European Journal of Immunology

Amplification of cytokine production through synergistic activation of NFAT and AP-1 following stimulation of mast cells with antigen and IL-33

Marcus V. Andrade[1,2], Shoko Iwaki[1], Catherine Ropert[4], Ricardo T. Gazzinelli[4], Jose R. Cunha-Melo[3] and Michael A. Beaven[1]

[1] Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA
[2] Department of Internal Medicine, School of Medicine, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[3] Department of Surgery, School of Medicine, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil
[4] Laboratory of Immunopathology, René Rachou Institute, FIOCRUZ, Belo Horizonte, MG, Brazil

IL-33 is associated with atopic and autoimmune diseases and, as reported here, it interacts synergistically with Ag to markedly enhance production of inflammatory cytokines in rodent mast cells even in the absence of degranulation. Investigation of the underlying mechanisms revealed that synergy in signaling occurred at the level of TGF-β-activated kinase 1, which was then transmitted downstream through JNK, p38 MAP kinase, and AP-1. Stimulation of the Ca^{2+}/calcineurin/NFAT pathway by Ag, which IL-33 did not, was critical for the synergy between Ag and IL-33. For example, selective stimulation of the NFAT pathway by thapsigargin also markedly enhanced responses to IL-33 in a calcineurin-dependent manner. As indicated by luciferase-reporter assays, IL-33 failed to stimulate the transcriptional activities of NFAT and AP-1 but augmented the activation of these transcription factors by Ag or thapsigargin. Robust stimulation of NF-κB transcriptional activity by IL-33 was also essential for the synergy. These and pharmacologic data suggested that the enhanced production of cytokines resulted in part from amplification of the activation of AP-1 and NFAT as well as co-operative interactions among transcription factors. IL-33 may retune mast cell responses to Ag toward enhanced cytokine production and thus determine the symptoms and severity of Ag-dependent allergic and autoimmune diseases.

Keywords: Ag · Cytokine · IL-33 · Mast Cells · Signaling mechanisms

Supporting Information available online

Introduction

Mast cells participate in innate and adaptive immune responses as well as allergic and autoimmune diseases [1, 2]. Their best known role is mediating IgE-dependent allergic reactions through their ability to respond to allergens with release of inflammatory mediators via degranulation, production of inflammatory lipids, and synthesis of cytokines. These responses can be substantially enhanced by endogenous and exogenous factors such as Kit ligand (stem cell factor), adenosine, prostaglandin E_2, and pathogenic TLR ligands [3, 4] and it is now apparent that the responses of mast cells to allergens

Correspondence: Dr. Michael A. Beaven
e-mail: beavenm@nhlbi.nih.gov

are regulated by a variety of endogenous and exogenous ligands [1, 5, 6].

In cultured mast cells, Ag-induced aggregation of FcεRI through IgE results in the activation of tyrosine kinases such as Lyn, Fyn, and Syk. The ensuing tyrosine phosphorylation of adaptor proteins enables recruitment of other signaling molecules [2, 7, 8]. These molecules include PI3K, phospholipase (PL) Cγ1/2, and small GTPases including Ras and Rac. Further downstream, PLC-mediated increases in intracellular levels of free Ca^{2+} ($[Ca^{2+}]_i$) and activation of PKC provide necessary signals for degranulation [9, 10], whereas the activation of MAPK via GTPases is essential for engagement of transcription factors such as Elk, c-Jun, c-Fos, ATF-2, and cMyc that promote cytokine gene transcription [8]. Additional cytokine-related transcription factors, NFAT and NF-κB, are activated through the Ca^{2+}/calcineurin and PI3K/PKC-dependent pathways, respectively [4, 11]. The recruitment and extent of Ag-induced activation of transcription factors is enhanced on co-stimulation of mast cells with Kit ligand [12] or TLR ligands [4]. As a consequence, the production of cytokines is markedly augmented in a synergistic manner.

The recently described cytokine, IL-33, is also reported to stimulate cytokine production, but not degranulation or eicosanoid production in mast cells [13–16] and basophils [17–20], and may enhance FcεRI and G protein-coupled receptor-mediated cytokine production in mast cells and basophils [14, 21], although the mechanism of this enhancement is unknown. IL-33 accumulates in the affected tissues of patients with Crohn's disease, rheumatoid arthritis, atopic dermatitis, psoriasis, and anaphylactic reactions – afflictions thought to be associated with activation of mast cells [22–27]. In animal models, IL-33 is reported to exacerbate autoantibody-induced arthritis with enhanced autoantibody-mediated mast cell degranulation in synovial tissues [28] and to be essential for the late-phase inflammatory reaction during passive cutaneous anaphylaxis [29]. The IL-33 receptor, ST2, is a unique member of the Toll/interleukin-1 receptor (TIR) family of receptors [30] but operates in conjunction with the IL-1 receptor accessory protein [31–33]. ST2, in common with IL-1/IL-18 receptors and TLR, contains a TIR domain which enables recruitment of the TIR domain-containing adaptor protein, MyD88, and in turn IL-1 receptor-associated kinase (IRAK) and TNF receptor-associated factor 6 (TRAF6) [34, 35]. The engagement of TRAF6 leads to the activation of two key transcription factors. These include the dimeric AP-1 through the JNK pathway as well as NF-κB through dissociation of the inhibitor of the NF-κB complex (IKK) from this complex. However, some aspects of IL-33-mediated signaling remain unresolved. p38 MAP kinase and ERK 1/2 are also activated in mast cells by IL-33 [14, 36] in a MyD88-dependent manner [15], but the downstream consequences are unclear. Also, it is not known if IL-33 operates through TGF-β-activated kinase 1 (TAK1), which plays a pivotal role in the activation of the NF-κB, p38 MAP kinase, and JNK pathways in some types of cells when stimulated with agonists of other TIR family members including IL-1, IL-18, or TLR ligands [37–39]. Nevertheless, ST2-mediated signals appear to share the same general features as those mediated by IL-1 receptors and TLRs.

In investigating the mechanism of action of IL-33, and its similarities to that of TLR ligands, we found that as little as 10 pg/mL of IL-33 was capable of markedly amplifying production of inflammatory cytokines and chemokines when cells were stimulated with Ag along with IL-33. In contrast to Ag, IL-33 failed to induce a calcium signal for degranulation. Moreover, in the presence of low concentrations of both Ag and IL-33 mast cells exhibited no or minimal degranulation but produced inflammatory cytokines in amounts exceeding those achieved with optimal concentrations of either stimulant alone. The present studies were undertaken to examine the mechanisms underlying these phenomena with the expectation that the results would broaden our understanding how responses to Ag can be substantially retuned by other inflammatory factors.

Results

IL-33 interacts synergistically with Ag to enhance cytokine production in a MyD88-dependent manner

In contrast to Ag, IL-33 failed to stimulate degranulation in RBL-2H3 cells (rat basophilic leukemia mast cells) or BMMC (bone marrow-derived mast cells) (Fig. 1A and B) and enhanced degranulation to only a minimal or modest extent when added in combination with Ag (Fig. 1C). The most pronounced effect of IL-33 was its ability to synergistically augment production of cytokines by Ag in RBL-2H3 cells and BMMC. For example, production of TNF-α was markedly enhanced when cells were simultaneously stimulated with various concentrations of Ag and 1 ng/mL IL-33 (Fig. 2A and B). Synergy was apparent over a wide range of concentrations of IL-33 and a fixed concentration of Ag (3 ng/mL) and was evident with as little as 10 pg/mL IL-33 (Fig. 2C and D). IL-33 alone did not stimulate production of TNF-α in RBL-2H3 cells (Fig. 2C) and did so to a limited extent in BMMC (Fig. 2D). Therefore, IL-33 was capable of significantly amplifying production of TNF-α at concentrations (i.e. 10 pg/mL) below those required for stimulation of TNF-α production by IL-33 alone (i.e. 100 pg/mL in Fig. 2D). Such synergy was not due to priming of cells by IL-33 as synergy was not observed when cells were incubated with IL-33 for 16 h before stimulation with Ag in the absence of IL-33 as compared to simultaneous addition of IL-33 and Ag (compare "Before" columns with "Simultaneous" columns in Fig. 2E).

We next examined the effects of relatively low concentrations of IL-33 (70 pg/mL) and Ag (3 ng/mL) on production of a broad array of cytokines and chemokines in BMMC by the use of a multiplex assay system. These assays indicated that IL-33 alone induced production of TNF-α, IL-6, IL-13, MCP-1, MCP-3, MIP-1α either to the same extent or, in the case of IL-6 and IL-13, to a greater extent than Ag. However, both stimulants together interacted synergistically in stimulating production of (Fig. 3A–F) but they failed to stimulate production of IL-2 and RANTES when administered individually or in combination (data not shown). MyD88 was also examined, because of its known role in

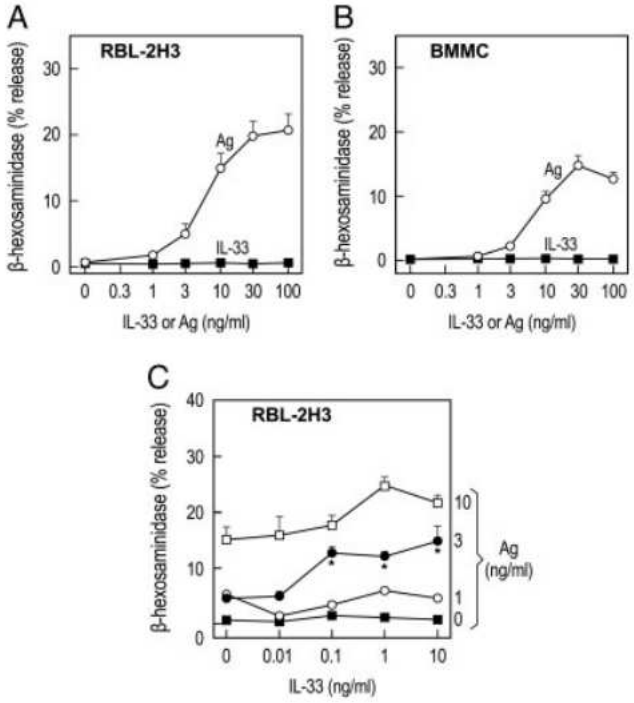

Figure 1. IL-33 fails to stimulate mast cell degranulation but modestly enhances Ag-induced degranulation. (A and C) RBL-2H3 cells and (B) BMMC, sensitized with Ag-specific IgE, were stimulated for 15 min with the indicated concentrations of either (A and B) IL-33 or Ag or (C) both simultaneously. The data show percentage release of cellular β-hexosaminidase into medium. Data show mean+SEM (triplicate cultures) and are representative of at least two independent experiments. Statistical differences ($p \geq 0.05$) were determined by ANOVA by use of the Prism software.

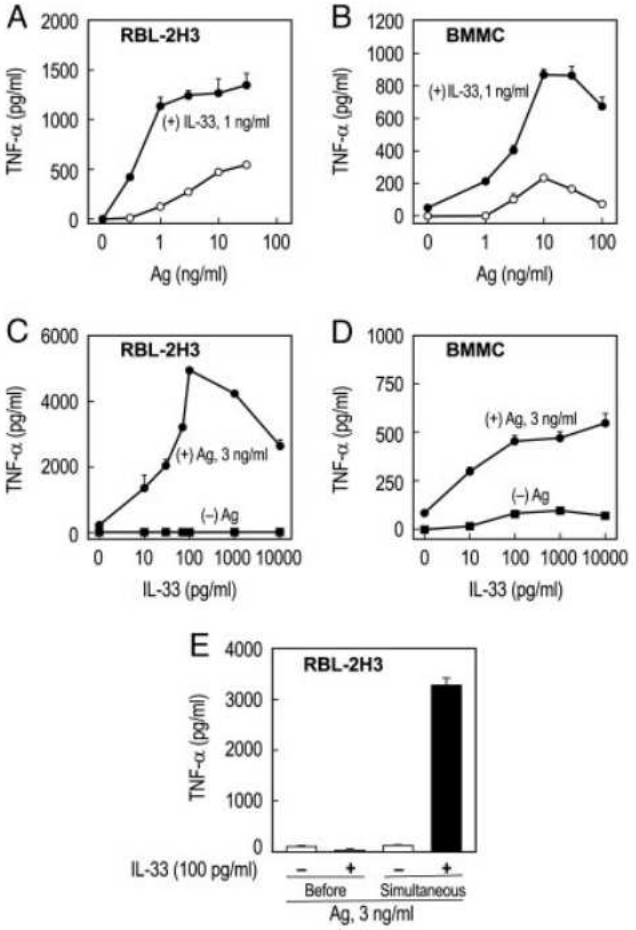

Figure 2. Ag and IL-33 interact synergistically in the production of TNF-α. Sensitized RBL-2H3 cells and BMMC were incubated for 3 h with (A and B) the indicated concentrations of Ag, in the absence or presence of IL-33, or (C and D) the indicated concentrations of IL-33, in the absence or presence of Ag before measurement of TNF-α in the medium. (E) Cells were exposed to IL-33 for 16 h and then washed before stimulation with 3 ng/mL Ag (Before); alternatively cells were incubated for 16 h without IL-33, washed, and then stimulated simultaneously with IL-33 and Ag for 3 h (Simultaneous). Data show mean+SEM (triplicate cultures) and are representative of at least two independent experiments. All differences were highly significant, $p \geq 0.001$ as determined by ANOVA by use of the Prism software.

IL-33-mediated signaling events, to verify that MyD88 was also essential for the synergy between IL-33 and Ag (Fig. 3G–I). In BMMC derived from WT mice, the stimulated production of TNF-α, IL-6, and IL-13 by optimal concentrations of IL-33 and Ag (10 and 20 ng/mL, respectively) was substantially enhanced on simultaneous addition of both stimulants. However, the responses to IL-33 and the synergy between IL-33 and Ag were no longer apparent in MyD88-deficient BMMC (Fig. 3G–I). The responses to Ag alone were not affected by the absence of MyD88.

The studies with BMMC indicated that synergistic production of cytokines was apparent at either low (3 ng/mL Ag and 70 pg/mL IL-33 in Fig. 3A–F) or high (20 ng/mL Ag and 10 ng/mL IL-33 in Fig. 3G–I) concentrations of Ag and IL-33. Subsequent experiments were conducted with the lower concentrations of stimulants because 70 pg/mL of IL-33 falls within the range concentrations normally found in synovial fluid from patients with rheumatoid arthritis [40] and less than optimal concentration of Ag (3 ng/mL) was the more likely situation in patients with atopic disease.

Synergistic interactions among signaling pathways

Others have reported that ST2 receptors are expressed in mouse BMMC [15, 16, 41] and we have verified by immunoblotting that both RBL-2H3 cells and BMMC express ST2 receptors (data not shown). Examination of early ST2-mediated signaling events in RBL-2H3 cells indicated that both IRAK1 and TAK1 were phosphorylated following stimulation with 70 pg/mL IL-33 but this was not so with 3 ng/mL Ag (Fig. 4A). Although the extent of IRAK1 phosphorylation under these conditions was modest, substantial phosphorylation was apparent at higher concentrations of IL-33 (data not shown). Of note though, the phosphorylation of IRAK1 was not enhanced by the combination of stimulants whereas the phosphorylation of TAK1 was enhanced several fold on co-stimulation (Fig. 4A). Although 3 ng/mL Ag did not induce detectable phosphorylation of TAK1, such phosphorylation was detectable at higher concentrations of Ag (e.g. 50 ng/mL as shown in Supporting Information Fig. S1). Collectively, the data in Figs. 3

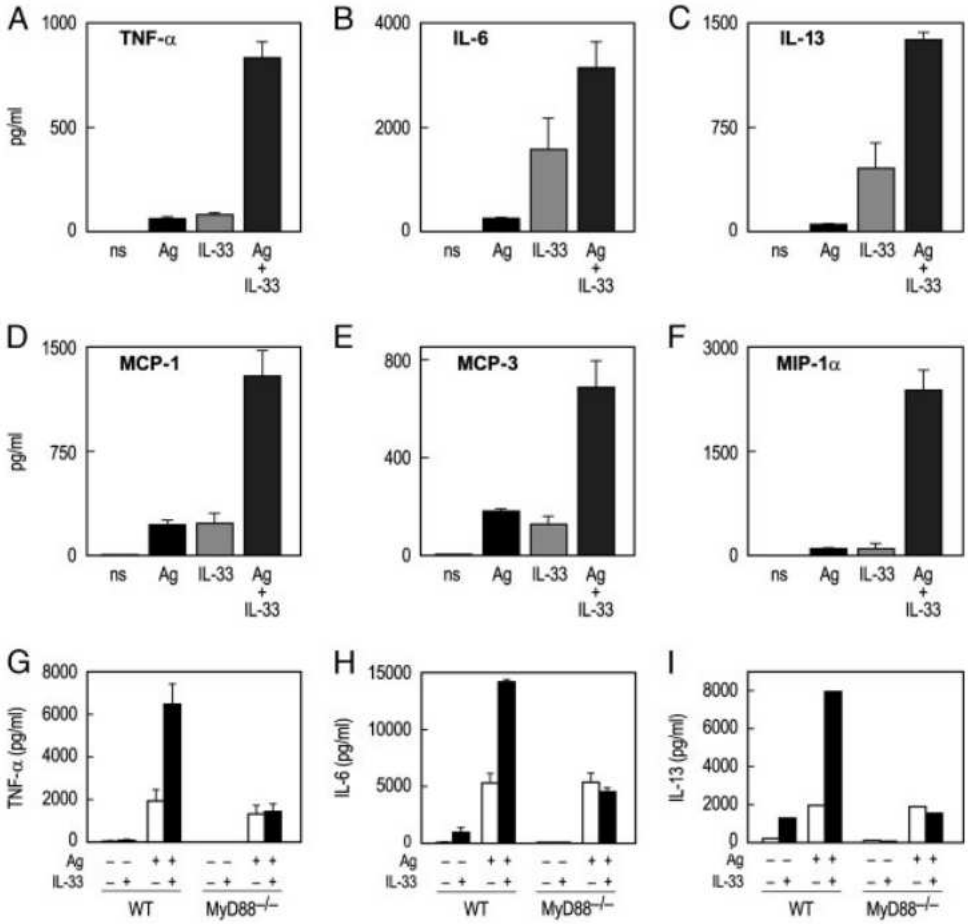

Figure 3. Ag and IL-33 interact synergistically in the production of several cytokines in WT but not in MyD88-deficient BMMC. (A–F) BMMC were sensitized with Ag-specific IgE before stimulation or not (ns) with 3 ng/mL Ag or 70 pg/mL IL-33, individually or in combination, for 3 h. The indicated cytokines or chemokines were assayed by use of the Procarta assay kit as described in the *Materials and methods* section. Data show mean +SEM (triplicate cultures) and are representative of at least two independent experiments. (G–I) BMMC derived from WT or MyD88 $^{-/-}$ mice were sensitized with Ag-specific IgE before stimulation with 20 ng/mL Ag, 10 ng/mL IL-33, or both in combination. Note that these concentrations were higher than those used in panels A–F. Media were assayed for TNF-α, IL-6, and IL-13 by ELISA 24 h later. Data show mean+SEM from three independent experiments, except for panel I, which shows mean values from one experiment. In all panels, the differences between the sum of responses to individual stimulants and the response to the combination of stimulants were statistically significant at the $p \leq 0.05$ (B and C) or $p \leq 0.01$ (A and D–H) level as determined by ANOVA by use of the Prism software.

and 4, and Supporting Information Fig. S1 are consistent with the notion that IL-33 operates via ST2 through MyD88, the IRAKs, and TAK1 and that TAK1 is a possible point of convergence of signals from ST2 and FcεRI.

As expected for FcεRI-mediated signaling events, Ag stimulation resulted in phosphorylation of Src kinase, Bruton's tyrosine kinase (Btk), protein kinase B (Akt), and PLCγ_2 (Fig. 4B). IL-33 also stimulated, although to a lower extent than Ag, phosphorylation of Src kinase and Akt, which is an indicator of PI3K activation, but not Btk or PLCγ_2. None of these phosphorylation events were significantly enhanced on co-stimulation of cells.

Several downstream signaling events were also enhanced on co-stimulation of cells with low concentrations of IL-33 and Ag (Fig. 4C and D). Low concentrations of Ag (3 ng/mL) stimulated activating phosphorylations of ERK and p38 MAP kinase (Fig. 4C) and minimally stimulated phosphorylation of JNK (detectable after longer exposure than shown in Fig. 4C). IL-33 (70 pg/mL) induced modest phosphorylation of all three kinases. However, phosphorylation of JNK and p38 MAP kinase, but not ERK, was markedly augmented on stimulation with both Ag and IL-33. Phosphorylation of the downstream targets of JNK and p38 MAP kinase, the transcription factors c-Jun and ATF-2, as well as the increase in levels of c-Jun that normally occurs after Ag stimulation [42] were either synergistically or additively enhanced by co-stimulation with IL-33 and Ag (Fig. 4C). Some enhancement of phosphorylation was observed throughout the NF-κB pathway to include IKKα/β, IκBα, and NF-κB even though Ag barely stimulated this pathway at low concentrations (Fig. 4D). The levels of IKKα and NF-κB protein remained unchanged but degradation of IκBα by the polyubiquitination pathway [43, 44] was apparent following stimulation by IL-33 alone or in combination with Ag (Fig. 4D).

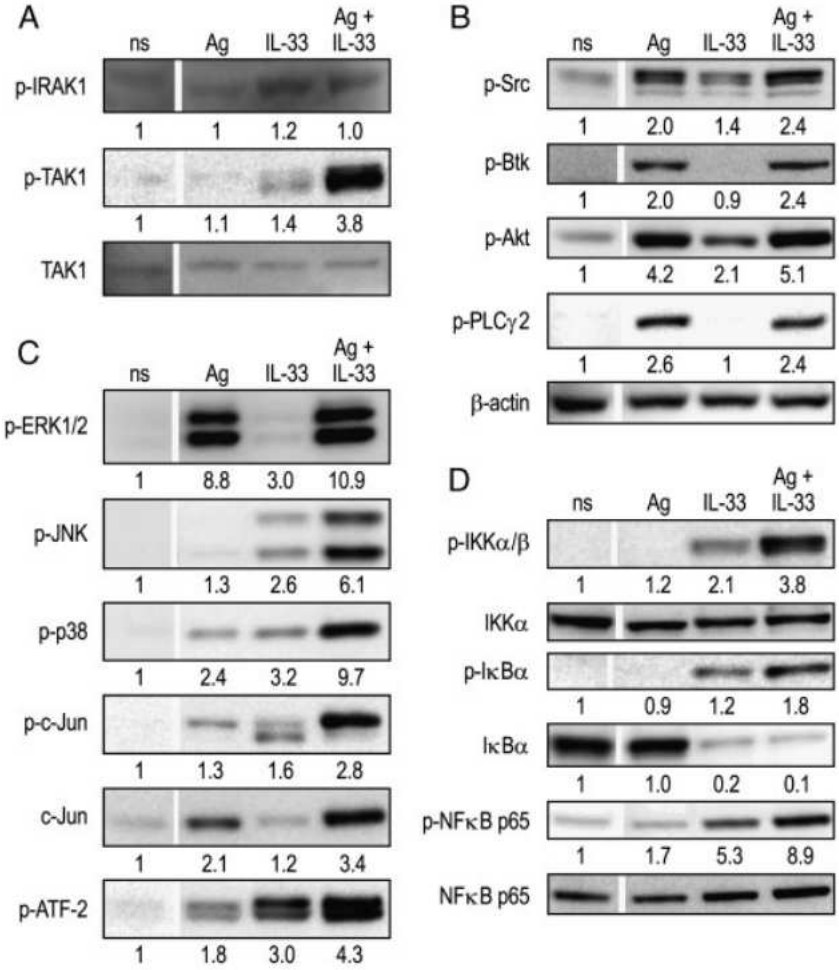

Figure 4. Pattern of phosphorylation of signaling proteins in RBL-2H3 cells in response to Ag and IL-33 individually or in combination. RBL-2H3 cells sensitized with Ag-specific IgE were stimulated or not (ns) with 3 ng/mL Ag, 70 pg/mL IL-33, or both for 30 min. (A and B) Western blots were prepared from cell lysates for detection of phosphorylated rat IRAK1 (Thr 209), TAK1 (Thr 187), pan Src (Tyr 416), Btk (Ser 180), Akt (Ser 473), and PLCγ2 (Tyr 1217) as well as TAK1 protein. (C and D) Western blots were prepared from cell lysates to detect downstream phosphorylations, which included phosphorylated rat ERK1/2 (Thr 202/Tyr 204), JNK (Thr 183/Tyr 185), p38 MAP kinase (Thr 180/Tyr 182), c-Jun (Ser 63), ATF-2 (Thr 69/71), and c-Jun protein. Blots were also probed for phosphorylated IKKα/β (Ser 176/180), IκBα (Ser 32), and NF-κB (Ser 536) and their protein counterparts. Data are representative of at least three independent experiments. The numeric values indicate average density of bands from these experiments after correction for their protein counterparts. The β-actin band is shown as an additional loading control. In this figure, images were cropped to allow direct visual comparison of bands from non-stimulated and stimulated cells from the same gel.

As in RBL-2H3 cells, the phosphorylation of TAK1 in BMMC was enhanced by simultaneous stimulation with 3 ng/mL Ag and 70 pg/mL IL-33 (Fig. 5). In contrast to Ag, IL-33 neither stimulated phosphorylation of Syk, which lies upstream of PLCγ, nor enhanced Syk phosphorylation by Ag (Fig. 5). The signaling pathways that lead to degranulation were not investigated in further detail because of the relatively modest effects of IL-33 on degranulation. With respect to pathways that regulate cytokine production, the phosphorylations of JNK, c-Jun, and ATF-2 were enhanced on costimulation with IL-33 and Ag but to a lesser extent than that observed in RBL-2H3 cells. There was no remarkable synergy in the phosphorylation of NF-κB but these and other experiments suggested that NF-κB was already optimally phosphorylated by IL-33. As described in a later section, the activation of NF-κB was further examined by luciferase-reporter assays.

Role of the Ca^{2+}/calcineurin/NFAT pathway in cytokine production

Even at high concentrations, IL-33 (10 ng/mL) failed to elicit an increase in $[Ca^{2+}]_i$ or alter the Ca^{2+}-response to Ag in RBL-2H3 cells or BMMC (Fig. 6A and B). The lack of a calcium response was consistent with the failure of IL-33 to induce phosphorylation of Syk (Fig. 5) and PLCγ (Fig. 4B) or degranulation (Fig. 1). An increase in $[Ca^{2+}]_i$ is a necessary signal not only for degranulation [10] but also for the activation of the calcineurin/NFAT pathway and cytokine production in Ag-stimulated mast cells [4]. Therefore, experiments were performed to determine whether or not activation of the Ca^{2+}/calcineurin/NFAT pathway acts in synergy with IL-33-activated Ca^{2+}-independent signaling pathways to enhance production of cytokines. Thapsigargin was tested as a potential selective activator of the calcineurin/NFAT

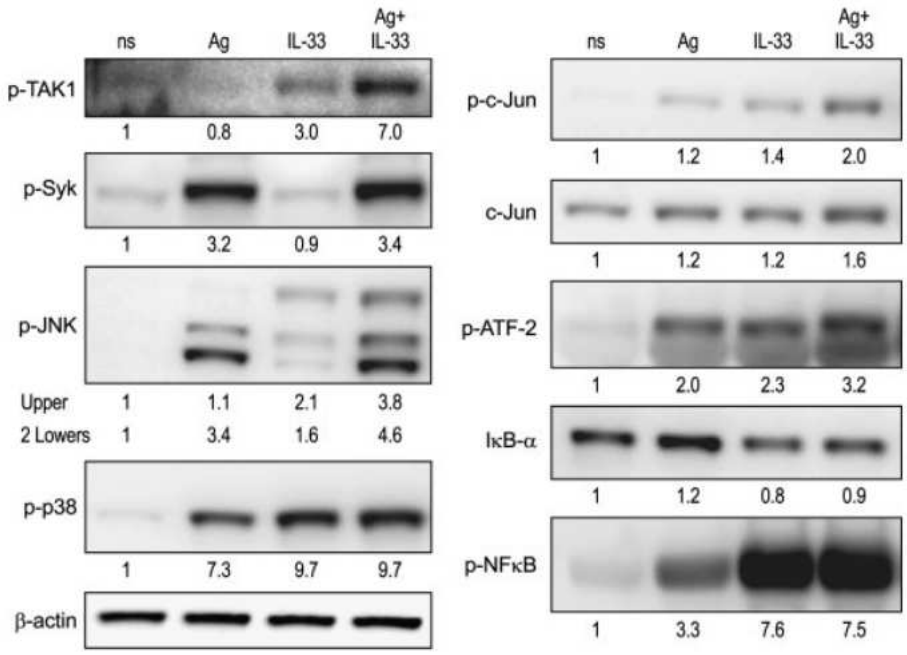

Figure 5. Pattern of phosphorylation of signaling proteins in BMMC following stimulation with Ag and IL-33 individually or in combination. Experiments were performed with sensitized BMMC as described for RBL-2H3 cells in Fig. 4, except that cells were stimulated for 10 min. Data are representative of at least two independent experiments and, as for Fig. 4, the numeric values indicate average density of bands from these experiments after correction for their protein counterparts. The β-actin band is shown as an additional loading control.

pathway because of its unique ability to increase $[Ca^{2+}]_i$ by blocking uptake into intracellular Ca^{2+}-stores [10]. A concentration of 30 nM thapsigargin was chosen as this causes little or no stimulation of phospholipid hydrolysis by PLC or PLD, phosphorylation of Akt and MAP kinases, or degranulation ([45] and our unpublished data). At this concentration, thapsigargin induced a substantial increase in $[Ca^{2+}]_i$ that was unaffected by the presence of IL-33 (Fig. 6C). Also, 30 nM thapsigargin stimulated production of TNF-α to the same extent as 3 ng/mL Ag and this production was enhanced by more than 20-fold upon costimulation with 70 pg/mL IL-33 (Fig. 6D). The production of TNF-α in response to Ag or thapsigargin, individually or in combination with IL-33, was inhibited by more than 95% by the calcineurin inhibitor, cyclosporin A (Fig. 6D). Therefore, both thapsigargin and Ag interact synergistically with IL-33 in a calcineurin-dependent manner. The presumed downstream activation of NFAT via Ca^{2+}/calcineurin [46] was verified by luciferase reporter assays as demonstrated in the next section.

Enhancement of transcriptional activities by IL-33

Luciferase reporter assays were used to investigate interactions of low concentrations of IL-33 (70 pg/mL), thapsigargin (30 nM), and Ag (3 ng/mL) at the level of gene transcription (Fig. 7). The action of thapsigargin was selective in that it stimulated NFAT activity (Fig. 7A) but minimally so NF-κB (Fig. 7B) and it failed to stimulate AP-1 (data not shown). In contrast, Ag stimulated all three transcription factors (Fig. 7C–E) as well as NFAT and AP-1 in combination (Fig. 7F). IL-33 stimulated NF-κB activity (Fig. 7B and D) but failed to stimulate NFAT transcriptional activity (Fig. 7A and C), as would be expected from the inability of IL-33 to generate a calcium signal. IL-33 also failed to stimulate AP-1 and NFAT/AP-1 activities (Fig. 7E and F). Nevertheless, IL-33 enhanced the activation of NFAT by thapsigargin (Fig. 7A) and Ag (Fig. 7C) and of AP-1 and NFAT/AP-1 by Ag (Fig. 7E and F). Additional studies with the calcineurin inhibitor, cyclosporin A, reaffirmed that IL-33 potentiated the coactivation of NFAT/AP-1 by Ag or thapsigargin and, moreover, this activation was completely blocked by cyclosporin A whatever the mode of stimulation (Fig. 7G–J).

These results demonstrated that NFAT and AP-1 interacted co-operatively and that activation of both these factors was further enhanced by IL-33 even though IL-33 itself activates neither transcription factor at low concentrations. These co-operative interactions likely occur at the level of gene transcription because regulatory signaling events upstream of NFAT, namely phosphorylation of PLCγ and calcium signal, are neither activated nor enhanced by IL-33. In contrast to NFAT and AP-1, synergy was not observed in the activation of NF-κB. The relatively robust activation of NF-κB by IL-33 was not further enhanced by either thapsigargin or Ag (Fig. 7B and D).

Pharmacologic evaluation of the roles of signaling pathways in cytokine production

The possibility that TAK1 (Fig. 4A) participates in the synergistic interaction of Ag and IL-33 was examined by knock down of TAK1 and by use of the irreversible fungal TAK1 inhibitor,

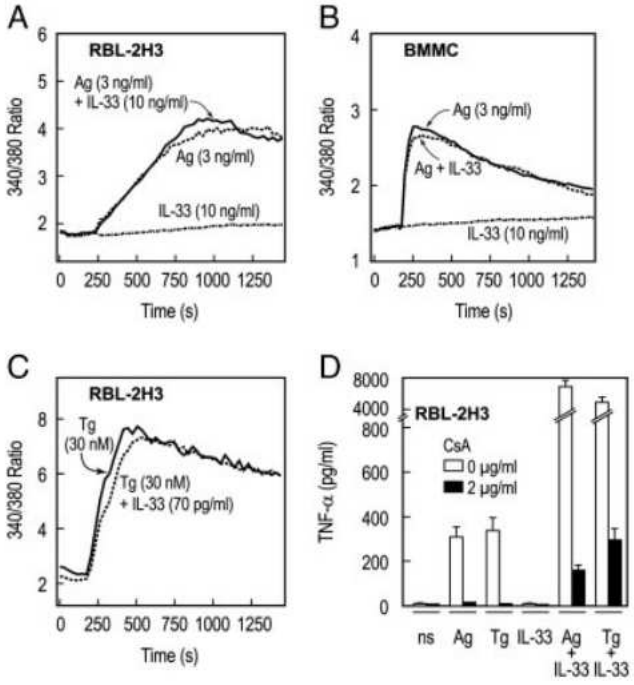

Figure 6. IL-33 fails to mobilize Ca^{2+} but mobilization of Ca^{2+} upon stimulation with Ag or thapsigargin markedly potentiates TNF-α production by IL-33 in a calcineurin-dependent manner. (A–C) RBL-2H3 cells or BMMC, sensitized with Ag-specific IgE, were stimulated with the indicated concentrations of Ag, IL-33, thapsigargin (Tg), or combinations thereof for measurement of changes in $[Ca^{2+}]_i$. (D) Sensitized cells were stimulated with 3 ng/mL Ag, 70 pg/mL IL-33, 30 nM thapsigargin, or the indicated combinations in the absence or presence of 2 μg/mL cyclosporin A (CsA). Cells were stimulated for (A–C) the indicated times or (D) 90 min. Data show mean+SEM from three separate cultures and are representative or two or more experiments.

5Z-7-oxozeaenol [47], in RBL-2H3 cells. The enhanced production of TNF-α was indeed much reduced in cells transfected with siRNA against TAK1 (Fig. 8A) or when cells were exposed to 100 nM 5Z-7-oxozeaenol (Fig. 8B). 5Z-7-Oxozeaenol also blocked Ag-induced TNF-α production (Fig. 8B) to indicate possible involvement of TAK1 in the responses to Ag as well as IL-33 and was equally effective in suppressing production of TNF-α and IL-6, although some decrease in degranulation was noted with high concentrations of 5Z-7-oxozeaenol (Supporting Information Fig. S2). In this series of experiments, expression of TAK1 was reduced by 62–69% in siRNA-transfected cells and the phosphorylation of TAK1 (Thr 187) by >60% in 100 nM 5Z-7-oxozeaenol-treated cells (data not shown). However, we were unable to obtain satisfactory blots with available antibodies against phosphorylated TAK1 (Thr 184) leaving the question unresolved as to why suppression of TAK1 activity by 5Z-7-oxozeaenol was more effective than knockdown with siRNA in suppressing TNF-α production in Ag-stimulated cells (Fig. 8A and B).

Studies with 5Z-7-oxozeaenol and other pharmacological inhibitors (identified in parentheses) of IκBα (Bay 11–7082, 10 μM), JNK (SB 202190, 10 μM), p38 MAP kinase (SP 600125, 20 μM), and calcineurin (cyclosporin A, 2 μg/mL) suggested that all of these signaling proteins as well as TAK1 regulated production of TNF-α to some degree in BMMC (Fig. 8C and D). However, the extent of regulation varied according to mode of stimulation. In Ag-stimulated BMMC, production of TNF-α was partially suppressed by inhibitors of TAK1, IκBα, and JNK and totally blocked by inhibitors of p38 MAP kinase and calcineurin (Fig. 8C). In IL-33-stimulated BMMC, TNF-α production was totally blocked by inhibition of TAK1, IκBα, and JNK and partially repressed by inhibition of p38 MAP kinase, and was unaffected by inhibition of calcineurin (Fig. 8D). These results suggested that p38 MAP kinase and calcinerin were the preeminent regulators in Ag-stimulated cells and TAK1, IκBα, and JNK were predominant in IL-33-stimulated BMMC. In co-stimulated BMMC, the results were a hybrid of those observed with the individual stimulants. Even though the inhibitors may have unintended and unknown consequences, it would appear that all five signaling molecules play substantial roles in regulating TNF-α production on costimulation with Ag and IL-33 (Fig. 8E).

Discussion

We find that low, clinically relevant concentrations of IL-33 substantially amplify Ag-induced production of chemokines and inflammatory cytokines in mast cells. In addition, Ag in combination with IL-33 elicits greater production of cytokines than is achieved with optimally effective concentrations of either stimulant alone (Figs. 2 and 3G–I). However, IL-33 failed to induce a calcium signal (Fig. 6) and degranulation (Fig. 1). Although the synergy in cytokine production was apparent over a wide range of concentrations of IL-33 and Ag, the underlying mechanisms were investigated at low concentrations of stimulants to reflect as far as possible conditions that might occur in allergic/inflammatory diseases.

At low concentrations, IL-33 by itself stimulated phosphorylation of TAK1, JNK, p38 MAP kinase, c-Jun, ATF-2, and robustly so components of the NF-κB pathway (Figs. 4 and 5) as would be expected of ST2-mediated responses. IL-33 also induced phosphorylation of Src kinase and Akt in common with Ag (Fig. 4B). In contrast to Ag, IL-33 failed to induce phosphorylation of LAT (data not shown), Syk (Fig. 5), Btk, and PLCγ (Fig. 4B), which is consistent with the lack of a calcium signal and degranulation. When IL-33 was used in combination with Ag, synergy in signaling was evident at the level of TAK1 (Fig. 4A). The enhanced signaling was then transmitted downstream through the JNK, p38 MAP kinase, c-Jun, and ATF-2 in RBL-2H3 cells (Fig. 4B), although this was less evident in BMMC (Fig. 5). The involvement of TAK1 and these downstream molecules in cytokine production was verified by the inhibitory effects of pharmacological inhibitors (Fig. 8). A pathway that is independent of the canonical IRAK/TRAF6/TAK1 signaling pathway has been detected in IL-33-stimulated basophils [18] but our pharmacological studies suggest that this is not the case in mast cells. In addition, TAK1 is critical for the activation of cells through IL-1R and TLRs [39, 48] but this has not been formally established in the case of ST2. Our studies with anti-TAK1 siRNA and

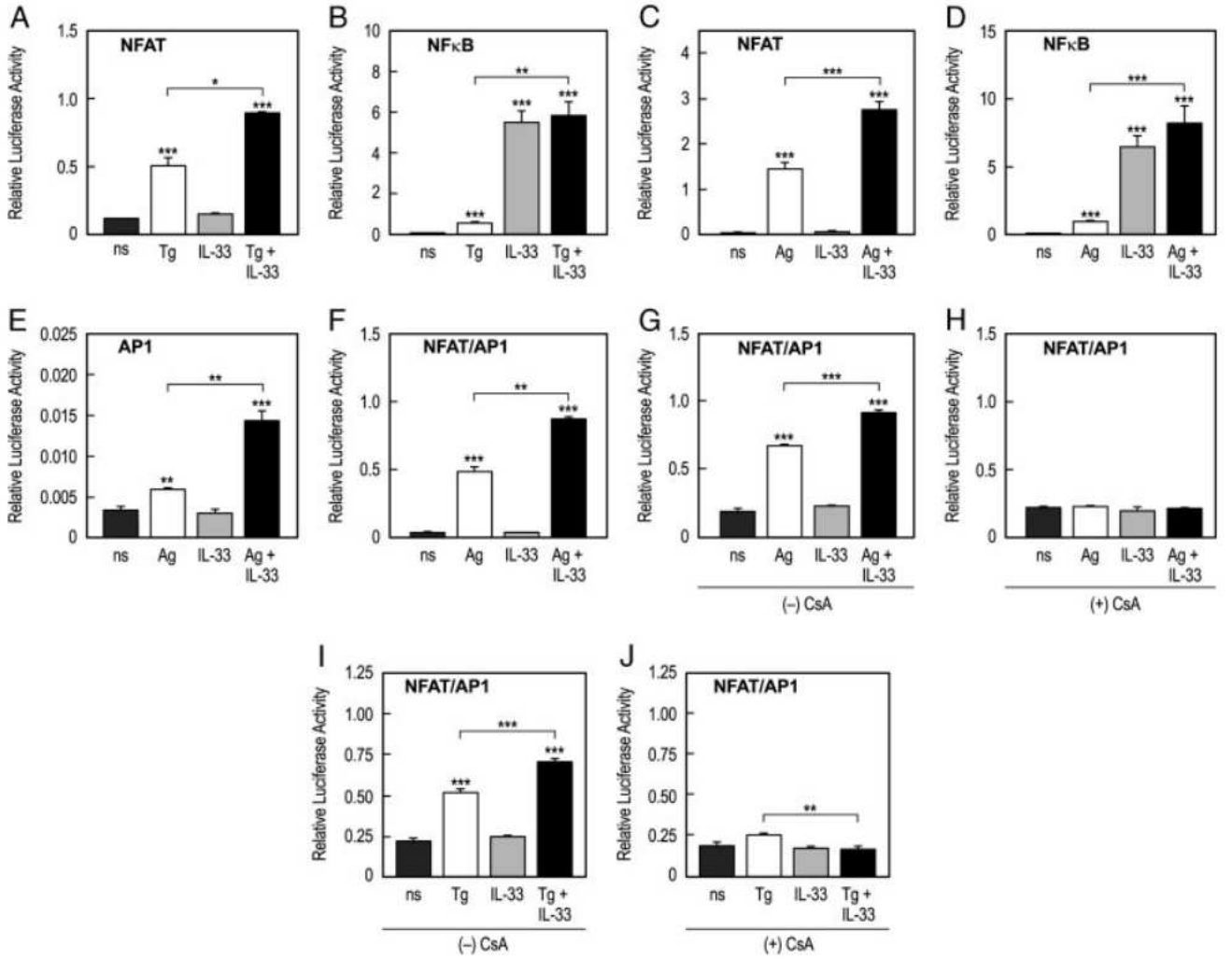

Figure 7. Stimulation of NFAT, NF-κB, and AP-1 transcriptional activity by thapsigargin, Ag, and IL-33 and the effects of cyclosporin A. (A–F) Sensitized RBL-2H3 cells were stimulated or not (ns) with 30 nM thapsigargin (Tg), 3 ng/mL Ag, 70 pg/mL IL-33, or combinations thereof. In an additional set of experiments, (G–J) run in parallel, cyclosporin A (CsA, 2 μg/mL) was added or not 10 min before addition of stimulants. Transcriptional activities were measured 90 min later by the dual luciferase reporter assay. Data show mean+SEM from three to five separate experiments and are expressed as the ratio of firefly/Renilla luminescence activities. Statistical differences ($p > 0.05$, $p > 0.01$, $p > 0.001$), as determined by ANOVA by use of the Prism software, between non-stimulated (ns) and stimulated samples and between the indicated stimulant and combination of stimulants are shown.

5Z-7-oxozeaenol (Fig. 8) suggest that TAK1 is indispensable for IL-33 activation of mast cells and for the synergy between IL-33 and Ag. Also, the studies with MyD88-deficient cells (Fig. 3G–I) and visualized phosphorylated proteins (Figs. 4 and 5) suggest that the MyD88/IRAK/TAK1 pathway is engaged in IL-33-stimulated mast cells as depicted in Fig. 9.

Nothing is known about the role of TAK1 in FcεRI-activated mast cells, although our data suggest engagement of TAK1 by FcεRI. Low concentrations of Ag failed to stimulate detectable phosphorylation of TAK1 (Fig. 4A), yet the same concentration of Ag substantially enhanced phosphorylation of TAK1 by IL-33 (Fig. 4A). Also, high concentrations of Ag alone can stimulate TAK1 phosphorylation (Supporting Information Fig. S1), indicating that strength of the Ag stimulus may be an important determinant. FcεRI-mediated activation of NF-κB is reported to be dependent on PI3K, phosphoinositide-dependent kinase, protein kinase B, and PKC upstream [11] and the Bcl10 (B-cell lymphoma 10) and Malt1 downstream [49]. However, Bcl10 and Malt1 regulate activation of TAK1 and the NF-κB pathway in T-cell receptor signaling [50] and TAK1 is essential for engagement of the p38 MAP kinase pathway but not for activation of NF-κB during B-cell receptor signaling [48]. Therefore, TAK1 appears to be engaged in T-cell and B-cell receptor signaling although the downstream consequences may differ. Further studies are necessary to define the signaling links between FcεRI and TAK1, if any, as well as the pathways activated through TAK1.

Synergy also appears to be highly dependent on co-operative interactions among transcription factors particularly between NFAT and AP-1. An essential role for NFAT in the activation of AP-1 (Fig. 7G–J) and production of TNF-α (Fig. 8) is indicated by the inhibitory actions of cyclosporin A. IL-33 activates neither of these transcription factors but it does enhance their activation by

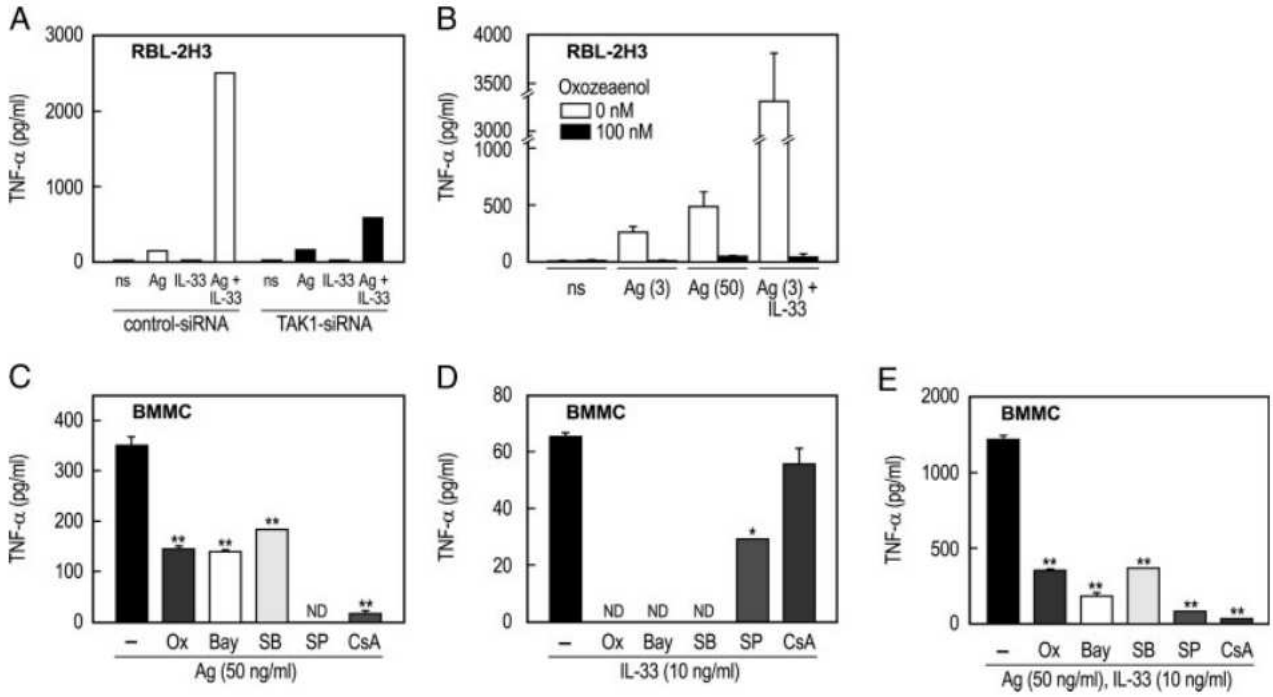

Figure 8. Suppression of TNF-α production by knock-down of TAK1 and pharmacologic inhibitors of various signaling proteins. (A and B) RBL-2H3 cells and (C–E) BMMC, sensitized with Ag-specific IgE, were stimulated with Ag and IL-33, individually or in combination, for 3 h for measurement of TNF-α by ELISA. (A) Cells were transfected with scrambled control siRNA or anti-TAK1 siRNA 48 h before stimulation with 3 ng/mL Ag or 70 pg/mL IL-33. (B–E) Cells were exposed to the inhibitors for 20 min before addition of stimulants. Cells were stimulated with 3 or 50 ng/mL Ag and 70 pg/mL IL-33 (B) or at the concentrations indicated (C–E). Data show mean + SEM of values from three separate cultures and are representative of two independent experiments. Statistical differences ($p \leq 0.05$, $p \leq 0.01$) between controls (no inhibitor) and inhibitor-treated samples were determined by ANOVA by use of the Prism software. ND indicates not detectable. Key for inhibitors in (C–E): Ox, 100 nM 5Z-7-oxozeaenol; Bay, 10 μM Bay 11-7082; 10 μM SB 202190; 20 μM SP 600125; CsA, 2 μg/mL cyclosporin A.

mechanisms that need to be clarified. For example, the NFAT pathway [4] is selectively activated by low concentrations of thapsigargin but this activation is augmented by IL-33 (Fig. 7). IL-33 also enhanced the Ag-induced activation of NFAT, AP-1, or NFAT/AP-1 in combination (Fig. 7). As a result, production of TNF-α is substantially enhanced whether cells are stimulated by thapsigargin or Ag in the presence of IL-33 (Fig. 6D). The relatively robust stimulation of NF-κB transcriptional activity by IL-33, however, was not further augmented by Ag. Examination of the genomic sequences by the rVISTA [51] and Mulan [52] programs indicated highly conserved putative binding sites for NFAT, AP-1, and NF-κB in the promoter regions of TNF-α and IL-13 genes and for AP-1 and NF-κB in the IL-6 gene. The AP-1- and NFAT-binding sites are located in close proximity at several sites in the TNF-α gene and synergistic cooperative interaction between AP-1 and NFAT is well documented [53]. In addition, NFAT and NF-κB show considerable functional overlap in co-ordinating induction of many cytokines [54]. Therefore, it seems possible that the combination of Ag and IL-33 brings into play a much more effective combination of transcription factors for production of least a subset of cytokines than do the individual stimulants.

The studies with pharmacologic inhibitors, in general, support the notion that the hybridization of the different signaling mechanisms for Ag and IL-33 results in robust augmentation of cytokine production (Fig. 8C–D). The studies indicate that the production of TNF-α by Ag is absolutely dependent p38 MAP kinase and the calcineurin/NFAT pathways and only partially dependent on the NF-κB, JNK, and TAK1 pathways, whereas the effects of IL-33 are absolutely dependent on the latter pathways. In contrast, the enhanced production of TNF-α by the combination of Ag and IL-33 becomes almost totally dependent on all pathways.

There are discrepancies in the literature with regard to IL-33 and degranulation. Our finding that IL-33 is unable to stimulate degranulation is consistent with the findings of other workers [13–16] with the exception of Melendez and co-workers who reported that IL-33 induces an increase in $[Ca^{2+}]_i$ and degranulation in mast cells [23]. We find, however, that in addition to RBL-2H3 cells and BMMC, cultured human mast cells exhibit no such responses to IL-33 under the exact conditions described by these workers (our unpublished data, Shoko Iwaki, Alasdair M. Gilfillan, and Michael A. Beaven). Therefore, we conclude that in the presence of low concentrations of Ag and IL-33, mast cells might undergo minimal degranulation but produce an abundance of inflammatory cytokines. As noted for basophils [17, 20], IL-33 may modestly enhance FcεRI-mediated degranulation in mast cells even though it is inactive by itself (Fig. 1). The reason for this enhancement is unclear. IL-33 had no palpable effect on FcεRI-mediated phosphorylation of PLCγ or calcium signal

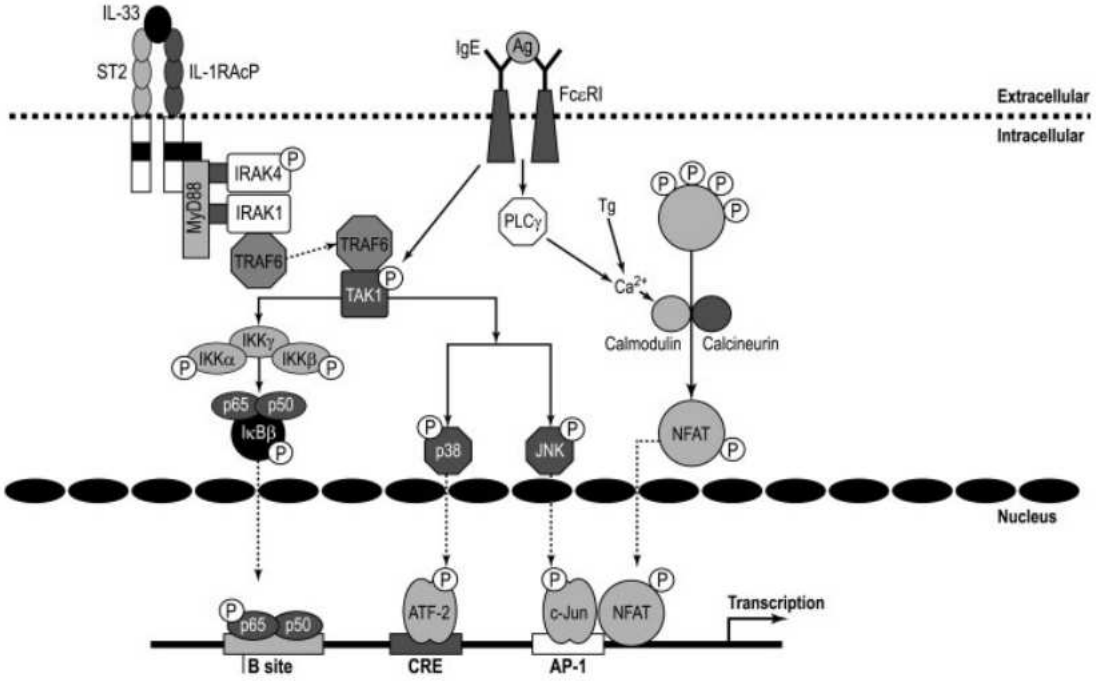

Figure 9. Proposed mechanisms for amplification of cytokine gene transcription on co-stimulation of mast cells with low concentrations of Ag (or thapsigargin) and IL-33. Phosphorylation of TAK1 by IL-33 via the ST2/IL-1 receptor accessory protein (IL-1RAcP) complex, MyD88, the IRAKs, and TRAF6 is amplified by Ag via FcεRI by an as yet undetermined mechanism. This results in enhanced phosphorylation of p38 MAP kinase and JNK and, in turn, phosphorylation of ATF-2 and c-Jun (Fig. 4). Enhancement of phosphorylation events throughout the NF-κB pathway were also observed (Fig. 4) but this did not result in detectable augmentation of NF-κB transcriptional activity as determined by luciferase assays (Fig. 7). Synergy was not apparent in the phosphorylation of PLCγ (Fig. 4B) and calcium mobilization, whether initiated by Ag or thapsigargin (Tg) (Fig. 6A–C), but amplification of NFAT activation was noted (Fig. 7). Studies with cyclosporin A, an inhibitor of calcineurin which dephosphorylates and hence activates NFAT, indicate that the Ca^{2+}/calcineurin/NFAT pathway is necessary for TNF-α-gene transcription (Fig. 6D) and the activation of NFAT/AP-1 (Fig. 7G–J) to suggest essential co-operative interactions between NFAT and AP-1. In total, the data suggest that Ag and IL-33 together recruit a more effective combination of transcription factors and an essential role for NFAT.

(Figs. 4B and 6A and B) and presumably the enhancement of degranulation was dependent on other pathways.

In conclusion, IL-33 strongly potentiates production of inflammatory cytokines by Ag, and vice versa, by mechanisms that involve synergistic interactions at the level of TAK1 and gene transcription (as depicted in Fig. 9). The synergistic pulse appears to propagate from TAK1 through the JNK/c-Jun and p38 MAP kinase/ATF-2 pathways. IL-33 is unable to initiate signals that are essential for mobilizing Ca^{2+}, degranulation, and activation of NFAT. However, the activation of NFAT by Ag or thapsigargin provides a strong signal for enhancing cytokine production possibly by acting in combination with other transcription factors such as AP-1. IL-33 may thus markedly amplify production of cytokines when mast cells are stimulated with low concentrations of Ag with possible implications in severity of mast cell-related diseases.

Materials and methods

Reagents

Reagents were obtained from the following sources: materials for culture medium from GIBCO/Invitrogen (Carlsbad, CA, USA) and MediaTech (Manassas, VA, USA) and fetal calf serum from HyClone/Thermo Scientific (Logan, VT, USA); the DNP human serum albumin (Ag) and Ag-specific IgE from Sigma-Aldrich (St. Louis, MO, USA); murine recombinant IL-33 from R&D Systems (Minneapolis, MN, USA); thapsigargin from Calbiochem (Gibbstown, NJ, USA); anti-TAK1 siRNA (ON-TARGETplus SMARTpool, Rat Map3k7) from Dharmacon/Thermo Scientific (Layfayette, CO, USA); all primary antibodies from Cell Signaling Technology (Danvers, MA, USA), except for antibodies against IRAK1 and ST2 from AnaSpec (San Jose, CA, USA) and R&D Systems, respectively; secondary antibodies from Sigma-Aldrich or GE Healthcare/Amersham (Piscataway, NJ, USA); enzyme inhibitors from Calbiochem except Bay 11-7082 and cyclosporin A from Biomol (Plymouth Meeting, PA, USA) and Sigma-Aldrich, respectively; Fura-2 AM ester from Molecular Probes/Invitogen (Eugene, OR, USA); ELISA kits from Invitrogen, except IL-33 from R&D systems; p-nitrophenyl N-acetyl-β-glucosaminide from Sigma-Aldrich; Procarta Cytokine Assay Kits from Panomics/Affymetrix (Santa Clara, CA, USA); and Dual-Luciferase Reporter Assay Systems for NFAT, AP-1, and NF-κB from Promega (Madison, WI, USA). The plasmid (NFAT/AP-1 – 3 luciferase) was obtained from Addgene (Addgene plasmid 11783) as provided by Anjana Rao and described in [55].

Isolation of BMMC from WT and MyD88 / mice

Mouse BMMC were generated from bone marrow of WT (C57BL/6) and MyD88 knockout mice by culture for 4–8 wk in RPMI 1640 medium supplemented with 10% fetal calf serum, 30 ng/mL of murine IL-3 (PeproTech, Rocky Hill, NJ, USA), 4 mM of glutamine, 25 mM HEPES, 1 non-essential amino acids, 50 μM of 2-mercaptoethanol, and 100 mM of sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. All the procedures were done under guidelines of the Institutional Animal Care and Use Committee from the National Institutes of Health, USA, and the approval of the Animal Care committee of the Federal University of Minas Gerais, Brazil. Cells were cultured at 37°C in 5% CO_2 incubator and medium was changed twice a wk. After 4 wk, the cell culture consisted of >90% mature BMMC, and thereafter (4–8 wk) used for experiments.

Cell culture and experimental conditions

Cells were sensitized overnight with anti-DNP IgE (50 ng/mL), either in suspension (BMMC) or in multiwell plates (RBL-2H3 cells), in complete growth medium supplemented with fetal calf serum (10% for BMMC, 15% for RBL-2H3 cells), glutamine, antibiotic, and antimycotic agents. For each experiment, cells were washed three times in the required medium. To measure degranulation, cells were transferred to 24-well plates (0.5 10^6 cells/0.5 mL/well) in a PIPES-buffered medium (119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 25.0 mM PIPES, 40 mM NaOH, 1 M $CaCl_2$, 10% w/v bovine serum albumin). For assay of cytokines by ELISA, cells were also transferred to 24-well plates at the same density but in complete growth medium. For immunoblotting, cells were plated in 60-mm^2 dishes (2.5 10^6 cells/5 mL). Cells were incubated for 10 min at 371C before addition of inhibitors or stimulants. Inhibitors were added 10 or 20 min as indicated before addition of stimulants.

Measurement of degranulation, cytokines, and chemokines

Degranulation was determined by a colorimetric assay of the granule marker, β-hexosaminidase, as previously described [9]. Following stimulation of cells, 10 μL of medium and cell lysates (in 0.1% Triton X) were transferred to 96-well plates for assay of β-hexosaminidase activity by measurement of release of p-nitrophenol from p-nitrophenyl N-acetyl-β-D-glucosaminide. Values were expressed as the percentage of intracellular β-hexosaminidase that was released into the medium. Statistical differences for these and all other assays were determined by ANOVA by use of the Prism software.

Individual cytokines were assayed by ELISA by use of commercial kits. Multiple cytokines was assayed simultaneously with the Procarta Protein Cytokine Assay Kit in individual cultures (5 10^5 cells/0.5 mL). This procedure involves separation of tagged antibody-coated beads. The cytokines and chemokines assayed included mouse IL-1α, -1β, -2, -3, -4, -5, -6, -9, -12p40, -12p70, -13, -17, and -23, TNF-α, TGF-β, INF-γ, GM-CSF, vascular endothelial growth factor, MIP-1α, eotaxin, MCP-1, MCP-3, keratinocyte-derived cytokine, RANTES, and interferon-γ-inducible protein 10. Media samples were collected 3 h after addition of Ag. The samples were processed in the NHLBI FACS Core facility.

Immunoblotting

Following stimulation, cells were washed twice with cold phosphate buffered saline and lysed in lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, 25 μg/mL aprotinin, 2 μg/mL pepstatin, protease inhibitor cocktail (1 tablet/10 mL, Roche Applied Science, Indianapolis, IN, USA)). Total protein content was assayed by use of the BCA™ kit (Thermo Scientific) and individual proteins were separated by SDS-PAGE. Blots were probed with the indicated primary and peroxidase-labeled secondary antibodies and visualized by chemiluminescence. Densitometry was performed with the Kodak 2000 program.

Measurement of Ca^2 $_i$

Changes in $[Ca^{2+}]_i$ were monitored by use of Fura-2 AM ester as described [56]. RBL-2H3 cells and BMMC were sensitized with IgE overnight as described above and then loaded with Fura-2 AM for 30 min at 371C in growth medium. Cells were placed in a 96-well black culture plates (10 000 cells/well) (CulturPlate-96 F, PerkinElmer Life Sciences, Waltham, MA, USA) in a PIPES-buffered medium (see previous section) containing 0.3 mM sulfinpyrazone after washing cells with the same medium. Fluorescence was measured at two excitation wavelengths (340 and 380 nm) and an emission wavelength of 510 nm in a Wallac Victor plate reader (PerkinElmer Life Sciences). The ratio of the fluorescence readings was calculated following subtraction of the autofluorescence of the cells.

Dual luciferase reporter assay

RBL-2H3 cells (2 10^6 cells) were transfected with 2 μg pGL4.10 luciferase vector with or without the designated constructs along with 0.5 μg pGL4.74TK Renilla luciferase as an internal control (see the section Reagents). Cells (2 10^5 cells) were incubated for 18 h in 24-well plates before addition of stimulants. Four hours later, cells were washed with PBS and lysed in 1 passive lysis buffer (Promega) for assay of firefly

and Renilla luciferase activities in the Wallac Victor multiplate reader. Values were calculated as the ratio of firefly/Renilla luminescence.

Acknowledgements: This work was supported by the Intramural Research Program, NHLBI, National Institutes of Health (M. V. A., S. I., and M. A. B.); National Institutes of Health grant RO1 TW006612 (GRIP) (M. V. A.); Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundaeão de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Brazil (M. V. A., C. R., R. T. G., J. R. C.-M.). M. V. A. performed much of this work while on leave from the Faculty of Medicine, Universidade Federal de Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. The authors also thank Dr. Mauro M. Texeira, Department of Biochemistry and Immunology, Federal University of Minas Gerais, Brazil, for suggestions and initial supply of IL-33, and Leigh Samsel, FACS Core Facility, National Heart, Lung and Blood Institute, for assistance with the Affymetrix cytokine assay system.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Beaven, M. A.,** Our perception of the mast cell from Paul Ehrlich to now. *Eur. J. Immunol.* 2009. **39**: 11–25.

2 **Kalesnikoff, J. and Galli, S. J.,** New developments in mast cell biology. *Nat. Immunol.* 2008. **9**: 1215–1223.

3 **Kuehn, H. S. and Gilfillan, A. M.,** G protein-coupled receptors and the modification of FcεRI-mediated mast cell activation. *Immunol. Lett.* 2007. **113**: 59–69.

4 **Qiao, H., Andrade, M. V., Lisboa, F. A., Morgan, K. and Beaven, M. A.,** FceRI and Toll-like receptors mediate synergistic signals to markedly augment production of inflammatory cytokines in murine mast cells. *Blood* 2006. **107**: 610–618.

5 **Galli, S. J., Kalesnikoff, J., Grimbaldeston, M. A., Piliponsky, A. M., Williams, C. M. and Tsai, M.,** Mast cells as "tunable" effector and immunoregulatory cells: Recent Advances. *Annu. Rev. Immunol.* 2005. **23**: 749–786.

6 **Gilfillan, A. M. and Tkaczyk, C.,** Integrated signalling pathways for mast-cell activation. *Nat. Rev. Immunol.* 2006. **6**: 218–230.

7 **Rivera, J. and Gilfillan, A. M.,** Molecular regulation of mast cell activation. *J. Allergy Clin. Immunol.* 2006. **117**: 1214–1225.

8 **Abramson, J. and Pecht, I.,** Regulation of the mast cell response to the type 1 Fcε receptor. *Immunol. Rev.* 2007. **217**: 231–254.

9 **Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F. and Beaven, M. A.,** Ca^{2+}-dependent and Ca^{2+}-independent isozymes of protein kinase C mediate exocytosis in antigen-stimulated rat basophilic RBL-2H3 cells: Reconstitution of secretory responses with Ca^{2+} and purified isozymes in washed permeabilized cells. *J. Biol. Chem.* 1993. **268**: 1749–1756.

10 **Ma, H. T. and Beaven, M. A.,** Regulation of Ca^{2+} signaling with particular focus on mast cells. *Crit. Rev. Immunol.* 2009. **29**: 155–186.

11 **Kalesnikoff, J., Baur, N., Leitges, M., Hughes, M. R., Damen, J. E., Huber, M. and Krystal, G.,** SHIP negatively regulates IgE+antigen-induced IL-6 production in mast cells by inhibiting NFκB activity. *J. Immunol.* 2002. **168**: 4737–4746.

12 **Hundley, T. R., Gilfillan, A. M., Tkaczyk, C., Andrade, M. V., Metcalfe, D. D. and Beaven, M. A.,** Kit and FcεRI mediate unique and convergent signals for release of inflammatory mediators from human mast cells. *Blood* 2004. **104**: 2410–2417.

13 **Allakhverdi, Z., Smith, D. E., Comeau, M. R. and Delespesse, G.,** Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. *J. Immunol.* 2007. **179**: 2051–2054.

14 **Iikura, M., Suto, H., Kajiwara, N., Oboki, K., Ohno, T., Okayama, Y., Saito, H., Galli, S. J. and Nakae, S.,** IL-33 can promote survival, adhesion and cytokine production in human mast cells. *Lab Invest.* 2007. **87**: 971–978.

15 **Ho, L. H., Ohno, T., Oboki, K., Kajiwara, N., Suto, H., Iikura, M., Okayama, Y. et al.,** IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcεRI signals. *J. Leukoc. Biol.* 2007. **82**: 1481–1490.

16 **Moulin, D., Donze, O., Talabot-Ayer, D., Mezin, F., Palmer, G. and Gabay, C.,** Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. *Cytokine* 2007. **40**: 216–225.

17 **Pecaric-Petkovic, T., Didichenko, S. A., Kaempfer, S., Spiegl, N. and Dahinden, C. A.,** Human basophils and eosinophils are the direct target leukocytes of the novel IL-1-family member IL-33. *Blood* 2009. **113**: 1526–1534.

18 **Kroeger, K. M., Sullivan, B. M. and Locksley, R. M.,** IL-18 and IL-33 elicit Th2 cytokines from basophils via a MyD88- and p38α-dependent pathway. *J. Leukoc. Biol.* 2009. **86**: 769–778.

19 **Smithgall, M. D., Comeau, M. R., Yoon, B. R., Kaufman, D., Armitage, R. and Smith, D. E.,** IL-33 amplifies both T_h1- and T_h2-type responses through its activity on human basophils, allergen-reactive T_h2 cells, iNKT and NK cells. *Int. Immunol.* 2008. **20**: 1019–1030.

20 **Suzukawa, M., Iikura, M., Koketsu, R., Nagase, H., Tamura, C., Komiya, A., Nakae, S. et al.,** An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. *J. Immunol.* 2008. **181**: 5981–5989.

21 **Silver, M. R., Margulis, A., Wood, N., Goldman, S. J., Kasaian, M. and Chaudhary, D.,** IL-33 synergizes with IgE-dependent and IgE-independent agents to promote mast cell and basophil activation. *Inflamm. Res.* 2010. **59**: 207–218.

22 **Carriere, V., Roussel, L., Ortega, N., Lacorre, D. A., Americh, L., Aguilar, L., Bouche, G. and Girard, J. P.,** IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc. Natl. Acad. Sci. USA* 2007. **104**: 282–287.

23 **Pushparaj, P. N., Tay, H. K., H'ng, S. C., Pitman, N., Xu, D., McKenzie, A., Liew, F. Y. and Melendez, A. J.,** The cytokine interleukin-33 mediates anaphylactic shock. *Proc. Nat. Acad. Sci. USA* 2009. **106**: 9773–9778.

24 **Xu, D., Jiang, H. R., Kewin, P., Li, Y., Mu, R., Fraser, A. R., Pitman, N. et al.,** IL-33 exacerbates antigen-induced arthritis by activating mast cells. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 10913–10918.

25 **Liew, F. Y., Pitman, N. I. and McInnes, I. B.,** Disease-associated functions of IL-33: the new kid in the IL-1 family. *Nat. Rev. Immunol.* 2010. **10**: 103–110.

26 **Smith, D. E.,** IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clin. Exp. Allergy* 2010. **40**: 200–208.

27 **Theoharides, T. C., Zhang, B., Kempuraj, D., Tagen, M., Vasiadi, M., Angelidou, A., Alysandratos, K. D. et al.,** IL-33 augments substance P-

induced VEGF secretion from human mast cells and is increased in psoriatic skin. *Proc. Natl. Acad. Sci. USA* 2010. **107**: 4448–4453.

28 **Xu, D., Jiang, H. R., Li, Y., Pushparaj, P. N., Kurowska-Stolarska, M., Leung, B. P., Mu, R. et al.,** IL-33 exacerbates autoantibody-induced arthritis. *J. Immunol.* 2010. **184**: 2620–2626.

29 **Hsu, C.-L., Neilsen, C. V. and Bryce, P. J.,** IL-33 is produced by mast cells and regulates IgE-dependent inflammation. *PLoS One* 2010. **5**: e11944.

30 **Arend, W. P., Palmer, G. and Gabay, C.,** IL-1, IL-18, and IL-33 families of cytokines. *Immunol. Rev.* 2008. **223**: 20–38.

31 **Chackerian, A. A., Oldham, E. R., Murphy, E. E., Schmitz, J., Pflanz, S. and Kastelein, R. A.,** IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. *J. Immunol.* 2007. **179**: 2551–2555.

32 **Ali, S., Huber, M., Kollewe, C., Bischoff, S. C., Falk, W. and Martin, M. U.,** IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. *Proc. Natl. Acad. Sci. USA* 2007. **104**: 18660–18665.

33 **Palmer, G., Lipsky, B. P., Smithgall, M. D., Meininger, D., Siu, S., Talabot-Ayer, D., Gabay, C. and Smith, D. E.,** The IL-1 receptor accessory protein (AcP) is required for IL-33 signaling and soluble AcP enhances the ability of soluble ST2 to inhibit IL-33. *Cytokine* 2008. **42**: 358–364.

34 **Gadina, M. and Jefferies, C. A.,** IL-33: a sheep in wolf's clothing? *Sci. STKE* 2007. **2007**: e31.

35 **Kakkar, R. and Lee, R. T.,** The IL-33/ST2 pathway: therapeutic target and novel biomarker. *Nat. Rev. Drug Discov.* 2008. **7**: 827–840.

36 **Schmitz, J., Owyang, A., Oldham, E., Song, Y., Murphy, E., McClanahan, T. K., Zurawski, G. et al.,** IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005. **23**: 479–490.

37 **Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z. and Matsumoto, K.,** The kinase TAK1 can activate the NIK-IκB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature* 1999. **398**: 252–256.

38 **Jiang, Z., Ninomiya-Tsuji, J., Qian, Y., Matsumoto, K. and Li, X.,** Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. *Mol. Cell Biol.* 2002. **22**: 7158–7167.

39 **Shim, J. H., Xiao, C., Paschal, A. E., Bailey, S. T., Rao, P., Hayden, M. S., Lee, K. Y. et al.,** TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes Dev.* 2005. **19**: 2668–2681.

40 **Matsuyama, Y., Okazaki, H., Tamemoto, H., Kimura, H., Kamata, Y., Nagatani, K., Nagashima, T. et al.,** Increased levels of interleukin 33 in sera and synovial fluid from patients with active rheumatoid arthritis. *J. Rheumatol.* 2010. **37**: 18–25.

41 **Moritz, D. R., Rodewald, H. R., Gheyselinck, J. and Klemenz, R.,** The IL-1 receptor-related T1 antigen is expressed on immature and mature mast cells and on fetal blood mast cell progenitors. *J. Immunol.* 1998. **161**: 4866–4874.

42 **Razin, E., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M. and Rivera, J.,** Protein kinase C-β and C-ε link the mast cell high-affinity receptor for IgE to the expression of c-fos and c-jun. *Proc. Natl. Acad. Sci. USA* 1994. **91**: 7722–7726.

43 **Su, T. T., Guo, B., Kawakami, Y., Sommer, K., Chae, K., Humphries, L. A., Kato, R. M. et al.,** PKC-β controls IκB kinase lipid raft recruitment and activation in response to BCR signaling. *Nat. Immunol.* 2002. **3**: 780–786.

44 **Hayden, M. S. and Ghosh, S.,** Shared principles in NF-κB signaling. *Cell* 2008. **132**: 344–362.

45 **Cissel, D. S., Fraundorfer, P. F. and Beaven, M. A.,** Thapsigargin-induced secretion is dependent on activation of a cholera toxin-sensitive and a phosphatidylinositol-3-kinase-regulated phospholipase D in a mast cell line. *J. Pharmacol. Exp. Ther.* 1998. **285**: 110–118.

46 **Mascarell, L. and Truffa-Bachi, P.,** New aspects of cyclosporin a mode of action: from gene silencing to gene up-regulation. *Mini Rev. Med. Chem.* 2003. **3**: 205–214.

47 **Ninomiya-Tsuji, J., Kajino, T., Ono, K., Ohtomo, T., Matsumoto, M., Shiina, M., Mihara, M. et al.,** A resorcylic acid lactone, 5Z-7-oxozeaenol, prevents inflammation by inhibiting the catalytic activity of TAK1 MAPK kinase kinase. *J. Biol. Chem.* 2003. **278**: 18485–18490.

48 **Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K. et al.,** Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nat. Immunol.* 2005. **6**: 1087–1095.

49 **Klemm, S., Gutermuth, J., Hültner, L., Sparwasser, T., Behrendt, H., Peschel, C., Mak, T. W. et al.,** The Bcl10-Malt1 complex segregates FcεRI-mediated nuclear factor-κB activation and cytokine production from mast cell degranulation. *J. Exp. Med.* 2006. **203**: 337–347.

50 **Sun, L., Deng, L., Ea, C. K., Xia, Z. P. and Chen, Z. J.,** The TRAF6 ubiquitin ligase and TAK1 kinase mediate IKK activation by BCL10 and MALT1 in T lymphocytes. *Mol. Cell* 2004. **14**: 289–301.

51 **Loots, G. G. and Ovcharenko, I.,** rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Res.* 2004. **32**: W217–W221.

52 **Loots, G. G. and Ovcharenko, I.,** Mulan: multiple-sequence alignment to predict functional elements in genomic sequences. *Methods Mol. Biol.* 2007. **395**: 237–254.

53 **Mácian, F., López-Rodriguez, C. and Rao, A.,** Partners in transcription: NFAT and AP-1. *Oncogene* 2001. **20**: 2476–2489.

54 **de Lumley, M., Hart, D. J., Cooper, M. A., Symeonides, S. and Blackburn, J. M.,** A biophysical characterisation of factors controlling dimerisation and selectivity in the NF-κB and NFAT families. *J. Mol. Biol.* 2004. **339**: 1059–1075.

55 **Macian, F., Garcia-Rodriguez, C. and Rao, A.,** Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. *EMBO J.* 2000. **19**: 4783–4795.

56 **Iwaki, S., Tkaczyk, C., Satterthwaite, A. B., Halcomb, K., Beaven, M. A., Metcalfe, D. D. and Gilfillan, A. M.,** Btk plays a crucial role in the amplification of FcεRI-mediated mast cell activation by Kit. *J. Biol. Chem.* 2005. **280**: 40261–40270.

Abbreviations: **Akt:** protein kinase B **Bcl10:** B cell lymphoma 10 **BMMC:** bone marrow–derived mast cells **$[Ca^{2+}]_i$:** concentration of free cytosolic Ca^{2+} **IKK:** inhibitor of the NF-κB kinase complex **IRAK:** IL-1 receptor-associated kinase **PL:** phospholipase **RBL-2H3:** rat basophilic leukemia mast cell **TAK1:** TGF-β-activated kinase 1 **TIR:** Toll/interleukin-1 receptor **TRAF6:** TNF receptor-associated factor 6

Full correspondence: Dr. Michael A. Beaven, Rm 8N109/Bldg. 10, National Institutes of Health, Bethesda, MD 20892-1760, USA
Fax: +1-301-402-0171
e-mail: beavenm@nhlbi.nih.gov

Received: 7/6/2010
Revised: 2/11/2010
Accepted: 15/12/2010
Accepted article online: 3/1/2011

