IL-33 Expands Suppressive CD11b+ Gr-1int and Regulatory T Cells, including ST2L + Foxp3+ Cells, and Mediates Regulatory T Cell-Dependent Promotion of Cardiac Allograft Survival

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*J Immunol* 2011; 187:4598-4610; Prepublished online 26 September 2011; doi: 10.4049/jimmunol.1100519
http://www.jimmunol.org/content/187/9/4598

**Supplementary Material** http://www.jimmunol.org/content/suppl/2011/09/27/jimmunol.1100519.DC1

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IL-33 Expands Suppressive CD11b+ Gr-1\textsuperscript{int} and Regulatory T Cells, including ST2L+ Foxp3\textsuperscript{+} Cells, and Mediates Regulatory T Cell-Dependent Promotion of Cardiac Allograft Survival


Interleukin-33 is an atypical IL-1 family member that facilitates type 2 responses typified by enhanced Th2 cell activity and the production of type 2-associated cytokines, particularly IL-4, IL-5, and IL-13 in vivo (1, 2). This contrasts to other IL-1 family cytokines, including IL-1β and IL-18, that promote type 1 responses mediated predominantly by Th1 cells and IFN-γ (3, 4). ST2 (also known as IL-1R-like-1), is a TLR/IL-1R superfamily member that, with the IL-1R accessory protein, constitutes the functional IL-33R (1, 5, 6). Like IL-33, ST2 is implicated in type 2 responses (1, 7). It is highly homologous with the IL-1R and exists in two forms, a soluble form (sST2), which acts as an IL-33 decoy receptor, and the signaling transmembrane form, ST2L (8).

ST2L is highly expressed on activated Th2 cells (9, 10) and mast cells (11). Functionally, IL-33 acts as a chemoattractant for Th2 cells (12) and drives their production of Th2-associated cytokines (1, 6, 13). IL-33 stimulates both human and mouse mast cells to produce IL-6, IL-8, and IL-13, especially with IgE cross-linking (14, 15).

In addition to its influence on mast cells and Th2 cells, IL-33 can promote Th2 responses by supporting the proinflammatory activities of other immune cells expressing ST2L, particularly eosinophils (16, 17) and basophils (18, 19). Recently, ST2L signaling has been found to be critical for expansion of newly identified IL-13-expressing innate immune cells, which are

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Received for publication February 23, 2011. Accepted for publication August 24, 2011.

This work was supported by National Institutes of Health Grants R01 AI60994 and P01 AI81678 (to A.W.T.), B.R.R. was supported by National Institutes of Health Training Grant T32 AI74490 and by an American Heart Association predoctoral fellowship (7070020). A.C. was supported by a Basic Science fellowship from the American Society of Transplantation and a Sunflowers for Holli fellowship from the American Liver Foundation. This work was also supported by a National Institutes of Health Pathway-to-Independence Career Development award (K99/R00 HL97155) and a Starzl Transplantation Institute Young Investigator grant (to H.R.T.).

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The online version of this article contains supplemental material.

Abbreviations used in this article: B6, C57BL/6; BM, bone marrow; DC, dendritic cell; FIB, B6 Foxp3-IREs-mRFP; Flt3L, flt3-like tyrosine kinase-3 ligand; int, intermediate; mDC, myeloid dendritic cell; MDSC, myeloid-derived suppressor cell; MST, mean survival time; nTreg, naturally occurring regulatory T cell; Qdot, quantum dot; sST2, soluble form of ST2; ST2L, signaling transmembrane form of ST2; Treg, regulatory T cell; WT, wild-type.

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instrumental in Th2-mediated responses to helminths (20, 21). Overall, studies on SI2-deficient animals and use of either ST2-specific Ab or sST2 to block IL-33 activity support a role for IL-33 in the facilitation of Th2 responses (2).

It is now evident that the immunological activities of IL-33 and ST2L are more diverse than proposed originally. Recently, functional analysis of IL-33-deficient mice has suggested that IL-33 amplifies both Th1 and Th2 responses, particularly by targeting innate immune cells in mucosal tissues (22). Moreover, IL-33 can augment inflammatory cytokine production, particularly TNF-α, IL-1β, and IL-6 by mouse macrophages (23). During sepsis, IL-33 administration does not facilitate a Th1-to-Th2 shift, but reduces systemic proinflammatory cytokines and targets ST2L-expressing neutrophils to facilitate their antibacterial responses (24). In addition, NK cells and NKT cells express ST2L and respond to IL-33 by producing IFN-γ (25). Furthermore, IL-33, highly expressed by epithelial and endothelial cells, may act as an “alarmin,” or endogenous danger signal that, during inflammation and tissue damage, is released to function as a general inflammatory mediator (22, 26). Thus, depending on the environment and cells targeted, IL-33 may act as a potent type 2 signal or generalized proinflammatory signal.

Notably, bone marrow (BM)-derived myeloid dendritic cells (mDCs) and endogenous CD11c+ dendritic cells (DCs) also express ST2L, especially when exposed to the antiproliferative/immunosuppressive agent rapamycin (27). IL-33 induces MAPK signaling in BM-derived mDCs (5) and facilitates their support of IL-4 and IL-13 production by Th cells in vitro (28). Mayuzumi et al. (29) reported recently that exogenous IL-33 promotes the generation of CD11c+CD11b+ mDCs in BM cell cultures. DCs from these IL-33-treated cultures were phenotypically and functionally immature and failed to respond to inflammatory stimuli, such as LPS (29). However, the impact of IL-33 on myeloid APC generation and function, especially in vivo, has not been well defined.

Although recognized as a potent facilitator of Th2-mediated inflammatory diseases, especially allergic hypersensitivity and arthritis (2, 30, 31), IL-33 also exhibits cardiovascular protective properties. Cardiac hypertrophy after pressure overload is worsened significantly when IL-33 activity is blocked or ST2 is absent (8). Likewise, systemic administration of IL-33 limits cardiovascular disease by inhibiting atherosclerosis development in apolipoprotein E−/− mice (32). Inhibition of atherosclerosis is mediated through the potent instigation of a Th1-to-Th2 switch (32). Recently, IL-33 delivery after experimental cardiac transplantation was shown to promote allograft survival, an effect presumed to result from a Th2 shift in immune reactivity (33). This interpretation would be consistent with past reports suggesting that reduction of Th1 responses by systemic administration of neutralizing IL-12 mAb prolongs allograft survival (34). However, there is also strong evidence that IL-4 and Th2 cytokines can promote graft dysfunction and rejection (35). Thus, the role of ST2 or IL-33 in immune responses to transplanted organs is unclear and warrants further examination.

We report the novel observation that IL-33, given to normal or cardiac-transplanted mice, while facilitating Th2 responses also expands suppressive CD4+ Foxp3+ regulatory T cells (Tregs), including a newly identified ST2L− subset. In addition, our data reveal the ability of IL-33 to modulate both CD11c+ DCs and particularly expand CD11b+ Gr-1− myeloid-derived suppressor cells (MDSCs). Specifically, IL-33 increases CD11b+ cells expressing intermediate (int) levels of Gr-1 (Gr-1int) that exhibit potent T cell suppressive capacity. Expansion of Tregs and MDSCs depends on recipient expression of SI2, but not host mast cells. Likewise, IL-33-mediated prolongation of graft survival requires recipient SI2 gene expression and Tregs, but not Gr-1− cells. As such, these findings establish a new immunomodulatory mechanism, beyond Th2 polarization, and involving Tregs, that underlies the immunosuppressive and heart graft-protective properties of IL-33.

Materials and Methods

Animals and cytokine administration

Male C57BL/6 (B6; H2Kb), BALB/c (H2Kq), and C3H/HeJ (C3H; H2Kc) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 Kitm1Cgn/Kitm1Cgn mice were kindly provided by Dr. Adriana T. Larregina (Department of Dermatology, University of Pittsburgh). BALB/c SI2−/− mice (7) were bred for experimental use at the University of Pittsburgh. B6 Foxp3-ires-mRFP (FIR) mice (36) were provided by Dr. Fadi Lakkis (Starzl Transplantation Institute and Department of Surgery, University of Pittsburgh). All mice were housed in the specific pathogen-free facility of the University of Pittsburgh School of Medicine with access to food and water ad libitum and used at 8–12 wk of age. Experiments were conducted under an institutional animal care and use committee-approved protocol and in accordance with National Institutes of Health guidelines.

Mice were injected i.p. for 10 consecutive days with recombinant mouse IL-33 (0.5 μg/d) or recombinant human fms-like tyrosine kinase-3 ligand (FLK; 10 μg/d) (37). Agen, Thousand Oaks, CA). Recombinant mouse IL-33 was produced as described (12). On day 11, positive selection of CD11c+ cells was performed on spleen cell preparations, as described (27). Splenic Ly6C+ cells or Ly6G+ cells were enriched as reported (38) by positive selection after staining with FITC-conjugated anti-Ly6C (clone R595) (Enzo Life Sciences, Plymouth Meeting, PA) was added on day 7 for 18 h plus 2 d, 75% of the culture supernatant was replaced with fresh cytokine-containing media. On day 4, nonadherent cells were discarded. Where indicated, 100 ng/ml TLR-grade LPS from Salmonella minnesota R595 (Enzo Life Sciences, Plymouth Meeting, PA) was added on day 7 for 18 h prior to cell harvesting. Nonadherent cells were then harvested on day 8 and assessed phenotypically by flow cytometry and functionally in MLR.

Flow cytometric analyses

Surface Ag expression by leukocytes was analyzed by flow cytometric analysis as described (5). Briefly, fluorophore-conjugated mAbs obtained from BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA), unless otherwise indicated, were used to stain splenocytes or BM-derived cells for CD3 (17A2), CD4 (L3T4), CD8 (53-6.7), CD11c (HL3), CD11b (M1/70), CD45 (30-F11), CD45R/B220 (RA3-6B2), CD86 (GL-1), F4/80 (BM8), Gr-1 (RB6-8C5), I-A/E (M5/114.15.2), Ly6G (1A8), Ly6C (H1K14), NK1.1 (PK136), or T1/ST2 (DJ8; MD Bioproducts, St. Paul, MN). After surface staining of freshly isolated splenocytes, fixation/permeabilization and intracellular staining with fluorophore-conjugated mAb to Foxp3 (FJK-16a) was also completed where indicated. Where designated, 4- to 5-h stimulation of splenocytes with PMA and ionomycin (Sigma) in the presence of GolgiPlug (BD Biosciences) was followed by surface and intracellular staining controls. Data were acquired with an LSR II or LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo 8.8.6 (Tree Star, Ashland, OR). Total splenocyte numbers were calculated by counting live cells, identified via trypan blue dye exclusion, and then multiplying the number of total live cells by the percentage of the indicated population falling in the appropriate gates, including live cell singlet and overall total live cell gates based off side scatter and forward scatter profiles.

Vascularized heart transplantation

Heterotopic (intra-abdominal) heart transplantation was performed with transfer of wild-type (WT) B6 hearts to WT or SI2−/− BALB/c mice, as described (39, 40). Briefly, hearts were transplanted into recipients through
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A

BALB/c St2

CD45

CD11b CD11c

PBS

IL-33

Flt3L

CD11b

Gr-1

CD11c

B

BALB/c St2

CD45

CD11b CD11c

CD11b

Gr-1

CD11c

C

D

% CD45^+ Cells

% CD45^+ CD11c^-

Total Splenocytes (x10^9)

Gr-1^hi

Gr-1^int

Gr-1^neg

CD86

I-A^b/I-E^d

CD11b^+ CD11c^-

CD11b^+ CD11c^+

CD11b^lo CD11c^+

CD11b^lo CD11c^-

% of Max.
anastomosis of the donor ascending aorta and pulmonary artery and recipient abdominal aorta and inferior vena cava. Recombinant mouse IL-33 (0.5 μg/d) in PBS or an equal volume of PBS alone was administered i.p. on days 0, 1, 3, 6, 10, 13, 15, and 17. All groups included four to eight mice. Where indicated, WT BALB/c recipients were administered anti-CD25 mAb (PC-61; BioXcell, West Lebanon, NH; 0.5 mg i.p. on day −4 and day 0 (41–43)) or anti–Gr-1 mAb (RB6-8C5; BioXcell; 0.5 mg i.v. on days −3, −2, and −1 (44)), or rat IgG isotype control (BioXcell; 0.5 mg i.p. on day −4 and day 0). Flow cytometry was performed daily by abdominal palpation and rejection defined by the complete cessation of cardiac contraction. Additional transplanted mice (n = 4) were euthanized at day 11 posttransplant and serum, allograft, native heart, and splenocytes harvested and analyzed by Luminex, histological/immunohistological staining, ELISPOT assay, and flow cytometric analysis.

**Histological and immunofluorescence assessment of heart allografts**

Hearts were harvested, bisected, and either embedded and snap frozen in OCT medium (Sakura Finetek USA, Torrance, CA) or fixed in 10% neutral buffered formalin. Formalin-fixed sections were processed, embedded in paraffin wax, and sections cut (5 μm) and stained with H&E or by standard immunohistochemical staining to distinguish CD3+ cells, as described (45). For confocal analysis, OTC-embedded tissue was sectioned (8 μm), adhered to slides, washed in PBS, then fixed in 2% paraformaldehyde in PBS. After washing in PBS, fixed tissue was permeabilized in 0.1% Triton X-100 (Sigma-Aldrich), washed, and blocked with 2% BSA in PBS. Staining was accomplished by first labeling sections with biotinylated mAb (I-Ad/I-Ed). Shaded histogram depicts isotype control. Data depict one experiment representative of three experiments performed. Percent suppression is presented and calculated by the equation 1 – [(cpm of T cells + stimulators + Ly6C/G cells)/(cpm of T cells + stimulators alone)] × 100.

**ELISPOT assay**

CD4+ cells from transplanted and naive BALB/c mice were isolated by negative depletion as described earlier, but with the addition of rat anti-mouse CD90 mAb (53-6.7; Becton Dickinson). Purified CD4+ T cells were incubated with CD3-depleted, gamma-irradiated B6, BALB/c, or C3H splenocytes (0.1 × 10^6 to 1 × 10^6 T cells + 2.5 × 10^5 APCs/well) in 96-well nitrocellulose-backed plates (Millipore, Bedford, MA) precoated with anti-IFN-γ, anti–IL-4, or anti–IL-5 mAb (BD Pharmingen). After 3 d, ELISPOT plates were developed by washing the wells and subsequent incubation with biotinylated anti-IFN-γ, anti–IL-4, or anti–IL-5 mAb secondary Ab, streptavidin–HRP, and AEC Substrate Solution (BD Biosciences). The spots were counted using an ELISPOT plate reader (CTL, Cleveland, OH).

**Luminex**

Serum levels of basic fibroblast growth factor, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p40/70), IL-13, IL-17, IFN-γ–produced protein 10 (CXCL10), keratinocyte-derived chemokine, MCP-1 (CCL2), monokine induced by IFN-γ (CXCL9), MIP-1α (CCL3), TNF-α, and VEGF were assessed via the Mouse Cytokine 20-Plex Panel (Invitrogen) and analyzed with a Bio-Plex reader by the University of Pittsburgh Cancer Institute Biomarkers Facility.

**Treg suppression assay**

FIR reporter mice were administered PBS or recombinant mouse IL-33 (0.5 μg/d, i.p., day 0 to day 10) and splenocytes isolated on day 11. After RBC lysis, splenocytes were isolated by negative depletion as described earlier. The CD4+ cells were blocked in 10% normal goat serum and then surface stained with allophycocyanin-conjugated Ab to CD4 and FITC–anti-ST2. A Becton Dickinson FACSAria was used for fluorescence detection and FlowJo software.

**Statistical analyses**

Results are expressed at means ± 1 SD. The significance of differences between means was determined using Student t test and Prism (GraphPad Software).
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IL-33 promotes the expansion of poorly stimulatory CD11b+ cells

Given the recent demonstration (29) that addition of IL-33 to mouse BM cell cultures results in the generation of “immature” mDCs that are unresponsive to TLR ligation, we first sought to define the influence of IL-33 on the differentiation and expansion of myeloid APCs in vivo. We examined WT or St2−/− BALB/c mice for modulation of CD11b+ and CD11c+ cell populations after administration of mouse recombinant IL-33 (0.5 μg/d i.p. for 10 d). In addition, we compared the influence of IL-33 on these populations to that of Flt3L (10 μg/d i.p. for 10 d), a potent hematopoietic growth factor that increases CD34+ stem cells and multiple myeloid cell populations, including mDCs in vivo (49). Consistent with the report of Mayuzumi et al. (29), IL-33 administration to WT BALB/c (Fig. 1) and B6 (Supplemental Fig. 1) mice increased the incidence (Fig. 1A, 1C, Supplemental Fig. 1) and absolute numbers (Fig. 1C) of CD11b+CD11c+ cells, but not CD11b+CD11c− cells. CD11b+CD11c+ cells in IL-33-treated mice were phenotypically less mature than corresponding populations from PBS- or Flt3L-treated mice (Fig. 1D). Consistent with this finding, purified CD11c+ splenocytes from IL-33–treated mice were poorer stimulators of normal allogeneic CD3+ T cells than those from PBS-treated animals (data not shown). These data are consistent with the capacity of IL-33 to promote the generation of poorly stimulatory CD11c+ cells in vitro (29).

We also observed a profound increase in CD11b+CD11c− cells in response to IL-33 administration (Fig. 1A, Supplemental Fig. 1). Expansion of CD11b+CD11c− cells by IL-33 was observed in both B6 and BALB/c mice and was dependent on St2 gene expression as it was not observed in St2−/− mice (Fig. 1B). However, lack of St2 did not result in global defects in myeloid lineage cell expansion, as Flt3L treatment of St2−/− mice facilitated the expansion of CD11b+CD11c+, CD11b+CD11c+, CD11b+CD11c+, and CD11b+Gr-1+ (Ly6C/Ly6G)+ cells (Fig. 1B). Notably, although IL-33 and Flt3L elicited a similar increase in frequency and overall number of CD11b+ cells (Fig. 1A, 1C), IL-33 preferentially increased CD11b+Gr-1− cells, whereas Flt3L selectively augmented CD11b+Gr-1+ cells in both WT mice (Fig. 1A) and St2−/− mice (Fig. 1B). The promotion of CD11b+Gr-1− cells by IL-33 did not require mast cells, which constitutively express high levels of ST2L (Supplemental Fig. 1). Specifically, after IL-33 treatment of Kit+Kit+/− mice that lack skin and tissue mast cells (50), the increased incidence of CD11b+Gr-1+ cells remained prominent (Supplemental Fig. 1).

In vitro, we replicated the reported (29) capacity of IL-33 to facilitate the generation of immature (MHC II−CD86−) CD11b+CD11c− mDCs that resist TLR4 ligation-induced maturation (Supplemental Fig. 2A, 2B). Addition of IL-33 together with GM-CSF and IL-4 to BM cell cultures also resulted in immature, maturation-resistant mDCs (Supplemental Fig. 2). mDCs from both IL-33–treated and Flt3L-treated mice did not upregulate surface CD86 after exposure to LPS (Supplemental Fig. 2B). Likewise, cells generated in IL-33–containing cultures exhibited poor T cell allostimulatory capacity, both before and after stimulation with LPS (Supplemental Fig. 2C). Notably, and similar to our in vivo observations, CD11b+CD11c− Gr-1− cells were increased in BM cell cultures exposed to IL-33 (Supplemental Fig. 2A). Thus, both in vitro and in vivo, IL-33 promoted the expansion of CD11b+CD11c− cells that were phenotypically immature and poorly stimulatory. In addition, IL-33 enhanced CD11b+Gr-1− cells.

IL-33 expands Ly6C+ and Ly6G+ myeloid cells

As we observed a significant increase in CD11b+Gr-1− cells concordant with an increase in poorly stimulatory CD11c+ cells, we hypothesized that IL-33 might also increase the generation of MDSCs in vivo. MDSCs are a rare, heterogeneous population of incompletely differentiated, immature myeloid and myeloid progenitor cells (reviewed in Refs. 51–53). Although heterogeneous, MDSCs are globally defined as CD11b+Gr-1−. The Gr-1 epitope is shared by both the cell surface glycoproteins Ly6C and Ly6G, and at least two subpopulations of MDSCs are recognized and defined by their levels of expression of these molecules (52). More specifically, monocytic MDSCs are CD11b+Ly6GloLy6C+ and granulocytic MDSCs are CD11b+Ly6G+Ly6C+ in tumor models. Monocytic MDSC populations have been described as F4/80+ and granulocytic MDSCs as F4/80−, thus distinguishing them from Gr-1−F4/80−Ly6C+ monocyte/macrophages (52). These MDSC subsets are thought to use different mechanisms to suppress T cell function (52).

Compared with control mice, those given IL-33 exhibited increases in several CD11b+ and F4/80-expressing subsets (Fig. 2A). CD11b+F4/80−, CD11b+F4/80+, and CD11b+F4/80− cells were all increased substantially after IL-33 administration. CD11b+F4/80− cells splenocytes from control mice were Ly6G−Ly6C+ and CD11c−, consistent with macrophages and mDCs (Fig. 2B). IL-33 administration promoted CD11b+F4/80+ cells, but also increased presumptive monocytes or DC precursors expressing CD11c+ and Ly6C (Fig. 2B). IL-33–treated mice also demonstrated a large increase in CD11b+F4/80− splenocytes (Fig. 2A). Assessment of Ly6G/C and CD11c expression (Fig. 2B) revealed that these cells were very similar to those from PBS-treated mice, consisting of Ly6G+Ly6C−CD11c+ cells. This phenotype is indicative of granulocytes and neutrophils and consistent with previous reports that IL-33 promotes their in vitro and in vivo expansion (2). A prominent population of CD11b+F4/80− cells was also increased profoundly after IL-33 administration (Fig. 2A). This population was composed of both Ly6G+ and Ly6C+ cells that differed from those in PBS-treated mice (Fig. 2B). Specifically, the CD11b+F4/80+ cells in IL-33–treated mice displayed a greater proportion of Ly6G+ cells, but had prominent populations of Ly6G−Ly6C+ and Ly6G−Ly6C− cells (Fig. 2B), potentially granulocytic or monocytic MDSCs, respectively. Thus, IL-33 administration modulates splenic myeloid populations favoring the expansion of CD11b+CD11c−F4/80−Ly6G+ and Ly6C+ cells, a phenotype associated with MDSCs (52).

IL-33-expanded Ly6C- and Ly6G-expressing cells exhibit increased suppressive function

Flt3L is known to expand MDSCs (51, 54, 55). To establish whether IL-33 could expand functional MDSCs, the two Gr−1− expressing populations, Ly6G+ and Ly6C+ cells, were enriched (Supplemental Fig. 3) from control, IL-33–treated, or Flt3L–treated BALB/c mice and assessed for their capacity to inhibit BALB/c CD11c+ splenocyte-induced proliferation of CD3+ allogeneic T cells (Fig. 2C). Both Ly6G+ and Ly6C-expressing populations from IL-33–treated or Flt3L–treated mice potentially suppressed T cell responses compared with the minimal suppression exhibited by those from control mice (Fig. 2C). In total, these data indicate that IL-33 administration increases CD11b+Gr-1−, particularly Gr-1− cells with potent T cell suppressive capacity.
IL-33 exhibits potent ability to increase suppressive CD4+ Foxp3+ cells, including a ST2L+ subset

The influence of IL-33 on lymphocyte populations was assessed 24 h after 10-d treatment of BALB/c and B6 mice with IL-33. IL-33 facilitates Th2 responses (56), and ST2L is a proposed marker of Th2 but not Th1 cell polarization (9). This is consistent with the significant enhancement of ST2L expression on both B6 and BALB/c CD4+ T cells after IL-33 administration (Fig. 3A, 3C, 3E). When the influence of IL-33 on CD4+ Foxp3+ cells was assessed, a previously unreported augmentation of both the incidence (Fig. 3A, 3C) and overall number of CD4+ Foxp3+ cells was observed (data not shown). This effect was dependent on
**FIGURE 3.** St2-dependent increase in suppressive CD4+ Foxp3+ cells, including a prominent ST2L+ subset, after IL-33 administration. IL-33–treated mice display an St2-dependent increase in splenic Foxp3+ CD4+ cells. A and B, Flow cytometric analysis of surface ST2L or intracellular Foxp3 expression by WT BALB/c St2++ (A) or BALB/c St2++ (B) CD4+ T cells after treatment with PBS or IL-33 (0.5 μg/d: day 0 to day 10). Data depict the percentage of CD45+ CD3+ CD4+-gated cells expressing ST2L and intracellular Foxp3 on day 11. Flow cytometric plots are from one experiment representative of three performed. C, Mean incidence for the indicated population after IL-33 (0.5 μg/d: day 0 to day 10) or Flt3L (10 μg/d: day 0 to day 10) administration to WT BALB/c mice. Data are from one experiment (n = 2 to 3 mice per group) that is representative of three performed. Error bars indicate mean + 1 SD. *p < 0.05 (Student t test). D, Coexpression of ST2L by BALB/c splenic CD4+ CD25+ T cells. Data represent the percentage of splenic CD45+ CD3+ CD4+-gated cells expressing CD25+ (left panels) and CD4+ CD25+-gated (right panels) cells expressing ST2L and intracellular Foxp3 on day 11 after PBS or IL-33 treatment. Representative flow cytometric plots from one experiment representative of four performed. E and F, IL-33 expands B6 CD4+ Foxp3+ ST2L+ and STL2+ T cells displaying suppressive capacity. E, Flow cytometric analysis of B6 splenic CD3+ CD4+ T cells after treatment with PBS or IL-33 as above. Data depict the percentage of CD45+ CD3+ CD4+-gated cells and are representative of three independent experiments performed. F, FACS was used to isolated viable ST2L+ and ST2L+ CD4+ Foxp3+ (RFP+) cells from PBS-treated or IL-33–treated B6 Foxp3-reporter mice. Graded numbers of Foxp3+ (RFP+) cells were assessed for their capacity to suppress CD3/CD28-stimulated proliferation of CFSE-labeled B6 CD4+ CD25+ T cells. Data depict the Foxp3+ CD4+-gated cell proliferation profile, percent divided, and division index as calculated via FlowJo Proliferation Platform. Data are from one experiment representative of two performed.
ST2L expression, as it was not observed in St2−/− mice (Fig. 3B).
It also did not depend on the presence of mast cells (Supplemental
Fig. 4). It has been demonstrated recently that administration of
Flt3L, which, like IL-33, expands CD11c+ and CD11b+ cells, also
expands Foxp3+ Tregs (57). When compared directly, IL-33
exhibited a similar capacity to increase the frequency (Fig. 3C)
and absolute number (data not shown) of CD4+ Foxp3+ cells to that
of Flt3L. Upon further examination, we also found that a prominent
subset of CD4+ Foxp3+ cells in IL-33–treated mice that expressed
ST2L, or membrane-bound ST2 (Fig. 3A, 3C, 3D, 3E). Notably,
the largest subset of CD4+ CD25+ cells in PBS-treated mice cells was
Foxp3+ and ST2L+ (Fig. 3D), indicating that a significant propor-
tion (40–50%) of naturally occurring regulatory T cells (nTregs)
are ST2L+. Viable Foxp3+ cells were isolated by FACS from PBS-
treated or IL-33–treated F1 mice and assessed for their capacity to
suppress T cell proliferation. Both CD4+ Foxp3+ ST2L− and CD4+
Foxp3+ ST2L+ cells from IL-33–treated mice demonstrated
pressive activity (Fig. 3F). ST2L+ Foxp3+ cells from PBS-treated
mice were similarly suppressive (data not shown). Thus, these
data identify a previously unreported capacity of IL-33 to expand
pressive CD4+ Foxp3+ T cells in vivo. Likewise, our data reveal
that ST2L, in addition to being expressed on Th2 cells (9), is found
on an IL-33–expanded subset of suppressive nTregs.

Increased expression of ST2 by cardiac allografts

As described above, St2 gene products, particularly ST2L, are in-
creased in cardiac hypertrophy and with, IL-33, may limit cardio-
vascular pathology (8, 58). Using an mAb detecting both sST2 and
ST2L, we examined whether mouse cardiac allografts displayed
modulation of ST2 by Qdot-based immunostaining of cryostat sec-
tions and observed profound upregulation of ST2 expression during
rejection (Fig. 4). Specifically, 10 d after their transplantation into
unmanipulated BALB/c recipients, B6 cardiac allografts exhibited
strongly ST2+ myocardium and CD31+ endothelium (Fig. 4). This
contrasted with normal B6 hearts and with BALB/c recipient native
hearts, both of which stained only weakly for ST2 in the myocar-
dium and did not express ST2 on CD31+ endothelium (Fig. 4). Thus,
upregulated ST2 in rejecting grafts is consistent with the reported
upregulation of ST2 during vascular pathology and suggests a po-
tential target by which IL-33 may facilitate cardiac allograft survival.

Prolongation of graft survival by IL-33 requires host ST2
expression

To determine the influence of IL-33 and ST2 on cardiac allograft
survival, groups of WT or St2−/− BALB/c mice were given heter-
eoletic B6 heart transplants (day 0) and either remained untreated
or received IL-33 (0.5 μg i.p. on days 0, 1, 3, 6, 10, 13, 15, and 17).
Allografts harvested from WT BALB/c recipients treated with
IL-33 exhibited markedly reduced mononuclear cell infiltration and
greater areas of normal myocardium (Fig. 5A, 5B). IL-33–treated
mice grafts were also less infiltrated by CD3+ T cells (Figs. 5C–5E).
Likewise, B6 allografts in WT BALB/c mice treated with IL-33
survived significantly longer (mean survival time [MST] =
29 d versus MST = 9 d for control recipients; p < 0.0001; Fig. 5D).
However, BALB/c St2−/− recipient mice, which lack functional
IL-33R due to the absence of ST2L, did not display any change in
graft survival compared with controls (MST = 11 d; Fig. 5D).
Thus, although heart grafts display upregulated ST2 expression, the
therapeutic effect of IL-33 required St2 expression by the recipient.

Not only Th2-type responses, but also intragraft Foxp3+ cells,
are increased by IL-33 monotherapy after cardiac
transplantation

Administration of IL-33 has been associated with enhanced sys-
temic Th2 cytokine levels and the functional activation of ST2L-
expressing cells, including Th2 cells, basophils, mast cells, and
eosinophils (2). Consistent with these observations, IL-33 treat-
ment after cardiac transplantation significantly increased serum
levels of IL-5 and IL-13 compared with those of naive BALB/c
mice and control graft recipients (Fig. 6A). IL-33 administration
also led to a significant decrease in circulating IL-6. Although
there was a trend toward increased IL-4 and decreased IFN-γ
levels in the circulation, the difference between IL-33–treated
and control mice was not significant (Fig. 6A). There was also no
significant difference, at day 11 posttransplant, in systemic CD4+
cell expression of IL-4 or IFN-γ determined by flow cytometry
(Fig. 6B) or ELISPOT analysis (data not shown). However,
increases in alloreactive CD4+ IL-5+ cells in IL-33–treated ani-
mals were detected (Fig. 6C). A significant reduction in the in-
cidence of splenic CD3+ CD8+ and CD8+ IFN-γ+ T cells was also
observed (Fig. 6D, 6E). Notably, although splenic numbers of
CD4+ Foxp3+ T cells did not differ between control and IL-33–
treated allograft recipients (Fig. 6B), transplants from IL-33–
treated animals displayed a marked increase in Foxp3+ cells (Fig.
6F). This increase was evident despite the overall decrease in
graft-infiltrating cells, including CD3+ T cells (Figs. 5A–C, Fig.
6F). A trend toward increased ST2+ cells was also observed in IL-
33–treated grafts, although differences between PBS-treated and
IL-33–treated grafts did not reach significance. Overall, IL-33 administration facilitated increased Th2-type cellular and systemic responses and increased Foxp3+ cells within cardiac allografts.

**IL-33 treatment increases CD11b+ Gr-1int cells in heart graft recipients**

Given our findings that IL-33 promoted the expansion of CD11b+ Gr-1int cells comprising Ly6G+ and Ly6C+ populations with the ability to suppress allogeneic T cell proliferation (Fig. 2C), we assessed whether IL-33 administration could promote a similar effect after cardiac transplantation. Both control and IL-33–treated graft recipients displayed an increase in splenic CD11b+ cells compared with naive BALB/c mice (Fig. 7A). However, whereas control graft recipients displayed a significant increase in the incidence of CD11b+ Gr-1hi cells (Fig. 7A, 7B), IL-33–treated hosts displayed large increases in Gr-1int cells among CD11b+ cells (Fig. 7A, 7B, 7D). As such, whereas both control and IL-33–treated graft recipients displayed a significant increase in the overall numbers of splenic CD45+ CD11b+ cells (Fig. 7A, 7C) compared with those of naive BALB/c mice, IL-33–treated animals displayed significant increases in CD11b+ Gr-1int cells compared with those of both naive and control graft recipients (Fig. 7A, 7B, 7D). Thus, both when given to normal mice and after cardiac transplantation, IL-33 modulates myeloid populations to enrich for CD11b+ Gr-1int cells.

**IL-33-mediated allograft survival is dependent on CD25+ Tregs**

To define whether IL-33–mediated prolongation of allograft survival depended on the presence of Tregs or MDSCs, BALB/c recipients were administered Abs established to selectively deplete these populations before B6 heart transplantation and subsequent IL-33 administration. IL-33 prolonged cardiac allograft survival significantly in mice treated with IgG or anti–Gr-1 mAb (Fig. 8). However, graft recipients depleted of CD25+ nTregs via anti-CD25 mAb administration displayed no therapeutic benefit from IL-33 administration (Fig. 8). Specifically, nTreg-depleted recipients displayed graft survival comparable with that of BALB/c recipients receiving no mAb and only PBS (Fig. 8). Thus, these data demonstrate the critical importance of nTregs, but not MDSCs, in the capacity of IL-33 to promote cardiac allograft survival.

**Discussion**

To our knowledge, we show for the first time that administering IL-33 to normal or heart-allografted mice facilitates myeloid APC expansion, including immature CD11b−CD11c+ DCs and to a greater extent suppressive CD11b+Gr-1int MDSCs. Furthermore, we report the novel finding that IL-33 has the capacity to expand functional CD4+ Foxp3+ Tregs, which mediate the capacity of IL-33 to promote cardiac allograft survival. Our findings thus broaden the range of in vivo immunoregulatory properties of IL-33. Excluding limited studies on peritoneal macrophages, the in vivo influence of IL-33 on myeloid APCs is poorly understood. IL-33 has been found to be similar to other hematopoietic growth factors, including GM-CSF or Flt3L, in its ability to sustain cell survival and promote CD11c+ mDC generation in BM culture (29). In these studies, IL-33 did not appear proinflammatory, as mDCs generated in IL-33 expressed little MHC or costimulatory molecules, but increased their PD-L1 and PD-L2 expression. IL-33–generated mDCs were poorly stimulatory and also displayed little TLR-induced upregulation of CD86 or proinflammatory cytokines.
In this study, we now demonstrate that IL-33 expands CD11b<sup>lo</sup> CD11c<sup>+</sup> DCs in vivo. However, whereas IL-33 displays similar capacity to Flt3L to expand mDCs in vitro (29), Flt3L is a more potent facilitator of CD11b<sup>lo</sup> CD11c<sup>+</sup> and CD11b<sup>hi</sup> CD11c<sup>+</sup> DC expansion in vivo. Consistent with the foregoing observation (29) that IL-33 expands poorly stimulatory DCs ex vivo, we show that CD11b<sup>lo</sup> CD11c<sup>+</sup> cells from IL-33–treated animals display decreased MHC class II and CD86. We did not observe increased plasmacytoid DCs (CD11b<sup>−</sup>CD11c<sup>lo</sup>B220<sup>+</sup>) after IL-33 administration (data not shown). Thus, both in vivo and ex vivo, IL-33 expands CD11b<sup>lo</sup> CD11c<sup>+</sup> mDCs.

In addition to CD11b<sup>lo</sup> CD11c<sup>+</sup> cells, we found that IL-33 mediates a previously unreported capacity to expand CD11b<sup>+</sup> CD11c<sup>+</sup> Gr-1<sup>+</sup> cells in vitro and in vivo. Given the growing consensus that CD11b<sup>+</sup> Gr-1<sup>+</sup> MDSCs play a major role in cancer progression (51, 52, 59) and the importance attributed to MDSCs in tolerance induction (44, 60), these cells have emerged as an immunoregulatory population of great interest. In the current studies, IL-33 preferentially expanded CD11b<sup>+</sup> CD11c<sup>+</sup> F4/80<sup>int</sup> Gr-1<sup>int</sup> cells compared with PBS or Flt3L treatment of mice. Both Ly6G<sup>+</sup> and Ly6C<sup>+</sup> suppressive CD11b<sup>+</sup> cells were evident in the IL-33–treated animals. Across multiple tumor models, it is the CD11b<sup>+</sup> Gr-1<sup>int</sup> population of MDSCs that consistently exhibits in vitro and in vivo suppressive capacity (61). Eosinophils (CD11b<sup>+</sup> Ly6C<sup>+</sup> Ly6G<sup>+</sup> F4/80<sup>−</sup>) are expanded by IL-33 administration (1), but do not suppress T cell proliferation (61). Furthermore, although IL-33 stimulates mast cells, and these have been implicated in mobilization of MDSCs during tumor development (62), our data suggest that mast cells do not mediate IL-33–induced expansion of CD11b<sup>+</sup> Gr-1<sup>int</sup> cells, as they were still greatly increased after IL-33 treatment of mast cell-deficient mice.

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**FIGURE 6.** IL-33–treated allograft recipients display a Th2-type systemic response and increased intragraft Foxp3<sup>+</sup> cells. A, Significantly decreased IL-6 and markedly increased circulating IL-5 and IL-13 levels observed in IL-33 recipients. The indicated serum cytokines were measured by Luminex assay on day 11 after transplantation of B6 hearts into WT BALB/c hosts, which also received PBS or IL-33 as described in the legend to Fig. 5. Comparison with normal B6 serum is also shown. Fibroblast growth factor, GM-CSF, IL-1α/β, IL-2, IL-17, CXCL1, MIP-1α, TNF-α, and VEGF were not detected or significantly altered from normal levels. Data shown are the means ± 1 SD, n = 3 to 4 mice per group. *p < 0.05 (Student t test). B, After their isolation on day 11 from BALB/c mice given B6 heart transplants alone (PBS) or with IL-33 monotherapy, splenocytes were stained and assessed by flow cytometry after stimulation with PMA/ionomycin in the presence of GolgiPlug. The graph depicts mean percent CD45<sup>+</sup> cells + 1 SD for cells in the indicated gate. n = 2 to 3 per group. *p < 0.05 (Student t test). C, Also on day 11, splenic CD4<sup>+</sup> cells were purified from BALB/c mice given B6 heart transplants alone (control PBS treatment) or with IL-33 monotherapy. The frequency of directly reactive CD4<sup>+</sup> IL-5–secreting T cells was determined 3 d after stimulation with CD3-depleted BALB/c (donor) or C3H (third party) splenocytes. Data are group means ± SD, with n = 3/group. *p < 0.05 (Student t test). D and E, Assessment of splenic CD45<sup>+</sup> CD8<sup>+</sup> T cells in IL-33–treated versus control heart graft recipients revealed reduced incidences of CD3<sup>+</sup> CD8<sup>+</sup> T cells, particularly CD8<sup>+</sup> IFN<sup>+</sup> cells. D, Representative flow plots from one animal representative of the three analyzed. E, Graph depicts mean percent CD45<sup>+</sup> cells + 1 SD for cells contained in the indicated gate. n = 3/group. *p < 0.05 (Student t test). F, Confocal analysis of staining for ST2 (red), Foxp3 (green), and Hoechst (blue) demonstrate that heart grafts from IL-33–treated recipients had increased Foxp3<sup>+</sup> cells. The graph presents the mean number + 1 SD of Foxp3<sup>+</sup> cells per ×40 field for each condition. Positive cells in five randomly selected ×40 fields from four individuals per group were quantified through use of the Image-based Tool for Counting Nuclei and ImageJ software. *p < 0.05 (Student t test).
High-dose GM-CSF subverts immune reactivity by increasing MDSCs (63). Likewise, Flt3L administration increases MDSCs in vivo (51, 54), and spleen cells from Flt3L-treated animals can induce tolerance across MHC barriers (64). Our findings provide new evidence that both Flt3L and IL-33 expand Ly6G+ and Ly6C+ cells with the capacity to suppress alloreactive T cells. However, it is currently unclear how IL-33 acts to promote several distinct myeloid cell populations. Potentially, by stimulating BM cell GM-CSF production (29, 65), IL-33 can act indirectly to promote mobilization of myeloid precursors. However, our current data demonstrate that IL-33 administration also facilitates immature mDCs and MDSCs in vivo. Likewise, we find that IL-33–stimulated BM cell cultures display expansion of immature mDCs, as previously observed (29), and also CD11b+ Gr-1+ cells. Notably, our data also show that addition of IL-33 to GM-CSF plus IL-4–stimulated cultures also generates mDCs with reduced maturity and resistance to proinflammatory stimuli. Thus, IL-33 also may also impede full myeloid cell differentiation.

As discussed earlier, MSDCs have been established as mediators of immune tolerance (44, 60), and we find IL-33 administration greatly increases splenic CD11b+ F4/80lo Gr-1int cells. On day 11, spleen cells from BALB/c mice given B6 heart grafts alone or with IL-33 monotherapy were compared by flow cytometry with normal animals or heart graft recipients receiving only PBS. A, The left panels depict the incidence of total CD45+ cells, and the right panels indicate the incidence of those from the CD11b+ CD11c- gate. Data are representative flow plots from one animal representative of the three to four assessed for each group. B, Data represent the mean frequency of the indicated population in CD45+ CD11b+ CD11c- gated cells (n = 3 to 4 per group). Error bars indicate 1 SD. *p < 0.05 (Student t test). C and D, IL-33 increases the number of splenic CD11b+F4/80lo Gr-1int cells. Data indicate the calculated total splenic numbers for the indicated populations (n = 3 to 4 per group). Error bars indicate 1 SD. *p < 0.05 (Student t test).
rejection and dysfunction (35). In particular, IL-5–secreting CD4+ T cells and eosinophils promote rejection of fully MHC-mismatched heart allografts in recipients depleted of CD8+ T cells (66). Also, whereas IL-4 and IL-13 are necessary for neonatal tolerance (67), they are not required for transplant tolerance in adult animals (68). Notably, IL-13 is implicated in tolerance, particularly by targeting myeloid cells (69) and activating the suppressive function of MDSCs (13, 51). Thus, although MDSCs do not appear fundamental to IL-33 protection of cardiac allografts, the role of IL-13 in IL-33–mediated increases in functional MDSCs and poorly stimulatory mDCs warrants further examination.

Most consistent with the capacity of IL-33 to prolong cardiac allograft survival is our demonstration of its capacity to increase suppressive CD4+ Foxp3+ cells, including a prominent ST2+ subset. Heart grafts from IL-33–treated animals have increased Foxp3+ cells compared with those from control animals, and depletion of nTregs from recipients prior to transplantation ablated any therapeutic benefit of IL-33 monotherapy. From our findings, it follows that, although IL-33 supports Th2 responses, it targets nTregs to mediate protection of cardiac allografts during acute rejection.

To date, the impact of IL-33 has focused mainly on CD4+ Th2 cells. Polarized Th2 cells express ST2L and migrate to IL-33, which stimulates their IL-5 and IL-13 expression (1, 12). Th2 polarization in the presence of Ag and IL-33 results in Th2 cells with suppressive capacity, thus, IL-33 not only supports the induction of Th2 responses but also mediates the expansion of functional Foxp3+ Tregs.

Numerous transplant models have shown the importance of nTregs in limiting alloreactivity and supporting tolerance-inducing regimens (42, 70). It has been proposed that Tregs may traffic to the graft site and inhibit inflammatory responses (42), although definitive evidence for this mechanism is lacking. It is well understood that Tregs can directly suppress CD4+ and CD8+ T cell function, or indirectly through their actions on DC/APC function and interleukin-1 family. Numerous transplant models have shown the importance of nTregs in limiting alloreactivity and supporting tolerance-inducing regimens (42, 70). It has been proposed that Tregs may traffic to the graft site and inhibit inflammatory responses (42), although definitive evidence for this mechanism is lacking. It is well understood that Tregs can directly suppress CD4+ and CD8+ T cell function, or indirectly through their actions on DC/APC function and interleukin-1 family.

In summary, our data show that, in addition to facilitating Th2 responses, IL-33 expands functional MDSCs and suppressive CD4+ Foxp3+ Tregs, including those expressing ST2L. Importantly, we also establish that the prolongation of cardiac allograft survival by IL-33 requires recipient Tregs. As such, these findings reveal previously undefined functions of this IL-1 family member and identify a specific mechanism whereby IL-33 targets recipient nTregs to prolong organ allograft survival.

Acknowledgments

We acknowledge Lisa Mathews for excellent technical assistance and Miriam Freeman for assistance in manuscript preparation. We also acknowledge Dr. Giorgio Raimondi for valuable advice on completion of the bead-based Treg suppression assay.

Disclosures

The authors have no financial conflicts of interest.

References


Supl. Fig. 1
Supl. Fig. 3
Supl. Fig. 4
Supplemental Figure Legends

SUPPLEMENTAL FIGURE 1. IL-33-promoted expansion of MHC<sub>lo</sub> CD11c<sup>+</sup> and CD11b<sup>+</sup> cells is independent of mast cells. WT or mast cell-deficient Kit<sup>+/</sup>/Kit<sup>Sl/d</sup>B6 mice were given IL-33 (0.5 μg/d i.p.) or Flt3L (10 μg/d i.p.) for 10 d. On d 11, total splenocytes were isolated and underwent fluorescence staining and flow analysis. Increased CD11b<sup>+</sup> Gr-1<sup>mod</sup> splenocytes were observed following IL-33 administration to WT or Kit<sup>+/</sup>/Kit<sup>Sl/d</sup>B6 mice. Data represent total CD45<sup>+</sup> gated cells and numbers on dot plots indicate % of gated cells. Data are from 1 animal from each group and are representative of the 2-4 mice analyzed.

SUPPLEMENTAL FIGURE 2. IL-33 promotes the expansion of poorly-stimulatory, MHC<sub>lo</sub> CD11c<sup>+</sup> and CD11b<sup>+</sup> Gr-1<sup>+</sup> cells in vitro. BM cells cultured for 8 d with GM-CSF (GM; 1000 U/ml) and IL-4 (1000 U/ml), GM, IL-4 and IL-33 (25 ng/ml), or IL-33 alone were subjected to immunofluorescent staining and flow cytometric analysis. A, Addition of IL-33 to GM and IL-4 resulted in CD11b<sup>+</sup> CD11c<sup>+</sup> mDC displaying low CD86 and MHC (I-A<sub>b</sub>). BM cell exposure to IL-33 alone supported both CD11b<sup>+</sup> CD11c<sup>+</sup> CD86<sub>lo</sub> MHC<sub>lo</sub> cells and CD11c<sub>lo</sub> CD11b<sup>+</sup> Gr-1<sup>+</sup> cells. Data represent total viable cells based on SSC by FSC and numbers on plots indicate % of gated cells. Data are from 1 experiment representative of 3 performed. B-C, BM cell cultures were stimulated for 18 h with 100 ng/ml of LPS and analyzed by B flow cytometry or C used to stimulate BALB/c CD3<sup>+</sup> cells for 72 h in MLR. B, CD11c<sup>+</sup> CD11b<sup>+</sup> in cultures treated with IL-33, whether alone or with GM and IL-4, displayed reduced levels of I-A<sub>b</sub> and CD86, which were poorly
upregulated following LPS exposure. CD11c+CD11b+ cells from IL-33-stimulated GM and IL-4 cultures displayed similar characteristics. However, CD11c+CD11b+ cells from GM-CSF cultures alone or with IL-33 where both I-Ablo and CD86lo and LPS non-responsive. C, IL-33 containing cultures displayed a reduced allostimulatory capacity, even following LPS exposure. Mean 3[H]-thymidine incorporation ± SD is shown for 1 experiment representative of 3 performed. * = p < 0.05 measured by Student’s t-test.

SUPPLEMENTAL FIGURE 3. Enrichment of Ly6G+ or Ly6C+ splenocytes. BALB/c mice were administered IL-33 (0.5 μg/d) or Flt3L (10 μg/d) i.p. for 10 d (d0-d10). On d11, Ly6G+ and Ly6C+ splenocytes were isolated by immunobead selection. Flow cytometric analysis of A the starting total splenocyte population and B the Ly6G- and C the Ly6C-enriched populations for CD11b, Ly6G, and Ly6C expression. Data are from 1 experiment and representative of at least 2 performed.

SUPPLEMENTAL FIGURE 4. Mast cell-deficient mice display increased ST2L+ CD4+ cells and CD4+ Foxp3+ T cells following IL-33 administration. Like WT BALB/c mice, IL-33-treated WT or Kit+/Kit−B6 mice display increased splenic ST2L+ and Foxp3+ CD4+ cells. A-B, Flow plots depict the % of CD45+ CD3+ CD4+ gated cells following IL-33 administration (0.5 μg/d: d0-d10) and staining for ST2L surface expression A or intracellular Foxp3 B on d11. Representative flow cytometric plots from 1 animal representative of at least 2 analyzed.