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Caspase-1, Caspase-8, and Calpain Are Dispensable for IL-33 Release by Macrophages

Tatsukuni Ohno,* Keisuke Oboki,* Naoki Kajiwara,†* Eiichi Morii,† Katsuyuki Aozasa,† Richard A. Flavell,‡ Ko Okumura,§ Hirohisa Saito,‡§ and Susumu Nakae‡§¶

In addition to IL-1 and IL-18, IL-33 was recently identified as a member of the IL-1 cytokine family. rIL-33 can promote production of Th2-type cytokines by Th2 cells and mast cells in vitro. Administration of rIL-33 to mice results in increases in IgE secretion and eosinophilic inflammation. However, the precise immune cell source of IL-33 remains unclear. Moreover, although recombinant pro-IL-33 is cleaved by recombinant caspase-1 in vitro, as are pro-IL-1β and pro-IL-18, the involvement of caspase-1 in pro-IL-33 cleavage remains controversial. In this study, we show that mouse peritoneal macrophages, but not splenic dendritic cells, produced IL-33 upon stimulation with LPS. Likewise, mouse bone marrow cell-derived cultured mast cells also produced a small, but significant amount of IL-33 via FcεRI cross-linking, but not in response to stimulation with LPS. To our surprise, IL-33 release was found even in caspase-1-deficient, caspase-8 inhibitor-treated, and calpain inhibitor-treated macrophages. These observations suggest that caspase-1, caspase-8, and calpain-independent IL-33 production by macrophages and/or mast cells may contribute to the pathogenesis of Th2-type allergic inflammation. The Journal of Immunology, 2009, 183: 7890–7897.

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Materials and Methods

**Mice**

BALB/c mice (CLEA Japan) and BALB/c-casparase-1"/" (20) were housed under specific pathogen-free conditions in our institute (National Research Institute for Child Health and Development), and the animal protocols were approved by the Institutional Review Board of the National Research Institute for Child Health and Development.

**Cells**

For purification of CD4^+ T cells, mouse spleen and inguinal, axillary, brachial, and submaxillary lymph node lymph samples were harvested and pooled. The pooled cells were incubated with biotinylated anti-mouse B220 (RA3-6B2), CD8 (53-6,7), CD11b (M1/70), CD11c (HLC), CD32 (P6C6.5), CD49b (DX5), CD119 (c-kit, 2B8), FcεRIα (MAR-1), Gr-1 (RB6-8C5), Ter119, and yδTCL (GL3) mAbs at 4°C for 20 min. These Abs were obtained from eBioscences and BD Biosciences. After washing, the cells were incubated with Streptavidin Particles Plus-DM (BD Biosciences). Then CD4^+ T cells (>95%) were isolated by negative selection using a BD IMag system (BD Biosciences).

For purification of peritoneal macrophages and splenic DCs, mouse peritoneal exudate cells (PECs) and spleen cells were collected and incubated with anti-mouse CD16/CD32 mAb (93; eBiosciences) at 4°C for 20 min. The PECs were then incubated with biotinylated anti-mouse F4/80 mAb (BM8; eBiosciences) at 4°C for 45 min, and then stimulated with and without LPS for 24 h. For immunoprecipitation, F4/80^+ peritoneal macrophages (>92%) and CD11c^+ splenic DCs (>92%) were isolated by positive selection using a MACS system (Miltenyi Biotec). Then CD4^+ T cells, mouse spleen and inguinal, axillary, brachial, and submaxillary lymph node lymph samples were harvested and pooled. The pooled cells were incubated with biotinylated anti-mouse B220 (RA3-6B2), CD8 (53-6,7), CD11b (M1/70), CD11c (HLC), CD32 (P6C6.5), CD49b (DX5), CD119 (c-kit, 2B8), FcεRIα (MAR-1), Gr-1 (RB6-8C5), Ter119, and yδTCL (GL3) mAbs at 4°C for 20 min. These Abs were obtained from eBioscences and BD Biosciences. After washing, the cells were incubated with streptavidin-coated microbeads (Miltenyi Biotec) at 4°C for 4°C for 20 min. Spleen cells were incubated with CD11c-Microbeads (Miltenyi Biotec) at 4°C for 20 min. F4/80^+ peritoneal macrophages (>92%) and CD11c^+ splenic DCs (>92%) were isolated by positive selection using a MACS system (Miltenyi Biotec).

For collection of thiglycollate (TGC)-induced mouse peritoneal macrophages (TGC macrophages), mice were i.p. injected with 5 ml of 2% TGC (Nissui). Three days after injection, PECs were collected.

**Mouse BMCMCs were generated, as described elsewhere (11). In brief, mouse femoral bone marrow cells were cultured in the presence of 10 ng/ml rmIL-7 (PeproTech) for 6–8 wk, at which time flow cytometry showed the cells to be a >98% c-kit^+ FcɛRI^+ population. Before using the cells, rmIL-3 was removed by washing.

**Cell culture**

CD4^+ T cells were cultured with plate coated anti-CD3 mAb (1 μg/ml) (145-2C11; eBioscience), F4/80^+ macrophages, CD11c^+ DCs, or TGC macrophages were stimulated with 100 ng/ml LPS (Salmonella enterica serotype typhimurium; Sigma-Aldrich), 100 ng/ml rmIL-3 (PeproTech) for 6–8 wk, at which time flow cytometry showed the cells to be a >98% c-kit^+ FcɛRI^+ population. Before using the cells, rmIL-3 was removed by washing.

**RT-PCR**

IL-33 mRNA expression was analyzed by RT-PCR. The PCR primers were 5'-gagatgccaaacgaagc-3' and 5'-tcgaggagagtacg-3' for mIL-33, and 5'-gcectgctgaaaactc-3' and 5'-tagctgctagcctt-3' for murine GAPDH. The PCR conditions were (94°C for 30 s, 55°C for 30 s; 72°C for 60 s) × 35 cycles (IL-33) or 25 cycles (GAPDH).

**ELISA/ELISPOT**

For IL-33 detection, Nunc-Immuno plates (Nunc) for ELISA and MultiScreen-IP plates (MAIPS4510; Millipore) for ELISPOT were coated with anti-mIL-33 polyclonal Ab (R&D Systems; 1 μg/ml in PBS) as a capture Ab at 4°C overnight. After blocking with PBS containing 1% BSA (ELISA) or 10% FCS (ELISPOT), samples, and rmIL-33 as a standard cytokine, the cells were incubated at room temperature (r.t.) for 2 h for ELISA or cultured at 37°C for 24 h for ELISPOT. After washing the wells, biotinylated anti-mouse/human IL-33 mAb (Nessy-2; Alexis Biochemicals, 400 ng/ml in PBS containing 1% BSA) as a detection Ab was applied and incubated at r.t. for 1 h for ELISA and ELISPOT. Then, after washing the wells, HRP-conjugated streptavidin (BD Biosciences) was added to the wells at r.t. for 1 h. Tetramethylbenzidine (eBioscience) and 3-aminio-9-ethylcarbazole (Sigma-Aldrich) were used as substrates in ELISA and ELISPOT, respectively. For ELISPOT, positive spots on Ab-coated MultiScreen-IP plates (MAIPS4510; Millipore) were analyzed with NIH Image software. For IL-1α, IL-1β, and IL-6 detection, rmIL-1α DuoSet (R&D Systems) and mIL-1β and mIL-6 ELISA sets (eBioscience) were used for ELISA and ELISPOT.

**Immunocytochemistry**

Immunocytochemistry for IL-33 was conducted by the polymer-immuno-complex method (DakoCytomation), according to the manufacturer’s instructions. Briefly, cytospin samples were fixed with 4% formaldehyde at 4°C for 20 min and then blocked with peroxidase-blocking solution (DakoCytomation). The resultant complex was mixed with mouse anti-human/mouse IL-33 mAb (Nessy-2; Alexis Biochemicals) and EnVision detection reagent (DakoCytomation) at r.t. for 1 h, followed by addition of normal mouse serum and further incubation at r.t. for 1 h. Cytospin samples were incubated with the resultant complex at r.t. for 1 h, and then the IL-33/anti-IL-33/EnVision complex was detected with diaminobenzidine.

**Immunoprecipitation and Western blot analysis**

Cells that were stimulated with and without 100 ng/ml LPS or lugs that were harvested from mice at 6 h after inhalation of LPS (10 μg in 20 μl of sterile, pyrogen-free 0.9% NaCl (saline)) or saline were homogenized in cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS, 250 mg of lung tissue/ml) at 4°C for 30 min. Then, after centrifugation (16,000 × g) at 4°C for 30 min, the supernatants were collected. For immunoprecipitation, the lysates were incubated with anti-mIL-33 mAb (10 μg; Nessy-1; Alexis) or anti-mIL-1β mAb (2 μg; B122; BD Biosciences) at 4°C for 12 h. The immunoprecipitants, lung and cell lysates, mIL-33, and mIL-1β were analyzed by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (In Vitrogen). The membranes were blocked and then incubated consecutively with anti-mIL-33 mAb (4G4; MBL) and HRP-conjugated anti-mouse IgG (Amersham) for IL-33, and anti-mIL-1β (166926; R&D Systems) and anti-rat IgG (Amersham) for IL-1β. The immunoreactive proteins were visualized with ECL reagents (ECL Western Blotting Detection Reagents; Amersham).

**Cell survival**

Cell viability was assessed using the MEBCYTO-Apoptosis Kit (MBL) with flow cytometry or a colorimetric lactate dehydrogenase (LDH) assay kit (CytoTox 96; Promega).

**Multiple simultaneous Tag (MUSTag) assay**

For modified immune PCR, called MUSTag technology, anti-mIL-1β/anti-mIL-6 polyclonal Ab (R&D Systems) and anti-mIL-33 polyclonal Ab (R&D Systems), as detection Abs, were conjugated with DNA. Briefly, the detection Abs (15 μg/ml) were incubated with 100 μl of the DNA-conjugated Abs was determined by fluorescence microscopy. The biotinylated double-strand MUSTag DNA fragment was designed as follows: 5’-[biotin]-CAGCTGTTACTCGTTCAAGGATACGCTATGACGCGCCCTTCCTCTATCAGTGTACCAATTACGATCTGGACACCTCTTGAGTGCACTGAGACCGCTCATCCTGGTCGACCCACGCGGTTGC-3’, the first box = EcoRI restriction site, the second box = MUSTag forward priming site, and the third box = MUSTag reverse priming site.

MUSTag assays were performed according to the manufacturer’s instructions (Synthera Technologies). Briefly, Maxisorp Immuno-Module 96-well plates (Nalge Nunc International) were coated with 50 μl/well anti-mIL-1β mAb (2 μg/ml; 30311; R&D Systems) or anti-human/mouse IL-33 mAb (5 μg/ml; Nessy-1; Alexis Biochemicals) in 50 mM sodium carbonate buffer (pH 9.6) at 4°C overnight. After blocking with a blocking buffer (Synthera Technologies) for 1 h at r.t., serially diluted standard rmIL-1β (R&D Systems) or rmIL-33 (PeproTech; 10 ng/ml to 0.64 pg/ml) in sample dilution buffer (Synthera Technologies) and samples (50 μl/well) were
incubated at r.t. for 1 h. After washing with wash buffer (Synthera Technologies), DNA-conjugated anti-mIL-1β or anti-mIL-33 polyclonal Ab in the dilution buffer (8 ng/ml, 30 µl/well) was added, followed by incubation at r.t. for 1 h. After washing, 7.5 U/ml EcoRI (30 µl/well; Nippon Gene) in digestion buffer was added, followed by incubation at r.t. for 15 min. A total of 3 µl of the solution in each well was subjected to real-time quantitative PCR (qPCR) analyses.

qPCR analyses were performed using the Mx3005P Real-Time PCR System (Agilent). The PCR mixtures consisted of 10 µl of 2× SYBR Premix EX Taq (Takara Bio), 100 nM MUSTag primers (Synthera Technologies), 0.4 µl of 50× 6-carboxy-X-rhodamine Reference Dye II (Takara Bio), 3 µl of each sample, and sterile distilled water added to a final volume of 20 µl. The temperature program for PCR was as follows: 1) 95°C for 10 s; 2) (95°C for 5 s) 60°C for 20 s. The fluorescence intensity was measured at the end of each cycle using excitation/emission filters of 492/516 nm for SYBR Green I and 585/610 nm for 6-carboxy-X-rhodamine (passive reference). The threshold cycle ( Ct ) values were calculated from amplification plots using MXPro qPCR software version 3.20 (Agilent) with adaptive baseline and amplification-based threshold algorithms, and further analyses were conducted with GraphPad Prism version 4.03 (GraphPad). Each standard was assayed in triplicate, and the mean Ct values with SD were plotted against the log of the concentration of each recombinant enzyme. The standard curves were fit to the following sigmoidal four-parameter logistic equation:

\[
Ct = C_{min} + \frac{C_{max} - C_{min}}{1 + 10^{\left(\log EC_{50} - \log X\right) \cdot Hill\ Slope}}
\]

(1)

where X is the enzyme concentration, Ct is the corresponding Ct value, C max is the theoretical maximum Ct value (the minimum response), C min is the theoretical minimum Ct value (the maximum response), EC 50 is the concentration causing 50% of the maximum response, and Hill Slope is the slope of the linear portion of the sigmoidal curve. In all curve fittings, each C max parameter was fixed to the mean Ct value of the blank concentration, and then C min, EC 50, and Hill Slope were obtained by a nonlinear least-squares method. The concentrations of IL-33 and IL-1β in unknown samples were calculated from the respective standard curves.

Statistics

An unpaired Student’s t-test, two tailed, was used for statistical evaluation of the results.

Results

IL-33 mRNA is expressed in mouse macrophages, DCs, and BMCMCs

Macrophages, DCs, and mast cells express TLRs. These immune cells are considered to be important for host defense against various pathogens by activating innate immune responses via TLRs. TLRs are also important for the elicitation of acquired immune responses; for example, TLR signals contribute to, but are not essential for, the development of Th2-associated allergic responses (21). Thus, we used RT-PCR to examine IL-33 mRNA expression in those cells after LPS stimulation. Resting mouse F4/80 + peritoneal macrophages, but not CD11c + splenic DCs or BMCMCs, constitutively expressed IL-33 mRNA (Fig. 1). After PMA plus ionomycin stimulation, IL-33 mRNA was increased in CD11c + splenic DCs and BMCMCs, but not in F4/80 + peritoneal macrophages (Fig. 1). After LPS stimulation, IL-33 mRNA was up-regulated in F4/80 + peritoneal macrophages and CD11c + splenic DCs, but not in BMCMCs (Fig. 1). However, BMCMCs expressed IL-33 mRNA after addition of monomeric IgE (22) and rmIL-33, and also after IgE/Ag stimulation (Fig. 2). IL-33 mRNA was not observed to be expressed by CD4 + T cells under any conditions (Fig. 1).

IL-33 protein is produced by mouse macrophages, but not by DCs

In contrast to IL-33 mRNA expression, IL-33 protein levels were always below the limit of detection by ELISA in the culture supernatants of F4/80 + peritoneal macrophages and CD11c + splenic DCs after stimulation with LPS or PMA plus ionomycin (Fig. 3), even though IL-6 production was detected in the same supernatants (data not shown). However, IL-33 protein was detected in whole-cell lysates of F4/80 + peritoneal macrophages, but not of CD11c + splenic DCs, after LPS and PMA plus ionomycin stimulation (Fig. 3). To detect released IL-33 protein, we next established an IL-33-specific MUSTag assay system (limit of detection: 50 pg/ml). The data show representative results from at least two to three independent experiments. Semi-quantitative PCR analysis was performed using the serially diluted cDNA templates during the exponential phase of PCR amplification.

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### FIGURE 1. IL-33 mRNA expression in macrophages, DCs, and BMCMCs after LPS stimulation. Mouse splenic CD4 + T cells, CD11c + DCs, peritoneal F4/80 + macrophages (Mφ), and BMCMCs were stimulated for 1 h with plate-coated anti-CD3 mAb (1 µg/ml), 100 ng/ml LPS, or 0.1 µg/ml PMA plus 1 µg/ml ionomycin. –, Indicates no stimulation. IL-33 mRNA expression was determined by RT-PCR. GAPDH mRNA expression was used as an internal control. Densitometric analysis was performed using NIH Image software, and IL-33 mRNA expression was normalized against GAPDH mRNA expression (the value of IL-33 mRNA expression/the value of GAPDH mRNA expression × 100). The data show representative results from at least two to three independent experiments. Semi-quantitative PCR analysis was performed using the serially diluted cDNA templates during the exponential phase of PCR amplification.

### FIGURE 2. IL-33 mRNA expression in BMCMCs. Mouse BMCMCs were stimulated for 1 h with various concentrations of monomeric IgE (SPE-7), LPS, or rmIL-33 or 0.1 µg/ml PMA plus 1 µg/ml ionomycin. –, Indicates no stimulation. Mouse BMCMCs were sensitized overnight with 1 µg/ml anti-DNP IgE (SPE-7). After washing, IgE-sensitized BMCMCs were cultured for 1 h with various concentrations of DNP-HSA. IL-33 mRNA expression was determined by RT-PCR. GAPDH mRNA expression was used as an internal control. Densitometric analysis was performed using NIH Image software, and IL-33 mRNA expression was normalized against GAPDH mRNA expression (the value of IL-33 mRNA expression/the value of GAPDH mRNA expression × 100). The data show representative results from at least two to three independent experiments. Semi-quantitative PCR analysis was performed using the serially diluted cDNA templates during the exponential phase of PCR amplification.
>0.64 pg/ml rmIL-33), which is much more sensitive than general ELISA (limit of detection: 30 pg/ml rmIL-33). Nevertheless, as shown in Fig. 3, any IL-33 that was present in the culture supernatants (Sup) and whole-cell lysates (WCL; 5 × 10^6 macrophages or 1 × 10^6 DCs in 100 μl of lysis buffer) were determined by ELISA. Data show the mean ± SEM (n = 3). *p < 0.05 vs medium.

Because IL-33 is localized in the nucleus as an intracellular NF (19), it has been unclear whether pro-IL-33 is cleaved and/or released. We next performed Western blot analysis for detection of processed-form IL-33. As shown in Fig. 4A, >1.2 ng of rmIL-33 was detectable with our Western blot system. However, both pro-IL-33 (32 kDa) and processed-form IL-33 (18 kDa) in whole-cell lysates or culture supernatants of LPS-stimulated F4/80 peritoneal macrophages (1 × 10^6 cells purified from pooled PECs from 20 mice) were below the limit of detection with Western blot analysis even after immunoprecipitation using anti-IL-33 Ab (data not shown). It has been shown that IL-33 mRNA is constitutively expressed in the lungs of mice (1). In support of this, pro-IL-33 (a 31-kDa protein), but not processed-form IL-33 (18 kDa), was detected in whole-lung homogenates from naive wild-type mice by the Western blot analysis (Fig. 4B). Pro-IL-33 expression was increased in whole-lung homogenates from LPS-inhaled wild-type mice in comparison with saline-inhaled wild-type mice by Western blot analysis, but processed-form IL-33 expression could not be detected in those homogenates by Western blot analysis irrespective of LPS treatment (Fig. 4B) or even by the Western blot analysis after immunoprecipitation using anti-IL-33 Ab (data not shown). Likewise, even though pro-IL-33 (32-kDa) expression was increased in whole-cell lysates, but not culture supernatants, of TGC macrophages after stimulation with LPS, processed-form IL-33 was below the limit of detection in both samples by Western blot analysis after immunoprecipitation using anti-IL-33 Ab (Fig. 4C). In contrast, released-form IL-1β could be detected under similar cell culture conditions by Western blot analysis after immunoprecipitation using anti-IL-1β Ab (Fig. 4D). However, in comparison with the amount of pro-IL-1β in whole-cell lysates of LPS-treated TGC-macrophages, the amount of released-form IL-1β in the culture supernatants was very small (Fig. 4D). These observations suggest that detection of low levels of processed-form IL-33, like released-form IL-1β, was difficult with our Western blot system.

Therefore, we established an IL-33-specific ELISPOT system to detect IL-33-releasing cells. Consistent with the IL-33 levels detected in whole-cell lysates by ELISA, ELISPOT analysis found...
that F4/80<sup>+</sup> peritoneal macrophages and TGC-induced macrophages, but not CD11c<sup>+</sup> splenic DCs, released IL-33 at significant levels after LPS or PMA plus ionomycin stimulation (Fig. 5A and data not shown). These findings suggest that macrophages, but not DCs, are a potential source of IL-33.

**Caspase-1, caspase-8, and calpain are dispensable for IL-33 release by macrophages**

It was reported that recombinant pro-IL-33 was cleaved by recombinant caspase-1 in vitro (1), suggesting that in vivo release of IL-33, as well as IL-1ß and IL-18, is mediated by caspase-1. In contrast, as reported by Carriere et al. (19), the biological involvement of caspase-1 in pro-IL-33 cleavage remains controversial. To clarify this issue, we performed IL-33-specific ELISPOT assay using F4/80<sup>+</sup> peritoneal macrophages from caspase-1-deficient mice. Both the ELISA and ELISPOT assays showed that release of IL-1ß, but not IL-1α, by caspase-1-deficient macrophages after LPS stimulation was profoundly decreased in comparison with that by wild-type macrophages (Fig. 5A). In contrast, interestingly, we found that even caspase-1-deficient macrophages were able to release a significant amount of IL-33 after LPS or PMA plus ionomycin stimulation (Fig. 5A). Parallel experiments showed reduced IL-1ß release, but normal IL-33 release by caspase-1-deficient peritoneal macrophages after LPS stimulation (Fig. 5A). Meanwhile, the cell viability assessed by annexin V and propidium iodide (PI) staining and the LDH activity in the culture supernatants were not affected by the addition of LPS (Fig. 5, B and C). Accordingly, both the IL-33 and IL-1ß detected in the ELISPOT assay appeared to have been largely released by live cells rather than by apoptotic/necrotic/dead cells. Thus, these observations suggest that release of IL-33 is independent of caspase-1.

Like caspase-1, caspase-8 is involved in the cleavage of pro-IL-1ß under certain conditions (18). Therefore, we used a caspase-8 inhibitor to examine whether caspase-8 is involved in IL-33 release by LPS-stimulated macrophages. Although both the ELISA-determined levels of IL-1ß in the culture supernatants and the ELISPOT-determined number of IL-1ß-releasing macrophages were reduced in the presence of the caspase-8 inhibitor after LPS stimulation, the number of IL-33-releasing macrophages was not affected (Fig. 6).

Similar to IL-33, IL-1α, but not IL-1ß or IL-18, is localized in the nucleus (17). Pro-IL-1α is cleaved by calcium-dependent activation of a membrane-associated cysteine proteinase, calpain (16,
17). Indeed, in the presence of calpain inhibitors, the levels of IL-1α in the culture supernatants and the number of IL-1α-releasing macrophages were reduced in ELISA and ELISPOT, respectively, after LPS stimulation (Fig. 7). In contrast, the number of IL-33-releasing macrophages detected by ELISPOT was not influ-
enced by the addition of calpain inhibitors to the culture (Fig. 7). Taken together, these observations indicate that neither caspase-1, caspase-8, nor calpain is essential for IL-33 release by macro-
phages and suggest that the mechanism of cleavage of pro-IL-33 is different from the cleavage mechanisms for pro-IL-1α, pro-IL-1β, and pro-IL-18.

**IL-33 production is induced in BMCMCs after IgE/Ag stimulation**

As shown in Figs. 1 and 2, the expression of IL-33 mRNA was up-regulated in BMCMCs upon stimulation with monomeric IgE, rmIL-33, or PMA plus ionomycin, but not LPS, and after IgE/Ag-FcεRI cross-linking. However, IL-33 protein in the culture supernatants was below the limit of detection by ELISA under all conditions, whereas IL-6 in the supernatants was detectable in the whole-cell lysates of BMCMCs after stimulation with monomeric IgE, LPS, or rmIL-33. We have now demonstrated that IL-33 mRNA was constitutively expressed in resting mouse F4/80+ peritoneal macrophages and up-regulated in those cells after LPS or PMA plus ionomycin stimulation. In contrast to the resting bone marrow-derived DCs reported by Schmitz et al. (1), CD11c+ DCs freshly isolated from mouse spleen did not express IL-33 mRNA. However, as in the case of F4/80+ peritoneal macrophages, IL-33 mRNA expression was strongly induced in peritoneal macrophages, rather than DCs or mast cells, after PMA plus ionomycin stimulation.

**Discussion**

IL-33 can promote Th2 cell and mast cell activation, suggesting that it contributes to the development of such Th2 cytokine- and mast cell-associated disorders as allergic diseases (1, 7–11). Indeed, administration of rIL-33 results in dramatic pathological changes in mice, as follows: splenomegaly, elevated numbers of blood eosinophils, increased serum IgE and IgA levels, and IL-5 and IL-13 levels, and eosinophil-dominant inflammation in the gut and lung (1). Thus, it is clear that IL-33, like IL-1β and IL-18, acts as a proinflammatory cytokine in immune responses. In contrast, the precise immune cell source of IL-33 remains poorly understood, although IL-33 mRNA was increased in the lungs of mice in an OVA-induced asthma model (13).

Schmitz et al. (1) demonstrated that LPS-stimulated bone marrow-derived macrophages and resting bone marrow-derived DCs expressed IL-33 mRNA in mice. We have now demonstrated that IL-33 mRNA was constitutively expressed in resting mouse F4/80+ peritoneal macrophages and up-regulated in those cells after LPS or PMA plus ionomycin stimulation. In contrast to the resting bone marrow-derived DCs reported by Schmitz et al. (1), CD11c+ splenic DCs freshly isolated from mouse spleen did not express IL-33 mRNA. However, as in the case of F4/80+ peritoneal macrophages, IL-33 mRNA expression was strongly induced in CD11c+ splenic DCs by LPS or PMA plus ionomycin stimulation. We also found that mouse BMCMCs expressed IL-33 mRNA upon stimulation with monomeric IgE and rmIL-33, but not LPS, and after IgE/Ag cross-linking. Thus, these observations suggest that macrophages, rather than DCs or mast cells, may be a producer of IL-33 during bacterial infections.

Schmitz et al. (1) reported that recombinant pro-IL-33 was cleaved by recombinant caspase-1 in vitro. In contrast, Carriere et al.
It was recently reported that pro-IL-33 was released by necrotic cells in an apoptosome-dependent manner (24, 25). We also confirmed that IL-33 was not detected in the culture supernatants from that setting (Fig. 9B). Such treatments induced profound PI-positive necrosis (Fig. 9A). In addition, pro-IL-33, but not a protease-cleaved form of IL-33, was detected in the culture supernatants from that setting (Fig. 9B). The number of IL-33-positive F4/80+ peritoneal macrophages was dramatically increased after PMA plus ionomycin stimulation (13.4-fold increase vs the control condition, medium alone) by ELISPOT (Fig. 9C). Compared with this, the proportion of PI-positive necrotic macrophages was increased after PMA plus ionomycin stimulation (2.8-fold increase vs the control condition, medium alone; Fig. 9C). Although we cannot rule out the possibility that pro-IL-33 is released only by necrotic cells, our observations suggest that it is released by live cells in addition to necrotic cells.

In our present study, we also clearly demonstrated for the first time that neither caspase-1, caspase-8, nor calpain is essential for IL-33 release by F4/80+ peritoneal macrophages after LPS stimulation. That is, whereas IL-1β release or IL-1α release was significantly impaired in caspase-1-deficient, caspase-8 inhibitor-treated, or calpain inhibitor-treated macrophages after stimulation, respectively, IL-33 release was unaffected by these treatments. It is also known that caspase-1-deficient mice show significantly reduced IL-1β and IL-18 release, but they still release significant amounts of IL-1β and IL-18. In our present study, we demonstrated that macrophages and mast cells are potential producers of IL-33, and that neither caspase-1, caspase-8, nor calpain is essential for the release of IL-33.
IL-33. These observations imply that caspase-1- and calpain-independent IL-33 production by macrophages and/or mast cells may contribute to the pathogenesis of certain infections and/or Th2-type allergic inflammation.

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Disclosures

The authors have no financial conflict of interest.

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