TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Decreased frequency of peripheral $CD4^+CD161^+$ Th_{17}-precursor cells in kidney transplant recipients on long-term therapy with Belatacept

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Keywords
Belatacept, $CD4^+CD161^+$, kidney, soluble CD30, transplantation, Th_{17}.

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Conflicts of Interest
The authors have no conflicts of interest to disclose.

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Received: 15 November 2011
Revised requested: 28 December 2011
Accepted: 16 January 2012
Published online: 20 February 2012

doi:10.1111/j.1432-2277.2012.01441.x

Summary

Clinical trials have pointed out the promising role of co-stimulation blocker Belatacept for improvement of graft function and avoidance of undesired side-effects associated with calcineurin-inhibitors (CNI). However, due to the worldwide limited availability of appropriate patients, almost no data exist to assess the effects of sustained application of this immunomodulator on the recipient's immune system. The aim of this study was to reveal specific alterations in the composition of immunologic subpopulations potentially involved in development of tolerance or chronic graft rejection following long-term Belatacept therapy. For this, peripheral lymphocyte subsets of kidney recipients treated with Belatacept ($n = 5$; average 7.8 years) were determined by flow-cytometry and compared with cells from matched patients on CNI ($n = 9$) and healthy controls ($n = 10$). T cells capable of producing IL-17 and serum levels of soluble CD30 were quantified. Patients on CNI showed a higher frequency of $CD4^+CD161^+$ Th_{17}-precursors and IL-17-producing $CD4^+$ T cells than Belatacept patients and controls. Significantly higher serum levels of soluble CD30 were observed in CNI patients, indicating a possible involvement of the CD30/CD30L-system in Th_{17}-differentiation. No differences were found concerning $CD4^+CD25^+CD127^{low}FoxP3^+$ regulatory T cells. In conclusion, patients on therapy with Belatacept did not show a comparable Th_{17}-profile to that seen in individuals with chronic intake of CNI. The distinct effects of Belatacept on Th_{17}-immunity might prove beneficial for the long-term outcome following kidney transplantation.

Introduction

Recent developments of effective immunosuppressive protocols successfully have lowered the incidence of acute rejection (AR) episodes and improved short-term outcome after kidney transplantation (Tx) [1,2]. Nevertheless, long-term graft and patient survival have not equally benefited from these advancements: chronic allograft dysfunction (CAD) remains a strong hindrance for long-term graft acceptance [3,4]. The etiology of CAD is complex and still not fully understood [5], both immunological and nonimmunological factors account for its development. Meanwhile the latter remain difficult to control, immunological factors such as the incidence of AR or the degree of HLA-matching have been successfully addressed [6].

Calcineurin-inhibitors (CNI) show excellent results in the early-phase after organ Tx as they effectively control AR and thus in clinical practice still present one of the most preferred immunosuppressive medications [7]. Nevertheless, they are associated with undesirable side effects and chronic use of CNI is a major contributing factor to CAD [8]. For this reason, other drugs such as the T-cell co-stimulation blocker Belatacept (LEA29Y) have been

developed [9]. Belatacept is a fusion protein composed of the Fc-fragment of a human IgG1 immunoglobulin linked to the extracellular domain of CTLA-4. Its mechanism of action is to bind to the co-stimulatory ligands CD80/CD86 to inhibit their interaction with the co-stimulatory receptor CD28 and thus block the second signal necessary to activate T cells, which plays an important role in graft rejection [10]. In kidney Tx, use of Belatacept has shown superior renal function and similar patient/graft survival as conventional protocols based on CNI, but without showing the typical signs of CNI toxicity [11,12].

Although the theory of co-stimulation blockade is well established, the exact mechanism of Belatacept-mediated immunomodulation remains incompletely characterized. So far, it has been reported that Belatacept treatment does not alter the frequency or function of regulatory T cells (T_{regs}), but rather results in enhanced intragraft recruitment of T_{regs} [13,14]. As T_{regs} play an important role in transplantation tolerance [15,16], increasing the relative frequency of these cells within the graft might have an impact on the incidence of acute rejection and/or graft survival. Nevertheless, the use of Belatacept as maintenance therapy has not successfully induced tolerance following organ transplantation, yet [12].

Most recently, focus in transplant immunology is increasingly placed on a lately discovered T-helper cell population with the ability to secrete interleukin-17 (IL-17): the Th_{17}-lineage [17]. These cells seem to be involved in autoimmune and inflammatory diseases and thus might also play an important role for the development of CAD [18]. They originate from $CD4^+CD161^+$ Th_{17}-precursor cells [19], but only little is known about the exact role of this T-cell subset. Due to their known properties, they might have a considerable influence on the development of tolerance: Th_{17}-cells appear to be resistant to suppression by T_{regs} and even may convert from T_{regs} under inflammatory conditions [20–23]. Overall, the capacity of Th_{17}-cells to cause allograft rejection is becoming increasingly clear [24]. Whether Belatacept might influence this Th_{17}/T_{reg}-axis has not been investigated yet.

Thus, the aim of the present study was to analyze the impact of long-term therapy with Belatacept following kidney transplantation on the composition of various T-cell and non-T-cell subsets potentially involved in mechanisms leading to transplantation tolerance or chronic allograft dysfunction.

Material and methods

Patients and immunosuppression

All patients ($n = 14$) received primary kidney allografts from living or deceased donors at Hannover Medical School, Germany, between July 2000 and October 2003. Detailed patient characteristics and transplantation information are summarized in Table 1. The Belatacept group consisted of $n = 5$ patients and obtained induction therapy with two 20 mg doses of anti-CD25-antibody Basiliximab (Simulect®, Novartis, Basel, Switzerland) on days 0 and 4 of transplantation combined with a triple immunosuppressive regimen made up of Belatacept (Bristol-Myers Squibb, New York, USA; amendment to trial IM103-100), mycophenolate mofetil and steroids. The transplant controls (CNI-group, $n = 9$) consisted of kidney transplanted patients closely matched in age, current serum creatinine and time after transplantation. They similarly received Basiliximab administration, but immunosuppressive therapy was based on a calcineurin-inhibitor (Table 1). The

Table 1. Demographic and clinical data of transplanted patients.

#	Age	Sex	Donor	Mismatch	Acute rejection (time after Tx)	Immunosuppression
1	48	m	LRD	1-1-1	No	Bela, MMF, Pred
2	49	m	DD	1-2-1	No	Bela, MMF, Pred
3	35	m	DD	1-1-0	Banff 1 (month 5)	Bela, MMF, Pred
4	38	f	DD	0-0-0	No	Bela, MMF, Pred
5	52	m	DD	0-2-1	No	Bela, MMF, Pred
6	20	f	LRD	0-0-0	No	CyA, MMF, Pred
7	41	m	LRD	1-1-1	No	CyA, MMF, Pred
8	46	m	LRD	2-2-2	No	CyA, MMF, Pred
9	37	f	DD	0-1-0	Borderline (month 4)	CyA, MMF, Pred
10	66	m	DD	1-1-1	No	CyA, MMF, Pred
11	58	f	DD	1-1-2	No	CyA, MMF, Pred
12	27	m	LUD	0-0-1	No	CyA, Aza, Pred
13	23	f	LRD	0-0-0	No	Tacro, Aza, Pred
14	59	f	LUD	0-2-1	Banff 1 (month 6)	Tacro, MMF

LRD = living related donor, LUD = living unrelated donor, DD = deceased donor, Bela = Belatacept, MMF = Mycophenolate mofetil, Pred = Prednisolon, CyA = Cyclosporine, Aza = Azathioprine, Tacro = Tacrolimus.

daily exposure to mycophenolate mofetil and steroids was comparable to the Belatacept group (1.2 ± 0.4 vs. 1.4 ± 0.4 g and 4.7 ± 0.9 vs. 4.5 ± 1.1 mg, respectively). There were no significant differences for covariates as duration of pretransplant dialysis or treatment with immunosuppressive drugs as part of the therapy of the causal nephropathy between the groups. All patients gave written informed consent to participate in this study. The protocol was approved by the local ethical commission.

Kidney biopsy and acute rejection

Upon clinical presumption of acute graft rejection such as insufficient decline or sudden rise of serum creatinine, patients received kidney biopsy. Biopsy-proven AR was diagnosed histologically according to the latest Banff classification [25]. Borderline (BL) changes were regarded and treated as rejection if associated with a creatinine increase of ≥20% compared with previous values. All cases of rejection were successfully treated by three high-dose steroid pulses.

Sample collection and cell isolation

Heparinized blood samples were obtained during routine blood withdrawal. All samples were processed within 3 h. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Ficoll solution. PBMCs were frozen in supplemented RPMI 1640 containing 20% FCS and 10% DMSO and stored in liquid nitrogen until further analysis in batch. Serum samples were collected from the same preparations and stored at −20 °C until use. Specimens of healthy controls (HC, $n = 10$) were procured in the same manner.

Flow-cytometry

PBMCs were thawed, cultured overnight in supplemented RPMI 1640 at 5% CO_2, and then analyzed by multi-color flow-cytometry. $CD4^+CD25^+CD127^{low}FoxP3^+$ regulatory T cells were measured as previously described [26] using a BD FACSCalibur. In brief, cell surface staining was performed using the monoclonal antibodies (mAb) CD4-FITC (clone: RPA-T4), CD25-PE (clone: M-A251; all BD Biosciences, San Jose, USA) and CD127-PE-Cy5 (clone: eBioRDR5; eBioscience, San Diego, USA). Intracellular staining of FoxP3 was performed according to the manufacturer's instructions (FoxP3 Flow Kit, clone: 206D; isotype control: MOPC-21; BioLegends, San Diego, USA). Further T-cell and non-T-cell populations were analyzed by cell surface staining, applying the following mAb and using a BD LSR-II flow-cytometer: CD3-PE-Cy7 (clone: SK7 (Leu4)), CD4-V450 (clone: RPA-T4), CD8-APC (clone: RPA-T8), CD16-FITC (clone: 3G8), CD19-APC (clone: HIB19), CD28-FITC (clone: CD28.2), CD56-V450 (clone: B159) and CD161-PE (clone: DX12; all BD Biosciences).

Measurement of IFN-Y and IL-17 production

PBMCs were thawed, cultured overnight in supplemented RPMI 1640 at 5% CO_2, and stimulated using phorbol 12-myristate 13-acetate (PMA, 1 ng/ml) and ionomycin (1 μg/ml) for 6 h. Brefeldin A was added at 10 μg/ml to prevent cytokine secretion (all Sigma-Aldrich, Germany). The cells were then stained for CD4 (FITC, clone: RPA-T4). Intracellular staining for interferon-Y (Alexa 647; clone: 4S.B3) and interleukin-17A (Alexa 647; clone: BL168) was performed according to the manufacturer's instructions (all BioLegends, San Diego, USA). An appropriate control using an IgG1-antibody (Alexa 647; clone: MOPC-21) was performed. Flow-cytometric analysis was performed using a BD FACSCalibur.

Soluble CD30 enzyme-linked immunosorbent assay

Serum samples were thawed and immediately used for detection of soluble CD30 (sCD30) by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, San Diego, USA). In brief, samples were incubated with a HRP-conjugate on microwell plates precoated with anti-sCD30-antibody. Following 3 h of incubation, wells were washed and TMB-substrate solution was added. Approximately 10 min. thereafter, color development was stopped by adding a stop solution. Absorbance was read using a spectrophotometer at 450 nm wavelength.

Statistical analysis

Clinical and flow-cytometric data were statistically analyzed applying *PASW Statistics 18.0* (IBM, USA). The correlation of parameters was investigated by *Spearman's Correlation Test*. The *Kruskal–Wallis- or Mann–Whitney-U-test* were applied as appropriate. Differences were regarded statistically significant with $P < 0.05$.

Results

Demographic and clinical data

Successful kidney transplantation was performed in all cases ($n = 14$) with initial graft function. Due to matching of patients, both the Belatacept and the CNI groups showed similar recipient age (44.4 ± 7.4 vs. 41.9 ± 16.7 years; Table 1) and graft function as indicated by serum creatinine (99.8 ± 26.9 μmol/l vs. 102.3 ± 17.9

μmol/l) at the time of investigation. In addition, all patients were included at comparable time points after transplantation (94.6 ± 6.1 months vs. 84.9 ± 16.8 months). No augmented incidence of bacterial or viral infections was found in either group. There were no significant differences in the total number of HLA-mismatches (HLA-A, -B, -DR loci) between Belatacept- vs. CNI-treated patients (2.4 ± 1.5 vs. 2.3 ± 2.0) with identical incidence of biopsy proven AR (∼20%). Furthermore, the current number of peripheral blood lymphocytes was comparable between both transplant groups (1.7 ± 0.5 and 2.2 ± 1.0 for Belatacept and CNI groups, respectively) and to healthy controls (1.9 ± 0.4 cells/μl blood × 10^3).

Comparable frequencies of T_{regs} and other common T-cell/non-T-cell subsets in long-term transplant recipients

In general, comparable frequencies of conventional T-cells ($CD3^+$, $CD4^+$, and $CD8^+$) were observed in both transplant groups. The relative numbers of $CD3^+$ and $CD8^+$ T cells were significantly higher than in healthy individuals. Furthermore, both the CNI- and Belatacept-treated kidney graft recipients showed a significantly decreased frequency of $CD3^-CD19^+$ B cells compared with the control group (Table 2).

Concerning regulatory T cells, patients on long-term Belatacept treatment showed slightly elevated—but not to a significant extent—frequencies of the conventional $CD4^+CD25^+CD127^{low}FoxP3^+$ T_{regs} as compared with the other two groups. Likewise by trend, the subset of $CD3^+CD4^-CD8^-$ seemed decreased in Belatacept patients. Both transplant groups showed an increased frequency of $CD3^+CD8^+CD28^-$ cells compared with healthy individuals, but due to the small number of cases again this did not reach statistical significance.

Regarding NK and NKT cells, the latter as well as $CD3^-CD56^{bright}$ cells were comparable in all groups. As the only notable difference between the transplant recipients, a significantly reduced frequency of $CD3^-CD56^{dim}$ NK cells was observed in patients on CNI-therapy (Table 2).

Decreased frequency of $CD4^+CD161^+$ Th_{17}-precursor cells in long-term Belatacept-treated kidney recipients

Recently, a new T-helper cell subset with the ability to secrete IL-17 has been described. These so called Th_{17}-cells have been reported to develop from a $CD4^+$ precursor population expressing CD161. We thus determined the frequency of $CD4^+CD161^+$ cells within the peripheral blood of transplant recipients on different maintenance regimens and healthy controls applying flow-cytometry. Highest frequency of $CD4^+CD161^+$ cells was found in patients with long-term CNI-therapy followed by healthy controls. Individuals receiving an immunosuppressive protocol based on Belatacept showed a significantly reduced number of $CD4^+CD161^+$ cells. This was observed regarding the relative frequency among $CD4^+$ T cells (Fig. 1) as well as the absolute number of cells within the blood (583.4 ± 252.2 cells/μl vs. 204.2 ± 33.7 cells/μl vs. 279.3 ± 118.4 cells/μl blood for CNI, Belatacept and HC, respectively).

Table 2. Comparison of various T-cell and non-T-cell subsets.

	CNI (n = 9)	Bela (n = 5)	HC (n = 10)	P-value
Conventional T-cell subsets				
$CD3^+$	86.8 ± 3.2	80.8 ± 9.8	70.6 ± 5.6	P = 0.001
$CD3^+$ $CD4^+$	42.6 ± 14.2	44.2 ± 4.6	45.1 ± 8.1	P = 0.715
$CD3^+$ $CD8^+$	36.0 ± 9.8	32.2 ± 7.1	21.7 ± 5.3	P = 0.015
Unconventional T-cell subsets				
$CD4^+$ $CD25^+$ $CD127^{low}$ $FoxP3^+$	2.8 ± 0.8*	3.9 ± 1.5*	2.9 ± 1.2*	P = 0.189
$CD3^+$ $CD8^+$ $CD28^-$	19.9 ± 12.7	17.9 ± 7.4	10.0 ± 7.1	P = 0.122
$CD3^+$ $CD4^-$ $CD8^-$	5.9 ± 2.5	2.9 ± 1.3	4.2 ± 3.4	P = 0.153
NK-cell subsets				
$CD3^-$ $CD56^{dim}$	6.0 ± 0.4	11.6 ± 7.7	12.3 ± 4.4	P = 0.030
$CD3^-$ $CD56^{bright}$	0.8 ± 0.4	0.6 ± 0.3	0.7 ± 0.4	P = 0.800
NKT-cells				
$CD3^+$ $CD56^+$	8.4 ± 6.1	6.5 ± 1.7	5.6 ± 3.1	P = 0.657
B-cells				
$CD3^-$ $CD19^+$	5.0 ± 3.2	5.3 ± 2.7	11.4 ± 3.8	P = 0.002

CNI = calcineurin-inhibitor, Bela = Belatacept, HC = healthy control; data are presented as mean percentage ± SD of total lymphocytes if not stated otherwise.

Analysis was performed on gated lymphocytes as identified by forward and side scatter characteristics.

*Data are presented as mean percentage ± SD of $CD4^+$ cells.

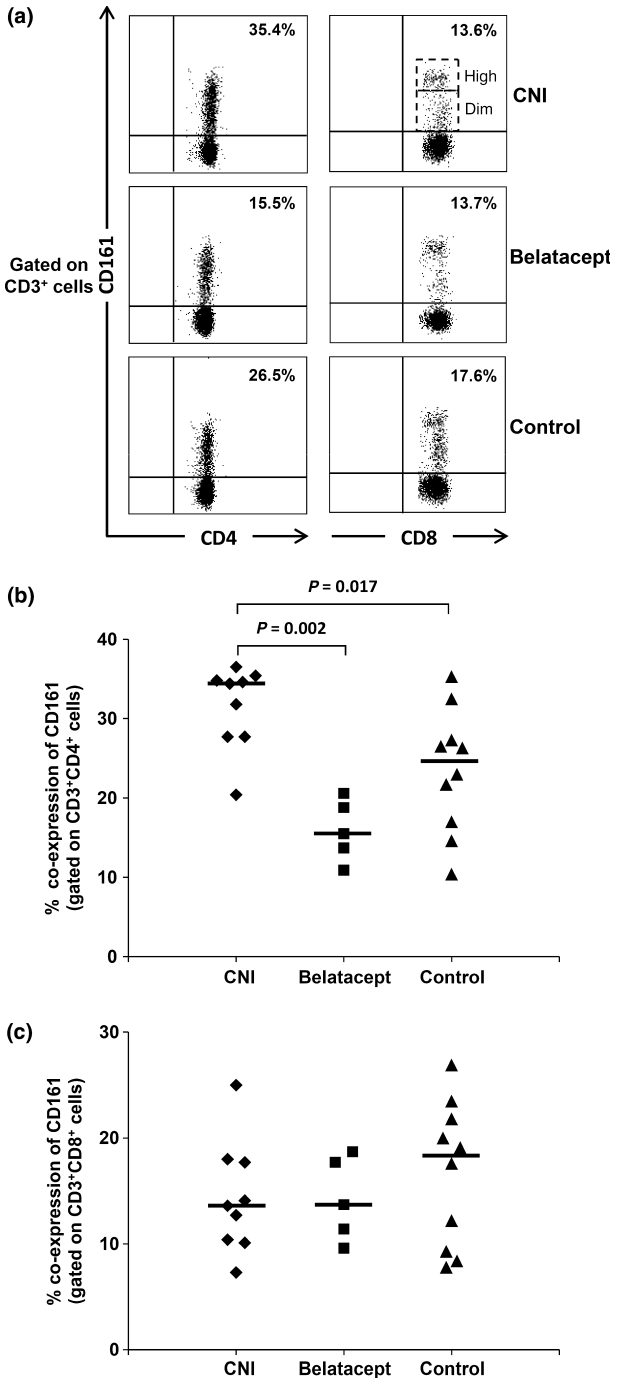

Figure 1 Decreased frequency of $CD4^+CD161^+$ Th_{17}-precursor cells in long-term Belatacept-treated kidney recipients. (a) Dotplots with representative staining patterns of PBMCs from one kidney transplant recipient receiving CNI, one individual on Belatacept-treatment as well as a healthy control. Cells were stained for CD3, CD4, CD8 and CD161. The frequencies of $CD4^+CD161^+$ and $CD8^+CD161^+$ cells determined by flow-cytometry are shown (numbers indicate percentage of $CD3^+$ cells). The latter was divided in $CD161^{dim}$ and $CD161^{high}$ subsets as indicated by the dashed boxes. Frequencies of (b) $CD3^+CD4^+$ and (c) $CD3^+CD8^+$ cells co-expressing CD161 are shown for each group. Individual results of flow-cytometry for patients receiving CNI (diamonds, $n = 9$) or Belatacept-treatment (squares, $n = 5$) as well as healthy controls (triangles, $n = 10$) are presented as percentage. The median within each group is indicated.

Th_{17}-cells also may arise from a $CD8^+$ population. No differences among the groups were found concerning the frequency of $CD8^+CD161^+$ cells. Even subdivision of the $CD8^+$ population into $CD161^{high}$ and $CD161^{dim}$ cells (Fig. 1) resulted in comparable subset frequencies among all groups ($3.4 \pm 1.4\%$ vs. $2.5 \pm 2.5\%$ vs. $3.4 \pm 3.6\%$ for $CD8^+CD161^{high}$ and $11.1 \pm 5.8\%$ vs. $11.9 \pm 5.2\%$ vs. $12.2 \pm 6.3\%$ for $CD8^+CD161^{dim}$ of total $CD3^+$ cells for CNI, Belatacept and HC, respectively).

Increased frequency of IL-17 producing $CD4^+$ T cells in long-term CNI-treated kidney recipients

Th_{17}-cells have the ability to secrete IL-17. We thus determined the frequency of $CD4^+IL\text{-}17^+$ and $CD4^+IFN\text{-}\gamma^+$ cells within the peripheral blood of transplant recipients on different maintenance regimens and healthy controls applying flow-cytometry. Lowest frequency of $CD4^+IL\text{-}17^+$ cells was found in patients with long-term Belatacept therapy followed by healthy controls. Individuals receiving an immunosuppressive protocol based on CNI showed a significantly increased number of $CD4^+IL\text{-}17^+$ cells (Fig. 2). No differences between the groups were observed regarding the frequency of IFN-γ producing $CD4^+$ cells.

This finding is in line with our results on the frequency of $CD4^+CD161^+$ cells. Thus, a significant correlation of the frequency of IL-17 producing $CD4^+$ T cells and the frequency of $CD4^+CD161^+$ cells was observed ($r_s = 0.699$, $P < 0.001$). The latter did not correlate with the frequency of IFN-γ producing cells within the $CD4^+$ subset ($r_s = 0.308$, $P = 0.143$).

Increased serum levels of sCD30 in long-term CNI-treated kidney recipients

The basic rationale behind the intake of immunosuppressive medication following organ transplantation is to prevent T-cell activation to avoid graft rejection. The serum level of soluble CD30 has been shown to reflect the degree of T-cell activation. As CNI and Belatacept have different mechanisms of action, we compared the serum levels of sCD30 as a measurement of the state of T-cell activation. We found that transplanted individuals receiving long-term Belatacept treatment showed comparable levels of sCD30 as to healthy controls nontransplanted and without the dependence on immunosuppression. In contrast, determination of sCD30 in kidney recipients with long-term CNI treatment revealed significantly increased serum levels, indicating a higher degree of

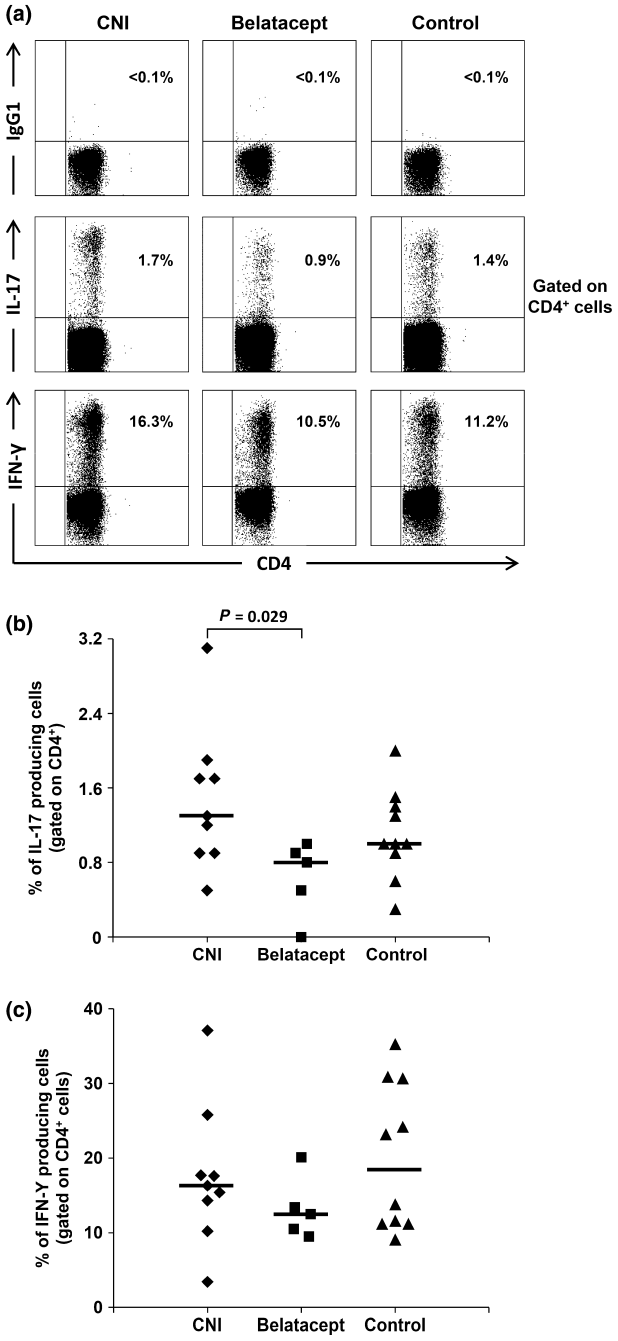

Figure 2 Increased frequency of IL-17 producing $CD4^+$ T-cells in long-term CNI-treated kidney recipients. (a) Dotplots with representative staining patterns of PBMCs from one kidney transplant recipient receiving CNI, one individual on Belatacept treatment as wells as a healthy control. Cells were stained for CD4 combined with IL-17 or IFN-γ following stimulation with PMA/ionomycin for 6 h. The frequencies of $CD4^+IL\text{-}17^+$ and $CD4^+IFN\text{-}\gamma^+$ cells determined by flow-cytometry are shown (numbers indicate percentage of $CD4^+$ cells). An appropriate control using an IgG1-antibody was performed. Frequencies of (b) IL-17 and (c) IFN-γ producing cells within the $CD4^+$ population are shown for each group. Individual results of flow-cytometry for patients receiving CNI (diamonds, $n = 9$) or Belatacept treatment (squares, $n = 5$) as well as healthy controls (triangles, $n = 10$) are presented as percentage. The median within each group is indicated.

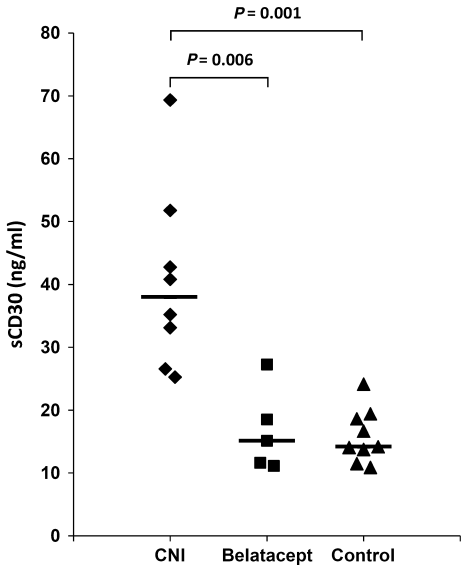

Figure 3 Increased serum levels of sCD30 in long-term CNI-treated kidney recipients. The levels of soluble CD30 were determined in the serum of patients receiving CNI (diamonds, $n = 8$) or Belatacept treatment (squares, $n = 5$) as well as in healthy controls (triangles, $n = 9$) by ELISA. Results for each individual are presented as ng/ml. The median within each group is indicated.

T-cell activation than observed for the other two groups (Fig. 3).

T-cell activation via the CD30/CD30L system has been shown to be involved in Th_{17} differentiation. Our finding is in line with this observation: the serum level of sCD30 was found to correlate strongly with the frequency of $CD4^+CD161^+$ cells ($r_s = 0.924$, $P < 0.001$) as well as the frequency of IL-17 producing $CD4^+$ T-cells ($r_s = 0.778$, $P = 0.002$). No correlation with the frequency of IFN-γ producing cells was observed ($r_s = 0.467$, $P = 0.108$).

Discussion

Most studies available investigating the effects of different immunosuppressive regimens focus on the early course after transplantation. In the present study, we have analyzed the effects of chronic application of different immunosuppressive drugs (CNI versus Belatacept) in long-term kidney transplant recipients. Comparing the lymphocyte subset composition in these patients, the most striking differences were found in $CD4^+CD161^+$ Th_{17}-precursor cells. Although this subset was significantly expanded in CNI-treated patients, the frequency of $CD4^+CD161^+$ cells

in patients on Belatacept was comparable to healthy controls or even somewhat reduced.

Patients receiving Belatacept or CNI did not show significant differences in the number of rejection episodes or the levels of serum creatinine, suggesting a similar clinical course in the two cohorts. Surprisingly, however, the level of soluble CD30 which is regarded as associated with the degree of immune activation [27] and a known predictive serum marker for graft rejection [28,29] and outcome after kidney transplantation [30] was significantly different in the two groups of patients. Although the level found in Belatacept-treated patients was the same as in healthy controls, sCD30 concentration was clearly elevated in the CNI-group. We assume that this difference is attributable to the different mechanisms of action of these two drugs. Belatacept blocks the second signal necessary to activate T cells by binding to the B7-ligands CD80˙CD86, and thus inhibiting the interaction with the co-stimulatory molecule CD28 [9,10]. The latter is a ''key costimulator'' and signals initiated by CD28/B7-ligand interaction are required to involve further co-stimulatory pathways in the activation process. For example, expression of the co-stimulatory molecule CD30 has been shown to depend on CD28-signaling [31]. It is conceivable that CD30 will be upregulated in long-term transplanted patients due to chronic challenge of their immune system by donor antigen. We assume that the absence of increased levels of sCD30 in Belatacept-treated patients reflects effective inhibition of CD28/B7–ligand interaction, thereby preventing CD28-induced upregulation/activation of CD30. Following this hypothesis, increased levels of sCD30 found in CNI-treated recipients may result from the fact that CD28-associated signaling pathways can cause substantial T-cell activation/proliferation even in the absence of IL-2 and thus are unaffected by CNI [32], which prevent T-cell activation via reduction of IL-2 synthesis (signal 3) [33].

We further found that patients on long-term Belatacept therapy showed reduced frequencies of the $CD4^+CD161^+$ subset in comparison with CNI-treated individuals. Concerning the Th_{17}-pathway, it has been proposed that human Th_{17}-cells with the ability to secrete IL-17 originate from $CD4^+CD161^+$ precursors [19,20]. This is consistent with our finding of a direct correlation of $CD4^+CD161^+$ cells and IL-17-producing $CD4^+$ cells in the peripheral blood of investigated individuals. In accordance with this, a reduced frequency of IL-17- producing $CD4^+$ T cells was observed in Belatacept-treated recipients compared with the CNI-group. Meanwhile, no differences were found for $CD8^+CD161^{dim}$ and $CD8^+CD161^{high}$ cells. These findings clearly indicate again that chronic use of both CNI and Belatacept has varying immunologic effects, resulting in different Th_{17}-profiles in treated individuals.

Recent data from mouse models suggest that the CD30/CD30L system might be involved in Th_{17}-differentiation (27). Based on these observations, it would be interesting to know whether there could be a direct relationship between the high levels of sCD30 found in CNI-treated patients and their expanded $CD4^+CD161^+$ T-cell population. CD30L is mainly expressed on APCs, but can be induced on T cells and has the capacity to transduce signals after binding of sCD30 [34]. Thus, it might be possible that high levels of sCD30 molecules found in CNI-treated patients could trigger signals resulting in expansion of the IL-17-producing $CD161^+$ subset. It should be noted, however, that the number of patients in this study is too small to draw definite conclusions on a possible mechanistic link between Th_{17}-cells and CD30/CD30L signaling pathways.

Concerning a possible impact of Belatacept on the Th_{17}/T_{reg}-axis, we further analyzed the frequency of T_{regs} in this context. It has previously been shown that co-stimulation via CD28 is essential for expansion and function of human T_{regs} [35], and thus chronic intake of Belatacept might have a negative impact. Yet, this was not confirmed in humans so far: the frequency or function of regulatory T cells was not seen to be altered by use of Belatacept, but rather resulted in an enhanced intragraft recruitment of these cells [13,14]. The present data are consistent with these findings. In comparison with healthy controls and long-term CNI patients, the frequency of T_{regs} rather seemed slightly elevated in individuals receiving the T-cell co-stimulation blocker Belatacept—although this did not reach statistical significance. This is further supported by the report that the balance of Th_{17}/T_{regs} might be influenced by indoleamine 2,3-dioxygenase (IDO) as it has been shown that the latter can activate regulatory T cells and block their conversion into Th_{17}-like T cells [36]. Recently, in kidney transplant recipients under Belatacept therapy, elevated levels of IDO-expressing $CD16^+$ peripheral T cells, as well as intragraft T_{regs}, were observed compared with CNI patients [37].

If the mechanistic link between Th_{17}-differentiation and the CD30/CD30L system can be confirmed in humans, one could further postulate from the results shown that the long-term use of Belatacept might prove beneficial for the contribution of Th_{17}-lineage to the development of CAD after kidney Tx as these cells seem to be involved in mechanism that can cause allograft rejection [22,24]. However, our results and the conclusions drawn stand in contrast to the recent report of Bouguermouh *et al.* who have found that use of CTLA4-Ig facilitates Th_{17}-differentiation [38]. But the latter finding is of limited value for interpretation of the presented results as it solely relies on *in vitro* observations compared

to our *in vivo* investigations in this rare group of long-term transplanted patients. Furthermore, they have observed that re-stimulation with CD28-antibody significantly enhanced the production of IL-17 and thus CD28 co-stimulation did not inhibit fully differentiated Th_{17}-cells.

Describing alterations in the composition of peripheral lymphocyte subsets in transplanted patients, effects resulting from confrontation of their immune system with antigens of the graft need to be differentiated from effects resulting from immunosuppressive treatment. For the presented collective, we found that kidney transplantation in CNI- and Belatacept-treated patients was associated with a significant decrease in the frequency of $CD19^+$ B-cells as well as increase in $CD8^+$ T-cells. The latter mainly could be addressed to the $CD3^+CD8^+CD28^-$ subset. Both findings are consistent with previous reports [39,40]. As these effects were not observed in healthy controls, we assume that the alloantigens of the graft are the main driving force behind these subset shifts.

Finally, according to the lately founded tolerance consortia, NK cells and B cells seem to be of particular importance for development of tolerance [41]. Meanwhile, no differences between the two groups of long-term graft recipients monitored in this study were observed for B cells (both showed decreased frequencies compared with healthy controls); the frequency of $CD3^-CD56^+$ NK-cells was significantly lower in patients receiving CNI. The impairment of this cell type by CNI has recently been extensively described [42], and according to our data, seems to preferably affect the $CD3^-CD56^{dim}$ subset.

In summary, we have shown that long-term treatment with CNI- and Belatacept-based regimens results in different compositions of T-cell subpopulations potentially involved in mechanisms leading to transplantation tolerance or chronic allograft dysfunction. Patients on CNI presented with a significantly increased peripheral $CD4^+CD161^+$ T-cell population as well as an increased frequency of IL-17 producing $CD4^+$ cells. The fact that these patients also showed significantly higher serum levels of sCD30 might indicate a possible role of the CD30/CD30L system on Th_{17}-differentiation. Patients receiving Belatacept to block co-stimulation via the CD28/B7-ligand pathway did not show this shift toward Th_{17}. Thus, the latter finding in combination with prevention of chronic CNI toxicity might positively influence the long-term outcome following kidney transplantation. After recent approval of Belatacept by the FDA [43], further research investigating the clinical impact as well as the exact mechanisms of action responsible for this underlying phenomenon might be realized in the near future.

Authorship

FWRV: designed study, performed research, collected/analyzed data, wrote the paper. KT: performed research, collected data, revised manuscript. SK: performed research, collected data. JK: contributed patient material, revised manuscript. RS: analyzed data, revised manuscript. TB: contributed patient material, provided clinical data, revised manuscript.

Funding

This work was supported by grants of the German Research Foundation (DFG, SFB 738). The contents of this article are the sole responsibility of the authors.

Acknowledgements

The authors thank the nurses of the clinical study team for their kind support.

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