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IL-33 is an Unconventional Alarmin That Stimulates IL-2 Secretion by Dendritic Cells To Selectively Expand IL-33R/ST2⁺ Regulatory T Cells

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IL-33 is a recently characterized IL-1 family member that is proposed to function as an alarmin, or endogenous signal of cellular damage, as well as act as a pleiotropic cytokine. The ability of IL-33 to potentiate both Th1 and Th2 immunity supports its role in pathogen clearance and disease immunopathology. Yet, IL-33 restrains experimental colitis and transplant rejection by expanding regulatory T cells (Treg) via an undefined mechanism. We sought to determine the influence of IL-33 on hematopoietic cells that drives Treg expansion and underlies the therapeutic benefit of IL-33 administration. In this study, we identify a feedback loop in which conventional mouse CD11c⁺ dendritic cells (DC) stimulated by IL-33 secrete IL-2 to selectively expand IL-33R(Th2⁻)⁻ suppressive CD4⁺Foxp³ Treg. Interestingly, this occurs in the absence of classical DC maturation, and DC-derived (innate) IL-2 increases ST2 expression on both DC and interacting Treg. ST2⁺ Treg represent an activated subset of Foxp³ cells, demonstrated to be ICOS⁺CD44⁺ compared with their ST2⁻ counterparts. Furthermore, although studies have shown that IL-33–exposed DC promote Th2 responses, we reveal that ST2⁺ DC are required for IL-33–mediated in vitro and in vivo Treg expansion. Thus, we have uncovered a relationship between IL-33 and innate IL-2 that promotes the selective expansion of ST2⁺ Treg over non-Treg. These findings identify a novel regulatory pathway driven by IL-33 in immune cells that may be harnessed for therapeutic benefit or for robust expansion of Treg in vitro and in vivo. The Journal of Immunology, 2014, 193: 4010–4020.

Interleukin-33 is a barrier tissue-expressed IL-1 family member (1) that when released targets IL-1R-like 1, more commonly referred to as ST2, to promote Th2 responses in T cells, mast cells, eosinophils, basophils, and innate lymphoid cell populations (2–6). Thus, the ST2/IL-33 axis plays a protective role in immunity against parasitic infection (7), but may exacerbate pathologic conditions such as asthma (8), arthritis (9), eosinophilic pericarditis (10), and dermatitis (11). Recently, pleiotropic functions of IL-33 have emerged, with several reports supporting a role for IL-33 in promoting Th1 immune responses and antiviral immunity (12–14).

Although implicated in both Th1 and Th2 immunity, its capacity to limit Th1 and Th17 responses in vivo (15, 16) suggests a potential regulatory role for IL-33 and ST2 in controlling inflammation (6). Likewise, in mouse models of cardiac transplantation, IL-33 administration prolongs fully MHC-mismatched allograft survival by reducing Th1-mediated IFN-γ production (in favor of elevated IL-4, IL-5, IL-10, and IL-13) and increasing Foxp³ regulatory T cells (Treg) and CD11b⁺Gr-1⁺ myeloid-derived suppressor cell systemically and in the graft (17–19). Our group has demonstrated that prolongation of cardiac allograft survival by IL-33 monotherapy is dependent on Foxp³ Treg (18). Despite indications that IL-33 expands Treg (18), the precise characterization of IL-33–expanded Treg and elucidation of the mechanisms contributing to their expansion are lacking. Given the ability of IL-33 to target CD11c⁺ dendritic cells (DC) (18, 20–22), and the critical role of DC in Treg homeostasis and expansion (23, 24), we tested the hypothesis that DC serve a critical function in orchestrating Treg expansion mediated by IL-33.

In the current study, we identified a new functional interrelation between IL-33 and IL-2 mediating selective Treg expansion. Specifically, although IL-33 fails to induce classical DC maturation, it stimulates IL-2 production by CD11c⁺ DC that is critical for expanding ST2-expressing Treg during interactions with CD4⁺ T cells. In connection, a significant reduction in ST2 expression on DC in the absence of IL-2 may dampen DC responsiveness to IL-33. We further demonstrate that ST2 on DC is critical for IL-33–mediated Treg expansion, as use of ΔαβΔγ⁻⁻ DC in vitro and depletion of CD11c⁺ DC in vivo inhibit this function of IL-33. Collectively, our findings establish an important immunoregulatory function of IL-33 carried out through a novel mechanism of CD11c⁺ DC-mediated Treg expansion. These data highlight the IL-33/ST2 axis as a potential target for development of therapeutic modalities aimed at promoting immune regulation or expansion of Treg.

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Abbreviations used in this article: BM, bone marrow; CTV, CellTrace Violet; DC, dendritic cell; DT, diphertheria toxin; mu, mouse; Treg, regulatory T cell; WT, wild-type.

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Materials and Methods

Animals and IL-33 administration

Male C57BL/6 (B6; H2Kb), BALB/c (H2Kb), B6.FVB-Tg(ligand-DTR/EGFP)57Lan/J (CD11c-DTR), and B6.129P2-Ildm1Hor/J (IL-2−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/c ST2−/− mice were obtained from A. McKenzie (Medical Research Council Laboratory of Molecular Biology, University of Cambridge, Cambridge, U.K.) and bred for experimental use (25). C57BL6Il-33tm1Flv/J (Foxp3-ires-nRFP [FIR]) reporter mice (26) for breeding were graciously provided by F. Lakis (Starz Transplantation Institute, University of Pittsburgh, Pittsburgh, PA). All mice were housed in the specific-pathogen-free facility at the University of Pittsburgh School of Medicine. Recombinant mouse (mu) IL-33 (BioLegend, San Diego, CA) was dissolved in PBS and injected i.p. (0.5 μg/mouse; for 10 d). Control animals received PBS alone.

Experiments were conducted under an Institutional Animal Care and Use Committee–approved protocol and in accordance with National Institutes of Health guidelines. Animals were fed a diet of Purina rodent chow (Ralston Purina, St. Louis, MO) and received food and water ad libitum.

Propagation, purification, and treatment of CD11c+ DC

CD11c+ bone marrow (BM)–derived DC were propagated from total BM cells using recombinant mu GM-CSF (1000 U/ml; R&D Systems, Minneapolis, MN) alone or in combination with recombinant mu IL-4 (1000 U/ml; Schering-Plough, Kenilworth, NJ) and harvested on day 7, as described (18, 27, 28). Briefly, culture medium supplemented with GM-CSF and IL-4 was replenished every 2 d, and CD11c+ cells were purified using magnetic beads (Miltenyi Biotec, Auburn, CA). Purified CD11c+ cells were cultured overnight in complete RPMI 1640 media alone or complete media supplemented with either 100 ng/ml LPS (Enzo Life Sciences, Farmingdale, NY) or 20 ng/ml IL-33.

T cell isolation and MLR

T cells were purified from spleens of wild-type (WT) B6, B6 FIR, or BALB/c mice. RBC-lysed, single-cell suspensions were incubated with anti-CD45R/B220, anti-CD16/CD32, anti–TER–119, anti–I-A/I-E, anti-CD11b, and anti-Ly6G/Ly6C obtained from BD Biosciences. For CD4+ or CD8+ T cells, anti-CD4 or anti-CD8a was included, respectively. Ab-bound cells were eliminated using Dynabeads (Life Technologies) following the manufacturer’s instructions. Control, LPS, or IL-33 DC were used as stimulators in 5-d MLR of CellTrace Violet (CTV; Life Technologies)-labeled CD4+CD25− cells (Fig. 2A) from untreated and IL-33–treated mice revealed CTV labeling of CD4+CD25− cells at a 1:10 DC:T cell ratio. Recombinant human IL-2 (50 U/ml; PeproTech, Rocky Hill, NJ), IL-33 (10 ng/ml), or anti-IL-2 (10 μg/ml; JES6-1A12; BioLegend) were added to the MLR, as indicated in the figures and figure legend.

Suppression assays

Bulk CD4+ T cells were purified from PBS- or IL-33–treated, B6 FIR mice and stained for flow sorting (FACSARia; BD Biosciences, San Jose, CA) of RFP(Foxp3)+ CD4+ ST2+ or ST2− Treg. Alternatively, splenic B6 FIR CD4+ T cells were cultured with IL-33–exposed BALB/c DC, and Treg were sorted after 5 d. Purity was consistently >92%. Sorted Treg were tested for their ability to suppress CD3/CD28 T-activator bead (5 × 10⁵; Life Technologies)-induced, CTV-labeled B6 CD4+ or CD8+ T cell proliferation at a ratio of 8:1 T effector to Treg (1 × 10⁵ × 10⁵). Where indicated, recombinant mu IL-12 (5 ng/ml; BioLegend) alone or in combination with IL-33 (10 ng/ml) was used to induce IFN-γ in CD8+ T cell cultures. Cultures were harvested on day 4 for flow cytometric analysis of proliferation and IFN-γ by intracellular flow analysis.

Flow cytometric analysis

Flow cytometry data were acquired using a LSRRfortessa (BD Biosciences) and analyzed using FlowJo v10 (Tree Star, Ashland, OR). Intracellular staining was carried out using Foxp3/transcription factor–staining buffer set (eBioscience, San Diego, CA). All fluorochrome-conjugated Abs were purchased from BioLegend, eBioscience, or BD Biosciences, except ST2 (MD Bioproducts, St. Paul, MN).

Cytokine quantitation

 Supernatants from CD11c+ cells cultured for 18 h were harvested and IL-2 quantified using IL-2 Ready-SET-Go! (eBioscience) kit, according to the manufacturer’s instructions. Other cytokines were measured in DC supernatants per manufacturer’s instructions. IL-17A, and IL-5 were quantitated in supernatants from MLR using ELISA Max kits (BioLegend), according to the manufacturer’s instructions.

BM chimeras and diphtheria toxin administration

Recipient B6 mice were given antibiotic-treated (Sulfatrim; Hi-Tech Pharmacal, Amityville, NY) water for 7 d prior to irradiation. Chimeric mice were generated by irradiating WT B6 recipient mice with two doses (900 cGy and 400 cGy) in a span of 6 h. Two hours after the second radiation dose, mice received 1 × 10⁶ syngeneic WT or CD11c-DTR B6 BM. Mice were continued on antibiotic-treated water for 7 d. For depletion of CD11c+ cells (starting 5 wk post-BM transplant), diphtheria toxin (DT; Sigma-Aldrich, St. Louis, MO) was administered (125 ng/mouse) every other day for 10 d (a total of 6 doses), and mice were euthanized 2 d after the last dose. Mice were also treated with PBS or IL-33 (0.5 μg/mouse; for 10 d) starting 1 d after the first DT treatment and ending 1 d after the last DT treatment. Spleens were harvested 1 d after the final treatment with IL-33, and total splenocytes were stained for flow cytometric analysis.

Statistics

Statistical significance was determined by Student’s t test and one- or two-way ANOVA, where appropriate, using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA). A p value <0.05 was considered significant. All experiments were carried out independently for a minimum of three times, unless indicated otherwise in the figure legends. All bar graphs represent statistical mean ± SD.

Results

IL-33 expands a ST2+ subset of CD4+CD25+ Foxp3+ cells in the thymus and spleen

We reported previously that IL-33 systemically expands functionally suppressive Foxp3+ Treg that prolong experimental cardiac allograft survival (17, 18). Our precise characterization of the impact of IL-33 on Treg populations in the thymus and spleen now reveals that administration of IL-33 profoundly modulates Treg populations, particularly an ST2+ (IL-33Rα) subset, in both primary and secondary lymphoid organs (Fig. 1). Interestingly, ST2+ Foxp3+ cells were present at a low frequency in naive, unmanipulated mice, comprising ~10% of CD3+CD4+CD25+ cells found in both the thymus and spleen (Fig. 1A). Administration of IL-33 expanded CD4+CD25+ cells, particularly ST2+Foxp3+ cells, and in the spleen they approach a proportion similar to that of ST2+Foxp3+ cells (Fig. 1A). Interestingly, IL-33 decreases the total number of cells in the thymus while increasing the total number of splenocytes (Fig. 1B), corresponding to a significant increase in total CD4+Foxp3+ cells, including ST2+Foxp3+ cells in the spleen (Fig. 1C). These data substantiate that ST2+Foxp3+ cells are an existing subset of Treg and are expanded following delivery of IL-33.

Phenotypic analysis of CD4+ Foxp3+ compared with Foxp3− cells (Fig. 2A) from untreated and IL-33–treated mice revealed expression of classical Treg markers on ST2+Foxp3+ cells, including PD-1, CTLA-4, LAG-3, OX-40, LAP (TGF-β), CD39, CD73, CD103, and GARP (Fig. 2B). Several distinguishable characteristics of ST2+ Treg relative to their ST2− counterparts, especially following IL-33 administration, included higher GATA-3, ICOS, CD44, and CD69, with corresponding low expression of CD62L (Fig. 2B). Thus, ST2+Foxp3+ cells, while sharing expression of classical Treg markers, are distinct from their ST2− Foxp3+ counterparts and display markers consistent with activated Treg (29, 30).

IL-33–expanded Treg regulate effector T cell responses

We next tested the suppressive function of Treg from PBS- and IL-33–treated mice, including a comparative analysis of the ST2− and ST2+ subsets. We found that a Treg:T effector ratio, in which conventional Treg (control ST2−) failed to significantly suppress effector T cell proliferation, IL-33–expanded Treg exhibit significant suppressive capacity against both CD4+ and CD8+ T cells (Fig. 3A). Although ST2− Treg consistently proved more potent than ST2− Treg, we did not observe a significant difference between the ST2− and ST2+ subsets isolated from IL-33–treated mice.
Although much has been elucidated on the role of IL-33 and ST2 in promoting Th2 responses, work with our collaborators revealed that IL-12 induces ST2 expression on CD8<sup>+</sup> T cells, thus promoting IL-33–augmented IFN-γ production (13). Our present data recapitulate these observations (Fig. 3B). However, we also reveal that, although Treg from IL-33–treated mice did not inhibit CD8<sup>+</sup> T cell IFN-γ driven by IL-12 alone, they potently suppressed IL-33–augmented CD8<sup>+</sup> T cell IFN-γ expression (Fig. 3B). ST2<sup>+</sup> Treg reduced IFN-γ production by ∼50% and displayed a trend toward greater suppressive capacity relative to ST2<sup>−</sup> Treg from IL-33–treated mice (Fig. 3B). In contrast, ST2<sup>−</sup> Treg from PBS-treated mice did not significantly impact IFN-γ expression at ratios examined (data not shown). In total, these data establish that IL-33–expanded Treg are effective T cell suppressors and, to our knowledge, are the first to make the important observation that IL-33–expanded Treg can limit IL-33–augmented IFN-γ production by CD8<sup>+</sup> T cells.

IL-33–exposed DC promote Th2 cytokine responses and proliferation of ST2<sup>+</sup>Foxp3<sup>+</sup> Treg in vitro

Administration of Flt3 ligand in mice augments naturally occurring Treg through a profound expansion of DC (21, 24). Yet, when we directly compared administration of IL-33 to Flt3 ligand, IL-33 did not drastically augment CD11c<sup>+</sup> cell numbers, despite its ability to increase the incidence of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg (18). Thus, our past findings do not support increased DC numbers as a mechanism driving an increase in Treg following IL-33 delivery. However, DC express ST2 (20, 21, 27), and, therefore, we sought to determine whether IL-33 expansion of Treg is mediated instead through a direct impact of IL-33 on DC function.

Consistent with previous observations (21), DC exposed to IL-33 promoted Th2 responses in CD4<sup>+</sup> T cells, manifested in increased secretion of IL-5, but not IFN-γ (Th1) or IL-17A (Th17) (Fig. 4A). Interestingly, we observed IL-33–exposed DC also promoted the proliferation of ST2<sup>+</sup>Foxp3<sup>+</sup> Treg (Fig. 4B). Although TLR4 and ST2 are both part of the TLR/IL-1R family, the function of IL-33–exposed DC was distinct relative to DC exposed to LPS, which augmented IFN-γ and IL-17A levels, but failed to increase IL-5 (Fig. 4A) or proliferation of ST2<sup>+</sup> Treg (Fig. 4B). ST2<sup>+</sup>Foxp3<sup>+</sup> T cells expanded in vitro by IL-33–exposed DC expressed high levels of ICOS and CD44 (Fig. 4C), a phenotype that matches splenic ST2<sup>+</sup>Foxp3<sup>+</sup> Treg expanded by IL-33 administration in vivo (Fig. 2B). Ex vivo expanded ST2<sup>+</sup> Treg were functionally suppressive (Fig. 4D). Confirming that ST2 expression on DC was central to this phenomenon, ST2<sup>−/−</sup> DC were poor stimulators of ST2<sup>+</sup> Treg proliferation (Fig. 4E) compared with WT (ST2<sup>+/+</sup>) DC. This limited proliferation of ST2<sup>+</sup> Treg occurred despite the addition of exogenous IL-33 in the MLR, and the presence of ST2<sup>−/−</sup> CD4<sup>+</sup> T cells, which could potentially respond to IL-33 directly. Overall, these data support IL-33 targeting DC as a critical mechanism of ST2<sup>+</sup> Treg expansion.

IL-33 stimulates IL-2 production by CD11c<sup>+</sup> DC

Several groups have described limited phenotypic changes associated with the Th2-promoting capacity of DC exposed to IL-33 (21, 22). However, mechanisms underlying the ability of IL-33–exposed DC to influence T cell responses are unknown. We characterized DC phenotype and cytokine production following overnight culture with IL-33, and compared these findings with DC exposed to LPS. In stark contrast to stimulation with LPS, exposure of BALB/c DC to IL-33 had a subtle impact on surface marker expression, as we observed a small, but significant increase in CD86 and programmed death ligand-1 (PD-L1), but no significant change in MHC class II (I-A<sup>B</sup>), CD40, CD80, or programmed death ligand-2 (PD-L2) (Fig. 5A). Similar results were observed when DC were propagated from B6 mice (data not shown). Furthermore, unlike LPS, IL-33 did not induce significant production of proinflammatory cytokines (Fig. 5B), indicating that IL-33 fails to induce robust DC maturation. In fact, even when DC were pre-exposed to IL-33, they maintained their ability to respond to subsequent stimulation with LPS (Fig. 5C).

Past investigation revealed that IL-2 signaling promotes ST2 expression on CD4<sup>+</sup> T cells (31), and a growing body of literature suggests that pathogen-exposed DC are an unappreciated, yet
significant source of IL-2 for T cells (32, 33). We now make the important observation that IL-33 stimulated a 5- to 6-fold increase in IL-2 secretion compared with levels produced by control DC (Fig. 5D, Supplemental Fig. 1). This finding was substantiated using IL-2−/− DC (Fig. 5D) and DC propagation conditions with and without IL-4 (Supplemental Fig. 1). Interestingly, IL-33 did not increase CD25 expression on DC above levels on control DC, whereas LPS induced a 2.5-fold increase in its expression (Fig. 5E). Collectively, these data indicate that by maintaining low CD25 expression and significantly increasing IL-2 production, DC exposed to IL-33 provide a significant source of IL-2 to support ST2+ Treg expansion.

CD11c+ DC production of IL-2 is necessary for IL-33–mediated proliferation of ST2+ Treg

The role of IL-2 in driving T cell–mediated immune responses is well established (34–36). More recently, DC delivery of IL-2 to Treg within the synapse has been suggested to facilitate Treg function and expansion (37, 38). Our data to this point argue that enhanced production of IL-2 by DC in response to IL-33 may be a critical mechanism through which IL-33 promotes ST2+ Treg proliferation. To test this, we added a neutralizing Ab against IL-2 to the MLR and observed complete inhibition of ST2+ Treg proliferation driven by IL-33–exposed DC (Fig. 6A, 6B). Importantly, IL-33–DC (and control DC + IL-33) demonstrated a preferential stimulation of ST2+Foxp3+ cells (Fig. 6C) over ST2−Foxp3+ cells, and all Foxp3+ T cells. To confirm that IL-2 produced by IL-33–exposed DC was critical for ST2+ Treg expansion, we repeated the experiment with DC propagated from IL-2−/− mice and observed that, unlike WT DC, IL-33–exposed IL-2−/− DC did not expand ST2+ Treg beyond that of their untreated counterparts (Fig. 6D). Interestingly, IL-2−/− DC also showed reduced expression of ST2 and CD25 (Supplemental Fig. 2). Overall, these data indicate that innate IL-2 signaling in DC is necessary for steady-state expression of ST2 and, presumably, their subsequent ability to respond to IL-33 with IL-2 production that drives selective expansion of ST2+ Treg.

In both control DC and IL-33–exposed DC cultures, exogenous IL-2 alone did not significantly expand ST2+ Treg, nor did it augment IL-33–mediated ST2+ Treg expansion (Fig. 6A, 6B).
Instead, the addition of exogenous IL-2 did display a trend toward increasing the incidence of ST2⁺ non-Treg (Fig. 6C). Addition of IL-33 to control DC cultures, however, profoundly increased ST2⁺ Treg (Fig. 6), and this effect was ablated by addition of anti–IL-2 Abs (Fig. 6). Thus, treatment of DC with IL-33, either before or during culture with CD4⁺ T cells, supports the selective expansion of suppressive ST2⁺ Treg over ST2⁻ Treg and non-Treg.

Following IL-33 administration (Fig. 2B) or culture with IL-33–exposed DC (Fig. 4C), ICOS⁺CD44⁺ST2⁺ Treg are the dominant expanded Treg population. However, the addition of anti–IL-2 reduced ICOS⁺CD44⁺ST2⁺ Treg incidence to the same level of that of control DC alone (Supplemental Fig. 3A), and similar results were observed for CD44⁺ST2⁺ Treg (Supplemental Fig. 3B). Correspondingly, unlike WT DC, IL-33–exposed IL-2⁻/⁻ DC failed to significantly increase either CD44⁺ or ICOS⁺ ST2⁺ Treg (Supplemental Fig. 3C). These in vitro data further support a mechanism in which IL-2 production by IL-33–exposed DC underlies ICOS⁺CD44⁺ ST2⁺ Treg proliferation and correlates with our ex vivo analysis of ST2⁺ Treg following IL-33 administration (Fig. 2B). These data lend strong support for DC as the critical mediators of IL-33–induced expansion of ST2⁺ Treg in vivo.

CD11c⁺ DC mediate expansion of ST2⁺ Treg by IL-33 in vivo

The ability of IL-33 to increase Treg in vivo is dependent on expression of ST2 (18), and the present data reveal that IL-33–exposed DC expand ICOS⁺CD44⁺ST2⁺ Treg in vitro that parallel those expanded by IL-33 in vivo. The failure of ST2⁻/⁻ DC to expand ST2⁺ Treg suggests DC may promote this phenomenon in vivo. To test this, we generated BM chimeras whereby...
WT B6 mice were reconstituted with BM from CD11c-DTR mice, or WT B6 as controls. Recipient mice were subsequently administered diphtheria toxin (DT) to effectively deplete CD11c+ DC concurrently during IL-33 administration (Fig. 7A). Nonirradiated controls, mice reconstituted with WT B6 BM, and mice reconstituted with CD11c-DTR BM all show significant expansion of ST2+ Treg following IL-33 treatment as compared with PBS-treated controls (Fig. 7B). As reported previously (23), administration of DT alone to CD11c-DTR chimeric mice reduced the frequency of total CD4+Foxp3+ Treg (Fig. 7B). Although depletion of CD11c+ cells alone did not modulate incidence of existing ST2+Foxp3+ cells, IL-33 administration failed to expand ST2+
Treg when CD11c+ cells were ablated concurrently (Fig. 7B). These critical data are in accordance with our findings in vitro and again reveal that, although T cells, including Treg, may express ST2, IL-33 does not facilitate expansion of ST2+ Treg in the absence of CD11c+ DC that can respond to IL-33 (Fig. 8).

**Discussion**

IL-33 is expressed by epithelial and endothelial cells in most organs (1, 39, 40), and proinflammatory cytokines or TLR ligands increase its expression in tissues and myeloid cells (14, 41–44). Any means supporting IL-33 secretion are uncertain, yet unprocessed IL-33 is functional when released during necrosis (6, 45). Thus, IL-33 is described as an alarmin, or endogenous factor activating immune responses when released from damaged tissue (14, 46–48). DC, expressing TLR/IL1R family members such as TLR4 and ST2 (20, 21, 27), are well equipped to recognize alarmins, such as IL-33 or high-mobility group box 1, which, like LPS, targets TLR4 (49). Both LPS and high-mobility group box 1 mature DC into potent immunostimulatory cells that provide the ample MHC, costimulatory molecules, and IL-1β, -6, -23, and IL-12p70 needed for Th1 and Th17 polarization (50). Likewise, it has been recently established that IL-12p70 potently inhibits Treg proliferation (51). Thus, IL-33 is an unconventional alarmin that fails to induce classical DC maturation or support Th1 immunity. Although we substantiated a capacity of IL-33–exposed DC to generate Th2 responses (21), we establish that IL-33–exposed DC support expansion of ST2+Foxp3+ Treg. In total, these data suggest IL-33 is an unconventional alarmin that preserves DC in a relatively immature state, but induces functional changes mediating their ability to polarize naive T cells into Th2 effectors and expand Treg.

DC have the ability to produce IL-2 following TLR stimulation (32, 33), and our present analysis revealed significantly elevated IL-2 production by DC following overnight stimulation with IL-33. When DC were generated from BM in GM-CSF and IL-4, IL-33 stimulated more IL-2 production by DC compared with LPS, whereas LPS induced increased IL-2 compared with IL-33 when DC were propagated using GM-CSF alone. Nonetheless, IL-33 induced a significant increase over control DC in both culture conditions, and the overall fold increase was similar in DC from cultures with GM-CSF with or without IL-4. Also, whereas we observed lower IL-2 produced by DC generated in GM-CSF alone in response to LPS compared with amounts previously reported (52), our studies used 100-fold less LPS (100 ng/ml versus 10 μg/ml). In total, these data demonstrate that IL-33 stimulates DC IL-2 secretion in vitro and suggest that DC signaling induced by IL-4, or closely related IL-13, may shape ST2 signaling to promote IL-2 secretion.

IL-33 initiates ST2 signaling that leads to activation of NFAT and AP-1 (53, 54), two transcription factors that are critical for IL-2 production (36). Interestingly, NFAT family members have also recently been implicated in the regulation of monocytic and macrophage TLR-mediated responses (55). As ST2 is a member of the TLR/IL-1R superfamily, our observation that IL-33, like LPS, induces IL-2 is consistent with a common signaling pathway shared between TLR4 and ST2 (7). However, IL-33 induces IL-2 production, yet fails to initiate the profound DC maturation triggered by LPS exposure. Given these different functional outcomes, it will be important to complete future studies identifying overlap...
FIGURE 6. CD11c+ DC-derived IL-2 promotes selective expansion of ST2+ Treg following IL-33 exposure. CD11c+ (A–C) BALB/c, (D) WT B6, or IL-2−/− DC were cultured overnight in media alone or supplemented with IL-33 (20 ng/ml). DC were cultured in MLR with CTV-labeled (A–C) B6 FIR or (D) BALB/c CD4+ T cells for 5 d. Some wells were supplemented with IL-33 (10 ng/ml), neutralizing IL-2 Ab (10 μg/ml), IL-2 (50 U/ml), or a combination, as indicated. After 5 d, cells were harvested and stained for flow cytometric analysis. Representative flow plots and an average of (A) Foxp3 and (B) Foxp3 and ST2 expression on CD4+ cells or (C) ST2 expression versus CTV on CD4+ Foxp3+ (top panels) and CD4+Foxp3− (bottom panels) cells. Results in (A–C) were averaged from n = 4 independent experiments. (D) Representative flow plots of ST2 expression versus CTV on CD4+Foxp3+ cells from cultures with WT B6 or IL-2−/− DC and averaged from n = 3 independent experiments. The graph represents the fold change in
and divergence in NFAT, AP-1, and other pathways in DC involved in TLR–IL-1R family signaling following IL-33 stimulation to that generated by TLR ligands, such as LPS.

IL-2 is known to induce ST2 expression by CD4+ T cells, especially in conjunction with IL-33 (31, 56). Moreover, IL-2 is both important for Th2 differentiation (57) and critical for Treg development (35, 37, 58). We demonstrate that neutralization of IL-2 in the presence of IL-33–exposed DC completely blocks ST2+ Treg expansion. Because activated T cells are a major source of IL-2 (34), the importance of DC-derived IL-2 in ST2+ Treg expansion was established using DC generated from IL-2−/− mice. Significantly, IL-33–exposed IL-2−/− DC failed to induce proliferation of ST2+ Treg over that of untreated DC, which is in contrast to their WT counterparts. The importance of IL-2 derived from IL-33–exposed DC in selective ST2+ Treg expansion is further supported by our observation that addition of exogenous IL-2 alone to control or IL-33–exposed DC–T cell cocultures failed to significantly augment ST2+ Treg proliferation. In total, our data support DC delivery of IL-2 to CD25 high Treg, potentially into the DC:Treg synapse (38), as a driving factor underlying their ability to expand ST2+ Treg over other T cell subsets during interactions with the bulk CD4+ T cell population. However, additional experimentation is necessary to precisely define whether ST2-mediated cross-presentation of IL-33 by DC plays any role in the expansion of ST2+ Treg or how the function and homeostasis of ST2+ Treg are impacted by IL-33 once innate IL-2 has initiated their expansion.

A related and intriguing finding was that LPS induced a prominent increase in CD25 expression on DC, whereas IL-33 did not. Presumably, this permits maximum availability of innate IL-2 to target local cells. Furthermore, IL-2−/− DC themselves exhibit reduced ST2 expression, supporting a mechanism in which innate IL-2 facilitates both autocrine and local cell expression of ST2. Based on these data, we propose a regulatory loop in which proliferating ST2+ cells with IL-33 DC versus control DC for WT and IL-2 KO. Black boxes on flow plots indicate populations used to generate corresponding graphs. *p < 0.05, **p < 0.01, ***p < 0.001. AU, arbitrary units for fold change reported in graphs in (C) and (D).
FIGURE 8. IL-33 stimulates IL-2 secretion by CD11c+ DC that drives ST2 expression and ST2+ Treg expansion. LPS signaling through TLR4 induces classical DC maturation characterized by upregulation of costimulatory molecules and production of proinflammatory cytokines such as IL-12 that drive Th1 immune responses in naive CD4+ T cells. DC exposed to LPS secrete little IL-2, but upregulate CD25 to deplete available IL-2. A baseline level of IL-2 secretion is required for expression of ST2 on DC in the steady state, which may control their ability to respond to IL-33. Exposure of DC to IL-33 enhances their production of IL-2, but does not alter expression of costimulatory molecules or proinflammatory cytokine production. CD25 levels remain constant, thus allowing for maximum bioavailability of IL-2 for T cells. DC-derived IL-2 promotes ST2 expression and expansion of ICOS+ CD44high CD44high Foxp3+ Treg, which constitutively express high levels of CD25. IL-2 may also support ST2 expression on naive CD4+ T cells and promote the development of Th2 responses by IL-33–exposed DC.

inmate IL-2 regulates ST2 expression on local leukocytes to promote Th2 responses and preferentially expand ST2+ Treg (Fig. 8).

We previously demonstrated that administration of IL-33 requires recipient ST2 expression and Treg to promote heart transplant survival (18). The present examinations reveal that ST2+ Treg are present in the primary and secondary lymphoid organs of naive mice and profoundly expand after IL-33 administration with the assistance of DC. A population of ST2-expressing Treg was shown recently to infiltrate damaged muscles and facilitate their repair (59). Thus, ST2+ Treg may possess unique biologic functions in addition to their demonstrated suppressive capacity. Other groups have described CD44high and ICOS+high Treg subsets that exhibit enhanced suppressor function compared with their corresponding counterparts (27, 28). We found that ST2+ Treg exhibited the greatest suppressor activity against CD8+ effector T cell function, including an ability to dampen IL-33–augmented CD8+ T cell IFN-γ production. Therefore, in circumstances in which IL-33, released as an alarm, may act on other ST2+ leukocytes and promote disease immunopathology (8–11), consumption of IL-33 by ST2+ Treg may be an important regulator of IL-33–associated inflammatory responses. Given the ability of IL-33–exposed DC to selectively expand ST2+ Treg over non-Treg, it is easy to envision the development of IL-33–exposed DC as a means to facilitate Treg expansion to support efforts for their use as regulatory cell therapy to prevent graft-versus-host disease following hematopoietic stem cell transplant or support induction of transplantation tolerance (60, 61).

This study makes several new observations regarding IL-33, DC, and Treg immunobiology. We identify an unappreciated mechanism of IL-33–mediated immune regulation in which ST2+ Treg are expanded through IL-33–driven production of IL-2 by CD11c+ DC. The result of this newly established IL-33/IL-2 axis in CD11c+ DC is the ability to preferentially expand an activated subset of suppressive Foxp3+ Treg expressing ST2. Finally, our demonstration, both in vitro and in vivo, that IL-33–exposed DC potently and selectively expand ST2+ Treg sets the stage to develop and potentially harness this new knowledge in ongoing experimental and clinical attempts to apply the regulatory capacity of both DC and Treg.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. BM-derived DC generated in the absence of IL-4 secrete IL-2 in response to IL-33. (A) BALB/c BM-derived DC were generated in 7 d culture using GM-CSF alone, and purified CD11c+ cells were cultured for 18 h in medium alone or supplemented with IL-33 (20 ng/ml) or LPS (100 ng/ml) Supernatants were harvested for IL-2 quantitation by ELISA. (B) Fold change in IL-2 production induced by IL-33 compared to control DC with GM-CSF alone or GM-CSF + IL-4 (using data from Fig. 5) was calculated. Data represents (A) triplicate wells from one experiment representative of three independent experiments, or (B) an average of three independent experiments. ***p < 0.001.
Supplemental Fig. 2

Supplemental Figure 2. Innate IL-2 signaling in CD11c⁺ DC promotes ST2 and CD25 expression. WT B6 or IL-2 KO BM-derived CD11c⁺ DC were generated in 7 d culture, cultured overnight in media alone, and then stained for flow analysis of ST2 and CD25 expression. Representative flow plots and histograms depicting ST2 and CD25 expression on CD11c⁺ cells. Results were averaged from n = 3 independent experiments. *p < 0.05, **p < 0.01.
Supplemental Figure 3. IL-33-exposed DC expand ICOS<sup>hi</sup>CD44<sup>hi</sup> ST2<sup>+</sup>Foxp3<sup>+</sup> Treg. (A,B) BALB/c or (C) WT or IL-2 KO B6 CD11c<sup>+</sup> DC were cultured in media alone or supplemented with IL-33 (20 ng/ml). After 18 h, DC were harvested, washed, and cultured in MLR with allogeneic CD4<sup>+</sup> T cells for 5 days. Some wells were supplemented with IL-33 (10 ng/ml), neutralizing IL-2 antibody (10 µg/ml), recombinant human IL-2 (50 U/ml), or a combination as indicated. After 5 d, cells were harvested and stained for flow cytometric analysis of (A,C) ICOS or (B,C) CD44 expression on CD4<sup>+</sup>Foxp3<sup>+</sup>-gated cells. Data are representative of and averaged from n = 4 (A,B) or n = 3 (C) independent experiments. For (C), the frequency of ICOS<sup>hi</sup>ST2<sup>+</sup> or CD44<sup>hi</sup>ST2<sup>+</sup> cells with IL-33 DC relative to Control DC was calculated to generate the graph of Fold Change. AU = arbitrary units. *p < 0.05, **p < 0.01, ***p < 0.001.