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Inducible IL-33 Expression by Mast Cells Is Regulated by a Calcium-Dependent Pathway

Chia-Lin Hsu and Paul J. Bryce

IL-33 is an IL-1 family cytokine that displays dual functions: a cytokine via its receptor, T1/ST2, or a chromatin-binding factor within the nucleus. Functionally, it promotes Th2-associated immunity by enhancing the activation and survival of several cell types. However, the pathways regulating IL-33 expression are still unclear. Although several cells display constitutive expression of IL-33, we showed previously that mast cells expressed low levels of IL-33 constitutively but that IL-33 was induced upon IgE-mediated activation. This was mediated via a calcium-dependent mechanism. In this study, we define the pathway through which this inducible IL-33 is regulated. Importantly, this pathway does not alter expression in cells with high constitutive IL-33 expression, such as epithelial cells or fibroblasts. Our data show that, upstream of calcium, inhibition of PI3K and Sphk activity decreases inducible IL-33 expression to IgE/Ag activation. Additionally, expression of Sphk1 short hairpin RNA prevents upregulation of IL-33 expression. Downstream of calcium, NFAT activity is necessary and sufficient for inducible IL-33 expression. We also demonstrate calcium-dependent transcription from two regions of the IL-33 gene that contain putative NFAT-binding sites, one upstream of exon 1 and one upstream of the start site. Interestingly, we show that blocking other calcium pathways, including inositol triphosphate receptor, or NF-kB inhibits IgE-driven IL-1ß, another IL-1 family cytokine, but it has no influence on inducible IL-33 expression. In summary, our data demonstrate cell-specific differences in the regulation of IL-33 expression and define a pathway critical for the expression of inducible IL-33 by mast cells upon their activation. The Journal of Immunology, 2012, 189: 3421–3429.

Interleukin-33 (IL-1F11, NF-HEV) belongs to the IL-1 cytokine family, which also includes IL-1α, IL-1β, IL-1R antagonist, and IL-18 (1). It was originally described as a nuclear located transcriptional regulator that associated with chromatin via a helix-turn-helix-like motif in its N terminus (2, 3). However, in 2005, IL-33 was reported as the ligand for the receptor T1/ST2 (DER4, Fit-1) (1). Since then, T1/ST2, in association with the IL-1R accessory protein (4, 5), has been shown to facilitate a diverse range of immunoregulatory influences in response to IL-33, which include stimulating Th1/Th2-associated (1, 6–8) and proinflammatory cytokine production (9), as well as survival (6, 8), migration (6, 10), and adhesion (6, 8) of T1/ST2-expressing cells. Furthermore, IL-33 has become increasingly established as functionally important in a variety of Th2-associated immune responses, including allergic airway inflammation (11–13), helminth expulsion (14), allergic conjunctivitis (15), and anaphylaxis (16). However, many of these studies used administration of exogenous rIL-33, and consequently very little is known about the pathways that regulate IL-33 expression.

Several cell types have been shown to exhibit constitutive expression of IL-33; these include endothelial cells, epithelial cells, fibroblasts, and pancreatic stellate cells (17–20). In these studies, IL-33 was present predominantly within the cell nucleus. In the context of regulation, IL-33 levels were increased in endothelial cells when they became super confluent, but they were then downregulated upon proinflammatory cytokine stimulation (17). Conversely, IL-33 expression in pancreatic stellate cells was upregulated upon stimulation by either TNF-α or IL-1ß, and also in fibroblasts by the combination of TLR3 stimulation and TGF-β (18). Similarly, TLR3 stimulation increased IL-33 expression in mucosal epithelial cells; this TLR3 stimulation was shown to be NF-kB dependent, as a potent NF-kB pathway inhibitor, BAY 11-7082, blocked this increased expression (21).

More recently, IL-33 has been found to be expressed in an inducible fashion by some immune cells, including dendritic cells (22), monocytes/macrophages (23–26), and mast cells (25, 27). In these studies, whereas IL-33 is induced upon cell activation, constitutive expression of IL-33 appears to be low or absent. For example, our previous work demonstrated that whereas unstimulated mast cells possess very low basal IL-33 expression, Ag-mediated cross-linking of IgE on FcεRI led to significant increases in mRNA and protein (27). Interestingly, similar to TLR3, the IL-33 receptor T1/ST2 also signals via NF-kB (as well as MAPKs) (1), and yet IL-33 did not promote further IL-33 in mast cells, despite being a potent inducer of several other mast cell-derived cytokines (27–29). Instead, IL-33 expression in mast cells was calcium sensitive, as EDTA addition prevented IgE-mediated expression and ionomycin was sufficient to induce expression. However, the molecular mechanism behind this calcium-sensitive inducible IL-33 expression in mast cells remains unclear, and we therefore wanted to elucidate this pathway. Although there are many pathways that can lead to calcium mobilization in mast cells
upon cross-linking of FcRI, two major pathways that have been shown to regulate mast cell responses are 1) sphingosine-1-phosphate (SIP1) generated via the sphingosine kinases (Spkks), and 2) inositol triphosphate (IP_3) binding to its receptor, IP_3R (30). Furthermore, mast cells express two isoforms of Spk, Spk1 and Spk2, that have been shown to exert differential effects on cytokine production and degranulation of mast cells (31). This is regulated by PI3K, where PI3K is required for full activation of Spk1 but not for Spk2 (32).

In this study, we show that inducible IL-33 expression by mast cells upon cross-linking of FcRI is dependent on a PI3K-Sphk1/Sphk2 pathway. Furthermore, NF-κB is required for this IL-33 expression but is required for concurrent IL-1β expression. We also identify two regulatory regions upstream of the il33 transcription start site that contain putative Nfat binding sites and support calcium-driven transcription. However, in cell types that exhibit constitutive expression, activation of this calcium-dependent pathway had little to no effect on IL-33 mRNA levels, suggesting that IL-33, in addition to having dual functions, also possesses dual pathways that regulate its expression.

**Materials and Methods**

**Cell lines and reagents**

N1N1-dimethyl-n-erythro-sphingosine (DMS) was obtained from Bionol International. 2-Aminooxyethyldiphenyl borate (2APB), LY294002, cytochimerin, mouse IgG (SP-7 clone), and DNP-human serum albumin (DNP-HSA) were obtained from Sigma-Aldrich. Wortmannin, BAY 11-7085, and inhibitor of NFAT-calcinucin association-6 (INCA-6) were obtained from Calbiochem. W146 (trithioacetate salt) and JTE-013 were purchased from Cayman Chemical. TaqMan gene expression primers and probes were obtained from Applied Biosystems. Primers were obtained from Invitrogen. pLG.3-basic and pLG.3-control (Invitrogen) and JTE-013 were purchased from Cayman Chemical. TaqMan gene expression primers and probes were obtained from Applied Biosystems. Primers were obtained from Invitrogen. pLG.3-basic and pLG.3-control plasmid, lactate dehydrogenase (LDH) assay, and Dual-Glo luciferase assay were obtained from Promega. Fluoro-4 was obtained from Invitrogen. Anti-mouse CD16/32 (2.4G2), allophycocyanin-anti-mouse CD117 (c-Kit), PE-anti-mouse FcεRI (MAR-1), and FITC-anti-γ2A were obtained from BD Biosciences. Allophycocyanin-anti-rat IgG (Fab′)2 was from eBioscience. pGFP-V-RS plasmids with or without scrambled, Spk1, or Spk2 short hairpin RNA (shRNA) were purchased from OriGene Technologies. Amma mouse dendritic cell transfection buffer was obtained from Lonza. Monoclonal anti-mouse IL-33 Ab (clone 396118) and anti-mouse CD16/32 Ab were purchased from eBioscience or R&D Systems. Fixation and permeabilization buffers were from eBioscience or R&D Systems. The mouse fibroblast cell line, NIH3T3, was provided by Dr. Christian Strehl (Northwestern University). The mouse endothelial cell line HEVα was provided by Dr. Joan Cook-Mills (Northwestern University). The mouse mast cell line MC/9 and the mouse intestinal epithelial cell line CMT93 were purchased from the American Type Culture Collection.

**Bone marrow-derived mast cell generation and activation**

Bone marrow-derived mast cells (BMMCs) were obtained by flushing bone marrow from femurs using complete RPMI 1640 media. 10% FBS, penicillin/streptomycin, l-glutamine, and nonessential amino acids. Cells were then cultured in mast cell growth media (complete RPMI 1640 media plus 30 ng/mL recombinant mouse IL-3) for 5 wk. Mast cell phenotype was determined by flow cytometry for c-Kit/FcεRI+ cells (>95%). Mast cells were primed with 1 μg/mL DNP-specific IgE overnight and then activated with 0.5 μg/mL DNP-HSA or 0.25 μM ionomycin for 4 h for gene expression or 24 h for protein expression. In some experiments, mast cells were pretreated with inhibitors 30 min prior to DNP-HSA or ionomycin activation.

**Real-time RT-PCR**

RNA from treated cells was generated by an RNasy RNA isolation kit (Qiagen). cDNA was synthesized by qScript cDNA SuperMix (Quantab Bioscience). Gene expression was determined by real-time PCR using an ABI 7500 thermal cycler (Applied Biosystems) and specific TaqMan probes (Applied Biosystems) for each gene of interest. β-actin expression was used as an internal control, and changes in the threshold cycle values were determined, as previously described (33).

**Intracellular staining**

BMMCs or MC/9 cells were activated with IgE/DNP or 1 μM ionomycin with or without inhibitor treatment for 24 h. Cells were stained with allophycocyanin-anti-mouse CD117 following by fixing and permeabilizing with fixation and permeabilization buffer (eBioscience or R&D Systems). Cells were then stained with monoclonal anti-mouse IL-33 or isotype Ab, followed by FITC-anti-rat IgG2a or allophycocyanin-anti-rat IgG (Fab′)2.

**Calcium mobilization assay**

BMMCs were primed with 1 μg/mL DNP-specific IgE overnight. Cells were then incubated with calcium-free buffer (HBSS, 2 mM proenecid, and 0.1% BSA) containing 1.5 μM fluo-4 for 30 min and transferred to calcium-containing buffer (HBSS, 2 mM proenecid, 0.1% BSA, and 1.8 mM calcium). Cells were treated with different inhibitors, and calcium flux was measured 2 min prior to 0.5 μg/mL DNP-HSA or DMSO stimulation. Cells were also stimulated with 0.25 μM ionomycin to confirm the ability to induce calcium flux in the presence or absence of inhibitors. Calcium flux was analyzed by flow cytometry. The effect of all inhibitors on calcium flux upon IgE activation of mast cells is shown in Supplemental Fig. 1.

**Construction of il33 regulatory region reporter assays**

Putative Nfat binding sites were determined using Patch public 1.0 software (BioBase). The region between −1000 and −1 of il33 upstream of exon 1 (il33-exon) was cloned from C57BL/6 genomic DNA using the following primers: forward, 5′-ACGAAATCTTTTGTACGATGC-3′, reverse, 5′-AACACAGTGCGCTTCAG-3′. HindIII and XhoI restriction enzyme sites were introduced to the ends of il33-exon using the following primers: forward, 5′-CTCGAGACAATCTTTAGCAG-3′, reverse, 5′-AAGCTTGACAGGCGCTTCAG-3′. The region between −1000 to +100 within il33 intron 1 relative to the ATG transcriptional start site (il33-ATG) was cloned from C57BL/6 genomic DNA using the following primers: forward, 5′-AGGTTTCTCTGCTCGTCG-3′, reverse, 5′-AAGGACCGAGGCTTCGCTC-3′. HindIII and XhoI restriction enzyme sites were introduced to the ends of il33-ATG by the following primers: forward, 5′-CTCGAGACAATCTTTAGCAG-3′, reverse, 5′-AAGCTTGACAGGCGCTTCAG-3′. The region upstream of il33-exon and il33-ATG were cloned into the pGL3-basic plasmid (Promega) using HindIII and XhoI.

**BMMC transfection**

BMMCs were electroporated by Amaxa Nucleofector II (Lonza) using mouse dendritic cell buffer (Lonza) and program C-005. Cells were suspended in mast cell culture medium with 30 ng/mL IL-3 immediately after electroporation.

**Luciferase assays**

BMMCs (1 x 10^5) were cotransfected with 1 μg of il33-exon, il33-ATG, or pGL3-basic (mock) with 0.8 μg pRLSV40 plasmid (Promega) to control for efficiency of transfection. Twenty-four hours after transfection, cells were lysed with lysis buffer (Promega) and luciferase activity was analyzed using the Dual-Glo luciferase assay system (Promega). The firefly luciferase activity was normalized to the Renilla luciferase activity. The firefly/Renilla ratio was expressed as the relative luciferase unit. The fold induction above the control group (unstimulated) was determined based on changes in the relative luciferase unit values.

**shRNA plasmid transfection**

BMMCs (2 x 10^5) were electroporated with 4 μg scrambled shRNA-, Spk1 shRNA-, or Spk2 shRNA-expressing plasmids that coexpressed enhanced GFP (eGFP) as indicated by the Amaxa system, as previously described. Three days after transfection, cells were split into two wells (1 mL/well) with an extra 30 ng/mL IL-3. Seven days after transfection, mast cells were primed with DNP-IgE overnight and stimulated with DNP-HSA for 24 h. IL-33 expression after IgE-mediated activation was determined in eGFP cells versus eGFP cells by intracellular flow staining.

**NFAT overexpression**

A plasmid expressing a constitutively active (ca)NFAT-c1 mutant that coexpressed eGFP was a gift from Dr. Neil Clipstone (Loyola University, Chicago, IL) and was previously described (34). BMMCs were transfected with 200 ng caNFAT-c1 plasmid or control plasmid by the Amaxa system as previously described. IL-33 protein expression in eGFP cells versus eGFP cells was analyzed by intracellular staining 24 h after transfection.
**Results**

IL-33 expression is induced by calcium in mast cells but not in several other cell types

Because we previously demonstrated that ionomycin was sufficient to upregulate IL-33 gene expression in mast cells (27), we first explored whether IL-33 gene expression was calcium dependent in other cell types that had been previously reported to express IL-33. A murine fibroblast cell line (NIH3T3), endothelial cell line (HEVa), and mucosal epithelial cell line (CMT93) were tested, in addition to an immortalized murine mast cell line (MC/9) and primary BMMCs that served as positive controls. Cells were stimulated with 0.25 μM ionomycin for 4 h and IL-33 mRNA expression was analyzed by real-time PCR. We found that, as predicted, resting mast cells (both BMMCs and MC/9) express very low levels of IL-33 compared with the fibroblast, endothelial, or epithelial cells (Fig. 1A). Upon ionomycin stimulation, however, IL-33 expression was significantly increased in mast cells and HEVa endothelial cells, but not in either fibroblasts or epithelial cells. Interestingly, the relative expression levels were higher in epithelial and endothelial cells than in fibroblasts, as seen from the $2^{-ΔΔCT}$ values. Intracellular staining showed that the IL-33 produced by mast cells was present in both the nucleus and cytoplasm, although predominantly in the cytoplasm (Fig. 1B), which is in contrast to the predominantly nuclear IL-33 localization in endothelial or epithelial cells (17, 19) but is similar to IL-33 localization seen in monocytes (23). These results indicated that the regulation of IL-33 by calcium mobilization is cell type dependent and that the localization of IL-33 in mast cells after activation is predominantly cytoplasmic.

Sphk/SIP regulates IL-33 gene expression in mast cells

Because calcium was necessary to induce IL-33 expression in mast cells upon FcεRI cross-linking (27), we wanted to determine which pathways immediately downstream of FcεRI were required for calcium-mediated upregulation of IL-33. Because both IP$_3$R and Sphks are downstream of FcεRI signaling and were previously shown to regulate the expression of several cytokines from mast cells (35), we tested whether activation of either of these signaling mediators was necessary. BMMCs were primed with DNP-specific IgE overnight and treated with different doses of DMS, a potent inhibitor of the Sphks (36), or 2APB, an inhibitor of several calcium-dependent pathways, including IP$_3$R, 30 min prior to DNP-HSA activation. The blockade of IgE-mediated calcium flux by DMS is shown in Supplemental Fig. 1A and by 2APB in Supplemental Fig. 1B. Although DMS can also inhibit protein kinase C activity (37), we used a relatively low concentration of DMS that has been shown to inhibit Sphk function without significantly affecting protein kinase C activity (38). Cells were harvested 4 h after stimulation and IL-33 expression was analyzed. At the concentrations used, both inhibitors did not affect cell viability, as determined by an LDH release assay (data not shown). We found that IL-33 gene expression was inhibited by DMS and not by 2APB (Fig. 2A), indicating a role for the calcium activation pathway involving Sphks, but not IP$_3$R or the other pathways affected by 2APB. DMS decreased IL-33 gene expression in a dose-dependent manner (Fig. 2B), whereas 2APB did not affect IL-33 gene expression at any dose investigated (data not shown). DMS had no effect on ionomycin-stimulated IL-33 gene expression, as expected, because ionomycin induces calcium mobilization directly and does not require Sphk activation (Fig. 2C). In contrast to IL-33, IL-1β was inhibited by 2APB but not by DMS (Fig. 2D), confirming that IP$_3$R activation from FcεRI is required for expression of some cytokines, including other IL-1 family members. We conclude that IL-33 gene expression is therefore likely regulated by Sphk-S1P–induced Ca$^{2+}$ mobilization.

Autocrine effects of S1P regulate IL-33 gene expression in mast cells

S1P generated downstream of Sphk activation has been shown to be released by mast cells and act via S1P receptors expressed on the surface of mast cells in an autocrine manner to further increase calcium signaling (39). Mast cells express two S1P receptors,
S1PR1 and S1PR2 (31); we therefore used two specific inhibitors of S1PR1 and S1PR2 (W146 and JTE-013, respectively) to determine whether such autocrine effects through the S1P receptors influenced IL-33 gene expression. BMMCs were treated and activated as previously described and cell viability was determined by an LDH assay. IL-33 gene expression was inhibited by both W146 and JTE-013 (Fig. 3), although the highest dose of JTE-013 did slightly decrease mast cell viability, as determined by LDH release (data not shown). IL-6 secretion was also inhibited by both W146 and JTE-013 (data not shown), in agreement with previously published findings (40). These data indicate that S1P-mediated autocrine effects are also important for IL-33 expression.

**PI3K is important for IL-33 gene expression in mast cells**

Because mast cells have been shown to express two different Sphks, Sphk1 and Sphk2 (41), and PI3K is required for Sphk1 activation upon FceRI cross-linking but not for Sphk2 activation (32), we next investigated the effects of two inhibitors of PI3K activity, LY294002 and wortmannin. BMMCs were treated, activated, and analyzed as previously described, and the effects on IgE-mediated calcium flux by wortmannin is shown in Supplemental Fig. 1C and by LY294002 in Supplemental Fig. 1D. Both PI3K inhibitors suppressed IL-33 gene expression in a dose-dependent manner in response to IgE/DNP activation (Fig. 4A) without inducing cell death (data not shown). Supporting this result, we also determined that IL-33 protein induction in mast cells was inhibited by PI3K inhibition upon IgE/DNP stimulation using intracellular staining (Fig. 4B). However, ionomycin stimulation bypassed the inhibitory effects of blocking PI3K and induced IL-33 expression even in the presence of the PI3K inhibitors (Fig. 4C). Accordingly, IL-33 expression by mast cells upon activation of FceRI is dependent on PI3K, again supporting a role for Sphk1.

**Sphk1, but not Sphk2, shRNA inhibited IL-33 expression upon IgE/DNP stimulation**

DMS, the Sphk inhibitor we used in Fig. 2, is not selective for either Sphk1 or Sphk2 and so does not distinguish which Sphk was required for the calcium-mediated inducible IL-33 expression from mast cells, whereas inhibition of PI3K may influence other signaling pathways. To better define the role of Sphk1 over Sphk2, we therefore used shRNA knockdown of Sphk1 or Sphk2 in BMMCs using plasmids that expressed scrambled, Sphk1, or Sphk2 shRNA with eGFP coexpression to detect shRNA induction in transfected cells. Plasmid-transfected cells were gated on GFP+ cells. Although scrambled shRNA or Sphk2 shRNA had no effect on IL-33 induction, cells that expressed Sphk1 shRNA failed to upregulate IL-33 upon IgE/DNP activation (Fig. 5), indicating that Sphk1 was required for IL-33 expression in mast cells.

**NFAT regulates IL-33 gene expression in mast cells**

Because NFAT is a critical calcium-sensitive transcription factor downstream of calcium induction, we next wanted to examine the role of NFAT in the induction of IL-33 expression. BMMCs were pretreated with INCA-6, which is a specific peptide that interrupts the interaction between NFAT and calcineurin, and CsA; BMMCs were then activated by IgE/Ang or ionomycin as previously described. The absence of effect on IgE-mediated calcium flux by LY294002 and wortmannin. BMMCs were treated, activated, and analyzed as previously described, and the effects on IgE-mediated calcium flux by wortmannin is shown in Supplemental Fig. 1C and by LY294002 in Supplemental Fig. 1D. Both PI3K inhibitors suppressed IL-33 gene expression in a dose-dependent manner in response to IgE/DNP activation (Fig. 4A) without inducing cell death (data not shown). Supporting this result, we also determined that IL-33 protein induction in mast cells was inhibited by PI3K inhibition upon IgE/DNP stimulation using intracellular staining (Fig. 4B). However, ionomycin stimulation bypassed the inhibitory effects of blocking PI3K and induced IL-33 expression even in the presence of the PI3K inhibitors (Fig. 4C). Accordingly, IL-33 expression by mast cells upon activation of FceRI is dependent on PI3K, again supporting a role for Sphk1.

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**FIGURE 2.** IL-33 expression in mast cells is regulated by Sphk-induced calcium mobilization. BMMCs were pretreated with 10 μM DMS, 50 μM 2APB, or both for 30 min prior to activation by IgE/DNP (black bar). IL-33 (A) and IL-1β (D) gene expression was analyzed by real-time RT-PCR. (B) IL-33 expression was inhibited by DMS in a dose-dependent manner. (C) IL-33 expression was analyzed in BMMCs pretreated with 10 μM DMS and activated with IgE/DNP (black bar) or 0.25 μM ionomycin (gray bar). Data are from three independent experiments (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 3.** S1PR-mediated autocrine effects are required for IL-33 gene expression in mast cells. BMMCs were pretreated with different doses of W146 or JTE-013 30 min prior to activation by IgE/DNP. IL-33 gene expression was analyzed by real-time RT-PCR. Data represented five individual experiments, n = 3. *p < 0.05.
CsA is shown in Supplemental Fig. 1E and by INCA-6 in Supplemental Fig. 1F. IL-33 gene expression was significantly decreased in a dose-dependent manner by both inhibitors (Fig. 6A) without influencing cell viability (data not shown). INCA-6 and CsA also inhibited IL-33 gene expression upon ionomycin activation (Fig. 6B) as predicted, because NFAT activation lies downstream of calcium mobilization. Using intracellular staining, we observed that CsA and INCA-6 also blocked the upregulation of intracellular IL-33 protein in response to ionomycin (data not shown). Furthermore, to determine whether NFAT activity is sufficient to induce IL-33 expression, we overexpressed a caNFAT mutant (caNFAT-c1) in BMMCs. This caNFAT-c1 mutant contains an IRES-GFP, allowing for selection of transfected cells based on GFP expression. Overexpression of active NFAT was sufficient to increase IL-33 protein expression (Fig. 6C). These results indicate that IL-33 gene expression by mast cell is regulated by activation of the Ca\(^{2+}\)-sensitive transcription factor NFAT.

NF-κB is dispensable for IL-33 gene expression in mast cells

Although it is well established that calcium induces NFAT activation, calcium can also induce NF-κB activation (42). Additionally, the expression of constitutive IL-33 has been shown to be NF-κB driven in mucosal epithelial cells (21), and the other IL-1 family cytokines, IL-1β and IL-18, are regulated by NF-κB (43). We therefore wanted to examine whether NF-κB was also needed for IL-33 expression by mast cells. Pretreatment of BMMCs with BAY 11-7085, the NF-κB inhibitor previously shown to block IL-33 expression in mucosal epithelial cells (21), 30 min prior to activation by cross-linking of the Fc\(\varepsilon\)RI did not affect IL-33 expression (Fig. 7A). Conversely, IL-1β expression was significantly inhibited in a dose-dependent manner (Fig. 7B). Taken together, these findings further support differences between the pathways regulating constitutive and inducible IL-33, as well as different pathways in the regulation of the IL-1 family cytokines upon Fc\(\varepsilon\)RI-mediated activation of mast cells.

Regulatory regions of \textit{il33} support calcium-dependent transcription

We next wanted to determine whether there were any regulatory regions of \textit{il33} that specifically conferred responsiveness to ionomycin. The ATG start codon of \textit{il33} is located at exon 2 (44); it has been previously shown that some genes can contain regulatory regions within the intronic region between exon 1 and exon 2 when the ATG is located at exon 2 (45, 46). For this reason, we
cloned both the upstream region from exon 1 of *il33* (bp 21600 to 21 from the start of exon 1; *il-33-exon) and the intron 1 of *il33* (bp 21000 to +100 from the ATG starting site of exon 2; *il-33-ATG*) into the pGL.3-basic luciferase plasmid (Promega) and investigated the ability of these regions to support luciferase expression that would indicate which region contained regulatory potential. BMMCs were transfected using Amaxa Nucleofector II with cotransfection of a *Renilla*-expressing control plasmid (pGL.3-RNL) to control for efficiency. Transfected cells were activated with 0.25 μM ionomycin for 24 h in the presence of vehicle, 10 μM INCA-6, or 1.5 μM CsA, and luciferase activity was analyzed. Interestingly, both *il-33-exon* (Fig. 8A) and *il-33-ATG* (Fig. 8B) supported ionomycin-induced transcription and this was inhibited by blockade of NFAT activation, suggesting that these regions may contain NFAT regulatory sites. Indeed, using Patch public 1.0 software (Biobase), we identified several putative NFAT binding sites (TTTCC, GGGAA, TGCTGA, or TCAGCA) within these two regions.

**Discussion**

IL-33 is being increasingly recognized as regulating immune responses in infection and allergic, autoimmune, and chronic inflammatory diseases from both patient studies and animal models (13). Consequently, the IL-33/ST2 pathway may have potential as a therapeutic target in various diseases (47). Despite this wealth of information on its functions, how IL-33 expression is regulated has remained unclear. Knowing the mechanisms of regulation may help target IL-33 in specific therapeutic ways.

From several studies that have investigated the cellular expression of IL-33, there would appear to be two prevailing findings. In some cells, such as epithelial cells, endothelial cells, and fibroblasts, IL-33 is constitutively expressed at high basal levels and is located within the nuclear compartment (17, 20, 21, 48). Conversely, in mast cells (as well as dendritic cells and monocytes or macrophages) IL-33 is induced upon cellular activation and, from the studies that have explored cellular location, this expression would seem to be predominately cytoplasmic (22–27). In our previous study, we saw no evidence for cleavage of IL-33 in mast cells (27), and so the cytoplasmic location is rather surprising, as full-length IL-33 has a chromatin-binding domain (3), likely responsible for its nuclear localization in most cells. We did determine that the intracellular detection of IL-33 in mast cells did not require bre-
felin A, and so IL-33 was likely not being transported via the Golgi apparatus, a finding that is consistent with IL-33 lacking a signal peptide sequence required for this pathway (49). However, mast cells are well recognized for containing several unique granules and vesicles, and thus one possibility is that IL-33 is exported via one of these unique mechanisms. However, despite this remaining area of query, the inducible nature of IL-33 expression in mast cells has allowed us to define the necessary pathways that lead to IL-33 transcription in these cells.

Interestingly, our findings seem to support divergent signaling mechanisms for regulating the expression of inducible versus constitutive IL-33. Previous findings had demonstrated the importance of TLR3-mediated NF-κB in regulating IL-33 levels in cell types with constitutive expression (18, 21). However, TLR3 stimulation failed to induce IL-33 in mast cells (data not shown) while the induction of IL-33 that did occur in response to FcεRI-mediated activation was not influenced by NF-κB inhibition. Conversely, IL-1β expression that occurred upon activation was highly NF-κB dependent. Consequently, our data would suggest that the expression of the IL-1 family members that occurs in mast cells is dependent on different signaling pathways mediated downstream of FcεRI.

To our knowledge, our study represents the first to define the cellular signaling pathways required for induction of IL-33 in inflammatory cells and has defined this as being regulated via NFAT. Interestingly, IL-33 itself has also been suggested to require calcium for its effects on mast cells because calcium chelation diminished IL-33–driven activation (16). However, the effect of IL-33 on many mast cell–derived cytokines, as well as prostaglandins and leukotrienes, were predominantly NF-κB dependent. Interestingly, IL-5 and IL-13 were not NF-κB dependent, suggesting that alternative signaling pathways from T1/ST2 regulate these cytokines. We previously demonstrated that IL-33 activation did not increase IL-33 expression and that T1/ST2 is actually shed from the mast cell surface upon FcεRI-mediated activation (27). Consequently, although signaling from T1/ST2 may be capable of inducing a calcium-associated response, this seems insufficient to activate the pathway needed for inducible IL-33 in mast cells. Therefore, whereas mast cells are capable of upregulating IL-33 upon Ag-triggered activation, this is unlikely to serve any autocrine or paracrine amplification influences due to reduction of surface ST2 expression and the lack of NFAT-mediated signals if binding was to occur. Andrade et al. (50) have demonstrated that IL-33 treatment delivered concurrently with Ag activation leads to an altered or stronger mast cell response, including NFAT activation. This was also dependent on NF-κB, being a direct influence of IL-33 and ST2 on NFAT. Consequently, we postulate a dual role for IL-33 whereby the release of constitutive IL-33 from epithelium or other cells during Ag exposure may enhance immediate inflammatory responses, whereas mast cell–derived IL-33 occurs later and mediates an alternative function, such as the recruitment of inflammatory cells after FcεRI-mediated activation that we previously demonstrated in a model of anaphylaxis (27).

In defining the pathway from FcεRI to IL-33 expression, we chose to specifically explore the S1P and IP3 pathways owing to the extensive evidence for these being critical pathways involved in IgE-mediated activation (35). However, other pathways may also contribute to mobilizing calcium in mast cells. The calcium

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**FIGURE 8.** Two regulatory regions of il33 support calcium-dependent transcription. The region between -1600 and -1 of il33 upstream according to exon 1 (il33-exon) and the region between -1000 and +100 within il33 intron 1 according to ATG transcriptional start site (il33-ATG) were cloned into pGL3-basic luciferase reporter plasmid. BMMCs were transfected with pGL3-basic (mock), il-33-exon (A), or il-33-ATG (B) together with pGL3-RNL as a transfection efficiency control. Cells were activated with 0.25 μM ionomycin for 24 h in the presence of vehicle, 10 μM INCA-6, or 1.5 μM CsA after transfection. Luciferase activity was analyzed 24 h after stimulation. Data represent four to five independent experiments. **p < 0.01, ***p < 0.001.

**FIGURE 9.** Pathway for inducible expression of IL-33 in mast cells. IL-33 expression in mast cells upon cross-linking of IgE receptors is regulated by a PI3K-Sphk1-S1P-NFAT pathway. NF-κB is dispensable for the expression of IL-33 and, instead, regulates IL-1β. S1P receptor also participates in regulating IL-33 expression.
release-activated calcium (CRAC) channel is a store-operated channel and is opened by the emptying of internal calcium stores (51). It has been shown that CRAC channels could prolong cytosolic increase of calcium and regulate degranulation, chemokine, and cytokine secretion in mast cells (52, 53). Our findings would support this pathway as not being necessary because 2APB, which can activate CRAC channels at concentrations of <10 μM or inhibit at >50 μM (54), did not alter IL-33 expression at the 50 μM dose upon IgE/DNP activation. Indeed, 2APB has been shown to exert potent effects independently of IP3R (54, 55). However, the lack of effects of 2APB on expression of IL-33 would suggest that these alternative pathways most likely do not participate.

Interestingly, in addition to divergence in the critical transcription factors, induction of IL-33 and IL-1β was also divergent at the level of the proximal signaling pathways required, as IL-33 was sensitive to the Sphk inhibitor DMS, whereas IL-1β was blocked by 2APB inhibition of the IP3/IP3R pathway.

Furthermore, our findings suggest that induction of IL-33 is dependent on Sphk1 rather than Sphk2. However, some contrasting results for the roles of Sphk1 and Sphk2 in mast cells have been reported, which may relate to the approach taken. In BMMCs, Sphk1 has been proposed to regulate Ag-induced calcium mobilization, degranulation, and migration (41); furthermore, Sphk1-deficient mice show less responsiveness in a systemic anaphylactic model (36). In contrast, Sphk2, and not Sphk1, was shown to modulate calcium influx and downstream signaling in fetal liver-derived mast cells (57). In adult mice, it has been suggested that compensatory pathways exist in the Sphk single knockout (41). We observed no differences in IL-33 expression in mast cells derived from bone marrow of either the Sphk1 or Sphk2 knockout (a gift from Dr. Juan Rivera, National Institutes of Health) (Supplemental Fig. 2), suggesting that such compensation may also impact IL-33. However, we think that the combination of evidence demonstrating the requirement for the Sphks generally and also PI3K and shRNA supports our conclusion that Sphk1 is mostly likely the critical pathway to IL-33 expression.

Our data also demonstrate that NFAT activity is important for IL-33 expression. Using bioinformatics-based analysis, we identified two potential regulatory regions, located upstream of exon 1 and within intron 1, which contained putative NFAT binding sites. Interestingly, although we show that both regions support ionomycin-driven transcription, the region upstream of il33 exon 1 had a higher capability to induce luciferase activity compared with the intron 1, suggesting that this region may be a primary regulatory region of IL-33 expression whereas intron 1 may play some enhancing role. Mast cells are known to express four members of the NFAT family (NFAT1–4) (58) and different NFAT members have been shown to modulate unique mast cell responses (59, 60). The authors have no financial conflicts of interest.

References


