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


IL-33 administration is associated with facilitation of Th2 responses and cardioprotective properties in rodent models. However, in heart transplantation, the mechanism by which IL-33, signaling through ST2L (the membrane-bound form of ST2), promotes transplant survival is unclear. We report that IL-33 administration, while facilitating Th2 responses, also increases immunoregulatory myeloid cells and CD4+ Foxp3+ regulatory T cells (Tregs) in mice. IL-33 expands functional myeloid-derived suppressor cells, CD11b+ cells that exhibit intermediate (int) levels of Gr-1 and potent T cell suppressive function. Furthermore, IL-33 administration causes a S12-dependent expansion of suppressive CD4+ Foxp3+ Tregs, including an ST2L+ population. IL-33 monotherapy after fully allogeneic mouse heart transplantation resulted in significant graft prolongation associated with increased Th2-type responses and decreased systemic CD8+ IFN-γ responses. Also, despite reducing overall CD3+ cell infiltration of the graft, IL-33 administration markedly increased intra-graft Foxp3+ cells. Whereas control graft recipients displayed increases in systemic CD8+ IFN-γ+ cells, IL-33–treated recipients exhibited increased CD11b+ Gr-1int cells. Enhanced ST2 expression was observed in the myocardium and endothelium of rejecting allografts, however the therapeutic effect of IL-33 required recipient ST2 expression and was dependent on Tregs. These findings reveal a new immunoregulatory property of IL-33. Specifically, in addition to supporting Th2 responses, IL-33 facilitates regulatory cells, particularly functional CD4+ Foxp3+ Tregs that underlie IL-33–mediated cardiac allograft survival. The Journal of Immunology, 2011, 187: 000–000.

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 mediated by Th2 cells, IL-13, and IL-4, that promote type 2-associated cytokines, particularly IL-5, IL-4, IL-5, and IL-13 in vivo (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 mediated by Th2 cells, IL-13, and IL-4, that promote type 2-associated cytokines, particularly IL-5, IL-4, IL-5, and IL-13 in vivo (1, 2). 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...
instrumental in Th2-mediated responses to helminths (20, 21). Overall, studies on ST2-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 (30). 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 ST2, but not host mast cells. Likewise, IL-33–mediated prolongation of graft survival requires recipient ST2 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 (H2Kb), and C3H/HeJ (C3H; H2Kk) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 Kit+Kit+ or Kit−/− mice were kindly provided by Dr. Adriana T. Larregina (Department of Dermatology, University of Pittsburgh). BALB/c ST2−/− 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-approvzed 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 (FLK3L; 10 μg/d (37); Amgen, 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 no. HK1.4) or PE-conjugated anti-Ly6G (RB6-8C5) and then immunomagnetic purification using anti-FITC or PE microbeads (Miltenyi Biotec, Auburn, CA).

BM cell culture

BM cells were differentiated from freshly isolated or cryopreserved cells for 8 d in the indicated combinations of recombinant mouse GM-CSF (1000 U/ml; R&D Systems, Minneapolis, MN), recombinant mouse IL-4 (1000 U/ml; R&D Systems), and recombinant mouse IL-33 [25 ng/ml (29)]. Every 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 BioSource (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), CD68 (GL-1), F4/80 (BM8), Gr-1 (RB6-8C5), I-Ai-I (M511/4/15.2), Ly6G (1A8), Ly6C (HK1.4), NK1.1 (PK136), or T1/ST2 (D8); MD Bioproducts, St. Paul, MN). After surface staining of freshly isolated splenocytes, fixation/permeabilization and intracellular staining with fluorochrome-conjugated mAb to Foxp3 (FJK-16a) was also completed where indicated. Where designated, 4- to 5-h stimulation of splenocytes with Concanavalin A (Con A) or phorbol 12-myristate 13-acetate (PMA) and ionomycin was followed by permeabilization and intracellular staining with fluorochrome-conjugated mAbs to T-bet (B9.6), IL-4 (11B11), IFN-γ (XM14.1) as described (5, 39). Appropriately conjugated, isotype-matched IgGs served as 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 by trypan blue dye exclusion, and then multiplying the number of total live cells by the frequency 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 ST2−/− BALB/c mice, as described (39, 40). Briefly, hearts were transplanted into recipients through...
anatomosis of the donor ascending aorta and pulmonary artery and re- cipient 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 0 and day 0 (41–43)], 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 days 0, 1, 3, and 6). Graft function was assessed 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 immunofluorescent 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, and 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, OCT-embedded tissue was sectioned (8 μm), adhered to slides, washed in PBS, then fixed in 2% paraformaldehyde. After washing in PBS, fixed tissues were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich), washed, and blocked with 2% BSA in PBS. Staining was accomplished by first layering sections with purified rat anti-mouse ST2 (D18; MD Bioproducts) and subsequent probing with Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch). After overnight blocking with unconjugated goat anti-rat Ab (Jackson ImmunoResearch), sections were stained with polyclonal rabbit anti-Foxp3 (Novus Biologicals, Littleton, CO) then Alexa Fluor 488-conjugated goat anti-rabbit IgG (Molecular Probes/Invitrogen) as a secondary Ab to detect Foxp3. Samples were counterstained with 1% bismarckzamid (Hoechst; Sigma-Aldrich). Optical sections (0.4 μm) were generated using an Olympus Fluoview 1000 scanning confocal microscope (Olympus America, Lehigh Valley, PA). Images were all taken with a 40× objective lens (Olympus UPLSAPO). Images were analyzed with ImageJ software (NIH, Washington, DC).

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, La Jolla, CA) where p < 0.05 was considered significant. GraphPad Prism 5.0C software package (GraphPad Software) was used to generate survival curves, and the significance of differences in graft survival between groups was determined by Kaplan–Meier analysis and the log-rank test.

Results

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
IL-33 EXPANSION OF MDSC AND Treg PROMOTES ALLOGRAFT SURVIVAL

A

BALB/c St2<sup>+/+</sup>

PBS

IL-33

Flt3L

CD11b<sup>+</sup> CD11c<sup>-</sup>

CD45<sup>+</sup>

CD11b<sup>+</sup> CD11c<sup>-</sup>

CD11b<sup>+</sup> CD11c<sup>-</sup>

Gr-1

B

BALB/c St2<sup>−/−</sup>

PBS

IL-33

Flt3L

CD11b<sup>+</sup> CD11c<sup>-</sup>

CD45<sup>+</sup>

CD11b<sup>+</sup> CD11c<sup>-</sup>

CD11b<sup>+</sup> CD11c<sup>-</sup>

Gr-1

C

PBS

IL-33

Flt3L

% CD4<sup>+</sup> Cells

% CD4<sup>+</sup> CD11c<sup>-</sup> CD4<sup>+</sup> CD11c<sup>-</sup>

Total Splenocytes (×10<sup>7</sup>)

D

CD86

I-A<sup>b</sup>/I-E<sup>d</sup>

CD11b<sup>+</sup> CD11c<sup>-</sup>

CD11b<sup>+</sup> CD11c<sup>-</sup>

CD11b<sup>-</sup> CD11c<sup>-</sup>

Gr-1<sup>hi</sup>

Gr-1<sup>int</sup>

Gr-1<sup>neg</sup>

% of Max.

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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+ 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 mice and IL-33 plus GM-CSF and IL-4 culture conditions 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 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+ Ly6G+ CD11c− F4/80− Ly6G− and Ly6C− cells, a phenotype associated with MDSCs (52).

**IL-33–expanded Ly6G− 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 potentely 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.

**FIGURE 1.** IL-33 promotes the expansion of CD11b+ CD11c+ MHC1− and CD11b+ Gr-1− cells in vivo. WT (St2+/+, A) or St2−/− BALB/c (B) mice were given IL-33 (0.5 µg/d) or Flt3L (10 µg/d) i.p. for 10 d (day 0 to day 10). On day 11, total splenocytes were isolated, stained as described in Materials and Methods, and analyzed by flow cytometric analysis. A and B, St2−/− mice were treated with St2+ (left panels) or St2− (right panels) cells from WT BALB/c or St2+/+ (A) or St2−/− (B) mice, and numbers on dot plots indicate percentage of gated cells. Data are from one experiment representative of three performed and depict one animal representative of the indicated treatment group. C, Mean incidence (top panels) for the indicated populations calculated across three independent experiments (n = 7 to 9 per group). Total splenic numbers (bottom panels) for the indicated populations from one experiment representative of the three independent experiments preformed (n = 2 to 3 per group). Error bars indicate 1 SD. *p < 0.05 (Student t test). D, Decreased MHC class II and CD86 on CD11c+ and CD11b+ cells from mice treated with IL-33 or Flt3L. Freshly isolated total splenocytes were assessed for CD86 and MHC class II (I-Aβ/I-Eβ). Shaded histogram depicts isotype control. Data depict one experiment representative of three experiments performed.
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.
When compared directly, IL-33-treated or IL-33–treated FIR mice and assessed for their capacity to increase the frequency and absolute number (data not shown) of CD4+ Foxp3+ cells to that exhibited a similar capacity to increase CD4+ Foxp3+ T cells in vivo. Likewise, our data reveal that ST2L+ mice were similarly suppressive (data not shown). Thus, these data identify a previously unreported capacity of IL-33 to expand suppressive nTregs.

Increased expression of ST2 by cardiac allografts

As described above, ST2 gene products, particularly ST2L, are increased in cardiac hypertrophy and, with IL-33, may limit cardiovascular 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 sections 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 myocardium 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 potential 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 heterotopic 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 mouse grafts were also less infiltrated by CD3+ T cells (Fig. 5C). 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−/− recipients 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 systemic 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 treatment 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 animals were detected (Fig. 6C). A significant reduction in the incidence 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-1^int cells in heart graft recipients**

Given our findings that IL-33 promoted the expansion of CD11b+ Gr-1^hi 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-1^int cells (Fig. 7A, 7B), IL-33–treated hosts displayed large increases in Gr-1^int cells among CD11b+ cells (Fig. 7A, 7B, 7D). As such, whereas both control and IL-33–treated graft recipients exhibited 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-1^int 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-1^int 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 nTregs 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^lo CD11c+ DCs and to a greater extent suppressive CD11b+ Gr-1^int 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\textsuperscript{lo} CD11c\textsuperscript{+} 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\textsuperscript{lo} CD11c\textsuperscript{+} and CD11b\textsuperscript{hi} CD11c\textsuperscript{+} DC expansion in vivo. Consistent with the foregoing observation (29) that IL-33 expands poorly stimulatory DCs ex vivo, we show that CD11b\textsuperscript{lo} CD11c\textsuperscript{+} cells from IL-33–treated animals display decreased MHC class II and CD86. We did not observe increased plasmacytoid DCs (CD11b\textsuperscript{−} CD11c\textsuperscript{lo} B220\textsuperscript{+}) after IL-33 administration (data not shown). Thus, both in vivo and ex vivo, IL-33 expands CD11b\textsuperscript{lo} CD11c\textsuperscript{+} mDCs.

In addition to CD11b\textsuperscript{lo} CD11c\textsuperscript{+} cells, we found that IL-33 mediates a previously unreported capacity to expand CD11b\textsuperscript{+} CD11c\textsuperscript{2} Gr-1\textsuperscript{+} cells in vitro and in vivo. Given the growing consensus that CD11b\textsuperscript{+} Gr-1\textsuperscript{−} 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\textsuperscript{+} CD11c\textsuperscript{lo} F4/80\textsuperscript{int} Gr-1\textsuperscript{int} cells compared with PBS or Flt3L treatment of mice. Both Ly6G\textsuperscript{+} and Ly6C\textsuperscript{+} suppressive CD11b\textsuperscript{+} cells were evident in the IL-33–treated animals. Across multiple tumor models, it is the CD11b\textsuperscript{+} Gr-1\textsuperscript{int} population of MDSCs that consistently exhibits in vitro and in vivo suppressive capacity (61). Eosinophils (CD11b\textsuperscript{+} Ly6C\textsuperscript{−} Ly6G\textsuperscript{+} F4/80\textsuperscript{lo}) 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\textsuperscript{+} Gr-1\textsuperscript{−} cells, as they were still greatly increased after IL-33 treatment of mast cell-deficient mice.

**FIGURE 6.** IL-33–treated allograft recipients display a Th2-type systemic response and increased intragraft Foxp3\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} 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\textsuperscript{+} CD8\textsuperscript{+} T cells in IL-33–treated versus control heart graft recipients revealed reduced incidences of CD3\textsuperscript{+} CD8\textsuperscript{+} T cells, particularly CD8\textsuperscript{+} IFN-γ cells. D, Representative flow plots from one animal representative of the three analyzed. E, Graph depicts mean percent CD45\textsuperscript{+} 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\textsuperscript{+} cells. The graph presents the mean number ± 1 SD of Foxp3\textsuperscript{+} 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 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+ Gr-1+ 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+ CD11c+ F4/80+ Gr-1+ 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).

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As discussed earlier, MSDCs have been established as mediators of immune tolerance (44, 60), and we find IL-33 administration greatly increases splenic CD11b+ Gr-1+ MDSCs in normal and transplanted mice. However, depletion of Gr-1+ cells prior to transplantation and IL-33 administration did not impede the capacity of IL-33 to prolong cardiac allograft survival. Thus, although this is a newly identified immunoregulatory capacity of IL-33, it does not appear to underlie the capacity of IL-33 to suppress acute allograft rejection.

It has been suggested that IL-33 prolongs cardiac allograft survival by promoting Th2 responses (33). We find that administration of IL-33 concurrent with cardiac allotransplantation increases systemic levels of IL-5 and IL-13, increases IL-5+ CD4+ cells, and decreases CD8+ IFN-γ+ T cells. Reduction of intragraft IFN-γ by systemic neutralization of IL-12, and concomitant increases in IL-4 and IL-10, inhibit indirect T cell responses (to alloantigen presented by recipient Dcs on self MHC) resulting in prolonged allograft survival across minor Ag mismatches, but not full MHC barriers (34). Thus, there is some support for Th2 cytokine-mediated prolongation of graft survival. However, there is also evidence that IL-4 and Th2 cytokines can promote graft
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 ST2L+ 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 that express IL-5, but not IL-4 (31). We now demonstrate that a significant subset (40–50%) of CD4+ CD25+ nTregs express ST2L, the membrane-bound form of ST2, and both ST2L+ and ST2L– Foxp3+ cells are significantly expanded by IL-33. CD4+ Foxp3+ cells from IL-33–treated mice exhibited suppressive capacity, thus, IL-33 not only supports the induction of Th2 responses but also mediates the capacity to expand functional Foxp3+ Tregs.

Numerous transplant models have shown the importance of nTregs in limiting alloreactivity and supporting tolerance-inducing regimens (42, 70). It will be of interest to establish the environment in which a significant subset (40–50%) of CD4+ CD25+ nTregs express ST2L, presumably ST2L on recipient nTregs. Importantly, we found that CD4+ Foxp3+ Tregs, including those expressing ST2L. In particular, targeting CD4+ Foxp3+ cells from IL-33–treated mice exhibited suppressive capacity, thus, IL-33 not only supports the induction of Th2 responses but also mediates the capacity to expand functional Foxp3+ Tregs.

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Disclosures

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