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The impact of belatacept on the phenotypic heterogeneity of renal T cell-mediated alloimmune response: the critical role of maintenance treatment and inflammatory load

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Abstract

Belatacept offers superior long-term outcome relative to calcineurin inhibitor (CNI)-based immunosuppression. However, the higher frequency of early T cell-mediated rejection (TCMR) in belatacept-treated patients hampered the widespread adoption of costimulation blockade. Here, we applied gene expression analysis and whole slide inflammatory cell quantification to assess the impact of belatacept on intragraft immune signature. We studied formalin-fixed, paraffin-embedded renal biopsies from 92 patients stratified by histopathologic diagnosis (TCMR, borderline changes, or normal) and immunosuppression regimen (belatacept, CNI). An interaction model was built to explore maintenance treatment-dependent expression level changes of immune response-related genes across diagnostic categories of normal, borderline changes, and TCMR. Ninety-one percent of genes overexpressed in TCMR showed significant correlation with whole-section inflammatory load. There were 27 genes that had a positive association with belatacept treatment. These were mostly related to myeloid cells and innate immunity. Genes negatively associated with costimulation blockade (n=14) could be linked to B cell differentiation and proliferation. We concluded that expression levels of genes characteristic of TCMR are strongly interconnected with quantitative changes of the biopsy inflammatory load. Our results might suggest differential involvement of the innate immune system, and an altered B cell engagement during TCMR in belatacept-treated patients relative to CNI-treated referents.

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Author contribution

DD, FV, and ZGL participated in research design. SC and FV provided clinical data. DD, AC, and HJ participated in the performance of the research. DD, JRG, and CB participated in data analysis. DD and ZGL wrote the paper. FV, JRG, and CB contributed to the final form of the manuscript with editing, formatting, and content-related suggestions.

Disclosure

The authors declare no conflicts of interest.

Data availability statement

The data that supports the findings of this study are available in the supplementary material of this article.

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Keywords

rejection; costimulation; belatacept

Introduction

The introduction of calcineurin inhibitors (CNIs) ushered a new era of renal transplantation by dramatically improving short-term allograft survival[1, 2]. However, CNIs also became an additional contributor to late allograft loss due to their deleterious toxicity profile[3]. Since the late 1990s, there have been ongoing efforts to minimize CNI use or replace them with less toxic agents such as mycophenolate mofetil (MMF) with or without mechanistic target of rapamycin (mTOR) inhibitors and steroid[4, 5]. The most recent and, so far, most promising attempt was the introduction of belatacept[6] (a second generation CTLA4-Ig). Belatacept acts by blocking the CD28:CD80/86 costimulatory pathway, a critical second signal needed for T-lymphocyte activation. Belatacept-based protocols offer better overall graft survival and improved long-term renal function relative to CNI-based protocols. This survival benefit is attributed mainly to decreased cardiac and renal toxicity[7–9]. However, the higher rate of acute T cell-mediated rejection (TCMR) during the first post-transplant year in belatacept-treated patients (also known as belatacept-resistant rejection, BRR) is considered a significant drawback.

Previous studies implicated different lymphocyte populations in BRR. Besides CD4+/CD57+/PD-1- cells[10], mature, highly differentiated effector memory T cells (T_{EMRA})[11–13], Th_{17} cells[14] and antigen-primed Tfh cells[15] were all suggested to play a role in BRR. Despite the significant efforts towards exploring the immunophenotype of BRR, transcriptome profiling studies of renal transplant biopsies from patients treated with belatacept are scarce. In addition, most of the previous molecular phenotyping studies relied on microarray technology or quantitative reverse transcription PCR, which used fresh, frozen, or RNAlater preserved tissues[16–18]. These technologies are less reliable on formalin-fixed, paraffin-embedded samples (FFPE)[19] which significantly limits their applicability to routinely archived renal allograft biopsies. Furthermore, the representativeness of a separate tissue core dedicated for transcriptome analysis is uncertain since it is processed “blindly”, without morphologic evaluation.

Gene expression directly assessed via oligonucleotide probes developed by Nanostring[20] has the potential to circumvent some of these limitations and make archived samples readily accessible for gene expression analysis[21, 22]. The platform has been validated on FFPE human renal transplant biopsies[23, 24]. Here, we utilized it to characterize the immune signature of renal transplant biopsy samples from patients treated with either belatacept-based or CNI-based immunosuppression. Using FFPE material for gene expression studies allows direct correlation between morphology and gene expression profiling; therefore, we combined transcript abundance analysis with quantitative immunohistochemistry to correlate the biopsy inflammatory load with the transcriptomic profile. Our aim was to explore the effect of belatacept treatment on the immunome across the diagnostic spectrum of T cell-mediated alloimmune responses.

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Material and methods

Patient selection

This was a retrospective, descriptive study approved by the institutional review board at University of California, San Francisco (UCSF, IRB approval number: 13–11762). First, we retrieved all available protocol and for-cause biopsies of patients who were treated with belatacept-based immunosuppressive regimens at UCSF between 2003 and 2017. Of these, all biopsies were enrolled except those from patients with history of BK polyoma virus nephropathy or those with isolated antibody-mediated rejection (AMR). If multiple biopsies were available from the same patient, the first biopsy with a histological diagnosis of BL changes or TCMR was enrolled. The final selection consisted of 49 biopsies from distinct patients. The biopsies represented a range of morphological diagnoses from normal, through borderline (BL) changes to TCMR with or without AMR.

For the control group, another 49 samples were chosen from a pool of protocol and for-cause biopsies that were obtained from recipients treated with a CNI-based regimen at UCSF between 2009 and 2016. These were selected to match the inflammatory load of the biopsies in the belatacept-treated group on a one-to-one basis. Inflammation was assessed semi-quantitatively in the cortical, non-scarred interstitium per the Banff 2019 scheme. Besides the exclusion criteria above, patients with documented non-compliance were also disqualified in the CNI group.

Immunohistochemistry and inflammatory cell quantitation

Three micrometer-thick sections immediately adjacent to sections cut for gene expression profiling were used to digitally quantify the inflammatory cell load in cases that had enough tissue in the FFPE tissue block after cutting the RNA curls (n = 83). In brief, after deparaffinization and low pH antigen retrieval, sections were incubated with anti- CD45 antibody (1:2000, clone: PD7/26 + 2B11, Dako-Agilent) followed by antigen visualization with Bond Polymer Refine Detection Kit (Leica Biosystems). Whole-slide digital images (WSDI) (Aperio ScanScope XT, Leica Biosystems) of the CD45-stained slides were analyzed by the Cell Count module of the Definiens Tissue Studio software. Inflammatory cell count (ICC) was defined by the average number of CD45-positive cells per 1 mm^2 tissue area. For each WSDI, two inflammatory cell count metrics were generated: whole section ICC and cortical ICC. Cortical ICC measured the inflammatory cell density only in the cortex while perivascular lymphoid aggregates, large vessel lumina, the renal capsule, and non-renal tissue were excluded from the analysis.

RNA isolation and gene expression analysis

RNA was isolated by Purelink FFPE RNA isolation kit (Invitrogen) from four 10 μm-thick FFPE sections consecutive to the section used for immunohistochemistry. RNA yield (ng/μl) and RNA purity (260/280 ratio) was measured on the Nanodrop 1000 platform (Thermo Fisher Scientific). The median RNA yield per FFPE block was 31.54 ng/μl (IQR = 23.47), with a median 260/280 value of 1.89 (IQR = 0.21).

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Expression analysis of 756 immune response-related genes was performed on a Nanostring nCounter Sprint instrument, based on the manufacturer's recommendations. Our gene panel (Supplemental Table 1) consisted of Nanostring's Cancer Immune v1.1 oligonucleotide set (n = 730) in addition to a custom panel of 26 probes and 40 housekeeping genes. Internal quality control (QC) metrics of the platform were used with the default settings[23]. Samples that failed to detect at least 55 % of the target genes above the background noise level were omitted from further analysis. The background noise threshold was determined to be 20 counts (two standard deviations above the average count of a negative-control probe set). Three samples from patients treated with belatacept were excluded from further analysis due to failure to detect a sufficient number of gene targets above background. Consequently, their inflammatory load-matched counterparts in the CNI-treated group were also dropped from the study. Raw counts of all non-housekeeping genes for samples that were included in the final dataset (n = 92) are shown in Supplemental Table 2.

Statistics

Statistical analyses of the clinical, laboratory, and histological data between the treatment cohorts were performed using SPSS 25.0 software. Continuous variables were expressed as medians with inter-quartile range (IQR). Mann-Whitney U-test, Chi-square test, and Spearman's rank order were used where applicable. P-values less than 0.05 were considered significant. For the analysis of the gene expression dataset the nSolver software (v4.0) with the Advanced Analysis module (AAM), and "R" software environment (v3.6.1) were used. Only those genes were considered in the analysis, the raw transcript count of which passed the low-count gene test with default settings of AAM (raw count number < 20, observation frequency: 1).

Next, the geNorm algorithm[25] was utilized for gene count normalization based on a positive-control probe set (to adjust for technical variation) and the housekeeping genes (to adjust for variation in RNA input). To obtain information on the overall structure of the gene expression dataset principal component analysis and permutational multivariate analysis of variance (PERMANOVA), were applied (by using "prcomp", and "adonis" functions of "R", respectively).

Differential expression (DE) analyses between the diagnostic groups and between the treatment groups were performed in AAM using a false discovery rate of 5 %, as determined by the Benjamini-Hochberg method. Our power calculation showed that 46 experimental subjects in each treatment group is sufficient to reject the null hypothesis (no difference between the expression profile of belatacept and CNI-treated patients) with a probability of 0.92 using exact test[26] and an adjusted p-value of 0.05 (using the Benjamini-Hochberg method). We supposed that the total number of genes for testing would be 800, and the top 20 genes would be differentially expressed. Also, the desired fold change was at least 2 and we expected the top 20 DE genes to have an average count of 500 with a maximum dispersion of 0.5.

Lastly, we built a linear model to understand how the expression level of the genes in our panel depends on the interaction of maintenance treatment with histological diagnosis. All six diagnosis/treatment groups were included. The model was fitted by using the "glm"

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function of "R". In brief, the dependent variable of the model for a particular gene was its log_2 transformed transcript count. The independent variables were: histological diagnosis (Normal, BL changes, and TCMR), maintenance treatment (CNI-, or belatacept-based), log_2ICC, and induction treatment (anti-thymocyte globulin or anti-IL-2Rα antibody), as we hypothesized that the latter two also had an effect on the expression of the genes in our panel. P-values were adjusted for false discovery rate of 5 % by the Benjamini-Hochberg method[27].

Results

Clinical and histological characteristics

There were 46 samples each in the belatacept-, and in the CNI-based treatment groups (Figure 1). There were no significant differences between the groups for subject characteristics except for maintenance treatment (by design) and induction treatment (Table 1): the proportion of patients who received anti-thymocyte globulin (ATG) treatment in the belatacept group was significantly higher than that of the CNI group. Thirty-four patients in the belatacept group never received CNI pre-biopsy, while nine were converted from a CNI-based regimen to a belatacept-based one before the biopsy (drug overlap period was two to four weeks). The median time to biopsy from CNI cessation was seventy-nine days (interquartile range: seventy-two days). Of these, five biopsies showed TCMR while four displayed borderline changes. In addition, three patients had been receiving belatacept, MMF/MPA, and CNI in combination posttransplant, with CNI withdrawal at 30, 45 and 120 days before the biopsy, all of which demonstrated TCMR.

By study design, the treatment groups comprised the same number of samples with normal morphology (n = 13), BL changes (n = 15), and TCMR (n = 18). Banff scores were comparable between the two treatment groups (Table 1). Antibody-mediated rejection (C4d positive and negative combined) as defined by the Banff 2019 classification was present besides TCMR in three CNI-treated, and four belatacept-treated patients (p = 1.000).

Next, belatacept-, and CNI-treated patients were combined per diagnostic group to compare cortical ICC amongst histological diagnoses. Pairwise comparisons (Figure 2A) revealed significant differences in median cortical ICC values between biopsies showing TCMR and normal ($p < 0.0005$), TCMR and BL changes ($p < 0.0005$), and BL changes and normal morphology ($p = 0.023$). In contrast, cortical inflammatory cell count was comparable between the treatment groups of a given diagnostic group (Figure 2B).

Correlation between inflammatory load and gene expression

We evaluated the relationship between *PTPRC* gene transcript abundance and whole section ICC. As we used CD45 immunohistochemistry to detect inflammatory cells in the biopsies, this was a direct correlation between the RNA and protein product abundance of a gene. We found that *PTPRC* counts showed excellent correlation with log_2 transformed whole section ICC (Spearman's rho = 0.918, $p < 0.0005$, Figure 3). In addition, normalized *PTPRC* mRNA counts significantly predicted whole section inflammatory cell count/mm2 ($p < 0.0005$).

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Next, we evaluated the pairwise correlations between whole section log_2ICC and each gene that was significantly differentially expressed in TCMR cases relative to biopsies with normal morphology. We found that 375 genes (91 %) showed significant correlation with whole section log_2ICC (Supplemental Table 3). Of these, 69 % had a Spearman's rho > 0.7. The genes with the strongest correlation were: *IL2RG, PIK3CG, CCR5, ITGAL, SELPLG, ITGB2, CD3E, SPN, CCL5, TNFAIP3*. The correlation between *PTPRC* counts and whole section log_2ICC was ranked seventeenth. Furthermore, all genes that were on our panel out of the 50 that have been previously reported to be characteristic of TCMR[28] had a correlation coefficient at least 0.730 ($p < 0.0005$), with the most highly correlated being *CD96, CD3D, CD84, TAP1, CD8A, SH2D1A, BTLA, LAG3, IL12RB1, ICOS* (Spearman's rho ≥ 0.859, $p < 0.0005$).

Principal component analysis (PCA) of the gene expression dataset

Principal component for all 92 cases was based on 601 genes that met the RNA QC criteria (see "Material and Methods"). The first principal component explained 64 % of the total variance in the gene expression dataset (Supplemental table 4), and set cases with TCMR apart from subjects with normal morphology irrespective of the treatment group they belonged to (Figure 4). Biopsy samples displaying BL changes fell in between normal and TCMR groups. Separation according to histological diagnosis was confirmed by PERMANOVA, ($F(5, 86) = 18.423$, $p = 0.001$, $R^2 = 0.52$). In addition, there was maintenance treatment-dependent clustering of TCMR (but not normal or BL changes) cases along the second principal component, that explained 6 % of the total variance (second principal component, Supplemental table 4). Principal component loadings for each gene are shown in Supplemental table 5.

Differential expression across categories of histological diagnosis

In biopsy samples with BL changes there were 321 genes that showed a significantly higher expression level relative to samples with normal morphology when patients with belatacept-, and CNI-based maintenance treatments were combined by diagnostic group (Supplemental Table 6). Of these, 188 displayed more than two-fold change. Among the top 10 transcripts by fold change were genes associated with costimulatory signaling (*CD80, ICOS, SLAMF7*, and *SH2D1B*), markers of kidney injury (*LCN2, LTF*), a chemokine and a chemokine receptor (*CCL4, CCR7*), a member of TNF signaling (*TNFRSF17*), and a natural killer (NK)-cell activating receptor (*KLRC2*).

Four-hundred sixty-five genes were significantly differentially expressed in the TCMR group (maintenance treatments combined) relative to the normal group, out of which 329 showed at least a two-fold increase (Supplemental Table 7). The top 10 transcripts by fold change included chemokines and chemokine receptors (*CXCL-9, CXCL-11*, and *CCR7*), positive and negative modulators of T-cell signaling (*PD-1, CTLA-4, LAG-3, ICOS*, and *SLAMF-7*), and T-cell markers (*CD8A, CD7*). Sixty-eight percent of the genes that had higher transcript abundance in cases with TCMR were also characterized by a higher transcript count in cases with BL changes (Figure 5). Only 9 genes - mostly known to be expressed constitutively by the renal tubular epithelium (e.g. *MME, DPP4*) or regulated

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negatively by IFN-y (*APOE*) – had a significantly lower expression by at least two-fold in the TCMR cases relative to the normal samples.

Differential expression between belatacept-, and CNI-treated patients

There was a significant difference between the belatacept treated group and the CNI-treated group for the expression level of fifty-seven genes. Of these, nine genes showed at least a 2-fold increase in the belatacept group: genes related to phagocyte trafficking (S100A8), innate immune response regulation (MEFV, LILRA5, LAIR2), pattern recognition (LY96), phagocyte/NK-cell effector function (FCGR3A), and T-effector function (GZMB, LAG3, JAK3). Only eight genes had significantly lower level of expression in the belatacept group, however, none of them reached a two-fold difference (Figure 6, Supplemental table 8).

Interaction between belatacept treatment and histological diagnosis in a linear model on gene expression level changes

We identified 41 genes whose expression level change among diagnostic categories was different in the belatacept group relative to the CNI group (Figure 6, Supplemental Table 8). Of these 41 genes, 27 had a significant, positive slope coefficient for the interaction term between histological diagnosis and maintenance treatment. These genes had a higher predicted transcript abundance change from normal to BL changes and/or from BL changes to TCMR in the belatacept group relative to the CNI group. Six out of the 10 genes with the highest coefficient value were also expressed at higher levels in belatacept treated patients relative to baseline of patients treated with CNI: *CD16, S100A8*, *CCL8, CD20*9, *LY96, and CFB*. The remaining genes among the group of 10 with the highest coefficient value were also related to phagocyte trafficking (*CCL2, CXCL11*) or pattern recognition (*MRC1, MSR1)*.

Fourteen genes were characterized by a significant, negative slope coefficient for the interaction term in the linear model (Supplemental Table 8). These genes were characterized by a lower increase in the predicted transcript count from normal through BL changes to TCMR in the belatacept treatment group relative to the CNI group. The genes with the strongest negative association with costimulation blockade were: B-cell associated cell surface receptors (*CD20, TNFRSF13C, CD79A, CD19, FCGRB2*), helper T-lymphocyte associated chemokine/chemokine receptor (*CCL17, CCR6*), *CD40LG* (T-cell costimulatory molecule), *IL29* (IFN type III cytokine), and *TLR10* (an anti-inflammatory pattern-recognition receptor).

Discussion

In this retrospective study we quantitatively measured the inflammatory load and the expression of 756 key genes of the immune response in biopsies from belatacept-, and CNI-treated patients. The study design had two key strengths. One, the biopsies represented the full spectrum of the morphological changes under costimulation blockade from Normal to TCMR. Two, the severity of inflammation in the control CNI group matched the severity of inflammation in the biopsies of the belatacept-treated patients. Therefore, differences, if any,

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in the gene expression between the two groups could not be attributed to differences in the inflammatory burden.

First, we demonstrated that the severity of inflammation (as expressed by ICC) strongly correlated with the expression level of genes most characteristic of TCMR[28]. While this is not an unexpected finding, such remarkable concordance between quantitative histologic and molecular characteristics has not been reported before[29–31] and could potentially be attributed to two factors unique to our study design. On one hand, immunohistochemistry combined with whole slide image analysis provided a precise quantitative metric for the inflammatory load that is well-suited for correlation analysis with transcriptomics data. On the other hand, the same tissue block was used for histologic assessment and also for gene expression analysis. Our results show that this approach is suitable of mitigating sampling bias between different tissue interrogation techniques and can improve cross-platform data integration.

Second, we showed that belatacept treatment has a detectable effect on the expression level of a set of genes in the panel: PCA separated treatment groups in the TCMR category, and we found a positive association between belatacept treatment and the expression level of innate immune response-related genes based on the differential expression analysis between maintenance treatment groups. In addition, PCA also showed that the major driver of variance in the gene expression dataset was the histological diagnosis, as it was also confirmed by the differential expression analysis across histological categories. As both histological diagnosis and maintenance treatment showed an impact on gene expression, we built a linear model to assess how these two factors interact with each other, i.e., how belatacept treatment modifies the effect of histological diagnosis on the expression pattern of the gene panel relative to CNI treatment. The model was adjusted for ICC, as we proved that ICC has a fundamental impact on transcript abundance of the genes in the panel. In addition, our model also included the type of induction regimen as a covariate, since significantly more patients received ATG in the belatacept group.

Based on our linear model, belatacept treatment had an impact on the transcript abundance change of 41 genes across diagnostic categories of normal, through BL changes to TCMR. The genes that showed a positive association with costimulation blockade were mostly innate immune response-related, including key cell surface receptors of phagocytes (*CD16, CD14, CD68, CD209, TLR4, MRC1, MSR1*). Most of these genes also showed significant overexpression in the belatacept groups relative to the CNI group. Since the amount of inflammation was comparable between the two treatment groups, this finding suggests that the innate immune compartment had an enhanced involvement in belatacept-treated patients relative to CNI-treated referents. A potential explanation for this could be that belatacept and calcineurin inhibitors have different impact on the cellular elements of the innate immune system. Calcineurin inhibitors have a well-documented regulatory effect on the monocyte/macrophage cell line: they reduce cytokine production via ERK phosphorylation[32], downregulate production of IL-6 and TNF-α after toll-like receptor stimulation *in vitro*[33], and impair phagocytosis functionality[34]. However, the effect of belatacept on innate immune system is less well studied and understood. Although it has been shown that abatacept, a first generation CTLA4-Ig, modulates pro-inflammatory macrophage responses[35], Mayer *et al*

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found that belatacept does not impair the stimulatory capacity of dendritic cells[36]. While our results raise the possibility of the enhanced engagement of the innate immune system in costimulation blockade-resistant rejection, further studies will be needed to explore the exact role of such a phenomenon in the development of BRR.

The fourteen genes with a significant, negative association with belatacept treatment were mostly related to B-cell activation (*CD19, CD79A, CD20*), B-cell proliferation, and adaptive immune response (*CD40LG*). Not all of these genes proved to be also significantly differentially expressed in belatacept-treated patients relative to their CNI-treated counterparts. However, the fact that their expression level increase from normal through BL changes to TCMR was negatively affected by costimulation blockade, aligns well with previous *in vitro, in vivo*, and clinical studies[37, 38] that showed that belatacept has an improved control of the humoral alloimmune response relative to CNI treatment. An important gene in this respect might be *CD40LG* that is expressed by antigen-activated helper T cells. It binds its receptor *CD40* on antigen-stimulated B cells and plays a pivotal role in Ig isotype switching[39], thus it is fundamental in B cell differentiation and proliferation. We found *CD40LG* to have a significant negative association with belatacept treatment. This is a novel finding and most probably a direct effect of belatacept since the promoter region of the *CD40LG* gene contains a *CD28* response element[40]. Although our study explored gene expression changes in the graft and not in lymph nodes, negative association of *CD40LG* expression with belatacept-treatment can potentially indicate a mechanism of action with which belatacept interferes with the development of DSAs.

A recently published transcriptomic analysis of belatacept-, and CNI-treated patients[41] did not reveal differentially expressed genes between the two treatment groups. The discrepancy between the results of the present and the cited study is probably in part because of the broader panel of target genes used in the current work that allowed a more in-depth exploration of innate immune responses and the myeloid lineage in particular. In addition, we were able to detect subtle but potentially important differences between the treatment groups due to the larger set of samples evaluated and a one-to-one subject matching based on the severity of inflammation. Markers of certain T cell populations previously reported to be of importance in BRR[9, 13, 14] were comparable between the treatment groups. Since these prior studies used different modalities (RNA microarray, flow cytometry etc.) to examine different types of biospecimens (peripheral blood, RNA-later preserved renal tissue) it is challenging to interpret the lack of differential expression of certain genes in our study that represents a limitation of our work.

In addition, due to the retrospective nature of the study, patients were enrolled from different belatacept clinical trials and non-trial setting, therefore they did not receive the drug according to a single standardized protocol. While 74 % of subjects in the belatacept group never received CNI between the time of transplantation and the biopsy, 26 % did. However, even these patients were CNI-free for a median time of 2.5 months prebiopsy, which suggests that the effects on gene expression described here are belatacept-related. On the other hand, the CNI cohort featured heterogeneity for additional immunosuppressive drugs (MMF, mTOR inhibitors, and steroids). These factors made it difficult to decipher the relevance of the unexpected finding of comparable renal function between the treatment

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groups. It should, however, be pointed out that there were major differences between our study and the BENEFIT trial that established the superiority of belatacept over CNI treatment in regards to renal function[7–9]. First, our study looked at eGFR data at different time points posttransplant (depending on the time of the biopsy) while the BENEFIT trial compared patients uniformly at 12 months posttransplant. Second, all but one patient received tacrolimus-based immunosuppression in our CNI-group while the active comparator arm in the BENEFIT trial was administered cyclosporine. Third, in 72 % of patients in our study the eGFR values were registered during an ongoing T cell-mediated alloimmune process unlike in the BENEFIT trial. Because of these, our data on eGFR cannot be used to compare the effect of CNI and belatacept on renal function.

In summary, our results demonstrated the increased expression of myeloid cell-related genes in belatacept-treated patients relative to patients on a CNI-based maintenance regimen. Further studies are needed to better understand the potential clinical implications of this finding especially for prospective clinical trials with costimulation blockers directed against T cell surface receptors that may have a limited effect in regulating innate immune cells[42]. We also proved that FFPE-derived RNA profiling in renal transplant biopsies is suitable for targeted gene expression analysis in close context of morphological alterations. Using the same biopsy core for histological and molecular assessment can facilitate better stratification of cases for molecular studies and enables the transplant research community to link molecular features directly to histological alterations in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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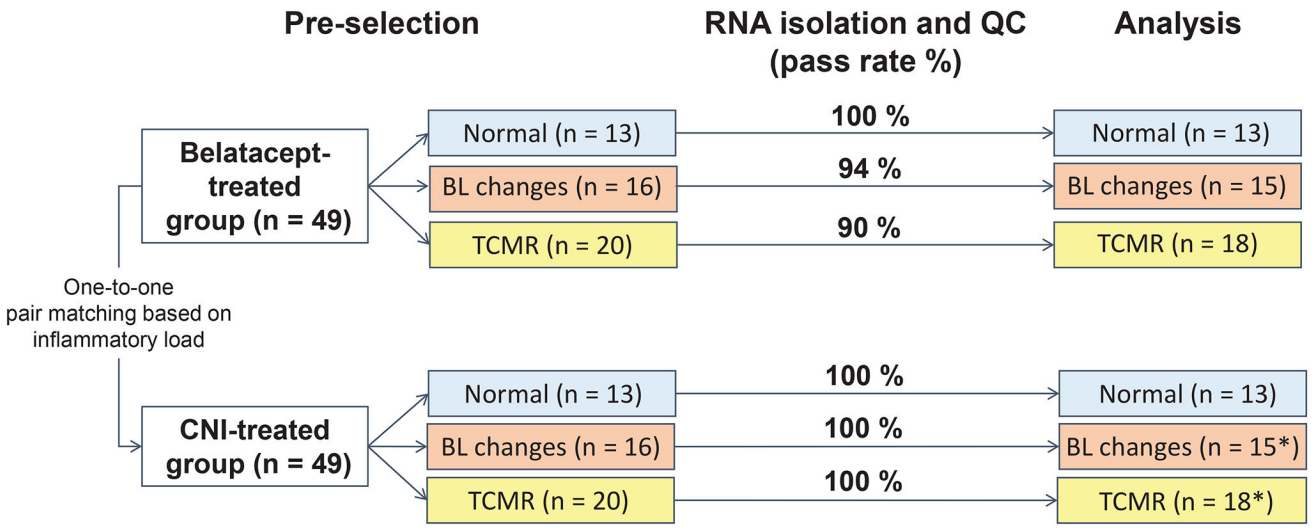

Figure 1.

Study design: Patients in the calcineurin inhibitor (CNI)-based treatment group were matched to patients in the belatacept treatment group on a one-to-one basis, based on inflammatory load. Pass rate column shows the percentage of samples that met the RNA quality control (QC) criteria. Note: although all samples in the CNI-based treatment group passed the RNA QC, one sample in the BL changes and two in the TCMR groups were excluded from the gene-expression analysis to maintain the one-to-one matching between the two treatment groups (asterisks).

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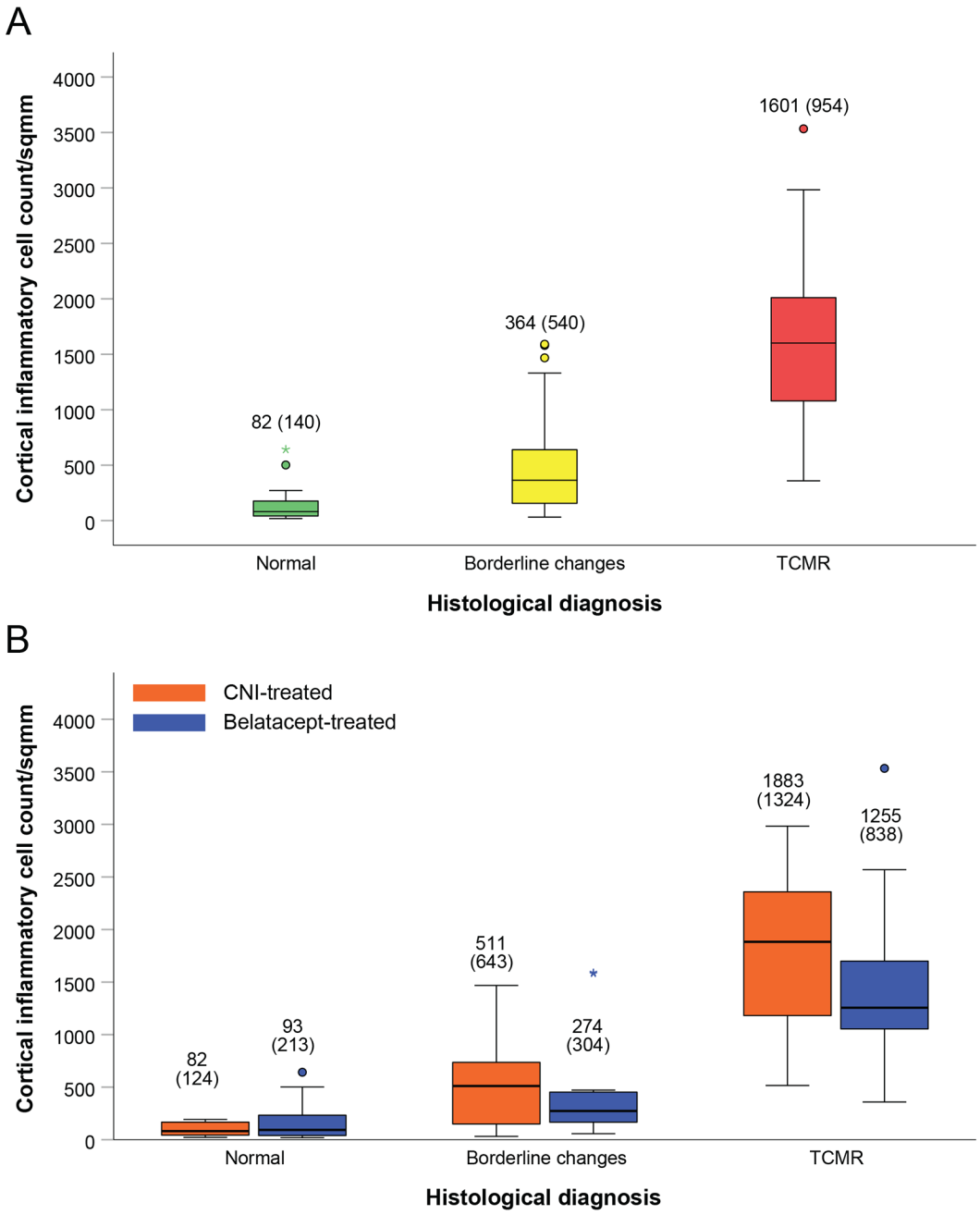

Figure 2.

Cortical inflammatory cell counts (cICC) of the study groups. Pairwise comparisons between the diagnostic categories (i.e. the combination of treatment groups by histological diagnosis) revealed significant differences in median cICC values between biopsies showing T cell-mediated rejection (TCMR) and normal ($p < 0.0005$), TCMR and borderline (BL) changes ($p < 0.0005$), and BL changes and normal morphology ($p = 0.023$) (Panel A). There was no significant difference between the treatment groups in the diagnostic categories of normal ($p = 0.671$), BL changes ($p = 0.792$) and TCMR ($p = 0.202$) (Panel B). Median cICC values for each group is on top of the boxplot, interquartile ranges are in brackets.

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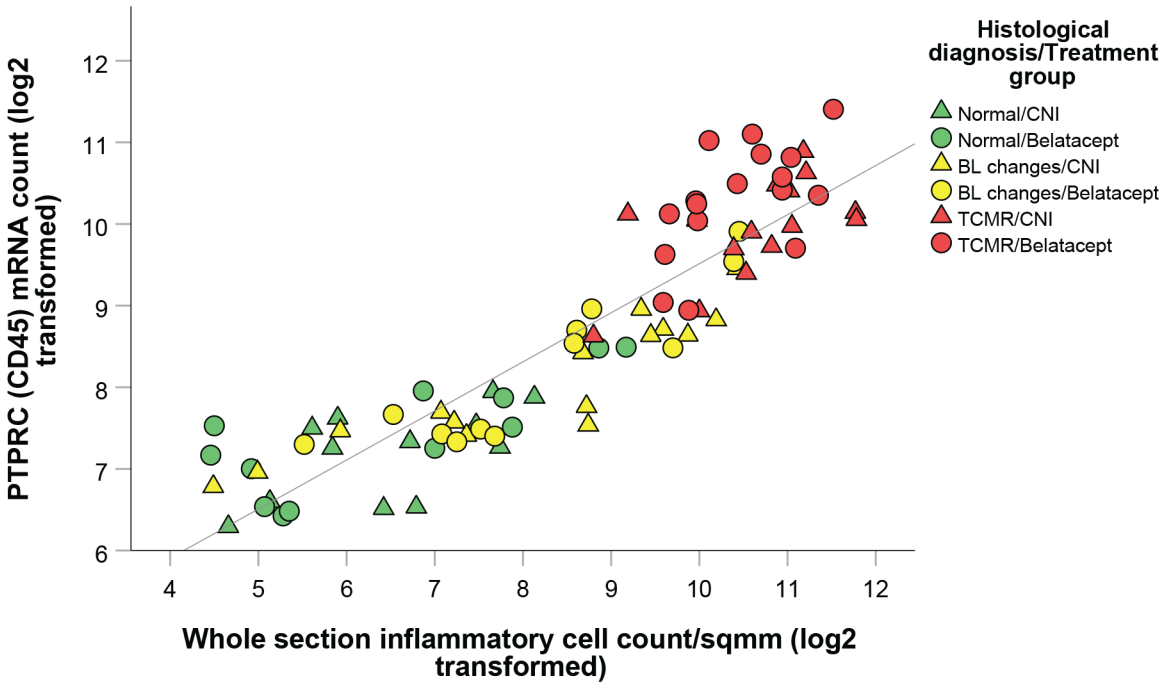

Figure 3.

There was an excellent correlation between *PTPRC* gene transcript abundance, and whole section inflammatory cell count/mm2 (ICC, Spearman's rho = 0.918, $p < 0.0005$). Whole section ICC was calculated by digitally identifying and counting CD45 antigen positive cells on whole slide digital images. Normalized PTPRC mRNA count also significantly predicted whole section ICC ($F(1, 81) = 369.031$, $p < 0.0005$). The former variable accounted for 82 % of the variation in the latter variable, with adjusted $R^2 = 81.8\%$. The regression equation was: $\log_2 ICC = -3.233 + (1.364 \times \log_2 PTPRC)$, where ICC is whole section inflammatory cell count and *PTPRC* is normalized PTPRC transcript count.

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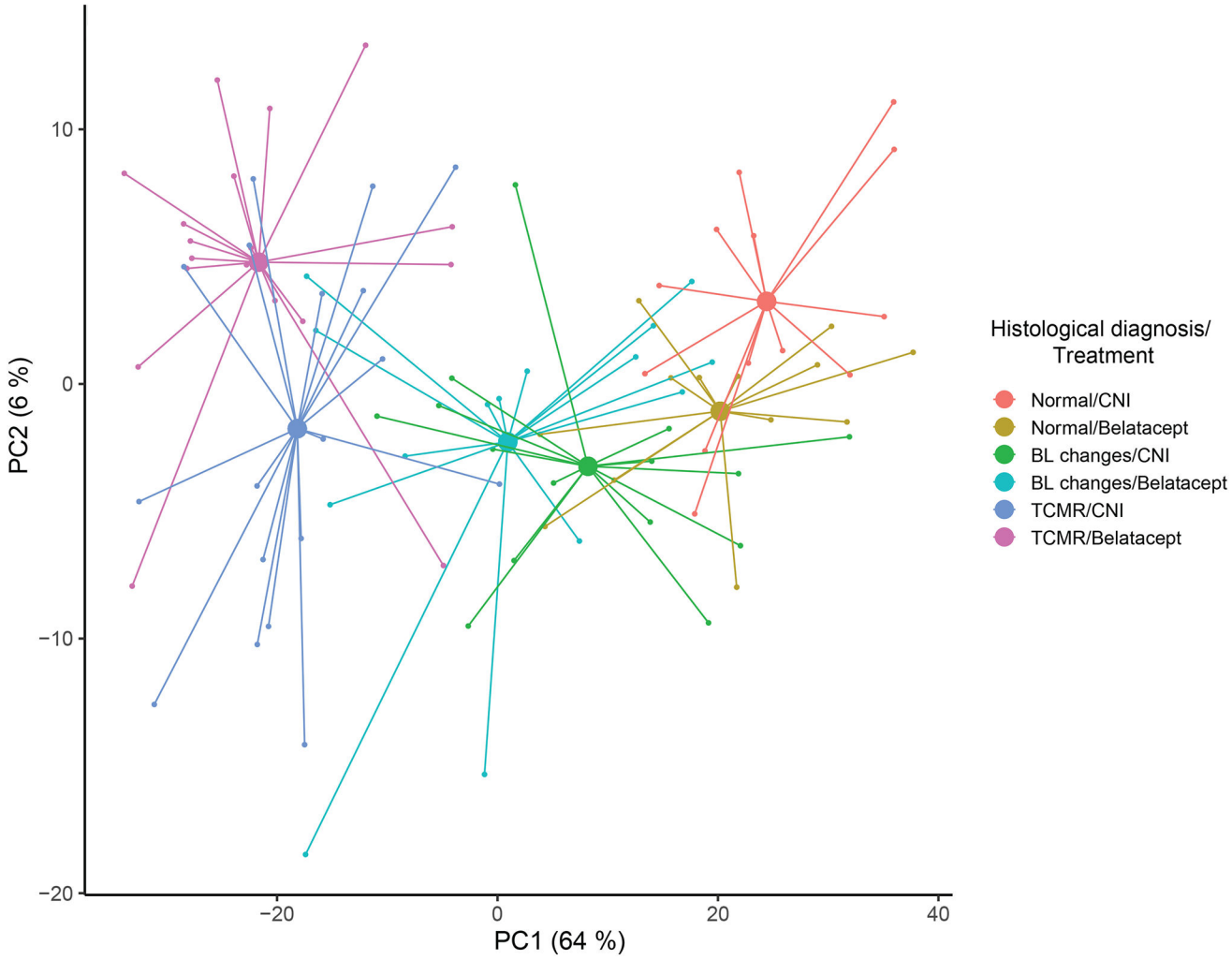

Figure 4.

Principal component analysis shows that histological diagnosis explains the most variance in the gene expression dataset (principal component 1, 64 %). Subjects with T cell-mediated rejection (TCMR) were segregated from subjects with normal morphology irrespective of the treatment group they belonged to ($p = 0.001$ by PERMANOVA). Biopsy samples displaying borderline changes fell in between the two groups. Principal component 2 separates the TCMR samples based on treatment group. Each small node represents a distinct sample, while the large nodes are the mean centroids of a given treatment/diagnostic group.

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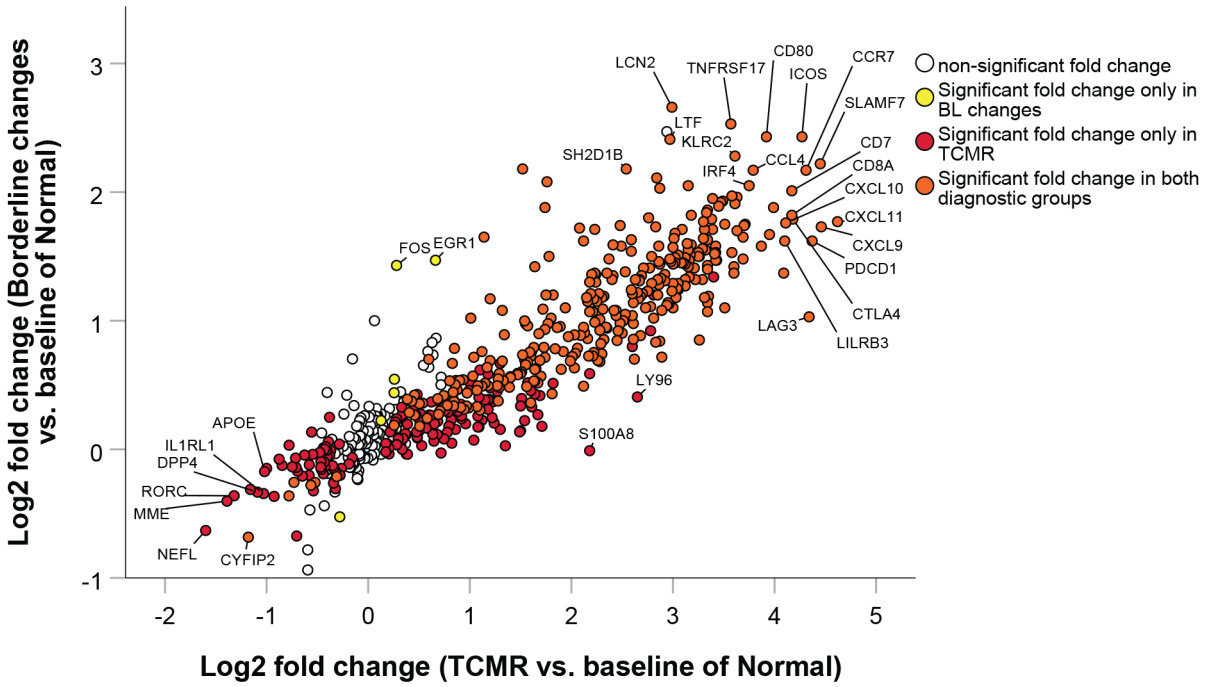

Figure 5.

Log_2fold change in intragraft gene expression in subjects with borderline (BL) changes (y-axis) or with T cell-mediated rejection (TCMR) (x-axis) relative to the baseline of patients with normal biopsy morphology. Genes that were significantly differentially expressed in both models are highlighted in orange, those that were only significant in BL changes vs normal are highlighted in yellow, genes only significant in TCMR vs normal are highlighted in red, while genes that did not show significant change in the models are left blank.

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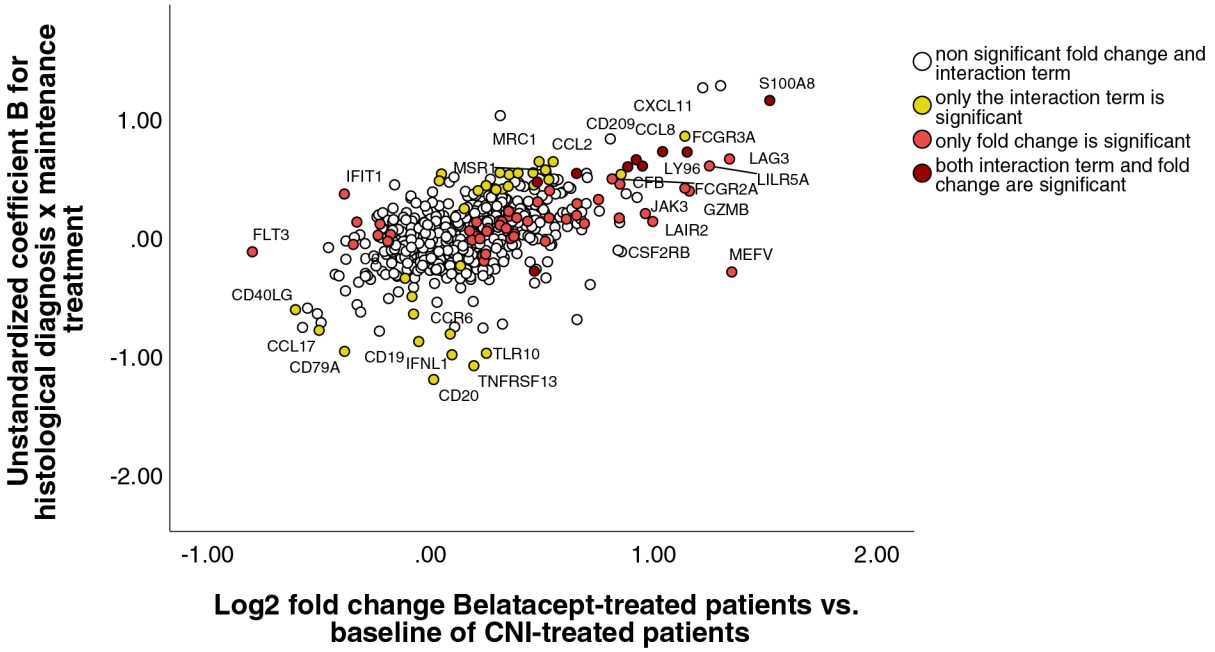

Figure 6.

Coefficient B for the interaction between histological diagnosis and maintenance treatment on gene expression (y-axis) plotted against $\log_2$fold change in gene expression in subjects treated with belatacept relative to the baseline of patients who received a CNI-based regimen (x-axis). Those genes whose transcript abundance change across diagnostic categories was significantly associated with belatacept treatment (i.e. the slope coefficient for the interaction term between histological diagnosis and maintenance treatment in the general linear model was significant) and were also significantly differentially expressed in belatacept-treated patients *vs* baseline of CNI-treated patients are highlighted with red. Those that were characterized by a significant slope coefficient only are highlighted in yellow, while genes that showed only significant differential expression are orange. Genes with non-significant differential expression and slope coefficient are left blank.

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Table 1.

Clinical, treatment, and pathologic characteristics of the two maintenance treatment groups.

	CNI-based maintenance treatment (n = 46)	Belatacept-based maintenance treatment (n = 46)	p-value
Recipient age (years)	42.50 (28.50)	53 (20.50)	0.051
Recipient female gender (n)	22	13	0.085
Form of transplantation (n)			
deceased donor	27	22	0.577
living related	10	13	
living unrelated	9	11	
Recipient race, non-black (n)	36	41	0.259
HLA mismatch (HLA-A, -B, -C, DR)			
> 0	44	43	1.000
cPRA			
> 0 %	26	20	0.286
DSA			
negative (n)	32	33	1.000
positive (class I, class II, or both)	4	3	
Time from transplantation to biopsy (months)	6.5 (2.63)	6 (5.25)	0.101
Induction therapy			
Ant-thymocyte globulin	23	36	***0.009***
Anti-IL-2Rα antibody	23	10	
Maintenance therapy (in addition to either CNI or CTLA4-Ig)			
MMF/MPA or mTOR inhibitor (n)	46	44	0.495
Glucocorticoids	41	39	0.758
eGFR^{l} level (ml/min/1.73 m^2)			
All patients per treatment group	60 (22)	50.5 (22.5)	0.086
Only patients with Normal biopsy morphology	71 (29)	68 (33)	0.960
Only patients with borderline changes	60 (5)	51 (22)	0.101
Only patients with TCMR	47 (31.5)	40 (23.25)	0.214
Histological diagnoses			
Normal	13	13	1.000
Borderline changes	15	15	
TCMR	18	18	
grade I	16	12	0.228
grade II or III	2	6	
accompanying active ABMR	3	4	1.000
Banff scores			
glomerulitis (g)	0 (0)	0 (0.25)	0.199
peritubular capillaritis (ptc)	0 (0)	0 (1)	0.183

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	CNI-based maintenance treatment (n = 46)	Belatacept-based maintenance treatment (n = 46)	p-value
interstitial inflammation in non-scarred cortex (i)	1 (2)	1 (2)	0.603
interstitial inflammation in scarred cortex (i-IF/TA)	0 (0)	0 (0)	0.325
total inflammation (ti)	1 (2)	1 (3)	0.366
tubulitis (t)	1 (2)	1 (2)	0.967
atrophic tubulitis (at)	0 (1)	1 (1)	0.157
vasculitis (v)	0 (0)	0 (0)	0.135
glomerular double contouring (cg)	0 (0)	0 (0)	0.317
interstitial fibrosis (ci)	0 (0.25)	0 (1)	0.451
tubular atrophy (ct)	1 (1)	1 (1)	0.836
intimal fibrosis (cv)	0 (0)	0 (1)	0.075
arteriolar hyalinosis (ah)	0 (0)	0 (0)	0.882

[a] calcineurin inhibitor,

[b] interquartile ranges are in brackets,

[c] human leukocyte antigen,

[d] calculated panel reactive antibody titer,

[e] donor-specific antibody,

[f] anti-interleukin 2 receptor alpha antibody,

[g] mycophenolate mofetil,

[h] mycophenolic acid,

[i] mechanistic target of rapamycin,

[j] estimated glomerular filtration ratio,

[k] T cell-mediated rejection,

[l] antibody-mediated rejection