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## High CTLA-4 Expression on Th17 Cells Results in Increased Sensitivity to CTLA-4 Coinhibition and Resistance to Belatacept

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### Abstract

The CD28/CTLA-4 blocker belatacept selectively inhibits alloreactive T cell responses but is associated with a high incidence of acute rejection following renal transplantation, which led us to investigate the etiology of belatacept resistant graft rejection. T cells can differentiate into functionally distinct subsets of memory T cells that collectively enable protection against diverse classes of pathogens and can cross-react with allogeneic antigen and mediate graft rejection. Th17 cells are a pro-inflammatory CD4<sup>+</sup> lineage that provides immunity to pathogens and are pathogenic in autoimmune disease. We found that Th1 and Th17 memory compartments contained a similar frequency of divided cells following allogeneic stimulation. Compared to Th1 cells, Th17 memory cells expressed significantly higher levels of the coinhibitory molecule CTLA-4. Stimulation in the presence of belatacept inhibited Th1 responses but augmented Th17 cells due to greater sensitivity to coinhibition by CTLA-4. Th17 cells from renal transplant recipients were resistant to ex vivo CD28/CTLA-4 blockade with belatacept, and an elevated frequency of Th17 memory cells was associated with acute rejection during belatacept therapy. These data highlight important differences in costimulatory and coinhibitory requirements of CD4<sup>+</sup> memory subsets, and demonstrate that the heterogeneity of pathogen-derived memory has implications for immunomodulation strategies.

### Introduction

During a secondary T cell response, memory T cells maintain the functional and phenotypic properties that reflect their priming conditions (1). Recent studies have shown that pathogen-primed memory T cells can cross-react with alloantigen (2, 3) and that alloreactive T cells are inherently more polyspecific for peptide:MHC than conventional T cells (4, 5), suggesting that the alloreactive memory T cell pool reflects the pathogen-specific stimulation history of an individual. The heterogeneity of T cell memory recall responses is critically important for transplant recipients who receive lifelong immunosuppression to prevent T cell mediated graft rejection.

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#### Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

The recently approved CTLA-4 Ig derivative belatacept inhibits graft-specific immune responses by blocking CD28/CTLA-4 signals on T cells, and offers significantly improved long-term graft function and fewer toxicities compared to calcineurin inhibitors. However, belatacept is associated with a high incidence of pathologically severe acute rejection within one year of transplantation (6). While the mechanism of this rejection is currently unknown, the kinetics and severity of this phenomenon suggests that a CD28/CTLA-4 blockade resistant population of T cells mediates this rejection.

Although classically studied CD4<sup>+</sup> Th1 responses are known to depend on CD28 signals for optimal secondary recall responses (2, 7), the costimulation requirements of Th17 cells are less understood. Intriguingly, recent studies have suggested differences in the costimulation signals that mediate differentiation of naïve Th0 cells into Th1 or Th17 cells (8–13). While this work has focused on cosignaling during primary differentiation into Th17 cells, little is known about the costimulation requirements of memory Th17 cells during subsequent recall responses.

In this study we investigated the relative contribution of Th17 cells to alloreactivity and their susceptibility to costimulation blockade with belatacept. We demonstrate that Th17 memory cells express high levels of the coinhibitory receptor CTLA-4, which results in resistance to belatacept and is associated with rejection in renal transplant recipients. This study demonstrates that the costimulatory requirements of CD4<sup>+</sup> Th1 and Th17 subsets are distinct, and highlights the differential susceptibilities of heterogeneous microbe-elicited memory populations to immunomodulation with costimulation blockade.

## Materials and Methods

### Human Study Approval

Healthy donor peripheral blood mononuclear cells (PBMC) and patient PBMC and lymph node samples were isolated following protocols approved by the Emory University Institutional Review Board (IRB #00006248).

### Human Alloreactive Proliferation Assay

Monocyte-derived dendritic cells (MDDCs) were derived from  $3 \times 10^6$  fresh PBMC in a 6 well plate in RPMI supplemented with 10% human AB serum (Mediatech, VA), 2.4 mM L-glutamine. Non-adherent lymphocytes were washed off 4 hours later, and adherent cells were cultured with 50 ng/mL of IL-4 and 100  $\mu$ g/mL of GM-CSF (R&D Systems) for 5–7 days at 37 °C. Responders were derived from healthy donor fresh PBMC CFSE labeled with 5  $\mu$ M CFSE (Invitrogen) for 3 min and co-cultured with allogeneic MDDC at a 3:1 ratio in 96 well flat-bottomed plates for 4 d at 37 °C. Some cultures were restimulated with 30 ng/mL PMA and 400 ng/mL Ionomycin (Sigma) for 4 h, and 10  $\mu$ g/mL GolgiStop (BD Biosciences) was added for the final 3 h. To determine frequency of divided CD4<sup>+</sup> fractions in response to allogeneic stimulation, cells were gated on CD4<sup>+</sup>CD45RA<sup>+</sup>CFSE<sup>low</sup> or CD4<sup>+</sup>CD45RA<sup>-</sup>CFSE<sup>low</sup>, followed by either IFN- $\gamma$ <sup>+</sup> or CCR6<sup>+</sup>IL-17<sup>+</sup> as described. To determine the effect of belatacept following allogeneic stimulation, cells were first gated on CD4<sup>+</sup>CD45RA<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (CD45RA<sup>+</sup> Th1), CD4<sup>+</sup>CD45RA<sup>-</sup> IFN- $\gamma$ <sup>+</sup> (CD45RA<sup>-</sup> Th1), or

CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 (CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17) followed by CFSE<sup>low</sup> divided cells. The effect of belatacept on CD4<sup>+</sup> subsets following allogeneic stimulation was calculated as  $(1 - (\% \text{ CFSE}^{\text{low}} \text{ with belatacept} / \% \text{ CFSE}^{\text{low}} \text{ with no treatment})) \times 100$ .

### Human Polyclonal Stimulation and Costimulation Blockade

Fresh or frozen PBMC from healthy donors cells were cultured in 96 well flat-bottomed plates in RPMI supplemented with 10% human AB serum (Mediatech, VA) and 2.4 mM L-glutamine. Frozen PBMC were rested overnight before stimulation. Cells were stimulated with either 1 µg/mL (PBMC) or 2 µg/mL (lymph node T cells) functional grade anti-CD3 (OKT3; eBiosciences) in the presence of belatacept (100 µg/mL; Bristol-Myers Squibb, NY) or human IgG1-Fc control (BioXCell, Lebanon, NH), or with anti-CD3/CD28 Dynabeads (Invitrogen) in the presence of 10 µg/mL anti-CTLA-4 (BN13; BioXCell, Lebanon, NH) or mouse IgG1 (BioXCell, Lebanon, NH), as indicated. Cells were washed twice with media and restimulated with PMA/Iono for 4 h as described above. CD4<sup>+</sup> T cell subsets were defined by the following gating strategy: CD45RA<sup>+</sup> Th1, CD4<sup>+</sup>CD45RA<sup>+</sup>IFN-γ<sup>+</sup>; CD45RA<sup>-</sup> Th1, CD4<sup>+</sup>CD45RA<sup>-</sup> IFN-γ<sup>+</sup>; CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17, CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>+</sup>IL-17<sup>+</sup>. The change in frequency of CD4<sup>+</sup> populations was calculated as  $(\% \text{ Cytokine}^+ \text{ Blockade} / \% \text{ Cytokine}^+ \text{ IgG}) \times 100$  of the indicated population.

### Surface and Intracellular Staining of Healthy Donor and Patient Cells

Fresh PBMC were isolated from normal healthy donors under IRB approval using CPT tubes and plated at  $1 \times 10^5$ /well in round-bottom 96 well plates. PMA/Iono and GolgiStop were added for 4 h as described above. Surface staining was performed for 20 min at 23°C using the following antibodies: CD4 (L200 or RPA-T4), CD8 (RPA-T8), CD14 (M5E2), CD19 (HIB19), CD28 (CD28.2) or IgG1κ, CD45RA (MEM-56 or HI100), and CCR6 (TG7/CCR6) or IgG2bκ. Cells were prepared for intracellular staining following manufacturer's protocol (BD Biosciences Fix/Perm Kit) and stained with the following antibodies: CTLA-4 (CD152, BN13) or IgG2aκ, IFN-γ (45.B3), and IL-17A (eBio64DEC17). All panels depict CD4<sup>+</sup>CD45RA<sup>+</sup> or CD4<sup>+</sup>CD45RA<sup>-</sup> populations. For CTLA-4 surface capture assay, anti-CTLA-4 PE was added for the final 3 h of PMA/Iono stimulation, and cells were prepared for intracellular flow cytometry. Samples were analyzed using an LSR II flow cytometer (BD Biosciences), and data was analyzed using FlowJo software (Treestar, San Carlos, CA).

### Transplant Recipient Cohort Selection

Patients were selected as rejectors if an episode of acute cellular rejection was diagnosed by renal biopsy following transplantation (1–6 months post-transplant for belatacept-treated patients, mean 2.7 months; or 1–14 months post-transplant for tacrolimus-treated patients, mean 5.8 months). Stable patients were free from rejection episodes through the time of assessment (mean time of assessment 9.5 months). Belatacept stable, belatacept rejectors, and tacrolimus rejectors were not significantly different for gender or age ( $59 \pm 3.0$ ,  $49 \pm 5.4$ ,  $54 \pm 4.9$  y.o., respectively), were all first time transplant recipients, were similarly HLA mismatched, and had comparable common indications for transplantation.

## Renal Transplant Recipient PBMC and LN cells

PBMC and LN cells from patients were cryopreserved at  $-80^{\circ}\text{C}$  in RPMI with 40% FBS and 10% DMSO.  $1 \times 10^6$  thawed PBMC were cultured in 96 well round-bottom plates and rested overnight in RPMI with 10% human AB serum (for PBMC; Mediatech, VA) or 10% FBS and 20 U/mL IL-2 (for LN cells; eBiosciences) and 2.4 mM L-glutamine. Cells were washed twice with media before stimulation with either anti-CD3 for 3 d (LN cells, as described above) or PMA/Iono for 4 h (PBMC). Patient PBMC were defined by the following gating strategy: Th1 Memory,  $\text{CD4}^+\text{CD45RA}^-\text{CCR6}^- \text{IFN-}\gamma^+$ , Th17 Memory,  $\text{CD4}^+\text{CD45RA}^-\text{CCR6}^+\text{IL-17}^+$  LN  $\text{CD4}^+$  T cell subsets were defined by the following gating strategy:  $\text{CD45RA}^+$  Th1,  $\text{CD3}^+\text{CD4}^+\text{CD45RA}^+\text{IFN-}\gamma^+$ ;  $\text{CD45RA}^-$  Th1,  $\text{CD3}^+\text{CD4}^+\text{CD45RA}^- \text{IFN-}\gamma^+$ ;  $\text{CD45RA}^-$  Th17,  $\text{CD3}^+\text{CD4}^+\text{CD45RA}^-\text{IL-17}^+$  The effect of belatacept was calculated as described for healthy donors.

## Statistics

Proliferation, cytokine production, and population frequencies between groups were compared using Mann-Whitney non-parametric (two-tailed) analysis. All analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA). Significance was determined as \* $p < 0.05$ , \*\* $p < 0.005$ , \*\*\* $p < 0.001$ . Data shown and described depict average  $\pm$  SEM.

## Results

### Alloreactive Th17 memory cells are resistant to belatacept

Th17 memory cells are important for fungal and extracellular bacterial immunity (14, 15), and can be potent drivers of autoimmune disease (16, 17). However, their involvement in alloreactivity is not well defined (18). We stimulated CFSE-labeled responders with allogeneic monocyte-derived dendritic cells (MDDC) followed by brief PMA/Iono restimulation to identify alloreactive Th1 and Th17 cells. Alloreactive  $\text{CD45RA}^-$  Th1 cells were found in the  $\text{CCR6}^-$  and  $\text{CCR6}^+$  compartments ( $13.8 \pm 2.85\%$  and  $15.7 \pm 2.08\%$  of  $\text{CFSE}^{\text{lo}}$ , respectively), while  $\text{CD45RA}^-$  Th17 cells were exclusively found within the  $\text{CCR6}^+$  fraction (Figure 1A–B)(19). We found that  $2.78 \pm 0.478\%$  of total alloreactive  $\text{CCR6}^+$  memory cells were Th17 cells (Figure 1B). After 4 days of allogeneic stimulation,  $\text{CD45RA}^+$  Th1,  $\text{CD45RA}^-$  Th1 and  $\text{CD45RA}^- \text{CCR6}^+$  Th17 cells contained similar frequencies of divided cells ( $7.95 \pm 2.05\%$   $\text{CD45RA}^+$  Th1,  $7.66 \pm 1.66\%$   $\text{CD45RA}^-$  Th1,  $11.8 \pm 3.2\%$   $\text{CD45RA}^- \text{CCR6}^+$  Th17, Figure 1C–D). This demonstrates that like Th1 cells, Th17 cells can participate in alloreactive responses.

The addition of belatacept to allogeneic co-cultures resulted in the inhibition of  $\text{CD45RA}^+$  Th1 and  $\text{CD45RA}^-$  Th1 cells ( $72.4 \pm 5.46\%$  and  $82.0 \pm 10.5\%$  of no treatment, respectively, Figure 1C and 1E). In contrast, we found that  $\text{CD45RA}^- \text{CCR6}^+$  Th17 cells were augmented by belatacept in each pair evaluated ( $127 \pm 11.8\%$  of no treatment, Th17 vs.  $\text{CD45RA}^+$  Th1  $p = 0.0079$ , Th17 vs.  $\text{CD45RA}^-$  Th1,  $p = 0.0079$ , Figure 1C and 1E), suggesting that Th17 cells might differentially utilize on the CD28/CTLA-4 pathway compared to other  $\text{CD4}^+$  memory subsets.

### Th17 memory cells significantly upregulate the coinhibitor CTLA-4

Multiple studies have indicated that CD4<sup>+</sup> Th1 memory recall responses require CD28 (2, 7), while less is known about the costimulation requirements during recall of Th17 memory cells. Because belatacept inhibits both CD28 and CTLA-4 signals by binding to their common ligands CD80/CD86 on APCs, we next investigated the CD28/CTLA-4 costimulatory pathways on Th1 and Th17 cells.

Nearly all resting naïve CD45RA<sup>+</sup> and memory CD45RA<sup>-</sup> CD4<sup>+</sup> cells were CD28<sup>+</sup> and expressed high levels of CD28 (Figure 2A). CTLA-4, which is also blocked by belatacept, is maintained in intracellular vesicles that rapidly flux to and from the cell surface during activation. Staining for total intracellular CTLA-4 revealed that nearly half of resting memory CD4<sup>+</sup> T cells expressed low levels of CTLA-4, and that the level of expression rose after restimulation (Figure 2B). Th17 memory cells expressed significantly more CTLA-4 than Th1 memory, as the majority of IL-17<sup>+</sup> cells ( $64.4 \pm 2.55\%$ ) were CTLA-4<sup>high</sup> compared to a small fraction of IFN- $\gamma$ <sup>+</sup> cells ( $24.1 \pm 3.33\%$ )(Figure 2B–C)

We next sought to determine if the differences in the expression of CTLA-4 between Th1 and Th17 memory cells were reflected in CTLA-4 levels on the cell surface. Using a modified staining protocol to capture rapidly endocytosed surface CTLA-4, we found that very few resting naïve and memory cells expressed surface CTLA-4, while restimulated memory cells upregulated CTLA-4 (Figure 2D). Strikingly, significantly more Th17 memory cells ( $84.8 \pm 2.25\%$ ) were CTLA-4<sup>+</sup> compared to Th1 memory cells ( $37.0 \pm 2.77\%$ ) (Figure 2D). Furthermore, Th17 memory cells expressed significantly more CTLA-4 than Th1 (Figure 2E). These data are the first demonstration that Th1 and Th17 memory cells differ significantly in their expression of CTLA-4 following restimulation.

### CD45RA<sup>-</sup> Th17 cells are uniquely sensitive to CTLA-4 coinhibition

We hypothesized that both alloreactive and non-alloreactive Th17 memory cells would be resistant to belatacept due to relatively greater sensitivity to signaling through coinhibitory CTLA-4. Following polyclonal stimulation with soluble anti-CD3, CD45RA<sup>+</sup> and CD45RA<sup>-</sup> Th1 cells were inhibited by belatacept ( $53.2 \pm 7.53\%$  and  $87.11 \pm 4.66\%$  of IgG-Fc, respectively, Figure 3A–B). In contrast, CD45RA<sup>-</sup> Th17 cell frequencies were significantly augmented ( $130 \pm 9.5\%$  of IgG-Fc,  $p = 0.0001$  vs CD45RA<sup>+</sup> Th1 and CD45RA<sup>-</sup> Th1, Figure 2A–B). Both CD45RA<sup>-</sup> Th1 and CD45RA<sup>+</sup> Th1 cells were inhibited over a range of belatacept concentrations in a dose-dependent manner, while CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 cells were augmented over a range of belatacept doses (Supplemental Figure 1). Together, these results demonstrate that the relative contribution of CD28/CTLA-4 signaling is distinct between Th1 and Th17 cells.

To directly test whether CD45RA<sup>-</sup> Th17 cells are more sensitive to coinhibitory signals through CTLA-4 we next interrogated the function of the CTLA-4 pathway on Th17 cells. While both CD45RA<sup>+</sup> and CD45RA<sup>-</sup> Th1 responses were augmented in the presence of anti-CTLA-4, the CD45RA<sup>-</sup> Th17 cell population was increased to a significantly greater degree than the CD45RA<sup>-</sup> Th1 population ( $168 \pm 15.0\%$  vs  $118.5 \pm 8.10\%$  of IgG,  $p =$

0.0071, Figure 3C–D). Taken together, these results demonstrate that CD45RA<sup>−</sup> Th17 cells are more sensitive to coinhibition than CD45RA<sup>−</sup> Th1 cells.

### **Renal transplant recipient-derived lymph node CD45RA<sup>−</sup> Th17 cells are resistant to belatacept**

Belatacept has been recently approved for post transplant immunosuppression in renal transplant recipients, but it has been associated with more frequent and severe early acute rejection episodes compared to calcineurin inhibitor based regimens (6). The critical site of action of belatacept on primary and memory T cell activation is likely to be the site of T cell:APC interaction in secondary lymphoid tissue (20), leading us to investigate the effect of belatacept on Th1 and Th17 cells derived from lymph nodes in a cohort of patients who received renal allografts.

Lymph node cells harvested from renal transplant recipients prior to the induction of immunosuppressive therapy were stimulated in the presence of CD28/CTLA-4 blockade with belatacept. Similar to T cells in the peripheral blood of healthy donors (Figure 3A–B), belatacept inhibited the proliferation of CD45RA<sup>+</sup> Th1 cells ( $69.1 \pm 3.43\%$  of IgG-Fc) and modestly reduced the frequency of CD45RA<sup>−</sup> Th1 cells ( $94.2 \pm 2.08\%$  of IgG-Fc, Figure 4A–B). Lymph node-derived CD45RA<sup>−</sup> Th17 cells, in contrast, were augmented in the presence of belatacept compared to control Ig molecule ( $115.5 \pm 2.43\%$  of IgG-Fc,  $p = 0.0049$  vs CD45RA<sup>−</sup> Th1, Figure 4A–B). Thus, in contrast to CD45RA<sup>+</sup> and CD45RA<sup>−</sup> Th1 cells, CD45RA<sup>−</sup> Th17 cells from the secondary lymphoid organs of transplant recipients are also augmented in the presence of belatacept.

### **An elevated Th17 memory cell frequency correlates to rejection during belatacept therapy in renal transplant recipients**

We hypothesized that acute cellular rejection that occurs in patients treated with belatacept might be associated with elevated Th17 memory cell frequencies. To investigate this possibility, we studied a cohort of renal transplant recipients treated with standard immunosuppressive regimens containing either belatacept or the calcineurin inhibitor tacrolimus. Belatacept treated patients were classified based on whether they experienced biopsy-proven acute cellular rejection early following transplantation or were stable (described in Methods). Tacrolimus-treated patients were similarly classified as rejectors by the occurrence of biopsy-proven acute cellular rejection.

At baseline, the frequencies of CCR6<sup>−</sup> Th1 and CCR6<sup>+</sup> Th17 memory cells were not significantly different between each of these three groups (Figure 5A–B). We next investigated the Th1 and Th17 memory cell frequencies at 1 month post-transplant for belatacept stable patients and the time of rejection in belatacept or tacrolimus rejectors. The frequency of Th1 memory cells was not significantly different between belatacept-treated patients who were stable or rejecting, nor were they different between patients rejecting while being treated with belatacept vs those rejecting on tacrolimus (Figure 5C–D). In contrast, the frequency of Th17 memory cells was significantly elevated in belatacept rejectors ( $2.80 \pm 0.593\%$ ) compared to stable belatacept-treated patients ( $0.801 \pm 0.203\%$ ,  $p = 0.0286$ ) and tacrolimus rejectors ( $1.26 \pm 0.261\%$ ,  $p = 0.0286$ , Figure 5C–D). These data

demonstrate that acute rejection episodes in belatacept treated patients are associated with an increased Th17 memory population relative to rejection in tacrolimus-treated patients.

## Discussion

In this study, we show that Th17 memory cells, which are elicited by microbes such as *Candida* and *Staphylococcus* (14, 15, 18), are a component of the alloreactive memory T cell compartment. As a population, a similar fraction of Th1 and Th17 cells divide in response to allogeneic stimulation. This observation provides a new dimension to previous descriptions of alloreactive heterologous immunity (2), including a recent study that found that a significant portion of viral specific memory CD4<sup>+</sup> and CD8<sup>+</sup> T cells are alloreactive (3). This finding is also congruous with recent work indicating that alloreactive T cells inherently recognize multiple distinct peptide:MHC complexes, a property termed polyspecificity (4, 5). Our study supports the idea that the alloreactive memory T cell pool is comprised of microbe-elicited cells possessing both the Th1 and Th17 phenotype.

The observation that Th17 cells from renal transplant recipients are resistant to belatacept coupled with the association of elevated Th17 memory cell frequencies with acute cellular rejection strongly suggests that Th17 memory cells play a role in clinical belatacept-resistant graft rejection. Indeed, Th17 cells are known to be potent mediators of pathologic immune responses in a number of autoimmune diseases, and the CTLA-4 Ig derivative abatacept has been reported to be ineffective or even exacerbate the Th17-mediated autoimmune diseases MS, IBD, and SLE (21–23). Thus, this finding provides a potential explanation for immunomodulation of pathologic immune responses in both transplantation and autoimmunity.

While offering insight into Th17 cell biology in both healthy donors and renal transplant recipients, this study does not exclude the possibility that cellular subsets other than Th17 memory cells also play a role in belatacept resistant graft rejection. Similarly, the activation and effector function of belatacept-resistant Th17 cells may secondarily drive the recruitment and activation of additional cell subsets that may also participate in allograft rejection. A recent study by Vondran *et al.* found that patients with stable renal allograft function who were treated with belatacept for nearly eight years had diminished Th17 cell frequencies compared to patients treated with CNIs (24). In conjunction with our study, these data suggest that elevated Th17 frequencies are not associated with long-term treatment with belatacept in the absence of acute rejection but are increased in the setting of acute rejection. Future studies that provide careful monitoring of peripheral blood and graft-infiltrating cells prior to episodes of rejection in belatacept-treated individuals would help determine if Th17 memory cell frequencies can be used in as a predictive tool in the clinic.

Our data demonstrate that high expression of coinhibitory CTLA-4 on Th17 cells results in an unexpected augmentation of CD45RA<sup>-</sup>Th17 cells in the presence of belatacept, in contrast to inhibition of CD45RA<sup>+</sup> and CD45RA<sup>-</sup> Th1 cells. Th17 cells are known to be maintained at relatively low frequencies in vivo, even at sites of inflammation (11, 19), and high expression of the coinhibitor CTLA-4 is likely a mechanism that contributes to this phenomenon. The correlation of a higher expression level with greater augmentation in the

context of selective CTLA-4 blockade strongly suggests that CTLA-4 is acting in a cell intrinsic mechanism on Th1 and Th17 populations. However, recently several groups have implicated cell extrinsic mechanisms of action for CTLA-4 (25–27), and we cannot definitively exclude additional cell extrinsic action in our experiments. Additional studies will be needed to determine mechanistic basis for the increased CTLA-4 expression in Th17 cells.

These data highlight an important caveat for the clinical use of belatacept for the prevention of organ transplant rejection. Most of the acute rejection has occurred early following transplantation, and thus the use of Th17-inhibiting adjunct therapies during this window offers one potential solution to prevent rejection and maintain the long-term advantages of belatacept. As well, therapies that specifically target alloreactive Th17 responses might be particularly valuable in conjunction with belatacept. For example, the IL-12/23 blocker ustekinumab, currently approved to treat psoriasis, might offer such a solution due to the importance of IL-23 for Th17 maintenance. Recently, the use of inhibitors of the bromodomain and extra-terminal domain (BET) family of chromatin remodelers have been demonstrated to suppress human Th17 cells and autoimmune pathology in a murine model (28).

In conclusion, the CD28/CTLA-4 costimulation blocker belatacept is the first advance in post-transplant immunosuppression in many years. The paradoxical increase in pathologically severe acute rejection episodes is instructive of the need to develop higher resolution understanding of the functional characteristics of pathologic T cell subsets in order to more effectively modulate pathological T cell responses.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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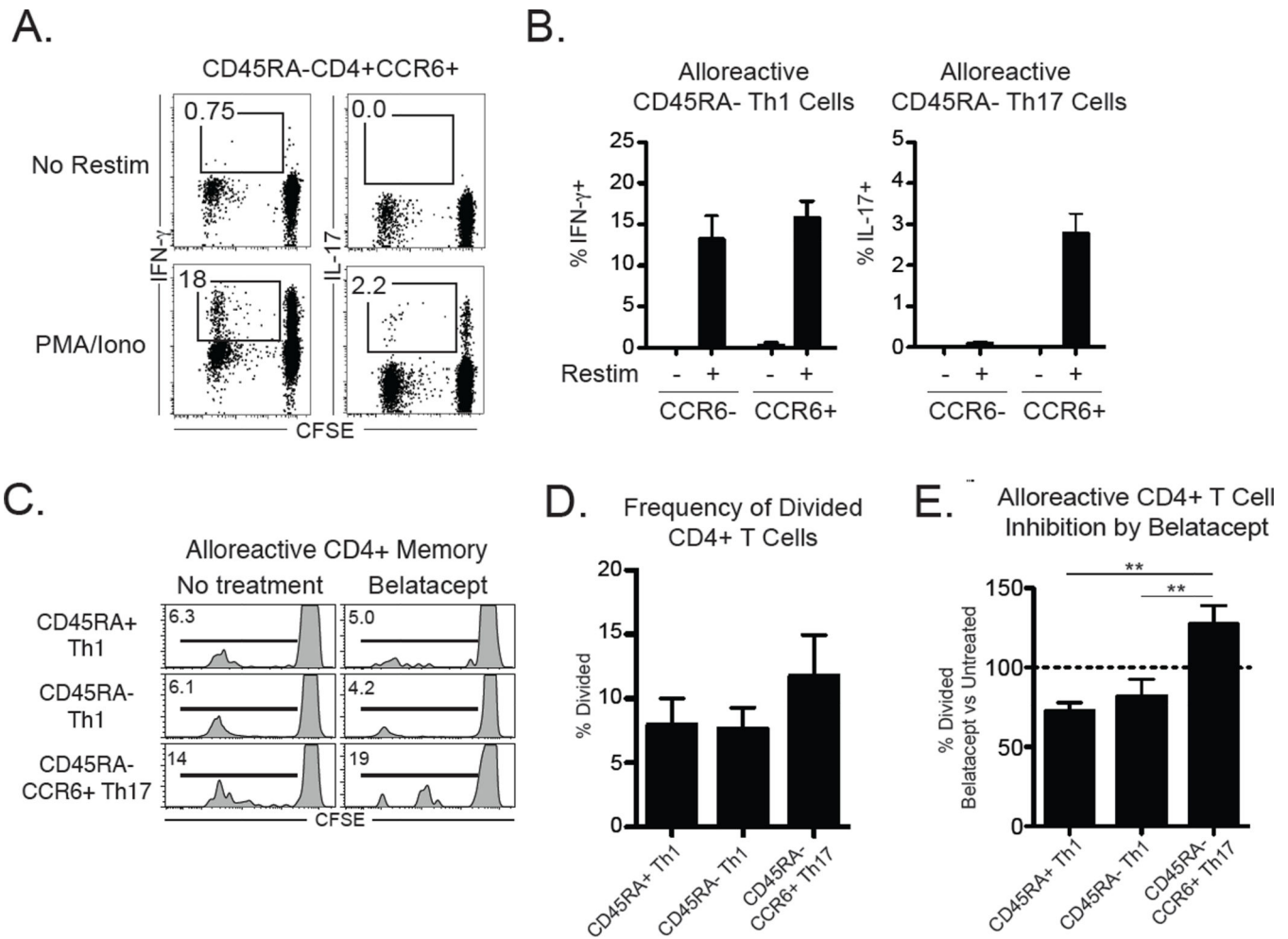
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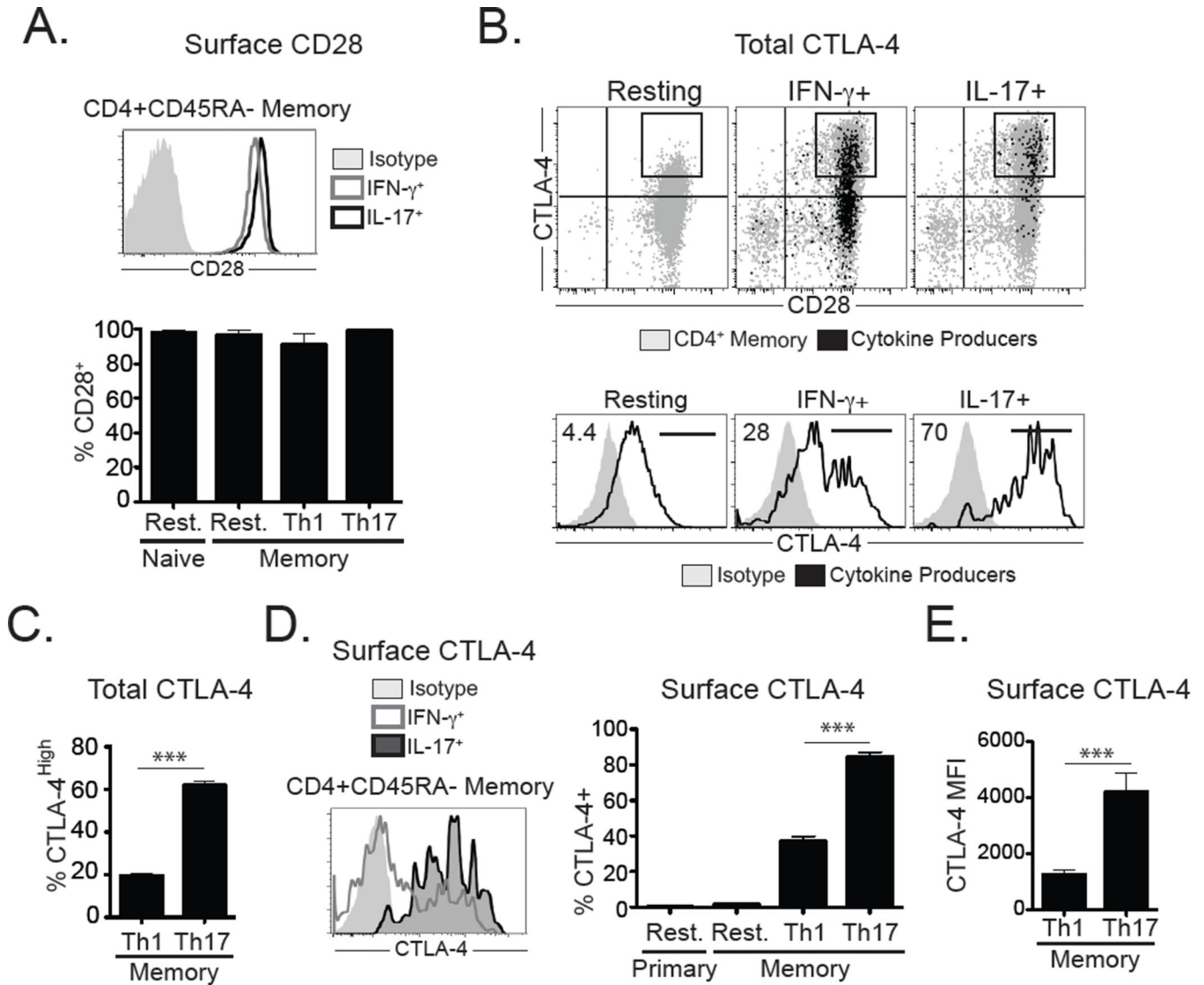
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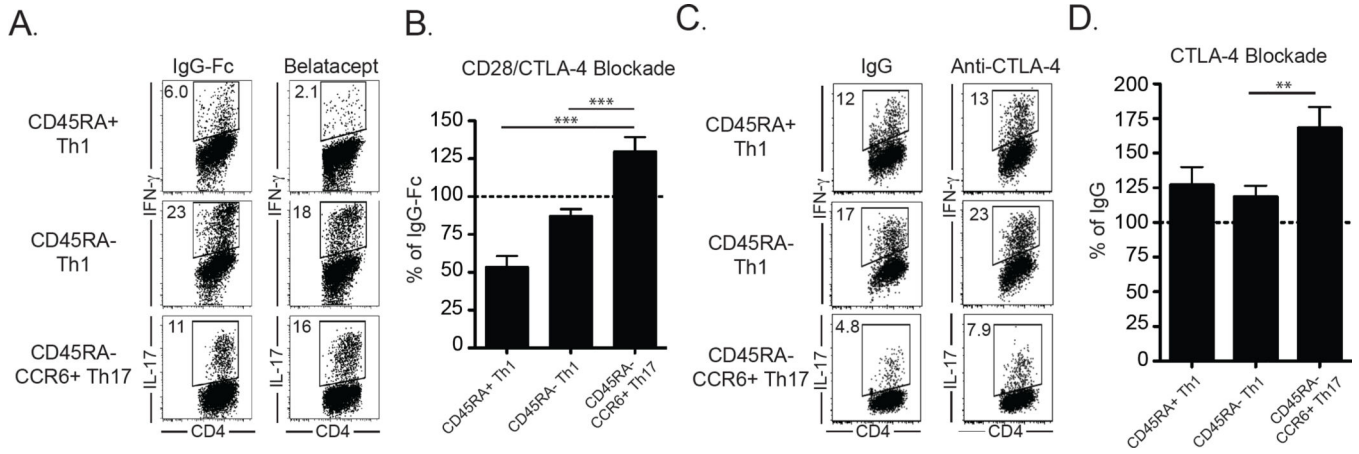
**Figure 1. CD45RA<sup>-</sup> Th17 Cells are Alloreactive and Resistant to Belatacept**

CFSE labeled T cell responders were stimulated with allogeneic MDDCs for 4 d followed by brief restimulation with PMA/Iono or not. Data depicted are from five independent stimulator/responder pairs. (A–B) Cells were gated on CD4<sup>+</sup>CD45RA<sup>+</sup>CFSE<sup>low</sup> or CD4<sup>+</sup>CD45RA<sup>-</sup>CFSE<sup>low</sup>, followed by either IFN- $\gamma$ <sup>+</sup> or IL-17<sup>+</sup> as described. (A) Representative frequencies of Th1 and Th17 cells among CD45RA<sup>+</sup>CCR6<sup>+</sup>CFSE<sup>low</sup> cells with and without PMA/Iono restimulation. (B) Summary frequencies of Th1 and Th17 cells within the CD45RA<sup>-</sup>CCR6<sup>-</sup> and CD45RA<sup>-</sup>CCR6<sup>+</sup> subsets. (C–E) Cells were first gated on CD4<sup>+</sup>CD45RA<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (CD45RA<sup>+</sup> Th1), CD4<sup>+</sup>CD45RA<sup>-</sup>IFN- $\gamma$ <sup>+</sup> (CD45RA<sup>-</sup> Th1), or CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>+</sup>IL-17<sup>+</sup> (CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17), followed by CFSE<sup>low</sup> divided cells. (C) Concatenated histograms depicting the frequency of CFSE<sup>low</sup> alloreactive CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1, and CD45RA<sup>-</sup> CCR6<sup>+</sup> Th17 cells in the absence (left column) or presence (right column) of belatacept following allogeneic co-culture. (D) Frequency of divided CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1 and CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 cells (CD45RA<sup>+</sup> Th1/Th17  $p = 0.310$ , CD45RA<sup>-</sup> Th1/Th17  $p = 0.421$ ). (E) Relative frequency of divided CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1, and CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 following allogeneic stimulation in the presence of belatacept compared to no treatment (CD45RA<sup>+</sup> Th1/Th17  $p = 0.0079$ , CD45RA<sup>-</sup> Th1/Th17  $p = 0.0079$ ).



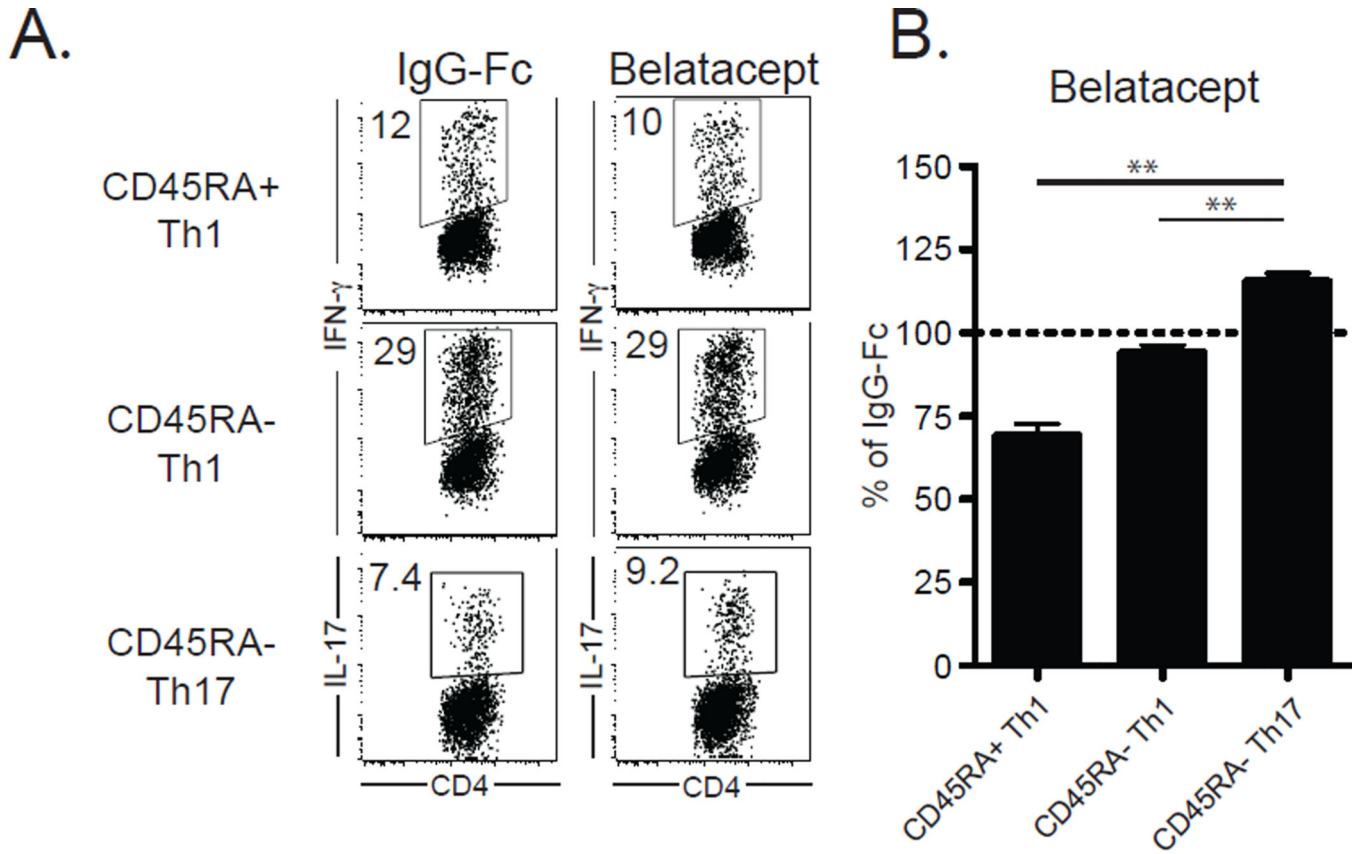
**Figure 2. Th17 Memory Cells Express High Levels of CTLA-4**

Peripheral blood T cells were stimulated briefly with PMA/Iono and analyzed by flow cytometry. CD4<sup>+</sup> T cell subsets were defined by the following gating strategy: Primary Th1, CD4<sup>+</sup>CD45RA<sup>+</sup>IFN- $\gamma$ <sup>+</sup>; Memory Th1, CD4<sup>+</sup>CD45RA<sup>-</sup> IFN- $\gamma$ <sup>+</sup>; Memory Th17, CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>+</sup>IL-17<sup>+</sup>. (A) Representative histogram (top) and summary data (bottom) of surface CD28<sup>+</sup> CD4<sup>+</sup> populations resting or restimulated with PMA/Iono (p = 0.092, n = 10). (B) Top, CD28 and total CTLA-4 expression on resting and restimulated Th1 and Th17 memory cells. Quadrant gate depicts CD28<sup>+</sup> and CTLA-4<sup>+</sup> population, square gate defines CTLA-4<sup>high</sup> population. Bottom, total CTLA-4 expression on resting and restimulated Th1 and Th17 memory cells. Gate depicts CTLA-4<sup>high</sup> expression (n = 8). (C) Frequency of CTLA-4<sup>high</sup> cells among Th1 and Th17 memory cells (p = 0.0009, n = 8). (D) Representative frequency (left) and summary frequencies of surface CTLA-4<sup>+</sup> CD4<sup>+</sup> populations (p < 0.0001, n = 9). (E) Mean fluorescence intensity of CTLA-4 on restimulated CD4<sup>+</sup> IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup> memory cells (p < 0.0001, n = 9).



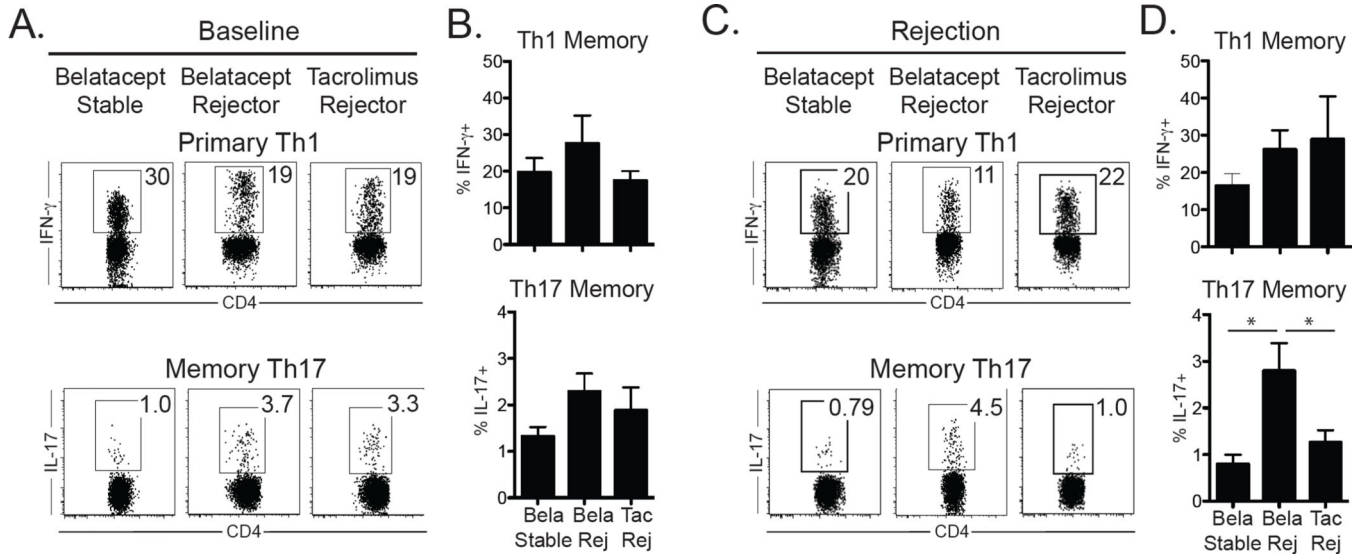
**Figure 3. CD45RA<sup>-</sup> Th17 Cells are Resistant to Inhibition by Belatacept and More Dependent on Coinhibition by CTLA-4**

Peripheral blood T cells from healthy donors were stimulated with anti-CD3 in the presence of blocking molecules or relevant control for 3 d followed by brief restimulation with PMA/Iono. CD4<sup>+</sup> T cell subsets were defined by the following gating strategy: CD45RA<sup>+</sup> Th1, CD4<sup>+</sup>CD45RA<sup>+</sup>IFN- $\gamma$ <sup>+</sup>; CD45RA<sup>-</sup> Th1, CD4<sup>+</sup>CD45RA<sup>-</sup> IFN- $\gamma$ <sup>+</sup>; CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17, CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>+</sup>IL-17<sup>+</sup>. (A) Representative frequency of CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1, and CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 after stimulation with anti-CD3 and belatacept or IgG-Fc control. (B) Relative change in frequencies of CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1, and CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 cells after anti-CD3 stimulation and treatment with belatacept compared to IgG-Fc among multiple individuals (CD45RA<sup>+</sup> Th1/Th17  $p = 0.0006$ , CD45RA<sup>-</sup> Th1/Th17  $p = 0.0006$ ,  $n = 7$ ). (C) Representative frequency of CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1, and CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 cells after stimulation with anti-CD3/CD28 beads and anti-CTLA-4 or IgG control. (D) Relative change in frequencies of CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1, and CD45RA<sup>-</sup>CCR6<sup>+</sup> Th17 cells after stimulation with anti-CD3/CD28 beads and anti-CTLA-4 or IgG control (CD45RA<sup>+</sup> Th1/Th17  $p = 0.0152$ , CD45RA<sup>-</sup> Th1/Th17  $p = 0.0152$ ,  $n = 6$ ).



**Figure 4. Renal Transplant Recipient Lymph Node CD45RA<sup>-</sup> Th17 Cells are Resistant to Belatacept**

T cells from explanted renal transplant recipient lymph nodes were stimulated with anti-CD3 for 3 d in the presence of belatacept or IgG-Fc control followed by brief restimulation with PMA/Iono (n = 5/group). CD4<sup>+</sup> T cell subsets were defined by the following gating strategy: CD45RA<sup>+</sup> Th1, CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>+</sup>IFN- $\gamma$ <sup>+</sup>; CD45RA<sup>-</sup> Th1, CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>-</sup>IFN- $\gamma$ <sup>+</sup>; CD45RA<sup>-</sup> Th17, CD3<sup>+</sup>CD4<sup>+</sup>CD45RA<sup>-</sup>IL-17<sup>+</sup> (A) Representative frequency and (B) summary data of CD45RA<sup>+</sup> Th1, CD45RA<sup>-</sup> Th1, and CD45RA<sup>-</sup> Th17 cells after proliferation and brief restimulation (CD45RA<sup>+</sup> Th1/Th17 p = 0.0049, CD45RA<sup>-</sup> Th1/Th17 p = 0.0049).



**Figure 5. Renal Transplant Patients Experiencing Acute Rejection on Belatacept-based Regimens Have Elevated Frequencies of Th17 Memory Cells**  
 Peripheral T cells from renal transplant patients classified as Belatacept Stable, Belatacept Rejectors, or Tacrolimus Rejectors (as described in the Methods) were stimulated for 4 h with PMA/Iono (n = 4–8/group). CD4<sup>+</sup> T cell subsets were defined by the following gating strategy: Th1 Memory, CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>-</sup>IFN- $\gamma$ <sup>+</sup>, Th17 Memory, CD4<sup>+</sup>CD45RA<sup>-</sup>CCR6<sup>+</sup>IL-17<sup>+</sup> (A) Representative frequencies from a single patient in each group and (B) summary frequencies of Th1 memory cells (Bela Stable/Bela Rejector p = 0.755, Bela Rejector/Tac Rejector p = 0.612) and Th17 memory cells (Bela Stable/Bela Rejector p = 0.106, Bela Rejector/Tac Rejector p = 0.400) collected at baseline. (C) Representative frequencies of a single patient in each group and (D) summary frequencies of Th1 memory cells (Bela Stable/Bela Rejector p = 0.200, Bela Rejector/Tac Rejector p = 0.886) and Th17 memory cells (Bela Stable/Bela Rejector p = 0.0286, Bela Rejector/Tac Rejector p = 0.0286) collected at 1 month following transplantation in Bela Stable or at the time of rejection in Belatacept and Tacrolimus Rejectors.