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Title: Type 2 Innate Lymphoid Cells Treat and Prevent GI Tract GvHD Through Enhanced Accumulation of Myeloid Derived Suppressor Cells

Authors: Danny W. Bruce¹, Heather E. Stefanski⁷, Benjamin G. Vincent^{1,3,4}, Trisha A. Dant⁵, Shannon Reisdorf¹, Hemamalini Bommasamy¹, David A. Serody¹, Justin E. Wilson⁴, Karen P. McKinnon^{1,5}, Warren D. Shlomchik⁸, Paul M. Armistead^{1,2}, Jenny YP Ting^{1,4}, John T Woosley⁶, Bruce R. Blazar⁷, Dietmar M. W. Zaiss⁹, Andrew N. J. McKenzie¹⁰, James M. Coghill^{1,3}, and Jonathan S. Serody^{1,3,5} *

Affiliations:

¹Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

²Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

³Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

⁴Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

⁵Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

⁶Department of Laboratory Medicine and Pathology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

⁷Department of Pediatrics and University of Minnesota Cancer Center, Minneapolis, MN 55455, USA.

⁸Department of Medicine and Cancer Center of the University of Pittsburgh, Pittsburgh, PA 15213, USA.

⁹Centre for Immunity, Infection and Evolution, and the Institute for Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, Edinburgh EH9 3FL, UK.

¹⁰The MRC Laboratory of Molecular Biology, Cambridge CB2 0QH, UK.

* Corresponding Author: JSS, Room 5012, Marsico Hall, University of North Carolina School of Medicine, Chapel Hill, North Carolina 7599-7295, E-mail: Jonathan_Serody@med.unc.edu

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One Sentence Summary: Adoptive transfer of donor ILC2 cells effectively prevents and treats acute GvHD of the lower GI tract by increasing the number of GI tract myeloid-derived suppressor cells.

Abstract:

Acute graft-versus-host disease (aGvHD) is the most common complication for patients undergoing allogeneic stem cell transplantation. Despite extremely aggressive therapy targeting donor T cells, patients with grade III or greater aGvHD of the lower GI tract, who do not respond to therapy with corticosteroids, have a dismal prognosis. This has increased interest in the function of local immune and non-immune cells in regulating the inflammatory process in the GI tract during aGvHD. Here, we demonstrate using murine models of allogeneic bone marrow transplantation, that type 2 innate lymphoid cells (ILC2s) in the lower GI tract are sensitive to conditioning therapy and show very limited ability to repopulate from donor bone marrow. Infusion of donor ILC2s was effective in reducing the lethality of aGvHD and in treating lower GI tract disease. This was associated with reduced donor pro-inflammatory Th1 and Th17 cells, accumulation of donor myeloid derived suppressor cells (MDSCs) mediated by ILC2 production of IL-13, improved GI tract barrier function and a preserved graft-versus-leukemia (GvL) response. Infusion of donor ILC2s to restore gastrointestinal tract homeostasis may be critical for optimal treatment of severe lower GI tract aGvHD.

Introduction

Allogeneic stem cell transplant (allo-SCT) has the potential to provide curative therapy for patients with high risk acute leukemia, lymphoid malignancies, and other malignant diseases (1-3). Despite improvements in HLA-typing and stem cell donor selection, GvHD remains the major complication of allo-SCT with incidence of acute GvHD (aGvHD) ranging from 30-80% and accounting for 15-30% of mortality of transplant recipients (4, 5). Grade III-IV aGvHD involving the lower gastrointestinal tract is the most common cause of morbidity and mortality from aGvHD. The administration of corticosteroids is the standard approach for the treatment of patients with grade II-IV aGvHD with approximately 70% of patients treated responding (6). However, the long-term survival of patients with corticosteroid non-responsive aGvHD involving the lower GI tract is dismal with less than 20% of those patients alive one year after diagnosis (7). Clearly, new forms of therapy are needed for the treatment of patients with corticosteroid non-responsive aGvHD of the lower GI tract.

Research over the past forty years has primarily focused on the role of donor derived T cells in the pathogenesis of aGvHD (8). Work from both pre-clinical transplant models and from clinical transplant studies has indicated a critical role for T cells, specifically Th1/Tc1 T cells, in the pathophysiology of aGvHD. Thus, the treatment of aGvHD has almost entirely focused on targeting donor T cells. However, despite highly potent therapy targeting T cells such as alemtuzumab, outcome for patients with corticosteroid non-responsive aGvHD has not improved (7). This has led to increasing interest in the role of other pro-inflammatory immune cells, such as macrophages, neutrophils and B lymphocytes in the pathophysiology of aGvHD, and the local function of anti-inflammatory immune and non-immune cells (9, 10).

A second group of immune cells that diminish the effector function of pro-inflammatory immune cells may be critical to the immune response during aGvHD. FoxP3-expressing regulatory T cells (T_{regs}) limit the expansion and effector function of donor T cells. Infusion of donor T_{regs} has been shown to be an effective prophylactic approach for the prevention of aGvHD (11). At this time, it is not clear if the infusion of T_{regs} can effectively treat on-going aGvHD. Our group has shown that IL-13 activated bone marrow myeloid-derived suppressor cells (MDSC) used at the time of bone marrow transplant in pre-clinical models inhibited GvHD lethality (12). However, their ability to treat active aGvHD is quite modest. Thus, despite intense research evaluating the function of immune cells that diminish effector T cell function, there is not a currently identified population of cells that has significant activity treating active aGvHD.

Over the past decade, a number of researchers have identified populations of innate immune cells (ILC) critical for rapid mucosal immune responses (13, 14). The initial ILC described four decades ago was the NK cell. Recently, multiple populations of ILCs that generate IFN- γ (ILC1), IL-5 and IL-13 (ILC2) and IL-17 and/or IL-22 (different subpopulations of ILC3 cells) have been described (15). ILC2s, previously termed nuocytes or innate helper type 2 cells, express GATA-3 and ID-2 similar to Th2 cells. They generate substantial type-2 cytokines. ILC2s, which respond to IL-25, are critical to the anti-helminth immune response and play an important role in allergen-induced inflammation (16-20). The role of innate cells in the biology of aGvHD has been evaluated recently. Hanash et al demonstrated that ILC3 cells in the GI tract were not sensitive to conditioning therapy but were decreased in mice with acute GvHD. The loss of ILC3 cells was associated with decreased generation of IL-22, impaired epithelial barrier function, and diminished numbers of intestinal stem cells (21). In humans, patients with decreased numbers of circulating CD69⁺ ILC2 and ILC3 cells had an increased risk of aGvHD (22).

We hypothesized that unlike ILC3 cells, there was a population of innate lymphoid cells that was sensitive to conditioning therapy. Here, we demonstrate that ILC2s in the GI tract but not in the lung are highly sensitive to conditioning therapy prior to allo-SCT and, more importantly, that there is quite limited repopulation of ILC2 cells in the GI tract from donor bone marrow. Infusion of IL-33 activated donor ILC2 cells significantly reduced GI tract associated aGvHD mortality, improving GvHD scores and associated weight loss in two separate murine models. Additionally, ILC2 treatment of recipients with established aGvHD significantly increased survival. Finally, we provide a novel mechanism by which ILC2s recruit donor myeloid-derived suppressor cells (MDSCs) to the lower GI tract to mitigate aGvHD. These studies strongly suggest that expanded ILC2s may be a potent cellular therapy for the treatment of lower GI tract aGvHD.

Results

Type 2 innate lymphoid cells are radiation sensitive

To evaluate the effect of conditioning therapy on ILCs, we irradiated B6D2 mice and quantitated GI tract ILCs. ILC3s in the lamina propria (LP) of the colon but not the mesenteric lymph node (MLN), isolated 24 hours after lethal irradiation were relatively resistant to radiation (Figure 1, A and B) (13, 21, 23). In contrast, CD4⁺ T cells in the LP and lung were radiation sensitive. Unlike ILC3s, ILC2s from the LP of the colon were highly

sensitive to radiation (Figure 1B). Similar to ILC3s, ILC2s in the MLN and spleen were exquisitely sensitive to radiation (Figure 1B, Supplemental Figure 1A). Interestingly, lung ILC2s were relatively resistant to radiation therapy (Figure 1B). We also examined the effects of chemotherapy on ILC2s and found that they are significantly reduced within 24 hours of cyclophosphamide treatment in the LP and MLN (Figure 1C).

Next, we evaluated the reconstitution of murine donor and recipient ILC2s four weeks after allo-SCT. Over this time period, there were extremely limited numbers of donor and/or recipient ILC2s in the LP of recipient mice that received irradiation and bone marrow without donor T cells, with a decrease in ILC2s in the LP at day 28 compared to day 1 (Figure 1D). By comparison, there were approximately 20-fold greater numbers of donor and/or host ILC2s in the lung on day 28 post-transplantation (Figure 1D). Thus, ILC2s in the GI tract, but not the lung, are sensitive to conditioning therapy and have extremely limited repopulation kinetics from donor bone marrow in the first 4 weeks post-transplant.

Co-infusion of ILC2s reduces aGvHD and increases recipient survival

To evaluate the function of *ex vivo* ILC2s in GvHD biology, murine ILC2s were expanded as previously described (24), and their phenotype verified by flow cytometry (Supplemental Figure 2, A, B, and C) and transcriptome analysis (Supplemental Figure 3, A and B; Supplemental Table 1). Next, we performed survival studies in a murine model of allo-SCT with co-infused ILC2s. Splenic donor C57BL/6 (B6) T cells with a 1:1 ratio of ILC2 (BM, T cells + WT ILC2) or without ILC2s (BM + T cells) were infused into lethally irradiated haploidentical recipients on day 0. Co-transplantation of a single dose of ILC2s significantly improved survival with 70% of recipients surviving 60 days post-transplant (Figure 2A). There was a significant reduction in both the clinical score and weight loss in mice receiving ILC2s (Figure 2, B and C). On day 20, there was a decrease in pathology score in the colon and spleen (Figure 2D), with a non-significant decrease in the ileum and no difference in the lung and liver (Supplemental Figure 4A). Using a 2:1 ratio of ILC2s to T cells, we repeated the survival studies in a B6 into BALB/c transplant model. There was a significant improvement in survival (Figure 2E) and clinical GvHD score (Supplemental Figure 4B).

Treatment of aGVHD with ILC2s

While the use of ILC2s to prevent aGvHD would be beneficial, clinically it would be more impactful to use these cells to treat active aGvHD. ILC2s were infused 7 days after transplant, a time when donor T cells have initiated inflammation in GvHD target organs. Infusion of a single dose of ILC2s significantly improved survival over untreated B6D2 recipients (Figure 2F). In addition, there was a significant reduction in clinical GvHD score in recipients of ILC2s on day 7 (Figure 2G). We repeated our day 7 treatment experiments in the complete MHC mismatch model and found significantly improved survival with ILC2 infusion (Supplemental Figure 4C). These findings indicate that a single infusion of ILC2s can significantly improve survival after the onset of GvHD.

ILC2s migrate to the GI tract and produce Th2 inducing cytokines

We were interested in determining if ILC2s migrated to GvHD target organs after allo-SCT. For this, we infused B6-GFP⁺ ILC2s into lethally irradiated B6D2 mice. While at day 12 post-BMT only limited GFP signal was found in the LP, we observed large clusters of GFP⁺ ILC2s in Peyer's patches (PPs) of the colon and small bowel (Figure 3A). Evaluation of GFP⁺ ILC2s by flow cytometry confirmed that IL-33 activated cells had a surface phenotype similar to *ex vivo* expanded ILC2s (Figure 3B). Greater than 90% of donor GFP⁺ ILCs in the GI tract expressed IL-13 (Figure 3D), while IL-5 expression varied with more than 40% of ILC2s in the GI tract expressing IL-5 (Figure 3C). These findings indicate that ILC2s migrate to the GI tract, maintain their phenotype, and produce ILC2 signature cytokines.

Infused ILC2s reduce GI tract T cell burden and limit Th1 and Th17 induction

We evaluated the effect of the infusion of ILC2s on the generation of pro-inflammatory donor T cells in GvHD target organs. Co-infusion of ILC2s reduced the accumulation of donor T cells in PP, the colon and small bowel as determined by GFP intensity (Figure 4A). In addition, there was significantly less GFP in tissue homogenates (Figure 4B). To confirm these findings, we analyzed T cell infiltration into the lungs, liver, spleen, MLN and colonic LP by flow cytometry (Figure 4C). Recipients of ILC2s had a reduction in donor lymphocytes in the LP of the colon (Figure 4D), with significant reduction in donor CD8⁺ T cells (Figure 4E). There were significant reductions in the total number of LP CD4⁺ and CD8⁺ T cells that expressed IFN- γ and CD4⁺ T cells that generated IL-17A (Figure 4F). Infusion of ILC2 cells did not lead to a significant difference in the quantity of IFN- γ producing donor CD4⁺ or CD8⁺ T cells in other GvHD target organs (Supplemental Figure 5, A and B).

The decrease in IFN- γ producing CD4⁺ and CD8⁺ T cells in the colon was not associated with an increase in T cells generating IL-10 or an increase in FoxP3 expressing regulatory T cells (T_{regs}) (Supplemental Figure 5, C and D). These data indicate that co-infusion of ILC2s is associated with a reduction in the number of pro-inflammatory donor T cells generating IFN- γ and/or IL-17A in the GI tract.

ILC2 suppression of aGvHD is independent of increased Th2 differentiation

Conceivably, ILC2s could induce donor Th2 polarization, which would mitigate aGvHD (25). To assess for Th2 cells after ILC2 infusion, we analyzed IL-4 production by donor T cells (Supplemental Figure 5E). We did not observe an increase in IL-4 producing donor T cells in recipients of ILC2s in the colon (Supplemental Figure 5F). To verify that the mechanisms behind ILC2 suppression of aGvHD are independent of the generation of Th2 cells, we performed survival studies using T cells from STAT6^{-/-} mice (26). Recipients of STAT6^{-/-} T cells alone developed aGvHD with all recipients dying before day 30; however, 80% of recipients given STAT6^{-/-} T cells with ILC2 cells survived long term with significantly reduced clinical scores by day 20 post-BMT (Supplemental Figure 5, G and H). The ability of ILC2 cells to suppress aGvHD does not require the generation of donor Th2 cells.

ILC2 infusion enhances LP myeloid cells

We observed an increase in donor myeloid cells in the lower GI tract in recipients of WT ILC2s (Supplemental Figure 6, A and B). We analyzed donor myeloid cells for expression of CD45, CD11b, Gr-1, Ly-6C and Ly-6G, a phenotype that is consistent with myeloid-derived suppressor cells (MDSC), which have been shown to suppress aGvHD (Figure 5A)(12, 27). We found a significant increase in donor MDSCs in the LP of colon and small bowel in ILC2 treated recipient mice (Figure 5B). However, MDSCs were not increased in the lung or liver of recipients of ILC2 cells (Supplemental Figure 6C). To test whether the function of ILC2s was dependent on MDSCs, we depleted myeloid cells using an anti-Gr-1 mAb in transplanted mice receiving either BM and T cells with or without donor ILC2 cells. Depletion of Gr-1⁺ cells reduced the median survival of control BM and T cell recipients from 25 to 20 days (Figure 2A compared to Figure 5C). In contrast, depletion of Gr-1⁺ cells completely ablated the beneficial effect of ILC2s, with no difference being observed in survival between mice receiving donor T cells with anti-Gr-1 antibody, or donor ILC2s with T cells and anti Gr-1 antibody (Figure

5C). The ability of anti-Gr-1 mAb to abrogate the beneficial effects of donor ILC2 cells was dependent on the ability of ILC2 cells to prevent aGvHD as infusion of anti-Gr-1 mAb had no impact on the outcome of recipient mice receiving only donor bone marrow cells (Fig 5c). There was a similar loss of efficacy of ILC2 infusion after depletion of Ly-6C⁺ cells (Supplemental Figure 6D). Thus, the ability of ILC2s to prevent aGVHD is dependent on the presence of Gr-1⁺ cells.

IL-13 is required for ILC2 induced donor MDSC suppression of inflammatory T cells

Next, we performed survival studies to test the ability of IL-13^{-/-} ILC2s to suppress aGvHD(12). Compared to the infusion of BM + T cells alone, there was no significant improvement in median survival for mice receiving BM, T cells and IL-13^{-/-} ILC2s (Figure 6A). When compared to mice that received WT ILC2 treatment, only 25% of IL-13^{-/-} ILC2 recipients survived long term as compared to 75% survival when WT ILC2 are given (Figure 6A). To further investigate the role of ILC2 derived IL-13 suppression of aGvHD, we evaluated IFN- γ and IL-17A expression by donor CD4⁺ and CD8⁺ T cells in the colon. The decrease in T cells generating IFN- γ and/or IL-17A observed in mice receiving WT ILC2s was lost after the infusion of IL-13^{-/-} ILC2s (Figure 6B compared to Figure 4F). In addition, IL-13^{-/-} ILC2s failed to enhance donor MDSCs in GvHD target organs (Figure 6C and Supplemental Figure 6E). These data indicate a critical role for IL-13 production by ILC2 cells to suppress donor T cell production of the pro-inflammatory cytokines IFN- γ and IL-17A.

To evaluate how ILC2 cells impact the presence of MDSCs, we co-cultured expanded ILC2s with BM derived MDSCs (BM MDSC) and quantified MDSCs 72 hours later. BM MDSCs cultured with congenic ILC2s showed significantly enhanced survival over MDSCs cultured alone, MDSCs cultured in the presence of IL-13, and those cultured with IL-7 and IL-33 (Figure 6D). However, BM MDSCs co-cultured with WT ILC2s survived significantly better than those cultured with IL-13 deficient ILC2s. To evaluate if ILC2s and MDSCs require cell-to-cell contact, we cultured both populations in plates across a semi-permeable membrane. Enhanced survival of MDSCs by ILC2s was abrogated in the absence of cell-to-cell contact (Figure 6D).

ILC2 infusions affect lower GI tract permeability

Previous investigators have demonstrated a critical role for ILC2s in the maintenance of epithelial integrity(13). Six days after BMT, we found there was no difference in intestinal permeability when comparing

non-irradiated age matched mice to BMT recipients of BM only, BM and T cells or BM, + T cells + WT ILC2 (Figure 7A). On days 12 and 20 post-transplant, recipients of donor T cells had significantly enhanced epithelial permeability compared to healthy B6D2 mice as well as recipients of BM only (Figure 7, B and C). ILC2 treated mice had significantly improved epithelial integrity compared to control mice and showed no statistical difference compared to untreated B6D2 or recipients of bone marrow alone (Figure 7, B and C). ILC2 infusion is associated with improved intestinal barrier function in the setting of reduced lower GI tract aGvHD.

Activated ILC2s produce amphiregulin in response to IL-33 (Supplemental Table 1), which promotes wound healing and epithelial barrier repair (15). We evaluated the ability of amphiregulin deficient ILC2s (Areg^{-/-} ILC2) to protect against aGvHD mortality. Infusion of Areg^{-/-} ILC2 cells with donor T cells and BM led to diminished overall survival compared to recipients of WT ILC2 cells (Figure 7D and Figure 2A). However, the kinetics of the response differed from that found using IL-13^{-/-} ILC2 cells with mortality occurring later post-transplant (Figure 7D and Figure 6A). The infusion of Areg^{-/-} ILC2s failed to increase epithelial barrier integrity (Figure 7E). Thus, the generation of amphiregulin by ILC2 cells is critical to the maintenance of the epithelial barrier in the GI tract mediated in part by ILC2 cells.

ILC2 cells do not inhibit the Graft-versus-Leukemia (GvL) response

The anti-tumor response mounted by donor T cells is key to the clinical efficacy of allo-SCT. We tested the GvL response by giving allogeneic bone marrow transplant recipients, GFP-expressing blast crisis chronic myelogenous leukemia (BC-CML) tumor cells (28). Recipients of donor T cells had significantly fewer tumor cells in the spleen compared to mice receiving TCD bone marrow alone and this effect was independent of the infusion of donor ILC2 cells (Figure 8A).

Comparison of ILC2 to other cell therapy treatments for GvHD

Multiple cell populations have been generated in an attempt to mitigate the incidence and/or severity of aGvHD. Two populations that have recently been evaluated by our group are natural T_{regs} and IL-13 MDSCs (12, 29). To compare the function of ILC2 cells with these other immunosuppressive cell populations, we infused T_{regs} or ILC2 cells with donor T cells, TCD bone marrow and luciferase expressing P815 (luc-P815) tumor cells. Mice that received BM and luc-P815 without T cells showed extensive tumor growth in the spleen, MLN and

inguinal lymph nodes. Mice that received T cells efficiently eliminated tumor cells when ILC2 cells were given (Figure 8A). In agreement with a previous report (30), infusion of 1:1 ratio of T_{regs} to splenic T cells led to suppression of aGvHD but with the loss of the graft-versus-tumor effect critical for allo-SCT (Figure 8B). When we evaluated a lower dose of T_{regs}, (1:4 ratio) we found that the GvL response was preserved but that the majority of mice succumbed to GvHD (Fig 8B). These findings indicate that the survival of B6D2 recipient mice transplanted with B6 donor T cells and P815 tumor cells is significantly improved with co-administration of ILC2 cells compared to a 1:1 or 1:4 ratio of T_{con}:T_{reg} cells (Figure 8C).

When given as treatment, MDSCs fail to suppress aGvHD (27). ILC2s and MDSCs were given on day 7 following allo-SCT in mice that received BM and splenic T cells. When used therapeutically, MDSCs did not improve survival or clinical score compared to mice receiving donor T cells alone (Figure 8, D and E). However, there was a significant improvement in the survival of mice receiving donor ILC2 cells with a significant reduction in clinical score (Figure 8, D and E). Thus, ILC2 cells *in vivo* do not inhibit the GvL response found after the infusion of T_{reg} cells and have significantly improved function to treat aGvHD compared to MDSCs.

Human ILC2 Cell expansion

While these findings indicate a critical role for the administration of donor ILC2 cells to control lower GI tract GvHD, for clinical translation, we needed to expand human ILC2 (hILC2) cells *ex vivo*. We isolated CD34⁺ cord blood cells and cultured these cells with IL-33 and IL-2 with/without IL-25. Culture with IL-2/IL-33 alone led to 2.5-3 log expansion of CRTH2⁺, CD161⁺ lineage negative hILC2 cells that peaked on day 18 (Figure 8F, Supplemental Figure 6F). The addition of IL-25 allowed for the persistent expansion of hILC2 cells through day 21. Thus, human ILC2 cells can be expanded *ex vivo* from CD34⁺ cord blood progenitors.

Discussion

Steroid refractory aGvHD of the lower GI tract is the most common cause of morbidity and mortality in patients that develop aGvHD after stem cell transplantation. Here, we demonstrate that the administration of donor ILC2s at the time of allo-SCT and up to one week after transplant significantly improved survival and clinical GvHD scores. ILC2s did not abrogate the anti-tumor GvL response, which may not be surprising given

their primary function in the lower GI tract, which is not the site of tumor growth in our models or for patients undergoing allo-SCT. Thus, ILC2 infusions represent a novel approach to therapy for lower GI tract GvHD.

One complication of allo-SCT is the incomplete reconstitution of immune cells from donor bone marrow. Some cell populations, such as B-1 lymphocytes, are generated preferentially from fetal liver and/or bone marrow and not adult bone marrow cells(31). In contrast, ILC2s in the GI tract, which have been shown to derive from adult bone marrow precursors, have extremely slow reconstitution kinetics after bone marrow transplantation. We find significantly decreased numbers of both donor and host ILC2s with a greater decrease at day 28 compared to day 1 after allo-SCT. These findings support clinical data regarding the paucity of circulating ILC2s 12 weeks after allogeneic stem cell transplantation (32) . There are at least two potential hypotheses for the impaired recovery of ILC2 cells from donor bone marrow after allogeneic bone marrow transplantation. First, despite their generation from bone marrow cells during development, ILC2 precursor cells may not be effectively generated from adult bone marrow precursor cells. In support of this hypothesis, a recent publication demonstrated poor recovery in patients with the common γ -chain deficiency or JAK3 mutation of ILC cells, other than ILC1 cells, after stem cell transplantation. Interestingly, the impaired recovery of ILCs after stem cell transplantation was much greater in patients receiving non-myeloablative conditioning therapy. The impaired recovery of ILC cells after transplant was not associated with an increased risk of infectious diseases suggesting that ILC cells may not be critical in the presence of T and B cells for the control of infectious pathogens (33). Our findings suggest that ILC cells may only be critical in specific uncommon settings such as assisting in the control of the persistent inflammation in the GI tract during severe aGvHD. Thus, a substantial number of patients after allogeneic SCT may function normally in the absence of a sufficient number of ILC2 cells. A second hypothesis is that ILC2 precursor cells are present in the donor bone marrow population but that cellular plasticity and the significant pro-inflammatory environment after allogeneic bone marrow transplantation in the lower GI tract converts these ILC2 precursor cells to ILC1 and/or ILC3 cells (34-36). Both of these mechanisms could contribute to the poor recovery of ILC2 cells in mice after allogeneic BMT.

ILC2s have been found to impact the presence of alternatively activated macrophages (37). We are the first to demonstrate a role for ILC2s in the presence of MDSCs, which required ILC2 production of IL-13. Previous studies have shown that IL-13 is capable of activating MDSCs through activation of STAT1 and STAT6, this leads to upregulation of arginase and INOS, which are critical to the immunosuppressive function of MDSCs (38,

39). Stromal cell production of IL-13 has been demonstrated to be important in this process(40). Here we demonstrate that ILC2s can maintain the viability of MDSCs *in vitro*. This process requires IL-13 generated by ILC2s and cell-to-cell contact between MDSCs and ILC2s. How this functions *in vivo* is a current focus.

IL-33, which is critical for the generation of ILC2 cells, is a ligand for the IL-33 receptor also termed ST2. Previous work evaluating biomarkers as predictors of outcome for patients undergoing allogeneic stem cell transplantation has found that elevation of ST2 was associated with a lack of therapeutic response for GVHD on day 28 post-transplant and increased mortality six months after transplantation (41). In those evaluations, ST2 levels were greater for patients undergoing myeloablative compared to reduced intensity conditioning regimens. These data were confirmed in a study of recipients of cord blood transplantation (42). We have shown that increased generation of IL-33 post-transplantation is associated with increased severity of GvHD in murine models (43), and our group and another have found that blocking the interaction of IL-33 with the IL-33 receptor (ST2) diminished aGvHD (44). We hypothesize that the increased levels of ST2 post-transplant are, in part, due to the loss of ILC2 cells in the GI tract that would respond to IL-33. Persistent inflammation in the GI tract would lead to increased production of soluble ST2, which would be greater after myeloablative conditioning therapy due to the greater loss of ILC2 cells from the increased dose of chemotherapy or irradiation. Our data would suggest that infusion of donor ILC2 cells, by markedly decreasing the accumulation of IFN- γ and IL-17 producing T cells in the GI tract, diminishes this pro-inflammatory environment. We hypothesize that ST2 levels would significantly decrease after ILC2 infusion, which is currently being evaluated.

AREG is a member of the epithelial growth factor receptor family and plays a critical role in wound healing after injury(45). Basophils, mast cells, CD4⁺ T cells, eosinophils, dendritic cells in addition to ILC2 cells can generate AREG. In some specific instances, FoxP3⁺ T_{regs} that express the IL-33 receptor (ST2) can generate AREG. Our data is consistent with a critical role for the production of AREG by ILC2 cells in their function to promote lower GI tract barrier function. However, we did not determine the relative contribution of ILC2 cells to the generation of AREG in the lower GI tract post-transplant. Future work is required to determine if AREG production by these other immune cells (and by non-immune cells) is critical to promoting GI tract epithelial integrity after the development of acute GvHD.

There are several additional questions that remain to be addressed from our findings. It is not clear the mechanism for the tissue-specific effects of conditioning therapy on ILC2 cells found in the lung compared to

those in the GI tract. This could be related to differences in the microbiota at each site. Alterations in the microbiome after conditioning therapy could activate ILC2 cells in the GI tract leading to enhanced depletion of cycling ILC2 cells after conditioning therapy. Furthermore, there are clear differences in the supporting immune and non-immune cells at each site that also could play a role in the cell cycle status of ILC2 cells in the lung compared to the GI tract. Finally, how ILC2 and ILC3 cells interact during aGvHD is not clear. As our hypothesis focused on the effects of conditioning therapy on regulatory immune populations in the GI tract, and since ILC3 cells in the GI tract are not depleted by conditioning therapy, we have not performed evaluations focusing on these cells.

In conclusion, we demonstrate that restoration of ILC2s can treat GI tract aGvHD, is dependent on the generation of IL-13 and amphiregulin by the ILC2s, and unlike T_{regs} does not impact the GvL response. These findings provide strong pre-clinical rationale for testing the infusion of ILC2 cells to treat patients with steroid non-responsive lower GI tract GvHD.

Materials and Methods

Mice

C57BL/6J (termed B6), BALB/cJ, B6.129S2 STAT6^{-/-} (termed STAT6^{-/-}) and C57BL/6J × DBA/2 F1 (termed B6D2) were purchased from The Jackson Laboratory. The generation of enhanced GFP-expressing C57BL/6 mice has been described previously (46). C57BL/6 IL-13 eGFP and mice C57BL/6 Areg^{-/-} mice have been described previously (24, 47). Donor and recipient mice were age-matched males between 8 and 16 wk. All experiments were performed in accordance with protocols approved by the University of North Carolina Institutional Animal Care and Use Committee.

Isolation and expansion of murine ILC2s and MDSCs

10 to 12 week old B6 mice were given 0.4 μg of recombinant mouse IL-17E/IL-25 (R&D Systems, Minneapolis, MN) by IP injection for 4 days. On day 5, cells were isolated from the MLN and peritoneum by peritoneal lavage using RPMI with 10% fetal bovine serum (Complete media). ILC2s were isolated by negative selection with a MACs column using biotinylated antibodies [(Anti-CD8 α (53-6.7), anti-CD4 (RM 4.4), anti-CD3 ϵ (145-2C11), anti- $\gamma\delta$ TCR (UC7-13DS), anti-TER119 (TER-119), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-NK1.1 (PK136)

(eBioscience, San Diego CA), anti-CD11c (N418), anti-CD19 (MB19-1), anti-Ly6G (1A8) and anti-CD49b (DX5) (BioLegend, San Diego CA] and Streptavidin Microbeads (Miltenyi 130-048-101). Cells were cultured for six days in complete media with rIL-7 and rIL-33 (10ng/ml) (PeproTech, Rocky Hill, NJ) changing the media every 2 days. ILC2 activation was evaluated using flow cytometry on day 6 by IC staining of IL-5 and IL-13. For BM-MDSCs, freshly isolated CD90 depleted BM was plated at 3.0×10^5 cells/ml and incubated 4 days with 250U/ml recombinant GM-CSF (315-03) and 100ng/ml G-CSF (250-05) (PeproTech, Rocky Hill, NJ). On day 4, 80ng/ml recombinant IL-13 (210-13) (PeproTech, Rocky Hill, NJ) was added to the cultures. After 4 days, CD11b⁺ cells were isolated by magnetic bead separation using the Miltenyi CD11b⁺ selection kit (130-049-601) (Miltenyi Biotec, Bergisch Gladbach, Germany). CD11b⁺ cells were analyzed for expression of GR-1; approximately 96% of the live cells were CD11b⁺/GR-1⁺. For ILC2/MDSC co-culture experiments, ILC2s and MDSCs were plated at a 1:1 ratio at a density of 2.0×10^5 cells/ml, in conventional 24 well plates or in 5.0 μ M filter transwell plates, with either 10ng/ml IL-7 and IL-33 or 80ng/ml IL-13 for 3 days before quantitation of live MDSCs by flow cytometry.

RNA seq evaluations

RNA was extracted from cultured ILC2s using the RNEasy mini kit (Qiagen) and mRNA sequencing libraries were prepared according to the TruSeq protocol (Illumina). For mRNA-sequencing, samples were processed as previously described by The Cancer Genome Atlas(48). Bases and QC assessment of sequencing were generated by CASAVA 1.8. QC-passed reads were aligned to the murine reference genome using MapSplice (49). Aligned reads were sorted and indexed using SAMtools, and then translated to transcriptome coordinates and filtered for large inserts and zero mapping quality using UBU v1.0 (<https://github.com/mozack/ubu> website). For the reference transcriptome, UCSC murine knownGenes (50) was used, with genes located on non-standard chromosomes removed. The abundance of transcripts was then estimated using an Expectation-Maximization algorithm implemented in the software package RSEM (51) v1.1.13. Estimated counts were normalized to the upper quartile(52) prior to comparison of expression across protocols. Normalized counts were converted to expression rank values for comparison with microarray data. RNA-Seq raw data files will be submitted to the NCBI GEO archive.

Comparison of ILC2 mRNA-seq with published microarray data

Murine ILC gene signatures and raw microarray data were extracted from Robinette et. al. and relevant NCBI GEO datasets(53). Microarray data were processed in R (<http://www.R-project.org>, R Development Core Team, Vienna, Austria) to determine transcript relative expression values using the Robust Multichip Average algorithm implemented in the *oligo* package(54). Transcript-level inference of relative expression was done by cross-referencing normalized probe signal values with the 'mogene10sttranscriptcluster' database (Affymetrix). Normalized signal values were converted to expression rank values for comparison with mRNA-seq data. Spearman rank correlations between gene expression values of ILC-associated genes for measured ILC2 (n = 6) and published ILC1 (n = 2, 12 comparisons), ILC2 (n = 2, 12 comparisons), and ILC3 (n = 8, 48 comparisons) were calculated and used as a statistic. Significance between sample groups was evaluated using the Kruskal-Wallis test. Mean rank expression values for each gene from each published group were plotted against mean rank expression values in the cultured ILC2s. Significance was tested using the 'cor.test' module in R (method = 'spearman').

Transplantation models

Total T cells were isolated using a Cedarlane T cell recovery column kit (Cedarlane Laboratories, Burlington, NC), followed by antibody depletion using PE-conjugated anti-mouse B220 (RA3-B62) and anti-mouse CD25 (3C7) Abs (eBioscience, San Diego, CA) and magnetic bead selection using anti-PE beads (130-0480801) Miltenyi Biotec, Cambridge, MA). T cell depleted (TCD) bone marrow was prepared as described previously (55). The day prior to transplantation, recipient mice received either 950 cGy (B6D2) or 800cGy (BALB/c) of total body irradiation. For the B6, IL-13^{-/-}, Areg^{-/-} and STAT6^{-/-} into B6D2 or B6 into BALB/c transplants, recipients were intravenously injected with either 4 × 10⁶ T cells and 3 × 10⁶ TCD BM cells, or 5 × 10⁵ total T cells and 5 × 10⁶ TCD BM cells, respectively. For ILC2 treatment groups, B6D2 or BALB/c recipients also received 4 × 10⁶ or 1 × 10⁶ CD90⁺ ILC2s respectively. Recipients were monitored twice a week and scored for clinical GvHD symptoms (designated as "Clinical Score") using a semi-quantitative scoring system as previously described (56, 57); animals were coded for these evaluations. The sample size was chosen for the effect size needed based on our previous experience with sample sizes needed to demonstrate a significant difference in GvHD scoring between control and treated groups. Investigators were blinded to the clinical score and the pathological

evaluations. For the scoring evaluation experiments, the inclusion of 9-12 recipients provided a power of 90% to detect a difference of 14 days in the median GvHD score of ≥ 5 with an alpha error of < 0.05 between control and treated groups. For all experiments, a control group received TCD bone marrow alone without additional T cells, which controlled for the presence of T cells in the marrow inoculum and potential infectious complications during aplasia. MDSC depletion was performed by IP injecting recipients twice weekly starting on day 7 with 200 μ g anti-GR-1 antibody (RB6-8C5) or anti-Ly-6G antibody (Mons-1;BioXCell, West Lebanon, NJ) until the termination of the experiment.

Evaluation of radiation and chemotherapy sensitivity

B6D2 mice received 950 cGy of total body irradiation or cyclophosphamide (200mg/kg) (Santa Cruz, sc-219703, Dallas TX.) by intraperitoneal injection. 24 hours or 28 days later cells were isolated from the LP, MLN and spleen for evaluation by flow cytometry. Quantitation of ILCs was performed using the absolute event count of live cells gated as lineage negative and CD90⁺. Expression of ICOS⁺ or ROR γ t⁺ were used to enumerate ILC2 or ILC3 cells, respectively.

Fluorescence stereo-microscopy and GFP quantification

Organs from anesthetized animals were imaged with a Zeiss SteReo Lumar V12 microscope with GFP bandpass filter (Carl Zeiss MicroImaging, Inc.) at room temperature. AxioVision (Carl Zeiss) software was used to determine GFP intensities. Control and treatment recipient organs were imaged using the identical magnification (mag) and exposure (exp) times on day +12: PP-exp 200 ms, mag 40x. Organs from recipient animals were homogenized and absolute GFP levels were determined by ELISA (Cell Biolabs, AKR-121). No GFP is found in mice receiving sham treatment. Detailed experimental procedures were conducted as described previously (55).

Cell isolation from GvHD target organs

Spleen, liver, lung, MLN and colon were excised and weighed. LP lymphocytes were isolated using the Miltenyi LP dissociation kit (130-097-410) as per the manufacturer's instructions. Livers and lungs were digested in a solution of 1 mg/ml collagenase A (Roche) and 75 U of DNase I (Sigma-Aldrich, St. Louis, MO) in RPMI 1640/5% newborn calf serum. Digested tissues were treated with ACK lysis buffer to remove RBCs and were passed

through 100- μ m pore size cell strainers. Leukocytes were collected at the interface of a 40%/80% Percoll (Sigma-Aldrich) gradient in RPMI 1640/5% newborn calf serum. The pelleted cells were washed in PBS/2% FBS. Spleens and MLN were teased apart, treated with ACK lysis buffer, and washed in PBS/2% FBS.

Graft-versus-leukemia analysis

BMT were performed as described and at the time of transplant B6D2 recipients were given 2.5×10^4 GFP⁺BC-CML or luciferase-expressing P815 (Luc-P815 tumor cells (ATCC TIB-64) (Lentivirus BLIV101PA-1, System Biosciences, Mountain View CA). Recipients of BC-CML tumor were euthanized 21 days post-BMT and splenocytes were evaluated for GFP expression by flow cytometry. Regulatory T cells were isolated from B6 splenocytes using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (130-091-041) (Miltenyi Biotec, Bergisch Gladbach, Germany). Recipients of Luc-P815 were monitored for survival and tumor infiltration via luciferase imaging on the IVIS Kinetic Optical System. Mice received 3 mg D-Luciferin (PerkinElmer) 10 minutes prior to imaging. These cell lines were validated by MHC typing, GFP or luciferase expression and growth parameters.

Flow cytometry analysis

ILC2s were evaluated by flow cytometry for lineage markers (88-7772), and CD90 (HLS51), ICOS (7E.17G9), CD127 (A7R34), T1/ST2 (RMST2-2), Sca-1 (D7), CD44 (IM7), CD25 (PC61.5), GITR (DTA-1) and intracellular staining for IL-4 (11B11), IL-13 (eBio13A), IL-5 (TRFK5), IFN- γ (XMG1.2), and IL-22 (1H8PWSR). T cells were evaluated by surface staining of H2K^d (34-1-2S), CD4 (GK1.5), CD8 (53-6.7) and intracellular staining for IFN- γ (XMG1.2), IL-17 (ebio17B7), IL-4 (11B11), IL-10 (JESS-16E3) and FoxP3 (FJK-16s). For analysis of MDSCs, donor cells were stained for the surface markers: CD45 (30-F11), CD11b (M1/70), CD11c (N418), GR-1 (RB6-8C5), Ly-6C (HK1.4) and Ly-6G (1A8) (eBioscience, San Diego CA). Sample acquisition was performed using a BD FACs CANTO or BD LSR Fortessa (BD Bioscience, East Rutherford NJ.).

FITC-Dextran permeability assay

Intestinal permeability was assessed by luminal enteral administration of FITC-dextran 4000 (Sigma), a non-metabolizable macromolecule that is used as a permeability probe. Mice were fasted for 4 hours prior to gavage with FITC-dextran (40mg/100g of body weight). After 4 hours, whole blood was collected by cardiac puncture

and FITC-dextran measurements were performed in triplicate by fluorimeter using a PerkinElmer “EnSpire” multimode reader. Dilutions of FITC-dextran in PBS were used as a standard curve and absorption of 50 μ l of serum or standard was measured at 488nm.

Expansion of human ILC2s

Isolated CD34⁺ cells from frozen umbilical cord blood cells (UCB) (NHLBI Umbilical Cord Blood Unit) were expanded with (50 ng/ml) IL-6, Flt3L, SCF, TPO (PeproTech, Rocky Hill, NJ) and 750 nM SR1 (Sigma-Aldrich, St. Louis, MO) for 15 days. Cells were then cultured in 24 well plates with 0.5×10^6 /ml with 100U/ml of IL-2, 50ng/ml of IL-25 and 50ng/ml of IL-33 (PeproTech, Rocky Hill, NJ), with RPMI containing 10% human Ab serum (Valley Biomedical, Winchester, VA) and 1% penicillin and streptomycin (Thermo Fisher Scientific, Waltham, MA). Cytokines were replenished every three days. On days 5, 10, 15, 20, and 25, cells were harvested and flow cytometry was performed. Human ILC2s were characterized as CRTH2⁺ (BM16) CD161⁺ (HP-3G10) and T cell (OKT3), B cell (HIB19), monocyte (P67.6) lineage-negative cells. All antibodies were purchased from eBioscience, San Diego, CA.

Statistical Evaluations

Survival differences were evaluated using a Mantel–Cox log-rank test. Survival curves were generated using the Kaplan–Meier method. Differences in GvHD clinical and pathology scores were determined using a two way ANOVA, with Bonferroni correction for repeated measures of multiple comparisons. Statistical analysis of RNA-seq data described above. Unless otherwise noted in the figure legends, all other continuous variables were compared using the two tailed Student’s *t* test with Welch’s correction. A *p* value of ≤ 0.05 was considered statistically significant.

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AUTHOR CONTRIBUTIONS

J.S.S. conceived of the project and directed the research; D.W.B. designed all murine experiments and evaluated the data; H.E.S. designed the experiments for the expansion of hILC2; B.G.V. and D.A.S performed analysis of epigenetic studies; and D.W.B. and J.S.S. wrote the manuscript.

1. Kersey JH. The role of allogeneic-cell transplantation in leukemia. *N Engl J Med.*363(22):2158-9.
2. Horwitz ME. Reduced intensity versus myeloablative allogeneic stem cell transplantation for the treatment of acute myeloid leukemia, myelodysplastic syndrome and acute lymphoid leukemia. *Curr Opin Oncol.*23(2):197-202.
3. Faber EA, Jr., and Vose JM. The role of hematopoietic stem cell transplant in follicular lymphoma. *J Natl Compr Canc Netw.*8(3):337-44; quiz 45.
4. Dignan FL, Clark A, Amrolia P, Cornish J, Jackson G, Mahendra P, Scarisbrick JJ, Taylor PC, Hadzic N, Shaw BE, et al. Diagnosis and management of acute graft-versus-host disease. *Br J Haematol.*158(1):30-45.
5. Schroeder MA, and DiPersio JF. Mouse models of graft-versus-host disease: advances and limitations. *Dis Model Mech.*4(3):318-33.
6. Bolanos-Meade J, Logan BR, Alousi AM, Antin JH, Barowski K, Carter SL, Goldstein SC, Hexner EO, Horowitz MM, Lee SJ, et al. Phase 3 clinical trial of steroids/mycophenolate mofetil vs steroids/placebo as therapy for acute GVHD: BMT CTN 0802. *Blood.* 2014;124(22):3221-7; quiz 335.
7. Xhaard A, Rocha V, Bueno B, de Latour RP, Lenglet J, Petropoulou A, Rodriguez-Otero P, Ribaud P, Porcher R, Socie G, et al. Steroid-refractory acute GVHD: lack of long-term improved survival using new generation anticytokine treatment. *Biol Blood Marrow Transplant.* 2012;18(3):406-13.
8. Gatti RA, Kersey JH, Yunis EJ, and Good RA. Graft-versus-host disease. *Prog Clin Pathol.* 1973;5(1-18).
9. Hulsdunker J, and Zeiser R. Insights into the pathogenesis of GvHD: what mice can teach us about man. *Tissue Antigens.* 2015;85(1):2-9.
10. Magenau J, Runaas L, and Reddy P. Advances in understanding the pathogenesis of graft-versus-host disease. *Br J Haematol.* 2016;173(2):190-205.
11. Coghill JM, Sarantopoulos S, Moran TP, Murphy WJ, Blazar BR, and Serody JS. Effector CD4+ T cells, the cytokines they generate, and GVHD: something old and something new. *Blood.*117(12):3268-76.
12. Highfill SL, Rodriguez PC, Zhou Q, Goetz CA, Koehn BH, Veenstra R, Taylor PA, Panoskaltzis-Mortari A, Serody JS, Munn DH, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. *Blood.* 2010;116(25):5738-47.
13. Klose CS, and Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nature immunology.* 2016;17(7):765-74.
14. Rivera A, Siracusa MC, Yap GS, and Gause WC. Innate cell communication kick-starts pathogen-specific immunity. *Nature immunology.* 2016;17(4):356-63.
15. Walker JA, Barlow JL, and McKenzie AN. Innate lymphoid cells--how did we miss them? *Nature reviews Immunology.* 2013;13(2):75-87.
16. Anthony RM, Rutitzky LI, Urban JF, Jr., Stadecker MJ, and Gause WC. Protective immune mechanisms in helminth infection. *Nature reviews Immunology.* 2007;7(12):975-87.
17. Kim HY, DeKruyff RH, and Umetsu DT. The many paths to asthma: phenotype shaped by innate and adaptive immunity. *Nature immunology.* 2010;11(7):577-84.
18. Palm NW, Rosenstein RK, and Medzhitov R. Allergic host defences. *Nature.* 2012;484(7395):465-72.
19. Pulendran B, and Artis D. New paradigms in type 2 immunity. *Science.* 2012;337(6093):431-5.
20. Saenz SA, Siracusa MC, Monticelli LA, Ziegler CG, Kim BS, Brestoff JR, Peterson LW, Wherry EJ, Goldrath AW, Bhandoola A, et al. IL-25 simultaneously elicits distinct populations of innate lymphoid cells and multipotent progenitor type 2 (MPPtype2) cells. *The Journal of experimental medicine.* 2013;210(9):1823-37.
21. Hanash AM, Dudakov JA, Hua G, O'Connor MH, Young LF, Singer NV, West ML, Jenq RR, Holland AM, Kappel LW, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity.* 2012;37(2):339-50.
22. Munneke JM, Bjorklund AT, Mjosberg JM, Garming-Legert K, Bernink JH, Blom B, Huisman C, van Oers MH, Spits H, Malmberg KJ, et al. Activated innate lymphoid cells are associated with a reduced susceptibility to graft-versus-host disease. *Blood.* 2014;124(5):812-21.
23. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, Angelosanto JM, Laidlaw BJ, Yang CY, Sathaliyawala T, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nature immunology.* 2011;12(11):1045-54.

24. Neill DR, Wong SH, Bellosi A, Flynn RJ, Daly M, Langford TK, Bucks C, Kane CM, Fallon PG, Pannell R, et al. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature*. 2010;464(7293):1367-70.
25. Krenger W, Snyder KM, Byon JC, Falzarano G, and Ferrara JL. Polarized type 2 alloreactive CD4+ and CD8+ donor T cells fail to induce experimental acute graft-versus-host disease. *Journal of immunology*. 1995;155(2):585-93.
26. Zhang Y, Zhang Y, Gu W, and Sun B. TH1/TH2 cell differentiation and molecular signals. *Adv Exp Med Biol*. 2014;841(15-44).
27. Messmann JJ, Reisser T, Leithauser F, Lutz MB, Debatin KM, and Strauss G. In vitro-generated MDSCs prevent murine GVHD by inducing type 2 T cells without disabling antitumor cytotoxicity. *Blood*. 2015;126(9):1138-48.
28. Matte-Martone C, Venkatesan S, Tan HS, Athanasiadis I, Chang J, Pavisic J, and Shlomchik WD. Graft-versus-leukemia (GVL) against mouse blast-crisis chronic myelogenous leukemia (BC-CML) and chronic-phase chronic myelogenous leukemia (CP-CML): shared mechanisms of T cell killing, but programmed death ligands render CP-CML and not BC-CML GVL resistant. *Journal of immunology*. 2011;187(4):1653-63.
29. Coghill JM, Fowler KA, West ML, Fulton LM, van Deventer H, McKinnon KP, Vincent BG, Lin K, Panoskaltsis-Mortari A, Cook DN, et al. CC chemokine receptor 8 potentiates donor Treg survival and is critical for the prevention of murine graft-versus-host disease. *Blood*. 2013;122(5):825-36.
30. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, Salomon BL, and Cohen JL. Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. *J Clin Invest*. 2003;112(11):1688-96.
31. Esplin BL, Welner RS, Zhang Q, Borghesi LA, and Kincade PW. A differentiation pathway for B1 cells in adult bone marrow. *Proc Natl Acad Sci U S A*. 2009;106(14):5773-8.
32. Mjosberg JM, Trifari S, Crellin NK, Peters CP, van Drunen CM, Piet B, Fokkens WJ, Cupedo T, and Spits H. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CCR2 and CD161. *Nature immunology*. 2011;12(11):1055-62.
33. Vely F, Barlogis V, Vallentin B, Neven B, Piperoglou C, Perchet T, Petit M, Yessaad N, Touzot F, Bruneau J, et al. Evidence of innate lymphoid cell redundancy in humans. *Nat Immunol*. 2016.
34. Bjorklund AK, Forkel M, Picelli S, Konya V, Theorell J, Friberg D, Sandberg R, and Mjosberg J. The heterogeneity of human CD127(+) innate lymphoid cells revealed by single-cell RNA sequencing. *Nat Immunol*. 2016;17(4):451-60.
35. Lim AI, Menegatti S, Bustamante J, Le Bourhis L, Allez M, Rogge L, Casanova JL, Yssel H, and Di Santo JP. IL-12 drives functional plasticity of human group 2 innate lymphoid cells. *J Exp Med*. 2016;213(4):569-83.
36. Ohne Y, Silver JS, Thompson-Snipes L, Collet MA, Blanck JP, Cantarel BL, Copenhaver AM, Humbles AA, and Liu YJ. IL-1 is a critical regulator of group 2 innate lymphoid cell function and plasticity. *Nat Immunol*. 2016;17(6):646-55.
37. Bouchery T, Kyle R, Camberis M, Shepherd A, Filbey K, Smith A, Harvie M, Painter G, Johnston K, Ferguson P, et al. ILC2s and T cells cooperate to ensure maintenance of M2 macrophages for lung immunity against hookworms. *Nat Commun*. 2015;6(6970).
38. Hix LM, Karavitis J, Khan MW, Shi YH, Khazaie K, and Zhang M. Tumor STAT1 transcription factor activity enhances breast tumor growth and immune suppression mediated by myeloid-derived suppressor cells. *J Biol Chem*. 2013;288(17):11676-88.
39. Gibrilovich DI, Ostrand-Rosenberg S, and Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol*. 2012;12(4):253-68.
40. Condamine T, and Gibrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. *Trends Immunol*. 2011;32(1):19-25.
41. Vander Lugt MT, Braun TM, Hanash S, Ritz J, Ho VT, Antin JH, Zhang Q, Wong CH, Wang H, Chin A, et al. ST2 as a marker for risk of therapy-resistant graft-versus-host disease and death. *N Engl J Med*. 2013;369(6):529-39.
42. Ponce DM, Hilden P, Mumaw C, Devlin SM, Lubin M, Giral S, Goldberg JD, Hanash A, Hsu K, Jenq R, et al. High day 28 ST2 levels predict for acute graft-versus-host disease and transplant-related mortality after cord blood transplantation. *Blood*. 2015;125(1):199-205.
43. Reichenbach DK, Schwarze V, Matta BM, Tkachev V, Lieberknecht E, Liu Q, Koehn BH, Pfeifer D, Taylor PA, Prinz G, et al. The IL-33/ST2 axis augments effector T-cell responses during acute GVHD. *Blood*. 2015;125(20):3183-92.

44. Zhang J, Ramadan AM, Griesenauer B, Li W, Turner MJ, Liu C, Kapur R, Hanenberg H, Blazar BR, Tawara I, et al. ST2 blockade reduces sST2-producing T cells while maintaining protective mST2-expressing T cells during graft-versus-host disease. *Sci Transl Med*. 2015;7(308):308ra160.
45. Zaiss DM, Gause WC, Osborne LC, and Artis D. Emerging functions of amphiregulin in orchestrating immunity, inflammation, and tissue repair. *Immunity*. 2015;42(2):216-26.
46. Panoskaltsis-Mortari A, Price A, Hermanson JR, Taras E, Lees C, Serody JS, and Blazar BR. In vivo imaging of graft-versus-host-disease in mice. *Blood*. 2004;103(9):3590-8.
47. Zaiss DM, Yang L, Shah PR, Kobie JJ, Urban JF, and Mosmann TR. Amphiregulin, a TH2 cytokine enhancing resistance to nematodes. *Science*. 2006;314(5806):1746.
48. Weinstein JN, Collisson EA, Mills GB, Shaw KR, Ozenberger BA, Ellrott K, Shmulevich I, Sander C, and Stuart JM. The Cancer Genome Atlas Pan-Cancer analysis project. *Nat Genet*. 2013;45(10):1113-20.
49. Wang K, Singh D, Zeng Z, Coleman SJ, Huang Y, Savich GL, He X, Mieczkowski P, Grimm SA, Perou CM, et al. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. *Nucleic Acids Res*. 2010;38(18):e178.
50. Karolchik D, Barber GP, Casper J, Clawson H, Cline MS, Diekhans M, Dreszer TR, Fujita PA, Guruvadoo L, Haeussler M, et al. The UCSC Genome Browser database: 2014 update. *Nucleic Acids Res*. 2014;42(Database issue):D764-70.
51. Li B, and Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*. 2011;12(323).
52. Bullard JH, Purdom E, Hansen KD, and Dudoit S. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. *BMC Bioinformatics*. 2010;11(94).
53. Robinette ML, Fuchs A, Cortez VS, Lee JS, Wang Y, Durum SK, Gilfillan S, and Colonna M. Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nature immunology*. 2015;16(3):306-17.
54. Carvalho BS, and Irizarry RA. A framework for oligonucleotide microarray preprocessing. *Bioinformatics*. 2010;26(19):2363-7.
55. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, West ML, Burgents JE, Blazar BR, and Serody JS. Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. *Blood*. 2010;115(23):4914-22.
56. Fulton LM, Carlson MJ, Coghill JM, Ott LE, West ML, Panoskaltsis-Mortari A, Littman DR, Blazar BR, and Serody JS. Attenuation of acute graft-versus-host disease in the absence of the transcription factor RORgammat. *Journal of immunology*. 2012;189(4):1765-72.
57. van Den Brink MR, Moore E, Horndasch KJ, Crawford JM, Hoffman J, Murphy GF, and Burakoff SJ. Fas-deficient lpr mice are more susceptible to graft-versus-host disease. *Journal of immunology*. 2000;164(1):469-80.

Figure 1

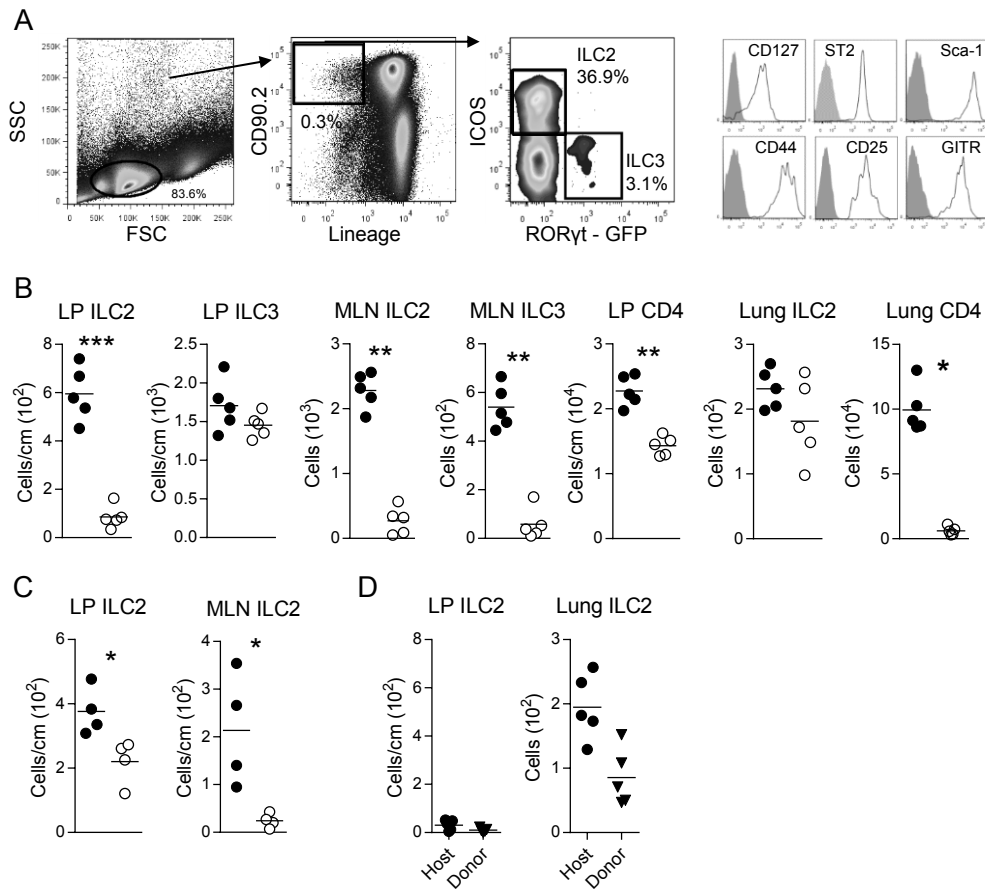


Figure 1. ILC2s are sensitive to radiation and chemotherapy conditioning. (A) Flow cytometry gating scheme used to define ILC2 and ILC3 cell subsets in mesenteric lymph node (MLN). (B) Quantitation by flow cytometry of innate lymphoid and CD4+ T cells in the lamina propria (LP), MLN, or lung in mice without irradiation (white bars) or 24 hours after receiving radiation (950cGy) (grey bars). These represent 3 independent experiments, n=5 for each group. (C) Quantitation of ILC2s in the LP and MLN in mice receiving no therapy (white bars) or 24 hours after receiving 200 mg/kg of cyclophosphamide (grey bars). These represent two independent experiments, n=4 for each. (D) Quantitation of host and donor derived ILC2s in the LP and lung 28 days post- BMT, mean \pm SEM. These represent two independent experiments, n=5. Statistical analysis by student's *t*-test with Welch's correction, ** p<0.01, * p<0.05.

Figure 2

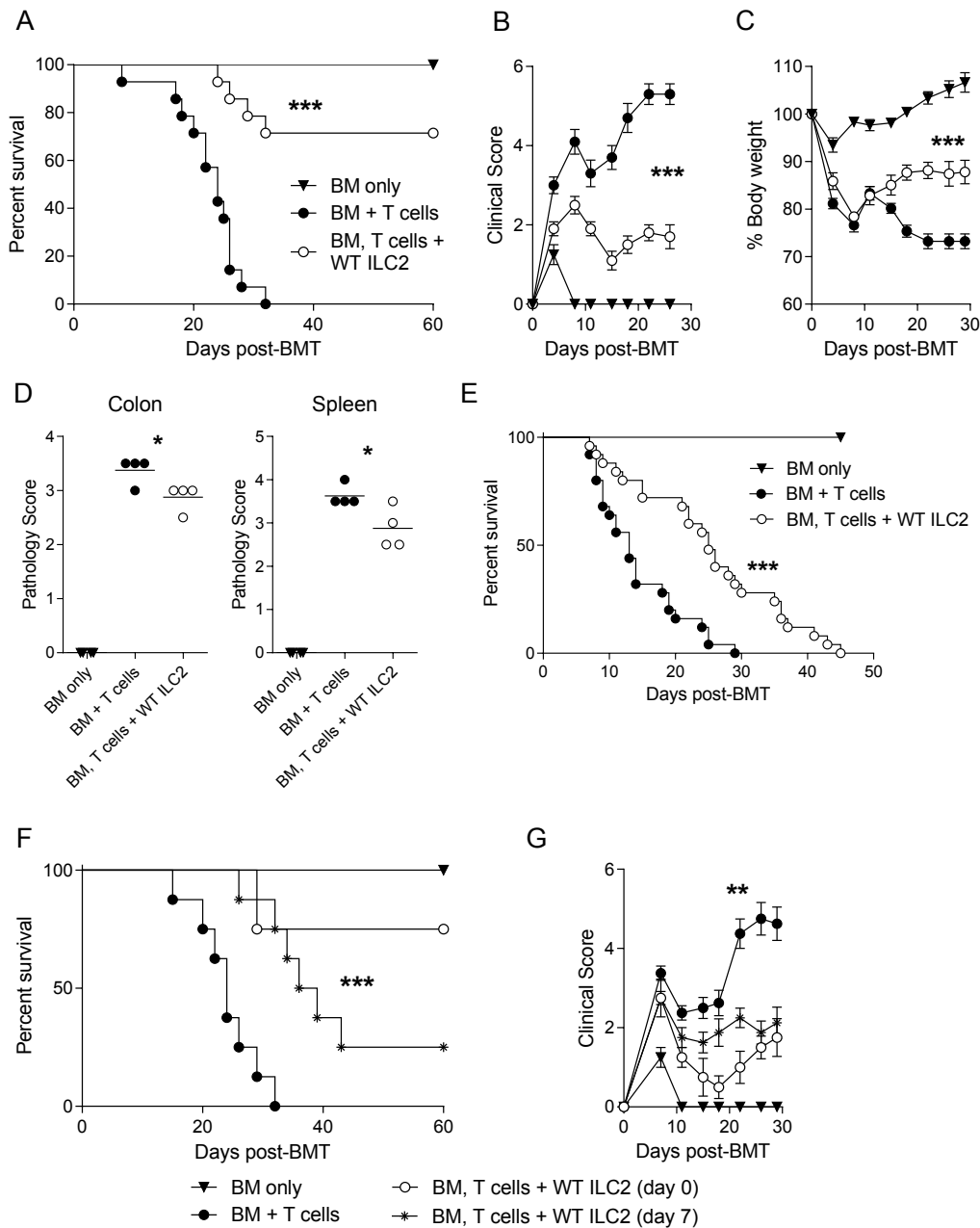


Figure 2. Co-transplantation of donor ILC2s reduces aGvHD incidence. Lethally irradiated B6D2 mice received TCD BM (BM only), BM plus total splenic T cells (BM + T cells) or BM plus T cells with activated ILC2s (BM, T cells + WT ILC2). **(A)** Kaplan-Meier plot of survival following allo-SCT, one representative of 3 experiments shown (n=8 each experiment), Log-rank (Mantel-cox) test, *** p<0.001. **(B)** Clinical score and **(C)** percentage of body weight change post-transplantation, analyzed by 2 way ANOVA, with Bonferroni correction for repeated measures of multiple comparisons, *** p<0.001. **(D)** Pathology scores of histological evaluations of GvHD target organs, 20 days post BMT (n=4 mice per group), student's *t*-test with Welch's correction, * p<0.05. **(E)** Kaplan-Meier plot for irradiated BALB/c mice that received TCD BM (BM only), BM plus splenic T cells (BM

+ T cells) or BM plus T cells with ILC2s (BM, T cells + WT ILC2). This represents 2 independent experiments, n=14. Log-rank (Mantel-cox) test, *** p<0.001. **(F)** Kaplan-Meier plot for B6D2 recipients that received BM alone (BM only), BM and splenic T cells (BM + T cells) and WT ILC2s either at the time of transplant [BM, T cells + WT ILC2 (Day 0)] or 7 days after transplant [BM, T cells + WT ILC2 (Day 7)], represents two experiments, n = 12 total in each group, Log-rank (Mantel-cox) test, *** p<0.001. **(G)** Clinical score of recipients from (Figure 2F), analyzed by 2-way ANOVA, with Bonferroni correction for repeated measures of multiple comparisons, ** p<0.01.

Figure 3

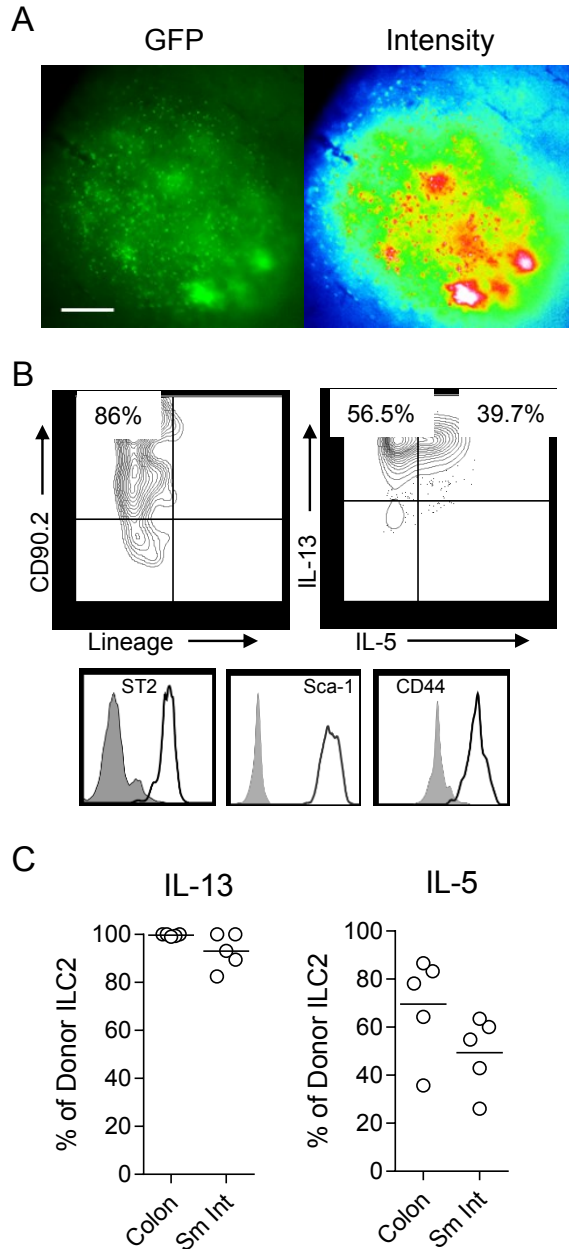


Figure 3. ILC2 evaluation in tissues post-transplant. (A) Fluorescence microscopy of B6-GFP ILC2s in Peyer's patches 12 days after BMT, GFP imaging (left) and signal intensity (right), 40x magnification, exposure 200 msec. These data represent 3 experiments, n=6 each. (B) Flow cytometry plots of B6-GFP ILC2 phenotype in the LP of BMT recipients 12 days after transplant, gated first as GFP⁺. (C) Percentage of B6-GFP ILC2s expressing IL-13 and IL-5, average ± SEM. These represent 3 independent experiments, n=5 each.

Figure 4

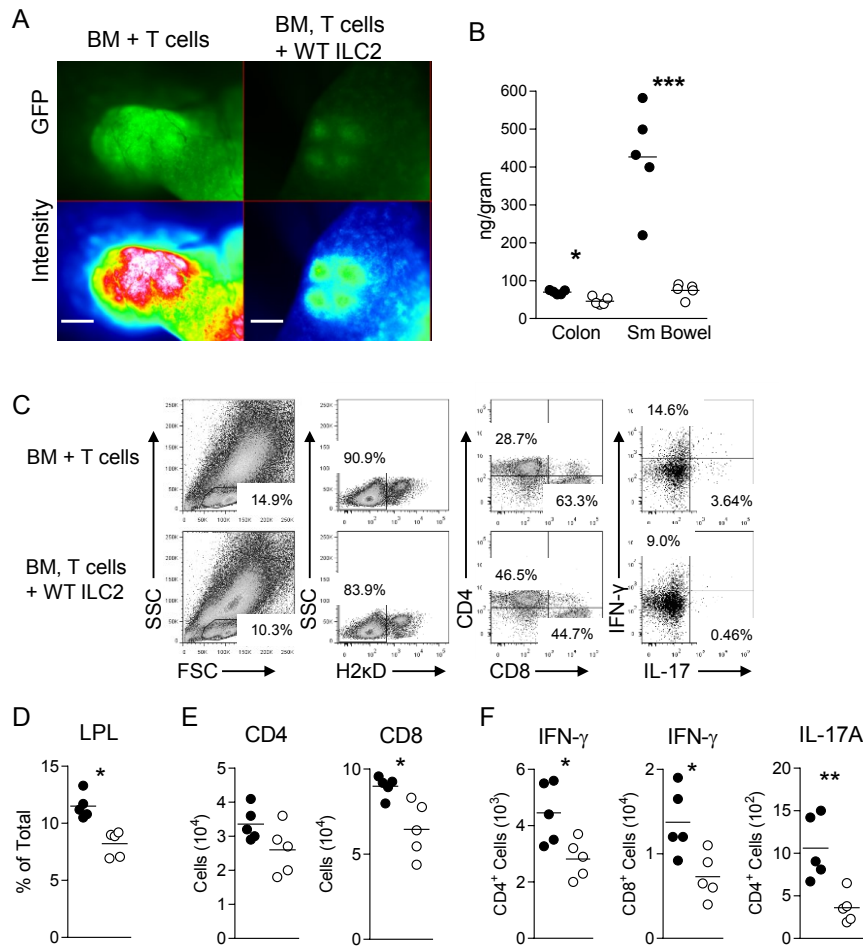


Figure 4. Co-transplantation of WT ILC2s reduces pro-inflammatory donor T cells numbers in the GI tract.

Donor T cells were evaluated in the GI tract 12 days post-transplant using GFP⁺ splenic T cells alone (BM + T cells) or GFP⁺ T cells and WT ILC2s (BM, T cells + WT ILC2). **(A)** Fluorescence microscopy of B6-GFP donor T cells in Peyer's patches, GFP imaging (top row) and signal intensity (bottom row), 40x magnification, exposure 200 msec. **(B)** ELISA quantification of GFP in tissue homogenates from BM + GFP⁺ T cells (black dot) and BM, GFP⁺ T cells + WT ILC2 (open circle) normalized to grams of tissue. Represents three experiments (n=5 each), student's *t*-test with Welch's correction, * p<0.05, **p<0.01. Donor T cells from BMT were evaluated by flow cytometry 12 days after BMT. **(C)** Density plots and gating scheme for the evaluation of donor T cells and intracellular cytokine expression from colon. **(D)** Percentage of donor T cells in the LP BM + T cells (black dots) and BM, T cells + WT ILC2 (open circles). **(E)** The total number of CD4⁺ and CD8⁺ donor T cells in the LP. **(F)** Total number of IFN- γ producing donor CD4⁺ and CD8⁺ T cells and the number of donor CD4⁺ T cells producing IL-17A in the LP. These data represent two independent experiments (n=5), student's *t*-test with Welch's correction, * p<0.05, **p<0.01.

Figure 5

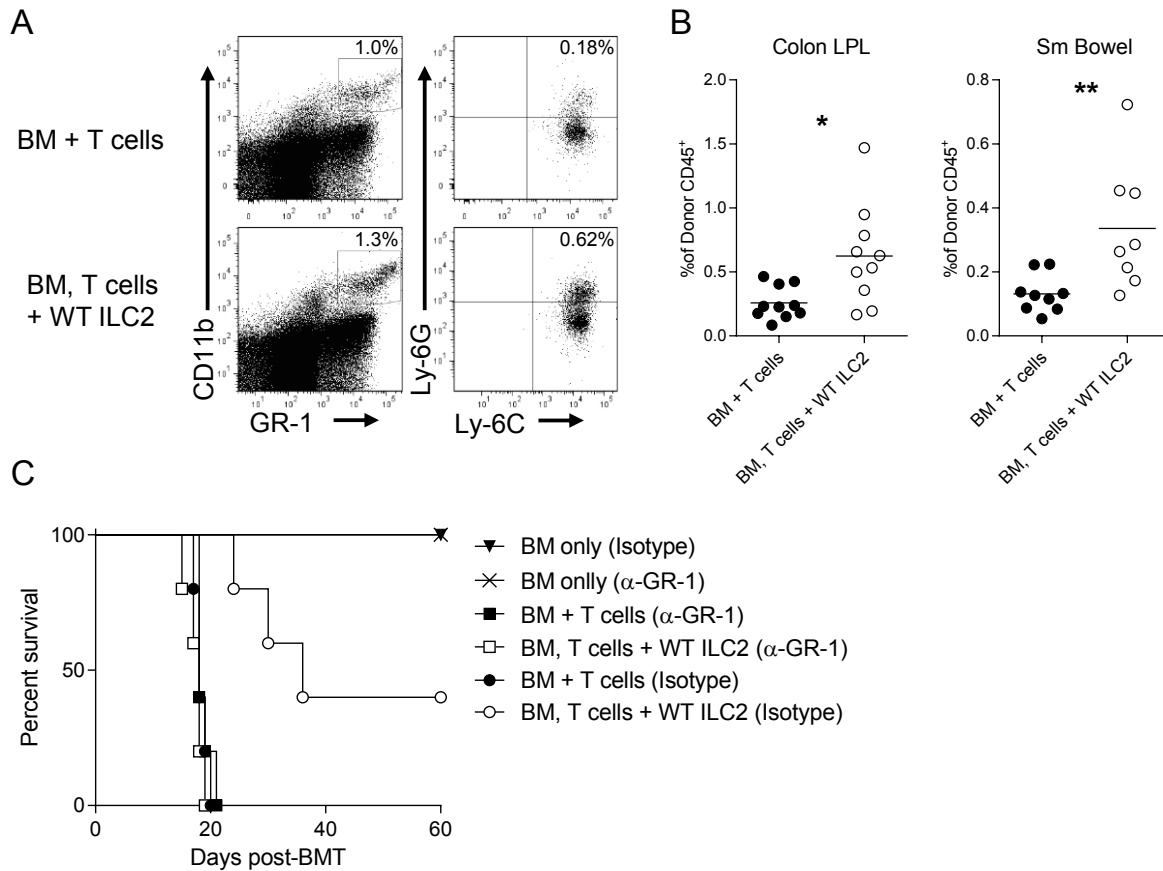


Figure 5. Co-transplantation of WT ILC2s increases MDSC numbers in the GI tract. (A) Representative density plots of CD11b⁺/GR-1⁺/Ly-6C⁺/Ly-6G⁺ MDSCs (gated on donor CD45⁺ cells) 12 days after BMT. (B) Frequencies of MDSCs as a percentage of CD45⁺ granulocytes in the colon and small bowel of BMT recipients 12 days post-transplant. These represent 2 independent experiments, bar graphs are average \pm SEM, student's *t*-test with Welch's correction, * *p* < 0.05, ** *p* < 0.01. (C) Kaplan-Meier plot showing results of Gr-1 depletion. Lethally irradiated B6D2 mice (950cGy) received 3.0×10^6 TCD BM (BM only), BM plus 4.0×10^6 total splenic T cells (BM + T cells) or BM plus T cells with 4.0×10^6 IL-33 activated ILC2s (BM, T cells + WT ILC2). One group additionally received 200 μ g anti-GR-1 (α -GR-1) with another group receiving isotype control antibody twice weekly beginning 7 days post-transplant. One representative of 2 independent experiments shown, *n* = 6 per group.

Figure 6

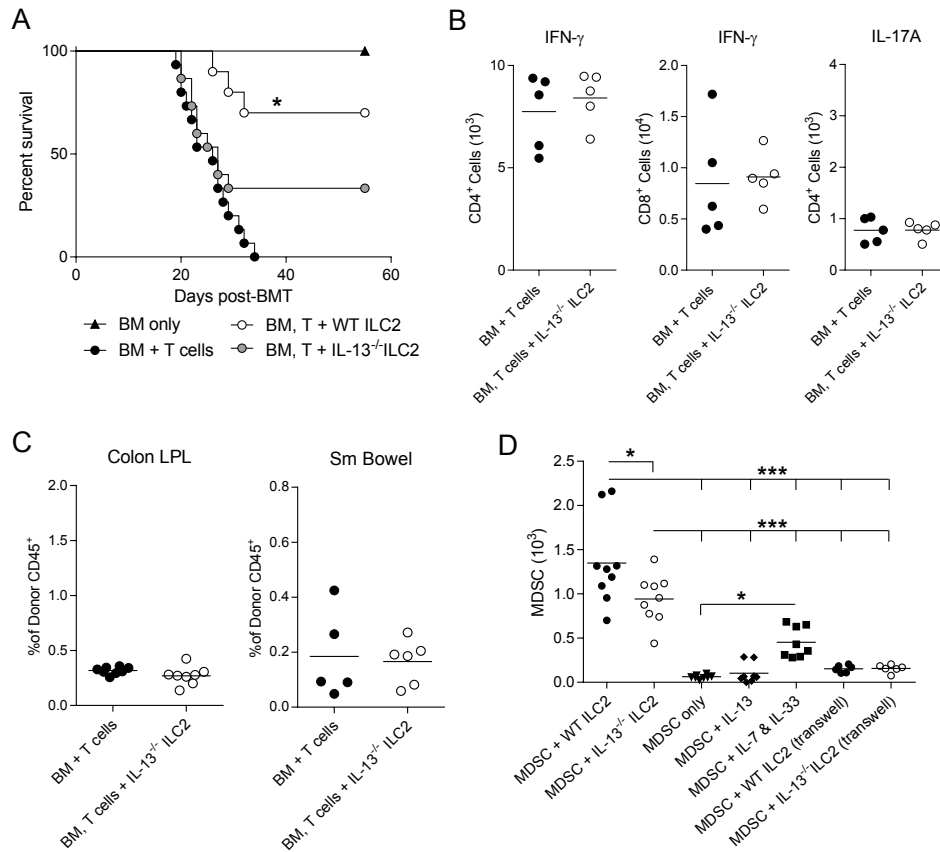


Figure 6. Reduced efficacy of ILC2s in the absence of IL-13. B6D2 recipients of B6 TCD BM (BM only), BM plus splenic T cells (BM + T cells) or BM plus T cells with cultured IL-13^{-/-} ILC2 (BM, T cell + IL-13^{-/-} ILC2) were evaluated for **(A)** Kaplan-Meier plot of survival following allo-SCT, one representative of 2 combined experiments shown (n=8 per group in each experiment). WT ILC2 group shown represents one experiments from figure 2A. Immune infiltrates were evaluated in GvHD target organs by flow cytometry 12 days after allo-SCT with or without IL-13^{-/-} ILC2. **(B)** Total number of IFN- γ producing donor CD4⁺ and CD8⁺ T cells and the number of donor CD4⁺ T cells producing IL-17A in the colon LP. These data represent two independent experiments, mean \pm SEM (n=5 each). **(C)** Frequencies of CD11b⁺/GR-1⁺/Ly-6C⁺/Ly-6G⁺ MDSCs (gated on donor CD45⁺ cells) MDSCs in the colon and small bowel of BMT recipients of IL-13^{-/-} ILC2s 12 days post-transplant. These represent 2 independent experiments, mean \pm SEM. **(D)** BM MDSC were co-cultured for 72 hours with WT or IL-13^{-/-} ILC2 (1:1), MDSC alone or with either IL-13 (80 ng/ml) or IL-7 & IL-33 (10 ng/ml each). BM MDSC were co-cultured WT or IL-13^{-/-} ILC2 (1:1) in transwell assays, alone, or in the presence of IL-13 only. These represent 2 independent experiments, mean \pm SEM, 1-way ANOVA with Bonferroni's correction for multiple comparisons, * p<0.05, ** p<0.01, *** p<0.001.

Figure 7

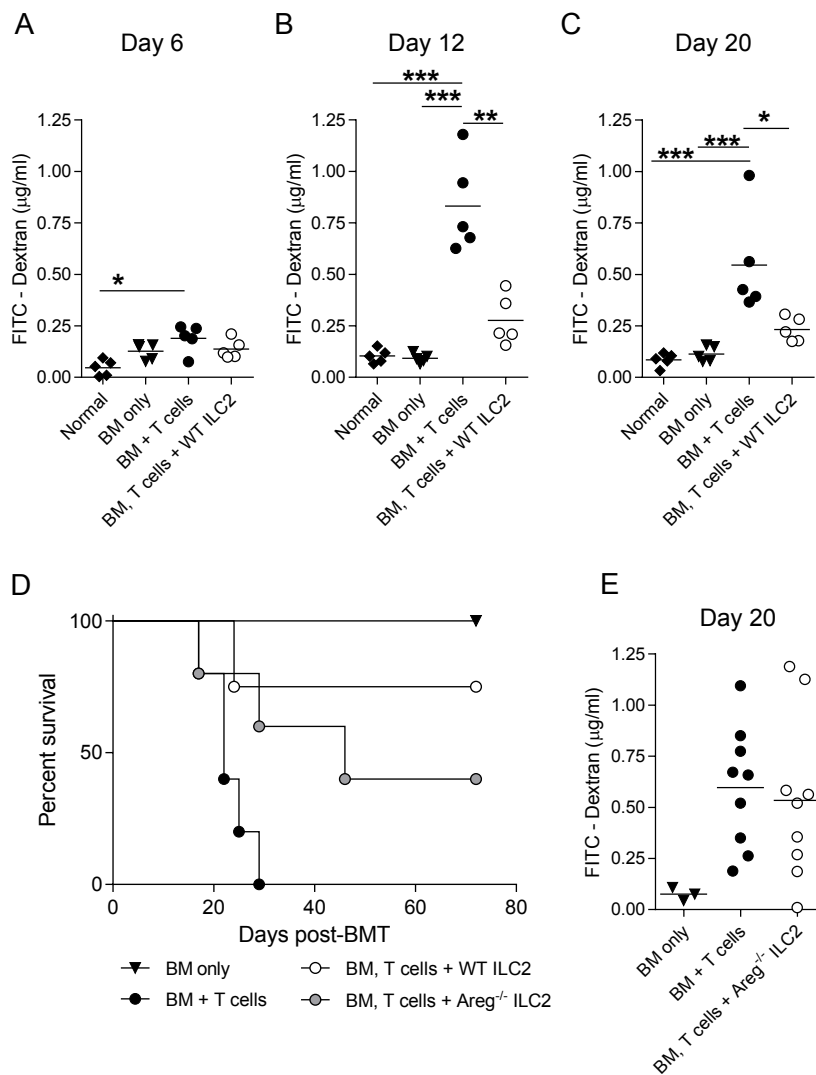


Figure 7. ILC2 treatment improves intestinal barrier function and does not abrogate the GvL response.

Quantification of FITC-dextran in the serum of BMT recipients (**A**) 6 days, (**B**) 12 days, and (**C**) 20 days after transplant. One representative of 2 independent experiments shown, mean \pm SEM (n=5 per group). Statistical analysis by 1-way ANOVA with Bonferroni's correction for multiple comparisons; * p<0.05, ** p<0.01, *** p<0.001. (**D**) Kaplan-Meier plot of B6D2 recipients of B6 TCD BM (BM only), BM plus splenic T cells (BM + T cells) or BM plus T cells with cultured Areg^{-/-} ILC2 (BM, T cell + Areg^{-/-} ILC2) were evaluated for survival following allo-SCT, one representative of 2 experiments shown (n=5 per group in each experiment). WT ILC2 group shown represents one experiments from figure 2A. (**E**) Quantification of FITC-dextran in the serum of BMT recipients 20 days after transplant. One combined representative of 2 independent experiments shown, mean \pm SEM (n=5 per group).

Figure 8

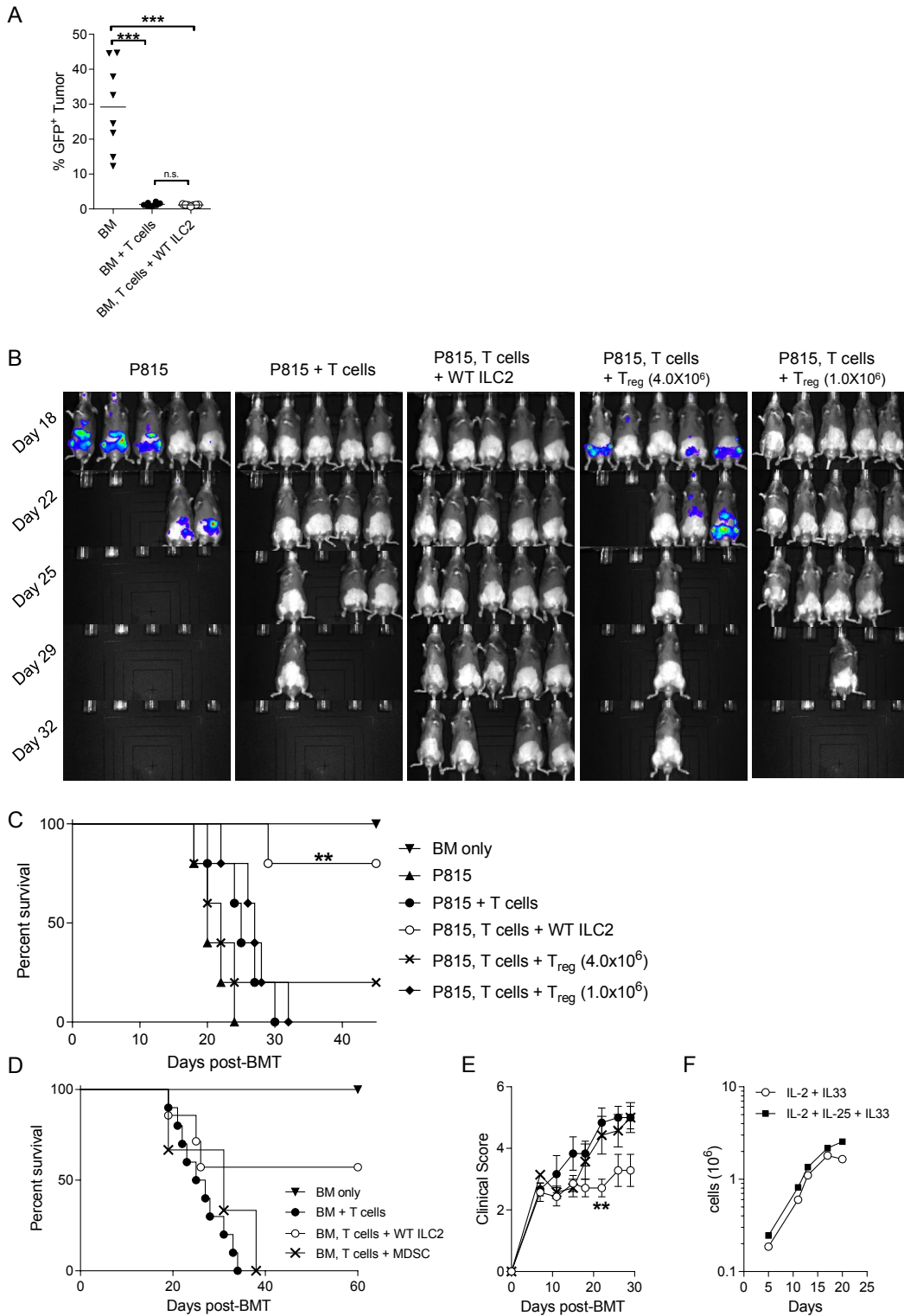


Figure 8. Comparison of ILC2s and other cellular therapies for GvHD. (A) Percentage of GFP⁺ BC-CML tumor in host spleen 21 days after BMT in the B6 into B6D2 model. Represents two independent experiments (n=8 each). Analysis by student's *t*-test with Welch's correction, *** p<0.001. (B) IVIS imaging of Luc-P815 tumor in B6D2 BMT recipients 15 days after transplant comparing recipients of BM and Luc-P815 cells (BM only), BM

with splenic T cells and Luc-P815 cells (BM + T cells), BM, splenic T cells, T_{regs} and Luc-P815 cells (BM, T cells + T_{reg}) and BM, splenic T cells, WT ILC2 and Luc-P815 cells (BM, T cells + WT ILC2); represents two independent experiments n = 5 in each group. **(B)** Kaplan-Meier plot comparing the survival of the allo-SCT recipient's described in (A). Log-rank (Mantel-cox) test, ** p<0.01. **(D)** Kaplan-Meier plot comparing the survival of recipients of allo-SCT (B6 into B6D2 model) that received BM alone (BM only), BM and splenic T cells (BM + T cells), and those that received BM and splenic T cells at the time of transplant with either WT ILC2 (BM, T cells + WT ILC2) or BM MDSC (BM, T cells + MDSC) 7 days after BMT. Represents 2 combined experiment, (n=10 or more per group). Log-rank (Mantel-cox) test. **(E)** Clinical scores of recipients from survival study in (g), ** p<0.01 by 2-way ANOVA, with Bonferroni correction for repeated measures of multiple comparisons. **(F)** Expansion of human ILC2s (hILC2) from SR-1-stimulated CD34⁺ cord blood cells in the indicated cytokines. hILC2s were enumerated as CRTH2⁺/CD161⁻/Lin⁻ cells (Supplemental Figure 6F) from days 3-21, n=3.