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T-cell phenotype in protocol renal biopsy from transplant recipients treated with belatacept-mediated co-stimulatory blockade

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Abstract

Background. Belatacept is thought to disrupt the interaction between CD80/86 and CD28, thus preventing T-cell activation by blocking the co-stimulatory second signal. However, the consequences on the T-cell profile in human renal transplant cases have not been determined.

Methods. In this study, we analysed intra-graft levels of the mRNAs for Treg (FOXP3), cytotoxic CD8 T cells (Granzyme B), Th1 (INFγ, Tbet), Th2 (GATA3) and Th17 (RORγt and IL-17) in protocol biopsies obtained 12 months after renal transplantation in recipients treated with Belatacept or calcineurin inhibitor (CNI).
Results. Only the intra-graft abundance of FOXP3 mRNA was significantly lower ($P < 0.001$) in the Belatacept group than the CNI group.
Conclusions. These results are in agreement with *in vitro* data suggesting that CD28 is a major co-stimulatory signal of both Tregs development and peripheral homeostasis but contrast with clinical trials showing a better 1-year graft function and a lower incidence of chronic allograft nephropathy in patients receiving Belatacept than patients treated with CNI. They suggest that immune benefits induced by Belatacept are not mediated by Treg expansion and that FOXP3 is not by itself a prognostic marker of long-term graft function in a non-inflammatory context. These results have to be, however, considered as preliminary since the size of our study population is limited.

Keywords: Belatacept; T-cell phenotype; Treg

Introduction

Belatacept, a recombinant protein containing the ectodomain of CTLA4, is thought to inhibit the interaction between CD80/86 and CD28, thus preventing T-cell activation by blocking the co-stimulatory second signal [1,2]. Such blockade results in T-cell anergy and tolerance of experimental organ transplantation [3]. These preliminary findings have led to a Phase II clinical trial in human renal transplantation combining short-term induction with basiliximab and maintenance therapy with Belatacept [4]. This study revealed effective immunosuppression in patients given Belatacept; renal function was better and the incidence of chronic allograft nephropathy lower in patients treated with Belatacept than those given cyclosporin A [4].

Contrasting with these clinical findings, recent analyses of the T-cell alloimmune response in renal recipients treated with immunosuppressive drugs including Belatacept indicate that this immunosuppressive regimen may have detrimental consequences on regulatory T cells (Treg) number and function [5,6]. More recently, it has been shown that CD28 co-stimulation down-regulates the CD4+ Th17 pathway and that CTLA4-Ig facilitates both murine and human Th17 differentiation [7], a T-cell subset involved in allograft rejection and vasculopathy [8]. Therefore, the consequences of the use of Belatacept for Th17 polarization need to be determined.

It is likely that the outcome of the allograft response—rejection or tolerance—after transplantation is in part determined by the level of expression of alloaggressive and graft-protecting T cells within the allograft or in the urine of renal transplant recipients [9–12]. Therefore, protocol biopsies may be very valuable as they may provide a window into pathogenetic mechanisms that cannot be investigated if the graft is only examined after dysfunction occurrence. In particular, real-time PCR and microarrays could be used with protocol biopsies for molecular studies of cells infiltrating allografts; this approach may provide data predictive of graft outcome [13,14].

To determine the T-cell immune profile observed in renal transplant recipients treated with Belatacept, we analysed samples obtained during protocol biopsy 12 months after renal transplantation in patients treated with Belatacept and cyclosporin A [calcineurin inhibitor (CNI)] as controls; in particular, we assayed mRNAs for Treg (FOXP3), cytotoxic CD8 T cells [Granzyme B (GB)], Th1 (INFγ,T Bet), Th2 (GATA3) and two molecular markers of the Th17 pathway, RORγt and IL-17. Our results clearly show that only FOXP3 was expressed significantly more weakly in the group of patients treated with Belatacept than in the group treated with CNI.

Materials and methods

Biopsies and patients

Patients receiving a primary renal transplant from a deceased donor and included in the Phase II and III clinical trials of Belatacept were enrolled in this study. The protocol of the Phase III study is depicted in Figure 1. Details of the study and the outcome of the previous Phase II trial are described elsewhere [4]. The protocol for the Phase III trial was similar to that for Phase II with two differences: the low-intensity Belatacept group was given an additional Belatacept infusion on Day 4 and maintenance administration of Belatacept for all patients was 5 mg/kg every 4 weeks.

Histological data were obtained from protocol biopsies performed 12 months post-transplant in Henri Mondor Renal Transplant Center. Eighteen biopsies were obtained from the 18 patients on Belatecept-based immunosuppression and 13 biopsies from 13 patients on cyclosporin A-based immunosuppression. All biopsies were evaluated by routine light microscopy and scored using the Banff 07 classification [15].

RNA isolation and quantification by real-time quantitative RT-PCR

We assayed all the biopsy specimens included in this study for intra-renal mRNAs analysis for GB, FOXP3, Tbet, RORγt, GATA3, INFγ and IL-17. Total tissue RNA was purified using the RNeasy kit (Qiagen SA), and we used nanodrop technology to determine RNA quantities. The integrity of the total RNA preparations was checked using the RNA 6000 Pico Assay kit with an Agilent 2100 Bioanalyser, and the RNA Integrity Number was calculated by the Agilent Bioanalyser Expert software. All cDNA analysed was assayed by relative quantification using real-time PCR in an ABI Prism 7900 using SYBR Green. The housekeeping gene GAPDH was used as a control and for normalization of data. GAPDH mRNA levels were similar in all groups (data not shown). Primer and probe sequences and PCR parameters are indicated in Table 1. First-strand cDNA was synthesized in RT samples, in a reaction mix containing: total RNA isolated from biopsies, 16 U/μL M-MLV reverse transcriptase (Gibco-BRL, Life Technologies, Cergy-Pontoise, France), 4 μM Oligo-(dT) 12–18 (Amersham-Pharmacia Biotech, Saclay, France) and 0.8 mM mixed dNTP (Amersham-Pharmacia Biotech, Saclay, France). Dilution series of calibrator cDNA were used to establish a standard curve for target genes and the housekeeping gene; Ct values were plotted against the log cDNA concentration added. We used the resulting linear graphs to determine the differences in Ct values for each sample, expressed as a relative percentage of mRNA present in the reference calibrator dilution, according to the $\Delta\Delta$Ct method ($2^{-\Delta\Delta Ct}$).

Only samples meeting the following criteria were considered for analysis: (i) housekeeping gene expressed at a sufficient level (crossing point <31 cycles) and (ii) at least one other cDNA analysed (FOXP3, GB, IFNγ, Tbet, GATA3 and RORγt) detected. In order to decrease the potential impact of low abundance mRNA, the sensitivity of SYBR green was checked twice. First, efficiency of the amplification for each primers set using a positive control at different concentration and, next, all samples that were not amplified (or are amplified at late cycle of Q-PCR) by

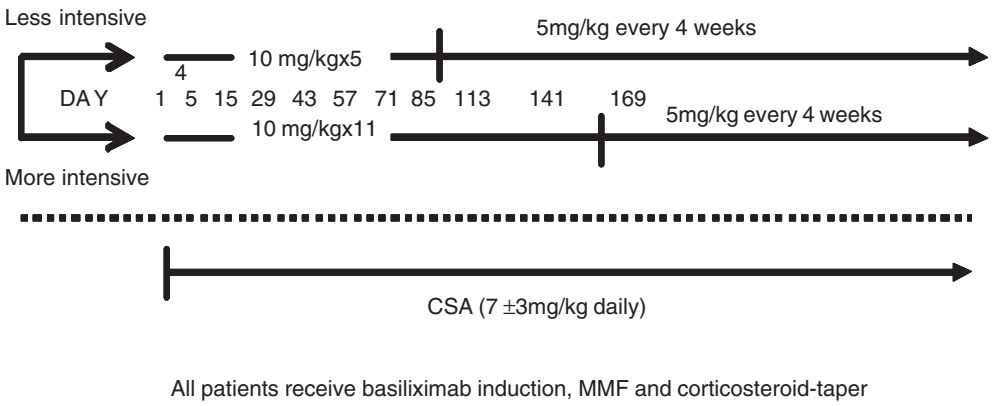

Fig. 1. Clinical trial protocol and dosing regimen. Patients were randomized to receive an intensive Belatacept regimen, a less-intensive Belatacept regimen or cyclosporin A for maintenance immunosuppression. Both Belatacept regimens included an early phase (10 mg/kg of body weight), which was longer with more frequent administration in the intensive regimen, and a late phase (5 mg/kg of body weight at 4-week intervals). All patients received induction therapy with basiliximab, mycophenolate mofetil and a corticosteroid taper.

the housekeeping gene (GAPDH) due to the low abundance of RNA we discarded from our study.

Immunohistological analysis

For CD3 immunohistochemistry, sections of kidney biopsy were embedded in paraffin and cut. The sections were then treated with proteinase-K (20 mg/mL; Sigma-Aldrich, Milan, Italy) for 10 min at 37°C, subjected to microwaves (twice for 15 min in citrate buffer 10 mM [pH 6] at an operating frequency of 2450 MHz and power output of 600 W) and incubated in citrate buffer (15 min). The sections were incubated with mouse anti-CD3 antibodies diluted at 1:500, then with biotinylated sheep anti-mouse IgG [Chemicon International, Temecula, CA] and goat anti-rabbit IgG [Vector Laboratories, Burlingame, CA] and finally with avidin–biotin peroxidase complex solution. The reactions were developed with diaminobenzidine. The sections were then counterstained with Harris hematoxylin (Biooptica, Milan, Italy). The total number of positive cells in 1 mm^2 was counted for quantitative analysis.

Flow cytometry

Flow cytometry was performed with a Coulter FC500 within 24 h from the staining. Listmode parameters were analysed and stored on the software CXP™ (Beckman-Coulter) by direct antibody staining of 100 µL of whole blood (collected in EDTA, red cells lysed and washed). Staining antibodies were purchased from Beckman-Coulter (PE-labelled anti-CD25, FITC-labelled anti-CD4; eBioscience, Hatfield, UK). For CD86 expression, a double labelling comprising of CD14-ECD (Beckman-Coulter, Hailey, FA, USA) and CD86-PE (eBioscience, Hatfield, UK) was performed. The median fluorescence intensity of CD86 was measured in monocytes which were defined according to two criteria: position in the forward and size scatter analysis and expression of CD14.

Statistical analysis

Results are presented as medians and interquartile ranges. Non-parametric tests were used for non-normal distributions of mRNA concentrations. We used the Mann–Whitney test to compare mRNA concentrations between the two groups. A P-value < 0.05 was considered to be statistically significant.

Results

Demographic and clinical data for the transplant patients included in this study are given in Table 2. The groups

Table 1. Sequences of oligonucleotide primers and FRET probes used for the quantification of target genes by quantitative PCR

			Applied Biosystems 7900 HT	
Gene	Sequence reference	Sense	Sequence	Amplicon
IFNγ	NM_000619 GI:56786137	5′	TGG CTT AAT TCT CTC GGA AAC G	22
		3′	AAA AGA GTT CCA TTA TCC GCT ACA TC	26
Tbet	NM_013351 GI:7019548	5′	TGA GGT GAA CGA CGG AGA GCC A	22
		3′	GGT AGA AGC GGC TGG GAA CAG GA	23
GB	NM_004131 GI:32483414	5′	GGG GAA GCT CCA TAA ATG TCA CCT TG	26
		3′	GCT TCA CCT GGG CCT TGT TGC TAG	24
FOXP3	NM_014009 GI:31982942	5′	TCC ACA ACA TGC GAC CCC CTT TCA	24
		3′	ACA GCC CCC TTC TCG CTC TCC A	22
IL-17	NM_002190 GI:45768845	5′	ATC TCC ACC GCA ATG AGG AC	25
		3′	GTG GAC AAT CGG GGT GAC AC	20
RORγt	NM_005060 GI:48255916	5′	CTC CAT CTT TGA CTT CTC CCA CTC CCT A	28
		3′	CAC ATG CTG GCT ACA CAG GCT C	22
GATA3	NM_002051 GI:50541957	5′	GCC CCT CAT TAA GCC CAA GCG A	22
		3′	CAG GGG TCT GTT AAT ATT GTG AAG CTT GTA GT	32
GAPDH	NM_002046 GI:7669491	5′	GGA TTT GGT CGT ATT GGG CGC	21
		3′	GTT CTC AGC CTT GAC GGT GC	20

Table 2. Demographic and clinical data for transplant patients in the Phase II and III trials

	Belatacept (n = 18)	CNI (n = 12)	P-value
Age (years)	50.4 ± 11	49.2 ± 9	ns
Male sex (%)	64	71	ns
Type of donor DD/LD (%)	95/5	100/0	0.19
Creatinine 1 month post-transplant	1.5 ± 0.8	1.6 ± 0.9	0.12
Creatinine 6 months post-transplant	1.45 ± 0.6	1.8 ± 0.6	0.04
Creatinine 1 year post-transplant	1.4 ± 0.7	1.8 ± 0.7	0.03
PBAR (%)	9	8	ns
Graft loss (%)	0	0	ns

DD, deceased donor; LD, living donor; BPAR, biopsy-proven acute rejection.

of patients treated with Belatacept and CNI did not differ according to age, sex, type of donor, incidence of acute rejection, percentage of graft loss or serum creatinine level 1 month post-transplant. Creatinine levels 6 months and 1 year post-transplant were significantly lower in the Belatacept group (P = 0.04 and P = 0.03, respectively).

Similarly, histological analysis of the 1-year protocol biopsy did not reveal any statistical difference between the two groups for tubulitis (t), interstitial infiltrate (i), glomerulitis (g), interstitial fibrosis (ci), tubular atrophy (ct), vascular fibrous thickening (cv), arteriolar hyalinosis (ah) or peritubular capillaritis (ptc). The interstitial fibrosis/tubular atrophy (IF/TA) score was similar for both groups. The global inflammatory score (i + t + g) also did not differ significantly between the two groups (Table 3). All samples analysed were negative for Cd4 immunostaining.

CD3 immunostaining did not reveal significant differences between the two groups: 2.7±1.3 cells/mm^2 in the Belatacept group and 3.2±2.1 cells/mm^2 in the CNI group.

We next analysed the expression of markers for the Th1 (INFγ and Tbet), Th2 (GATA3), Treg (FOXP3) and Th17 (IL-17 and RORγt) and cytotoxic CD8 (GB) T-cell subsets in the 1-year renal biopsies from patients treated with Belatacept (n = 17) and CNI (n = 18) (Figure 2). As assessed using the Mann–Whitney test, only the level of mRNA for FOXP3 differed significantly between the two groups: it was significantly less abundant in the Belatacept group than the CNI group. The expression of GB was higher in the CNI group than in the Belatacept group, although the difference was not statistically different. The median values in the Belatacept and CNI groups, respectively, were FOXP3, 0.185 and 1.785 (P < 0.001); GB, 0.215 and 0.441 (P = 0.06); IFNγ, 1.204 and 0.97 (P = 0.59); GATA3, 0.370 and 0.493 (P = 0.25); RORγt, 1 and 1.237 (P = 0.2); IL-17, 0.001 and 0.002 (P = 0.67); and Tbet, 0.545 and 0.540 (P = 0.8).

We next performed a Tregs count based on flow cytometry data from the peripheral blood. By analysing the percentage of CD25high in CD4+ T cells in 10 patients from both groups. The percentage of CD25high in CD4+ T cells was lower in the Belatacept group (3.1 ± 2.3% *vs* CsA = 4.7 ± 1.9%), although the difference did not reach statistical significance (P = 0.06) (Figure 3A). We finally analysed residual CD86 expression on circulating monocytes in three patients from the CsA group and three patients from the Belatacept group (pre- and post-dose). A double labelling comprising of CD14-ECD and CD86-PE was performed and we analysed the mean fluorescence intensity of CD86 measured in monocytes (see the Materials and methods section). Our results did not show a significant difference in CD86 expression between patients from the Belatacept (pre-dose) and the CsA group but a significant decrease in CD86 expression after administration of Belatacept. However, we observed a substantial free CD86 expression, suggesting an incomplete blockage of this signalling pathway (Figure 3B).

Table 3. Histological findings for 1 year post-transplant protocol biopsies in transplant patients in the Phase II and III trials

Histological parameter	Belatacept (n = 18)	CNI (n = 13)	P-value
t	0.76 ± 0.71	0.84 ± 0.9	ns
i	0.86 ± 0.61	0.90 ± 0.4	ns
g	0.1 ± 0.3	0.2 ± 0.4	ns
t + i + g	0.51 ± 0.54	0.64 ± 0.50	ns
IF/TA grade (I/II/III) (%)	73/22/5	65/20/15	ns
ptc (%)	<10	<10	ns
cg	0	0.2	ns
ci	1.6 ± 1.2	1.80 ± 0.9	ns
ct	1.30 ± 1.7	1.68 ± 0.51	ns
cv	0.92 ± 0.58	1.10 ± 0.86	ns
ah	1.05 ± 0.3	0.9 ± 0.7	ns
C4d staining (%)	0	0	ns

Mean values are reported ± standard deviation.
t, tubular infiltrate; i, interstitial infiltrate; g, glomerulitis; cg, allograft glomerulopathy; ct, tubular atrophy; cv, vascular fibrous thickening; ah, arteriolar hyalinization; IF/TA, interstitial fibrosis/tubular atrophy; ptc, peritubular capillaritis

Discussion

Here, we report the first description of the T-cell phenotype in protocol biopsies performed 12 months post-transplant in kidney recipients treated with Belatacept-mediated costimulatory blockade. The only difference we found between the Belatacept and control (cyclosporin A-treated) groups was that the intra-graft level of FOXP3 mRNA was significantly lower in the Belatacept group. No difference was observed in the level expression of markers for the Th1, Th2, Th17 and cytotoxic T-cell subsets. It has been

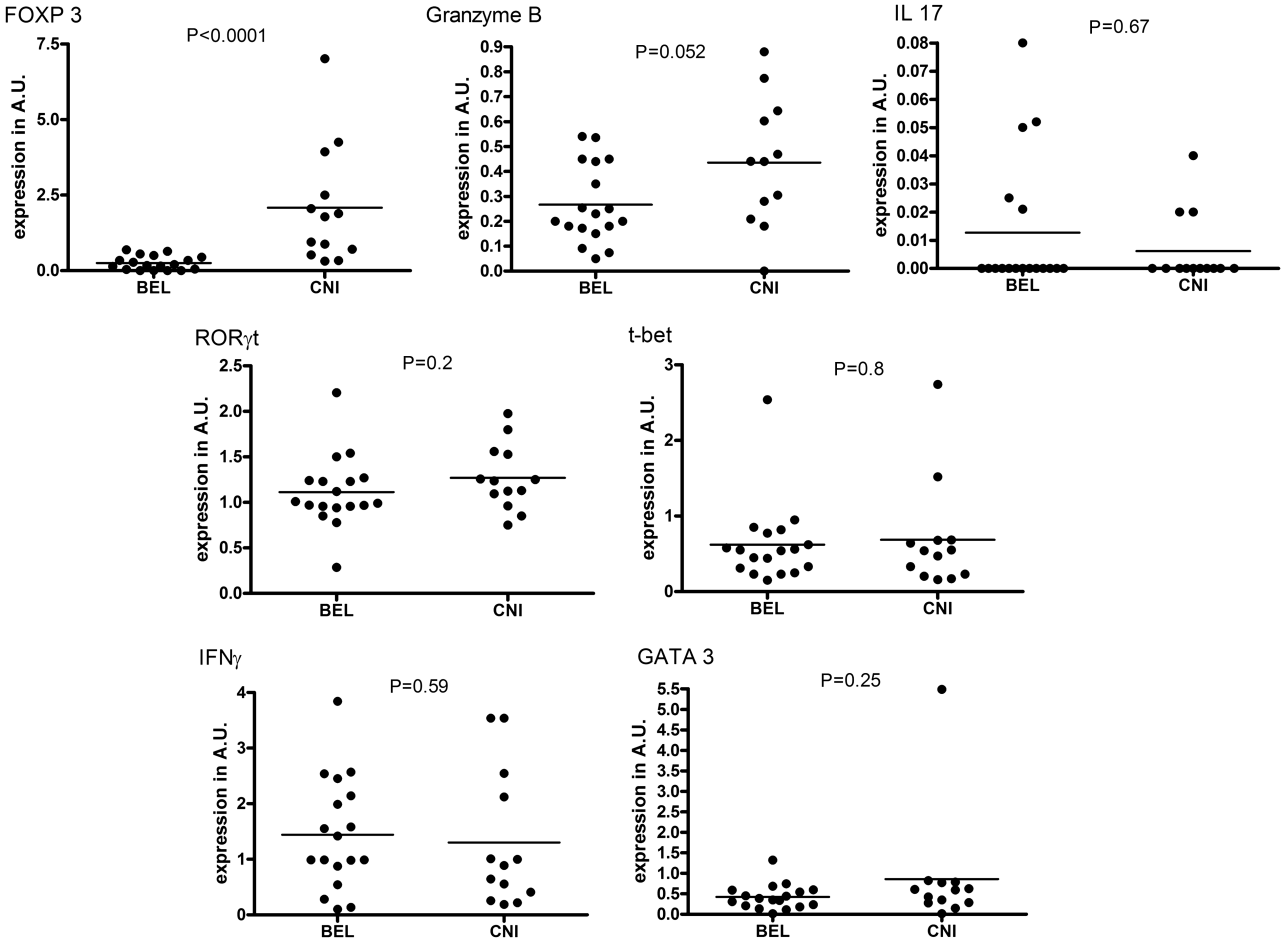

Fig. 2. Expression levels of seven genes (encoding FOXP3, GB, IFNγ, Tbet, GATA3, IL17 and RORγt) in 1-year protocol biopsy samples from patients treated with Belatacept and CNI.

previously shown that the anti-CD25 monoclonal therapy led to a profound, but transient, reduction in Treg [5]. Thus, it might be hypothesized that reduction of CD25+FOXP3+ cell is in part related to the induction protocol. However, the effect on FOXP3 is presumably a direct effect of Belatacept because both groups received the same induction with the anti-CD25, basiliximab.

Intra-graft expression of FOXP3 is reduced more strongly by Belatacept than CNI, a well-known inhibitor of both Treg expansion and function *in vivo* and *in vitro* [16,17]. These results are in agreement with previous experimental work showing that CD28 is a unique co-stimulatory signal promoting both thymic development and peripheral homeostasis of Tregs [18,19]. Moreover, CD28 maintains a stable pool of peripheral Tregs by supporting both their renewal and their survival, possibly by increasing TCR signalling strength and promoting IL-2 production by conventional T cells [19]. Thus, by blocking the CD28-B7 pathway, CTLA4-Ig is likely to affect Tregs number and function. Contrasting with these experimental findings, recent clinical trials found that Belatecept had no short-term or long-term effect on circulating Treg numbers or function [5,6]. These contradictory findings may be a consequence of the inability of Belatacept at the dose administered to block CD28-B7 fully as suggested by CD86 residual expression in patients treated with Belatacept or induced by a CD28-independent adaptive Tregs pathway mechanism [20]. However, the conclusions of these clinical reports are mainly supported by peripheral FOXP3 monitoring [peripheral blood leucocytes (PBL)] and it is now established that the gene expression profile in PBL does not represent intra-renal conditions [21]. Nevertheless, whereas intra-graft FOXP3 gene expression correlates with anti-donor immune responses in the graft, this is not necessarily true for peripheral FOXP3 mRNA levels [22].

Tregs play a crucial role in the control of innate and adaptive immune responses. There have been several reports mainly during the last decade on the potential interest of molecular analysis of a single gene expression to predict clinical outcome from pathogenic conditions. In the field of renal transplantation, such an objective has focused on FOXP3 monitoring in blood, urine, or the transplant itself to predict graft outcome in different contexts. It appears now quite clearly that FOXP3 expression correlates well with scarring and inflammation. In this context, FOXP3-positive cells appear to represent a mechanism for stabilizing this inflammatory process by controlling 'spaces' in the immune response [23]. The prognostic value of FOXP3 expression on graft outcome is still controversial. While some reports have suggested a positive correlation [24,25], a large

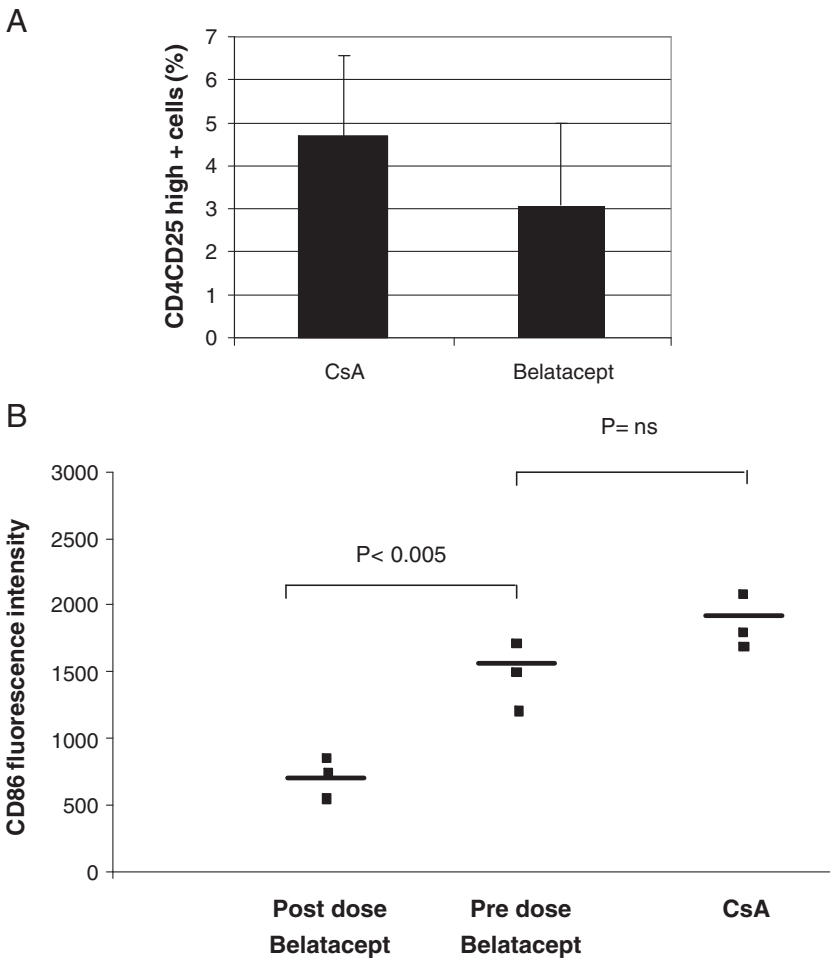

Fig. 3. (**A**) Percentage of CD4+CD25bright cells in patients from the Belatacept (n = 10) and CsA (n = 10) groups. (**B**) Median fluorescence intensity of CD86 expression on monocytes from three treated patients from the CsA and Belatacept groups.

study performed in biopsies for cause find a lack of association between FOXP expression and graft outcome [26]. In fact and according to a most recent report [27], it seems that FOXP3 expression could only correlate with graft outcome in a short period of time following an acute inflammatory episode such as acute [11,28], subclinical [12] or borderline [13] rejection. By contrast, the predictive value of FOXP3 expression in protocol biopsies has weakly been analysed with controversial results [22,29]. In our study, we showed that, in renal protocol biopsies from patient treated with Belatacept-mediated co-stimulatory blockade, expression of FOXP3 was significantly lower than that in patients treated with CNI, despite a better graft function and outcome. According to previous studies, our results suggest that, in a non-inflammatory context as observed in protocol biopsy, FOXP3 expression do not correlate with clinical condition. The rate of chronic allograft nephropathy is lower and the glomerular filtration rate better in the Phase II and III clinical trials of Belatacept than in patients treated with CNI [4,30], suggesting that potential immune benefits induced by Belatacept in these trials are perhaps not mediated by Treg expansion. It has recently been shown that patients treated with CTLA4-Ig display higher soluble HLA-G plasma concentrations, a naturally tolerogenic molecule in human, mainly secreted by myeloid dendritic cells [31]. Such mechanism is likely to play a preponderant role in allogeneic response in these patients.

We also analysed markers of other T-cell subsets including those of the Th1, Th2 and Th17 pathways. Indeed, CTLA-4/B7 interactions enhance human naïve CD4+ T-cell differentiation into IL-17- and IL-22-producing cells [7]. However, the amounts of Th17 markers, including IL-17 and RORγT, an orphan nuclear receptor which promotes mouse and human Th17 differentiation [32], were not higher in the group of patients receiving Belatacept than in the reference group. Expression of IL-22 in the serum of patients did not differ significantly between the two groups (data not shown). Again, this contrast between experimental findings and human studies suggests incomplete CD28/B7 blockade at the dose of Belatacept administered. The expression of GB, a marker of cytotoxic CD8 T cells, was higher in the CNI group, although the difference did not reach statistical significance. It has recently been shown that graft expression of GB was an accurate marker not only for acute but also for chronic rejection [16,17]. Whether the level of expression of GB will influence the long-term incidence of IF/TA and cellular- as well as humoral-mediated chronic rejection remains to be determined.

Conclusion

In summary, the T-cell immune profile observed in protocol biopsies from kidney transplant recipients treated with Belatacept-mediated co-stimulatory blockade was similar to that in patients treated with conventional therapy including CNI; the only significant difference was lower FOXP3 expression in the Belatacept group. These results are in agreement with *in vitro* data suggesting that CD28 is a major co-stimulatory signal for both Tregs development and peripheral homeostasis. They contrast with clinical findings from both Phase II and III clinical trials of Belatacept showing a better 1-year graft function and a lower incidence of chronic allograft nephropathy, suggesting that Belatacept-induced immunomodulation is mediated by a Treg-independent pathway. Lack of nephrotoxic drugs such as CNI and perhaps tolerance induction mediated by HLA-G are more likely to account for these results. These results also suggest that FOXP3 is not by itself a prognostic marker of long-term graft function in a non-inflammatory context. Our results should be interpreted as preliminary since the size of our study population is limited. They have to be confirmed by extensive data including more patients from the Belatacept trials.

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