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Supplemental Information

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

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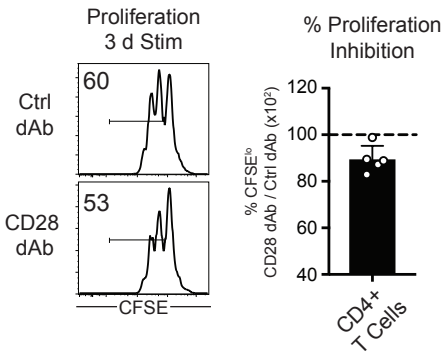

Figure S1. CD28 blockade inhibits CD4⁺ T cell proliferation, related to Figure 1. CFSE dilution of Th1 and Th17 cells after 3 days in culture with anti-CD3 and anti-CD28 dAb or control dAb. Each data point represents an individual human donor. Data are represented as mean ± SEM. dAb, domain antibody.

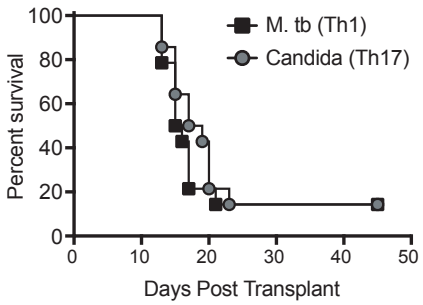

Figure S2. Th1 and Th17 polarized mice reject skin grafts with similar kinetics following control dAb treatment, related to Figure 1. Graft survival of mice polarized to Th1 or Th17 cells and treated with control dAb. Data shown are 9-10 mice/group from 2 independent experiments.

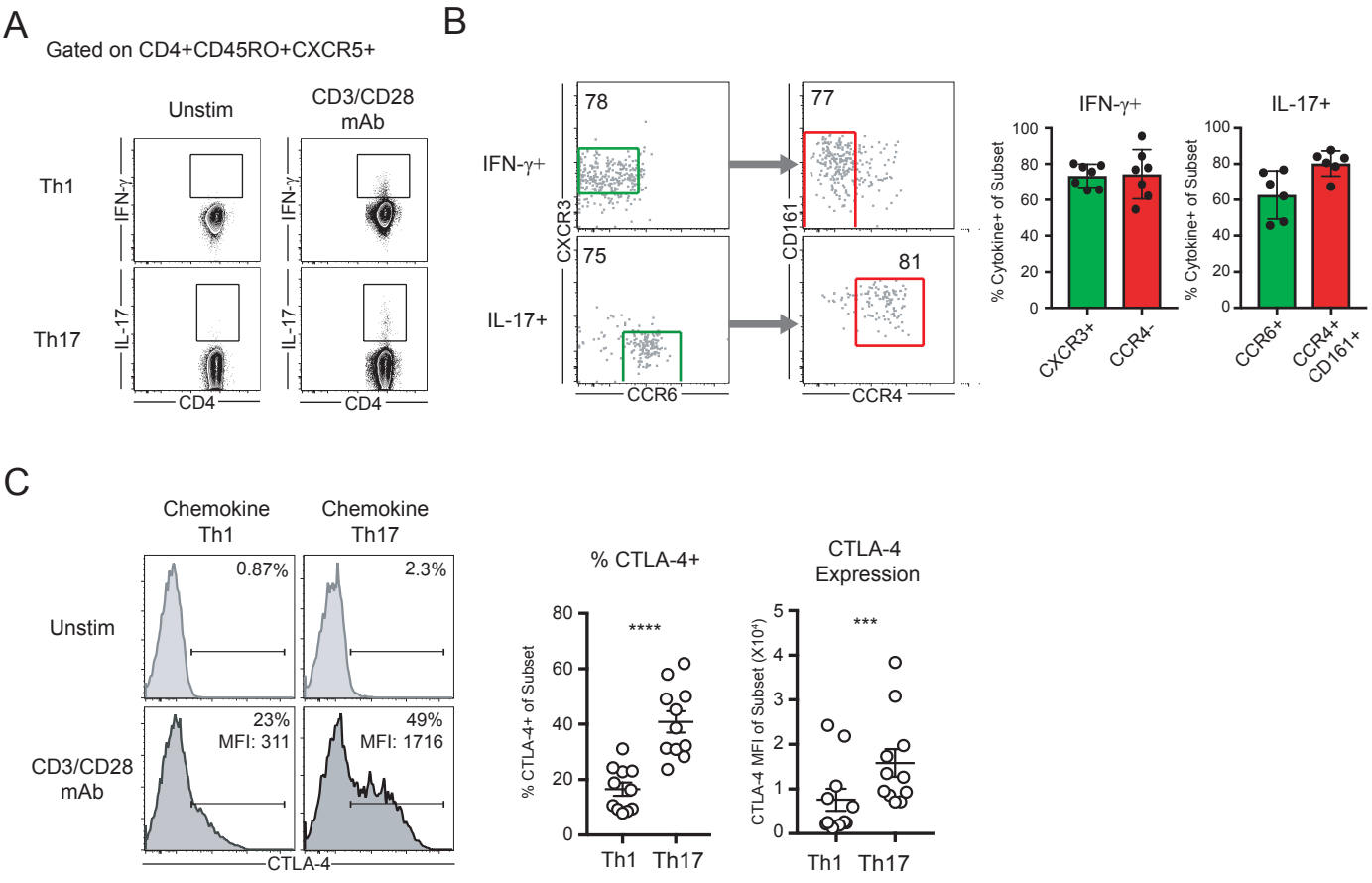

Figure S3. Human Chemokine Th17 cells express high levels of CTLA-4 relative to Chemokine Th1 cells, related to Figure 2. (A) Representative gating of cytokine producing Th1 and Th17 cells following anti-CD3/anti-CD28 mAb stimulation. (B) Representative gating and summary data of chemokine expression 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.

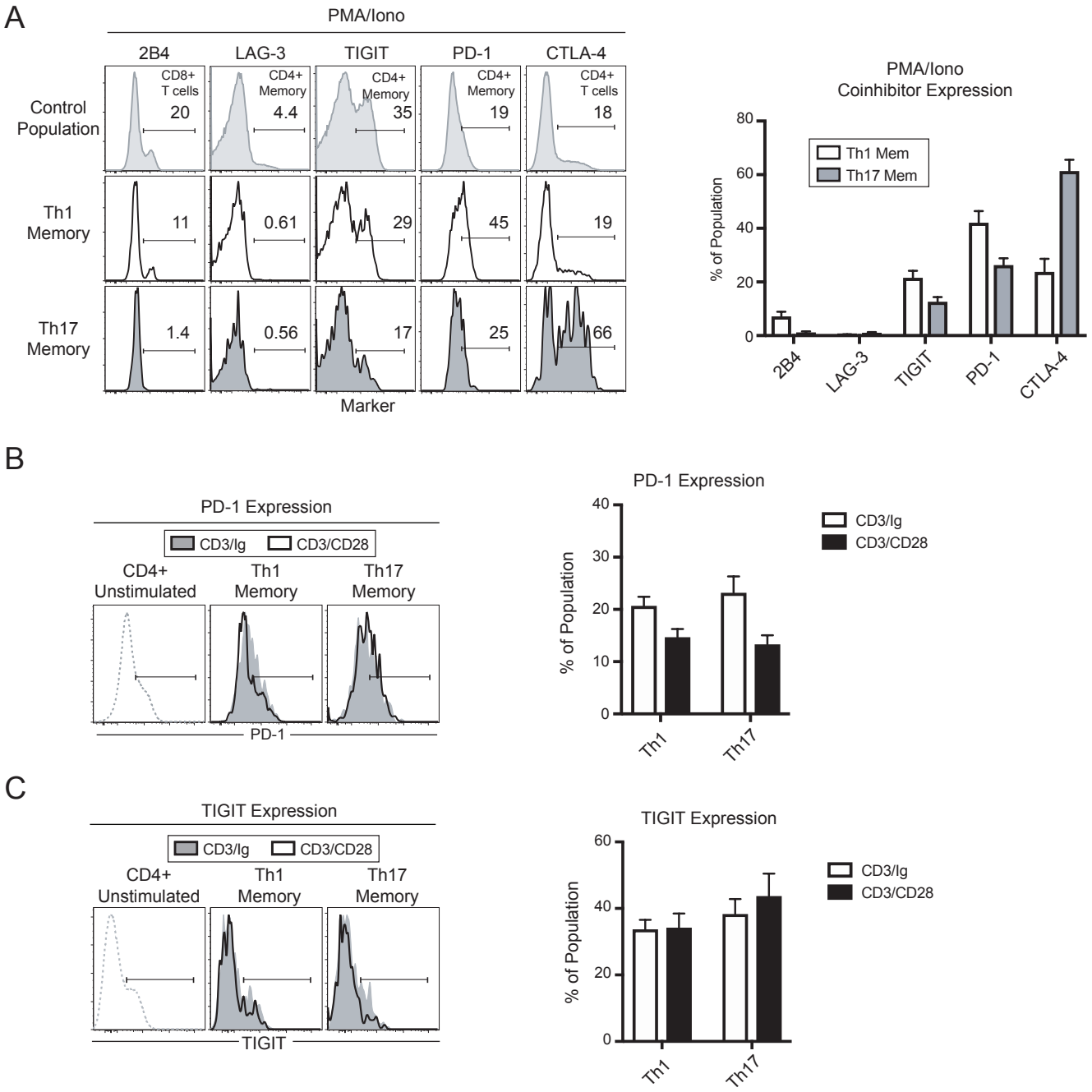

Figure S4. Coinhibitory receptor expression on Th17 cells are not impacted by CD28 cosignaling, related to Figure 3. (A) Representative flow plots and summary data depicting expression of coinhibitory receptors following PMA/Iono on denoted control populations (selected because of clear positive expression) 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 *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.