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ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease

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Abstract

Graft-versus-host disease (GVHD) remains a devastating complication after allogeneic hematopoietic cell transplantation (HCT). We previously identified high plasma soluble

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J.Z. and A.M.R. designed and performed research, analyzed data, and wrote the paper; B.G. and W.L. performed research; M.J.T. contributed new mice and provided intellectual input; C.L. graded and scored GVHD histopathology; R.K. and H.H. provided essential materials and provided intellectual input; B.R.B. was involved in data discussions and manuscript editing; I.T. designed and performed research, analyzed data, and was involved in data discussions; S.P. conceived the project, designed experiments, analyzed data, and wrote the paper.

Conflict of Interest:

Dr. Paczesny has a patent on "Methods of detection of graft-versus-host disease" licensed to Viracor-IBT Laboratories. Otherwise, the authors have no other relevant conflicts of interest to declare.

suppression of tumorigenicity 2 (sST2) as a biomarker of the development of GVHD and death. sST2 sequesters interleukin (IL)-33, limiting its availability to T cells expressing membrane-bound ST2 (mST2) [Th2 cells and ST2⁺FoxP3⁺regulatory T cells]. Here, we report that blockade of sST2 in the peri-transplant period with a neutralizing monoclonal antibody (anti-ST2 mAb) reduced GVHD severity and mortality. We identified intestinal stromal cells and T cells as major sources of sST2 during GVHD. ST2 blockade decreased systemic interferon- γ , IL-17, and IL-23 but increased IL-10 and IL-33 plasma levels. ST2 blockade also reduced sST2 production by IL-17-producing T cells while maintaining protective mST2-expressing T cells, increasing the frequency of intestinal myeloid-derived suppressor cells, and decreasing frequency of intestinal CD103 dendritic cells. Finally, ST2 blockade preserved graft-versus-leukemia activity in a model of GFP+MLL-AF9 acute myeloid leukemia. Our findings suggest that ST2 is a therapeutic target for severe GVHD, and that the ST2/IL-33 pathway could be investigated in other T-cell mediated immune disorders with loss of tolerance.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is an essential therapeutic modality for patients with hematological malignancies and other blood disorders. The most common indications for allo-HCT are acute myeloid leukemias and myelodysplastic syndromes. In these patients, the beneficial effects of allo-HCT are based on immune-mediated elimination of leukemic cells via the graft-versus-leukemia (GVL) activity of donor T cells, the most validated immunotherapy to date (1–3). Unfortunately, donor T cells also mediate damage to normal host tissues, potentially leading to graft-versus-host disease (GVHD) (4, 5). GVHD remains the major complication of allo-HCT and is associated with high mortality, morbidity, and healthcare costs. Current strategies to control GVHD rely on global immunosuppression, for which little progress has been made since the introduction of calcineurin inhibitor-based regimens in the mid-1980s. Despite standard prophylaxis with these regimens, acute and chronic GVHD still develop in approximately 40–60% of allo-HCT recipients (6–8). In addition, non-selective immunosuppression approaches can decrease GVL activity, increasing the risk of leukemia relapse (3, 9). Therefore, new approaches are needed to prevent GVHD without diminishing GVL efficacy.

We recently reported that high plasma levels of suppression of tumorigenicity 2 (ST2) at day 14 post-HCT is a prognostic biomarker for the development of GVHD and death (10). ST2, also known as interleukin (IL)-33 receptor (IL-33R), is the newest member of the IL-1 receptor family, and its only known ligand is IL-33 (11). Due to alternative splicing, ST2 has two main isoforms: a membrane-bound form (mST2) and a soluble form (sST2) (12). mST2 consists of three extracellular immunoglobulin domains and an intracellular toll-like receptor domain, which associates with the IL-1R accessory protein to induce MyD88-dependent signaling. ST2 is expressed on various innate and adaptive immune cell types and drives the production of type 2 cytokines, which are responsible for protective type 2 inflammatory responses in infection and tissue repair as well as detrimental allergic responses (11, 13–17). sST2 lacks the transmembrane and intracellular toll-like receptor domains and functions only as a decoy receptor to sequester free IL-33 (17–19).

As a reflection of the role of the IL-33/ST2 signaling pathway in allogeneic reactions, sST2 concentrations are increased in acute cardiac allograft rejection (20) and treatment with IL-33 prolongs allograft survival via the expansion of T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (21, 22). sST2 levels are also increased in patients with active inflammatory bowel disease (23, 24), a condition similar to gastrointestinal (GI) GVHD. sST2 increase has been suggested to represent a mechanism by which intestinal inflammatory pathogenic responses are perpetuated by limiting IL-33–driven ST2⁺ Treg accumulation and function in the intestine (25). Although both pro-inflammatory and anti-inflammatory roles have been reported for IL-33 (11), in the disease models mentioned above, IL-33 has had a clear anti-inflammatory role particularly via signaling through the membrane-bound mST2 on Tregs that results in an up to 20% greater steady-state level of total Tregs in the gut (25). In our study, due to the similarities with the colitis models, namely the elevated plasma level of the IL-33 decoy receptor, sST2, and because the GI tract is the main GVHD target organ, we hypothesized that sST2 has a pro-inflammatory role due to its decoy activity and IL-33 plays an anti-inflammatory role via an increase in ST2⁺ Tregs and MDSCs in the GI tract.

Whether sST2 is a key player in the development of GVHD or only a circulating molecule indicating increased GVHD risk has remained unclear. Furthermore, it was unclear if sST2 could be drug-targetable and therefore employed to alleviate GVHD. In the present study, we investigated the effects of sST2 blockade using anti-ST2 monoclonal antibody (mAb) on GVHD severity and mortality in a clinically relevant model of HCT and GVL effects against retrovirally transduced GFP⁺MLL-AF9 acute myeloid leukemia. We also tested the hypotheses that during GVHD the ratio of sST2 to mST2 is increased and that the major source of sST2 is the GI tract. Therefore, blocking the excess sST2 with anti-ST2 mAb would inhibit its decoy activity and release free IL-33 to bind mST2 receptor on mST2-expressing T cells [Th2 cells and ST2⁺FoxP3⁺ Tregs] that we found to be protective in our GVHD model. As no anti-ST2 mAb specific to the soluble form was available to us, we used the full-length anti-ST2 mAb available from Centocor (CNT03914) (26) and tested several doses and schedules to identify a treatment course that would inhibit the sST2 without inhibiting mST2. Our results indicate that anti-ST2 mAb represents a therapeutic modality for the safe and efficient targeting of sST2 to control severe GVHD. Our findings also suggest that sST2 secreted by intestinal stromal/endothelial cells and intestinal alloreactive T cells limits the local and systemic expansion and function of mST2-expressing cells, particularly Th2 cells and ST2⁺FoxP3⁺ Tregs, by antagonizing IL-33 activity and reducing its bioavailability. Because aberrant ST2/IL-33 signaling has been linked to many human diseases, the results of this study may have broad implications in other T cell-mediated immune disorders.

RESULTS

Similar to GVHD patients, experimental models of allo-HCT show increased plasma concentrations of sST2 before GVHD onset

To determine whether sST2 might contribute to GVHD similarly to observations in patients, we first assessed the kinetics of plasma sST2 in a minor histocompatibility antigen (miHA)-

mismatched model of allo-HCT and a human-to-mouse xenogeneic model. Donor T cells derived from C57BL/6 (B6) mice or human T cells were transplanted into irradiated miHA-mismatched C3H.SW or xenogeneic NOD-*scid*IL2Rgamma^{null} (NSG) mice, respectively, to induce GVHD. Mice receiving syngeneic T cells or irradiation only were used as controls. As expected, all allogeneic/xenogeneic recipient mice receiving donor T cells developed severe GVHD, with approximately 80% dying of GVHD. By contrast, mice receiving syngeneic cells did not develop any clinical signs of GVHD. Enzyme-linked immunosorbent assay (ELISA) analysis showed that the plasma sST2 concentration was significantly increased in mice receiving allogeneic/xenogeneic HCT but not syngeneic or irradiation only controls by day 10 and day 21 after transplantation, mimicking the kinetics observed in patients (10) (Fig. 1A and 1C).

sST2 can be blocked by a neutralizing anti-ST2 mAb, leading to a decrease in pro-inflammatory and increase in anti-inflammatory cytokine plasma levels and decreased acute GVHD severity and mortality

Given the high levels of circulating sST2, we hypothesized that sST2 blockade can ameliorate GVHD severity by blocking its decoy receptor activity and thus releasing free IL-33 that will be used by mST2-expressing T cells. We used a monoclonal antibody targeting murine ST2 (anti-ST2 mAb) (CNT03914) or an appropriate control isotype Ab (IgG) (26). We used the miHA model B6→C3H.SW, as it is most clinically relevant not only because approximately 80% of HCTs performed today in the US are 8/8 MHC-matched (the Center for International Blood and Marrow Transplant Research (CIBMTR) data as a personal communication) but also because the GVHD is both CD8 and CD4 dependent (5). The anti-ST2 mAb and IgG control (both at 100 µg/dose) were administered to mice via intra-peritoneal injection every other day from day -1 to day +9 after HCT. Anti-ST2 mAb blockade strongly attenuated GVHD and increased survival (Fig. S1A). Histopathologic scores in the small intestine, large intestine, and liver (primary GVHD target organs) were improved in anti-ST2 mAb-treated mice, suggesting that ST2 blockade alleviated GVHD severity in the main target organs (Fig. S1B). We then evaluated a shorter schedule of anti-ST2 mAb treatment with only two doses administered on day -1 and day +1 of HCT. Transient blockade of ST2 in the peri-transplant period was sufficient to provide long-lasting protection against GVHD (Fig. 1B). We next tested ST2 blockade in the human-to-mouse xenogeneic GVHD model, and the results showed alleviation of GVHD and improved survival (Fig. 1D). Systemic production of the inflammatory cytokines IFN-γ, IL-17, and IL-23 was decreased, whereas the release of anti-inflammatory cytokines IL-10 and IL-33 was increased in the plasma (Fig. 1E).

The GI tract is the major sST2-producing organ during GVHD

To understand the basis for the effects of the sST2 blockade, we determined the source of sST2 following allogeneic HCT. At day 10 post-HCT, pre-onset of GVHD, quantitative mRNA expression of both sST2 and mST2 was analyzed in the spleen, small intestine, large intestine, skin, BM, lung, heart [as a representative organ for the endothelium and a known source of sST2 (20, 27)], liver, and peripheral blood. The small and large intestines were by far the largest producers of sST2 even compared to the heart (Fig. 2A left), and strikingly, they also showed the lowest levels of mST2 expression (Fig. 2A middle). Therefore, the

sST2/mST2 ratio was increased in the GI tract of mice that developed GVHD (Fig. 2A right).

Intestinal stromal and endothelial cells are a major source of sST2 that is neutralized by anti-ST2 mAb, and ST2^{-/-} deficient recipients exhibit less severe GVHD

sST2 can be produced by a number of different cell types, and we found that sST2 is produced highly in the intestine. Therefore, we investigated the cellular source of sST2 in the intestine. The intestinal stromal cells that are CD45⁻Epcam⁻ and endothelial cells that are CD45⁻Epcam⁻CD146⁺ were major producers of sST2 during GVHD, whereas epithelial cells that are CD45⁺Epcam⁺ did not produce sST2 (Fig. 2B). In addition, myeloid cells (CD45⁺TCRβ⁻) produced only a small amount of sST2. To confirm that the host-derived origin of sST2 production is necessary for GVHD development, we used B6 ST2^{-/-} mice as recipients and showed that sST2 deficiency in recipients reduced the GVHD score and prolonged the survival of the C3H.SW→B6 model (Fig. S2). Furthermore, anti-ST2 mAb blockade decreased sST2 production by stromal cells compared to that in IgG control-treated animals (Fig. 2C). These data suggest that production of sST2 by host stromal cells plays an important role in GVHD and that anti-ST2 blockade can diminish this production.

Intestinal T cells are the other major cellular source of sST2 during GVHD, and T cell production of sST2 is decreased by ST2 blockade

Strikingly, while determining the source of intestinal sST2, we discovered that T cells, mostly CD4⁺ T cells, produced sST2 at the transcript (Fig. 2D) and protein levels (Fig. 2E). Secretion of sST2 by T cells significantly increased during GVHD progression (Fig. 2D). We next hypothesized that anti-ST2 mAb treatment would reduce the production of sST2 by alloreactive T cells in targets organs. Indeed, intestinal sST2 production by T cells was decreased in anti-ST2 mAb-treated animals (Fig. 2E). To explore further which T cell subsets produce sST2 and express mST2 during *in vitro* differentiating conditions, we measured sST2 production and mST2 expression in the CD4 subsets, Th1, Th2, and Th17, as well as CD8 subsets, Tc1, Tc2, and Tc17. Th17 and Tc17 cells were found to be strong producers of sST2 (Fig. 2F left) and to express only low levels of mST2 protein (Fig. S3). Similar results were observed in human T cell subsets (Fig. 2F right).

ST2 deficiency reduces the ratio of sST2-secreting T cells to mST2-expressing T cells

Whole transcriptome analysis of mesenteric lymph node (MLN) T cells comparing anti-ST2 mAb-treated mice vs. IgG control-treated mice showed that anti-ST2 treatment modulated gene expression of helper T cell cytokines (Fig. 3A). To further assess the effects of ST2 blockade on the helper T cell compartment, we examined the sST2-secreting/mST2-expressing T cell balance at the protein level by flow cytometry. ST2 blockade decreased the percentages of Th1 cells and pathogenic Th17 cells (Fig. 3B and 3C). To verify the role of donor sST2 in GVHD, we next used ST2^{-/-} donor T cells in recipients of allo-HCT. Given that ST2^{-/-} donor T cells are incapable of producing sST2, they had a protective effect on GVHD severity and increased survival (Fig. 3D). Recipients of ST2^{-/-} donor T cells showed lower frequencies of IFN-γ/Tbet-producing T cells (Fig. 3E) and IL-17/RORγt-producing T

cells (Fig. 3F), and less proliferation of IFN- γ ⁺IL-17⁺ pathogenic Th17 cells, as measured by Ki67, at day 10 post-transplantation in the GI tract (Fig. 3G).

At the same time, ST2 blockade increased the percentages of Th2 cytokine IL-4 and Th2 transcription factor GATA3 in T cells (Fig. 3H), as well as increased the frequency of FoxP3⁺ Tregs (Fig. 3J). Transient ST2 blockade maintained mST2 expression on GATA3⁺ Th2 cells (Fig. 3H) and FoxP3⁺ Tregs (Fig. 3J). IL-4⁺/GATA3⁺-producing Th2 cells were increased (Fig. 3I) as well as total FoxP3⁺ Tregs and IL-10-producing T cells (Fig. 3K) when ST2^{-/-} donor T cells were used as the graft source, confirming the negative impact of wild-type donor T cells on GVHD via production of sST2. We next specifically investigated the impact of ST2⁺FoxP3⁺ Tregs in GVHD. For this, we used the B6→C3H.SW model with ST2^{-/-} donor Tregs (ratio of Tregs/Tconv of 1/10) and demonstrated that recipients of wild-type donor Tregs had less severe GVHD and improved survival compared to recipients of ST2^{-/-} Tregs (Fig. 3L). These results suggest, similarly to the observed colonic inflammation (25), that wild-type donor Tregs have a better suppressive capacity than ST2^{-/-} Tregs and that mST2 expression on Tregs is important for GVHD protection.

ST2 deficiency induces expansion of tolerogenic MDSCs and inhibits immunogenic CD103⁺ dendritic cells

Because IL-33 has been shown to induce expansion of MDSCs that have a potent T cell suppressive function (21, 22), we explored the effects of ST2 deficiency on intestinal antigen-presenting cell subsets. First, 99% of the antigen-presenting cell populations found in the intestine at day 10 post-HCT are of donor origin (Fig. S4, A and B). Second, ST2 blockade elicited expansion of intestinal MDSCs (CD45.1⁺ MAC-1⁺Gr-1⁺) in anti-ST2 mAb-treated mice (Fig. 4A). Recipients receiving ST2^{-/-} donor T cells also showed significantly increased frequencies of intestinal MDSCs. In addition, given that intestinal CD103⁺ dendritic cells have been shown to generate α 4 β 7 gut-tropic effector T cells in the intestine and MLNs (28), we measured the frequencies of these cells in our GVHD model with and without treatment. The frequencies of CD103⁺ dendritic cells were reduced following ST2 blockade in the GI tract (Fig. 4C) and MLNs (Fig. S5). Similar results were observed in mice receiving ST2^{-/-} donor T cells (Fig. 4D). The total CD11c⁺ dendritic cells from treated animals showed reduced expression of MHC class II and costimulatory molecules (CD40, CD80, CD86) on their surface as compared to the control group (Fig. 4E). Mast cells that express mST2 have been shown to play a major role in supporting regulatory T cells in several diseases including GVHD (29, 30). However, using the classical c-kit and Fc ϵ RI markers, we could not identify intestinal mast cells during GVHD (Fig. S4C).

ST2 blockade preserves substantial antitumoral cytotoxicity and GVL activity

Due to the strong effect of anti-ST2 mAb blockade on not only stromal/endothelial cells but also on T cells, it was crucial to verify that the T cell antitumoral cytotoxicity and GVL activity were preserved. One indication that GVL activity was preserved was the upregulation of cytokines and cytolytic molecules that have been implicated in antitumoral or GVL activity, such as IL-27 (31, 32), IL-18 (33), IL-9 (34, 35), type I IFNs (36), and granzyme A (37), in T cells from the MLNs in anti-ST2 mAb-treated vs. non-treated animals (Fig. 5A). *In vitro* cytolytic assays were performed against syngeneic tumors (A20

lymphoma cell line and MLL-AF9 eGFP⁺ leukemic cells) after stimulation of allo-antigen with a mixed lymphocyte reaction. Addition of anti-ST2 mAb did not decrease antitumoral cytotoxicity (Fig. 5B). Furthermore, due to two major limitations of current GVL models, one being the use of models that overestimate CD4-dependent pathways relative to those observed clinically and the second being the use of cell lines that are extremely sensitive to GVL activity (5), we developed primary retrovirally induced MLL-AF9 eGFP⁺ leukemic cells on the C3H.SW background. The phenotype of the leukemic cells in this model is eGFP⁺, CD3⁻, B220⁻ and Mac-1^{hi}Gr-1^{hi} and is based on previous reports (38, 39). Our results indicate that administration of anti-ST2 mAb over a short course (2 days, Fig. 5C) or a long course (6 days, Fig. 5D) or ST2^{-/-} donor T cells (Fig. 5E) preserved substantial GVL activity and resulted in significantly improved leukemia-free survival.

DISCUSSION

Pharmacological interventions are required to harness the therapeutic potential of sST2 inhibition. Here, we report that inhibition strategies using anti-ST2 mAb (26) reduced the severity of acute GVHD as well as GVHD mortality in the B6→C3H.SW and human T cell→NSG experimental murine model. Transient ST2 inhibition achieved with two doses of mAb was sufficient to provide long-lasting protection against GVHD. As hypothesized, blockade of the decoy receptor decreased circulating IFN- γ , IL-17, and IL-23 levels and released systemic IL-10 and IL-33 levels.

IL-33 has a paradoxical role in immune responses depending on the inflammatory environment and cell types involved. For example, it has recently been shown that IL-33 can increase the function of colonic ST2⁺ Tregs in a colitis model (25) and can exacerbate allergic bronchoconstriction via activation of ST2⁺ mast cells in a mouse model of allergy (40). IL-33 has also been shown to synergize with IL-12 to activate natural killer (NK) and natural killer T (NKT) cells and to enhance their IFN- γ production (41). A recent study showed that exogenous IL-33 administration during the cytokine storm worsened GVHD (42). In our models, adding exogenous IL-33 (five injections in the peri-transplantation period) had no worsening or protective effect on GVHD (Fig. S6). This might be due to the fact that in our minor mismatched models, lower levels of inflammatory cytokines are secreted in response to conditioning and alloreactivity as compared to levels in major mismatched models. On the contrary, we demonstrated an increase in systemic IL-10 and IL-33 that indirectly inhibited the expansion of pathogenic T cells and the production of inflammatory cytokines such as IFN- γ , IL-17, and IL-23. We also demonstrated that anti-ST2 mAb formed a stable complex with sST2 in circulating blood that could be released by immunodepletion of the immune complexes confirming that anti-ST2 mAb can inhibit sST2 specifically (Fig. S7). Importantly, the significant increases in plasma sST2 levels in minor histocompatibility antigen-mismatched allogeneic-HCT and human-to-mouse xenogeneic experimental models by day 10 and day 20 (time of human T cell engraftment) after transplantation, respectively, mimicked the kinetics observed in patients (10). These kinetics in plasma sST2 as well as plasma inflammatory and anti-inflammatory cytokines may have important clinical implications. The ability to identify high-risk patients by measuring plasma concentrations of sST2 and other systemic cytokines as early as day 14 after transplantation, before the development of GVHD, may permit more stringent monitoring

and preemptive interventions based on these markers and the use of a GVHD-specific inhibitor.

Although it was previously shown using the technologies available at the time that activated CD4⁺ T cells, but not resting T cells, might produce sST2 while expressing low levels of mST2 (43), this has never been demonstrated in the context of diseases via extensive analysis of all T cell subsets. We have shown here that there is a differential balance of sST2 secretion vs. mST2 expression in alloreactive T cells. Indeed, with increasing severity of GVHD, more pathogenic T cells (Th17 and Tc17) secrete sST2 and express less mST2, possibly explaining why elevation of plasmatic sST2 is specific to alloreactivity. This study also emphasizes that Th17 and Tc17 cells are seen mainly in the intestine during GVHD and are important players in GVHD development. We have clearly shown that transient ST2 blockade blocked sST2 specifically in the plasma and target organs, particularly in the GI tract, while maintaining the mST2 expression on T cells, particularly Th2 and ST2⁺ Tregs.

We also found that ST2 blockade not only decreased the expression of the Th1 transcription factor Tbet and the corresponding inflammatory cytokine IFN- γ , but also increased the production of the Th2 transcription factor GATA3 and Th2 cytokine IL-4, skewing the Th1/Th2 balance toward a Th2 phenotype, which protects against severe GVHD. In addition, we and others have previously shown that the ratio of FoxP3-expressing Tregs to conventional T cells is significantly decreased in severe GVHD (44–46). Remarkably, ST2 blockade also increased the frequency of functional FoxP3⁺ Tregs in the spleen and gut, and decreased the percentage of pathogenic Th17 cells, without impairing the ST2⁺FoxP3⁺ Tregs that we showed are crucial for protection against GVHD. Because the anti-ST2 Ab used in our study is a full-length Ab that potentially inhibits both the soluble and membrane-bound forms, we verified that the inhibitory effect was limited to the soluble form by measuring the frequency of ST2⁺ Tregs after treatment. In accordance with the findings of a recent study showing that ST2⁺ Tregs have a better suppressive capacity than Tregs not expressing mST2 and are better able to prevent colonic inflammation (25), we confirmed that this is true in intestinal GVHD as well. Indeed, ST2^{+/+} Tregs more effectively protected against GVHD than ST2^{-/-} Tregs. However, we demonstrated that although ST2^{+/+} Tregs have an important protective role in GVHD, the role of sST2 secretion by alloreactive conventional T cells (Tcons) is predominant because HCT with ST2^{-/-} donor T cons with or without ST2^{+/+} donor Tregs (Tregs/Tconv ratio of 1/10) resulted in less severe GVHD and improved survival in both cases (Fig. S8 and Fig. 3D). Together, our results indicate that high levels of sST2 production by T cells during GVHD may represent a mechanism to further perpetuate pathogenic responses by limiting IL-33-driven Treg accumulation.

T cells subsets are regulated by antigen-presenting cells. Given the role of the ST2/IL-33 pathway in MDSCs and mast cells (21, 22, 29, 30), we explored their respective frequencies as well as that of CD103⁺ dendritic cells in the intestine and MLNs during GVHD with and without ST2 deficiency. ST2 deficiency (ST2 blockade or knockout) induces expansion of tolerogenic MDSCs and a decrease in CD103⁺ dendritic cells. Furthermore, the total CD11c⁺ dendritic cells from treated animals expressed lower levels of MHC class II and costimulatory molecules (CD40, CD80, CD86) on their surface as compared to the control group. The potential mechanisms responsible for these changes need to be further explored.

The GI tract has been shown to be the sentinel site for GVHD (47), and this may be due to the presence of large numbers of nonhematopoietic stromal cells that can act as antigen-presenting cells within this target organ (48). GVHD of the GI tract affects up to 60% of HCT recipients, and the GI tract is also the GVHD target organ associated with the highest mortality rate (49, 50). Consistent with the tropism of GVHD for the GI tract, we found that the intestine was indeed the major source of sST2, particularly the stromal cells and endothelial cells of the GI tract, which are classically damaged during conditioning by irradiation or chemotherapy. We further confirmed the importance of host sST2 in GVHD based on the observation of less severe GVHD in recipient ST2^{-/-} mice. Importantly, we also demonstrated that intestinal T cells are another major source of sST2. This mechanism whereby sST2 is secreted by mainly CD4⁺ T cells (mostly Th17 cells) may explain the specificity of sST2 immune functions during alloreactivity as well as the quite dramatic protective effect of anti-ST2 blockade on GVHD severity and mortality. Indeed, our findings highlight the therapeutic potential of targeting sST2 as a new strategy for controlling GVHD following allo-HCT that could be applied in a number of other diseases with elevated sST2 that are commonly due to T cell dysregulation in immune responses.

Finally, ST2 blockade retained substantial GVL activity. The reasons might be that: (1) Tbet⁺ and IFN- γ ⁺ CD4 T cells are suppressed to a lesser extent after ST2 blockade (Fig. 3A); (2) anti-ST2 mAb may target more specifically sST2-producing alloreactive T cells that are not implicated in GVL activity; and (3) cytokines and cytolytic molecules related to antitumoral or GVL activity are up regulated in mice treated with anti-ST2 mAb (Fig. 5A).

Several limitations to the present study should be noted. First, although ST2 blockade ameliorates GVHD mortality in correlation with an increase in systemic IL-33, *ex vivo* systemic injections of IL-33 did not lead to improvement in the treated animals, suggesting that endogenous produced IL-33 and exogenously administered IL-33 have different (i) circulating doses (in the pg/mL range vs. ng/mL range, respectively), (ii) pharmacokinetics, and (iii) binding properties *in vivo*. Second, the anti-ST2 mAb from Centocor recognizes both sST2 and mST2, which may target the beneficial effect of ST2⁺ Tregs. Although we have demonstrated that (i) sST2 during GVHD was produced by the key T cell players, (ii) that ST2⁺ Tregs were not decreased, and (iii) that the net result of ST2 blockade was GVHD alleviation, the development of monoclonal antibodies or small inhibitory molecules targeting only sST2, leaving mST2 intact, would further strengthen the results found in our study. Furthermore, a ST2-Fc fusion protein has been developed and used with some success to inhibit ST2/IL33 signaling *in vitro* (51) and *in vivo* (42). Monoclonal antibodies will need to be humanized for use in clinical trials. Third, we have only shown the protective effect of ST2 blockade when used as a prophylactic treatment; thus, further experiments are needed to show the effects of ST2 blockade in models in which acute GVHD has already started to develop and in chronic GVHD models.

In sum, our findings identify intestinal alloreactive T cells as an important source of the decoy receptor for IL-33 that can be blocked with two doses of anti-ST2 mAb in the peri-transplant period without inhibiting the beneficial mST2 expression on Th2 cells and Tregs. This study offers new perspectives on the translation of drug-targetable biomarkers for selectively and safely treating GVHD and other T cell-mediated human disorders.

MATERIALS AND METHODS

Study design

This study was designed to inhibit the interaction between IL-33 and sST2, the decoy receptor for IL-33, to release free IL-33 as a proof-of-principle demonstration of a drug targetable biomarker. We assessed the potential of sST2 blockade in multiple experimental models of GVHD. We used a monoclonal antibody targeting murine ST2 (anti-ST2 mAb) from Centocor (CNT03914), which was available to us through a Material Transfer Agreement or an appropriate control isotype Ab (IgG) with different doses and schedules. We evaluated the therapeutic effect of anti-ST2 mAb in GVHD by monitoring GVHD clinical scores, histopathological GVHD scores, and survival. We also measured the increases in production of systemic anti-inflammatory cytokines IL-10 and IL-33 and the decreases in systemic pro-inflammatory cytokine production by ELISA. We then investigated the source of sST2 during GVHD by analyzing sST2 secretion in different organs. We further assessed the ratio of sST2-secreting T cells to mST2-expressing T cells, mostly FoxP3⁺ Tregs. We also compared the protective effects of mST2-expressing Tregs and ST2^{-/-} Tregs against GVHD. Finally, we generated a model of retrovirally transduced GFP⁺MLL-AF9 acute myeloid leukemia to assess the effects of ST2 blockade on GVL activity. All experiments were replicated at least 3 times.

Mice

Balb/c (H-2^d), B6 (H-2^b, CD45.2⁺), B6 (C57BL/6.Ptprca, H-2^b, CD45.1⁺), C3H.SW (H-2^b, CD45.2⁺), NOD-*scid*/IL2Rgamma^{null} (NSG) mice were from the Jackson Laboratories. B6 (ST2^{-/-}, H-2^b, CD45.2⁺) mice were provided by Dr. Andrew McKenzie from University of Cambridge, UK. B6 (ST2^{-/-}, H-2^b, CD45.1⁺) were bred in the mouse breeding facility at Indiana University School of Medicine. Animal protocols were approved by Institutional Animal Care and Use Committee at Indiana University School of Medicine.

Induction and assessment of GVHD

Mice underwent allo-HCT as previously described (52). Briefly, in miHA-mismatched GVHD models (B6→C3H.SW and C3H.SW→B6), C3H.SW or B6 recipients mice received 1100 cGy and 1250 cGy total body irradiation (¹³⁷Cs source) at day -1. Then recipient mice were injected intravenously with T cell depleted (TCD) bone marrow cells (5 × 10⁶) plus splenic T cells (2 × 10⁶ for C3H.SW, 3 × 10⁶ for B6) from either syngeneic or allogeneic donors at day 0. T cells from donor mice were enriched by either murine Pan T Cell Isolation Kit (Miltenyi) and T cell depleted bone marrow (TCD BM) was prepared with CD90.2 Microbeads (Miltenyi). For adoptive transfer models (B6→C3H.SW), WT and ST2^{-/-} B6 donor total T cells or Tregs were purified by murine Pan T Cell Isolation Kit and murine CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi). Irradiated C3H.SW recipient mice were injected intravenously with TCD bone marrow cells (5 × 10⁶) and indicated number of T cells in different experiments. In the xenogeneic GVHD model (human T cells→NSG mice), irradiated (350 cGy) NSG mice were transplanted with human total T cells from PBMCs (2.5 × 10⁶) at day 0. PBMCs were prepared from human PB Leukopacks from healthy donors that were purchased from the Central Indiana Blood Center under an institutional review board-approved protocol. PBMCs were isolated within 24 h

after blood draw by Ficoll (GE Healthcare,) density gradient centrifugation. Human total T cells were purified using the human Pan T Cell Isolation Kit (Miltenyi). Mice were housed in sterilized microisolator cages and maintained on acidified water (pH < 3) for 3 weeks as described previously (53). NSG mice were maintained on food supplemented with Uniprim and acid water during the whole time course of the experiment. Survival was monitored daily. Clinical GVHD scores were assessed weekly as described previously (54). According to animal protocols approved by the Institutional Review Board, mice were euthanized when the clinical score achieved 6.5.

Enzyme-linked immunosorbent assay

Concentrations of ST2 (both murine and human), IL-17A, IL-23, and IL-33 in mouse plasma and culture supernatants were measured by Quantikine ELISA Kits (R&D Systems). IFN- γ and IL-10 were measured by the DuoSet ELISA Kit (R&D Systems).

Anti-ST2 mAb treatment

The anti-ST2 mAb was from Centocor, a pharmaceutical company of Johnson and Johnson (CNT03914). This chimeric antibody contains a mouse IgG1 Fc, and it recognizes the full length extracellular domain of membrane bound ST2 (26). Anti-ST2 mAb or IgG control antibody were given to recipient mice receiving allo-HCT mice by intraperitoneal (IP) injection at day -1 and day 1 post-HCT, 100 μ g/mouse. In some experiments, mice received 6 doses of anti-ST2 mAb every other day from day -1 to day +9. In the xenogeneic GVHD model, NSG mice received 4 doses of both anti-human (R&D Systems, Clone 97203) and anti-mouse ST2 (CNT03914) mAbs every other day from day -1 to day 5 (IP, 100 μ g/mouse).

Histopathological analysis of GVHD

Specimens of liver and intestine were made of formalin-preserved tissue by the Pathology Department at Indiana University Medical Center. Slides were coded without reference to prior treatment and examined in a blinded fashion by Dr. Liu (University of Florida). A semi-quantitative scoring system was used to assess the following abnormalities known to be associated with GVHD (55, 56). After scoring, the codes were broken and data were compiled.

Isolation of intestinal cells

Single cell suspensions were prepared from intestines as described, with modifications (57). Briefly, intestines were flushed with PBS to remove fecal matter and mucus. Fragments (<0.5 cm) of intestines were digested in 10 mL of DMEM containing 2 mg/mL collagenase type B (Roche), 10 μ g/mL DNase I (Roche), and 4% BSA (Sigma) at 37 °C with shaking for 90 min. The digested mixture was then diluted with 30 mL of plain DMEM, filtered through 70- μ m strainers, and centrifuged at 850 $\times$ g for 10 min. Cells pellets were suspended in 5 mL 80% Percoll (GE Healthcare), overlaid with 8 mL 40% Percoll, and spun at 2000 rpm for 20 min at 4°C without braking. Enriched lymphocytes were collected from the interface. Stromal, endothelial, epithelial, and CD45⁺TCR- β ⁻ cells were sorted directly from single cell suspensions without Percoll separation.

Flow cytometric analysis and cell sorting

All antibodies (Table S4) and reagents for flow cytometry were purchased from eBioscience unless stated otherwise. The cells were pre-incubated with purified anti-mouse CD16/CD32 mAb for 10–20 minutes at 4°C to prevent non-specific 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. FoxP3/Transcription Factor Staining Buffer Set and Fixation and Permeabilization Kit were used for intracellular transcription factor and cytokine staining. For cytokine staining, cells were restimulated with phorbol myristate acetate (PMA; 50 ng/mL), ionomycin (1 µg/mL; Sigma-Aldrich), and Brefeldin A for 4–6 hours prior to any staining.

Cell sorting

MLN T cells [Fixable viability dye (FVD)⁻CD90.2⁺CD3⁺] from treated C3H.SW recipients mice receiving allo-HCT were sorted for nanostring analysis. Total T cells (FVD⁻CD45⁺TCR-β⁺), CD4⁺ T cells, CD8⁺ T cells, CD45⁺TCR-β⁻ cells, stromal cells (FVD⁻CD45⁻Epcam⁻), epithelial cells (FVD⁻CD45⁻Epcam⁺), and endothelial cells (FVD⁻CD45⁻Epcam⁻CD146⁺) were sorted from single cell suspensions of intestine from treated C3H.SW recipient mice for quantitative reverse-transcription polymerase chain reaction (RT-PCR) or western blot analysis. Cell sorting was performed on BD FACSAria (BD Bioscience) or iCyt Reflection (Sony Biotechnology) in the flow cytometry core facility at Indiana University School of Medicine.

Nanostring analysis

Sorted MLN T cells from either IgG control or anti-ST2 mAb treated GVHD mice were directly lysed by RTL buffer (QIAGEN) on ice. The cell concentration for lysis was $0.2 \times 10^4 - 1 \times 10^4$ cells/µL with a total of 5 µL by RTL buffer. Lysis samples were frozen in liquid nitrogen immediately then stored at -80°C or on dry ice. Nanostring analysis was performed with the nCounter® Analysis System at NanoString Technologies. nCounter® Mouse Immunology Kit, which includes 561 immunology-related mouse genes, was used in our study.

Quantitative RT-PCR

Total RNA from spleen, small intestine, large intestine, skin, bone marrow, lung, heart, and peripheral blood were isolated using the RNeasy Plus Mini Kit (QIAGEN). cDNA was prepared with SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative real-time polymerase chain reaction (PCR) was performed using SYBR Green PCR mix on an ABI Prism 7500HT (Applied Biosystems). Thermocycler conditions included 2-minute incubation at 50°C, then 95°C for 10 minutes; this was followed by a 2-step PCR program: 95°C for 5 seconds and 60°C for 60 seconds for 40 cycles. β-Actin was used as an internal control to normalize for differences in the amount of total cDNA in each sample. The primer sequences were as follows: actin forward: 5'-CTCTGGCTCCTAGCACCATGAAGA-3' (58) actin reverse: 5'-GTAAACGCAGCTCAGTAACAGTCCG-3' mST2 forward: 5'-AAGGCACACCATAAGGCTGA-3' mST2 reverse: 5'-TCGTAGAGCTTGCCATCGTT-3'

sST2 forward: 5'-TCGAAATGAAAGTTCCAGCA-3' (25) sST2 reverse: 5'-TGTGTGAGGGACACTCCTTAC-3'.

Two-color western blots

Sorted cells were lysed by RIPA Buffer (Pierce Biotechnology) with Pierce Phosphatase Inhibitor Mini Tablets (Pierce Biotechnology) and Protease Inhibitor Cocktail Tablets (Roche). Samples were boiled, electrophoretically separated, and transferred on Immobilon-FL® PVDF membrane (Millipore). Blots were blocked in Odyssey® Blocking Buffer (LI-COR) for 1 hour at room temperature and incubated with specific primary antibodies: biotinylated anti-mouse ST2 monoclonal antibody (DJ8; mdBioproducts) and anti-β-Actin monoclonal antibody (926–42212, LI-COR) at 4°C overnight. IRDye® 800CW Streptavidin (926–32230, LI-COR) and IRDye® 680RD goat anti-mouse IgG polyclonal antibodies (926–68070, LI-COR) were used as secondary detection antibodies for ST2 and β-actin, respectively. Fluorescence from blots was then developed by Odyssey® CLx Imaging Systems (LI-COR) according to the manufacturer's instructions.

T cell differentiation

Total CD4⁺ or total CD8⁺ T cells were purified from B6 spleens via magnetic isolation beads (Miltenyi Biotec). These 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). Both the CD4⁺ and CD8⁺ cells were polarized toward either Th1/Tc1 (1 ng/mL IL-2, 20 ng/mL IL-12), Th2/Tc2 (20 ng/mL IL-4), or Th17/Tc17 (4 ng/mL TGF-β, 10 ng/mL IL-6, 10 ng/mL IL-1β, and 20 ng/mL IL-23) conditioning in complete media. On day 3, the cells were expanded with fresh media in the presence of additional cytokines at the same concentration as day 0 for Th1/Tc1, Th2/Tc2, and Th17/Tc17 cells. On day 5, the cells were stimulated with 10 µg/mL of both anti-CD3 and anti-CD28 as well as with PMA (50 ng/mL) and ionomycin (1 µg/mL) overnight. The next day the supernatant was collected for ELISA analysis. Cytokines and antibodies were purchased from R&D Systems (IL-1β, IL-2, IL-4, IL-6, IL-12, IL-23, and TGF-β) and eBioscience (anti-CD3, anti-CD28). Human T cells were purified from PBMCs of healthy donors and activated with anti-CD3/CD28 microbeads (anti-CD3/ICOS for Th17/Tc17) from Life Technologies. Both the CD4⁺ and CD8⁺ cells were polarized toward either Th1/Tc1 (1 ng/mL IL-2, 20 ng/mL IL-12, and 10 µg/mL anti-IL-4), Th2/Tc2 (20 ng/mL IL-4 and 10 µg/mL anti-IFN-γ), or Th17/Tc17 (4 ng/mL TGF-β, 10 ng/mL IL-6, 10 ng/mL IL-1β, 20 ng/mL IL-23, 10 µg/mL anti-IL-4, and 10 µg/mL anti-IFN-γ) conditioning in complete media. On day 3, the cells were expanded with fresh media in the presence of additional cytokines at the same concentration as day 0 for Th1/Tc1, Th2/Tc2, and Th17/Tc17 cells. On day 7, the cells were stimulated with 10 µg/mL of both anti-CD3 and anti-CD28 as well as with 50 ng/mL PMA.

Generation of MLL-AF9-eGFP leukemic cells

The retroviral vector containing the MLL-AF9 eGFP cDNA construct (38) was generously provided by Dr. Reuben Kapur and used to generate retroviral supernatants by transit transfection of Phoenix Eco Cell Line (ATTC) using FuGENE 6 Transfection Reagent (Promega). Eighteen hours before transfection, Phoenix Eco cells were seeded in gelatin-coated 100-mm plates (8×10^6 /plate). After gently aspirating off cell culture medium, cells

were transfected with a mixture of 20 µg of DNA and 60 µl of transfection reagent (3 µL/µg DNA) in 5 mL of plain DMEM. Six to eight hours later, 3 mL of DMEM supplemented with 15% heat inactivated FBS were added to plate. After 18 hours of incubation, the medium was replaced with 8 mL of IMDM (Life Technologies) supplemented with 10% FBS, 100 U/mL penicillin and streptomycin, and 1 mM sodium pyruvate. After a 24-hour incubation, retroviral supernatants were collected and filtered through 0.45-µm filters. Freshly-prepared retroviruses were used to transduce c-kit⁺ cells that were magnetically enriched from Balb/c or C3H.SW BM cells using the CD117 MicroBead Kit (Miltenyi) and pre-stimulated for 48 hours with 10 ng/mL IL-3, 10 ng/mL IL-6, and 20 ng/mL SCF (all from Peprotech). After two consecutive 24-hour infections in non-tissue culture plates pre-coated with retronectin (59), cells were collected and their infection efficiency was determined by eGFP expression using flow cytometry. Approximately 1×10^6 of cells, 8% of which were eGFP⁺, were injected into lethally irradiated Balb/c or C3H.SW mice intravenously via the tail vein. Mice were monitored daily and checked weekly for leukemia development through total blood cell and platelet counts (Hemavet 1700, Drew Scientific). At day 35, mouse peripheral blood showed high leukocyte counts, anemia, and low platelet counts (leukemia symptoms). Mice were subsequently euthanized and bone marrow cells were analyzed by flow cytometry, which showed that all bone marrow cells were eGFP⁺, CD3⁻, B220⁻ and Mac-1^{hi}Gr-1^{hi}.

***In vitro* cytotoxicity assay**

The A20 cell line expressing E2-Crimson fluorescent protein was generously provided by Dr. Helmut Hanenberg. Cytotoxicity assays were performed as previously described (34, 35), with some modifications. Briefly, T cells were primed in a mixed lymphocyte reaction. E2-Crimson A20 or eGFP-expressing MLL-AF9 cells were incubated with syngeneic Balb/c or allogenic B6 T cells at different ratios as indicated. After 8 hours of incubation at 37°C, cells were analyzed by flow cytometry.

Induction and assessment of GVL effect

C3H.SW mice were lethally irradiated (1100 cGy) 1 day before BM transplantation. Recipient mice were injected intravenously with 5×10^6 B6 or C3H.SW BM cells and 2×10^6 enriched B6 or C3H.SW splenic T cells and 10^4 MLL-AF9 cells on day 0. Mice were treated intraperitoneally with anti-ST2 antibody or isotype IgG control at day -1 and day +1 or 6 doses of anti-ST2 mAb every other day from day -1 to day +9, as described above. Mice were monitored daily for survival and leukemia development and weekly for GVHD score. 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 of 6.5. Cells from peripheral blood, BM, spleen, and liver were analyzed by flow cytometry.

Statistics

Log-rank test was used for survival analysis. Differences between two groups were compared using unpaired t test using GraphPad Prism software, version 6.05. Error bars in graphs represent mean $\pm$ standard error of the mean (SEM). Differences between three or more groups were compared using one-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad Prism software, version 6.05. Values of p less than .05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. ST2 blockade and GVHD

(A) Irradiated C3H.SW mice (1100 cGy) were transplanted with syngeneic (●) or allogeneic B6 (■) BM cells (5×10^6) and splenic purified T cells (2×10^6). Soluble ST2 concentrations in plasma collected at indicated times post-HCT from C3H.SW recipients (unit: ng/mL) ($p=0.0001$; $n=10-12$; t-test). The data are from 4 independent experiments. (B) Clinical scores of GVHD and survival curves for C3H.SW mice receiving syngeneic (●) or allogeneic B6 cells and treated with anti-mouse ST2 antibody (○) or IgG control antibody (●) at day -1 and day +1 post-HCT. The data are from 3 independent experiments (p values for GVHD scores are in Table S1, $p=0.0256$ for survival analysis; $n=15-23$ per group; t-test for GVHD score and Log-rank test for survival analysis). (C) Irradiated NSG mice (350 cGy) received 2.5×10^6 T cells purified from PBMCs of healthy donors (■). The control group was irradiated without receiving human T cells (●).

Human soluble ST2 concentrations in plasma collected at indicated times post-HCT from NSG recipient mice with or without engrafted human T cells (unit: pg/mL). The data are from 3 independent experiments ($p=0.0028$; $n=7-9$ per group; t-test). (D) Clinical scores of GVHD and survival curves for NSG mice receiving human T cells and treated with anti-human and anti-mouse ST2 antibodies (○) or IgG control antibody (●) every other day from day -1 to day +5 (4 doses) (p values for GVHD scores are in Table S1, $p=0.0329$ for survival analysis; $n=10$ per group; t-test for GVHD score and Log-rank test for survival analysis). (E) IFN- γ , IL-17, IL-23, IL-10, and IL-33 concentrations in plasma collected every 5 days post-HCT from the B6→C3H.SW model (unit: pg/mL). The data are from 3 independent experiments. Syngeneic group (●); allogeneic groups treated with anti-ST2 (○) or IgG control (●) (p values are in Table S1; $n=3-9$ per group, t-test).

Fig. 2. Soluble ST2 (sST2) and membrane ST2 (mST2) expression during GVHD

(A) In the B6→C3H.SW model, mRNA expression of sST2 and mST2 in different organs (spleen, small intestine, large intestine, skin, bone marrow, lung, heart, liver, and peripheral blood) of C3H.SW recipients mice at day 10 post-allogeneic HCT. The data are from 4 independent experiments (p values are in Table S2; n=4; one-way ANOVA). (B) sST2/actin mRNA expression in intestine or intestinal cell subsets (epithelial cells, stromal cells, endothelial cells, or non-T hematopoietic cells) from C3H.SW recipients mice at day 10 post-allogeneic HCT (n=5). The data are from 2 independent experiments with 2–3 pooled mice. (C) Western blot analysis of sorted intestinal stromal cells from IgG control or anti-ST2 antibody treated C3H.SW recipients mice at day 10 post-allogeneic HCT [M=marker

(kDa)] (left). The red box indicates the lack of sST2 protein present after anti-ST2 treatment. The bar graph shows the sST2/actin ratio in IgG control or anti-ST2 treated mice (n=6). The data are from 2 independent experiments with 3 pooled mice. **(D)** sST2/actin expression on sorted intestinal stromal cells and T cells from C3H.SW mice at day 10 and day 28 post-allogeneic HCT. The data are from 2 independent experiments with 2–3 pooled mice (p=0.0120; n=5; t-test). **(E)** Western blot analysis of sorted intestinal T cells from IgG control or anti-ST2 antibody-treated C3H.SW recipients 10 days post-allogeneic HCT [M=marker (kDa)]. The red box indicates the lack of sST2 protein present after anti-ST2 treatment. The unmodified blots are in Fig. S9. The bar graph shows the sST2/actin ratio in CD4 T cells from IgG control or anti-ST2 treated mice. The data are from 2 independent experiments with 3 pooled mice (p=0.0032; n=6; t-test). **(F)** sST2 secretion by both murine and human in vitro differentiated T cell subsets. The data are from 3–4 independent experiments (p values are in Table S2; n=3–4; t-test). The unmodified blots are in Fig. S9.

Fig. 3. ST2 deficiency and T cell populations during GVHD

(A) Transcriptome analysis of T cell related genes in mesenteric lymph node (MLN) T cells from anti-ST2-treated vs. IgG-treated C3H.SW recipient mice at day 10 post-allogeneic HCT. MLN T cells from 4 mice in each group were pooled for analysis. (B and C) Flow cytometric analysis of transcription factor and cytokine production by donor-derived CD4⁺ splenic T cells from IgG-treated or anti-ST2-treated C3H.SW recipients mice at day 10 after allogeneic HCT. The bar graphs show the percentages of cells expressing IFN- γ or Tbet (p=0.0150 for IFN- γ , p=0.0055 for Tbet; n=5; t-test) (B) and IL-17/IFN- γ or ROR γ t (p=0.0003 for IL-17/IFN- γ and p=0.0075 for ROR γ t; n=4-5; t-test) (C). The data are from 2 independent experiments. Gating strategy for (B) and (C) found in Fig. S10. (D) Survival curves for C3H.SW recipient mice receiving either only 5×10^6 wild type (WT) B6 BM cells (●) or 2×10^6 WT (●) or ST2^{-/-} B6 T cells (○) (p=0.0289; n=6-10; Log-rank test). The data are from 2 independent experiments. (E-G) Flow cytometric analysis at day 10 after allogeneic HCT shows percentages of intestinal CD4⁺ T cells expressing IFN- γ and Tbet (p=0.0173 for IFN- γ and p=0.0320 for Tbet; n=4; t-test) (E) and IL-17/IFN- γ , and ROR γ t (p=0.0273 for IL-17/IFN- γ and p=0.0273 for ROR γ t; n=4-5; t-test) (F) as well as Ki67 proliferation staining of cells expressing both IL-17 and IFN- γ (p=0.0088; n=4; t-test) (G). The data are from 2 independent experiments. (H) The bar graphs show the percentages of T cells expressing IL-4 or GATA3 from IgG-treated or anti-ST2-treated C3H.SW recipients mice at day 10 after allogeneic HCT, and the flow cytometry plots show mST2

expression on GATA3 T cells after IgG or anti-ST2 treatment. The data are from 2 independent experiments ($p=0.0049$ for IL-4 and $p=0.0252$ for GATA3; $n=6$; t-test). **(I)** IL-4 and GATA3-expressing T cells from C3H.SW recipient mice receiving WT or ST2^{-/-} B6 T cells at day 10 after allogeneic HCT ($p=0.0032$ for IL-4 and $p=0.0253$ for GATA3; $n=4$; t-test). The data are from 2 independent experiments. **(J)** The bar graphs show the percentages of intestinal T cells expressing FoxP3 from IgG-treated or anti-ST2-treated C3H.SW recipients mice at day 10 after allogeneic HCT, and the flow cytometry plots show mST2 expression on FoxP3 T cells after IgG or anti-ST2 treatment ($p=0.0087$; $n=5$; t-test). The data are from 2 independent experiments. **(K)** FoxP3 and IL-10 expressing T cells from C3H.SW recipient mice receiving WT or ST2^{-/-} B6 T cells at day 10 after allogeneic HCT. The data are from 2 independent experiments ($p=0.0459$ for FoxP3 and $p=0.0471$ for IL-10; $n=4-5$; t-test). **(L)** Survival curves for C3H.SW recipients mice transplanted with 5×10^6 B6 TCD BM cells plus either 2×10^5 WT (●) or ST2^{-/-} B6 (○) regulatory T cells (Tregs) with 2×10^6 WT B6 conventional T cells. (◆ TCD BM only). The data are from 2 independent experiments ($p=0.043$; $n=5-10$ per group; Log-rank test). Flow cytometric gating strategies are in Fig. S10.

Fig. 4. ST2 deficiency and antigen-presenting cells during GVHD

Flow cytometric analysis of intestinal myeloid-derived suppressor cells (MDSCs = MAC-1⁺Gr-1⁺ cells), CD103⁺ dendritic cells, and CD11c⁺ total dendritic cells in the B6 (CD45.1⁺)→C3H.SW (CD45.2⁺) model at 10 days post-allogeneic HCT. **(A)** Donor CD45.1⁺ MDSCs in IgG control or anti-ST2-treated C3H.SW recipients ($p=0.0247$; $n=4$; t-test). The data are from 2 independent experiments. **(B)** Donor CD45.1⁺ MDSCs in C3H.SW recipients receiving WT or ST2^{-/-} B6 T cells ($p=0.0277$; $n=3$; t-test). **(C)** Donor CD45.1⁺CD103⁺ dendritic cells in IgG control or anti-ST2-treated C3H.SW recipients ($p=0.0012$; $n=8$; t-test). The data are from 4 independent experiments. **(D)** Donor CD45.1⁺CD103⁺ dendritic cells in C3H.SW recipients receiving WT or ST2^{-/-} B6 T cells ($p=0.0244$; $n=3$; t-test). **(E)** Expression of MHC-II and co-stimulation molecules on CD11c⁺ total dendritic cells from IgG control and anti-ST2-treated mice, representative flow cytometry histograms (top panels) and bar graphs (bottom panels) of mean fluorescence intensity (MFI) ($p=0.0391$ for MHC II, $p=0.0469$ for CD40, $p=0.0154$ for CD80, and $p=0.0263$ for CD86; $n=3$; t-test). Flow cytometric gating strategies are in Fig. S11.

Fig. 5. ST2 deficiency and GVL activity

(A) Transcriptome analysis of anti-tumor related genes in MLN T cells from anti-ST2-treated vs. IgG-treated C3H.SW recipient mice at day 10 post-allogeneic HCT. MLN T cells from 4 mice in each group were pooled for analysis. (B) In vitro cytotoxic T lymphocyte assay with A20 and MLL-AF9 retrovirally induced acute myeloid leukemia in the presence of IgG control and anti-ST2 mAb (5 μg/mL) (n=3). The data are from 3 independent experiments. (C) Survival curves of C3H.SW mice receiving 10⁴ GFP⁺ MLL-AF9 leukemic cells with syngeneic HCT C3H.SW→C3H.SW (●) or allogeneic HCT (B6→C3H.SW) treated with IgG control (●) or anti-ST2 mAb (○) (p=0.010; n=5–10 per group; Log-rank test). (D) Survival curves of C3H.SW mice receiving 10⁴ GFP⁺ MLL-AF9 leukemic cells with syngeneic HCT C3H.SW→C3H.SW (●) or allogeneic HCT (B6→C3H.SW) treated with 6 doses (100 μg/dose, every other day from day -1 to day +9) of IgG control (●) or anti-ST2 mAb (□) (p=0.0357; n=4–5 per group; Log-rank test). (E) Survival curves of C3H.SW mice receiving 10⁴ GFP⁺ MLL-AF9 leukemic cells with WT (●) or ST2^{-/-} (▲) B6 T cells (p=0.0203; n=6–10 per group; Log-rank test).