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ST2/MyD88 deficiency protects mice against aGVHD and spares T-regulatory cells

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Abstract

Acute graft-versus-host disease (aGVHD) hinders the efficacy of allogeneic hematopoietic cell transplantation (HCT). Plasma levels of soluble ST2 (sST2) are elevated in human and murine aGVHD and correlated to type 1 T cells response. Membrane-bound ST2 (ST2) signals through the adapter protein MyD88. The role of MyD88 in T cells during aGVHD has yet to be elucidated. We found that knocking out MyD88 in the donor T cells protected against aGVHD independent of IL-1R and TLR4 signaling in two murine HCT models. This protection was entirely driven by MyD88$^{-/-}$ CD4 T cells. Transplanting donor MyD88$^{-/-}$ conventional T cells (Tcons) with WT or MyD88$^{-/-}$ regulatory T cells (Tregs) lowered aGVHD severity and mortality. Transcriptome analysis of sorted MyD88$^{-/-}$ CD4 T cells from the intestine ten days post-HCT showed lower levels of *Il1rl1* (gene of ST2), *Ifng*, *Csf2*, *Stat5*, *Batf*, and *Jak2*. Transplanting donor ST2$^{-/-}$ Tcons with WT or ST2$^{-/-}$ Tregs showed a similar phenotype with what we observed when using donor MyD88$^{-/-}$ Tcons. Decreased ST2 was confirmed at the protein level with less secretion of sST2 and more expression of ST2 compared to WT T cells. Our data suggests that Treg suppression from lack of MyD88 signaling in donor Tcons during alloreactivity uses the ST2 but not the IL-1R or TLR4 pathways, and ST2 represents a potential aGVHD therapeutic target sparing Tregs.

INTRODUCTION

Allogeneic HCT (HCT) is a validated curative therapy for patients with hematological malignancies and nonmalignant diseases. However, acute graft-versus-host disease (aGVHD) is a leading complication of HCT, restricting its effectiveness. Gastrointestinal damage from alloreactive T cells is the most correlated to aGVHD-related mortality (1, 2). Damage caused during aGVHD to the gastrointestinal tract leads to release of alarmins and products of commensal bacteria as well as production of pro-inflammatory cytokines (3, 4). To help mediate this response, many cells release decoy receptors that do not signal, one of

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AUTHOR CONTRIBUTIONS

B.G., H.J. and J.Y. designed and performed research, analyzed data, and wrote the paper; J.Z. and A.R. designed and performed research; J.E. and K.C. performed research; S.P. conceived the project, designed experiments, analyzed data, and edited the paper.

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which is soluble ST2 (sST2) (5). Plasma levels of sST2 are elevated in human (6–13) and murine (14, 15) aGVHD and parallel type 1 T cell responses, which drive aGVHD (16).

During conditioning for HCT, systemic IL-33 levels are increased due to its release primarily by non-hematopoietic cells (17). This is followed by an increase of sST2 post-HCT, mediated primarily by the donor T cells (14). We recently reported that ST2 blockade using a neutralizing anti-ST2 antibody can attenuate aGVHD by decreasing sST2 secreted by T cells while maintaining membrane-bound ST2 (ST2) signaling in T cells (14). Both sST2 and ST2 bind IL-33, with sST2 sequestering free IL-33, preventing signaling, and IL-33/ST2 signaling through the adapter protein myeloid differentiation primary response gene 88 (MyD88) (18). ST2 pathway activation helps drive type 2 responses (19) and enhance Treg immunosuppressive function (20–22), which both prevent aGVHD (15, 23, 24). Indeed, ST2 blockade using a neutralizing anti-ST2 antibody during aGVHD increases systemic levels of IL-33, leading to more IL-33 availability for regulatory T cells (Tregs) (14). This suggests that there is a balance between IL-33, sST2-secreting T cells, and membrane-bound ST2 T cells that regulates inflammation during aGVHD.

The IL-1 receptor (IL-1R) superfamily and toll-like receptor (TLR) family, except TLR3, also signal through MyD88 (25, 26). MyD88 was first identified in antigen presenting cells (APCs) (27, 28). However, in a murine model, MyD88 signaling in host hematopoietic cells has been shown to be completely dispensable for aGVHD induction (29). In T cells MyD88 is also expressed (28), but its function is less well understood. In CD4 T cells stimulated with ovalbumin or a peptide to the haplotype $H2^b$, MyD88 deficiency decreases T cell proliferation and type 1 cytokine production through loss of IL-1 receptor (IL-1R) signaling, which is dependent on Tregs being present (30). In aGVHD loss of IL-1R signaling in donor T cells ameliorated disease (31). The role of MyD88 signaling in donor T cells during alloreactivity is not known. We hypothesized that loss of MyD88 in the donor T cells would alleviate aGVHD through IL-1R, TLR4, sST2, or a combination of these.

METHODS

Mice.

Boy/J (C57BL/6.Ptprca, $H\text{-}2^b$, CD45.1) and C57BL/6 ($H\text{-}2^b$, CD45.2) mice were purchased from the *In Vivo* Therapeutics Core at the Indiana University School of Medicine. BALB/c ($H\text{-}2^d$, CD45.2) and C3H.SW ($H\text{-}2^b$, CD45.2) were bred at the Indiana University School of Medicine. B6.B10ScN-$Tlr4^{lps\text{-}del}$/JthJ ($TLR4^{-/-}$) mice were purchased from Jackson Laboratory (Bar Harbor, ME). $MyD88^{-/-}$ mice provided by Dr. Steve Kunkel and (University of Michigan, Ann Arbor, MI), $ST2^{-/-}$ mice provided by Dr. Andrew McKenzie (University of Cambridge, Cambridge, UK), and $IL\text{-}1R^{-/-}$ mice provided by Dr. Travis Jerde (Indiana University Purdue University of Indianapolis, Indianapolis, IN) were backcrossed on a C57BL/6 background for at least 10 generations. C57BL/6 $ST2^{-/-}MyD88^{-/-}$ mice were generated in house by crossing C57BL/6 $ST2^{-/-}$ and C57BL/6 $MyD88^{-/-}$. Loss of MyD88 and ST2 were verified by PCR. The Institutional Animal Care and Use Committee approved all animal protocols.

aGVHD induction and assessment.

Mice underwent HCT as previously described (14). Briefly, in a major MHC-mismatched model (B6 → BALB/c), BALB/c recipient mice received 900 cGy of total body irradiation (^{137}Cs as source) at day −1. In a miH-mismatched aGVHD model (B6 → C3H.SW), C3H.SW recipient mice received 1100 cGy of total body irradiation at day −1. Then, recipient mice were injected intravenously with WT B6 T cell–depleted (TCD) BM cells (5×10^6) plus WT, MyD88$^{-/-}$, IL-1R$^{-/-}$, TLR4$^{-/-}$, ST2$^{-/-}$, or ST2$^{-/-}$MyD88$^{-/-}$ splenic total T cells, or CD4$^+$, CD8$^+$ T cells (1×10^6 for BALB/c and 2×10^6 for C3H.SW, unless indicated otherwise) from either syngeneic or allogeneic donors at day 0. Splenic T cells from donor mice were enriched using the murine Pan T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA), and TCD BM was prepared with CD90.2 Microbeads (Miltenyi). Purity of T cell isolation from spleen was confirmed via flow cytometry to be >97%. Depletion of T cells from BM was confirmed via flow cytometry to be <0.1%. For some experiments, donor T cells were first labeled with CFSE before injection. In adoptive transfer models, wild-type, MyD88$^{-/-}$ and ST2$^{-/-}$ B6 total donor T cells or Tregs were purified using the murine Pan T Cell Isolation Kit and murine CD4$^+$CD25$^+$ Regulatory T Cell Isolation Kit (Miltenyi). The mice were housed in sterilized microisolator cages and maintained on acidified water (pH <3) for 3 weeks. Survival was monitored daily and clinical GVHD scores were assessed weekly (14).

ELISA.

We measured concentrations of murine plasma IFN-γ using DuoSet Kit and sST2 using Quantikine Kit (R&D Systems, Minneapolis, MN) according to manufacturer's protocols.

Isolation and sorting of intestinal CD4 T cells.

We prepared single-cell suspensions of mononuclear cells from small intestines as previously described (14). Briefly, small intestines were flushed with cold PBS to remove mucus and feces. The intestines were cut into <0.5 cm fragments and digested in 10 mL of DMEM containing 4% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), 2 mg/mL collagenase type B (Roche, Indianapolis, IN), and 10 µg/mL DNase I (Roche) at 37°C with shaking (250rpm) for 90 minutes. The digested mixture was diluted with 30 mL DMEM, filtered through a 100 µm strainer, and centrifuged for 10 minutes at 850*g*. The cells were resuspended in 5 mL of 80% Percoll (GE Healthcare, Little Chalfont, United Kingdom) and overlaid with 8 mL of 40% Percoll. The cells were spun at 4°C for 20 minutes at 800*g* without braking. The interface, which contains the live mononuclear cells was collected and washed twice with PBS. Live CD4$^+$ T cells (Fixed Viability Dye$^-$CD90.2$^+$CD4$^+$; all from eBioscience, Waltham, MA) were stained with fluorescent antibodies and sorted on the BD FACSAria (BD Pharmingen, San Diego, CA).

Flow cytometry analysis.

All antibodies and reagents for flow cytometry were purchased from eBioscience, unless stated otherwise. Single cell suspensions were preincubated with purified anti-mouse CD16/CD32 mAb for 10 to 20 min at 4°C to prevent nonspecific binding of 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 mg/ml; Sigma-Aldrich), and brefeldin A for 4 to 6 hours before any staining. Staining antibodies against mouse antigens included: anti-CD45.1, anti-CD45.2, anti-CD90.2, anti-CD4, anti-CD8, anti-CD62L, anti-CD44, anti-Foxp3, anti-IL-4, anti-IFNγ, anti-IL-17, and anti-GM-CSF.

Nanostring analysis.

Sorted intestinal CD4 T cells were prepared and analyzed as previously described (14). Briefly, sorted intestinal CD4+CD127$^+$CD25$^-$ Tcons from either recipients of WT or MyD88$^{-/-}$ allogeneic donor T cells were directly lysed in RLT buffer (Qiagen, Hilden, Germany) on ice. Cell concentration for each sample was 2×10^3 cells/μL. Preparation of samples for analysis was then performed according to the Nanostring Technologies protocol for gene expression. Plates were run on the nCounter SPRINT ProfilerAnalysis System and the data analysis using nSolver 3.0. The nCounter Mouse Immunology Kit, which includes 561 immunology-related mouse genes, was used in the study.

Quantitative RT-PCR.

Total RNA from sorted intestinal T cells (Fixed Viability Dye$^-$CD3$^+$, all from eBioscience), were isolated using the RNeasy Plus Mini Kit (Qiagen). Complementary DNA (cDNA) was prepared with the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA). Using an ABI Prism7500HT (Applied Biosystems, Foster City, CA), quantitative real-time PCR was performed with the SYBR Green PCR mix. 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′-CTCTGGCTCCTAGCACCATGAAGA-3′; actin reverse, 5′- GTAAAACGCAGCTCAGTAACAGTCCG-3′; mST2 forward, 5′-AAGGCACACCATAAGGCTGA-3′; mST2 reverse, 5′-TCGTAGAGCTTGCCATCGTT-3′; sST2 forward, 5′-TCGAAATGAAAGTTCCAGCA-3′; sST2 reverse, 5′-TGTGTGAGGGACACTCCTTAC-3′.

Western Blot.

Tcons, CD8$^+$ T cells, and Tregs were isolated from WT B6 spleens using CD4 microbeads and CD8 microbeads (both from Miltenyi), respectively, following manufacturer's protocols. Purities of CD4 and CD8 T cells after selection were >95%. Sorted cells were lysed in RIPA buffer (Pierce Biotechnology, Waltham, MA) with Pierce Phosphatase Inhibitor MiniTablets (Pierce Biotechnology) and Protease Inhibitor Cocktail Tablets (Roche). Samples were boiled, electrophoretically separated, and transferred on Immobilon-FL polyvinylidene difluoride membranes (MilliporeSigma, Burlington, MA). The blots were blocked with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature and incubated with specific primary antibodies: rabbit MyD88 mAb (D80F5, Cell Signaling Technology, Danvers, MA) ST2 mAb (Dj8, MD bioproduct) and anti–β-actin mAb (LI-COR), both at 4°C overnight. IRDye 800CW goat anti-rabbit (LI-COR) and IRDye 680RD goat anti-mouse IgG polyclonal antibodies (LI-COR) were used as secondary detection antibodies for MyD88, 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.

Immunosuppression assays.

The suppressive capacities of WT and MyD88$^{-/-}$ Tregs were assessed with a CFSE inhibition assay as previously published (83). Briefly, CD25 depleted total T cells (Tcons) were isolated from WT or MyD88$^{-/-}$ mice splenocytes. Antigen presenting cells (APC) were isolated from WT mice splenocytes. Tcons were labeled with CFSE (Invitrogen) and plated together with WT or MyD88$^{-/-}$ Tregs at different ratios in the presence of anti-CD3 (0.25 μg/ml) and APC. After 4 days, proliferation of Tcons was measured by flow cytometry.

Statistical Analysis.

Log-rank test was used for survival analysis. Differences between two groups were compared using 2-tailed unpaired t tests or Mann-Whitney U test. Bonferroni correction was used when comparing multiple groups. All statistical analyses were performed using GraphPad Prism, version 7.02. Data in graphs represent mean ± standard error of the mean (SEM). P values less than 0.05 were considered significant.

RESULTS

MyD88$^{-/-}$ T cells reduce aGVHD severity and mortality in multiple murine models

First, we tested whether loss of MyD88 affected normal splenic T cells in naïve mice. We found no difference in splenic T cell numbers; CD4/CD8 frequency; or naïve, memory, and effector frequencies (Fig. 1A). Ability to polarize toward Th1, Th2, or Th17 cells *in vitro* was also not affected by the absence of MyD88 as shown by IFN-γ, IL-4, or IL-17 production, respectively (Fig. 1B). To explore the role of MyD88 signaling in the donor T cells *in vivo* following HCT, we used two clinically relevant murine allo-HCT models: a MHC-major mismatch model (MH) C57BL/6, H-2^b → BALB/c, H-2^d and a minor histocompatibility antigen mismatch (miH) model C57BL/6, H-2^b → C3H.SW, H-2^b, as shown in the upper panel of Fig.1C and 1D. In both models splenic T cells were isolated and bone marrow cells were depleted of T cells. In the MH model, C57BL/6 → BALB/c, mice receiving WT T cells quickly developed and succumbed to severe aGVHD (median survival time: 14 days). However, mice receiving MyD88$^{-/-}$ T cells had decreased aGVHD scores and mortality (median survival time: >30 days) compared to mice receiving WT T cells (Fig. 1C). Using the miH model, C57BL/6 → C3H.SW, we observed a similar decrease in aGVHD mortality (median survival time: WT - 43 days; MyD88$^{-/-}$ - >60 days; Fig. 1D). These results show that signaling through MyD88 in the donor T cells is critical in the pathogenesis of aGVHD.

Loss of IL-1R and TLR4 on T cells, both upstream of MyD88, does not alleviate aGVHD

To elucidate the mechanism as to why MyD88$^{-/-}$ donor T cells induce less severe aGVHD, we targeted upstream receptors of MyD88. The upstream receptors for MyD88 include the IL-1 receptor superfamily and the toll-like receptor (TLR) family, with the exception of TLR3 (25, 26). One group has shown that MyD88$^{-/-}$ CD4 T cells produce less IFN-γ and proliferate less than WT CD4 T cells after immunization and this was due to defective IL-1R signaling (30). Another group found that, in an aGVHD model, recipients of IL-1R$^{-/-}$ T cells survived longer than recipients of WT T cells (31). Thus, we next asked whether the phenotype observed using MyD88$^{-/-}$ donor T cells is mediated through IL-1R. In our models, we found no difference between groups in clinical score or survival from mice receiving WT or IL-1R$^{-/-}$ donor T cells in either the MH model (median survival time: WT - 14 days; IL-1R$^{-/-}$ - 28 days; Fig. 2A) or the miH model (median survival time: WT - 43 days; IL-1R$^{-/-}$ - 39 days; Fig. 2B). Another group showed that recipients of TLR4$^{-/-}$ BM and T cells together reduced aGVHD severity compared to WT recipients through defective donor antigen presenting cells (APC) response, but did not test whether TLR4$^{-/-}$ T cell response compared to WT was also affected (32). Thus, we asked whether loss of TLR4 on the donor T cells could affect aGVHD severity and mortality. Recipients of TLR4$^{-/-}$ donor T cells both models did not reduce aGVHD severity and mortality (MH model median survival time: WT - 10 days; TLR4$^{-/-}$ - 9 days; Fig. 2C; miH model median survival time: WT - 18 days; TLR4$^{-/-}$ - 33 days; Fig. 2D). These data show that IL-1R and TLR4 signaling in donor T cells are not necessary for aGVHD induction.

Transplantation of donor MyD88$^{-/-}$ CD4 T cells, but not CD8 T cells, reduces aGVHD severity independent of intrinsic MyD88 signaling

MyD88 signaling in T cells has been characterized in both the CD4 and the CD8 compartments (28, 30, 33, 34). However, the importance of MyD88 signaling in donor CD4 T cells, CD8 T cells, and Tregs in the context of aGVHD has not been studied. CD4 T cons, CD8 T cells, and Tregs isolated from the spleen of naïve WT mice expressed MyD88, with CD4 T cons cells expressing approximately 3 times more MyD88 than CD8 T cells (Fig. 3A). To determine if MyD88 in CD4 T cells, CD8 T cells, or both is important for aGVHD development, we isolated WT CD4, WT CD8, MyD88$^{-/-}$ CD4, and MyD88$^{-/-}$ CD8 T cells from naïve mice. Transplanting MyD88$^{-/-}$ CD4 T cells with WT or MyD88$^{-/-}$ CD8 T cells increased the survival of the recipient mice compared to transplanting WT CD4 T cells with MyD88$^{-/-}$ CD8 T cells (miH model median survival time: MyD88$^{-/-}$ CD8 – 36 days; MyD88$^{-/-}$ CD4 - >60 days; Fig. 3B,C), the latter showing a similar phenotype to total WT T cell recipients. These data show that MyD88 signaling in CD4 T cells, but not CD8 T cells, is needed for optimal aGVHD induction. GM-CSF expression by T cells has been implicated in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, through a STAT5 dependent mechanism (35, 36). Recently, GM-CSF was also implicated in promoting aGVHD through a BATF-dependent mechanism (37). We found that production of GM-CSF in the intestine 10 days post-HCT is significantly decreased in both transplanted MyD88$^{-/-}$ CD4 T cell groups compared to transplanted WT CD4 T cell groups (Fig. 3D).

The CD4 T cell compartment consists of both pro-inflammatory conventional T cells (Tcons) and anti-inflammatory Tregs. MyD88$^{+/+}$ Tregs prolong allograft survival in both organ transplantation and chronic GVHD through a cell-intrinsic mechanism (38). Intrinsic MyD88 signaling in CD4 T cells has also been implicated in mounting a proper antiviral response (39). We next explored the cell-intrinsic role of MyD88$^{-/-}$ Tregs in aGVHD. After singly transplanting Treg-depleted WT or MyD88$^{-/-}$ Tcons and co-transplanting WT Tcons with WT or MyD88$^{-/-}$ Tregs, we did not observe differences in either aGVHD severity or mortality (MH model median survival time: WT Tcon - 10 days; MyD88$^{-/-}$ Tcon - 10 days; WT Tregs - 26 days; MyD88$^{-/-}$ Tregs - 24 days) in both matched groups, which means the intrinsic MyD88 signaling in donor T cells does not impact aGVHD (Fig. 3E, F).

MyD88 Tcons require the presence of Tregs for alleviation of aGVHD

Immunization of CD4 specific MyD88$^{-/-}$ mice has been shown to decrease IFN-γ production by CD4 T cells compared to WT CD4 T cells; however, IFN-γ levels after immunization were the same between WT and MyD88$^{-/-}$ CD4 T cells when Tregs were absent (30). As no differences were observed in both single Tcons-transplanted groups, we next sought to reveal whether the presence of Tregs is necessary for protection when using MyD88$^{-/-}$ donor Tcons in the MH model. Indeed, as shown in Figures 3G and H, use of MyD88$^{-/-}$ Tcons with WT or MyD88$^{-/-}$ Tregs, led to aGVHD amelioration. These data show that loss of extrinsic MyD88 signaling in Tcons in the presence of Tregs reduces aGVHD severity and mortality.

MyD88$^{-/-}$ donor T cells do not have defects in their proliferation, apoptosis, and migration markers but show decreased intestinal T cell infiltration, and increased Th2 and Treg frequencies following allo-HCT

To determine if the donor MyD88$^{-/-}$ T cells had a defect in proliferation or apoptosis markers following allo-HCT, we stained CD45.1 WT T cells and CD45.2 MyD88$^{-/-}$ T cells with carboxyfluorescein succinimidyl ester (CFSE) right before transplantation and injected a mixture of these cells at a 1:1 ratio into lethally irradiated BALB/c WT recipients. At day 3 post-HCT, we did not observe a difference in proliferation between groups from donor cells collected in the small intestine (Fig. 4A). We then isolated small intestinal lymphocytes at day 5 post HCT to test for differences in apoptosis, measured using annexin V. We found no differences between groups in apoptosis of T cells in the small intestine at day 5 post-HCT (Fig. 4B). Next, we isolated T cells from WT or MyD88$^{-/-}$ mice and transplanted them respectively into lethally irradiated BALB/c recipients, together with TCD WT bone marrow cells. We compared the expression of migration markers CCR5 and $\alpha4\beta7$ as well as the quantity of infiltrated lymphocytes that distributed in the intestinal lamina propria at day 10 post-HCT. We found that although the frequencies of both CCR5^{+} T cells and $\alpha4\beta7^{+}$ T cells were not different between WT and MyD88$^{-/-}$ T cells-transplanted groups, the absolute numbers of both CCR5^{+} T cells and $\alpha4\beta7^{+}$ T cells were significantly decreased in the MyD88$^{-/-}$ T cells-transplanted group than in the WT T cells-transplanted group in our MH model (Fig. 4C, D, E). Interestingly, compared to the WT T cells-transplanted group, both IL-4 and IL-10-produced intestinal CD4^{+} T cells were significantly increased in MyD88$^{-/-}$ T cells-transplanted group. What's more, the frequencies of intestinal Tregs and IL-10-produced Tregs were also, as expected, significantly increased in MyD88$^{-/-}$ T cells-transplanted group at day 10 post-HCT (Fig. 4F, G). Taken together, the above data suggest that decreased infiltrated T cells and increased inhibitory cytokines expression in CD4 T cells and increased Tregs may collaboratively contribute to the improved aGVHD in MyD88$^{-/-}$ T cells-transplanted group, rather than due to differences in T cells expansion or apoptosis.

ST2/MyD88 signaling in Tcons is necessary for aGVHD development

Transcriptome analysis from day 10 post-HCT in the MH model comparing WT or MyD88$^{-/-}$ sorted CD4^{+}CD127^{+}CD25^{-} Tcons recovered from the intestines showed that MyD88$^{-/-}$ CD4 Tcons express lower levels of genes involved in the inflammatory response, including *Il1rl1* (gene of ST2), *Ifng*, *Batf*, *Csf2* (gene of GM-CSF), *Stat5*, and *Jak2* (Fig. 5A). MyD88$^{-/-}$ T cells recovered from the intestine at day 10 post-HCT expressed less sST2 and more ST2 compared to WT T cells (Fig. 5B and C). Systemic levels of sST2 and IFN-γ in recipients of MyD88$^{-/-}$ T cells were also decreased at days 5 and 10 post-HCT compared to recipients of WT T cells (Fig. 5D and E). These data confirm that alloreactive T cells in the intestines produce sST2, as we previously suggested (14). We hypothesized that the protective phenotype observed when transplanting MyD88$^{-/-}$ Tcons in the presence of Tregs was mediated by a lack of ST2 signaling on donor Tcons. Recipients of ST2$^{-/-}$ Tcons with either WT or ST2$^{-/-}$ Tregs had lower aGVHD score and mortality compared to recipients of WT Tcons with either WT or ST2$^{-/-}$ Tregs (Fig. 5F and G).

To further show the importance of ST2/MyD88 signaling in donor T cells during aGVHD, we generated ST2$^{-/-}$MyD88$^{-/-}$ double knockout mice (DKO) by crossing ST2$^{-/-}$ and MyD88$^{-/-}$ mice. In the MH model, recipients of the donor DKO T cells had lower disease severity, resulting in fewer deaths compared to mice receiving WT cells (median survival time: WT ~ 9 days; DKO > 30 days (Fig. 6A and B). Although the recipients of DKO T cells did exhibit less disease severity compared to recipients of MyD88$^{-/-}$ T cells, survival between these groups was not different. Serum levels of sST2 and IFN-γ were also significantly lower at day 10 post-HCT in recipients of DKO T cells (Fig. 6C and D). We also tested for systemic levels of IL-6 and TNFα but we saw no difference between recipients of WT and DKO T cells (data not shown). To determine if DKO Tcons have increased susceptibility to Treg-mediated suppression, we performed a suppressive assay. We did not see any impairment of Treg suppressive capability when Tregs were either WT or DKO or when Tcons were WT or DKO (Fig. 6E).

DISCUSSION

Previous studies using MyD88$^{-/-}$ T cells have shown that MyD88 is necessary for optimal CD4 and CD8 T cell responses *in vivo* (30, 33, 39, 40). We found no differences in the ability of T cells to produce IFN-γ, IL-4, or IL-17 under Th1, Th2, or Th17 polarizing conditions, respectively, when using αCD3/αCD28 polyclonal stimulation. However, during antigen-specific responses, T cells may require MyD88 for optimal differentiation of Th1 and Th17 cells (28, 30, 33, 39, 40). In the context of aGVHD, we show here that MyD88 in T cells is necessary for optimal allo-response. Indeed, loss of MyD88 in the donor T cells leads to decreased aGVHD severity and mortality in two different allogeneic murine models:

a MH model (C57BL/6, H-2^b → BALB/c, H-2^d) and a miH model (C57BL/6, H-2^b → C3H.SW, H-2^b). It has been shown that diminished Th1 and Th17 responses due to loss of MyD88 are a product of loss of IL-1R signaling on CD4 T cells (30). As well, WT T cells upregulate IL-1R on their surface by day 3 post-HCT and transplanting IL-$1R^{-/-}$ donor T cells alleviated aGVHD in MH model (C57BL/6 → BALB/c) (31). However, we have found that IL-$1R^{-/-}$ donor T cells have no significant loss of effector function, as aGVHD was not attenuated in our two models. Our results are more in accordance with what has been shown examining MyD88 signaling in T cells in response to viral infection (33, 39, 40). After transfer of IL-$1R^{-/-}$ or $MyD88^{-/-}$ T cells into $RAG^{-/-}$ mice and infecting with vaccinia virus, mice with IL-$1R^{-/-}$ CD8 T cells were able to respond to the infection normally while mice with $MyD88^{-/-}$ CD8 T cells mounted a reduced response (40). Similar results were found during LCMV infection in CD8 T cells (33). Looking at WT, $MyD88^{-/-}$, and IL-$1R^{-/-}$ CD4 T cells in absence of CD8 T cells in response to LCMV infection, WT and IL-$1R^{-/-}$ mice developed wasting disease and had lower virus levels while $MyD88^{-/-}$ mice did not develop wasting disease and had higher virus levels due to failure to induce LCMV-specific CD4 T cell response. This response was not due to impaired APC function (39). Our results also are in accordance with a clinical trial that observed no difference in aGVHD outcomes in HCT patients when using prophylactic treatment with IL-1 receptor antagonist, an IL-1R inhibitor (41). This discrepancy in our results along with the results in the literature looking at anti-viral response and a clinical trial with those shown others could be due to the difference in models. One used an immunization model, while our models and others use alloresponses and viral responses for stimulation, respectively. It is possible that the different use of antigens could impact which receptors become upregulated on T cells. IL-1R is upregulated on 2W:I-A^b tetramer-positive T cells but not 2W:I-A^b tetramer-negative T cells after antigen stimulation. The immunization model also does not take into account the presence of a variety of other molecules that would be present in a diseased state. PAMPs, DAMPs, and alarmins released during viral infection or during conditioning for allo-transplant but not during immunization could impact T cell response to IL-1 signaling. A difference in the microbiota of the recipient mice could also explain this difference. Recent work has shown that the makeup of the intestinal microbiome can affect aGVHD severity (42–46). The mice from commercial vendors which are purchased for experiments can have significantly different microbiotas which can impact immune response (47). Aberrant IL-1/IL-1R signaling has been shown to alter the microbiota in mice (48). We purchased the BALB/c mice in our experiments from The Jackson Laboratory while the other group purchased their BALB/c mice from Charles River, Harlan, or from the local stock of the animal facility at Freiburg University Medical Center (31). It is possible that the difference in phenotype we saw compared what has been published is in part due to differences in intestinal microbiota of the recipients.

During pre-HCT conditioning, the integrity of intestinal mucosa is impaired, and the intestinal epithelial tight junctions will become more permeable. A very recent research has indicated that the intestinal epithelial barrier loss is a crucial driver of the GVHD propagation (49). With the damage of intestinal epithelial barrier, broad range of DAMPs, PAMPs and alarmins will be released and sensed by kinds of immune cells, among of which LPS, with other bacterial products, is secreted and then recognized by TLR4 expressed immune cells. Activated TLR4 transducts the signaling downstream via MyD88 adaptor. TLR4 is found on both human and murine CD4 T cells, but its function is not well understood. One study showed that only naïve murine T cells and not activated T cells express TLR4 (50). However, TLR4 on human T cells was only detected in activated CD4 T cells (51). In a murine model of EAE, TLR4$^{-/-}$ T cells transferred into RAG1$^{-/-}$ followed by EAE induction did not produce disease (52). However, in a spontaneous model of colitis, IL-10$^{-/-}$TLR4$^{-/-}$ T cells transferred into RAG1$^{-/-}$ mice accelerated disease progression (53). During aGVHD, we found that TLR4 signaling in donor T cells was not necessary. A lack of TLR4 expression on activated T cells could explain why we didn't see any difference when using TLR4$^{-/-}$ donor T cells, but we did not test for TLR4 surface expression post-HCT. Our data contrasts that found by others, who found that using TLR4$^{-/-}$ as donors does protect against aGVHD (32). We believe this difference is caused by the use of TLR4$^{-/-}$ BM and TLR4$^{-/-}$ T cells together compared to our use of WT BM and TLR4$^{-/-}$ T cells. As donor dendritic cells are present in the BM during transplantation and as it is well documented that TLR4 stimulation of dendritic cells triggers their maturation and cytokine expression, we believe that the protective phenotype observed is caused by TLR4$^{-/-}$ on these dendritic cells. A limitation of this work is that we did not go through the full range of MyD88-depedent TLRs. It is possible that other MyD88-dependent TLRs on donor T cells are important for aGVHD development (54). For example, TLR2 signaling has been shown to promote a Th17 response and that loss of TLR2 on CD4 T cells ameliorated EAE (55); however, loss of TLR2 on the donor T cells as well as TLR2 blockade using an anti-TLR2 blocking antibody did not reduce aGVHD severity or mortality (56).

MyD88 signaling has been shown to be important in both macrophages and T cells in responses to viruses, as MyD88$^{-/-}$ T cells show impaired anti-viral clearance (33, 39, 40); however, its role in different subsets of T cells *in vivo* during aGVHD is not known. We first compared the expression of MyD88 in three major T cell subpopulations. Western blot data indicated that CD4^{+} T cons have the highest expression of MyD88, nearly three times more than CD8^{+} T cells or Tregs. When transplanting WT CD4 T cells with MyD88$^{-/-}$ CD8 T cells, we observed no difference in aGVHD severity or mortality. When transplanting MyD88$^{-/-}$ CD4 T cells with WT CD8 T cells, we observed a decrease in aGVHD severity and an increase in survival. This is in accordance with the findings that MyD88$^{-/-}$ CD4 T cells have impaired function during coronavirus encephalomyelitis while MyD88$^{-/-}$ CD8 T cells appear normal (57). Transcriptome analysis using Nanostring of CD4^{+} T cells from the intestine 10 days post-HCT also showed that genes responsible for a potent type 1 response to be downregulated in mice receiving MyD88$^{-/-}$ T cells. Interestingly, GM-CSF production was lower in MyD88$^{-/-}$ CD4 T cells but not in MyD88$^{-/-}$ CD8 T cells. It has recently been shown that loss of GM-CSF in the donor T cells attenuates aGVHD and that GM-CSF production in the donor T cells is mediated through basic leucine zipper transcription factor, ATF-like (BATF) signaling (37). Our Nanostring data suggests that loss of MyD88 impacted BATF expression, as BATF expression is much lower in MyD88$^{-/-}$ CD4 T cells than in WT CD4 T cells. Exploration of MyD88/BATF/GM-CSF regulation would help to understand how MyD88 affect GM-CSF production.

The CD4 T cell compartment consists of both Tcons and Tregs, with type 1 Tcons promoting aGVHD and Tregs alleviating aGVHD. We showed here that transplantation of

WT or MyD88$^{-/-}$ Tcons without Tregs demonstrated no difference in aGVHD severity or mortality. However, in a colitis model in which naïve WT or MyD88$^{-/-}$ CD4^{+} CD45RB^{+} T cells were transplanted into RAG1$^{-/-}$ recipients, MyD88$^{-/-}$ cells were unable to induce severe colitis as compared to WT cells (58). The discrepancy observed could be due to the difference in pro-inflammatory cytokines observed between models. This colitis model is dependent on IL-17 production from Th17 cells, and MyD88$^{-/-}$ CD4 T cells did indeed produce less IL-17. A difference in the Th1 cytokine IFN-γ was not observed (58). Our aGVHD model with this dose of donor T cells is mainly dependent on IFN-γ as we did not detect any Th17 cells in the intestine in our models, and IFN-γ was significantly lower in the plasma of MyD88$^{-/-}$ recipients. It has been shown that Treg-specific MyD88$^{-/-}$ cells have no impairment of suppressive capability compared to WT Treg (30). But, it has also been shown that MyD88$^{-/-}$ Tregs during skin transplantation and cGVHD are deficient in their suppressive capabilities (38). MyD88$^{-/-}$ Tregs also protect less against colitis compared to WT Tregs (58); however, we found that transplantation with donor WT Tcons and either WT or MyD88$^{-/-}$ Tregs did not alleviate aGVHD. The difference in our data could be due to the kinetics of disease. In the skin transplant model, Treg frequencies were similar early after transplant and only started to decrease after 21 days post-skin transplant (38). In the colitis model, a difference in disease severity using MyD88$^{-/-}$ Tregs compared to WT Tregs was not observed until 9 weeks post-transplant into RAG1$^{-/-}$ mice (58). In our aGVHD model, we start seeing severe aGVHD as early as 10 days post-HCT. In the immunization model, the authors waited only seven days before measuring Tcons proliferation and pro-inflammatory cytokine production (30). We cannot eliminate the possibility based off our data that, in a slower disease progression setting, MyD88$^{-/-}$ Tregs do indeed develop a suppressive defect. Therefore, the importance of MyD88 signaling in Tregs may be highly disease and time dependent.

It has been shown that naïve CD4 T cells require MyD88 signaling through the IL-1R in order to overcome Treg-mediated suppression for induction of a Th1 response (30). While our data suggests that IL-1R signaling in T cells is not required for aGVHD induction, left open was the possibility that MyD88 signaling in Tcons is required for Treg-mediated suppression in aGVHD. Indeed, when transplanting MyD88$^{-/-}$ Tcons with Tregs, we did observe a decrease in aGVHD severity and mortality. Interestingly, this phenotype did not depend on MyD88 in the Tregs, as transplanting WT or MyD88$^{-/-}$ Tregs with MyD88$^{-/-}$ Tcons showed no difference in aGVHD severity or mortality. We believe that this may be caused by loss of signaling through soluble factors, such as IL-6 or TNFα, that act directly or indirectly through or on MyD88. Deficiency of IL-1β/MyD88 signaling has already been mentioned, but IL-6 has also been implicated in Tcons resistance to Treg-mediated suppression (59–61). It has been suggested that this is due to blocking of Treg-mediated inhibition of IL-2Rα on Tcons (61). We did not check for IL-2Rα expression on Tcons during our experiments. Loss of IL-6-produced T cells, but not bone marrow cells or non-hematopoietic cells, also prevents aGVHD mortality in a murine model, although the mechanism behind this remains unexplored (62). Interestingly, similar to our data with MyD88$^{-/-}$ donor T cells, it was also found that the absence of IL-6 did not affect the expansion of T cells. As IL-6 is known to be upregulated by multiple TLR/MyD88 signaling pathways, it is possible that reduced IL-6 in MyD88$^{-/-}$ donor T cells could explain our phenotype. However, we did not check for IL-6 production in T cells in our models. IL-6 signaling is known to activate STAT3 (63). It is also possible that the absence of MyD88 signaling reduces phosphorylated STAT3 levels in the Tcons, which has been shown to be important in Tcons for their resistance to Treg suppression (64). Although STAT3 is not classically thought to be downstream of MyD88, it has recently been shown that activation of TLR4 through MyD88, TLR7, or TLR9 directly leads to phosphorylation of STAT3 (65–67). Indeed, pSTAT3 Y705 is increased significantly in patient CD4 T cells before onset of aGVHD (68). IL-7 and IL-15 may also play a role in Tcons-resistance to Tregs during aGVHD (69–71). Adoptive transfer of T cells into lymphopenic hosts, as would be after irradiation in our aGVHD models, leads to increased availability of IL-7 and IL-15 for the transferred T cells. Although a link between IL-7 and MyD88 has yet to be made, it has been shown that IL-15 promotes MyD88 expression in T cells (72). How IL-15 causes MyD88 upregulation and the effect of MyD88 upregulation by IL-15 has yet to be explored. Tcons could be using these pathways mentioned involving MyD88 to redundantly prevent their Treg-mediated suppression, which would explain why loss of IL-1R or TLR4 alone was insufficient.

A common convergence of all these pathways is the phosphatidylinositol-3 kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) pathway. This pathway helps control many cellular processes, such as proliferation, survival, migration, and metabolism (73). It has been suggested that hyper-activation of PI3K leads to Tcons resistance to Treg-mediated suppression (74). Indeed, in murine models that have genetic deficiencies in proteins that negatively regulate PI3K signaling, Tcons are more resistant to Treg-mediated suppression (75, 76). Several cytokine receptors, TNF receptors, TLRs, and T cell costimulatory receptors have been shown to activate PI3K signaling (77). While MyD88 has not been implicated in all these pathways, we suspect that loss of MyD88 may affect enough pathways to prevent hyper-activation of PI3K/ATK/mTOR signaling, thus rendering Tcons susceptible to Treg-mediated suppression (Fig. 7). Direct targeting of mTOR using rapamycin has been extensively studied in GVHD and is given to patients routinely as a prophylaxis, with some studies suggesting efficacy as a treatment option of aGVHD (78). Recently, in a murine model of aGVHD, direct pan-PI3K inhibition using a small molecule inhibitor prevented severe aGVHD development, in part through controlling T cell activation (79). However, how pan-PI3K inhibition works on Tcons and Tregs specifically was not studied nor the direct mechanism of how pan-PI3K inhibition of T cells prevented severe aGVHD development.

ST2 on T cells has been found primarily on the Th2 and Treg subsets. ST2 is a member of the IL-1R superfamily and signals through MyD88 IL-33/ST2 signaling enhances Th2 and Treg activity through increased IL-5 and IL-13 production in Th2 cells (19, 80–82) and increased *Foxp3* expression in Tregs (21). A soluble form of ST2, sST2, sequesters free IL-33 and does not signal. Recently, we have shown that T cells, specifically type 1 and type 17 T cells, can produce sST2 (14). We and others have shown that total ST2$^{-/-}$ T cells ameliorate aGVHD (14, 17). We also find here that Tcons have much higher expression of ST2 than CD8^{+} T cells, but an equivalent expression with Tregs. It should be noticed that both MyD88 and ST2 are simultaneously highly expressed in CD4^{+} Tcons rather than the Tregs, which may means a parallel function in Tcons. Due to the similarities of expression, we asked whether the phenotype of MyD88$^{-/-}$ Tcons in the presence of WT or MyD88$^{-/-}$ Tregs observed above would be paralleled when using ST2$^{-/-}$ Tcons and WT or ST2$^{-/-}$ Tregs. Indeed, transplanting ST2$^{-/-}$ Tcons alleviated aGVHD in a similar manner regardless of using WT or ST2$^{-/-}$ Tregs, suggesting that ST2/MyD88 signaling is required for Tcons to overcome Treg-mediated suppression. We did notice a small but non-statistically significant difference in aGVHD severity and mortality when using ST2$^{-/-}$ Tregs with WT or ST2$^{-/-}$ Tcons compared to using WT Tregs with WT or ST2$^{-/-}$ Tcons, suggesting that loss of ST2 on Tregs may impact their suppressive capabilities. This would be in line with the higher expression of ST2 as compared to MyD88 on Tregs (Fig 3A) and what has previously been shown (20, 21, 83). In our hands, an *in vitro* suppression assay did not show any differences in suppression by Tregs when either Tcons or Tregs lost both ST2 and MyD88 signaling. This possible suggests that the presence of the allo-response and/or pro-inflammatory environment during aGVHD allows Tcons to resist suppression. We also show that isolated MyD88$^{-/-}$ CD4 T cells from the intestine at day 10 post-HCT express less sST2 and more ST2 compared to WT CD4 T cells. However, whether MyD88 signaling is directly important in sST2 expression by CD4 T cells or whether the decrease in sST2 expression is due to a decrease in Th1 response is not clear. Loss of MyD88 signaling leading to lower sST2 expression would partially explain the protective phenotype against aGVHD we observed when we transplanted MyD88$^{-/-}$ donor T cells as compared with transplanting WT donor T cells (Fig. 7). There is evidence that STAT3 and ERK signaling, both of which can be activated through MyD88, influence ST2 proximal promoter activity (84), which has been suggested to promote sST2 production (85). We have not tested for STAT3 or ERK activity in our models. As well, the increase in ST2 expression may be a compensatory mechanism by the CD4 T cells trying to overcome the loss of MyD88.

We and others have attempted to look for ST2 expression via flow cytometry on Th1 during aGVHD settings without success. However, recent reports have shown that ST2 can indeed be present on Th1 cells (17, 86, 87). ST2 signaling on Th1 cells helps clear LCMV infection through increased IFN-γ production and is dependent on T-bet and STAT4 (86). The effect of IL-33 on Th1 differentiation was also seen by using an OVA-immunization murine model as well as human *in vitro* cell cultures (87). The expression of ST2 on the surface only occurred during times of inflammation (86). During aGVHD, IL-33 administration during peak inflammatory response (days 3–7 post-HCT) enhanced aGVHD severity and mortality (17), while IL-33 administration during the peri-transplant period ameliorated aGVHD through enhanced ST2^{+} Treg response (88). This suggests that ST2 may be only transiently expressed on Th1 cells, while it is more stably expressed on Th2 cells and Tregs at later time points during aGVHD. Although an inflammatory response is clearly occurring during aGVHD, perhaps this transient expression is the reason that we were not able to detect ST2 in our aGVHD model, as we’ve only looked for ST2 expression via flow cytometry after day 10 post-HTC. Further work needs to be done to assess a potential role of ST2/MyD88 signaling in promoting a Th1 response early during aGVHD.

A weakness of our mouse model is that the ST2$^{-/-}$ mouse we use has a loss of both the membrane and soluble forms of ST2. It is therefore difficult to determine whether sST2 production by Tcons or if indeed ST2 is present on Th1 cells and loss of ST2 on these cells is more important in the phenotype we observed. Development of distinct sST2$^{-/-}$ and membrane ST2$^{-/-}$ mice could really help answer these questions.

We conclude that loss of ST2/MyD88 protects mice from fatal aGVHD through lower sST2 production by Tcons while also sparing Treg function. The results of our study confirm that ST2 represents an aGVHD therapeutic target.

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Abbreviations used in this paper:

aGVHD	acute graft-versus-host disease
APC	antigen presenting cell
BM	bone marrow
CFSE	carboxyfluorescein succinimidyl ester
cGy	centigray
DKO	ST2$^{-/-}$MyD88$^{-/-}$ double knockout
HCT	allogeneic hematopoietic cell transplantation
MH	major histocompatibility model
miH	minor histocompatibility model
sST2	soluble ST2
ST2	membrane-bound ST2
TCD	T cell-depleted
Tcon	conventional T cell
Treg	regulatory T cell
WT	wild-type

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Key Points

1. MyD88 deficiency in donor Tcons with Tregs protected against alloreactivity and aGVHD
2. This protective effect uses ST2 but not the IL-1R or TLR4 pathways

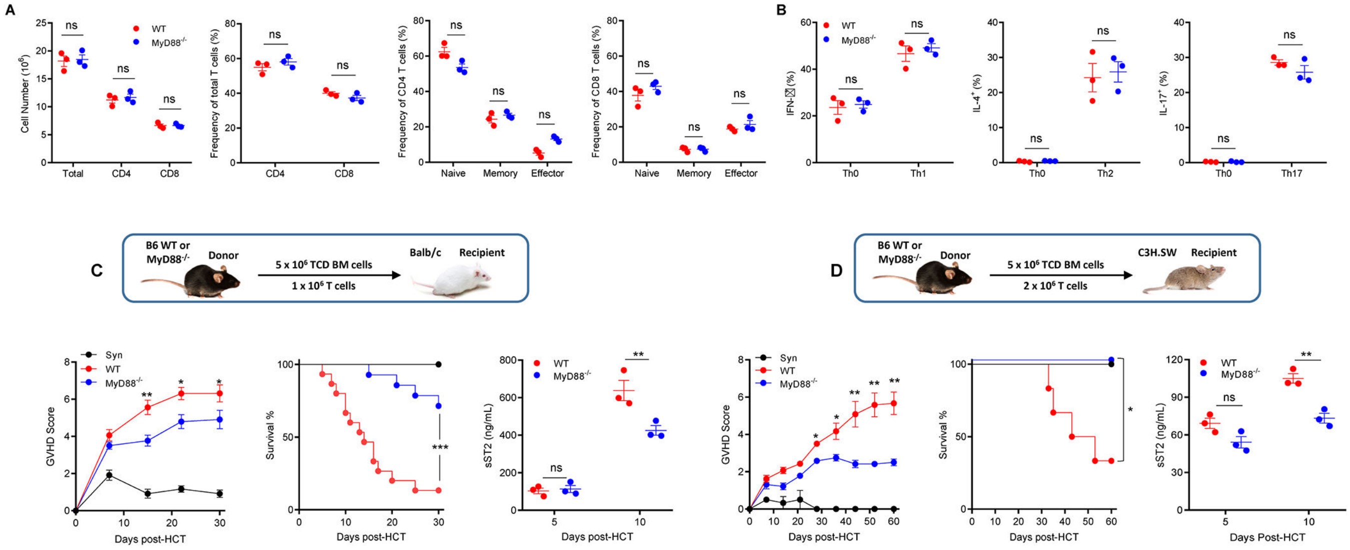

Figure 1.

Both morbidity and mortality of aGVHD are significantly reduced using MyD88$^{-/-}$ donor T cells in murine models. (**A**) Cell numbers and frequencies of total CD4 and CD8 T cells (Left), naïve (CD62L^{+}CD44^{-}), memory (CD62L^{+}CD44^{+}), and effector (CD62L^{-}CD44^{+}) CD4 or CD8 populations (Right) harvested from spleen of WT or MyD88$^{-/-}$ mice (mean ± SEM; n=3). (**B**) CD4 T cells were stimulated with plate-bound αCD3 (2 μg/ml) and soluble αCD28 (5 μg/ml) under Th0 (no additional cytokines), Th1 (20 ng/ml IL-12, 2 ng/ml IL-2), Th2 (20 ng/ml IL-4), or Th17 (4 ng/mL TGF-β, 20 ng/ml IL-6) conditions for 5 days. Graphs show frequency of IFN-γ (Left), IL-4 (Middle), and IL-17 (Right) expressions (mean ± SEM; n=3). (**C**) Lethally irradiated BALB/c mice (900 cGy) were given 5 × 10^{6} T cell depleted bone marrow (TCD-BM) cells and 1 × 10^{6} donor T cells from WT or MyD88$^{-/-}$ B6 mice for allogeneic transplant or WT BALB/c TCD-BM and donor T cells from WT BALB/c mice for syngeneic transplant. (Left) GVHD score and (Middle) survival; BALB/c → BALB/c (n=5), B6 WT (n=15), or B6 MyD88$^{-/-}$ total T cells (n=15) groups. (Right) Kinetics of plasma levels of sST2 in BALB/c mice collected at days 5 and 10 post-HCT (mean ± SEM, n=3). (**D**) Lethally irradiated C3H.SW mice (1100 cGy) were given 5 × 10^{6} TCD-BM cells and 2 × 10^{6} donor T cells from WT or MyD88$^{-/-}$ B6 mice for allogeneic transplant or WT C3H.SW TCD-BM and donor T cells from WT C3H.SW mice for syngeneic transplant. (Left) GVHD score and (Middle) survival; C3H.SW → C3H.SW (n=5), B6 WT (n=6), or B6 MyD88$^{-/-}$ total T cells (n=6). (Right) Kinetics of plasma levels of sST2 in BALB/c mice collected at days 5 and 10 post-HCT (mean ± SEM, n=3). The ns means no significant, $*p < 0.05$, $**p < 0.01$

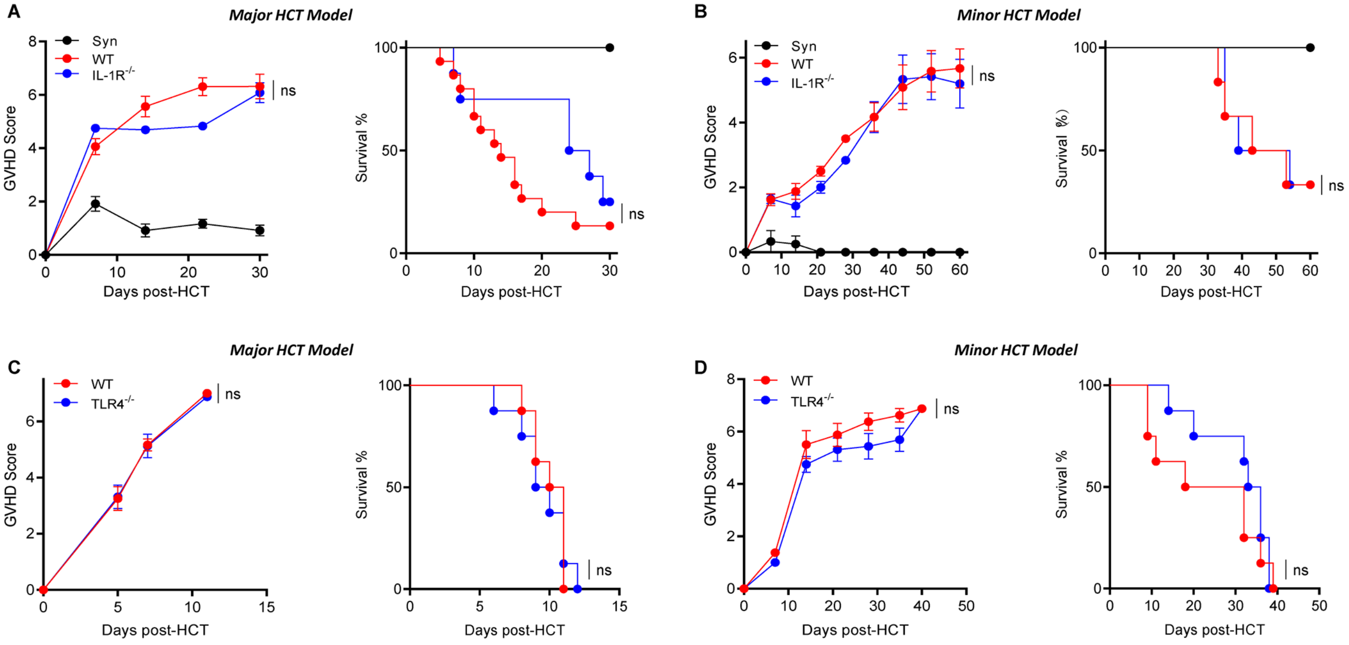

Figure 2.

MyD88 signaling on donor T cells through IL-1R or TLR4 does not affect aGVHD severity or mortality. (**A**) Lethally irradiated BALB/c mice (900 cGy) were given 5×10^6 TCD-BM cells and 1×10^6 donor T cells from WT or IL-1R$^{-/-}$ B6 mice for allogeneic transplant or WT BALB/c TCD-BM and donor T cells from WT BALB/c mice for syngeneic transplant. (Left) GVHD score and (Right) survival; BALB/c → BALB/c (n=5), B6 WT (n=15), or B6 IL-1R$^{-/-}$ total T cells (n=8). (**B**) Lethally irradiated C3H.SW mice (1100 cGy) were given 5×10^6 TCD-BM cells and 2×10^6 donor T cells from WT or IL-1R$^{-/-}$ B6 mice for allogeneic transplant or WT C3H.SW TCD-BM and donor T cells from WT C3H.SW mice for syngeneic transplant. (Left) GVHD score and (Right) survival; C3H.SW → C3H.SW (n=5), B6 WT (n=6), or B6 IL-1R$^{-/-}$ total T cells (n=6). (**C**) Lethally irradiated BALB/c mice (900 cGy) were given 5×10^6 TCD-BM cells and 1×10^6 donor T cells from WT or TLR4$^{-/-}$ B6 mice for allogeneic transplant. (Left) GVHD score and (Right) survival; B6 WT (n=8) or B6 TLR4$^{-/-}$ total T cells (n=8). (**D**) Lethally irradiated C3H.SW mice (1100 cGy) were given 5×10^6 TCD-BM cells and 2×10^6 donor T cells from WT or TLR4$^{-/-}$ B6 mice for allogeneic transplant. (Left) GVHD score and (Right) survival; B6 WT (n=8) or B6 TLR4$^{-/-}$ total T cells (n=8). The ns means no significant.

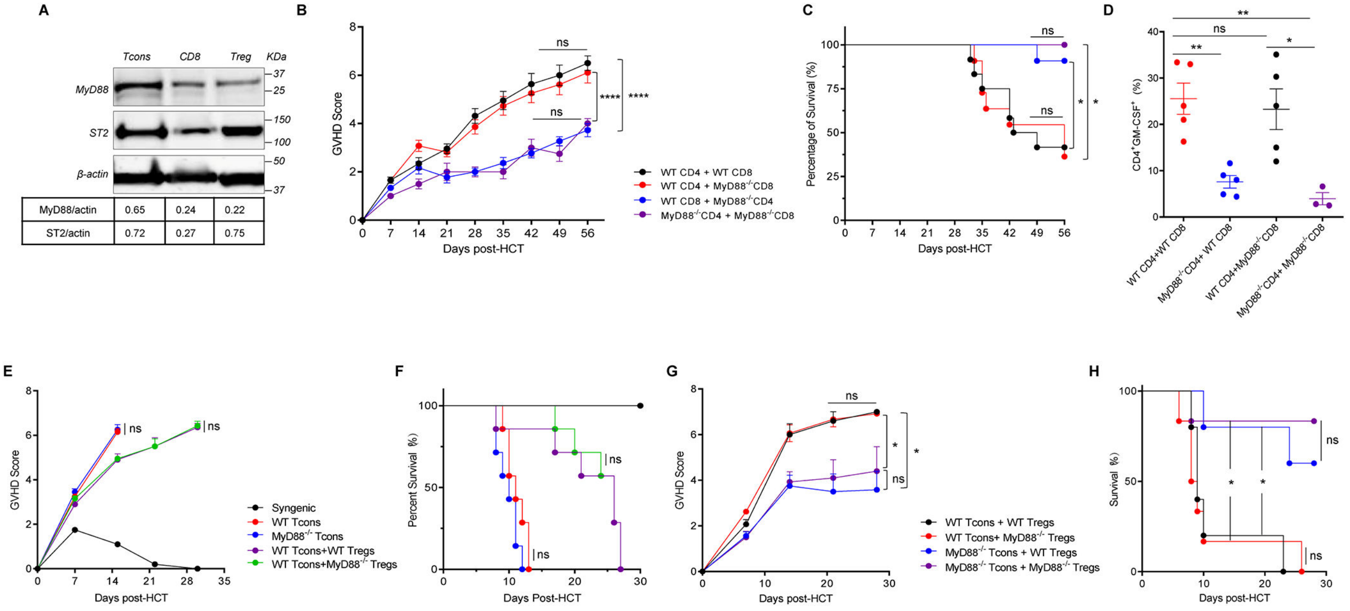

Figure 3.

MyD88$^{-/-}$ CD4 Tcons in the presence of Tregs alleviates aGVHD severity and mortality. (**A**) Western blot of MyD88 from freshly isolated Tcons, CD8 T cells and Tregs from a WT B6 spleen. Lethally irradiated C3H.SW mice (1100 cGy) were respectively given 5×10^6 TCD-BM cells and a mixture (WT CD4 + WT CD8 or WT CD4+MyD88$^{-/-}$ CD8 or MyD88$^{-/-}$ CD4 + WT CD8 or MyD88$^{-/-}$ CD4 + MyD88$^{-/-}$ CD8) of 2×10^6 donor T cells. (B)The GVHD score and (C) survival are real-time monitored; WT CD4 + WT CD8 (n=13), WT CD4+MyD88$^{-/-}$ CD8 (n=13), MyD88$^{-/-}$ CD4 + WT CD8 (n=13) and MyD88$^{-/-}$ CD4 + MyD88$^{-/-}$ CD8 (n=4) (**D**) T cells were harvested from the intestine at day 10 post-HCT and stained for live GM-CSF produced CD4 T cells. Lethally irradiated BALB/c mice (900 cGy) were given 5×10^6 TCD-BM cells with 1×10^6 donor Tcons without Tregs from WT or MyD88$^{-/-}$ B6 mice for allogeneic transplant or WT BALB/c TCD-BM and 1×10^6 donor T cells from WT BALB/c mice for syngeneic transplant; or with a 5:1 mixture of WT Tcon + WT or MyD88$^{-/-}$ Tregs totaling 1×10^6 donor T cells from B6 mice for allogeneic transplant. (**E**) The GVHD score and (**F**) survival are real-time monitored; BALB/c → BALB/c (n=5), B6 WT Tcons (n=7), B6 MyD88$^{-/-}$ Tcons (n=7), B6 WT Tcons and B6 WT Tregs (n=7), B6 WT Tcons and B6 MyD88$^{-/-}$ Tregs (n=7). Lethally irradiated BALB/c mice (900 cGy) were given 5×10^6 TCD-BM cells and a 10:1 mixture of WT or MyD88$^{-/-}$ Tcons + WT or MyD88$^{-/-}$ Tregs totaling 1×10^6 donor T cells from B6 mice for allogeneic transplant. (**G**) GVHD score and (**H**) survival; WT or MyD88$^{-/-}$ Tcons + either WT or MyD88$^{-/-}$ Tregs (all groups n=6). The ns means no significant, *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

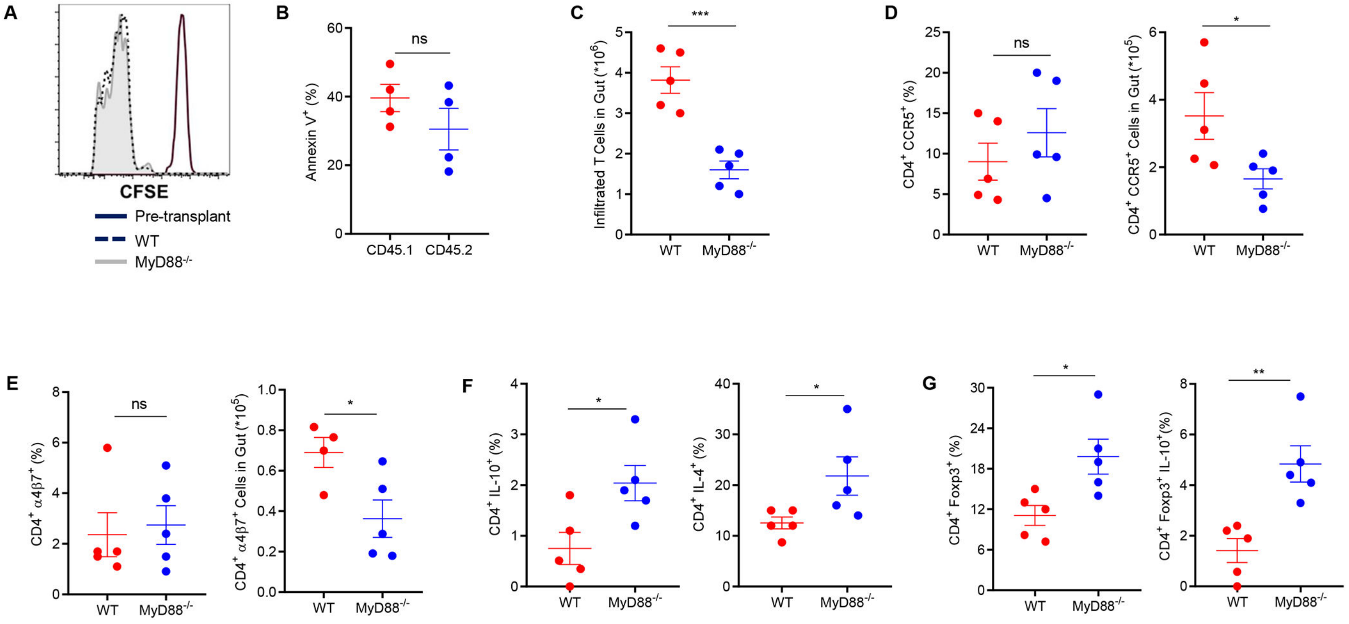

Figure 4.

MyD88$^{-/-}$ donor T cells do not have defects in proliferation or apoptosis, but a significant increase of Tregs and the production of IL-4 and IL-10 during aGVHD. (**A, B**) Lethally irradiated BALB/c mice (900 cGy) were given 5×10^6 TCD-BM cells and 1×10^6 mixture of CFSE labeled donor T cells from WT CD45.1 or MyD88$^{-/-}$ CD45.2 (1:1) B6 mice. (**A**) Proliferation of CFSE labeled T cells from WT CD45.1 or MyD88$^{-/-}$ CD45.2 donors harvested from the intestine at day 3 post-HCT. (**B**) Annexin V staining of T cells from WT CD45.1 or MyD88$^{-/-}$ CD45.2 donors harvested from the intestine at day 5 post-HCT (mean ± SEM, n=4). (**C-G**) Lethally irradiated BALB/c mice (900 cGy) were given 5×10^6 TCD-BM cells and 1×10^6 donor T cells from WT or MyD88$^{-/-}$ B6 mice respectively. (**C**) Quantity of the infiltrated small intestinal lamina propria lymphocytes in both groups (mean ± SEM, n=5). (**D** and **E**) Expressions of CCR5 and α4β7 in CD4^{+} T cells and absolute numbers of CCR5 and α4β7 positive CD4^{+} T cells were detected in infiltrated small intestinal lamina propria lymphocytes (mean ± SEM, n=5). (**F**) Percentages of IL-4 and IL-10-produced CD4^{+} T cells in the gut were detected at day 10 post-HCT (mean ± SEM, n=5). (**G**) Frequencies of the Tregs and IL-10-produced Tregs in the gut were also detected at day 10 post-HCT (mean ± SEM, n=5). The ns means no significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

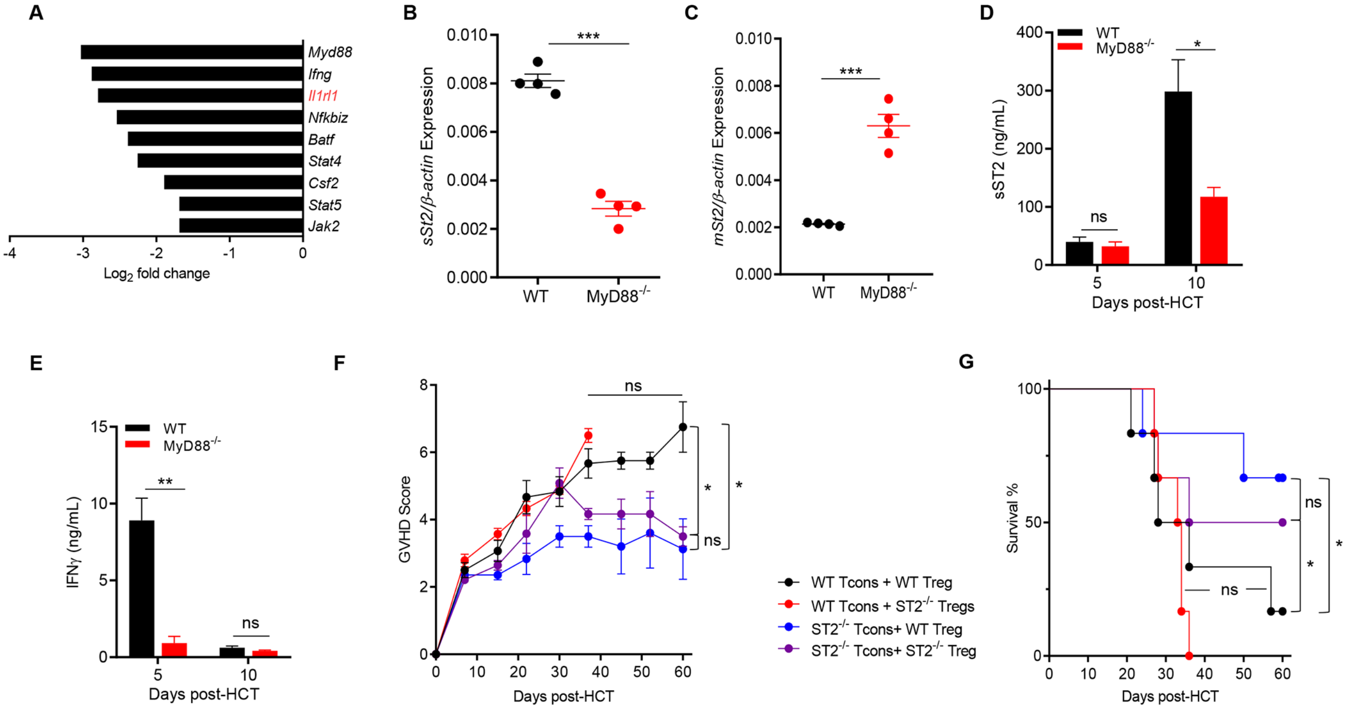

Figure 5.

MyD88$^{-/-}$ Tcons are more susceptible to Treg-mediated suppression through loss of ST2. (**A**) Transcriptome analysis comparing intestinal WT and MyD88$^{-/-}$ CD4 T cells harvested 10 days post-HCT (n=2 per group). Lethally irradiated BALB/c mice (900 cGy) were given 5 × 10^6 TCD-BM cells and 1 × 10^6 donor T cells from WT or MyD88$^{-/-}$ B6 mice for allogeneic transplant. (**B** and **C**) Relative expressions of sST2 and mST2 from WT or MyD88$^{-/-}$ T cells harvested from the intestine 10 days post-HCT (mean ± SEM, n=4). (**D** and **E**) Kinetics of plasma levels of sST2 and IFN-γ in BALB/c mice collected at days 5 and 10 post-HCT (mean ± SEM, n=3). Lethally irradiated C3H.SW mice (1100 cGy) were given 5 × 10^6 TCD-BM cells and a 10:1 mixture of WT or ST2$^{-/-}$ Tcon + either WT or ST2$^{-/-}$ Tregs totaling 2 × 10^6 donor T cells from WT or ST2$^{-/-}$ B6 mice for allogeneic transplant. (**F**) GVHD score and (**G**) survival were real-time monitored; WT or ST2$^{-/-}$ Tcons + either WT or ST2$^{-/-}$ Tregs (all groups n=6). The ns means no significant,*p < 0.05, **p < 0.01, ***p < 0.001.

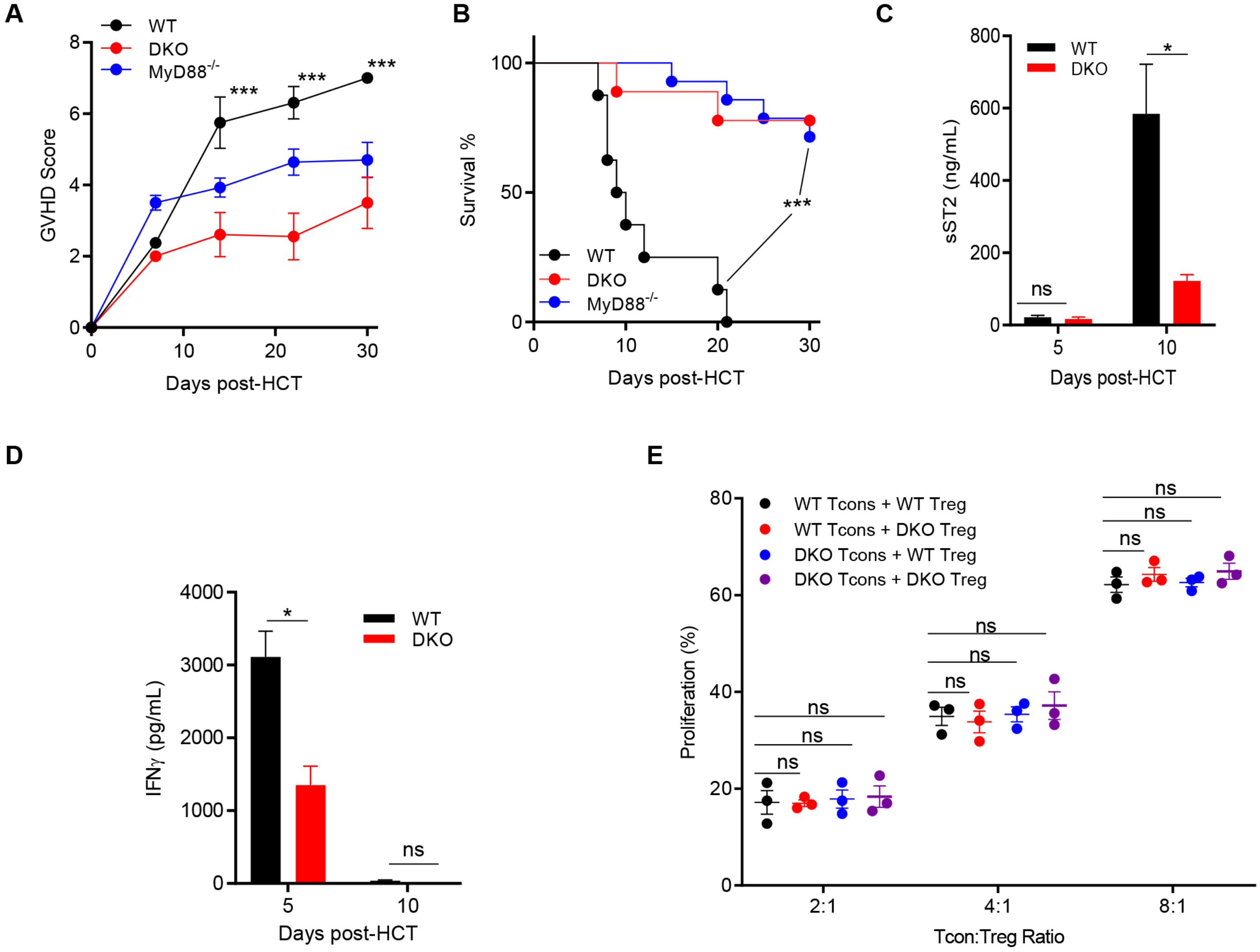

Figure 6.

ST2$^{-/-}$MyD88$^{-/-}$ DKO T cells reduce aGVHD severity and mortality similarly to MyD88$^{-/-}$ T cells. Lethally irradiated BALB/c mice (900 cGy) were given 5 × 10^6 TCD-BM cells and 1 × 10^6 donor T cells from WT, MyD88$^{-/-}$, or DKO B6 mice for allogeneic transplant. (**A**) GVHD score and (**B**) survival were real-time monitored (all groups n=8). (**C**) Kinetics of plasma levels of sST2 and IFN-γ (**D**) in BALB/c mice collected at days 5 and 10 post-HCT (mean ± SEM, n=3). (**E**) Total Tcons (TCRβ$^+$CD25$^-$) and Tregs (TCRβ$^+$CD4$^+$CD25$^+$) from the spleen of naïve WT and DKO mice were harvested via FACs sorting. CFSE-labeled WT or DKO Tcons were plated with WT or DKO Tregs at a 2:1, 4:1, or 8:1 ratio and stimulated with plate-bound αCD3 (2 μg/mL) and αCD28 (5 μg/mL) for 72 hours. Suppression was measured by lack of CFSE dilution. The ns means no significant, *p < 0.05, ***p < 0.001. p values in **A** and **B** are comparing WT and DKO groups.

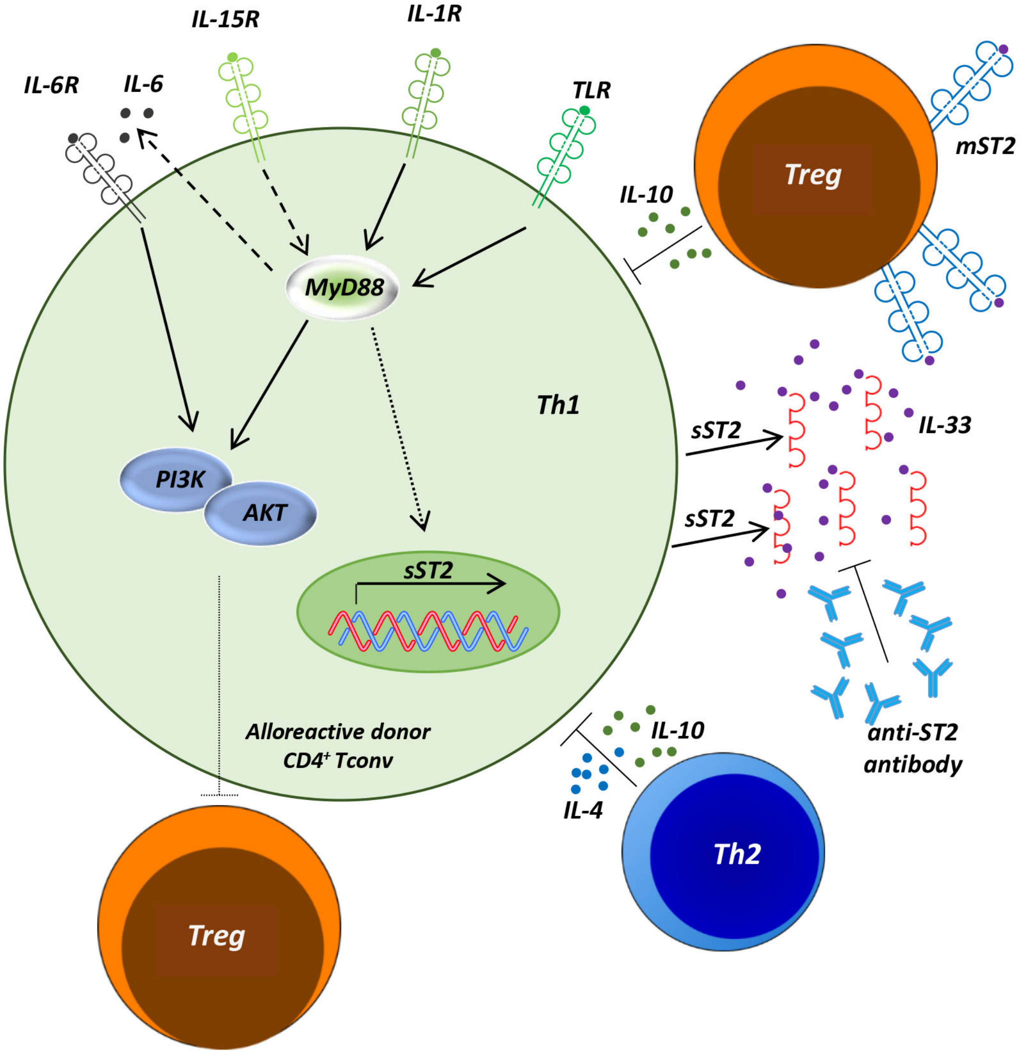

Figure 7.

Schema of possible mechanisms of action of MyD88$^{-/-}$ donor T cells during aGVHD. Various receptor signaling pathways have been shown to use MyD88 as an adaptor protein, including all TLRs except TLR3, the IL-1 superfamily of receptors, and recently the IL-15 receptor. We propose a two-pronged approach as to how MyD88 Tcons are able to resist Treg-mediated suppression. First, signaling through MyD88 activates the PI3K/AKT pathway. Shortly after conditioning for transplant, the damage caused by the conditioning leads to release of various DAMPs, PAMPs, and alarmins that can activate TLR and IL-1R superfamily signaling. The loss of lymphocytes after conditioning also causes excessive IL-15 to be available. IL-6 is produced and released through IL-1R and TLR signaling and can bind to the IL-6R on other Tcons. Both IL-6 signaling and MyD88 signaling can activate the PI3K/AKT pathway. Through a yet to be defined mechanism, others have proposed that hyper-activation of PI3K/AKT signaling promotes resistance of Tcons to suppression by Tregs. Second, we show that sST2 production in Tcons is reduced in MyD88$^{-/-}$ Tcons; however, how MyD88 regulates sST2 production is still unknown. sST2 released by Tcons can bind free IL-33, preventing IL-33/ST2 signaling on Tregs and Th2. IL-33/ST2 signaling on Tregs and Th2 has been shown by numerous groups to promote Treg function. Blocking sST2 with a neutralizing antibody has been shown to increase Treg frequency and ameliorate experimental aGVHD. We hypothesize that MyD88$^{-/-}$ Tcons have less PI3K/AKT activation and secrete less sST2, allowing the Tcons to be better repressed by Treg cells. Solid lines: direct effect; Dashed lines: indirect effect; dotted lines: proposed effect.