T Cell Activation with CD28

- CD28

AKT

Days

Less CTLA-4 Coinhibition

Augmented Response

Th1

Th17

More CTLA-4 Coinhibition

AKT

Inhibited Response

CD28

CTLA-4

Days
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 vs. 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 to 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 of 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, Krummey et al., 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 vs. Th17 cells. We utilized an anti-CD28 domain antibody (dAb) to selectively inhibit CD28 on Th1 vs. Th17 cells, which revealed that Th1 cells are susceptible while 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 to 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 vs. Th17 cells that results in disparate responses to immunomodulation.
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 vs. CTLA-4 signals were primarily responsible for this observation. Using a novel anti-CD28 domain antibody (dAb), which is comprised of 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). While 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), 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 vs. Th17 cells.

We performed the same assay with CFSE-labeled PBMC to assess the impact of anti-CD28 dAb on proliferation in Th1 vs. Th17 cells. The presence of anti-CD28 dAb resulted in less proliferation of the total CD4+ T cells in culture (Figure S1). Interestingly, Th1 vs. Th17 cells proliferated to similar degrees in the presence of the anti-CD28 dAb vs 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 vs. Th17-polarized mice treated with control dAb were not significantly different (Th1 15d, Th17 17d) (Figure S2). In contrast, we observed a differential effect of anti-CD28 dAb on graft survival in Th1 vs. Th17-polarized mice. While the majority of Th1-polarized mice maintained their grafts long-term (Figure 1E), 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., 1996, Walunas et al., 1994, Lindsten et al., 1993, Krummel and Allison, 1995), leading us to hypothesize that differential sensitivity to CD28-mediated AKT signals in Th1 vs. Th17 cells could result in the CD28 blockade-resistant phenotype of observed in Th17 cells relative to Th1 cells. In support of this, we found that CD28 blockade with anti-CD28 dAbs resulted 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 to 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 CXCR3
[hi]CCR6
[lo] and CCR4
[lo] (Figure S3A-B). Th17 cells, in contrast, were CCR6
[hi]CXCR3
[lo] and CCR4
[hi]CD161
[hi] (Figure S3A-B). 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 (Figure 2D-F). 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 to 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 while 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 (Figure 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 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). While CTLA-4 expression was slightly higher on Th17 cells compared to Th1 cells in the presence of CD3 stimulation alone, 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 vs. 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 vs. 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 vs. 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 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 CXCR3$^{hi}$CCR6$^{b}$ and CCR4$^{b}$ or CCR6$^{hi}$CXCR3$^{lo}$ and CCR4$^{hi}$CD161$^{hi}$, 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 (Figure 4B-C). Among Thy1.1$^+$ chemokine Th17 cells, however, overexpression
of either FOXO1 or FOXO3 resulted in significant inhibition of CTLA-4 expression
(Figure 4B-C). 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 more sensitive to CD28/AKT signaling in Th17 as compared to 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 Ctl4 gene expression (Teft et al., 2006, Finn et al., 1997, Walunas et al., 1996, Walunas et al., 1994, 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
provides evidence that CD28-dependent CTLA-4 is acting 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. While
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 this study**

While 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, while 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.
**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.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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Figure Legends

Figure 1. Th17 cells are resistant to selective CD28 blockade. (A) Schematic of peripheral blood mononuclear cell (PBMC) 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 d 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 2 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.

Figure 2. CTLA-4 expression on human Th17 cells is uniquely sensitive to AKT inhibition. (A) Expression of CTLA-4 on Th1 vs. 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 vs. 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 vs. 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<sup>hi</sup>CCR4<sup>lo</sup> “chemokine” Th1 cells or CCR6<sup>hi</sup>CCR4<sup>hi</sup>CD16<sup>+</sup>CD1<sup>+</sup> “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 depicts 6 individual donors. Statistical analyses performed using (C-D) Student’s t-test (two-tailed) or (F) two-way ANOVA with Tukey’s multiple comparison test. Summary data depicts mean ± SEM. Significance is defined as *p<0.05, ***p<0.001, ****p<0.0001.

Figure 3. High Th17 CTLA-4 expression is dependent on CD28 signals. (A) Representative flow cytometry and summary data of frequency of Th1 vs. Th17 cells after 3 day culture with anti-CD3 and agonistic anti-CD28 mAb or control IgG. (B) Expression of CD69 on Th1 vs. 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 vs. Th17 cells activated with beads coated in anti-CD3 or anti-CD3/anti-CD28. (D) Expression of CTLA-4 on Th1 vs. Th17 cells activated with anti-CD3/IgG relative to anti-CD3/agonistic anti-CD28. See also Figure S4. (A, D) Each data point represents an individual human donor. (B) Summary data represents 7 individual human donors. (C) Summary data represents 9 individual human donors. Statistical analyses performed using (A, D) Student’s t-test (two-tailed) or (B-C) two-way ANOVA with Sidak’s multiple comparison test. Summary data depicts mean ± SEM. mAb, monoclonal antibody. Significance is defined as *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

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 vs. 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 vs. 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 depicts mean ± SEM. Significance is defined as **p<0.01, ****p<0.0001.
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Figure 1

A

PBMC Anti-CD3 CD28 dAb

B

Bulk CD4+ T Cells CD28 dAb / Ctrl

C

Th1 Th17

CD28 dAb

D

% Divided CD28 dAb / Ctrl dAb

E

CD28 dAb Treatment Murine Skin Graft Survival

CD28 dAb 3 d Stimulation

PBMC Anti-CD3 CD28 dAb

Control dAb

3 days

IFN-γ Th1 IL-17 Th17

5 h PMA/Iono

% Divided

CD4+ T Cell Number

CD28 dAb / Ctrl dAb (x102)

Percent survival

Days Post Transplant

Th1 Polarized

Th17 Polarized
Figure 2

**A**
CTLA-4 Expression
CD28 dAb

**B**
Bulk CD4+ T Cell Memory

**C**
Th1
Th17

**D**
AKT IV Inhibitor

**E**
CXCR5+CD4+ T Cell Memory

**F**
AKT-1/2 Inhibitor

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STATISTICAL SIGNIFICANCE

* p < 0.05
** p < 0.01
*** p < 0.001
**** p < 0.0001
Figure 3

A

![Graph showing CTLA-4 and CD69 expression](image)

B

![Graph showing CD69 expression](image)

C

![Graph showing CTLA-4 expression](image)

D

![Graph showing CTLA-4 expression](image)
Figure 4

A

Transfection → Anti-CD3/Anti-CD28 Stimulation

Empty Vector Thy1.1

FOXO1 Thy1.1

FOXO3 Thy1.1

B

|                | Chemokine Th1 | Chemokine Th17 |
|----------------|---------------|----------------|
| Unstim         | 3.3%          | 3.1%           |
| Empty Vector   | 25%           | 31%            |
| FOXO1          | 8.7%          | 6.3%           |
| FOXO3          | 11%           | 11%            |

C

|                | Chemokine Th1 | Chemokine Th17 |
|----------------|---------------|----------------|
| CTLA-4 MFI / EV (10^2) |
| EV             |               |               |
| FOXO1          |               |               |
| FOXO3          |               |               |
• 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.