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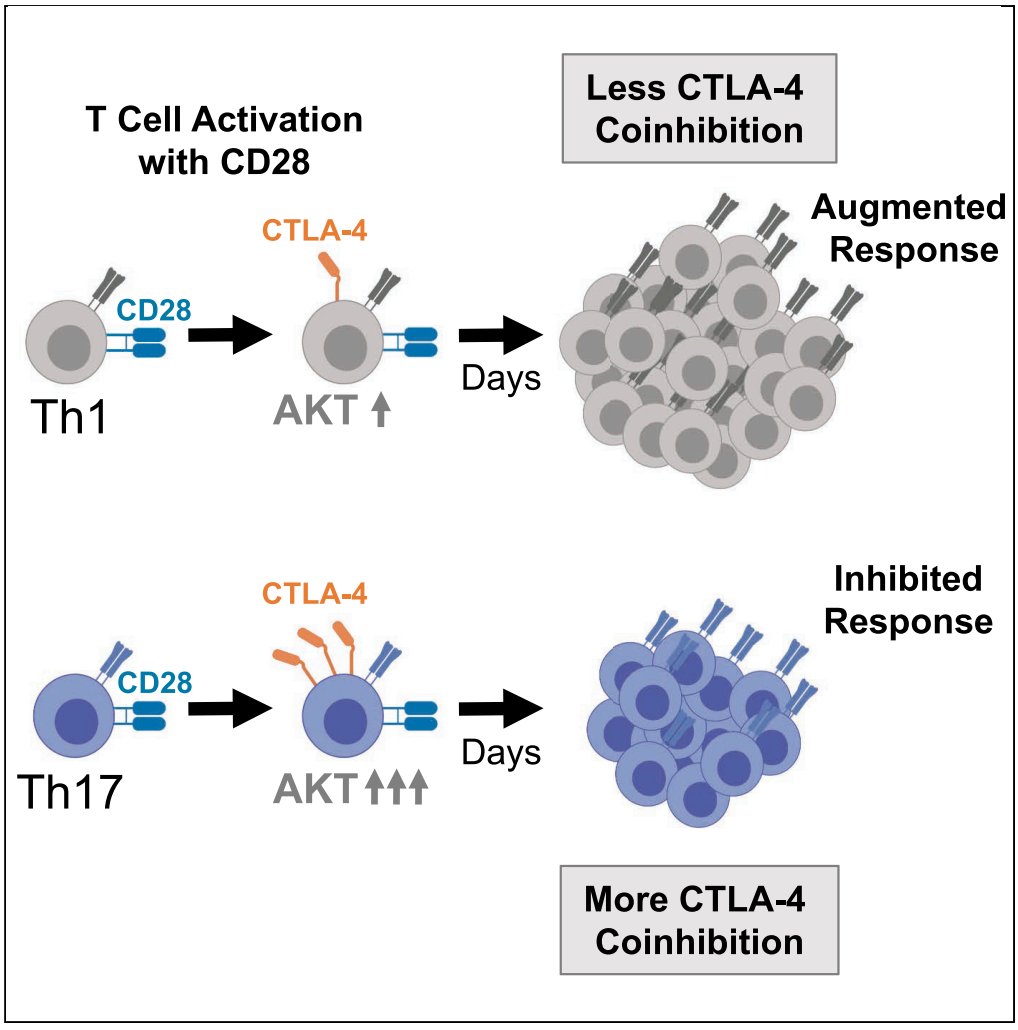

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HIGHLIGHTS

CD28 blockade resulted in augmentation of human Th17 cells relative to Th1 cells

Th17 polarized mice exhibited graft rejection in the presence of CD28 blockade

A significant portion of Th17 cell CTLA-4 expression was induced by CD28 ligation

Overexpression of FOXO1 or FOXO3 inhibited Th17 cell CTLA-4 expression

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CD28-Dependent CTLA-4 Expression Fine-Tunes the Activation of Human Th17 Cells

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SUMMARY

Previous work has demonstrated that Th17 memory cells but not Th1 cells are resistant to CD28/CTLA-4 blockade with CTLA-4 Ig, leading us to investigate the individual roles of the CD28 and CTLA-4 cosignaling pathways on Th1 versus Th17 cells. We found that selective CD28 blockade with a domain antibody (dAb) inhibited Th1 cells but surprisingly augmented Th17 responses. CD28 agonism resulted in a profound increase in CTLA-4 expression in Th17 cells as compared with Th1 cells. Consistent with these findings, inhibition of the CD28 signaling protein AKT revealed that CTLA-4 expression on Th17 cells was more significantly reduced by AKT inhibition relative to CTLA-4 expression on Th17 cells. Finally, we found that FOXO1 and FOXO3 overexpression restrained high expression of CTLA-4 on Th17 cells but not Th1 cells. This study demonstrates that the heterogeneity of the CD4$^+$ T cell compartment has implications for the immunomodulation of pathologic T cell responses.

INTRODUCTION

CD4$^+$ T helper (Th) cells differentiate into subsets that can both provide immunity against distinct classes of microbes and mediate pathogenic immune responses, including autoimmunity and transplant rejection. A variety of cosignaling receptors are recognized to play distinct roles in the activation and differentiation of specific Th subsets. The prototypic cosignaling pathway on T cells is CD28/CTLA-4, in which costimulatory CD28 and coinhibitory CTLA-4 receptors on T cells compete for the same ligands, CD80 and CD86, on antigen-presenting cells. Recently the cosignaling requirements of Th17 cells have garnered clinical interest, as this population is the target of a number novel therapeutics for autoimmune diseases such as multiple sclerosis (MS), inflammatory bowel disease (IBD), and systemic lupus erythematosus (SLE) (McGeachy and Cua, 2008; Sallusto and Lanzavecchia, 2009; Tsokos, 2011; Miossec et al., 2009).

Clinical CD28/CTLA-4 blockade with CTLA-4 Ig (abatacept and its close derivative belatacept) is used to treat autoimmune disease and to prevent graft rejection following renal transplantation. Interestingly, Th17 cells are resistant to CD28/CTLA-4 blockade with CTLA-4 Ig *in vitro* relative to Th1 cells (Krummey et al., 2014a; Bouguermouh et al., 2009), and CTLA-4 Ig and its derivatives have shown limited efficacy in clinical trials of MS, IBD, and SLE (Merrill et al., 2010; Sandborn et al., 2012; Linsley and Nadler, 2009). Investigations involving the CD28 pathway on Th17 cells in several experimental systems have demonstrated variable effects of CD28 on Th17 cells relative to Th1 cells (de Wit et al., 2011; Santarlasci et al., 2012; Paulos et al., 2010). Recently, our group showed that human and murine Th17 cells express significantly more CTLA-4 than Th1 cells (Krummey et al., 2014a, 2014b). However, mechanistic explanation of these observations and their relationship to Th17 cell resistance to CTLA-4 Ig is lacking.

In this study, we sought to understand the potential link between the observation that Th17 cells are relatively resistant to CTLA-4 Ig and the differential expression of CTLA-4 on Th1 versus Th17 cells. We utilized an anti-CD28 domain antibody (dAb) to selectively inhibit CD28 on Th1 versus Th17 cells, which revealed that Th1 cells are susceptible, whereas Th17 cells are resistant to CD28 blockade. This effect was mimicked by pharmacologic AKT inhibition, which revealed that Th17 cell activation is relatively resistant to AKT inhibition compared with Th1 cells. We found that the mechanism underlying this resistance is the fact that agonism of CD28 strongly induced CTLA-4 expression on Th17 but not Th1 cells and that the transcription factors FOXO1 and FOXO3 controlled high expression of CTLA-4 on Th17 cells. This report reveals a critical difference in the CD28 pathway on Th1 versus Th17 cells that results in disparate responses to immunomodulation.

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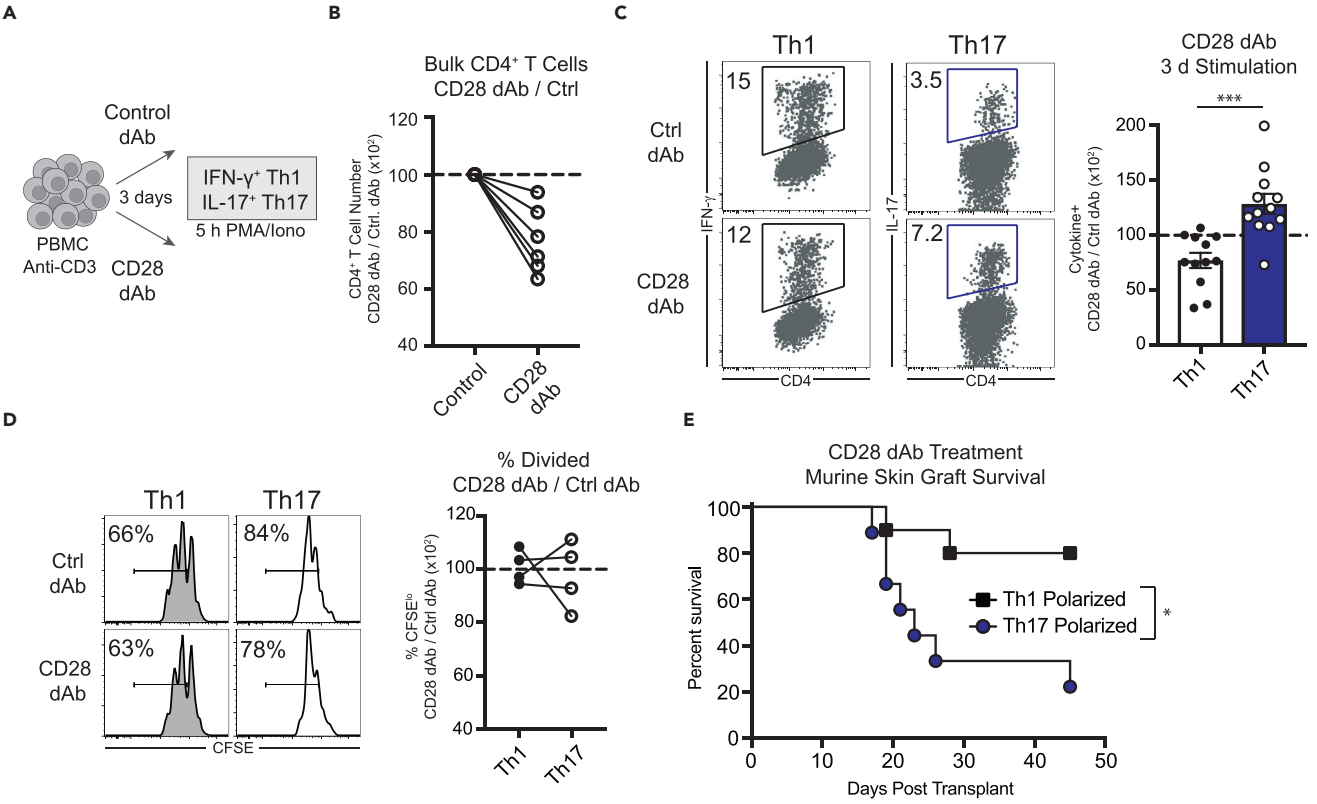

Figure 1. Th17 Cells Are Resistant to Selective CD28 Blockade

(A) Schematic of peripheral blood mononuclear cell (PMBC) stimulation and assessment of Th1 and Th17 populations.

(B) Frequency of bulk human CD4$^+$ T cells after 3 days of culture with anti-CD3 and blocking anti-CD28 domain antibody (dAb), normalized to control dAb.

(C) Left panel, representative flow cytometry data depicting frequencies of human Th1 and Th17 cells following 3 days in culture in the presence of either control or anti-CD28 dAb (left). Right panel, summary data depicting the ratio of the frequency of Th1 and Th17 cells in anti-CD28 dAb cultures relative to control. See also Figure S1.

(D) Representative flow cytometry and summary data depicting CFSE dilution of human Th1 and Th17 cells after 3 days in culture following stimulation with anti-CD3 in the presence of anti-CD28 dAb or control dAb.

(E) Graft survival of mice containing donor-reactive cells polarized to either Th1 or Th17 and treated with anti-CD28 dAb. See also Figure S2.

(B–D) Each data point in the summary data represents an individual human donor. (E) Data shown represent 9–10 mice/group compiled from two independent experiments ($p = 0.015$). Statistical analysis performed using (C) Student's t test (two-tailed) and (E) log rank (Mantel-Cox) test. Summary data depict mean ± SEM. dAb, domain antibody. All summary data depict the mean ± standard deviation. Significance is defined as *$p < 0.05$, ***$p < 0.001$.

RESULTS

Human Th17 Cells Are Resistant to Selective CD28 Blockade

We have previously shown that human Th17 cells are more resistant to the CD28/CTLA-4 blocker CTLA-4 Ig (belatacept) relative to Th1 cells (Krummey et al., 2014a). We questioned whether differences in CD28 versus CTLA-4 signals were primarily responsible for this observation. Using a novel anti-CD28 domain antibody (dAb), which comprises a Vκ single antigen-binding site and a mutated "silent" Fc domain (lulizumab [Suchard et al., 2014]), we selectively inhibited CD28 signals during polyclonal stimulation with anti-CD3 (Figure 1A) (see Transparent Methods section within Supplemental Information). Anti-CD28 dAb treatment of bulk CD4$^+$ T cell cultures resulted in reduced numbers of CD4$^+$ T cells after 3 days (Figure 1B). The frequency of Th1 cells within those cultures (as measured by IFN-γ secretion following restimulation with PMA/ionomycin) was significantly inhibited by CD28 blockade (Figure 1C), whereas the frequency of Th17 cells was not inhibited by CD28 blockade (Figure 1C). These results suggest that CD28 signaling leads to distinct functional outcomes in Th1 versus Th17 cells.

We performed the same assay with Carboxyfluorescein succinimidyl ester (CFSE)-labeled peripheral blood mononuclear cells (PBMCs) to assess the impact of anti-CD28 dAb on proliferation in Th1 versus Th17 cells.

The presence of anti-CD28 dAb resulted in less proliferation of the total $CD4^+$ T cells in culture (Figure S1). Interestingly, Th1 versus Th17 cells proliferated to similar degrees in the presence of the anti-CD28 dAb versus control dAb (Figure 1D). This suggests that the ability of the anti-CD28 dAb to inhibit Th17 cells occurs at the level of activation and does not affect cells once they begin to proliferate.

Murine Th17 Cells Mediate Graft Rejection in the Presence of CD28 Blockade

To assess the impact of CD28 blockade on Th17 cells *in vivo*, we utilized an antigen-specific model of skin graft rejection that relies on $CD4^+$ T cells polarized to Th1 or Th17 *in vivo* (Krummey et al., 2014b). In this model, graft survival times in Th1-polarized versus Th17-polarized mice treated with control dAb were not significantly different (Th1, 15 days; Th17, 17 days) (Figure S2). In contrast, we observed a differential effect of anti-CD28 dAb on graft survival in Th1 versus Th17-polarized mice. The majority of Th1-polarized mice maintained their grafts long-term (Figure 1E), whereas the majority of Th17-polarized animals rejected their grafts by day 30 (MST 23 d, Figure 1E). These results demonstrate that the presence of Th17 immunity confers resistance to CD28 blockade *in vivo*, similarly to our observations of *in vitro* stimulated of human T cells.

CTLA-4 Expression on Human Th17 Cells Is Uniquely Sensitive to AKT

CTLA-4 expression has been shown to be dependent on CD28 signals in bulk T cells (Finn et al., 1997; Walunas et al., 1994, 1996; Lindsten et al., 1993; Krummel and Allison, 1995), leading us to hypothesize that differential sensitivity to CD28-mediated AKT signals in Th1 versus Th17 cells could result in the CD28 blockade-resistant phenotype observed in Th17 cells relative to Th1 cells. In support of this, we found that CD28 blockade with anti-CD28 dAbs resulted in a greater inhibition of CTLA-4 on Th17 cells relative to Th1 cells (Figure 2A).

We next investigated whether intracellular signals downstream of CD28, which are transmitted through PI3K-Akt-mTOR axis (Hedrick et al., 2012; Powell et al., 2012), could inhibit CTLA-4 expression. We treated cells with an AKT phosphorylation inhibitor (AKT IV) (Kau et al., 2003; Lee et al., 2009) or DMSO vehicle control and activated them in the presence of CD3/CD28 monoclonal antibodies (mAbs). AKT inhibition significantly reduced both CD69 and CTLA-4 expression on bulk $CD4^+$ populations compared with vehicle control (Figure 2B). AKT inhibition resulted in a relatively greater inhibition of IFN-γ-producing Th1 cells relative to IL-17-producing Th17 cells (Figure 2C). On the other hand, AKT inhibition resulted in a relatively less fold reduction of CTLA-4 expression on cytokine-producing Th1 cells relative to Th17 cells (Figure 2D).

In addition to defining Th1 and Th17 cells by cytokine production, surface markers have also been used to define these populations (Maggi et al., 2010; Annunziato et al., 2007; Singh et al., 2016). Following stimulation with anti-CD3/anti-CD28, the majority of cytokine-producing Th1 cells were CXCR3hiCCR6lo and CCR4lo (Figures S3A and S3B). Th17 cells, in contrast, were CCR6hiCXCR3lo and CCR4hiCD161hi (Figures S3A and S3B). We explored differences in CTLA-4 expression between these "chemokine" Th1 and Th17 cells identified using these alternate definitions. Similar to Th17 cells defined by their IL-17 secreting ability, chemokine Th17 cells expressed significantly higher levels of CTLA-4 than chemokine Th1 cells (Figure S3C). In chemokine Th1 and Th17 cells, we found that AKT inhibition with an additional AKT phosphorylation inhibitor (AKT-1/2) (Zhao et al., 2008) resulted in greater fold reduction of CTLA-4 expression on Th17 cells relative to Th1 cells (Figures 2D–2F). Together, these results demonstrate that Th17 cells are uniquely sensitive to CD28/AKT signaling, as blockade of this pathway results in both diminished CTLA-4 expression and a greater efficiency of activation relative to Th1 cells. This interpretation is supported by the finding that CD28 blockade results in less activation, as measured by cytokine production, of Th1 cells compared with Th17 after 3-day culture (Figure 1B).

Human Th17 CTLA-4 Expression Is Dependent on CD28 Signals

To directly assess the functional impact of CD28 agonism on Th1 and Th17 cells, we stimulated T cells in the presence of anti-CD28 mAb or control IgG. We found that, although CD28 agonism resulted in a modest increase in the frequency of Th1 cells (Figure 3A), it resulted in a significant decrease in the frequency of Th17 cells (Figure 3A).

Given the findings that Th17 CTLA-4 expression is relatively dependent on AKT signaling (Figures 2D and 2F) and that the frequency of Th17 cells is reduced in the presence of strong CD28 cosignaling (Figure 3A), we hypothesized that coinhibitory CTLA-4 expression on Th17 cells could be driven by CD28 signaling. To

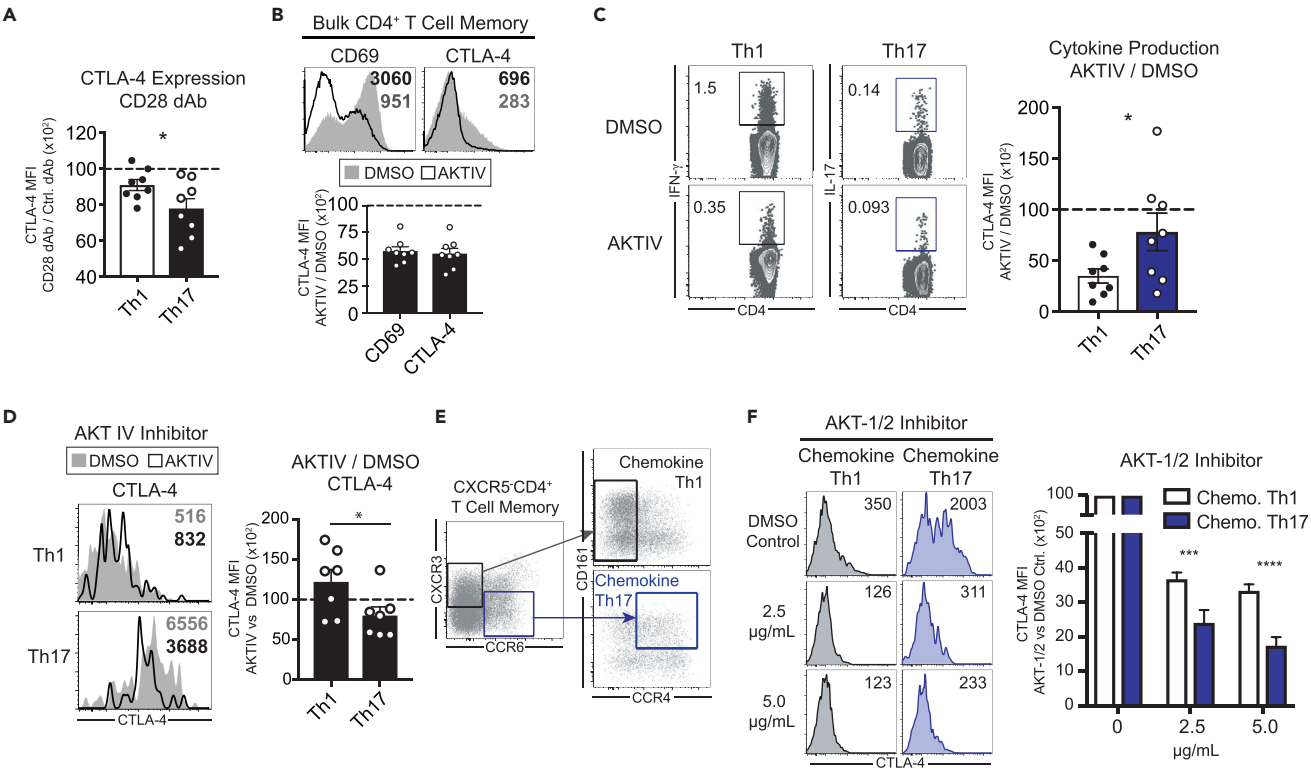

Figure 2. CTLA-4 Expression on Human Th17 Cells Is Uniquely Sensitive to AKT Inhibition

(A) Expression of CTLA-4 on Th1 versus Th17 cells following activation with anti-CD3 and blocking anti-CD28 dAb or control dAb.

(B) Expression of CD69 and CTLA-4 on human bulk CD4$^+$ memory T cells activation with anti-CD3/anti-CD28 in the presence or absence of the AKT inhibitor AKTIV.

(C) Frequency of Th1 versus Th17 memory cells activation with anti-CD3/anti-CD28 in the presence or absence of the AKT inhibitor AKTIV.

(D) Expression of CTLA-4 on human Th1 versus Th17 memory T cells activation with anti-CD3/anti-CD28 in the presence or absence of the AKT inhibitor AKTIV.

(E) Gating strategy for identifying CXCR3^{hi}CCR4^{lo} "chemokine" Th1 cells or CCR6^{hi}CCR4^{hi}CD161^{hi} "chemokine" Th17 cells. See also Figure S3.

(F) Expression of CTLA-4 on chemokine Th1 or Th17 cells following activation with anti-CD3/anti-CD28 mAbs in the presence of the AKT inhibitor AKT-1/2. Summary data depict six individual donors.

Statistical analyses performed using (C and D) Student's t test (two-tailed) or (F) two-way ANOVA with Tukey's multiple comparison test. Summary data depict mean ± SEM. Significance is defined as *p < 0.05, ***p < 0.001, ****p < 0.0001.

address this question, we purified CD4$^+$ T cells to remove CD80/86-bearing APCs within peripheral blood leukocytes and stimulated CD4$^+$ T cells in the presence of beads coated in either anti-CD3 or anti-CD3/CD28 mAb. We found that Th1 and Th17 memory cells were similarly activated by anti-CD3 stimulation as measured by CD69 expression (Figure 3B). CD28 agonism, however, resulted in slightly greater CD69 expression on both Th1 and Th17 cells (Figure 3B). CTLA-4 expression was slightly higher on Th17 cells compared with Th1 cells in the presence of CD3 stimulation alone, whereas the expression of CTLA-4 was dramatically increased on Th17 cells by CD28 agonism (Figure 3C). In contrast, Th1 memory cells did not significantly upregulate CTLA-4 following CD28 agonism (Figure 3C). Thus, CD28 agonism resulted in a relatively greater fold increase in the expression (MFI) of CTLA-4 in Th17 versus Th1 cells (Figure 3D). CD28 agonism did not significantly alter the expression of 2B4, TIGIT, TIM-3, or PD-1 (Figure S4). Thus, these results demonstrate that CD28 stimulation results in different levels of CTLA-4 upregulation on Th1 versus Th17 cells.

FOXO1 and FOXO3 Control CTLA-4 Expression in Human Th17 Cells

We next assessed whether the terminal signaling component of the CD28/AKT pathway, the transcription factors FOXO1 and FOXO3, impacts CTLA-4 expression in Th1 versus Th17 cells. We overexpressed FOXO1 or FOXO3 by transfecting human PBMC with vectors containing FOXO1 or FOXO3 along with the surface maker Thy1.1 as a tag to allow for detection of transfected cells (Figure 4A). CD4$^+$ T cells

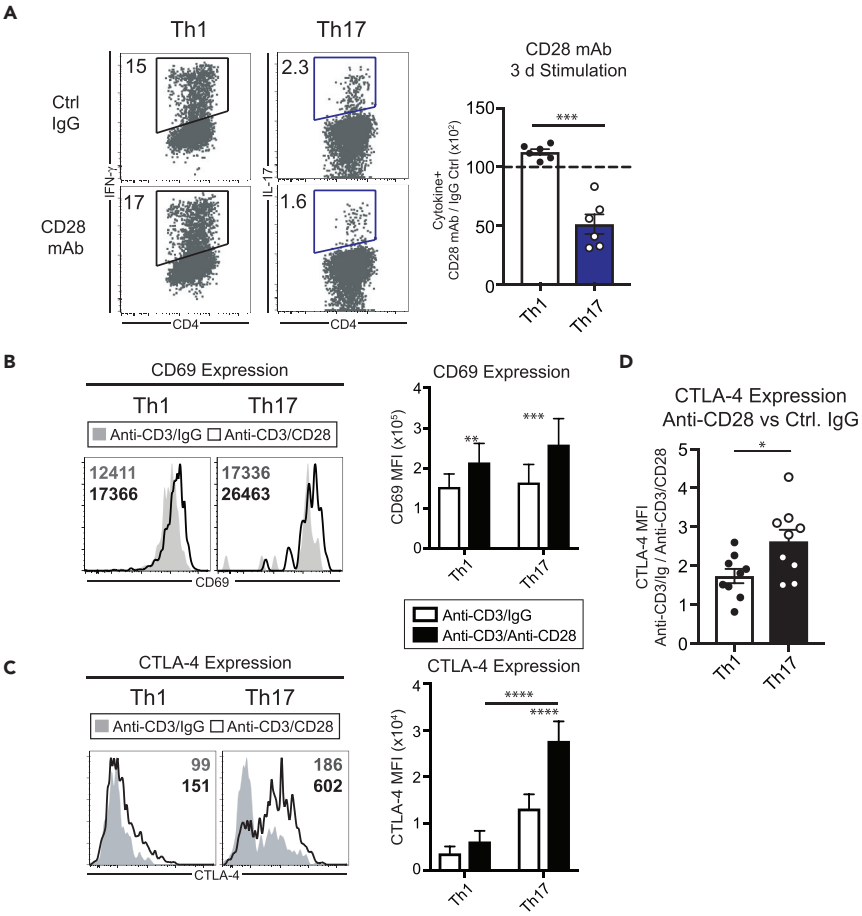

Figure 3. High Th17 CTLA-4 Expression Is Dependent on CD28 Signals

(A) Representative flow cytometry and summary data of frequency of Th1 versus Th17 cells after 3 days in culture with anti-CD3 and agonistic anti-CD28 mAb or control IgG.

(B) Expression of CD69 on Th1 versus Th17 cells activated with beads coated in anti-CD3/isotype control IgG or anti-CD3/agonistic anti-CD28 mAb.

(C) Expression of CTLA-4 on Th1 versus Th17 cells activated with beads coated in anti-CD3 or anti-CD3/anti-CD28.

(D) Expression of CTLA-4 on Th1 versus Th17 cells activated with anti-CD3/IgG relative to anti-CD3/agonistic anti-CD28. See also Figure S4.

(A and D) Each data point represents an individual human donor. (B) Summary data represent seven individual human donors. (C) Summary data represent nine individual human donors. Statistical analyses performed using (A and D) Student's t-test (two-tailed) or (B and C) two-way ANOVA with Sidak's multiple comparison test. Summary data depict mean ± SEM. mAb, monoclonal antibody. Significance is defined as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

were transfected with FOXO1 and FOXO3 vectors (or empty vector control) and stimulated with anti-CD3/anti-CD28 mAbs, and transfected Th1 and Th17 cells were identified by gating on Thy1.1 and CXCR3hiCCR6lo and CCR4lo or CCR6hiCXCR3lo and CCR4hiCD161hi, respectively. Results indicated that neither FOXO1 nor FOXO3 overexpression significantly impacted CTLA-4 expression on Thy1.1$^+$ chemokine Th1 cells relative to empty vector Th1 controls (Figures 4B and 4C). Among Thy1.1$^+$ chemokine Th17 cells, however, overexpression of either FOXO1 or FOXO3 resulted in significant inhibition of CTLA-4 expression (Figures 4B and 4C). These results demonstrate that overexpression of either FOXO1 or FOXO3 is sufficient to repress CTLA-4 expression in human Th17 cells.

DISCUSSION

Although Th17 cells have been shown to be resistant to CD28/CTLA-4 blockade with CTLA-4 Ig both *in vitro* and *in vivo* by our group and others, it was not clear from these studies whether this resistance is mediated through CD28, CTLA-4, or another signaling pathway. Here we provide evidence that CTLA-4 expression is

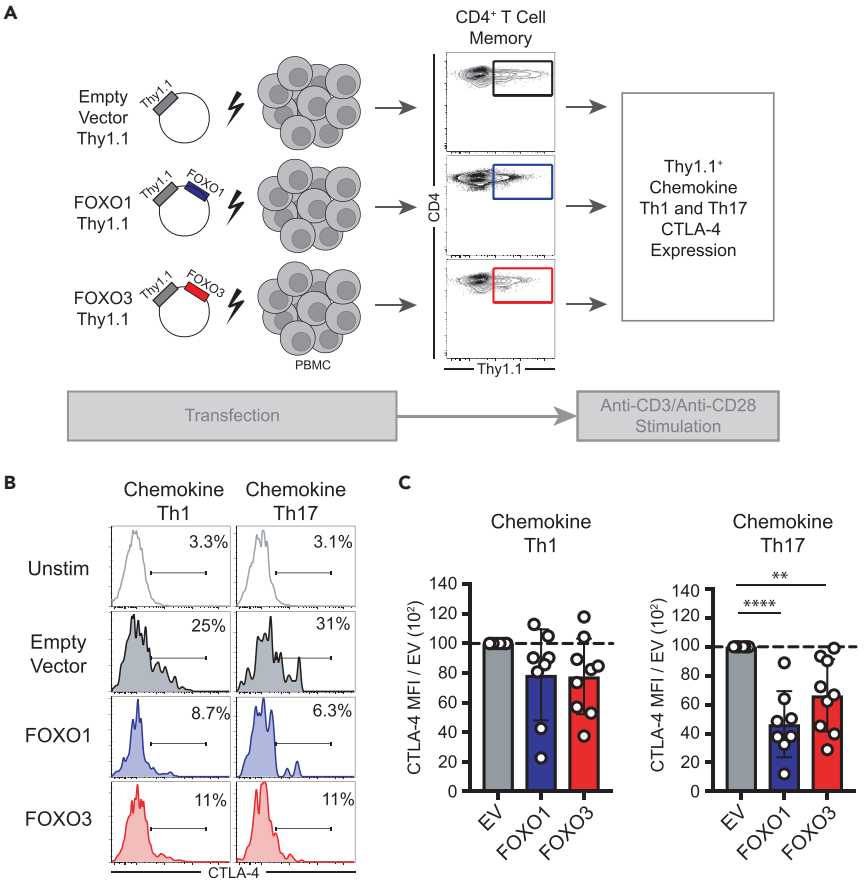

Figure 4. FOXO1 and FOXO3 Control CTLA-4 Expression in Human Th17 Cells

(A) Schematic of the experimental design in which vectors containing a Thy1.1 tag and either FOXO1 or FOXO3 were used to overexpress FOXO1 or FOXO3 in Th1 versus Th17 cells via transient transfection.

(B) Bulk CD4$^+$ T cells were transfected with FOXO1- or FOXO3-containing vectors (or empty vector controls) and were stimulated with anti-CD3/agonistic anti-CD28. Expression of CTLA-4 on Thy1.1$^+$ chemokine Th1 or Th17 cells was assessed.

(C) Summary data of the expression of CTLA-4 (MFI) on chemokine Th1 versus Th17 cells following transfection as in (B) relative to the CTLA-4 MFI in empty vector control Th1 or Th17 cells, respectively. Each data point represents an individual human donor.

Statistical analysis performed using one-way ANOVA with Dunnett's multiple comparisons test. Summary data depict mean ± SEM. Significance is defined as **$p < 0.01$, ****$p < 0.0001$.

more sensitive to CD28/AKT signaling in Th17 as compared with Th1 cells, resulting in stronger induction of this coinhibitory pathway following costimulatory CD28 signaling. Consistent with this finding, in a murine model of Th1 or Th17 polarized effector cells, Th17 polarized mice were not protected from graft rejection in the presence of CD28 blockade, providing an *in vivo* correlation with *in vitro* studies of human T cells. Thus, this work demonstrates how differences in the amount of CD28-dependent CTLA-4 expression serves as a feedback loop to fine-tune Th1 and Th17 cell responses.

The role for CD28-dependent CTLA-4 expression on Th17 cells offers a nuanced understanding of classic work defining this cosignaling pathway. Seminal studies demonstrated that CD28 signals are required for optimal *Ctla4* gene expression (Teft et al., 2006; Finn et al., 1997; Walunas et al., 1994, 1996; Lindsten et al., 1993; Krummel and Allison, 1995). It is important to note that these studies were conducted on bulk murine CD4$^+$ or CD8$^+$ T cell populations or T cell lines, in contrast to the primary human CD4$^+$ T cell subsets investigated in this study. Our findings provide evidence that subtle differences in sensitivity to the CD28 pathway can result in profound functional differences on specific CD4$^+$ T helper subsets, which cannot be appreciated in evaluation of bulk T cell populations. As evidence of this distinction, although we found that CD28 blockade inhibited the proliferation of bulk CD4$^+$ T cells, we did not find a difference

in the number of cell divisions among Th1 or Th17 cells treated with anti-CD28 dAb. This supports the notion that changes in the frequency of Th1 and Th17 cells under CD28 blockade reflects differences in activation rather than proliferation, in contrast to the impact of CD28 blockade on the proliferation of bulk $CD4^+$ T cells.

This study supports a role for cell intrinsic coinhibition by CTLA-4, as our data provide evidence that CD28-dependent CTLA-4 acts to diminish the number of polarized Th17 cells that become activated to produce cytokine or enter the cell cycle, thus paradoxically reducing the number and frequency of Th17 cells after CD28 ligation. Although multiple functional roles have recently been ascribed to CTLA-4, including cell extrinsic mechanisms (Corse and Allison, 2012; Wang et al., 2012; Qureshi et al., 2011), our results are consistent with previous findings that CTLA-4 signaling inhibits activation without inducing apoptosis (Krummel and Allison, 1995; Walunas et al., 1996). However, it is important to note that our results do not exclude the possibility of additional cell extrinsic functions of CTLA-4 on Th17 cells.

This study provides a link between functional data involving CD28/CTLA-4 blockade of Th1 and Th17 cells and investigations of FOXO1/FOXO3-mediated CTLA-4 expression (Powell et al., 2012). Mouse models of FOXO1 and dual FOXO1/FOXO3 germline deletion have diminished CTLA-4 expression on $CD4^+$ populations and established that FOXO1 and FOXO3 can bind to the *Ctla4* promoter (Kerdiles et al., 2010; Ouyang et al., 2010; Kim et al., 2013). In a study of the role of common gamma chain-induced T cell cytokine production, *Il-17* production by human Th17 cells is uniquely reliant on AKT, PI3K, and FOXO1 (Wan et al., 2011). However, a specific connection between AKT and CTLA-4 expression on human Th17 cells has not previously been shown. Further investigation is needed to uncover the transcriptional mechanism that enables FOXO3 to selectively repress *Ctla4* expression in Th17 cells, such as the possibility that epigenetic changes at the *Ctla4* locus play a role in these findings.

As clinical immunomodulatory drugs become more targeted to specific pathways on pathologic T cells, understanding the effect of these agents on individual T cell subsets is critical. This work provides an important example of how manipulation of a single receptor can have profound functional implications for shaping T cell responses in autoimmune disease and organ transplantation.

Limitations of the Study

Although we made efforts to evaluate the cell-intrinsic impact of CD28/CTLA-4 signals on $CD4^+$ T cells, a number of the *in vitro* assays and our *in vivo* experiments necessarily included the presence of additional cells. Thus, we cannot definitively rule out that the impact of CD28 blockade on Th1 and Th17 cells was partially the result of interaction with additional cell type(s). One additional limitation of the study is the difficulty in dissecting signaling mechanisms in primary human cells. More specifically, although we were able to show a differential impact of CD28 signaling and AKT inhibition on Th17 cells relative to Th17 cells, uncovering the precise differences in the signaling cascades that leads to these functional differences will require additional studies.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.100912.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.M.K. and M.L.F.; Methodology, S.M.K., C.R.H., and M.L.F.; Investigation, S.M.K., C.R.H., and D.L.; Formal Analysis, S.M.K.; Writing the manuscript – Original Draft, S.M.K.; Writing – Reviewing & Editing, M.L.F; Funding Acquisition, M.L.F.; Supervision, M.L.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

CD28-Dependent CTLA-4 Expression Fine-Tunes the Activation of Human Th17 Cells

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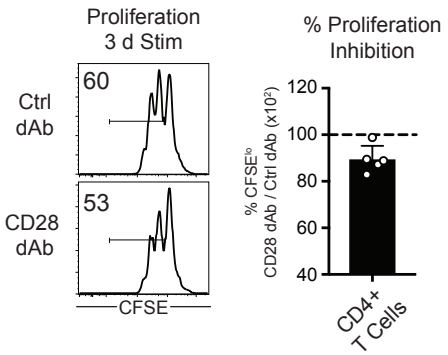

Figure S1. CD28 blockade inhibits CD4⁺ T cell proliferation, related to Figure 1. CFSE dilution of Th1 and Th17 cells after 3 days in culture with anti-CD3 and anti-CD28 dAb or control dAb. Each data point represents an individual human donor. Data are represented as mean ± SEM. dAb, domain antibody.

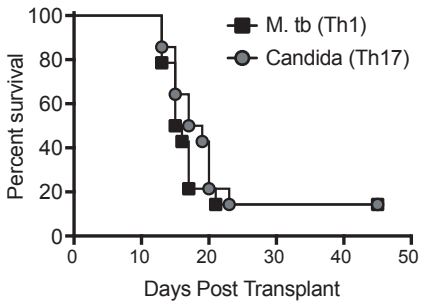

Figure S2. Th1 and Th17 polarized mice reject skin grafts with similar kinetics following control dAb treatment, related to Figure 1. Graft survival of mice polarized to Th1 or Th17 cells and treated with control dAb. Data shown are 9-10 mice/group from 2 independent experiments.

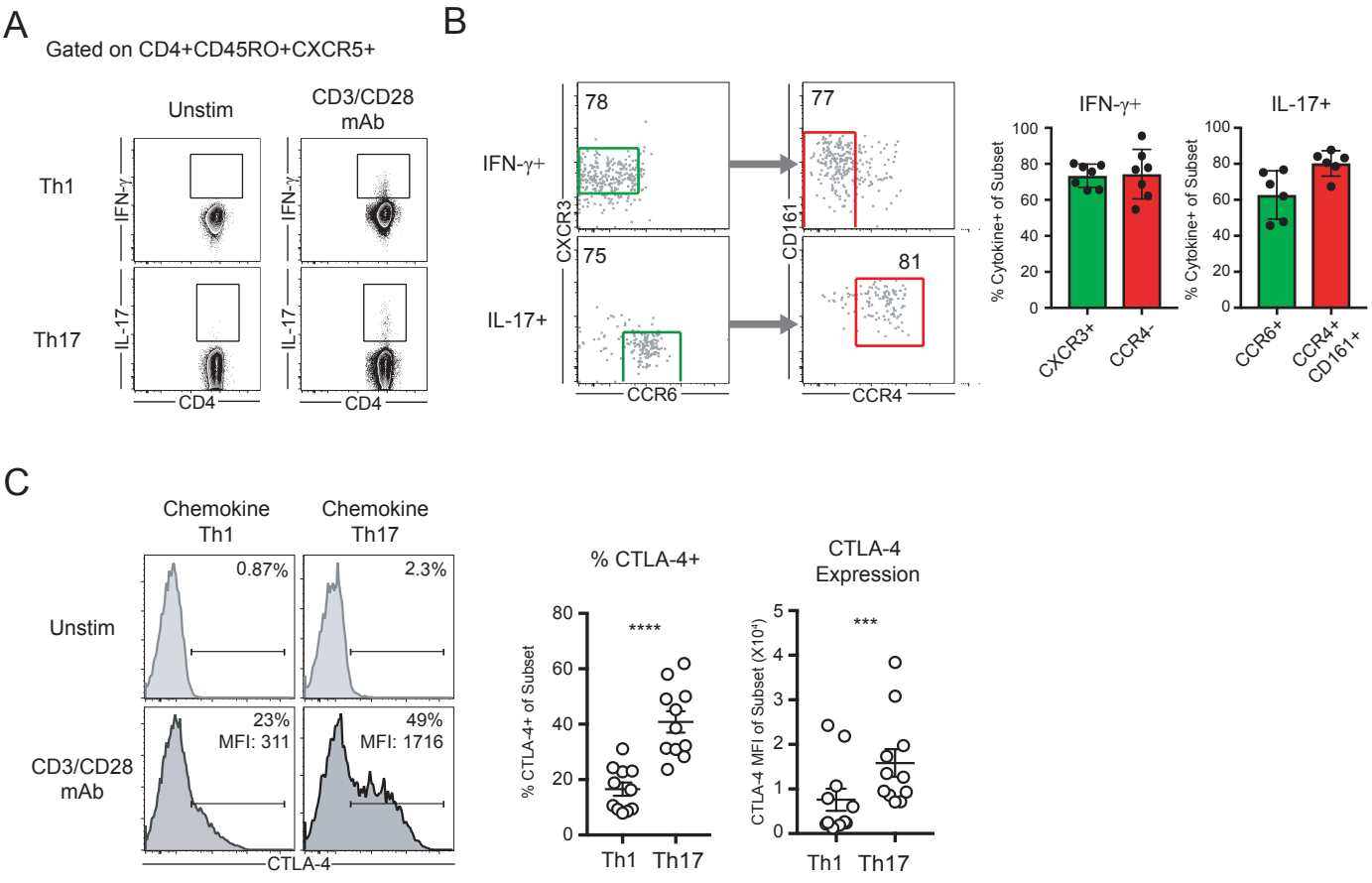

Figure S3. Human Chemokine Th17 cells express high levels of CTLA-4 relative to Chemokine Th1 cells, related to Figure 2. (A) Representative gating of cytokine producing Th1 and Th17 cells following anti-CD3/anti-CD28 mAb stimulation. (B) Representative gating and summary data of chemokine expression of cytokine-producing Th1 and Th17 cells. (C) Representative and summary data of CTLA-4 expression on Chemokine Th1 and Th17 cells. (C) Each data point represents an individual human donor. Summary data depicts mean ± SEM.

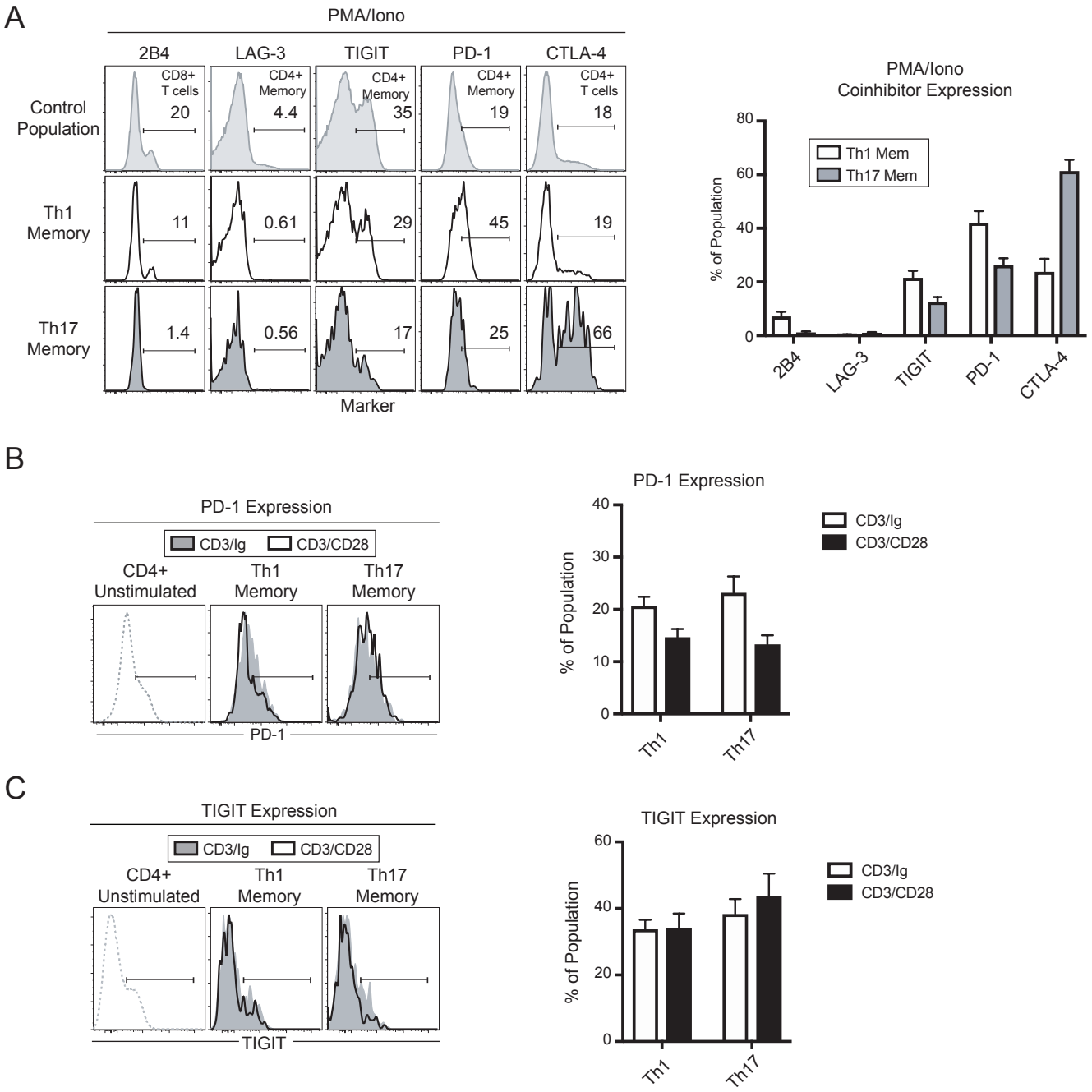

Figure S4. Coinhibitory receptor expression on Th17 cells are not impacted by CD28 cosignaling, related to Figure 3. (A) Representative flow plots and summary data depicting expression of coinhibitory receptors following PMA/Iono on denoted control populations (selected because of clear positive expression) or cytokine producing Th1 or Th17 cells. (B) Expression of PD-1 following stimulation with anti-CD3/IgG beads or anti-CD3/anti-CD28 mAb beads. (C) Expression of TIGIT following stimulation with anti-CD3/IgG beads or anti-CD3/anti-CD28 mAb beads. Summary data depicts (A) 7 or (B-C) 4 individual human donors. Summary data depicts mean ± SEM.

Transparent Methods

Contact For Reagent and Resource Sharing.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mandy L. Ford (mandy.ford@emory.edu). Plasmids generated in this study have been deposited to Addgene (pCMJJ4-FOXO1-IRES-Thy1.1 Cat# 132702, pCMJJ4-FOXO3-IRES-Thy1.1 Cat# 132703, pCMJJ4-Thy1.1 Cat# 132705). Human and mouse CD28 and control dAb reagents were provided through an MTA with Bristol-Myers Squibb. Please direct requests for this reagent to Ms. Diane Shevell (diane.shevell@bms.com).

Experimental Model and Subject Details.

***Animals*.** Male C57BL/6NCr mice were obtained from the National Cancer Institute Grantee Program (Charles River, Frederick, MD) at 6-8 weeks of age. Transgenic mOVA (Act-OVA) mice were a generous gift from Marc Jenkins, and were maintained in the Emory University vivarium (Ehst et al., 2003). Transgenic OVA-specific $CD8^+$ T cells (OT-I) (Hogquist et al., 1994) and $CD4^+$ T cells (OT-II) (Barnden et al., 1998) were purchased from Taconic Farms (Germantown, NY) and bred to $Thy1.1^+$ background at Emory University. All experiments were conducted in age-matched males randomly assigned to experimental groups. This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The protocol was approved by the Institutional Animal Care and Use Committee at Emory University. Each experiment was powered to detect a 50% difference in the incidence of graft rejection (power 0.8, alpha 0.05). All animals were housed in specific pathogen-free animal facilities at Emory University.

***Human Subjects*.** Fresh peripheral blood mononuclear cells (PBMC) were isolated from normal healthy donors using protocols approved by the Emory University Institutional Review Board. Informed consent was obtained for all subjects in accordance IRB protocols. The pool of healthy

donors used in this study was comprised of 31 individuals (15 females and 16 males) aged 19-61. Because comparisons were made between T cell populations within individual subjects, subjects were randomized to each experiment. Blood was collected using BD Vacutainer CPT tubes (BD Diagnostics). In some experiments, cells were frozen in 10% DMSO and 50% FBS and stored in liquid nitrogen. Cells were thawed and rested overnight at 37°C in 96-well plates before stimulation. Cells were cultured in RPMI 1640 supplemented with 10% FBS (Mediatech, VA), 2.4 mM L-glutamine, and 10 μM 2-mercaptoethanol (Sigma).

Method Details

Murine Skin Graft Model. For the induction of OVA-specific Th1 and Th17 populations *in vivo*, spleens from Thy1.1$^+$ OT-I and OT-II mice were processed to single-cell suspension and stained with mAbs for CD4, CD8α, Thy1.1, Vα2, and Vβ5 for flow cytometric analysis of T cell frequency. The frequency was assessed as the frequency of total Vα2$^+$Vβ5$^+$ cells in the CD8$^+$ or CD4$^+$ T cell gate for OT-I or OT-II mice, respectively. Cells were resuspended in PBS, and 1x10^6 OT-I and 10^6 OT-II were injected i.v. into naive C57BL/B6 recipients. For *C. albicans* immunization, an approximately 50 μL aliquot of *C. albicans* were grown as yeast for 16-18 h overnight at 30°C in 10 mL yeast extract/peptone/dextrose broth (Teknova) and then washed in 40 mL PBS and diluted 1:50 in RPMI 1640 with 10% FBS. Transition to hyphae was induced for 4–6 h at 37°C and monitored by light microscopy. Mice were immunized with 4x10^6 hyphae in IFA (ThermoFisher Scientific) mixed 1:1 in PBS and 100 mg $OVA_{323–339}$ peptide (ISQAVHAAHAEINEAGR; Genscript) in each hind footpad. *Mycobacterium* mice were immunized with Complete Freund's Adjuvant (ThermoFisher Scientific) containing 1 mg/ml heat-killed *Mycobacterium butyricum* diluted 1:1 in PBS and 100 mg $OVA_{323–339}$ peptide. Immunizations were performed the day following adoptive transfer to C57BL/6 recipients. Fourteen days later, full thickness tail and ear skins were transplanted onto the dorsal thorax of

recipient mice and secured with adhesive bandages. All surgery was performed under general anesthesia consisting of 4 μg fentanyl, 200 μg midazolam, and 70 μg haloperidol per mouse. Where indicated, mice were treated with 100 μg of anti-CD28 dAb (Bristol-Myers Squibb) on days 0, 2, 4, 6 post transplantation followed by 3 times per week until graft rejection.

T Cell Proliferation Assay. For the 3 day proliferation assay, $3x10^5$/mL PBMC were cultured with 1 μg/mL anti-CD3 (OKT3, eBiosciences) and either 10 μg/mL anti-CD28 mAb (clone CD28.2, BD Biosciences), mouse IgG1 (Biolegend), anti-CD28 dAb (Bristol-Myers Squibb), or non-specific dAb (Bristol-Myers Squibb) in 96-well flat-bottomed plates for 3 d at 37 °C. The anti-CD28 dAb is a Vκ domain antibody that selectively inhibits CD28 and lacks an F_c domain (Suchard et al., 2014). After 3 days, CountBrite beads were added (Invitrogen) and cells were transferred to 96 well U-bottom plates and stimulated with 30 ng/mL PMA and 400 ng/mL Ionomycin (Sigma) for 4 h. 10 μg/mL GolgiStop (BD Biosciences) was added for the final 3.5 h. In some experiments, PBMC were labeled with Cell Trace CFSE proliferation kit (Invitrogen) according to manufacturer's instructions prior to 3 day culture.

CD28 Blockade and AKT Inhibition Assays. For assessment of CTLA-4 expression following anti-CD28 dAb treatment, PBMC were incubated for 18 h with 1 μg/mL functional grade anti-CD3 (OKT3) and 10 μg/mL anti-CD28 dAb or control dAb in 96-well U-bottom plate. After 1 h, 10 μg/mL GolgiStop and 10 μL anti-CTLA-4 PE (BNI3) were added. Following stimulation, cells were washed in FACS buffer and stained with antibodies as described below. For AKT inhibition assays, PBMC were incubated for 18 h in media with 0.5 μg/mL AKT inhibitor IV (CalBioChem), 2.5 μg/mL or 5.0 μg/mL AKT-1/2 (CalBioChem) or matched concentrations of DMSO (Sigma). Cells were washed and then incubated with 1 μg/mL anti-CD3 and 10 μg/mL anti-CD28 mAb (CD28.2) for 5 h, with 10 μg/mL GolgiStop added for the final 4 h.

T Cell Stimulation Assays. Beads were prepared using Dynabeads M-450 Epoxy (Invitrogen) and conjugating 5 μg each of anti-CD3 (OKT3) with anti-CD28 (9.3, BioXCell) or human IgG-Fc

(BioXCell) according to manufacturer's instructions. $CD4^+$ T cells were isolated from PBMC using negative selection $CD4^+$ T cell isolation kit (Miltenyi). 1-5x10^5 $CD4^+$ T cells were stimulated using CD3/Ig or CD3/CD28 stimulation M450 beads. Cells were stimulated with beads at a 1:3 ratio for 5 hrs in a 96-well U-bottom plate, with 10 μg/mL GolgiStop (BD Biosciences) and anti-CTLA-4 PE for the final 4 h. For PMA/Iono, cells were stimulated with 30 ng/mL PMA and 400 ng/mL Ionomycin (Sigma) for 4-5 hrs, with 10 μg/mL GolgiStop (BD Biosciences) and anti-CTLA-4 PE added after 30-60 min.

***Flow Cytometry*.** Surface antigen staining was performed for 15-30 min at room temperature using the following antibodies: CD3, CD4, CD14, CD19, CCR4 (CD194), CCR6 (CD196), CXCR3 (CD197), CXCR5, CD161, CD69, CD45RA or CD45RO, PD-1, TIGIT, 2B4, and TIM-3. For cytokine analysis, cells were prepared for intracellular staining following manufacturer's protocol (BD Biosciences Fix/Perm Kit) and stained with antibodies to IFN-γ and IL-17A. Samples were acquired using an LSR II or Fortessa cytometer (BD Biosciences), and data was analyzed using FlowJo software (Flowjo LLC, San Carlos, CA). Th1 and Th17 cells were defined as antigen experienced $CD3^+CD4^+CD14^-CD19^-CD45RA^{lo}$ and IFN-γ^+ or IL-17^+. In some experiments, $CD45RO^{hi}$ was used instead of $CD45RA^{lo}$. Live/dead Aqua (Invitrogen) was used to exclude dead cells. In Figure 1C and Figure 3C, Th1 cells were defined as total $CD4^+$ cells to avoid confounding from CD45 isoform changes over 3 days. No $CD45RA^{hi}$ or $CD45RO^{lo}$ cells express IL-17.

FOXO Overexpression Assay. FOXO1 (NM_002015, Catalog no. RC200477) and FOXO3 (NM_001455, Catalog no. RG209846) transcripts were obtained from Origene (Rockville, MD) and expression plasmids were generated using the pCMJJ4-Thy1.1 vector (a gift from Joshy Jacob, Emory University), containing and IRES and Thy1.1 gene. For the generation of FOXO1-IRES-Thy1.1 construct, FOXO1 was amplified with primers containing Pac1 and BamH1 digestion sites. For FOXO3-IRES-Thy1.1, FOXO3 was amplified using the following primers

containing Pme1 digestion sites and bluntly ligated into the vector. Clone sequences were verified using universal primers CMV-For, IRES-R, and FOXO1-specific primer (5'-GCTCGGCGGGCTGGAAGAA-3') or FOXO3 specific primer (5'-ATAGTCGATTCATGCGGGTCCAGA-3'). Plasmids were transformed into One Shot Top10 Chemically Competent *E. Coli* (ThermoFisher) following manufacturer's instructions. MaxiPreps were performed using ZymoPure II MaxiPrep Kit (ThermoFisher) and quantified using a NanoDrop (ThermoFisher). All three plasmids were deposited in AddGene (Catalog numbers pending). For the overexpression assay, fresh PBMC were isolated as described above in CPT tubes and 8-10x10^6 fresh PBMC were transfected with 2.0 μg of either empty Thy1.1 vector, FOXO1-IRES-Thy1.1, or FOXO3-IRES-Thy1.1 using Nucleofector IIb (Lonza) program U-14 according to manufacturer's instructions. Transfected cells were rested in 3 mL of pre-warmed complete R10 media in 12-well flat bottom plates. After 14-18 h, cells were collected and live cells were isolated by underlaying 4 mL of Ficoll-Hypaque (GE Life Sciences) on cultured cells and spinning for 20 min at 400xg. The live cell layer at the interface was isolated in complete R10 and plated into a U-bottom 96 well plate. The cells were then stimulated for 5 h with 1 μg/mL functional grade anti-CD3 (OKT3) and 10 μg/mL CD28 mAb. After 1 hour, 10 μg/mL GolgiStop and 10 μL anti-CTLA-4 PE (BNI3) were added.

Quantification and Statistical Analysis

All data points represent individual donors, and where individual data points are not depicted the value of n is provided in the corresponding Figure Legend. All statistical analysis was performed using GraphPad Prism v7. The proliferation effect of anti-CD28 dAb was calculated as the % $CFSE^{lo}$ cells with CD28 dAb group / % $CFSE^{lo}$ in control dAb group for either $CD4^+$ T cell or Th1 and Th17 cells. The effect of anti-CD28 dAb or mAb was calculated as the % $cytokine^+$ cells treatment group / % $cytokine^+$ cells in control IgG group, with IFN-γ^+ for Th1 and IL-17^+ for Th17, respectively. The effect of AKT inhibition was determined by dividing the cytokine frequency or

marker expression in the AKT inhibitor group by the DMSO control group for either cytokine$^+$ Th1 and Th17 cells or chemokine defined Th1 or Th17 cells. The ratio of CTLA-4 following stimulation with anti-CD3/Ig or anti-CD3/CD28 beads was calculated as CTLA-4 MFI in anti-CD3/CD28 group / anti-CD3/Ig group. The impact of FOXO1 or FOXO3 overexpression on CTLA-4 expression was calculated by dividing the % CTLA-4$^+$ cells in each individual population by the % CTLA-4$^+$ cells in the EV-Thy1.1 group.

The frequencies of cytokine production and relative protein expression levels were assessed using Student's t-test (two-tailed). Comparisons of anti-CD3/IgG vs anti-CD3/anti-CD28 beads were performed using two-way ANOVA with Sidak's multiple comparison test. Comparisons AKT-1/2 treatments on chemokine Th1 and Th17 cell CTLA-4 expression were performed using two-way ANOVA with Tukey's multiple comparison test. Comparisons of FOXO1 and FOXO3 overexpression on Th1 and Th17 CTLA-4 expression were compared using one-way ANOVA with Dunnett's multiple comparisons test. Comparisons of graft survival performed using log-rank (Mantel-Cox) test. All summary data depict the mean ± standard deviation. Significance was determined as *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.