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ORIGINAL ARTICLE

T cell receptor beta chain (TCR-Vβ) repertoire of circulating CD4$^+$ CD25$^-$, CD4$^+$ CD25^{low} and CD4$^+$ CD25^{high} T cells in patients with long-term renal allograft survival

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Summary

The mechanisms underlying maintenance of renal allografts in humans under minimal or conventional immunosuppression are poorly understood. There is evidence that CD4$^+$ CD25$^+$ regulatory T cells and clonal deletion, among other mechanisms of tolerance, could play a key role in clinical allograft survival. Twenty-four TCR-Vβ families were assessed in CD4$^+$ CD25$^-$, CD4$^+$ CD25^{low} and CD4$^+$ CD25^{high} T cells from patients with long-term renal allograft survival (LTS), patients exhibiting chronic rejection (ChrRx), patients on dialysis (Dial) and healthy controls (HC) by flow cytometry. LTS patients presented a higher variability in their TCR-Vβ repertoire, such decreased percentage of Vβ2$^+$, Vβ8a$^+$ and Vβ13$^+$ in CD4$^+$ CD25^{low} and ^{high} compared with CD4$^+$ CD25$^-$ subset and increased Vβ4 and Vβ7 families in CD4$^+$ CD25^{high} T cells exclusively. Additionally, LTS patients, particularly those that were not receiving calcineurin inhibitors (CNI), had increased percentages of CD4$^+$ CD25^{high} T cells when compared with Dial ($P < 0.05$) and ChrRx ($P < 0.05$) patients. Our results suggest that a differential expression of particular TCR-Vβ families and high levels of circulating CD4$^+$ CD25^{high} T cells in long-term surviving renal transplant patients could contribute to an active and specific state of immunologic suppression. However, the increase in this T cell subset with regulatory phenotype can be affected by CNI.

Introduction

The mechanisms responsible for long-term allograft survival are not completely understood. Studies in animal models have shown a key role for regulatory T cells (Treg) in the establishment and maintenance of tolerance to allografts [1–3] and *in vitro* studies have illustrated the capacity of these cells to control responsiveness by down-regulating IL-2 production in both activated CD4$^+$ CD25$^-$ [4] and CD8$^+$ T cells [5].

In clinical transplantation, it has been reported that circulating CD25^{high} T cells from kidney transplant recipients on maintenance immunosuppression still exhibit regulatory activity against third party targets and diminished immune responsiveness toward donor antigens [6–8] and that these cells can be successfully expanded from a small volume of blood for subsequent functional analysis [9]. However, there is no consensus about changes in circulating Treg cells from transplant patients under different clinical conditions. Some authors have shown that the number of Treg cells in stable kidney transplant [10] and drug-free tolerant recipients [11] is similar to that of healthy controls, while others have described that a decreased percentage of these cells is associated with the appearance of acute [12] or chronic rejection [11,13]. We and others have previously found that kidney- [14] and lung-[13]

transplanted patients with long-term allograft survival exhibited increased percentages of circulating $CD4^+$ $CD25^+$ T cells compared with healthy individuals. Nevertheless, identification and quantification of circulating Treg populations has been proposed as a potential subrogate marker for induction of clinical transplantation tolerance [15].

T cells recognize antigens through T-cell receptor (TCR) leading to T-cell activation and selective clonal expansion [16,17]. In a previous work, we have demonstrated alterations in TCR-Vβ repertoire of transplanted patients with stable renal allograft survival with and without immunosuppression [18]. These alterations included augmented oligoclonality and diminished polyclonality, which could be attributed to expansion of Treg cells or deletion of alloreactive T cells.

Although it has been described that suppressive activity of Treg cells is nonspecific, their activation and expansion is antigen-specific [19]. Previous studies in mice have not shown differences in Vα and Vβ TCR repertoires in $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ populations [20], and TCR repertoires in Treg cells and $CD4^+$ $CD25^-$ were comparably diverse and partially overlapping, even if TCRs of Treg cells display a higher avidity for self-antigens [21]. In humans, the evaluation of the TCR repertoire has shown that circulating and thymus $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells share a similar and complex Vβ repertoire [22,23] and more recently that $CD4^+$ $CD25^{high}Foxp3^+$ T cells have a similar and overlapping TCR repertoire with $CD4^+$ $CD25^-$ T cells [24]. However, it is not known whether variations in TCR-Vβ repertoire in kidney transplant patients are restricted to a particular T-cell subset, such as $CD4^+$ $CD25^{high}$ T cells, thus comparison of TCR repertoire between effector and Treg cells may indicate whether a selective or nonrestricted TCR-Vβ. expression is involved in long-term allograft survival in kidney transplanted patients.

Using monoclonal antibodies for 24 different Vβ families, we analysed, by flow cytometry, the TCR-Vβ repertoire of circulating $CD4^+$ $CD25^-$, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ cells in patients with stable renal function after 10 or more years of transplantation and compared it with patients exhibiting chronic rejection, patients on dialysis and healthy controls. Results suggest that expression of particular VB families and circulating $CD4^+$ $CD25^{high}$ T cells could contribute to long-term survival of renal allografts, but this T-cell subset can be affected by the use of calcineurin inhibitors.

Materials and methods

Patients and controls

Peripheral blood was collected from 15 healthy individuals (HC), 10 patients on dialysis (Dial) who were in the waiting list for kidney transplantation, 15 patients with progressive deteriorating renal function and biopsy-proven chronic rejection at different times post-transplantation (ChrRx), and 16 patients >10 years after kidney transplantation with stable transplant function (serum creatinine <1.5 mg/dl) without clinical or laboratory evidence of graft rejection [long-term renal allograft survival (LTS) patients]. Two of these LTS patients suspended their immunosuppression 3 years earlier. HC individuals were gender- and age-matched with LTS patients.

All kidney transplants were performed by the Transplant Group of the Universidad de Antioquia and the Hospital San Vicente de Paul, Medellín, Colombia, between 1985 and 2004. The study protocol was approved by the Ethical Committee of the Universidad de Antioquia and all the samples used in this study were collected after an informed consent was obtained.

Human leukocyte antigen (HLA) class I and II typing of donors and recipients was performed by complement-dependent cellular cytotoxicity (CDC) or polymerase chain reaction sequence-specific primers (PCR-SSP) methods using reagents supplied by the Collaborative Transplant Study (Heildelberg, Germany). Demographic and clinical information of patients and controls is shown in Table 1.

Thirteen LTS patients had living-related donor (five HLA identical and eight HLA haploidentical) while in the ChrRx group, seven patients had living-related donor (four HLA haploidentical and three individuals with 2.0, 3.0 and 4.0 mismatches respectively). Eleven patients were recipients of deceased donor kidneys (mean HLA-A, -B and -DRβ mismatches were 1.5 for three LTS patients and 3.5 for eight ChrRx patients). As shown in Table 1, there was a great variation in immunosupression treatment, mainly in the ChrRx group.

Flow cytometry

For all analysis, EDTA-anticoagulated venous blood was used. Phenotypic characterization of $CD4^+$ T cell subsets according to CD25 expression and their TCR-Vβ repertoire was done by direct staining of whole-blood with the following fluorochrome-labeled monoclonal antibodies (mAb): Mouse anti-human CD4-FITC or PE (13B8.2, Immunotech Marseille, France), anti-CD127-PE (eBioRDR5, eBioscience, San Diego, CA, USA), anti-CD25-PECy-5 (M-A251, BD Pharmingen San Diego, CA, USA) and 24 different anti-TCR-Vβ FITC or PE-conjugated mAb (Immunotech, Marseille, France, and Pierce ENDOGEN, Rockford, IL, USA). Isotype controls were used for each of the 24 different combinations.

One-hundred microliters of whole-blood were incubated for 20 min with anti-CD25-PE-Cy5, anti-CD4-FITC

Table 1. Demographic and clinical information of patients and controls.

Study groups	Age at testing*	Gender	Type of donor: LRD					Years posttx*	HLA mm*	Creatinine*	Immunosuppression												
		M	F	I	H	O	Deceased				None	A + S	A + S + C	A + S + C + M	S + C + M	S + F + M	S + F + E	S + M + E	S + M	M + E
LTS	44 (36–51)	11	5	5	8	0	3	12 (11–14)	1 (0–3)	1.3 (1.1–1.4)	2	8	5	–	–	–	–	–	–	1
ChrRx	36 (27–52)	9	6	0	4	3	8	5 (3–10)	3 (3–4)	2.8 (2.5–3.4)	0	2	–	1	4	4	1	1	2	–
Dial	38 (21–56)	3	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
HC	43 (23–63)	11	4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

*Median (interquartile range).
Posttx, post-transplantation; LRD, living-related donor; I, HLA-identical; H, HLA-haploidentical; O, Other; mm, mismatch; A, azathioprine; S, steroids; C, Cyclosporin A; M, Mycophenolate Mofetil; F, FK506 (Tacrolimus); E, Everolimus; NA, not applicable.

or -PE and anti-TCR-Vβ-PE or -FITC respectively, according to the fluorochrome of the anti-CD4, at room temperature and darkness. Then, 100 µl of CAL-LYSE (CALTAG, Burlingame, CA, USA) solution were added for 10 min and finally, 1 ml of sterile de-ionized water was added. A total of 10^5 cells were analysed by flow cytometry (EPICS XL, Beckman-Coulter, Hialeah, FL, USA). For analysis of positive cells, the lymphocytes area was delimited over FSC versus SSC, then, $CD4^+$ cells were analysed in FL1 or FL2 (CD4-FITC or CD4-PE) versus FL3 (CD25-PE-Cy5). Thus, three different subsets of $CD4^+$ cells were defined, based on the CD25 expression ($CD25^-$, $CD25^{low}$ and $CD25^{high}$). Finally, Vβ staining was assessed for regions corresponding to $CD4^+$ $CD25^-$, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ subsets in FL1 versus FL2 (Vβ-FITC versus CD4-PE or CD4-FITC versus Vβ-PE). Human Treg cells have been defined considering the $CD4^+$ T cells expressing high levels of CD25 and the transcription factor, FOXP3. However, it has been also reported that expression of the IL-7 receptor alpha chain (CD127) is down-regulated on Treg cells and its expression inversely correlates with FOXP3 and CD25 expression [25,26]. Thus, to verify the phenotype of regulatory T cells defined previously as $CD4^+$ $CD25^{high}$ cells, some individuals in each group were also tested for CD127 expression in the $CD4^+$ $CD25^{high}$ cells. Autofluorescence background and nonspecific staining were determined by isotype-matched control mAb staining for the different combinations. The percentage and mean fluorescence intensity (MFI) of CD25 and the Vβ families were determined using the CYTOMATION SUMMIT 4850 INNOVATION DRIVE program (Cytomation, Inc., Fort Collins, CO, USA).

Statistical analysis

Differences in the percentage of $CD4^+$ $CD25^-$, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ cell subsets and MFI of CD25 among groups were determined by one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Comparison of the percentage of $CD4^+$ $CD25^{high}$ cells according to immunosuppression was done by unpaired *t*-test. Percentages and MFI of TCR-Vβ families within the $CD4^+$ $CD25^-$, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ cell subsets were compared by two-way ANOVA, with Bonferroni post-test. For all tests, $P < 0.05$ was considered significant and the analyses were performed using the statistical software GRAPHPAD PRISM 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Results

Frequency of circulating CD4+ T cell subsets and immunosuppression treatment effect on the percentages of circulating CD4+ CD25high cells

The expression of CD25 on circulating CD4+ T cells was used to determine the percentage of CD4+ CD25−, CD4+ CD25low and CD4+ CD25high cell subsets in HC, Dial, ChrRx and LTS individuals. LTS patients had higher percentage of CD4+ CD25− cells when compared with Dial patients ($P < 0.05$) (Fig. 1a) but there were not significant differences in the percentage of CD4+ CD25low (Fig. 1b) subsets among the study groups. LTS patients had also increased percentages of circulating CD4+ CD25high cells when compared with Dial ($P < 0.05$) and ChrRx ($P < 0.05$) patients (Fig. 1c). To corroborate the phenotype of the CD4+ CD25high cells as Treg cells initially defined according to a MFI of CD25 higher than 10^2 logarithms on dotplots, we determined expression of CD127 on CD4+ CD25high cells. We found that CD4+ CD25high Treg cells, previously defined according to high levels of CD25, expressed low levels of CD127 compared with CD4+ CD25− and CD4+ CD25low, which mainly express high levels of CD127 (data not shown). Variation in the percentages of circulating CD4+ CD25high cells in LTS patients suggest the existence of two groups, thus the LTS group was split according to whether they were receiving CNI treatment or not. Such of those LTS patients not receiving CNI (n-CNI) had increased percentage of CD4+ CD25high cells compared with LTS patients receiving CNI ($P < 0.05$) (Fig. 2).

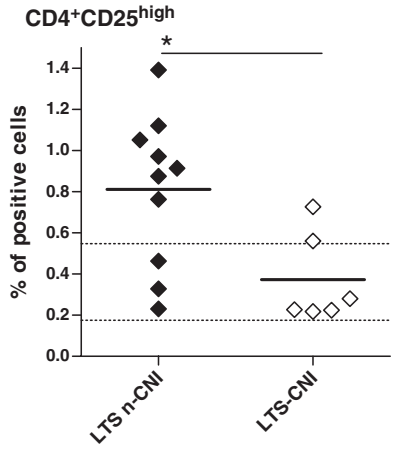

Figure 2 Analysis of CD4+ CD25high T cells according to immunosuppression. The percentage of circulating CD4+ CD25high T cells from kidney transplant patients that were not receiving CNI, LTS n-CNI (◆) and LTS-CNI (◇). *$P < 0.05$. *Dotted lines*, define the 95% confidence interval of the values found in the healthy control group.

Determination of TCR-Vβ repertoire of CD4+ CD25−, CD4+ CD25low and CD4+ CD25high T cell subsets in the study groups

First, we compared the TCR-Vβ repertoire of CD4+ CD25−, CD4+ CD25low and CD4+ CD25high cell subsets among the three groups of patients and controls. CD4+ CD25− cells had increased frequency of positive T cells to some Vβ families (Vβ2, Vβ8(a), Vβ13, Vβ17 and Vβ22) compared with the other families, but these increments were observed in all studied groups (Fig. 3a). In this subset, the number of Vβ2+ cells was higher in ChrRx patients compared with HC and Dial patients ($P < 0.05$ and $P < 0.001$ respectively) and in LTS patients compared with Dial patients ($P < 0.01$). The percentage of Vβ13+ cells was augmented in Dial patients compared with ChrRx, LTS patients and HC ($P < 0.001$, $P < 0.001$ and $P < 0.001$ respectively). Vβ8(a)+ cells were more frequent in Dial patients when compared with ChrRx ($P < 0.01$). The TCR-Vβ repertoire in the CD4+ CD25low subset only showed a lower percentage of Vβ6.7+ cells in ChrRx patients when compared with HC ($P < 0.05$) (Fig. 3b). The TCR-Vβ repertoire of CD4+ CD25high subset (Fig. 3c), showed an increased percentage of Vβ4+ and

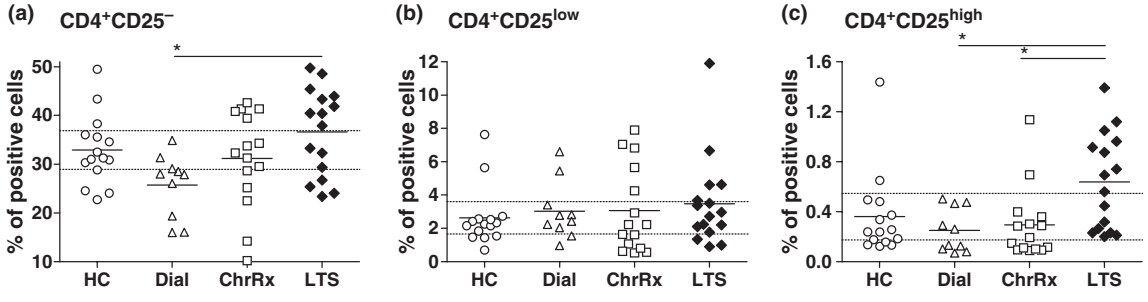

Figure 1 Frequency of circulating CD4+ T cell subsets. Percentage of (a) CD4+ CD25−, (b) CD4+ CD25low and (c) CD4+ CD25high cell subsets in healthy controls (HC) (○), Dial (△), ChrRx (□) and LTS (◆) patients. *$P < 0.05$. *Dotted lines*, lower and upper 95% confidence interval of the values found in the healthy control group.

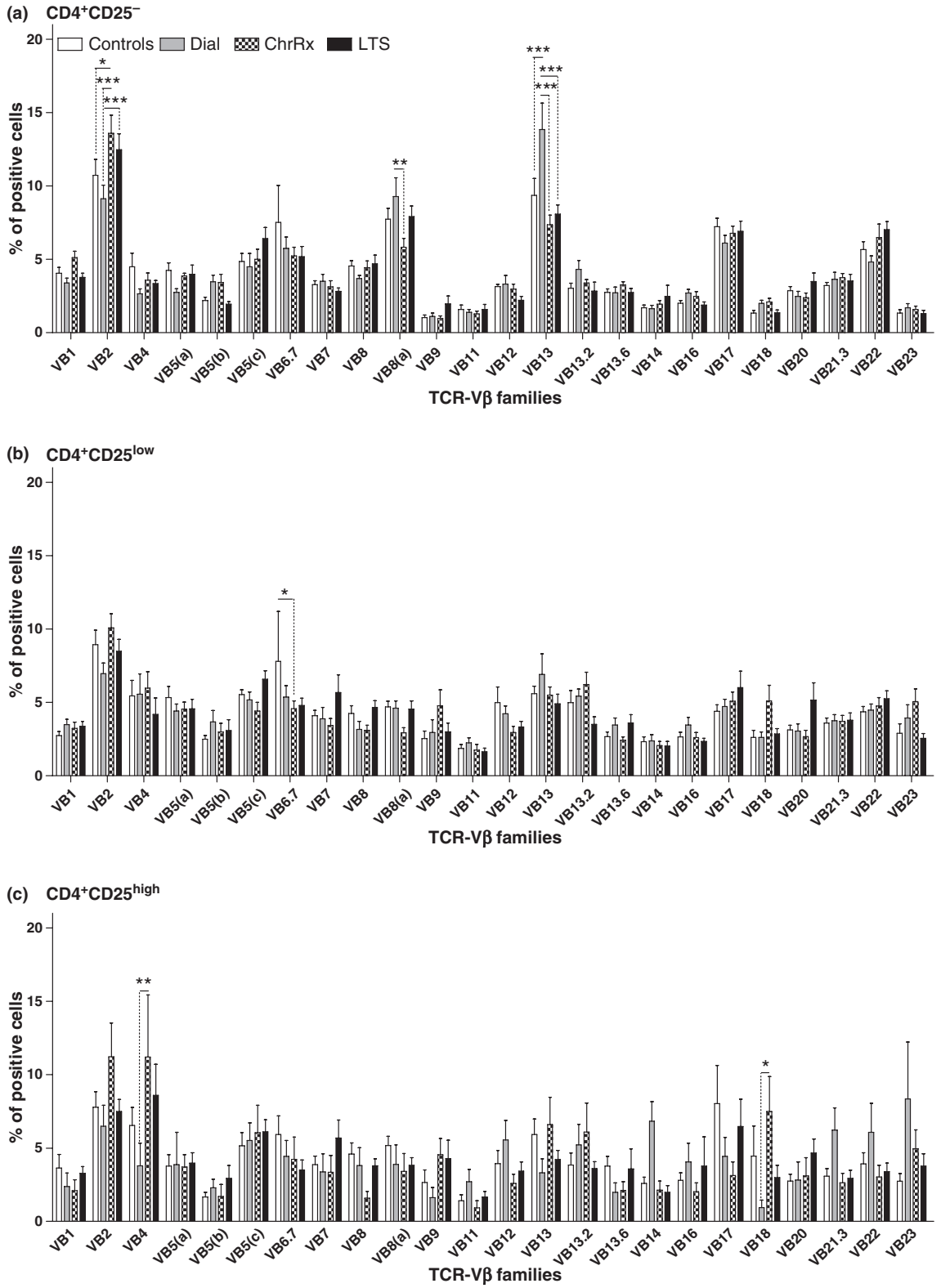

Figure 3 TCR Vβ diversity in CD4+ T cell subsets. TCR Vβ repertoire was determined by three-color flow cytometry with FITC, PE and Cy-Chrome-conjugated antibodies directed against CD4, CD25 and 24 Vβ families. The percentage of positive cells within the gates of (a) CD4+ CD25−, (b) CD4+ CD25low and (c) CD4+ CD25high cell subsets is shown for each Vβ family. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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$V\beta18^+$ cells in ChrRx patients compared with Dial patients ($P < 0.01$ and $P < 0.05$). There were not significant differences for the MFI of the 24 Vβ families within the different $CD4^+$ cells among the groups (data not shown). Thus, although most of Vβ families were similarly expressed in all studied groups, the percentage, but not the expression of some Vβ families, changed according to the $CD4^+$ subset and differed among groups.

Determination of TCR-Vβ repertoire of healthy controls, dialysis, ChrRx and LTS patients in T cells subsets

Healthy Controls subjects, Dial, ChrRx and LTS patients showed variable percentages of different Vβ families according to the $CD4^+$ T cell subset (Fig. 4). Controls exhibited an augmented percentage of $V\beta5(c)^+$ cells in $CD4^+$ $CD25^{low}$ subset compared with $CD4^+$ $CD25^{high}$ subset ($P < 0.05$) (Fig. 4a). In Dial patients, $CD4^+$ $CD25^-$ T cells showed higher percentage of $V\beta8(a)^+$ and $V\beta13^+$ cells compared with $CD4^+$ $CD25^{low}$ ($P < 0.05$ and $P < 0.001$) and $CD4^+$ $CD25^{high}$ ($P < 0.01$ and $P < 0.001$) T cells (Fig. 4b). Also, $CD4^+$ $CD25^{high}$ T cells showed increased percentage of $V\beta14^+$ and $V\beta23^+$ cells compared with $CD4^+$ $CD25^-$ ($P < 0.01$ and $P < 0.001$) and $CD4^+$ $CD25^{low}$ T cells (both $P < 0.05$). In ChrRx patients, $V\beta4^+$ cells were more frequent in $CD4^+$ $CD25^{high}$ subset compared with $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^{low}$ subsets ($P < 0.001$ and $P < 0.01$ respectively) and $V\beta18^+$ cells were also higher in the same subset compared with $CD4^+$ $CD25^-$ ($P < 0.01$) (Fig. 4c).

The TCR-Vβ repertoire of LTS patients showed several differences among $CD4^+$ subsets. There was a higher frequency of $V\beta2^+$ cells in $CD4^+$ $CD25^-$ subset compared with $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ subsets ($P < 0.001$ for both), while $V\beta4^+$ T cells were predominant in $CD4^+$ $CD25^{high}$ subset compared with $CD4^+$ $CD25^-$ cells ($P < 0.001$). $V\beta7^+$ cells exhibited a significant increase in $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ cells compared with $CD4^+$ $CD25^-$ subset ($P < 0.05$ for both). There was a rise in $V\beta8(a)^+$ and $V\beta13^+$ cells in $CD4^+$ $CD25^-$ subset compared with $CD4^+$ $CD25^{low}$ ($P < 0.01$ for both) and $CD4^+$ $CD25^{high}$ ($P < 0.001$ for both) subsets. Additionally, the percentage of $V\beta22^+$ cells was higher in $CD4^+$ $CD25^-$ T cells compared with $CD4^+$ $CD25^{high}$ T cell subset ($P < 0.01$) (Fig. 4d).

Discussion

Immunomodulation by Treg cells has been proposed as one of the most important mechanisms responsible for allograft acceptance in clinical transplantation [27]. Even though many types of regulatory or suppressor T cells have been described [28,29] and new phenotypic markers have been introduced, $CD4^+$ T cells that express high levels of IL-2 receptor α chain or CD25 ($CD4^+$ $CD25^{high}$) have been defined routinely as a subset of Treg cells in

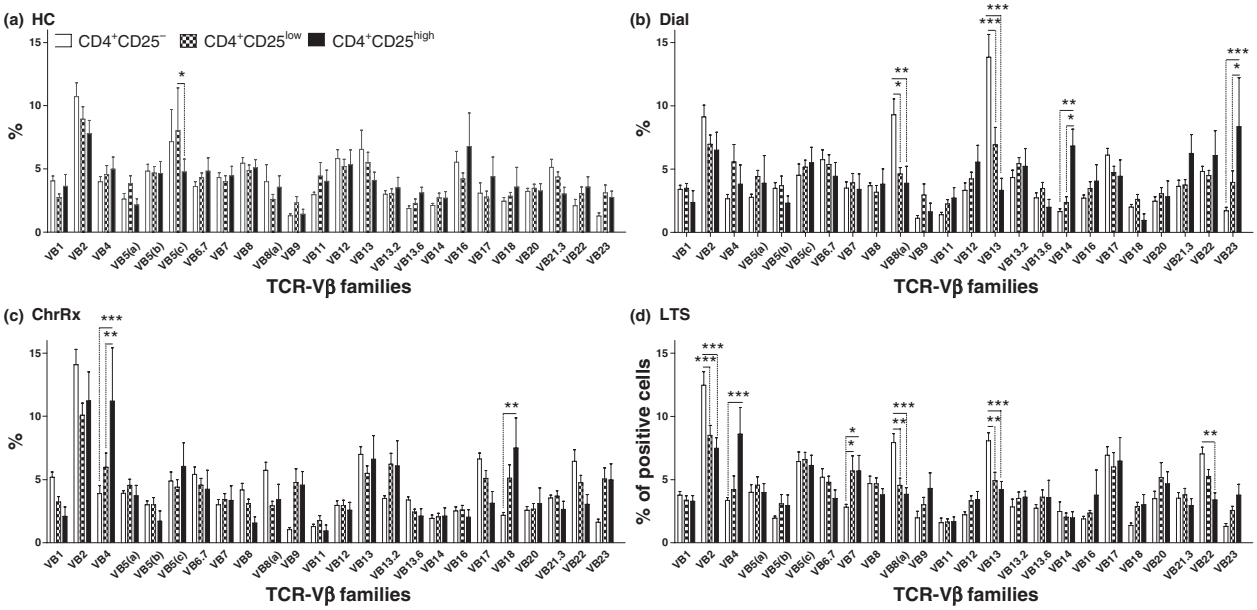

Figure 4 TCR Vβ diversity of $CD4^+$ T cell subsets in different group of transplanted patients and Controls. TCR Vβ diversity was determined by three-color flow cytometry with FITC, PE and Cy-Chrome-conjugated antibodies directed against CD4, CD25 and 24 Vβ families. The percentage of positive cells (*y*-axis) within the gates of $CD4^+$ CD25, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ cell subsets is shown for each Vβ family (*x*-axis) for (a) healthy controls (HC), (b) Dialysis (Dial), (c) chronic rejection (ChrRx) and (d) LTS patients. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

humans [30–33]. In this study, we attempted to characterize the TCR repertoire in $CD4^+$ $CD25^-$, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ subpopulations of transplanted patients under different graft outcomes.

Characterization of TCR repertoire has been important to identify changes in T cell responses during development of alloimmune response [34]. In animal models of allogeneic transplantation, the study of TCR Vβ repertoire has shown that the expansion of some selective Vβ families and highly altered repertoires can be associated with tolerance induction [35,36] and particularly with tolerogenic graft infiltrating lymphocytes [37]. In patients with graft rejection, clonal analyses have shown that graft infiltrating T cells can display either oligoclonal [38] or both oligoclonal and polyclonal patterns [38,39]. However, it has also been reported that peripheral blood T cells can exhibit clonal expansions and a skewed usage of TCR Vβ repertoires under the same clinical conditions [40]. In a previous report, we demonstrated that LTS patients, with and without immunosuppression, have an increased oligoclonality and decreased polyclonality, as compared with patients with well-functioning grafts at different times post-transplantation, patients with acute and chronic rejection, patients on dialysis and healthy controls [18].

Recently, Brouard *et al.* [41] described that drug-free operationally tolerant kidney recipients displayed a strongly altered TCR-Vβ usage in peripheral blood compared with healthy individuals, as well as a strong accumulation of Vβ transcripts in selected T cells, particularly in $CD8^+$ cells. Our previous findings by TCR-Vβ spectratyping of peripheral blood mononuclear cells showed that LTS patients have oligoclonal expression more frequently detected in Vβ2, Vβ7, Vβ8 and Vβ9 families, however, this study did not allow to establish if these changes were restricted to a particular T cell subset [18]. Using flow cytometry, other authors [22,23] have found in normal subjects that TCR-Vβ usage by $CD4^+$ $CD25^+$ and $CD4^+$ $CD25^-$ T cells is very similar. Interestingly, in both reports, Vβ2 is one of the most frequent Vβ families.

In our study, analysis of TCR-Vβ among different CD4 subsets, demonstrated that $CD4^+$ $CD25^-$ T cells had more altered TCR repertoire as compared with $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ cells. Amongst the variations found in $CD4^+$ $CD25^-$ cells there was an augmented percentage of $V\beta2^+$ cells in ChrRx and LTS patients, which could reflect the expansion of effector alloreactive clones in transplanted patients under persistent stimulation by the alloantigens present on the graft. In addition, the percentage of $V\beta13^+$ T cells was found to be increased in dialysis patients, but normal in the other groups. The significance of this finding is unknown but it may reflect the immune activation reported in those patients [42].

The analysis of the study groups, considering the TCR-Vβ repertoire of different CD4 cell subsets, showed that LTS patients exhibited more variations. These patients had decreased percentage of $V\beta2^+$, $V\beta8a^+$ and $V\beta13^+$ $CD4^+$ $CD25^{low}$ and $CD25^{high}$ cells, as compared with the $CD4^+$ $CD25^-$ subset. These changes may indicate a selective reduction of alloreactive clones in those patients. Contrariwise, Vβ4 and Vβ7 families were increased only on $CD4^+$ $CD25^{high}$ T cells from LTS patients suggesting that these families may be associated with the expansion of specific regulatory T cells in these patients.

Determination of the TCR repertoire by flow cytometry allows to identify simultaneously the TCR-Vβ repertoire in each cell subset and establish similarities or changes in TCR diversity [43]. Evaluation of TCR-Vβ repertoire in $CD4^+$ T cells of transplant patients has shown that its expression in $CD4^+$ $CD25^{high}$ cells is not modified after *in vitro* expansion [9], but to the best of our knowledge there are no comparisons of TCR-Vβ expression within the $CD4^+$ $CD25^-$, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ T cell subsets in patients with the characteristics described in our study. It would be important to confirm our findings using purified cell subsets and more analytical methods such as spectratyping and DNA sequencing.

The increased percentage of particular Vβ families in ChrRx and LTS patients could reflect a selective expansion of alloreactive and Treg cells as a consequence of persistent exposure to donor alloantigens. Other reports have demonstrated that age [44], autoimmune diseases [45,46] and viral infections [47,48] could also influence the TCR repertoire. We can not be sure whether these factors influenced our results; however, our healthy controls were matched by age with LTS patients and none of them have clinical autoimmune diseases.

We have previously reported that transplanted patients with long-term allograft survival have diminished expression of molecules involved in the activation of T cell (TCR α, β and ζ chains) and an augmented percentage of circulating $CD4^+$ $CD25^+$ T cells expressing CD69. We hypothesized that these patients could have defects in alloantigen recognition and signal transduction, suggesting a state of active anergy and also an active immunologic suppression [14]. In this study, we further discriminated $CD4^+$ cells in $CD4^+$ $CD25^-$, $CD4^+$ $CD25^{low}$ and $CD4^+$ $CD25^{high}$ T cells in LTS, ChrRx, Dial patients and HC. Results showed an increase in the percentage of circulating $CD4^+$ $CD25^{high}$ cells in LTS patients when compared with the other groups, suggesting that in LTS patients $CD4^+$ $CD25^{high}$ T cells could be involved in maintenance of allograft function. Of note, the two patients with more than 3 years without immunosuppression and excellent graft function, had the highest

percentage of $CD4^+$ $CD25^{high}$ T cells. Although it is not possible to conclude that a high level of circulating Treg cells is a subrogate marker for induction or establishment of clinical tolerance, our finding that patients with chronic rejection exhibited similar percentage of $CD4^+$ $CD25^{high}$ T cells in relation to healthy controls; suggest that Treg cells are effectively involved in the long-term outcome of renal transplants in LTS patients [49].

Accumulating evidence from different studies suggests that CNIs have a negative effect on the function of Treg cells [50–52]. We observed that our LTS patients who were not receiving CNI exhibited higher percentages of $CD4^+$ $CD25^{high}$ T cells when compared with patients under CNI treatment, supporting the hypothesis that CNI could affect the numbers of Treg cells in these patients. In agreement with our finding, it has been reported that treatment with CNIs [53–56] reduces the percentages of circulating Treg cells, although other investigators have suggested that CNIs may allow the expansion of this cell subset [57]. Functional assays of human Treg cells from transplant patients could be helpful to establish the precise role of CNI treatments in the properties and maintenance of Treg cells. However, the finding that CNIs reduce the levels of circulating Treg cells may suggest the possibility of avoiding long-term over-immunosuppression with CNIs.

Taken together, our results suggest that a differential expression of particular Vβ families and high levels of circulating $CD4^+$ $CD25^{high}$ T cells in long-term surviving renal transplant patients could contribute to an active and specific state of immunologic suppression. Also, non expansion of Treg cells in ChrRx patients could determine the poor allograft function in those patients. Our findings also imply that a regimen of immunosuppression with CNIs could be detrimental for maintenance of peripheral $CD4^+$ $CD25^{high}$ T cell in transplant patients.

Although we found differences in the percentage of $CD4^+$ $CD25^{high}$ cells in LTS patients as well as in the TCR repertoire, it would be important to study more patients with stable renal function under minimal or without any immunosuppression to more strongly sustain the validity of these results. Future studies with functional assays in those patients are necessary to ensure that the establishment of transplant tolerance in clinical transplantation is strongly influenced by circulating Treg cells.

Authorship

SYV: performed study, collected data, analysed data and wrote the paper. LFA: analysed renal biopsy specimens. LFG: supervised research, analysed data and revised the paper. CMA: designed research/study, supervised research, analysed data and revised the paper.

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