

CD57⁺ CD4 T Cells Underlie Belatacept-Resistant Allograft Rejection

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Belatacept is a B7-specific fusion protein used to prevent allograft rejection by blocking T cell costimulation. Generally efficacious, it fails to prevent acute rejection in a sizable minority of patients. In experimental models, memory T cells mediate costimulation blockade-resistant rejection (CoBRR), but this remains undefined in humans. To explore relationships between individual patients' immune cell phenotypes and CoBRR, we studied patients receiving belatacept or conventional calcineurin inhibitor-based immunosuppression. We identified a population of CD57⁺PD1⁻ CD4 T cells present prior to transplantation that correlated with CoBRR. Contrary to data recognizing CD57 as a marker of senescence on CD8 T cells, we discovered a nonsenescent, cytolytic phenotype associated with CD57 on CD4 T cells. Moreover, CD57⁺ CD4 T cells expressed high levels of adhesion molecules implicated in experimental CoBRR, were CD28⁻, expressed a transcriptional phenotype broadly defining allograft rejection and were shown to be present in rejecting human kidney allografts. These data implicate CD57⁺ CD4 T cells in clinical CoBRR. If prospectively validated, this characteristic could identify patients at higher risk for acute rejection on belatacept-based therapy.

Abbreviations: ACR, acute cellular rejection; BRR, belatacept-resistant rejection; CNI, calcineurin inhibitor; CoB, costimulation blockade; CoBRR, costimulation blockade-resistant rejection; DC, dendritic cell; IFN- γ , interferon γ ; IPA, ingenuity pathway analysis; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; TNF- α , tumor necrosis factor α ; VPD450, Violet Proliferation Dye 450

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Introduction

Kidney transplantation is a standard life-saving therapy, but organ survival necessitates the use of immunosuppressive drugs. These drugs render a transplant recipient immune compromised, and the degree of compromise, in large part, determines the clinical risk of the procedure. As such, clinical immunosuppression has evolved through the development of increasingly targeted drug therapies to successfully manipulate the immune response toward an allograft without overly impairing the recipient's protective immune capacity. Following kidney transplantation, most patients receive calcineurin inhibitors (CNIs; e.g. tacrolimus), which suppress T cell function through inhibition of a ubiquitous intracellular signaling pathway. This leads to very effective, nonspecific T cell immunosuppression and a substantial decrease in acute rejection rates that comes at the expense of impaired protective immunity, particularly to viruses and fungi (1–4); it also precipitates chronic CNI-associated nonimmune nephrotoxic and metabolic side effects (5,6). This dilemma has impelled the development of more specific, targeted therapeutics to prevent rejection without the complications observed with CNIs, the most prominent of which is belatacept.

Belatacept, a fusion protein targeting a specific extracellular costimulation pathway (the CD28-B7 receptor ligand pair), was developed as a potential replacement for CNIs. Belatacept binds to CD80 and CD86 with high affinity, preventing their binding to the critical T cell costimulatory receptor CD28—a mechanism now referred to as *costimulation blockade* (CoB) (7). TCR ligation in the absence of costimulation is generally ineffective in activating naïve, antigen-specific T cells, such that CoB substantially impairs *de novo* alloimmune responses. Antigen-experienced T cells often have reduced requirements for costimulation, and thus CoB can have a sustained inhibitory effect on new immune encounters without impairing previously established protective immunity.

Clinical trials evaluating the efficacy of belatacept-based immunosuppression demonstrated improved side effect profiles, graft function, and patient and graft survival up

to 5 years after transplant compared with patients receiving CNi-based immunosuppression (8–10). Unfortunately, patients treated with belatacept experienced more severe and higher rates of acute cellular rejection (ACR) compared with patients treated with CNIs (9,10). This CoBRR has limited the clinical use of belatacept despite its substantially reduced side effect profile. Given the highly specific nature of belatacept's action, we sought to investigate whether the immune cell profile of a patient at the time of transplant, particularly the degree to which the patient has progressed from a naïve to antigen-experienced cell phenotype, can be assessed to identify patients at risk of CoBRR. We found that the CD57⁺ CD4 T cell, a particular cell type that is atypical in healthy adults but common in patients with kidney failure, is associated with belatacept-resistant rejection (BRR) and worthy of further study.

Materials and Methods

Sample acquisition

Kidney allograft recipients receiving belatacept or tacrolimus according to labeled indication were enrolled in an institutional review board–approved tissue acquisition protocol at the Emory Transplant Center. Collection and use of patient blood samples for laboratory analysis was approved by the institutional review board at Emory University (approval IRB00006248). Written informed consent was received from participants prior to inclusion in each study. Peripheral blood mononuclear cells (PBMCs) were collected prior to transplantation and at multiple time points after transplantation. Patients were followed clinically and segregated by outcome for analysis. For this study, we performed a retrospective analysis of stored patient samples. All belatacept-treated patients enrolled who had not received a prior transplant and who had baseline samples available for analysis were included in this study. Within 7 months of transplantation, of the 14 patients receiving belatacept-based therapy, nine patients experienced ACR and five were rejection-free. Ten tacrolimus-treated patients, five that experienced ACR and five that were rejection-free, were selected and had demographics similar to the belatacept-treated patients for comparison. Patient demographics and additional supporting information may be found in the online version of this article.

Flow cytometry and intracellular cytokine staining

PBMCs obtained prior to drug administration were analyzed by flow cytometry to characterize the immune cell repertoire at baseline, interrogating for markers of memory, differentiation, activation, exhaustion, and senescence. Antibodies were used against CD2 (62300; BD Biosciences, San Jose, CA), CD3 (557943; BD Biosciences), CD4 (317435; BioLegend, San Diego, CA), CD8 (47-00878-42; eBioscience, San Diego, CA), LFA1 (563936; BD Biosciences), CD28 (302930; BioLegend), VLA4 (304314; BioLegend), CD57 (555619; BD Biosciences) and PD1 (329906; BioLegend). Intracellular staining for Ki67 (556026; BD Biosciences) and granzyme B (562462; BD Biosciences) was done using BD Biosciences Fixation/Permeabilization Solution Kit. Intracellular staining for interferon γ (IFN- γ ; 562392; BD Biosciences) and tumor necrosis factor α (TNF- α ; 340534; BD Biosciences) was done following 4-h stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A (Sigma-Aldrich, St. Louis, MO). All samples were run using BD Biosciences LSRFortessa and analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

Polymerase chain reaction

Analysis of patient telomere length was performed on QuantStudio (Life Technologies; Thermo Fisher Scientific, Waltham, MA) with iTaq Sybr (Bio-Rad, Hercules, CA). Reference DNA was generated by the combination of five healthy control participants and was used to produce a standard curve to determine relative telomere:single copy gene ratio (11,12).

Microarray

Peripheral blood obtained from five healthy control participants was processed and sorted on a BD FACSAria cell sorter (BD Biosciences) into CD3⁺CD4⁺CD8⁻CD57⁺PD1⁻ and CD3⁺CD4⁺CD8⁻CD57⁻PD1⁻ populations. Antibodies were used against CD3 (560366; BD Biosciences), CD4 (557852; BD Biosciences), CD8 (47-0088-42; eBioscience), CD57 (555619; BD Biosciences) and PD1 (329906; BioLegend). Total RNA was extracted from the sorted populations, and quality was assessed. Synthesis and amplification of cDNA was performed, and fragmented and biotinylated samples were hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA). The arrays were scanned, and probe intensity measurements were normalized across the samples using the robust multichip average algorithm (Gene Expression Omnibus accession GSE64805).

Immunohistochemistry

Kidney allograft biopsies were obtained per protocol time point or suspected rejection. A total of 36 biopsies were dual stained for anti-CD57 (DAKO, Carpinteria, CA) and anti-CD4 (DAKO). Blinded analysis was performed to determine cell density and localization.

Allogeneic mixed lymphocyte reaction

PBMCs were obtained by cytopheresis of healthy volunteers who gave their informed written consent. PBMCs were isolated by Ficoll-Paque density-gradient centrifugation (GE Healthcare, Little Chalfont, UK), and monocytes and lymphocytes were isolated by positive CD14 or CD3 selection (purity >95%; Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were then plated and cultured, as described previously, with 25 ng/mL recombinant human IL-4 (Immuno Tools, Friesoythe, Germany) and 1000 IU/mL recombinant human granulocyte-macrophage colony stimulating factor (Peprotech, Rocky Hill, NJ). At day 6, immature dendritic cells (DCs) were characterized by staining for CD14 and CD209 and stimulated for an additional 48 h with lipopolysaccharide (50 ng/mL; Sigma-Aldrich) (activated DCs). For proliferation assay, lymphocytes were stained with 1 μ M Violet Proliferation Dye 450 (VPD450; BD Biosciences) in Dulbecco's phosphate-buffered saline for 10 min at 37°C and washed two times in complete medium and added to activated allogeneic DCs (DCs:CD3⁺ T cells ratio 1:3) with or without belatacept (25 μ g/mL). After 7 days of coculture, VPD450 dilution and phenotype were analyzed by fluorescence-activated cell sorting. Antibodies were purchased from BD Biosciences (V450-labeled anti-CD14, APC-labeled anti-CD209, APC-Cy7-labeled anti-CD3, BUV395-labeled anti-CD4, PerCP-Cy5.5-labeled anti-CD8, BV605-labeled anti-CCR7, PE-labeled anti-CD57, APC-labeled anti-27, PE-Cy7-labeled anti-CD38) or from Miltenyi Biotec (fluorescein isothiocyanate-labeled anti-CD45RO). Flow cytometry data were recorded with an LSRFortessa cell analyzer (BD Biosciences) and analyzed with Diva software (BD Biosciences).

Statistics

Differences in percentage of CD57⁺PD1⁻ CD4 T cells were calculated using the Mann-Whitney test due to the bimodal distribution of this cell subset. For additional flow cytometry analyses, p-values were calculated using unpaired t-tests, linear regressions and one- and two-way ANOVA, and r values were computed using Pearson correlation coefficients. For the microarray, a paired t-test was used on the normalized expression values to find differentially expressed genes in CD57⁺ versus CD57⁻

cells. For immunohistochemistry, significant differences between stable and rejection outcomes were calculated using an unpaired t-test. A p-value of <0.05 was considered statistically significant.

Results

High prevalence of CD57⁺PD1⁻ CD4 T cells in peripheral blood prior to transplant is associated with belatacept-resistant rejection

It is known that the activation of antigen-experienced T cells is less costimulation-dependent relative to that of naïve cells (13,14), and molecules associated with antigen experience have been related to CoBRR (15–17). We

explored pretransplant expression of surface markers indicative of antigen experience and terminal differentiation, specifically PD1 and CD57. PD1 is upregulated following activation and maintains expression on exhausted T cells (18,19), and CD57 is a carbohydrate epitope present on replicative senescent cells (20–23). Although this has been well described in the context of CD8 T cells (24,25), our study identified a population of CD4 T cells in the peripheral blood of transplant patients receiving belatacept-based immunosuppression that were CD57⁺PD1⁻ and that correlated with BRR, regardless of memory status (Figure 1B). No significant differences in outcome were observed in any subset of PD1- or CD57-expressing CD8 T cells (Figure 1C).

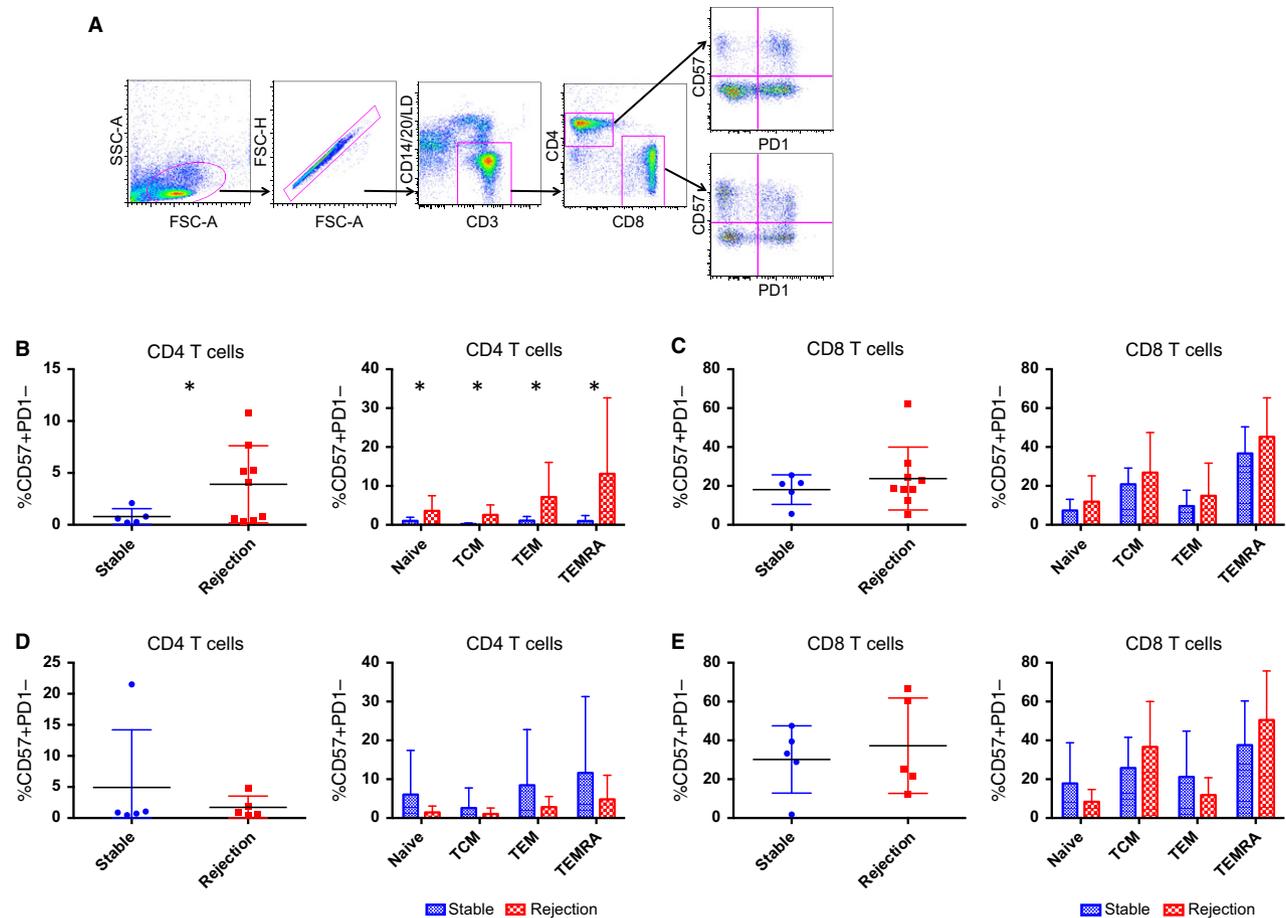


Figure 1: CD57⁺PD1⁻ CD4 T cells in peripheral blood associate with belatacept-resistant rejection. (A) Representative flow plot of the gating strategy to identify CD57⁺PD1⁻ T cells. (B) The left panel shows that baseline PBMCs from patients treated with belatacept who went on to experience acute cellular rejection had a significantly higher prevalence of CD57⁺PD1⁻ CD4 T cells compared with those from patients with stable outcome (p < 0.03). The right panel shows that CD57⁺PD1⁻ CD4 T cells were not segregated to terminally differentiated effector memory cells but were significantly increased in patients with rejection compared with patients with stable outcome in all memory compartments based on CD45RA and CCR7 expression (p < 0.03). (C) The left panel shows that the percentage of CD57⁺PD1⁻ CD8 T cells did not segregate with outcome in belatacept-treated patients. The right panel demonstrates that the lack of association was true regardless of memory subset. (D and E) Analysis of baseline PBMCs from patients treated with tacrolimus revealed no correlation between outcome and prevalence of CD57⁺PD1⁻ CD4 or CD8 T cells. PBMC, peripheral blood mononuclear cell; TCM, T central memory cells; TEM, T effector memory cells; TEMRA, T effector memory cells RA positive. *p < 0.03.

To distinguish whether this was specific to BRR or a characteristic of rejection in general, we studied patients treated with conventional tacrolimus-based immunosuppression. Using the same parameters, no significant difference between outcome and the expression of CD57 or PD1 was elucidated in CD4 or CD8 T cells (Figure 1D and E). These data demonstrate that CD57 expression on CD4 T cells in peripheral blood prior to transplant is associated with rejection specifically in belatacept-treated patients. We found this population of cells to be rare in healthy control participants but significantly increased in patients with kidney failure (Figure 2), increasing its unique relevance to kidney transplantation.

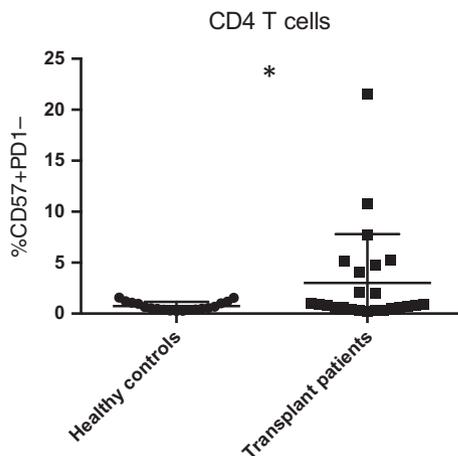


Figure 2: CD57⁺PD1⁻ CD4 T cells are atypical in healthy control participants. The frequency of CD57⁺PD1⁻ CD4 T cells was significantly increased in PBMCs of patients with kidney failure awaiting transplant compared with PBMCs of healthy participants ($p < 0.05$), highlighting the unique relevance of this cell type to kidney transplantation. PBMC, peripheral blood mononuclear cell. * $p < 0.05$.

CD57⁺ CD4 T cells are not senescent by traditional indices

CD57 has been described as a marker of immune cell senescence, a phenotype that would be unlikely to segregate with an active process like allograft rejection; therefore, we used independent assays to ascertain whether this was true in our cell population of interest. First, we performed polymerase chain reaction analysis of relative telomere length in the same patient cohort described above. Terminally differentiated cells that have undergone multiple divisions have shortened telomeres (26), and that should correlate with increased CD57 expression. In the patients treated with belatacept, expression of CD57 on CD4 T cells did not correlate with relative telomere length (Figure 3A). This lack of correlation indicated a possible nontraditional role of CD57 on CD4 T cells. Second, we looked at expression of Ki67, a protein strictly associated with cell proliferation (27). Increased expression of CD57 on CD4 T cells in belatacept-treated patients significantly correlated with increased Ki67 expression ($p < 0.01$) (Figure 3B), also inconsistent with cell senescence. The nonsenescent phenotype of CD57⁺ CD4 T cells in this setting contradicts the paradigm of CD57 expression and senescence.

The gene profile of CD57⁺PD1⁻ CD4 T cells is highly associated with allograft rejection

To gain insight into the function of CD57⁺PD1⁻ CD4 T cells, we performed a microarray to examine their gene expression profile. PBMCs from healthy control participants were sorted into CD57⁺PD1⁻ and CD57⁻PD1⁻ CD4 T cells and analyzed by microarray. A heat map of normalized gene expression revealed a distinct transcriptome associated with CD57⁺ compared with CD57⁻ CD4 T cells (Figure 4A). The most upregulated genes were indicative of a highly cytotoxic profile and included molecules involved in activation, adhesion, cell trafficking and cytotoxicity. We then imported the gene expression profiles into an independent ingenuity pathway analysis (IPA), an unsupervised analysis of >600 biological

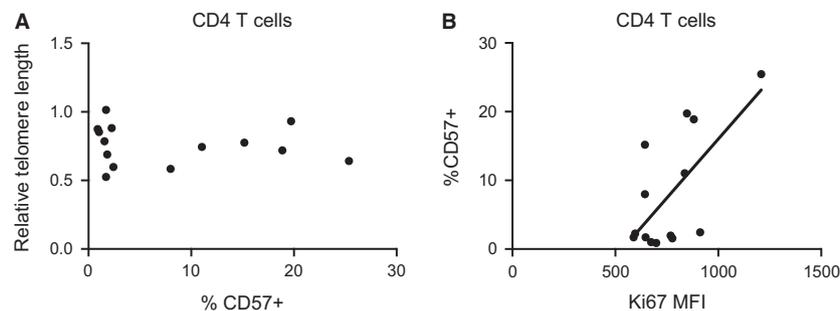


Figure 3: CD57⁺ CD4 T cells are not senescent based on traditional indices. (A) Relative telomere length of belatacept-treated patients was determined by polymerase chain reaction and did not correlate with CD57 expression on CD4 T cells in the same belatacept-treated patients. (B) Ki67 expression significantly correlated with increased expression of CD57 on CD4 T cells in belatacept-treated patients ($p < 0.01$, $r = 0.6704$). MFI, mean fluorescence intensity.

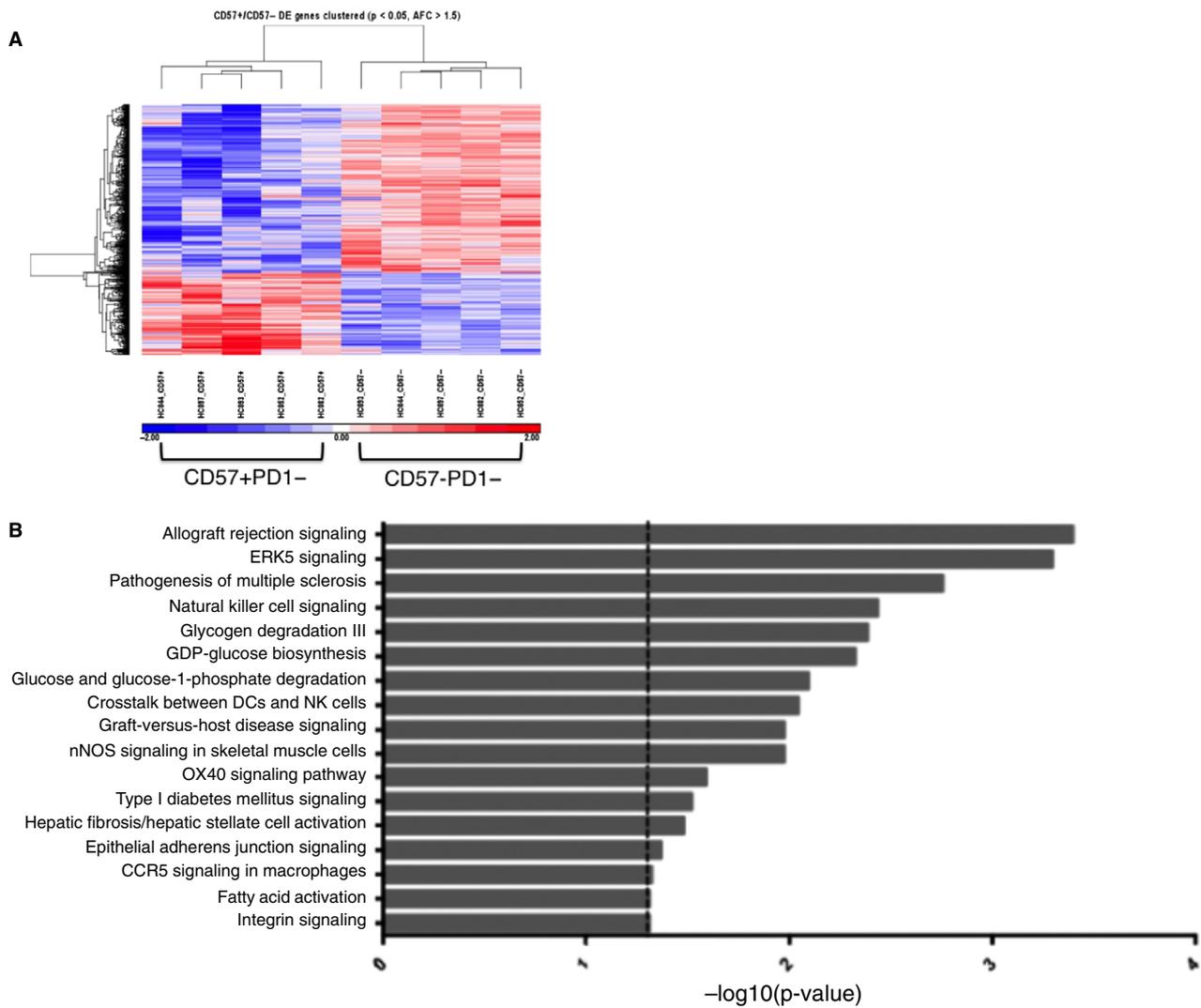


Figure 4: Genes involved in allograft rejection are upregulated in CD57⁺ CD4 T cells. (A) PBMCs from healthy control participants (n = 5) were sorted into CD57⁺PD1⁻ and CD57⁻PD1⁻ CD4 T cells. RNA was extracted, cDNA synthesis and amplification was performed, and samples were hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array. Clear segregation of gene expression was observed in CD57⁺ compared with CD57⁻ CD4 T cells. (B) Ingenuity pathway analysis deemed allograft rejection signaling the most closely associated signaling pathway with the genes upregulated in CD57⁺ compared with CD57⁻ CD4 T cells. AFC, absolute fold change; DC, dendritic cell; GDP, guanosine diphosphate; PBMC, peripheral blood mononuclear cell.

pathways. IPA remarkably deemed allograft rejection signaling as the most closely associated signaling pathway with the genes upregulated in CD57⁺ CD4 T cells (Figure 4B).

CD57⁺ cells have increased expression of adhesion molecules

Adhesion molecules, such as CD2 and LFA1, have also been implicated in CoBRR. Their selective elimination has been shown to substantially reduce the risk of CoBRR in animal models (15–17,28). To evaluate the relationship between expression of CD57 and CD2, LFA1 and VLA4, we performed additional phenotyping on base-

line PBMCs from transplant patients. CD2^{hi}CD4 T cells were shown to express high levels of LFA1 and VLA4, with CD57 present on cells that expressed high levels of these adhesion molecules (Figure 5A). Importantly, although all CD57⁺ cells expressed high levels of CD2, LFA1 and VLA4, the converse was not true, such that CD57 was a unifying marker of increased adhesion molecule expression (Figure 5B). These results suggested that CD57⁺PD1⁻ CD4 T cells may not only serve as a marker of rejection risk in the periphery but may play an active role in mediating rejection, prompting us to determine whether these cells were present in rejecting allografts.

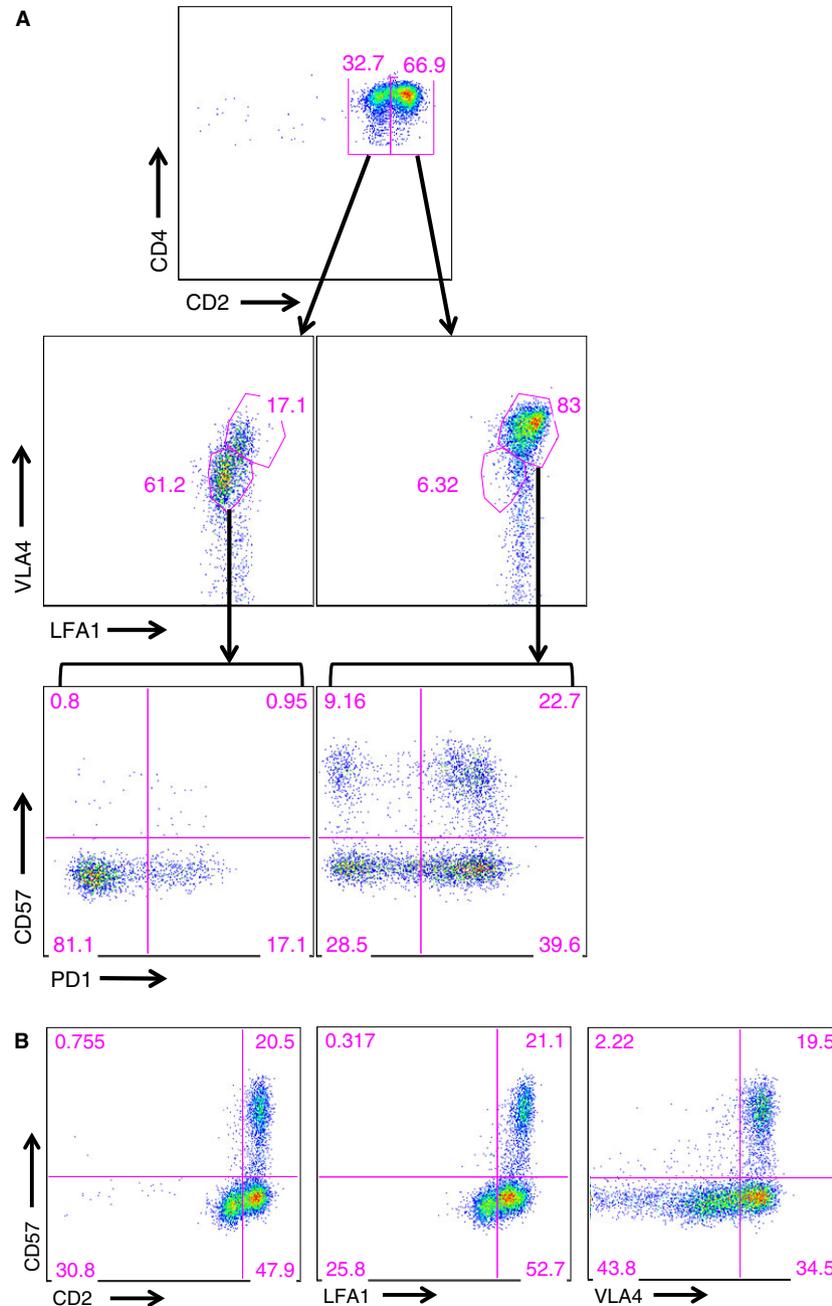


Figure 5: CD57 is present on cells with increased expression of other adhesion molecules implicated in CoBRR. (A) Representative flow cytometry plots of PBMCs from patients prior to transplantation demonstrating CD2^{hi} CD4 T cells also expressed high levels of adhesion molecules LFA1 and VLA4, whereas CD2^{lo} CD4 T cells expressed low levels of LFA1 and VLA4. CD57⁺ CD4 T cells were found exclusively in the CD2^{hi}LFA1^{hi}VLA4^{hi} subset. (B) Representative flow cytometry plots of PBMCs from the same patients prior to transplantation validated that all CD57⁺ CD4 T cells expressed high levels of CD2, LFA1, and VLA4. CoBRR, costimulation blockade-resistant rejection; PBMC, peripheral blood mononuclear cell.

There is an increased density of CD57⁺ T cells in rejecting kidney allografts

Clinical kidney allograft biopsies were obtained either for protocol surveillance or at the time of suspected rejection and were dual stained for CD4 and CD57 (Fig-

ure 6A). CD57⁺ CD4 T cells were present in rejecting kidney allografts at a higher density compared with stable kidney allografts (Figure 6B). In fact, patients with a higher percentage of circulating CD57⁺PD1⁻ CD4 T cells prior to transplantation also had an increased

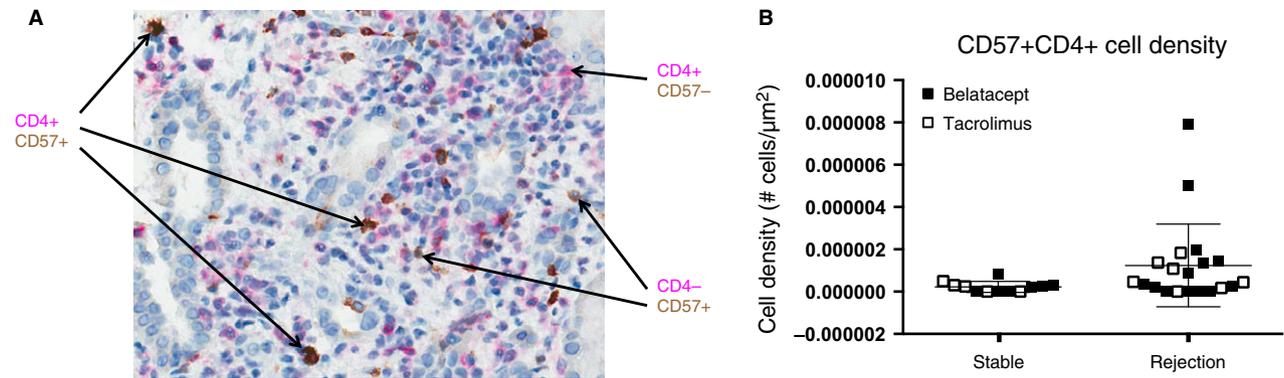


Figure 6: Increased density of CD57⁺ T cells in rejecting allografts. (A) A representative kidney allograft biopsy dual stained to show CD4 (in pink) and CD57 (in brown) expression demonstrates that CD57⁺ CD4 T cells were able to infiltrate kidney allografts. (B) Summary data of all biopsies from patients treated with belatacept (closed squares) and patients treated with tacrolimus (open squares) demonstrated that the density of CD57⁺ CD4 T cells in kidney biopsies collected at the time of rejection was greater than that of cells in patient biopsies obtained at a stable protocol time point ($p = 0.08$).

density of CD57⁺ CD4 T cells in rejecting allografts. Consequently, this population of cells is not only present in the peripheral blood but also is capable of infiltrating the graft, likely due to the increased expression of adhesion molecules.

CD57⁺ CD4 T cells are CD28⁻ and exhibit cytolytic properties

Multiple groups have shown that CD28 loss is associated with CoBRR (29,30), but loss of CD28 did not statistically associate with BRR in our study. Although we found an inverse correlation between CD57 and CD28 expression (Figure 7A), not all CD28⁻ cells were CD57⁺ (Figure 7B). *In vitro* studies revealed that CD57⁺PD1⁻ CD4 T cells are almost exclusively polyfunctional, producing both IFN- γ and TNF- α following stimulation with PMA and ionomycin (Figure 7C), whereas CD28⁻PD1⁻ CD4 T cells were largely incapable of producing either IFN- γ or TNF- α (Figure 7D). Furthermore, all CD57⁺ CD4 T cells expressed high levels of granzyme B compared with CD57⁻ CD4 T cells, which express low levels of granzyme B (Figure 7E). Although loss of CD28 perhaps identified cells indifferent to CD28⁻ B7 blockade by belatacept, CD57 expression identified those CD28⁻ cells capable of actively mediating BRR.

CD57⁺ CD4 T cells are resistant to the immunosuppressive effects of belatacept

To determine whether CD57⁺ CD4 T cells are resistant to the immunosuppressive effects of belatacept, CD3 T cells from healthy volunteers were labeled with VPD450 and cocultured with activated allogeneic DCs. After 7 days in culture in the presence or absence of belatacept, cell subset proliferation was assessed by flow cytometry. Both CD57⁺ and CD57⁻ CD4 T cells were able to proliferate in response to activated DCs in the absence of belatacept (Figure 8A). Although proliferation

of CD57⁻ CD4 T cells was significantly inhibited in the presence of belatacept, proliferation of CD57⁺ CD4 T cells remained uninhibited (Figure 8B and C), confirming that CD57⁺ CD4 T cells are activated in a costimulation-independent fashion.

Discussion

The search for a suitable alternative to CNI-based immunosuppression has become increasingly relevant as long-term outcomes and toxicities have gained prominence in clinical transplantation. Early results with belatacept, although encouraging with regard to graft function and off-target side effects, have been disappointing with regard to the ability to prevent early acute rejection. This has impelled an explicit look for mechanistic correlates of belatacept resistance and, in particular, a means of risk stratification for patients to guide immunosuppressive regimen choice. We chose to investigate variability in cell surface phenotype, recognizing that individual lymphocyte repertoire evolves considerably over time as a result of environmental pathogen exposure; these changes markedly alter cells' costimulation requirements, including expression of and dependence on CD28 (31–34). These characteristics have been shown experimentally to mollify the efficacy of CoB-based regimens. In this series of experiments, we showed this to be potentially relevant to humans and characterized a particular cell type that is prevalent in renal failure patients, the CD57⁺PD1⁻ CD4 T cell, the presence of which correlates with the risk of CoBRR. This cell type is present within rejecting allografts, and its characteristics relate to the known mechanisms of cellular allograft rejection and costimulation resistance. It is important to note that there are several mechanisms of allograft rejection, and the prevalence of CD57⁺PD1⁻ CD4 T cells demonstrates a

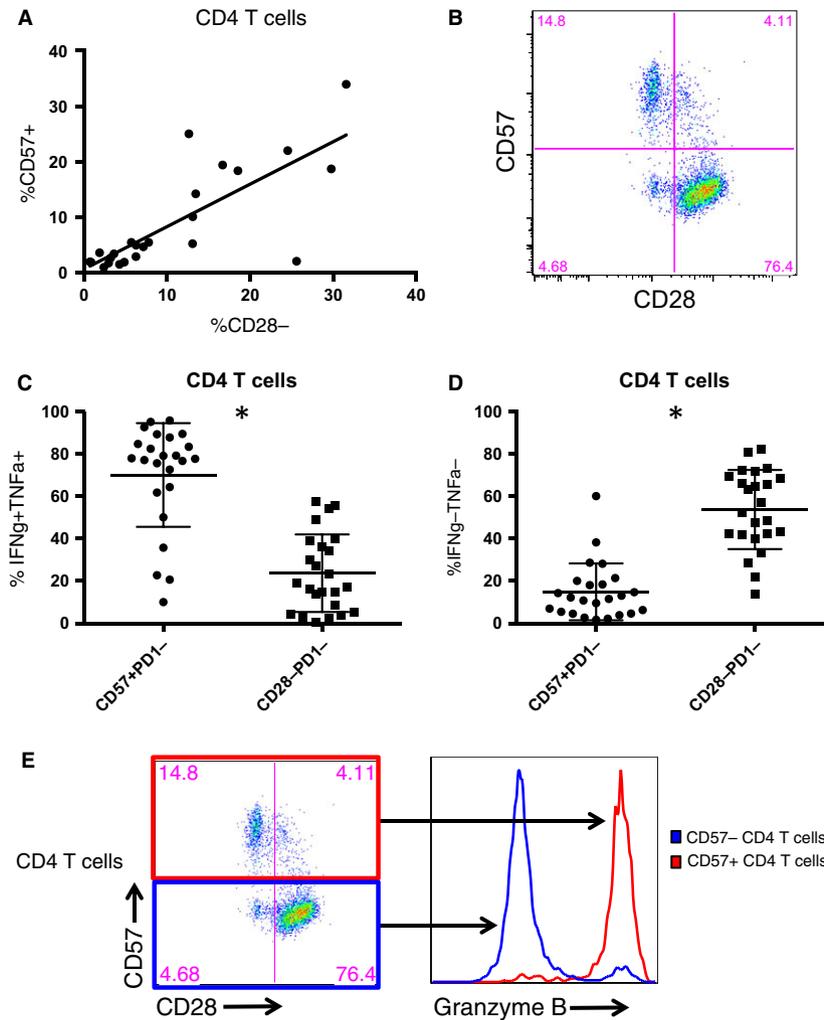


Figure 7: CD57⁺ CD4 T cells are polyfunctional with a cytolytic profile. (A) Summary data for all patient samples (n = 24) showed an inverse correlation of CD57 and CD28 expression on CD4 T cells at baseline, prior to transplantation ($p < 0.01$, $r = 0.7759$). (B) Representative flow plot demonstrating CD57 and CD28 were not expressed in a mutually exclusive manner. (C) Baseline PBMCs from the same patients were stimulated with PMA and ionomycin. Following stimulation, most CD57⁺PD1⁻ CD4 T cells produced both IFN- γ and TNF- α ($p < 0.01$). (D) Following stimulation with PMA and ionomycin, most CD28⁺PD1⁻ CD4 T cells from the same patients' baseline PBMCs were incapable of producing either IFN- γ or TNF- α ($p < 0.01$). (E) Representative flow and histogram demonstrating that CD57⁺ CD4 T cells expressed high levels of granzyme B (in red), whereas CD57⁻ CD4 T cells expressed low levels of granzyme B (in blue). IFN- γ , interferon γ ; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; TNF- α , tumor necrosis factor α .

potential additional, nonexclusive mechanism of CoBRR to be aware of in this population.

Although CD57⁺ CD4 T cells have been previously associated with cell senescence in HIV-infected patients (23,35), we have demonstrated that CD57 is a unique, unifying marker of human CD4 T cells that are enriched in patients with kidney failure and have a cytolytic profile characterized by high levels of adhesion molecules previously implicated in CoBRR. CD57⁺ CD4 T cells are capable of infiltrating allografts and producing multiple cytokines on stimulation. In this context, CD57 appears

not to be a marker of senescence but rather a potential surrogate indicator of patients' immune history as it relates to BRR. This cell population is consistent with that described previously as being prevalent in the kidney transplant patients (36). Enrichment in patients with renal failure is an additional novel observation of these studies, the mechanism of which deserves further study. Similarly, additional studies are necessary to completely define the mechanism by which these cells mediate CoBRR. Nevertheless, their association with and proximity to rejecting kidneys is clearly demonstrated in our studies.

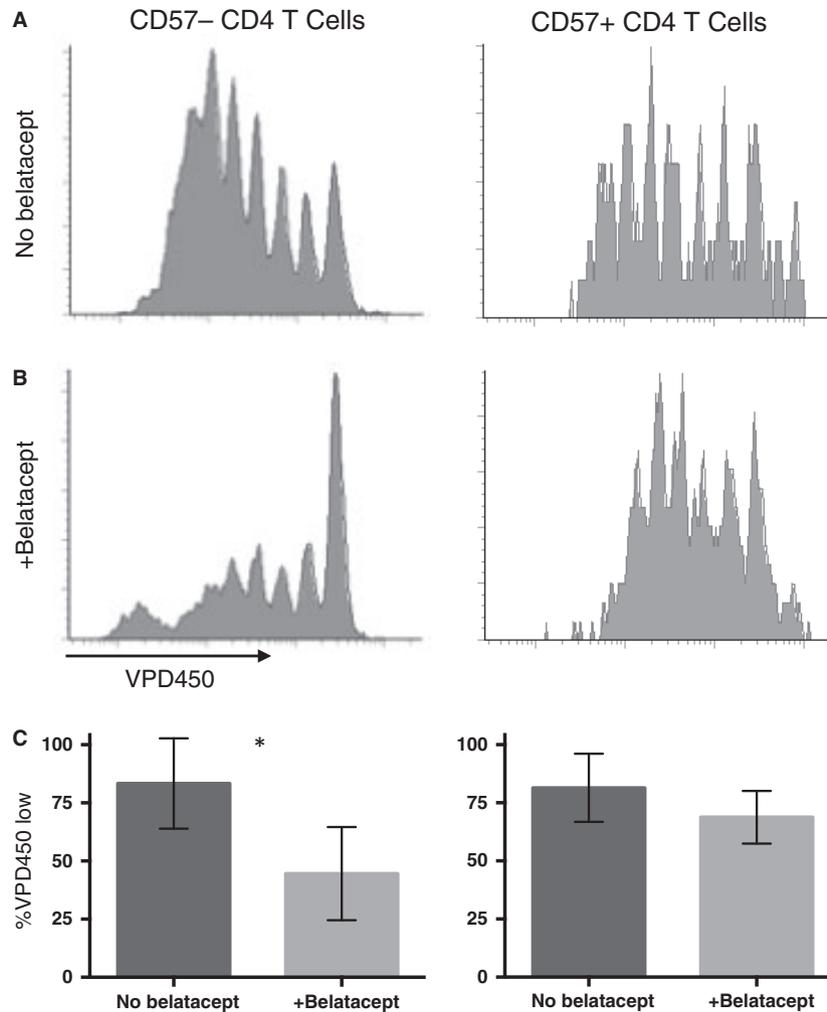


Figure 8: CD57⁺ CD4 T cells are resistant to *in vitro* immunosuppression with belatacept. (A) CD57⁻ (left panels) and CD57⁺ (right panels) CD4 T cells from healthy control participants were able to proliferate in response to activated allogeneic dendritic cells in the absence of belatacept. (B) Proliferation of CD57⁻ CD4 T cells was inhibited by belatacept, whereas CD57⁺ CD4 T cells retained the ability to proliferate in the presence of belatacept. (C) Summary data showed that proliferation of CD57⁻ CD4 T cells was significantly inhibited ($p < 0.03$) with the addition of belatacept, whereas no significant change in proliferation was observed in CD57⁺ CD4 T cells ($n = 4$). VPD450, Violet Proliferation Dye 450.

CD57⁺ CD4 T cells have been described previously in the context of transplantation. Legendre et al described a higher percentage of CD57⁺ (then called Leu7⁺) CD4 T cells in patients that experienced rejection compared with stable patients (36). That study, predating the tacrolimus era and combined with studies reported in this paper, strongly suggests this cell type is worthy of attention. Collectively, these data support the finding that CD57⁺ CD4 T cells play a role in allograft rejection, particularly in the absence of CNIs, and that prospective assessment of this population could be used to identify patients at particular risk for ACR, especially in CoB-based regimens.

Graft survival rates have improved in the past 20 years largely due to the development of targeted immunosup-

pressant drugs. Traditional immunosuppression management consists of universalized algorithms of serum drug levels and dosing regimens that are generally applied to transplant populations. With increasingly specific forms of immune manipulation, the opportunity for more personalized immune management becomes more evident and may become increasingly required. This is particularly true when selectively targeting a molecule that progressively diminishes in expression over a lifetime of immune perturbation. Although the increased risk of rejection associated with belatacept-based immunosuppression has proved a significant challenge for its clinical implementation, CD57⁺PD1⁻ CD4 T cells represent a potential therapeutic target and a practical screening tool to identify patients at higher risk for ACR on belatacept-based therapy. The ability to clearly and accurately define

markers of risk of rejection will help tailor immunosuppression therapies on an individual basis.

We acknowledge that these studies were performed in a small group of patients, albeit one that was very carefully monitored and controlled. Clinical intolerance of high rejection rates has prevented us from expanding the clinical cohort substantially. Because our clinical goal has been to provide adjuvant therapies to mitigate BRR, expanding this cohort has not been feasible. Additional studies are necessary to elucidate associations between CD57 and other costimulation molecules to potentially target and inhibit the activation of CD57⁺ CD4 T cells and phenotype these cells following transplantation. Nevertheless, we believe these observations to be sufficiently statistically supported and mechanistically plausible to warrant their reporting and to stimulate further study.

In summary, we found that expression of CD57 identifies a population of polyfunctional CD28⁻ cells with cytolytic potential. Although CD28 loss is intuitively necessary, it does not appear to be sufficient for BRR; therefore, CD28 loss may define belatacept indifference, but CD57 expression appears to identify cells mediating belatacept resistance. Importantly, this phenotype is present in the peripheral blood of patients awaiting transplant and assessing the prevalence of CD57⁺PD1⁻ CD4 T cells prior to transplantation may identify patients not suited for belatacept-based therapy.

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Disclosure

The authors of this manuscript have conflicts of interest to disclose as described by the *American Journal of Transplantation*. R. Townsend is employed by Bristol-Myers Squibb. The other authors have no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Figure S1: There was no artificial segregation of CD57+PD1– CD4 T cells ($p < 0.74$, left) or CD57+PD1– CD8 T cells ($p < 0.11$, right) prior to transplantation in patients that went on to receive belatacept compared to patients that went on to receive tacrolimus.

Figure S2: Gating strategy for cytokine staining represented in Figure 7C and D. All gates are made on unstimulated controls groups and the same gates are then applied to cells following stimulation. The first gate is made on lymphocytes, followed by single cells based on FSC-A by FSC-H. Then live CD3 T cells are gated on and segregated into CD4 or CD8 T cells. CD4 T cells were then segregated based on either CD57+PD1– or CD28-PD1– populations (outlined by black boxes). Indicated cells were then interrogated for their ability to produce cytokines IFN γ and TNF α following stimulation. All gates are established on non-stimulated controls. This gating strategy is one representative flow plot from a stimulated patient sample.

Table S1: This table illustrates patient demographics, cause of end stage renal disease (ESRD), the type and duration of pre-transplant dialysis received, EBV and CMV serology, percentage of CD57+PD1– CD4 T cells, whether they received a kidney from a deceased or living donor, whether patients received belatacept- or tacrolimus-based immunosuppression following transplantation and whether they experienced a rejection or stable outcome. In this sample set, there was no significant correlation between percentage of CD57+PD1– CD4 T cells and other patient demographics. HTN, hypertension; FSGS, focal segmental glomerulosclerosis; DMII, type 2 diabetes mellitus; PKD, polycystic kidney disease; HD, hemodialysis; PD, peritoneal dialysis; EBV, Epstein-Barr virus; CMV, Cytomegalovirus; DCD, deceased donor; LRD, living related donor; LUD, living unrelated donor; n/a, not available.