Interleukin-33 Is Biologically Active Independently of Caspase-1 Cleavage*

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The new interleukin (IL)-1 family cytokine IL-33 is synthesized as a 30-kDa precursor. Like pro-IL-1β, human pro-IL-33 was reported to be cleaved by caspase-1 to generate an 18-kDa fragment, which is sufficient to activate signaling by the IL-33 receptor T1/ST2. However, the proposed caspase-1 cleavage site is poorly conserved between species. In addition, it is not clear whether caspase-1 cleavage of pro-IL-33 occurs in vivo and whether, as for IL-1β, this cleavage is a prerequisite for IL-33 secretion and bioactivity. In this study, we further investigated caspase-1 cleavage of mouse and human pro-IL-33 and assessed the potential bioactivity of the IL-33 precursor. We observed the generation of a 20-kDa IL-33 fragment in cell lysates, which was enhanced by incubation with caspase-1. However, in vitro assays of mouse and human pro-IL-33 indicated that IL-33 is not a direct substrate for this enzyme. Consistently, caspase-1 activation in THP-1 cells induced cleavage of pro-IL-1β but not of pro-IL-33, and activated THP-1 cells released full-length pro-IL-33 into culture supernatants. Finally, addition of full-length pro-IL-33 induced T1/ST2-dependent IL-6 secretion in mast cells. However, we observed in situ processing of pro-IL-33 in mast cell cultures, and it remains to be determined whether full-length pro-IL-33 itself indeed represents the bioactive species. In conclusion, our data indicate that pro-IL-33 is not a direct substrate for caspase-1. In addition, our results clearly show that caspase-1 cleavage is not required for pro-IL-33 secretion and bioactivity, highlighting major differences between IL-1β and IL-33.

Interleukin (IL)2-33, the most recently described cytokine of the IL-1 family, is synthesized as a 30-kDa precursor. Human pro-IL-33, like pro-IL-1β, was reported to be cleaved by caspase-1 in vitro to generate an 18-kDa fragment, termed mature IL-33, which is sufficient to activate signaling by the IL-33 receptor T1/ST2 (1).

Caspase-1 is an endoproteinase that specifically cleaves Asp-Xaa bonds, where Xaa typically refers to a small, often hydrophobic residue (2–4). Caspase-1 activity absolutely requires the presence of an Asp residue at position −1 of the cleavage site. Consistently, replacement of Asp118 by other amino acids, such as Ala, was previously demonstrated to prevent caspase-1 cleavage of pro-IL-1β (2). Recombinant (r) mature IL-33 starts at Ser112 for human (h) IL-33 and at Ser109 for mouse (m) IL-33, neither of which corresponds exactly to the position of a potential caspase-1 cleavage site. Indeed, the N-terminal moiety of human pro-IL-33 sequence contains a single Asp at position 110, and the N-terminal portion of mouse pro-IL-33 contains an Asp at positions 88 and 106. In fact, the region located between amino acids 80 and 110 of pro-IL-33 is rather poorly conserved between species (5). In particular, no Asp residues can be consistently found at an identical position across species to hint at the presence of a conserved caspase-1 cleavage site. So far, caspase-1 cleavage of pro-IL-33 has not been investigated in any species other than human.

Expression of endogenous IL-33 has been described most extensively in endothelial cells, where essentially nuclear, full-length 30-kDa pro-IL-33 is detected (5–7). To date, only two studies have examined potential effects of caspase-1 activation on the processing and secretion of pro-IL-33 in living cells. In one study, stimulation of murine glial cultures with caspase-1 activators induced secretion of bioactive IL-33 into culture supernatants, but the size of the secreted protein was not assessed (8). It is thus not clear whether caspase-1 cleavage of pro-IL-33 occurs in mouse cells. In a second study, Western blot analysis revealed the presence of a 32-kDa protein and minor 17 and 20 kDa bands reacting with anti-IL-33 antibodies in the supernatants of THP-1 cells upon caspase-1 activation, suggesting secretion of full-length pro-IL-33 and of two potential cleavage products (9). Although this last observation suggests that some pro-IL-33 may be secreted, it is not known to what extent IL-33 secretion is dependent on caspase-1 cleavage. Finally, so far all studies reporting T1/ST2-mediated effects of IL-33 were performed using the recombinant mature form of IL-33, whereas potential bioactive activity of the full-length precursor form has not been tested. It thus remains to be shown whether, as for IL-1β, caspase-1 cleavage is indeed required for IL-33 bioactivity. In the present study, we thus further investigated caspase-1 cleavage of mouse and human pro-IL-33 in vitro and in cultured cells and assessed the potential bioactivity of the IL-33 precursor.

EXPERIMENTAL PROCEDURES

Materials—Cell culture reagents were obtained from Invitrogen. rmIL-33 and rhIL-33 produced in Escherichia coli were obtained from Alexis Corp. (Lausen, Switzerland). Soluble
Cloning of Mouse and Human Pro-IL-33 and Pro-IL-1β Expression Vectors—An IMAGE expressed sequence tag clone containing the cDNA encoding the full-length mouse pro-IL-33 (GenBank™ accession number BC003847) in the pCMV-Sport6 vector was used as expression vector for full-length mouse pro-IL-33 in 293T human embryonic kidney cells. Mutagenesis of Asp to Ala in mouse D88A and D106A pro-IL-33 mutants was performed using the QuickChange kit (Stratagene, La Jolla, CA) and appropriate primer pairs, according to the manufacturer’s instructions. The cDNA corresponding to the full-length human pro-IL-33 (GenBank™ accession number AY905581) was amplified by reverse transcription-PCR using total RNA extracted from cultured rheumatoid arthritis synovial fibroblasts (forward primer, 5'-gctctagacctatatgcct-3') and reverse primer, 5'-acctgctttggacctgg-3') and cloned into the pCAGGS vector (10) for expression in 293T cells. For expression in THP-1 cells, full-length human pro-IL-33 cDNA was cloned into the pWPI lentiviral vector (11). Viral particles were produced in 293T cells using a standard protocol as described (12). The cDNA corresponding to full-length human pro-IL-1β (GenBank™ accession number NM_000576) was amplified by reverse transcription-PCR using RNA extracted from phorbol 12-myristate 13-acetate (PMA)-stimulated THP-1 cells (forward primer, 5'-gggatcctaattgaggttga-3'; and reverse primer, 5'-gccccgattggagttttcgg-3') and cloned into the pCDNA3.1+ vector (Invitrogen) for expression in 293T cells. The sequence of the entire coding region was verified before use for all constructs.

Cell Culture, Transfection, and Lentiviral Infection—Human embryonic kidney 293T cells were cultured in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) supplemented with 1-glutamine, streptomycin, penicillin, and 10% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO2. For transfection, 293T cells were plated at a density of 100,000 cells/ml and transfected 24 h after seeding with 1 μg/ml pro-IL-33 or pro-IL-1β expression vector, or empty vectors as a control, by calcium phosphate precipitation (13). THP-1 cells were cultured in RPMI 1640 medium (4.5 g/liter glucose) supplemented with 10 mM Hepes, 1 mM sodium pyruvate, 5×10⁻⁵ M β-mercaptoethanol, streptomycin, penicillin, and 10% fetal calf serum. For viral infection, THP-1 cells were plated at a density of 250,000 cells/well and infected with freshly produced lentiviruses encoding hIL-33 and green fluorescent protein (GFP), or GFP alone as a control, using a double infection protocol (12). P815 cells were cultured in Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose) supplemented with 6% fetal calf serum, 10 mM Hepes, 10 mM minimum Eagle’s medium nonessential amino acids, penicillin, and streptomycin. Primary mouse bone marrow-derived mast cells (BMMC) were generated as described (14) from wild-type and T1/ST2 knockout BALB/c mice (15) and used after 10 weeks of culture.

In Vitro Protein Synthesis—Wild-type and D88A and D106A mutant mouse pro-IL-33 as well as human pro-IL-33 and pro-IL-1β were synthesized in vitro using the PURExpress in vitro protein synthesis kit (New England BioLabs, Ipswich, MA). This kit contains a cell-free coupled transcription/translation system reconstituted from purified components and is thus essentially free of any contaminant proteases. To generate templates for the in vitro protein synthesis, the various cDNAs were amplified from theabove described expression vectors using appropriate primers to insert a T7 promoter and an E. coli ribosome entry site upstream of the gene-specific sequence, according to the manufacturer’s instructions. The sequence of all PCR products was verified before use. Protein synthesis was carried out using 250 ng of DNA template in a final volume of 25 μl. Reactions without cDNA were set up as negative controls.

In Vitro Caspase-1 Assay—Transfected 293T cells were used 72 h after transfection. Cells were lysed in caspase assay buffer (10 mM Hepes, pH 7.5, 0.1% CHAPS, 2 mM EDTA, 10% glycerol, 10 mM dithiothreitol) by brief sonication on ice and stored at −80°C until used. Cell lysates (10 μl) were incubated for 6 h at 37°C without or with 2 units of recombinant active human caspase-1 (ALX-201-056, Alexis Corp.). In vitro synthesized proteins (0.7 μl) were incubated in 20 μl of caspase buffer for 6 h at 37°C without or with 2 units of recombinant active human caspase-1. Reactions were stopped by the addition of Laemml buffer, and samples were resolved on SDS-PAGE and analyzed by Western blotting.

Caspase-1 Activation in THP-1 Cells—Lentivirally transduced THP-1 cells were stimulated for 3 h with 0.5 μM PMA in complete RPMI 1640 medium and left to adhere overnight. Cells were then switched to Opti-MEM (Invitrogen) and stimulated or not with 50 μg/ml lipopolysaccharide (LPS) for 6 h, as described previously (16). Culture supernatants were removed, and cell lysates were prepared by freeze-thawing after addition of fresh Opti-MEM. Supernatant and cell lysate proteins were concentrated by methanol precipitation, resuspended in Laemmli buffer, and resolved by SDS-PAGE, and analyzed by Western blotting.

Western Blotting—Samples were fractionated by SDS-PAGE and transferred to a Porablot membrane (Macherey-Nagel, Düren, Germany). The membrane was blocked in 0.05% Tris-buffered saline, Tween 20, containing 5% bovine serum albumin or 5% Blotto (Santa Cruz Biotechnology, Santa Cruz, CA), and immunoblotting was performed with one of the following antibodies, as indicated: biotinylated monoclonal anti-IL-33 antibody (Nessy-1, 1:1,000, Alexis Corp.); biotinylated polyclonal anti-IL-33 antibody (BAF3626, 1:2,000), polyclonal anti-hIL-1β antibody (AF3625, 1:1,000), and monoclonal anti-hIL-1β (MAB201, 1:1,000, R&D Systems); or the D116 antibody specific for mature 17-kDa hIL-1β (1:1,000, Cell Signaling Technology, Danvers, MA). Immunoreactive bands were visualized by ECL (Amersham Biosciences) using appropriate horseradish peroxidase-coupled secondary reagents.

Immunofluorescence—Lentivirally transduced THP-1 cells were seeded in chamber slides, primed with 0.5 μM PMA, and stimulated or not with 50 μg/ml LPS as described above. The cells were fixed (phosphate-buffered saline, 4% paraformaldehyde; 10 min at room temperature), permeabilized (phosphate-buffered saline, 0.1% Triton X-100; 10 min), and blocked (phosphate-buffered saline, 1% bovine serum albumin, 10% normal

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goat serum; 1 h) before staining with a monoclonal anti-hIL-33 antibody (ALX-804-726, 1:100, Alexis Corp.) and detection with a Texas Red-labeled anti-mouse IgG secondary antibody. To assess staining specificity, negative controls were performed in the absence of the primary antibody. Slides were mounted using a 4’,6-diamidino-2-phenylindole-containing mounting medium (Vector Laboratories), and images were acquired on an LSM510 Meta confocal microscope (Carl Zeiss AG, Feldbach, Switzerland).

**Purification of Mouse Pro-IL-33—293T cells** were seeded in 10-cm Petri dishes and transiently transfected with the mouse pro-IL-33 expression vector. Cells were harvested 72 h after transfection in TNT (50 mM Tris, pH 7.4, 150 mM NaCl, 0.5% Triton X-100) supplemented with a protease inhibitor mixture (Roche Diagnostics) and lysed by brief sonication on ice. Alternatively, transfected cells were used to prepare nuclear extracts following standard procedures (17). Briefly, cells were lysed in sucrose buffer on ice, and nuclei were pelleted, resuspended in low salt buffer, and lysed by the addition of high salt buffer. Mouse pro-IL-33 was affinity-purified from TNT total cell extracts or from nuclear extracts using mouse sST2-Fc and the Seize X protein G immunoprecipitation kit (Pierce) and eluted with a sucrose buffer on ice, and nuclei were pelleted, resuspended in low salt buffer, and lysed by the addition of high salt buffer.

**Bioactivity of Purified and in Vitro Translated Mouse Pro-IL-33—Bioactivity of purified mouse pro-IL-33 or of in vitro synthesized wild-type, D88A, and D106A mouse pro-IL-33 was assessed using P815 mastocytoma cells and BMMC in vitro** synthesized wild-type, D88A, and D106A mouse pro-IL-33 were preincubated with sST2-Fc (50 μg/ml) before culture supernatants were harvested. To assess specificity of the observed effects, rIL-33 or in vitro synthesized proteins were preincubated with sST2-Fc (50 μg/ml) for 15 min at 37 °C before addition to the cells. Alternatively, BMMC generated from wild-type or T1/ST2 knock-out mice were incubated without or with purified pro-IL-33 (10 μg of 200 μl of total eluate), rIL-33 (1 ng/ml), or LPS (1 μg/ml) for 24 h before culture supernatants were harvested. Levels of IL-6 in P815 and BMMC culture supernatants were assessed using an enzyme-linked immunosorbent assay DuoSet kit (R&D Systems).

**Statistical Analysis**—The significance of differences was assessed by analysis of variance. A difference between experimental groups was considered significant when the p value was <0.05.

**RESULTS**

**Generation of a 20-kDa Fragment of IL-33 in Cell Lysates is Enhanced in the Presence of Caspase-1**—A total cell lysates of 293T cells overexpressing mouse pro-IL-33 (mIL-33) or of control 293T cells transfected with empty vector (control) were either left untreated (no rIL) or incubated for 6 h at 37 °C in the presence (Csp) or absence (37 °C) of recombinant human active caspase-1. Samples were analyzed by Western blotting with a polyclonal anti-mIL-33 antibody (BAF3626). B, total cell lysates of 293T cells overexpressing D88A mutant mouse pro-IL-33, D106A mutant mouse pro-IL-33, or wild-type mouse pro-IL-33 (WT) were incubated for 6 h at 37 °C in the absence or presence of recombinant human active caspase-1. C, total cell lysates of 293T cells overexpressing human pro-IL-33 (hIL-33) or of control 293T cells transfected with empty vector (control) were either left untreated or incubated for 6 h at 37 °C in the absence or presence of recombinant human active caspase-1. Samples were analyzed by Western blotting with a polyclonal anti-hIL-33 antibody (AF3625). A–C, the positions of full-length pro-IL-33 (pro), rIL-33 (rec), and the 20-kDa fragment (arrow) are indicated on the left. Molecular mass markers are indicated on the right. D, total cell lysates of 293T cells overexpressing human pro-IL-1β (hIL-1β) were incubated for 6 h at 37 °C in the absence or presence of recombinant human active caspase-1. Samples were analyzed by Western blotting with the D116 antibody, which specifically recognizes the cleaved 17-kDa form of IL-1β (arrow).

IL-33 was overexpressed in 293T cells, and total cell lysates were incubated with recombinant human active caspase-1 in vitro. Western blot analysis revealed the presence of a major band corresponding to the 30-kDa full-length form of pro-IL-33, even after incubation with caspase-1 (Fig. 1A). In addition, we detected a less intense band at 20 kDa, the abundance of which was increased in the presence of active caspase-1. No band corresponding to the size of rmIL-33 was detected either in the absence or presence of caspase-1. Similar results were obtained also upon incubation of the cell lysates with recombinant active mouse caspase-1 and using two different anti-mIL-33 antibodies (BAF3626 and Nessy-1) for detection (Fig. 1A and data not shown).

In an attempt to identify which of the two N-terminal Asp residues might represent a potential caspase-1 cleavage site in mouse pro-IL-33, we generated two mutant forms of mouse pro-IL-33, D88A and D106A, in which either Asp<sup>88</sup> or Asp<sup>106</sup> was replaced by Ala. Surprisingly, neither of these two mutations interfered with the generation of the 20 kDa band, which was detectable already in untreated cell lysates and slightly enhanced in the presence of caspase-1 (Fig. 1B).

Similarly, lysates of 293T cells overexpressing human pro-IL-33 contained essentially the 30-kDa full-length form of pro-IL-33, even after incubation with caspase-1, as well as a minor band at 20 kDa (Fig. 1C). Again, similar results were obtained using two different anti-hIL-33 antibodies (AF3625 and ALX-804-726) for detection (Fig. 1C and data not shown).
For both mIL-33 and hIL-33, the observed 20-kDa fragment was of higher molecular mass than 18-kDa rIL-33. This 20 kDa band was generally detectable already before caspase-1 treatment, although its abundance was variable in different cell lysate preparations (Fig. 1 and data not shown). However, the 30-kDa full-length pro-IL-33 remained the most abundant form detected in all preparations, even in the presence of caspase-1. Finally, lysates of 293T cells overexpressing pro-IL-1β were used as a positive control for efficient caspase-1 activity in our experimental conditions, and indeed, pro-IL-1β was cleaved efficiently into its 17-kDa mature form (Fig. 1D).

IL-33 Is Not a Direct Substrate for Caspase-1—The presence of the 20 kDa band in untreated cell lysates as well as in cells transfected with mutant pro-IL-33 suggested that generation of this IL-33 fragment is not mediated directly by caspase-1. In fact, 293T cell lysates contain a number of proteases that might be involved in IL-33 processing. We thus sought to assay caspase-1 cleavage of pro-IL-33 in a protease-free system. To this end, we synthesized pro-IL-33 using an in vitro transcription/translation system reconstituted from purified and recombinant components, which is essentially protease-free. Interestingly, when incubating in vitro synthesized mouse (Fig. 2A) or human (Fig. 2B) pro-IL-33 with recombinant human active caspase-1, we could not observe any caspase-1-dependent cleavage of pro-IL-33, which was recovered exclusively in its 30-kDa full-length form, even after 6 h of incubation. In contrast, using the same experimental conditions, human pro-IL-1β was totally cleaved to generate 17-kDa mature IL-1β (Fig. 2C). Taken together, these observations demonstrate that in contrast to pro-IL-1β, pro-IL-33 is not a direct substrate for caspase-1.

IL-33 Is Released in Its Precursor Form after Activation of Endogenous Caspase-1 in THP-1 Cells—We next investigated the effect of caspase-1 activation on pro-IL-33 processing and secretion in living cells. For these experiments, we used THP-1 cells because caspase-1 activation and IL-1β secretion have been described extensively in these cells, in response to PMA and LPS, in well established experimental conditions (16). In our hands, THP-1 cells did not express endogenous pro-IL-33 as assessed by reverse transcription-PCR and Western blotting (Fig. 3B and data not shown). We thus used a lentiviral vector to express human pro-IL-33 in these cells. Following lentiviral transduction, expression of pro-IL-33 was observed essentially in the nuclei of THP-1 cells, with and without LPS stimulation (Fig. 3A and data not shown). We monitored potential pro-IL-33 cleavage and secretion upon activation of endogenous caspase-1 in response to LPS in PMA-primed THP-1 cells (16). Cleavage and secretion of endogenous IL-1β were monitored in parallel in the same samples as a positive control. Western blot analysis revealed the presence of 30-kDa full-length pro-IL-33 as well as the 20-kDa fragment in cell lysates of THP-1 cells transduced with the IL-33 expression vector (Fig. 3B). The abundance of the 20-kDa fragment was not enhanced in LPS-treated cells. In addition, no band corresponding to the size of rhIL-33 was detected either in the absence or presence of LPS. Interestingly, we observed release of full-length pro-IL-33 into culture supernatants specifically in LPS-stimulated THP-1 cells. LPS-induced secretion of mature 17-kDa IL-1β, as an indication of effective caspase-1 activation, was easily observed in both IL-33-expressing and control THP-1 cells (Fig. 3C).

Pro-IL-33 Exerts T1/ST2-dependent Biological Effects on Mast Cells—Because we observed secretion of IL-33 in its pro form, we next investigated whether full-length pro-IL-33, like pro-IL-1α, might be able to activate its cognate cell surface receptor. We thus purified mouse pro-IL-33 from transfected 293T total cell lysates or nuclear extracts using ST2-Fc/protein G beads. Although pro-IL-33 readily bound to these beads, it proved very difficult to elute efficiently using elution conditions.

![FIGURE 2. IL-33 is not a direct substrate for caspase-1. A, in vitro synthesized mouse pro-IL-33 (mIL-33) or a control in vitro synthesis reaction performed without cDNA (no DNA) was incubated for 6 h at 37°C in the absence (37°C) or presence (Csp 37°C) of recombinant human active caspase-1. Samples were analyzed by Western blotting with a polyclonal anti-mlL-33 antibody (BAF3626). B, in vitro synthesized human pro-IL-33 (hIL-33) or a control in vitro synthesis reaction performed without cDNA was either left untreated (no ttt) or incubated for 6 h at 37°C in the absence or presence of recombinant human active caspase-1. Samples were analyzed by Western blotting with a polyclonal anti-hIL-33 antibody (AF3625). A and B, the positions of full-length pro-IL-33 (pro) and rIL-33 (rec) are indicated on the left. Molecular size markers are indicated on the right. C, in vitro synthesized human pro-IL-1β (hIL-1β) was either left untreated or incubated for 6 h at 37°C in the absence or presence of recombinant human active caspase-1. Samples were analyzed by Western blotting with the MAB201 antibody, which recognizes both pro-IL-1β and cleaved IL-1β (left panel) and with the D116 antibody, which specifically recognizes the cleaved 17-kDa form of IL-1β (right panel).]
Processing and Bioactivity of Prointerleukin-33

**FIGURE 3. IL-33 is released in its precursor form by LPS-activated THP-1 cells.** A, localization of IL-33 in PMA- and LPS-stimulated THP-1 cells expressing GFP alone (THP-1 pWPI, left panel) or human pro-IL-33 and GFP (THP-1 pWPI-hIL-33, right panels) was analyzed by immunocytochemistry (upper panels). Anti-IL-33 staining (red) was essentially detected in cell nuclei, which were visualized with 4',6-diamidino-2-phenylindole (DAPI; blue). Efficiency of lentiviral transduction was verified by monitoring GFP expression (lower panels). B, THP-1 cells expressing GFP (pWPI) or human pro-IL-33 and GFP (IL-33) were primed with PMA and stimulated (LPS) or not (C) with LPS for 6 h. Secreted (supernatant) and intracellular (lysate) proteins were analyzed by Western blotting using a polyclonal anti-hIL-33 antibody (AF3625). The positions of full-length pro-IL-33 (pro), rIL-33 (rec), and the 20-kDa fragment (arrow) are indicated on the left. Molecular mass markers are indicated on the right. C, the same samples as in B were analyzed by Western blotting with the D116 antibody, which specifically recognizes the cleaved 17-kDa form of IL-1β (arrow).

**FIGURE 4. Purified pro-IL-33 exerts T1/ST2-dependent biological effects on mast cells.** A, mouse pro-IL-33 was purified from total cell lysates or nuclear extracts of transfected 293T cells using ST2-Fc/protein G beads, as described under “Experimental Procedures.” Total cell lysate was used for purification (load, 10 μl of 500 μl), column flow-through (FT, 10 μl of 500 μl), first wash (wash, 10 μl of 500 μl), and eluted fractions 1–4 (el1–el4, 10 μl of 100 μl) were analyzed by Western blotting using the Nessy-1 anti-IL-33 antibody (left panel). Alternatively, 10 μl of ST2-Fc/protein G beads was incubated with 20 μl of total cell lysate and washed, and pro-IL-33 was eluted directly in Laemmli buffer (lb el, right panel). Lysate indicates the total cell lysate (1.5 μl). B, wild-type (WT) and T1/ST2 knock-out (ST2KO) BMMC were stimulated for 24 h with 10 μl of purified pro-IL-33, 1 μg/ml LPS, or 1 ng/ml rmIL-33 before IL-6 secretion was assessed in culture supernatants by enzyme-linked immunosorbent assay. Results shown are mean ± S.E. of culture triplicates. Similar results were obtained with eluates from three independent purifications. *, p < 0.05 versus control; & p < 0.05 versus wild-type BMMC with the same stimulation.

compatible with subsequent bioactivity tests, such as elution using a low pH (2.8) buffer (Fig. 4A, left panel) or, in contrast, a high pH (11.5) triethanolamine buffer (data not shown). Efficient recovery of pro-IL-33 could only be achieved using stringent denaturing conditions, as illustrated by elution in Laemmli buffer (Fig. 4A, right panel). In fact, control experiments indicated that pro-IL-33 exhibited considerable background binding to protein G beads even in the absence of ST2-Fc (data not shown), and this “stickiness” may thus account for our difficulties in recovering bound pro-IL-33. In addition, this observation indicates that binding of pro-IL-33 to ST2-Fc/protein G beads cannot be inferred to demonstrate specific binding of pro-IL-33 to ST2.

Despite this lack of specificity, we used binding of pro-IL-33 to ST2-Fc/protein G beads followed by acid elution as a tool to prepare protein fractions enriched in pro-IL-33. Full-length 30-kDa pro-IL-33 was the only form of IL-33 detectable in ST2-Fc/protein G beads eluates (Fig. 4A, right panel). Addition of eluted fractions to P815 mastocytoma cells induced IL-6 secretion, and this effect was prevented in the presence of sST2-Fc (data not shown). Consistently, eluted pro-IL-33 induced IL-6 secretion in wild-type BMMC but not in ST2 knock-out BMMC (Fig. 4B), further suggesting that pro-IL-33 exerts T1/ST2-dependent biological effects in mast cells. The responsiveness to LPS was similar in wild-type and ST2 knock-out BMMC, indicating that there is no intrinsic defect in the induction of IL-6 secretion by proinflammatory stimuli in ST2 knock-out cells.

In view of the difficulties encountered with the purification of pro-IL-33 from cell lysates, we used in vitro synthesized proteins as an alternative source of pro-IL-33 to characterize its biological effects further. In vitro synthesized mouse pro-IL-33 dose-dependently induced IL-6 production in P815 cells, and this effect was inhibited in the presence of sST2-Fc (Fig. 5A). The two mutant forms of mouse pro-IL-33, D88A and D106A, similarly induced IL-6 production in P815 cells, and their effect was also inhibited by sST2-Fc (Fig. 5B).

Finally, we investigated potential in situ processing of pro-IL-33 to shorter forms in the culture medium of P815 cells.
Processing and Bioactivity of Prointerleukin-33

In this study, we report generation of a 20-kDa fragment of IL-33 in cell lysates, which is enhanced by incubation with caspase-1 in vitro. The molecular mass of this fragment is higher than that of recombinant, so-called mature, IL-33. We further demonstrate that generation of this fragment is not mediated directly by caspase-1, which does not cleave pro-IL-33 in an in vitro system devoid of contaminating proteases. In addition, our results indicate that caspase-1 cleavage is not required for pro-IL-33 secretion and bioactivity. Although it is generally assumed that the IL-33 processing is regulated similarly to that of IL-1β, our data thus highlight major differences between these two cytokines.

We believe that the 20-kDa form of IL-33, which we observed using both the mouse and human proteins, in fact corresponds to the mature form of IL-33 initially described by Schmitz et al. (1) after incubation of human pro-IL-33 with caspase-1. In this early work, the molecular mass of this short IL-33 form was indeed not directly compared with that of rIL-33. Our data further suggest that the cleavage reported in this study was not caused directly by caspase-1, but rather was mediated by contaminating proteases, which are known to be present in reticu-locyte lysates (16). Caspase-1 has indeed been observed previously to mediate activation of other downstream proteases directly or indirectly (4, 18). In support of this hypothesis, we observed the presence of the 20-kDa form of IL-33 to a variable extent already in lysates of 293T cells, which do not express endogenous caspase-1,3 before addition of recombinant active caspase-1. Furthermore, generation of this fragment was still observed with the D88A and D106A mutant forms of mouse pro-IL-33, indicating that it was independent of the presence of the two potential caspase-1 cleavage sites present in the N-terminal moiety of mouse pro-IL-33. Finally, our observations fit the lack of a conserved region corresponding to a potential caspase-1 cleavage site between species. Interestingly, generation of this 20-kDa form of IL-33 was observed not only in vitro, but also in THP-1 cells. The mechanisms underlying its production as well as its functional relevance remain to be investigated.

Another interesting observation is the release of full-length pro-IL-33 into the supernatants of LPS-activated THP-1 cells, suggesting that pro-IL-33 can be secreted in the absence of caspase-1 processing. Consistent with our results, the presence of full-length pro-IL-33 in the supernatants of activated THP-1 cells has been detected previously (9). In this previous report, two shorter forms of IL-33 were also

These cells do not express caspase-1, as assessed by reverse transcription-PCR (data not shown), but we hypothesized that, after addition to the cultures, pro-IL-33 might be processed by other secreted or cell surface proteases. Indeed, we could detect processing of pro-IL-33 to shorter forms, in particular 28-, 20-, and 18-kDa fragments (Fig. 5C), although, after a 24-h incubation with P815 cells, the relative abundance of full-length pro-IL-33 and these shorter fragments was variable between experiments (Fig. 5C and data not shown). The presence of shorter forms was also observed when P815 cells were incubated with the D88A and D106A mutant forms of pro-IL-33 (data not shown). It thus remains to be determined which forms of IL-33 indeed correspond to biologically active molecules.

DISCUSSION

In this study, we report generation of a 20-kDa fragment of IL-33 in cell lysates, which is enhanced by incubation with caspase-1 in vitro. The molecular mass of this fragment is higher than that of recombinant, so-called mature, IL-33. We further demonstrate that generation of this fragment is not mediated directly by caspase-1, which does not cleave pro-IL-33 in an in vitro system devoid of contaminating proteases. In addition, our results indicate that caspase-1 cleavage is not required for pro-IL-33 secretion and bioactivity. Although it is generally assumed that the IL-33 processing is regulated similarly to that of IL-1β, our data thus highlight major differences between these two cytokines.

We believe that the 20-kDa form of IL-33, which we observed using both the mouse and human proteins, in fact corresponds to the mature form of IL-33 initially described by Schmitz et al. (1) after incubation of human pro-IL-33 with caspase-1. In this early work, the molecular mass of this short IL-33 form was indeed not directly compared with that of rIL-33. Our data further suggest that the cleavage reported in this study was not caused directly by caspase-1, but rather was mediated by contaminating proteases, which are known to be present in reticulocyte lysates (16). Caspase-1 has indeed been observed previously to mediate activation of other downstream proteases directly or indirectly (4, 18). In support of this hypothesis, we observed the presence of the 20-kDa form of IL-33 to a variable extent already in lysates of 293T cells, which do not express endogenous caspase-1,3 before addition of recombinant active caspase-1. Furthermore, generation of this fragment was still observed with the D88A and D106A mutant forms of mouse pro-IL-33, indicating that it was independent of the presence of the two potential caspase-1 cleavage sites present in the N-terminal moiety of mouse pro-IL-33. Finally, our observations fit the lack of a conserved region corresponding to a potential caspase-1 cleavage site between species. Interestingly, generation of this 20-kDa form of IL-33 was observed not only in vitro, but also in THP-1 cells. The mechanisms underlying its production as well as its functional relevance remain to be investigated.

Another interesting observation is the release of full-length pro-IL-33 into the supernatants of LPS-activated THP-1 cells, suggesting that pro-IL-33 can be secreted in the absence of caspase-1 processing. Consistent with our results, the presence of full-length pro-IL-33 in the supernatants of activated THP-1 cells has been detected previously (9). In this previous report, two shorter forms of IL-33 were also

3 D. Talabot-Ayer, C. Gabay, and G. Palmer, unpublished observations.
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observed in THP-1 supernatants. We cannot formally exclude from our data that shorter forms of IL-33 may also be released into THP-1 supernatants at concentrations falling below the detection limit of our Western blot analysis. Alternatively, full-length pro-IL-33 might be further processed within THP-1 culture supernatants by extracellular or cell surface proteases, as is the case with P815 cells. The selective release of pro-IL-33 by LPS-activated cells suggests (19). In addition to its specific release by activated cells, pro-IL-33 was also recently suggested to function as an alarmin upon leakage from damaged endothelial or epithelial cells, which constitutively express its nuclear pro form (7).

Finally, incubation with full-length pro-IL-33 induced T1/ST2-dependent biological effects in mast cells. However, because we observed variable extents of processing of wild-type and Asp-to-Ala mutant pro-IL-33 proteins in P815 culture medium during the time frame used for stimulation, it is not clear at this stage whether the full-length form itself and/or one or several cleavage products represent the truly bioactive species. Interestingly, in contrast to the various cell lysates we analyzed, P815 culture medium also contained an 18-kDa form of IL-33, which was similar in size to the recombinant protein. In any event, P815 cells do not express caspase-1, indicating that extracellular IL-33 processing is again caspase-1-independent. Taken together, our data clearly demonstrate that, in contrast to IL-1β, IL-33 bioactivity does not require activation by caspase-1 cleavage.

In conclusion, we describe the generation of a 20-kDa fragment of mIL-33 and hIL-33, which is different from rIL-33. This fragment is not produced by direct caspase-1 cleavage. In fact, our results indicate that pro-IL-33 is not a substrate for this enzyme. We also observed that pro-IL-33 is released in its full-length form by activated THP-1 cells and that addition of pro-IL-33 induces T1/ST2-dependent biological effects in mast cells. Taken together, our results clearly indicate that caspase-1 cleavage is not required for IL-33 secretion and bioactivity.

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