IL-33 is a chromatin-associated cytokine of the IL-1 family that has recently been linked to many diseases, including asthma, rheumatoid arthritis, atherosclerosis, and cardiovascular diseases. IL-33 signals through the IL-1 receptor-related protein ST2 and drives production of pro-inflammatory and T helper type 2-associated cytokines in mast cells, T helper type 2 lymphocytes, basophils, eosinophils, invariant natural killer T cells, and natural killer cells. It is currently believed that IL-33, like IL-1β and IL-18, requires processing by caspase-1 to a mature form (IL-33112–270) for biological activity. Contrary to the current belief, we report here that full-length IL-331–270 is active and that processing by caspase-1 results in IL-33 inactivation, rather than activation. We show that full-length IL-331–270 binds and activates ST2, similarly to IL-33112–270, and that cleavage by caspase-1 does not occur at the site initially proposed (Ser111), but rather after residue Asp178 between the fourth and fifth predicted β-strands of the IL-1-like domain. Surprisingly, the caspase-1 cleavage site (DGVD179G) is similar to the consensus site of cleavage by caspase-3, and IL-33 is also a substrate for this apoptotic caspase. Interestingly, we found that full-length IL-33, which is constitutively expressed to high levels by endothelial cells in most normal human tissues, can be released in the extracellular space after endothelial cell damage or mechanical injury. We speculate that IL-33 may function, similarly to the prototypical alarmins HMGB1 and IL-1α, as an endogenous danger signal to alert cells of the innate immune system of tissue damage during trauma or infection.

Cytokines of the IL-1 family play a major role in a wide range of inflammatory, infectious, and autoimmune diseases (1, 2). IL-33 [previously known as nuclear factor from high endothelial venule, or NF-HEV (3)] is the most recent addition to the IL-1 family (4, 5). Based on animal model studies and analyses of diseased tissues from patients, IL-33 has been proposed to represent a promising therapeutic target for several important diseases, including asthma and other allergic diseases (4), rheumatoid arthritis (5, 6), atherosclerosis (7), and cardiovascular diseases (8, 9). IL-33 has been shown to signal through the IL-1 receptor-related protein ST2 (4) and to drive production of cytokines [both pro-inflammatory and T helper type 2 (Th2)-associated cytokines] and chemokines in mast cells, Th2 lymphocytes, basophils, eosinophils, invariant natural killer (NK) T cells, and NK cells (4, 10–19). IL-33 signaling has also been shown to require the IL-1 receptor (IL-1R) accessory protein, IL-1RAcP, indicating that IL-33 shares with IL-1 not only structural homology but also signaling pathways (11, 14).

We initially discovered IL-33 as a nuclear factor abundantly expressed in endothelial cells of high endothelial venules in lymphoid organs (3, 5), but we (20) and others (21) have recently found that IL-33 is also constitutively expressed to high levels in the nucleus of endothelial cells in other human tissues. These observations indicated that IL-33 is widely expressed along the vascular tree and that endothelial cells constitute a major cellular source of IL-33 in most human tissues (20, 21). We also showed that nuclear IL-33 possesses transcriptional regulatory properties and associates with chromatin in vivo (5). Recently, we found that IL-33 mimics Kaposi sarcoma herpesvirus for attachment to chromatin, and docks, through a short chromatin-binding peptide, into the acidic pocket formed by the histone H2A-H2B dimer at the surface of the nucleosome (22). Together, our findings suggested IL-33 is a dual-function protein that may play important roles as both a cytokine and an intracellular nuclear factor (5, 22). A similar duality of function has previously been shown for IL-1α and chromatin-associated cytokine HMGB1. IL-1α, a cell-associated cytokine, exhibits potent pro-inflammatory cytokine activities mediated by the cell surface IL-1 receptors (1) but also functions extracellularly as by translocating to the nucleus and regulating transcription (23). HMGB1 regulates transcription and chromatin structure in the nucleus (24) but also functions extracellularly as a cytokine when secreted by activated macrophages during inflammation (25) or released by necrotic cells (26). IL-1α has also been shown to be released by dying cells (1, 27–29), and HMGB1 and IL-1α were thus defined as endogenous “danger signals” or “alarmins” that may alert the immune system after tissue damage during trauma or infection (30).

It is currently believed that IL-33, like classical IL-1 family members IL-1β and IL-18, requires maturation by caspase-1 for optimal biological activity. This is based on the initial report by Schmitz et al., who indicated caspase-1 cleaves IL-33 after residue Ser111, resulting in the production of mature IL-33112–270 (4). Most of the studies that have been published to date used a recombinant IL-33 protein corresponding to this mature form. Here, we show that full-length IL-331–270 is biologically active, and that cleavage by caspase-1 does not occur at the site initially proposed but rather after residue Asp178 within the IL-1-like domain. Consequently, caspase-1 processing results in inactivation of IL-33, rather than activation. We also demonstrate that full-length IL-331–270 can be released in the extracellular space after endothelial cell damage or injury. We discuss the possibility that IL-33 may function as an endogenous danger signal, like the prototypical alarmins IL-1α and HMGB1.

### Results

The 20–22 kDa Caspase-1 Cleavage Product of IL-33 Does Not Correspond to the IL-1-Like Domain. As previously reported by Schmitz et al. (4), we confirmed that in vitro translated human IL-33 can be cleaved by caspase-1 to a 20–22 kDa form, as determined by SDS/PAGE (Fig. L4). As expected, pro-IL-1β was also cleaved by caspase-1 to the IL-1β mature form. Cleavage was abrogated in both cases by the caspase-1 inhibitor Ac-YVAD-CHO. (Fig. L4). We initially discovered IL-33 as a nuclear factor abundantly expressed in endothelial cells of high endothelial venules in lymphoid organs (3, 5), but we (20) and others (21) have recently found that IL-33 is also constitutively expressed to high levels in the nucleus of endothelial cells in other human tissues. These observations indicated that IL-33 is widely expressed along the vascular tree and that endothelial cells constitute a major cellular source of IL-33 in most human tissues (20, 21). We also showed that nuclear IL-33 possesses transcriptional regulatory properties and associates with chromatin in vivo (5). Recently, we found that IL-33 mimics Kaposi sarcoma herpesvirus for attachment to chromatin, and docks, through a short chromatin-binding peptide, into the acidic pocket formed by the histone H2A-H2B dimer at the surface of the nucleosome (22). Together, our findings suggested IL-33 is a dual-function protein that may play important roles as both a cytokine and an intracellular nuclear factor (5, 22). A similar duality of function has previously been shown for IL-1α and chromatin-associated cytokine HMGB1. IL-1α, a cell-associated cytokine, exhibits potent pro-inflammatory cytokine activities mediated by the cell surface IL-1 receptors (1) but also functions extracellularly as by translocating to the nucleus and regulating transcription (23). HMGB1 regulates transcription and chromatin structure in the nucleus (24) but also functions extracellularly as a cytokine when secreted by activated macrophages during inflammation (25) or released by necrotic cells (26). IL-1α has also been shown to be released by dying cells (1, 27–29), and HMGB1 and IL-1α were thus defined as endogenous “danger signals” or “alarmins” that may alert the immune system after tissue damage during trauma or infection (30).

It is currently believed that IL-33, like classical IL-1 family members IL-1β and IL-18, requires maturation by caspase-1 for optimal biological activity. This is based on the initial report by Schmitz et al., who indicated caspase-1 cleaves IL-33 after residue Ser111, resulting in the production of mature IL-33112–270 (4). Most of the studies that have been published to date used a recombinant IL-33 protein corresponding to this mature form. Here, we show that full-length IL-331–270 is biologically active, and that cleavage by caspase-1 does not occur at the site initially proposed but rather after residue Asp178 within the IL-1-like domain. Consequently, caspase-1 processing results in inactivation of IL-33, rather than activation. We also demonstrate that full-length IL-331–270 can be released in the extracellular space after endothelial cell damage or injury. We discuss the possibility that IL-33 may function as an endogenous danger signal, like the prototypical alarmins IL-1α and HMGB1.
The size of the N-terminal fragment generated after caspase-1 processing indicated that the cleavage must occur within the IL-1-like domain. Inspection of this region revealed the existence of a potential cleavage site (DGVDI78G), similar to the consensus site of cleavage by caspase-3, and located between the fourth and fifth predicted β-strands of the IL-1-like domain (Fig. 2A). The possibility that caspase-1 may cleave IL-33 at this position was further supported by the observation that an in vitro translated fragment corresponding to IL-33 residues 1–178 co-migrated on SDS/PAGE with the 20–22 kDa caspase-1 cleavage product (Fig. 2B). To provide definitive proof for caspase-1 cleavage of IL-33 after residue Asp178, a single mutation was introduced that replaced Asp178 by an alanine. As shown in Fig. 2C, this mutation totally prevented the processing of full-length IL-33 by caspase-1. We concluded that caspase-1 cleaves IL-331–270 at the DGVDG site. Mutation of Asp178 to alanine also abrogated cleavage of IL-33 by caspase-3 (Fig. 2D), indicating that caspase-1 and caspase-3 cleave IL-331–270 at the same site within the IL-1-like domain. We then asked whether cleavage of IL-33 at this site occurs in cells undergoing apoptosis.

WT IL-33 and the IL-33D178A mutant were expressed in U2OS epithelial cells (Fig. S2), and apoptosis was induced by treatment with the DNA-damaging agent doxorubicin. Western blot analysis revealed that WT IL-33, but not the IL-33D178A mutant, is cleaved during doxorubicin-induced apoptosis and that cleavage can be prevented by pretreatment with the pan-caspase inhibitor Z-VAD-fmk (Fig. 2E). We next looked at endogenous native IL-33. As endothelial cells constitute a major cellular source of IL-33 in human tissues (20, 21), we selected human primary endothelial cells as a cellular system for these experiments. Western blot analysis revealed that endogenous IL-33 migrates as a 30–31 kDa band, the identity of which was validated after knockdown of IL-33 expression with specific siRNAs (Fig. 2F). Treatment of the endothelial cells with the apoptosis-inducing agent staurosporine resulted in a complete maturation of endogenous IL-33 that was prevented by pretreatment with the pan-caspase inhibitor Z-VAD-fmk (Fig. 2G). We concluded that native IL-33 is processed by endogenous caspases during apoptosis in primary human endothelial cells.

**Full-length IL-331–270 Binds and Activates the IL-33 Receptor ST2.** As our data indicated that, unlike IL-1β and IL-18, cleavage of IL-33 by caspase-1 does not lead to the production of the mature IL-1-like domain, we then asked whether, similarly to the IL-1α precursor (1, 31), full-length IL-331–270 may possess biological activity. We first tested the capacity of full-length IL-331–270 to bind to the ST2 receptor in pull-down experiments. We found that a human ST2-Fc fusion protein precipitates in vitro synthesized full-length IL-331–270, similarly to the isolated IL-1-like domain IL-33112–270 (Fig. 3A). In contrast, the amino-terminal
part of IL-33, IL-33<sub>1-111</sub>, did not bind to the ST2-Fc chimera protein. These results indicated that full-length IL-33<sub>1-270</sub> specifically binds to the ST2 receptor. Binding of full-length IL-33 to ST2 was also observed using endogenous native IL-33 obtained from endothelial cells extracts (Fig. 3B), indicating the natively folded forms behave similarly to the in vitro generated forms regarding ST2 binding.

We next investigated the capacity of full-length IL-33<sub>1-270</sub> to signal through ST2 by using a previously described NFrkB-dependent reporter assay (4, 14). HEK293T cells, transiently transfected with an expression vector for human ST2 together with an NFrkB-GFP reporter gene construct, were stimulated with full-length IL-33<sub>1-270</sub>, IL-33<sub>1-111</sub>, or IL-33<sub>112-270</sub>. As previously reported (4, 14), a small population of cells expressed GFP in the absence of IL-33 stimulation. However, stimulation of cells with full-length IL-33<sub>1-270</sub> or the IL-1-like domain IL-33<sub>112-270</sub> but not IL-33<sub>1-111</sub>, led to a significant increase in the number of GFP<sup>+</sup> cells (Fig. 3C). Quantification of the results by FACS analysis revealed that full-length IL-33<sub>1-270</sub> and the IL-1-like domain IL-33<sub>112-270</sub> possess a similar capacity to activate the NFrkB-GFP reporter gene construct (Fig. 3D). We concluded that, similarly to IL-33<sub>112-270</sub>, full-length IL-33<sub>1-270</sub> is able to bind and activate the ST2 receptor.

The 2 Caspase-1 Cleavage Products, IL-33<sub>1-178</sub> and IL-33<sub>179-270</sub>, Do Not Activate ST2. As we found caspase-1 processing of IL-33 occurs within the IL-1-like domain, we predicted the 2 caspase-1 cleavage products did not activate the ST2-dependent reporter gene. These results were confirmed using another bioassay, IL-33-dependent secretion of IL-6 by the mast cell line MC/9 (32). Full-length IL-33 significantly induced IL-6 secretion by MC/9 cells whereas the 2 caspase-1 cleavage products had no effect (Fig. 4C). We con-
cell scraping, a process that mimics the transient sublethal

Pull-down of full-length IL-331–270 with ST2-Fc fusion protein. Full-length dependent NF_

C

and flow cytometry (Methods). The percentage increase in GFP

microscopy (Materials and Methods). Cells were analyzed for GFP expression by fluorescence microscopy (A) and flow cytometry (B). The percentage increase in GFP⁺ cells is shown (Below). Results are shown as means and SDs of 3 independent transfection experiments.

cluded that, unlike full-length IL-331,270, the 2 caspase-1 cleavage products, IL-331–178 and IL-33112–270, do not possess biological activity, indicating that caspase-1 processing inactivates IL-33.

IL-33 is Released Extracellularly After Endothelial Cell Damage or Injury. IL-33 and the alarmin HMGB1 are both chromatin-associated cytokines, and we then studied the possibility that full-length IL-33, similarly to HMGB1 (26), may be released after cell damage or necrosis. We first tested the effect of mechanical injury on primary human endothelial cells that express high levels of endogenous IL-33 both in vivo (20, 21) and in culture (Fig. 5A). Mechanical wounding of endothelial cells by cell scraping, a process that mimics the transient sublethal

membrane disruptions that are observed in cells subjected to mechanical forces in vivo, has previously been shown to result in the release of growth factors such as basic FGF (33). Interestingly, we found that full-length IL-33 was released from endothelial cells mechanically wounded by cell scraping (Fig. 5B). HMGB1 was similarly released under these conditions. Scratching of endothelial monolayers by tracing lines with a surgical scalpel, a needle, or a pipette tip has been widely used as an in vitro model of wound healing. Using this model, we confirmed that IL-33, like HMGB1, is released into the supernatant after mechanical injury of endothelial cells. In contrast, IL-33 and HMGB1 were not released extracellularly in the absence of endothelial cell damage (Fig. 5B). We then tested the effect of necrosis induced by several cycles of freezing and thawing (26), and found IL-33 was released by necrotic cells and its levels in the supernatant increased with the number of freezing-thawing cycles (Fig. 5C). Finally, full-length IL-33 and HMGB1 were also detected in the medium of endothelial cells treated with non-ionic detergents Nonidet P-40 and Triton X-100, which provoke damage to the cell membrane (Fig. 5D). Together, these data demonstrate that IL-33, like HMGB1, can be released in the extracellular space after endothelial cell damage or mechanical injury.

Discussion

In the present study, we demonstrate that IL-33 does not require maturation for binding and activation of the IL-33 receptor ST2, and that, contrary to the current belief, processing by caspase-1 results in IL-33 inactivation, rather than activation. Indeed, we unexpectedly discovered that cleavage of IL-33 by caspase-1 does
not occur at the site initially proposed (Ser111), but rather at a site located in the middle of the IL-1-like domain (DGVDVGD176G).

To the best of our knowledge, IL-33 is the first member of the IL-1 family shown to be inactivated after maturation by caspase-1. IL-33 thus differs from IL-1β and IL-18, which require maturation by caspase-1 for liberation of their mature biologically active forms (1, 2); from IL-1 family member IL-1F7, which requires caspase-1 processing for translocation to the nucleus (34); and from IL-1α, which is not a substrate for caspase-1 but is cleaved by calpain to release a C-terminal 17 kDa form with biological activity (1). IL-33 is therefore very unique in the IL-1 family, in terms of processing and biologically active domains. As our data convincingly demonstrate caspase-1 cleaves IL-33 at position D178 but not at position S111, and no non-specific band. (C) Higher amounts of IL-33 and HMGB1 were released in the supernatants after endothelial cell damage induced by repeated cycles of freezing and thawing. (D) IL-33 and HMGB1 were also released in the supernatants after treatment of the endothelial cells for 5 min at 37 °C with non-ionic detergents 0.2% Nonidet P-40 and 0.2% Triton X-100.

Materials and Methods

Plasmid Constructions and Protein Production. IL-33 deletion mutants were amplified by PCR using the human IL-33/NF-HEV cDNA (NM.000576.2) as a template. The PCR fragments thereby obtained were cloned into plasmid pcDNA3.1A/myc-his (Invitrogen). The IL-33D178A mutant was generated by PCR and cloned into the same expression vector. IL-33-Δ270 and IL-33-Δ178 deletion mutant were also cloned into plasmid pcDNA3 (Invitrogen) for use in experiments presented in Figs. 2, 38, and 58. Human IL-1β (NM.000576.2) was amplified by PCR and cloned into vector pcDNA3.1/myc-his. All primer sequences are available upon request. Pro-IL-1β, full-length IL-33-Δ270, IL-33Δ178, and IL-33 deletion mutants were synthesized in vitro in rabbit reticulocyte lysate using the TNT-T7 kit with (fluorescent protein) or without (unlabeled protein) the FluoroTect GreenLys labeling system according to the manufacturer’s instructions (Promega).

In Vitro Caspase Cleavage Assays. In vitro translated fluorescent or unlabeled proteins (5 μg lysate) were incubated with various concentrations of recombinant caspase-1 (Sigma) or caspase-3 (Calbiochem) in 14 μL assay buffer (Calbiochem) for 2 h at 37 °C. The resulting cleavage products were analyzed by SDS/PAGE and fluorography (Typhoon 9400 fluorimeter; GE Healthcare) or Western blot. In some experiments, caspases 1 and 3 were preincubated for 20 min at 37 °C with 100 μM of their respective inhibitors, Ac-YVAD-CHO and Ac-DEVD-CHO (Calbiochem).

Western Blot and Pull-down Assays. Proteins were fractionated by SDS/PAGE, electroblotted, and detected with mAbs to myc-epitope tag (9E10, 1:1,000; Sigma), IL-33 (Nessy-1, 1:1,000; Alexis Biochemicals; 305B, 1:1,000; Alexis Biochemicals), or PARP (1:2,000; BD PharMingen), or rabbit antiserum to IL-33 (Nter (IL-33-Δ178: 1:400) (3, 5), IL-33-Cter (AT-110, no. 210–447, 1:1,000; Alexis Biochemicals), or HMGB1 (Ab18256, 1:200; Abcam), followed by HRP-conjugated goat anti-mouse or anti-rabbit IgG (1:10,000; Promega), and finally an enhanced chemiluminescence kit (GE Healthcare). Pull-down assays with human ST2-Fc chimera protein (1 μg; R&D Systems) were performed as previously described (4), using in vitro translated IL-33 proteins (25 μL lysate) or endothelial cells.
endothelial cell freeze-thaw extracts (3.5 × 10^6 cells) containing endogenous native IL-33.

**Receptor Gene Assays and ELISA.** HEK293T cells (1.5 × 10^5 cells/well in 12-well plates) were transfected with 2 μg pFN-Px8-hrGFP (Stratagene) reporter plasmid and 1 μg of pEF-BOS-hST2 (provided by S. Tominaga, Tochigi, Japan), using a phosphate calcium precipitation method. One day after transfection, cells were stimulated for 16 h with in vitro translated full-length IL-33 or deletion mutants (12.5 μL lysate/well). Cells were then analyzed for GFP expression by fluorescence microscopy (Eclipse TE300 microscope; Nikon) and flow cytometry (FACScan, Cellquest Software; Becton Dickinson). DuoSet IL-6 ELISA assays (R&D Systems) were performed as described (32) using MC/9 mast cells (ATCC; 4 × 10^4 cells/well in 96-well plates) stimulated for 40 h with in vitro translated full-length IL-33 or deletion mutants (10 μL lysate/well).

**Mammalian Cell Culture and Induction of Apoptosis or Cell Damage.** Human HEK293T and U2OS epithelial cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin (all from Invitrogen). Human umbilical vein endothelial cells (Praxcell) were cultured in endothelial cell growth medium (Promocell), supplemented with 20% fetal calf serum (Invitrogen) and heparin (Sigma), and used at confluence in all experiments. Knockdown of IL-33 expression with ON-TARGET plus SMARTpool siRNA duplexes (Dharmacon) was performed as described (22). For induction of apoptosis, cells were incubated with 1 μM staurosporine (Sigma) for 3 h (endothelial cells) or 2 μM doxorubicin (Sigma) for 24 h (U2OS cells), with or without pretreatment with 50 μM pan-caspase inhibitor Z-VAD-fmk (Calbiochem) for 16 h (endothelial cells) or 24 h (U2OS cells). In other experiments, cell damage was induced by repeated cycles of freezing and thawing (26), mechanical scraping from the substratum (33), or mechanical wounding of monolayers by tracing lines with a surgical scalpel. Supernatants were prepared from treated and untreated cells in Opti-MEM serum-free media (Invitrogen), by spinning the cells or cell lysates at 16000 g for 5 min. In some cases, supernatants were concentrated by TCA or filtration using Vivaspin columns (Sartorius).

**ACKNOWLEDGMENTS.** We thank Drs. Tominaga and Yanagisawa (Tochigi, Japan) for the gift of hST2 expression vector. This work was supported by grants from the Commission Nationale pour le Cancer (Equipe labellisée Ligue 2009), ANR-Program Blanc “Cuboidale,” and MAIN European Network of Excellence (FP6-502935).

11:483–486.
23. Borchardt C, et al. (2004) The precursor form of IL-1alpha is an intracellular proinflamma-
27. Chen CJ, et al. (2007) Identification of a key pathway required for the sterile inflam-
Fig. S1. Reactivity of IL-33 antibodies with the different parts of the protein. The IL-33 mAb Nessy-1 recognizes the C-terminal part of IL-33 (aa 179–270) whereas the IL-33 mAb 305B recognizes the middle part of IL-33 (aa 112–178) and the IL-33 Nter polyclonal antiserum recognizes the IL-33 N terminus (aa 1–15). IL-33 deletion mutants containing a myc-epitope tag at their C terminus were generated by in vitro translation and analyzed by Western blot with anti-myc, IL-33 Nter, Nessy-1, or 305B antibodies. RRL, un-programmed rabbit reticulocyte lysate.
Fig. S2. The IL-33D178A mutant protein localizes to the nucleus similarly to WT IL-33. IL33 (1–270) and IL-33D178A proteins were ectopically expressed in human epithelial cell lines U2OS (A) and HEK293T (B), and their subcellular localization was analyzed by immunofluorescence staining with IL-33 mAb 3058. DNA was counterstained with DAPI.