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ORIGINAL ARTICLE

Variation in numbers of $CD4^+CD25^{high}FOXP3^+$ T cells with normal immuno-regulatory properties in long-term graft outcome

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Summary

Chronic rejection (CR) is a major cause of long-term graft loss that would be avoided by the induction of tolerance. We previously showed that renal transplant patients with CR have lower numbers of peripheral $CD4^+CD25^{high}$ T cells than operationally tolerant patients, patients with stable graft function and healthy volunteers (HV). We explored here the profile of $CD4^+CD25^{high}$ blood T cells in these patients focusing on their expression of the regulatory T cells (Treg) gene Forkhead Box P3 (FOXP3) and their suppressive function. We show that CR is associated with a decreased number of $CD4^+CD25^{high}$ $FOXP3^+$T cells with normal regulatory profile, whereas graft acceptance is associated with $CD4^+CD25^{high}FOXP3^+$T cell numbers similar to HVs. These data suggest that Treg numbers, rather than their intrinsic suppressive capacity, may contribute to determining the long-term fate of renal transplants.

Introduction

Despite improvements in immunosuppressive therapy over the last decade, chronic graft injury remains a major cause of graft loss in the long term [1–3]. A portion of such injury is thought to arise from late allo-immune chronic rejection (CR) [4]. CR is characterized by progressive renal dysfunction and is diagnosed on a histological basis by the presence of transplant glomerulopathy and/or interstitial fibrosis, tubular atrophy and/or vascular occlusive changes [4–6]. Moreover, CR is only poorly influenced by currently used immunosuppressors, and long-term exposure to immunosuppression (IS) leads to post-transplant infections [7], malignancies and lymphoproliferative diseases [8,9], all of which contribute to the high level of morbidity and mortality observed in transplant patients [10]. Thus, ways of inducing long-term allograft acceptance in the absence of IS, i.e. transplant tolerance, are being actively sought after. Although tolerance to allografts is difficult to induce in the clinical setting, some rare patients display well-functioning grafts despite having stopped their immunosuppressive medication, a state referred to as operational tolerance [11].

In rodents, there is a mounting evidence that transplant tolerance is driven at least in part by $CD4^+CD25^+$ cells with suppressive activity, so-called regulatory T cells (Treg) [12]. In humans, these cells have been shown to play a key role in controlling autoimmunity [13] and allergic diseases [14]. Both a decrease in Treg number with normal regulatory function [15,16] and a deficit in Treg function have been described in the induction or evolution of these various pathologies [17]. Nevertheless, relatively little is known thus far of the Treg population in human transplant recipients and whether Tregs influence long-term graft acceptance or rejection. A study of direct pathway alloresponses in stable kidney recipients by

Game *et al.* using mixed lymphocyte culture, limiting dilution assay, and IFNg ELISPOT before and after the depletion of $CD4^+CD25^+$ cells revealed no preferential regulation to donor antigen when compared with third party. The authors suggested that regulation by $CD4^+CD25^+$ cells does not account for the direct pathway hyporesponsiveness that occurs in the majority of renal transplant recipients [18]. On the other hand, Velthuis *et al.* studied the presence and function of $CD4^+CD25^{bright+}$ Tregs in 33 renal transplant recipients more than 5 years after transplantation. They concluded that in a subset of CNI-free kidney allograft recipients a long time after transplantation, functional $CD4^+CD25^{bright+}$ Tregs are present in the peripheral blood and are at least partially responsible for the state of proliferative donor nonresponsiveness [19].

In a previous study [20], we reported for the first time that patients with CR display a lower number of $CD4^+CD25^{high}$ blood T cells than patients with long-term stable graft function and healthy volunteers (HV), but that these cells expressed normal levels of CTLA4, GITR, CCR4 and CD103 markers associated with Treg activity [21,22]. Operationally tolerant patients, on the other hand, had numbers similar to HVs, indicating that operational tolerance is not characterized by an increase in peripheral blood Treg numbers. Here, we further explored the profile of $CD4^+CD25^{high}$ blood T cells in renal transplant patients with late graft acceptance or rejection, focusing on their expression of the Treg master gene Forkhead Box P3 (FOXP3) as well as their suppressive function.

Materials and methods

Patients

Peripheral blood samples were collected from 44 adult renal transplant recipients and 10 normal adult controls. The protocol was approved by the University Hospital Ethical Committee. All patients gave informed consent. The clinical history of the patients is outlined in Table 1. Note that because of the rarity of some patients (those with operational tolerance patients) and because the experiments span several years, the different analyses were not performed on all patients.

Drug-free operationally tolerant recipients (DF-Tol)

Drug-free operationally tolerant recipients ($n = 7$) were patients with a stable kidney graft function (blood creatinemia <150 μmol/l and proteinuria <1 g/24 h) in the absence of IS for at least 1 year (range: 2–17). Immunosuppressive treatment was stopped due to noncompliance ($n = 5$), post-transplant lymphoproliferative disorder ($n = 1$) or calcineurin inhibitor toxicity ($n = 1$). The clinical parameters of these patients have been described in detail elsewhere [11]. Because of their normal and stable

Table 1. Patient demographics and clinical data: recipients with chronic rejection (CR) and patients with long-term graft survival with (Sta) or without immunosuppression (IS) (drug-free operationally tolerant patients) were matched for age. Cockroft creatinine clearance and proteinuria were measured on the day of blood sampling. Data are presented as median (range).

Group	CR	Stable	Operationally tolerant
n	22	15	7
Recipient age in years: mean ± SD (range)	51.9 ± 13 (28–76)	50.4 ± 8.9 (33–66)	54.6 ± 16.3 (38–76)
Gender ratio (M:F)	7:15	6:9	5:2
Cockroft Creatinine clearance ml/min: mean ± SD (range)	26.4 ± 13.9 (10.4–69.3)	75.2 ± 35.9 (46.9–135)	66.3 ± 23 (38–97)
Proteinuria g/24 h: mean ± SD (range)	2.1 ± 2.2 (0.12–7.75)	0.1 ± 0.2 (0–0.48)	0.4 ± 0.5 (0–1)
Time post-transplantation inyrs: mean ± SD (range)	7.3 ± 4.4 (2–17)	7.6 ± 3.3 (4–17)	15.4 ± 6.1 (5–33)
C4d+ biopsy	Yes 11/20 NA 2/22	NA	NA
Circulating HLAAb	Yes 14/22	0/22	Yes 1/6 NA1/7
Transplant Glomerulopathy	Yes 20/22	NA	NA
Banff c grade	Ib-IIIb	NA	NA
IS protocol	Anti-metabolite: MMF: 17/22 CNI: CsA: 22/22 Steroids: 8/22	Anti-metabolite: Aza: 2/15 MMF: 11/15 CNI: CsA: 15/15 Steroids: 1/15	None for 8.9 ± 5.3 years (2–17)

NA, not applicable; Aza, azathioprine; MMF, mycophenolate mofetil; CNI, calcineurin inhibitor; CsA, cyclosporin A.

graft function and in the light of recommendations made by the University Hospital Ethical Committee and the Committee for the Protection of Patients from Biological Risks, no biopsies were taken from these patients (Table 1).

Patients with stable graft function under standard IS (Sta) (n = 15)

Stable patients had stable graft function under standard IS with a creatinemia <150 μM/l and proteinuria <1 g/24 h for at least 3 years. No biopsies were available for these patients as they presented no deterioration of graft function (Table 1).

Patients with CR (n = 22)

These patients exhibited a progressive deterioration of their renal function under standard IS with a proteinuria >1 g/24 h and a creatinemia >150 μM/l. CR, including chronic active antibody-mediated rejection and T-cell mediated CR, was diagnosed on a graft biopsy according to the updated Banff classification criteria [4,5]. CR was defined by histological signs of chronic allograft arteriopathy (arterial intimal fibrosis with mononuclear cell infiltration) and/or transplant glomerulopathy with glomerular double contours. Twenty of 22 patients had a transplant glomerulopathy, nine of 22 had an active humoral component as demonstrated by the presence of C4d together with circulating anti-HLA antibodies.

Healthy volunteers

Age-matched HVs (n = 10; mean age: 47.4 ± 7.5 years; range: 36–61; sex 2 M/8 F) were included as controls (Table 1). All had a normal blood formula and no infectious or other concomitant pathology for at least 6 months prior to the study.

Materials

Isolation and purification of blood cell subsets from PBMC

Venous blood samples were collected in EDTA test tubes and processed for analysis within 6 h. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll hypaque gradient (Eurobio, Les Ulis, France). $CD4^+CD25^{high}$ and $CD4^+CD25^-$ T cells were either sorted with microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) or using a high throughput FACSAria cytofluorometer (BD Biosciences) using CD25-alexa 647, CD4-FITC, and CD127-PE antibodies (BD Biosciences). Purity was systematically >95%.

Reagents and FACS analysis

All patients and healthy individuals were first examined for complete blood count and screened for the presence of $CD4^+CD25^{high}$ T cells by flow cytometry as described elsewhere [20]. Antibodies were purchased from Becton Dickinson (San Jose, CA, USA) (CD25-PE and PC5) Beckman Coulter (Marseille, France) (CD4-FITC) and BD Biosciences (Mountain View, CA, USA) (CD3-PC7 and PC5, CD4-APC). Staining for intracellular FOXP3 protein was performed on PBMC with the PE-conjugated FOXP3 (PCH101) antibody according to the manufacturer's instructions (eBioscience, Clinisciences, San Diego, CA, USA). Peripheral $CD4^+CD25^{high}$ T cells were analyzed by direct whole blood staining (Beckman Coulter Marseille, France). Flow cytometry was performed on a FACSCalibur with CELL QUEST PRO software (BD Biosciences).

$CD4^+CD25^{high}$ T cell proliferation

Experiments were performed on seven Sta (mean age:51.7 ± 7.2 years; range: 45–63; sex: 4 M/3 F; Cockroft creatinine clearance 67.2 ± 12 ml/min; proteinuria: 0.1 ± 0.2 g/24 h), nine CR (mean age: 51.9 ± 14 years; range: 28–75; sex 2 M/7 F; Cockroft creatinine clearance 33.1 ± 17.4 ml/min; proteinuria: 2.4 ± 2.2 g/24 h), five DF-Tol (mean age: 52.8 ± 16 years; range: 38–76; sex: 4 M/1 F; Cockroft creatinine clearance 67.5 ± 26.7 ml/min; proteinuria: 0.4 ± 0.5 g/24 h) and eight HV (mean age: 48.7 ± 8.8 years; range: 36–61; sex 2 M/6 F). Proliferation assays were performed by stimulating $CD4^+CD25^{high}$ T cells with autologous irradiated (35 Gy) PBMC. Autologous-irradiated PBMC (20 000 cells) were cocultured for 72 h with $CD4^+CD25^{high}$ T cells (20 000 cells) in duplicate wells in round bottom 96-well plates previously coated overnight at 4°C with 1 μg/ml CD3 monoclonal antibody (mAb) (Orthoclone OKT3®, Janssen-Cilag, Neuss, Germany). Cells were pulsed for the final 8 h of culture with 1 μCi per well of [3H] thymidine (Amersham Biosciences, Buckinghamshire, UK). Cells were harvested and counted in a scintillation counter.

Autologous $CD4^+CD25^{high}$ T cell suppressive activity [*autologous mixed lymphocyte reaction* (*MLR*)]

Experiments were performed on seven Sta (mean age: 51.7 ± 7.2 years; range: 45–63; sex: 4 M/3 F; Cockroft creatinine clearance 67.2 ± 12 ml/min; proteinuria: 0.1 ± 0.2 g/24 h), nine CR (mean age: 51.9 ± 14 years; range: 28–75; sex 2 M/7 F; Cockroft creatinine clearance 33.1 ± 17.4 ml/min; proteinuria: 2.4 ± 2.2 g/24 h) five DF-Tol (mean age: 52.8 ± 16 years; range: 38–76; sex:

4 M/1 F; Cockroft creatinine clearance 67.5 ± 26.7 ml/min; proteinuria: 0.4 ± 0.5 g/24 h) and eight HV (mean age: 48.7 ± 8.8 years; range: 36–61; sex 2 M/6 F). Patients were included according to the number of available PBMC. $CD4^+CD25^-$ T cells (20 000 cells) were stimulated in the presence of 20 000-irradiated autologous PBMC in duplicate in round bottom 96-well plates previously coated overnight at 4°C with 1 μg/ml CD3 mAb (Orthoclone OKT3®). $CD4^+CD25^{high}$ T cells were added at $CD25^-$: $CD25^{high}$ T-cell ratios ranging from 1:1 to 1:4. Cultures were performed in medium alone or in the presence of recombinant human IL2 (Proleukin®, Chiron Corporation, Emeryville, CA, USA), anti-IL10 (R&D Systems, Abingdon, UK) or anti-TGFβ (clone 2 G7) at a final concentration of 100 U/ml, 1 μg/ml, and 10 μg/ml, respectively. After 72 h of coculture, cells were pulsed for 8 h with 1 μCi per well of [3H] thymidine (Amersham Biosciences). Cells were harvested and counted in a scintillation counter. Culture medium consisted of RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 1% nonessential amino acids, 1 mM sodium pyruvate and 10% heat-inactivated human serum (Sigma, St Louis, MO, USA). Absence of activated cells within the $CD4^+CD25^{high}$ population was confirmed by their inability to proliferate or produce IFNγ, or absence of expression of CD127.

Cytokine production

IFNγ, IL1β, IL2, IL4, IL5, IL6, IL8, IL10, TNFα and TNFβ protein levels were measured in supernatants of $CD4^+CD25^-$ MLRs before and after addition of $CD4^+CD25^{high}$ T cells (ratio 1:1; stimulation with irradiated autologous PBMC cells and CD3 mAb) after 24 h of culture, using a multiplex fluorescent bead immunoassay, the FlowCytomix Multiplex human Th1/Th2 Kit II (Bender MedSystems, Tebu-bio, France). Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) according to the manufacturer's instructions. For each cytokine, the minimum detectable level was indicated by the manufacturer (BD Biosciences).

Statistical analysis

Data were presented as mean ± SD. The comparisons among the three groups of patients were performed by using a nonparametric one-way ANOVA Kruskal–Wallis' test and Dunn's test for multiple comparisons. The Mann–Whitney test was used for comparisons between two groups. Values of $P < 0.05$ were considered as significant.

Results

Contrasting peripheral blood $CD4^+CD25^+FOXP3^+$ T cell numbers in long-term graft acceptance versus rejection: decreased numbers in CR

We previously showed that patients with CR have lower $CD4^+CD25^{high}$ blood T-cell numbers than patients with drug-free operational tolerance and HVs, but that these cells display the same phenotype as the two other groups in terms of expression of the classical Treg markers CTLA4, GITR, CCR4 and CD103 [20]. Given that CD25 is also a marker of activated T cells; here, we additionally analyzed the number of cells co-expressing the Treg master gene FOXP3. We found a decrease in the number of $CD4^+$ T cells co-expressing $CD25^+$ and FOXP3 in CR patients (CR: 10.4 cells/μl of blood) compared with operationally tolerant patients (DF-Tol: 26.5 cells/μl of blood, $P < 0.01$) healthy individuals (HV: 24.3 cells/μl of blood, $P < 0.007$) (Fig. 1a) and patients with stable graft function (Sta: 18.2 cells/μl of blood, $P < 0.01$). Nevertheless, no difference was observed in actual FOXP3 expression within the purified $CD4^+CD25^{high}$ blood T-cell population itself (Fig. 1b), suggesting that the difference observed within the $CD4^+$ T-cell population simply reflects a difference in the corresponding cell numbers between the different groups.

Thus, patients with CR display fewer $CD4^+CD25^{high}$-$FOXP3^+$ blood T cells, but these cells have the same amount of intracellular FOXP3 as those from operationally tolerant patients, patients with stable graft function under standard IS and healthy individuals.

Normal capacity of peripheral blood $CD4^+CD25^{high}$ T cells from patients with CR to suppress proliferation of autologous $CD4^+CD25^-$ T cells

A lack of viable donor cells for the patients included for study precluded the possibility of testing anti-donor specific responses. We thus used a conventional approach to examine the functional regulatory capacity of $CD4^+CD25^{high}$ T cells of the different patients by assessing their ability to suppress the proliferation and cytokine production of anti-CD3-stimulated autologous $CD4^+CD25^-$ T cells. $CD4^+CD25^{high}$ T cells were purified either using immuno-magnetic beads, a technique routinely used to purify potent Treg [23], or by high throughput cell sorting with a FACSAria cytofluorometer using the CD4, CD25, and CD127 markers. Lack of CD127 expression has recently been identified as a hallmark of Treg [24]. As shown in Fig. 2, the level of proliferation of the $CD4^+CD25^-$ and $CD4^+CD25^{high}$ T cell populations did not significantly differ between the different groups of patients. A strong $CD4^+CD25^-$ T cell

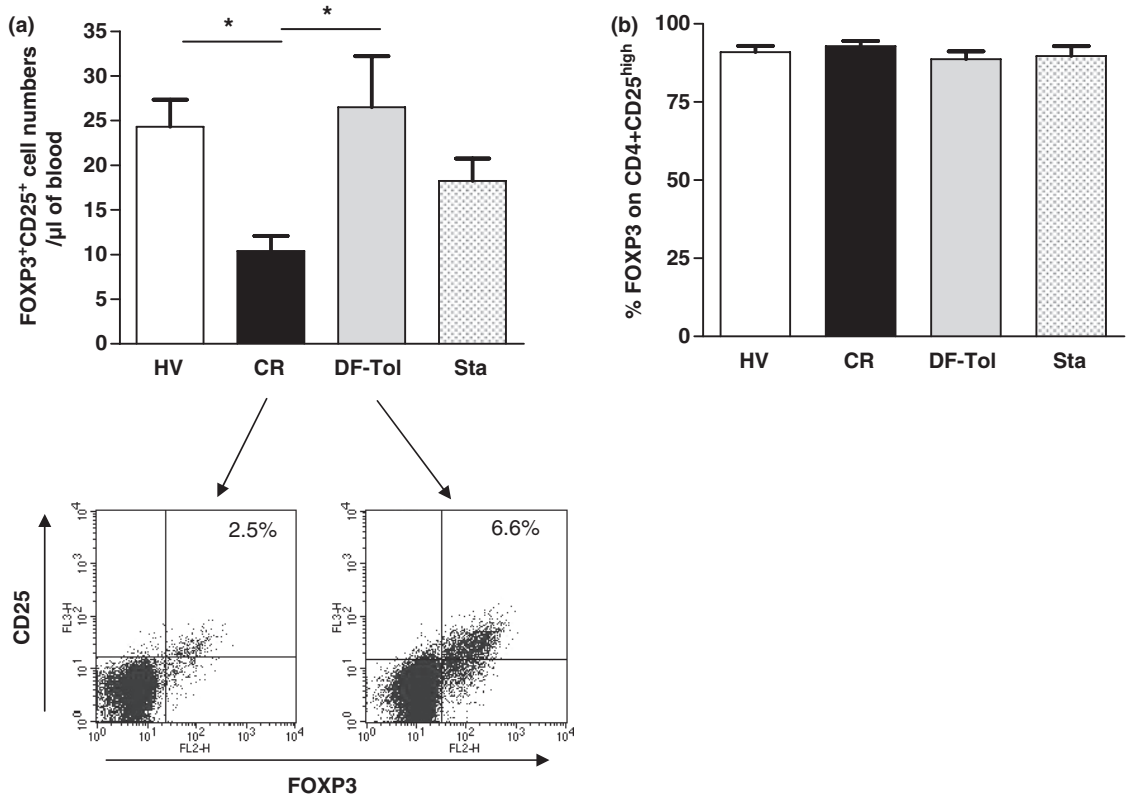

Figure 1 Forkhead Box P3 (FOXP3) expression by FACS analysis: FOXP3 protein was measured on $CD4^+CD25^+$ T cells by intracellular staining. (a) $CD4^+$ T cells were double stained with anti-CD25 and anti-FOXP3 antibodies. Absolute numbers of $CD25^+FOXP3^+$ T cells were calculated for healthy individuals (HV, $n = 10$), patients with chronic rejection (CR, $n = 22$), patients with drug-free operational tolerance ($n = 7$) and patients with stable long-term graft survival under immunosuppression (Sta, $n = 15$). (b) Percentage of FOXP3 protein expression by $CD4^+CD25^{high}$ T cells. *, indicates $P < 0.05$.

proliferative response was obtained by day 3 of activation whereas the $CD4^+CD25^{high}$ population of the different patient groups poorly proliferated in the same culture conditions. $CD4^+CD25^{high}$ unresponsiveness was reversed in all cases by addition of IL2, suggesting a state of anergy (Fig. 2).

Analysis of the global percentage of inhibition for each group revealed no statistically significant variations, with an average of 67 ± 11.1% for HVs, 69.7 ± 7.4% for operationally tolerant patients, 78.6 ± 13.7% for patients with stable graft function under standard IS and 65.6 ± 19.5% for CR patients (Fig. 3a). Thus, on a per cell basis (effector:responder ratio of 1:1), $CD4^+CD25^{high}$ T cells from patients with CR display no intrinsic deficit in their suppressive properties compared with the same cells from kidney transplant recipients with long-term graft survival and HVs. Moreover, operationally tolerant patients display no increase in suppressive capacity compared with the HVs and the different groups of transplant patients.

The inhibitory capacity of the $CD4^+CD25^{high}$ T cells was dose-dependent (almost maximal suppression at a 1:1 ratio; Fig. 3b), and independent of IL10 and TGFβ, as blocking antibodies had no effect (Fig. 3c). Finally, this suppressive activity was stable over time (Fig. 4).

Normal capacity of peripheral blood $CD4^+CD25^{high}$ T cells from patients with CR to suppress IFNγ production by autologous $CD4^+CD25^-$ T cells

We next analyzed the ability of $CD4^+CD25^{high}$ T cells to suppress cytokine production by $CD4^+CD25^-$ responder cells in the different groups of patients. After 24 h of culture, $CD4^+CD25^{high}$ T cells from the different groups of patients produced no detectable levels of the cytokines analyzed. Similarly, no detectable levels of IL12p70, IL2, IL4, IL5 and TNFβ were produced by $CD4^+CD25^-$ T cells. On the other hand, IL8, IL10, IL1β, IL6 and TNFα were variably secreted by $CD4^+CD25^-$ T cells, but this production was unaltered by coculture with the $CD4^+CD25^{high}$ population in the different groups (*data not shown*).

Whereas $CD4^+CD25^{high}$ T cells produced undetectable levels of the Th1 cytokine IFNγ, the amount of IFNγ secreted by the $CD4^+CD25^-$ T-cell population did not

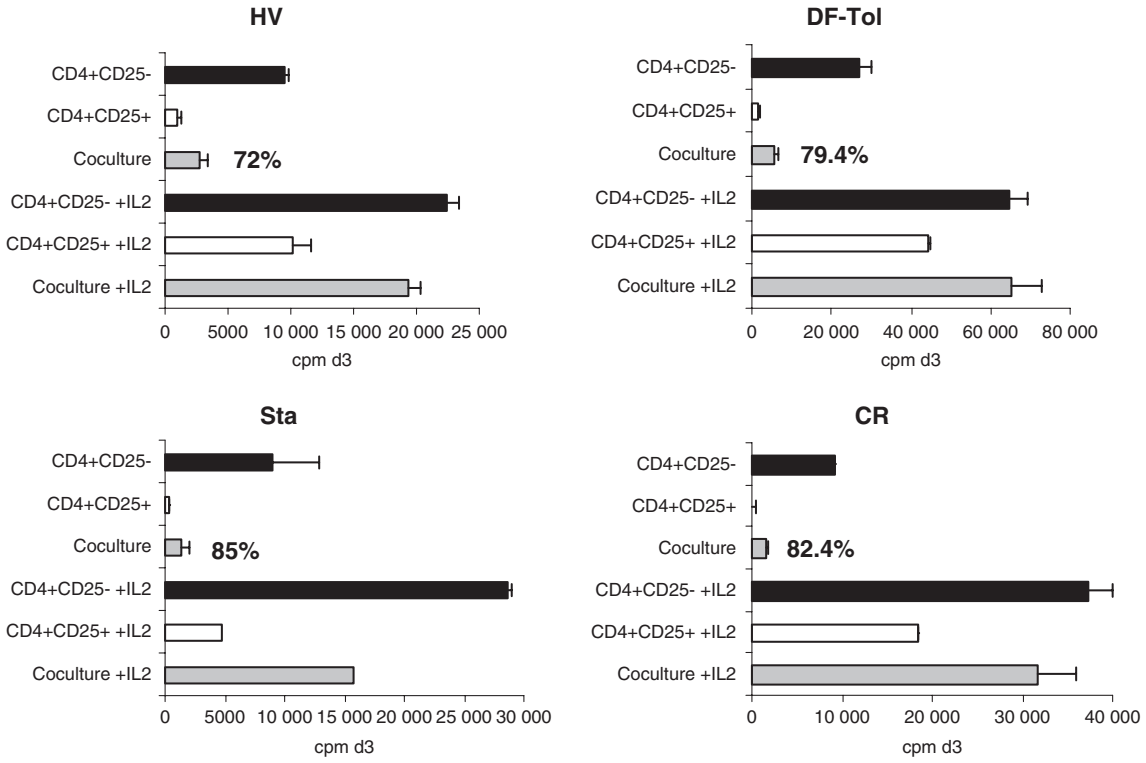

Figure 2 $CD4^+CD25^{high}$ T-cell suppressive activity in kidney recipients and healthy volunteers (HV). The ability of $CD4^+CD25^{high}$ T cells freshly isolated from HV, patients with chronic rejection, drug-free operationally tolerant patients and patients with stable renal function under standard immunosuppression (Sta), to suppress $CD4^+CD25^-$ T-cell responders was assessed (autologous mixed lymphocyte reaction). Responder cells were activated in the presence of 20 000-irradiated (35 Gy) autologous peripheral blood mononuclear cells plus anti-CD3 monoclonal antibody (1 μg/ml) (see Methods for details). $CD4^+CD25^{high}$ T cells were added (ratio 1:1) to $CD4^+CD25^-$ T-cell responder cells and [3H] thymidine incorporation was measured after 72 h. When indicated, IL2 was added at 100 U/ml. Each proliferation assay was carried out in duplicate (SD). Each experiment is representative of all the experiments in the different groups of patients and HVs. Percentages indicate inhibition of proliferation.

differ between patients with CR (mean: 182.73 pg/ml; range: 18–604) and those with stable graft function under standard IS (mean: 187.65 pg/ml; range: 53–322) (Fig. 5). Finally, $CD4^+CD25^{high}$ T cells from patients with CR were able to inhibit the secretion of IFNγ, which typically occurs following $CD4^+CD25^-$ T-cell activation, to the same extent as those from patients with stable graft function under IS and HVs (Fig. 5).

Discussion

There is now compelling evidence that $CD4^+CD25^{high}$ T cells may be involved in regulating the immune system and particularly in determining transplant outcome [12]. We have previously shown that patients with CR display lower numbers of $CD4^+CD25^{high}$ blood T cells compared with operationally tolerant patients and healthy individuals, but that these cells express normal levels of the typical Treg markers CTLA4, GITR, CCR4 and CD103 [20]. In the present study, we further investigated the FOXP3 profile and the suppressive function of peripheral blood $CD4^+CD25^{high}$ T cells in patients with long-term kidney graft acceptance or rejection compared with HVs.

Forkhead Box P3 has been shown to play a major role in the differentiation of $CD4^+CD25^{high}$ Tregs and to be expressed constitutively at high levels in these cells [25]. Mutations in the FOXP3 gene are associated with autoimmune pathologies in humans [26] and its role in transplantation has now been clearly demonstrated [27]. However, some recent evidence suggests a transient expression of FOXP3 in human activated nonregulatory $CD4^+$ T cells while it is stably expressed in natural $CD4^+CD25^+$ Treg cells [28–31]. Nevertheless, this temporary expression in T effector cells was insufficient to suppress expression of reported targets of FOXP3 repressor activity, including CD127, IL-2, and IFNgamma. Thus, expression of FOXP3 would be a normal consequence of $CD4^+$ T-cell activation and, in humans, it can no longer be used as an exclusive marker of nTregs.

We report here that, when assessed on a per cell basis, the level of FOXP3 protein in peripheral blood $CD4^+CD25^{high}$ T cells is equivalent between renal trans-

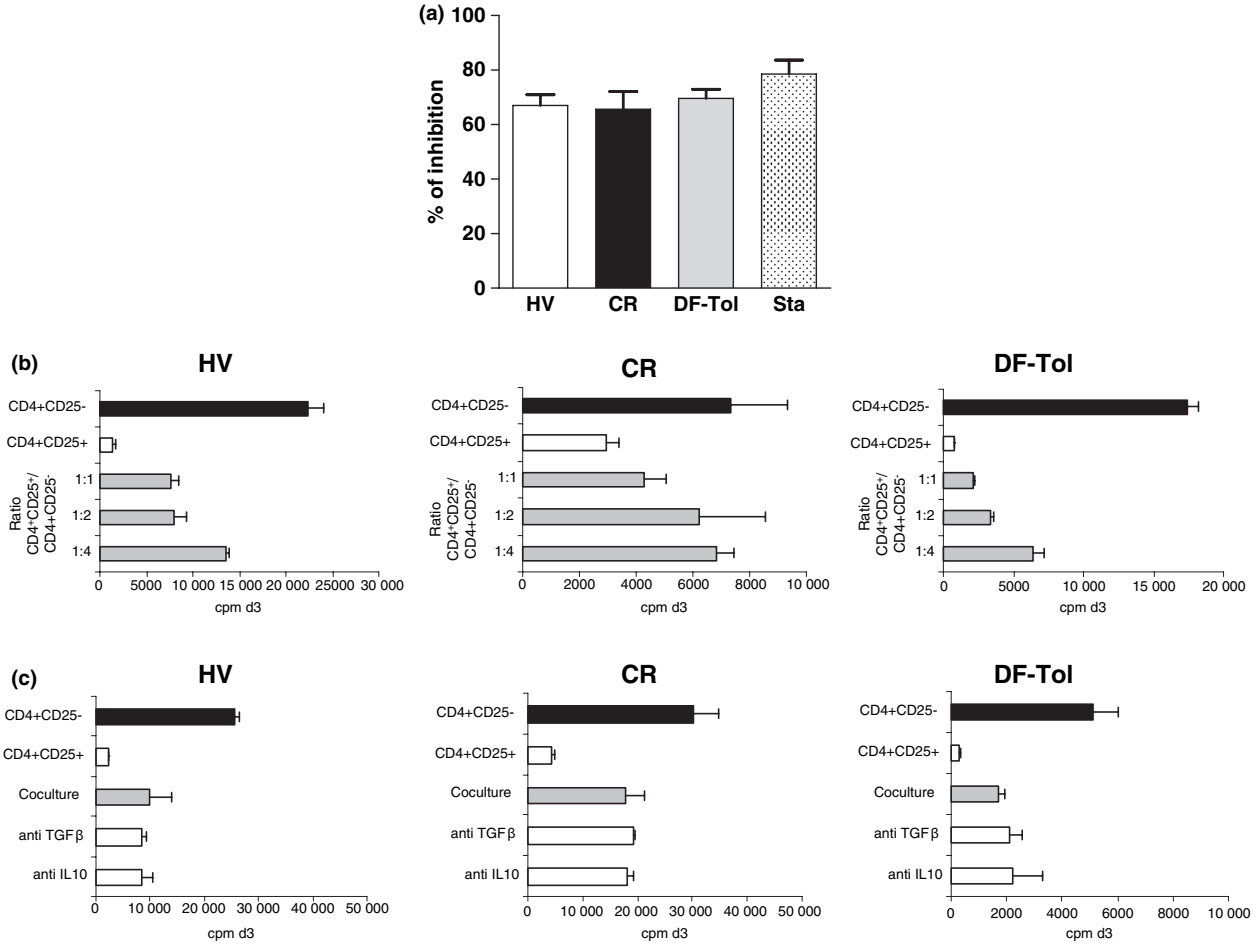

Figure 3 $CD4^+CD25^{high}$ T-cell suppressive activity in autologous mixed lymphocyte reaction. (a) Average inhibition of proliferation in healthy volunteers (HV) (n = 8), Drug-free operationally tolerant recipients (n = 5), standard immunosuppression (n = 7), and chronic rejection (n = 9) with a 1:1 ratio of $CD4^+CD25^{high}$/$CD4^+CD25^-$ T cells. (b) Dose-dependant suppression. $CD4^+CD25^-$ cells and $CD4^+CD25^{high}$ T cells were cultured at various ratios, with anti-CD3 and irradiated autologous peripheral blood mononuclear cells. In the coculture, the number of $CD4^+CD25^-$ responder cells was constant whereas the number of $CD4^+CD25^{high}$ T cells varied by serial twofold dilutions. [3H] thymidine incorporation was measured after 72 h. (c) Effect of the addition of anti-IL10 (1 μg/ml) and anti-TGFβ (10 μg/ml) antibodies to the coculture. Each proliferation assay was carried out in duplicate (SD).

plant patients with long-term graft acceptance or rejection and HVs, but that patients with CR display fewer $FOXP3CD4^+CD25^{high}$ T cells, suggesting that Treg numbers in the periphery may influence long-term graft outcome.

Because both deficient numbers [15,16] and function [17,32,33] of $CD4^+CD25^+$ T cells have been reported in the induction or evolution of different pathologies, we next investigated whether the decreased numbers of $CD4^+CD25^{high}FOXP3^+$ T cells in patients with CR is additionally associated with impaired suppressive properties. Given the lack of viable donor cells, which precluded the possibility of testing anti-donor-specific responses, we used a conventional approach to examine the regulatory capacity of $CD4^+CD25^{high}$ T cells of these patients by assessing their ability to suppress the proliferation and cytokine production of stimulated autologous $CD4^+CD25^-$ T cells *in vitro*. As could be expected for Treg, the $CD4^+CD25^{high}$ T cells from the different groups of patients were unresponsive to anti-CD3 and autologous PBMC activation, unless IL2 was present, in contrast to their $CD4^+CD25^-$ counterparts that proliferated vigorously. Although in humans activated T cells can also express the FOXP3 marker [34,35], the $CD4^+CD25^{high}$ T cells we isolated from the different patient groups displayed all the classical functional properties of regulatory cells (low proliferation, absence of IFNγ production and inhibition of proliferation and IFNg production by $CD4^+CD25^-$ effectors in response to stimulation). To further ensure the selection of Treg without contaminating

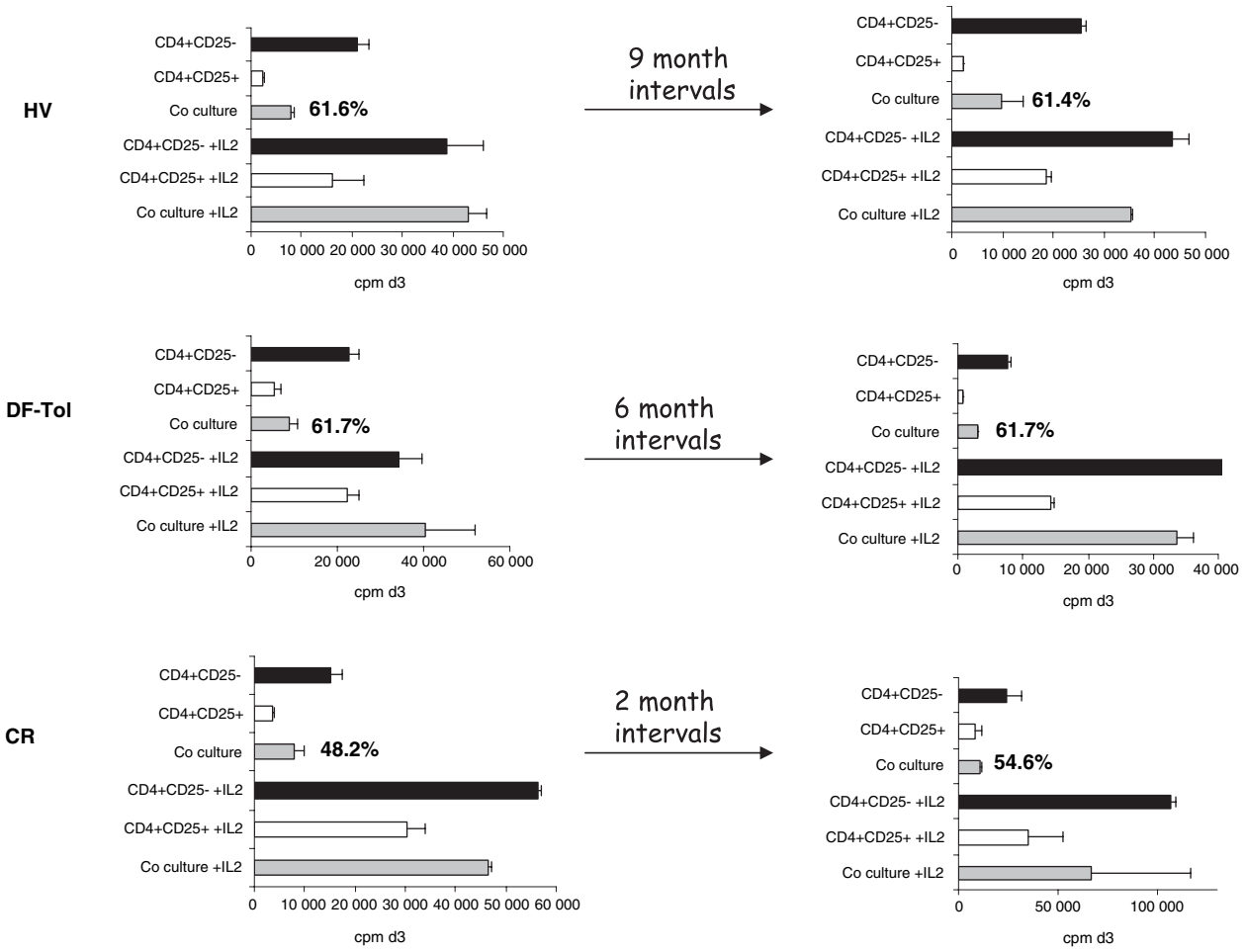

Figure 4 Stability of the suppressive activity of $CD4^+CD25^+$ T cells over time. The ability of $CD4^+CD25^{high}$ T cells, freshly isolated from healthy volunteers (HV), standard immunosuppression, drug-free operationally tolerant recipients (DF-Tol) and chronic rejection (CR) recipients, to suppress $CD4^+CD25^-$ T cells responders was assessed over time, as described in the legend to Figure 2. Graphs show one HV with a 9-month interval, one DF-Tol with a 6 month interval and one CR with a 2-month interval. Each experiment is representative of all experiments in the different groups of patients. Percentages indicate inhibition of proliferation. Each proliferation assay was carried out in duplicate (SD).

effectors, the $CD4^+CD25^{high}$ T cells were additionally selected on the basis of their absence of CD127 expression, a characteristic of human Treg [24]. The lack of effect of IL10 and TGFβ-blocking antibodies on the suppressive function of the $CD4^+CD25^{high}$ T cells used in our assay indicates that these were indeed natural Tregs that have been shown to nonspecifically suppress the proliferation of $CD4^+CD25^-$ T cells in a contact-dependant manner [36]. This is further supported by the finding that $CD4^+CD25^{high}$ T cells had no effect on the production of IL10 by $CD4^+CD25^-$ T cells in the different groups of patients.

Our results show that despite being present at lower numbers in CR patients, $CD4^+CD25^{high}$ $FOXP3^+$ T cells from these patients did not display an intrinsic defect in their suppressive properties, as shown by their ability to suppress both the proliferation and IFNγ production of autologous $CD4^+CD25^-$ T cells in the same way as those from patients with stable, well-functioning grafts under standard IS, operationally tolerant patients and HVs. Thus, despite previous reports of Treg function being influenced by the immunosuppressive regimen [37–39], our results show that the suppressive activity of the $CD4^+CD25^{high}FOXP3^+$ T cells studied here was not influenced by presence or absence of IS. However, we could not exclude that CR patients may have fewer Tregs because they received higher doses of immuno-suppressive drugs. Unfortunately, the analyses of Treg numbers had not been performed before transplantation to determine whether patients with low numbers of Tregs would be prone to develop CR. Moreover, our previous data in patients with renal insufficiency showing normal Treg

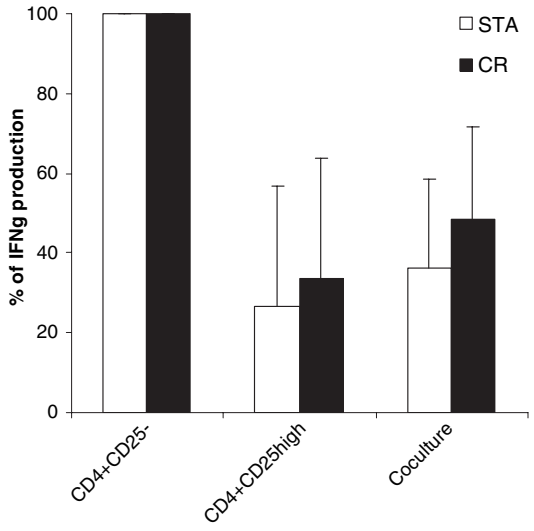

Figure 5 Interferon γ (IFNγ) production in culture supernatants from stable patients and patients with chronic rejection. Cytokines were measured by cytometric bead array (see Methods for details) after 24 h of culture in supernatants from $CD4^+CD25^-$ T cells, $CD4^+CD25^{high}$ T cells and from the coculture (ratio 1:1) following stimulation with irradiated autologous peripheral blood mononuclear cells and with CD3 monoclonal antibody. The percentage of IFNγ production in supernatants was calculated with the quantity of IFNγ produced by $CD4^+CD25^-$ cells being represented as 100%.

numbers suggest that the change in circulating T regulatory cell numbers in CR recipients is not a consequence of renal impairment [20].

Finally, these data also show that kidney transplant patients with operational tolerance not only display normal numbers of circulating $CD4^+CD25^{high}$ T cells as we reported previously [20], but also their natural $CD4^+CD25^{high}FOXP3^+$ blood T cells exhibit normal suppressive properties *in vitro*. Thus, operational tolerance is not characterized by increased natural Treg activity. Nevertheless, this does not exclude a role for other types of Treg in maintaining tolerance. For example, recent data suggest that two distinct classes of human $CD4^+$ Tregs may participate in kidney transplant tolerance: $CD4^+TGF\beta1^+CD25^{+/-}$ T cells that are mainly localized to the tubulo-interstitium of the allograft and absent from the circulation [40], and $CD4^+CD25^+FOXP3^+$ natural Tregs that can be found in lymphoid aggregates within the accepted allograft [40] as well as in the periphery [23].

On the other hand, it may not be absolute Treg numbers that are important, rather ratios of Treg to T effectors. This is corroborated by several reports suggesting that graft outcome may be linked to a disruption of the Treg/T effector ratio [41], a concept also reported in operational tolerance [40]. Along these lines, we previously showed that in addition to displaying lower Treg numbers, patients with CR display more $CD8^+CD28^-CD27^-$ T cells with a cytotoxic and apoptotic phenotype, whereas a normal ratio of these cells was observed in patients with operational tolerance [42]. The latter data, together with those described here, suggest that an imbalance between $CD4^+CD25^{high}$ Tregs and effector $CD8^+CD28^-CD27^-$ T cells may favor graft failure [41] whereas a balance similar to that observed in HVs may favor graft acceptance. Prospective studies are now necessary to determine whether patients who eventually develop CR are predisposed to do so because of peripheral blood T-cell imbalances at the time of transplantation.

To conclude, our data show that CR is associated with a decrease in $CD4^+CD25^{high}$ T-cell numbers but that these cells display normal immuno-regulatory properties. Graft acceptance on the other hand is associated with $CD4^+CD25^{high}$ T-cell numbers similar to HVs. Thus, Treg numbers or potentially the balance of Treg to T effectors may contribute to determining the long-term fate of renal transplants.

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Authorship

CB: performed research/study and wrote the paper. MR: performed research/study. MG: collected patients. SL: performed research/study. AM: analyzed histological data. LB: sorted cells. MH: sorted cells. JA-C: did English correction. J-PS: designed research/study. SB: designed research/study and wrote the paper.

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