Granzyme B is not required for regulatory T cell–mediated suppression of graft-versus-host disease

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Regulatory T (T_{reg}) cells can suppress a wide variety of immune responses, including antitumor and alloimmune responses. The mechanisms by which T_{reg} cells mediate their suppressive effects depend on the context of their activation. We previously reported that granzyme B is important for T_{reg} cell–mediated suppression of antitumor immune responses. We therefore hypothesized that granzyme B may likewise be important for suppression of graft-versus-host disease (GVHD). We found that allogeneic mismatch induces the expression of granzyme B in mixed lymphocyte reactions and in a model of graft-versus-host disease (GVHD). However, wild-type and granzyme B–deficient T_{reg} cells were equally able to suppress effector T (T_{eff}) cell proliferation driven by multiple stimuli, including allogeneic antigen-presenting cells. Surprisingly, adoptive transfer of granzyme B–deficient T_{reg} cells prevented GVHD lethality, suppressed serum cytokine production in vivo, and prevented target organ damage. These data contrast strikingly with our previous study, which demonstrated that granzyme B plays a nonredundant role in T_{reg} cell–mediated suppression of antitumor responses. Taken together, these findings suggest that targeting specific T_{reg} cell–suppressive mechanisms, such as granzyme B, may be therapeutically beneficial for segregating GVHD and graft-versus-tumor immune responses. (Blood. 2010;115: 1669-1677)

Introduction

$CD4^+Foxp3^+$ regulatory T (T_{reg}) cells play an indispensable role in maintaining peripheral tolerance to self-antigens by suppressing effector immune responses. Mice or humans with a deficiency of T_{reg} cells, induced by antibody-mediated[1,2] or toxin-mediated[3,4] depletion or by mutations[5] and deletions[6,7] of the lineage specification factor Foxp3, manifest severe autoimmune disease. In addition to preventing autoimmunity, T_{reg} cells can also suppress immune responses generated against tumor cells,[8,9] alloantigens,[10] allergens,[11-13] and microbial antigens.[14,15]

Several mechanisms have been proposed to explain how T_{reg} cell–mediated suppression of effector immune responses occurs. In certain model systems, T_{reg}-cell secretion of anti-inflammatory cytokines, such as transforming growth factor-β and interleukin-10 (IL-10), has been shown to be required for suppressive function.[16-18] In other experimental settings, contact-dependent mechanisms, such as interactions between CTLA-4 on T_{reg} cells and CD80/CD86 on antigen-presenting cells (APCs), have also been reported.[19-21] Because of the variety of animal models, in vitro activation methods, and readouts for suppression, rigorously defining nonredundant T_{reg}-suppressive mechanisms has been challenging and controversial. It is probable that T_{reg} cells use multiple mechanisms depending on the context in which they are activated in vivo.[22]

Our group previously demonstrated that human regulatory T cells can use the perforin/granzyme pathway to suppress effector T (T_{eff})–cell proliferation and kill autologous immune cells.[23,24] These findings were subsequently extended to a murine tumor challenge model, where we showed that adoptively transferred granzyme B– and perforin-deficient T_{reg} cells were defective in their ability to inhibit antitumor responses.[25] In that study, we reported that *Gzmb*$^{-/-}$ mice in the 129/SvJ background have markedly improved survival (compared with strain-matched wild-type [WT], *Prf1*$^{-/-}$, and other *Gzm*$^{-/-}$ mice) after intravenous challenge with a variety of tumor cell lines.[25] RMAS lymphoma and B16 melanoma cells, both derived from C57Bl/6 mice, are mismatched with 129/SvJ mice across minor histocompatibility barriers; MB0 cells, an acute myeloid leukemia cell line generated via retroviral transduction of bone marrow cells, are syngeneic to 129/SvJ mice. These findings suggested an immunoregulatory role for granzyme B in tumor clearance, and we hypothesized that granzyme B plays a nonredundant role in T_{reg} cell–mediated suppression of the antitumor immune response. Flow-cytometric studies confirmed that granzyme B was expressed in T_{reg} cells harvested from the tumor microenvironment. Further, using bioluminescence imaging, we demonstrated that adoptive transfer of T_{reg} cells into *Gzmb*$^{-/-}$ RMAS-tumor-bearing hosts restored tumor burden in a granzyme B– and perforin-dependent manner. Taken together, we attributed the improved survival of *Gzmb*$^{-/-}$ mice after tumor challenge to defective T_{reg}-cell function in these hosts, and we concluded that granzyme B is important for T_{reg} cell–mediated suppression of antitumor responses.

In this study, we examined the role of granzyme B in T_{reg}-cell function within the context of another mouse model of alloimmunity, graft-versus-host disease (GVHD). We initially hypothesized that granzyme B would be important for the suppression of GVHD.

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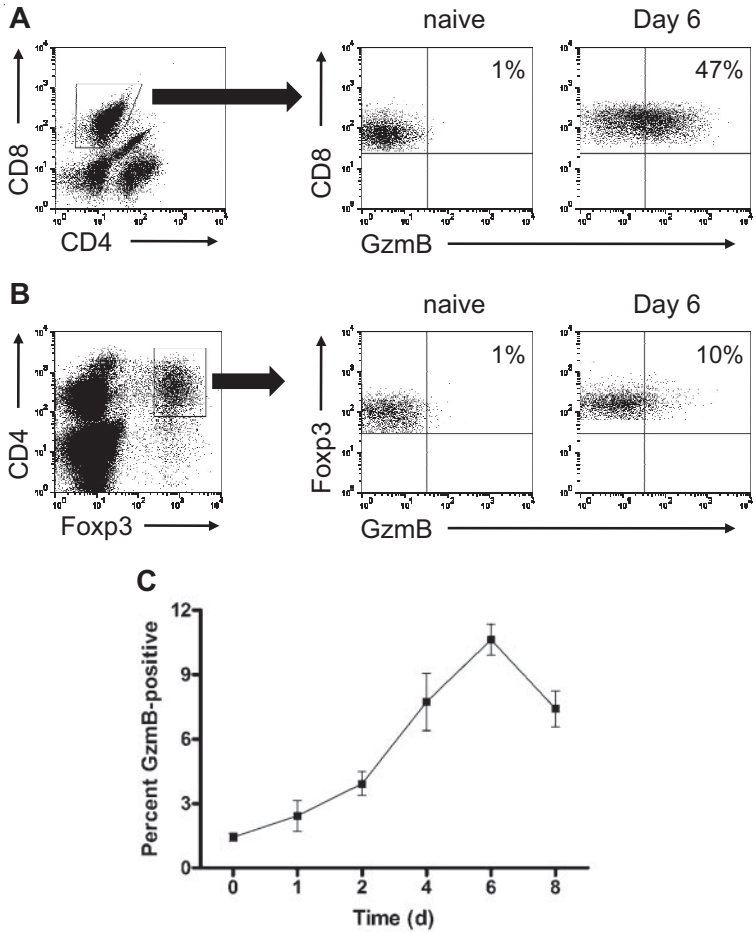

Figure 1. Alloactivated T_{reg} cells express granzyme B during in vitro MLRs. The 129/SvJ WT (H-2K^b) splenocytes were cultured with irradiated (2000 cGy) Balb/c WT (H-2K^d) splenocytes in complete medium supplemented with 50 U/mL IL-2. At various time points, splenocytes were harvested and analyzed by flow cytometry for granzyme B expression. (A) Representative flow plots of granzyme B expression, gated on naive and day 6 MLR-stimulated CD8$^+$ T cells. (B) Representative flow plots of granzyme B expression, gated on naive and day 6 MLR-stimulated CD4$^+$Foxp3$^+$ T_{reg} cells. (C) Summary graph of percentage granzyme B–expressing T_{reg} cells (n = 3 independent MLR cultures per time point).

However, using several readouts of suppressive function in vitro and in vivo, we unexpectedly found that granzyme B was not required for suppression of GVHD, even though T_{reg} cells up-regulate granzyme B in this model. Taken together with previously reported findings from our tumor challenge studies (as well as other allograft models), these data suggest that the use of granzyme B as a T_{reg}-suppressive mechanism is context-dependent and could potentially be exploited to segregate GVHD and graft-versus-tumor effects.

Methods

Mice

WT 129/SvJ (H-2^b) and Balb/c mice (H-2^d) were obtained from The Jackson Laboratory. Foxp3-ires-GFP (FIG) reporter mice have been previously described.[26] Targeted FIG 129/SvJ ES clones were a generous gift from Talal Chatila (University of California–Los Angeles). FIG mice were rederived in the 129/SvJ background and were bred with Gzmb$^{-/-}$ mice to generate granzyme B–deficient FIG mice. All mice were maintained in specific pathogen–free housing, and all experiments were conducted in accordance with institutional animal care and use guidelines under appropriate protocols approved by the Washington University School of Medicine.

Antibodies and reagents

Antibodies used include CD4 (RM4-5), CD8 (53-6.7), CD25 (7D4), CD16/32 (2.4G2; BD Biosciences), Foxp3 (FJK-16s; eBioscience), H-2K^b (CTKb), and granzyme B (GB12; Invitrogen). Serum cytokines were analyzed using the Bio-Plex Pro T_h1/T_h2 Mouse Panel Kit, according to the manufacturer's instructions (Bio-Rad). Samples were analyzed on a Bio-Plex 200 Workstation. CD3/CD28 Dynabeads were obtained from Invitrogen. Endotoxin-free recombinant human IL-2 was obtained from Chiron and was stored at −80°C after reconstitution.

Cell isolation and stimulation

All cells were cultured in K10 media (RPMI 1640, 10% fetal calf serum, 10mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, 1% nonessential amino acid, 1% sodium pyruvate, 1% L-glutamine, 1× penicillin/streptomycin, 0.57μM β-mercaptoethanol). One-way mixed lymphocyte reactions (MLRs) were prepared by culturing 4×10^6 129/SvJ responder splenocytes with 4×10^6 irradiated (2000 cGy) Balb/c stimulator cells in 6-well plates with 5 mL of media supplemented with 50 U/mL IL-2. At indicated time points, cells were harvested for analysis. CD4$^+$ and CD8$^+$ splenic T cells were purified using the Pan T cell isolation kit, followed by cell separation on the AutoMACS, according to the manufacturer's instructions (Miltenyi Biotec). CD4$^+$CD25$^-$ T_{eff} cells were negatively selected using the Pan T cell isolation kit supplemented with biotinylated anti-CD8 and anti-CD25 antibodies. Bone marrow cells and splenic APCs were depleted of T cells using CD90.2 Microbeads. T_{reg} cells were purified using the CD4$^+$CD25$^+$ regulatory T-cell isolation kit. For purification of CD4$^+$Foxp3$^+$ T_{reg} cells, splenocytes from FIG mice were surface stained with anti-CD4 and CD4$^+$GFP$^+$ cells were isolated on a Reflection (iCyt) cell sorter (routinely ≥ 95% pure).

Suppression assays

Concanavalin A (ConA)–based T_{reg} suppression assays were performed as described.[6] Briefly, 2×10^4 CD4$^+$CD25$^-$ T_{eff} cells were stimulated for 72 hours with 2 μg/mL ConA in the presence of 8×10^4 T cell–depleted,

irradiated (2000 cGy) APCs and a 1:2 titration of WT or Gzmb$^{-/-}$ T_{reg} cells in 96-well round-bottomed plates. Cocultures were pulsed with 1 μCi per well of [^{3}H]thymidine for the final 8 to 16 hours. Allogeneic T_{reg} suppression assays were performed as described.[27] Briefly, 10^5 CD4$^+$CD25$^-$ T_{eff} cells were mixed with a 1:2 titration of WT or Gzmb$^{-/-}$ T_{reg} cells from 129/SvJ mice and stimulated for 5 days with 10^5 irradiated (2000 cGy) Balb/c APCs in 96-well round-bottomed plates. Cocultures were pulsed with 1 μCi per well of [^{3}H]thymidine for the final 8 to 16 hours. All data shown are mean [^{3}H]thymidine incorporation in triplicate cultures. For ex vivo suppression assays, purified CD4$^+$CD25$^-$ T_{eff} cells were washed with phosphate-buffered saline, resuspended at 10^6 cells/mL, and then labeled at 37°C for 15 minutes with 300nM CellTrace Far Red DDAO-SE (Invitrogen). Labeling reactions were stopped with RPMI 1640 media containing 10% fetal bovine serum. A total of 4×10^4 labeled T_{eff} cells were cultured in the presence or absence of 4×10^4 CD3/CD28 Dynabeads and indicated numbers of fluorescence-activated cell sorter (FACS)–purified WT or Gzmb$^{-/-}$ CD4$^+$GFP$^+$ GVHD-activated T_{reg} cells. After 3 days of culture in 96-well round-bottomed plates, cells were harvested for flow cytometric analysis.

Intracellular staining and flow cytometry

A total of 10^6 cells were washed and resuspended in staining buffer (phosphate-buffered saline, 0.5% bovine serum albumin, 0.5mM ethylenediaminetetraacetic acid). Samples were labeled with primary-conjugated antibodies against cell-surface markers, fixed and permeabilized (Foxp3 staining kit; eBioscience), and stained with primary-conjugated anti–granzyme B antibody and anti-Foxp3 antibody. Sample data were acquired on a Cytek-modified FACScan (BD Biosciences) flow cytometer and analyzed with FlowJo (TreeStar) software.

GVHD model

For flow cytometric analyses and cell sorting of GVHD-activated T_{reg} cells, Balb/c hosts were given total body irradiation (900 Gy) and injected with donor cells via the tail vein within 24 hours. All mice received 2×10^6 bone marrow cells from 129/SvJ WT mice and 2×10^6 T cells from 129/SvJ WT mice, WT FIG mice, or Gzmb$^{-/-}$ FIG mice. Splenocytes were harvested at indicated time points and stained for flow cytometric analysis or cell sorting. For GVHD survival studies and serum cytokine analyses, Balb/c hosts were given total body irradiation and injected intravenously with donor cells within 24 hours. Mice received 2×10^6 T cell–depleted bone marrow cells only, bone marrow cells with 4×10^5 CD25-depleted CD4$^+$ and CD8$^+$ T_{eff} cells, or bone marrow and T_{eff} cells with either WT or Gzmb$^{-/-}$ T_{reg} cells at the indicated ratios. Survival and appearance of mice were monitored daily. Mice were bled for serum cytokine analysis 7 days after transplantation. Some mice were killed 7 days after transplantation for GVHD histopathology analysis.

GVHD histopathology

Seven days after transplantation, tissues (lung, liver, and gut) were harvested, fixed immediately in 10% buffered formalin, and then embedded in paraffin. Sections were cut and stained with hematoxylin and eosin for histologic evaluation of GVHD. As previously reported, a semiquantitative scoring system was used to assess abnormalities associated with GVHD or allograft rejection.[28] The scoring system designated 0 as normal, 0.5 as focal and rare, 1.0 as focal and mild, 2.0 as diffuse and mild, 3.0 as diffuse and moderate, and 4.0 as diffuse and severe. Scores were added to provide a total score for each specimen.

Results

Granzyme B is expressed in alloactivated T_{reg} cells in vitro and in vivo

To determine whether T_{reg}-cell expression of granzyme B is induced by allogeneic mismatch, we established 1-way MLRs and used flow-cytometric analysis to monitor the kinetics of granzyme B expression in alloactivated T_{reg} cells in vitro. The 129/SvJ bulk splenocytes were cultured with irradiated Balb/c stimulator splenocytes in 6-well plates, and cells were harvested for analysis over an 8-day time course. Under these conditions, there was robust induction of granzyme B in effector CD8$^+$ T cells on day 6, shown in Figure 1A, which is consistent with our previously reported observations.[29] The proportion of granzyme B–expressing CD4$^+$Foxp3$^+$ T_{reg} cells increased during alloactivation, peaking on day 6 at approximately 10%, and began to decline on day 8 (Figure 1B-C). This proportion is low relative to the percentage of granzyme B$^+$ CD8$^+$ T cells; however, it demonstrates that signals generated during allogeneic mismatch can result in T_{reg}-cell expression of granzyme B independently of tumor-derived signals.

Next, we used a fully mismatched GVHD mouse model to determine whether T_{reg} cells acquire granzyme B during alloactivation in vivo. In this model, Balb/c mice are lethally irradiated on day −1 and reconstituted with 2×10^6 bone marrow cells and 2×10^6 T cells from 129/SvJ WT mice on day 0. On days 3 to 6

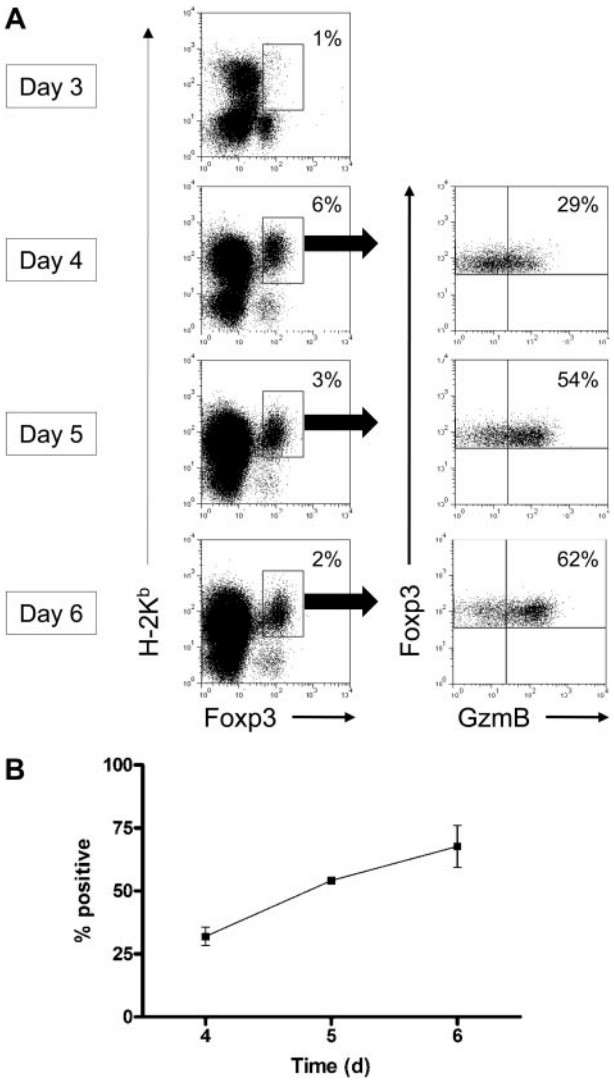

Figure 2. Alloactivated T_{reg} cells express granzyme B in a mouse model of GVHD in vivo. Lethally irradiated Balb/c mice were reconstituted with 2×10^6 bone marrow cells and 2×10^6 T cells derived from 129/SvJ WT mice. Splenocytes were harvested from recipient mice on days 3 to 6 for flow cytometric analysis of granzyme B expression in donor-derived T_{reg} cells. (A) Representative flow plots, gated on H-2K^{b+}Foxp3$^+$ T_{reg} cells, are shown. (B) Summary graph of percentage granzyme B–expressing T_{reg} cells is shown (n = 3 mice per time point).

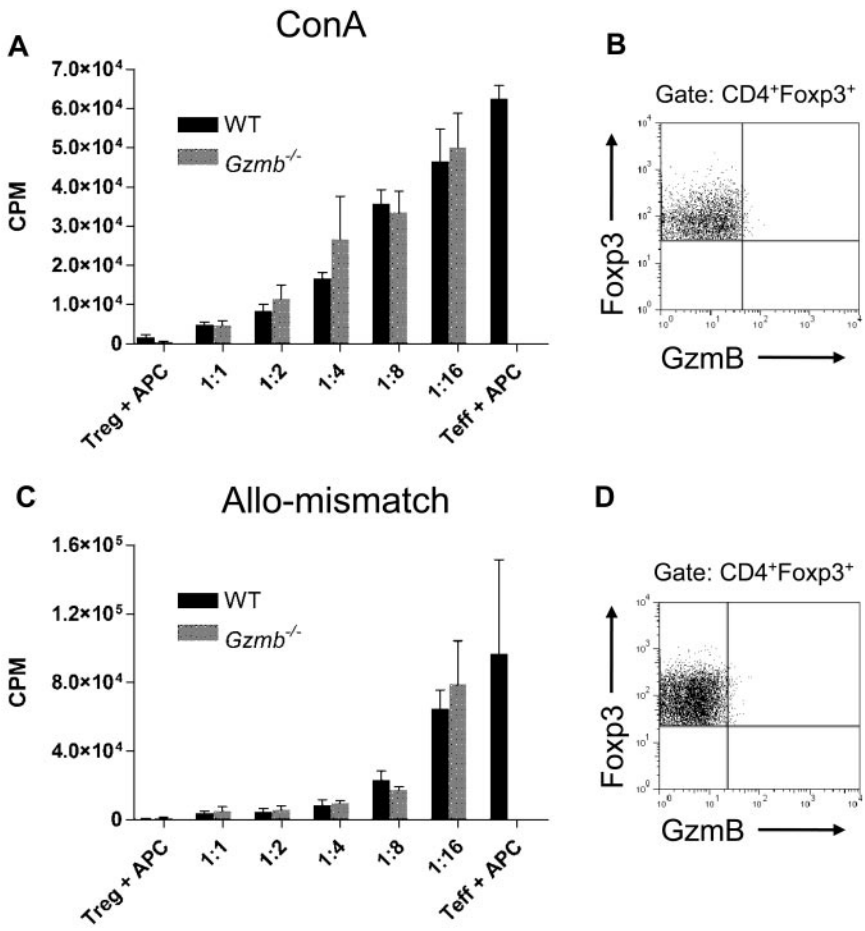

Figure 3. Granzyme B is not required for T_{reg} cell–mediated suppression of ConA-activated and alloactivated T_{eff}-cell proliferation in vitro. (A) Suppression of $CD4^+CD25^-$ T_{eff}-cell proliferation stimulated by ConA in the presence of syngeneic T cell–depleted, irradiated APCs. (B) Representative flow plot of granzyme B expression, gated on wild-type T_{reg} cells cultured for 3 days with ConA-activated T_{eff} cells under maximal suppression conditions (1:1). (C) Dose-dependent suppression of 129/SvJ $CD4^+CD25^-$ T_{eff}-cell proliferation stimulated by fully mismatched Balb/c T cell–depleted APCs. (D) Representative flow plot of granzyme B expression, gated on wild-type T_{reg} cells cultured for 5 days with alloactivated T_{eff} cells under maximal suppression conditions (1:1). All data shown are representative of 3 independent experiments.

after transplantation, mice were killed and splenocytes were analyzed for granzyme B expression within the donor T_{reg}-cell ($H\text{-}2K^{b+}Foxp3^+$) compartment. On day 3 after transplantation, there were very few donor-derived T_{reg} cells in the spleen (Figure 2). By day 4, however, there was a substantial migration and/or expansion of donor-derived splenic T_{reg} cells, and granzyme B protein was detected in approximately 30% of the gated cells. That proportion continued to rise throughout the 6 days of in vivo alloactivation, and by day 6, more than 60% of donor-derived T_{reg} cells were granzyme B–positive. Therefore, T_{reg} cells express granzyme B protein during in vitro and in vivo alloactivation across major histocompatibility barriers.

Granzyme B is not required for T_{reg}-cell function in classic suppression assays

We performed in vitro T_{reg} suppression assays to interrogate the role of granzyme B in T_{reg} cell–mediated inhibition of $CD4^+CD25^-$ T_{eff}-cell proliferation. First, we used the lymphocyte mitogen ConA as a polyclonal stimulator of T-cell proliferation. Consistent with previously reported findings, ConA-stimulated WT T_{reg} cells cultured with APCs are hypoproliferative and can suppress the proliferation of T_{eff} cells in a dose-dependent manner (Figure 3A).[6] *Gzmb*$^{-/-}$ T_{reg} cells were also able to suppress ConA-driven T_{eff}-cell proliferation.

Because we observed T_{reg} cell expression of granzyme B in MLRs, we used allogeneic mismatch to drive T_{eff}-cell proliferation in these suppression assays. T_{eff} cells were cultured with irradiated Balb/c stimulator APCs and were assayed for thymidine incorporation after culturing for 5 days. In concordance with reported observations, WT T_{reg} cells also suppressed T_{eff}-cell proliferation in a dose-dependent manner, and *Gzmb*$^{-/-}$ T_{reg} cells were equally capable of inhibiting this allogeneic effector function (Figure 3C).[27,30]

To determine whether granzyme B was expressed under experimental conditions where T_{reg} cells are actively suppressing ConA- or allo-mismatch–driven T_{eff}-cell proliferation, we examined these T_{reg} cells for granzyme B expression. At a 1:1 T_{reg}/T_{eff} cell ratio, where proliferation is maximally suppressed after the 3 (ConA) or 5 (allo-mismatch) days of culture, cells were harvested and analyzed by flow cytometry. In both assays, granzyme B was not detected in the gated $CD4^+Foxp3^+$ T_{reg}-cell population (Figure 3B,D). In particular, for the allo-mismatched T_{reg} suppression assay, the absence of granzyme B–expressing T_{reg} cells contrasts with our MLR expression studies, where we found a small proportion of granzyme B–positive T_{reg} cells (Figure 1C). This is probably the result of differences in cell populations in culture. Whereas the MLRs contained unfractionated splenocytes in both the responder and stimulator populations, the allogeneic T_{reg} suppression assay cocultures contained only purified responder T_{reg}- and T_{eff}-cell populations, along with mismatched T cell–depleted stimulator APCs. The absence of accessory cells in the T_{reg} suppression assay cocultures, which could produce soluble factors and/or cell contact-mediated signals, may potentially account for the differential expression of granzyme B under the 2 allogeneic culture conditions. Together, these data demonstrate that granzyme B is not required for T_{reg} cell–mediated suppression of T_{eff}-cell proliferation in vitro.

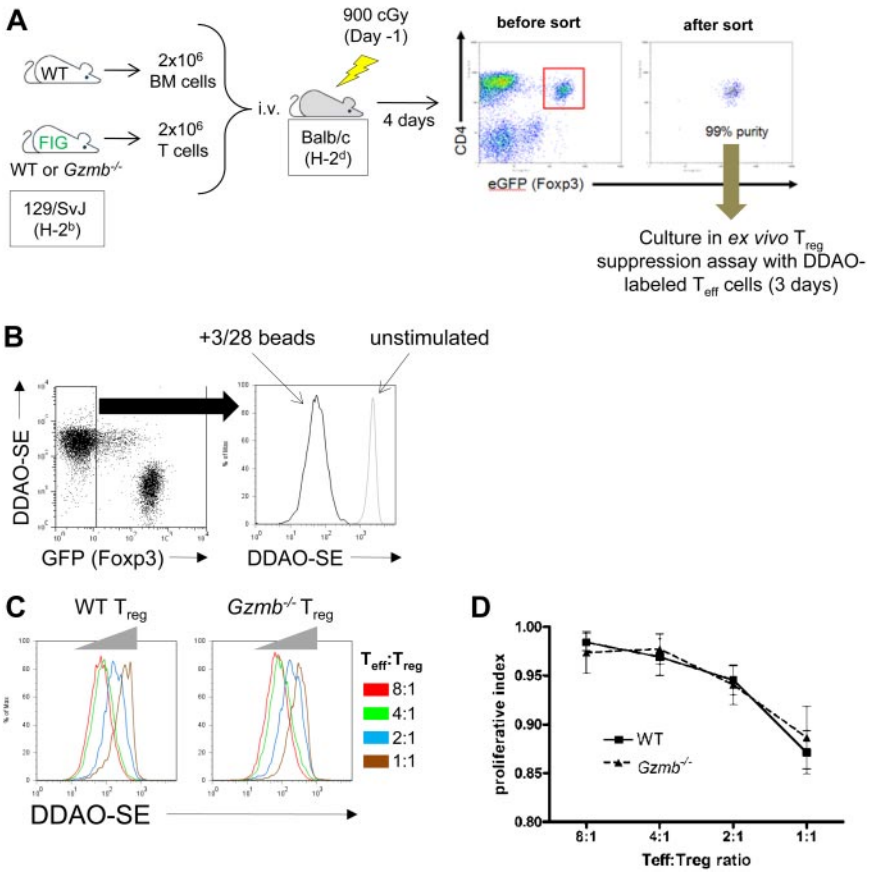

Figure 4. In vivo alloactivated T_{reg} cells do not require granzyme B to suppress T_{eff}-cell proliferation ex vivo. (A) Experimental protocol for generation of WT and *Gzmb*$^{-/-}$ GVHD-activated T_{reg} cells: 2×10^6 T cells from 129/SvJ WT or *Gzmb*$^{-/-}$ FIG reporter mice were injected intravenously in lethally irradiated (900 cGy) Balb/c hosts, together with 2×10^6 bone marrow cells from WT (non-FIG) mice. Four days after transplantation, splenic WT or *Gzmb*$^{-/-}$ CD4$^+$GFP$^+$ T_{reg} cells were sort-purified and cultured ex vivo with DDAO-SE–stained CD4$^+$CD25$^-$ T_{eff} cells for 3 days. T cells were either unstimulated or stimulated with CD3/CD28 beads. (B) Representative flow plot and histogram of T_{eff}-cell proliferation (ie, loss of DDAO-SE staining) in the presence or absence of CD3/CD28 beads. (C) Dose-dependent inhibition of T_{eff}-cell proliferation mediated by wild-type or *Gzmb*$^{-/-}$ GVHD-activated T_{reg} cells. (D) Summary graph of normalized data from 3 independent experiments.

In vivo–activated T_{reg} cells purified from mice with GVHD suppress T_{eff}-cell proliferation ex vivo

Because no granzyme B was detectable in functional T_{reg} cells from classic suppression assay cocultures, it remained unclear whether alloactivated T_{reg} cells require granzyme B for its function under the particular conditions when granzyme B is expressed. To address this experimentally, we rederived Foxp3 reporter mice in the 129/SvJ background from targeted embryonic stem cell clones that were engineered by Haribhai et al where a bicistronic FIG construct was targeted to the endogenous Foxp3 locus.[26] Consistent with their published findings, GFP fluorescence accurately reported Foxp3 expression (supplemental Figure 1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). FIG mice were bred with strain-matched *Gzmb*$^{-/-}$ mice to homozygosity. Analysis of these strains confirmed that T_{reg}-cell numbers were not affected by granzyme B deficiency (supplemental Figure 2). These WT and *Gzmb*$^{-/-}$ FIG mice would allow for T_{reg} cells to be alloactivated in vivo in mismatched hosts and subsequently FACS-purified for assays directed at interrogating the requirement for granzyme B in T_{reg}-suppressive function.

In Figure 2, we showed that more than 25% of donor-derived T_{reg} cells express granzyme B 4 days after transplantation in a mouse model of GVHD. We used this same model but instead transferred 2×10^6 WT or *Gzmb*$^{-/-}$ FIG T cells into lethally irradiated Balb/c hosts, together with 2×10^6 WT bone marrow cells (Figure 4A). On day 4 after transplantation, Balb/c hosts were killed and WT or *Gzmb*$^{-/-}$ CD4$^+$GFP$^+$ donor-derived FIG T_{reg} cells were FACS-purified. Notably, there was no difference in spleen cellularity or in the proportion of donor-derived WT or *Gzmb*$^{-/-}$ CD4$^+$Foxp3$^+$ T_{reg} cells, suggesting that *Gzmb*$^{-/-}$ T_{reg} cells have no intrinsic defect in survival or proliferative capacity compared with WT Treg cells (data not shown). These alloactivated T_{reg} cells were then cocultured with DDAO-SE–stained CD4$^+$CD25$^-$ T_{eff} cells and stimulated ex vivo with anti-CD3/CD28 beads. After 72 hours, T_{eff}-cell proliferation was analyzed by flow cytometric analysis of DDAO-SE fluorescence gated on GFP$^-$ effector cells. T_{eff} cells cultured in the absence of anti-CD3/CD28 beads retained DDAO-SE staining, whereas T_{eff} cells stimulated with the beads lost DDAO-SE fluorescence by orders of magnitude (Figure 4B). Coculturing T_{eff} cells with a 1:2 serial titration of WT GVHD-activated T_{reg} cells resulted in the dose-dependent suppression of T_{eff}-cell proliferation (Figure 4C). Similarly, a 1:2 titration of *Gzmb*$^{-/-}$ GVHD-activated T_{reg} cells inhibited T_{eff}-cell proliferation. These data were summarized by deriving a proliferative index, calculated by normalizing the loss of DDAO-SE fluorescence in T_{reg}/T_{eff} cell cocultures to the maximal loss of fluorescence observed in T_{eff} cells stimulated by anti-CD3/CD28 beads in the absence of T_{reg} cells (Figure 4D). These data demonstrate that granzyme B is not required for T_{reg} cell–mediated suppression of T_{eff}-cell proliferation, even under conditions where T_{reg} cells acquire granzyme B protein via endogenous signals generated during alloactivation in vivo.

Adoptive transfer of Gzmb$^{-/-}$ T_{reg} cells rescues reconstituted hosts from lethal GVHD equivalently to recipients of WT T_{reg} cells

In addition to inhibiting T_{eff}-cell proliferation, T_{reg} cells have been reported to suppress a variety of effector functions, resulting in diverse physiologic outcomes depending on the model being studied. The ability of donor-type WT T_{reg} cells to protect hosts

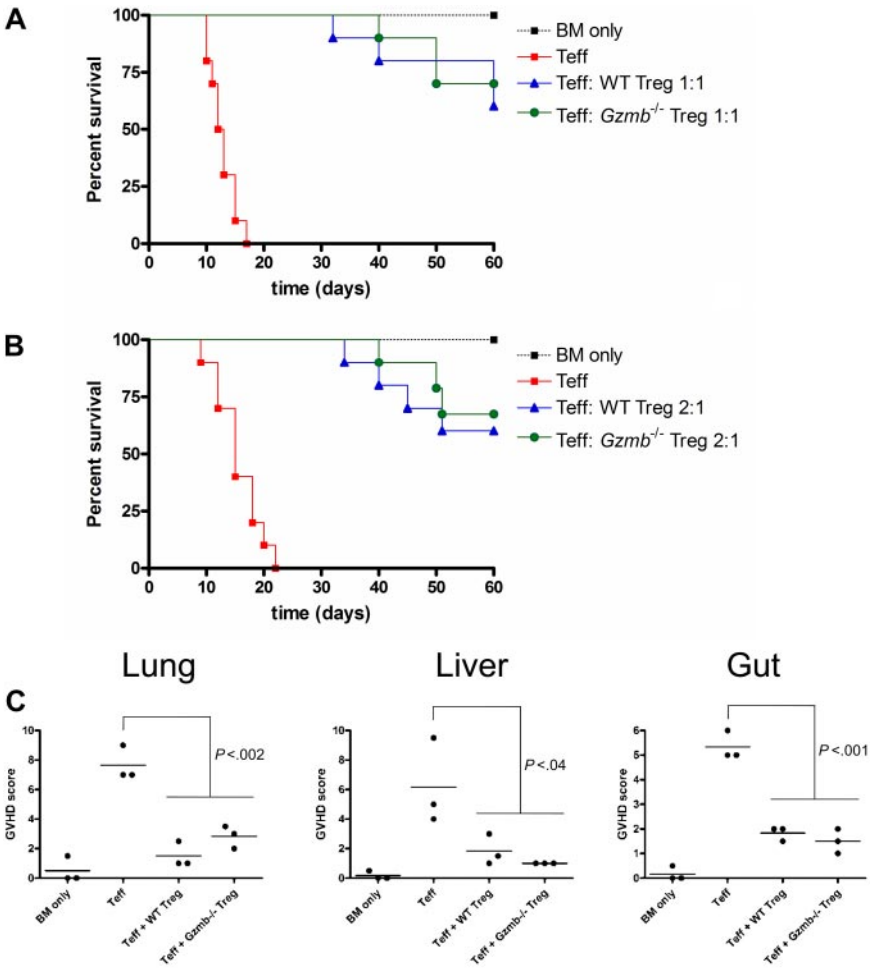

Figure 5. T_{reg} cells do not require granzyme B to rescue hosts from GVHD lethality or to prevent GVHD target organ damage. Lethally irradiated (900 cGy) Balb/c mice received 2×10^6 129/SvJ TCD BM cells with or without 4×10^5 129/SvJ CD25$^-$ T_{eff} cells (both CD4$^+$ and CD8$^+$) and either (A) 4×10^5 or (B) 2×10^5 wild-type or *Gzmb*$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} cells. Kaplan-Meier survival curves of recipient mice, pooled from 2 independent experiments (n = 10 mice per group), are shown. (C) Seven days after transplantation, 3 mice per experimental group (as outlined in panel A) were killed, and portions of lung, liver, and gut were prepared for histopathologic analysis. There was no statistically significant difference between groups receiving WT or *Gzmb*$^{-/-}$ T_{reg} cells.

from acute GVHD lethality has been demonstrated by multiple groups.[27,30-32] To determine whether there is a granzyme B–dependent component to this survival phenotype, we adopted an acute GVHD mouse model in which lethally irradiated Balb/c mice were reconstituted with T cell–depleted bone marrow cells only, bone marrow cells with CD25-depleted CD8$^+$ and CD4$^+$ T_{eff} cells, or bone marrow cells transferred with T_{eff} cells and either WT or *GzmB*$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} cells at a 1:1 ratio (Figure 5A). Recipient mice were monitored for survival and GVHD morbidity over a 60-day period. All mice receiving only T cell–depleted bone marrow cells survived with no signs of acute GVHD during this period. Mice receiving bone marrow cells with T_{eff} cells died with 100% penetrance within the first 3 weeks after transplantation. These mice exhibited classic signs of acute GVHD, such as weight loss, hunching, fur ruffling, and diarrhea. GVHD lethality and morbidity were significantly reduced by cotransfer of either WT or *Gzmb*$^{-/-}$ T_{reg} cells, and the latency of lethality was prolonged by approximately 2 weeks. A lower T_{reg} cell dose in this mouse model produced similar findings (Figure 5B). Furthermore, histopathologic scoring of GVHD severity in relevant target organs (eg, lung, liver, and gut) revealed that adoptive transfer of either WT or Gzmb$^{-/-}$ T_{reg} cells was equally able to prevent target organ damage in this model (Figure 5C). These data suggest that granzyme B is not required within the donor T_{reg} compartment for rescuing hosts from acute lethal GVHD.

Inhibition of GVHD-associated serum cytokine production by adoptively transferred T_{reg} cells is granzyme B–independent

Aberrant cytokine production is a central component to the pathophysiology of GVHD and has previously been shown to be mitigated by the transfer of T_{reg} cells.[30] Thus, we measured serum levels of cytokines commonly associated with GVHD in our mouse model, where transfer of T_{eff} and T_{reg} cells at a 1:1 ratio suppressed GVHD lethality irrespective of whether the transferred T_{reg} cells were WT or *Gzmb*$^{-/-}$. Seven days after conditioned Balb/c hosts were transplanted with bone marrow and/or T cells as described in Figure 5, the mice were bled and sera were analyzed using cytokine bead arrays. Shown in Figure 6A, mice receiving CD25$^-$ T_{eff} cells with no T_{reg} cells had significantly higher amounts of serum IL-2 relative to recipients of T cell–depleted bone marrow cells alone, and cotransfer of WT or *Gzmb*$^{-/-}$ T_{reg} cells restored IL-2 levels to near baseline levels. Suppression of IL-4 (Figure 6B), IL-5 (Figure 6C), granulocyte-macrophage colony-stimulating factor (Figure 6E), and interferon-γ production (Figure 6F) occurred in mice receiving T_{reg} cells independently of granzyme B genotype. There was no statistically significant difference in IL-10 levels (Figure 6D) across the 4 experimental groups, which is expected given that IL-10 is an anti-inflammatory cytokine that has been shown to play a role in T_{reg} cell–mediated suppression of GVHD lethality.[27] In addition, there was also no difference in TNF-α levels across these groups (data not shown). Taken together with the GVHD survival data and proliferation assays, these findings demonstrate that

Gzmb$^{-/-}$ T_{reg} cells have no defect in suppressing lethality caused by GVHD, partly because their capacities to inhibit cytokine production and block T_{eff}-cell proliferation remain intact.

Discussion

Based on our previous data, we hypothesized that granzyme B would be important for suppression of alloimmune responses in MLRs and in a mouse model of GVHD. In this report, we showed that T_{reg} cells do indeed up-regulate granzyme B protein in both settings. However, granzyme B was not required for the suppression of T_{eff}-cell proliferation driven by either ConA or mismatched APCs in vitro, and no granzyme B was detected in the T_{reg} compartment even when T_{eff}-cell proliferation was maximally suppressed. This contrasts with our finding that granzyme B protein was detected in a small proportion of MLR-activated T_{reg} cells. The absence of accessory responder and stimulator cells in the suppression assay cocultures, which were present in the MLRs and could produce soluble molecules and/or cell contact-mediated signals, may be partially responsible for the differential expression of granzyme B in these experiments. These data also suggest that coligation of the T-cell receptor and traditional costimulatory molecules, although sufficient to activate other T-cell effector functions (ie, proliferation), is insufficient to induce expression of granzyme B in T_{reg} cells.

Because the signals that are required for granzyme B induction in T_{reg} cells have yet to be defined in vitro, we alloactivated T_{reg} cells in vivo, using endogenous signals generated during GVHD to arm T_{reg} cells with granzyme B. The use of FIG mice allowed for the purification of viable activated T_{reg} cells based on expression of Foxp3, the most definitive marker that distinguishes between T_{reg} and activated T_{eff} cells. We found that these purified cell populations did not require granzyme B to suppress T_{eff}-cell proliferation ex vivo. Because of technical limitations, we were unable to purify sufficient numbers of in vivo–activated T_{reg} cells to determine whether *Gzmb*$^{-/-}$ T_{reg} cells were defective in suppressing other aspects of T_{eff} cell function, such as cytotoxicity, in ex vivo assays. We also attempted to measure suppression of T_{eff}-cell expansion in vivo by measuring the loss of carboxyfluorescein succinimidyl ester in labeled T_{eff} cells that were cotransferred with or without WT or *Gzmb*$^{-/-}$ T_{reg} cells. However, because the injected cells were distributed systemically, we were unable to collect enough events to determine whether there was a significant difference between mice that received WT or *Gzmb*$^{-/-}$ T_{reg} cells.

Although a reductionist approach for characterizing T_{reg}-cell function in suppression assays has been helpful in elucidating candidate molecules and pathways that are important for suppressive function in vitro, it remains to be determined whether these same molecules and pathways are relevant in vivo.[33] It is therefore important to functionally validate candidate molecules involved in suppression within the physiologic context of an appropriate disease model. To that end, we adopted an acute GVHD mouse model that was previously shown to be mitigated by the transfer of donor-type T_{reg} cells.[27] Using multiple physiologic readouts, including survival, cytokine production, and target organ damage, we unexpectedly found that *Gzmb*$^{-/-}$ T_{reg} cells had no defect in their ability to ameliorate GVHD. Taken together, these results show that granzyme B is not required for T_{reg} cell–mediated suppression of GVHD lethality and effector functions across major histocompatibility barriers.

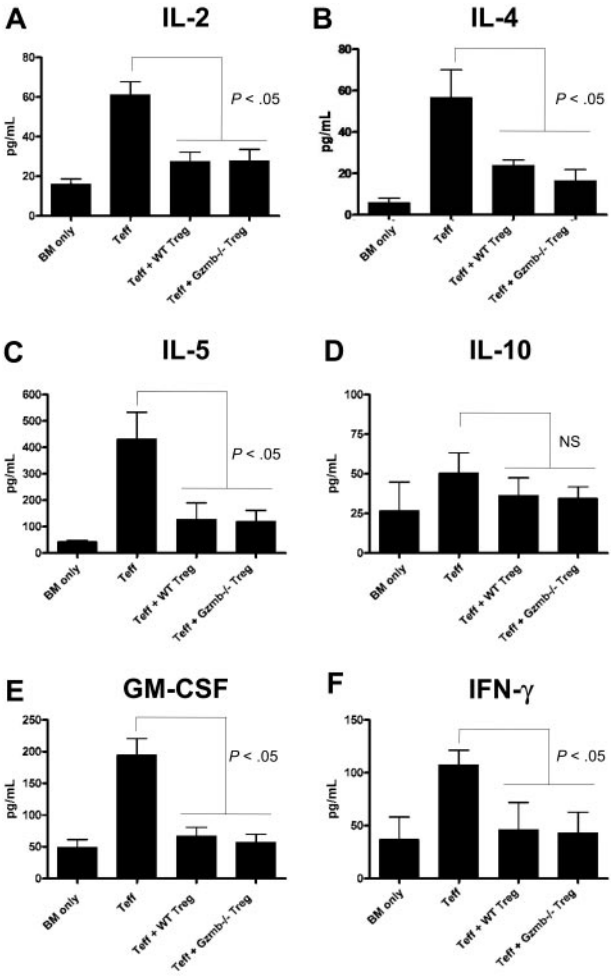

Figure 6. T_{reg} cells do not require granzyme B to suppress production of GVHD-associated cytokines in vivo. Lethally irradiated (900 cGy) Balb/c mice received 2×10^6 129/SvJ TCD BM cells with or without 4×10^5 129/SvJ CD25$^-$ T_{eff} cells (both CD4$^+$ and CD8$^+$) and 4×10^5 wild-type or *Gzmb*$^{-/-}$ CD4$^+$CD25$^+$ T_{reg} cells. Seven days after transplantation, serum was harvested and analyzed via cytometric bead array for the production of (A) IL-2, (B) IL-4, (C) IL-5, (D) IL-10, (E) granulocyte-macrophage colony-stimulating factor, and (F) interferon-γ. There was no statistically significant difference between groups receiving WT or *Gzmb*$^{-/-}$ T_{reg} cells.

These observations are different from our reported findings on T_{reg}-cell function in the setting of tumor challenge, where granzyme B and perforin play nonredundant roles in T_{reg} cell–mediated suppression of antitumor immune responses.[25] Granzyme B–dependent T_{reg} function has also been confirmed by others in different systems. Gondek et al reported that T_{reg} cell–mediated inhibition of T_{eff}-cell proliferation in vitro required granzyme B but not perforin,[34] whereas Zhao et al demonstrated that activated T_{reg} cells can suppress B-cell proliferation by killing antigen-presenting (but not bystander) B cells in a perforin- and granzyme B–dependent manner.[35] Gondek et al later validated their in vitro findings in a skin allograft model by showing that hosts reconstituted with granzyme B–deficient T_{reg} cells were unable to establish long-term tolerance to skin allografts.[36]

This presents a dichotomy in the mechanisms used by alloactivated T_{reg} cells to suppress effector immune responses, even though allogeneic T-cell activation is a shared feature of all of these models. In the tumor model, the perforin/granzyme pathway is a nonredundant component of T_{reg}-cell function, although others have shown that additional molecules, such as CTLA-4 and GITR,

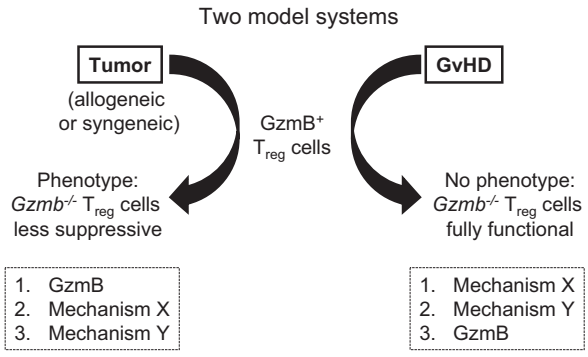

Figure 7. Model-dependent role of granzyme B in T_{reg} cell–mediated suppressive function. Schematic illustrating the differential phenotypes observed with *Gzmb*$^{-/-}$ Treg cells in suppressing antitumor responses and GVHD.

also play important roles.[21,37] In the GVHD model, the contribution of granzyme B to T_{reg} cell–suppressive function may potentially be masked by additional mechanisms that are activated under these specific conditions (Figure 7). A recent study of a fully mismatched pancreatic islet allograft model showed that T_{reg} cell–mediated inhibition of dendritic cell migration to the graft occurred in a transforming growth factor-β– and IL-10–dependent manner, raising the possibility that these classic T_{reg}-associated cytokines could be involved in other alloimmune settings such as GVHD.[38] However, it is clear from multiple studies that not all allogeneic mouse models are equivalent; the experimental context in which T_{reg}-cell activation occurs may play a critical role in defining the particular mechanism(s) operative in that context.[33,39,40]

Finally, the differential requirement of granzyme B for T_{reg}-cell function in GVHD and tumor models also raises the potential of targeting specific T_{reg}-suppressive mechanisms for therapeutic benefit. Allogeneic bone marrow transplantation is a major treatment modality for hematologic malignancies, but the graft-versus-tumor effect mediated by donor lymphocyte populations against mismatched host tumor cells often comes at the expense of GVHD.[41,42] We previously demonstrated that disarming granzyme B in T_{reg} cells results in enhanced tumor clearance, but our findings in this report demonstrate that T_{reg} cells lacking granzyme B are still able to prevent GVHD lethality. Moreover, Edinger et al demonstrated that transfer of T_{reg} cells rescues hosts from GVHD while preserving graft-versus-tumor activity.[30] Based on our studies, we hypothesize that different suppressive mechanisms used by alloactivated and tumor-activated T_{reg} cells may explain this phenotype. We attempted to test this hypothesis using bioluminescence imaging to serially monitor the clearance of allogeneic tumors in our GVHD model system, but we were limited by the current sensitivity and specificity of the tumor clearance assay. However, it is possible that the inhibition of T_{reg}-suppressive molecules, such as granzyme B, may allow for the segregation of GVHD and graft-versus-tumor activity within the same host. Adoptive T_{reg}-cell immunotherapy, along with modulation of T_{reg}-cell function, holds therapeutic promise and should be an area of continued investigation.

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Authorship

Contribution: S.F.C. and X.C. performed experiments; S.F.C., X.C., A.H., T.A.F., and T.J.L. contributed to experimental design and data analysis; and S.F.C. and T.J.L. wrote the paper.

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