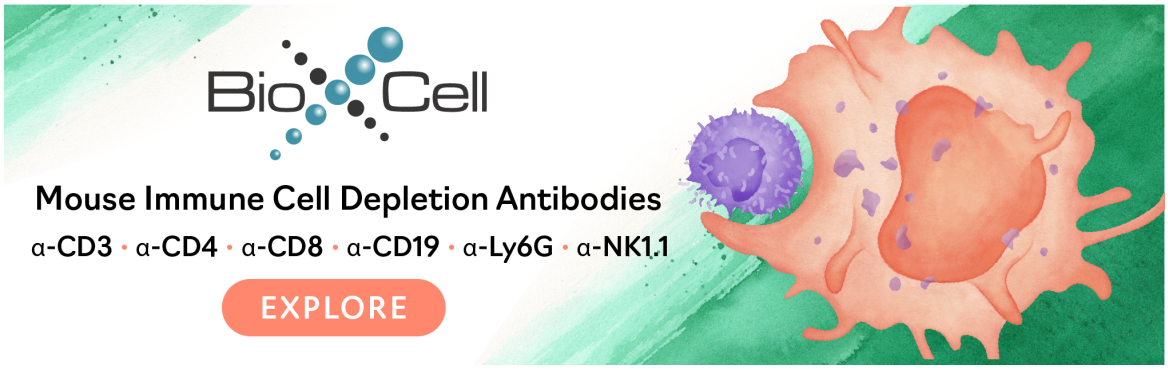

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CD8$^+$ Foxp3$^+$ Regulatory T Cells Are Induced during Graft-versus-Host Disease and Mitigate Disease Severity

Amy J. Beres,*,† Dipica Haribhai,‡ Alexandra C. Chadwick,§ Patrick J. Gonyo,§ Calvin B. Williams,‡ and William R. Drobyski*,†,¶

Regulatory T cells (Tregs), in particular CD4$^+$ Foxp3$^+$ T cells, have been shown to play an important role in the maintenance of tolerance after allogeneic stem cell transplantation. In the current study, we have identified a population of CD8$^+$ Foxp3$^+$ T cells that are induced early during graft-versus-host disease (GVHD), constitute a significant percentage of the entire Treg population, and are present in all major GVHD target organs. These cells expressed many of the same cell surface molecules as found on CD4$^+$ Tregs and potently suppressed in vitro alloreactive T cell responses. Induction of these cells correlated positively with the degree of MHC disparity between donor and recipient and was significantly greater than that observed for CD4$^+$-induced Tregs (iTregs) in nearly all tissue sites. Mice that lacked the ability to make both CD8$^+$ and CD4$^+$ iTregs had accelerated GVHD mortality compared with animals that were competent to make both iTreg populations. The absence of both iTreg populations was associated with significantly greater expansion of activated donor T cells and increased numbers of CD4$^+$ and CD8$^+$ T cells that secreted IFN-γ and IL-17. The presence of CD8$^+$ iTregs, however, was sufficient to prevent increased GVHD mortality in the complete absence of CD4$^+$ Tregs, indicating at least one functional iTreg population was sufficient to prevent an exacerbation in GVHD severity, and that CD8$^+$ iTregs could compensate for CD4$^+$ iTregs. These studies define a novel population of CD8$^+$ Tregs that play a role in mitigating the severity of GVHD after allogeneic stem cell transplantation. ***The Journal of Immunology*****, 2012, 189: 464–474.**

Graft-versus-host disease (GVHD) is the major complication associated with allogeneic stem cell transplantation and is attributable, in large part, to an imbalance between the effector and regulatory arms of the immune system (1). A preponderance of evidence in experimental murine models and humans indicates that there is a progressive loss of regulatory T cells (Tregs) during GVHD (2–5). This decline in Treg numbers unleashes cytotoxic T cells and proinflammatory cytokine pathways that subsequently mediate pathological damage. Conversely, the adoptive transfer of Tregs at the time of transplantation can enhance overall survival and abrogate GVHD lethality (6–10), providing confirmation that these cells play a central role in the maintenance of transplantation tolerance. The most well-characterized population of Tregs in GVHD biology has been CD4$^+$ T cells, which express the Foxp3 transcription factor (11). This population is comprised of two major subsets that have been termed natural Tregs (nTregs) and induced Tregs (iTregs), based on the unique ontological and developmental characteristics that are specific for each cell population (12). The majority of experimental murine bone marrow (BM) transplantation (BMT) studies have focused on the role of nTregs, whereas the contribution of iTregs to the prevention of GVHD lethality is still largely unclear. CD4$^+$ iTregs that are in vivo derived have been identified in GVHD recipients (13, 14), but their ability to mitigate graft-versus-host reactivity has not been critically examined. Analysis of this population has also been confounded by the presence of nTregs in most experimental models of GVHD, which has limited the ability to isolate the effects of these cells. Studies in other inflammatory disease models, however, have provided strong evidence that these two populations have nonredundant, complementary roles in maintaining immunological tolerance (15, 16), indicating that Tregs are not a monolithic population, but constitute a heterogeneous population of cells with differing specificities and functions.

The premise that Tregs constitute a heterogeneous population has been bolstered by the identification of a population of CD8$^+$ Foxp3$^+$ T cells in autoimmune disorders and after allergen exposure (17–20). These cells, which express many of the cell surface molecules such as GITR, CD103, and CTLA-4 commonly found on classical CD4$^+$ Tregs, have also been shown to suppress immune responses in vitro (21). The potential importance of this cell population is highlighted by their more recent identification in humans who received stem cell transplants for autoimmune disorders and diabetes, where they were found to correlate in an inverse manner with the level of ongoing inflammation (22, 23). Furthermore, these cells have been detected in tumor-bearing animals along with biopsies from patients with cancer, where they have been implicated in suppressing the host immune response against the underlying malignancy (24, 25). Whether these cells are present or have any functional role in allogeneic stem cell transplantation or, more specifically, GVHD biology is not known. In the current study, we demonstrate that CD8$^+$ Foxp3$^+$

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; BMT, BM transplantation; EGFP, enhanced GFP; GVHD, graft-versus-host disease; iTreg, induced regulatory T cell; nTreg, natural regulatory T cell; Treg, regulatory T cell.

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Tregs are induced early during the course of GVHD and constitute a significant percentage of the entire Treg population. Moreover, these cells play a role in preventing GVHD-mediated lethality and are able to complement CD4$^+$ iTregs, establishing them as a novel Treg population in GVHD biology.

Materials and Methods

Mice

C57BL/6 (B6) (H-2^b), BALB/c (H-2^d), FVB/N (H-2^q), B6.SJL (CD45.1), B6.PL (Thy1.1$^+$), B6.129S7-Rag-1 (B6 Rag-1), C.B10-H2^b/LilMcd (BALB.B) (H-2^b), and B6.C-H2bm1/Byj (bm1) (H-2K^{bm1}) mice were bred in the Animal Resource Center at the Medical College of Wisconsin or purchased from The Jackson Laboratory (Bar Harbor, ME). Foxp3^{EGFP} mice and Foxp3$^{\Delta EGFP}$ in which there is mutation in the Foxp3 coding region that renders the Foxp3 gene nonfunctional were bred at Medical College of Wisconsin and have been previously described (26). The latter mice were reconstituted with 40–60 $\times$ 10^6 spleen cells from B6.SJL Foxp3^{EGFP} (CD45.1) animals 1–2 d after birth to prevent the development of lethal autoimmunity. All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care-accredited Biomedical Resource Center of the Medical College of Wisconsin. Experiments were all carried out under protocols approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Mice received regular mouse chow and acidified tap water ad libitum.

Bone marrow transplantation

BM was flushed from donor femurs and tibias with DMEM (Life Technologies-BRL, Carlsbad, CA) and passed through sterile mesh filters to obtain single-cell suspensions. Host mice were conditioned with total body irradiation administered as a single exposure at a dose rate of 900–1100 cGy using a Shepherd Mark I Cesium Irradiator (J.L. Shepherd and Associates, San Fernando, CA). Irradiated recipients received a single i.v. injection in the lateral tail vein of BM with or without added spleen or purified T cells. Mice were weighed two to three times per week and were euthanized when they attained predefined morbidity criteria. In experiments that used Foxp3$^{\Delta EGFP}$ mice as donors, CD45.2$^+$ CD4$^+$ enhanced GFP (EGFP)$^-$ or CD45.2$^+$ CD8$^+$ EGFP$^-$ T cells were sorted from the spleens of reconstituted animals prior to transplantation.

Cell sorting and flow cytometry

Spleen cells were collected from Foxp3^{EGFP} and/or Foxp3$^{\Delta EGFP}$ mice and sorted on a FACSAria II (BD Biosciences, Mountain View, CA). Sort purity was typically 98–99%. Spleen, liver, lung, and colon cells from transplant recipients were labeled with mAb conjugated to FITC, PE,

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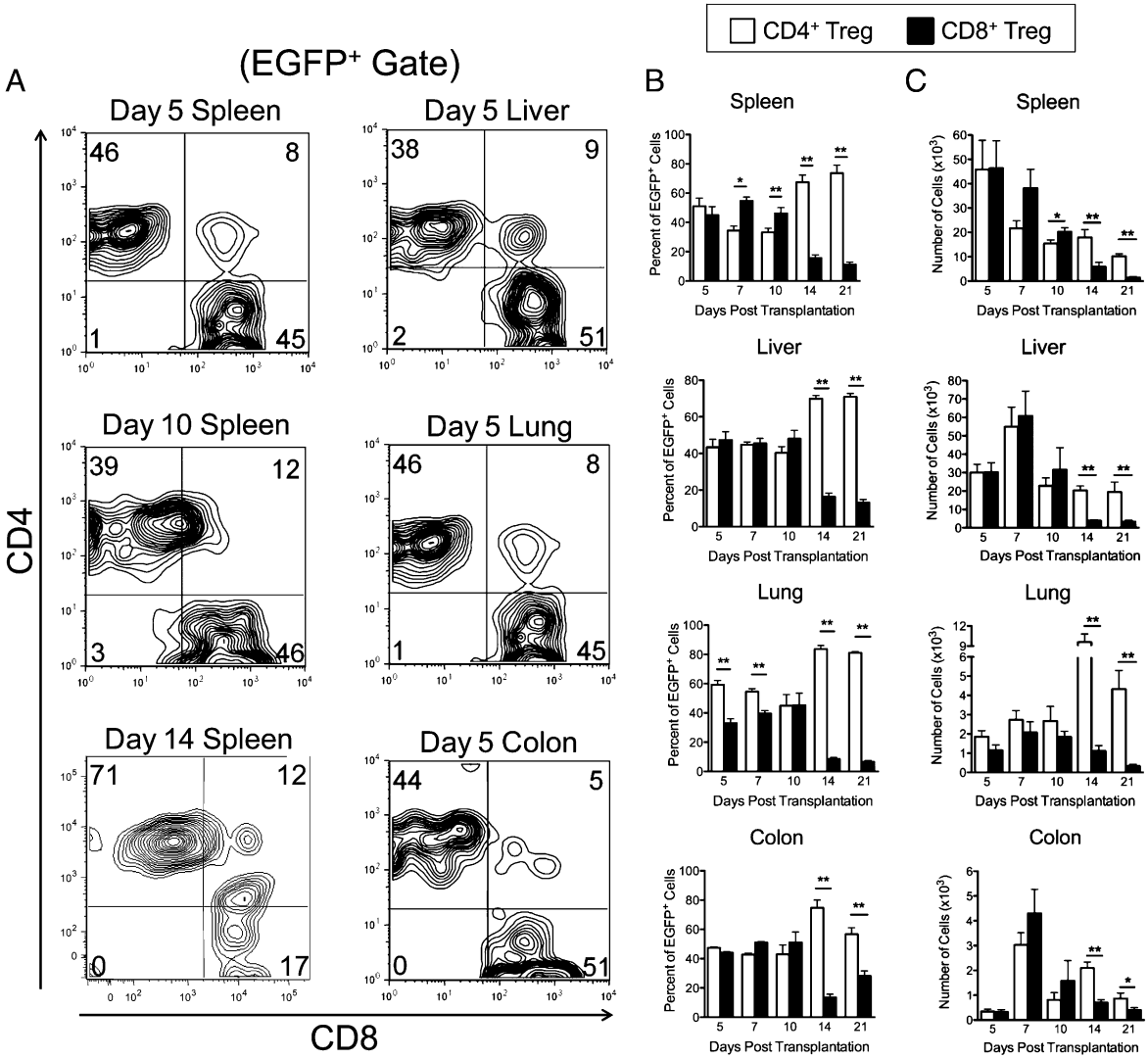

FIGURE 1. CD8$^+$ Foxp3$^+$ T cells are detectable in GVHD target organs. Lethally irradiated (900 cGy) BALB/c mice were transplanted with 10 $\times$ 10^6 B6 Foxp3^{EGFP} BM and spleen cells (adjusted to yield a αβ T cell dose of 0.6 $\times$ 10^6). Mice (n = 10–15 per group) were euthanized on day 5, 7, 10, 14, or 21 posttransplantation. (**A**) Representative contour plots gated on EGFP$^+$ cells depicting the percentage of CD4$^+$ and CD8$^+$ Tregs in the spleen, liver, lung, and colon at specified time points posttransplantation. (**B** and **C**) Relative and absolute numbers of CD4$^+$ (□) and CD8$^+$ (■) Tregs are shown at the indicated time points in the specified GVHD target organs. Data are shown as the mean ± SEM and are the cumulative results from four independent experiments. *$p < 0.05$, **$p < 0.01$.

BD Horizon 450 (V450), BD Horizon 500 (V500), PE-Cy7, PE-Texas Red, or allophycocyanin that were obtained from BD Biosciences (San Diego, CA), or Brilliant Violet 421 from BioLegend (San Diego, CA). Cells were analyzed on a FACSCalibur or LSRII flow cytometer with CellQuest or FACSDiva software (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Cell isolation

To isolate lamina propria lymphocytes, pooled colons were incubated in HBSS buffer (Life Technologies-BRL) supplemented with 2% FBS, EDTA (0.05 mM), and 15 μg/ml DTT (Invitrogen, Carlsbad, CA) at 37°C for 30 min and subsequently digested in a solution of collagenase D (1 mg/ml; Roche Diagnostics, Mannheim, Germany) in DMEM with 2% FBS for 75 min at 37°C. The resulting cell suspension was then layered on a 44/67% Percoll gradient (Sigma-Aldrich, St. Louis, MO). Liver and lung lymphocytes were isolated by collagenase D digestion, followed by layering on a Percoll gradient.

Mixed lymphocyte culture

Thy1.2$^+$ cells (1×10^5) were purified from B6 spleens using the MACS system (Miltenyi Biotec, Auburn, CA) and cocultured with 5×10^4 BALB/c dendritic cell-enriched stimulator cells in U-bottom microwell plates (BD Biosciences, Lincoln Park, NJ) at 37°C. Stimulator cells were obtained by digestion of spleens with collagenase D (1 mg/ml), followed by positive selection of CD11c$^+$ cells using the MACS system. Nonirradiated stimulator cells were then seeded into microwell plates. Flow-sorted CD4$^+$ or CD8$^+$ Tregs, or activated CD8$^+$EGFP$^-$ T cells were added at varying ratios to wells containing T and dendritic cells. One microcurie of [^{3}H]thymidine was added to triplicate wells for the final 12–18 h prior to harvest. Thymidine incorporation was assessed using a Wallac 1450 Microbeta liquid scintillation counter (Perkin Elmer, Shelton, CT). Control wells consisted of responders, stimulators, and Tregs alone.

Intracellular cytokine staining

Lymphocytes isolated from spleen, liver, lung, and colon were stimulated with 50 ng/ml PMA (Sigma-Aldrich) and 750 ng/ml ionomycin (Calbiochem, La Jolla, CA) for 1 h and then incubated with GolgiStop (BD Pharmingen) for an additional 4 h. Cells were surface stained with Brilliant Violet 421 anti-CD3, allophycocyanin anti-CD8, and PE-Texas Red anti-CD4, and then intracellularly stained with PE-labeled Ab to IL-17 and Alexa Fluor 700-labeled Ab to IFN-γ. All Abs were obtained from BD Biosciences with the exception of Brilliant Violet 421, which was obtained from BioLegend.–

Statistics

Group comparisons of Tregs and T cell populations, intracellular cytokine staining, and thymidine incorporation were performed using the Mann-Whitney *U* test. Survival curves were constructed using the Kaplan-Meier product limit estimator and compared using the Gehan Wilcoxon test. A p value ≤ 0.05 was deemed to be significant in all experiments.

Results

CD8$^+$ Foxp3$^+$ Tregs are induced early during GVHD

During the course of studies designed to define the functional role of various CD4$^+$ Treg populations in GVHD biology, we identified a population of CD8$^+$ T cells that expressed Foxp3 and were present in recipients undergoing GVHD. To further examine this observation, lethally irradiated BALB/c mice were transplanted with MHC-incompatible marrow grafts from B6 Foxp3^{EGFP} animals. A serial time course analysis revealed the emergence of CD8$^+$ Foxp3$^+$ T cells within 5 d posttransplantation in GVHD target tissues (i.e., spleen, liver, colon, and lung) (Fig. 1A, Supplemental Fig. 1). The percentage of these cells was no different from that of CD4$^+$ Tregs for the first 10 d posttransplantation, with the exception of the spleen, where an increased frequency of CD8$^+$ Tregs was observed, and the lung, where the converse was present (Fig. 1B). Overall, absolute numbers of CD4$^+$ and CD8$^+$ Foxp3$^+$ T cells, however, were similar in all tissue sites over this time frame (Fig. 1C). This was noteworthy given that the CD4$^+$ Treg pool that derives from the marrow graft is composed of both nTregs and iTregs, whereas the bulk of CD8$^+$ Tregs (henceforth referred to as CD8$^+$ iTregs) is presumably induced due to the negligible expression of Foxp3 on CD8$^+$ T cells in the spleen, thymus, and BM (Supplemental Fig. 2). Thereafter, the absolute number of both CD4$^+$ and CD8$^+$ Treg populations declined with time in most tissues, although the rate of decline was more precipitous for CD8$^+$ iTregs. Consequently, the absolute number of CD8$^+$ iTregs was significantly lower than CD4$^+$ Tregs at days 14 and 21 posttransplantation (Fig. 1C). Notably, CD8$^+$ iTregs were not detected beyond 4 wk in any tissue site (data not shown), indicating that these Foxp3-expressing T cells had limited in vivo

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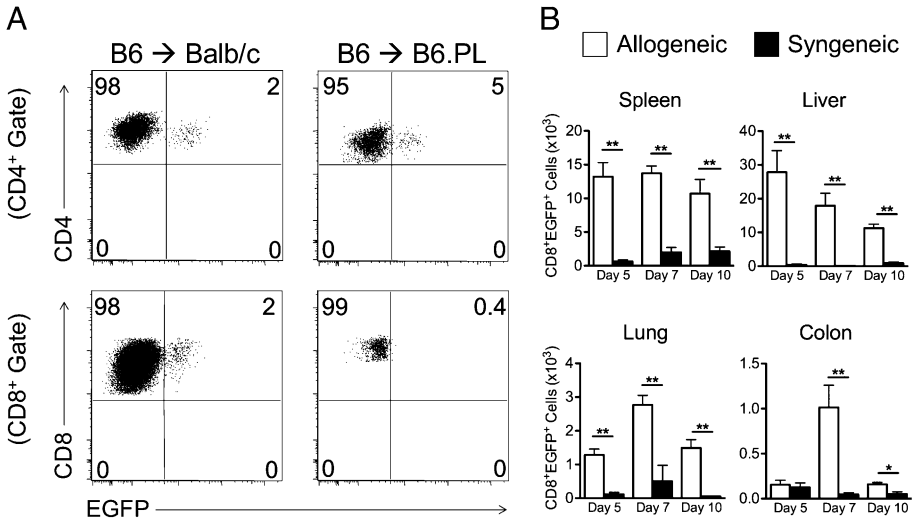

FIGURE 2. Induction of CD8$^+$ Tregs is not attributable to homeostatic expansion. Lethally irradiated BALB/c (□, allogeneic) or B6.PL mice (■, syngeneic) (n = 11 per group) were transplanted with B6 Foxp3^{EGFP} BM (10×10^6) and spleen cells (adjusted to yield a αβ T cell dose of 0.6×10^6). Mice were euthanized at the indicated days posttransplantation. (**A**) Representative dot plot showing the percentages of CD4$^+$ Foxp3$^+$ and CD8$^+$ Foxp3$^+$ T cells in the spleen 5 d posttransplantation for recipients of allogeneic and syngeneic marrow grafts. For recipients of syngeneic marrow grafts, donor cells were identified by gating on Thy1.2$^+$ (B6) cells. (**B**) Absolute numbers of CD8$^+$ Foxp3$^+$ T cells in GVHD target organs at the specified time points posttransplantation. Data are shown as the mean ± SEM and are the cumulative results from three independent experiments. *$p < 0.05$, **$p < 0.01$.

persistence in GVHD. CD8$^+$ Tregs were induced in a different MHC-mismatched strain combination, indicating that the results were not strictly model dependent (Supplemental Fig. 3). The emergence of CD8$^+$ iTregs in GVHD animals was not attributable to homeostatic expansion occurring in a posttransplant lymphopenic environment, because mice transplanted with syngeneic marrow grafts did not have significant induction of these cells in any tissue sites (Fig. 2).

Augmented induction of CD8$^+$ iTregs during the early stages of GVHD

nTregs constitute the majority of the Treg pool in the periphery, as iTregs have been estimated to comprise only 4–15% of total CD4$^+$ Tregs in various studies (16, 27). Therefore, we reasoned that the majority of CD4$^+$ Tregs that were present in the spleen and tissue sites of GVHD recipients early after transplantation were most likely nTregs given that ~10% of CD4$^+$ T cells in the marrow graft inoculum expressed Foxp3. Because the total number of CD4$^+$ and CD8$^+$ Tregs was equivalent early posttransplantation, we hypothesized that there might be disparate induction of CD8$^+$ and CD4$^+$ Tregs during GVHD. To directly address this issue, we performed experiments in which mice were transplanted with B6 Rag-1 BM plus CD4$^+$ Foxp3^{EGFP-} and CD8$^+$ Foxp3^{EGFP-} T cells so that only iTregs could reconstitute in these animals. Examination of Treg reconstitution in the spleen and GVHD target organs 5, 10, and 14 d posttransplantation revealed an increased percentage of CD8$^+$ iTregs in the spleen, liver, and lung, whereas there was no difference in the colon (Fig. 3A, 3B). We also observed an increased absolute number of CD8$^+$ iTregs in the spleen and liver at all time points, and in the lung on day 5 (Fig. 3C). The increase in CD8$^+$ iTregs, relative to CD4$^+$ iTregs, was not due to an overall increase in the total number of CD8$^+$ T cells as there was no significant difference in the absolute number of CD4$^+$ and CD8$^+$ T cells at any of these time points (Supplemental Fig. 4). These data indicated that the induction of CD8$^+$ Tregs from the conventional T cell compartment exceeded that of CD4$^+$ Tregs during the early stages of GVHD.

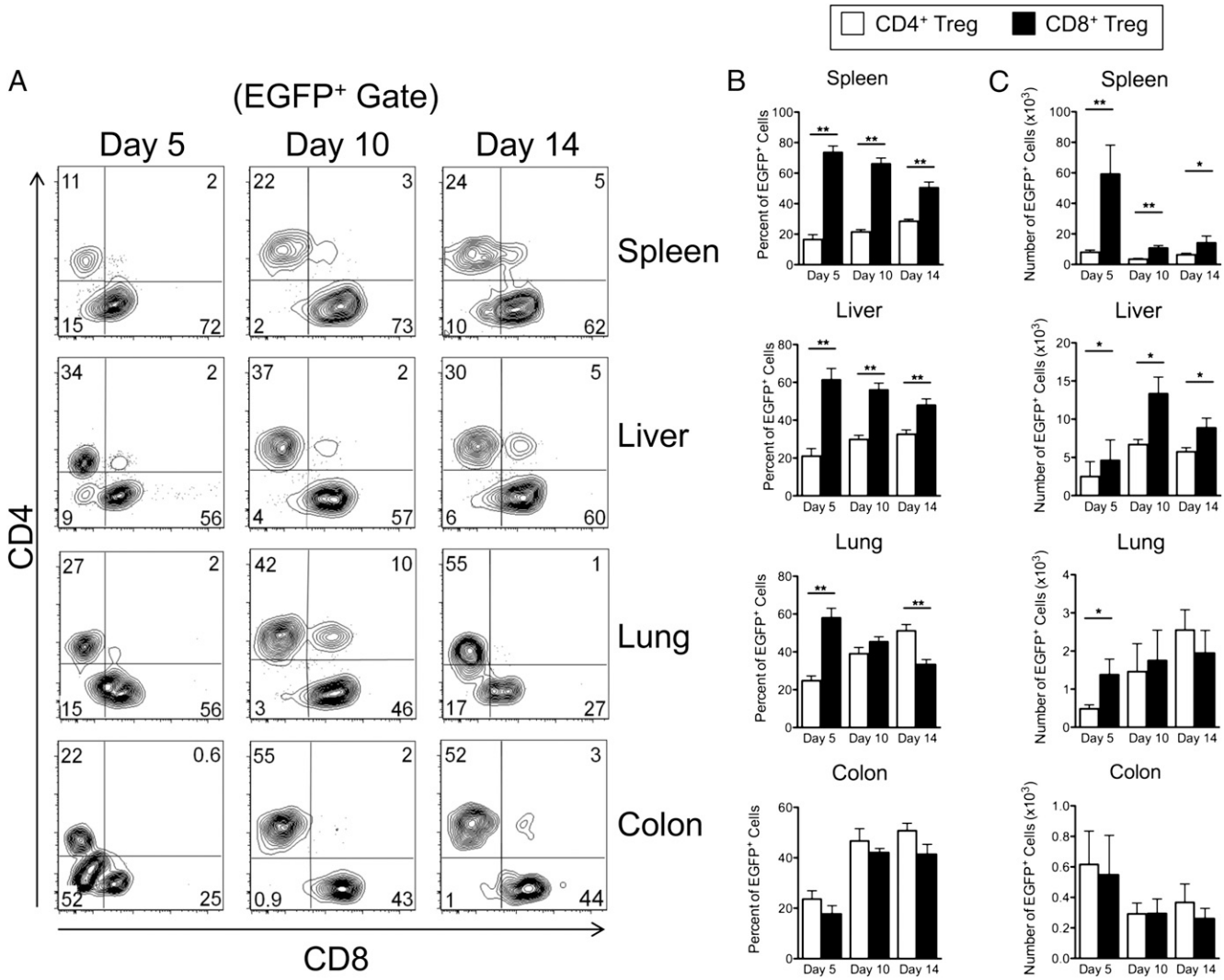

FIGURE 3. In vivo induction of CD8$^+$ Tregs is augmented during the early stages of GVHD. Lethally irradiated BALB/c mice were transplanted with B6 Rag-1 BM along with 0.3×10^6 CD4$^+$EGFP$^-$ and 0.15×10^6 CD8$^+$EGFP$^-$ T cells from Foxp3^{EGFP} animals. Mice (n = 7–11 per group) were euthanized at 5, 10, or 14 d posttransplantation. (**A**) Representative contour plots gated on EGFP$^+$ cells depicting CD4$^+$ and CD8$^+$ iTregs in the specified tissue sites. (**B** and **C**) Relative and absolute numbers of CD4$^+$ (□) and CD8$^+$ (■) iTregs in the spleen, liver, lung, and colon at the defined time points posttransplantation are depicted. Data are presented as the mean ± SEM and are the cumulative results from three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$.

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The magnitude of CD8+ Treg induction correlates positively with the degree of MHC disparity

The induction of Foxp3 expression in conventional T cells is dependent upon cell activation (28–30). T cell activation is also a prominent component of GVHD due to the recognition of alloantigens expressed by host APCs (31–33). We therefore examined whether the degree of MHC disparity between donor and recipient was a factor that modulated the magnitude of CD8+ Treg induction. To address this question, we performed transplant studies using donor/recipient strain combinations in which there were differing degrees of MHC disparity. Specifically, in addition to a MHC-disparate model, we employed murine models in which mice were MHC matched and differed only at multiple minor histocompatibility Ags (B6→BALB.B), or had an isolated MHC class I mismatch (B6→bm1). The dose of T cells administered to recipients was sufficient to induce lethal GVHD in all strain combinations (data not shown). These results demonstrated that CD8+ iTregs were detectable in recipients of MHC-matched, minor Ag-mismatched grafts (Fig. 4A). The percentage of total CD8+ T cells that expressed Foxp3 and the overall number of CD8+ iTregs were significantly less than observed in MHC-mismatched recipients at days 5 and 10 posttransplantation (Fig. 4B, 4C). Moreover, the percentage of CD8+ iTregs was much lower than CD4+ Tregs (Fig. 4A), in contrast to what was observed in BALB/c recipients of B6 marrow grafts where percentages tended to be equivalent (Fig. 1). We also observed that there was only a negligible CD8+ iTreg population in recipients of class I-mismatched grafts where a 3-aa difference distinguishes donor from host (Fig. 4). To confirm that the absence of CD8+ Treg induction in bm1 recipients could not be ascribed to lack of CD4+ T cells in the inoculum, we repeated studies with the inclusion of mature donor CD4+ T cells in the graft. Under these conditions where CD4+ T cells do not contribute to an antihost response, CD8+ iTregs were still not detectable (data not shown). Collectively, these results indicated that the induction of CD8+ Tregs during GVHD directly correlated with the degree of MHC disparity between donor and recipient.

CD8+ Tregs suppress alloreactive T cell responses in vitro and GVHD in vivo

The significant increase in CD8+ Treg numbers in GVHD recipients led us to examine whether these cells were functionally

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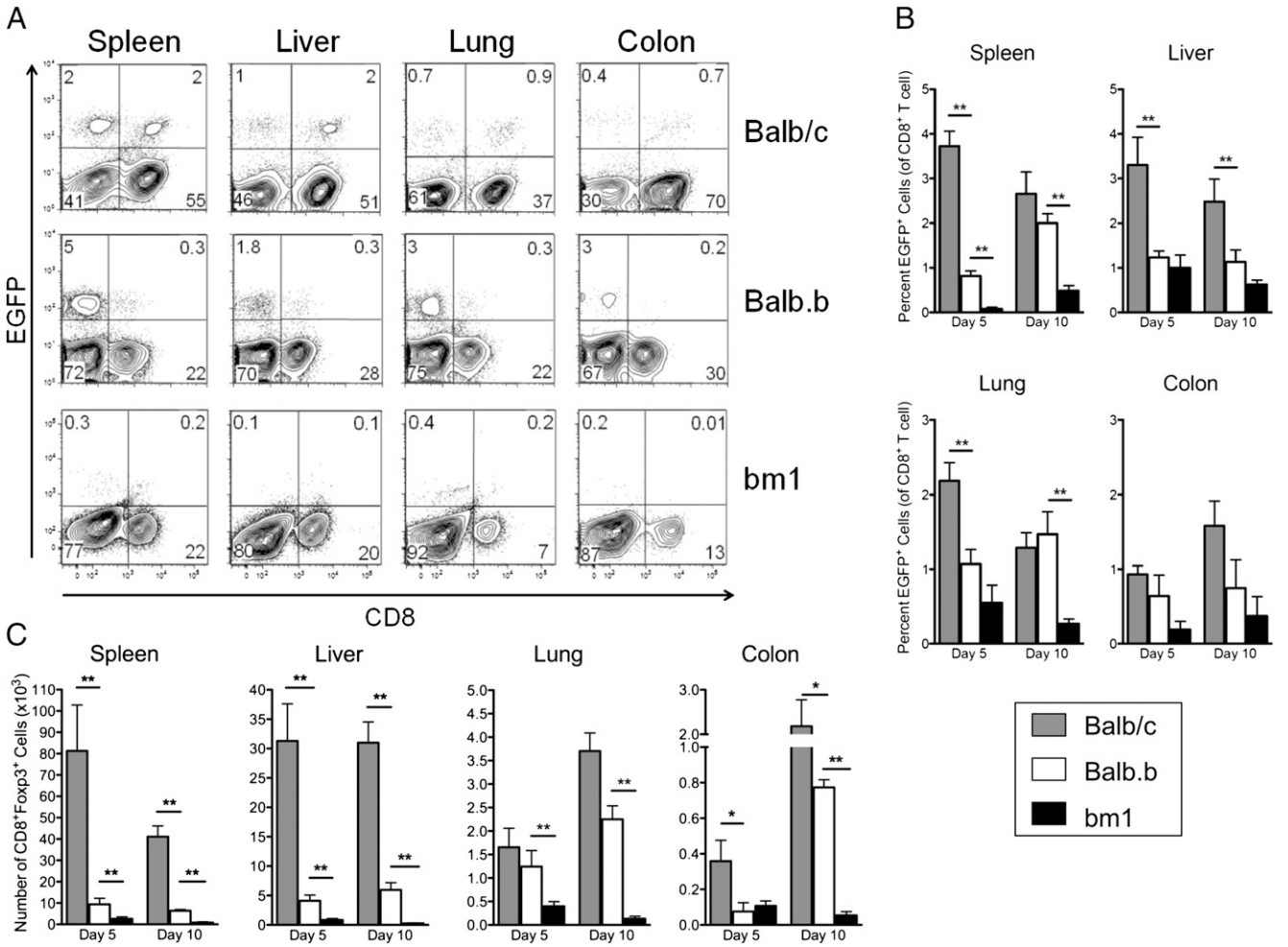

FIGURE 4. CD8+ Foxp3+ Treg induction correlates positively with the degree of MHC disparity. Lethally irradiated BALB/c (n = 14–15, gray bars), BALB.B (n = 6–15, white bars), or bm1 (n = 6–9, black bars) mice were transplanted with Foxp3^{EGFP} BM and 0.6×10^6 αβ T cells, 10×10^6 unfractionated splenocytes, or 2×10^6 purified CD8+ T cells, respectively. Mice were euthanized at 5 or 10 d posttransplantation. (**A**) Representative contour plots (gated on live cells) depicting CD8+ EGFP+ Tregs in the specified tissue sites 5 d posttransplant. (**B**) Percentage of total CD8+ T cells that were also Foxp3+ in the spleen, liver, lung, and colon. (**C**) Absolute number of CD8+ Foxp3+ Tregs in GVHD target organs at defined time points posttransplantation in the specified models. Data are presented as the mean ± SEM and are the cumulative results from three independent experiments. $*p < 0.05$, $**p < 0.01$.

suppressive. To address this question, we first sorted CD8$^+$ Foxp3^{EGFP+} T cells from the spleen and liver of GVHD mice 5 d post-BMT. Phenotypic characterization of in vivo-derived CD8$^+$ CD3$^+$ Tregs demonstrated that they expressed many of the same surface Ags expressed on CD4$^+$ Tregs (e.g., GITR, CD44, CTLA-4, CD25), with the exception that there was higher expression of CD103 (Fig. 5A). Notably, CD8$^+$ Tregs induced during GVHD expressed the CD8 β-chain, indicating that they were not CD8αα cells that have been shown to have suppressive properties in other murine model systems (34–36). We then tested whether these cells were able to inhibit alloreactive T cell proliferation in an in vitro suppression assay. These studies demonstrated that CD8$^+$ Foxp3$^+$ T cells derived from GVHD mice suppressed alloreactive T cell proliferation in a dose-dependent manner and that suppression was no different from that observed with sorted CD4$^+$ Foxp3$^+$ T cells derived from the same tissue sites (Fig. 5B). CD8$^+$ Foxp3^{EGFP-} T cells that did not undergo conversion also suppressed T cell responses, but this was significantly less than CD8$^+$ Foxp3$^+$ cells at nearly all effector:Treg ratios.

To determine whether CD8$^+$ iTregs had a suppressive role in preventing GVHD mortality in vivo, we employed donor mice that have a mutation in the Foxp3 coding region (Foxp3$^{\Delta EGFP}$ mice), which renders the protein nonfunctional such that these cells cannot become suppressive Tregs in vivo (26). CD4$^+$ and CD8$^+$ Foxp3$^{\Delta EGFP-}$ T cells (CD45.2$^+$) were isolated from the spleens of these mice and employed in transplantation experiments to determine the relative role of CD4$^+$ and CD8$^+$ iTregs in GVHD. Of note, iTregs constitute the only Treg populations in these studies because there are no transferred nTregs in the grafts. Lethally irradiated BALB/c mice were transplanted with B6 Rag-1 BM alone or together with CD4$^+$EGFP$^-$ and CD8$^+$ EGFP$^-$ T cells from Foxp3^{EGFP} mice (EGFP), CD4$^+$EGFP$^-$ T cells from Foxp3$^{\Delta EGFP}$ and CD8$^+$EGFP$^-$ T cells from Foxp3^{EGFP} animals (CD4Δ), CD4$^+$EGFP$^-$ from Foxp3^{EGFP} mice and CD8$^+$ EGFP$^-$ T cells from Foxp3$^{\Delta EGFP}$ mice (CD8Δ), or CD4$^+$EGFP$^-$ and CD8$^+$EGFP$^-$ T cells from Foxp3$^{\Delta EGFP}$ (CD4/8Δ). We observed that there was no difference in overall survival between mice that were transplanted with CD4Δ or CD8Δ marrow grafts compared with animals transplanted with T cells that were fully competent to differentiate into both CD4$^+$ and CD8$^+$ iTregs (Fig. 5C). Mice that were cotransplanted with both CD4$^+$ Foxp3$^{\Delta EGFP-}$ and CD8$^+$ Foxp3$^{\Delta EGFP-}$ T cells (CD4/8Δ grafts), however, had significantly worse survival ($p = 0.01$ compared with GVHD control). These data indicated that, in the absence of nTregs, at least one functional iTreg population was necessary to prevent an acceleration of GVHD-associated mortality. Furthermore, CD8$^+$ iTregs could compensate for the absence of CD4$^+$ iTregs (i.e., there was no difference in survival between GVHD and CD4Δ groups, but worse survival in the CD4/8Δ group), because otherwise one would have expected survival in animals transplanted with both CD4Δ and CD4/8Δ marrow grafts to be equivalent, and inferior to GVHD control mice.

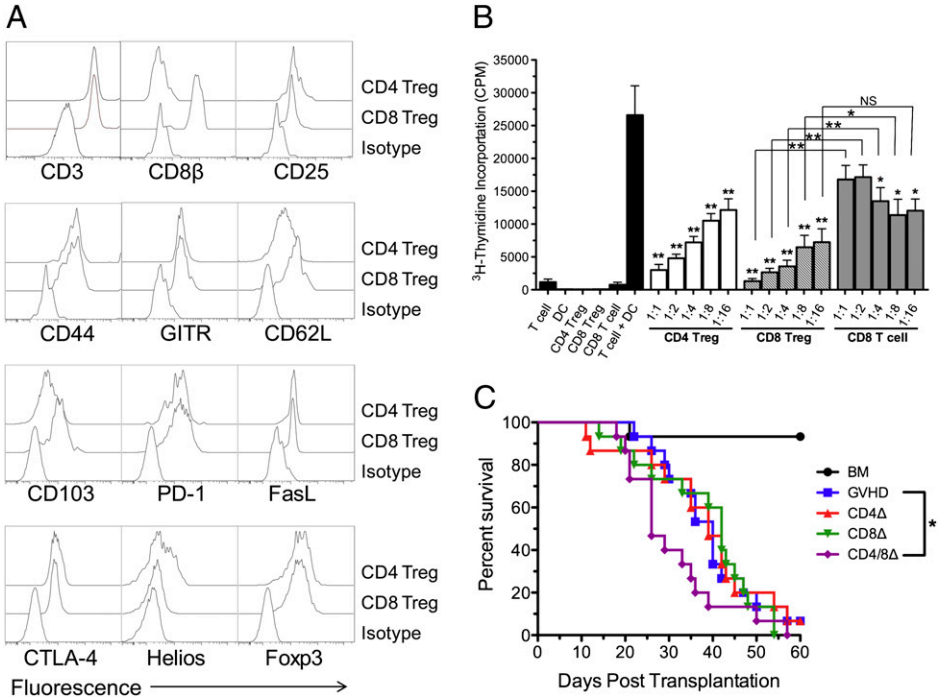

FIGURE 5. In vivo-induced CD8$^+$ Tregs are suppressive and mitigate the severity of GVHD. Lethally irradiated BALB/c mice were transplanted with Foxp3^{EGFP} BM and spleen cells. Mice ($n = 30$) were euthanized on day 5 posttransplantation, and spleens and livers were FACS sorted to purify CD4$^+$ and CD8$^+$ donor-derived EGFP$^+$ Tregs. (**A**) Cell surface expression of CD3, CD8β, CD25, CD44, GITR, CD62L, CD103, PD-1, and FasL, and intracellular expression of CTLA-4, Helios, and Foxp3 on in vivo-derived CD4$^+$ and CD8$^+$ Tregs. Isotype control-stained cells are shown for comparison. Data are presented as representative histograms from three independent experiments. (**B**) Purified B6 Thy1.2$^+$ T cells (1×10^5) were cultured with BALB/c CD11c$^+$ dendritic cells (5×10^4) in the presence of varying ratios of donor-derived CD4$^+$ Foxp3$^+$ Tregs (white bars), CD8$^+$ Foxp3$^+$ Tregs (hatched bars), or CD8$^+$ Foxp3$^-$ T cells (gray bars) from GVHD mice for 5 d in triplicate wells. Control wells are depicted as black bars. Data are presented as mean cpm ± SEM and are the cumulative results from three experiments. (**C**) Lethally irradiated BALB/c mice ($n = 15$ per group) were transplanted with B6 Rag-1 BM alone (BM, black line) or together with either 0.3×10^6 CD4$^+$EGFP$^-$ and 0.15×10^6 CD8$^+$EGFP$^-$ T cells from Foxp3^{EGFP} mice (EGFP, blue line), 0.3×10^6 CD4$^+$EGFP$^-$ T cells from Foxp3$^{\Delta EGFP}$ and 0.15×10^6 CD8$^+$EGFP$^-$ T cells from Foxp3^{EGFP} animals (CD4Δ, red line), 0.3×10^6 CD4$^+$EGFP$^-$ from Foxp3^{EGFP} mice and 0.15×10^6 CD8$^+$EGFP$^-$ T cells from Foxp3$^{\Delta EGFP}$ mice (CD8Δ, green line), or 0.3×10^6 CD4$^+$EGFP$^-$ and 0.15×10^6 CD8$^+$EGFP$^-$ T cells from Foxp3$^{\Delta EGFP}$ animals (CD4/8Δ, purple line). Overall survival is depicted and represents cumulative results from three independent experiments. There was no difference in survival between mice transplanted with either CD4Δ or CD8Δ and CD4/8Δ marrow grafts. *$p < 0.05$, **$p < 0.01$.

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The absence of CD4+ or CD8+ iTregs results in an increase in the corresponding iTreg population

Given that we observed inferior survival only in transplant recipients that were incapable of making both CD4+ and CD8+ iTregs, we examined Treg reconstitution to determine whether the absence of either iTreg population during GVHD resulted in a compensatory increase in the corresponding Tregs. Animals that lacked the ability to make CD8+ iTregs had an increased percentage of CD4+ iTregs in the spleen and lung compared with GVHD control animals (EGFP) (Fig. 6A). Also, mice that were unable to reconstitute CD4+ iTregs had increased frequencies of CD8+ iTregs in the spleen, liver, and lung. No differences were observed in the colon for either iTreg population. To determine whether increased frequencies resulted in an absolute increase in iTregs, we also quantified absolute numbers of these cells in all tissue sites. We observed that mice that had the inability to make CD8+ iTregs had a significantly greater absolute number of CD4+ iTregs than animals that were functionally competent to make both Treg populations in the spleen and lung (Fig. 6B). Similarly, the absence of CD4+ iTregs was accompanied by more CD8+ iTregs in spleen, liver, and lung. Thus, frequencies and absolute numbers were concordant in all tissue sites. We observed small percentages/numbers of CD4+ and CD8+ iTregs in mice transplanted with CD4/8Δ grafts, which we attributed to minor contamination of the sort population with B6.SJL Foxp3^{EGFP} cells that were used to rescue B6 Foxp3$^{\Delta EGFP}$ mice at birth from lethal autoimmunity. Overall, these results indicated that there was a compensatory increase in iTregs in several GVHD target organs when mice were unable to reconstitute the corresponding iTreg population. This was a potential explanation for why the absence of a single iTreg population alone did not lead to an increase in GVHD mortality (Fig. 5C).

The absence of functional CD8+ and CD4+ iTregs results in enhanced donor T cell expansion and increased proinflammatory cytokine production

To determine why mice that lacked both CD4+ and CD8+ iTregs had accelerated mortality, whereas mice that were deficient in only one iTreg population did not, we examined what effect the absence of these populations had on donor T cell expansion and inflammatory cytokine production. To address this issue, animals were transplanted as in Fig. 5C and then euthanized 2 wk post-transplantation for analysis. Examination of spleen and GVHD target organ tissues revealed that there were significantly greater absolute numbers of activated donor CD4+ CD44^{high} CD62L^{low} and CD8+ CD44^{high} CD62L^{low} T cells within the spleen, lung, and liver of mice that were functionally deficient in both iTreg populations compared with animals that were competent to reconstitute both populations (Fig. 7A, 7B). On average, this amounted to a 2- to 4-fold increase in these tissue sites. An absence of CD8+ iTregs alone had more modest effects, but still resulted in increased numbers of activated CD4+ T cells in the spleen and lung, and donor CD8+ T cells in the spleen. The absence of CD4+ iTregs had no deleterious effect on donor T cell expansion in any tissue site when compared with GVHD controls.

The absolute number of CD4+ and CD8+ T cells that secreted IFN-γ was significantly higher (~2- to 4-fold) in the spleen, liver, and lung in animals that were unable to reconstitute both iTreg populations (Fig. 8A, 8B). These animals also had increased numbers of CD4+ IL-17+ T cells in the liver and lung, and CD8+ IL-17+ T cells in the lung (Fig. 8C, 8D). The absence of CD8+ iTregs alone resulted in a significant increase in the number of CD4+ IFN-γ+ T cells in the spleen and lung, CD8+ IFN-γ+ T cells in the spleen, and CD4+ IL-17+ T cells in the lung. Conversely, we observed an increase only in CD4+ IFN-γ+ T cells in the lung of mice that were functionally incapable of making CD4+ iTregs. Collectively, these data demonstrated that the absence of both CD4+ and CD8+ iTregs resulted in increased expansion of activated donor T cells, as well as a significant increase in the absolute number of CD4+ and CD8+ T cells that were capable of secreting the proinflammatory cytokines, IFN-γ and IL-17.

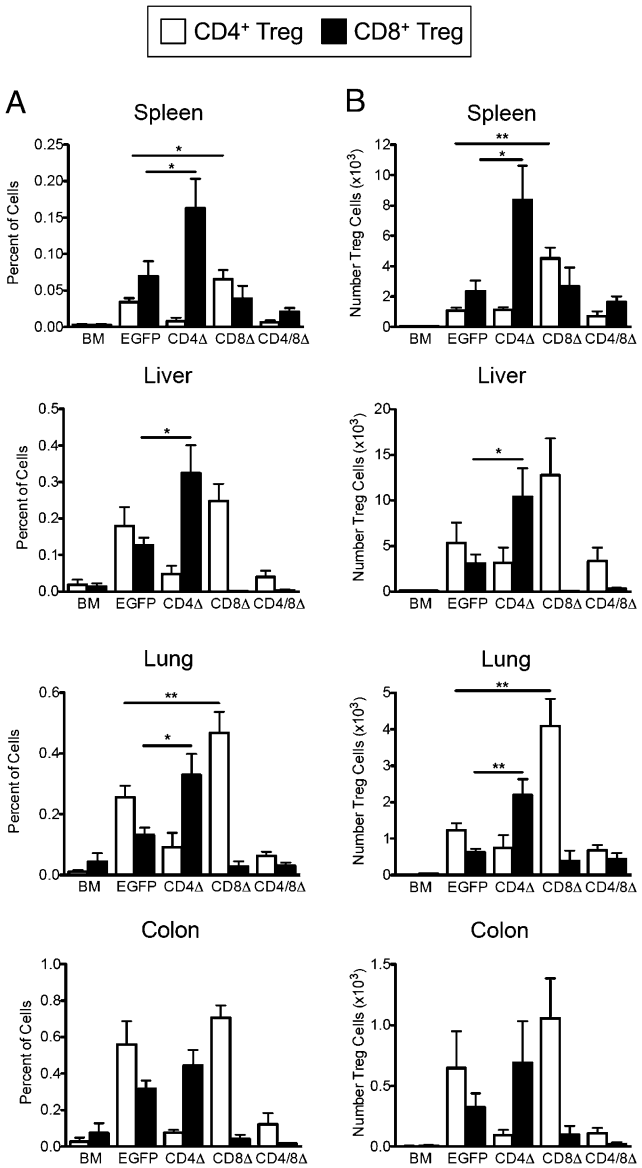

FIGURE 6. CD8+ and CD4+ iTreg reconstitution during GVHD. Lethally irradiated BALB/c mice (n = 13–14 per group) were transplanted with B6-Rag-1 BM alone (BM) or together with either 0.3 × 10^6 CD4+EGFP− and 0.15 × 10^6 CD8+EGFP− T cells from Foxp3^{EGFP} mice (EGFP), 0.3 × 10^6 CD4+EGFP− T cells from Foxp3$^{\Delta EGFP}$ and 0.15 × 10^6 CD8+EGFP− T cells from Foxp3^{EGFP} animals (CD4Δ), 0.3 × 10^6 CD4+ EGFP− from Foxp3^{EGFP} mice and 0.15 × 10^6 CD8+EGFP− T cells from Foxp3$^{\Delta EGFP}$ mice (CD8Δ), or 0.3 × 10^6 CD4+EGFP− and 0.15 × 10^6 CD8+EGFP− T cells from Foxp3$^{\Delta EGFP}$ animals (CD4/8Δ). Mice were euthanized at day 14 posttransplantation. (**A** and **B**) Percentage and absolute numbers of CD4+EGFP+ (□) and CD8+EGFP+ (■) iTregs in the spleen, liver, lung, and colon are depicted. Data are presented as mean ± SEM and are the cumulative results of three independent experiments. *$p < 0.05$, **$p < 0.01$.

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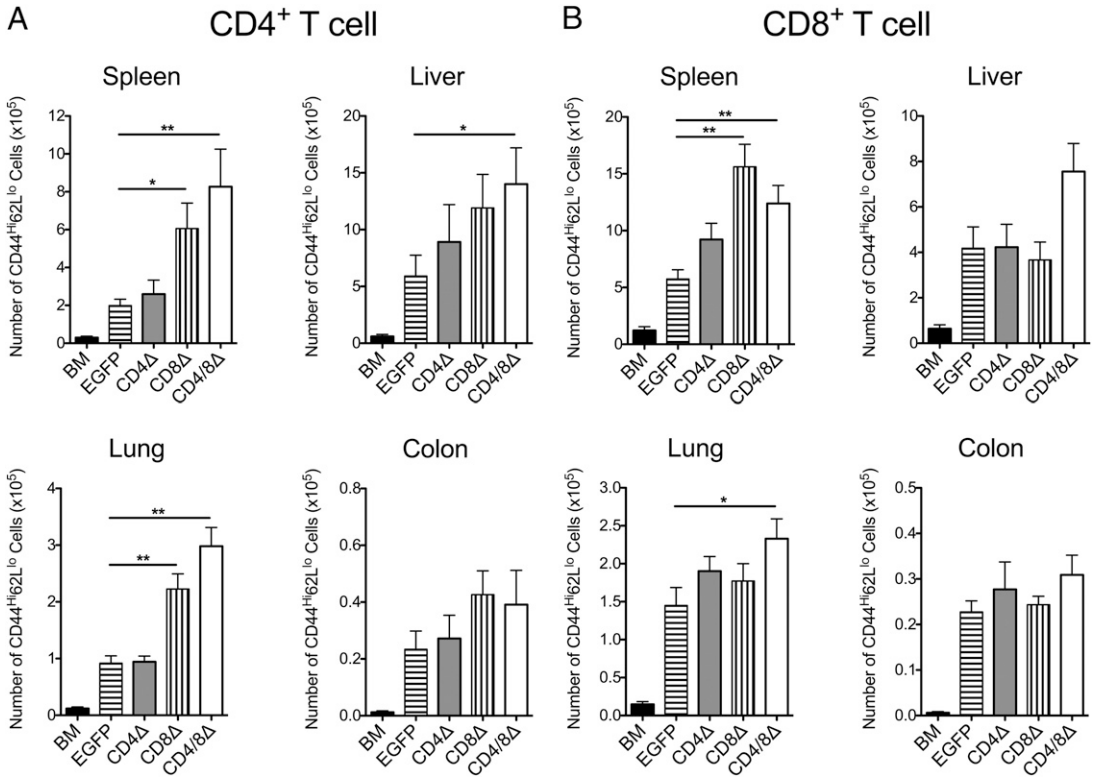

FIGURE 7. Increased donor T cell expansion in GVHD target organs in the absence of functional $CD8^+$ and $CD4^+$ iTregs. Lethally irradiated BALB/c mice (n = 13–14 per group) were transplanted with B6-Rag-1 BM alone (BM, black bar) or together with either 0.3×10^6 $CD4^+EGFP^-$ and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{EGFP}$ mice (EGFP, horizontally striped bar), 0.3×10^6 $CD4^+EGFP^-$ T cells from $Foxp3^{\Delta EGFP}$ and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{EGFP}$ animals (CD4Δ, grey bar), 0.3×10^6 $CD4^+EGFP^-$ from $Foxp3^{EGFP}$ mice and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{\Delta EGFP}$ mice (CD8Δ, vertically striped bar), or 0.3×10^6 $CD4^+EGFP^-$ and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{\Delta EGFP}$ animals (CD4/8Δ, white bar). Mice were euthanized at day 14 posttransplantation. (**A** and **B**) The absolute numbers of $CD4^+CD44^{high}CD62L^{low}$ (A) and $CD8^+CD44^{high}CD62L^{low}$ (B) T cells in the defined tissue sites is depicted. Data are the cumulative results of three independent experiments and are presented as the mean ± SEM. $*p < 0.05$, $**p < 0.01$.

The absence of $CD8^+$ iTregs alone also had more pronounced effects on T cell expansion and cytokine production than the isolated absence of $CD4^+$ iTregs.

Discussion

Understanding the role of specific Treg populations for the prevention of GVHD is of increasing importance given that Treg therapy is now in the early stages of implementation in BM transplant recipients. In initial studies, unselected $CD4^+$ $CD25^+$ T cells have been administered to allogeneic BMT recipients with the ultimate goal that this approach will attenuate GVHD severity (37, 38). To date, whereas cells have been able to be administered safely, it is not clear how potent they are in abrogating GVHD severity. Moreover, whether this is the optimal Treg population and to what extent various Treg subpopulations cooperate to maintain immune tolerance are undefined (39). It is against this backdrop that we examined whether other Treg populations might be important in the regulation of GVHD. The studies described in this work now define $CD8^+$ $Foxp3^+$ T cells as another Treg population that plays a role in the maintenance of transplantation tolerance in allogeneic stem cell transplant recipients.

$CD8^+$ Tregs were induced early during GVHD and constituted a sizable percentage of the total Treg pool within the first 10 d posttransplantation. This was surprising given that generation of these cells derives from the conventional T cell compartment because, in contrast to $CD4^+$ Tregs, constitutive Foxp3 expression on $CD8^+$ T cells in the thymus and in the peripheral T cell compartment of healthy mice is negligible (Supplemental Fig. 2). In fact, the absolute number of $CD8^+$ iTregs was significantly greater than $CD4^+$ iTregs when the contributions of both were comparatively analyzed in the absence of $CD4^+$ nTregs. Induction was not due to homeostatic expansion occurring in a lymphopenic environment, but rather was the consequence of alloreactivity (Fig. 2). We observed that there was a correlation between the degree of MHC disparity between donor and host, and the magnitude of $CD8^+$ Treg induction. A component of this was attributable to the increased percentage of total $CD8^+$ T cells early posttransplantation in recipients of completely MHC-mismatched grafts (i.e., B6→BALB/c). However, even after correcting for that factor, there was still a significantly higher percentage of $CD8^+$ Tregs in most GVHD target tissues when compared with animals that were reconstituted with MHC-matched, minor Ag-mismatched, or class I-mismatched grafts (Fig. 4). The explanation for this observation is not entirely clear, but may be due, in part, to a higher precursor frequency for alloantigens in the setting of a complete MHC mismatch because Foxp3 expression requires activation to be induced (20). Alternatively, it is possible that the strength of the T cell activation signal is more robust in this setting, which serves to enhance $CD8^+$ Treg induction. It should be noted, however, that there have been a number of reports in which $CD4^+$ iTreg generation unexpectedly did not occur despite strong immunological stimuli (11, 40–42). Consequently, the conditions that lead to the generation of iTregs in specific contexts are still incompletely understood.

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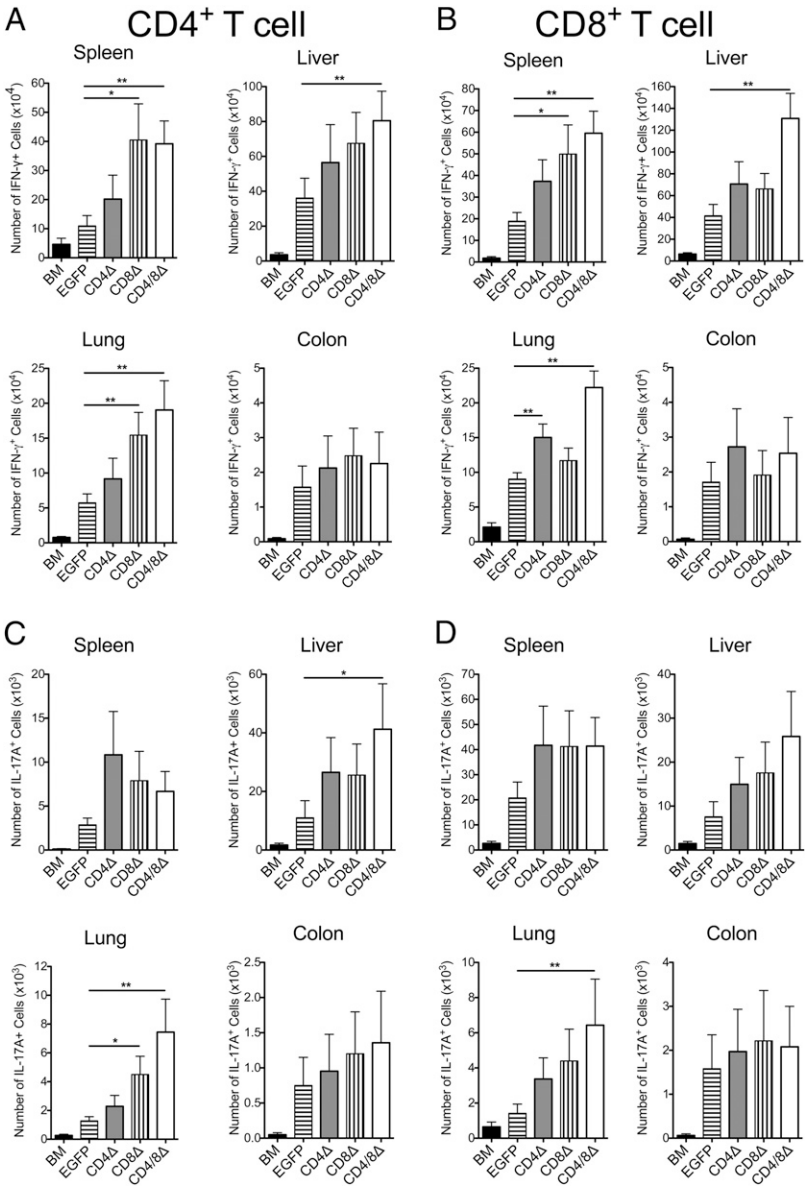

FIGURE 8. Increased numbers of IFN-γ– and IL-17–secreting T cells in GVHD target organs in the absence of functional $CD8^+$ and $CD4^+$ iTregs. Lethally irradiated BALB/c mice (n = 13–14 per group) were transplanted with B6-Rag-1 BM alone (BM, black bar) or together with either 0.3×10^6 $CD4^+EGFP^-$ and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{EGFP}$ mice (EGFP, horizontally striped bar), 0.3×10^6 $CD4^+EGFP^-$ T cells from $Foxp3^{\Delta EGFP}$ and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{EGFP}$ animals (CD4Δ, grey bar), 0.3×10^6 $CD4^+EGFP^-$ from $Foxp3^{EGFP}$ mice and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{\Delta EGFP}$ mice (CD8Δ, vertically striped bar), or 0.3×10^6 $CD4^+EGFP^-$ and 0.15×10^6 $CD8^+EGFP^-$ T cells from $Foxp3^{\Delta EGFP}$ animals (CD4/8Δ, white bar). Mice were euthanized at day 14 posttransplantation. (**A** and **B**) The absolute numbers of IFN-γ–producing $CD4^+$ (A) and $CD8^+$ (B) T cells in the specified tissue sites as determined by intracellular staining. (**C** and **D**) The absolute number of IL-17–secreting $CD4^+$ (C) and $CD8^+$ (D) T cells in the specified tissue sites is shown. Data are the cumulative results of three independent experiments and are presented as the mean ± SEM. $*p < 0.05$, $**p < 0.01$.

$CD8^+$ iTregs were found to be equally suppressive to $CD4^+$ Tregs when tested in vitro and expressed many of the same cell surface markers that defined the $CD4^+$ Treg compartment. To determine whether these cells had functional activity to suppress GVHD in vivo, we employed a murine BMT model in which the only Treg populations that can be generated are iTregs, as there were no transferred nTregs in the marrow graft. This allowed us to make valid comparisons between $CD4^+$ and $CD8^+$ iTregs. Under these conditions, we observed that mice that lacked both iTreg populations had accelerated mortality that was attributable to GVHD, whereas animals that could make either iTreg population had survival commensurate with that of GVHD control mice. Hence, at least one functional iTreg population was required to mitigate GVHD severity. $CD8^+$ iTregs could also compensate for the complete absence of $CD4^+$ iTregs, as mice transplanted with grafts deficient in $CD4^+$ iTregs had survival that was comparable to that of animals that could reconstitute both iTreg populations. This was evidence that $CD8^+$ iTregs had suppressive capability in vivo. This was further substantiated by the fact that mice that were selectively deficient in $CD8^+$ iTregs had significant increases in donor T cell expansion and proinflammatory cytokine production in some GVHD target organs (Figs. 7, 8). For the most part, enhanced immune reactivity in the absence of $CD8^+$ iTregs was observed in the spleen and the lung, raising the question as to whether these cells may have an important and preferential regulatory role within these tissue sites.

These results indicate that there appear to be compensatory regulatory mechanisms that are operative during GVHD. When $CD8^+$ iTregs were absent in recipient animals, there was a corresponding increase in the absolute number of $CD4^+$ iTregs in nearly all tissue sites (Fig. 6). We observed similar findings when $CD4^+$ iTregs were unable to be generated, although not to the same extent as for $CD8^+$ iTregs. We would speculate therefore that these reciprocal increases are an explanation for the fact that only in the absence of both iTreg populations was there an increase in GVHD lethality. The question then arises as to whether $CD4^+$ and $CD8^+$ iTregs have redundant or unique roles in the maintenance of transplantation tolerance. Prior studies have demonstrated that $CD4^+$ Tregs constitute a heterogeneous population that can be distinguished, in part by phenotypic differences that alter migration and function (43–45). Thus, it is not out of the question to postulate that $CD8^+$ iTregs may also have a functional niche that distinguishes them from $CD4^+$ Tregs. We observed that the absolute numbers, temporal appearance,

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and tissue localization differed between $CD4^+$ and $CD8^+$ iTregs, which is circumstantial evidence that they may have divergent roles in suppressing the graft-versus-host response. In this regard, it will be of interest to determine whether $CD8^+$ iTregs employ similar mechanistic pathways as $CD4^+$ Tregs to suppress GVHD. The latter cells have been shown to suppress immune responses through a variety of mechanisms that include the production of immune suppressive cytokines such as IL-10, inhibition of dendritic cell function, modulation of effector T cells, and secretion of cytotoxic molecules such as granzymes (46). Consequently, there may not be only one mechanistic pathway that is employed by $CD8^+$ iTregs.

A suppressive role for $CD8^+$ iTregs was noteworthy given that these cells were not detected in mice beyond 4 wk post-transplantation. Why $CD8^+$ iTregs appear to have limited persistence in vivo during GVHD is not clear. Because these cells were continuously exposed to recipient alloantigens, the absence of a persistent antigenic stimulus to enforce $CD8^+$ Treg induction does not appear to be a plausible explanation for this observation. One possible explanation is that the proinflammatory milieu may adversely impact the continued induction and survival of $CD8^+$ iTregs. In previous studies (13), we have shown that $CD4^+$ iTreg reconstitution is modest during GVHD in comparison with nTreg regeneration. Similar results have been reported by Bucher et al. (14), who also noted a very low frequency of $CD4^+$ iTregs in the spleen and colon of GVHD animals in which nTregs were not administered in the graft. However, in vivo-derived $CD4^+$ iTreg reconstitution can be enhanced by blockade of proinflammatory cytokines such as IL-6 and IL-21 (13, 14) with a corresponding reduction in GVHD severity. IL-6 exposure has also been shown to result in methylation of the upstream Foxp3 enhancer and in repression of Foxp3 expression, both of which inhibit Treg development and function (47). Alternatively, the inflammatory milieu could alter the availability of TGF-β, which is necessary for continued expression of Foxp3 in $CD4^+$ Tregs (48). In that regard, stability of Foxp3 expression is necessary for functional activity, and loss of expression has been shown to be one explanation for why in vitro differentiated $CD4^+$ iTregs have reduced in vivo survival (49). Thus, instability of Foxp3 expression could be another potential explanation for why $CD8^+$ iTregs are not detectable beyond 4 wk. Finally, it should be noted that these explanations are not mutually exclusive and could all be operative to some extent in limiting the persistence of these cells in GVHD recipients.

In summary, these studies define $CD8^+$ $Foxp3^+$ Tregs as a novel population of Tregs that emerge during the course of GVHD and serve to mitigate disease severity after allogeneic stem cell transplantation. These results also serve to highlight the heterogeneity that exists within the Treg population in GVHD biology. Understanding the complexity that is resident within these $CD4^+$ and $CD8^+$ Treg populations will be critical to the successful application of Treg therapy into the clinic.

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Disclosures

The authors have no financial conflicts of interest.

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