

Targeting of B and T lymphocyte associated (BTLA) prevents graft-versus-host disease without global immunosuppression

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Graft-versus-host disease (GVHD) causes significant morbidity and mortality in allogeneic hematopoietic stem cell transplantation (aHSCT), preventing its broader application to non-life-threatening diseases. We show that a single administration of a nondepleting monoclonal antibody specific for the coinhibitory immunoglobulin receptor, B and T lymphocyte associated (BTLA), permanently prevented GVHD when administered at the time of aHSCT. Once GVHD was established, anti-BTLA treatment was unable to reverse disease, suggesting that its mechanism occurs early after aHSCT. Anti-BTLA treatment prevented GVHD independently of its ligand, the costimulatory tumor necrosis factor receptor herpesvirus entry mediator (HVEM), and required BTLA expression by donor-derived T cells. Furthermore, anti-BTLA treatment led to the relative inhibition of CD4⁺ forkhead box P3⁻ (Foxp3⁻) effector T cell (T eff cell) expansion compared with precommitted naturally occurring donor-derived CD4⁺ Foxp3⁺ regulatory T cell (T reg cell) and allowed for graft-versus-tumor (GVT) effects as well as robust responses to pathogens. These results suggest that BTLA agonism rebalances T cell expansion in lymphopenic hosts after aHSCT, thereby preventing GVHD without global immunosuppression. Thus, targeting BTLA with a monoclonal antibody at the initiation of aHSCT therapy might reduce limitations imposed by histocompatibility and allow broader application to treatment of non-life-threatening diseases.

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Abbreviations used: aHSCT, allogeneic hematopoietic stem cell transplantation; BLI, bioluminescence imaging; BMC, BM cell; BTLA, B and T lymphocyte associated; Foxp3, forkhead box P3; GVHD, graft-versus-host disease; GVT, graft versus tumor; HVEM, herpesvirus entry mediator; MCMV, murine CMV; TCD-BM, T cell-depleted BMC; T eff cell, CD4⁺Foxp3⁻ effector T cell; T reg cell, CD4⁺Foxp3⁺ regulatory T cell.

Replacement of an abnormal lymphohematopoietic system by allogeneic hematopoietic stem cell transplantation (aHSCT) from a healthy donor is an effective treatment for many disorders of the hematopoietic system (Sykes and Nikolic, 2005; Copelan, 2006). Induction of a mixed hematopoietic donor-host chimerism can induce long-lasting tolerance to foreign tissues without the need for life-long immunosuppressive therapy (Kawai et al., 2008). aHSCT therapy has been improved by better donor identification (Petersdorf et al., 2004), more tolerable conditioning regimens (McSweeney et al., 2001), and enhanced supportive care. However, significant treatment-related morbidity and mortality from chemotherapy, radiotherapy,

infections, and graft-versus-host disease (GVHD) remain significant clinical problems. Therefore, aHSCT is commonly indicated only for treatment of conditions where other treatment options are far inferior or lacking.

Costimulatory molecules of the CD28 and TNF families regulate GVHD, with inhibitory and activating receptors either decreasing or increasing its severity (Tamada et al., 2000; Blazar et al., 2003; Xu et al., 2007). B and T lymphocyte associated (BTLA) is an inhibitory immunoglobulin superfamily receptor, whose ligand is the TNF receptor herpesvirus entry mediator

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(HVEM) and which has only been examined in a nonirradiated model of chronic allostimulation without classical GVHD where donor cells lacking BTLA failed to persist (Hurchla et al., 2007). The role of BTLA in aHSCT using irradiated recipients, in which clinical symptoms and pathology similar to human GVHD develop, has not been examined.

RESULTS AND DISCUSSION

To determine the role of BTLA in the development of GVHD, we first examined WT and BTLA^{-/-} donor mice (Watanabe et al., 2003) using a nonlethal parent-into-irradiated F1 model of aHSCT (Stelljes et al., 2008). In this model, GVHD results from partial MHC mismatch between H-2^b haplotype donor cells and lethally irradiated H-2^{b/d} haplotype recipients. BM and splenocytes from WT or BTLA^{-/-} mice on the C57BL/6 background were transferred into lethally irradiated CB6F1 recipients (Fig. 1 a). Transplantation of WT donor cells into CB6F1 recipients caused body weight loss of ~30% and clinical scores (Cooke et al., 1996) of ~3 that persisted for >40 d. BTLA^{-/-} and WT donor cells caused similar GVHD, suggesting that BTLA does not normally regulate GVHD in this model. To test whether BTLA

expressed by recipient mice might regulate GVHD in this model, we used BTLA^{-/-} CB6F1 hosts as recipients of BTLA^{-/-} BM and splenocytes (Fig. S1 a). BTLA^{-/-} donor cells induced similar GVHD in BTLA^{+/-} and BTLA^{-/-} hosts, which is comparable to GVHD by WT donor cells (Fig. 1 a). Collectively, these data suggest that BTLA does not normally regulate GVHD.

Because BTLA generates inhibitory signals and functions in autoimmunity (Watanabe et al., 2003), malaria infection (Lepenies et al., 2007), and intestinal inflammation (Steinberg et al., 2008), we wondered whether harnessing the inhibitory effects of BTLA on the immune response by forced engagement would attenuate GVHD. To test this, we compared the effects of an agonistic nondepleting anti-BTLA monoclonal antibody (Hurchla et al., 2005; Lepenies et al., 2007), 6A6, administered at the time of aHSCT (Fig. 1 b) with an isotype control antibody, PIP, that recognizes bacterial GST (Gronowski et al., 1999). Mice treated with PIP showed similar progression of GVHD (Fig. 1, a and b), with clinical scores between 3 and 4 persisting for >140 d (Fig. S1 b). GVHD was associated with thickening of the lamina propria and muscularis, with severe inflammation and ulceration of

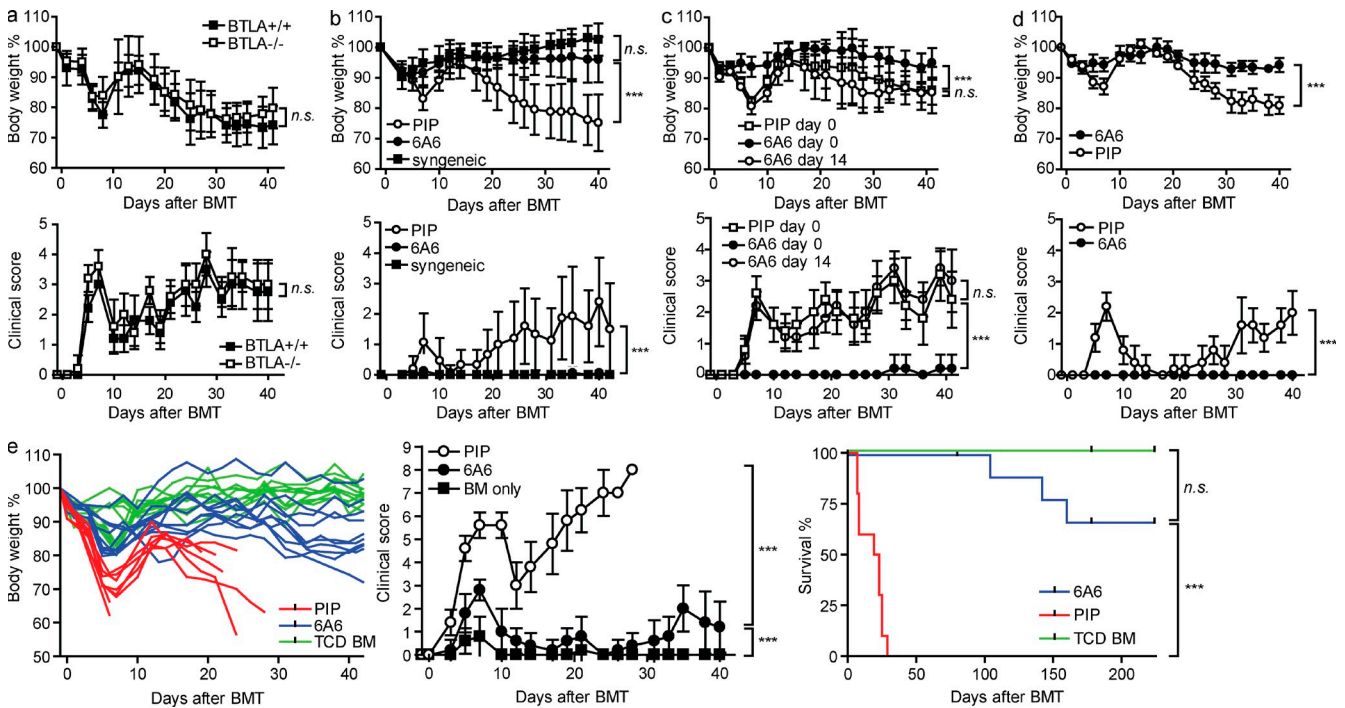


Figure 1. Anti-BTLA treatment permanently prevents GVHD. (a) Lethally irradiated CB6F1 mice received BMC and splenocytes from C57BL/6 WT (closed squares, *n* = 5) or BTLA^{-/-} (open squares, *n* = 5) donors. (b) Lethally irradiated CB6F1 mice received BMC and splenocytes from syngeneic donors (closed squares, *n* = 10), C57BL/6 mice and antibodies PIP (open circles, *n* = 15), or 6A6 (closed circles, *n* = 15). Shown are cumulative data from three independent experiments. (c) Lethally irradiated CB6F1 mice received BMC and splenocytes from C57BL/6 mice plus control antibody PIP (open circles, *n* = 5) or 6A6 (closed circles, *n* = 5) on the day of BMT or 6A6 14 d after BMT (open squares, *n* = 5). (d) Lethally irradiated CB6F1 mice received BMC and splenocytes from C57BL/6 HVEM^{-/-} mice and control antibody PIP (open circles, *n* = 5) or 6A6 (closed circles, *n* = 5). (e) Lethally irradiated BALB/c mice received TCD-BM alone (green lines and closed squares, *n* = 10) or in combination with splenocytes from C57BL/6 mice and control antibody PIP (red lines and open circles, *n* = 10) or 6A6 (blue lines and closed circles, *n* = 10) on the day of BMT. Shown are cumulative data from two independent experiments. Weight and clinical score data shown are mean ± SD. Data are representative of two independent experiments with five mice per group or cumulative data from independent experiments as indicated. P-values of >0.05 are considered not significant (n.s.). ***, *P* < 0.001.

the colon (Stelljes et al., 2008; Fig. S1 c, bottom). In contrast, a single treatment of 10 $\mu\text{g/g}$ body weight of 6A6, given at the time of aHSCT, prevented GVHD completely, with weight loss and GVHD similar to the syngeneic control group (Fig. 1 b) for 140 d after aHSCT (Fig. S1 b). Furthermore, 6A6-treated mice had no histological evidence of GVHD in the colon (Fig. S1 c, top). Thus, a single administration of anti-BTLA antibody eliminates weight loss, histological changes, and clinical signs of GVHD.

We next asked if 6A6 acted by simply depleting donor cells that express BTLA. As a positive control, we included a depleting murine anti-BTLA antibody, 6F7 (Hurchla et al., 2005; Truong et al., 2009). CFSE-labeled donor cells were transferred into WT recipients that also received PIP, 6A6, or 6F7 antibody. 2 d after transfer, we found similar numbers of CFSE⁺ cells in mice that received either PIP or 6A6 antibody (Fig. S1 d, left) and no significant differences between numbers of CD19⁺, CD4⁺, or CD8⁺ lymphocytes (Fig. S1 d, right). Treatment with 6F7 caused a significant depletion of CFSE⁺ lymphocytes, particularly from the CD19⁺ cell population (Fig. S1 d). Furthermore, surface-bound 6A6 was detectable on live donor-derived cells *in vivo* up to 7 d after transfer (Fig. S1 e). In addition, 6A6 treatment was unable to prevent GVHD caused when BTLA^{-/-} donors were used as a source of BM for aHSCT (Fig. S1 f). Thus, 6A6 does not deplete lymphocytes (Hurchla et al., 2007; Lepenies et al., 2007) but requires the expression of BTLA on donor cells to prevent GVHD.

Next, we asked if 6A6 could reverse established GVHD. We compared immediate with delayed administration of 6A6 (Fig. 1 c). Again, immediate 6A6 administration prevented GVHD. In contrast, there was no statistical difference in weight loss or clinical scores between mice that received 6A6 14 d after aHSCT and with mice that received PIP (Fig. 1 c).

6A6 binds to a region of BTLA that is involved in interactions with HVEM (Hurchla et al., 2005). Thus, 6A6 might prevent GVHD by preventing HVEM and BTLA interactions, thus blocking costimulatory signaling to donor cells (Xu et al., 2007). Although our data indicated that host BTLA is not involved (Fig. S1 a), we wished to test this possibility independently. Transfer of HVEM^{-/-} donor cells caused induction of GVHD when administered with PIP (Fig. 1 d). The severity of GVHD caused by HVEM^{-/-} donor cells was somewhat less than that caused by WT donor cells (Fig. 1 b), consistent with a study which found that HVEM and LIGHT are costimulatory in promoting GVHD (Xu et al., 2007). However, 6A6 also prevented GVHD caused by HVEM^{-/-} donor cells (Fig. 1 d). These results indicate that 6A6 prevents GVHD in a manner that is independent of HVEM, suggesting it acts directly through BTLA.

Because the parent-into-irradiated F1 model of aHSCT does not result in lethal GVHD, we wished to test the potency of the anti-BTLA treatment in a model of complete MHC mismatch (H-2^b into H-2^d) that results in lethal GVHD in untreated mice (Lu et al., 2001; Edinger et al., 2003b). Control mice developed severe GVHD with pronounced weight loss and clinical scores of >6 (Fig. 1 e, left and middle) and died

within 30 d after aHSCT from severe GVHD (Fig. 1 e, right). Although mice that received the anti-BTLA treatment were not fully protected from GVHD, with clinical scores ranging from 1 to 3 (Fig. 1 e, middle) and slightly more weight loss than the control group that had received T cell-depleted BM cells (BMCs [TCD-BMs]) alone (Fig. 1 e, left), 70% of recipients treated with 6A6 survived for >200 d after aHSCT.

Although the precise molecular targets of BTLA signaling are still obscure (Gavrieli and Murphy, 2006; Wu et al., 2007), BTLA engagement by HVEM can inhibit T cell proliferation *in vitro* (Sedy et al., 2005) and promote tolerance induction *in vivo* (Liu et al., 2009). Therefore, we asked if anti-BTLA treatment alters donor T cell proliferation or IL-2 production *in vivo*. CFSE-labeled donor splenocytes were transferred into CB6F1 recipients that were treated with PIP, 6A6, or 6F7. Donor T cell proliferation was assessed after aHSCT and, by 3 d after aHSCT, reduced CFSE levels suggested that proliferation had occurred (Fig. 2 e). CD4⁺ T cells had reduced proliferation after treatment with 6A6 and 6F7 antibodies 3 d after aHSCT (Fig. 2 e, middle), and the total accumulation of donor CD4⁺ T cells was significantly reduced compared with control ($P < 0.0018$ and $P < 0.0083$; Fig. 2 b). In contrast, CD8⁺ T cell proliferation and accumulation were not significantly affected by 6A6 treatment ($P < 0.8489$; Fig. 2, a and e, left). As 6F7 treatment leads to partial depletion of cells, we also assessed the level of annexin V binding, which is an indicator of early apoptosis. Mice treated with 6F7, but not PIP or 6A6, showed increased binding of annexin V on CD4⁺ and CD8⁺ T cells 7 d after aHSCT, which was not observed on day 3 (Fig. S1 g).

Although accumulation of CD4⁺ T cells was lower in 6A6-treated than in PIP-treated mice, the production of IL-2 7 d after aHSCT was not statistically different (Fig. S2, a and b). A small but significant reduction in IFN- γ and IL-4, but not IL-17, production was observed in 6A6-treated mice compared with controls (Fig. S2, a and b). In summary, 6A6 administered at the time of aHSCT reduced the proliferation and accumulation of donor-derived CD4⁺Foxp3⁻ effector T cells (T eff cells) without inducing anergy, inhibiting IL-2 production or causing major alterations in cytokines.

These effects were suggestive of the actions of CD4⁺Foxp3⁺ regulatory T cells (T reg cells) expressing the transcription factor forkhead box P3 (Foxp3; Hori et al., 2003). T reg cells have recently been reported to play a significant role in regulating GVHD (Taylor et al., 2002; Edinger et al., 2003b), and there are ongoing clinical trials aimed directly at the use of T reg cells as an intervention in human GVHD (NCI clinical trial NCT00725062).

To determine whether 6A6 treatment influences T reg cells, we measured the accumulation and proliferation of donor-derived T eff cells after aHSCT (Fig. 2, c and e, right). In PIP-treated recipients that developed GVHD, significantly fewer T reg cells were detected 7 d ($P < 0.0393$) after aHSCT when compared with recipients that received either anti-BTLA treatment 6A6 or 6F7 (Fig. 2 c). Therefore, anti-BTLA treatment inhibits the proliferation of T eff cells yet

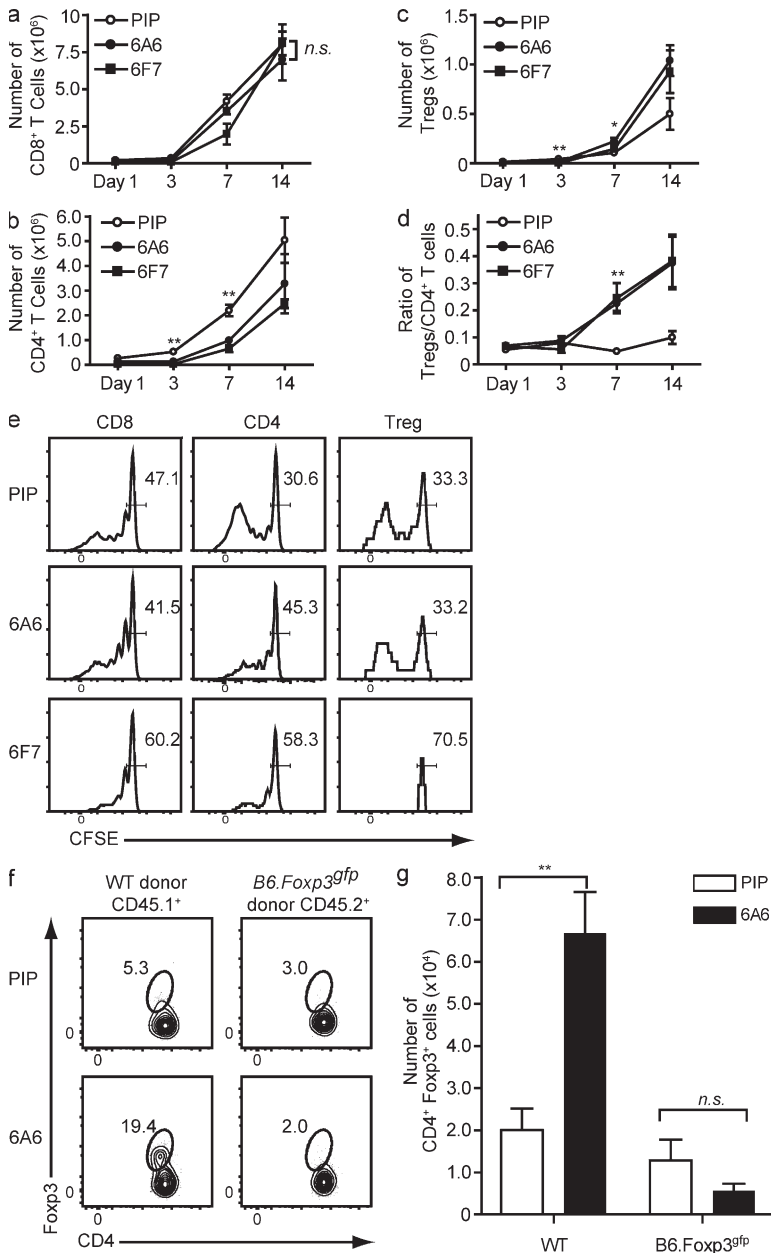


Figure 2. Anti-BTLA treatment allows for the expansion of preexisting donor-derived T reg cells by inhibiting T eff cell proliferation. (a–e) Lethally irradiated CB6F1 mice received a CFSE-labeled graft from B6.SJL donors and were treated with control antibody PIP (open circles), 6A6 (closed circles), or 6F7 (closed squares; *n* = 3 per group). The number of CD8⁺ T cells (a), CD4⁺Foxp3⁻ (b), and CD4⁺ Foxp3⁺ (c) was calculated from absolute numbers of live splenocytes, and the percentage of the lymphocyte population was assayed by flow cytometry. (d) The ratio of total CD4⁺Foxp3⁺/CD4⁺Foxp3⁻ cells was calculated at the indicated time points by dividing the number of T reg cells in c by the number of T eff cells in b. (e) CFSE intensity of CD8⁺, CD4⁺Foxp3⁻, and CD4⁺Foxp3⁺, 3 d after aHSCT with PIP, 6A6, or 6F7 treatment. (f and g) Lethally irradiated CB6F1 mice received aHSCT from B6.SJL mice along with purified CD4⁺ Foxp3⁻ T cells from B6.Foxp3^{gfp} mice with control antibody (PIP) or 6A6 (*n* = 5 per group). After 7 d, splenocytes were assayed by flow cytometry to determine the relative frequency of CD4⁺Foxp3⁺ cells among CD4⁺ cells (f) or the absolute number of CD4⁺Foxp3⁺ T reg cells (g). Statistical comparisons in a–d are between the PIP- and 6A6-treated groups at the indicated time point, and the data are displayed as mean ± SEM. Shown are representative data from two independent experiments with three to five mice per group. P-values of >0.05 are considered not significant (n.s.). *, 0.01 < *P* < 0.05; **, 0.001 < *P* < 0.01.

allows the accumulation of T reg cells, resulting in an increased T reg/T eff cell ratio (*P* > 0.0028; Fig. 2 d). This result is in agreement with the demonstration that T reg cells maintain low levels of BTLA after activation, in contrast to conventional T cells which up-regulate BTLA expression upon activation (Fig. S2 c; Hurchla et al., 2005). Thus, 6A6 treatment increases the numbers and the frequency of donor-derived T reg cells after aHSCT.

6A6 treatment could increase T reg cell frequency either by inducing Foxp3 expression in naive donor CD4⁺ T cells (Chen et al., 2003) or by causing in vivo expansion of preexisting donor T reg cells relative to T eff cells. To distinguish these alternatives, we used B6.Foxp3^{gfp} reporter mice (Fontenot et al., 2005). We performed mixed aHSCT with WT and Foxp3^{gfp}

mice as donors, using purified GFP-negative cells from Foxp3^{gfp} mice to remove preexisting T reg cells from the donor population. In this mixed aHSCT setting, 6A6 treatment similarly decreased the accumulation of T eff cells in both populations, but T reg cells expanded only from preexisting T reg cells (Fig. 2, f and g; and Fig. S2, e and f). Therefore, the donor T reg/T eff cell ratio increased only among the WT donor T cells and not in the Foxp3^{gfp} donor T cells, as assessed by intracellular staining for endogenous Foxp3 (Fig. 2 f and Fig. S2 g). In addition, donor-derived CD4⁺ T cells, which were originally isolated from B6.Foxp3^{gfp} mice as negative for GFP expression, remained negative for Foxp3 as assessed by the Foxp3–GFP reporter (Fig. S2 e, right).

To further characterize the conditions necessary for the expansion of T reg cells, we evaluated what effects anti-BTLA treatment had on T reg cells in the steady state and whether the presence of alloantigen or HVEM on host tissue is required. When unmanipulated B6.Foxp3^{gfp} mice received 6A6, the frequency of T eff cells, T reg cells, or the resulting ratio did not change (Fig. S3, a–d). Similarly, although syngeneic aHSCT of CB6F1 resulted in a small decrease of T eff cells 7 d after transplant, no major accumulation of T reg cells and no increased T reg/T eff cell ratio was observed (Fig. S3, e–h). When HVEM^{-/-} donors and MHC-mismatched HVEM^{-/-} recipients were used, the ratio of T reg/T eff cells increased by inhibiting the accumulation of T eff cells

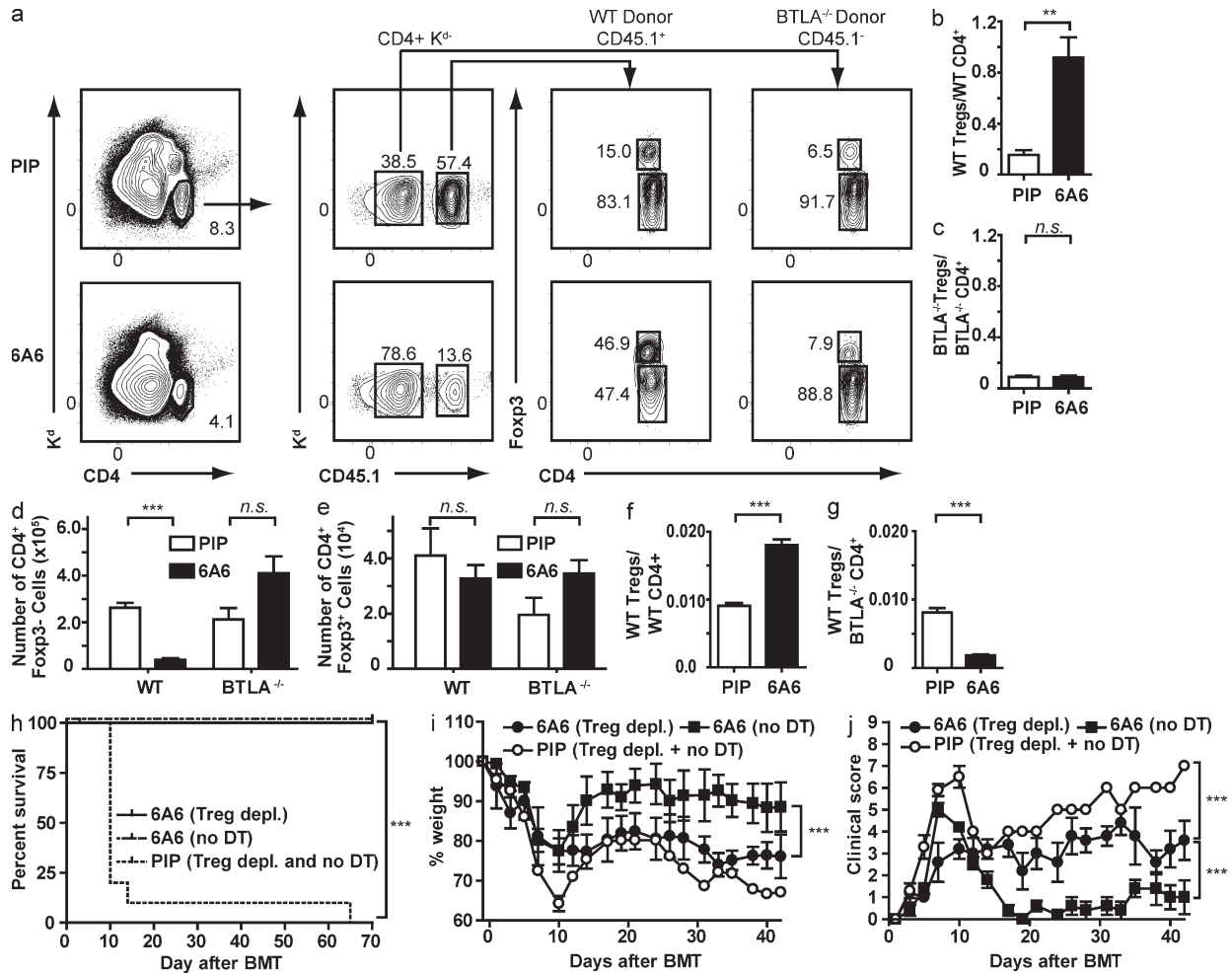


Figure 3. Direct engagement of BTLA on T eff cells leads to an increased frequency of CD4⁺Foxp3⁺ cells. Lethally irradiated CB6F1 mice received a 1:1 mixed aHSCT with WT-B6.SJL and B6 BTLA^{-/-} donor cells (a–e) with either control antibody (PIP) or 6A6 (*n* = 5 per group). After 7 d, splenocytes were analyzed by flow cytometry. (a) Shown are FACS plots to identify donor cells as H-2K^d- and CD4⁺ (left). Intracellular Foxp3 was detected among CD4⁺ T cells (right) gated on WT-B6.SJL (CD4⁺CD45.1⁺ H-2K^d-) or B6 BTLA^{-/-} (CD4⁺CD45.1⁻ H-2K^d-) donor cell populations as indicated. Numbers represent the percentage of cells within the indicated gates. The ratios of the number of WT T reg/WT T eff cells (b) and KO T reg/ KO T eff cells (c) are shown, as well as the total number of T eff cells (d) and T reg cells (e) from the indicated donors after treatment with control antibody (PIP, open bars) or 6A6 (closed bars). (f and g) Lethally irradiated BALB/c mice received a 1:1 mixture of purified WT-B6.SJL and BTLA^{-/-} CD4⁺ cells and T reg cells from B6. Foxp3^{DTR} mice, with either control antibody PIP or 6A6 (*n* = 3–4 per group). After 7 d, splenocytes were analyzed by flow cytometry to determine the ratio of WT T reg/WT T eff cells (f) or WT T reg/BTLA^{-/-} T eff cells (g). Data are displayed as mean ± SEM. Shown are representative data from two independent experiments. (h–j) Recipient BALB/c mice received TCD-BM and splenocytes from T reg cell ablated or unmanipulated Foxp3^{DTR} mice and PIP or 6A6 as indicated. Survival curves (h), weight curves (i), and clinical scores (j) are shown for 6A6-treated mice that received a graft from either untreated (no DT) Foxp3^{DTR} donors (dashed line or filled squares; *n* = 5) or a graft from DT-treated (T reg depl.) Foxp3^{DTR} donors (solid line or filled circles; *n* = 5) and PIP-treated mice that received a graft from DT-treated or untreated (T reg depl. and no DT) Foxp3^{DTR} donors (dotted line or open circles; *n* = 10). Data are shown as mean ± SD from two independent experiments. P-values of >0.05 are considered not significant (n.s). **, 0.001 < *P* < 0.01; ***, *P* < 0.001.

(Fig. S3, i–l) as observed before (Fig. 2, d and g). Collectively, these results suggest that 6A6 has no effect in the steady state, does not involve HVEM, and requires allostimulation to increase the T reg/T eff cell ratio.

Because BTLA is expressed on both T eff and T reg cells, we tested whether the increased T reg/T eff cell ratio was the result of a decrease in T eff cell expansion, an increased proliferation of T reg cells, or both by performing a mixed aHSCT using congenically marked WT BTLA^{+/+} and BTLA^{-/-} donors. In this setting, the frequency of T reg cell within the

CD4⁺BTLA^{-/-} population did not change in the presence of 6A6 (7.9 ± 2 vs. 7.3 ± 2%; Fig. 3, a and c), whereas the T reg cell frequency within the CD4⁺ WT population increased more than threefold in the presence of 6A6 (48 ± 10 vs. 14.4 ± 5.5%; Fig. 3, a and b).

Importantly, 6A6 treatment decreased T eff cell numbers more than fivefold from 2.6 × 10⁵ ± 4.6 × 10⁴ cells in the control group to 3.9 × 10⁴ ± 1.4 × 10⁴ (*P* < 0.0001; Fig. 3 d), indicating that WT-BTLA^{+/+} T eff cells are the main target of the anti-BTLA treatment. WT and BTLA^{-/-} T reg cells

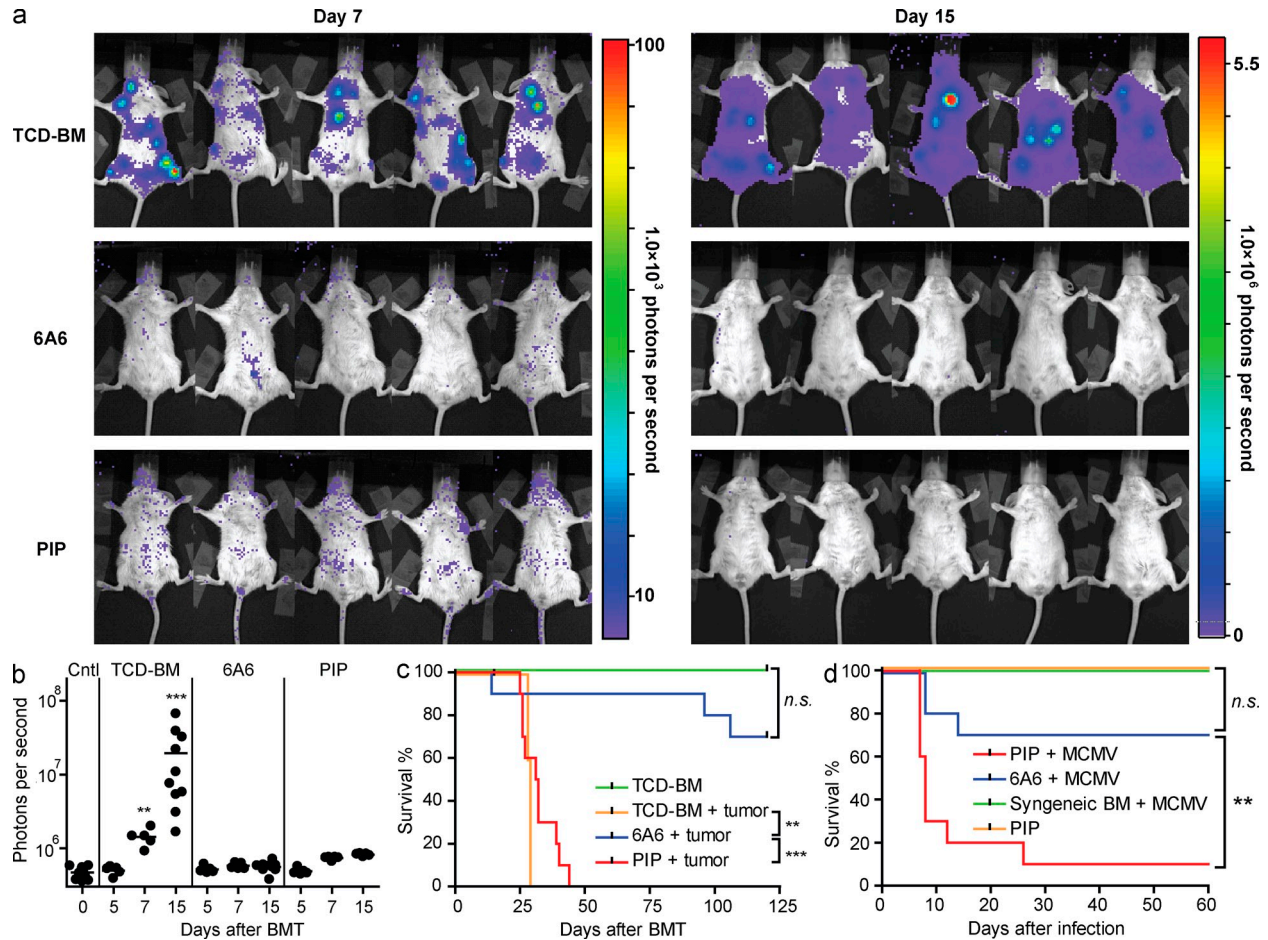


Figure 4. Anti-BTLA treatment does not lead to global immunosuppression. (a) Representative images of A20-Luc tumor cell localization 7 d (left) and 15 d (right) after aHSCT in lethally irradiated BALB/c mice that had received A20-Luc lymphoma cells with TCD-BM alone ($n = 10$) or together with splenocytes from C57BL/6 mice and control antibody PIP ($n = 10$) or 6A6 ($n = 10$). (b) Shown are units of photons per second for individual animals from panel a for days 5 ($n = 5$), 7 ($n = 5$), and 15 ($n = 10$) after aHSCT, as well as unmanipulated BALB/c mice (day 0, $n = 12$) for comparison. Asterisks indicate statistically significant differences between TCD-BM and 6A6 groups. Shown are cumulative data from two independent experiments, with each point representing the BLI signal from an individual mouse. Horizontal lines represent the mean BLI signal. Vertical lines serve to separate experimental groups. (c) Survival data of mice from a and from mice that received TCD-BM alone without tumor for comparison. Shown are cumulative data from two independent experiments. (d) CB6F1 BMC and splenocytes from syngeneic (green line; $n = 10$) or C57BL/6 donors and antibodies PIP (red line; $n = 10$) or 6A6 (blue line; $n = 10$) were infected with MCMV 4 wk after aHSCT and were monitored for survival. PIP-treated uninfected mice (orange line, $n = 10$) served as controls. Shown are cumulative data from two independent experiments. Data are shown as mean \pm SD from two independent experiments. P-values of >0.05 are considered not significant (n.s.), $**$, $0.001 < P < 0.01$; $***$, $P < 0.001$.

do not expand significantly in the presence of $BTLA^{-/-}$ T eff cells (Fig. 3 e), confirming our observation that T reg cells expand only when T eff cells express BTLA and are inhibited by 6A6. To exclude any indirect effects on host tissues, we cotransferred purified populations of WT T reg cells together with WT or $BTLA^{-/-}$ T eff cells into BALB/c recipients, which express an allele of BTLA not recognized by 6A6 (Fig. 3, f and g; and Fig. S4; Hurchla et al., 2005). The WT T reg/WT T eff cell ratio increased after 6A6 treatment ($P < 0.0004$; Fig. 3 f) as observed before (Fig. 2 g and Fig. 3 b). In contrast, when $BTLA^{-/-}$ T eff cells were present the WT T reg/ $BTLA^{-/-}$ T eff cell ratio did not increase (Fig. 3 g), indicating that the expansion of T reg cells is caused by 6A6 reducing the expansion of T eff cells. In this setting, the WT

T reg/ $BTLA^{-/-}$ T eff cell ratio even decreased (Fig. 3 g and Fig. S4 i), suggesting that low expression levels of BTLA on T reg cells inhibits their expansion when proliferation of $BTLA^{-/-}$ T eff cells is not restricted.

To ask whether the inhibition of T eff cells had an effect on GVHD in the absence of T reg cells, we used C57BL/6 $Foxp3^{DTR}$ mice as donors in the lethal model of GVHD. Pretreatment with diphtheria toxin reduced T reg cells by $>95\%$ in $Foxp3^{DTR}$ donors. 90% of control animals died within 14 d of aHSCT, irrespective of whether or not the BM graft contained T reg cells (Fig. 3 h). All mice that were treated with the 6A6 antibody survived beyond day 70 (Fig. 3 h). Mice that received a graft with T reg cells and 6A6 eventually recovered to $\sim 90\%$ of their starting weight (Fig. 3 i) and developed

low-grade GVHD with scores ranging from 1 to 3, as seen before (Fig. 1 e). Mice that received a T reg cell–depleted graft and 6A6, however, recovered only 70–80% of their starting weight and had more severe GVHD scores ranging from 3 to 5 (Fig. 3 j). These data highlight that forced inhibition of T eff cells through BTLA (Fig. 3 d) is sufficient to prevent lethality. Nonetheless, the relative expansion of T reg cells (Fig. 3, b and f) bears biological significance, as they are required to protect the host from clinical GVHD (Fig. 3, i and j).

Because engagement of BTLA by 6A6 inhibited T eff cell proliferation, we wondered whether this treatment allowed for antigen-specific responses to tumors and pathogens. First, we examined whether graft-versus-tumor (GVT) activity was maintained after 6A6 treatment (Fig. 4, a–c). We used a model of minimal residual disease after aHSCT that utilizes bioluminescence imaging (BLI) of luciferase-expressing A20–Luc leukemia cells (H-2^d) in vivo (Edinger et al., 2003a). In this model, GVT activity requires cytolytic activity of T cells to control the tumor (Edinger et al., 2003b). Transplantation of A20–Luc leukemia cells with TCD-BM alone resulted in progressive tumor growth (Fig. 4, a and b). In mice that had received a T cell–containing graft with or without the 6A6 antibody, however, tumor was never detectable by BLI, suggesting that GVT effects were intact. Although all control mice died before day 45 of severe GVHD, 90% of 6A6-treated mice survived beyond day 90 (Fig. 4 c). To exclude any antibody-mediated anti-tumor effects, we confirmed that the 6A6 antibody does not bind to A20 cells, which was expected because of their BALB/c origin (Fig. S2 d). To document initial tumor engraftment, we quantified the total tumor number A20–GFP cells in the BM by flow cytometry 7 d after aHSCT (Fig. S3 m).

We next examined immune responses to murine CMV (MCMV; Nguyen et al., 2008) 4 wk after aHSCT. All animals that had undergone syngeneic aHSCT survived for >60 d after infection (Fig. 4 d). Of the mice in the allogeneic group that had received the PIP antibody, 70% died within 10 d of infection compared with 20% of the 6A6-treated mice. Thus, although 6A6 treatment prevents GVHD, it allows for resistance to MCMV, unlike PIP-treated control recipients.

To test the immune response against bacterial infection, animals were infected with the intracellular bacterium *Listeria monocytogenes*. 3 d after infection, animals that had received the PIP antibody had either higher ($P = 0.0005$ for liver and $P = 0.1816$ for spleen) or lower *L. monocytogenes* organ burdens ($P = 0.0227$ for liver and $P = 0.0059$ for spleen; Fig. S3, n–p), as has been described previously (Miura et al., 2000). In contrast, anti-BTLA–treated mice controlled infection similarly to both control groups, indicating an intact innate immune response (Fig. S3, n–p). These preliminary results suggest that 6A6–treated mice were similarly resistant to *L. monocytogenes* infection to unmanipulated mice.

In summary, this study demonstrates that the anti-BTLA antibody 6A6 administered at the time of aHSCT prevents GVHD without the need for additional immunosuppressive therapy. The mechanism of action is through direct engagement of BTLA on donor T cells, selectively inhibiting T eff

cells and allowing the relative expansion of naturally occurring T reg cells. Once established, this new balance of T eff cells is sufficient to prevent GVHD permanently while allowing for intact responses to viral and bacterial pathogens, as well as GVT effects. Thus, BTLA may represent a novel therapeutic target in prevention of human GVHD. Increasing the safety of aHSCT could potentially allow its application more widely as a tolerogenic therapy in treatment of autoimmune disorders or solid organ transplantation, for which it is currently performed only experimentally (Sykes and Nikolic, 2005; Kawai et al., 2008).

MATERIALS AND METHODS

Mice and BM transplantation. B6.SJL-Ptprca Pep3b/BoyJ (B6.SJL), C57BL/6, and C57BL/6 × BALB/c F1 (CB6F1) mice were obtained from The Jackson Laboratory or bred in our facility. *BTLA*^{−/−} (Watanabe et al., 2003), *Hvem*^{−/−} (Wang et al., 2005), *Foxp3*^{3^{flp}} (Fontenot et al., 2005), and *Foxp3*^{DTR} (Kim et al., 2007) mice were backcrossed to C57BL/6 for at least nine generations. *HVEM*^{−/−} H2-K^{b/d} or H2-K^{d/d} were obtained by crossing *HVEM*^{−/−} mice on a B6 (H2-K^{b/b}) background to BALB/c (H2-K^{d/d}) mice. The resulting *HVEM*^{+/-} H2-K^{d/b} F1 mice were intercrossed to obtain *HVEM*^{−/−} H2-K^{b/d} or H2-K^{d/d} F2 recipients. Mice were 12–18 wk old and sex matched for all experiments. Mice were bred and maintained in our specific pathogen-free animal facility according to institutional guidelines with protocols approved by the Animal Studies Committee of Washington University.

Cell transplantation and assessment of GVHD. Mice received transplants according to a standard protocol as previously described (Stelljes et al., 2008). In brief, BMCs were harvested by flushing tibia and femurs of donor mice. For the nonlethal parent-into-F1 model of GVHD, CB6F1 (H-2^{b/d}) recipients were lethally irradiated with 9 Gy total body irradiation using a ¹³⁷Cs source at a dose rate of ~70 cGy/min and reconstituted with 2×10^7 BMCs and 10^7 splenocytes from syngeneic (H-2^{b/d}) or parental C57BL/6 donors (H-2^b). For the lethal model of GVHD, BALB/c (H-2^d) recipients were lethally irradiated with 8 Gy total body irradiation using a ¹³⁷Cs source at a dose rate of ~70 cGy/min and reconstituted with 2×10^7 TCD-BM alone or an additional 10^7 splenocytes from allogeneic C57BL/6 donors (H-2^b). To obtain TCD-BM, cells were depleted of CD4⁺ and CD8⁺ cells by magnetic depletion (Miltenyi Biotec) according to the manufacturer's recommendation. In experiments where a T reg cell–depleted graft from *Foxp3*^{DTR} donors was used, recipients received an intraperitoneal injection of 20 μg diphtheria toxin on the day of BMT. GVHD was monitored by calculating the loss in total body weight. Body weights were measured before transplantation and three times a week after transplantation. Clinical GVHD intensity was scored by assessing weight loss, posture, activity, fur texture, and skin integrity (Cooke et al., 1996). Histopathologic analyses of the bowel were performed on hematoxylin and eosin–stained tissue.

Administration of antibody. In some experiments, mice received at the time of aHSCT, unless otherwise noted, a single intraperitoneal injection of 10–20 μg/g body weight of anti-BTLA antibodies 6A6 and 6F7, whose properties we have previously published in detail (Hurchla et al., 2005). In brief, the IgG hamster monoclonal antibody 6A6 is specific for the C57BL/6 allele of BTLA and does not deplete BTLA-expressing cells in vivo (Hurchla et al., 2007; Lepenies et al., 2007). The IgG1κ mouse monoclonal anti-BTLA antibody 6F7 recognizes all known alleles of BTLA (Hurchla et al., 2005) and has been shown to deplete BTLA-expressing cells in vivo. The IgG1 hamster monoclonal anti-GST antibody PIP (Gronowski et al., 1999) was used as an isotype control.

Cell purification and depletion. To obtain purified populations of CD4⁺ *Foxp3*^{3^{flp}}-negative cells or CD4⁺ *Foxp3*^{3^{flp}}-positive cells in the indicated experiments, *Foxp3*^{3^{flp}} splenocytes were stained with CD4⁺ and the desired population was purified by cell sorting on the MoFlo cytometer (Dako).

Purification of C57BL/6 CD4⁺ and BTLA^{-/-} CD4⁺ cells was obtained from splenocytes that were depleted of CD8⁺ and B220⁺ cells by magnetic depletion (Miltenyi Biotech) according to the manufacturer's recommendation. *Foxp3^{DTR}* mice received intraperitoneal injections for 2 d of 20 µg diphtheria toxin per day before harvesting BM and splenocytes for aHSCT.

CFSE labeling and flow cytometry. Cells were labeled with CFSE (Sigma-Aldrich) by being incubated for 8 min at 25°C with 1 µM CFSE at a density of 40 × 10⁶ cells per ml in PBS. Labeling was quenched by incubation of cells for 1 min with an equal volume of FCS and cells were washed twice with media containing 10% (vol/vol) FCS. 50 × 10⁶ total cells per mouse were injected intravenously. Single cell suspensions from spleens were analyzed by flow cytometry using the following antibodies for detection: K⁴-FITC (SF1-1.1), CD4-PECy7, APC, PerCPy5.5, v450 (RM4-5) and PE (GK1.5), CD8-v450 (53-6.7), anti-Armenian and Syrian hamster IgG cocktail-PE, CD19-APC (1D3), and annexin V-PE (BD); and CD45.1-PECy7 and APC (A20), CD45.2-APC-eFluor780 (104), CD8-APC Alexa Fluor 750 (53-6.7), CD4-APC Alexa Fluor 750 (RM4-5), BTLA-bio (6F7), and SA-v450 (eBioscience). Intracellular Foxp3 was detected using the Mouse Regulatory T cell staining kit (eBioscience) with Foxp3-PE or APC (FJK-16s). For intracellular cytokine staining, splenocytes were first restimulated with PMA/ionomycin for 4 h and were stained with antibodies to surface markers, followed by fixation with 2% formaldehyde for 15 min at room temperature. Cells were then washed once in 0.05% saponin and stained with anti-cytokine antibodies (anti-IL-17 FITC, IL-2 PE, IFN-γ PE-Cy7, and IL-4 APC) in 0.5% saponin. All flow cytometry data were collected on a FACSCanto II (BD) and were analyzed with FlowJo software (Tree Star, Inc.).

Tumor model and assessment of GVT effects. As a tumor challenge, 2 × 10⁴ A20-Luc or A20-GFP were administered intravenously together with the donor graft as indicated. Imaging was done as previously described (Rehemtulla et al., 2000; Edinger et al., 2003a). In brief, D-Luciferin (Biosynth AG) was reconstituted in 0.9% sodium chloride (Baxter) to a concentration of 15 mg/ml, filtered (0.2 µm), and frozen at -80°C until use. Mice were given intraperitoneal injections at a dose of 150 mg/kg and allowed to remain active in the cage for 5 min to allow circulation of luciferin. Using the Xenogen IVIS 200 system (Caliper Life Sciences) with attached anesthesia chamber, the animals were then anesthetized with 2% isoflurane for 5 min and subsequently transferred to the imaging chamber where they continued to receive a regulated flow of isoflurane through the manifold's nose cones. The Living Image software program (Caliper Life Sciences) was used to obtain and analyze data. For all experiments, a 60-s exposure time was used. To detect A20-GFP in the BM, single cell suspensions from both femurs were analyzed by flow cytometry using the antibody CD19-APC (1D3; BD). Cells that expressed high levels of CD19 and GFP were considered to be A20-GFP cells.

Cell lines. The BALB/c B cell lymphoma cell line A20 was obtained from the American Type Culture Collection. To generate luciferase-expressing A20 cells (A20-Luc), a 1995-bp HindIII-BamHI fragment from pGL4.23[luc2/minP] vector (Promega) containing a minimal promoter, a Luc2 coding sequence, and a SV40 late poly(A) signal was cloned into the HindIII and BamHI sites of the pcDNA3.1⁺ mammalian expression vector (Invitrogen) to generate pcDNA3.1⁺-Luc2. For stable transfections, 10 × 10⁶ A20 cells/ml in complete IMDM supplemented with 10% fetal calf serum and with 30 µg/ml PvuII-linearized pcDNA3.1⁺-Luc2 were electroporated at room temperature in 0.4-ml aliquots in 0.4-cm cuvettes in a Gene Pulser (Bio-Rad Laboratories) at 240 V and 960 mF. After electroporation, cells were cultured for 24 h in IMDM supplemented with 10% fetal calf serum and then selected in the presence of 800 µg/ml geneticin. To generate GFP-expressing A20 cells, a retroviral reporter vector (Ranganath et al., 1998) was used that contains the coding sequence of herpes simplex virus 1 thymidine kinase, followed by an internal ribosomal entry site and GFP (HSV1-TK-IRES-GFP-RV). The retroviral vector was packaged in Phoenix A cells. Before infection, the A20 cell line was stimulated with 5 µg/ml LPS (Sigma-Aldrich) for 24 h. For infection, cells were cultured in retroviral supernatant supplemented with 8 µg/ml polybrene

(Sigma-Aldrich) and 5 µg/ml LPS and spun at 930 rcf for 1 h before being cultured at 37°C for 24 h. 72 h after infection, cells were sorted for high GFP expression on a FACSAria II (BD) and stable GFP expression of >95% was confirmed several times by flow cytometry at later time points.

Models of infectious disease. For MCMV infection, mice were infected with Smith strain MCMV 4 wk after aHSCT. Virus preparation and administration was performed as described previously (Krug et al., 2004). In brief, a salivary gland stock of MCMV was prepared from BALB/c mice, with a titer of 6.75 × 10⁶ PFU/ml. Mice were infected intraperitoneally with a low dose of virus (10⁴ PFU/mouse) and then monitored for survival. For *L. monocytogenes* infection, mice were infected intravenously with 2.5 × 10⁴ *L. monocytogenes* (strain EGD; gift from E.R. Unanue, Washington University School of Medicine, St. Louis, MO) 3 mo after aHSCT. To determine organ *L. monocytogenes* burden at day 3 after infection, spleens and livers were homogenized in PBS plus 0.05% Triton X-100. Serial dilutions of homogenate were plated on brain heart infusion agar, and bacterial CFUs were assessed after overnight growth at 37°C. Small portions of spleen and liver were also fixed in 10% formalin and stained with hematoxylin and eosin.

Statistical analysis. A Student's unpaired two-tailed *t* test with a 95% confidence interval was used for statistical analyses of body weight data, clinical scores, and cell numbers. For analyses of survival data the log-rank test was used. A Mann-Whitney unpaired two-tailed Student's *t* test with a 95% confidence interval was used for statistical analyses of bioluminescence data in Fig. 4. Statistical analyses were done using Prism 4 (GraphPad Software, Inc.). FACS data are expressed as means ± SEM. All other data are presented as means ± SD. All experiments have been repeated at least once with three to five mice per group, unless stated otherwise.

Online supplemental material. Fig. S1 demonstrates that BTLA expression by recipient tissue does not promote GVHD and 6A6 antibody does not deplete lymphocytes. Fig. S2 shows that 6A6 treatment does not lead to donor T eff cell anergy and expands preexisting T reg cells. Fig. S3 shows that 6A6 inhibition of T eff cells and expansion of T reg cells requires allostimulation but not HVEM expression by donor or host. Fig. S4 shows that 6A6 alters the T reg cell/effector CD4⁺ T cell ratio by directly inhibiting BTLA-expressing T cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20102017/DC1>.

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