JEM

SUPPLEMENTAL MATERIAL

Albring et al., http://www.jem.org/cgi/content/full/jem.20102017/DC1

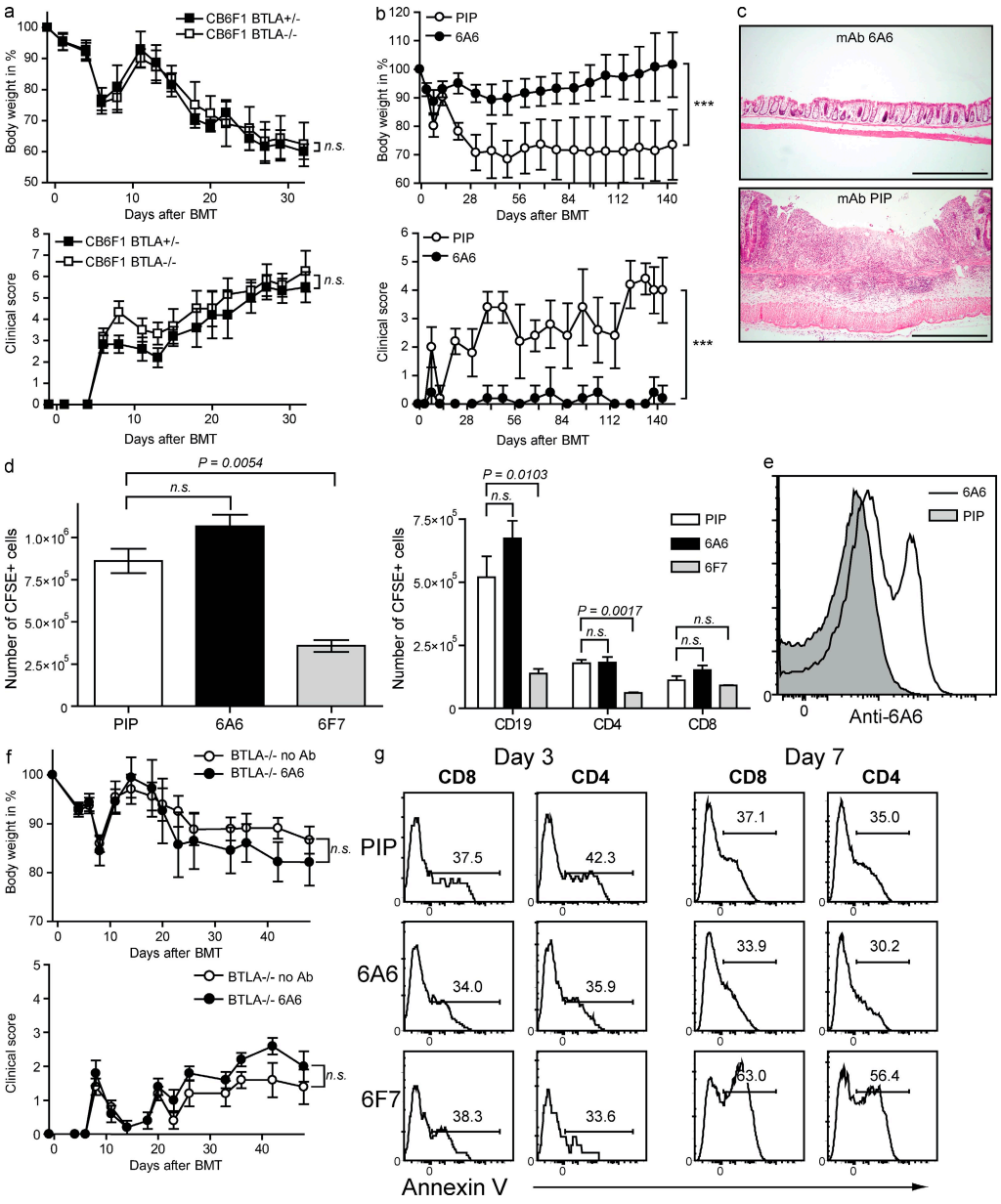

Figure S1. BTLA expression by recipient tissue does not promote GVHD and 6A6 antibody does not deplete lymphocytes. (a) Lethally irradiated CB6F1 BTLA$^{+/-}$ (n = 5, closed squares) or CB6F1 BTLA$^{-/-}$ (n = 5, open squares) mice received 2.0 × 10^7 BMC and 1.0 × 10^7 splenocytes from C57BL/6 BTLA$^{-/-}$ donors. Data shown are mean ± SD (n = 5). (b) In one experiment of Fig. 1 b, the mice were followed for 143 d. Data shown are mean ± SD (n = 5 per group). (c) Histopathology of the colon 143 d after BMT of animals in Fig. 1 b that received a single injection of 6A6 (top) or the control antibody PIP (bottom). Bars, 500 µm. (d) Lethally irradiated C57BL/6 mice received 5.0 × 10^6 CFSE-labeled splenocytes from B6.SJL mice together with a single intraperitoneal injection of 400 µg of either control antibody PIP or anti-BTLA antibodies 6A6 or 6F7. After 2 d, splenocytes were stained for CD4, CD8α, CD19, and anti-hamster (for 6A6 and PIP). Shown are numbers of either all donor CFSE$^+$ cells (d, left) or CD19$^+$, CD8$^+$, and CD4$^+$ subsets of CFSE$^+$ cells (d, right) recovered from mice that had received control antibody PIP (open bars), 6A6 (filled bars), or 6F7 (shaded bars). Data shown are mean ± SEM (n = 3). (e) Lethally irradiated CB6F1 (CD45.1$^-$) mice received 5.0 × 10^7 splenocytes from B6.SJL (CD45.1$^+$) mice together with a single intraperitoneal injection of 400 µg of either 6A6 or PIP. After 7 d, splenocytes were stained for CD45.1 and secondary anti-hamster antibody. The histogram shows anti-hamster reactivity (i.e., 6A6$^+$) within the CD45.1$^+$ population from mice that received 6A6 (bold line) or PIP (shaded fill). (f) aHSCTs were performed as described in Fig. 1 b, except that BTLA$^{-/-}$ mice were used as donors. Data shown are mean ± SD (n = 5 per group). (g) Lethally irradiated CB6F1 mice received 2.0 × 10^7 CFSE-labeled BMCs and 1.0 × 10^7 splenocytes from C57BL/6 SJL mice together with either a single 400 µg intraperitoneal injection of control antibody PIP, 6A6, or 6F7 on the day of aHSCT. Spleens were harvested 3 and 7 d after aHSCT and stained for either CD8$^+$ or CD4$^+$ lymphocytes and their ability to bind annexin V. Shown is the percentage of cells that are annexin V$^+$. The data are representative of two experiments with three mice per group. P-values of >0.05 are considered not significant (n.s). ***, P < 0.001.

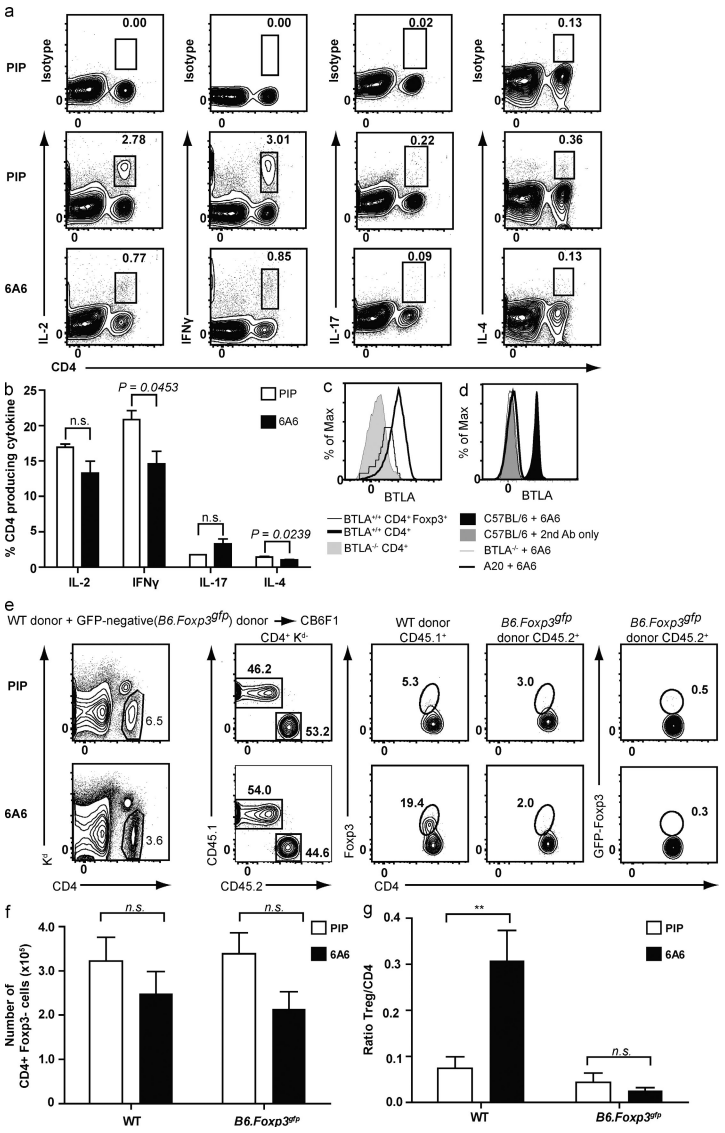

Figure S2. 6A6 treatment does not lead to donor T eff cell anergy and expands preexisting T reg cells. (a and b) Lethally irradiated CB6F1 mice received 2.0 × 10^7 BMC and 1.0 × 10^7 splenocytes from B6.SJL mice and either control antibody PIP or 6A6. 7 d later, aHSCT splenocytes were harvested and stimulated with PMA/ionomycin for 5 h. After restimulation, cells were stained for CD4 and IL-2, IFN-γ, IL-17, and IL-4 or isotype controls for the cytokines. (a) Plots show either isotype control (top row) or production of the indicated cytokine (bottom two rows) by $CD4^+$ cells for control and 6A6-treated mice. Cells were gated on $CD4^+$ cells, and cells producing the indicated cytokine are shown as a percentage of all cells. (b) The percentage of $CD4^+$ cells from panel a producing the indicated cytokine are shown as mean ± SEM (n = 3). The data are representative of two experiments with three or five mice per group. (c) BTLA expression on donor T cells 7 d after aHSCT into a lethally irradiated Balb/c host. Shown are histograms of BTLA expression WT $CD4^+$ T cells (thick line), $BLTA^{-/-}$ $CD4^+$ T cells (filled gray), and $BTLA^{+/+}$ $CD4^+$ $Foxp3^+$ cells (thin line). These data are representative of two experiments with three to four mice per group. (d) Cells were stained for CD19 and with unconjugated 6A6 anti-BTLA antibody, followed by a secondary anti–Armenian and –Syrian hamster IgG cocktail. Shown are histograms of $CD19^+$ cells from C57BL/6 splenocytes (filled black) as a positive control, C57BL/6 splenocytes stained with secondary Ab only (filled gray), and BALB/c $BTLA^{-/-}$ splenocytes (thin line) as negative controls, as well as A20 lymphoma cells (thick line). (e–g) Lethally irradiated CB6F1 ($CD45.2^+$ H-$2K^{d+}$) mice received 2.0 × 10^7 BMC and 1.0 × 10^7 WT B6.SJL ($CD45.1^+$ H-$2K^{d-}$) splenocytes, along with 1.0 × 10^6 purified $CD4^+$ Foxp3-negative T cells from *B6.Foxp3^{gfp}* mice ($CD45.2^+$ H-$2K^{d-}$) with a single 400-µg intraperitoneal injection of either control antibody PIP or 6A6. After 7 d, splenocytes were stained for CD45.1, CD45.2, H-$2K^d$, CD4, and intracellular expression of Foxp3. Foxp3 expression was also determined by GFP expression. (e) Donor cells were identified by the lack of H2-K^d and the expression of CD4, and CD45.2 or CD45.1 was used to determine the origin of the donor. Expression of intracellular Foxp3 or GFP of $CD4^+$ T cells from the WT donor ($CD45.1^+$) and $B6.Foxp3^{gfp}$ ($CD45.2^+$) is shown. (f) Number of $CD4^+FoxP3^-$ cells from WT donors (left) or *B6.Foxp3^{gfp}* donors (right) that received either control antibody PIP (open bars) or 6A6 (filled bars). (g) The ratio of T reg/T eff cells of each donor, either WT (left) or *B6.Foxp3^{gfp}* (right), that received control antibody PIP (open bars) or 6A6 (filled bars). Data shown are mean ± SEM (n = 5). The data are representative of two experiments with five mice per group. P-values of >0.05 are considered not significant (n.s). **, 0.001 < P < 0.01.

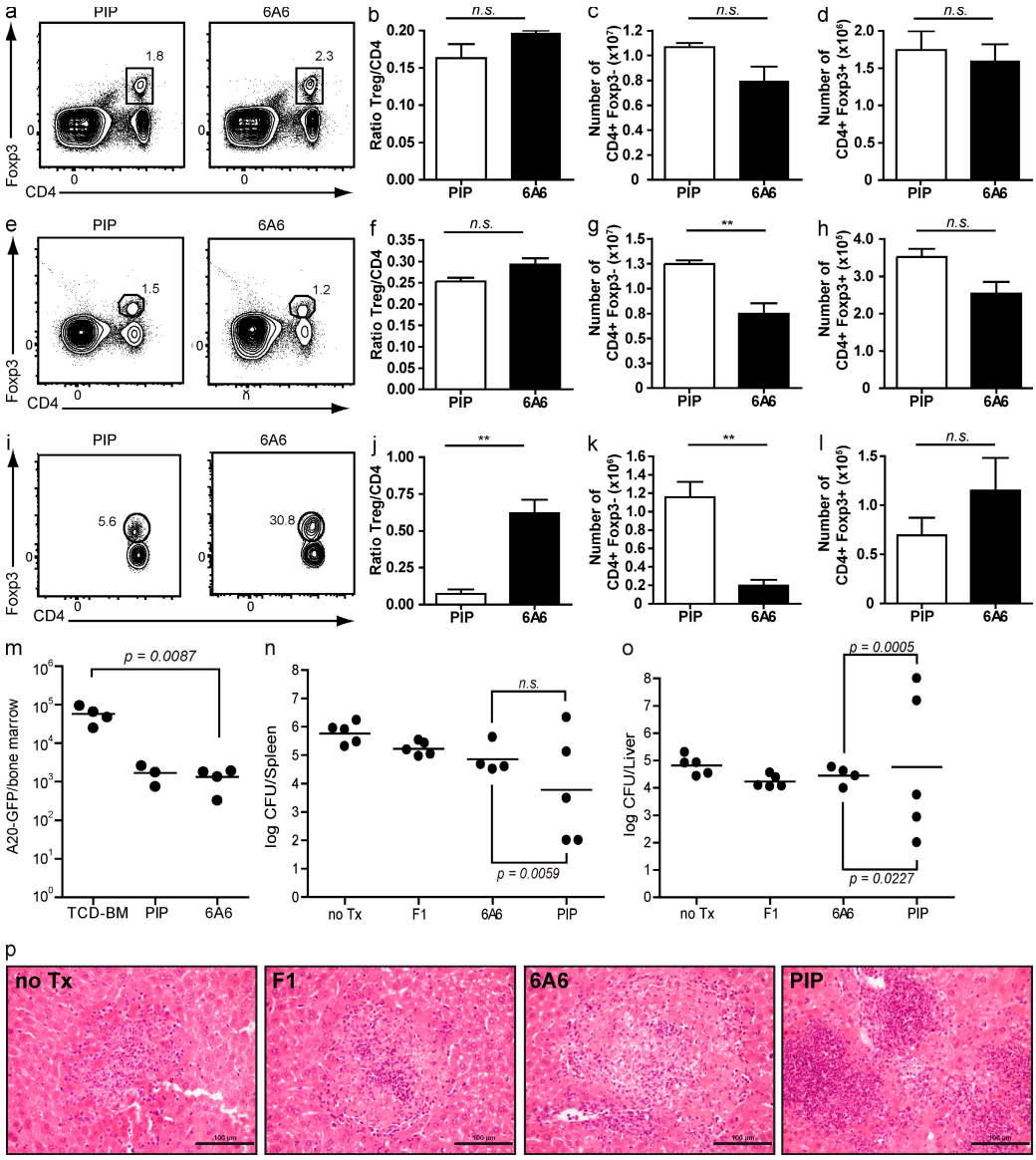

Figure S3. 6A6 inhibition of T eff cells and expansion of T reg cells requires allostimulation but not HVEM expression by donor or host. (a–d) *B6.Foxp3*^{gfp} mice were given an intraperitoneal injection of 400 µg of either control antibody PIP or 6A6 and splenocytes were harvested 6 d later. (e–h) Lethally irradiated CB6F1 mice received 2.0 × 10^7 BMC and 1.0 × 10^7 CB6F1 splenocytes with a single intraperitoneal 400-µg injection of either control antibody PIP or 6A6. Splenocytes were harvested 7 d after aHSCT. (i–l) Lethally irradiated F2 HVEM$^{-/-}$ recipients received 2.0 × 10^7 BMC and 1.0 × 10^7 C57BL/6 HVEM$^{-/-}$ (H2-K$^{b/b}$) splenocytes. Splenocytes were harvested 7 d after aHSCT (a, e, and i) and stained for CD4, and expression of the Foxp3-GFP reporter (a) or intracellular expression of Foxp3 (e and i) was assessed. FACS plots showing the percentage of all (a and e) or of CD4$^+$ donor (i) cells that express CD4 and Foxp3 after control antibody PIP (left) or 6A6 treatment (right). (b, f, and j) Ratio of T reg/T eff cell; (c, g, and k) number of CD4$^+$ Foxp3$^-$ cells; (d, h, and l) number of CD4$^+$ Foxp3$^+$ cells. The data in a–h are from one experiment with five mice per group, and the experiments in i-l show pooled data from two independent experiments with four mice per condition. Bar graphs are the mean ± SEM. (m) Lethally irradiated BALB/c mice received 2 × 10^4 A20-GFP lymphoma cells with 2.0 × 10^7 TCD-BM alone (n = 4) or in combination with 1.0 × 10^7 splenocytes from C57BL/6 mice together with either a single 400-µg intraperitoneal injection of control antibody (PIP; n = 3) or 6A6 (n = 4) on the day of aHSCT. 5 d after aHSCT the BM of both femurs was analyzed by FACS. Shown are total numbers of CD19$^+$ GFP$^+$ cells recovered from the BM, with each point representing data obtained from an individual mouse. Horizontal lines represent the mean cell count. (n–p) Lethally irradiated CB6F1 mice received 2.0 × 10^7 BMC and 1.0 × 10^7 splenocytes from syngeneic CB6F1 mice (F1; n = 5) or C57BL/6 mice and either control antibody PIP (n = 5) or the antibody 6A6 (n = 4). Unmanipulated age-matched CB6F1 mice (no Tx; n = 5) served as controls. 3 mo later, aHSCT mice were infected intravenously with 2.5 × 10^4 *L. monocytogenes*. 3 d later, mice were sacrificed and CFUs in spleen (n) and liver (o) were determined. Each point represents CFUs obtained from an individual mouse. Horizontal lines represent the mean CFUs. (p) Representative histopathology of the liver 3 d after infection with *L. monocytogenes*. For the PIP control, the liver of an animal with high *L. monocytogenes* burden is depicted. Bars, 100 µm. Data are from one experiment with five mice per group. P-values of >0.05 are considered not significant (n.s). **, 0.001 < P < 0.01.

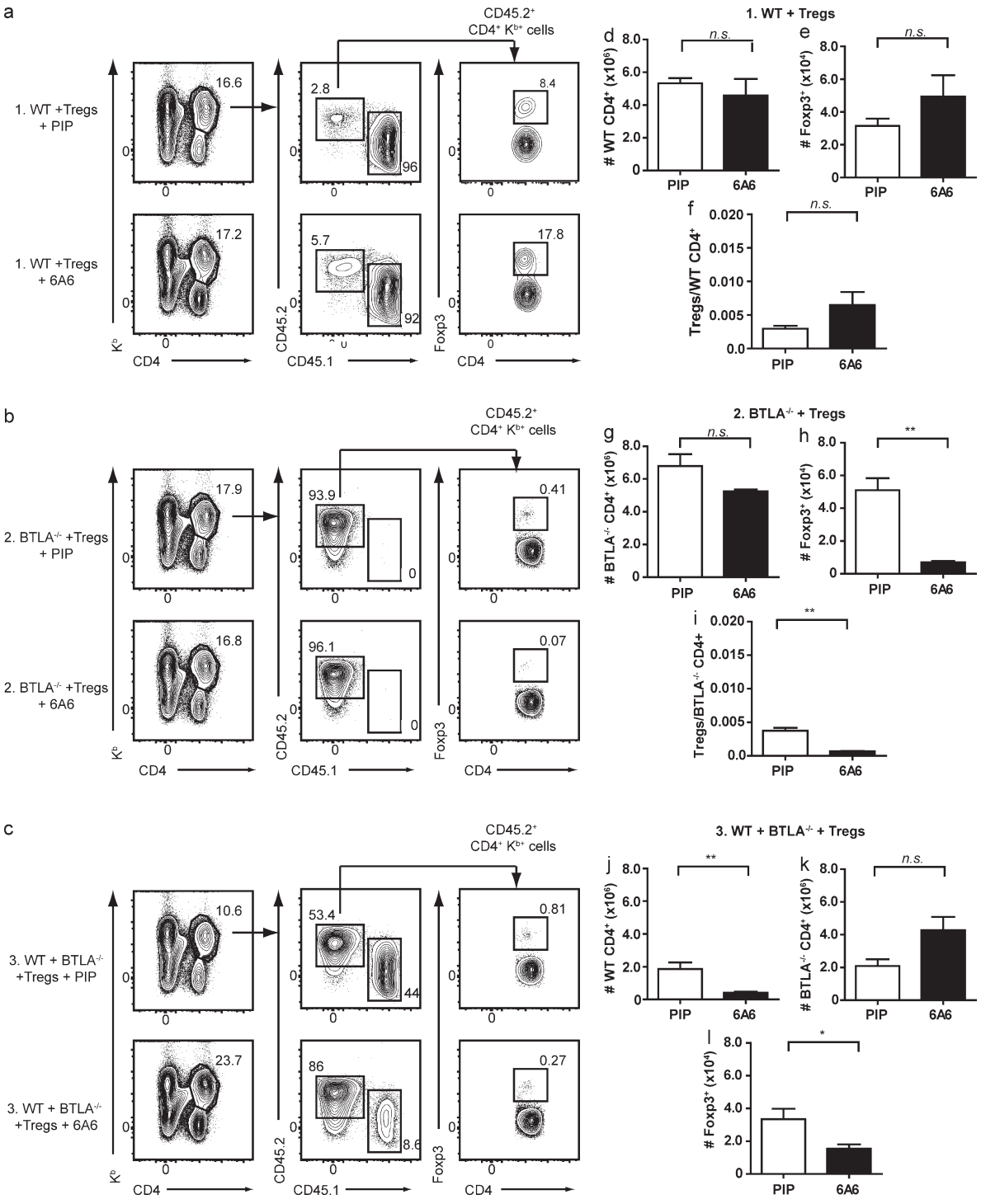

Figure S4. 6A6 alters the T reg/T eff cell ratio by directly inhibiting BTLA-expressing T cells. Lethally irradiated BALB/c mice received 2 × 10^5 T reg cells purified from *B6.Foxp3*^{gfp} mice along with CD4+ cells. Group 1 (a and d–f), 5 × 10^6 purified WT B6.SJL cells; group 2 (b and g–i), 5 × 10^6 BTLA$^{-/-}$ CD4+ cells; group 3 (c and j–l), a 1:1 mixture of 2.5 × 10^6 B6.SJL CD4+ T cells and 2.5 × 10^6 BTLA$^{-/-}$ CD4+ T cells. Each group received either a single 400-µg intraperitoneal injection of control antibody PIP or 6A6. After 7 d, splenocytes were stained for FACS and gated on H-2K^b and CD4 to identify donor cells, and then on CD45.1 to identify WT cells or on CD45.2 to identify BTLA$^{-/-}$ and T reg cells. The T reg cells were separated from the BTLA$^{-/-}$ cells on the basis of GFP Foxp3 expression (a–c, right). The number of WT CD4+ (d and j) and BTLA$^{-/-}$ CD4+ (g and k) was determined in each condition. The resulting ratio of T reg/WT T eff cell (f) or T reg cell/BTLA$^{-/-}$ CD4+ (i) was then calculated. Bar graphs are the mean ± SEM. The data are representative of two independent experiments with three to four mice per group. P-values of >0.05 are considered not significant (n.s). *, 0.01 < P < 0.05; **, 0.001 < P < 0.01.