Donor-type $CD4^+CD25^+$ Regulatory T Cells Suppress Lethal Acute Graft-Versus-Host Disease after Allogeneic Bone Marrow Transplantation

Petra Hoffmann,[1] Joerg Ermann,[1] Matthias Edinger,[2]
C. Garrison Fathman,[1] and Samuel Strober[1]

[1]*Division of Immunology and Rheumatology and* [2]*Division of Bone Marrow Transplantation, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305*

Abstract

Acute graft-versus-host disease (aGVHD) is still a major obstacle in clinical allogeneic bone marrow (BM) transplantation. $CD4^+CD25^+$ regulatory T (T_{reg}) cells have recently been shown to suppress proliferative responses of $CD4^+CD25^-$ T cells to alloantigenic stimulation in vitro and are required for ex vivo tolerization of donor T cells, which results in their reduced potential to induce aGVHD. Here we show that $CD4^+CD25^+$ T cells isolated from the spleen or BM of donor C57BL/6 (H-2^b) mice that have not been tolerized are still potent inhibitors of the alloresponse in vitro and of lethal aGVHD induced by C57BL/6 $CD4^+CD25^-$ T cells in irradiated BALB/c (H-2^d) hosts in vivo. The addition of the $CD4^+CD25^+$ T_{reg} cells at a 1:1 ratio with responder/inducer $CD4^+CD25^-$ T cells resulted in a >90% inhibition of the mixed leukocyte reaction and marked protection from lethal GVHD. This protective effect depended in part on the ability of the transferred $CD4^+CD25^+$ T cells to secrete interleukin 10 and occurred if the T_{reg} cells were of donor, but not host, origin. Our results demonstrate that the balance of donor-type $CD4^+CD25^+$ T_{reg} and conventional $CD4^+CD25^-$ T cells can determine the outcome of aGVHD.

Key words: immune regulation • T lymphocytes • IL-10 • mixed leukocyte reaction • alloimmunity

Introduction

GVHD remains a major complication after allogeneic bone marrow transplantation (BMT),* thereby preventing the widespread use of this therapeutic approach for the treatment of malignant and nonmalignant diseases (1, 2). Acute GVHD (aGVHD) is initiated by alloreactive donor T cells that recognize MHC class I and II molecules on the surface of host cells as well as peptides presented by them. The infiltration of several target organs such as gut, liver, and skin by donor leukocytes including T cells is thought to be one of the key processes in the early phase of aGVHD (3). The activation and expansion of the donor T cells, leading to the secretion of proinflammatory cytokines and the recruitment of additional inflammatory effector cells to these sites, further damages the affected tissues (4–6).

The most effective means to avoid GVHD is T cell depletion of the bone marrow (BM) graft (7, 8). However, such a pretreatment of the donor inoculum has been shown in numerous studies to be associated with a higher incidence of graft failure and a higher risk for opportunistic infections, tumor relapse, and secondary lymphoproliferative diseases (9–11). Therefore, recent approaches have aimed at the induction of alloantigen-specific immune tolerance of the donor T cell population before transplantation (12, 13).

Recently, $CD4^+CD25^+$ T cells with immunoregulatory potential have been isolated from the thymus and periphery of mice, rats, and humans, and have been shown to share similar characteristics and functions (14–16). They have been shown in various mouse models to prevent the development of experimental autoimmune diseases such as diabetes and inflammatory bowel disease (17–20), and regulate the homeostatic proliferation of the peripheral T cell pool (21). In vitro they suppress the activation and proliferation of cocultured conventional $CD4^+$ and $CD8^+$ T cells in re-

Address correspondence to Samuel Strober, Division of Immunology and Rheumatology, Department of Medicine, CCSR Building, 2215-C, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305. Phone: 650-725-6500; Fax: 650-725-6104; E-mail: sstrober@stanford.edu

**Abbreviations used in this paper:* aGVHD, acute GVHD; APC, allophycocyanin; BM, bone marrow; BMT, BM transplantation; CTLA, CTL-associated antigen; PB, peripheral blood; TBI; total body irradiation; TCD, T cell–depleted; T_{reg}, regulatory T; WT, wild-type.

sponse to polyclonal stimuli in a direct, cell contact–dependent way by inhibiting their IL-2 production (22–24).

Although stimulation via their TCR appears to be a prerequisite for the suppressor function of $CD4^+CD25^+$ T cells, the exact mechanism of suppression is still unknown (25). Several groups have demonstrated that $CD4^+CD25^+$ T cells secrete the immunosuppressive cytokine IL-10 upon activation in vitro (26, 27). However, blocking IL-10 did not abrogate suppression in vitro in proliferation assays (22, 28, 29). In vivo, IL-10 production by $CD4^+CD25^+$ T cells seemed to be essential in some (30, 31), but not in other, systems (32). Another cytokine implicated in the suppressor function of $CD4^+CD25^+$ T cells is TGF-β (26, 30, 33) and in particular, surface-bound TGF-β (26). Finally, it has been controversial whether and how the high expression levels of CTL-associated antigen (CTLA)-4 on $CD4^+CD25^+$ T cells are related to their suppressor function (24, 34, 35).

Human $CD4^+CD25^+$ regulatory T (T_{reg}) cells isolated from peripheral blood (PB) have been shown to suppress alloresponses in the MLR (27, 36), and two previous reports have indicated a role for murine $CD4^+CD25^+$ T cells in tolerance induction to alloantigens. Taylor et al. (37) showed in an allogeneic BMT model that the depletion of $CD25^+$ cells from the donor $CD4^+$ T cell population completely abrogated ex vivo tolerization to host alloantigens and resulted in a loss of protection against GVHD. Kingsley et al. (31) demonstrated that the pretreatment of donor mice with alloantigen and anti-CD4 antibodies led to the generation of alloantigen-specific $CD4^+CD25^+$ T cells that could prevent the rejection of skin allografts. However, in both studies only tolerized, but not untreated, $CD4^+CD25^+$ T cells demonstrated an alloantigen-specific immunoregulatory activity that resulted in diminished GVHD induction and allograft acceptance, respectively.

We have previously shown that NK T cells, another T cell subpopulation with regulatory potential, suppress GVHD in a murine model of allo-BMT across a complete MHC class I and II barrier (38). Here, we used the same model to investigate the capacity of $CD4^+CD25^+$ T cells to suppress or prevent aGVHD after allogeneic transplantation. The results clearly show that freshly isolated, unmanipulated $CD4^+CD25^+$ T cells from the spleen or BM of naive donor animals can suppress lethal GVHD induced by coinjected $CD4^+CD25^-$ T cells. We also demonstrate that IL-10 production by transplanted $CD4^+CD25^+$ T cells is necessary for full protection. Finally, we provide evidence that the $CD4^+CD25^+$ T_{reg} cells had to be of donor origin to convey protection from lethal GVHD.

Materials and Methods

Animals. Male C57BL/6 (H-$2K^b$) and BALB/c (H-$2K^d$) wild-type (WT) mice were obtained from the breeding facility of the Department of Comparative Medicine (Stanford University School of Medicine, Stanford, CA). Male B6.129P2-*Il10*^{tm1Cgn} (B6.IL-$10^{-/-}$) mice were purchased from The Jackson Laboratory. All mice were 6–12-wk-old. The care of all experimental animals was in accordance with institutional guidelines.

Antibodies and FACS®. The following reagents were used for flow cytometric analysis: unconjugated anti-CD16/32 (2.4G2), allophycocyanin (APC)–anti-TCRαβ (H57-597), FITC– and PE–anti-CD4 (RM4-5), biotinylated anti-CD25 (7D4), PE–anti-CD25 (PC61), FITC–anti-CD44 (IM7), FITC–anti-CD45RB (16A), FITC–anti-CD62L (MEL-14), PE–anti-NK1.1 (PK136), PE-anti–CTLA-4 (4F10), PE-hamster IgG (A19-3), and FITC-anti–H-$2K^b$ (AF6-88.5; all from BD Biosciences). Streptavidin–Texas red conjugate was purchased from Caltag. All stainings were performed in PBS/1% calf serum in the presence of purified anti-CD16/32 at saturation to block unspecific staining via FcRII/III. Propidium iodide was added before analysis to exclude dead cells. All analyses were done on a modified dual laser FACSVantage™ (Becton Dickinson) with FlowJo® software (Becton Dickinson) for data analysis. For intracellular staining of CTLA-4, cells were stained for surface markers, washed twice, fixed, and permeabilized using the cytofix/cytoperm kit from BD Biosciences, and then stained with either PE-anti–CTLA-4 or PE-hamster IgG as recommended in the kit manual.

Cell Isolation and Sorting. Single cell suspensions were prepared from spleens, washed twice, and filtered through a fine nitex membrane. The samples were then enriched for either $CD4^+$ cells with anti-CD4 magnetic microbeads or $CD25^+$ cells with PE–anti-CD25 Ab and anti-PE magnetic beads using the MidiMACS® system (Miltenyi Biotec). After staining with anti-CD4 FITC and anti-CD25 PE as required, cells were FACS® sorted into $CD4^+CD25^-$ and $CD4^+CD25^+$ populations on a modified dual laser FACSVantage™. For the preparation of purified BM $TCR\alpha\beta^+NK1.1^-CD4^+CD25^-$ and $TCR\alpha\beta^+NK1.1^-CD4^+CD25^+$ cells, BM cells were obtained from the femur and tibia, and single cell suspensions were prepared and filtered through nitex. Cells were enriched for $CD4^+$ cells as described above. Enriched cells were stained with APC–anti-TCRαβ, PE–anti-NK1.1, FITC–anti-CD4, and biotin–anti-CD25 plus streptavidin–Texas red conjugate, and then FACS® sorted on a modified dual laser FACSVantage™. For the preparation of PB T cells, PBMC were isolated from PB by density centrifugation on Lympholyte M (Cedarlane Ltd.), washed twice in RPMI 1640 medium (Bio Whittaker), and stained with PE–anti-CD4 and APC–anti-TCRαβ. For the preparation of T cell–depleted (TCD) BM, BM cells, obtained as described above, were stained with biotin–anti-Thy1.2 (5a-8; Caltag) and streptavidin magnetic beads, and then passed over two consecutive MACS® LS separation columns (Miltenyi Biotec). TCD BM contained <0.01% T cells as determined by staining with APC–anti-TCRαβ.

GVHD Model. For BMT, BALB/c hosts were given total body irradiation (TBI; 800 cGy) from a 200-Kv x-ray source and injected with donor cells via the tail vein within 24 h. All mice received 2×10^6 TCD BM cells for reconstitution with or without T cells as indicated in the text and figures. Mice were kept on antibiotic water (25 μg/ml neomycin/0.3 U/ml polymyxin B; Sigma-Aldrich) for the first 28 d. The survival and appearance of mice were monitored daily and body weight was measured weekly. 100 d after transplantation, lineage-specific chimerism of transplanted animals was measured by staining PBMC with FITC-anti–H-$2K^b$ and cell lineage markers, as listed above.

Histopathology. Histopathological specimens from the skin, liver, and large intestine were obtained on days 49 and 100 after transplantation, fixed in formalin, and embedded into paraffin

blocks. Tissue sections were stained with hematoxylin and eosin, and examined at ×400.

MLR and Polyclonal Stimulation Assays. Cultures were set up in triplicates in 96-well round-bottom plates (Costar) in a total volume of 200 μl. Cells were cultured in RPMI 1640 medium with 10% FCS, 10 mM Hepes, 1% nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from GIBCO BRL), and 5×10^{-5} M 2-mercaptoethanol (Sigma-Aldrich). Fixed numbers of responder cells and irradiated allogeneic stimulator cells (10^5 cells, respectively) were mixed with variable numbers of $CD4^+CD25^+$ T cells to obtain the ratios indicated in the text and figures. After lysis of red blood cells, splenocytes were T cell depleted with anti-Thy1.2 magnetic beads using the MidiMACS® system and irradiated with 3,000 cGy before they were used as stimulator cells. Proliferation was assessed after 5 d by pulsing the cells with 1 μCi/well [^{3}H]thymidine (Amersham Biosciences) for the last 16 h. Cells were harvested onto filter membranes using a Wallac harvester (Perkin-Elmer) and the amount of incorporated [^{3}H]thymidine was measured with a Wallac Betaplate counter (PerkinElmer).

The culture conditions for the suppression assays with polyclonal anti-CD3 stimulation differed as follows: 5×10^4 TCD irradiated splenocytes were incubated with 2.5×10^4 syngeneic FACS®-sorted $CD4^+CD25^-$ and/or 2.5×10^4 $CD4^+CD25^+$ T cells in the presence of 0.5 μg/ml anti-CD3 (145-2C11; BD Biosciences). Proliferation was measured as described above after 72 h of incubation.

Statistical Analysis. Differences in the survival of the groups of hosts given BM transplants were analyzed using the log-rank test. Differences in the proliferation of responder cells were analyzed using the two-tailed Student's *t* test.

Results

Freshly Isolated Splenic $CD4^+CD25^+$ T_{reg} Cells Suppress the Proliferation of $CD4^+CD25^-$ T Cells after Allogeneic Stimulation In Vitro. Recently, several investigators have demonstrated that purified, but otherwise nontreated, $CD4^+CD25^+$ T cells isolated from the PB of normal human volunteers are able to suppress allospecific responses of $CD4^+CD25^-$ T cells in vitro in the MLR (27, 36). Similar results were obtained in the murine system using a partially MHC class II–mismatched strain combination (37). To show that this also holds true in a situation of complete MHC class I and II mismatch, we analyzed the ability of freshly isolated C57BL/6 $CD4^+CD25^+$ T_{reg} cells (H-2^b) to suppress the proliferative response of syngeneic $CD4^+CD25^-$ T cells in vitro to allogeneic BALB/c (H-2^d) stimulator cells. Highly purified $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were prepared from splenocytes in a two-step procedure combining immunomagnetic beads for enrichment and flow cytometry for the separation and final purification of the $CD25^+$ and $CD25^-$ subpopulations of CD4 T cells. Sorted populations routinely showed a purity of >96% on reanalysis (Fig. 1 A).

10^5 sorted C57BL/6 $CD4^+CD25^-$ T cells, cultured for 5 d with 10^5 irradiated TCD BALB/c splenocytes as stimulators, showed a strong proliferative response (mean incorporation of 22,000 ± 8,610 cpm; Fig. 1 B). This could be suppressed in a dose-dependent way by the addition of increasing numbers of C57BL/6 $CD4^+CD25^+$ T cells to the constant number of responder and stimulator cells in the cultures, resulting in a >90% reduction of the response at a $CD25^+$:$CD25^-$ T cell ratio of 1:1 (mean 1,450 ± 600 cpm, $P = 0.005$; two-tailed Student's *t* test) and a >50% reduction (mean 8,200 ± 1,300 cpm, $P = 0.03$) at a 1:4 ratio. In addition, sorted C57BL/6 $CD4^+CD25^+$ T cells did not proliferate in response to BALB/c stimulator cells. When 10^5 $CD4^+CD25^-$ T cells were added to the cultures instead of $CD4^+CD25^+$ T cells, no decrease in proliferation occurred (unpublished data). Thus, increasing the total

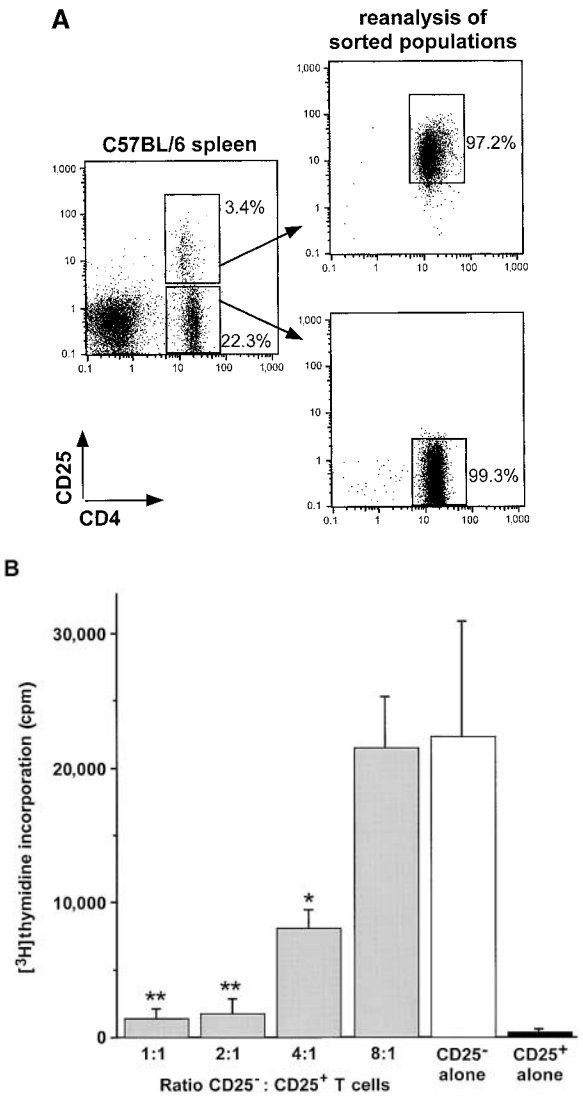

Figure 1. (A) Composition of the $CD4^+$ T cell population in the spleen of an adult C57BL/6 mouse and purity of the FACS®-sorted $CD25^-$ and $CD25^+$ subpopulations. (B) Dose-dependent suppression of the alloresponses of C57BL/6 $CD4^+CD25^-$ T cells to BALB/c stimulator cells by C57BL/6 $CD4^+CD25^+$ T cells. Cultures were set up with 10^5 sorted C57BL/6 $CD4^+CD25^-$ T cells and 10^5 irradiated BALB/c stimulator cells plus variable numbers of C57BL/6 $CD4^+CD25^+$ T cells to obtain the indicated ratios. The bars represent the means of triplicate values and the brackets indicate the SDs. **, $P < 0.01$; *, $P < 0.05$ (Student's *t* test). One of two experiments with similar results is shown.

number of cells in the cultures could not account for the observed suppression. These results clearly demonstrate that freshly isolated, untreated $CD4^+CD25^+$ T cells are not only hyporesponsive to a complete class I– and II–mismatched alloantigen, but are also able to suppress the alloresponse of cocultured $CD4^+CD25^-$ T cells.

Splenic $CD4^+CD25^+$ T_{reg} Cells Suppress Lethal aGVHD Induced by $CD4^+CD25^-$ T Cells after Allogeneic Transplantation. aGVHD, induced by the transplantation of C57BL/6-derived splenocytes into lethally irradiated BALB/c hosts, is initiated predominantly by alloreactive $CD4^+$ donor T cells (39). To see whether naive $CD4^+CD25^+$ T cells could suppress aGVHD induced by $CD4^+CD25^-$ T cells, we coinjected the two subpopulations at a 1:10 and 1:1 ratio with TCD BM into BALB/c hosts within 24 h after lethal TBI (800 cGy; Fig. 2 A). All mice that received 4.5×10^5 $CD4^+CD25^-$ T cells and TCD BM developed signs of aGVHD (diarrhea, weight loss, and hunched posture) shortly after transplantation, and all died within 29 d. Mice given only TCD BM cells appeared healthy and 100% of the animals survived for at least 100 d. At a ratio of 1:10, which is similar to the percentage of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells in the spleen of normal C57BL/6 mice (Fig. 1 A), no protective effect of the $CD4^+CD25^+$ T cells could be seen. At a ratio of 1:1 of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, however, the recipients were clearly protected from lethal aGVHD and 93% survived for 100 d ($P < 0.0001$; log-rank test). Animals receiving 9×10^5 $CD4^+CD25^-$ T cells instead of the mixture of 4.5×10^5 $CD4^+CD25^-$ and 4.5×10^5 $CD4^+CD25^+$ T cells, died even more quickly (by day 8 after transplant) than those receiving a single dose of 4.5×10^5 $CD4^+CD25^-$ T cells. Occasionally, recipients of $CD25^+$ and $CD25^-$ T cells at a 1:1 ratio showed mild signs of GVHD at ~4–5 wk after transplantation and their mean body weights during that time were lower than those of the control group given TCD BM alone (Fig. 2 B). However, after 7–8 wk, the differences in mean body weights between the two groups were no longer significant. In contrast, the mean body weights of the animals given only $CD4^+CD25^-$ T cells dropped markedly within the first 1–3 wk after transplantation. We checked surviving animals that had been transplanted with $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells at a 1:1 ratio after 100 d for donor cell chimerism of their PBMC. All animals showed complete donor chimerism of B cells, macrophages, and granulocytes, and 11 out of 15 animals also displayed complete chimerism of T cells, with the remaining recipients showing >85% of donor-derived T cells (unpublished data). We killed animals that had received TCD BM either alone or with a 1:1 mixture of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells at days 49 and 100 after allotransplantation for histopathological examination of GVHD target organs (large intestine and skin). Although all tissues examined on day 100 were free of any signs of GVHD in both groups, T cell recipients showed mild signs of GVHD in skin and gut on day 49, including a mononuclear cell infiltrate in the dermis, apoptotic intestinal crypt cells, and polymorphonuclear and mononuclear cell infiltrates in the lamina propria (unpublished data).

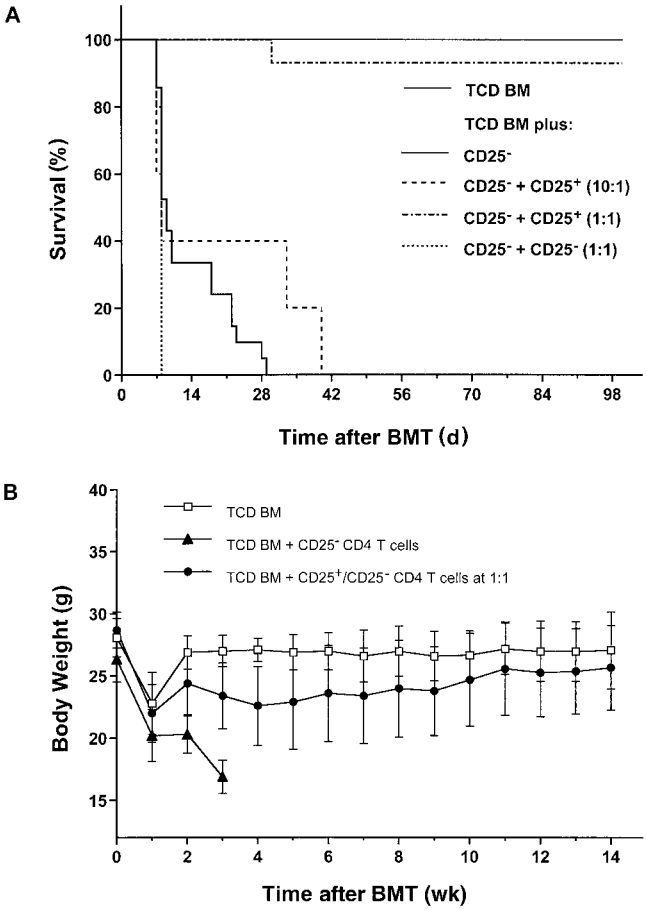

Figure 2. (A) Lethal GVHD of BALB/c recipients induced by C57BL/6 $CD4^+CD25^-$ T cells can be suppressed by coinjected C57BL/6 $CD4^+CD25^+$ T cells. BALB/c mice received 800 cGy TBI, 2×10^6 C57BL/6 TCD BM cells, and 4.5×10^5 C57BL/6 splenic $CD4^+CD25^-$ T cells ($CD25^-$) with variable numbers of C57BL/6 splenic $CD4^+CD25^+$ ($CD25^+$) or $CD4^+CD25^-$ T cells ($CD25^-$) to obtain the indicated ratios. Combined data from four independent experiments with 10–21 animals per group are shown. (B) Mean body weights and SDs of BALB/c hosts (10 animals per group) after BMT. BALB/c mice received 800 cGy lethal TBI and either 2×10^6 C57BL/6 TCD BM cells alone (□) or TCD BM plus 4.5×10^5 C57BL/6 $CD4^+CD25^-$ T cells (▲), or TCD BM plus a 1:1 mixture of 4.5×10^5 C57BL/6 $CD4^+CD25^+$ and 4.5×10^5 $CD4^+CD25^-$ T cells (●).

$CD4^+CD25^+$ T_{reg} Cells Are Highly Enriched within the BM T Cell Population. We previously showed that BM NK T cells, another T cell subpopulation with regulatory potential, are potent suppressors of lethal GVHD (38). Although NK T cells are rare among T cells in secondary lymphoid organs such as the spleen, they are highly enriched within BM T cells. Unseparated BM T cells are only very weak inducers of GVHD and even after the removal of the NK T cell subpopulation, the remaining $CD4^+$ BM T cells are still not as potent in GVHD induction as their counterparts from PB or spleen (39). This prompted us to look for $CD4^+CD25^+$ T_{reg} cells in the BM of untreated C57BL/6 mice. Five-color FACS® analysis revealed that ~30% of

$TCR\alpha\beta^+$ cells in the BM were $CD4^+$ and of these $CD4^+$ T cells, ~18% coexpressed NK1.1 (Fig. 3 A). None of the latter cells expressed CD25 (unpublished data). However, 41% of the remaining $NK1.1^-CD4^+$ T cell population expressed CD25. In comparison, $CD4^+TCR\alpha\beta^+NK1.1^-$ gated spleen cells contained only 13% $CD25^+$ cells. Additional analysis revealed that both $CD4^+CD25^+$ T cells from the BM and spleen shared the phenotype $CD44^{int}$, $CD45RB^{low}$, intracellular CTLA-4^+, and bimodal expression of CD62L (Fig. 3 B).

To clarify whether $CD4^+CD25^+$ T cells in BM are also functionally comparable to $CD4^+CD25^+$ T_{reg} cells from other lymphoid organs, we purified $TCR\alpha\beta^+NK1.1^-CD4^+CD25^+$ cells from the BM of C57BL/6 animals and examined their proliferative response to polyclonal activation via CD3 in the presence of autologous APC as well as their suppressive potential toward $CD4^+CD25^-$ T cells from the spleen. As shown in Fig. 4 A, sorted BM $TCR\alpha\beta^+NK1.1^-CD4^+CD25^+$ T cells did not proliferate when stimulated via their TCR. In addition, they suppressed the proliferation of cocultured splenic $CD4^+CD25^-$ T cells (32,700 vs. 5,150 cpm in the absence and presence of $CD4^+CD25^+$ BM T cells, respectively; $P < 0.001$). When tested for their capacity to suppress the MLR using C57BL/6 responder T cells and BALB/c stimulator cells, >98% suppression was observed at a ratio of 1:1 of splenic responder to BM T_{reg} cells compared with cultures without BM T_{reg} cells (Fig. 4 B). Thus, BM $TCR\alpha\beta^+NK1.1^-CD4^+CD25^+$ T cells showed the typical behavior of $CD4^+CD25^+$ T_{reg} cells from the spleen.

We determined whether BM $CD4^+CD25^+$ T cells can also suppress aGVHD after allogeneic transplantation. Lethally irradiated BALB/c hosts were transplanted with C57BL/6 TCD BM cells and 10^5 $CD4^+CD25^-$ T cells from C57BL/6 PB with or without an equal number of $NK1.1^-CD4^+CD25^+$ T cells from C57BL/6 BM. Our previous studies showed that sorted BM $TCR\alpha\beta^+$ T cells (total T cells) can suppress GVHD induced by PB T cells (38). As shown in Fig. 4 C, all animals that received TCD BM alone survived without any signs of GVHD, whereas 88% of the animals that also received PB T cells died from GVHD. In comparison, the survival of the group given PB $CD4^+CD25^-$ and BM $CD4^+CD25^+$ T cells at a 1:1 ratio was significantly increased with only 17% of the animals dying during the observation period of 100 d ($P = 0.019$). Mice receiving $CD4^+CD25^-$ instead of $CD4^+CD25^+$ BM T cells with PB T cells were not protected (71% of the animals died; $P = 0.19$). These results confirmed that BM $CD4^+CD25^+$ T cells are not only phenotypically, but also

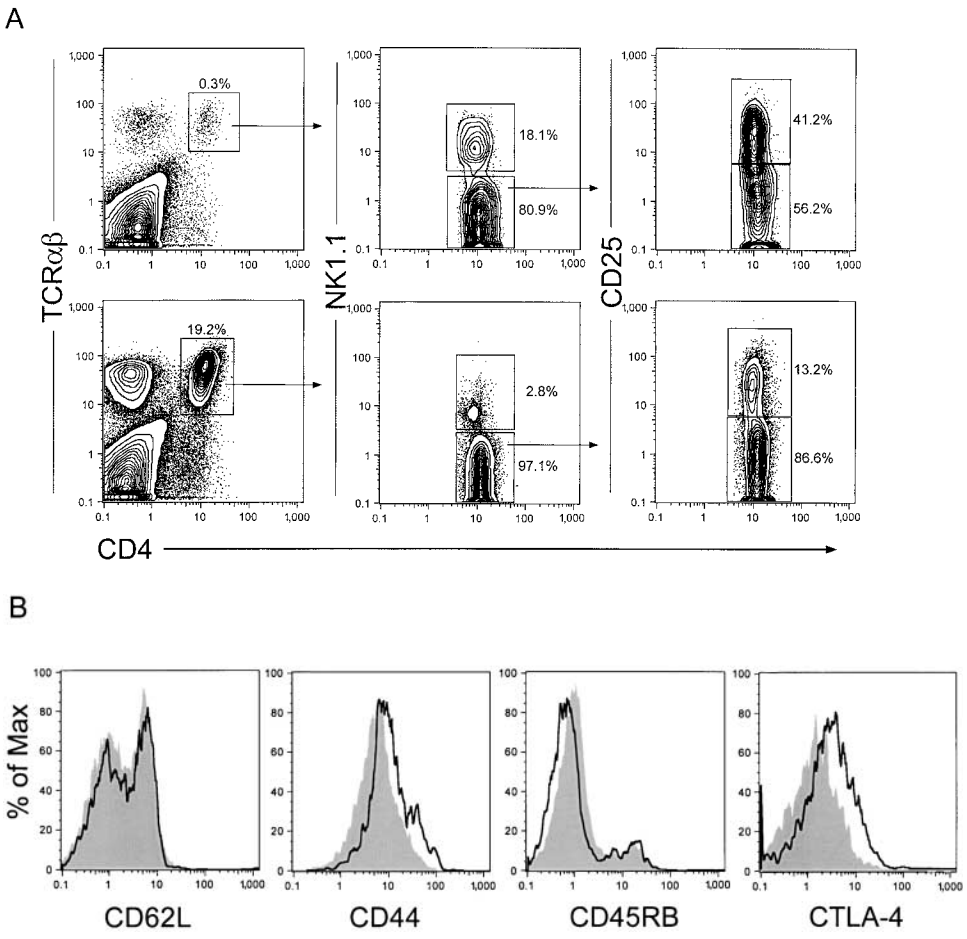

Figure 3. Phenotypic characterization of $CD4^+CD25^+NK1.1^-$ T cells from C57BL/6 BM and spleen. (A) Proportion of $CD4^+CD25^+NK1.1^-$ cells among $TCR\alpha\beta^+$ cells in C57BL/6 BM (top) and spleen (bottom). (B) Surface expression of CD62L, CD44, and CD45RB, and intracellular/surface expression of CTLA-4 by $CD4^+CD25^+NK1.1^-$ T cells in C57BL/6 BM (bold line) and spleen (filled).

functionally comparable to $CD4^+CD25^+$ T_{reg} cells from the spleen.

$CD4^+CD25^+$ T_{reg} Cells from IL-$10^{-/-}$ Animals Suppress the Proliferation of WT $CD4^+CD25^-$ T Cells to Alloantigen In Vitro, but Do Not Convey Full Protection from Lethal GVHD In Vivo. Recently, it has been shown that IL-10 plays an important role in the suppression of some experimental autoimmune diseases and the facilitation of tolerance to alloantigens in vivo by $CD4^+CD25^+$ T_{reg} cells (30, 31, 40). However, this is in contrast to results obtained in vitro, where attempts to block the suppression of $CD25^-$ by $CD25^+$ T cells with anti–IL-4 or anti–IL-10 antibodies have failed (22, 26–28). To further clarify the role of IL-10 production by T_{reg} cells in the suppression of alloresponses in vitro and in vivo, we first compared the capacity of splenic $CD4^+CD25^+$ T_{reg} cells from WT and IL-$10^{-/-}$ mice to suppress the proliferative response of WT splenic $CD4^+CD25^-$ T cells to alloantigen in vitro. As shown in Fig. 5 A, the proliferation of $CD4^+CD25^-$ T cells from WT C57BL/6 animals in response to irradiated TCD BALB/c splenocytes was suppressed in a comparable dose-dependent way by both WT and IL-10–deficient T_{reg} cells, resulting in a >50% reduction in proliferation at a 1:4 ratio of $CD25^+$ and $CD25^-$ T cells ($P < 0.05$), and a >90% reduction ($P < 0.001$) at a ratio of 1:1. These data clearly in-

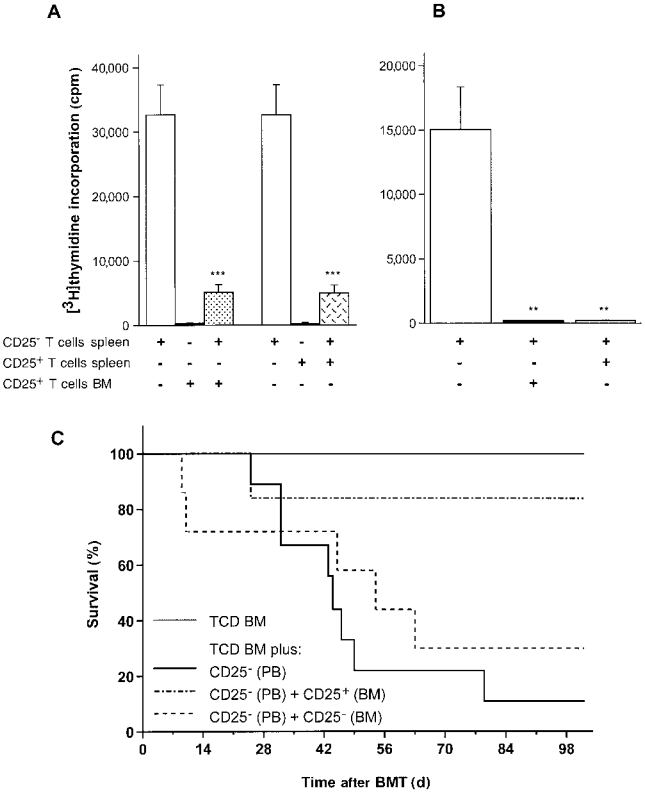

Figure 4. Suppressive effect of $NK1.1^-CD4^+CD25^+$ T cells from the BM of C57BL/6 mice. (A) Suppression of the proliferation of 2.5×10^4 C57BL/6 splenic $CD4^+CD25^-$ T cells, stimulated with soluble anti–CD3-Ab in the presence of autologous APC by 2.5×10^4 C57BL/6 $NK1.1^-CD4^+CD25^+$ T cells from BM, or $CD4^+CD25^+$ T cells from the spleen. The bars represent the means of triplicate values and the brackets indicate the SDs. ***, $P < 0.001$ (Student's *t* test). One of two experiments with similar results is shown. (B) Suppression of the alloresponse of C57BL/6 $CD4^+CD25^-$ T cells to BALB/c stimulator cells by C57BL/6 BM $CD4^+CD25^+$ T cells. Cultures were set up with 10^5 sorted splenic C57BL/6 $CD4^+CD25^-$ T cells and 10^5 irradiated BALB/c stimulator cells plus 10^5 C57BL/6 BM or splenic $CD4^+CD25^+$ T cells. The bars represent the means of triplicate values and the brackets indicate the SDs. **, $P < 0.01$ (Student's *t* test). One of two similar experiments is shown. (C) BALB/c recipients of C57BL/6 $CD4^+CD25^-$ PB T cells can be rescued from lethal aGVHD by the coinjection of C57BL/6 $NK1.1^-CD4^+CD25^+$, but not $NK1.1^-CD4^+CD25^-$, BM T cells. BALB/c mice received 800 cGy TBI, 2×10^6 C57BL/6 TCD BM cells, and 10^5 $CD4^+CD25^-$ PB T cells with or without an equal number of $CD4^+CD25^+NK1.1^-$ or $CD4^+CD25^-NK1.1^-$ BM T cells. Combined data from two independent experiments with six to nine mice per group are shown.

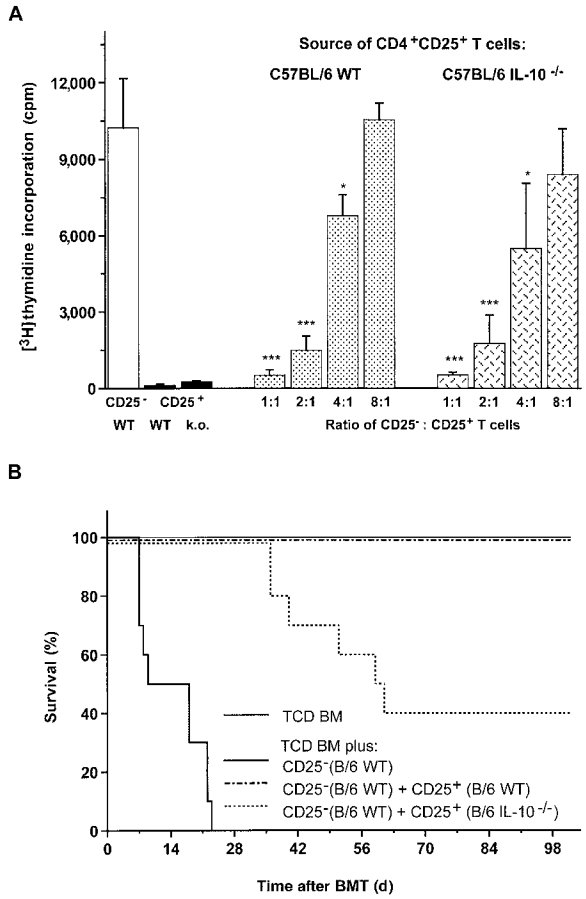

Figure 5. Functional comparison of $CD4^+CD25^+$ T cells from C57BL/6 WT and C57BL/6 IL-$10^{-/-}$ animals. (A) Dose-dependent suppression of the alloresponses of C57BL/6 WT $CD4^+CD25^-$ T cells to BALB/c stimulator cells by C57BL/6 WT or IL-$10^{-/-}$ $CD4^+CD25^+$ T cells. Cultures were set up with 10^5 $CD4^+CD25^-$ T cells, 10^5 BALB/c stimulator cells, and variable numbers of $CD4^+CD25^+$ T_{reg} cells to obtain the indicated ratios. The bars represent the means of triplicate values and the brackets indicate the SDs. ***, $P < 0.001$; *, $P < 0.05$ (Student's *t* test). One of two experiments with similar results is shown. (B) Protection of BALB/c hosts from lethal aGVHD by $CD4^+CD25^+$ T cells from C57BL/6 WT and IL-$10^{-/-}$ animals. BALB/c mice received 800 cGy TBI, 2×10^6 C57BL/6 TCD BM cells, and 4.5×10^5 C57BL/6 WT $CD4^+CD25^-$ T cells with or without 4.5×10^5 $CD4^+CD25^+$ T cells from either C57BL/6 WT- or IL-10–deficient mice. Combined data from two independent experiments with 10 animals per group are shown.

dicate that IL-10 production/secretion by T_{reg} cells is not involved in the direct suppression of alloreactivity in vitro.

We then explored the requirement for T_{reg} cell–derived IL-10 in the suppression of lethal GVHD by coinjecting splenic $CD4^+CD25^-$ T cells from C57BL/6 WT mice with splenic $CD4^+CD25^+$ T cells from IL-$10^{-/-}$ or WT animals into lethally irradiated BALB/c hosts. Mice receiving additional IL-10–deficient T_{reg} cells had a longer median survival time than those receiving WT $CD4^+CD25^-$ T cells alone (45 vs. 15 d, respectively; $P < 0.0001$), but 60% of the mice eventually died within 60 d after transplant. These latter mice never regained the baseline body weight for animals receiving WT T_{reg} cells as noted in Fig. 2 B, and continued to show clinical signs of GVHD, including diarrhea. In contrast, mice that received additional WT $CD4^+CD25^+$ T cells were completely protected from lethality (100% survival for >100 d) and returned to a normal appearance after mild and transient signs of aGVHD (Fig. 5 B). These results demonstrate that protection from lethal GVHD in vivo is partially dependent on IL-10 production by donor $CD4^+CD25^+$ T_{reg} cells.

Responder-type, but Not Stimulator-type, T_{reg} Cells Inhibit Alloreactive $CD25^-$ T Cells In Vitro in MLR. It has been shown that $CD4^+CD25^+$ T cells need to be activated via their T cell receptor to be functional in vitro, i.e., suppress the response of $CD4^+CD25^-$ or $CD8^+$ T cells to antigenic or polyclonal stimuli (22, 23, 28). However, the nature of antigens recognized by $CD4^+CD25^+$ T cells and the prerequisites necessary for their activation, especially in the response to alloantigens, are still only poorly understood. To address this question we cocultured C57BL/6 splenic $CD4^+CD25^-$ T cells and irradiated BALB/c stimulator cells in the presence of either C57BL/6 or BALB/c splenic $CD4^+CD25^+$ T cells at a 1:1 ratio. Whereas C57BL/6 T_{reg} cells suppressed the proliferation of the C57BL/6 $CD4^+CD25^-$ T cells by >95%, BALB/c-derived T_{reg} cells slightly enhanced the proliferation of the $CD4^+CD25^-$ T cells (Fig. 6 A). A lack of suppression was also obtained when C57BL/6 $CD4^+CD25^+$ T cells were cocultured with BALB/c $CD4^+CD25^-$ T cells as responder and C57BL/6 stimulator cells. Dieckmann et al. (41) obtained comparable results with human allogeneic $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells, if the stimulator cells and the $CD4^+CD25^+$ T cells originated from the same donor.

Donor-type, but Not Host-type, T_{reg} Cells Protect from Lethal GVHD. In light of the in vitro data described above, we determined whether T_{reg} cells had to be of donor origin or whether host-type T_{reg} cells were capable of preventing lethal GVHD. To answer this question, we isolated $CD4^+CD25^-$ T cells from the spleens of C57BL/6 mice and coinjected them with either C57BL/6 or BALB/c splenic $CD4^+CD25^+$ T cells into lethally irradiated BALB/c hosts. As expected, all animals receiving only C57BL/6 $CD4^+CD25^-$ T cells developed severe GVHD and died by day 23, whereas 100% of the animals receiving both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from C57BL/6 mice were protected from lethal GVHD and survived for >100 d ($P < 0.0001$; Fig. 6 B). In contrast, all recipients of

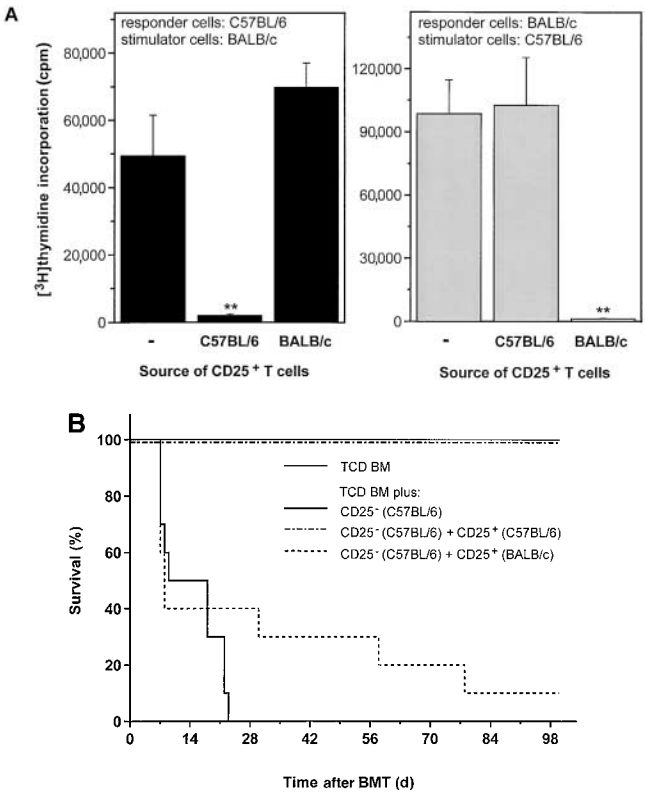

Figure 6. (A) Comparison of the suppressive effects of responder- and stimulator-type $CD4^+CD25^+$ T cells on the alloresponses of BALB/c or C57BL/6 $CD4^+CD25^-$ T cells in the MLR. Cultures were set up with 10^5 $CD4^+CD25^-$ responder T cells and equal numbers of each of the remaining cell populations. The bars represent the means of triplicate values and the brackets indicate the SDs. **, $P < 0.01$ (Student's *t* test). One of two experiments with similar results is shown. (B) Comparison of the protective effect of $CD4^+CD25^+$ T cells from C57BL/6 and BALB/c animals in lethal aGVHD of BALB/c hosts induced by C57BL/6 $CD4^+CD25^-$ T cells. BALB/c mice received 800 cGy TBI, 2×10^6 C57BL/6 TCD BM cells, and 4.5×10^5 C57BL/6 $CD4^+CD25^-$ T cells with or without 4.5×10^5 $CD4^+CD25^+$ T cells from either C57BL/6 or BALB/c animals. Combined data from two independent experiments with 10 animals per group are shown.

C57BL/6 $CD4^+CD25^-$ T cells and BALB/c $CD4^+CD25^+$ T cells developed severe signs of GVHD and almost all failed to regain their baseline body weight after the initial decline. They continued to show clinical signs of GVHD, including diarrhea, hunched back, and ruffled fur, and 90% of the animals died of the disease within 78 d (Fig. 6 B). Thus, survival of the latter group was not significantly different from the group given C57BL/6 $CD4^+CD25^-$ T cells alone ($P = 0.28$). This clearly shows that transferred host-type T_{reg} cells may delay the onset of lethal GVHD, but are ultimately unable to protect the recipients.

Discussion

Several recent studies have shown that human $CD4^+CD25^+$ T cells isolated from the PB, umbilical cord blood, thymus, or tonsils of healthy volunteers can suppress alloresponses of conventional $CD4^+$ and $CD8^+$ T

cells in vitro (16, 29, 42). In the current murine study, $CD4^+CD25^+$ T cells from the spleen of C57BL/6 mice showed significant suppression of the MLR of $CD4^+CD25^-$ T cells toward BALB/c stimulator cells, even at a ratio of 1:4 of $CD25^+/CD25^-$ cells. Other reports demonstrated that the induction of tolerance to alloantigen in vivo, either in neonatal mice by the injection of semiallogeneic spleen cells within 24 h after birth (43), or in adult mice by the treatment with anti-CD4 Ab followed by the injection of allogeneic blood (31), depends on the presence of $CD4^+CD25^+$ T cells. Furthermore, $CD4^+CD25^+$ T cells isolated from mice tolerized by either treatment displayed a strong, alloantigen-specific suppressive activity, as shown in vitro and by long-term acceptance of allografts in vivo. In both cases, however, $CD4^+CD25^+$ T cells isolated from unprimed animals showed no alloantigen-specific regulatory function either in vitro or in vivo. Taylor et al. (37) recently reported that the induction of tolerance to alloantigens ex vivo by the costimulatory blockade of either the CD40/CD40L or the CD28/CTLA-4/B7 pathway also depends on the presence of $CD4^+CD25^+$ T cells. Whole $CD4^+$ cells, but not $CD4^+CD25^-$ cells, cultured under blocking conditions for 9 d in vitro had a reduced capacity to induce lethal GVHD in a C57BL/6→B6.C-$H2^{bm12}$/KhEg transplantation model. However, freshly isolated $CD4^+CD25^+$ T cells, when injected with an equal number of naive, unseparated $CD4^+$ T cells, only marginally prolonged the survival time of the recipients compared with animals transplanted with only $CD4^+$ cells without any rescue from lethal GVHD. This is in contrast to our current findings in the murine C57BL/6→BALB/c transplantation model that shows significant protection from GVHD lethality by unmanipulated donor-type $CD4^+CD25^+$ T cells. We were able to rescue >90% of BALB/c recipients given a high dose of 4.5×10^5 sorted C57BL/6-derived $CD4^+CD25^-$ T cells from lethal GVHD by coinjecting the animals with an equal number of freshly isolated $CD4^+CD25^+$ T cells from the C57BL/6 donors. This is particularly striking, as we have shown that as few as 0.25×10^5 $CD4^+$ T cells, transplanted with 1.5×10^6 TCD BM cells, caused severe GVHD and the death of half of the animals within 70 d (39). There are, however, several differences between the experimental GVHD models applied by Taylor et al. (37) and by us, such as differences in the preparatory regimen (sublethal vs. lethal irradiation), in strain disparity (partial mismatch in MHC class II vs. complete MHC class I and II mismatch), and in the numbers and phenotype of the transplanted cells that could very well account for the different outcome of the experiments.

After allogeneic transplantation, aGVHD is characterized by donor leukocyte infiltrates in the gut and liver (39). Recruitment, activation, and expansion of mature donor T cells at these sites is regarded as one of the key processes during the initiation phase of GVHD that can lead to severe tissue damage, multiorgan failure, and death of the transplanted hosts (3). Using FACS® analysis and immunohistochemistry, we have demonstrated the presence of both $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ donor T cells (and/or their progeny) in GVHD target tissues (gut and liver) 5 d after cotransfer into lethally irradiated hosts (unpublished data). This indicates that $CD4^+CD25^+$ T cells do not prevent $CD4^+CD25^-$ T cells from entering GVHD target organs, but rather comigrate and then restrict the local expansion and/or tissue-damaging proinflammatory activity of the $CD4^+CD25^-$ T cells at these sites. This interpretation is supported by two studies showing that $CD4^+CD25^+$ T cells regulate the expansion of $CD4^+CD25^-$ T cells after cotransfer into lymphocyte-deficient mice (40) and, although proliferating to a certain degree after transfer, $CD4^+CD25^+$ T cells do so without loss of their suppressive function (44).

Because $CD4^+CD25^+$ T cells suppress GVHD, it is important to determine whether they also suppress the graft-versus-tumor activity of cotransplanted T cells that is desirable after clinical, allogeneic BMT. Accordingly, we recently performed a separate study in which C57BL/6 $CD4^+CD25^+$ T cells were used to suppress GVHD in BALB/c hosts bearing the BALB/c-derived B cell lymphoma, BCL_1 (unpublished data). All hosts given C57BL/6 TCD BM cells alone died from progressive tumor growth, whereas hosts given donor TCD BM cells with donor $CD4^+$ and $CD8^+$ T cells all died early from aGVHD. In contrast, all animals given the latter donor cells along with donor T_{reg} cells survived without tumor growth or GVHD. In this model, tumor eradication has been previously shown to be mediated by donor $CD8^+$ T cells via the perforin and FasL pathways (45).

We have previously shown that NK T cells, another T cell subpopulation with regulatory potential that is characterized by the coexpression of NK and T cell markers, can prevent lethal GVHD in the C57BL/6→BALB/c transplantation model (38). Although very rare among T cells in the periphery (2–5% of T cells in the spleen), these cells are highly enriched within the BM T cell population, where they represent 25–30% of the TCR$\alpha\beta^+$ cells (38). Although a high dose of BM T cells failed to induce GVHD in mice, we could induce GVHD after removal of the $NK1.1^+$ T cell subpopulation (38). However, the $NK1.1^-$ T cells isolated from PB were far more potent in inducing lethal GVHD compared with equal numbers from the BM. The results presented here, showing that up to 40% of the $NK1.1^-CD4^+$ T cells within the BM of C57BL/6 mice actually belong to the $CD25^+$ T_{reg} cell pool, as judged from their phenotype as well as their functional activity in vitro and in vivo, could explain the poor capacity of BM $CD4^+/CD8^+NK1.1^-$ T cells to induce GVHD and the even lesser ability of purified BM $CD4^+NK1.1^-$ T cells (38, 39). The absolute number, however, of $CD4^+CD25^+$ T cells in the spleen is still five- to eightfold higher than that in the BM based on the percentage among nucleated cells in these organs and the estimated total number of 3.2×10^8 nucleated cells in the BM of adult mice (46).

It has clearly been shown in the murine as well as the human system that the suppressive effect of $CD4^+CD25^+$ T cells on cocultured $CD4^+CD25^-$ T cells in vitro requires

cell contact and does not involve soluble mediators (22, 27–29, 36). In contrast, there are several studies documenting a role for IL-10 and TGF-β in the regulatory function of $CD4^+CD25^+$ T cells in vivo (31, 32, 40, 47). We did not determine in this study whether WT T_{reg} cells secreted IL-10 in vitro when stimulated by alloantigen. However, using $CD4^+CD25^+$ T cells from IL-$10^{-/-}$ mice, we were unable to detect any dependency of the suppressive effect of these cells on alloreactive $CD4^+CD25^-$ T cells in vitro, which is in agreement with previous reports (27, 29). When transferred into lethally irradiated hosts with WT $CD4^+CD25^-$ T cells, however, $CD4^+CD25^+$ T_{reg} cells from IL-$10^{-/-}$ mice conveyed a significantly reduced protection from GVHD compared with that found after the transfer of WT $CD4^+CD25^+$ T_{reg} cells. Although the MLR only measures the proliferative response of the target $CD4^+CD25^-$ T cells and its immediate suppression, the GVHD assay measures a complex series of downstream immune events that culminate in tissue injury. It is possible that $CD4^+CD25^+$ T_{reg} cells, in addition to their direct suppressive effect, transform the target T cells via IL-10 into T_{reg} cells that could then inhibit these downstream immune events. This form of "infectious tolerance" has been reported in several other systems (48, 49). Thus, the cell contact–dependent mechanism of suppression measured in vitro may just be the earliest of a cascade of events triggered by T_{reg} cells in vivo.

When we studied the suppression of proliferation after polyclonal T cell stimulation in vitro, we found full cooperation between $CD4^+CD25^-$ T cells from C57BL/6 and $CD4^+CD25^+$ T cells from BALB/c animals, and vice versa, resulting in the complete suppression of the proliferative response. However, when we cultured MHC mismatched $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells in the presence of APC from the same mouse strain as the $CD4^+CD25^+$ T cells, no suppression of the proliferative response occurred. Similarly, host-type $CD4^+CD25^+$ T cells were unable to protect recipients from lethal GVHD induced by allogeneic $CD4^+CD25^-$ T cells. All three findings are in accordance with the notion that T_{reg} cells have to be stimulated via their T cell receptor to be fully functional but once activated, their suppressive effect is antigen nonspecific, i.e., donor regulatory cells require activation via allorecognition of stimulator or host cells to suppress (22, 36, 50).

In conclusion, we have demonstrated that freshly isolated $CD4^+CD25^+$ T cells from unprimed animals can rescue recipients from lethal GVHD induced by $CD4^+CD25^-$ T cells after allogeneic transplantation. In addition, our findings indicate that protection from lethality is partially dependent on IL-10 produced by transplanted $CD4^+CD25^+$ T cells and that only donor-type $CD4^+CD25^+$ T cells are capable of preventing the death of the host animals.

This work was supported by National Institutes of Health grants R01 HL58520, R01 CA92225, R01 CA65237, and P01 HL57443. P. Hoffmann is the recipient of a fellowship from the Clinique La Prairie Research Foundation.

Submitted: 12 March 2002
Revised: 6 June 2002
Accepted: 10 June 2002

References

1. Klingebiel, T., and P.G. Schlegel. 1998. GVHD: overview on pathophysiology, incidence, clinical and biological features. *Bone Marrow Transplant.* 21:S45–S49.
2. Deeg, H.J., and R. Storb. 1984. Graft-versus-host disease: pathophysiological and clinical aspects. *Annu. Rev. Med.* 35: 11–24.
3. Snider, D., and H. Liang. 2001. Early intestinal Th1 inflammation and mucosal T cell recruitment during acute graft-versus-host reaction. *J. Immunol.* 166:5991–5999.
4. Ferrara, J.L., R. Levy, and N.J. Chao. 1999. Pathophysiologic mechanisms of acute graft-vs.-host disease. *Biol. Blood Marrow Transplant.* 5:347–356.
5. Hill, G.R., and J.L. Ferrara. 2000. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood.* 95:2754–2759.
6. Shlomchik, W.D., M.S. Couzens, C.B. Tang, J. McNiff, M.E. Robert, J. Liu, M.J. Shlomchik, and S.G. Emerson. 1999. Prevention of graft versus host disease by inactivation of host antigen-presenting cells. *Science.* 285:412–415.
7. Ho, V.T., and R.J. Soiffer. 2001. The history and future of T-cell depletion as graft-versus-host disease prophylaxis for allogeneic hematopoietic stem cell transplantation. *Blood.* 98: 3192–3204.
8. Sprent, J., M. Schaefer, D. Lo, and R. Korngold. 1986. Properties of purified T cell subsets. II. In vivo responses to class I vs. class II H-2 differences. *J. Exp. Med.* 163:998–1011.
9. Voltarelli, J.C., S. Corpuz, and P.J. Martin. 1990. In vitro comparison of two methods of T cell depletion associated with different rates of graft failure after allogeneic marrow transplantation. *Bone Marrow Transplant.* 6:419–423.
10. Burnett, A.K., I.M. Hann, A.G. Robertson, M. Alcorn, B. Gibson, I. McVicar, L. Niven, S. Mackinnon, H. Hambley, A. Morrison, et al. 1988. Prevention of graft-versus-host disease by ex vivo T cell depletion: reduction in graft failure with augmented total body irradiation. *Leukemia.* 2:300–303.
11. Hertenstein, B., W. Hampl, D. Bunjes, M. Wiesneth, C. Duncker, U. Koszinowski, H. Heimpel, R. Arnold, and T. Mertens. 1995. In vivo/ex vivo T cell depletion for GVHD prophylaxis influences onset and course of active cytomegalovirus infection and disease after BMT. *Bone Marrow Transplant.* 15:387–393.
12. Zeller, J.C., A. Panoskaltsis-Mortari, W.J. Murphy, F.W. Ruscetti, S. Narula, M.G. Roncarolo, and B.R. Blazar. 1999. Induction of $CD4^+$ T cell alloantigen-specific hyporesponsiveness by IL-10 and TGF-β. *J. Immunol.* 163:3684–3691.
13. Blazar, B.R., P.A. Taylor, R.J. Noelle, and D.A. Vallera. 1998. CD4(+) T cells tolerized ex vivo to host alloantigen by anti-CD40 ligand (CD40L:CD154) antibody lose their graft-versus-host disease lethality capacity but retain nominal antigen responses. *J. Clin. Invest.* 102:473–482.
14. Stephens, L.A., and D. Mason. 2000. CD25 is a marker for $CD4^+$ thymocytes that prevent autoimmune diabetes in rats, but peripheral T cells with this function are found in both $CD25^+$ and $CD25^-$ subpopulations. *J. Immunol.* 165:3105–

3110.
15. Itoh, M., T. Takahashi, N. Sakaguchi, Y. Kuniyasu, J. Shimizu, F. Otsuka, and S. Sakaguchi. 1999. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J. Immunol.* 162: 5317–5326.
16. Stephens, L.A., C. Mottet, D. Mason, and F. Powrie. 2001. Human CD4(+)CD25(+) thymocytes and peripheral T cells have immune suppressive activity in vitro. *Eur. J. Immunol.* 31:1247–1254.
17. Salomon, B., D.J. Lenschow, L. Rhee, N. Ashourian, B. Singh, A. Sharpe, and J.A. Bluestone. 2000. B7/CD28 costimulation is essential for the homeostasis of the $CD4^+CD25^+$ immunoregulatory T cells that control autoimmune diabetes. *Immunity*. 12:431–440.
18. Sakaguchi, S. 2000. Animal models of autoimmunity and their relevance to human diseases. *Curr. Opin. Immunol.* 12: 684–690.
19. Singh, B., S. Read, C. Asseman, V. Malmstrom, C. Mottet, L.A. Stephens, R. Stepankova, H. Tlaskalova, and F. Powrie. 2001. Control of intestinal inflammation by regulatory T cells. *Immunol. Rev.* 182:190–200.
20. Shevach, E.M., R.S. McHugh, C.A. Piccirillo, and A.M. Thornton. 2001. Control of T-cell activation by $CD4^+$ $CD25^+$ suppressor T cells. *Immunol. Rev.* 182:58–67.
21. Annacker, O., O. Burlen-Defranoux, R. Pimenta-Araujo, A. Cumano, and A. Bandeira. 2000. Regulatory CD4 T cells control the size of the peripheral activated/memory CD4 T cell compartment. *J. Immunol.* 164:3573–3580.
22. Thornton, A.M., and E.M. Shevach. 1998. $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188:287–296.
23. Piccirillo, C.A., and E.M. Shevach. 2001. Cutting edge: control of $CD8^+$ T cell activation by $CD4^+CD25^+$ immunoregulatory cells. *J. Immunol.* 167:1137–1140.
24. Ermann, J., V. Szanya, G.S. Ford, V. Paragas, C.G. Fathman, and K. Lejon. 2001. CD4(+)CD25(+) T cells facilitate the induction of T cell anergy. *J. Immunol.* 167:4271–4275.
25. Read, S., and F. Powrie. 2001. CD4(+) regulatory T cells. *Curr. Opin. Immunol.* 13:644–649.
26. Nakamura, K., A. Kitani, and W. Strober. 2001. Cell contact–dependent immunosuppression by $CD4^+CD25^+$ regulatory T cells is mediated by cell surface–bound transforming growth factor β. *J. Exp. Med.* 194:629–644.
27. Levings, M.K., R. Sangregorio, and M.G. Roncarolo. 2001. Human $CD25^+CD4^+$ T regulatory cells suppress naive and memory T cell proliferation and can be expanded in vitro without loss of function. *J. Exp. Med.* 193:1295–1302.
28. Takahashi, T., Y. Kuniyasu, M. Toda, N. Sakaguchi, M. Itoh, M. Iwata, J. Shimizu, and S. Sakaguchi. 1998. Immunologic self-tolerance maintained by $CD25^+CD4^+$ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. *Int. Immunol.* 10:1969–1980.
29. Ng, W.F., P.J. Duggan, F. Ponchel, G. Matarese, G. Lombardi, A.D. Edwards, J.D. Isaacs, and R.I. Lechler. 2001. Human CD4(+)CD25(+) cells: a naturally occurring population of regulatory T cells. *Blood.* 98:2736–2744.
30. Asseman, C., S. Mauze, M.W. Leach, R.L. Coffman, and F. Powrie. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J. Exp. Med.* 190:995–1004.
31. Kingsley, C.I., M. Karim, A.R. Bushell, and K.J. Wood. 2002. CD25(+)CD4(+) regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. *J. Immunol.* 168:1080–1086.
32. Suri-Payer, E., and H. Cantor. 2001. Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4(+)CD25(+) T cells. *J. Autoimmun.* 16:115–123.
33. Asano, M., M. Toda, N. Sakaguchi, and S. Sakaguchi. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J. Exp. Med.* 184:387–396.
34. Takahashi, T., T. Tagami, S. Yamazaki, T. Uede, J. Shimizu, N. Sakaguchi, T.W. Mak, and S. Sakaguchi. 2000. Immunologic self-tolerance maintained by $CD25^+CD4^+$ regulatory T cells constitutively expressing cytotoxic T lymphocyte–associated antigen 4. *J. Exp. Med.* 192:303–310.
35. Read, S., V. Malmstrom, and F. Powrie. 2000. Cytotoxic T lymphocyte–associated antigen 4 plays an essential role in the function of $CD25^+CD4^+$ regulatory cells that control intestinal inflammation. *J. Exp. Med.* 192:295–302.
36. Jonuleit, H., E. Schmitt, M. Stassen, A. Tuettenberg, J. Knop, and A.H. Enk. 2001. Identification and functional characterization of human $CD4^+CD25^+$ T cells with regulatory properties isolated from peripheral blood. *J. Exp. Med.* 193:1285–1294.
37. Taylor, P.A., R.J. Noelle, and B.R. Blazar. 2001. $CD4^+$ $CD25^+$ immune regulatory cells are required for induction of tolerance to alloantigen via costimulatory blockade. *J. Exp. Med.* 193:1311–1318.
38. Zeng, D., D. Lewis, S. Dejbakhsh-Jones, F. Lan, M. Garcia-Ojeda, R. Sibley, and S. Strober. 1999. Bone marrow $NK1.1^-$ and $NK1.1^+$ T cells reciprocally regulate acute graft versus host disease. *J. Exp. Med.* 189:1073–1081.
39. Zeng, D., P. Hoffmann, F. Lan, P. Huie, J. Higgins, and S. Strober. 2002. Unique patterns of surface receptors, cytokine secretion, and immune functions distinguish T cells in the bone marrow from those in the periphery: impact on allogeneic bone marrow transplantation. *Blood.* 99:1449–1457.
40. Annacker, O., R. Pimenta-Araujo, O. Burlen-Defranoux, T.C. Barbosa, A. Cumano, and A. Bandeira. 2001. $CD25^+$ $CD4^+$ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J. Immunol.* 166:3008–3018.
41. Dieckmann, D., H. Plottner, S. Berchtold, T. Berger, and G. Schuler. 2001. Ex vivo isolation and characterization of $CD4^+CD25^+$ T cells with regulatory properties from human blood. *J. Exp. Med.* 193:1303–1310.
42. Taams, L.S., J. Smith, M.H. Rustin, M. Salmon, L.W. Poulter, and A.N. Akbar. 2001. Human anergic/suppressive CD4(+)CD25(+) T cells: a highly differentiated and apoptosis-prone population. *Eur. J. Immunol.* 31:1122–1131.
43. Gao, Q., T.M. Rouse, K. Kazmerzak, and E.H. Field. 1999. $CD4^+CD25^+$ cells regulate CD8 cell anergy in neonatal tolerant mice. *Transplantation.* 68:1891–1897.
44. Gavin, M.A., S.R. Clarke, E. Negrou, A. Gallegos, and A. Rudensky. 2002. Homeostasis and anergy of $CD4^+CD25^+$ suppressor T cells in vivo. *Nat. Immunol.* 3:33–41.
45. Lan, F., D. Zeng, P. Huie, J.P. Higgins, and S. Strober. 2001. Allogeneic bone marrow cells that facilitate complete chimerism and eliminate tumor cells express both CD8 and T-cell antigen receptor-αβ. *Blood.* 97:3458–3465.
46. Opstelten, D., and D.G. Osmond. 1983. Pre-B cells in

mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation of cytoplasmic mu-chain-bearing cells in normal mice. *J. Immunol.* 131:2635–2640.
47. Cottrez, F., S.D. Hurst, R.L. Coffman, and H. Groux. 2000. T regulatory cells 1 inhibit a Th2-specific response in vivo. *J. Immunol.* 165:4848–4853.
48. Groux, H., A. O'Garra, M. Bigler, M. Rouleau, S. Antonenko, J.E. de Vries, and M.G. Roncarolo. 1997. A $CD4^+$ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis. *Nature.* 389:737–742.
49. Cobbold, S., and H. Waldmann. 1998. Infectious tolerance. *Curr. Opin. Immunol.* 10:518–524.
50. Thornton, A.M., and E.M. Shevach. 2000. Suppressor effector function of $CD4^+CD25^+$ immunoregulatory T cells is antigen nonspecific. *J. Immunol.* 164:183–190.