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A Randomized Controlled Clinical Trial Comparing Belatacept With Tacrolimus After De Novo Kidney Transplantation

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Abbreviations page:

(S)AE, (serious) adverse event; AR, acute rejection; BPAR, biopsy-proven acute rejection; C_0, predose concentration; CDC, cross match-dependent cytotoxicity; CMV, cytomegalovirus; CNI, calcineurin inhibitor; DSA, donor-specific anti-HLA antibodies; EBV, Epstein-Barr virus; EMRA, end-stage terminally differentiated memory; GrB, granzyme B; HIV, human immunodeficiency virus; HR, hazard ratio; MFI, median fluorescence intensity; MMF, mycophenolate mofetil; MPA, mycophenolate acid; PRA, panel reactive antibodies; RCT, randomized controlled trial; SDC, Supplemental Digital Content(http://links.lww.com/TP/B427); TDM, therapeutic drug monitoring

Abstract

Background

Belatacept, an inhibitor of the CD28-CD80/86 co-stimulatory pathway, allows for calcineurin-inhibitor free immunosuppressive therapy in kidney transplantation but is associated with a higher acute rejection risk than ciclosporin. Thus, no biomarker for belatacept-resistant rejection has been validated. In this randomized controlled trial, acute rejection-rate was compared between belatacept- and tacrolimus-treated patients and immunological biomarkers for acute rejection were investigated.

Methods

Forty kidney-transplant recipients were 1:1 randomized to belatacept or tacrolimus combined with basiliximab, mycophenolate mofetil and prednisolone. The 1-year incidence of biopsy-proven acute rejection was monitored. Potential biomarkers, namely $CD8^+CD28^-$, $CD4^+CD57^+PD1^-$ and $CD8^+CD28^{++}$ EMRA T cells were measured pre and posttransplantation and correlated to rejection. Pharmacodynamic monitoring of belatacept was performed by measuring free CD86 on monocytes.

Results

The rejection incidence was higher in belatacept-treated than tacrolimus-treated patients: 55% *vs.* 10%; p = 0.006. All 3 graft losses, due to rejection, occurred in the belatacept group. Although 4 of 5 belatacept-treated patients with >35 cells $CD8^+CD28^{++}$ EMRA T cells/µL rejected, median pretransplant values of the biomarkers did not differ between belatacept-treated rejectors and nonrejectors. In univariable Cox regressions, the studied cell subsets were not associated with rejection-risk. CD86 molecules on circulating monocytes in belatacept-treated patients were saturated at all time points.

Conclusions

Belatacept-based immunosuppressive therapy resulted in higher and more severe acute rejection compared to tacrolimus-based therapy. This trial did not identify cellular biomarkers predictive of rejection. In addition, the CD28-CD80/86 co-stimulatory pathway appeared to be sufficiently blocked by belatacept and did not predict rejection.

Introduction

Belatacept, an inhibitor of the CD28-CD80/86 co-stimulatory pathway, has the potential to improve long-term outcomes of kidney transplantation.[1-5]Seven year follow-up of the BENEFIT study demonstrated a higher patient and graft survival, as well as better graft function in patients who were treated with belatacept as compared to ciclosporin.[1]Nonetheless, the higher incidence and severity grade of acute rejection (AR) that have been observed among belatacept-treated patients remain a concern.[6-9] Up until now, belatacept has not been compared head-to-head with tacrolimus in randomized-controlled trials (RCTs) in kidney transplantation without the use of lymphocyte-depleting therapy.[10-12] Observations made in uncontrolled studies suggest that the performance of belatacept in terms of preventing acute rejection as compared with tacrolimus may be inferior.[13, 14]

Identification of patients' pretransplantation who will develop AR during belatacept treatment would greatly help to personalize immunosuppressive therapy and maximize the potential of the drug. Experimental studies in rhesus macaques and *ex vivo* studies using human lymphocytes have demonstrated that antigen-experienced, cytotoxic $CD28^{-}CD8^{+}$ T cells are not dependent on co-stimulatory signaling via CD80/86 and are therefore less susceptible to the immunosuppressive effects of belatacept.[15-17] Recently, Espinosa and colleagues suggested that patients with a high frequency of cytotoxic $CD57^{+}PD1^{-}CD4^{+}$ T cells were at increased risk of AR during belatacept treatment.[18] A preliminary study in nonhuman primates suggested another biomarker for AR under belatacept, namely $CD28^{++}$ end-stage differentiated (EMRA) $CD8^{+}$ T cells that rapidly downregulate CD28 after kidney transplantation.[19] Biomarkers such as these may help in risk stratification and a more rational use of belatacept, but require prospective validation.

Alternatively, therapeutic drug monitoring (TDM) of belatacept therapy may improve outcomes. Because serum belatacept concentrations tend to vary little between individual patients, pharmacokinetic TDM is currently not recommended.[5, 20] However, pharmacodynamic TDM of belatacept is feasible. *Ex vivo* flowcytometric measurement of CD86 occupancy on monocytes by belatacept reflects effector T cell function,[21] demonstrating the potential of TDM to improve outcomes of belatacept therapy. However, no data from prospective clinical trials is available to provide guidance in this respect.

Here, the results of a RCT are reported in which forty patients were randomized to receive either belatacept- or tacrolimus-based immunosuppressive therapy after de novo kidney transplantation. The primary aims of this RCT were to compare the AR rate between belatacept and tacrolimus-treated patients and to identify biomarkers that were predictive of AR.

Materials and Methods

Refer to Supplemental Digital Content (SDC, http://links.lww.com/TP/B427), Materials and Methods for additional and detailed information.

Study design

This was an investigator-initiated, prospective, randomized-controlled, parallel group, open-label, single-center, clinical trial. Adult patients (≥18 years) who were scheduled to receive a single-organ, blood group AB0-compatible kidney from a living donor at the Erasmus MC, Rotterdam, the Netherlands, were eligible for participation. Historical and current cross-match-dependent cytotoxicity tests were negative. Table 1 lists the inclusion and exclusion criteria in detail. The study was approved by the institutional review board of the Erasmus MC (Medical Ethical Review Board number 2012-421) and was registered in the Dutch national trial registry (http://www.trialregister.nl/trialreg/index.asp; number NTR4242, registered October 2013).

Written informed consent was obtained from all patients before inclusion and randomization. The study was carried out in compliance with the Good Clinical Practice guidelines (http://apps.who.int/prequal/info_general/documents/gcp/gcp1.pdf) and the Declaration of Istanbul.[22]

Randomization procedure and intervention

Enrolled patients were randomly assigned on a 1:1 basis by 1 of the coordinating investigators (G.N.G. or D.A.H.) to either receive tacrolimus (Prograf®; Astellas Pharma, Leiden, the Netherlands) or belatacept (Nulojix®; Bristol Myers-Squibb, New York City, NY). Randomization was performed by use of 40 sealed, opaque, sequentially numbered envelopes containing treatment allocation. The random allocation sequence was generated by an independent biostatistician by use of a random number generator. Before the start of the study, it was determined that 20 patients would be allocated to each treatment arm. Data were collected and monitored by the coordinating investigators in a hospital-based electronic study database.

Tacrolimus was dosed based on bodyweight (a dose of 0.2 mg/kg/day in 2 equally divided doses, rounded off to the nearest 0.5 mg) per the package insert (https://www.astellas.us/docs/prograf.pdf). Thereafter, the tacrolimus dose was adjusted based on whole-blood predose concentrations. The tacrolimus target predose concentrations were as follows: 10-15 ng/mL (weeks 1 and 2), 8-12 ng/mL (weeks 3 and 4) and 5-10 ng/mL from week 5 onwards. Belatacept was dosed per the Less-Intensive (LI) regimen as described previously.[6, 7] Belatacept was administered intravenously in a dose of 10 mg/kg on the day of transplantation (day 0) and on days 4, 15, 30, 60 and 90 after transplantation. Thereafter, the dose was reduced to 5 mg/kg and given as monthly infusions up until month 12 after transplantation (end of study). Additional treatment is discussed in the SDC, http://links.lww.com/TP/B427.

Safety

Refer to SDC, http://links.lww.com/TP/B427 Material and Methods for data collection on (serious) adverse events.

Primary end points

The overall aim of this trial was to determine the effect of belatacept and tacrolimus-based immunosuppressive regimens on alloreactivity after kidney transplantation. The primary endpoint of the study presented here was the incidence of biopsy-proven acute rejection (BPAR) within the first year after transplantation. BPAR-rates were compared between belatacept- and tacrolimus-treated patients. We postulated that the incidence of BPAR would be higher among belatacept-treated patients[7] and that BPAR-biomarkers could be identified. All kidney transplant biopsies were obtained for cause and no protocol biopsies were obtained. Refer to SDC, http://links.lww.com/TP/B427 Materials and Methods for BPAR scoring system.

Pretransplant circulating frequencies of $CD8^+CD28^-$, $CD4^+CD57^+PD1^-$ and end-stage terminally differentiated memory (EMRA) $CD8^+CD28^{++}$ T cells, as well as their intracellular expression of a Granzyme B (GrB: an important cytotoxic protease during acute rejection) were measured as immunological primary end points.[19, 23, 24] These cell subsets were also measured posttransplantation, during acute rejection before additional anti-rejection therapy was given, or 3 months after transplantation in nonrejecting belatacept-treated patients. Free CD86 expression on circulating $CD14^+$ monocytes were determined pretransplantation as a predictor for rejection; and before every dose of belatacept administered after transplantation as a pharmacodynamic drug monitoring tool. A for belatacept competitive monoclonal antibody was used (clone HA5.2B7, Beckman Coulter, Brea, CA). In patients who rejected, the free CD86 expression was also assessed before additional anti-rejection therapy was given. Refer to SDC, http://links.lww.com/TP/B427Material and Methods for detailed information about our laboratory studies, including detection methods for DSA.

No formal statistical power calculation for the present study was performed, because 1) when the study was designed, it was unclear what the difference would be between belatacept and tacrolimus-treated patients in terms of BPAR, as only data from the BENEFIT and BENEFIT-EXT, in which the comparator was ciclosporin, were available at the time;[6, 7] 2) there were no published data available regarding the studied biomarkers and their association with BPAR that could serve for such a power calculation; and 3) because of financial constraints, we chose to conduct the present randomized controlled clinical trial with a limited number of patients in both arms.[25] In our view, the present trial should therefore be regarded as a pilot study. It may serve as the basis for a larger study by providing the data needed to perform a statistical power calculation.

Statistical analyses

Additional information is depicted in SDC, http://links.lww.com/TP/B427 Materials and Methods. Percentages and counts are given for categorical variables, and medians plus ranges for continuous variables, unless otherwise specified. Continuous variables were compared between the belatacept and the tacrolimus group or between belatacept-treated rejectors and nonrejectors using the Mann-Whitney U test, and categorical variables using the Fisher's exact test. Patient and death-censored graft survival, as well as death-censored BPAR-free survival were compared between the belatacept and tacrolimus group using the log-rank test. All included patients were analyzed per the intention-to-treat principle.

To determine if high numbers of cytotoxic $CD4^{+}CD57^{+}PD\text{-}1^{+}$, $CD8^{+}CD28^{-}$, or $CD8^{+}CD28^{++}$ EMRA T cells, as well as CD86 molecules/monocyte were risk factors for BPAR, univariable Cox regression analyses were performed with death-censored BPAR-free survival as the dependent variable. Independent variables included the cell types after log transformation (to

ensure approximately normal distribution of these variables), treatment arm, age, gender, ethnicity, HLA mismatches, HLA-DR mismatches, highest PRA, and cytomegalovirus (CMV) serostatus. Independent variables with a $p<0.10$ in the univariable analyses were intended to be included in a multivariable Cox regression analysis to predict BPAR.

Repeated measurements of CD86 occupancy on monocytes over time were compared between the study groups using a linear mixed model. To ensure a normal distribution of the model residuals, the dependent variable in the model was log transformed. Predictors were the values of CD86 molecules/monocyte pretransplantation, time point after transplantation (coded as categorical variable), treatment arm (belatacept or tacrolimus) and an interaction effect of time point and treatment arm to account for different trends over time between groups. The dependent variable was the value of CD86 molecules/monocyte after transplantation at a given time point. A random intercept was included in the linear mixed model to account for the within-subject correlations.

All tests were 2-tailed and statistical significance was defined as a p value <0.05. Bonferroni's correction for multiple testing was applied when necessary.[26] Statistical analyses were performed using IBM SPSS version 21 (SPSS Inc., Chicago, IL)

Results

Patients

Between October 1st, 2013 (first patient, first visit) and February 26th, 2015 (last patient, first visit) 280 patients were screened, of whom 88 were eligible for participation (Figure 1). Forty-eight patients did not wish to participate. Major reasons were fear of acute rejection and inconvenience of the monthly belatacept infusions.Forty patients were randomized and included

in the intention-to-treat analysis. The baseline characteristics of these patients are described in Table 2. Seventeen (85%) patients in the belatacept and 19 (95%) in the tacrolimus group completed the 1-year follow-up period (last patient, last visit occurred on February 19th, 2016).

Patient and graft survival

Patient survival was 95% in the tacrolimus group and 100% in the belatacept group (p = 0.32). One patient, randomized to the tacrolimus group, died 294 days after transplantation because of traumatic head injury. Three graft losses, all in the belatacept group, occurred on days 12, 59 and 161 after transplantation, resulting in a 1-year death-censored graft-survival of 85% in the belatacept group *vs.* 100% in the tacrolimus group (p = 0.08). All 3 graft losses were the result of glucocorticoid-resistant acute rejection (Banff type IIB in 2 cases and type III in the third patient[23]).

Biopsy-proven acute rejection

In total, 29 for cause biopsies were performed in the belatacept group and 10 in the tacrolimus group in 14 and 6 patients, respectively, p = 0.015. The incidence of BPAR was higher among the belatacept-treated patients than in the tacrolimus-treated patients: n = 11 (55%) *vs*. n = 2 (10%), respectively; p = 0.006 (Table 3). The death-censored BPAR-free survival was significantly lower in the belatacept-treated patients than in the tacrolimus-treated patients, (p = 0.002; Figure 2). Median time to rejection of patients who experienced AR was 56 (3–120) days in the belatacept group and 81 (10–152) days in the tacrolimus group. BPAR was of a more severe histological grade in the belatacept than in the tacrolimus group (p = 0.003; Table 3).

A detailed overview of the clinical course of the individual patients is depicted in Figure 3. In the belatacept group, n = 10 patients (50%) were treated for BPAR with pulse

methylprednisolone therapy. Six patients (30%) received additional treatment with alemtuzumab, which is the preferred T cell depleting antibody in our center.[27] In retrospect, and after revision by the second pathologist, 1 more patient in the belatacept group (case no. 13) was diagnosed as suffering from rejection but he was not treated with additional anti-rejection therapy. This patient had a so-called isolated v-lesion and despite not treating him, his graft function has remained excellent to the present day. After exclusion of this case, the BPAR rate was still significantly higher in the belatacept group than in the tacrolimus group. Nine patients (45%), all suffering from BPAR, were converted from belatacept to tacrolimus.

In the tacrolimus group, n = 2 patients were treated for BPAR: in 1 case with methylprednisolone pulse therapy only, in the other, additional treatment with alemtuzumab was given. Five patients (2 in the belatacept and 3 in the tacrolimus arm) received methylprednisolone for suspected rejection (For details see Figure 3 legend).

Safety

In total, 205 AEs occurred in the belatacept group (mean 10.3 per patient) and 238 in the tacrolimus group (mean 11.9 per patient); p = 0.41 (Table S1, SDC, http://links.lww.com/TP/B427). Of these, 22 and 35, respectively, were judged to be serious (means per patient 1.1 and 1.8, respectively; p = 0.15), excluding BPAR, graft loss, and death.

eGFR, excluding graft losses, was not different between belatacept-treated and tacrolimus-treated patients 12 months after transplantation (Table S2, SDC, http://links.lww.com/TP/B427): 54 (28–89) and 50 (33–84) mL/min per 1.73m^2, respectively; p = 0.57. Median protein/creatinine ratio was 13.2 (5.7–343.8) mg/mmol in the belatacept group

and 9.0 (5.3–43.5) mg/mmol in the tacrolimus group; p = 0.44. Additional routine measurements are depicted in Table S2, SDC, http://links.lww.com/TP/B427.

For the on-therapy analysis on month 12; graft function before, during and after BPAR in the belatacept group; the incidence of DSA and non-DSA; and pharmacokinetic drug monitoring, refer to SDC, Results and Tables S3-5, SDC, http://links.lww.com/TP/B427.

Immunological primary end-points (biomarkers)

Three potential biomarkers for (belatacept-resistant) rejection were measured pretransplantation, namely $CD8^+CD28^-$ T cells, $CD4^+CD57^+$ $PD1^-$ T cells, and $CD8^+CD28^{++}$ EMRA T cells. There were no significant differences in the numbers or percentages of these cells at baseline between the tacrolimus and belatacept groups (Table 4). The limited number of patients experiencing BPAR in the tacrolimus group (n = 2) precluded a meaningful statistical comparison between rejectors and nonrejectors in this group. Gating strategies, pretransplant numbers and percentages of the above-mentioned cell subsets are depicted for future rejectors and nonrejectors in the belatacept group (see Table S6, SDC, http://links.lww.com/TP/B427; Figure 4), and no statistically significant differences were observed. Intracellular Granzyme B (GrB) expression was measured in the cell subsets (Figure 4A). Next, we analyzed whether high numbers or proportions of these cell types increased BPAR risk within the first 12 after transplantation by conducting univariable Cox regression analyses (Table 5):

1) $CD8^+CD28^-$ T cells

$CD8^+CD28^-$ T cells are mostly effector-memory cytotoxic T cells that produce large amounts of proinflammatory cytokines,[15-17] and are not susceptible to co-stimulation blockade by belatacept. Almost 70% (31–89%) of $CD8^+CD28^-$ T cells produced GrB. Higher numbers and

proportions of pretransplant $CD8^+CD28^-$ T cells (irrespective of their intra-cellular GrB expression) did not significantly increase BPAR risk in the first 12 months after transplantation (Hazard Ratio [HR] 1.06; 95%-CI 0.61 to1.83 and HR 1.05; 95%-CI 0.50 to 2.20, respectively; Table 5).

2) $CD4^+CD57^+PD1^-$ T cells

Next, pretransplant $CD4^+CD57^+PD1^-$ T cells were compared between rejecting and nonrejecting belatacept-treated patients. These cells were recently described as being cytolytic, $CD28^-$, and to be associated with belatacept-resistant rejection.[18] The proportion of pretransplant $CD4^+CD57^+PD1^-$ T cells was low (<2% of the $CD4^+$ T cell population in most patients). Approximately 24% (1–74%) of these cells were GrB positive. Neither the absolute number nor the proportion of these cell predicted BPAR (HR 0.89; 95%-CI 0.58 to 1.27, and HR 0.90; 95%-CI 0.59 to 1.38, respectively; Table 5).

3) $CD8^+CD28^{++}$ EMRA T cells

Finally, $CD8^+CD28^{++}$ EMRA T cells were analyzed as high numbers of these cells predicted belatacept-resistant rejection in primates.[19] It was postulated that these cells rapidly down-regulate their surface CD28 expression after transplantation, making them resistant to co-stimulatory blockade.[19] Circa 3% (0–3%) of these cells expressed intracellular GrB. The absolute numbers or proportions of pretransplant $CD28^{++}$ cells within the $CD8^+$ EMRA T cell population did not increase BPAR risk (HR 0.86; 95%-CI 0.58 to 1.27, and HR 1.23; 95%-CI 0.64 to 2.33, respectively; Table 5) Interestingly, from the 5 patients with >35 $CD8^+CD28^{++}$ EMRA T cells/µL, 4 were rejectors and only 1 was a nonrejector (Figure 4B). In the tacrolimus group the n=2 rejectors had <10 $CD8^+CD28^{++}$ EMRA T cells/µL pretransplantation.

The above-mentioned cell surface biomarkers were also measured in belatacept-treated patients during acute rejection and before additional anti-rejection therapy was given, and were compared with the month 3 samples from patients who remained rejection-free (Figure S1, SDC, http://links.lww.com/TP/B427). No statistically significant differences were observed between rejecting and nonrejecting belatacept-treated patients.

The only significant risk factor for rejection in this study population was the use of a belatacept-based immunosuppressive regimen (HR 7.2; 95%-CI 1.6 to 32.6; p = 0.01) compared to tacrolimus-based therapy (Table 5). Since no other variable significantly influenced acute rejection risk and the sample size was small, no multivariable Cox regression analysis was conducted.

Pharmacodynamic monitoring of belatacept

The pharmacodynamic effect of belatacept was monitored by measuring free CD86 molecules on circulating monocytes. CD86 was saturated by belatacept at all time points, in both rejectors as nonrejectors. Moreover, pretransplantation CD86 molecules/monocyte were not predictive for BPAR (HR 0.33, 95%-CI 0.1-2.2). For details about CD86-expression on monocytes in belatacept- and tacrolimus-treated patients, refer to SDC, Results and Figure S2, SDC, http://links.lww.com/TP/B427.

Discussion

In this RCT, a belatacept-based and a tacrolimus-based immunosuppressive regimen without lymphocyte-depleting induction therapy were compared head-to-head for the first time in de novo kidney transplantation. The results of this trial demonstrate that belatacept is not as potent as tacrolimus in preventing rejection.

In comparison to the 1-year results of the BENEFIT-trial where ciclosporin was used as comparator,[7] we found a more pronounced difference in both BPAR incidence and severity. Ninety-one percent of BPAR in the belatacept group was classified as type II (or higher),[28] while in the BENEFIT-trial this was 69%. The use of lymphocyte-depleting therapy to treat rejection was comparable: *circa* 50% of BPAR in the BENEFIT-trial *vs.* 55% in this study. The incidence of graft loss caused by BPAR was higher in this study than in the BENEFIT-trial: 3 of 11 *vs.* 2 of 39 rejecting patients, respectively.

This larger difference in rate and severity of BPAR is not explained by dissimilarities between study groups. In the present study 1) there were no transplantations with deceased donors; 2) there were no patients with a PRA >30%; and 3) the proportion of Caucasians was larger. All 3 characteristics are associated with a lower BPAR risk.[29-35] In contrast, the proportion of preemptive transplantations was high in our study (55% of included patients), which may have led to the inclusion of patients with a more potent immune system.[36-38] Another explanation for the higher BPAR-rate could be that in this study TDM for MPA was performed, whereas this was not the case in the BENEFIT-trial. It is therefore, theoretically possible that belatacept-treated patients in BENEFIT were exposed to higher MPA concentrations.[1] However, we feel that this is an unlikely explanation as ciclosporin lowers exposure to MPA, whereas tacrolimus does not have such an effect.[39]

Our findings are in line with the higher BPAR rates observed in large retrospective studies and a small cohort study comparing belatacept to tacrolimus.[13, 14, 18]Wen et al, conducted a retrospective cohort study using registry data of a time period of 3 years, and compared 1-year clinical outcomes between belatacept- and tacrolimus-treated adult kidney transplant recipients.[39] Although the incidence of BPAR was not as high as in the present trial, Wen et al, also observed

significantly higher BPAR rates among belatacept-treated patients as compared with tacrolimus-treated patients who would have been eligible for participation in the BENEFIT-study: 15% of patients treated with belatacept and lymphocyte depleting antibody therapy, *versus* 23% of patients treated with belatacept without lymphocyte depleting antibody therapy, *versus* 6% of tacrolimus-treated patients. Nonetheless, it is important to stress that the higher incidence of BPAR in the present study should be interpreted with caution, because the study here included limited numbers of patients, had limited statistical power and may therefore be a chance finding.

In this study, no suitable pretransplant biomarker was found to predict belatacept-resistant BPAR.[15, 16, 18, 19] The first potential biomarker, pretransplant $CD8^+CD28^-$ T cell number, seemed a logical choice as these highly cytotoxic cells lack surface CD28 and are therefore not susceptible to belatacept.[15-17] Possible explanations for the observation that these cells were not associated with BPAR may be that 1) even though these cells are highly cytotoxic, they lack proliferative capacity,[40] and 2) the CD28-CD80/86 pathway is not the sole mediator of belatacept-resistant rejection. Targeting other co-stimulatory pathways, like CD40-40L, simultaneously with belatacept, might be more efficient to prevent BPAR.[41, 42] Preliminary data from Cortes-Cerisuelo et al, suggest that not the lack of CD28 on these cells before transplantation, but the potential to down-regulate CD28 after donor antigen stimulation is associated with BPAR in belatacept-treated patients.[43]

The second biomarker, pretransplant $CD4^+CD57^+PD1^-$ T cell number, was associated to belatacept-resistant rejection in an observational cohort study.[18] These findings were not confirmed here. Apart from differences in study design, the dissimilarities in study populations may explain this discrepancy.[23-26] Our study population 1) was mostly Caucasian; 2) received mostly preemptive transplants; and 3) was shorter on dialysis. These factors have, however, not

been associated with CD57 expression, and the proportions of $CD4^{+}CD57^{+}PD1^{-}$ T cells were similar pretransplantation. Age and CMV status, which influence these proportions[40, 44-48], were also comparable (data not shown).

The final biomarker, $CD8^{+}CD28^{++}$ EMRA T cell number, showed potential to predict BPAR under belatacept, even though the group medians did not differ between rejectors and nonrejectors. One of the 9 nonrejectors and 4 of the 11 rejectors had high numbers of these cells pretransplantation (>35 cells/μL). In-depth analysis of the antigen-specificity of these $CD8^{+}CD28^{++}$ EMRA T cells in larger studies seems warranted.[49]

Pharmacodynamic drug monitoring in the form of measuring free, non-belatacept bound CD86 molecules on circulating monocytes was not useful to predict BPAR under belatacept therapy, since free molecules were not higher in rejectors pretransplantation, and followed the same dynamics in rejectors as in nonrejectors.

Limitations of this study are the small sample size and the resulting increased chance of type II errors. The increased rejection risk among belatacept-treated patients therefore needs to be confirmed in larger RCTs. Ideally, such trials will also include biomarker studies and analyze pretransplant donor-specific immunity. Also, research on regulatory T cells would be of interest since blockade of CD80/86 leads to anergic T cells,[50] which consequently may fail to activate regulatory T cells via CD28. Studies on antigen-specific biomarkers, such as the IFNγ Elispot assay, would also be useful to study in larger, prospective trials.[51, 52]

In conclusion, this small RCT showed that belatacept-based immunosuppressive therapy results in a significantly higher rejection-rate and severity compared with standard, tacrolimus-

based therapy. The biomarker data were not informative as there were no differences in pretransplant cellular biomarkers between rejectors and nonrejectors. Belatacept adequately blocked the CD28-CD80/86 co-stimulatory pathway in all patients, making insufficient saturation an unlikely explanation for this higher rejection risk.

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Figure legends

Figure 1: Trial flowchart.

All patients who were included in the study were randomized, underwent transplantation and received at least 1 dose of belatacept or tacrolimus.

CDC, cytotoxicity dependent cross-match; CNI, calcineurin inhibitor; EBV, Epstein Barr Virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; MGUS, monoclonal gammopathy of unknown significance; PRA, panel reactive antibodies

Figure 2: Biopsy-proven acute rejection (BPAR)-free survival.

The time to first BPAR is depicted for the belatacept (dotted line) and the tacrolimus (solid line) group. In the tacrolimus group 1 patient died 294 days after transplantation due to traumatic head injury.

Figure 3: Clinical outcomes.

Each line represents the posttransplant course of the 20 individual belatacept- and 20 individual tacrolimus-treated patients (separated by the bold dotted line). Time of BPAR (✳), anti-rejection therapy (methylprednisolone intravenously [◆] or alemtuzumab subcutaneously [+]), switch to tacrolimus (|), development of donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA) (▲), graft loss (✕) and death (#) are shown.

In the belatacept group, n = 1 (5%) patient (no. 15) received methylprednisolone for presumed rejection pending the results of a kidney biopsy. Biopsy revealed an alternative diagnosis namely ascending urinary tract infection. In 1 other case, methylprednisolone was administered for suspected rejection (no. 14). A biopsy was not performed because of a

coagulation disorder. In the tacrolimus group, n = 3 (15%) patients received methylprednisolone for presumed rejection pending biopsy results. In all 3 cases an alternative diagnosis was made: acute tubular necrosis in 2 patients (no. 12 and 14) and an ascending urinary tract infection in 1 case (no. 16). One belatacept-treated patient (no. 13) was not treated for rejection, because the diagnosis of vascular rejection (isolated v-lesion) was only made in retrospect after revision of the biopsy.

Figure 4: $CD8^+CD28^-$, $CD4^+CD57^+PD1^-$ and $CD8^+CD28^{++}$ EMRA T cells pretransplantation.

$CD4^+$ and $CD8^+$ T cells were gated from 7-AAD negative $CD3^+$ lymphocytes (based on forward and sideward scatter) and EMRA T cells were gated as $CCR7^-$ and $CD45RO^-$ T cells. Typical examples are given for nonrejectors and rejectors in the belatacept group for $CD8^+CD28^-$, $CD4^+CD57^+PD1^-$ and $CD8^+CD28^{++}$ EMRA T cells and their intracellular Granzyme B expressions (A). The absolute numbers and percentages of $CD8^+CD28^-$, $CD4^+CD57^+PD1^-$ and $CD8^+CD28^{++}$ EMRA T cells are presented for nonrejectors and rejectors (B).

Figure 1

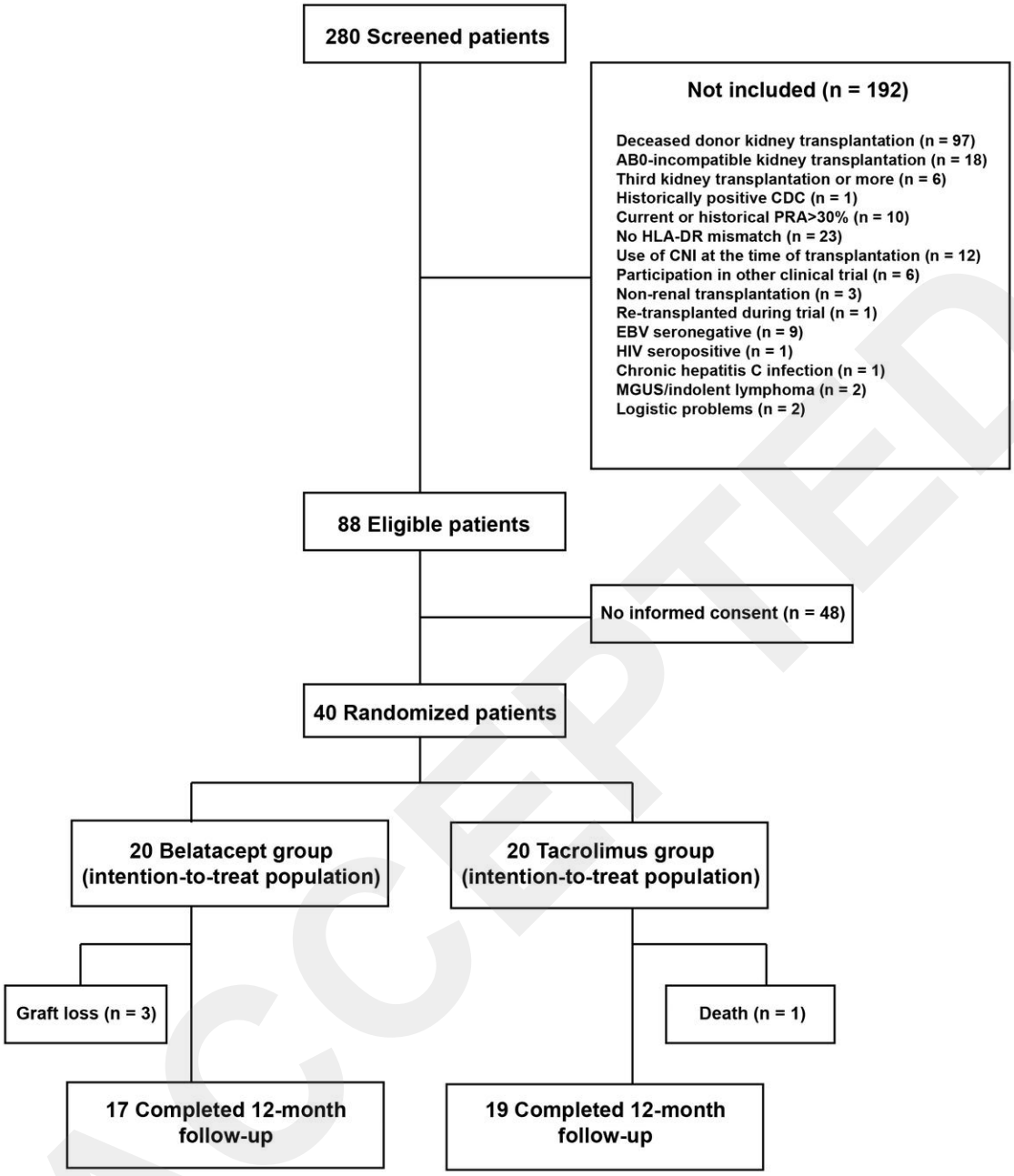
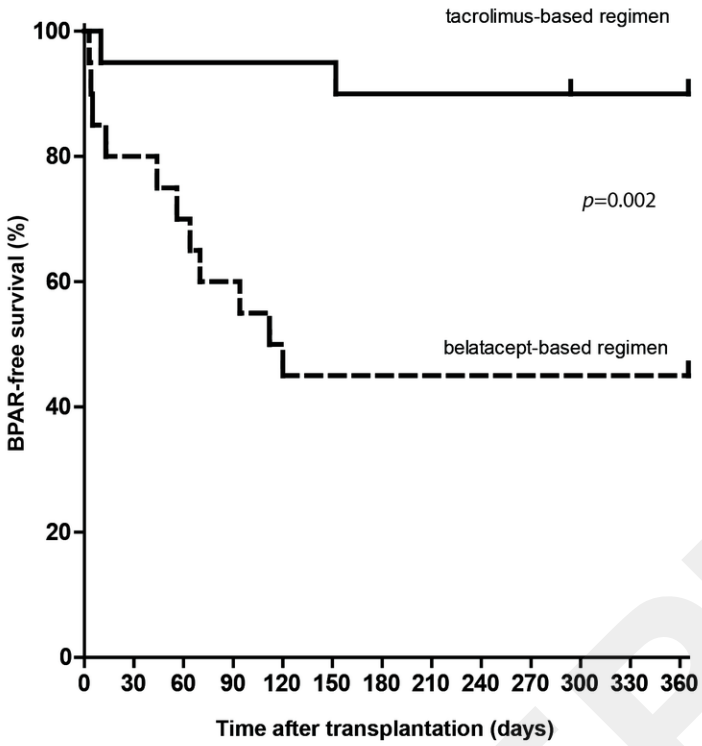


Figure 2



Days after transplantation	0	3	4	5	10	13	44	56	64	70	94	112	120	152	294	365
Belatacept-based regimen (No. at risk)	20	20	19	18	18	17	16	15	14	13	12	11	10	10	10	9
Tacrolimus-based regimen (No. at risk)	20	20	20	20	19	19	19	19	19	19	19	19	19	19	18	17

Figure 3.

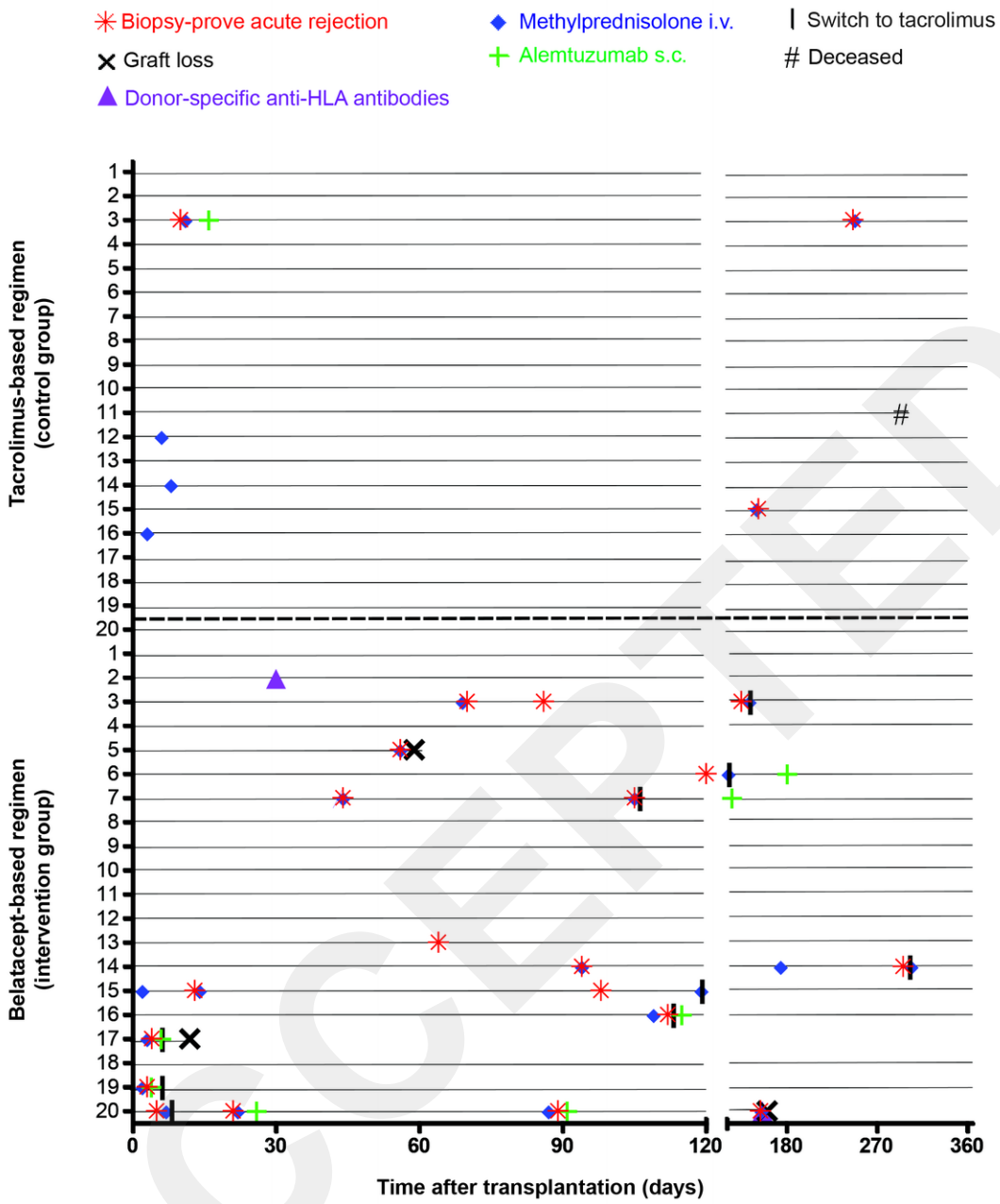
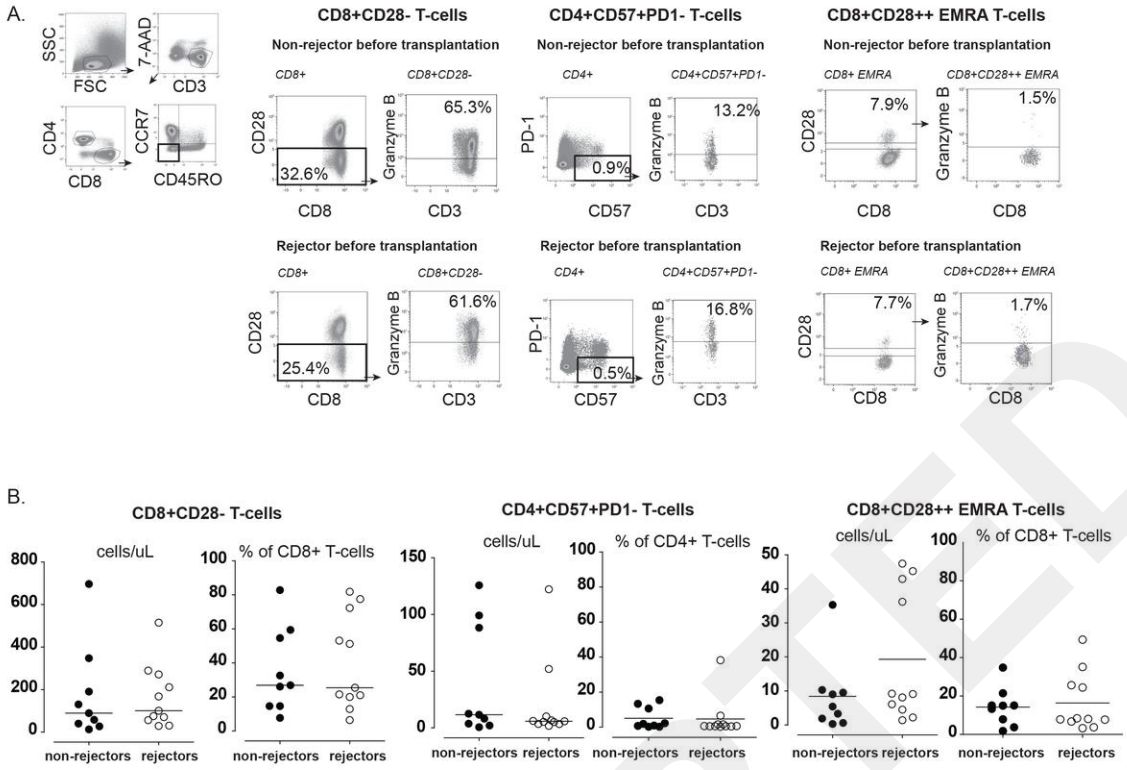


Figure 4



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SUPPLEMENTAL DIGITAL CONTENT

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Belatacept Causes a Higher Incidence of Acute Rejection Compared with Tacrolimus After de novo Kidney Transplantation: A Randomized Controlled Trial

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SUPPLEMENTAL MATERIALS AND METHODS

Detailed additionalMaterials and Methods

Additional (immunosuppressive) treatment – detailed information

The additional immunosuppressive therapy was identical in both groups and consisted of basiliximab (Simulect; Novartis Pharma B.V., Arnhem, the Netherlands) in a dose of 20 mg administered intravenously on day 0 (immediately before kidney transplant reperfusion) and day 4 after transplantation. Patients also received a starting dose of 1000 mg mycophenolate mofetil (MMF; CellCept; Roche Pharmaceuticals, Woerden, the Netherlands) twice daily aiming for plasma mycophenolic acid (MPA) predose concentrations between 1.5 and 3.0 mg/L. In addition, all patients received prednisolone in a dose of 50 mg twice daily intravenously on days 0–3, followed by 20 mg orally once daily (on days 4–14), after which the dose was tapered to 5 mg at month 3 after transplantation. Patients continued to receive 5 mg of prednisolone for the rest of the first posttransplant year.

All patients received trimethoprim/sulfamethoxazole prophylaxis for Pneumocystis Jirovecii pneumonia for at least 3 months. Patients receiving a kidney from a cytomegalovirus (CMV)-positive donor and patients who were seropositive for CMV received prophylaxis with valganciclovir for a duration of 6 months.

Additional antirejection therapy consisted of 3 doses of 1000 mg methylprednisolone intravenously for 3 consecutive days. In case of glucocorticoid-resistant rejection, lymphocyte-depleting therapy with 1 dose of 30 mg of alemtuzumab was administered subcutaneously.[1]

Primary end points – BPAR scoring methods

BPAR was scored as part of routine clinical care by a renal pathologist (M.C.C.) per the Banff '15 classification using 2 μm paraffin sections stained for HE, PAS, Jones and immunohistochemistry for C4d on 4 μm sections. After the completion of the study, all biopsies were reviewed again in a blinded fashion by 2 pathologists (M.C.C. and J.v.d.T.) per the Banff '15 classification.[2] In case of discrepancy, biopsies were reviewed and consensus was reached.

Safety

Data on clinical outcomes and (serious) adverse events [(S)AEs] were collected for safety and included patient- and graft survival, estimated GFR (eGFR), proteinuria, and development of donor-specific anti-HLA antibodies (DSA). DSA were retrospectively measured in patient sera 1 day before transplantation, and 1, 6 and 12 months after transplantation. In addition, we monitored delayed graft function, malignancies, (opportunistic) infections, posttransplant diabetes mellitus (PTDM), neurologic events, and acute tacrolimus-induced nephrotoxicity. PTDM was defined as the need for glucose-lowering medical therapy that persisted after month 3 posttransplantation in a patient not needing such treatment pretransplantation. Acute tacrolimus nephrotoxicity was defined as any ≥15% increase of serum creatinine with a return to baseline after tacrolimus dose reduction and after exclusion of other causes of renal transplant function deterioration.

Routine laboratory investigations included blood glucose, glycated hemoglobin (HbA1c), thrombocytes, leucocytes, hemoglobin (Hb), mean corpuscular volume (MCV), low-density lipoproteins (LDL), high-density lipoproteins (HDL) and triglycerides. Blood pressure and body weight were measured at every visit to the outpatient clinic.

Laboratory Studies – detailed information

Blood samples were collected on days 0 (pretransplant), 4, 30, 90, and months 6 and 12. Serum was collected on days 0, 15, 30, and months 6 and 12. Blood and sera were also collected during clinically suspected rejection, before additional antirejection therapy was given. In addition, blood and urine samples were collected on a routine basis as part of routine clinical care. Proportions of $CD8^+CD28^-$, $CD4^+CD57^+PD1^-$ and $CD8^+CD28^{++}$ EMRA T cells were determined pretransplantation (1 day before transplantation) and posttransplant (3 months after transplantation or during rejection) on thawed isolated peripheral blood mononuclear cells.

Absolute numbers of cells in blood

The Becton & Dickinson (BD Biosciences, San José, CA) multi-test 6-color, CD14 FITC (Serotec, Kidlington, United Kingdom) and TruCount Tubes were used to measure absolute numbers of $CD3^+$ T cells, $CD4^+$ T-helper cells, $CD8^+$ cytotoxic T cells, and $CD14^+$ monocytes. Absolute numbers were measured in 50 µL blood in the presence of 0.5 mL BD Pharm Lyse. All proportions of subsets measured in PBMCs (see below) were calculated back to these absolute numbers.

Flow cytometry of cytotoxic T cells in peripheral blood mononuclear cells (PBMCs)

Using the Ficoll density method, PBMCs were isolated and stored at -190°C before further characterization. T cells were identified by CD3 (AF700, BD), CD4 (V450, BD) and CD8a (APC-eF780, eBioscience). The immuno-regulatory receptor PD-1 (PE, BioLegend), the cytotoxic marker CD57 (FITC, BD), and the co-stimulatory molecule CD28 (APC, BD) were determined on $CD4^+$ and $CD8^+$ T cells. EMRA $CD8^+$ T cells were defined by $CD8^+$ $CCR7^-CD45RO^-$, using CCR7 (PE, BD) and CD45RO (PE-Cy7, BD). Intracellular expression of GrB (PE-CF594, BD) was also assessed.

Saturation of CD86 on monocytes and B cells in blood

The surface expression of free CD86 on $CD14^+$ monocytes was assessed using the Lyse-Wash method per the manufacturer's instruction. Cells were surface-stained in 100 µL blood and erythrocytes were subsequently lysed in 2 mL BD FACS Lysing solution, and washed away before measurement. Monoclonal antibodies used were the leukocyte marker CD45 PerCP (BD); CD19 PE-Cy7 (BioLegend); CD14 FITC (Serotec); and the for belatacept competitive binder of the co-stimulatory molecules of the CD28-pathway, CD86 PE (clone HA5.2B7 Beckman Coulter, Brea, CA).[3] Numbers of CD86 molecules per monocyte were calculated by using QuantiBrite beads per manufacturer's manual (BD).

Detection of serum DSA

Using the Luminex single antigen bead assay (Thermo Fisher Scientific, Waltham, MA) as previously described,[4] the development of DSA was determined by measuring the presence of DSA against HLA class I and II before and at different set time points after transplantation in serum. The MFI cut-off for positivity was 1000.

Panel reactive antibodies

Sera were tested for HLA-antibody specificities by standard National Institutes of Health (NIH) complement-dependent microlymphocytotoxicity test (LCT) using a panel of 54 donors yielding a measurement of the PRA (Panel Reactive Antibody). Ifsamples tested positive using a Human Linker for Activation of T cell ELISA (LAT) or a Complement-Dependent Cytotoxicity Crossmatch(CDC), HLA antibodies were specified with Luminex single antigen test (LABScreen SA, One Lambda Inc., Canoga Park, CA, USA).

Statistical analyses – additional information

Patient, graft and biopsy-proven acute rejection (BPAR)-free survival were defined as 1) time from transplantation to mortality, 2) time from transplantation to transplant nephrectomy, reinitiation of dialysis or (preemptive) retransplantation, and 3) time from transplantation to the diagnosis of BPAR, respectively, or as the end of the 12-month follow-up period, whichever came earlier.

In addition to intention-to-treat analyses, on-therapy analyses were conducted and included evaluable patients who were still on their assigned regimen 12 months after transplantation.

Categorical variables (+ reference groups) in the univariable Cox regression analyses included treatment arm (belatacept *vs.* tacrolimus), gender (female *vs.* male), ethnicity (noncaucasian *vs.* Caucasian), HLA mismatches (4 or more *vs.* less than 4), HLA-DR mismatches (2 *vs.* 1), highest PRA, and CMV serostatus (positive *vs.* negative).

SUPPLEMENTAL RESULTS

On-therapy analysis

The on-therapy analysis at month 12 revealed that eGFR and protein/creatinine ratios were similar between nonrejecting tacrolimus and belatacept-treated patients: median eGFR 57 (45-89) and 58 (37-84) mL/min per 1.73m^2, respectively (SDC, Table 3). Graft-loss censored median eGFR in belatacept-treated patients that suffered from rejection (n = 7) was 36 (28-76) mL/min per 1.73m^2 at month 12, which was lower than in the nonrejecting belatacept group, p = 0.001.

Graft function in time in belatacept-treated rejectors

The graft function before, during and after BPAR (after additional antirejection therapy) is displayed in SDC, Table 4, for the belatacept-group. Before and after BPAR the highest eGFR is depicted for each patient. It should be noted 6 patients had a decrease in eGFR after BPAR was diagnosed (including 3 graft losses), 2 patients had a similar eGFR after treatment for BPAR, and 3 patients had an improved eGFR.

Donor-specific and nondonor-specific anti-HLA antibodies (DSA and non-DSA)

None of the patients had DSA pretransplantation. During the first posttransplant year, 2 patients developed DSA, both in the belatacept group (Figure 3 and SDC, Table 5). One month after transplantation, patient no. 2 in the belatacept group developed DSA against HLA-DQ2 (Median Fluorescence Intensity [MFI] 3787; most likely C1q-negative[5], but these disappeared hereafter without additional therapy and no AR occurred. Patient no. 20 in the belatacept group developed DSA during her 4th rejection episode (right before losing her graft), which were also detectable in the cross match-dependent cytotoxicity test, against HLA-A1 (MFI 18000), B8

(MFI 22700), DR3 (MFI 11000), DR52 (MFI 5500) and DQ2 (MFI 16500) (SDC, Table 5). At this time, she had already been switched to a tacrolimus-based regimen and had been treated with methylprednisolone and alemtuzumab (Figure 3).

Two and 3 patients, in the belatacept and tacrolimus group, respectively, had nondonor specific anti-HLA antibodies (non-DSA) pretransplantation. In both the belatacept and the tacrolimus group 2 patients developed non-DSA after transplantation (SDC, Table 5).

Pharmacokinetic drug monitoring

SDC, Table 2 depicts belatacept doses, tacrolimus doses and predose concentrations (C_0), MMF doses and mycophenolic acid (MPA) C_0, and prednisolone doses. MPA C_0 were not different between the belatacept and tacrolimus groups after 12 months: 2.30 (0.99–3.54) and 1.83 (0.57–3.67) mg/mL, respectively; $p = 0.25$. Also, prednisolone doses were similar between the belatacept and tacrolimus group in month 12; $p = 0.59$.

Pharmacodynamic drug monitoring

The number of belatacept-free CD86 molecules on monocytes was calculated by measuring the MFI of bound anti-CD86-PE antibodies. These antibodies bind to CD86 molecules to the same epitope but with lower affinity than belatacept, which allows for measurement of free CD86 molecules.[3] A typical example is depicted for the MFIs of CD86-PE on monocytes for a patient treated with belatacept and a patient treated with tacrolimus (SDC, Figure 2A). As evidenced by a linear mixed model, belatacept significantly decreased free CD86 molecules on monocytes at different time points after transplantation compared to tacrolimus (SDC, Figure 2B). Free CD86 molecules/monocyte were 5.9-fold (95%-CI 5.4 to 7.7-fold) higher on day 4 and 5.3-fold (95%-CI 4.0 to 7.0-fold) higher 1 month after transplantation in

tacrolimus-treated patients compared to belatacept-treated patients, p <0.0001. Hereafter the difference in free CD86 molecules/monocyte between the belatacept- and tacrolimus-treated patients reduced, because almost half of the belatacept-treated patients had been converted to tacrolimus-based therapy. In these patients (n = 8), free CD86 expression returned to baseline 3–5 months after conversion (SDC, Figure 2C). Pretransplant values for (future) rejectors and nonrejectors in the belatacept group were significantly different: 753 (428 – 928) free CD86 molecules/monocyte *versus* 882 (528 – 1528) cells/monocyte, respectively, p = 0.04 (SDC, Figure 2D). However, the pretransplant values showed a great overlap between rejectors and nonrejectors, and the numbers of pretransplant CD86 molecules on monocytes were not associated with acute rejection risk (Table 5). No significant differences between (future) rejectors and nonrejectors were observed in posttransplant dynamics of free CD86 molecules/monocyte (SDC, Figure 2E).

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SUPPLEMENTAL TABLES

SDC, Table 1: Adverse events, intention-to-treat analysis*

	Belatacept group (n = 20)	**Tacrolimus group (n= 20)**	**p**
Blood or lymphatic system	0.75 (0.97)	1.00 (0.92)	0.22
• Leucopenia	7	7	
• Anemia	6	10	
• Thrombocytopenia	1	1	
• Other	1	2	
Bleeding and thrombotic events	0.30 (0.57)	0.40 (0.60)	0.52
• Major bleeding	0	2	
• Minor bleeding	4	2	
• Thrombosis	2	4	
Cancer	0	0	-
Cardiovascular	0.95 (0.83)	1.20 (0.83)	0.33
• Acute coronary syndrome / myocardial ischemia	1	1	
• Cardiac decompensation / volume overload	2	3	
• Hypertension	12	17	
• Other	4	3	
Gastrointestinal	0.65 (0.67)	0.60 (1.00)	0.40
• Diarrhea	2	4	
• Other	11	8	
Infection	2.25 (1.86)	1.90 (1.83)	0.46
• Opportunistic infection	0.45 (0.69)	1.90 (1.83)	0.57
▪ BKV	2	1	
▪ CMV	1	2	
▪ EBV	1	0	
▪ HSV	0	1	
▪ VZV	0	0	
▪ Fungal	5	2	
• Other infection	1.80 (1.70)	1.60 (1.64)	0.61
▪ Urinary tract infection	20	14	
▪ Upper respiratory tract infection	8	4	
▪ Pneumonia	2	0	
▪ Gastrointestinal infection	1	2	
▪ Other	5	12	
Locomotor system disorder	0.25 (0.55)	0.20 (0.52)	0.70
Metabolism or nutrition	1.75 (1.16)	2.00 (1.56)	0.84
• Posttransplant diabetes mellitus	1	7	

• Hypo- / hyperglycemic dysregulation	4	9	
• Calcium disorder (hypo- / hypercalcemia)	6	3	
• Potassium disorder (hypo- / hyperkalemia)	6	9	
• Hypophosphatemia	6	6	
• Dyslipidemia	8	4	
• Liver enzyme abnormality	3	1	
• Other	1	1	
Nervous system	0.50 (1.00)	0.65 (0.88)	0.36
• CVA/TIA	1	0	
• Tremor	2	8	
• Headache	1	1	
• Other	6	4	
Skin-related disorders	0.15 (0.37)	0.30 (0.47)	0.26
Surgical or procedural complication	0.10 (0.31)	0.20 (0.52)	
• Acute tubular necrosis	1	2	
• Delayed graft function	1	1	
• Renal infarction	0	1	
• Other	0	0	
Tacrolimus-induced nephrotoxicity	0.05 (0.22)	0.40 (0.60)	-
Urological complication	0.55 (0.76)	0.60 (0.88)	0.96
• Hydronephrosis	1	4	
• Urinary leakage	2	1	
• Other	8	7	
Wound-related problem	0.15 (0.37)	0.25 (0.44)	0.44
• Wound infection	2	3	
• Other	1	2	
Other	1.80 (1.44)	2.20 (2.04)	0.63
Total	10.25 (4.18)	11.90 (5.43)	0.41

* Meannumber of adverse events (+standard deviation) per patient are depicted for both treatment groups for the different categories of adverse events. Numbers of adverse events per subcategory are depicted per treatment group.

BKV, BK virus; CMV, cytomegalovirus; CVA, cerebrovascular accident; EBV, Epstein-Barr virus; HSV, herpes simplex virus; N/A, not applicable; VZV, varicella zoster virus; TIA, transient ischemic attack.

SDC, Table 2: Clinical outcomes, intention-to-treat analysis*

	Belatacept group (n = 20)						Tacrolimus group (n = 20)						
	n	**M3**	**n**	**M6**	**n**	**M12**	**n**	**M3**	**n**	**M6**	**n**	**M12**	**p**[†]
Blood pressure													
• Systolic / diastolic (mmHg)	18	137 (98 - 167) / 83 (40 - 94)	17	138 (93 - 181) / 80 (50 - 109)	17	147 (106 - 165) / 81 (50 - 85)	20	144 (108 – 178) / 85 (59 – 98)	20	138 (96 – 184) / 84 (55 – 95)	19	145 (110 – 170) / 85 (45 – 97)	0.64 / 0.42
Kidney function													
• Creatinine (µmol/L)	18	127 (73 – 276)	17	114 (74 – 219)	17	128 (71 – 207)	20	122 (64 – 242)	20	126 (61 – 179)	19	126 (79 – 179)	0.80
• eGFR (mL/min)	18	52 (18 – 72)	17	62 (26 – 88)	17	54 (28 – 89)	20	50 (23 – 80)	20	53 (33 – 85)	19	50 (33 – 84)	0.57
• Protein/Creatinine ratio (mg/mmoL)	18	19.3 (5.2 – 443.2)	17	18.2 (5.8 – 87.7)	17	13.2 (5.7 – 343.8)	20	15.3 (7.3 – 115.0)	20	12.1 (4.2 – 209.6)	19	9.0 (5.3 – 43.5)	0.44
Glucose metabolism													
• Glucose (mmol/L)	18	5.6 (4.7 – 9.4)	17	5.5 (2.9 – 13.7)	17	5.6 (2.9 – 13.7)	20	6.2 (3.7 – 10.7)	20	6.6 (4.7 – 13.5)	19	6.1 (4.3 – 26.7)	0.06
• HbA1c (mmol/mol)	6	36 (29 – 74)	3	37 (33 – 50)	5	41 (33 – 49)	6	48 (37 – 67)	5	42 (30 – 73)	10	46 (33 – 75)	0.31
Lipids													
• Cholesterol total (mmol/L)	18	4.6 (2.9 – 7.5)	16	4.7 (3.0 – 6.9)	16	4.7 (3.4 – 7.2)	20	4.5 (2.9 – 6.5)	20	4.5 (3.2 – 5.9)	19	4.7 (3.1 – 6.9)	0.55
• Triglycerides (mmol/L)	18	2.1 (1.1 – 4.1)	16	1.9 (1.1 – 4.0)	16	2.2 (1.2 – 3.2)	20	2.0 (0.8 – 5.3)	20	1.8 (0.7 – 4.2)	19	1.6 (0.9 – 5.9)	0.13
• HDL-cholesterol (mmol/L)	18	1.1 (0.7 – 3.0)	16	1.2 (0.9 – 3.1)	16	1.2 (0.8 – 3.5)	20	1.3 (0.8 – 2.7)	20	1.2 (0.6 – 2.8)	19	1.4 (0.8 – 3.4)	0.66
• LDL-cholesterol (mmol/L)	18	3.0 (1.2 – 5.3)	16	2.8 (1.0 – 4.9)	16	2.8 (1.3 – 5.3)	20	2.4 (1.2 – 4.4)	20	2.6 (1.2 – 4.3)	19	2.7 (1.2 – 4.3)	0.30
Hematology													

• Hemoglobin (mmol/L)	18	7.2 (5.0 – 9.5)	17	7.6 (6.3 – 9.6)	17	8.2 (7.0 – 9.9)	20	7.5 (6.5 – 9.4)	20	7.7 (6.2 – 10.5)	19	8.4 (6.5 – 10.5)	0.85
• MCV (fL)	18	96 (89 – 100)	17	93 (88 – 98)	17	92 (83 – 97)	20	94 (69 – 106)	20	90 (68 – 102)	19	88 (72 – 108)	0.20
• Thrombocytes (×10^9/L)	17	222 (162 – 401)	17	232 (119 – 477)	17	214 (138 – 394)	20	231 (148 – 495)	20	235 (131 – 457)	19	245 (163 – 380)	0.21
• Leucocytes (×10^9/L)	18	6.3 (1.0 – 15.5)	17	6.9 (1.9 – 11.1)	17	6.4 (2.2 – 17.4)	20	5.9 (1.3 – 11.8)	20	7.4 (1.7 – 14.2)	19	8.4 (4.0 – 12.0)	0.12
Pharmacokinetics													
• Belatacept dose (mg)	16	800 (575 – 938)	11	400 (300 – 45)	10	381 (300 – 450)	-	N/A	-	N/A	-	N/A	N/A
• Tacrolimus dose (mg)	2	10.0 (10.0 – 10.0)	6	5.5 (3.5 – 10.0)	7	5.0 (3.0 – 8.0)	20	4.0 (2.0 – 8.0)	20	4.0 (2.0 – 6.0)	19	4.0 (2.5 – 7.0)	0.19
• Tacrolimus concentration (ug/L)	2	2.2 (1.5 – 5.5)	6	5.8 (4.2 – 8.3)	7	7.2 (4.5 – 8.6)	20	7.0 (4.1 – 10.7)	20	6.3 (2.6 – 9.9)	19	6.8 (4.4 – 13.3)	0.53
• Mycophenolate mofetil dose (mg)	18	1000 (500 – 2000)	17	1000 (500 – 2000)	17	1000 (500 – 2000)	20	1000 (500 – 2000)	19	1000 (0 – 2000)	18	1000 (0 – 2000)	0.47
• Mycophenolate acid concentration (mg/mL)	17	3.04 (0.52-10.00)	16	2.45 (0.98 – 5.21)	17	2.30 (0.99 – 3.54)	20	2.53 (1.03 – 10.00)	19	1.69 (0.96 – 4.24)	18	1.83 (0.57 – 3.67)	0.25
• Prednisone dose (mg)	18	5.0 (5.0 – 10.0)	17	5.0 (5.0 – 10.0)	17	5.0 (5.0 – 10.0)	20	5.0 (5.0 – 10.0)	20	5.0 (5.0 – 10.0)	19	5.0 (2.5 – 10.0)	0.59

* Censored for graft loss and death; † Comparison between patients from the belatacept group and the tacrolimus group 12 months after transplantation

Target tacrolimus C0 of 5 – 10 ng/mL were achieved in 75%, 85% and 95% of patients in the tacrolimus group 3, 6 and 12 months after transplantation, respectively. Target MPA C0 of 1.5 – 3.0 mg/mL were achieved in 45%, 40% and 40% of patients in the tacrolimus group respectively 3, 6 and 12 months after transplantation, and in 30%, 40% and 60% of patients in the belatacept group respectively 3, 6 and 12 months after transplantation.

Data present medians (plus ranges).

BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high density lipoproteins; LDL, low density lipoproteins; M3, 3 months after transplantation; M6, 6 months after transplantation, M12, 12 months after transplantation; MCV, mean corpuscular volume

SDC, Table 3: Graft function 12 months after transplantation

	Belatacept nonrejectors (n=9)	**Belatacept rejectors, censored for graft loss (n=8)**	**Belatacept rejectors, including graft loss (n=11)**	**Tacrolimus (n=19)**	**Tacrolimus nonrejectors (n=17)**
Creatinine (µmol/L)	106 (71-143)	163 (93-207)	-	126 (79-179)	119 (79-178)
eGFR (mL/min)	57 (45-89)	36 (28-76)	34 (0-76)	50 (33-84)	58 (37-84)
Protein/Creatinine ratio	11.4 (7.9-25.0)	12.2 (5.7-343.8)	-	9.0 (5.3-43.5)	9.0 (5.3-43.5)

Data are medians (plus ranges). Graft function was compared between 1) the belatacept-treatedrejectors and belatacept-treated nonrejectors and 2) the tacrolimus-treated and belatacept-treated nonrejectors, using the Mann-Whitney U test. Creatinine concentrationat month 12 was significantly higher and eGFR at month 12 was consequently significantly lower in belatacept-treated rejectors than in belatacept-treated nonrejectors, both p=0.001. These parameters did not differ between nonrejecting belatacept-treated and tacrolimus-treated patients at month 12.

In the group of "Belatacept rejectors, including graft loss" the 3 patients that lost their grafts were set to an eGFR of zero on month 12. Creatinine and Protein/Creatinine ratio were not calculated for this group, since these could not be determined for the 3 patients after graft loss.

eGFR, estimated glomerular filtration rate

SDC, Table 4: Response to antirejection therapy in belatacept-treated rejectors

	Creatinine			eGFR		
No. (Patient)	Best before	BPAR	Best after	Best before	BPAR	Best after
3	84	132	93	59	35	52
5	89	698	N/A	56	5	0
6	155	211	136	39	27	45
7	148	188	164	45	34	40
13	89	107	93	80	65	76
14	109	148	110	72	50	71
15	227	279	145	25	19	41
16	106	210	152	62	28	41
17	305	807	N/A	14	5	0
19	325	367	161	18	16	41
20	162	175	N/A	33	30	0

Patient numbers are the same depicted as in Figure 3. For detailed clinical course per patient, please refer to this figure. Creatinine and estimated glomerular filtration rates (eGFR) are given for the 10 belatacept-treated rejectors before, during and after rejection (when applicable, before second rejection episodes). Both before and after rejection the highestmeasuredeGFRs are depicted. Patients no. 6, 16 and 19 were switched to tacrolimus (almost) immediately after rejection occurred. Patients no. 5 lost her graft immediately after rejection, and patients no. 17 and 20 were switched to tacrolimus, but still lost their grafts thereafter (eGFRs after rejection were set to zero). Patients no.7, 14 and 15 were switched to tacrolimus after a second episode of acute rejection. Patient no. 13 was diagnosed with BPAR after revision of the biopsy, and was therefore not treated with additional antirejection therapy. This patient had an isolated v-lesion which may explain the excellent outcome despite no treatment. Finally, patient no. 3 was switched after his third rejection episode.

N/A, not applicable

SDC, Table 5: Anti-HLA antibodies in serum

		Belatacept group (n = 20)	Tacrolimusgroup (n = 20)	p
Donor-specific	*Preexistent*	-	-	-
	De novo	2 (10%) *[Patients no. 2 and 20]*	-	0.49
Nondonor-specific	*Preexistent*	2 (10%) *[Patients no. 2 and 12]*	3 (15%) *[Patients no. 3, 11 and 12]*	1.00
	De novo	2 (10%) *[Patients no. 7 and 20]*	2 (10%) *[Patients no. 6 and 20]*	1.00

Patient numbers are the same as in Figure 3. None of the patients had donor-specific antihuman leukocyte antigen (HLA) antibodies (DSA) pretransplantation. During the first posttransplant year, 2 patients developed DSA, both in the belatacept group. Patient no. 2 in the belatacept group developed DSA against HLA-DQ2 (MFI 3787) 1 month after transplantation, but this disappeared hereafter without additional therapy and no acute rejection occurred. Patient no. 20 in the belatacept group had DSA, which were also detectable in the cross match-dependent cytotoxicity test, against HLA-A1 (MFI 18,000), -B8 (MFI 22700), -DR3 (MFI 11000), -DR52 (MFI 5500) and -DQ2 (MFI 16500) during her 4th rejection episode right before losing her graft. Now, she was already switched to a tacrolimus-based regimen and had been treated with multiple methylprednisolone and alemtuzumab gifts (Figure 3).

Two and 3 patients, in the belatacept and tacrolimus group, respectively, had nondonor-specific anti-HLA antibodies (non-DSA) pretransplantation. Patient no. 2 in the belatacept group had non-DSA against DR1 that remained present after transplantation, without clinical consequences. Patient no. 12 in the belatacept group had non-DSA against HLA-Dp11 which disappeared after transplantation. No rejection occurred. Patients no. 3, 11 and 12 in the tacrolimus group had non-DSA pretransplantation against HLA-B76, -DP1, and -DP; -DR4; and -B15; respectively. Only patient no. 3 suffered from an acute rejection Banff type 2B. No serum was available from this patient at the time of rejection, but in sera from month 1 to 12 no non-DSA were detected. Also in the other 3 patients, preexistent anti-HLA antibodies disappeared after transplantation.

Two patients in the belatacept group (no. 7 and no. 20) developed non-DSA. Patient no. 7 in the belatacept group developed non-DSA against HLA-DQ3 (measured on day 30) before he was diagnosed with an acute Banff type 2B rejection 44 days after transplantation. These non-DSA were also positive during rejection. After treatment with methylprednisolone they were no longer detectable and remained so throughout follow-up. Patient no. 20 in the belatacept group developed non-DSA against HLA-A24, -A68, and -DQ3 simultaneously with DSA. Two patients in the tacrolimus group (no. 6 and no. 20) developed non-DSA, without clinical consequences in the first year after transplantation against HLA-DP14 and HLA-A24, respectively.

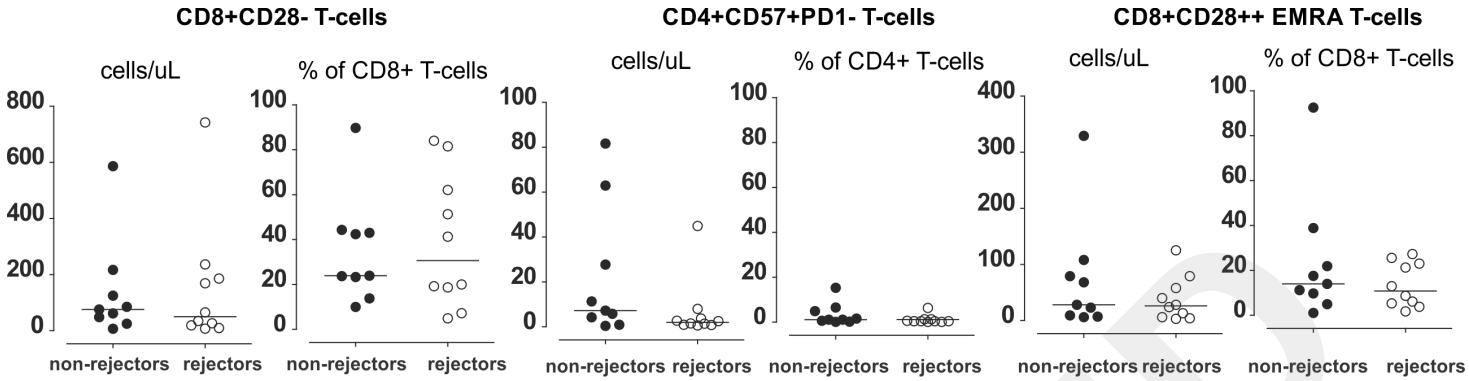
SDC, Table 6: Baseline characteristics of (future) rejectors and nonrejectors in the belatacept group

	Belatacept (n= 20)		p
	rejectors (n= 11)	nonrejectors (n= 9)	
Age at transplantation (years)	47 (25-76)	60 (40-74)	0.41
Male / female	7 (64%) / 4 (36%)	7 (78%) / 2 (22%)	0.49
Ethnicity			0.07
• Caucasian	11 (100%)	6 (67%)	
• African	-	2 (22%)	
• Asian	-	1 (11%)	
Body weight (kg)	83.3 (63.5 - 111.4)	76.0 (56.6 - 98.6)	0.26
HLA A mismatch (mean ± SD)	1.0 (± 0.6)	1.1 (± 0.8)	0.84
HLA B mismatch (mean ± SD)	1.4 (± 0.5)	1.2 (± 0.4)	0.63
HLA DR mismatch (mean ± SD)	1.2 (± 0.4)	1.0 (± 0.5)	1.00
Current PRA (%)	0 (0 - 4)	0 (0 - 5)	0.55
Peak PRA (%)	4 (0 - 6)	4 (0 - 5)	0.37
CMV status at transplantation			0.37
• Donor + / Recipient -	1 (9%)	2 (22%)	
• Donor + / Recipient +	2 (18%)	2 (22%)	
• Donor - / Recipient -	6 (55%)	1 (11%)	
• Donor - / Recipient +	2 (18%)	4 (44%)	
Donor age at transplantation (years)	60 (43 - 69)	53 (24 – 71)	0.33
Related / unrelated donor	4 (36%) / 7 (64%)	2 (22%) / 7 (78%)	0.64
Cause of end-stage renal disease			0.90
• Diabetes mellitus	1 (9%)	2 (22%)	
• Hypertension	-	2 (22%)	
• IgA nephropathy	-	1 (11%)	
• Polycystic kidney disease	2 (18%)	1 (11%)	
• Obstructive nephropathy	2 (18%)	1 (11%)	
• Unknown	3 (27%)	2 (22%)	
• Other	3 (27%)	0 (0%)	
Renal replacement therapy			0.37
• None (preemptive)	7 (64%)	3 (33%)	
• Hemodialysis	3 (27%)	4 (44%)	
• Peritoneal dialysis	1 (9%)	2 (22%)	
Time on dialysis therapy (days)	560 (147-2633)	425 (123-2782)	1.00
Number of kidney transplantation			1.00
• First	10 (91%)	9 (100%)	
• Second	1 (9%)	-	

Continuous variables are presented as medians (plus ranges) and categorical variables as numbers (plus percentages), unless otherwise specified

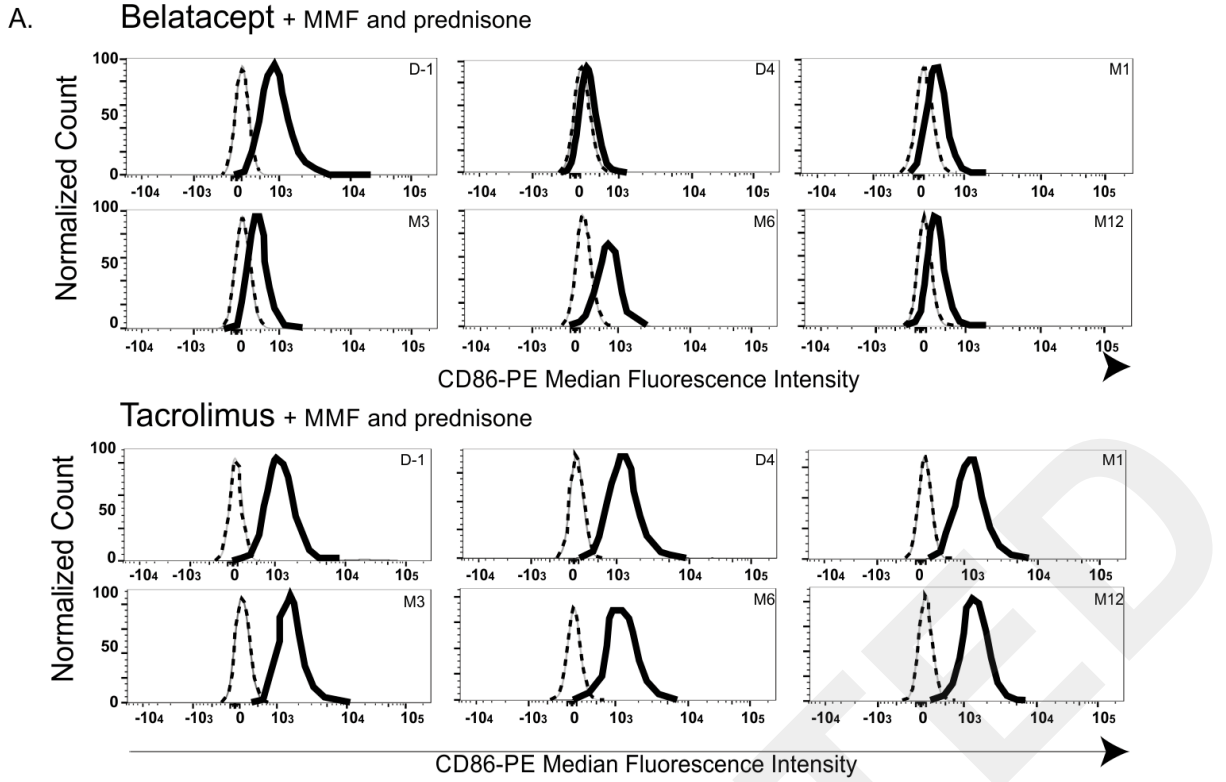
BPAR, biopsy-proven acute rejection; CMV, cytomegalovirus; HLA, human leukocyte antigen; PRA, panel reactive antibodies; SD, standard deviation.

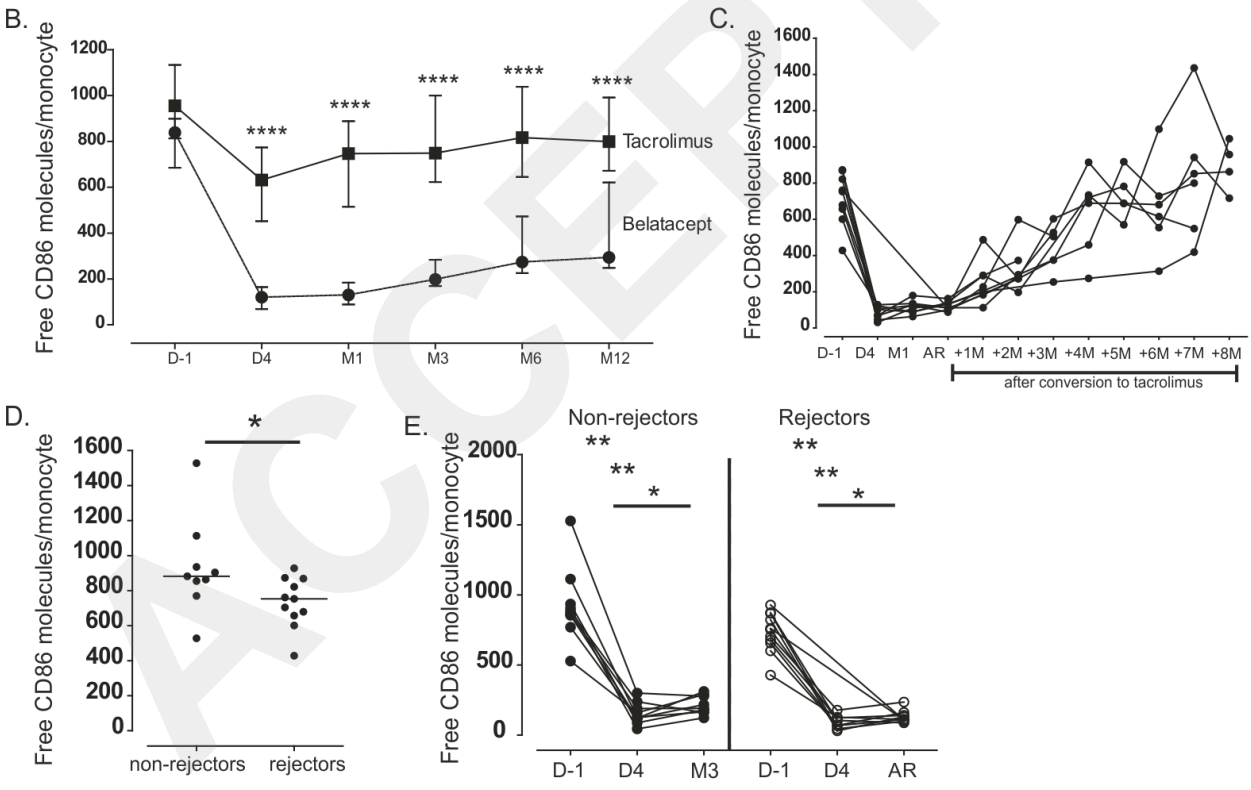
SUPPLEMENTAL FIGURES



SDC, Figure 1. CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T cells during rejection or 3 months after transplantation. CD4+ and CD8+ T cells were gated from 7-AAD negative CD3+ lymphocytes (based on forward and sideward scatter) and EMRA T cells were gated as CCR7- and CD45RO- T cells (See Figure 4). The absolute numbers and percentages of CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T cells are presented for nonrejectors 3 months after transplantation and for rejectors during acute rejection before additional antirejection therapy was given.

N.B.: From 1 rejector no materials were obtained during rejection, because biopsy-proven acute rejection was diagnosed in retrospect after revision by a second pathologist.





SDC, Figure 2. Pharmacodynamic drug monitoring of belatacept.
The median fluorescence intensity (MFI) of CD86 was assessed on circulating monocytes in belatacept and tacrolimus-treated patients using a competitive monoclonal antibody (clone HA5.2B7, solid line) with an IgG control (dotted line) (A). Free CD86 molecules per monocyte were calculated from MFIs (medians + interquartile ranges) and compared between the belatacept (triangles) and tacrolimus (squares) group on different time points in an intention-to-treat analysis using a linear mixed model (B). Free CD86 molecules/monocyte in tacrolimus-treated patients compared to belatacept-treated patients were 5.9-fold (95% CI 4.5 to 7.7-fold) higher on day 4; 5.3-fold (95% CI 4.0 to 7.0-fold) higher on month 1; 3.7-fold (95% CI 2.8 to 4.8-fold) higher on month 3; 2.6-fold (95% CI 2.0 to 3.4-fold) higher on month 6; and 2.1-fold (95% CI 1.6 to 2.8-fold) on month 12. Free CD86 molecules/monocytes were measured in n = 8 patients which were converted to a tacrolimus-based therapy after acute belatacept-resistant rejection (C). Numbers of free CD86 molecules/monocytes pretransplantation were compared between nonrejectors (n = 9) and rejectors (n = 11) in the belatacept group (D), as well as CD86 molecules/monocyte on day 4 and month 3 or during rejection after transplantation (E).
AR, acute rejection; D-1, 1 day pretransplantation; D4, 4 days after transplantation; M1, 1 month after transplantation; M3, 3 months after transplantation; M6, 6,
months after transplantation; M12, twelve months after transplantation; MMF, mycophenolate mofetil
* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$
N.B.: In (D) black lines represent the medians; the upper and lower border of the boxes represent the 25th and 75^{th} percentiles; the error lines represent 10th and 90th percentiles.