Published in final edited form as:

J Immunol. 2021 April 01; 206(7): 1668–1676. doi:10.4049/jimmunol.2000736.

Functional Characteristics and Phenotypic Plasticity of CD57+PD1– CD4 T Cells and Their Relationship with Transplant Immunosuppression

Brian I Shaw*, Jaclyn R Espinosa*, Linda Stempora*, Allison Miller*, Bartley Adams*, Allan D Kirk*

*Department of Surgery, Duke University, Durham, USA

Abstract

Costimulation blockade (CoB)-based immunosuppression offers the promise of improved transplantation outcomes with reduced drug toxicity. However, it is hampered by early acute rejections, mediated at least in part by differentiated, CoB resistant T cells, such as CD57+PD1– CD4 T cells. Herein, we characterize these cells pre-transplant, determine their fate post-transplant, and examine their proliferative capacity *in vitro* in humans. Our studies show that CD57+PD1– CD4 T cells are correlated with increasing age and CMV infection pre-transplant, and persist for up to one year post-transplant. These cells are replication incompetent alone but proliferated in the presence of unsorted PBMCs in a contact independent manner. When stimulated, cells sorted by CD57/PD1 status upregulate markers of activation with proliferation. Up to 85% of CD57+PD1– cells change expression of CD57/PD1 with stimulation, typically, upregulating PD1 and downregulating CD57. PD1 upregulation is accentuated in the presence of rapamycin but prevented by tacrolimus. These data support a general theory of CoB resistant cells as antigen-experienced, costimulation-independent cells and suggest a mechanism for the synergy of belatacept and rapamycin, with increased expression of the activation marker PD1 potentiating exhaustion of CoB resistant cells.

Introduction

Kidney allotransplantation is the preferred method of renal replacement therapy, with superior short- and long-term outcomes compared to dialysis(1). Optimal long-term outcomes in kidney transplantation are hampered by necessary but imperfect immunosuppression regimens. For the vast majority of patients, these regimens contain calcineurin inhibitors (CNIs)¹ such as tacrolimus, which is nephrotoxic(2), requires drug monitoring due to differential metabolism(3), and is associated with post-transplant malignancies (4). More recently, the modified CTLA4-Ig fusion protein belatacept has been approved for maintenance immunosuppression in kidney transplantation as a CNI alternative (5, 6). Belatacept works through the mechanism of costimulation blockade (CoB)² by

Corresponding Author: Brian I Shaw, Phone: 919-613-6713, Fax: 919-613-9191, brian.shaw@duke.edu.

¹-CNI - Calcineurin Inhibitor

²-CoB – Costimulation Blockade

blocking the interactions between CD28 on T cells and CD80/86 on antigen presenting cells(7, 8).

Although belatacept-based regimens have been shown to avoid the toxicities of CNIs, their widespread adoption has been hampered by higher rates of early acute rejection, indicating a need to pair belatacept with some adjuvant therapy, ideally one that facilitates the mechanisms of CoB (6, 9). The origins of these early CoB-resistant rejections (CoBRR)³ remain incompletely defined, highly relevant to the continued use of belatacept, and likely based in the mechanisms distinguishing CNI and CoB-based immunosuppressive approaches. In general, T cell receptor signaling is required for T cell activation. As CNIs effectively inhibit this fundamental requirement, they are highly effective in preventing T cell-mediated alloimmunity. In counter-distinction, CD28-CD80/86 interactions, facilitate (but do not initiate) the effects of the TCR's primary signaling event(10). Thus, belatacept targets an important, but relatively subordinate signaling event. Importantly, once a T cell has been activated, its dependence on CD28 engagement decreases, and in humans, CD28 expression is markedly reduced during the course of activation-induced cell differentiation, suggesting that costimulation becomes increasingly subordinate with progressive maturation(11). In counter-distinction, activated T cells become increasingly dependent on pathways governed by the mechanistic target of rapamycin (mTOR)⁴ to respond metabolically to exogenous cytokines and proliferate. (12) This pathway is targeted clinically by the drug rapamycin.

Several surface molecules are known to provide evidence of a T cell's relative maturation; among them, PD1 and CD57 have been shown to correlate with prior antigen exposure and subsequent activation-induced senescence, respectively (13). These markers have also been shown to associate with impaired viral immunity, suggesting that their acquisition has functional importance in shaping an immune response (13–16). We have shown that CD57+PD1– CD4 T cells are enriched in many patients with end stage renal disease (ESRD)⁴ and associate with the post-transplant risk for CoBRR. Moreover, these cells generally lack CD28, are present on histologic sections of rejecting grafts, and contain a transcriptional profile that is associated with allograft rejection (14, 15). Additional research has identified other subsets of mature T cells associated with CoBRR, suggesting that multiple mature cell types may contribute to this rejection phenotype and support the overarching hypothesis that repertoire maturation status influences the efficacy of CoB-based approaches (16, 17). Given these findings, the relative make-up of a transplant recipient's T cell repertoire would be anticipated to influence the effectiveness of CNI- or belatacept-based immunosuppression.

In the present study, we have endeavored to better define the characteristics of CD57+PD1– CD4+ T cells—cells known to be CoB resistant. We find that these cells have substantial CoB-independent activity and persist as a stable feature of the T cell repertoire in kidney transplant recipients despite ongoing CNI-based immunosuppression. Additionally, we show

³.CoBRR – Costimulation Blockade Resistant Rejection

⁴.ESRD – End Stage Renal Disease

that CD57+PD1[−] acquire PD1 with proliferation and that this acquisition is enhanced by rapamycin, but not tacrolimus.

Methods

Patients

We studied consented patients with ESRD who were awaiting transplantation (n=39) and kidney transplant recipients (n=46) who had available pre-, and when applicable, post-transplant PBMCs acquired through participation in Institutional Review Board-approved sample acquisition and storage protocols (Pro00057497 and Pro00030485). Clinical data were abstracted from existing clinical databases and patient charts. To examine the natural history of cell populations, patients were chosen who were on CNI-based immunosuppression, did not receive lymphocyte depletion as part of their post-transplant therapy and were not on a belatacept-based regimen. Additionally, we collected PBMC samples from healthy controls for *in vitro* assays enrolled under an Institutional Review Board approved blood acquisition protocol (Pro00062495). For all samples, peripheral blood was collected by venipuncture and isolated by ficoll hypaque density gradient. Cells were then cryopreserved in fetal-calf serum (FCS, Corning, Corning, NY) with 10% DMSO.

Flow Cytometry

We performed multiparameter flow cytometric analysis of patient samples using our phenotypic panel that allowed for identification of CD57+PD1[−] T cells. We did this cross-sectionally for all patients prior to transplant, and longitudinally for those patients who were transplanted. In these and subsequent flow cytometry assays the antibodies used included the following: BD Biosciences (CD2 PE CF594, 562300; CD3 Alexa 700 557943, CD4 BUV395, 563550; CD4 PE-Cy7, 557852; CD8 APC, 555369; CD11a BV421, 563936; CD14 BV510, 563079; CD20 BV510, 563067; CD28 BV711, 56313; CD28 PE-Cy5, 555730; CD57 FITC, 555619; CCR7 [CD197] PE-Cy 7, 557648; PD1[CD279] PE, 563245; Granzyme B PE-CF594, 562462), Biolegend (CD49d PE Cy7, 304314; PD1 [CD279] PE 329906) eBiosciences (CD8 APC-Alexa780, 47-0088-42; CD40 PerCP eFluor710, 46-0409-42) Miltenyi (KLRG1 APC, 130-103-639) and Invitrogen/Thermo Fisher (CD45RA QDot 655, Q10069; Live/Dead Aqua, L34957). Data were acquired on a custom BD LSR Fortessa Flow Cytometer and analyzed using FlowJo 9/10 (TreeStar/Becton Dickinson, Ashland, OR; Gating Strategy Supplemental Figure 1 A & B).

Cell Sorting and Proliferation Experiments

Cryopreserved PBMC from pre-transplant patients were obtained from cryostorage, thawed in R10 Media [Consisting of RPMI 1640 supplemented with Pen-Strep at 100U/mL, L-Glutamine at 2mM (all Gibco, Gaithersburg, MD) and 10% FCS], and rested overnight in R10. The cells were then labeled with violet proliferation dye (VPD; BD Biosciences, San Jose, CA) using the manufacturer's protocol. A magnet-based pan-T cell isolation kit (Miltenyi Biotech, Auburn, CA) was utilized to isolate T cells from PBMC. The isolated T cells were then labeled using CD8 APC (555369; BD Biosciences), CD57 FITC (555619; BD Biosciences) and PD1 PE (560795; BD Biosciences) fluorochrome conjugated monoclonal antibodies. We sorted the CD8 negative fraction of cells into four populations:

CD57+PD1⁻, CD57+PD1⁺, CD57-PD1⁻, and CD57-PD1⁺ using a Moflo Astrios EQ cell sorter (Beckman Coulter, Indianapolis, IN). Cells were then incubated in R10 in flat bottom plates with some wells coated with purified OKT3 CD3 antibody at 0.5ug/mL (eBioscience, San Diego, CA). In some conditions, we co-cultured the sorted, VPD-labeled T cells with unlabeled unsorted PBMC from the same cell donor (referred to as “bulk cells”) at a fixed ratio. We also added soluble CD28 at 2ug/mL to some conditions (eBiosciences, San Diego, CA). We added immunosuppression to some conditions using tacrolimus at 10ng/uL (Astellas, Tokyo, JP), rapamycin at 10ng/uL (Pfizer, New York, NY), belatacept at 125ug/mL (Bristol-Myers Squibb, New York, NY), adalimumab at 100ng/mL (R&D Systems, Minneapolis, MN) or tocilizumab at 50ug/mL (Genentech, South San Francisco, CA). After 96 hours of incubation at 37C in R10, cell supernatants were reserved for cytokine detection (see below) and cells themselves were stained and analyzed by flow cytometry using a BD LSR Fortessa Flow Cytometer (Gating Strategy Supplemental Figure 1C). For samples in which a permeable cell well insert was used, sorted cells were placed on the apical side of the insert and bulk PBMC below at an excess number on the basolateral side the insert in a 24 well plate (Costar, Corning, NY) with a 1.0µm pore insert (Falcon, Corning, NY). These conditions were stimulated using CD3/28 beads (Dynabeads, ThermoFisher, Waltham, MA).

Cytokine Detection

Secreted cytokines were assessed from some supernatants using Luminex analysis. Briefly, we utilized a Human 25-Plex Luminex panel (LHC0009M, ThermoFisher, Waltham, MA; Cytokines included: GM-CSF, IFN- α , IFN- γ , IL-1 β , IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNF- α , Eotaxin, IP-10, MCP-1, MIG, MIP-1 α , MIP-1 β , RANTES) to interrogate supernatants collected from proliferation assays described above. The supernatants were clarified by centrifugation, diluted 1:5, and analyzed on a Luminex 200 xPONENT 4.2 using standard techniques.

Statistical Analyses

Patient demographics were collected and standard summary statistics generated. Univariable correlations of patient demographics with CD57+PD1⁻ cell percentages were performed by linear regression. All parameters collected were included in an initial multivariable linear regression model and a final model was created by stepwise-backward selection with a cutoff of $p=0.10$. Continuous variables were compared by Wilcoxon-Mann Whitney U tests or T-tests and categorical variables by either Chi² or Fisher Exact tests as appropriate.

With sorted cell subsets, we compared proliferation across different treatments using a Friedman test with a Dunn's post-test comparing stimulated conditions (with or without immunosuppression) to the unstimulated condition. Linear regression models were used to compare change in cell subsets with varying amounts of proliferation among sorted cell subsets. The baseline percentage of the sorted cell subset was defined as an individual sample's frequency of that subset in unstimulated conditions without immunosuppression. Principal component analysis (PCA)⁵ was used to visualize the relationships of changes in

⁵.PCA – Principal Component Analysis

cell marker expression. The PCA was developed using percentage of proliferating cells, change in CD28 expression, change in CD57 expression, and change in PD1 expression to develop the components. We also developed heatmaps to depict change in amount of proliferation and CD57/PD1 status as they were represented in the principal components (i.e. what changes were contributing to the principal components). Multivariable regression was used to determine the effect of different immunosuppressive agents on changes in cell subsets.

Differences in levels of analytes in the Luminex data were compared by a Friedman test with a Dunn's post-test comparing conditions with immunosuppression to the stimulated, non-immunosuppressed, condition. All statistical tests were performed in STATA 15 (StataCorp, College Station, TX) and PRISM (GraphPad, San Diego, CA).

Results

CD57+PD1– CD4 T cells are associated with age and CMV status and persist following transplantation

We first examined 85 patients with ESRD who were awaiting a kidney transplant. Patient demographics are summarized in Table I. Univariable linear regression showed that increasing age and CMV positivity were positively correlated with frequency of CD57+PD1– CD4 T cells prior to transplantation ($p=0.02$, $R^2=0.06$ for age and $p=0.029$, $R^2=0.06$ for CMV; Table II). Backward-stepwise regression yielded the same result, with only age and CMV status correlated with frequency of CD57+PD1– CD4 T cells (Table II).

Transplanted patients were followed longitudinally after transplant, providing an opportunity to examine the cell prevalence in individuals with restored renal function. Tracking CD57+PD1– CD4 T cells over time, we saw a small, transient, but statistically significant decrease in the proportion of these cells immediately after transplantation, perhaps related to the induction immunotherapy utilized in transplantation, with rebound to the pre-transplant level within 3 months (Figure 1A). A similar pattern was seen when examining only the T effector-memory CD45RA+ (Temra) subset of CD57+ PD1– CD4 T cells (Figure 1B), and when surveying adhesion markers known to be highly expressed on CD57+PD1– CD4 T cells—CD2, LFA1 and VLA4 (14)—with a decrease immediately after transplant and rebound to pre-transplant levels within 3 months (Figure 1D–F). We also noted a transient decrease in the proportion of CD28-CD57+ cells (Figure 1C).

CD57+PD1– CD4 T cells require help to proliferate in culture

To evaluate the proliferative capacity of CD57+PD1– T cells, we sorted T cells into four subsets based on CD57/PD1 expression: CD57+PD1–, CD57+PD1+, CD57–PD1– and CD57–PD1+. Focusing on CD57+PD1– cells, we showed that these cells were unable to proliferate with CD3 stimulation, in contrast to CD57–PD1– cells which proliferated well. However, these sorted CD57+PD1– CD4 T cells acquired proliferative capacity when CD3 stimulation was provided in the presence of bulk, unsorted PBMC (Figure 2). This culture arrangement provided CD57+ cells access to a more physiologic environment with monocytes and other lymphocyte lineages and their attendant cell-cell and soluble

supporting factors, and allowed for the direct study of stimulated, proliferating CD57+PD1– CD4 T cells. Of note, these cells also proliferated when in co-culture with bulk, unsorted PBMC when a semi-permeable well insert was placed between the sorted population and the bulk cells in the presence of bead stimulation (Figure 2B).

We further examined the effect of immunosuppression on these cell subsets. Again, in the presence of our heterogeneous unsorted PBMC (“bulk cells”), each CD57/PD1 fraction had proliferative capacity. All subsets proliferated to varying degrees with CD3 and CD3/CD28 stimulation and were inhibited variably by belatacept, rapamycin, and tacrolimus immunosuppression. Notably, the CD57–PD1–, CD57+PD1– and CD57–PD1+ populations proliferated similarly, while CD57+PD1+ cells were most sensitive to immunosuppression, with even belatacept significantly inhibiting proliferation (Figure 3A). These experiments demonstrated that many cell populations typically thought of as not having proliferative capacity are likely able to proliferate in more physiologically supportive environments.

We further characterized the supernatants from these cultures and noted that several canonical pro-inflammatory cytokines (interferon gamma, tumor necrosis factor-alpha [TNF- α], IL-6, and IL12) were present both in cultures without immunosuppression, or with belatacept (data not shown). Therefore, we attempted to arrest proliferation of CD57+PD1– cells by using clinically available, therapeutic monoclonal antibodies that block either TNF- α or IL-6 signaling. However, blocking these pathways either alone or in concert with belatacept did not consistently decrease the proliferative capacity of CD57+PD1– CD4 T cells (Figure 3B).

CD57+PD1– T cell populations acquire PD1 with proliferation

We next turned our attention to analyzing the effects of stimulation on these sorted cell subsets, identifiable as a VPD labeled cell fraction to distinguish them from bulk cells. Using unstimulated, un-immunosuppressed conditions as a baseline, we calculated the absolute percent change in CD57, PD1, and CD28 expression of the sorted cells by subtracting the baseline percentage from the stimulated samples. CD57 and PD1 expression within each of the four sorted subsets changed substantially upon stimulation, such that 85% of CD57+PD1– cells moved to a different status after stimulation. The most frequent change in expression was the acquisition of PD1. This is consistent with what we observed longitudinally *in vivo*: though the frequency of CD57+PD1– cells changed early in the post-transplant course it rebounded quickly, consistent with a cell subset with the potential to vary the expression of its surface markers. Changes in CD57 and PD1 expression were proportional to proliferation (Supplemental Figure 2) and, when controlling for immunosuppression, were greater with rapamycin in the CD57+PD1– and CD57–PD1+ subsets.

We next analyzed change in CD57, PD1, and CD28 status, and proliferating cells using PCA in unstimulated and CD3 stimulated samples. We first examined the characteristics of the condition represented by PCA. As shown in Figure 4A, cells segregated both by stimulation (with CD3 stimulated cells assigned higher values on component 1) and by CD57 status (across component 2). We next examined how changes in surface marker expression were captured by the PCA. As depicted in Figure 4b, we saw that there was more proliferation at

higher values of component 1. Figure 4c shows that CD57⁺ and CD57⁻ cells diverge with proliferation. Following stimulation, CD57⁺ tend to become CD57⁻ while CD57⁻ cells tend to become CD57⁺ (albeit at a much lower rate). Both CD57⁺ and CD57⁻ cells gained PD1 at high levels of proliferation. With proliferation, CD57⁺ cells tended to stay CD57⁺ and CD57⁻ cells tended to stay CD57⁻. That is, both proliferation and stability of CD57 expression are positively associated with PD1 acquisition.

Rapamycin increases acquisition of PD1 by CD57+PD1– T cells

We also performed linear regressions to directly examine the change in CD57, PD1, and CD28 expression among the four sorted subsets (Supplemental Figure 3). These data confirmed that in all 4 sorted cell populations we saw an increase in PD1⁺ cells with increased proliferation. In multivariable regression, which included immunosuppression, rapamycin was independently associated with an increase in PD1⁺ cell frequency in both the CD57+PD1– and the CD57–PD1+ subsets (Supplemental Table 1; Figure 5), meaning the frequency of PD1⁺ cells was greater than would be expected for a given degree of proliferation with rapamycin.

Discussion

CoBRR remains a significant hurdle to the widespread adoption of CoB-based immunosuppression regimens. One population of cells thought to mediate CoBRR is CD57+PD1– CD4 T cells(14). In the present study, we demonstrate that this cellular phenotype is prevalent among ESRD patients, is associated with age and CMV status, and persists over time after transplantation. This persistence is relevant to the clinical practice of late conversion from a CNI to belatacept, and suggests why conversion within the first 6 months after transplantation is often unsuccessful(18).

CD57⁺ CD4 T cells have been studied most extensively in CMV and HIV(19). Similar to these prior studies, we note that increasing age and CMV status are associated with an expansion of CD57+PD1– CD4 T cells. In HIV, there is a differential expansion of CD57+ CD4 T cells after viral acquisition which correlates with disease control (20). Though transplantation is a major immunologic event, we find few differences in the frequency of the CD57+ PD1– CD4 T cell population after transplantation, except transiently during early time points of higher dose induction immunosuppression. This suggests that the factors necessary to create this pool of cells are likely established before transplantation and are not modified by events associated with transplantation, such as restoration of renal function. We posit that, CD57+PD1– CD4 T cells are not dependent on uremia, dialysis or other proinflammatory events seen in transplant recipients, but rather are exemplary of an intrinsic quality of a patient's immune system, such as prior viral exposure.

CD57⁺ CD4 T cells are known to have a very low proliferative capacity *in vitro* (21). Indeed, we find that CD57+PD1– CD4 T cells are unable to proliferate when isolated in culture. However, we show that CD57+ PD1– CD4 T cells are able to proliferate well with anti-CD3 stimulation when that stimulation occurs in the presence of unsorted cells, as would be available *in vivo*. The requirement for this heterogeneous population of unsorted PBMC to aid in the proliferation of CD57⁺ T cells suggests that the proliferative capacity of

these cells has perhaps been underestimated. Additionally, we show that cell-cell contact is not necessary for this proliferative capacity and that it does not depend exclusively on certain canonical pro-inflammatory cytokines (TNF- α and IL-6), two pathways that could readily be inhibited in the clinic. Further investigation to determine the factors necessary for CD57+ cell stimulation are ongoing.

Though PD1 is most well known as a marker of T cell exhaustion, (22) it has also been recognized as a marker of activation (23, 24). Regardless, chronically high levels of PD1 have been shown to weaken anti-viral responses (22, 25), and similarly been shown to be critical in facilitating alloantigen-specific, activation induced cell death (AICD)⁶ in experimental transplant models (26, 27). PD1 ligand is also known to be upregulated in transplanted allografts, and PD1-PD1 ligand interactions have been shown to be critical in eliminating alloantigen specific cells from the allograft and facilitating allospecific tolerance (28, 29). That rapamycin facilitates the expression of PD1 and tacrolimus does not is of more than academic interest, as these two agents have both been utilized as clinical adjuvants for use with belatacept (18, 30, 31). The opposing effects of rapamycin and tacrolimus with respect to PD-1 expression suggest a mechanism by which CD57+CD4 T cells are gradually eliminated from the post-transplant repertoire in the presence of rapamycin (32), but not CNI-treated patients.

Clinically, patients treated with CoB based regimens have superior renal function to those on traditional CNI based regimens. Indeed, this advantage is evident in spite of CoBRR episodes, and patients treated with CoB are less likely to develop donor specific antibody regardless of this proclivity to early acute rejection (6, 9). In contrast, early rejection episodes under CNI-based regimens have been shown to correlate with graft loss and alloantibody formation (33). In the present study we show that proliferating CD57+ PD1-CD4 T cells rapidly acquire PD1 with proliferation. We posit that this property makes responding T cells more likely to undergo AICD and less likely to sustain a donor-specific response. This drive toward a characteristic exhausted phenotype may, in part, explain why patients treated with CoB who experience rejection do not have worse graft outcomes, and why early CoBRR is typically short lived. Indeed, previous murine experiments showed increases in AICD of allospecific T will in mice treated with CoB and rapamycin compared with CoB alone or CoB with CNI (26, 27), and pre-clinical studies have favored a combination of rapamycin with belatacept (34).

Additional evidence for the hypothesis that PD1 acquisition by CD57+ CD4 T cells decreases their function is found in the HIV literature. Here, the degree of expansion of CD57+ CD4 T cells is inversely correlated with viral load in HIV “elite controllers” (individuals who have become infected but maintain very low viral loads for long period of time). Moreover, these cells augment the ability of cytotoxic CD8 T cells to inhibit viral replication *in vitro* (20). More recently it has been demonstrated that CD57+ CD4 T cells from elite controllers are more polyfunctional and less likely to express PD1 than those from non-controlling HIV infected individuals (35). This suggests that PD1 acquisition among CD57+ CD4 T cells is deleterious to their function.

⁶.AICD – Activation Induced Cell Death

We also noted that the acquisition of PD1 is enhanced when cells proliferate in the presence of rapamycin. Rapamycin is an inhibitor of mTOR and has myriad effects on cellular metabolism, cell cycle, immune activation and protein synthesis (36). Few studies have specifically examined the effects of rapamycin on PD1 expression and little is known especially in the context of stimulation (37). Previously, we have shown that a regimen of rapamycin and belatacept, in conjunction with induction using alemtuzumab (a CD52-specific monoclonal antibody) has excellent results with little rejection and many patients able to be weaned to belatacept alone therapy (31,38). These data support our finding that rapamycin treatment may be synergistic with CoB: either through elimination of CoB resistant cells or rendering these cells more senescent/sensitive to apoptosis through PD1 acquisition (24).

As transplant recipients experience cancer at a higher rate than the general population experience with PD1 blockade to treat malignancy in transplant recipients is growing (39). A recent case report showed successful treatment of melanoma in a kidney transplant recipient with pembrolizumab and rapamycin as maintenance immunosuppression after a rejection episode. Similar to our results, the authors in this study also showed that rapamycin allowed for activation of CD4 T cells (as determined by CD25 expression and IFN- γ secretion) but not proliferation. Further, rapamycin prevented rejection after a single rejection episode was treated with steroids (40).

In sum, CD57+PD1- CD4 T cells may represent one of many possible phenotypes that are situated between naivete and exhaustion, allowing for costimulation independence while maintaining effector function. Moreover, these cells require assistance from other cell types for proliferation, suggesting that bystander effects from local inflammation—related to transplantation itself or infection—facilitate their allospecific activity and potentiate subsequent rejection episodes. This phenomenon has been anecdotally observed in CoB sensitive patients off of all immunosuppression who reject in the setting of new viral infections (31).

Our study has several important limitations. First, aspects of it are performed *in vitro* on sorted cell populations. This may not accurately recapitulate the complex interface of the allograft and recipient immune system *in vivo*. However, these conditions give the greatest precision to test specific hypotheses. Additionally, our cultures of CD57+PD1- CD4 T cells with “bulk” unsorted PBMC do not allow analysis of the exact proliferative milieu necessary for CD57+PD1- CD4 T cells but rather demonstrate how these cells respond in a generally replete inflammatory environment. Clinical experience suggests, however, that many different inflammatory states may be of importance with regards to CoB and therefore it is reasonable to treat this as an exemplary condition. Moreover, this arrangement allows for the examination of the proliferative behavior of otherwise non-proliferating cell subsets. Additionally, stimulation with CD3/CD28 is different than stimulation with alloantigen. Finally, CD57+PD1- T cells are an uncommon cell population which limits their study and makes examining multiple replicates more difficult.

In conclusion, we present evidence that CD57+PD1- CD4 T cells persist over time after transplantation and that this population continues to have a vital proinflammatory phenotype

inconsistent with terminal senescence. Additionally, we show that CD57+PD1[−] CD4 T cells are replication incompetent in the absence of bulk unsorted PBMC, but with help, are able to proliferate in the presence of belatacept. Finally, we show that CD57+PD1[−] CD4 T cells acquire PD1 with proliferation and that this acquisition is enhanced with rapamycin treatment. These data provide a mechanistic basis for the clinical benefit observed with the co-administration of belatacept and rapamycin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We would like to thank the patients that generously donated samples for this study, the Substrate Services Core Research Support (SSCRS) facility for processing samples, and the Surgical Center for Outcomes Research (SCORE) for supplying clinical data. We would like to acknowledge the NIH (NIH R01 FD003539) and Bristol-Myers Squibb for their financial support of this project.

References

1. Axelrod DA, Schnitzler MA, Xiao H, Irish W, Tuttle-Newhall E, Chang S-H, Kasiske BL, Alhamad T, and Lentine KL. 2018. An economic assessment of contemporary kidney transplant practice. *Am. J. Transplant* 18: 1168–1176. [PubMed: 29451350]
2. Naesens M, Kuypers DRJ, and Sarwal M. 2009. Calcineurin inhibitor nephrotoxicity. *Clin. J. Am. Soc. Nephrol* 4: 481–508. [PubMed: 19218475]
3. Tavira B, Diaz-Corte C, Coronel D, Ortega F, and Coto E. 2014. Pharmacogenetics of tacrolimus: from bench to bedside? *Nefrologia*. 34: 11–17. [PubMed: 24463861]
4. Sprangers B, Nair V, Launay-Vacher V, Riella LV, and Jhaveri KD. 2017. Risk factors associated with post-kidney transplant malignancies: an article from the Cancer-Kidney International Network. *Clin. Kidney. J* 11: 315–329. [PubMed: 29942495]
5. Vincenti F, Larsen C, Durrbach A, Wekerle T, Nashan B, Blanche G, Lang P, Grinyo J, Halloran PF, Solez K, Hagerty D, Levy E, Zhou WJ, Natarajan K, Charpentier B, for the Belatacept Study Group. 2005. Costimulation blockade with belatacept in renal transplantation. *N. Engl. J. Med* 353: 770–781. [PubMed: 16120857]
6. Vincenti F, Rostaing L, Grinyo J, Rice K, Steinberg S, Gaite L, Moal M-C, Mondragon-Ramirez GA, Kothari J, Polinsky MS, Meier-Kriesche H-U, Munier S, and Larsen CP. 2016. Belatacept and Long-Term Outcomes in Kidney Transplantation. *N. Engl. J. Med* 374: 333–343. [PubMed: 26816011]
7. Linsley PS, Wallace PM, Johnson J, Gibson MG, Greene JL, Ledbetter JA, Singh C, and Tepper MA. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science*. 257: 792–795. [PubMed: 1496399]
8. Larsen CP, Pearson TC, Adams AB, Tso P, Shirasugi N, Strobert E, Anderson D, Cowan S, Price K, Naemura J, Emswiler J, Greene J, Turk LA, Bajorath J, Townsend R, Hagerty D, Linsley PS, and Peach RJ. 2005. Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties. *Am J Transplant*. 5: 443–453. [PubMed: 15707398]
9. Vincenti F, Charpentier B, Vanrenterghem Y, Rostaing L, Bresnahan B, Darji P, Massari P, Mondragon-Ramirez GA, Agarwal M, Di Russo G, Lin CS, Garg P, and Larsen CP. 2010. A Phase III Study of Belatacept-based Immunosuppression Regimens versus Cyclosporine in Renal Transplant Recipients (BENEFIT Study). *Am J Transplant*. 10: 535–546. [PubMed: 20415897]
10. Chen L, and Flies DB. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat. Rev Immunol* 13: 227–242. [PubMed: 23470321]
11. Mou D, Espinosa J, Lo DJ, and Kirk AD. 2014. CD28 negative T cells: is their loss our gain? *Am. J. Transplant* 14: 2460–2466. [PubMed: 25323029]

12. Zeng H, and Chi H. 2017. mTOR signaling in the differentiation and function of regulatory and effector T cells. *Curr. Opin. Immunol* 46: 103–111. [PubMed: 28535458]
13. Larbi A, and Fulop T. 2013. From “truly naïve” to ‘exhausted senescent’ T cells: When markers predict functionality. *Cytometry* 85: 25–35. [PubMed: 24124072]
14. Espinosa J, Herr F, Tharp G, Bosinger S, Song M, Farris AB, George R, Cheeseman J, Stempora L, Townsend R, Durrbach A, and Kirk AD. 2016. CD57(+) CD4 T Cells Underlie Belatacept-Resistant Allograft Rejection. *Am. J. Transplant* 16: 1102–1112. [PubMed: 26603381]
15. George RP, Mehta AK, Perez SD, Winterberg P, Cheeseman J, Johnson B, Kwun J, Monday S, Stempora L, Warsaw B, and Kirk AD. 2016. Premature T Cell Senescence in Pediatric CKD. *J. Am Soc. Nephrol* 28: 359–367. [PubMed: 27413076]
16. Mathews DV, Wakwe WC, Kim SC, Lowe MC, Breeden C, Roberts ME, Farris AB, Strobert EA, Jenkins JB, Larsen CP, Ford ML, Townsend R, and Adams AB. 2017. Belatacept-Resistant Rejection Is Associated With CD28+ Memory CD8 T Cells. *Am. J. Transplant* 17: 2285–2299. [PubMed: 28502128]
17. Cortes-Cerisuelo M, Laurie SJ, Mathews DV, Winterberg PD, Larsen CP, Adams AB, and Ford ML. 2017. Increased Pretransplant Frequency of CD28 +CD4 +T EM Predicts Belatacept-Resistant Rejection in Human Renal Transplant Recipients. *Am. J. Transplant* 17: 2350–2362. [PubMed: 28502091]
18. Adams AB, Goldstein J, Garrett C, Zhang R, Patzer RE, Newell KA, Turgeon NA, Chami AS, Guasch A, Kirk AD, Pastan SO, Pearson TC, and Larsen CP. 2017. Belatacept Combined With Transient Calcineurin Inhibitor Therapy Prevents Rejection and Promotes Improved Long-Term Renal Allograft Function. *Am. J. Transplant* 17: 2922–2936. [PubMed: 28544101]
19. Booiman T, Wit FW, Girigorie AF, Maurer I, De Francesco D, Sabin CA, Harskamp AM, Prins M, Franceschi C, Deeks SG, Winston A, Reiss P, Kootstra NA, on behalf of The Co-morBidity in Relation to Aids (COBRA) Collaboration. 2017. Terminal differentiation of T cells is strongly associated with CMV infection and increased in HIV-positive individuals on ART and lifestyle matched controls. *PLoS ONE* 12: e0183357–17. [PubMed: 28806406]
20. Johnson S, Eller M, Teigler JE, Maloveste SM, Schultz BT, Soghoian DZ, Lu R, Oster AF, Chenine A-L, Alter G, Dittmer U, Marovich M, Robb ML, Michael NL, Bolton D, and Streeck H. 2015. Cooperativity of HIV-Specific Cytolytic CD4 T Cells and CD8 T Cells in Control of HIV Viremia. *J. Virol* 89: 7494–7505. [PubMed: 25972560]
21. Palmer BE, Blyveis N, Fontenot AP, and Wilson CC. 2005. Functional and Phenotypic Characterization of CD57 +CD4 +T Cells and Their Association with HIV-1-Induced T Cell Dysfunction. *J. Immunol* 175: 8415–8423. [PubMed: 16339584]
22. Barber DL, Wherry EJ, Masopust D, Zhu B, Allison JP, Sharpe AH, Freeman GJ, and Ahmed R. 2006. Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature* 439: 682–687. [PubMed: 16382236]
23. Hong JJ, Amancha PK, Rogers K, Ansari AA, and Villinger F. 2013. Re-evaluation of PD-1 expression by T cells as a marker for immune exhaustion during SIV infection. *PLoS ONE* 8: e60186. [PubMed: 23555918]
24. Petrovas C, Casazza JP, Brenchley JM, Price DA, Gostick E, Adams WC, Precopio ML, Schacker T, Roederer M, Douek DC, and Koup RA. 2006. PD-1 is a regulator of virus-specific CD8+ T cell survival in HIV infection. *J. Exp. Med* 203: 2281–2292. [PubMed: 16954372]
25. Radziejewicz H, Ibegbu CC, Fernandez ML, Workowski KA, Obideen K, Wehbi M, Hanson HL, Steinberg JP, Masopust D, Wherry EJ, Altman JD, Rouse BT, Freeman GJ, Ahmed R, and Grakoui A. 2007. Liver-infiltrating lymphocytes in chronic human hepatitis C virus infection display an exhausted phenotype with high levels of PD-1 and low levels of CD127 expression. *J. Virol* 81: 2545–2553. [PubMed: 17182670]
26. Li Y, Li XC, Zheng XX, Wells AD, Turka LA, and Strom TB. 1999. Blocking both signal 1 and signal 2 of T-cell activation prevents apoptosis of alloreactive T cells and induction of peripheral allograft tolerance. *Nat. Med* 5: 1298–1302. [PubMed: 10545997]
27. Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, Nuñez G, Tang A, Sayegh M, Hancock WW, Strom TB, and Turka LA. 1999. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat. Med* 5: 1303–1307. [PubMed: 10545998]

28. Wang L, Han R, and Hancock WW. 2007. Programmed cell death 1 (PD-1) and its ligand PD-L1 are required for allograft tolerance. *Eur. J. Immunol* 37: 2983–2990. [PubMed: 17899549]
29. Keir ME, Liang SC, Guleria I, Latchman YE, Qipo A, Albacker LA, Koulmanda M, Freeman GJ, Sayegh MH, and Sharpe AH. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med* 203: 883–895. [PubMed: 16606670]
30. Ferguson R, Grinyo J, Vincenti F, Kaufman DB, Woodle ES, Marder BA, Citterio F, Marks WH, Agarwal M, Wu D, Dong Y, and Garg P. 2010. Immunosuppression with Belatacept-Based, Corticosteroid-Avoiding Regimens in De Novo Kidney Transplant Recipients. *Am. J. Transplant* 11: 66–76. [PubMed: 21114656]
31. Kirk AD, Guasch A, Xu H, Cheeseman J, Mead SI, Ghali A, Mehta AK, Wu D, Gebel H, Bray R, Horan J, Kean LS, Larsen CP, and Pearson TC. 2014. Renal transplantation using belatacept without maintenance steroids or calcineurin inhibitors. *Am. J. Transplant* 14: 1142–1151. [PubMed: 24684552]
32. Xu H, Bendersky VA, Brennan TV, Espinosa JR, and Kirk AD. 2017. IL-7 receptor heterogeneity as a mechanism for repertoire change during postdepletional homeostatic proliferation and its relation to costimulation blockade-resistant rejection. *Am. J. Transplant* 14: 720–730.
33. Loupy A, Vernerey D, Tinel C, Aubert O, Duong van Huyen J-P, Rabant M, Verine J, Nochy D, Empana J-P, Martinez F, Glotz D, Jouven X, Legendre C, and Lefaucheur C. 2015. Subclinical Rejection Phenotypes at 1 Year Post-Transplant and Outcome of Kidney Allografts. *J. Am Soc Nephrol* 26: 1721–1731. [PubMed: 25556173]
34. Lo DJ, Anderson DJ, Weaver TA, Leopardi F, Song M, Farris AB, Strobert EA, Jenkins J, Turgeon NA, Mehta AK, Larsen CP, and Kirk AD. 2013. Belatacept and sirolimus prolong nonhuman primate renal allograft survival without a requirement for memory T cell depletion. *Am. J. Transplant* 13: 320–328. [PubMed: 23311611]
35. Phetsouphanh C, Aldridge D, Marchi E, Munier CML, Meyerowitz J, Murray L, Van Vuuren C, Goedhals D, Fidler S, Kelleher A, Klenerman P, and Frater J. 2019. Maintenance of Functional CD57+ Cytolytic CD4+ T Cells in HIV+ Elite Controllers. *Front. Immunol* 10: 1387–17. [PubMed: 31275323]
36. Zaza G, Granata S, Caletti C, Signorini L, Stallone G, and Lupo A. 2018. mTOR Inhibition Role in Cellular Mechanisms. *Transplantation* 102: S3–S16. [PubMed: 29369970]
37. Hurez V, Dao V, Liu A, Pandeswara S, Gelfond J, Sun L, Bergman M, Orihuela CJ, Galvan V, Padrón Á, Drerup J, Liu Y, Hasty P, Sharp ZD, and Curiel TJ. 2015. Chronic mTOR inhibition in mice with rapamycin alters T, B, myeloid, and innate lymphoid cells and gut flora and prolongs life of immune-deficient mice. *Aging Cell* 14: 945–956. [PubMed: 26315673]
38. Schmitz R, Fitch ZW, Xu H, Ghali A, Mehta AK, Guasch A, & Kirk AD 2020. Kidney Transplantation using Alemtuzumab, Belatacept and Sirolimus - Five-Year Follow-up. *Am. J. Transplant* 20: 3609–3619 [PubMed: 32515087]
39. Kumar V, Shinagare AB, Rennke HG, Ghai S, Lorch JH, Ott PA, and Rahma OE. 2020. The Safety and Efficacy of Checkpoint Inhibitors in Transplant Recipients: A Case Series and Systematic Review of Literature. *Oncologist* 25: 505–514. [PubMed: 32043699]
40. Esfahani K, Al-Aubodah T-A, Thebault P, Lapointe R, Hudson M, Johnson NA, Baran D, Bhulaiga N, Takano T, Cailhier J-F, Piccirillo CA, and Miller WH. 2019. Targeting the mTOR pathway uncouples the efficacy and toxicity of PD-1 blockade in renal transplantation. *Nat. Comm* 10: 4712–4719.

Key Points:

1. CD57+PD1– CD4 T cells can proliferate when in the presence of unsorted PBMC
2. Rapamycin increases expression of PD1 on stimulated CD57+PD1– CD4 T cells

Figure 1:

Clinical correlates of and changes to CD57+PD1- CD4 T cells after transplantation. **(A)** percent of CD57+PD1- CD4 T cells decrease initially and rebound after transplant. **(B)** Though the percentage of CD57+PD1- CD4 Temra did not change after transplant, expression of adhesion molecules CD28- **(C)** CD2+ **(D)**, LFA1+ **(E)**, and VLA4+ **(F)** all transiently decreased at 1 month and then rebounded. Note, lines in red are in the top 20% for a given marker.

Figure 2:

CD57+ PD1- CD 4 T cells do not proliferate with monoclonal Ab stimulation alone but do in the presence of bulk unsorted PBMC. **(A)** Proliferation of CD57+PD1- or CD57-PD1- sorted CD4 T Cells with CD3 stimulation alone. **(B)** Proliferation of CD57+PD1- or CD57-PD1- sorted CD4 T cells in the presence of bulk PBMC. Transwell condition separates bulk cells from sorted cells by a 1 μ m transwell pore. Proliferation measured by Violet Proliferation dye (VPD). Bulk cells are not excluded from these plots. Proliferating cells are a percent of VPD positive cells.

Figure 3:

CD57+ PD1- Cells proliferate efficiently in the presence of bulk cells, are not inhibited by belatacept. This proliferation cannot be arrest by blocking of other clinically relevant cytokines and is not contact dependent. (A) Each panel shows proliferation of 5 unique responders, with the indicated CD57/PD1 phenotype sorted VPD450 labeled, and returned to bulk culture. (B) Each panel shows proliferation of 3 unique responders, with indicated CD57/PD1 phenotype sorted VPD450 labeled and returned to bulk culture. N=2 for CD57+PD1+ panel due to cell number limitations. Comparisons between individual unstimulated samples and stimulated samples (with or without immunosuppression) by Dunn's post-test after a Friedman test between all groups. Significant ($P<0.05$) post tests shown.

Figure 4:

PCA plots examining for change in phenotype with proliferation. **(A)** Cells cluster by stimulation (no stimulation vs CD3) and CD57 status. **(B)** PCA plot where the coloring is varied by the percent (%) proliferating in each sample. As samples increase in proliferation, they move to the right on Component 1. **(C)** Change in CD57/PD1 status is visualized on the PCA with coloring varying with the absolute percent change in the cell phenotype. CD57+ and CD57- cells have distinct patterns of phenotype change that become more divergent with more proliferation.

Figure 5:

Rapamycin enhances acquisition of PD1 among certain sorted subsets. Both CD57+PD1- (A) and CD57-PD1+ (B) cells have increased acquisition of PD1 under rapamycin relative to their level of proliferation. This is shown by the rapamycin treated samples (red markers) appearing above the linear regression line. Linear regression with 95% CI plotted. Unadjusted R^2 and overall p-value shown.

Table I:

Overall Cohort Demographics

Characteristic	n=85
Age-Med(IQR)	50(42–61)
Sex(Female)-n(%)	28(33)
Race-n(%)	
Black	46(54)
White	35(41)
Other/Unknown	4(5)
Primary Renal Disease-n(%)	
Diabetes Mellitus	27(32)
Focal Segmental Glomerulosclerosis	6(7)
Glomerulonephritis	8(9)
Human Immunodeficiency Virus	3(4)
Hypertension	33(38)
IgA Nephropathy	5(6)
Polycystic Kidney Disease	5(6)
Systemic Lupus Erythematosus	7(8)
History of Immunosuppression-n(%)	25(29)
Duration of Dialysis-Med (IQR)	32(7–58)
CMV Status-n(%) [*]	61(73)
EBV Status-n(%) [*]	77(98)

^{*} Indicates missing data; n=2 for CMV and n=7 for EBV status

Table II:

Linear Regression Identifying Associations with CD57+PD1– CD4 T Cell Prevalence

Univariable Models			
Variable	Coefficient (SE)	p-value	R²
Age	0.093(0.41)	0.027	0.06
CMV Status (Reference=Negative)	2.78 (1.25)	0.029	0.06
EBV Status (Reference=Negative)	1.16(5.3)	0.83	0.0006
Prior IS Use (Reference=None)	−0.70(1.22)	0.57	0.004
Race (Reference=Non-Black)	1.15(1.11)	0.30	0.01
Sex (Reference=Male)	−1.02(1.18)	0.39	0.009
Full Multivariable Model			
Variable	Coefficient (SE)	p-value	R²
Age	0.10(0.05)	0.025	0.14
CMV Status	2.46(1.3)	0.063	
EBV Status	−2.5(5.4)	0.62	
Prior IS Use	0.75(1.27)	0.56	
Race	1.40 (1.19)	0.24	
Sex	−1.55(1.21)	0.21	
Final Multivariable Model			
Variable	Coefficient (SE)	p-value	R²
Age	0.09 (0.04)	0.036	0.11
CMV Status	2.52 (1.23)	0.044	

SE-Standard Error

Bolded p-values <0.05