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TRANSPLANTATION

Peri-alloHCT IL-33 administration expands recipient T-regulatory cells that protect mice against acute GVHD

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Key Points

- Peri-alloHCT IL-33 delivery prevents acute GVHD through MAPK-dependent expansion of radiation-resistant recipient ST2$^+$ Tregs.
- IL-33–expanded Tregs regulate myeloid cell differentiation and activation, and limit effector T-cell accumulation in GVHD-target tissue.

During allogeneic hematopoietic cell transplantation (alloHCT), nonhematopoietic cell interleukin-33 (IL-33) is augmented and released by recipient conditioning to promote type 1 alloimmunity and lethal acute graft-versus-host disease (GVHD). Yet, IL-33 is highly pleiotropic and exhibits potent immunoregulatory properties in the absence of coincident proinflammatory stimuli. We tested whether peri-alloHCT IL-33 delivery can protect against development of GVHD by augmenting IL-33–associated regulatory mechanisms. IL-33 administration augmented the frequency of regulatory T cells (Tregs) expressing the IL-33 receptor, suppression of tumorigenicity-2 (ST2), which persist following total body irradiation. ST2 expression is not exclusive to Tregs and IL-33 expands innate immune cells with regulatory or reparative properties. However, selective depletion of recipient Foxp3$^+$ cells concurrent with peri-alloHCT IL-33 administration accelerated acute GVHD lethality. IL-33–expanded Tregs protected recipients from GVHD by controlling macrophage activation and preventing accumulation of effector T cells in GVHD-target tissue. IL-33 stimulation of ST2 on Tregs activates p38 MAPK, which drives expansion of the ST2$^+$ Treg subset. Associated mechanistic studies revealed that proliferating Tregs exhibit IL-33–independent upregulation of ST2 and the adoptive transfer of *st2*$^+$ but not *st2*$^-$ Tregs mediated GVHD protection. In total, these data demonstrate the protective capacity of peri-alloHCT administration of IL-33 and IL-33–responsive Tregs in mouse models of acute GVHD. These findings provide strong support that the immunoregulatory relationship between IL-33 and Tregs can be harnessed therapeutically to prevent GVHD after alloHCT for treatment of malignancy or as a means for tolerance induction in solid organ transplantation. (*Blood*. 2016;128(3):427-439)

Introduction

Interleukin-33 (IL-33) is an IL-1 cytokine with poorly understood pleiotropic immunologic functions.[1,2] It is expressed in the nucleus of epithelial cells in the steady state and upregulated in other cell types during inflammation and stress.[2-5] Depending upon the immunological context into which it is released, IL-33 can exacerbate both type 1 and type 2 immunity. In quiescent environments, IL-33 acts on cells expressing the IL-33 receptor suppression of tumorigenicity-2 (ST2), such as eosinophils,[6] basophils,[7,8] mast cells,[9] type 2 innate lymphoid cells (ILC2),[10,11] and CD4$^+$ effector T cells,[12] to stimulate robust secretion of the type 2 cytokines IL-5 and IL-13. IL-33–driven type 2 responses are protective against parasites,[13] however, IL-33–supported type 2 inflammation is involved in pathogenic allergic and fibrotic diseases.[11,14-21] Conversely, IL-12, a proinflammatory cytokine produced rapidly by myeloid antigen-presenting cells after exposure to pathogens, induces the expression of ST2 on CD8$^+$, as well as natural killer (NK) and NKT cells.[22-24] IL-12 signaling increases expression of T-bet, a transcription factor required for induction of ST2 on both CD4$^+$ and CD8$^+$ T cells.[22,25] The induction of ST2 on these type 1 effectors enables IL-33 to increase their proliferation and augment their interferon γ (IFNγ) production, which supports viral clearance.[4,25]

Allogeneic hematopoietic cell transplantation (alloHCT) is a common therapeutic modality for the treatment of hematologic malignancies. Unfortunately, the development of acute graft-versus-host

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disease (GVHD) is a complication that impacts upwards of 50% of recipients.[26] Acute GVHD results from type 1–dominated donor alloimmune responses injuring the skin, liver, and gastrointestinal tract.[26,27] Although typically treatable with immunosuppressants, GVHD causes significant morbidity, and the 15% of patients with refractory disease have mortality rates approaching 90%.[27] Conditioning therapy, involving total body irradiation (TBI) and/or chemotherapy, is required to eliminate recipient lymphocytes and allow for engraftment of donor stem cells.[28] TBI, however, also causes barrier tissue damage and ultimately generates a proinflammatory environment in the gut that supports GVHD.[29] Using IL-33–[30] and ST2-deficient[31] mice, we revealed that IL-33 is elevated in GVHD-target barrier tissues by conditioning with TBI or chemotherapy and augments detrimental $ST2^+$ donor T-cell–mediated type 1 alloimmune responses that caused lethal acute GVHD.[32] Developing the means to counter the proinflammatory properties of IL-33 should lessen the burden of GVHD.

Regulatory T cells (Tregs) suppress the alloimmune responses underlying GVHD and adoptive transfer of Tregs has shown therapeutic efficacy in early-stage clinical trials.[33-36] In addition to its potent type 1– and type 2–promoting capacity, a growing body of research supports a role for IL-33 in Treg immunobiology.[37-42] Our group and others have described a subset of $Foxp3^+$ Tregs that express $ST2$[37-39,42] and expand in response to IL-33.[37-39,43] IL-33–expanded Tregs play a role in protecting murine heart allografts from both acute[39] and chronic[43] rejection. $ST2^+$ Tregs exhibit an activated phenotype compared with their $ST2^-$ counterparts, even in the steady state, and display a superior ability to suppress $CD8^+$ T-cell IFNγ production.[38] In nonlymphoid compartments, $ST2^+$ Tregs are present at increased frequencies and would be well positioned to respond to IL-33 released from damaged tissue.[37,42] Despite these interesting observations, there are no definitive studies examining the impact of both donor and host IL-33–responsive Tregs after alloHCT. Also, given the ability of IL-33 to profoundly expand Tregs,[37-39] we investigated whether expansion of recipient IL-33–responsive Tregs by peri-alloHCT administration of IL-33 could overcome the deleterious effects of IL-33 post-TBI and alloHCT and found this to be the case.

Methods

Mice

C57BL/6 (B6; H-2^b), BALB/c (H-2^d), BALB/c Thy1.1, BALB/c CD45.1, B6-Tg(Foxp3-DTR/EGFP)23.2Spar/Mmjax (Foxp3-DTR), and B6 Foxp3-IRES-mRFP (FIR) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or National Cancer Institute (NCI) Mouse Repository (Frederick, MD). BALB/c $st2^{-/-}$ mice were obtained from Dr Andrew N. J. McKenzie[31] (MRC Laboratory of Molecular Biology, Cambridge, United Kingdom).

TBI and pre-TBI Treg depletion studies

B6 mice were treated with phosphate-buffered saline (PBS) or 1 μg of recombinant mouse IL-33 (BioLegend, San Diego, CA) by intraperitoneal injection every day for 10 days. On day 11, mice received lethal TBI (1100 cGy). Mice were euthanized on day 2 or day 4 post-TBI for analysis (nonirradiated control mice were used as the day 0 time point). For Treg depletion studies, Foxp3-DTR mice received 15 ng/g diphtheria toxin (DT; Sigma-Aldrich, St. Louis, MO) on day −11, and continued every other day thereafter through day −1 concurrently with IL-33 administration (day −10 through day −1).

AlloHCT and GVHD

Female recipient mice were exposed to lethal TBI (B6, 1100 cGy; BALB/c, 700 cGy) 1 day prior to alloHCT. On day 0, recipient mice were given 1×10^7 T-cell–depleted (TCD) allogeneic bone marrow (BM) cells alone or with 2×10^6 CD90.1-purified splenic allogeneic T cells by IV injection. For IL-33 peri-alloHCT studies, recipient mice were treated with PBS or 1 μg per mouse IL-33 from day −10 through day +4. For Treg depletion studies, Foxp3-DTR recipient mice received DT as in the previous section beginning on day −11, and continued every other day thereafter through day +3 concurrently with IL-33 administration (day −10 through day +4). For Treg adoptive transfer GVHD studies, 2×10^6 $CD4^+CD25^+$ spleen and lymph node cells from wild-type (WT; $st2^{+/+}$) or $st2^{-/-}$ BALB/c mice were transferred with 1×10^7 BALB/c TCD-BM and 4×10^6 CD25-depleted $CD3^+$ T cells, at a ratio of 1 Treg-to-2 T effectors. Mouse survival and clinical GVHD score were assessed as described.[32,44,45]

Other detailed methods

All other methods are described in detail in the supplemental Methods (available on the *Blood* Web site).

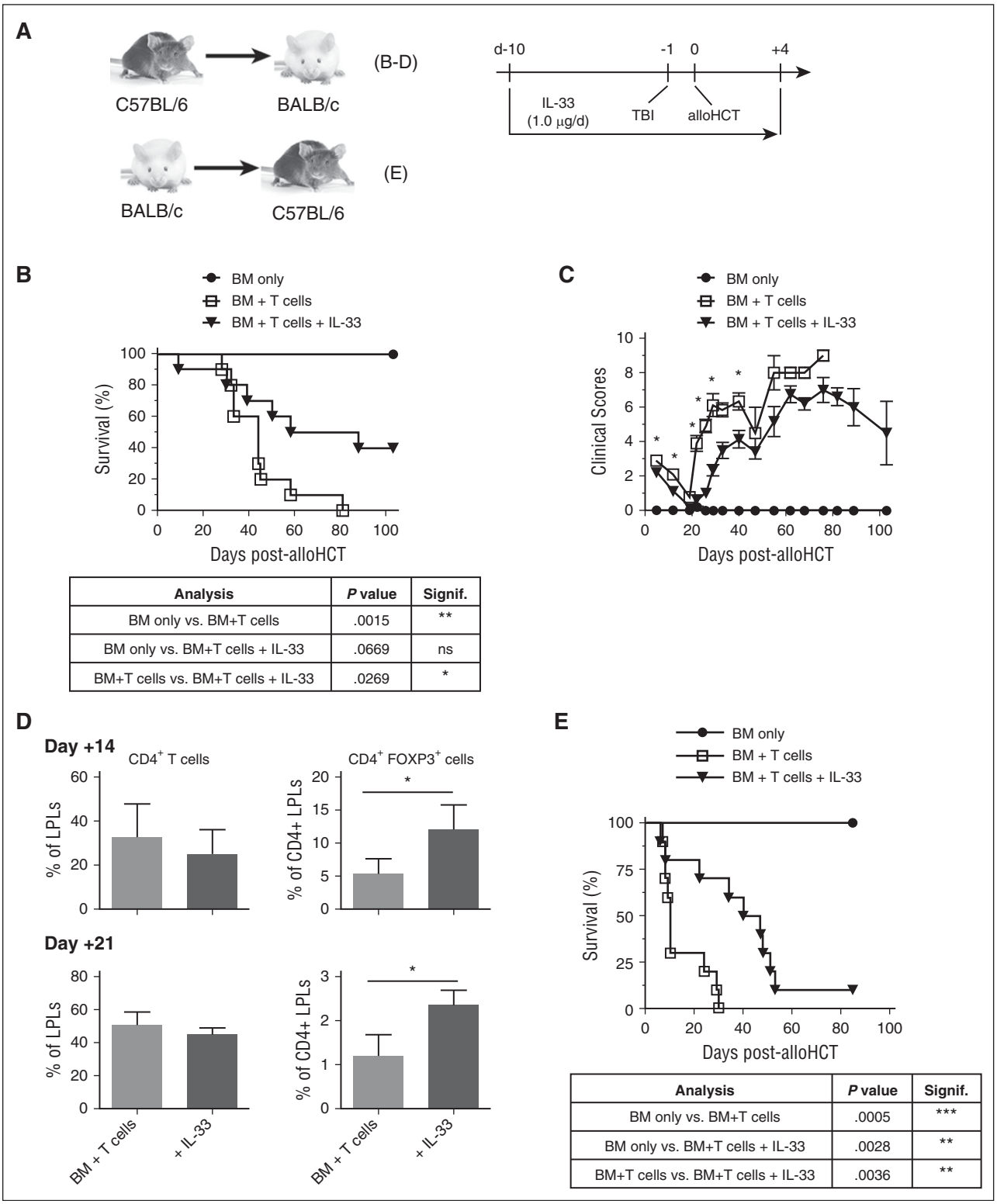
Results

Peri-alloHCT delivery of IL-33 to recipients protects against acute GVHD

We have demonstrated that recipient IL-33 released post-TBI and alloHCT promotes acute GVHD through stimulation of type 1 alloimmune responses.[32] The detrimental impact of released IL-33 was confirmed through alloHCT/GVHD experiments where administration of the IL-33 antagonist ST2-Fc, transplantation of $st2^{-/-}$ donor T cells, or transplant into $il33^{-/-}$ recipients, all prolonged survival and reduced type 1 alloimmune responses.[32] Yet, administration of IL-33 prolongs cardiac allograft survival, potentially through expansion of Tregs[39] or myeloid-derived suppressor cells (MDSCs),[42] or induction of type 2 responses.[43,46] Therefore, we tested whether peri-alloHCT conditioning of recipients with 1 μg of IL-33 (Figure 1A; day −10 through day +4) would protect against lethal acute GVHD. Compared with control mice, peri-alloHCT IL-33 administration prolonged BALB/c recipient mice survival (Figure 1B; mean survival time [MST] = 73 days for IL-33–treated mice vs MST = 44 days with vehicle alone; $P = .0269$). Approximately 50% of mice survived through day 100 post-alloHCT compared with vehicle-treated mice, which were all dead by day 80. Recipient protection from GVHD lethality was reflected in reduced clinical GVHD scores (Figure 1C). Assessment of GVHD-target tissues revealed that although the overall frequency of $CD4^+$ T cells in the colon lamina propria (LP) lymphocytes (LPLs) was not altered, an increased frequency of $CD4^+Foxp3^+$ cells in the LPLs was evident on day 14 and day 21 (Figure 1D). The benefits of peri-alloHCT IL-33 were also verified in studies where donor and recipient strains were reversed (BALB/c to B6; Figure 1E; MST = 43.5 days for IL-33–treated mice vs MST = 10 days with vehicle alone; $P = .0036$).

IL-33 expands $ST2^+$ Tregs and $CD11b^+F4/80^+Gr\text{-}1^{int}$ cells that persist following TBI

An ST2-expressing subset of $Foxp3^+$ Tregs, which undergoes expansion following administration of IL-33, has been identified.[38,39] Tregs also exhibit radiation resistance that elevates $Foxp3^+$ cell frequency within the $CD4^+$ T-cell population after exposure to TBI.[47] When we exposed mice to a lethal dose of TBI, we recapitulated these data[47] and established that the frequency of $CD4^+CD25^{hi}Foxp3^+$ Tregs increased relative to total $CD4^+$ T cells in the spleen through day +4 post-TBI (Figure 2A-C). The delivery of IL-33 for 10 days prior to TBI augmented this effect and an increased number of $CD4^+CD25^+$



Analysis	*P* value	Signif.
BM only vs. BM+T cells	.0015	**
BM only vs. BM+T cells + IL-33	.0669	ns
BM+T cells vs. BM+T cells + IL-33	.0269	*

Analysis	*P* value	Signif.
BM only vs. BM+T cells	.0005	***
BM only vs. BM+T cells + IL-33	.0028	**
BM+T cells vs. BM+T cells + IL-33	.0036	**

Figure 1. IL-33 conditioning peri-alloHCT protects against acute GVHD. (A) Indicated combinations of WT mice were administered IL-33 (1.0 μg per mouse per day) starting on day −10 prior to alloHCT. On day −1, recipient mice received lethal TBI (B-D, 700 cGy to BALB/c recipients; E, 1100 cGy to B6 recipients) followed by 1×10^7 WT allogeneic TCD-BM (B-D, B6; E, BALB/c) alone or with 2×10^6 allogeneic pan T cells (B-D, B6; E, BALB/c) on day 0. IL-33 was continued through day +4 posttransplant. PBS vehicle-treated mice were used as BM + T only controls. (B,E) Survival curves with statistical significances calculated by log-rank (Mantel-Cox) test (*$P < .05$, **$P < .01$). For panels B-C, n = 4 (BM only), 10 (BM + T cells), and 10 (BM + T cells + IL-33) and the depicted experiment is representative of 2 independent experiments completed. For panel E, n = 5 (BM only), 10 (BM + T cells), and (10 BM + T cells + IL-33). (C) Group means with SEM are depicted for clinical GVHD scores. Significant differences for each point with ≥3 mice per group were calculated between BM + T cells vs BM + T cells + IL-33 using an unpaired Student *t* test (*$P < .05$). (D) Average frequency and standard deviation (SD) of indicated colon LPL population. Three to 4 mice per group with statistical significance calculated between groups using an unpaired Student *t* test (*$P < .05$). d, day; ns, not significant.

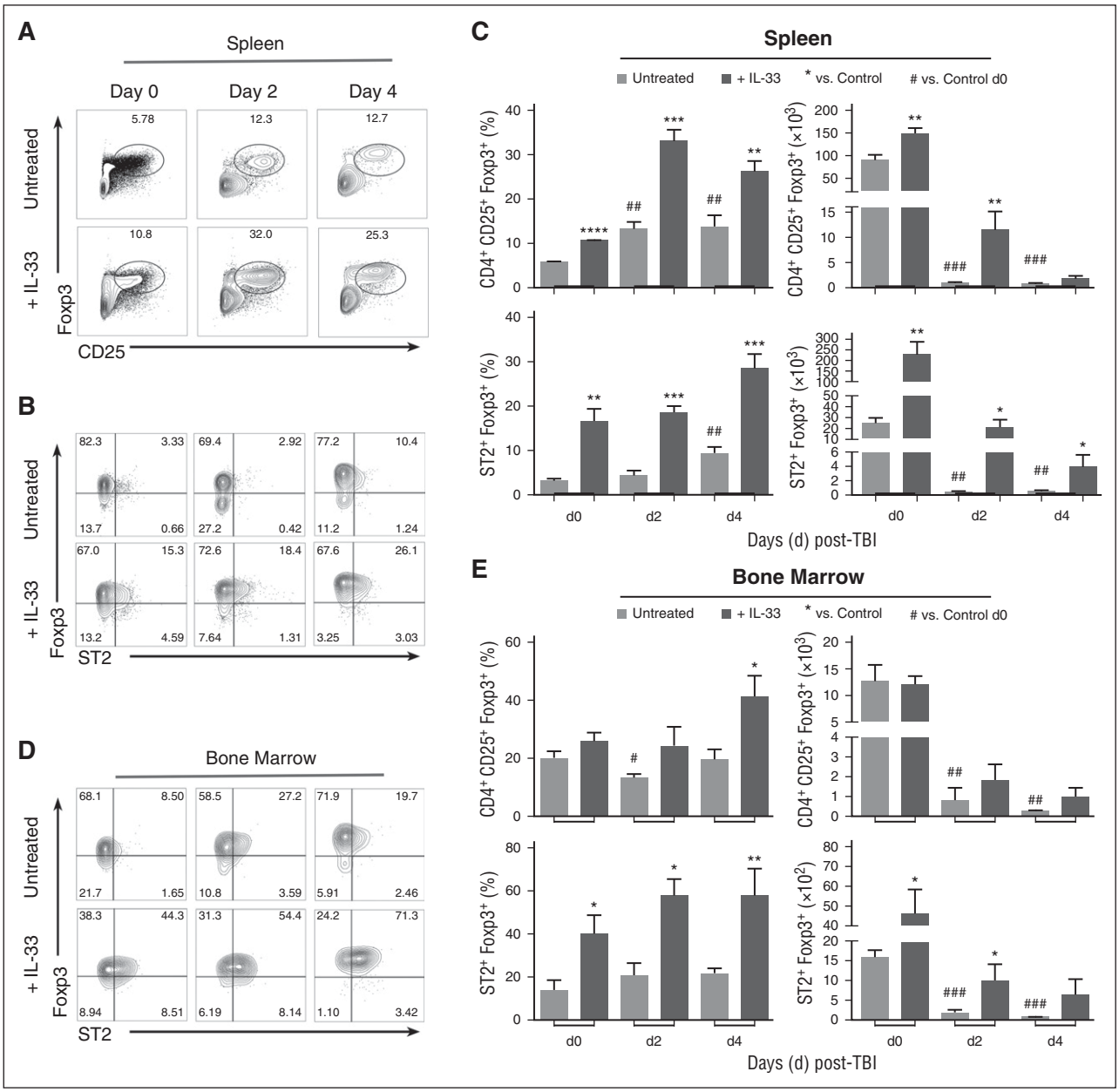


Figure 2. IL-33 and TBI expands ST2$^+$ Tregs that persist through day 4 post-TBI. B6 mice were administered IL-33 (1.0 μg per mouse per day) or PBS (control) for 10 days and then exposed to lethal TBI (1100 cGy). Mice were sacrificed on day 2 or day 4 post-TBI, and nonirradiated mice were sacrificed on day 0. (A) Representative contour plots of CD3$^+$CD4$^+$-gated splenocytes showing Foxp3 vs CD25 expression from control (PBS) and IL-33–treated mice before and after TBI. (B) CD4$^+$CD25$^+$-gated splenocytes showing Foxp3 vs ST2 expression. (C) Summary graphs for the frequency (%) and cell number with SD the mean for the indicated populations. Average from n = 3-4 mice per group, and statistical significance was calculated between each group using an unpaired Student t test (*$P < .05$, **$P < .01$, ***$P < .001$); *IL-33–treated vs control at each time point; #significance for day 2 or day 4 control vs control day 0. Data are representative of at least 4 independent experiments. (D) CD4$^+$CD25$^+$-gated BM cells showing Foxp3 vs ST2 expression. (E) Summary graphs for the frequency (%) and cell number of indicated BM populations. Statistical significances were calculated as in panel C. Three to 4 mice per group and data are representative of 2 independent experiments.

Tregs was observed in the spleen (Figure 2C) at day 0 and day 2 post-TBI. These increases were not found in the BM (Figure 2E). As expected,[38,39] IL-33 administration increased both the day 0 frequency and number of splenic ST2$^+$ Treg cells (Figure 2B-C). Similar increases in ST2$^+$ Tregs were observed in the BM (Figure 2D-E). ST2$^+$ Tregs displayed considerable resistance to TBI, as the frequency of viable ST2$^+$ Tregs remained increased in both locations (spleen; BM) in IL-33–treated mice through day +4 post-TBI (Figure 2). The frequency of ST2$^+$ Tregs was also increased in untreated mice spleens at day +4 post-TBI (Figure 2B-C).

In addition to driving Treg expansion, IL-33 administration increases the frequency of myeloid cells, including MDSCs[39,43] and macrophages.[39] We confirmed IL-33 expansion of splenic CD11b$^+$F4/80$^+$Gr-1^{int} cells (day 0; supplemental Figure 1A-B) that remain increased relative to control mice in number and frequency in the spleen through day 2 post-TBI (supplemental Figure 1A-B). Similar changes were not, however, observed in the BM (supplemental Figure 1C-D). The increase in splenic CD11b$^+$F4/80$^+$Gr-1^{int} cells was associated with a corresponding decrease in frequency of CD11b$^+$F4/80$^-$Gr-1^{hi} cells (supplemental Figure 1A-B). Expansion of the CD11b$^+$F4/80$^+$Gr-1^{int}

subset was dependent upon IL-33 administration, as TBI alone did not increase CD11b$^+$F4/80$^+$Gr-1^{int} cells, but instead skewed the frequency of CD11b$^+$ cells toward CD11b$^+$F4/80$^-$Gr-1^{hi} cells (supplemental Figure 1A-B).

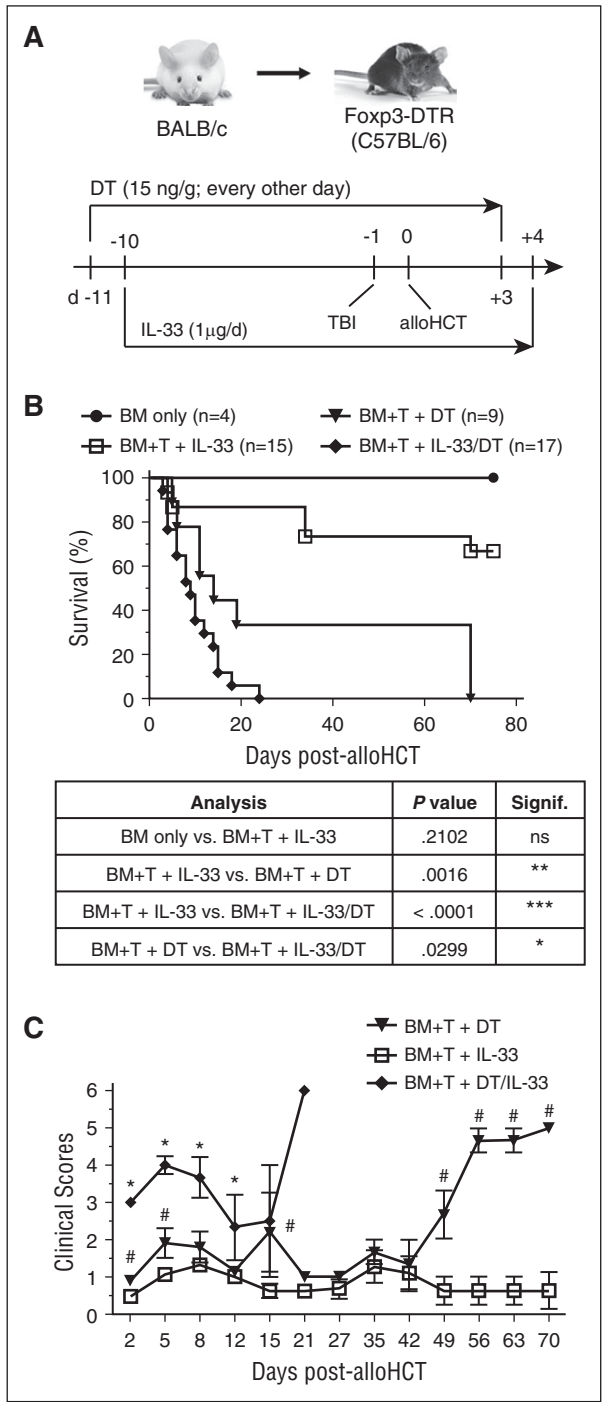
Tregs mediate the GVHD protection associated with peri-alloHCT delivery of IL-33

The capacity of IL-33 to prolong experimental cardiac allograft survival is dependent on Tregs, as antibody-mediated depletion of CD25$^+$ cells before transplantation and IL-33 administration eliminates this therapeutic effect.[39] To test the role of Tregs in protection against acute GVHD by peri-alloHCT delivery of IL-33, Foxp3-DTR recipient mice were administered DT concurrently during IL-33 administration (Figure 3A). Peri-alloHCT administration of IL-33 in Foxp3-DTR recipients resulted in ~70% of mice surviving through day 80 posttransplant, whereas IL-33 delivery concurrent with depletion of Tregs significantly accelerated acute GVHD lethality as compared with DT alone (MST = 14 days vs 9 days, $P < .03$; Figure 3B). Accelerated GVHD lethality in IL-33 delivery with DT was reflected in an early elevation in clinical score (Figure 3C). These findings support the capacity of IL-33–expanded recipient Tregs in controlling early IL-33–driven inflammatory events that lead to acute GVHD.

Tregs restrain myeloid cell expansion and polarization following IL-33 administration

In addition to suppressors of other T cells, Tregs are potential regulators of myeloid cell–mediated inflammation. Treg depletion results in macrophage polarization toward the proinflammatory M1 phenotype after myocardial infarction.[48] Likewise, Treg depletion causes accumulation of CD11b^{hi}Ly-6C^{hi} inflammatory monocytes in injured muscle.[49] Knowing that administration of IL-33 expands both splenic myeloid cells and Tregs and that Tregs are required for the therapeutic benefits of IL-33 against GVHD, we tested how Tregs influenced the myeloid compartment during IL-33 administration using Foxp3-DTR mice. Depletion of Tregs with DT or IL-33 administration alone increased splenocyte numbers compared with naive controls, and combined DT and IL-33 resulted in a threefold increase in total splenocytes (Figure 4A). Treg depletion by DT alone or with IL-33 was nearly complete (Figure 4B). As described in the preceding 2 paragraphs (supplemental Figure 1), IL-33 administration increased CD11b^{hi} cells, predominantly the F4/80$^+$Gr-1^{int} subset (Figure 4D). Depletion of Tregs, both alone or with IL-33, greatly augmented the frequency of CD11b$^+$F4/80$^-$Gr-1^{hi} cells (Figure 4D). Thus, IL-33 expanded potential monocytic MDSCs and macrophages (F4/80$^+$Gr-1^{int} cells), whereas Treg depletion instead promoted the expansion of neutrophils or granulocytic MDSCs (Gr-1^{hi}F4/80$^-$; Figure 4D). Confirming that these results were due to the elimination of Tregs and not as a result of DT toxicity, we verified that DT treatment of WT mice did not impact the myeloid or Treg compartments (supplemental Figure 2). When flow-sorted CD11b$^+$F4/80$^+$Gr-1^{lo} and CD11b$^+$F4/80$^-$Gr-1^{hi} cells were evaluated in a suppression assay, it was the Gr-1^{hi} subset that possessed ex vivo T-cell–suppressive capacity (Figure 4E).

This suppressive capacity was reduced by Treg depletion, particularly with concurrent IL-33 delivery (Figure 4E). The CD11b$^+$F4/80$^+$Gr-1^{lo} subset was not suppressive (Figure 4E). CD11b$^+$F4/80$^+$Gr-1^{lo} cells from IL-33–treated and Treg-depleted mice were compared by microarray to IL-33–treated and Treg-sufficient mice. Cells from Treg-depleted mice displayed a transcriptional profile consistent with active IFNγ signaling and M1 macrophage polarization[50] (increased STAT1, IRF1, IL-6, GBP3, -4, -7, and H2-A^b; Figure 4F). From these data, we concluded that Tregs, including IL-33–expanded Tregs, limit the frequency of CD11b$^+$F4/80$^-$Gr-1^{hi} neutrophils and support granulocytic MDSC suppression. They do not, however, impact on the frequency of CD11b$^+$F4/80$^+$



Analysis	*P* value	Signif.
BM only vs. BM+T + IL-33	.2102	ns
BM+T + IL-33 vs. BM+T + DT	.0016	**
BM+T + IL-33 vs. BM+T + IL-33/DT	< .0001	***
BM+T + DT vs. BM+T + IL-33/DT	.0299	*

Figure 3. Tregs mediate the GVHD-protective capacity of peri-alloHCT delivery of IL-33. (A) Foxp3-DTR B6 mice were administered 15 ng/g DT starting on day −11 (every other day) concurrently with daily IL-33 administration starting on day −10. On day −1, mice received lethal TBI (1100 cGy) followed by 1 × 10^7 WT BALB/c TCD-BM alone or with 2 × 10^6 BALB/c pan T cells on day 0. DT was continued through day +3 and IL-33 through day +4 posttransplant. (B) Resulting survival curves with statistical significances calculated by log-rank (Mantel-Cox) test (*$P < .05$, **$P < .01$, ***$P < .001$). (C) Recorded clinical scores are presented. Significant differences for each point with ≥3 mice were calculated between: (1) BM + T cells + IL-33 vs BM + T cells + DT/IL-33 (*$P < .05$) or (2) BM + T cells + DT vs BM + T cells + IL-33 (#$P < .05$) using an unpaired Student *t* test.

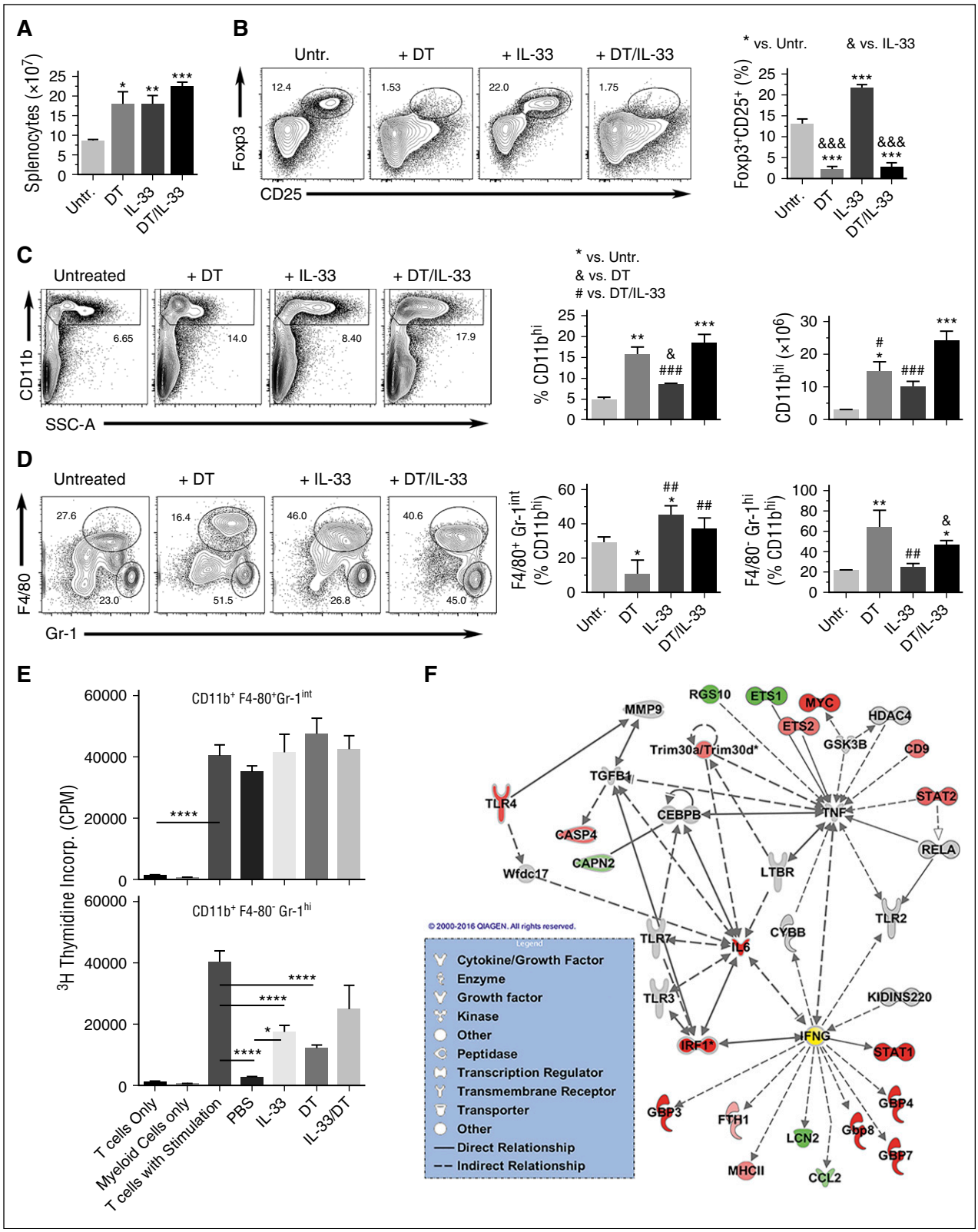


Figure 4. Tregs, including those expanded by IL-33, restrain the development of proinflammatory attributes by granulocytes and macrophages. Foxp3-DTR B6 mice were administered 15 ng/g DT on day −11 through day −1 (every other day) concurrently with IL-33 (day −10 through day −1). On day 0, spleens were harvested and splenocytes stained for multicolor flow cytometric analysis. (A) Assessed total splenocyte numbers for indicated groups (n = 3-4 mice per group): untreated (Untr.), DT only (DT), IL-33 only (IL-33), concurrent IL-33 and DT (DT/IL-33). (B) Representative contour plots and graphical analysis assessing Treg depletion during IL-33 treatment. Plots show Foxp3 vs CD25 expression on $CD4^+$-gated cells. Graph presents the average frequency of $CD25^+Foxp3^+$ cells. (C) Representative flow plots and graphical analysis of $CD11b^+$ cells in response to Treg depletion during IL-33 administration. (D) Representative flow plots and graphs showing F4/80 vs Gr-1 on $CD11b^+$ cells. All graphs depict averages and SD from 3 to 4 mice per group and are representative of 2 independent experiments. Indicated significant differences were calculated using an unpaired Student *t* test (*$P < .05$, **$P < .01$, ***$P < .01$). (E) $CD3^-CD11b^+F4/80^+Gr\text{-}1^{lo}$ and $CD3^-CD11b^+$ $F4/80^-$ $Gr\text{-}1^{hi}$ cells were flow sorted from the spleens of day 0 IL-33– or

macrophages, which are expanded by IL-33 and prone to M1 polarization in the absence of Tregs.

IL-33–expanded Tregs control myeloid cell activation and effector T-cell accumulation in GVHD-target tissue

Analysis of the myeloid cell compartment following IL-33 administration with Treg depletion showed that Tregs shape $CD11b^+$ myeloid cell expansion and influence their differentiation. Further analysis on day 7 post-alloHCT revealed that, although IL-33 did not significantly alter the frequency of splenic $CD11b^+F4/80$ or $CD11b^+Ly6G^{hi}$ cells over nontreated recipient mice (data not shown), IL-33 administration and associated Treg expansion supported a population of $F4/80^+$ $CD11b^+$ cells with phenotypically reduced stimulatory capacity (Figure 5A). IL-33 delivery with Treg depletion instead resulted in $F4/80^+CD11b^+$ cells consistent with M1 macrophages[50] as they displayed elevated surface expression of CD86 and major histocompatibility complex (MHC) class II ($I\text{-}A^b$) and intracellular IL-12p40 (Figure 5A). The extent of Treg expansion/deletion was confirmed in both the spleen and LP of the small intestine (SI), which is a key target tissue affected by GVHD (Figure 5B-C left panels). In the presence of Tregs, IL-33 treatment promoted accumulation of donor T cells in the spleen (Figure 5B right panel). In contrast, IL-33 administration with concurrent Treg depletion ablated donor T-cell splenic accumulation and permitted T-cell infiltration of the LP (Figure 5C right panel). These data indicate that IL-33 treatment expands Tregs that suppress macrophage maturation/M1 polarization, which may be important to reduce donor T-cell activation and exodus from the secondary lymphoid tissues. Likewise, IL-33–expanded Tregs protect against GVHD by suppressing effector T-cell infiltration of GVHD-target tissues.

IL-33–mediated ST2 signaling activates p38 MAPK to drive Treg expansion

Our investigations into the impact of IL-33 on Treg function revealed a selective expansion of $ST2^+Foxp3^+$ Tregs driven by IL-33–stimulated IL-2 production by $CD11c^+$ dendritic cells (DCs).[38] These findings did not probe whether IL-33 supported Treg induction or if its role was due to the expansion of thymic-derived Tregs. To address this point, we sorted $CD4^+CD25^+Foxp3^+$ Tregs and $CD4^+Foxp3^-CD62L^{hi}$ naive T cells (non-Tregs) from Foxp3-reporter mice. Culture with BALB/c $CD11c^+$ BM-derived DCs (BMDCs) showed that both IL-2 and IL-33 exhibit a similar capacity to expand Tregs (supplemental Figure 4A). Although roughly a third of the proliferating cells (CTV^{lo}) express ST2 in response to IL-2, ~70% of the proliferating Tregs expressed ST2 in cultures with IL-33 (supplemental Figure 4A). In contrast, the addition of IL-33 to non-Treg cultures did not induce Foxp3 expression or support ST2 expression on these cells (supplemental Figure 4B). These data highlight the critical role of IL-33 in expansion, but not induction, of Tregs as assessed in vitro in this assay.

To establish the functional relationship between IL-33 and ST2 on Tregs after BM transplantation, we infused sorted $ST2^+$ or $ST2^-$ Tregs into WT or $il33^{-/-}$ B6N mice 1 day after lethal irradiation. Recipient mice also received a syngeneic BM transplant to maintain IL-33^+ or IL-33 knockout (KO) conditions. These studies revealed that, whereas $ST2^+$ and $ST2^-$ Tregs display equal viability and both migrate to the spleen and BM (Figure 6A-C; supplemental Figure 5), $ST2^+$ Tregs are more proliferative in both locations (Figure 6; supplemental Figure 5). More importantly, these studies establish that proliferating Tregs express higher levels of ST2, $ST2^-$ Tregs can induce ST2, and increased ST2 expression on Tregs does not require IL-33 (Figure 6A-B). Finally, these examinations demonstrated that recipient IL-33 drives the proliferation of $ST2^+$ Tregs after transplantation (Figure 6; supplemental Figure 5).

We next sought to uncover the signaling pathways underlying $ST2^+$ Treg IL-33–induced proliferation. In mast cells[51,52] and basophils,[7] IL-33 activates NF-κB and MAPKs (Erk1/2, p38, and JNK) downstream of ST2. We also observed phosphorylation of p38 MAPK and downstream NF-κB signals (p-p65) in flow-sorted $ST2^+$ Tregs following IL-33 stimulation (Figure 6D). IL-33, however, did not cause phosphorylation of Erk1/2 or JNK (supplemental Figure 6). Although both are implicated in Treg proliferation,[53,54] IL-33 did not modify STAT5 or mammalian target of rapamycin–related S6 kinase phosphorylation (supplemental Figure 6). The importance of p38 activity to $ST2^+$ Treg proliferation was uncovered in subsequent studies utilizing a p38 MAPK-selective inhibitor and $CD4^+$ T-cell culture with allogeneic BMDCs. Although IL-33 induced a nearly threefold increase in proliferating $ST2^+$ Tregs stimulated with BMDCs compared with media alone, inhibition of p38 MAPK selectively blunted the proliferation of $ST2^+$, but not $ST2^-$, Tregs (Figure 6E-G). ST2 signaling resulted in NF-κB activation, however, NF-κB inhibition did not block $ST2^+$ Treg expansion (Figure 6D-E). These data identify IL-33–mediated activation of p38 as a pivotal mechanism for $ST2^+$ Treg expansion.

IL-33 responsiveness is critical for Treg-protective capacity against acute GVHD

We demonstrated that endogenous IL-33 is upregulated and released from GVHD-target tissue post-TBI, where its dominant function was to drive lethal alloreactive donor T-cell responses.[32] Although IL-33 expands Tregs[38,39,41] and supports Treg stability,[37] when physiologic ratios of donor Tregs to non-Tregs were transferred into alloHCT recipients, endogenous or exogenous IL-33 stimulation of Tregs posttransplant was insufficient to prevent lethal GVHD.[32] The data depicted in Figures 1-3 suggest that, by increasing recipient Tregs before alloHCT with exogenous IL-33, we can tip the immunologic balance to where IL-33–responsive Tregs counter IL-33–driven alloreactive responses supporting GVHD. We wanted to test this concept further and directly assessed whether adoptively transferred Treg responsiveness to IL-33 contributes to their therapeutic capacity. Following TBI, purified $st2^{+/+}$ (WT) or $st2^{-/-}$ $CD4^+CD25^+$ cells from BALB/c mice were adoptively transferred along with WT BALB/c donor TCD-BM and CD25-depleted T cells into WT B6 hosts (Figure 7). As expected,[55,56] WT $CD4^+CD25^+$ cells protected against acute GVHD

Figure 4 (continued) IL-33/DT-treated B6 Foxp3-DTR mice and assessed in an ex vivo suppression assay. Data represent the average and standard error of the mean (SEM) from 3 mice per group. Significant differences were calculated using an unpaired Student *t* test (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$). (F) Differential gene expression was also assessed by microarray between $CD3^-CD11b^+F4/80^+Gr\text{-}1^{lo}$ cell populations from day 0 IL-33– or IL-33/DT-treated B6 Foxp3-DTR mice (n = 3 mouse per group). Partek calculated fold change values and associated *P* and *q* values were assessed using Ingenuity Pathway Analysis (IPA) and identified IFNγ as an active upstream regulator in $CD3^-CD11b^+F4/80^+Gr\text{-}1^{lo}$ cells in the absence of Treg (*z* score, 2.137; *P*, 8.56E-06). The schematic is a graphical representation of the IFNγ signaling pathway in $CD3^-CD11b^+F4/80^+Gr\text{-}1^{lo}$ cells from IL-33–/DT- vs IL-33 only-treated mice. The level of upregulation is indicated by intensity of red color at that node. Gray nodes are part of network, but were not significantly modified between IL-33– or IL-33/DT-treated B6 Foxp3-DTR mice samples. Solid lines indicate direct relationships; dashed lines depict indirect relationships. Yellow color represents predicted upstream regulators. CPM, counts per minute; GSK, glycogen synthase kinase; LTBR, lymphotoxin β receptor; MMP, matrix metalloproteinase; SSC, side scatter; TLR, Toll-like receptor; TNF, tumor necrosis factor.

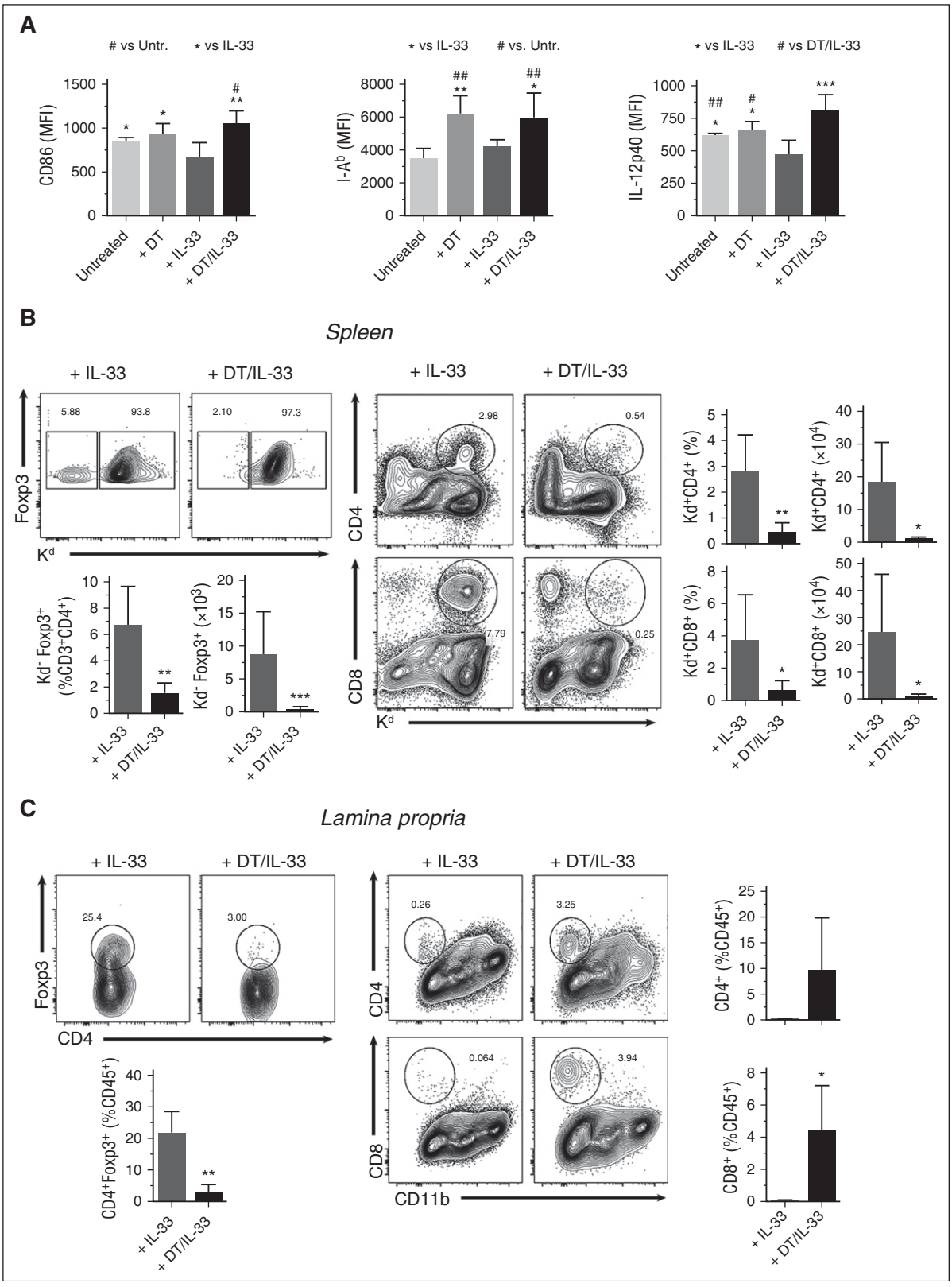


Figure 5. IL-33–expanded recipient Tregs are critical regulators of macrophage activation and accumulation of effector T cells in GVHD-target tissue. Foxp3-DTR B6 mice were administered 15 ng/g DT starting on day −11 (every other day) concurrently with daily IL-33 administration starting on day −10. On day −1, mice received lethal TBI (1100 cGy) followed by 1×10^7 WT BALB/c TCD-BM alone or with 2×10^6 BALB/c pan T cells on day 0. DT was continued through day +3 and IL-33 through day +4 posttransplant. On day 7 post-alloHCT, splenocytes and LPLs isolated from the SI were subjected to flow cytometry. (A) Flow cytometric analysis of $CD11b^+F4/80^+$-gated splenocytes presented as mean with SD for MFI of CD86, MHC class II/I-A^b, and IL-12p40 on $F4/80^+CD11b^+$ cells (n = 5 per group). (B-C) Flow cytometric and graphical analysis of spleen (B) and SI LPLs (C) on day 7 post-alloHCT from mice treated with IL-33 alone or in combination with DT. (B) Left panel, $CD4^+Foxp3^+$-gated cells. Right panel, $CD3^+$-gated cells. Analysis of $Foxp3^+$ Tregs and CD4 and CD8 effector T cells. Statistical significance between groups was calculated by Student *t* test. In panels B-C, $^*P < .05$, $^{**}P < .01$ for DT/IL-33 vs IL-33. MFI, mean fluorescence intensity.

compared with mice receiving BM and effector T cells alone (Figure 7B; $P < .001$ vs BM + T). Conversely, *st2*$^{-/-}$ CD4$^+$CD25$^+$ cells displayed reduced protective capacity relative to WT Tregs (Figure 7B). Specifically, where WT Tregs promoted long-term survival in 90% of recipients compared, only 10% of mice that received *st2*$^{-/-}$ cells (Figure 7B; $P = .0017$, *st2*$^{+/+}$ vs *st2*$^{-/-}$) were protected. These findings are consistent with critical, IL-33–mediated protective Treg functions after alloHCT.

Our studies demonstrate that IL-33 drives ST2$^+$ Treg proliferation ex vivo and in vivo. To characterize how a lack of IL-33–responsiveness impacts adoptively transferred Treg frequency, irradiated B6 recipients received CD4$^+$CD25$^+$ cells from *st2*$^{+/+}$ or *st2*$^{-/-}$ BALB/c mice, Th1.1$^+$ BALB/c TCD-BM, and CD25-depleted CD45.1$^+$CD3$^+$ T cells. Although overall spleen and SI LPLs numbers were unchanged (supplemental Figure 7), when splenocytes and LPLs from the recipients of *st2*$^{+/+}$ CD4$^+$CD25$^+$ cells were assessed, increased frequency of donor Tregs was present in the spleen and SI compared with recipients of *st2*$^{-/-}$ CD4$^+$CD25$^+$ cells (Figure 7D-E). Consistent with our observation that IL-33–expanded Tregs protect against GVHD by suppressing effector CD8$^+$ T-cell infiltration of GVHD-target tissues (Figure 5), mice receiving *st2*$^{-/-}$ Tregs also exhibited increased CD8$^+$ T cells in the SI (Figure 7D-E). These findings suggest an important role for the IL-33/ST2 axis in increasing the frequency of adoptively transferred Tregs, which may support their protection against GVHD.

Discussion

Since it was first described as a type 2 cytokine inducer a decade ago,[52] IL-33 has emerged as a pleiotropic IL-1 cytokine with the ability to augment type 1[4,22] responses and drive immune regulation.[37-41] Although mechanisms controlling IL-33 pleiotropy are unclear, its activity is controlled by both the regulated availability of ST2-expressing cells, as well as the presence of proinflammatory cytokines.[57,58] Recipient conditioning prior to alloHCT causes a breach in the intestinal epithelium that allows commensal bacteria to trigger proinflammatory cytokine secretion by local macrophages and DCs.[27] Recipient-derived IL-33 released into this inflammatory milieu augments the type 1 donor alloimmune responses causing lethal acute GVHD.[32] Data from solid organ transplant models, however, suggest that the regulatory properties of IL-33 can be harnessed to create a type 2/Treg-dominated environment that promotes allograft survival.[39,43,46] Findings in other rodent models indicate that IL-33–stimulated Tregs are important for control of immune-mediated inflammation of the gut, but their immunoregulatory capacity may be limited by exposure to IL-23.[37,59,60] These data fit with our current demonstration that recipient peri-alloHCT IL-33 treatment, well in advance of TBI-induced inflammation, provided Treg-mediated protection against acute GVHD. Likewise, in the absence of recipient Tregs, the effect of peri-alloHCT delivery mimicked that of post-alloHCT delivery,[32] in that it accelerated GVHD lethality (Figure 3). Importantly, we also provide novel mechanistic observations establishing that the Treg-protective capacities against GVHD require IL-33 signaling and identify that IL-33 activation of p38 MAPK mediates expansion of the ST2$^+$ subset of Tregs. In total, our current findings provide new appreciation for IL-33–responsive ST2$^+$ Tregs in the prevention of GVHD and establish a potential pathway for the application of IL-33 immunotherapy in alloHCT.

In addition to a subset of lymphoid[38,39] and tissue-resident Tregs,[37,41] ILC2,[61] as well as multiple myeloid cell subsets[7] constitutively express ST2. Proinflammatory stimuli can also induce the expression of functional IL-33 receptor on cytotoxic CD8$^+$ T cells[4,38] and Th1 cells.[25] In addition to ST2, the signaling transmembrane form, an IL-33-antagonistic soluble form (sST2), is generated by alternative splicing of messenger RNA.[62-66] In the clinical setting, sST2 upregulated during GVHD is associated with increasing disease severity,[67,68] and in mice, recent findings indicate that T cells, particularly the CD4$^+$ Th17 subset, are a significant source of sST2 during GVHD.[66] Our study, relying on depletion of Foxp3$^+$ Tregs, is limited in that it cannot definitively establish that the protective role of peri-alloHCT IL-33 resides in the ST2$^+$ subset of Tregs. However, we do reveal that among all the constitutive and potential ST2- and sST2-expressing cells of the body, Foxp3$^+$ Tregs are crucial for the protective effect of peri-alloHCT IL-33.

Much focus has been placed on the importance of Tregs to limit effector T-cell proliferation and proinflammatory cytokine production in response to donor antigens.[69,70] More recently, the ability of Foxp3$^+$ Tregs to control the inflammatory influence of myeloid cells has gained recognition.[48,49,71] In the current studies, macrophages were the primary population within the CD11b$^+$ myeloid cell compartment expanded by IL-33 administration. This effect is supported by IL-33–expanded Tregs, which restrain the accumulation of neutrophils and support granulocytic MDSC function. Treg depletion concurrent with IL-33 delivery results in macrophage activation and M1 polarization, reflected in a CD11b$^+$F4/80$^+$Gr-1^{int} cell gene expression profile consistent with IFNγ stimulation (Figure 4; supplemental Figure 3) and elevated macrophage expression of IL-12 and CD86 (Figure 5). Thus, our finding that a loss of Tregs results in increases in potentially detrimental M1-type macrophages parallel studies completed in models of sterile tissue damage.[48,49] Likewise, IL-12^{hi} and CD86^{hi} macrophages could act as instigators of type 1 donor effector T-cell activation and mediate observed T-effector exit from the spleen and infiltration of GVDH-target tissues.

We first described an expansion of Tregs by IL-33 delivery that was required for IL-33 prolongation of cardiac allograft survival.[39] Since then, reports of IL-33 contributing to Treg function and expansion in other models have grown considerably.[37,41,49] We now demonstrate that IL-33–driven Treg expansion is maintained post-TBI, consistent with Treg radiation resistance.[47] Effectively increasing the Treg-to-T-effector ratio is understood to be crucial for Treg control of alloimmune responses in the setting of transplantation and GVHD.[70,72] Increasing Tregs to adequate protective numbers via adoptive transfer suppresses the alloimmune responses underlying GVHD and has shown therapeutic efficacy in preclinical rodent studies and early-stage clinical trials. For instance, adoptive transfer of ex vivo–expanded Tregs reduced grade II-IV GVHD.[36,73] Low-dose IL-2 therapy is also being investigated as a therapeutic mechanism for in vivo Treg expansion to prevent acute GVHD following alloHCT.[74] We now demonstrate that pretransplant expansion of recipient Tregs via IL-33 delivery mediates protection against acute GVHD that is associated with reduced M1 macrophage generation and decreased T-effector frequency. Although not presently investigated, ST2$^+$ Tregs may also have IL-33–driven epithelial reparative capacities.[40] Thus, carefully orchestrating expansion of ST2$^+$ Tregs may be beneficial after alloHCT through both immune and nonimmune mechanisms. The findings of the present study, coupled with the recent work from our group[32] and others,[66,68] clearly establish the IL-33/ST2 axis, particularly as it relates to Treg immunobiology, as an attractive target for therapeutic intervention in the setting of alloHCT. We feel proper manipulation of the IL-33/ST2 axis after alloHCT will be critical to counter the IL-33–mediated detrimental type 1 alloimmune responses causing acute GVHD.[32] Given

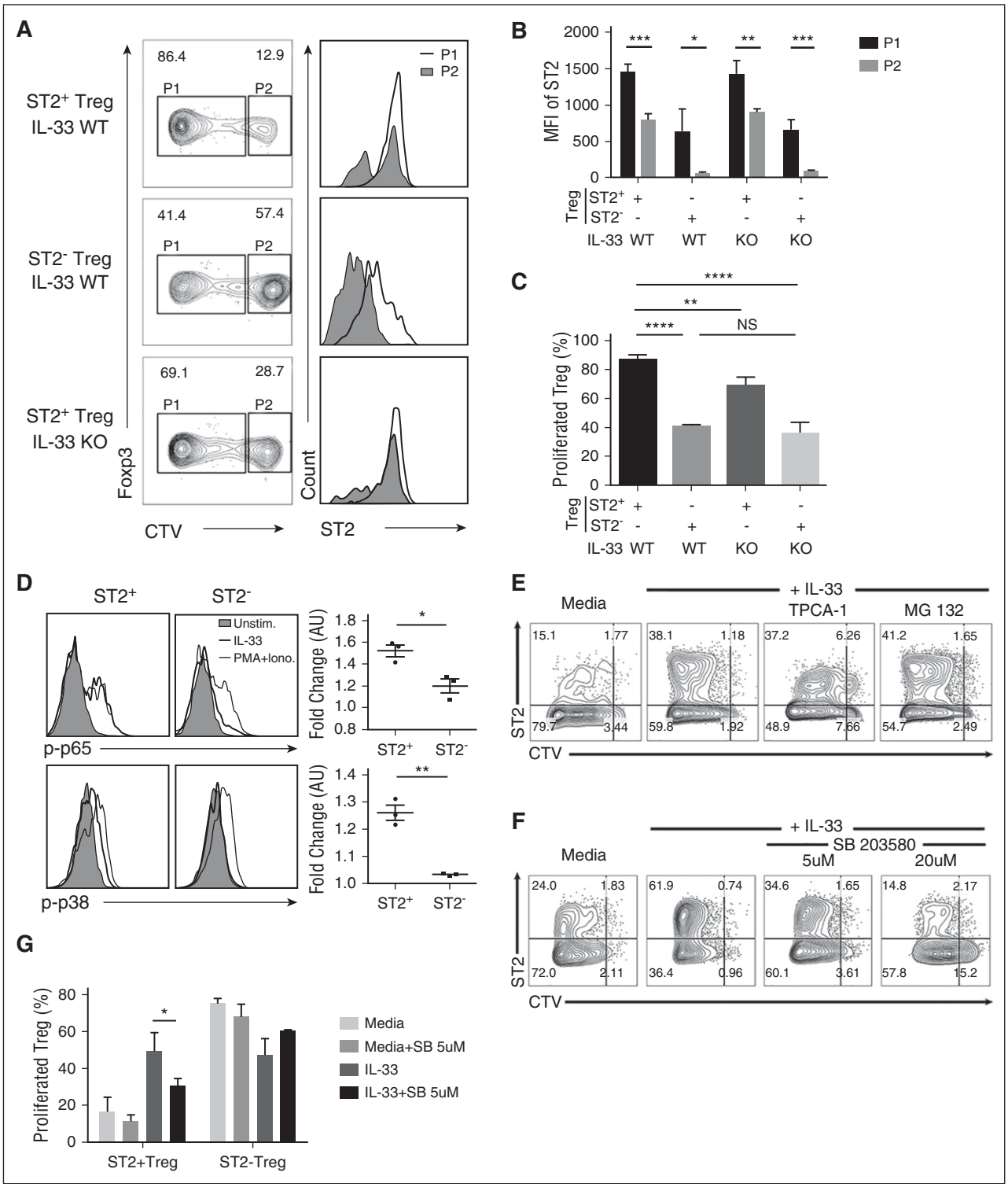


Figure 6. IL-33 mediates p38 MAPK-dependent signaling to promote the expansion of proliferating Tregs expressing ST2. (A) Sorted ST2$^+$ and ST2$^-$ CD4$^+$ Foxp3 (RFP)$^+$Thy1.1$^+$ cells from B6 OT-II FIR were labeled with CellTrace Violet (CTV), and infused into WT or *il33*$^{-/-}$ B6N mice that had been exposed to 1100 cGy 1 day prior. Recipient mice also received BM cells matched to recipient IL-33 status to create IL-33$^+$ or IL-33 KO conditions. On day 5 posttransplant, splenocytes were isolated and CD90.1$^+$ T cells assessed by flow cytometry for Foxp3 and ST2 expression, as well as proliferation (CTV dilution). Right flow plots, Proliferation and Foxp3 expression of the transferred CD90.1$^+$ Tregs. Left panel, The ST2 expression on proliferating (P1) vs nonproliferating (P2) CD90.1$^+$ cells. (B-C) Graphs present the average and SEM for (B) ST2 expression and (C) percentage proliferation (4 mice per group). Significant differences were calculated using unpaired Student *t* tests (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$). (D) CD4$^+$Foxp3(RFP)$^+$ Tregs were flow-sorted based on ST2 expression from B6 FIR mice and treated with IL-33 (100 ng/mL; bolded line) or (phorbol myristate acetate [PMA]/ionomycin; thin line) for 4 minutes before assessment for phospho-p38 or phospho-NF-κB p65 by flow cytometry. Representative histograms are presented. Unstimulated cells (filled histogram) served as a negative control. Graphs depict average calculated fold change in MFI of phospho-p38 or phospho-NF-κB p65 between IL-33–treated and untreated samples from 3 independent experiments. Statistical significance between groups calculated using the Student *t* test (*$P < .05$, **$P < .01$). (C) Bulk CD4$^+$ T cells cultured with BALB/c CD11c$^+$ BMDCs in media, stimulated with IL-33 alone, or IL-33 in combination with (E) the NF-κB inhibitors TPCA-1 or MG 132 or (F) the p38 MAPK inhibitor SB 203580. Flow plots depict ST2 expression vs CTV on CD3$^+$CD4$^+$Foxp3$^+$-gated cells. (G) Average of results from 3 independent experiments represented in panel F. Statistical significance between groups calculated using the Student *t* test (*$P < .05$, **$P < .01$). AU, arbitrary unit.

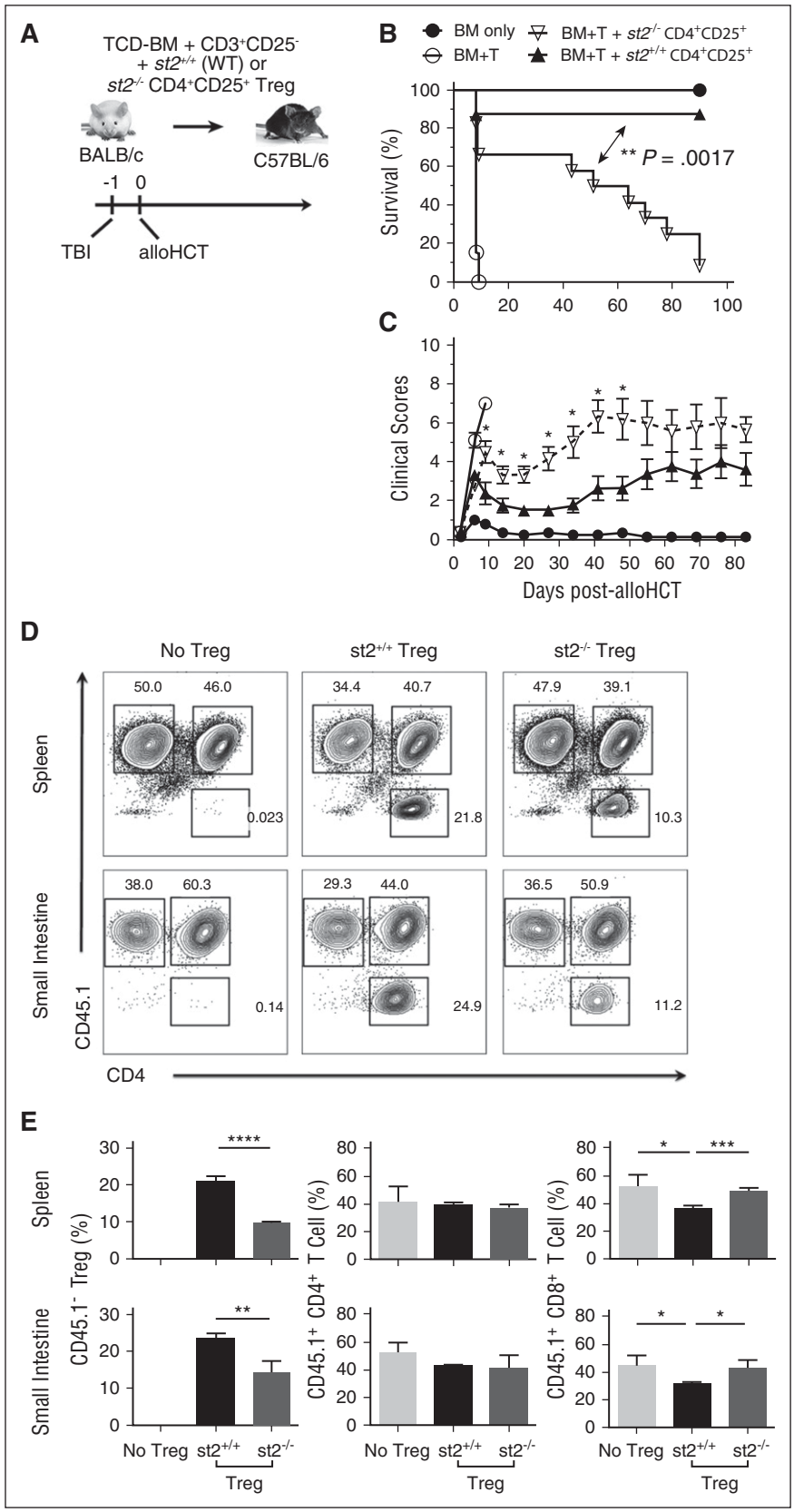


Figure 7. The IL-33–ST2 axis supports adoptively transferred Tregs and promotes their GVHD-protective capacity. (A) On day −1, WT B6 mice received lethal TBI (1100 cGy) followed by 1×10^7 WT BALB/c TCD-BM alone (n = 9) or with 4×10^6 BALB/c T cells (CD25-depleted) alone (BM+T; n = 13) or with 2×10^6 $CD4^+CD25^+$ Tregs from WT ($st2^{+/+}$; n = 8) or $st2^{-/-}$ (n = 12) BALB/c mice on day 0. Effective Treg-to-T-effector ratio was 1:2. (B) Survival is depicted with significant differences calculated using the log-rank (Mantel-Cox) test. (C) Clinical scores were also monitored and statistical differences determined between $st2^{-/-}$ Tregs vs $st2^{+/+}$ groups as in Figures 1 and 3. (D-E) B6 recipients irradiated as above received $CD4^+CD25^+$ T cells from $st2^{+/+}$ or $st2^{-/-}$ BALB/c mice, as well as $Thy1.1^+$ BALB/c TCD-BM, and CD25-depleted $CD45.1^+CD3^+$ T cells. (D) Representative flow plots depict CD45.1 and CD4 expression on K^{d+} $Thy1.1^-CD3^+$-gated splenocytes (top panels) or SI LPLs (bottom panels) at day 5 post-alloHCT. Full gating strategy is shown in supplemental Figure 7B. (E) Averages and SD are shown for the frequency of the indicated T-cell population in the spleen (top graphs) or SI LPLs (bottom graphs). Four mice per group and these data are representative of 2 independent experimental repeats. Statistical differences between groups were calculated using an unpaired Student *t* test (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$).

the pleiotropic nature of IL-33 as discussed, and the recent implication of sST2 as an inflammatory mediator underlying GVHD,[66] careful consideration must be given to appropriately shape donor and recipient immune responses to favor IL-33 immunoregulatory properties that can limit any detrimental IL-33 stimulation of the alloimmune responses causing GVHD.

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Authorship

Contribution: B.M.M. designed and performed experiments, analyzed data, and wrote the manuscript; D.K.R., X.Z., G.K.D., L.M., J.M.L., B.H.K., F.M.U., and D.P. designed and performed experiments, analyzed data, and edited the manuscript; C.J.F., M.J.S., and Q.L. discussed experimental design and data analysis; R.Z. edited the manuscript; and B.R.B. and H.R.T. designed and analyzed experiments and edited the manuscript.

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