 d ($n = 21$, data from the SRL and CsA groups were cumulated because no difference was recorded between the two groups at this time), and at 12 mo (data from SRL group [$n = 11$; □] and CsA group [$n = 10$; ■] are presented separately) after transplantation and C1H induction. Data are means ± SEM. °$P < 0.05$ *versus* healthy subjects, patient baseline, and CsA. (C) Representative FACS plots of CD25 expression on $CD3^+CD4^+$ cells from healthy subjects, patients at baseline and at 14 d after transplantation and C1H induction, and in patients who were on either SRL or CsA therapy and studied 12 mo after transplantation. $CD3^+CD4^+$ cells were designated $CD25^-$ when CD25 expression fell within the background staining that was observed using an isotype control, $CD25^{high}$ when CD25 expression exceeded that seen in the $CD4^-$ population, and $CD25^{low}$ when CD25 expression fell between these regions (26). (D through F) Using the gating shown in C, posttransplantation longitudinal analysis of the percentage of $CD3^+CD4^+$ that were $CD25^-$, $CD25^{low}$, or $CD25^{high}$ are shown. ○, SRL recipients ($n = 11$, $n = 10$ at 24 mo); ■, CsA recipients ($n = 10$). Data are means ± SEM. *$P < 0.05$ *versus* patient baseline (time 0); §$P < 0.05$ *versus* the SRL-treated group at the same time point.

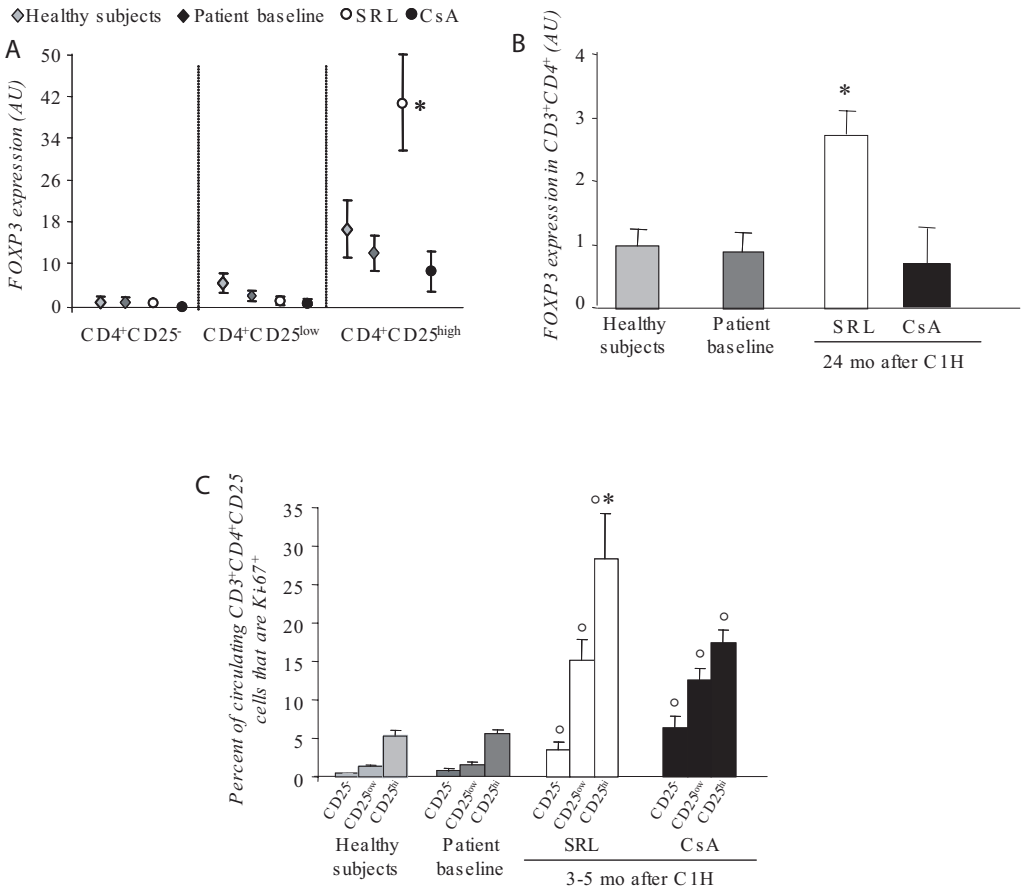

Figure 5. $CD3^+CD4^+CD25^{high}$ cells express FOXP3 and undergo homeostatic peripheral expansion in renal transplant patients after C1H induction. Both events are favored by SRL maintenance therapy. (A) FOXP3 mRNA expression in $CD3^+CD4^+CD25^-$, $CD3^+CD4^+CD25^{low}$, and $CD3^+CD4^+CD25^{high}$ cells in healthy subjects ($n = 11$), patients at baseline ($n = 21$), and SRL-treated patients ($n = 10$; ○) and CsA-treated patients ($n = 10$; ●) 24 mo after transplantation. (B) FOXP3 mRNA expression in positively selected $CD3^+CD4^+$ cells in healthy subjects ($n = 11$) and in patients at baseline ($n = 21$) and at 24 mo after transplantation (SRL [$n = 10$; □]; CsA [$n = 10$; ■]). Results are expressed as arbitrary units, taking the expression in $CD3^+CD4^+$ cells from a pool of healthy subjects as calibrator (arbitrary units = 1). Data are means ± SEM. *$P < 0.05$ *versus* healthy subjects, patients at baseline, and CsA-treated patients at the same time point. (C) Expression of the proliferation marker Ki-67 in $CD3^+CD4^+CD25^-$, $CD3^+CD4^+CD25^{low}$, and $CD3^+CD4^+CD25^{high}$ cells from healthy subjects and from patients at baseline and at 3 to 5 mo after transplantation (at the time of maximal expansion of $CD3^+CD4^+CD25^{high}$ cells; $n = 6$ for each group). Data are means ± SEM. °$P < 0.05$ *versus* the same CD25 subset from healthy subjects and patients at baseline; *$P < 0.05$ *versus* CsA-treated patients at the same time point.

patients, T cells that were studied at the same time points showed donor-specific hyporesponsiveness assayed with MLR and ELISPOT, compared with before transplantation (Figure 6, A and B). T cell response to PHA was normal (Figure 6B).

To clarify the role of Treg in these phenomena, we repeated ELISPOT assays at 24 mo after transplant and after $CD4^+CD25^{high}$ cell depletion by sorting. As shown in Figure 6C, in the sirolimus group, depletion of $CD4^+CD25^{high}$ cells was associated with a statistically significant ($P < 0.05$) increase in the frequency of IFN-γ–producing effector/memory cells to both donor and third-party antigens. The suppression ratios, defined as frequency after depletion minus frequency before depletion divided by frequency after depletion (33), were comparable for both anti-donor (0.43 ± 0.06) and anti–third-party (0.45 ± 0.06) response. To confirm that the $CD4^+CD25^{high}$ subset in sirolimus-treated patients had regulatory activity and to exclude any possible overlapping inhibitory effect of the concomitant immunosuppressive therapy on the function of effector T cells, we added sorted $CD4^+CD25^{high}$ cells, taken from these patients at 24 mo after transplantation, to $CD4^+CD25^{high}$-depleted PBMC from the same patients before surgery. Addition of $CD4^+CD25^{high}$ cells consistently reduced the IFN-γ frequencies to both donor and third-party antigens (Figure 6D). The amount of added Treg did correspond to the percentage of circulating $CD4^+CD25^{high}$ cells in patients before transplantation, which supports a potential clinical significance of our findings.

Conversely, in patients who were on CsA, $CD4^+CD25^{high}$ cell depletion had no effect on the frequencies of anti-donor IFN-γ–producing T cells that were taken at 24 mo after

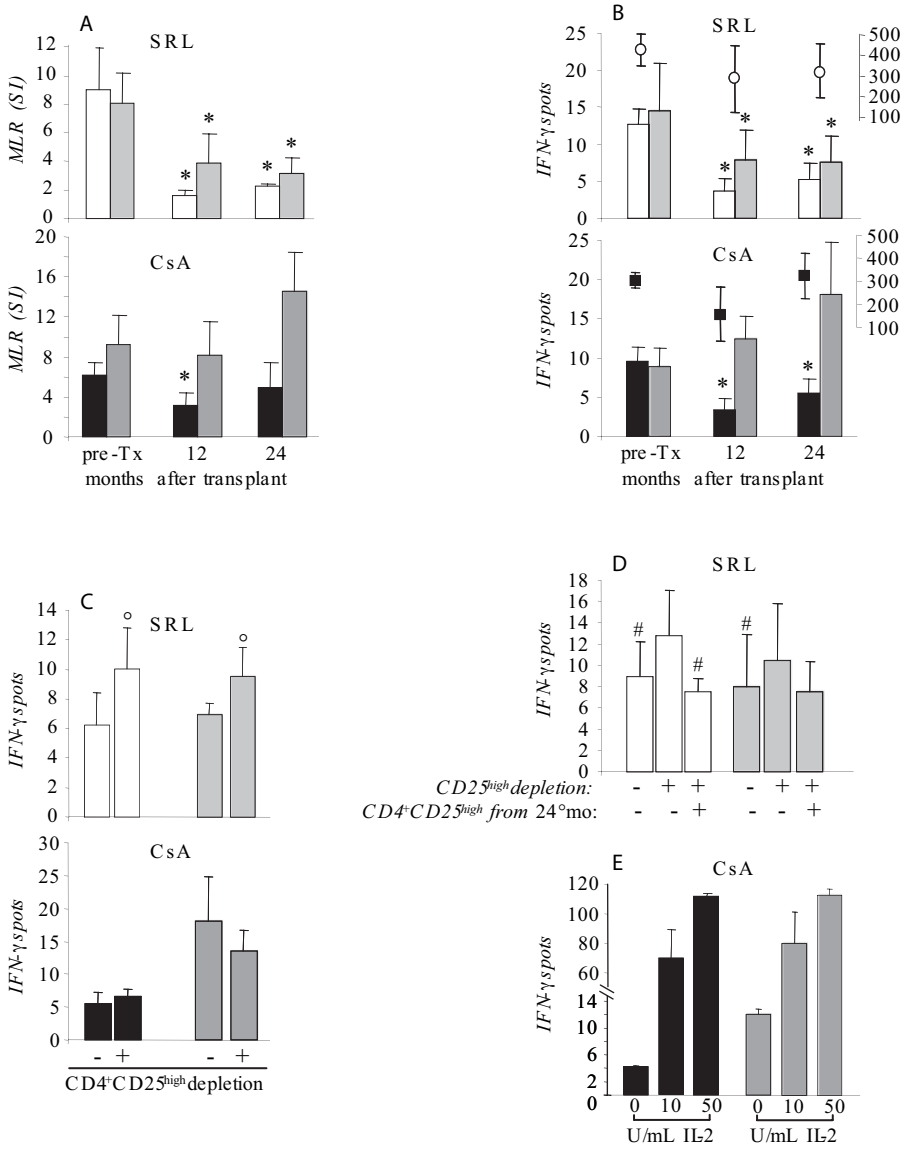

Figure 6. Functional assays on repopulating T cells after C1H induction in renal transplant patients. (A) MLR proliferative responses (stimulation index) to donor (□, SRL; ■, CsA) and third-party (▨) antigens of T cells from transplant patients who received SRL ($n = 6$) or CsA ($n = 6$) and for whom donor cells were available. Each patient was studied at baseline and at 12 and 24 mo after transplantation. (B) Enzyme-linked immunosorbent spot frequency of IFN-γ–producing activated/memory T cells (spots/300,000 peripheral blood mononuclear cells [PBMC]) to donor (□, SRL; ■, CsA) and third-party (▨) antigens from transplant patients who received SRL ($n = 6$) or CsA ($n = 6$). Each patient was studied at baseline and at 12 and 24 mo after transplantation. The responses to phytohemagglutinin (PHA), used as positive controls, are shown as ○ (SRL) or ■ (CsA) with respective Y-scale shown on the right. (C) Effect of $CD4^+CD25^{high}$ depletion by FACS sorting on the anti-donor (□, SRL; ■, CsA) and anti third-party (▨) frequencies of IFN-γ–producing cells. The number of spots/300,000 PBMC measured before (−) and after (+) $CD4^+CD25^{high}$ depletion in PBMC from renal transplant patients studied 24 mo after surgery (SRL, $n = 6$; CsA, $n = 6$) are shown. (D) Effect of the addition of autologous $CD4^+CD25^{high}$ to $CD4^+CD25^{high}$-depleted PBMC that were isolated at baseline on the frequencies of anti-donor (□) and anti third-party (▨) IFN-γ–producing T cells in SRL-treated patients. $CD4^+CD25^{high}$ cells were isolated by sorting from SRL patients at 24 mo after transplantation ($n = 3$), and 5000 cells were added to 300,000 pretransplantation $CD4^+CD25^{high}$-depleted PBMC from the same patients. (E) The frequencies of IFN-γ–producing cells in response to donor (■) and to third-party antigens (▨) in PBMC from CsA-treated patients studied 24 mo after transplantation ($n = 3$) in the absence (0) or in the presence of 10 or 50 U/ml IL-2. Data are means ± SEM. *$P < 0.05$ *versus* pretransplantation; °$P < 0.05$ *versus* predepletion (−); #$P < 0.05$ *versus* $CD25^{high}$-depleted PBMC (+/−).

transplantation (Figure 6C). These results indicate that Treg did not play a significant role in the hyporesponsiveness to donor alloantigens in CsA-treated patients. By contrast, addition of IL-2 to the ELISPOT assay increased the IFN-γ frequencies against donor antigens to reach anti–third-party values (Figure 6E). Thus, anergy rather than regulation con-

tributed to donor-specific hyporesponsiveness in CsA-treated patients (34).

Discussion

Here we demonstrated that in renal transplant recipients, $CD4^+CD25^{high}$ FOXP3-expressing Treg expand in the peripheral blood during immune reconstitution after profound T cell depletion with Campath-1H, provided that sirolimus and not CsA is given as maintenance immunosuppression. On the basis of these data, we hypothesized that lymphopenia and calcineurin-dependent signaling could be instrumental to achieving pro-tolerogenic Treg expansion in the clinical transplant setting.

Polyclonal and monoclonal anti–T cell antibodies have been used as an integral part of tolerance induction protocols in experimental transplantation (7,35). Their tolerogenic activity has been attributed primarily to the profound T cell depletion from the circulating pool *via* complement-dependent lysis or Fas/Fas ligand–mediated apoptosis (36,37), although emerging evidence suggests also a role for Treg expansion during lymphopenia-induced homeostatic proliferation (6,26,38,39). After anti–lymphocyte serum (ALS)-induced lymphopenia in C57/BL6 mice (5), the reduction of $CD4^+CD25^+$ Treg was smaller than that of $CD4^+CD25^-$ cells, which raised the concept that Treg may be resistant to ALS-depleting effects. At variance with ALS, we found that in renal transplant patients, Campath-1H did not selectively spare Treg but induced a profound and unselective depletion of $CD4^+CD25^-$, $CD4^+CD25^{low}$, and $CD4^+CD25^{high}$ subsets. These results are consistent with the observations that all $CD4^+$ T cell subsets, including $CD4^+CD25^+$, express at similar densities on their cell surface the CD52 target antigen of Campath-1H (40) and that Campath-1H, added *in vitro* to human blood, causes depletion of $CD4^+CD25^{high}$ cells (40).

As a part of homeostatic proliferation secondary to Campath-1H–induced lymphopenia, we documented a progressive increase of $CD4^+CD25^{high}$ T cells, which, however, was restricted to sirolimus-treated patients. $CD4^+CD25^{high}$ T cell expansion may result from two mutually nonexclusive and possibly complementary mechanisms. Antibody-induced lymphopenia may promote the selective homeostatic proliferation of naturally occurring $CD4^+CD25^{high}$ cells. Finding a higher expression of the proliferation marker Ki-67 in $CD4^+CD25^{high}$ cells than in $CD4^+CD25^{low}$ and $CD4^+CD25^-$ cells from renal transplant patients after Campath-1H would support this hypothesis. This possibility is also in line with data in genetically lymphopenic $Rag^{-/-}$ mice showing proliferation of adoptively transferred $CD4^+CD25^+$ but not $CD4^+CD25^-$ cells upon treatment with IL-2 (26). However, T cell depletion after Campath-1H infusion could stimulate the expansion of $CD4^+CD25^-$ cells that become activated and acquire high CD25 expression upon exposure to the lymphopenic environment, as observed in other studies in immunodeficient mice (38). Conversion between $CD4^+CD25^-$ and $CD4^+CD25^+$ phenotype also has been shown in immunocompetent wild-type mice after T cell depletion with ALS (5) and in ALS-stimulated human PBMC *in vitro* (41).

Data on the immune function of expanding cells after lymphopenia are controversial. $CD4^+CD25^+$ cells that were collected from mice after ALS inhibited *in vitro* $CD4^+$ and $CD8^+$ T cell proliferation against alloantigens and prolonged *in vivo* skin allograft survival upon adoptive transfer (5). On the contrary, studies in anti-CD4 plus anti-CD8 mAb–treated C57BL/6 mice documented that lymphopenia led to a skewing of the lymphocyte pool toward a higher percentage of effector/memory cells, which are prone to activate the immune response (42). Similarly, Pearl *et al.* (43) found that in renal transplant recipients who were given Campath-1H/deoxyspergualin induction therapy and no maintenance immunosuppression, postdepletion T cells were predominantly effector/memory T cells that expanded in the first month and were uniquely prevalent at the time of acute graft rejection episodes. Also in our patients, most residual T cells after Campath-1H actually expressed the $CD45RO^+$ memory-like marker. Nevertheless, a substantial proportion of cells that expanded from such remaining T cell pool, upon sirolimus maintenance immunosuppression, were $CD4^+CD25^{high}$ cells with regulatory phenotype and function. Indeed $CD4^+CD25^{high}$ cells that were taken from these patients did not express the CD69 activation marker but high levels of the Treg hallmark FOXP3. Moreover, they were hyporesponsive to alloantigens and capable of suppressing the alloreactive immune response of autologous effector $CD25^{-/low}$ T cells against donor antigens in co-cultures. Notably, Treg expansion did not cause a state of generalized immunosuppression, because T cells from these patients responded normally to a polyclonal T cell mitogenic stimulus. However, concomitant suppression of anti–third-party response was found, which likely reflects the alloantigen cross-reactivity of TCR from expanding Treg (44) or, alternatively, a phenomenon of bystander regulation (45).

At variance with the protocol of Pearl *et al.* (43), our patients were on maintenance immunosuppression with sirolimus and MMF, which would have been instrumental to favor Treg expansion and limit memory T cell proliferation and activation. This interpretation is in line with a number of published experimental pieces of evidence. In the presence of sirolimus, TCR-mediated stimulation of murine and human $CD4^+$ cells results in the generation of Treg that suppress syngeneic T cell proliferation *in vitro* (14,15) and prevent allograft rejection *in vivo* (14). Because of its favorable effects on Treg, sirolimus has been recently advocated as a pro-tolerogenic immunosuppressive drug (46). Moreover, MMF has been shown to prevent the differentiation of naïve T cells into memory cells and to inhibit memory cell proliferation both *in vitro* in human mixed lymphocyte cultures (47) and *in vivo* in F5 TCR transgenic mice (48).

Expansion of $CD4^+CD25^{high}FOXP3^+$ cells, however, was negligible in renal transplant patients who received the calcineurin inhibitor CsA along with MMF as maintenance therapy after Campath-1H induction. In this group, functional T cell studies excluded the presence of Treg that were capable of inhibiting anti-donor alloreactivity, because $CD4^+CD25^{high}$ depletion failed to reverse anti-donor T cell hyporesponsiveness. However, restoration of the immune response after addition of IL-2 to ELISPOT assays suggests that T cell anergy may have contributed to anti-donor hyporesponsiveness in the CsA-

treated group. We speculate that in patients who received Campath-1H and were maintained on CsA, donor reactive T cells that emerged during T cell reconstitution were primed *in vivo* by donor antigens from the kidney graft. However, the activation signaling through calcineurin/IL-2 was blocked by CsA, resulting in anergy instead of conversion into effector cells. This is in line with previous data in renal transplant patients on conventional immunosuppressive therapy including calcineurin inhibitors (34,49). Lack of development of $CD4^+CD25^{high}$ Treg with CsA could be explained by the fact that IL-2, whose generation is inhibited by CsA, promotes acquisition of CD25 molecules (16) and is a surviving factor for Treg *in vitro* (16) and *in vivo* (19). This interpretation is supported by findings that IL-2 administration to pediatric patients with sarcoma during immune reconstitution markedly increased Treg cell compartment in the peripheral blood as compared with patients who did not receive the cytokine therapy (26). *In vitro* evidence that CsA, by inhibiting calcineurin phosphatase-dependent NFAT translocation into the nucleus (50), suppresses FOXP3 promoter activity, mRNA, and protein expression in T cells (50) is also consistent with our *in vivo* data.

Conclusion

We have documented that in human renal transplantation, Campath-1H, *via* lymphocyte depletion, allows a subset of $CD4^+CD25^{high}$ FOXP3-expressing cells with regulatory activity to emerge, provided that sirolimus but not CsA is used as concomitant therapy. These cells did not seem to impair systemic immune competence to the extent that we found no difference in infectious episodes in the two groups (Table 3). Patients and graft outcome did not differ between the two study groups. However, GFR was numerically higher in sirolimus- than in CsA-treated patients, which may be related to differences in renal blood flow as a result of the vasoconstrictor effect of CsA. Nonetheless, we found that GFR values, at 24 mo after transplantation, strongly correlated with FOXP3 expression levels in $CD4^+$ cells ($P = 0.0087$, $r^2 = 0.78$), which suggests that the expansion of Treg in the sirolimus group contributed to maintain graft function, possibly by preventing the immune insult to the graft tissue. Whether in humans adaptive FOXP3-expressing Treg have functional regulatory capacity *in vivo*, as shown in rodents by adoptive transfer experiments, has not been documented yet. The results presented here provide the evidence, although indirect, that this may be the case.

Our findings create a case for launching a formal clinical trial to compare the best available anti-rejection treatment *versus* Campath-1H and low-dosage sirolimus and MMF, which seem to create the ideal environment for T cell regulation to occur. Whether this will eventually translate in a "tolerance permissive" state in human transplantation has to be established.

Acknowledgments

This study was partially supported by a grant from Associazione Ricerca Trapianti (Milan, Italy).

We thank Dr. Lorenzo Gallon (Northwestern University, Chicago, IL) for useful discussion during the development of the clinical design. We also thank Agustina Anabaya, Federica Arnoldi, Simona Gamba, Sergio Brescianini, Svitlana Yakymchuk, Emanuela Vergani, Emilia Camoni, and Franca Gamba for help in nursing assistance; Dr. Mario Bontempelli, Gianfranco Marchetti, Silvia Ferrari, Nadia Stucchi, Clara Petrò, and Elena Savoldelli for excellent technical assistance; and Dr. Annalisa Perna for helping to perform statistical analyses.

G.M. is a recipient of a fellowship from Associazione Ricerca Trapianti, and D.C. received a fellowship from Cav. Grana in memory of Libera Dossi Grana.

Disclosures

None.

References

1. Hariharan S, Johnson CP, Bresnahan BA, Taranto SE, McIntosh MJ, Stablein D: Improved graft survival after renal transplantation in the United States, 1988 to 1996. *N Engl J Med* 342: 605–612, 2000
2. Lechler RI, Sykes M, Thomson AW, Turka LA: Organ transplantation: How much of the promise has been realized? *Nat Med* 11: 605–613, 2005
3. Rocha PN, Plumb TJ, Crowley SD, Coffman TM: Effector mechanisms in transplant rejection. *Immunol Rev* 196: 51–64, 2003
4. Waldmann H, Adams E, Fairchild P, Cobbold S: Infectious tolerance and the long-term acceptance of transplanted tissue. *Immunol Rev* 212: 301–313, 2006
5. Minamimura K, Gao W, Maki T: CD4+ regulatory T cells are spared from deletion by antilymphocyte serum, a polyclonal anti-T cell antibody. *J Immunol* 176: 4125–4132, 2006
6. Vanasek TL, Nandiwada SL, Jenkins MK, Mueller DL: CD25+Foxp3+ regulatory T cells facilitate CD4+ T cell clonal anergy induction during the recovery from lymphopenia. *J Immunol* 176: 5880–5889, 2006
7. Bach JF: Induction of immunological tolerance using monoclonal antibodies: Applications to organ transplantation and autoimmune disease. *C R Biol* 329: 260–262, 2006
8. Morris PJ, Russell NK: Alemtuzumab (Campath-1H): A systematic review in organ transplantation. *Transplantation* 81: 1361–1367, 2006
9. Kaufman DB, Leventhal JR, Axelrod D, Gallon LG, Parker MA, Stuart FP: Alemtuzumab induction and prednisone-free maintenance immunotherapy in kidney transplantation: Comparison with basiliximab induction—Long-term results. *Am J Transplant* 5: 2539–2548, 2005
10. Vathsala A, Ona ET, Tan SY, Suresh S, Lou HX, Casasola CB, Wong HC, Machin D, Chiang GS, Danguilan RA, Calne R: Randomized trial of Alemtuzumab for prevention of graft rejection and preservation of renal function after kidney transplantation. *Transplantation* 80: 765–774, 2005
11. Knechtle SJ, Pirsch JD, H Fechner J Jr, Becker BN, Friedl A, Colvin RB, Lebeck LK, Chin LT, Becker YT, Odorico JS, D'Alessandro AM, Kalayoglu M, Hamawy MM, Hu H, Bloom DD, Sollinger HW: Campath-1H induction plus rapamycin monotherapy for renal transplantation: Results of a pilot study. *Am J Transplant* 3: 722–730, 2003
12. Wiederrecht GJ, Sabers CJ, Brunn GJ, Martin MM, Dumont FJ, Abraham RT: Mechanism of action of rapamycin: New insights into the regulation of G1-phase progression in eukaryotic cells. *Prog Cell Cycle Res* 1: 53–71, 1995
13. Grolleau A, Bowman J, Pradet-Balade B, Puravs E, Hanash S, Garcia-Sanz JA, Beretta L: Global and specific transla-

tional control by rapamycin in T cells uncovered by microarrays and proteomics. *J Biol Chem* 277: 22175–22184, 2002
14. Battaglia M, Stabilini A, Roncarolo MG: Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. *Blood* 105: 4743–4748, 2005
15. Valmori D, Tosello V, Souleimanian NE, Godefroy E, Scotto L, Wang Y, Ayyoub M: Rapamycin-mediated enrichment of T cells with regulatory activity in stimulated CD4+ T cell cultures is not due to the selective expansion of naturally occurring regulatory T cells but to the induction of regulatory functions in conventional CD4+ T cells. *J Immunol* 177: 944–949, 2006
16. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, Contag CH, Negrin RS: Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 108: 390–399, 2006
17. Bram RJ, Hung DT, Martin PK, Schreiber SL, Crabtree GR: Identification of the immunophilins capable of mediating inhibition of signal transduction by cyclosporin A and FK506: Roles of calcineurin binding and cellular location. *Mol Cell Biol* 13: 4760–4769, 1993
18. Malek TR, Bayer AL: Tolerance, not immunity, crucially depends on IL-2. *Nat Rev Immunol* 4: 665–674, 2004
19. Suzuki H, Kundig TM, Furlonger C, Wakeham A, Timms E, Matsuyama T, Schmits R, Simard JJ, Ohashi PS, Griesser H, *et al.*: Deregulated T cell activation and autoimmunity in mice lacking interleukin-2 receptor beta. *Science* 268: 1472–1476, 1995
20. Malek TR, Yu A, Vincek V, Scibelli P, Kong L: CD4 regulatory T cells prevent lethal autoimmunity in IL-2Rbeta-deficient mice. Implications for the nonredundant function of IL-2. *Immunity* 17: 167–178, 2002
21. Cattaneo D, Merlini S, Pellegrino M, Carrara F, Zenoni S, Murgia S, Baldelli S, Gaspari F, Remuzzi G, Perico N: Therapeutic drug monitoring of sirolimus: Effect of concomitant immunosuppressive therapy and optimization of drug dosing. *Am J Transplant* 4: 1345–1351, 2004
22. Perico N, Ruggenenti P, Gaspari F, Mosconi L, Benigni A, Amuchastegui CS, Gasparini F, Remuzzi G: Daily renal hypoperfusion induced by cyclosporine in patients with renal transplantation. *Transplantation* 54: 56–60, 1992
23. Gaspari F, Perico N, Ruggenenti P, Mosconi L, Amuchastegui CS, Guerini E, Daina E, Remuzzi G: Plasma clearance of nonradioactive iohexol as a measure of glomerular filtration rate. *J Am Soc Nephrol* 6: 257–263, 1995
24. Gallon L, Gagliardini E, Benigni A, Kaufman D, Waheed A, Noris M, Remuzzi G: Immunophenotypic analysis of cellular infiltrate of renal allograft biopsies in patients with acute rejection after induction with alemtuzumab (Campath-1). *Clin J Am Soc Nephrol* 1: 539–545, 2006
25. Baecher-Allan C, Brown JA, Freeman GJ, Hafler DA: CD4+CD25high regulatory cells in human peripheral blood. *J Immunol* 167: 1245–1253, 2001
26. Zhang H, Chua KS, Guimond M, Kapoor V, Brown MV, Fleisher TA, Long LM, Bernstein D, Hill BJ, Douek DC, Berzofsky JA, Carter CS, Read EJ, Helman LJ, Mackall CL: Lymphopenia and interleukin-2 therapy alter homeostasis of CD4+CD25+ regulatory T cells. *Nat Med* 11: 1238–1243, 2005
27. Bolton EM: Regulatory T cells in transplantation: Natural or induced? *Transplantation* 79: 643–645, 2005
28. Khattri R, Cox T, Yasayko SA, Ramsdell F: An essential role for Scurfin in CD4+CD25+ T regulatory cells. *Nat Immunol* 4: 337–342, 2003
29. Wu Y, Borde M, Heissmeyer V, Feuerer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, Mathis D, Benoist C, Chen L, Rao A: FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 126: 375–387, 2006
30. Lamioni A, Parisi F, Isacchi G, Giorda E, Di Cesare S, Landolfo A, Cenci F, Bottazzo GF, Carsetti R: The immunological effects of extracorporeal photopheresis unraveled: Induction of tolerogenic dendritic cells in vitro and regulatory T cells in vivo. *Transplantation* 79: 846–850, 2005
31. Sakaguchi S: Regulatory T cells: Key controllers of immunologic self-tolerance. *Cell* 101: 455–458, 2000
32. Newell KA, Larsen CP: Tolerance assays: Measuring the unknown. *Transplantation* 81: 1503–1509, 2006
33. Game DS, Hernandez-Fuentes MP, Lechler RI: Everolimus and basiliximab permit suppression by human CD4+CD25+ cells in vitro. *Am J Transplant* 5: 454–464, 2005
34. Ng WF, Hernandez-Fuentes M, Baker R, Chaudhry A, Lechler RI: Reversibility with interleukin-2 suggests that T cell anergy contributes to donor-specific hyporesponsiveness in renal transplant patients. *J Am Soc Nephrol* 13: 2983–2989, 2002
35. Makhlouf L, Grey ST, Dong V, Csizmadia E, Arvelo MB, Auchincloss H Jr, Ferran C, Sayegh MH: Depleting anti-CD4 monoclonal antibody cures new-onset diabetes, prevents recurrent autoimmune diabetes, and delays allograft rejection in nonobese diabetic mice. *Transplantation* 77: 990–997, 2004
36. Preville X, Flacher M, LeMauff B, Beauchard S, Davelu P, Tiollier J, Revillard JP: Mechanisms involved in antithymocyte globulin immunosuppressive activity in a nonhuman primate model. *Transplantation* 71: 460–468, 2001
37. Genestier L, Fournel S, Flacher M, Assossou O, Revillard JP, Bonnefoy-Berard N: Induction of Fas (Apo-1, CD95)-mediated apoptosis of activated lymphocytes by polyclonal antithymocyte globulins. *Blood* 91: 2360–2368, 1998
38. Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ: CD25- T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion. *J Immunol* 173: 7259–7268, 2004
39. Zelenay S, Lopes-Carvalho T, Caramalho I, Moraes-Fontes MF, Rebelo M, Demengeot J: Foxp3+ CD25- CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion. *Proc Natl Acad Sci U S A* 102: 4091–4096, 2005
40. Cox AL, Thompson SA, Jones JL, Robertson VH, Hale G, Waldmann H, Compston DA, Coles AJ: Lymphocyte homeostasis following therapeutic lymphocyte depletion in multiple sclerosis. *Eur J Immunol* 35: 3332–3342, 2005
41. Lopez M, Clarkson MR, Albin M, Sayegh MH, Najafian N: A novel mechanism of action for anti-thymocyte globulin: Induction of CD4+CD25+Foxp3+ regulatory T cells. *J Am Soc Nephrol* 17: 2844–2853, 2006
42. Wu Z, Bensinger SJ, Zhang J, Chen C, Yuan X, Huang X, Markmann JF, Kassaee A, Rosengard BR, Hancock WW, Sayegh MH, Turka LA: Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med* 10: 87–92, 2004
43. Pearl JP, Parris J, Hale DA, Hoffmann SC, Bernstein WB,

McCoy KL, Swanson SJ, Mannon RB, Roederer M, Kirk AD: Immunocompetent T-cells with a memory-like phenotype are the dominant cell type following antibody-mediated T-cell depletion. *Am J Transplant* 5: 465–474, 2005

44. Sanchez-Fueyo A, Sandner S, Habicht A, Mariat C, Kenny J, Degauque N, Zheng XX, Strom TB, Turka LA, Sayegh MH: Specificity of CD4+CD25+ regulatory T cell function in alloimmunity. *J Immunol* 176: 329–334, 2006
45. Bushell A, Jones E, Gallimore A, Wood K: The generation of CD25+ CD4+ regulatory T cells that prevent allograft rejection does not compromise immunity to a viral pathogen. *J Immunol* 174: 3290–3297, 2005
46. Blaha P, Bigenzahn S, Koporc Z, Schmid M, Langer F, Selzer E, Bergmeister H, Wrba F, Kurtz J, Kiss C, Roth E, Muehlbacher F, Sykes M, Wekerle T: The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. *Blood* 101: 2886–2893, 2003
47. Trzoukowski P, Zilvetti M, Friend P, Wood KJ: Recipient memory-like lymphocytes remain unresponsive to graft antigens after Campath-1H induction with reduced maintenance immunosuppression. *Transplantation* 82: 1342–1351, 2006
48. Quemeneur L, Beloeil L, Michallet MC, Angelov G, Tomkowiak M, Revillard JP, Marvel J: Restriction of de novo nucleotide biosynthesis interferes with clonal expansion and differentiation into effector and memory CD8 T cells. *J Immunol* 173: 4945–4952, 2004
49. Segundo DS, Ruiz JC, Izquierdo M, Fernandez-Fresnedo G, Gomez-Alamillo C, Merino R, Benito MJ, Cacho E, Rodrigo E, Palomar R, Lopez-Hoyos M, Arias M: Calcineurin inhibitors, but not rapamycin, reduce percentages of CD4+CD25+FOXP3+ regulatory T cells in renal transplant recipients. *Transplantation* 82: 550–557, 2006
50. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, Schmidt-Weber CB: Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* 176: 3593–3602, 2006