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

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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.

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.

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 (HVEM), and required BTLA expression by donor-derived T cells.
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 6A6 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

**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-2b haplotype donor cells and lethally irradiated H-2b/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

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**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 µg/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 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-2b into H-2b) 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–BMcs]) 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). In PIP-treated recipients that developed GVHD, significantly fewer T reg cell 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
mice as donors, using purified GFP-negative cells from Foxp3<sup>30b</sup> 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<sup>30b</sup> donor T cells, as assessed by intracellular staining for endogenous Foxp3 (Fig. 2 f and Fig. S2 g). In addition, donor-derived CD4<sup>+</sup> T cells, which were originally isolated from B6.Foxp3<sup>gfp</sup> 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<sup>30b</sup> 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<sup>−/−</sup> donors and MHC-mismatched HVEM<sup>−/−</sup> recipients were used, the ratio of T reg/T eff cells increased by inhibiting the accumulation of T eff cells

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<sup>+</sup> 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<sup>30b</sup> reporter mice (Fontenot et al., 2005). We performed mixed aHSCT with WT and Foxp3<sup>30b</sup>
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 × 10^5 ± 4.6 × 10^4 cells in the control group to 3.9 × 10^4 ± 1.4 × 10^4 (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 (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).

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do not expand significantly in the presence of BTLA<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> T eff cells were present the WT T reg/BTLA<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice as donors in the lethal model of GVHD. Pretreatment with diphtheria toxin reduced T reg cells by >95% in Foxp3<sup>−/−</sup> 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 ~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-2b) 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 e). 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.SJ–Pptra Peps3b/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), Hrappa–/– (Wang et al., 2005), Foxp3gfp (Fontenot et al., 2005), and Foxp3DTR (Kim et al., 2007) mice were backcrossed to C57BL/6 for at least nine generations. HVEM–/– H2-Kb/d or H2-Kb/d were obtained by crossing HVEM–/– mice on a B6 (H-2b) background to BALB/c (H-2Kd) mice. The resulting HVEM–/– H2-Kb/d F1 mice were intercrossed to obtain HVEM–/– H2-Kb/d or H2-Kb/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-2b) 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 × 107 BMCs and 107 splenocytes from syngeneic (H-2b) or parental C57BL/6 donors (H-2b). For the lethal model of GVHD, BALB/c (H-2a) 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 × 107 TCD-BM alone or an additional 107 splenocytes from allogeneic C57BL/6 donors (H-2a). 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 Foxp3DTR 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 IgG1k 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+ Foxp3gfp-negative cells or CD4+ Foxp3gfp-positive cells in the indicated experiments, Foxp3gfp splenocytes were stained with CD4+ and the desired
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. FlowJoT mice received intraperitoneal injections for 2 d of 20 μg diethylthiourea 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 × 10^6 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 × 10^6 total cells per mouse were injected intravenously. Single cell suspensions from spleens were analyzed by flow cytometry using the following antibodies for detection: K+–FITC (SF1–1.1), CD4–PECy7, APC, PerCP/Cy5.5, v50 (RM4–5) and PE (GK1.5), CD8–eFluor450 (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–v50 (eBioscience). Intracellular Foxp3 was detected using the Mouse Regulatory T cell staining kit (eBioscience) with Foxp3–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 × 10^4 A20–Luc or A20–GFP where 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/miniP] 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 × 10^6 A20 cells/ml in complete IMDM supplemented with 10% fetal calf serum and 30 μg/ml Puromycin were electroporated at room temperature in 0.4-μm aliquots in 0.4-μm 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 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–IRE–GFP–R). 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 FACSAnA 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 × 10^6 PFU/ml. Mice were infected intraperitoneally with a low dose of virus (10^4 PFU/mouse) and then monitored for survival. For L. monocytogenes infection, mice were infected intravenously with 2.5 × 10^4 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 energy and expands preexisting T reg cells. Fig. S3 shows that 6A6 inhibition of T eff cells and expansion of T reg cells requires allostimulus but not LIVEM 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.20102018/DC1.

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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 × 10⁷ BMC and 1.0 × 10⁷ splenocytes from C57BL/6 BTLA−/− donors. Data shown are mean ± SD (n = 5 per group). (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 × 10⁶ 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 × 10⁷ 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) aHSCs 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 × 10⁷ CFSE-labeled BMCs and 1.0 × 10⁷ 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 aHSC. Splenies were harvested 3 and 7 d after aHSC 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 \(2.0 \times 10^7\) BMC and \(1.0 \times 10^7\) 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. 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), BTLA\(^{-/-}\) CD4+ T cells (filled gray), and BTLA\(^{+/-}\) CD4+ Foxp3\(^+\) CD4+ 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\(^+-\)) mice received \(2.0 \times 10^7\) BMC and \(1.0 \times 10^7\) WT B6.SJL (CD45.1\(^+-\)) splenocytes, along with \(1.0 \times 10^6\) purified CD4+ Foxp3-negative T cells from B6.Foxp3\(^{-/-}\) mice (CD45.2\(^+-\)) 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 H-2K\(^d\) 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\(^{-/-}\) (CD45.2\(^+-\)) is shown. (f) Number of CD4+Foxp3\(^+\) cells from WT donors (left) or B6.Foxp3\(^{-/-}\) 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\(^{-/-}\) (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.Foxp3GFP 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-K10) 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 Treg/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 ± SEM. (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 Treg/T eff cell ratio by directly inhibiting BTLA-expressing T cells. Lethally irradiated BALB/c mice received 2 × 10⁵ T reg cells purified from B6.Foxp3<sup>+</sup> mice along with CD4<sup>+</sup> cells. Group 1 (a and d–f), 5 × 10⁶ purified WT B6.SJL cells; group 2 (b and g–i), 5 × 10⁶ BTLA<sup>−/−</sup> CD4<sup>+</sup> cells; group 3 (c and j–l), a 1:1 mixture of 2.5 × 10⁶ B6.SJL CD4<sup>+</sup> T cells and 2.5 × 10⁶ BTLA<sup>−/−</sup> CD4<sup>+</sup> 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<sup>+</sup> and CD4 to identify donor cells, and then on CD45.1 to identify WT cells or on CD45.2 to identify BTLA<sup>−/−</sup> and T reg cells. The T reg cells were separated from the BTLA<sup>−/−</sup> cells on the basis of GFP Foxp3 expression (a–c, right). The number of WT CD4<sup>+</sup> (d and j) and BTLA<sup>−/−</sup> CD4<sup>+</sup> (g and k) was determined in each condition. The resulting ratio of T reg/WT eff cell (f) or T reg cell/BTLA<sup>−/−</sup> CD4<sup>+</sup> (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.