Specifically differentiated T cell subset promotes tumor immunity over fatal immunity

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Allogeneic immune cells, particularly T cells in donor grafts, recognize and eliminate leukemic cells via graft-versus-leukemia (GVL) reactivity, and transfer of these cells is often used for high-risk hematological malignancies, including acute myeloid leukemia. Unfortunately, these cells also attack host normal tissues through the often fatal graft-versus-host disease (GVHD). Full separation of GVL activity from GVHD has yet to be achieved. Here, we show that, in mice and humans, a population of interleukin-9 (IL-9)–producing T cells activated via the ST2–IL-33 pathway (T9IL-33 cells) increases GVL while decreasing GVHD through two opposing mechanisms: protection from fatal immunity by amphiregulin expression and augmentation of antileukemic activity compared with T9, T1, and unmanipulated T cells through CD8x expression. Thus, adoptive transfer of allogeneic T9IL-33 cells offers an attractive approach for separating GVL activity from GVHD.

INTRODUCTION
One of the most validated immunotherapies to date, allogeneic hematopoietic cell transplantation (HCT), is a potentially curative option for high-risk hematological malignancies, including acute myeloid leukemia (AML), which affected >20,000 patients and led to >10,000 deaths in the United States alone in 2015 (American Cancer Society, 2015) and thus constitutes a critical unmet therapeutic need. Graft-versus-leukemia (GVL) reactivity requires donor T cell recognition of alloantigens on tumor cells (van den Brink and Burakoff, 2002; Warren and Deeg, 2013; Othus et al., 2015). Allogeneic–specific T cells can be generated without gene transfer and exhibit adequate T cell receptor affinity (Bachireddy et al., 2015; Cruz and Bollard, 2015; Dotti, 2015). Unfortunately, their reactivity to alloantigens in normal host tissues often leads to graft-versus-host disease (GVHD), a major cause of death after HCT. We previously showed that elevated plasma soluble stimulation 2 (sST2) is a risk factor of therapy-resistant GVHD and death (Vander Lugt et al., 2013). ST2 blockade reduces sST2–producing T cells while maintaining membrane ST2 (mST2)–expressing T helper type 2 (Th2) and mST2 FoxP3+ regulatory T (ST2+ T reg) cells during GVHD (Zhang et al., 2015). Adoptive cell transfer (ACT) of in vitro differentiatated total T2 cells (T cells containing only CD4+ cells differentiated under type 2 conditions [IL-4]) did not induce GVHD as severely as T1 cells (T cells containing CD4+ and CD8+ T cells differentiated under type 1 conditions [IL-12]); however, T2 cells did not show any antileukemic activity (Jung et al., 2003; Tawara et al., 2008). Thought to be associated with Th2 responses and arising from reprogrammed Th2 cells upon stimulation with TGF-β (Dardalhon et al., 2008; Veldhoen et al., 2008), Th9 cells (T cells containing only CD4+ cells differentiated under type 9 conditions [IL-4 + TGF-β]) were originally shown to be a subset of CD4 T cells that differed from Th2 cells in that Th9 cells produce IL-9 and little IL-4 and express the ETS transcription factor PU.1 (Chang et al., 2005, 2010). It has been shown that Th2 cells express mST2 (Löhnig et al., 1998; Xu et al., 1998), and the addition of IL-33 with TGF-β further increased mST2 expression on these cells (Blom et al., 2011). Reducing circulating sST2 driven by type 1 immune response with a neutralizing antibody led to protection against GVHD (Zhang et al., 2015) and increased mST2 expression on T reg cells, suggesting that ACT of mST2 expressing T cells represents a potential novel therapeutic approach to protect against GVHD. Thus, we were interested in IL-9–producing T cells because (a) ACT of these cells may protect against GVHD, similar to T2 cells or regulatory T cells; (b) IL-9 neutralization decreased the antitumor activity of T cells in melanoma models (Purwar et al., 2012); and (c) Th9 cells and IL-9–producing cytotoxic CD8 (Tc9) cells showed higher antitumor activity than Th1 and Tc1 cells in the same melanoma models (Lu et al., 2012, 2014). Whether or not Th9 and Tc9 (together T9 cells) express mST2, like T2, and how this ST2–IL-33 signaling affects T9 cells is unknown. In this study, we hypothesized that (a) the activation of T9 cells with
IL-33 during differentiation will enhance mST2 and IL-9 expression and (b) ACT of IL-33 activated T9 cells (T9IL-33) will decrease GVHD severity and possibly increase GVL activity.

RESULTS
ST2–IL-33 signaling increases mST2, IL-9, and PU.1 expression on T9 cells
To investigate the impact of ST2–IL-33 signaling on T9 differentiation, we polarized T cells from C57BL/6 mice into T9 cells in the presence (T9IL-33) or absence (T9) of IL-33. T9 cells expressed mST2 at the protein level, and mST2 protein expression on T9IL-33 cells was further increased on both CD4 and CD8 T cells (Fig. 1 A). PU.1 expression, a master transcription factor that promotes IL-9 production, was up-regulated in both CD4 and CD8 T cells upon addition of IL-33 during T9 differentiation. T9IL-33 cells have increased IL-9 expression in CD4 (>70% of total CD4 T cells expressing IL-9) and CD8 (>50% of total CD8 T cells expressing IL-9). IL-9 secretion from these cells was also increased. T9IL-33 cells did not secrete IFN-γ, IL-4, or ST2 in comparison with other differentiated T cell subsets (T1 and T2; Fig. 1 C; and Fig. S1 A and B). However, no expansion of T reg cells occurred during murine T9IL-33 differentiation in vitro (Fig. S1 C). Human T9 cells are poorly characterized. Our results demonstrate that, like murine T9IL-33 cells, differentiation of human T9 cells in the presence of IL-33 enhanced mST2 and PU.1 expressions as well as IL-9 expression and secretion by CD4 and CD8 T cells compared with other T cell subsets, including T9 cells (Fig. 1, D–F; and Fig. S1 D).

ACT of T9IL-33 cells reduces GVHD severity
We next evaluated the in vivo function of allogeneic T9IL-33 cells in comparison with freshly isolated unmanipulated T cells and T0 (without polarizing cytokines), T1, T2, T9, and T9IL-33 cells in a major histocompatibility antigen mismatch model of HCT. Mice receiving unmanipulated T, T0, or T1 cells showed severe GVHD and high mortality, whereas mice receiving T2 or T9 cells showed moderate GVHD with 40–60% survival. GVHD was almost completely abrogated in animals receiving T9IL-33 cells, with 100% survival in these mice (Fig. 1 G). Compared with the WT T9IL-33 group, ACT of T9IL-33 cells generated from ST2−/− or IL-9−/− T cells resulted in significantly more severe GVHD and reduced survival (Fig. 1 H). The role of the ST2–IL-33 axis was confirmed in a minor histocompatibility antigen model of GVHD (Fig. 1 I).

Pathological examination of the intestines during GVHD showed less tissue damage in mice that received T9IL-33 cells versus T1 cells (Fig. 2 A). To understand the mechanism responsible for the protective effect by T9IL-33 cells against lower intestinal epithelium damage during GVHD, we explored several possibilities. First, ex vivo analysis of T cells in the gut, the major GVHD target organ, 10 d after transplantation showed no difference in T cell proliferation between groups, as measured by carboxyfluorescein succinimidyl ester (CFSE) staining (Fig. 2 B), or the total number of gut-infiltrating T cells (Fig. 2 C). Second, we avoided possible differences in apoptosis and migration capacities (Waldman et al., 2006; Reshe夫 et al., 2012; Huang et al., 2015) between WT T9IL-33 and ST2−/− T9IL-33 cells in the intestine by measuring Annexin-V, α4β7, CRK (v-crk sarcoma virus CT10 oncogene homologue [avian], also called p38; Huang et al., 2015), and CCR5 expression (Fig. 2, D and E). Third, although ST2+ T reg cells and type 2 innate lymphoid cells (ILC2s) reduce GVHD severity (Munneke et al., 2014; Zhang et al., 2015), no difference in T reg frequency was observed after transfer of WT T9IL-33, T1, or ST2−/− T9IL-33 cells, and ILC2 cells were absent in the intestine of all HCT groups (Fig. 2, F and G). To confirm that the GVHD protective phenotype was not due to contaminating T reg cells in the graft and/or following homeostatic proliferation, we used the Foxp3-GFP reporter to differentiate total T cells toward T9IL-33 cells and then adoptively transferred either non–Foxp3-GFP–depleted T9IL-33 cells or Foxp3-GFP–depleted T9IL-33 cells (by cell sorting) to remove any Foxp3 T reg cells from the graft. We did not notice any expansion of Foxp3-GFP+ T reg cells ex vivo in the gut at day 14 or any differences in clinical scores for GVHD or in survival rates between the two groups (Fig. 2, H and I). These data indicate that ST2–IL-33 and IL-9 signaling are critical for T9IL-33 cell–mediated protection against GVHD, independent of T reg cells, and that this protection is not due to differences in proliferation, apoptosis, or migration of the T9IL-33 cells compared with other T cell subsets. As well, Foxp3-GFP depletion does not impact GVHD protection in mice that received T9IL-33 cells.

IL-33 induces amphiregulin (AREG) expression on T9 cells alleviating GVHD
We then used Transwell assays of T cells with allogeneic colonic epithelial cells and showed that WT T9IL-33 cells were able to maintain live colonic cells almost at the level of the control co-culture without T cells, whereas T1 and ST2−/− T9IL-33 cells (equivalent to T9 cells) killed ~70% and ~40% of the allogeneic colonic cells, respectively, in a contact-dependent manner (no differences in Transwell assays; Fig. 3 A). Next, we analyzed AREG, because its expression on mST2-expressing cells is involved in tissue repair via the gut-infiltrating T cells (Fig. 2 C). Second, we avoided possible differences in apoptosis and migration capacities (Waldman et al., 2006; Reshe夫 et al., 2012; Huang et al., 2015) between WT T9IL-33 and ST2−/− T9IL-33 cells in the intestine by measuring Annexin-V, α4β7, CRK (v-crk sarcoma virus CT10 oncogene homologue [avian], also called p38; Huang et al., 2015), and CCR5 expression (Fig. 2, D and E). Third, although ST2+ T reg cells and type 2 innate lymphoid cells (ILC2s) reduce GVHD severity (Munneke et al., 2014; Zhang et al., 2015), no difference in T reg frequency was observed after transfer of WT T9IL-33, T1, or ST2−/− T9IL-33 cells, and ILC2 cells were absent in the intestine of all HCT groups (Fig. 2, F and G). To confirm that the GVHD protective phenotype was not due to contaminating T reg cells in the graft and/or following homeostatic proliferation, we used the Foxp3-GFP reporter to differentiate total T cells toward T9IL-33 cells and then adoptively transferred either non–Foxp3-GFP–depleted T9IL-33 cells or Foxp3-GFP–depleted T9IL-33 cells (by cell sorting) to remove any Foxp3 T reg cells from the graft. We did not notice any expansion of Foxp3-GFP+ T reg cells ex vivo in the gut at day 14 or any differences in clinical scores for GVHD or in survival rates between the two groups (Fig. 2, H and I). These data indicate that ST2–IL-33 and IL-9 signaling are critical for T9IL-33 cell–mediated protection against GVHD, independent of T reg cells, and that this protection is not due to differences in proliferation, apoptosis, or migration of the T9IL-33 cells compared with other T cell subsets. As well, Foxp3-GFP depletion does not impact GVHD protection in mice that received T9IL-33 cells.

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Figure 1. IL-33 enhances mST2, IL-9, and PU.1 expression on T9 cells, and adoptive transfer of T9<sub>L33</sub> cells improves GVHD and survival after allo-HCT. (A) Representative histogram of mST2 expression on murine CD4 and CD8 T cell subsets after 5 d of differentiation (n = 5, from five independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05). (B) Representative histogram of mouse PU.1 expression in CD4 and CD8 T cell
be T cell suppressive—like T reg cells. We found that T9IL-33 cells could not suppress the function of T1 cells (Fig. 3 F). In addition, we did not observe a difference in the suppressive abilities of T reg cells with AREG blockade (Fig. 3 F), as previously shown by Arpaia et al. (2015) with genetic ablation of AREG. Thus, T9IL-33 cells do not behave like T reg cells, and they do not show a suppressive function toward other types of T cells. Ex vivo analysis of sorted T cells from intestine of HCT models also showed that AREG expression was greater in WT T9IL-33 cells than in T1 or ST2−/− T9IL-33 cells (Fig. 3 G). Furthermore, AREG blockade in HCT recipients receiving T9IL-33 increased the severity of GVHD and mortality in comparison with mice treated with isotype control (Fig. 3 H). AREG blockade did not change IFN-γ expression by gut-infiltrating T cells at day 10 after HCT (Fig. 3 I). These data are consistent with the in vitro data showing that T9IL-33 cells do not have a direct immunosuppressive effect with other T cells (Fig. S3 C). AREG expression by ex vivo isolated gut T cells at day 10 after HCT was not changed in mice receiving Foxp3-GFP-depleted or nondepleted T9IL-33 cells (Fig. 3 J). Plasma levels of ST2, TNF, and IFN-γ (Fig. 4, A and B) were lower in mice receiving T9IL-33 than in mice receiving T1 or T9 cells, suggesting a counterbalance of the excess of systemic ST2 generated during GVHD by mST2 on T9IL-33 cells. The increase in AREG (Fig. 3 B) is also dependent on IL-9 expression, as IL-9−/− T9IL-33 cells expressed very low levels of AREG compared with WT T9IL-33 cells (Fig. 4 C). The increased mST2 and decreased ST2 expression observed (Figs. 1 A and 2 I) was also dependent on IL-9 (Fig. 4 D). Although AREG blockade did not change the frequencies of IFN-γ− or IL-17-producing T cells, we observed a lower frequency of pathogenic cells producing IFN-γ and IL-17 in the gut at day 10 after HCT (Fig. 4 E). The levels of IFN-γ and IL-17 expression in T cells from the gut at day 10 after allo-HCT were also similar in C3H.SW mice receiving either allogeneic Foxp3-GFP–depleted or nondepleted T9IL-33 cells (Fig. 4 F). Human T9IL-33 cells also exhibited higher AREG expression than T1 cells (that do express ∼1%) and T9 cells (∼4%, significantly lower than ∼15% in T9IL-33 cells; Fig. 4 G). AREG blockade during co-culture with human allogeneic colonic epithelial cells decreased the protective effect of human T9IL-33 to levels seen with T9 cells (Fig. 4 H). Together, our data suggest that activation of the ST2–IL-33 pathway up-regulates AREG expression on both murine and human T9IL-33 cells and provides protection against epithelial damage during allo reaction, leading to a low degree of GVHD after ACT of T9IL-33 cells.

**T9IL-33 cells exhibit better antileukemic activity than T1 cells**

We next investigated whether ACT of T9IL-33 cells maintained antitumor activity. In recipients with lymphoma (A20), compared with syngeneic or allogeneic T cell subsets (unmanipulated T, T1, and T9), transfer of WT T9IL-33 cells resulted in milder GVHD without impairing antitumor activity, as more than 85% of these mice survived past 80 d after HCT and were GVHD/tumor free. In contrast, mice receiving unmanipulated T or T1 cells all died early of GVHD. In mice receiving T9 or ST2−/− T9IL-33 cells, GVHD onset was delayed and 60% died of tumor by day 60 (Fig. 5 A). The same trends were observed in recipients with mixed-lineage leukemia and AF9 fusion protein (MLL-AF9) AML in both major MHC and minor histocompatibility antigen HCT models (Fig. 5, B and C). Most mice receiving IL-9−/− T9IL-33 cells died of leukemia (Fig. 5 B). We then compared the transcriptomes of WT T9IL-33 versus ST2−/− T9IL-33 cells in CD8 and CD4 sorted populations and found higher expression of cytolytic molecules (granzyme a [Gzma], Gzmb, perforin [Prf1], and Fas) as well as markers of the T cell central memory phenotype (Cd62l and Cd27; Fig. 5 D, Fig. S2, and Table S2), which have been shown to correlate with higher antitumor activity (Klebanoff et al., 2016). Expression of cytolytic molecules was confirmed at the protein level (Fig. 5 E). Furthermore, ex vivo bone marrow (BM)—infiltrating CD8 T cells from T9IL-33 recipients expressed more Gzmb and Prf1 (Fig. 5 F). Total WT T9IL-33 cells showed more lysis against MLL-AF9 AML than total allogeneic T cells derived from unmanipulated T, T1, T9, ST2−/− T9IL-33, or syngeneic T9IL-33 cells (Fig. 6 A). Both sorted CD4 and CD8 cells from total allo geneic WT T9IL-33 cells showed higher antitumor activity (∼60% and 80% at a T/target ratio of 10:1, respectively) than CD4 and CD8 subsets after 5 d of differentiation (n = 5, from five independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01). (C) Representative plots of IL-9 and IFN-γ expression from in vitro differentiated murine cells and a bar graphs showing the frequency of IL-9− and IFN-γ−expressing T cells (n = 4, from four independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001). (D) mST2 expression on human CD4 and CD8 T cell subsets after 7 d of differentiation (n = 4, from four independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05, mean ± SEM). (E) Representative histogram of Pu.1 expression in human CD4 and CD8 T cell subsets after 7 d of differentiation (n = 3, from three independent experiments unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001). (F) Representative plots of IL-9 and IFN-γ expression in human T cells differentiated into T9 cells in the presence or absence of IL-33, and bar graphs showing the frequency of IL-9− and IFN-γ−expressing T cells (n = 4, from four independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.001). (G) Clinical scores of GVHD and survival curves for BALB/c mice transplanted with B6 or syngeneic BM cells and in vitro differentiated or freshly isolated syngeneic T cells (n = 12 each group, from two independent experiments unpaired t test; data are shown as mean ± SEM; **, P < 0.01; ***, P < 0.001). (H) Clinical scores of GVHD and survival curves for BALB/c mice receiving B6 or syngeneic BM cells and in vitro differentiated or freshly isolated syngeneic T cells (n = 24 per group, from three independent experiments, unpaired t test; data are shown as mean ± SEM; **, P < 0.01; ***, P < 0.001). (I) Clinical scores of GVHD and survival curves for C3H.SW mice receiving B6 or syngeneic BM cells and in vitro differentiated or freshly isolated syngeneic T cells (n = 7 per group, unpaired t test; data are shown as mean ± SEM; **, P < 0.01; ***, P < 0.001). For survival by log-rank test; **, P < 0.01; *** P < 0.001.
Figure 2. Impact of T1 versus T9IL-33 cells on gut pathology and effect of ST2–IL-33 signaling on gut T cell proliferation, viability, and migration, T reg expansion, and ILC2 expansion. (A) Pathology index of mouse intestines at day 10 after allo-HCT with either T1 or T9IL-33 cells (n = 3, from three independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05). Specimens were harvested, placed in 10% buffered formalin, embedded in paraffin, cut into 5-µm-thick sections, and stained with hematoxylin and eosin for histological examination. Slides were coded without reference to mouse type or prior treatment status and examined systematically by a single pathologist. (B) Representative plots of CFSE-labeled in gut T cells collected from mice on day 5 after all-HCT with syngeneic BALB/c T cells or allogeneic in vitro differentiated T cells. (C) Absolute counts of gut-infiltrating T cells from mice on day 10 after allo-HCT with syngeneic BALB/c T cells or allogeneic in vitro differentiated T cells (n = 3, mean ± SEM, from three independent experiments, unpaired t test; data are shown as mean ± SEM). (D) Representative plots of α4β7 and CRK (v-crk sarcoma virus CT10 oncogene homologue [avian], also called p38) and CCR5 in CD4 T cells infiltrating the gut at day 10 after HTC (n = 3, unpaired t test; data are shown as mean ± SEM). (E) Representative plots of Annexin V and fixable viability dye (FVD) staining of gut T cells at day 10 post-HTC (n = 3). (F) Representative plots of CD4 and FoxP3 and a bar graph showing the frequency of T reg cells (CD4+FoxP3+) in gut-infiltrating CD4 T cells at day 10 after HTC (n = 4, unpaired t test; data are shown as mean ± SEM). (G) Representative plots of mST2 and Gata3 in Lin−CD45+CD90.2+ cells (ILC2 markers, n = 4, unpaired t test; data are shown as mean ± SEM). (H) Ex
CD8 sorted cells from ST2<sup>−/−</sup> T<sub>9IL-33</sub> cells (320% and 55% at a T/target ratio of 10:1, respectively; Fig. 6 E, F). This anti-tumor effect was also dependent on IL-9, as IL-9<sup>−/−</sup> T<sub>9IL-33</sub> T cells had lower cytolytic activity than WT T<sub>9IL-33</sub> cells both in vivo (Fig. 5 B) and in vitro (Fig. 6 C). Importantly, AML cells do not express epidermal growth factor receptor (EGFR) and blocking AREG did not affect the killing of leukemic cells by T<sub>9IL-33</sub> cells, explaining AREG specificity for epithelial cells (Fig. 6 D). Compared with T9 and T1 cells, human T<sub>9IL-33</sub> cells showed enhanced production of Gzmb and Prf1 (Fig. 6 E) and enhanced cytolytic activity against MOLM14, an aggressive AML cell line with FLT3-ITD (fms-related tyrosine kinase 3 internal tandem duplication; Fig. 6 F). T<sub>9IL-33</sub> cells have a central memory phenotype (CD62L<sup>+</sup>CD44<sup>+</sup>KLRL1<sup>+</sup>) in vitro and ex vivo (Fig. 7, A and B) that is mediated through ST2–IL-33 signaling. In addition, short-term effector cells, defined as KLRL1<sup>+</sup>CD27<sup>−</sup> (Joshi et al., 2007), from CD8 WT T<sub>9IL-33</sub> cells expressed more Gzmb than CD8 ST2<sup>−/−</sup> T<sub>9IL-33</sub> cells in vitro (Fig. 7 C). Human T<sub>9IL-33</sub> cells also displayed enhanced central memory phenotypes (CD45RA<sup>−</sup>CCR7<sup>+</sup>; Sallusto et al., 1999) and (CD27<sup>−</sup> KLRL1<sup>+</sup>; Koch et al., 2008) compared with T1 and T9 cells (Fig. 7, D and E). Contact-dependent ST2–IL-33 signaling in CD4 T cell license CD8 T cells to express higher IL-9, PU.1, Gzmb, and PRf1 (Fig. 8, A and B). Contact-dependent ST2–IL-33 killing is significantly more effective when we block CD8<sup>+</sup>α<sup>−</sup> in vivo (Fig. 9, C and D). Blocking CD8<sup>+</sup>α<sup>−</sup> during human T<sub>9IL-33</sub> differentiation abolished their capacity to kill MOLM14 leukemic cells (Fig. 9 K). In summary, our data show that triggering mST2 on both murine and human T9 cells up-regulates CD8α expression and boosted the antileukemic activity of T<sub>9IL-33</sub> cells in vitro and in vivo.

**DISCUSSION**

Here, we show that the addition of IL-33 to IL-9–expressing CD4 (Th9) and CD8 (Tc9; together T9) cells enhanced their expression of mST2, IL-9, and PU.1. Compared with other T cell subsets that have been reported, T9 cells are not as well characterized. T9 cells arise after treatment with a cytokine combination of TGF-β1 and IL-4 (Dardalhon et al., 2008; Veldhoen et al., 2008). It has been shown that adding TGF-β1 to Th2 cells up-regulates mST2 expression (Blom et al., 2011). Consistent with this, we found that Th9 cells express higher levels of mST2. In addition, we show for the first time that Tc9 cells also have higher expression of mST2 and PU.1. PU.1 binds to the ST2 promoter and induces transcription of ST2 in mast cells and basophils (Baba et al., 2012), and it directly interacts with the IL-9 gene promoter, inducing IL-9 production (Chang et al., 2010) and inhibiting the production of Th2 cytokines (Chang et al., 2005). Although we did not study them here, other transcription factors and pathways such as BATF (Jabeen et al., 2013), IRF4 (Staudt et al., 2008; Taniyama et al., 2013), STAT5 (Olson et al., 2016), OX40/NF-κB (Xiao et al., 2012), and SIIRT1–mTOR–HIF1α (Wang et al., 2016) have been shown to be important for optimal Th9 function. Determining the importance of these transcription factors and their possible regulation by IL-33 could help to even further tailor and strengthen the activity of T<sub>9IL-33</sub> cells.

vivo expression of Foxp3-GFP in gut CD4 T cells collected on day 14 after allo-HCT with either Foxp3-GFP-depleted or nondepleted allogeneic T<sub>9IL-33</sub> cells (n = 9 per group, unpaired t test; data are shown as mean ± SEM).

3582 New T cell therapy in tumor and fatal immunity | Ramadan et al.
Acute GVHD is characterized by selective tissue damage to the mucosa, particularly of the gastrointestinal tract, and is driven mainly by donor T1 immune responses (van den Brink and Burakoff, 2002). ACT of in vitro T2 polarized allospecific donor T cells (Jung et al., 2003; Tawara et al., 2008) and Th2 and Th9 polarized cells in the presence of rapamycin (Mangus et al., 2013) decreased GVHD severity. Th2 cells showed a reduction in GVHD but were unable to control tumor cells in a murine model of L1210 leukemia, Jurkat T cell lymphoma, or metastatic breast cancer cell lines (Jung et al., 2013). Furthermore, in a phase 1 clinical trial, acute GVHD and overall survival were similar between Th2 and non-Th2 ACT recipients (Fowler et al., 2006). The impact of using both CD4+ and CD8+ T9 cells in GVHD and GVL had not been studied until now. We hypothesized that Th9 and Tc9 cells will behave like Th2 and Tc2 or T1 rapamycin in GVHD models while maintaining antileukemic activity in GVL models based on three studies showing that IL-9 and IL-9–producing T cells have potential as antitumoral therapies (Lu et al., 2012, 2014; Purwar et al., 2012). We used ACT because unlike many of the T cell subsets, it has been proven difficult to detect T9 cells in vivo, even in patients and models with Th2/Th9–driven diseases such as allergy (Kaplan et al., 2015; Kim et al., 2015). We nevertheless tried to enhance levels of in vivo murine T9 cells through the administration of TGF-β, IL-4, and IL-33 in donors or recipients, but we still observed a low frequency of T9 cells (unpublished data). One explanation for the difficulty in detecting these cells ex vivo is that T9 cells are short-lived. Another possibility is that T9 cells have high plasticity in vivo and become a different subset of T cell after injection. Further studies looking into the fate of adaptively transferred T9 and T9IL-33 cells are warranted to determine what is happening to these cells after transfer. Even though our data clearly show a reduction in GVHD morbidity and mortality in murine models when using T9IL-33 cells, similarly to Th2 cells, it is possible that this approach may not show efficacy in the clinic.

Mechanistically, our data show that mice receiving WT T9IL-33 have lower levels of systemic soluble ST2 (sST2) accompanied with low levels of inflammatory cytokines (IFN-γ and TNF). The lower levels of soluble sST2 that we found could help explain the decrease in GVHD severity, as we have previously shown that sST2 is correlated with GVHD severity (Vander Lugt et al, 2013) and lowering sST2 levels using a blocking antibody ameliorated GVHD while preserving signaling through mST2 on T reg cells (Zhang et al., 2015). Indeed, studies have shown that IL-33 activation of ST2+ T reg cells increases their suppressive function (Schiering et al., 2014; Zhang et al., 2015). Other studies have suggested that this increase in suppressive function of ST2+ T reg cells can be explained by either up-regulation of IL-10 (Vasanthakumar et al., 2015) or AREG, independent of TCR signaling (Arpaia et al., 2015). It has recently been shown that periodization of IL-33 expands recipient T reg cells that protect mice against acute GVHD (Matta et al., 2016). However, in vitro differentiation of T9IL-33 cells did not results in any increase in T reg frequency, and ACT of T9IL-33 cells did not lead to the accumulation of classical T reg cells or ST2+ T reg cells. These results suggest that in our models, the phenotype we see is not a bystander effect but more dependent on a direct effect of T9IL-33 cells on intestinal epithelial cells. IL-33 also promotes AREG expression on ILC2s, leading to intestinal tissue protection dependent on AREG–EGFR interactions (Monticelli et al., 2015). This AREG–EGFR interaction could explain...
Figure 4. Allogeneic T cell interaction with colonic epithelial cells and AREG blockade in mice and human. (A) sST2 concentration in plasma collected every 5 d after HCT from the BALB/c mice receiving $5 \times 10^6$ BM cells plus $10^6$ from (n = 4, from two independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01). (B) TNF and IFN-γ concentrations in plasma collected every 5 d after HCT from BALB/c mice receiving $5 \times 10^6$ BM cells plus $1 \times 10^6$ from syngeneic T cells (BALB/c mice) or T1, T9, or T9IL-33 T cells from B6 WT mice (n = 4, from two independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **. P < 0.01). (C) Ratio of AREG/actin mRNA expression in WT or IL-9−/− T9IL-33 cells. (D) Ratio of mST2/actin expression (left) and sST2/actin expression (right) in WT or IL-9−/− T9IL-33 cells (n = 3, from three independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01). (E) Ex vivo expression of IFN-γ and IL-17 in gut CD4 T cells collected on day 14 after allo-HCT with allogeneic T1, WT T9IL-33, or ST2−/− T9IL-33 cells (n = 4, from two independent experiments, unpaired t test; data are shown as mean ± SEM; **, P < 0.01; ***, P < 0.001). (F) Ex vivo expression of IFN-γ and IL-17 in gut CD4 T cells collected on day 14 after allo-HCT with T9 IL-33 cells from Foxp3-GFP reporter mice depleted or nondepleted of Foxp3 T reg cells (n = 3, unpaired t test; data are shown as mean ± SEM). (G) AREG expression in vitro differentiated human T1, T9, and T9IL-33 CD4 cells from healthy donors (n = 3, from three independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001). (H) Percentage of residual live human colonic cells after co-culture with T1, T9, T9IL-33 cells for 6 h in the presence of anti-AREG blocking antibody or isotype control and without T cells as control (n = 3, from three independent experiments, unpaired t test; data are shown as mean ± SEM; **, P < 0.01; ***, P < 0.001).
Figure 5. **T9IL-33 cells preserve GVL and antitumor activity.** (A) Survival curves for BALB/c mice receiving $10^4$ syngeneic lymphoma cell line A20 cells with syngeneic T cells or allogeneic in vitro differentiated cells ($n = 12$ mice per group, from two independent experiments; ***, $P < 0.0001$, log-rank test). Pie charts show cause of death. (B) Survival curves for BALB/c mice receiving $10^4$ cells of the syngeneic MLL-AF9 leukemic cell line with syngeneic T cells or allogeneic in vitro differentiated cells ($n = 12$ mice per group, from two independent experiments; **, $P < 0.01$; ***, $P < 0.001$, log-rank test). Pie charts show cause of death. (C) Survival curves for C3H.SW mice receiving $10^4$ MLL-AF9 leukemic cells with syngeneic T9IL-33 cells or allogeneic in vitro differentiated cells ($n = 14$ mice per group, from two independent experiments; ***, $P < 0.001$, log-rank test). Pie charts show cause of death. (D) Transcriptome analysis of Gzma, Gzmb, Prf1, Cd62l, Cd27, and Fas expression in sorted WT T9IL-33 versus ST2−/− T9IL-33 CD8 and CD4 cells ($n = 3$; see Fig. S2). (E) Representative plots of Gzmb and Prf1 expression in WT T9IL-33 and ST2−/− T9IL-33 cells gated on CD8, and bar graphs showing the frequency of GzmB+ and Prf1+ CD8 T cells ($n = 8$, from four independent experiments, unpaired $t$ test; data are shown as mean ± SEM; **, $P < 0.01$). (F) Representative plots of Gzmb and Prf1 expression in gated CD8 T cells from BM 28 d after adoptive transfer of allogeneic T cells with syngeneic MLL-AF9 cells. Bar graphs show the frequency of GzmB+ and Prf1+ CD8 T cells ($n = 4$, unpaired $t$ test; data are shown as mean ± SEM; ***, $P < 0.001$).
why ILC2s were associated with reduced susceptibility to GVHD in 51 acute leukemia patients, but their reconstitution after allo-HCT was slow (Munneke et al., 2014). ACT of T9IL-33 cells in our acute models of GVHD did not lead to the accumulation of ILC2s in the gut, again suggesting a direct effect of T9IL-33 on intestinal epithelial cells. We then showed that T9IL-33 cells have a direct contact–dependent effect on intestinal epithelial cells. AREG expression on T9IL-33 cells was the reason for their GVHD protective effect, as has been shown for T reg cells and ILC2s (Arpaia et al., 2015; Monticelli et al., 2015), but not other T cell subsets. Indeed, our data showed that T9IL-33 cells express high levels of AREG like T reg cells, and this expression was maintained on infiltrated T cells in the gut after allo-HCT. AREG expression on T9IL-33 cells was independent of Foxp3 expression in vitro and in vivo. Furthermore, blocking AREG on T9IL-33 cells reduced allogeneic mouse and human colonic epithelial survival in vitro and increased GVHD severity in vivo. This led to a reduction in the frequency of pathogenic Th1 cells in the intestine. This reduction of Th1 response ex vivo is mediated by high expression of mST2 and low levels of sST2, as we previously described (Zhang et al., 2015). Foxp3 deletion from T9IL-33 cell cultures before ACT did not have any impact on the protective effect of T9IL-33 cells in GVHD. Together,
our data show that in our models, the protective phenotype of intestinal epithelial cells from allopeuctivity is directly dependent on AREG-positive T9IL-33 cells. Therefore, possible avenues for exploring the ST2–IL-33 pathway as a target for GVHD treatment include blockade of sST2 through loss of IL-33 sequestration by sST2 (Zhang et al., 2015) and ACT of mST2-expressing T9IL-33 cells through protection by AREG–EGFR signaling.

Although new therapeutic approaches such as chimeric antigen receptor T cells have shown promise in acute and chronic lymphoid leukemias (Grupp et al., 2013; Porter et al., 2015), their use in AML requires subsequent HCT due to myelotoxicty to normal cells (Kenderian et al., 2015). Allogeneic specific T cells can be generated without gene transfer and exhibit adequate T cell receptor affinity (Bachireddy et al., 2015; Cruz and Bollard, 2015; Dotti, 2015), but unfortunately, their reactivity to alloantigens in normal host tissues often leads to GVHD. Furthermore, most experimental strategies to separate GVHD and GVL responses merely spare GVL function without enhancing it. Th1 and Tc1 cells have been regarded as most efficient in tumor rejection among the subsets of T cells (Dunn et al., 2006); however, ACT therapy of Tc1 had limited success, because high expression of T-bet led to a short life span (Joshi et al., 2007) and exhausted phenotype of these cells via up-regulation of KLRG1, PD-1, LAG3, and 2B4 (Crespo et al., 2013). One explanation for

Figure 7. **ST2–IL-33 signaling preserves central memory phenotype.** (A) Representative plots of CD62L+ and CD44+ (left) and CD27+ and KLRG1+ (right) cells, and bar graphs showing the frequency of CD44+CD62L+, CD27+, and KLRG1+ CD8 T cells from in vitro differentiated cells from WT T9IL-33 versus ST2+ T9IL-33 cells (n = 4, from four independent experiments, unpaired t test; data are shown as mean ± SEM; **, P < 0.01). (B) Representative plots of CD62L+ and CD44+ (left) and CD27+ and KLRG1+ (right) cells, and bar graphs showing the frequency of CD44+CD62L+ and CD27+ CD8 T cells from ex vivo BM collected on day 28 from mice receiving MLL-AF9 leukemic cells with WT T9IL-33 or ST2−/− T9IL-33 cells (n = 4, unpaired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01). (C) Representative plots of GzmB expression in WT or ST2−/− CD8+KLRG−1−CD27− short-term effector cells differentiated under T9IL-33 conditions, and a bar graph showing the frequency of GzmB expression in these cells (n = 3, unpaired t test; data are shown as mean ± SEM; ***, P < 0.001). (D and E) Representative plots of CD45RA− and CCR7+ (D) and CD27+ and KLRG1+ (E) cells, and bar graphs showing the frequency of CD45RA−CCR7+, CD27+, and KLRG1+ CD8 T cells from in vitro differentiated T1, T9, and T9IL-33 human cells (n = 3, from three independent experiments, paired t test; data are shown as mean ± SEM; *, P < 0.05; **, P < 0.01).
these differences between T1 and T9IL-33 cells might be due to the reduced IFN-γ production by T9IL-33 cells. The presence of high amounts of IFN-γ has been shown to up-regulate exhaustion markers and induce apoptosis of late effector memory phase T cells (Badovinac et al., 2000; Joshi et al., 2007). The GVL activity of T9IL-33 cells could also be explained by the persistence of their central memory phenotype, which has been shown to enhance antitumor activity (Klebanoff et al., 2005, 2016). Recently, it has been shown that IRF1–IL-21 signals and GITR signaling increases antitumor activity of T9 cells in melanoma models (Végran et al., 2014; Kim et al., 2015); however, we did not observe any difference at transcript levels of IL-21, IRF1, and GITR between CD4 and CD8 WT or ST2−/− T9IL-33 cells (Table S2). Further studies need to be completed to better understand how T9IL-33 cells maintain their central memory phenotype without becoming exhausted.

On CD8 T cells, ST2–IL-33 signaling drives protective antiviral responses (Bonilla et al., 2012). We showed here that ST2–IL-33 signaling enhanced antitumor activity via interactions between CD4 and CD8 T cells. The absence of CD4 T cells generates corrupt CD8 effector T cells (Ghosh et al., 2016). We showed here that Th9 cells license Tc9 for expression and production of ST2, IL-9, cytolytic molecules, and killing via a contact-dependent ST2–IL-33 pathway for optimal activity. Different mechanisms have been shown to explain the importance of CD4 T cells for antitumor activity of CD8 T cells. One of these mechanisms was the interaction between CD4 and CD8 via OX40 (Song et al., 2007). OX40 expression has been shown to be up-regulated on Th2 upon IL-33 activation (Murakami-Satsutani et al., 2014) and enhanced in Th9 cells (Xiao et al., 2012). We also noted significant up-regulation of OX40 (Tnfrsf4; Table S2) in both CD4 and CD8 WT T9IL-33 cells compared with CD4 and CD8 ST2−/− T9IL-33 cells. Furthermore, we observed an ST2–IL-33–dependent increase in CD8α expression on both CD4 and CD8 T9IL-33 cells. Mutation of the CD8α subunit leads to the deficiency of all CD8 T cell differentiation in the thymus (Kang et al., 2010) and impairs the development of cytotoxic T cell function (Fung-Leung et al., 1991) as well as antitumoral activity (de la Calle-Martin et al., 2001). Therefore, the T cell cytotoxic function is difficult to induce in absence of CD8α T cells, but it can be rescued by simultaneous removal of MHCII, consequently removing CD8α T cells and allowing for diminished competitiveness (Riddle et al., 2008). We found that blocking CD8α or use of CD8α KO animals completely abolished the antitumor activity of T9IL-33 cells. CD8α up-regulation has been associated with up-regulation of GzmK and massive infiltration of CD8 T cells in the stroma of patients with HPV1 oropharyngeal squamous cell carcinoma that correlated with prolonged survival (Jung et al., 2013). We also detected an increase in the production of GzmK correlated with CD8α expression on human Tc9IL-33 cells. There are no published data on GzmK expression in murine T cells and/or its relation with CD8α. Further studies of this important antitumoral mechanism are required. We demonstrated that

Figure 8. ST2–IL-33 signaling in CD4 impacts CD8 antitumor activity. (A) Granzyme B and perforin expression in CD8 T cells cultured with WT or ST2−/− CD4 cells together or through a Transwell under T9IL-33 conditions (n = 3, from three independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05). (B) Splenic CD4 and CD8 cells were either co-cultured together or separated in a Transwell under T9IL-33 conditions for 5 d. Representative plots and mean ± SEM of IL-9 expression in CD8 T cells (left), IL-9 secretion from total T9IL-33 (center), and PU.1 expression in CD8 T cells (right) either from co-culture (Co) or through Transwell (TW; n = 3, unpaired t test; data are shown as mean ± SEM, **, P < 0.01). (C) KLRG1 expression on purified CD8 T cells cultured together or through a Transwell with CD4 T cells. (D) Cytolytic assays of purified CD8 T cells differentiated into Tc9IL-33 cells or co-cultured in the presence of CD4 in MLR conditions (n = 4, from two independent experiments, unpaired t test; data are shown as mean ± SEM; *, P < 0.05).
knocking down IL-9 decreased antitumor activity both in vitro and in vivo through activation of ST2–IL-33 signaling. Expression of ST2–IL-33 increases the levels of IL-9, CD9α, and cytolytic molecule expression and preserves the central memory phenotype of these Tc9 cells, leading to increased antitumor activity of T9 cells.

We conclude that ST2–IL-33 activation of both murine and human IL-9–secreting T cells may serve as a new T cell therapy with dual opposing mechanisms: protecting normal tissues through up-regulation of AREG and mediating antileukemic activity via CD8α expression. Therefore, ACT of allogeneic T9IL-33 cells offers an attractive approach for separating GVL activity from GVHD.

**MATERIALS AND METHODS**

**Mice**

BALB/c (H-2b), C57BL/6 (B6, H-2b, CD45.2+), C57BL/6, Ptprc−/− (B6-129S1-Ptprc−/−, 129/Ptprc−/−), C3H.SW (H-2b, CD45.2+), and B6.129S2-Cd8atm1Mak/J (H-2b, CD45.2+) mice were purchased from The Jackson Laboratory. B6 ST2−/− (H-2b) mice were provided by A. McKenzie (University of Cambridge, Cambridge, England, UK), and B6 IL-9−/− (H-2b, CD45.2+) mice were provided by A. Rosenkranz (University of Graz, Graz, Austria). Animal protocols were approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine.

**T cell differentiation**

Total, CD4+, and CD8+ T cells were purified from spleens by magnetic bead selection (Miltenyi Biotec). Cells were plated at a concentration of 1 × 10⁶ cells/ml and cultivated with 1 μg/ml plate-bound anti-CD3 (2C11) and 5–10 μg/ml soluble anti-CD28 (37.51). CD4+ and CD8+ cells were polarized toward T0 (without cytokines), T1 (1 ng/ml IL-2 and 20 ng/ml IL-12), T2 (20 ng/ml IL-4), T9 (4 ng/ml TGF-β, 10 ng/ml IL-4, and 10 ng/ml IL-33) in DMEM supplemented with 10% FBS, 2 mM l-glutamine, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol (Life Technologies). On day 3, cells were expanded with fresh growth media in the presence of additional cytokines at the same concentrations as in day 0 medium. On day 5, cells were collected, washed, and prepared for phenotypic analysis or ACT into recipient mice.

Human T cells (CD4+ or CD8+) were purified from PBMCs of healthy donors and activated with anti–CD3/CD28 microbeads (Life Technologies). Both CD4+ and CD8+ cells were polarized toward T1 (1 ng/ml IL-2 and 20 ng/ml IL-12), T2 (20 ng/ml IL-4), T9 (4 ng/ml TGF-β, 10 ng/ml IL-4, and 10 ng/ml IL-33) in complete RPMI medium with 10% human AB serum. On day 3, cells were expanded with fresh medium in the presence of additional cytokines at the same concentrations as on day 0. On day 7, cells were collected, washed, and prepared for phenotypic analysis and in vitro assays.

**Induction and assessment of GVHD**

Mice underwent allo-BM transplantation as previously described (Zhang et al., 2015). In brief, BALB/c and C3H.SW recipients received 900 and 1100 cGy total body irradiation (137Cs source), respectively, on day -1. Recipient mice were injected intravenously with 5 × 10⁶ T cell–depleted (TCD) BM cells plus in vitro differentiated T0, T1, T2, T9, WT T9IL-33, ST2−/−T9IL-33, or IL-9−/− T9IL-33 T cells (1 × 10⁶ for BALB/c and 3 × 10⁶ for C3H.SW) from either syngeneic or allogeneic donors at day 0. TCD BM cells from donors were prepared with CD90.2 Microbeads (Miltenyi Biotec). Mice were housed in sterilized microisolator cages and maintained on acidified water (pH < 3) for 3 wk. Survival was monitored daily, and clinical GVHD scores were assessed weekly as de-
scribed previously (Cooke et al., 1996). Mice were euthanized when the clinical scores reached 6.5, in accordance with animal protocols approved by the institutional review board.

**Induction and assessment of the GVL effect**

BALB/c or C3H.SW mice were lethally irradiated (900 or 1,100 cGy, respectively) on day −1. Recipient mice were injected intravenously on day 0 with 5 × 10^6 syngeneic or allogeneic TCD BM cells and in vitro differentiated syngeneic T9^IL-33^ cells or T0, T1, T9, or T9^IL-33^ from WT B6 mice, or T9^IL-33^ from ST2^−/−^ B6 mice at 1 × 10^6 T cells for BALB/c recipient mice or 3 × 10^6 T cells for C3H.SW recipient mice. For GVL experiments, syngeneic GFP–MLL-AF9 leukemic cells generated from C3H.SW or BALB/c BM, as described previously (Zhang et al., 2015), were injected on day 0 at 1 × 10^4 GFP–MLL-AF9 cells for each recipient mouse. Mice were monitored daily for survival and leukemia development, and GVHD was scored weekly. We attributed death to leukemia based on a high percentage of eGFP+ cells and death to GVHD only if the mice had a low percentage of eGFP+ cells and a GVHD score ≥6.5. Cells from peripheral blood, BM, spleen, and liver were analyzed by flow cytometry.

**In vitro cytotoxicity assay**

Purified splenic T cells were primed in a mixed lymphocyte reaction (MLR) in the presence of polarizing cytokines (IL-4, TGF-β, and IL-33) for 5 d. Total T9^IL-33^ cells or sorted CD4 and CD8 from B6 WT T9^IL-33^, ST2^−/−^ T9^IL-33^, or C3H.SW T9^IL-33^ cultures were incubated with C3H.SW GFP–MLL-AF9 leukemic cells at different ratios. After 6 h, cells were washed, stained with viability dye, and analyzed by flow cytometry. Human T9 or T9^IL-33^ were labeled with 5 µM CFSE and co-incubated with MOLM14 leukemic cells labeled with 0.5 µM CFSE (Life Technologies). After 6 h, cells were washed and analyzed by flow cytometry. For imaging, cells were labeled with CD8^α, CD8^β, and SYTOX (S11348; ThermoFisher; SYTOX was added 5 min before acquisition). Images were acquired using Image Stream (Amnis) after 3 h of coincubation.

**Colonic epithelial cell apoptosis assay**

A BALB/c primary colonic epithelial cell line (BALB-5047; Cell Biological) was co-cultured together or separately (through a Transwell) with in vitro differentiated T9, WT T9^IL-33^, or ST2^−/−^ T9^IL-33^ cells at a ratio of 1:10 with anti-AREG or the appropriate isotype control. 6 h later, cells were washed, stained with viability dye and Annexin V, and analyzed by flow cytometry. In some experiments, colonic epithelial cells were co-cultured with T1 + T9^IL-33^ cells or T1 cells + in vitro polarized T reg cells purified magnetically using the CD4^+CD25^ regulatory T cell isolation kit from Miltenyi (130-091-041) at a ratio of 1:1.

Human T1, T9, or T9^IL-33^ cells were co-cultured with the primary colonic epithelial cell line (HNNC) in the presence of anti–human AREG or isotype control in the same conditions described above.

**In vivo treatment with anti-AREG**

C3H.SW recipients received 1,100 cGy total body irradiation (^137^Cs source) on day −1. On day 0, mice were injected intravenously with B6 TCD BM cells (5 × 10^6) plus 3 × 10^6 in vitro differentiated WT T9^IL-33^ cells. Mice received five doses of 100 µg anti-AREG or isotype control every other day from day −1 to day 7.

**CD8α blocking**

Anti-CD8α blocking antibody for mouse (53–6.7) or human (LT8) was added (both at 50 µg/ml) during differentiation of T9^IL-33^ cells or during coincubation with MLL-AF9 leukemic cells.

**Flow cytometry**

All antibodies (Table S1) and reagents for flow cytometry were purchased from eBioscience, unless otherwise stated. The cells were preincubated with purified anti–mouse CD16/CD32 mAb for 15 min at 4°C to prevent nonspecific binding of the antibodies. The cells were subsequently incubated for 30 min at 4°C with antibodies for surface staining. Fixable viability dye was used to distinguish live cells from dead cells. The FoxP3/transcription factor staining buffer set and fixation and permeabilization kit were used for intracellular staining. For cytokine staining, cells were restimulated with 10 µg/ml anti-CD3 for 4–6 h, and brefeldin A was added for the last 2 h of culture.

**MLR**

Purified splenic total T cells from B6 WT, ST2^−/−^, or CD8α^−/−^ mice were cultured with allogeneic TCD and irradiated splenocytes (3,000 cGy) from BALB/c or C3H.SW mice in the presence of T1 or T9^IL-33^ polarizing cytokines. On day 3, cells were expanded with fresh growth media in the presence of additional cytokines at the same concentrations as in day 0 medium. Splenic T cells from BALB/c or C3H.SW mice cultured under the same conditions were used as syngeneic controls.

**Isolation of intestinal cells**

Single-cell suspensions were prepared from intestines as described previously (Zhang et al., 2015). In brief, intestines were flushed with PBS to remove fecal matter and mucus. Fragments (<0.5 cm) of intestines were digested in 10 ml DMEM containing 2 mg/ml collagenase type B (Roche), 10 µg/ml deoxyribonuclease I (Roche), and 4% bovine serum albumin (Sigma-Aldrich) at 37°C with shaking for 90 min. The digested mixture was then diluted with 30 ml of plain DMEM, filtered through a 70-µm strainer, and centrifuged at 850 g for 10 min. The cell pellets were suspended in 5 ml of 80% Percoll (GE Healthcare), overlaid with 8 ml 40% Percoll, and spun at 2,000 rpm for 20 min at 4°C without braking. Enriched lymphocytes were collected from the interface. For sorting of intestinal stem cells, we did not perform Percoll separation.
Cell sorting

CD4+ or CD8+ T cells were harvested from in vitro differentiated WT T9IL-33 or ST2−/− T9IL-33 cells for quantitative RT-PCR and NanoString analysis. CD4+ T cells and CD8+ T cells were sorted from single-cell suspensions of intestine from GVHD mice at day 14 after transplantation of allogeneic T1, WT T9IL-33, or ST2−/− T9IL-33 cells. Cell sorting was performed using a BD FACSAria (BD Bioscience).

ACT of Foxp3-GFP T9IL-33 cells

Total splenic T cells from B6 Foxp3-GFP reporter mice were purified via magnetic bead selection (Miltenyi Biotec). Cells were plated at a concentration of 1×10^6 cells/ml and activated with 1 µg/ml plate-bound anti-CD3 (2C11) and 5–10 µg/ml soluble anti-CD28 (37.51). CD4+ and CD8+ cells were polarized toward T9 IL-33 cells as described before. 5 d later, cells were harvested and sorted to obtain Foxp3-GFP cells. Then, 3 × 10^6 Foxp3-GFP-depleted or nondepleted cells were injected with 5 × 10^6 TCD BM cells into lethally irradiated C3HLSW mice.

Histopathological analysis of GVHD

Specimens of liver and intestine were made of formalin-preserved tissue by the Pathology Department at Indiana University School, New Brunswick, NJ. A semiquantitative scoring system was used to assess abnormalities known to be associated with GVHD. After scoring, the codes were broken and the data were compiled.

T reg isolation

T reg cells were isolated magnetically from fresh splenocytes or in vitro polarized toward T reg cells (10 ng/ml IL-2 and 10 ng/ml TGF-β) using the CD4+CD25+ regulatory T cell isolation kit.

Quantitative RT-PCR

Total RNA was isolated from sorted cells using the RNeasy Plus Mini kit (QIAGEN). cDNA was prepared with SuperScript VILO cDNA Synthesis kit (Invitrogen). Quantitative RT-PCR was performed using SYBR green PCR mix on an ABI Prism 7500HT (Applied Biosystems). Thermocycler conditions included a 2-min incubation at 50°C and then 95°C for 10 min, followed by a two-step PCR program of 95°C for 5 s and 60°C for 60 s for 40 cycles. β-Actin was used as an internal control. The primer sequences were as follows: actin forward: 5′-CTCTGGCTTCTAGCACCATGAAGA-3′; reverse: 5′-GTAAAACGGCAGCTGATACGTCCG-3′; IL-2 forward: 5′-AAGGCACATCAAGGCTGA-3′; reverse: 5′-TCTGAGAAGCTGACCATC-3′; IL-9r forward: 5′-TTCGAGAAGTGAGCTTC-3′; reverse: 5′-GTGAAGATTCGAGCAGATTGAATG-3′; AREG forward: 5′-TCTGAGAAGTGAGCTTC-3′; reverse: 5′-GTGAAGATTCGAGCAGATTGAATG-3′; EGFR forward: 5′-TGGGATTATCCATGCGGAAGAC-3′; reverse: 5′-GAGGTTCCCAGCGGTCTCTCTCTCTCTCTCT-3′.

Nanostring

Sorted CD4+ or CD8+ T cells from WT or ST2−/− T9IL-33 cells were prepared for NanoString analysis as described previously (Zhang et al., 2015). In brief, RNA was isolated from sorted cells using the RNase Plus Mini kit (QIAGEN). NanoString analysis was performed with the nCounter Analysis System at NanoString Technologies. The nCounter Mouse Immunology kit, which includes 561 immunology-related mouse genes, was used.

ELISA

Concentrations of IFN-γ, TNF, IL-9, and IL-4 in the culture supernatant or plasma were measured with the Duoset ELISA kits; sST2 was measured with the Quantikine ELISA kit (R&D Systems).

Statistical analysis

A log-rank test was used for survival analysis. Differences between two groups were compared using two-tailed unpaired t tests using GraphPad Prism software (version 6.05). Data in graphs represent mean ± SEM. P-values less than 0.05 were considered significant.

Online supplemental material

Fig S1 shows that no expansion of T reg cells occurred during murine T9IL-33 differentiation in vitro. Fig. S2 shows the total number of transcripts reads in the transcriptome analysis for Gzma, Gzmb, Prf1, Fas, Cdc27, Cdc62l1, and Cdc8a in sorted WT T9IL-33 versus ST2−/− T9IL-33 CD8 and CD4. Table S1 lists all antibodies used for the flow cytometry analyses. Table S2, included in a separate PDF, provides the raw data of the transcriptome analysis on sorted CD8 and CD4 cells from WT T9IL-33 versus ST2−/− T9IL-33 cells.

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