Mast cells are critical for allergic and inflammatory responses in which the peptide substance P (SP) and the cytokine IL-33 are involved. SP (0.01–1 μM) administered together with IL-33 (30 ng/mL) to human cultured LAD2 mast cells stimulates a marked increase (P < 0.0001) in secretion of the proinflammatory cytokine IL-1β. Preincubation of LAD2 (30 min) with the SP receptor (NK-1) antagonists L-733,060 (10 μM) or CP-96345 (10 μM) inhibits (P < 0.001) secretion of IL-1β stimulated by either SP (1 μM) or SP together with IL-33 (30 ng/mL). Surprisingly, secretion of IL-1β stimulated by IL-33 is inhibited (P < 0.001) by each NK-1 antagonist. Preincubation with an antibody against the IL-33 receptor ST2 inhibits (P < 0.0001) secretion of IL-1β stimulated either by IL-33 or together with SP. The combination of SP (1 μM) with IL-33 (30 ng/mL) increases IL-1β gene expression by 90-fold in LAD2 cells and by 200-fold in primary cultured mast cells from human umbilical cord blood. The combination of SP and IL-33 increases intracellular levels of IL-1β in LAD2 by 100-fold and gene expression of IL-1β and procaspase-1 by fivefold and pro-IL-1β by twofold. Active caspase-1 is present even in unstimulated cells and is detected extracellularly. Preincubation of LAD2 cells with the natural flavonoid methoxyluteolin (100–1000 μM) inhibits (P < 0.0001) secretion and gene expression of IL-1β, procaspase-1, and pro-IL-1β. Mast cell secretion of IL-1β in response to SP and IL-33 reveals targets for the development of antiinflammatory therapies.

IL-1β | IL-33 | inflammation | mast cells | substance P

Substance P and IL-33 administered together stimulate a marked secretion of IL-1β from human mast cells, inhibited by methoxyluteolin

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Mast cells are immune cells that do not circulate but exist in vascularized tissues and have multiple diverse functions (1–3). Mast cells are best known for their critical role in allergic reactions (4–8) via activation by allergens of the high-affinity IgE receptor FceRI (9). Mast cells are also stimulated by the peptide substance P (SP) (10–12) initially characterized by Chang and Leeman (13) and shown to participate in inflammatory processes (14–17).

Mast cells, when stimulated, secrete preformed molecules stored in their granules that include histamine, tryptase (18), and many proinflammatory cytokines and chemokines synthesized de novo (19–22). Even though many immune cells secrete IL-1β (23), the ability of human mast cells to secrete IL-1β has not been previously investigated.

IL-33 is a member of the IL-1 family of cytokines and has emerged as an early warning sign (dubbed “alarmin”) (24) in autoimmune or inflammatory process (25–27). IL-33 is secreted by fibroblasts and endothelial cells (28). IL-33 augments the effect of IgE on the secretion of histamine from mast cells and basophils (24, 29) by “priming” them (30). We recently showed that stimulation of human mast cells by SP given together with IL-33 markedly increases secretion and gene expression of another proinflammatory cytokine, TNF (12). We also reported that this response is inhibited by the natural flavonoid methoxyluteolin (5,7,3',4'-tetramethoxyflavone) (12, 31, 32).

IL-1β is a key proinflammatory cytokine secreted mostly by macrophages that plays an important role in immune and inflammatory diseases (33). IL-1β is present in the cytoplasm in a biologically inactive form that requires activation via proteolytic cleavage by caspase-1. This protease is also present in the cytoplasm in a proform and is activated by the multiprotein complex known as “inflammasome” [Nod-like receptor pyrin domain containing protein 3 (NLRP3) and Apoptosis-associated speck-like protein containing CARD (ASC)] (34, 35).

The data presented in this report show that when SP and IL-33 are administered together a marked increase in the secretion of IL-1β from human cultured mast cells occurs. Preincubation with NK-1 antagonists inhibits not only the combined effect of SP and IL-33 but also the effect of IL-33 given alone. SP and IL-33, when administered together, also stimulate gene expression of pro-IL-1β and procaspase 1, components required for the synthesis of IL-1β. Both active caspase-1 and the mature form of IL-1β are present in unstimulated human mast cells. These effects are all inhibited by methoxyluteolin, which could be used for the treatment of inflammatory diseases.

Results

SP and IL-33 Administered Together Stimulate a Marked Secretion of IL-1β. Administration of SP (1 μM) and IL-33 (30 ng/mL) together are present in unstimulated human mast cells. These findings highlight the important role of SP and IL-33 in mast cell secretion of IL-1β and point to targets for the development of therapies for inflammatory diseases.

Significance

Mast cells are mandatory for allergic reactions and participate in inflammatory responses in which the peptide substance P (SP) and the cytokine IL-33 are involved. This report shows that SP administered together with IL-33 to cultured human mast cells causes a marked increase in the secretion and gene expression of IL-1β. These responses are mediated via the activation of the SP receptor NK-1 and the IL-33 receptor ST2 and can be inhibited by the natural flavonoid methoxyluteolin. These findings highlight the important role of SP and IL-33 in mast cell secretion of IL-1β and point to targets for the development of therapies for inflammatory diseases.

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Conflict of interest statement: T.C.T. is the recipient of US patent no. 7,906,153 covering the use of flavonoids in neuroinflammatory conditions.

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for 24 h stimulates a 100-fold (P < 0.01) increase in the secretion of IL-1β from LAD2 cells compared with unstimulated cells and a 10-fold increase compared with cells treated by IL-33 alone (Fig. 1A). In contrast, stimulation of LAD2 cells by SP (1 μM) alone for 24 h results in the secretion of 15 pg·10^{-6}·cells·mL^{-1}·IL-1β (P = 0.15), and stimulation by IL-33 (30 ng/mL) alone results in the secretion of 35 pg·10^{-6}·cells·mL^{-1}·IL-1β (P = 0.09), neither of which is significant (Fig. 1A).

LAD2 cells stimulated for 24 h either with known triggers of IL-1β secretion from macrophages [LPS (100 ng/mL), ATP (5 μM), nigericin (10 μM), TNF (50 ng/mL), IFN-γ (100 U), IgE (1 μg/mL)/anti-IgE (5 μg/mL), or their combinations as shown for 24 h] or their combinations as shown for 24 h (n = 3, **P < 0.01 and ***P < 0.0001 compared with unstimulated controls). The combination of SP and IL-33-stimulated secretion of IL-1β. LAD2 cells (1 × 10^5 cells per well) were preincubated with human IgE (1 μg/mL) overnight, and were stimulated the next day with anti-IgE (10 ng/mL) for 2 h and/or SP (1 μM) and IL-33 (30 ng/mL) for 24 h. Supernatant fluids were collected at the end of the incubation period and were assayed for IL-1β using ELISA (n = 3, **P < 0.01 and ***P < 0.001 compared to SP alone or to SP+IL-33, respectively). Conc, concentration.

![Fig. 1](image1.png)

Fig. 1. (A) SP and IL-33 stimulate IL-1β secretion. LAD2 cells (1 × 10^5 cells per well) were seeded in a 96-well culture plate and stimulated with LPS (100 ng/mL), ATP (5 μM), SP (1 μM), IL-33 (30 ng/mL), nigericin (10 μM), TNF (50 ng/mL), IFN-γ (100 U), IgE (1 μg/mL)/anti-IgE (5 μg/mL), or their combinations as shown for 24 h. Control cells were treated with culture medium only (n = 3, **P < 0.01 compared with unstimulated controls). (B) IgE/anti-IgE decrease the SP- and IL-33-stimulated secretion of IL-1β. LAD2 cells (1 × 10^5 cells per well) were seeded in a 96-well culture plate, were preincubated with human IgE (1 μg/mL) overnight, and were stimulated the next day with anti-IgE (10 ng/mL) for 2 h and/or SP (1 μM) and IL-33 (30 ng/mL) for 24 h. Supernatant fluids were collected at the end of the incubation period and were assayed for IL-1β using ELISA (n = 3, **P < 0.01 and ***P < 0.001 compared to SP alone or to SP+IL-33, respectively). Conc, concentration.

![Fig. 2](image2.png)

Fig. 2. Selection of the optimal doses to study IL-1β secretion stimulated by SP and IL-33 when administered in combination. (A and B) LAD2 cells (1 × 10^5 cells per well) were stimulated with SP (0.01–1 μM) (n = 3, **P < 0.05 and ***P < 0.0001 compared with SP alone) (A) or IL-33 (1–100 ng/mL) (B) and their combination as shown for 24 h (n = 3, **P < 0.001 and ****P < 0.0001 compared with SP alone). (C) Time-dependent study of IL-1β secretion. LAD2 cells (1 × 10^5 cells per well) were stimulated with the combination of SP (1 μM) and IL-33 (30 ng/mL) for 2–24 h. Supernatant fluids were collected at the end of the incubation period. IL-1β secretion was assayed using ELISA (n = 3, **P < 0.01). Conc, concentration.
this combination was selected for further studies. A time-course study showed that 24 h of stimulation yields the highest amount of IL-1β secretion (Fig. 2C), but some secretion of IL-1β was measurable even at 2 h of stimulation, illustrating the rapidity of the response.

**SP and IL-33 Administered Together Induce IL-1β Gene Expression.** LAD2 and primary human umbilical cord blood mast cells (hCBMCs) were stimulated with SP and IL-33 for 6 h, and the expression of the IL-1β gene was measured. SP (1 μM) and IL-33 (30 ng/mL) administered together for 6 h increase IL-1β gene expression, as measured by qPCR, by 90-fold ($P < 0.0001$) in LAD2 cells and by 200-fold ($P < 0.0001$) in hCBMCs (Fig. 3).

**NK-1 Receptor Antagonists Inhibit Secretion of IL-1β Stimulated by SP and IL-33.** Preincubation of LAD2 cells with each of the neurokinin 1 (NK-1) receptor antagonists [L-733,060 (10 μM) or CP-96345 (10 μM)] for 30 min and followed by stimulation for 24 h with SP (1 μM) alone, IL-33 (30 ng/mL) alone, or their combination significantly ($P < 0.0001$) inhibits the secretion of IL-1β (Fig. 4). Surprisingly, the antagonists also ($P < 0.001$) inhibit the effect of

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**Fig. 3.** SP and IL-33 markedly enhance IL-1β gene expression and secretion. LAD2 cells (1 x 10⁶ cells per well) (A) and hCBMCs (0.3 x 10⁶ cell per well) (B) were seeded in a 12-well culture plate and were stimulated with SP (1 μM), IL-33 (30 ng/mL), or their combination for 6 h. IL-1β mRNA expression levels were measured by qRT-PCR and were normalized to human GAPDH endogenous control ($n = 3$, ****$P < 0.0001$).

**Fig. 4.** NK-1 receptor antagonists inhibit IL-1β secretion. (A) LAD2 cells were pretreated with NK-1R antagonists L-733,060 (10 μM) ($n = 3$, **$P < 0.01$ and ****$P < 0.0001$ compared with IL-33 alone) and CP-96345 (10 μM) ($n = 3$, ****$P < 0.0001$ compared with SP and IL-33) for 30 min and then were stimulated with SP (1 μM), IL-33 (30 ng/mL), or their combination for 24 h. Antibody against ST2 inhibits IL-1β secretion. (B) LAD2 cells (1 x 10⁶ cells per well) were seeded in a 96-well culture plate and were preincubated with an antibody against ST2, the IL-33 receptor (ST2, 0.3–10 μg/mL), or a nonspecific antibody not recognizing ST2 (IgG control) (0.3–10 μg/mL) for 2 h and then were stimulated with IL-33 (30 ng/mL) for 24 h ($n = 3$, **$P < 0.01$, ****$P < 0.0001$ compared with IL33 alone). (C) LAD2 cells were preincubated with anti-ST2 neutralizing antibody (3 ng/mL) or IgG control (3 ng/mL) for 2 h and then were stimulated with SP (1 μM), IL-33 (30 ng/mL), or their combination for 24 h. Collected supernatant fluids were assayed by IL-1β ELISA ($n = 3$, ****$P < 0.0001$ compared with SP and IL-33). Conc, concentration.
IL-33 when given alone (Fig. 4A), suggesting an interaction between NK-1 and ST2.

An Antibody Against ST2 Inhibits Secretion of IL-1β Stimulated by the Combination of SP and IL-33. LAD2 cells were pretreated with an anti-ST2 antibody (3 μg/mL) or with a nonspecific antibody that does not recognize ST2 (3 μg/mL) for 2 h and then were stimulated for 24 h with IL-33 (30 ng/mL) alone (Fig. 4B) or with either SP (1 μM) alone (Fig. 4C) or the combination of both SP and IL-33 (Fig. 4C). Preincubation with the anti-ST2 antibody inhibits (P < 0.0001) secretion of IL-1β in response to either IL-33 alone or the combination of both SP and IL-33 but not in response to stimulation by SP alone (Fig. 4C).

The Combination of SP and IL-33 Stimulates Synthesis of pro-IL-1β. IL-33 (30 ng/mL) alone or in combination with SP (1 μM)
stimulates pro-IL-1β protein expression (Fig. 5A) but does not affect the inflammasome protein (NLRP3 or ASC) levels (Fig. 5A). An important finding is that active forms of caspase-1 (p20 kDa) and IL-1β (p17 kDa) are present even in unstimulated LAD2 cells (Fig. 5A).

The Combination of SP and IL-33 Increases Caspase-1 Gene Expression and Activity. The combination of SP (1 μM) and IL-33 (30 ng/mL) significantly (P < 0.01) increases caspase-1 gene expression by fivefold in both LAD2 cells (Fig. 5B) and primary hCBMCs (Fig. 5C). The presence of active caspase-1 in unstimulated and stimulated LAD2 cells was also investigated using the FLICA assay. Untreated control LAD2 cells make up over 5% of active caspase-1+ cells (Fig. 6A). The percentage of active caspase-1+ cells does not change after stimulation with SP (1 μM) alone, IL-33 (30 ng/mL) alone, or their combination (Fig. 6B–D). The combination of SP and IL-33 increases caspase-1 activity in the culture medium by twofold (Fig. 6F).

Methoxyluteolin Inhibits the Secretion of IL-1β. Preincubation with methoxyluteolin significantly (P < 0.01) inhibits the secretion (Fig. 7B) and intracellular levels (Fig. 7A) of IL-1β (Fig. 7).
Preincubation with the known inflammasome inhibitors [the selective irreversible caspase-1 inhibitor AC-YVAD-CMK (YVAD 50 μM), glybenclamide (GLY 50 μM), or methoxyluteolin (MET 50 μM), and then were stimulated with the combination of SP (1 μM) and IL-33 (30 ng/mL) for 6 h. The gene expression of the inflammasome components NLRP3 (A) and ASC (B) and caspase-1 gene expression (C) were measured by qRT-PCR and normalized to human GAPDH endogenous control (n = 3, *P < 0.05 and **P < 0.01 compared to SP and IL-33).

**Fig. 9.** Methoxyluteolin decreases caspase-1 gene expression. LAD2 cells (1 × 10^6 cells per well) were seeded in a 12-well culture plate, were preincubated with AC-YVAD-CMK (YVAD 50 μM), glybenclamide (GLY 50 μM), or methoxyluteolin (MET 50 μM), and then were stimulated with the combination of SP (1 μM) and IL-33 (30 ng/mL) for 6 h. The gene expression of the inflammasome components NLRP3 (A) and ASC (B) and caspase-1 gene expression (C) were measured by qRT-PCR and normalized to human GAPDH endogenous control (n = 3, *P < 0.05 and **P < 0.01 compared to SP and IL-33).

**Methoxyluteolin Inhibits Gene and Protein Expression of IL-1β.** Methoxyluteolin completely (P < 0.05) inhibits gene (Fig. 8A) and protein (Fig. 8B) expression of IL-1β. The inflammasome inhibitors partially reduce gene (Fig. 8A) and protein (Fig. 8B) expression of IL-1β. Expression of the inflammasome proteins NLRP3 and ASC is not affected (Fig. 8B).

**Methoxyluteolin Inhibits Gene Expression of Procaspase 1 and pro-IL-1β.** Only methoxyluteolin, but not the inflammasome inhibitors, significantly (P < 0.05) inhibits protein expression not only of IL-1β but also of procaspase 1 and pro-IL-1β (Fig. 8B), components necessary for the synthesis of IL-1β. Only methoxyluteolin also inhibits caspase-1 gene expression (Fig. 9C). Gene expression of the inflammasome proteins NLRP3 and ASC is not affected (Fig. 9A and B).

**Methoxyluteolin Inhibits Procaspase 1 Activity.** Pretreatment with either methoxyluteolin or the inflammasome inhibitors has no effect on the percentage of active caspase-1^+^ cells (Fig. 10.A–E). Only methoxyluteolin inhibits caspase-1 activity in the culture medium (Fig. 10F).

**Discussion**

Human mast cells synthesize and secrete 1,000-fold more IL-1β than controls in response to the combination of SP and IL-33 as early as 2 h. This impressive amount contrasts starkly with previous reports that unstimulated human foreskin skin mast cells produced a threefold increase in the secretion of IL-1β in response to phorbol 12-myristate 13-acetate (PMA) but not to IgE receptor cross-linking (37). PMA plus the ionophore A23187 also stimulated a sixfold increase in IL-1β secretion from the immature mast cell leukemic (HMC-1) cell line (38).

The effects we report occur via activation of the high-affinity NK-1 receptor previously shown to be expressed by human mast cells (12). The smallest amount of SP required for a response was 0.1 μM (Fig. 2), but we chose to use 1 μM for maximal effect, as we previously showed for TNF secretion (12). One explanation for the use of this seemingly high amount of SP in our experiments is that SP may be degraded during the assay procedure by chymase secreted from human mast cells, as reported previously (39).

Preincubation with the two different SP receptor antagonists L-733,060 or CP-96345 significantly inhibits IL-1β secretion stimulated by the combined administration of SP and IL-33 and also inhibits IL-1β secretion in response to IL-33 alone, suggesting that there may be some receptor–receptor interaction between NK-1 and ST2. The same SP receptor antagonists also inhibited the secretion of TNF from human cultured mast cells in response to IL-33 (12). The notion of NK-1–ST2 interactions is consistent with our previous finding that NK-1 coimmunoprecipitated with ST2 (12).

Here we report that IL-33 by itself and in combination with SP stimulates gene expression and synthesis not only of IL-1β, but also of pro-IL-1β and caspase-1. It is known that IL-1β is present in the cytoplasm in a biologically inactive form (pro-IL-1β) and is activated via proteolytic cleavage by caspase-1, which also is present in a proform (procaspase 1) and is activated by the NLRP3 inflammasome (34, 35). NLRP3 activation requires two signals (Fig. 1) (40, 41). Signal 1 can be pathogen-associated molecular patterns, environmental agents (silica, asbestos), or endogenous danger signals (27, 42) that induce the transcriptional factor NF-κB, which then stimulates gene expression of the NLRP3 components and pro-IL-1β (Fig. 11). Signal 2 can be reactive oxygen species, potassium ion influx, or calcium ions influx (43) and initiates the recruitment and assembly of the adaptor ASC together with the NLRP3 protein and procaspase-1, subsequently cleaving the inactive procaspase-1 into active caspase-1. When activated, caspase-1 then cleaves pro-IL-1β...
Neither stimulation or inhibition of LAD2 cells has an effect on the number of cells positive for Caspase-1 (A-E)

Methoxyluteolin inhibits Caspase-1 activity in the supernatant fluid from stimulated LAD2 cells

**Fig. 10.** (A–E) Methoxyluteolin and inflammasome inhibitors do not decrease the percentage of active caspase-1–expressing human mast cells. LAD2 cells (0.25 × 10^6 cells per well) were preincubated with AC-YVAD-CMK (YVAD, 50 μM), glybenclamide (GLY, 50 μM), or methoxyluteolin (MET, 50 μM) and then were stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 24 h. Stimulated cells were incubated with the FLICA caspase-1 probe for 1 h, and active caspase-1+ cells were assessed by flow cytometry. Each panel is representative of three experiments. (F) Methoxyluteolin decreases caspase-1 activity. LAD2 cells (0.5 × 10^5 cells per well) were preincubated with AC-YVAD-CMK (YVAD, 50 μM), glybenclamide (GLY, 50 μM), or methoxyluteolin (MET, 50 μM) and then were stimulated with SP (1 μM) and IL-33 (30 ng/mL) for 24 h. After stimulation, 50 μM YVAD-AFC substrate was added to cells and incubated for 2 h. Caspase-1 activity in the supernatant fluids was determined by measuring fluorescence at 405 nm. Fold-increase in caspase-1 activity was determined by comparing the values to the untreated control samples (n = 3, **P < 0.01 compared with SP and IL-33).

Neither stimulation or inhibition of LAD2 cells has an effect on the number of cells positive for Caspase-1 (A-E)

The fact that methoxyluteolin significantly inhibits the impressive gene expression and synthesis of IL-1β reported here is noteworthy. It was previously reported that the methoxyluteolin structural analog luteolin (5,7,3',4'-tetrahydroxyflavone) inhibited the limited IL-1β secretion from HMC-1 cells stimulated by PMA and the ionophore A23187 (38). The precise biochemical step(s) by which methoxyluteolin exerts its inhibitory effect on IL-1β secretion and synthesis is not presently known. We had previously reported that methoxyluteolin inhibits NF-κB activation stimulated by SP in human mast cells (31). Other potential sites of action of methoxyluteolin are presented in Fig. 11. Methoxyluteolin could inhibit the release of procaspase-1 and/or pro-IL-1β from the nucleus since flavonoids have been reported to enter the nucleus (63). Another possibility is that methoxyluteolin could inhibit how the secretory vesicles fuse with the plasma membrane to secrete IL-1β (Fig. 11). One study using cultured rat basophil leukemia (RBL-1) cells reported that a number of polyphenolic compounds, including luteolin, interfered with this secretory process (64). The concentrations (1–100 μM) shown to be effective in this report have previously been reported to inhibit human cultured mast cells (31, 32). The smallest concentration of luteolin that could be attained in the blood of individuals on ordinary dietary intake was estimated to be 0.1 μM (65), which is 10 times higher than the smallest concentration (1 μM) showing significant inhibitory activity in our studies. This higher concentration could be reached by administration of dietary supplements containing pure luteolin or methoxyluteolin.

In conclusion, IL-33 by itself, and more impressively SP and IL-33 administered together, are identified as stimuli of IL-1β into the active mature IL-1β, which is then secreted extracellularly (Fig. 11) (43, 44). The fact that unstimulated mast cells contain pre-IL-1β and active caspase-1 implies that mast cells can respond rapidly to danger signals (27) without requiring induction of their respective genes. The results reported are of additional importance given that none of the known triggers of the NLRP3 inflammasome (i.e., LPS, ATP, TNF, and IFN-γ) can stimulate IL-1β secretion from cultured human mast cells.

As reported here, stimulation of mast cells by SP and IL-33 alone or in combination does not affect the gene or protein expression of NLRP3 and ASC proteins. These findings raise the possibility that the NLRP3 inflammasome (signal 2) may not be required for the synthesis of IL-1β in human mast cells, at least in response to SP and IL-33 (Fig. 11).

IL-1β is known to be involved in the regulation of the innate immune response (33, 45) and is mandatory for autoimmune diseases known as “cryopyrin-associated periodic syndromes” (CAPS) (25, 26, 28, 46, 47) IL-1β could also be important in diseases involving mast cells and SP (22, 48) and may be involved in multiple other diseases that involve mast cells, including asthma (49), rheumatoid arthritis (50), multiple sclerosis (51), and psoriasis (52–54). In fact, gene expression and activity of caspase-1 were reported to be increased in lesional psoriatic epidermis (55, 56).

Even though anti–IL-1β antibody, soluble IL-1R, and IL-1RA are available for the treatment of inflammatory diseases involving IL-1β (57, 58), there are no specific clinically available drugs that could prevent IL-1β synthesis and secretion from any cell type, including mast cells (59–62). Hence, it is clinically desirable to develop molecules that could prevent the secretion of IL-1β, especially from mast cells.
Fig. 11. Diagrammatic representation of the stimulatory effect of SP and IL-33 on IL-1β synthesis and secretion and the proposed point of inhibition of methoxyluteolin. Our evidence indicates that (1) SP and IL-33 activate their respective receptors and stimulate synthesis of pro-caspase-1 and pro-IL-1β, possibly via NF-κB, activation, which is inhibited by methoxyluteolin. (2) Procaspase-1 and pro-IL-1β are released from the nucleus, a process that could also be inhibited by methoxyluteolin. (3) In the cytoplasm, caspase-1, which is already active, converts pro-IL-1β to active IL-1β. (4) Some of the pro-caspase-1 is converted to caspase-1, but this may be a minor contribution since the NLRP3 inflammasome does not seem to be involved. (5) IL-1β and active caspase-1 are then secreted extracellularly, a process that is also inhibited by methoxyluteolin. Orange boxes and ovals indicate facts supported by our findings. Open boxes and ovals indicate pathways not supported by our data. The (7) attached to methoxyluteolin indicates possible points of inhibition.

Materials and Methods

SP, LPS, ATP, and AC-YVAD-CMK were purchased from Sigma-Aldrich. Recombinant human IL-33, recombinant human TNF, and recombinant human IFN-γ were obtained from R&D Systems. Nigericin was purchased from Enzo Life Sciences, and glibenclamide was purchased from InvivoGen. Human recombinant IgE and anti-IgE were purchased from EMD Millipore. Recombinant human IL-33, recombinant human TNF, and recombinant human IFN-γ were obtained from Santa Cruz Biotechnology. Tetramethoxyflavone inhibitor PS 341 was obtained from Tocris Biosciences. Tetramethoxyflavone was obtained from Pharma Science Nutrients (Hangzhou Skyherb Technologies Co., Ltd.). The RNeasy Mini Kit was from Qiagen, Inc., and the iScript cDNA synthesis kits were purchased from Bio-Rad. Tagman gene-expression primers/assays for NLRP3 (Hs00918082_m1), PYCARD (Hs01547324_gH), CASP1 (Hs00354836_m1), IL1B (Hs01555410_m1), and GAPDH (the endogenous control) (4310884E) were purchased from Applied Biosystems. ELISA kits for IL-1β (DY201), IL-1β (DY3625), and TNF (DY210) were purchased from R&D Systems. Rabbit anti-human primary antibodies for NLRP3, pro-IL-1β, caspase-1, and β-actin were purchased from Cell Signaling Technology, and mouse anti-human ASC and cleaved IL-1β were obtained from Santa Cruz Biotechnology. The FLICA Caspase-1 Activity Assay was purchased from ImmunoChemistry Technologies, and Ac-YVAD-AFC was obtained from Santa Cruz Biotechnology.

Culture of Human Mast Cells. LAD2 cells, derived from a human mast cell leukemia (66), were kindly supplied by Dr. A. Kirshenbaum (NIH, Bethesda) and were cultured in StemPro-34 medium (Invitrogen) supplemented with 100 U/mL penicillin/streptomycin and 100 ng/mL recombinant human stem cell factor (rHSCF; StemGen), kindly supplied by Swedish Orphan Biovitrum AB. Cells were maintained at 37°C in a humidified incubator at an atmosphere of 95% O2/5% CO2. LAD2 cells double within 2 wk in the presence of 100 ng/mL stem cell factor (SCF), showing slow proliferation rates. Even though LAD2 cells are an immortalized proliferating cell line, this cell culture closely resembles CD34+-derived primary human mast cells due to its ability to respond to SCF and express functional FcRI receptors (66). The LAD2 mast cells have been repeatedly shown to behave like immature primary human mast cells (31, 67, 68). Our results were also validated using primary hCBMCs (12, 69) which give more reproducible results than normal primary mast cells derived from skin (70). Mononuclear cells were isolated by layering heparin-treated cord blood onto Lymphocyte Separation Medium (MP Biomedicals). CD34+ progenitor cells were isolated by positive selection of AC133 (CD133+ cells using magnetic cell sorting (CD133 Microbead Kit; Miltenyi Biotech). For the first 6 wk, CD34+ progenitor cells were cultured in Iscove’s modified Dulbecco’s medium (Life Technologies) supplemented with 1% insulin-transferrin-selenium, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. After 6 wk, the cells were cultured in Iscove’s modified Dulbecco medium supplemented with 10% FBS, 50 ng/mL IL-6, 0.1% β-mercaptoethanol, 1% penicillin/streptomycin, and 100 ng/mL rhSCF. These hCBMCs were cultured for at least 12 wk before being used for experiments.

Cell viability was measured by Trypan blue (0.4%) exclusion (11) or by propidium iodide at all SP and IL-33 concentrations tested.

Mast Cell Treatments. LAD2 cells and/or hCBMCs were stimulated with various concentration of SP (0.01–1 μM; Sigma-Aldrich) and IL-33 (1–30 ng/mL; R&D Systems) alone or in combination. In some experiments LAD2 cells were stimulated with human IgE (1 μg/mL; EMD Millipore) overnight and then were triggered with anti-IgE (10 ng/mL; Life Technologies). In other experiments, LAD2 cells were pretreated with the NK-1 antagonists L-733,060 (10 μM; Sigma-Aldrich) and CP-96345 (10 μM; Tocris Biosciences), a goat anti-human ST2-neutralizing antibody (0.3 μg/mL–10 μg/mL; R&D Systems), or nonspecific goat anti-human IgG antibody (0.3 μg/mL–10 μg/mL; R&D Systems), and the proteasome inhibitor PS 341 (1–50 μM; Tocris Biosciences); these agents were washed off after preincubation was competed. Methoxyluteolin (1–100 μM) was obtained (98.5% purity) from Hangzhou Skyherb Technologies Co., Ltd., and was used during the preincubation period and also during stimulation. Silencer Select siRNA targeting either NK-1 or ST2 receptors, as well as control scramble siRNA (10–100 nM; Life Technologies) were used in Lipofectamine RNAiMAX and Opti-MEM medium (Life
Technologies) to treat LAD2 cells for 72–96 h to inhibit gene expression of respective receptors.

**IL-1β and TNF Assays.** LAD2 cells (1 × 10^6 cells/0.5 mL in each well) were treated with various concentration of SP (0.01–1 μM) and IL-33 (30 ng/mL) for 24 h. Control cells were treated with the same volume of culture medium. Supernatant fluids were collected and assayed using IL-1β and TNF DuoSet ELISA kits (R&D Systems). These ELISA kits had 6.3% cross-reactivity with human recombinant pro-IL-1β according to the manufacturer’s instructions.

**RNA Isolation and qRT-PCR.** Mast cells were stimulated with SP (1 μM, 6 h), IL-33 (30 ng/mL), or their combination. Total mRNA was extracted for a NeoEasy Mini kit (Qiagen Inc.) in accordance with the manufacturer’s instructions. An iScript cDNA synthesis kit (Bio-Rad) was used for reverse-transcription of each mRNA sample. qRT-PCR was performed using Taqman gene-expression assays for IL-1β, NLRP3, ASC, and caspase-1 (Applied Biosystems). Samples were run at 45 cycles using a real-time PCR system (7300; Applied Biosystems). Relative mRNA levels were determined from standard curves run with each experiment. The mRNA gene expressions were normalized to GAPDH endogenous control (Applied Biosystems).

**Western Blot Analysis.** LAD2 cells (1 × 10^5 cells) were preincubated with glybenclamide (50 μM), AC-YVAD-CMK (50 μM), or methlut (50 μM) for 2 h and then were stimulated with SP (1 μM), IL-33 (30 ng/mL), or their combination for 24 h. The reaction was stopped by the addition of ice-cold PBS. Cells were washed once with PBS and then were lysed using protein lysis radio-immunoprecipitation (RIPA) buffer (Sigma-Aldrich) in the presence of protease and phosphatase inhibitor mixtures (Thermo Fisher Scientific, Inc.). Total protein concentration was determined by the bicinchoninic acid assay (Thermo Fisher Scientific, Inc.) method using BSA as the standard. The total cellular proteins (20-μg aliquots) were separated using 4–20% Mini Protein TGX gels (Bio-Rad) under SDS denaturing conditions and were electro-transferred onto PVDF membranes (Bio-Rad). Blocking was carried out with 5% BSA in Tris-buffered saline containing 0.05% Tween-20. The membranes were probed with the following primary antibodies at 1:1,000 dilutions: NLRP3, pro-caspase-1, β-actin (Cell Signaling Technology), ASC, and cleaved IL-1β (Santa Cruz Biotechnology). For the loading control β-actin was probed. For detection, the membranes were incubated with the appropriate secondary HRP-conjugated antibody (Cell Signaling Technology) at a 1:1,000 dilution, and the blots were visualized with enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.).

**FLICA Caspase-1 Activity Assay.** LAD2 cells (2.5 × 10^5) were treated with SP (1 μM), IL-33 (30 ng/mL), or their combination for 24 h, centrifuged at 500 × g for 5 min, and washed in Apoptosis Wash Buffer (ImmunoChemistry Technologies). Cells were resuspended in 300 μL of 1× FLICA-YVAD (ImmunoChemistry Technologies) fluorescent caspase-1–binding probe and were incubated for 1 h at 37 °C. Propidium iodide staining was used as negative control. Following the incubation, unreacted substrate was removed by washing the cells with Apoptosis Wash Buffer. Finally, cells were resuspended in 300 μL of Apoptosis Wash Buffer for flow cytometry analysis. Active caspase-1 was determined using a FACSCalibur flow cytometer (BD Biosciences).

**Caspase-1 Activity Assay.** LAD2 cells (5 × 10^5) were stimulated with SP (1 μM), IL-33 (30 ng/mL), or their combination for 24 h in black 96-well plates (Corning, Inc.). After stimulation, 50 μM YVAD-AFC was added to cells and incubated for 2 h at 37 °C (AC-YVAD-AFC, Santa Cruz Biotechnology). Fluorescence was measured by an Excitation wavelength of 405 nm and an emission wavelength of 505 nm. The medium background was subtracted from raw fluorescent values, and then the values were normalized to control untreated cells.

**Statistics.** All experiments were performed in triplicate and were repeated at least three times (n = 3). Data are presented as mean ± SD. Results were analyzed using the unpaired, two-tailed, Student's t test. Significance of comparisons between conditions is denoted by *P < 0.05, **P < 0.1, ***P < 0.001, and ****P < 0.0001.

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