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Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells

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Abstract

IL-33 (or IL-1F11) was recently identified as a ligand for the previously orphaned IL-1 family receptor T1/ST2. Previous studies have established that IL-33 and T1/ST2 exert key functions in Th2 responses. In this study, we demonstrate that IL-33 induces the production of pro-inflammatory mediators in mast cells. IL-33 dose and time-dependently stimulated IL-6 secretion by P815 mastocytoma cells and primary mouse bone marrow-derived mast cells (BMMC). This effect was dependent on T1/ST2 binding. In addition, IL-33 also induced IL-1β, TNF-α, MCP-1, and PGD2 production in BMMC. By RNase protection assay, we demonstrated that IL-33 increased IL-6 and IL-1β mRNA expression. These effects of IL-33 appeared to occur independently of mast cell degranulation, The results of this study show for the first time that IL-33, a novel member of the IL-1 family of cytokines, stimulates the production of pro-inflammatory mediators by mast cells in addition to its effect on T helper 2 responses. These findings open new perspectives for the treatment of inflammatory diseases by targeting IL-33.

Keywords: Cytokine; Inflammation; Mast cells

1. Introduction

IL-33 (or IL-1F11) is a new cytokine belonging to the IL-1 family, which was recently identified as a ligand for the previously orphaned IL-1 receptor (IL-1R) family member T1/ST2 [1]. IL-33 is produced as a 30 kDa propeptide, like IL-1β and IL-18, and is cleaved by caspase-1 to generate mature 18 kD IL-33. In fact, pro-IL-33 had been previously described as a nuclear protein, NF-HEV (Nuclear Factor-High Endothelial Venules), and thus exhibits a sub-cellular localisation similar to that of the IL-1α precursor [2]. The IL-33 propeptide contains a nuclear localization sequence (NLS) and a homeodomain-like helix-turn-helix DNA-binding domain, and, like pre-IL-1α [3], nuclear pre-IL-33 might thus exert unique biologic activities independent of caspase-1 cleavage and cell surface receptor binding.

Mature IL-33 however mediates its biological effects via T1/ST2 binding by activating NF-κB and MAP kinases [1]. A number of studies have established T1/ST2 as a selective marker of both murine and human T helper (Th)2 lymphocytes and as an important effector molecule of Th2 responses (reviewed in [4]). IL-33 drives production of Th2-associated cytokines from *in vitro* polarized Th2 cells. *In vivo*, IL-33 injection induced the expression of IL-4, IL-5, and IL-13 and led to severe pathological changes in the lung and the digestive tract [1]. T1/ST2 is also highly expressed on mast cells. In fact, mast cells seem to be the

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only hematopoietic cell lineage which expresses T1/ST2 throughout differentiation, starting from the earliest detectable committed mast cell progenitor [5]. However, the importance of IL-33 and T1/ST2 signaling for mast cell function remains completely unknown.

The T1/ST2 receptor also exists as a short, soluble isoform (sST2), obtained by the use of differential mRNA processing. Soluble ST2 is identical with the extracellular region of the long T1/ST2 isoform except for 9 additional amino acids, which are present at the C terminus of the molecule. The expression of sST2 is induced in fibroblasts, macrophages and monocytes stimulated with lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α or IL-1, as well as in activated Th2 clones [4]. Serum concentrations of sST2 are elevated in patients suffering from various disorders associated with an abnormal Th2 response, such as asthma [6], as well as in inflammatory conditions that are mainly independent of a Th2 response. Indeed, increased levels of sST2 were observed in sera from patients suffering from septic shock, trauma [4,7], systemic lupus erythematosus [8] and in the synovial fluid of patients with rheumatoid arthritis, when compared to osteoarthritis patients [9]. In addition, mast cells have been recognized as important mediators of the pathogenesis of inflammatory diseases including ulcerative colitis, Crohn's disease [10], and arthritis [11]. These observations suggest that, like other members of the IL-1/IL-1R families, IL-33 and T1/ST2 might be involved in the disease process in inflammatory disorders involving mast cell activation.

In this study, we investigated the effects of IL-33 on cytokine and chemokine production in P815 mastocytoma cells and in primary murine bone marrow derived mast cells *in vitro*.

2. Materials and methods

2.1. Materials

Cell culture reagents, Trizol and dNTP were obtained from Invitrogen Life Technologies (Basel, Switzerland). Recombinant mouse IL-33 produced in *Escherichia coli* was obtained from Alexis Corporation (Lausanne, Switzerland). The sST2/Fc fusion protein was obtained from Alexis Corporation (Lausanne, Switzerland) or from Amgen Inc. (Thousand Oaks, CA). IL-3 and stem cell factor (SCF) were purchased from Peprotech (Lucerne, Switzerland).

2.2. Cell culture

The mouse mastocytoma cell line P815 (a kind gift of Dr Paul Walker, Geneva) was cultured in DMEM High Glucose supplemented with 6% fetal calf serum, Hepes 10 mM, MEM Non essential amino acids 10 mM, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. For the experiments, cells were collected, centrifuged (250*g*, 5 min), and resuspended in fresh medium. Bone marrow-derived mast cells (BMMC)[2] were generated from bone marrow of 8-week-old C57BL/6 male mice as described [12]. Briefly, mice were sacrificed and intact femurs and tibias were removed. Sterile endotoxin-free RPMI-1640 medium was repeatedly flushed through the bone shaft using a syringe with a 25-G needle. The suspension of bone marrow cells was centrifuged at 250*g* for 5 min. Red blood cells were lysed on ice by incubation in Tris 20 mM/NH_4Cl 150 mM during 5 min. Cells were washed and cultured at a concentration of $0.5–1 \times 10^6$ cells/ml in RPMI-1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyruvate at 37 °C in a humidified atmosphere with 5% CO_2. A combination of SCF (50 ng/ml) and IL-3 (5 ng/ml) was added weekly to the culture medium for two weeks, and IL-3 (5 ng/ml) alone was added weekly during the following weeks. Nonadherent cells were transferred to fresh medium at least once a week. Cells were used after 5–7 weeks of culture, when a mast cell purity of >95% was achieved as assessed by toluidine blue staining and FACS analysis of c-Kit expression.

2.3. Evaluation of BMMC purity

For toluidine blue staining BMMC were centrifuged onto glass slides in a cytofuge, incubated for 5 min in a solution containing 1% toluidine blue in methanol, and washed with distilled water. The cells were then examined by light microscopy.

2.4. Analysis of c-Kit, T1/ST2 and IL-1R accessory protein (IL-1RAcP) expression by flow cytometry

Expression of c-Kit on BMMC was detected by flow cytometry on a FACScan (BD Biosciences, Heidelberg, Germany) using the PE-coupled rat monoclonal anti-c-Kit antibody 2B8 (BD Biosciences, dilution 1/100). Rat PE-coupled IgG2b was used as isotype control. Expression of T1/ST2 was detected using the FITC-coupled rat monoclonal anti-T1/ST2 antibody DJ8 (MD Biosciences, Morwell Diagnostics GmbH, Egg b. Zurich, Switzerland, dilution 1/100). Expression of IL-1RAcP was examined using the rat monoclonal anti-IL-1RAcP antibody M215 (Amgen Inc., dilution 1/100), followed by detection with a PE-coupled goat anti-rat Ig antibody (Southern Biotech, Birmingham, AL, dilution 1/800). For FACS analysis, 1×10^5 BMMC were incubated in 50 μl PBS containing 0.2% bovine serum albumin (BSA) and diluted antibodies for 30 min on ice. Before FACS analysis the BMMC were washed 3-times with PBS, 0.2% BSA.

[2] *Abbreviations used:* BMMC, bone marrow-derived mast cell; IL-1R, interleukin-1 receptor; IL-1RAcP, IL-1R accessory protein; MCP, monocyte chemoattractant protein; PGD2, prostaglandin D2; sST2, soluble ST2 receptor.

2.5. RNA extraction

Total RNA was extracted from 1×10^6 BMMC, P815 cells, 3T3 murine fibroblasts, ATDC5 mouse chondrocytes or from joints of mice with collagen induced arthritis [13] with TRIzol (Invitrogen) according to the manufacturer's protocol.

2.6. RT-PCR analysis of IL-1 receptor family members

Total RNA (3 μg) was digested with DNase I (Promega) and reverse-transcribed using AMV-RT (Promega) and random hexamer primers. PCR amplification (40 cycles for IL-1 receptor family members, 30 cycles for β-actin) was performed using TaqDNA polymerase (Qiagen) and the following primers: IL-1R accessory protein (IL-1RAcP) forward primer 5′-AAC CAT CGG TCA CTT GGT ATA AGG G-3′, IL-1RAcP reverse primer 5′-TTC ATC TGT TCC AAA GTG AGC TCG G-3′ (Genbank Accession No. NM_134103, product size: 586 bp), IL-1R type I (IL-1RI) forward primer 5′-GAG TTA CCC GAG GTC CAG TGG-3′, IL-1RI reverse primer 5′-TCC CTC CAA GAC CTC AGG CAA CAG-3′ (Genbank BC109135.2, product size: 791 bp), IL-18 receptor accessory protein (IL-18RAcP) forward primer 5′-CCC GGA AGT GCT AGA AGA CA-3′, IL-18RAcP reverse primer 5′-ACC CGC AGA GCC TTT TTG AC-3′ (Genbank BC120600.1, product size: 284 bp), T1/ST2 forward primer, common to the long and short isoforms, 5′-CCA TAA GGC TGA GAA GGA AA-3′, T1/ST2 long isoform specific reverse primer 5′-AAC AAA GTA CTC CAC AGA GT-3′ (Genbank NM_001025602.1, product size long isoform: 250 bp), T1/ST2 short isoform specific reverse primer 5′-TTG ATC ATG ATG GAT TCC CT-3′ (Genbank NM_010743.1, product size short isoform: 144 bp), IL-1R related protein 2 (IL-1Rrp2) forward primer 5′-AAA CAC CTA GCA AAA GCC CAG-3′, IL-1Rrp2 reverse primer 5′-AGA CTG CCC GAT TTT CCT ATG-3′ (Genbank BC117797.2 product size: 261 bp), β-actin forward primer 5′-CCA AGG CCA ACC GCG AGA AGA TGA C-3′, β-actin reverse primer 5′-AGG GTA CAT GGT GGT GCC GCC AGA C-3′ (Genbank NM_007393, product size: 586 bp). Annealing temperatures were 55 °C for the T1/ST2 isoforms and 60 °C for the other amplification products. The absence of DNA contamination in RNA preparations was verified by including RNA samples which had not been reverse-transcribed. PCR products were visualized on 2% agarose gels containing ethidium bromide.

2.7. RNase protection assay

RNase protection assay was performed on 5 μg of total RNA as described in the standard RiboQuant™ RNase protection assay protocol, using the multiprobe template set mCK-2b (BDBiosciences). The protected bands representing cytokine mRNA expression were quantified by phosphorimaging using a Cyclone Storage Phosphor System (PerkinElmer Life Sciences, Zaventem, Belgium) and normalized for expression of GAPDH mRNA.

2.8. Determination of cytokine levels

For determination of cytokine production, P815 cells and BMMC were plated in 96-well plates in 200 μl of complete culture medium at a density of, respectively, 20,000 and 100,000 cells per well. Cells were treated with the indicated concentrations of IL-33, IL-18 or IL-1β. In some experiments, sST2/Fc at indicated concentrations was added to IL-33 prior to stimulation. In addition, sTNFRII/Fc (Etanercept, generously provided by Wyeth, Zug, Switzerland) was used as a negative control. The blocking monoclonal anti-T1/ST2 antibody DJ8 (MD Biosciences) was added to the cells 1 h before stimulation with IL-33. The blocking monoclonal anti-IL-1Rrp2 antibody M616 (Amgen Corporation), and the blocking polyclonal anti-IL-1RI antibody (AF771, R&D Systems, Abington, UK) were used as negative controls. Levels of IL-6 and IL-1β in cell supernatants were assessed using enzyme-linked immunosorbent assay (ELISA) DuoSet kits from R&D Systems. For measurement of intracellular IL-1β levels, cells were lysed with 200 μl of complete culture medium containing 1% NP-40 (Sigma).

IL-6, IL-10, monocyte chemoattractant protein (MCP)-1, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-12p70, IL-2, IL-4 and IL-5 levels in culture supernatants were detected using the mouse Inflammation and Th1/Th2 cytokine CBA kits from BD Biosciences (San Jose, CA). Culture supernatants (50 μl) were mixed with 50 μl of the mixed capture beads and 50 μl of the mouse inflammation or Th1/Th2 PE detection reagent. The tubes were incubated at room temperature for 2 h in the dark, followed by a wash step. The samples were then resuspended in 400 μl of wash buffer before acquisition on the FACScan. The data were analyzed using the CBA software. Standard curves were generated for each cytokine using the mixed bead standard provided in the kit and the concentration of cytokine in the cell supernatant was determined by interpolation from the appropriate standard curve.

2.9. Determination of prostaglandin D2 levels

Prostaglandin (PG)D2 levels in culture supernatants of cells stimulated or not with IL-33 for indicated times were assayed using the PGD2 – MOX EIA kit from Cayman Chemical Company (Ann Arbor, MI).

2.10. Tryptase activity

BMMC degranulation was quantified by measuring tryptase activity in culture supernatants of cells stimulated or not with IL-33 (10 ng/ml) for 1 h using the Mast Cell Degranulation Assay Kit from Chemicon International

(Temecula, CA). The calcium ionophore A23187 (500 nM) was used as a positive control to induce degranulation.

3. Results

3.1. IL-33 induces IL-6 production by P815 mastocytoma cells

The P815 mastocytoma cell line was previously shown to express T1/ST2 [14]. We first examined whether IL-33 activates transcription factor pathways similar to those associated with intracellular IL-1 signaling. DNA-binding activities of NF-κB and AP-1 were assessed by EMSA using nuclear extracts from P815 cells stimulated with IL-33 for indicated times. As previously shown in BMMC [1], IL-33 induced NF-κB and AP-1 binding to DNA inP815 cells, which was maximal after 15 and 30 min of stimulation, respectively (data not shown).

To examine the biological effects of IL-33 on P815 cells, we then examined the secretion of IL-6 into culture supernatants. IL-33 dose dependently stimulated IL-6 production, and its effect was comparable to that of IL-1β (Fig. 1a). Heat-inactivated IL-33 did not induce any response, thus ruling out a non-specific effect due to LPS contamination. In fact, endotoxin levels in the recombinant mouse IL-33 preparation used were below 0.1EU/μg protein (<8 pg/μg protein), as measured by Limulus amebocyte lysate assay (Cambrex Bioscience). In addition, stimulation with LPS (up to 1 μg/ml) did not induce any IL-6 production in P815 cells (data not shown).

Levels of IL-6 were significantly induced after 6 h of culture with IL-33 and increased further during up to 72 h of stimulation (Fig. 1b). As IL-33 was recently shown to exert its effects by binding to cell surface T1/ST2, we examined whether incubation with the soluble extracellular domain of ST2 coupled to the Fc fragment of human IgG1

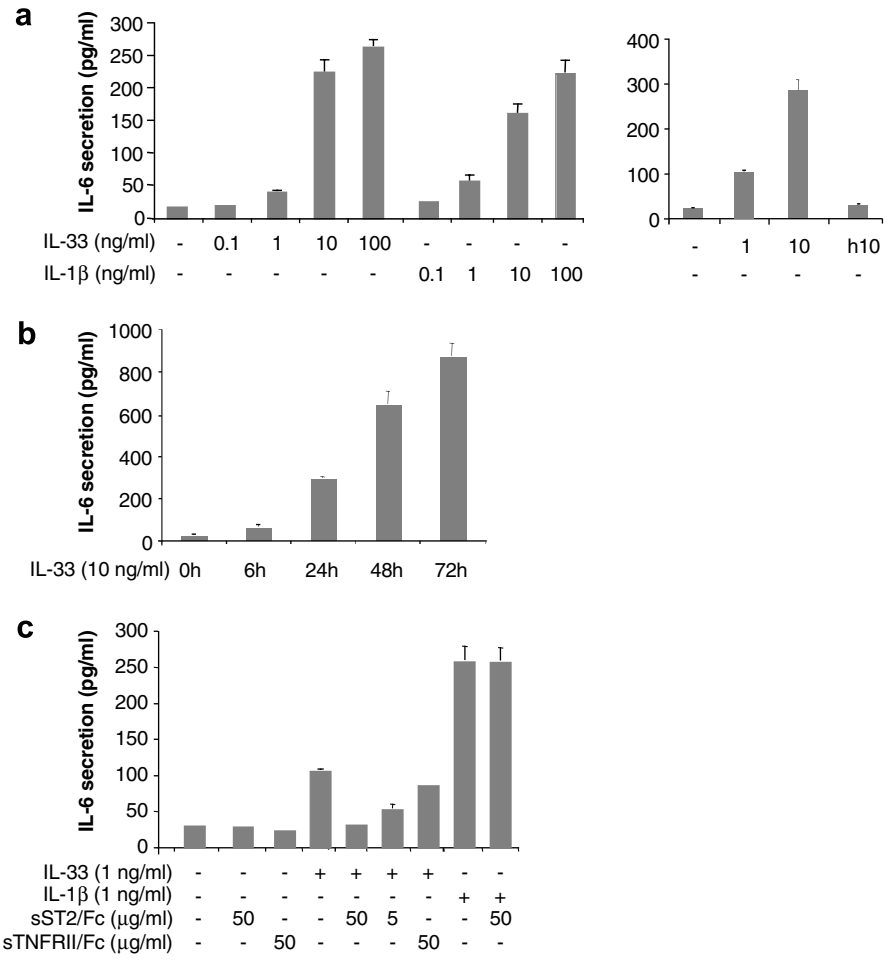

Fig. 1. IL-33 induces IL-6 production by P815 cells. (a, left panel) P815 cells were stimulated with IL-33 (0.1–100 ng/ml), or with IL-1β (0.1–100 ng/ml) for 48 h, before IL-6 levels were assessed by ELISA in culture supernatants. (a, right panel) P815 cells were stimulated with IL-33 (1 or 10 ng/ml), or with 10 ng/ml of heat inactivated IL-33 (h10) for 48 h, before IL-6 levels were assessed by ELISA in culture supernatants. (b) P815 cells were stimulated with IL-33 (10 ng/ml) for 6, 24, 48 or 72 h, before IL-6 levels were assessed by ELISA in culture supernatants. (c) Prior to stimulation IL-33 (1 ng/ml) was incubated with sST2/Fc (50 or 5 μg/ml) or with sTNFRII/Fc (50 μg/ml) for 15 min at 37 °C, then IL-33, sST2/Fc, sTNFRII/Fc, IL-33 and sST2/Fc or IL-33 and sTNFRII/Fc were added to P815 cells for 48 h. To control for the specificity of the effect of sST2/Fc, IL-1β (1 ng/ml) was incubated with sST2/Fc (50 μg/ml) for 15 min at 37 °C, then IL-1β (1 ng/ml) alone or IL-1β and sST2/Fc were added to p815 cells for 48 h. IL-6 levels were measured in culture supernatants by ELISA. Results are representative of at least two independent experiments and are expressed as means ± SEM.

(sST2/Fc) could interfere with the stimulatory effect of IL-33. Indeed, sST2/Fc inhibited the production of IL-6 induced by IL-33 in a dose dependent manner (Fig. 1c). In contrast, the extracellular domain of TNF receptor type II coupled to the Fc fragment of human IgG1 (sTNFRII/Fc, Etanercept), a specific TNF-α inhibitor used here as a negative control, did not interfere with IL-33 activity. Furthermore, the inhibitory effect of sST2/Fc was specific for IL-33, since sST2/Fc did not interfere with the induction of IL-6 production induced by IL-1β in P815 cells.

3.2. IL-33 induces IL-6 production by BMMC

Bone marrow derived mast cells were used to confirm the results obtained with P815 mastocytoma cells on primary mast cell cultures. The purity of BMMC was >95% as assessed by toluidine blue staining and FACS analysis of c-Kit expression (Fig. 2a and b). As previously reported [5], BMMC co-expressed high levels of c-Kit and T1/ST2 (Fig. 2b). BMMC also expressed IL-1RAcP, the co-receptor required for IL-33, IL-1, IL-F6, IL-1F8, and IL-1F9

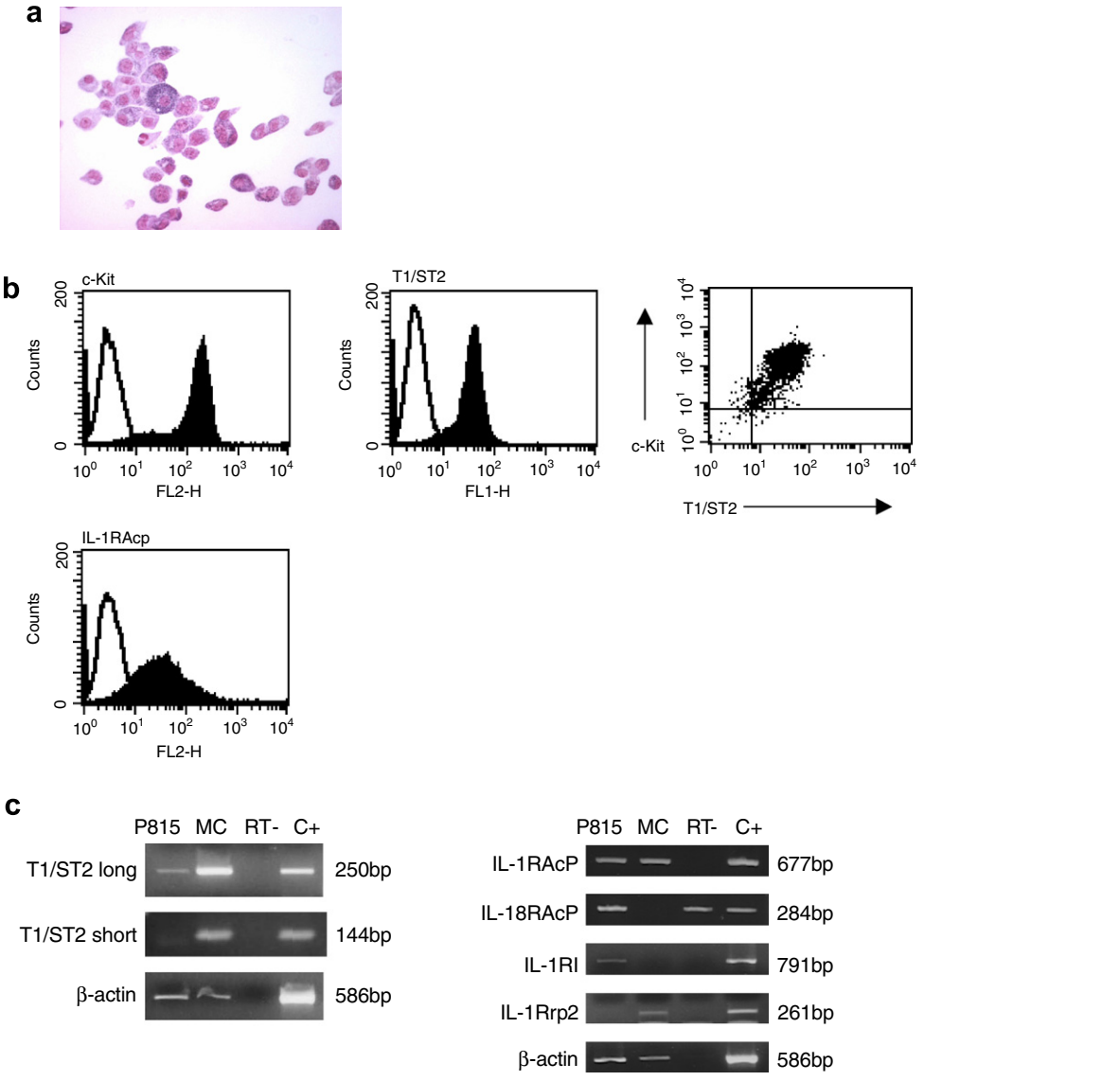

Fig. 2. Characterization of BMMC purity and expression of IL-1R family members. (a) Evaluation of BMMC purity by toluidine blue staining (original magnification 63×) (b) FACS analysis of cell surface expression of c-Kit, T1/ST2 and IL-1RAcP on BMMC. Anti-c-Kit staining (upper left panel, open line: isotype control, full line: anti c-Kit staining), anti-T1/ST2 staining (upper middle panel, open line: control, full line: anti-T1/ST2 staining), and double staining for c-Kit and T1/ST2 (upper right panel) are shown. BMMC co-express high levels of both receptors. Anti-IL-1RAcP staining (lower left panel, open line: control staining with secondary antibody alone, full line: anti-IL-1RAcP staining) shows cell surface expression of this co-receptor on BMMC (c) RT-PCR analysis of mRNA expression of IL-1 receptor family members. Expression of mRNA for the long and short T1/ST2 isoforms (left panels), IL-1RAcP, IL-18RAcP, IL-1RI and IL-1Rrp2 (right panels) was investigated in P815 cells and in BMMC (MC). Total RNA from ATDC5 mouse chondrocytes was used as a positive control for T1/ST2 long and short isoform and β-actin mRNA expression (left panels). Total RNA from mouse 3T3 fibroblasts was used as a positive control for IL-1RI, IL-1RAcP and β-actin mRNA expression (right panel). Total RNA from mouse joints was used as a positive control for IL-1Rrp2 expression. Non-reverse transcribed BMMC RNA was used as a negative control.

signaling. We next examined mRNA expression of different IL-1 family receptors, including the long and short isoforms of T1/ST2, IL-1RAcP, IL-18RAcP, IL-1RI and IL-1Rrp2 in P815 and BMMC by RT-PCR (Fig. 2c). As expected, mRNA encoding the long isoform of T1/ST2 was detected both in P815 cells and BMMC (Fig. 2c, left panel). BMMC, but not P815 cells, also expressed mRNA encoding the short soluble sST2 isoform. In addition, both P815 and BMMC expressed mRNA encoding IL-1RAcP (Fig. 2c, right panel). IL-18RAcP mRNA was detected in P815 cells and BMMC, while low levels of IL-1RI mRNA were present in P815, but not in BMMC. Finally, BMMC also expressed mRNA encoding IL-1Rrp2, the receptor for IL-1F6, IL-1F8, and IL-1F9.

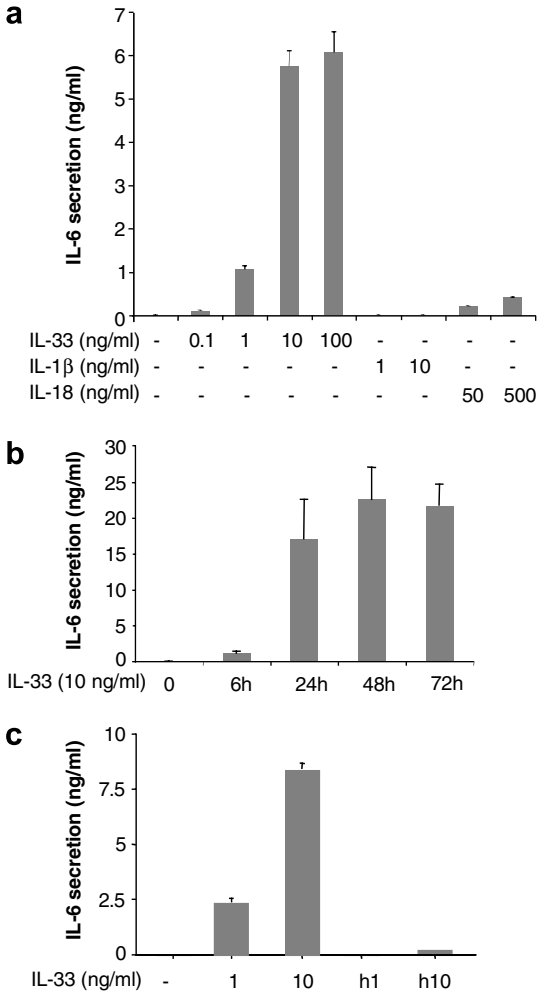

Fig. 3. IL-33 induces IL-6 production by BMMC. (a) BMMC were stimulated with IL-33 (100 pg/ml to 100 ng/ml), IL-1β (1 or 10 ng/ml), or IL-18 (50 or 500 ng/ml) for 24 h, before IL-6 levels were assessed by ELISA in the culture supernatants. (b) BMMC were stimulated with IL-33 (10 ng/ml) for 6, 24, 48 or 72 h, before IL-6 levels were assessed in cell supernatants by ELISA. (c) BMMC cells were stimulated with IL-33 (1 or 10 ng/ml), or with 1 or 10 ng/ml of heat inactivated IL-33 (h1, h10) for 24 h, before IL-6 levels were assessed by ELISA in culture supernatants. Results are representative of three independent experiments obtained with different batches of BMMC and are expressed as means ± SEM.

IL-33 at picomolar concentrations stimulated IL-6 production in BMMC with a maximum of efficacy at 10 ng/ml (Fig. 3a). To investigate the effects of other IL-1 family members, BMMC were cultured in the presence of IL-1β and IL-18. Consistent with low IL-18RAcP mRNA expression and absence of IL-1RI mRNA, incubation with IL-18 (50 or 500 ng/ml) exerted only a weak effect on BMMC, whereas IL-1β at doses up to 100 ng/ml (Fig. 3a and data not shown) was devoid of stimulatory activity. The effect of IL-33 on IL-6 release by BMMC was observed after 6 h with a maximal effect after 24 h (Fig. 3b). Heat inacti-

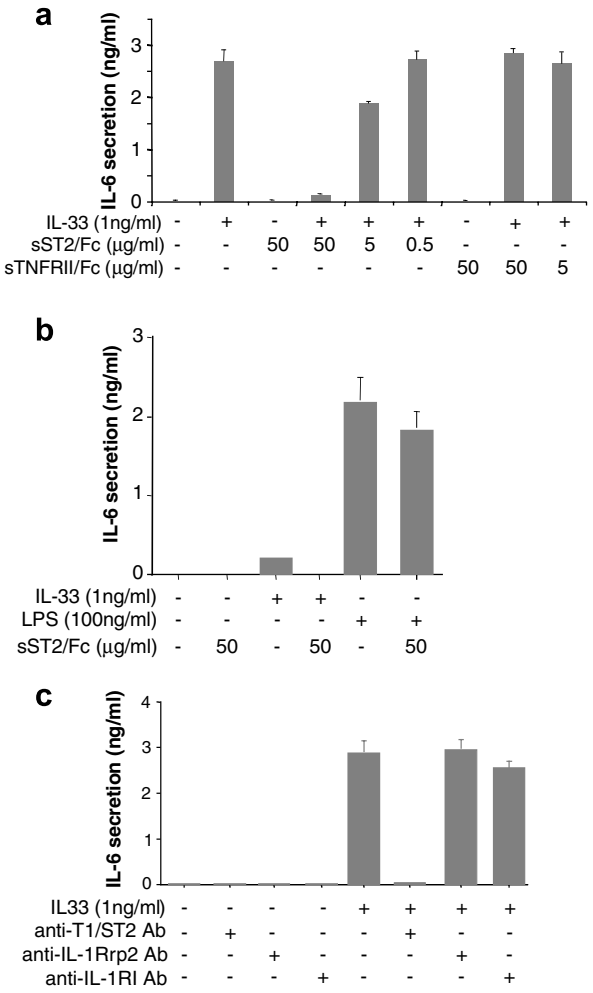

Fig. 4. IL-33 induced IL-6 production in BMMC is inhibited by sST2/Fc and anti-T1/ST2 antibodies. (a) Prior to stimulation, IL-33 (1 ng/ml) was incubated with sST2/Fc (50, 5 or 0.5 μg/ml) or sTNFRII/Fc (50 or 5 μg/ml) for 15 min at 37 °C, then IL-33, sST2/Fc, sTNFRII/Fc, IL-33 and sST2/Fc or IL-33 and sTNFRII/Fc were added to BMMC for 24 h. IL-6 levels were measured by ELISA. (b) BMMC were preincubated with sST2/Fc (50 μg/ml) for 1 h prior to stimulation with LPS (100 ng/ml) for 24 h or stimulated with IL-33 (1 ng/ml) alone or in combination with sST2/Fc (50 μg/ml) for 24 h. IL-6 levels were measured in culture supernatants by ELISA. (c) BMMC were preincubated with the anti-T1/ST2 antibody DJ8 (10 μg/ml), or with an anti-IL-1Rrp2 antibody (10 μg/ml) or an anti-IL-1RI antibody (10 μg/ml) which were used as negative controls, 1 h before stimulation with IL-33 (1 ng/ml) for 24 h. IL-6 levels were measured in culture supernatants by ELISA. Results are expressed as means ± SEM.

vation abolished the stimulatory effect of IL-33 on IL-6 production by BMMC (Fig. 3c).

Like in P815 mastocytoma cells, preincubation with sST2/Fc inhibited the effects of IL-33 on IL-6 production in BMMC, whereas sTNFRII/Fc did not interfere with IL-33 activity (Fig. 4a). It has been previously reported that pre-treatment with sST2/Fc inhibits cytokine production induced by LPS in macrophages [15–17]. We thus examined whether sST2/Fc could also affect the response to LPS in BMMC. However, as shown in Fig. 4b, LPS strongly induced IL-6 release by BMMC and this effect was not affected by preincubation of the cells with sST2/Fc.

Finally, we assessed the effect of a blocking anti-T1/ST2 antibody on IL-6 release induced by IL-33 in BMMC. Preincubation of BMMC with the monoclonal anti-T1/ST2 antibody DJ8 abolished the effect of IL-33, while control anti-IL-1Rrp2 and anti-IL-1RI blocking antibodies did not interfere with IL-33 activity (Fig. 4c). The specificity of the anti-T1/ST2 antibody DJ8 was further assessed using mouse chondrogenic ATDC5 cells, in which both IL-1β and IL-33 induce IL-6 secretion. In these cells, the anti-T1/ST2 antibody DJ8 (10 μg/ml) and the anti-IL-1RI antibody (10 μg/ml) completely abolished the effect of the corresponding receptor ligand, without affecting the response to the other cytokine (data not shown).

3.3. IL-33 induces IL-1β production by BMMC

The effect of IL-33 on the expression of several pro-inflammatory cytokines in BMMC was examined by RNase protection assay with a multiprobe set (Fig. 5a). In addition to IL-6 mRNA induction (16-fold), IL-1β mRNA was also induced in response to IL-33 (150-fold). In contrast, IL-33 did not stimulate the mRNA expression of other IL-1 family members such as IL-1α, IL-1Ra and IL-18. The stimulatory effect of IL-33 on IL-1β production was confirmed at the protein level in BMMC cell lysates by ELISA (Fig. 5b, left panel). In addition, we also observed an increase in IL-1β protein levels in BMMC culture supernatants, indicating that IL-33 induced not only IL-1β production, but also its secretion (Fig. 5b, left panel). IL-1β secretion was observed at 6 h with a peak at 24 h after IL-33 stimulation (Fig. 5b, right panel).

3.4. Effects of IL-33 on production of various cytokines and pro-inflammatory mediators by mast cells

To investigate the effects of IL-33 on the production of additional cytokines and chemokines in BMMC, we quantified the levels of TNF-α, MCP-1, IL-10, IFNγ, IL12p70, IL-2, IL-4 and IL-5 in BMMC cells supernatants using a BD cytometric bead array. We found that IL-4, IL-5, IFN-γ and IL12p70 were below the detection limit

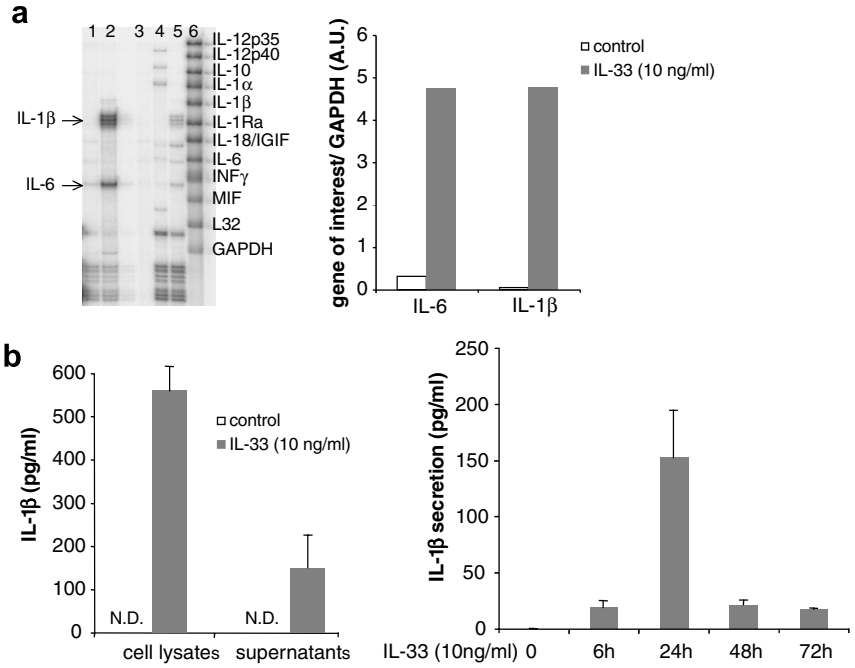

Fig. 5. IL-33 induces IL-1β production by BMMC. (a) RNA from BMMC stimulated or not with IL-33 for 6 h was extracted and RNase protection assay was performed (left panel). Lane 1: Unstimulated BMMC. Lane 2: BMMC stimulated for 6 h with IL-33 (10 ng/ml). Lane 3: tRNA. Lane 4: BD Biosciences positive control. Lane 5: Collagen-induced arthritic joint (positive control). Lane 6: Undigested probe. Densitometric quantification of IL-6 and IL-1β mRNA expression normalized to GAPDH mRNA in control (open columns) and IL-33 stimulated (gray columns) cells (right panel). Results are representative from two experiments performed on different batches of BMMC. (b) BMMC were stimulated with IL-33 (10 ng/ml) for 24 h. IL-1β levels were measured in cell lysates and culture supernatants by ELISA (left panel). BMMC were stimulated with IL-33 (10 ng/ml) for 6, 24, 48 or 72 h, before IL-1β secretion was measured in BMMC supernatants by ELISA (right panel). Results are representative of two independent experiments obtained with different batches of BMMC and are expressed as means ± SEM. N.D., not detectable.

(20 pg/ml) for this assay both in resting and in stimulated BMMC supernatants. Stimulation of BMMC with IL-33 for 24 h increased TNF-α (28-fold) and MCP-1 (4-fold) secretion (Fig. 6a), induced IL-2 secretion (unstimulated BMMC supernatants: < 20 pg/ml; IL-33 stimulated BMMC supernatants: $196 + 26$ pg/ml, $p < 0.001$), but left IL-10 production unchanged (Fig. 6a). Furthermore, IL-33 also dose-dependently induced the production of PGD2 by BMMC, with a maximal effect after 24 h stimulation (Fig. 6b).

Finally, we assessed a potential effect of IL-33 on BMMC degranulation. However, no change in granule number or localization was observed after IL-33 stimulation of BMMC as assessed by light microscopy after toluidine blue staining, whereas ionomycin and PMA induced degranulation in the same experimental conditions (data not shown). In addition, IL-33 did not increase tryptase activity in culture supernatants (Fig. 6c), which was enhanced in the same culture conditions by the calcium ionophore A23187 used as a positive control to induce degranulation.

4. Discussion

The results of this study show for the first time that IL-33, a novel member of the IL-1 family of cytokines, stimulates the production of pro-inflammatory mediators, including IL-6, IL-1β, TNF-α, MCP-1 and PGD2, by mast cells. The effect of IL-33 appeared to be independent of mast cell degranulation, although we cannot exclude that IL-33 could modulate degranulation in a more physiological context, such as after sensitization by IgE for instance.

IL-33 has been recently identified as a ligand for the previously orphaned receptor T1/ST2 [1]. Consistently, the effects of IL-33 on mast cells were specifically inhibited by soluble ST2 or by preincubation of the cells with a blocking anti-T1/ST2 antibody. The T1/ST2 receptor

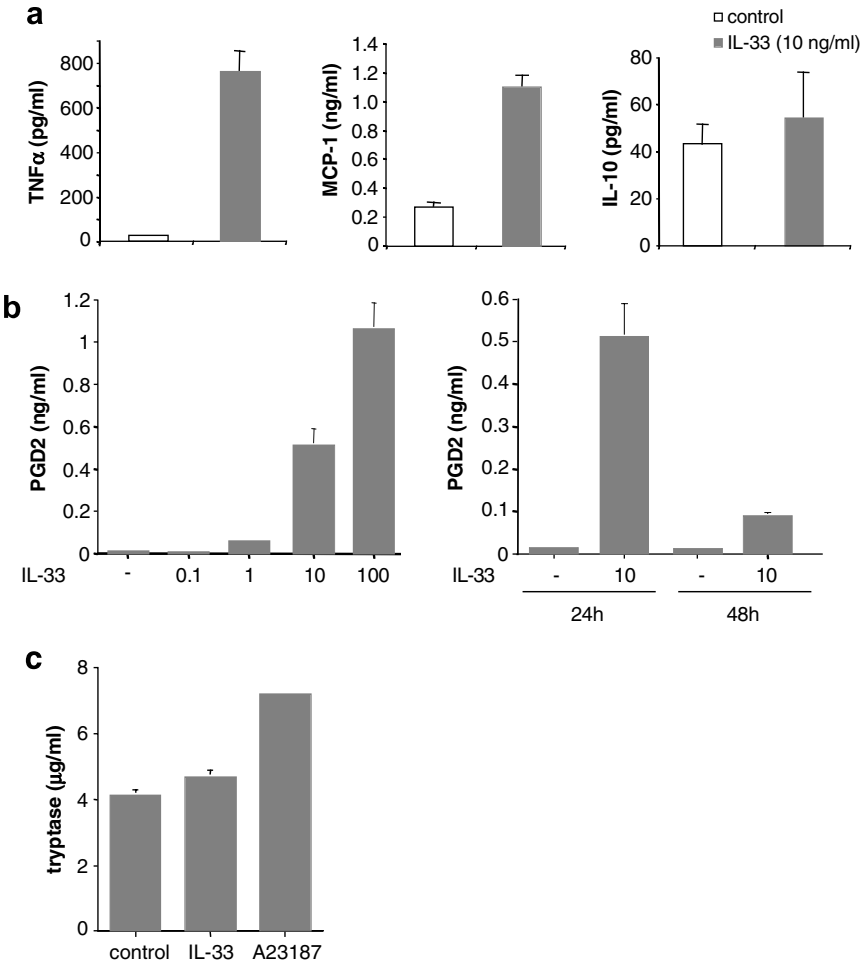

Fig. 6. Effect of IL-33 on the secretion of various pro-inflammatory mediators by BMMC. (a) BMMC were stimulated (gray columns) or not (open columns) with IL-33 (10 ng/ml) for 24 h. BMMC supernatants were then incubated with cytokine bead arrays and analyzed by flow cytometry. Stimulation with IL-33 induced secretion of TNF-α and MCP-1. IL-10 levels were not modified. (b) BMMC were stimulated with IL-33 (100 pg/ml to 100 ng/ml) for 24 h (left panel) or with IL-33 (10 ng/ml) for 24 or 48 h (right panel). PGD2 production was measured by EIA in culture supernatants. (c) BMMC were treated with IL-33 (10 ng/ml) or with the calcium ionophore A23187 (500 nM) for 1 h before tryptase activity in culture supernatants was assessed using a colorimetric assay. Results are expressed as means ± SEM.

shares common features with other IL-1 receptors, including three extracellular immunoglobulin domains and an intracellular Toll-like IL-1 receptor (TIR) domain [18]. Furthermore, we recently observed that IL-1RAcP acts as the co-receptor for IL-33 (GP, DTA and CG, manuscript in preparation). Post receptor signaling pathways induced upon IL-33 binding to T1/ST2 and IL-1RAcP are similar to those induced by IL-1 after binding to IL-1RI and IL-1RAcP, or to those stimulated by IL-1F6, IL-1F8, and IL-1F9 following their binding to IL-1Rrp2 and IL-1RAcP. IL-33 signaling stimulates the recruitment of adaptor molecules and kinases, including MyD88, TRAF6, and IRAK4 and results in the activation of NF-κB and MAPK signaling pathways [1]. Similarly, in mast cells, we observed induction of NF-κB and AP-1 DNA binding activity by IL-33 and production of pro-inflammatory mediators. Interestingly, among the various IL-1 family of cytokines tested, the response of primary mast cells was restricted to IL-33, since these cells do not express the signaling IL-1 receptor and did not respond to stimulation with IL-1β, whereas IL-18 exerted only mild effects. The lack of effect of IL-1β indicates that despite the presence of the common receptor chain IL-1RAcP and common intracellular signaling pathways, the biologic activities of IL-1 family members are modulated in certain cell types according to the expression of their specific receptors.

The role of mast cells is not limited to IgE-mediated allergic responses. Mast cells are also sensitive to IgG, complement, specific pathogen-associated patterns, and are able of phagocytosis, intracellular killing and antigen presentation. Mice deficient in mast cells are more susceptible to septic peritonitis [19] and these cells play also a critical role in the clearance of intestinal parasitic infection [20]. Moreover mast cells may also contribute to non-infectious inflammatory conditions such as in allergic reactions. In addition, their role in inflammatory arthritis has been assumed for many years as they are present in substantial numbers in the synovial membrane of rheumatoid arthritis patients as well as in the joints of mice with experimental arthritis [21]. Furthermore, c-Kit deficient mice lacking mast cells were protected of serum transfer-induced arthritis, thus suggesting that these cells participate in the pathogenesis of joint inflammation [11,22]. While IL-33 has been previously described to induce essentially Th2 responses [1], the observed effects of IL-33 on secretion of IL-6, IL-1β, TNF-α and PGD2 suggest that, in addition, this cytokine may also enhance the pro-inflammatory activities of mast cells in various conditions, including arthritis. Some of the pro-inflammatory effects of mast cells are mediated through the rapid release of TNF-α, as well as through the secretion of other inflammatory mediators. In particular, the production of IL-1β by mast cells was recently shown to play a central role in the initiation of joint inflammation in a model of autoantibody induced-arthritis [23]. IL-1β secretion is a tightly regulated, caspase-1-dependent process, which is controlled independently of IL-1β mRNA and protein production [24]. Activation of caspase-1 is regulated by a cytosolic protein complex called the inflammasome. Our data indicate that IL-33 induces not only the synthesis, but also the secretion of IL-1β by BMMC. However, to date, there is no information available concerning potential expression and activity of inflammasome components in mast cells. Pro-IL-18, like the IL-1β and IL-33 precursors, is also cleaved by caspase-1 to generate the mature form of the protein. Interestingly, mast cell chymase has been reported to cleave pro-IL-18 into a biologically active form distinct from the mature protein obtained upon caspase-1 cleavage [25]. By analogy, one could infer the possibility of caspase-1 independent cleavage of pro-IL-1β or pro-IL-33 by mast cell specific proteases to yield biologically active forms of these cytokines. Finally, IL-33 induced the secretion of MCP-1, which is known to play a major role for instance in mast cell dependent recruitment of monocytes to lesions during allergic inflammation [26,27].

Interestingly, it has been previously reported that administration of sST2 decreases collagen-induced arthritis in mice [16]. Similarly, sST2 has also been described to exert anti-inflammatory effects in a mouse model of septic shock [15] and in two different models of ischemia-reperfusion injury [28,29]. At the time, the mechanism proposed to explain this effect was the direct inhibition of macrophage activation by sST2 via a putative sST2 receptor expressed at the macrophage surface. However, in the present study, sST2 did not affect the activation of BMMC by LPS, suggesting that expression of this sST2 receptor may be restricted to particular cell types. Moreover, following the identification of IL-33 as the bona fide ligand for T1/ST2, neutralization of IL-33 activity by sST2 certainly needs to be considered as an alternative explanation for the observed *in vivo* effects of sST2.

In conclusion, this study shows for the first time that IL-33, a novel member of the IL-1 family of cytokines, stimulates the production of pro-inflammatory mediators by mast cells in addition to its effects on Th2 responses. These findings open new perspectives for the treatment of inflammatory diseases by targeting IL-33.

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