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

Jilu Zhang,1,2,3,4 Abdulraouf M. Ramadan,1,2,3,4 Brad Griesenauer,1,2,3,4 Wei Li,1,2,3,4 Matthew J. Turner,2,5,6 Chen Liu,7 Reuben Kapur,2 Helmut Hanenberg,1,2,8 Bruce R. Blazar,9 Isao Tawara,10 Sophie Paczesny1,2,3,4*

Graft-versus-host disease (GVHD) remains a devastating complication after allogeneic hematopoietic cell transplantation (HCT). We previously identified high plasma soluble suppression of tumorigenicity 2 (sST2) as a biomarker of the development of GVHD and death. sST2 sequesters interleukin-33 (IL-33), limiting its availability to T cells expressing membrane-bound ST2 (mST2) [T helper 2 (Th2) cells and ST2+FoxP3+ regulatory T cells]. We report that blockade of sST2 in the peritransplant 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 the frequency of intestinal CD103 dendritic cells. Finally, ST2 blockade preserved graft-versus-leukemia activity in a model of green fluorescent protein (GFP)–positive 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 through 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 health care 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 about 40 to 60% of allo-HCT recipients (6–8). In addition, nonselective 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 after HCT is a prognostic biomarker for the development of GVHD and death (10). ST2, also known as interleukin-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 (myeloid differentiation primary response gene 88)–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 through the expansion of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (21, 22). ST2 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 proinflammatory and anti-inflammatory roles have been reported for IL-33 (11), in the disease models mentioned above, IL-33 had a clear anti-inflammatory role particularly by signaling through the mST2 on Tregs that results in an up to 20% greater steady-state level of total Tregs in the gut (25). Here, due to the
Because aberrant ST2/IL-33 signaling has been linked to many human secreted by intestinal stromal/endothelial cells and intestinal allo-
resents a therapeutic modality for the safe and efficient targeting of
without inhibiting mST2. Our results indicate that anti-ST2 mAb rep-
and schedules to identify a treatment course that would inhibit sST2
in a minor histocompatibility antigen (miHA).

Whether sST2 is a key player in the development of GVHD or
only a circulating molecule indicating increased GVHD risk has re-
ained unclear. Furthermore, it was unclear whether sST2 could be
drug-targetable and therefore used to alleviate GVHD. Here, we in-
vestigated the effects of sST2 blockade using anti-ST2 monoclonal antibody (mAb) on GVHD severity and mortality in a clinically re-
levant model of HCT and the GVL effects against retrovirally trans-
duced green fluorescent protein (GFP)–positive 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 the mST2 receptor to mST2-expressing T cells [T helper 2 (T\textsubscript{2}r) cells and ST2 "FoxP3" T\textsubscript{reg}], that we found to be protective in our GVHD model. Because no anti-ST2 mAb specific to the sol-
uble form was available to us, we used the full-length anti-ST2 mAb
available from Centocor (CNT03914) and tested several doses and
schedules to identify a treatment course that would inhibit sST2
without inhibiting mST2. Our results indicate that anti-ST2 mAb rep-
resents 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 at-
tractive T cells limits the local and systemic expansion and function of
mST2-expressing cells, particularly T\textsubscript{2}r cells and ST2 "FoxP3" T\textsubscript{reg}, 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 trans-
planted into irradiated miHA-mismatched C3H.SW or xenogeneic
NOD-scidIL2R\textsuperscript{null} (NSG) mice, respectively, to induce GVHD. Mice
receiving syngeneic T cells or irradiation only were used as con-
trols. As expected, all allogeneic/xenogeneic recipient mice receiv-
ing donor T cells developed severe GVHD, with about 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 in syngeneic or irradiation-only controls, by day 10
and day 21 after transplantation, mimicking the kinetics observed in
patients (10) (Fig. 1, A and C).

sST2 can be blocked by a neutralizing anti-ST2 mAb, leading to a decrease in proinflammatory and an 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 recep-
tor activity and thus releasing free IL-33 that will be used by mST2-
expressing T cells. We used a mAb targeting murine ST2 (anti-ST2
mAb) (CNT03914) or an appropriate control isotype antibody [im-
munoglobulin G (IgG)] (26). We used the miHA model B6→C3H.
SW, as it is the most clinically relevant not only because about 80% of
HCTs performed today in the United States are 8/8 major histo-
compatibility complex (MHC)–matched [the Center for Interna-
tional Blood and Marrow Transplant Research (CIBMTR) data as
a personal communication] but also because 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 intraperitoneal injec-
tion every other day from day −1 to day +9 after HCT. Anti-ST2 mAb
blockade strongly attenuated GVHD and increased survival (fig. S1A
and table S3). Histopathological 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 evalu-
ated a shorter schedule of anti-ST2 mAb treatment with only two doses
administered on day −1 and day +1 of HCT. Transient blockade of sST2 in
the peritransplant period was sufficient to provide long-lasting pro-
tection against GVHD (Fig. 1B). We next tested ST2 blockade in the
human-to-mouse xenogeneic GVHD model, and the results showed al-
leviation of GVHD and improved survival (Fig. 1D). Systemic pro-
duction of the inflammatory cytokines interferon-γ (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 sST2 blockade, we
determined the source of sST2 after allo-HCT. At day 10 after HCT,
before the onset of GVHD, the quantitative mRNA expression of both
sST2 and mST2 was analyzed in the spleen, small intestine, large
intestine, skin, bone marrow (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 a major source of sST2 that is neutralized by anti-ST2 mAb, and
sST2−/−–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. 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 (T cell receptor β\textsuperscript{+})]
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 ST2 deficiency in recipients reduced the GVHD score and prolonged the survival of the C3H.SW→B6 model (fig. S2 and table S3). 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 T1, T2, and T17, as well as the CD8 subsets T cytotoxic 1 (Tc1), Tc2, and Tc17. Tc17 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 versus IgG control–treated mice showed that anti-ST2 treatment modulated the gene expression of T17 cell cytokines (Fig. 3A). To further assess the effects of ST2 blockade on the T17 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 T17 cell subsets and pathogenic T17 cells (Fig. 3B and C). To verify the role of donor ST2 in GVHD, we next used ST2−/− donor cells in recipients of allo-HCT. Given that ST2−/− donor cells are incapable of producing sST2, they had a protective effect on GVHD severity and increased survival (Fig. 3D). Recipients of ST2−/− donor cells showed lower frequencies of IFN-γ/Tbet−producing T cells (Fig. 3E) and IL-17/ROTY-producing T cells (Fig. 3F) and less proliferation of IFN-γ/IL-17 pathogenic T17 cells, as measured by Ki67, at day 10 after transplantation in the GI tract (Fig. 3G).

![Image](https://www.science translational medicine.org/)

**Fig. 1. ST2 blockade and GVHD.** (A) Irradiated C3H.SW mice (1100 cGy) were transplanted with syngeneic (■) or allogeneic B6 (■) BM cells (5 × 10⁶) and splenic purified T cells (2 × 10⁶). ST2 concentrations in plasma collected at the indicated times after HCT from C3H.SW recipients (ng/ml) (P = 0.0001, t test; n = 10 to 12). The data are from four 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 after HCT. The data are from three independent experiments (P values for GVHD scores are given in table S1; P = 0.026 for survival analysis, t test for GVHD score and log-rank test for survival analysis; n = 15 to 23 per group). (C) Irradiated NSG mice (350 cGy) received 2.5 × 10⁶ T cells purified from peripheral blood mononuclear cells (PBMCs) of healthy donors (■). The control group was irradiated without receiving human T cells (○). Human soluble ST2 concentrations in plasma collected at the indicated times after HCT from NSG recipient mice with or without engrafted human T cells (pg/ml). The data are from three independent experiments (P = 0.0028, t test; n = 7 to 9 per group). (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 (four doses) (P values for GVHD scores are given in table S1, P = 0.0329 for survival analysis, t test for GVHD score and log-rank test for survival analysis; n = 10 per group). (E) IFN-γ, IL-17, IL-23, IL-10, and IL-33 concentrations in plasma collected every 5 days after HCT from the B6→C3H.SW model (pg/ml). The data are from three independent experiments. Syngeneic group (■); allogeneic groups treated with anti-ST2 (○) or IgG control (●) (P values are given in table S1; t test; n = 3 to 9 per group).
At the same time, ST2 blockade increased the percentages of the T_{12} cytokine IL-4 and the T_{112} transcription factor GATA3 in T cells (Fig. 3H), as well as increased the frequency of FoxP3+ T_{regs} (Fig. 3J). Transient ST2 blockade maintained mST2 expression on GATA3+ T_{12} cells (Fig. 3H) and FoxP3+ T_{regs} (Fig. 3I). IL-4/GATA3–producing T_{12} cells (Fig. 3I) as well as total FoxP3+ T_{regs} and IL-10–producing T cells (Fig. 3K) were increased when ST2−/− donor T cells were used as the graft source, confirming the negative impact of wild-type donor T cells on GVHD through production of sST2. We next specifically investigated the impact of ST2+FoxP3 T_{regs} in GVHD. For this, we used the B6→C3H. SW model with ST2−/− donor T_{regs} [ratio of T_{regs}/T_{conv} (conventional T cells) of 1:10] and demonstrated that recipients of wild-type donor T_{regs} had less severe GVHD and improved survival compared to recipients of ST2−/− T_{regs} (Fig. 3L). These results suggest, similarly to the observed colonic inflammation (25), that wild-type donor T_{regs} have a better suppressive capacity than ST2−/− T_{regs} and that mST2 expression on T_{regs} 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 after 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 αβγΔεγ 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 after 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 cell numbers were increased with ST2 blockade, as shown in Fig. 4E. These results indicate that ST2 deficiency induces the expansion of tolerogenic MDSCs and inhibits immunogenic CD103+ dendritic cells, leading to enhanced T cell suppression and amelioration of GVHD.
cells from treated animals showed reduced expression of MHC class II and costimulatory molecules (CD40, CD80, and 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 T$_{reg}$ in several diseases including GVHD (29, 30). However, using the classical c-kit and FcRI 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 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 up-regulation of cytotoxic granules and cytolytic molecules that have been implicated in antitumoral activity (5), we developed primary retrovirally induced eGFP+ MLL-AF9 leukemic cells on the C3H.SW background. The phenotype of the leukemic cells in this model is eGFP+, CD3+CD8−, and MAC-1hiGr-1hi 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) of ST2−/− donor T cells (Fig. 5E) preserved substantial GVL activity and resulted in significantly improved leukemia-free survival.

Addition of anti-ST2 mAb did not decrease antitumoral cytotoxicity (Fig. 5B). Furthermore, because of two major limitations of current GVL models, the first 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, we developed primary retrovirally induced eGFP+ MLL-AF9 leukemic cells on the C3H.SW background. The phenotype of the leukemic cells in this model is eGFP+, CD3+, B220−, and MAC-1hiGr-1hi 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) of ST2−/− donor T cells (Fig. 5E) preserved substantial GVL activity and resulted in significantly improved leukemia-free survival.

**Fig. 4.** ST2 deficiency and antigen-presenting cells during GVHD. Flow cytometric analysis of intestinal 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 after allo-HCT. (A) Donor CD45.1+ MDSCs in IgG control– or anti-ST2–treated C3H.SW recipients (P = 0.0247, t test; n = 4). The data are from two independent experiments. (B) Donor CD45.1+ MDSCs in C3H.SW recipients receiving WT or ST2−/− B6 T cells (P = 0.0244, t test; n = 3). (C) Donor CD45.1+CD103+ dendritic cells (DC) in IgG control– or anti-ST2–treated C3H.SW recipients (P = 0.0012, t test; n = 8). The data are from four independent experiments. (D) Donor CD45.1+CD103+ dendritic cells in C3H.SW recipients receiving WT or ST2−/− B6 T cells (P = 0.0224, t test; n = 3). (E) Expression of MHC class II and costimulation 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 MFI (top panels), and bar graphs (bottom panels) of MFI (top panels), and bar graphs (bottom panels) of MFI (top panels), and bar graphs (bottom panels).
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 specifically inhibit sST2 (fig. S7). The significant increases in plasma sST2 levels in mHAg-mismatched allo-HCT and human-to-mouse xenograft experimental models by days 10 and 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 allow 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 through extensive analysis of all T cell subsets. We have shown here that there is a differential balance of sST2 secretion versus mST2 expression in alloreactive T cells. Indeed, with increasing severity of GVHD, more pathogenic T cells (T1117 and T17) secrete sST2 and express less mST2, possibly explaining why elevation of plasmatic sST2 is specific to alloreactivity. This study also emphasizes that T1117 and T17 cells are mainly seen in the intestine during GVHD and are important players in GVHD development. We have clearly shown that transient ST2 blockade specifically inhibited sST2 in the plasma and target organs, particularly in the GI tract, while maintaining the mST2 expression on T cells, particularly T112 and ST2+ Treg.

We also found that ST2 blockade not only decreased the expression of the T111 transcription factor Tbet and the corresponding inflammatory cytokine IFN-γ but also increased the production of the T12 transcription factor GATA3 and the T12 cytokine IL-4, skewing the T111/T12 balance toward a T12 phenotype, which protects against severe GVHD. In addition, we and others have previously shown that the ratio of FoxP3-expressing Treg to conventional T cells is significantly decreased in severe GVHD (44–46). ST2 blockade also increased the
frequency of functional FoxP3+ Tregs in the spleen and gut and decreased the percentage of pathogenic T$_{hTh}$ cells, without impairing the ST2+ FoxP3+ Tregs that we showed are crucial for protection against GVHD. Because the anti-ST2 mAb used in our study is a full-length antibody 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 Tconv is predominant because HCT with ST2−/− donor Tconv with or without ST2+/− donor Tregs (Treg/Tconv ratio of 1:10) resulted in less severe GVHD and improved survival in both cases (fig. S8, table S3, and Fig. 3D). Together, our results indicate that high levels of ST2 production by T cells during GVHD may represent a mechanism to further perpetuate pathogenic responses by limiting IL-33–driven Treg accumulation.

T cell 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, and 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 in 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. We also demonstrated that intestinal T cells are another major source of sST2. This mechanism whereby sST2 is secreted mainly by CD4+ T cells (mostly T$_{hTh17}$ cells) may explain the specificity of sST2 immune functions during alloreactivity as well as the marked 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 after allo-HCT, which could be applied in a number of other diseases with elevated systemic proinflammatory 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 Treg/Tconv and ST2−/− Treg/Tconv against GVHD. Finally, we generated a model of retrovirally transduced GEP+ MLL-AF9 acute myeloid leukemia to assess the effects of ST2 blockade on GVL activity. All experiments were replicated at least three times.

**Mice**

Balb/c (H-2d), B6 (H-2b, CD45.2+), B6 (C57BL/6. Ptprc-a, H-2b, CD45.1+), C3H.SW (H-2b, CD45.2+), and NSG mice were from The
Jackson Laboratory. B6 (ST2−/−, H-2b, CD45.2+) mice were provided by A. McKenzie from University of Cambridge, UK. B6 (ST2−/−, H-2b, CD45.1+) mice were bred in the mouse breeding facility at Indiana University School of Medicine. Animal protocols were approved by the Institutional Animal Care and Use Committee at Indiana University School of Medicine.

**Induction and assessment of GVHD**

The 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 recipient mice received 1100 and 1250 cGy of total body irradiation (137Cs as source) at day −1. Then, recipient mice were injected intravenously with T cell–depleted (TCD) BM 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 using the murine Pan T Cell Isolation Kit (Miltenyi), and TCD BM was prepared with CD90.2 Microbeads (Miltenyi). For adoptive transfer models (B6→C3H.SW), wild-type and ST2−/− B6 total donor T cells or Treg cells were purified using the 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 BM cells (5 × 10⁶) and the 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 total human T cells from PBMCs (2.5 × 10⁶) at day 0. PBMCs were prepared from human PB Leukopacks from healthy donors, which were purchased from the Central Indiana Blood Center under an Institutional Review Board–approved protocol. PBMCs were isolated within 24 hours after blood draw by Ficoll density gradient centrifugation (GE Healthcare). Total human T cells were purified using the human Pan T Cell Isolation Kit (Miltenyi). The mice were housed in sterilized microisolator cages and maintained on acidified water (pH <3) for 3 weeks as previously described (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 previously described (54). According to animal protocols approved by the Institutional Review Board, mice were euthanized when the clinical score achieved 6.5.

**ELISA**

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

**Anti-ST2 mAb treatment**

The anti-ST2 mAb was from Centocor, a pharmaceutical company of Johnson & Johnson (CNT03914). This chimeric antibody contains a mouse IgG1 Fc, and it recognizes the full-length extracellular domain of mST2 (26). Anti-ST2 mAb or IgG control antibody was given to recipient mice receiving allo-HCT mice via intraperitoneal injection at day −1 and day 1 after HCT, at 100 µg per mouse. In some experiments, the mice received six doses of anti-ST2 mAb every other day from day −1 to day +9. In the xenogeneic GVHD model, NSG mice received four doses of both anti-human (clone 97203, R&D Systems) and anti-mouse ST2 (CNT03914) mAbs every other day from day −1 to day 5 (intraperitoneally, 100 µg per 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. The slides were coded without reference to previous treatment and examined in a blinded fashion by C. Liu (University of Florida). A semiquantitative scoring system was used to assess abnormalities known to be associated with GVHD (55, 56). After scoring, the codes were broken and the data were compiled.

**Isolation of intestinal cells**

Single-cell suspensions were prepared from intestines as described, with modifications (57). Briefly, intestines were flushed with phosphate-buffered saline to remove fecal matter and mucus. Fragments (<0.5 cm) of intestines were digested in 10 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing collagenase type B (2 mg/ml) (Roche), deoxyribonuclease I (10 µg/ml) (Roche), and 4% bovine serum albumin (Sigma) at 37°C with shaking for 90 min. The digested mixture was then diluted with 30 ml of plain DMEM, filtered through 70-µm strainers, and centrifuged at 850g for 10 min. The cell pellets were suspended in 5 ml of 80% Percoll (GE Healthcare), overlaid with 8 ml of 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−epCAM+ cells were directly sorted 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 preincubated with purified anti-mouse CD16/CD32 mAb for 10 to 20 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 (FVD) was used to distinguish live cells from dead cells. The FoxP3/Transcription Factor Staining Buffer Set and the 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 to 6 hours before any staining.

**Cell sorting**

MLN T cells [FVD "CD90.2" "CD3"] from treated C3H.SW recipient mice receiving allo-HCT were sorted for nanosting analysis. Total T cells (FVD "CD45 "TCRβ"), CD4+ T cells, CD8+ T cells, CD45−epCAM− 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 in RLT buffer (Qiagen) on ice. The cell concentration for lysis was 0.2 × 10⁶ to 1 × 10⁶ cells/µl in a total volume of 5 µl with RLT buffer. Lysis samples were immediately frozen in liquid nitrogen and then stored at −80°C or in dry ice. Nanostring
Quantitative RT-PCR
Total RNA from spleen, small intestine, large intestine, skin, BM, lung, heart, and peripheral blood were isolated using the RNeasy Plus Mini Kit (Qiagen). Complementary DNA (cDNA) was prepared with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR was performed using SYBR Green PCR mix on an ABI Prism 7500HT (Applied Biosystems). Thermocycler conditions included 2-min incubation at 50°C, then at 95°C for 10 min; this was followed by a two-step PCR program: 95°C for 5 s and 60°C for 60 s 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'-CTCTGGCTCCAGGACATAAGA-3'; actin reverse, 5'-GAAGCAGCTGTAACACCTCG-3'; mST2 forward, 5'-AAAGGCAACATAGACCCTGA-3'; sST2 reverse, 5'-CTGTAAGCTTTGCGTACCTG-3'; sST2 forward, 5'-GTGTTAACATCTTAGCCACA-3' (25); sST2 reverse, 5'-TGGGTGAGGGA-CACTCCCTAC-3'.

Two-color Western blots
Sorted cells were lysed in RIPA (radioimmunoprecipitation assay) 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 polyvinylidene difluoride membranes (Millipore). The blots were blocked with Odyssey Blocking Buffer (LI-COR) for 1 hour at room temperature and incubated with specific primary antibodies: biotinylated anti-mouse ST2 mAb (D18, BD Bioproducts) and anti–β-actin mAb (926-42212, LI-COR), at 4°C overnight. IRDye 800CW streptavidin (926-32230, LI-COR) and IRDye 680RD goat antimouse 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 with the Odyssey CLx Imaging System (LI-COR) according to the manufacturer's instructions.

T cell differentiation
Total CD4+ or CD8+ T cells were purified from B6 spleens with magnetic isolation beads (Miltenyi). These cells were plated at a concentration of 1 × 10^6 cells/ml and activated with plate-bound anti-CD3 (2C11) (1 μg/ml) and soluble anti-CD28 (37.51) (5 to 10 μg/ml). Both the CD4+ and CD8+ cells were polarized toward either Th1/Tc1 [IL-2 (20 ng/ml), and anti–IFN-γ (10 μg/ml)], Th2/Tc2 [IL-4 (20 ng/ml) and anti–IL-12 (10 ng/ml), IL-6 (10 ng/ml), IL-1β (10 ng/ml), IL-23 (20 ng/ml), anti–IL-4 (10 μg/ml), and anti–IFN-γ (10 μg/ml)] conditioning in complete medium. On day 3, the cells were expanded with fresh medium in the presence of additional cytokines at the same concentration as on day 0 for Th1/Tc1, Th2/Tc2, and Th17/Tc17 cells. On day 7, the cells were stimulated with anti-CD3 and anti-CD28 (both 10 μg/ml) as well as with PMA (50 ng/ml).

Generation of MLL-AF9-eGFP leukemic cells
The retroviral vector containing the MLL-AF9 eGFP cDNA construct (38) was provided by R. Kapur and used to generate retroviral supernatants by transfection of Phoenix-Eco Cell Line (ATCC) using FuGene 6 transfection reagent (Promega). Eighteen hours before transfection, Phoenix-Eco cells were seeded in gelatin-coated 100-mm plates (8 × 10^5 per plate). After gently aspirating off the cell culture medium, the 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. After 6 to 8 hours, 3 ml of DMEM supplemented with 15% heat-inactivated fetal bovine serum (FBS) was added to the plates. After 18 hours of incubation, the medium was replaced with 8 ml of Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 10% FBS, penicillin and streptomycin (100 U/ml), 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 e-kit+ cells that were magnetically enriched from Balb/c or C3H.SW BM cells using the CD117 MicroBead Kit (Miltenyi) and were prestimulated for 48 hours with IL-3 (10 ng/ml), IL-6 (10 ng/ml), and stem cell factor (20 ng/ml) (all from PeproTech). After two consecutive 24-hour incubations in nontissue culture plates precoated with retronectin (59), the cells were collected and their infection efficiency was determined by eGFP expression, using flow cytometry. About 1 × 10^6 of cells, 8% of which were eGFP+, were injected into lethally irradiated Balb/c or C3H.SW mice intravenously through the tail vein. The 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). The mice were subsequently euthanized, and BM cells were analyzed by flow cytometry, which showed that all BM cells were eGFP+, CD3+, B220+, and MAC-1^hiGr-1^hi.

In vitro cytotoxicity assay
The A20 cell line expressing E2-Crimson fluorescent protein was provided by H. 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 alllogenic B6 T cells at different ratios as indicated. After 8 hours of incubation at 37°C, the 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, 2 × 10^6 enriched B6 or C3H.SW splenic T cells, and 10^6 MLL-AF9 cells on day 0. The mice were treated...
intrapерitoneally with anti-ST2 antibody or isotype IgG control at day −1 and day +1 or with six doses of anti-ST2 mAb every other day from day −1 to day +9, as described above. The mice were monitored daily for survival and leukemia development and weekly for GVHD score. We attributed death to leukemia on the basis of a high 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 with GraphPad Prism software, version 6.05. Error bars in graphs represent 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. P values less than 0.05 were considered significant.

**SUPPLEMENTARY MATERIALS**

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Materials and Methods

Fig. S1. Six dose ST2 blockade and GVHD.

Fig. S2. Host ST2 deficiency and GVHD.

Fig. S3. mST2 expression on T cell subsets.

Fig. S4. ST2 blockade and GVHD.

Fig. S5. MLN dendritic cells and GVHD.

Fig. S6. IL-33 administration and GVHD.

Fig. S7. Immune complex depletion.

Fig. S8. Analysis of CD103+ DCs.

Fig. S9. ST2 blockade and GVHD.

Fig. S10. Gating strategies of flow cytometric analysis for Fig. 3.

Fig. S11. Gating strategies of flow cytometric analysis for Fig. 4E.

Table S1. P values for Fig. 1.

Table S2. P values for Fig. 2.

Table S3. P values for figs. S1, S2, and S8.

Table S4. Antibodies for flow cytometric analysis.

**REFERENCES AND NOTES**


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ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease
Jilu Zhang, Abdulraouf M. Ramadan, Brad Griesenauer, Wei Li, Matthew J. Turner, Chen Liu, Reuben Kapur, Helmut Hanenberg, Bruce R. Blazar, Isao Tawara and Sophie Paczesny (October 7, 2015)
Science Translational Medicine 7 (308), 308ra160. [doi: 10.1126/scitranslmed.aab0166]

Blocking graft-versus-host disease
Bone marrow transplantation replaces unhealthy bone marrow with bone marrow from a healthy donor. However, the donor-derived immune cells may recognize the transplant recipient as foreign and attack, resulting in graft-versus-host disease (GVHD). Now, Zhang et al. report that blocking soluble suppression of tumorigenicity 2 (sST2), a plasma marker for GVHD, with a neutralizing antibody can reduce GVHD severity and mortality. The blockade decreased the production of proinflammatory cytokines and increased the frequency of anti-inflammatory molecules and cells while maintaining graft-versus-leukemia activity. These data suggest that targeting sST2 may help decrease GVHD after bone marrow transplantation.

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