Immunity
Article

Suppression of Interleukin-33 Bioactivity through Proteolysis by Apoptotic Caspases

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DOI 10.1016/j.immuni.2009.05.007

SUMMARY

Interleukin-33 (IL-33) is a member of the IL-1 family and is involved in polarization of T cells toward a T helper 2 (Th2) cell phenotype. IL-33 is thought to be activated via caspase-1-dependent proteolysis, similar to the proinflammatory cytokines IL-1β and IL-18, but this remains unproven. Here we showed that IL-33 was processed by caspases activated during apoptosis (caspase-3 and -7) but was not a physiological substrate for caspases associated with inflammation (caspase-1, -4, and -5). Furthermore, caspase-dependent processing of IL-33 was not required for ST2 receptor binding or ST2-dependent activation of the NF-κB transcription factor. Indeed, caspase-dependent proteolysis of IL-33 dramatically attenuated IL-33 bioactivity in vitro and in vivo. These data suggest that IL-33 does not require proteolysis for activation, but rather, that IL-33 bioactivity is diminished through caspase-dependent proteolysis within apoptotic cells. Thus, caspase-mediated proteolysis acts as a switch to dampen the proinflammatory properties of IL-33.

INTRODUCTION

Caspases are highly specific proteases that have been implicated in apoptosis and inflammation (Creagh et al., 2003; Martinon and Tschopp, 2004). Caspase-1, -4, and -5 are activated in response to pathogen products, such as lipopolysaccharide (LPS), that signal via members of the Toll-like receptor (TLR) and Nod-like receptor families (Martinon and Tschopp, 2004). Caspase-1 plays a critical role in the innate immune response to infectious agents through proteolytic processing of pro-interleukin-1β (pro-IL-1β) and pro-IL-18 to their mature forms (reviewed in Creagh et al. [2003]).

Recently, caspase-1 has also been implicated in the proteolytic maturation of the IL-1 family cytokine, IL-33 (also known as IL-1F11) (Schmitz et al., 2005). IL-33 is a ligand for the IL-1R family member ST2 (T1), which has previously been linked with maturation of T helper 2 (Th2) cells and negative regulation of IL-1R and TLR4 signaling (Xu et al., 1998; Meisel et al., 2001; Brint et al., 2004). Antagonistic antibodies against ST2 or IgG-ST2 fusion proteins lead to enhancement of Th1 cell responses and attenuation of Th2 cell-associated effects (Lohning et al., 1998; Xu et al., 1998).

Relatively little is known concerning the factors that stimulate IL-33 production and secretion. An artificially truncated form of this cytokine enhances production of Th2 cell cytokines from in vitro-polarized Th2 cells and suppresses Th1 cell cytokine production (Schmitz et al., 2005). Administration of the same truncated form of IL-33 in vivo induces expression of IL-4, IL-5, and IL-13 and also leads to eosinophilia, splenomegaly, and increased amounts of serum immunoglobulin E (IgE) and IgA (Schmitz et al., 2005; Chackerian et al., 2007). IL-33 is also a potent activator of eosinophils, basophils, and mast cells and can promote in vitro maturation of the latter from bone marrow precursors (Allakhverdi et al., 2007; Ali et al., 2007; Pecaric-Petkovic et al., 2009).

The role of caspase-1, or other inflammatory caspases, in the maturation of IL-33 remains enigmatic. High concentrations of caspase-1 are reported to cleave IL-33 in vitro, and this is proposed as a mechanism of activation of this cytokine, similar to IL-1β (Schmitz et al., 2005). However, the functional consequences of caspase-mediated proteolysis of IL-33 are not known because the activity of the full-length cytokine has not been investigated. Nor is it known whether IL-33 is cleaved by caspase-1 at physiological concentrations.

Here, we have examined the role of caspase-1, -4, and -5 in the maturation of IL-33. Surprisingly, we found little evidence that IL-33 is a physiological substrate for the inflammatory caspases. Rather, we showed that this cytokine was efficiently processed by caspases-3 and -7, proteases that are selectively activated during apoptosis. Furthermore, caspase-mediated proteolysis of IL-33 dramatically attenuated IL-33 biological activity in vitro and in vivo. Mutation of a single amino acid located within the caspase cleavage site of IL-33 also eliminated the biological activity of this cytokine, suggesting that this region

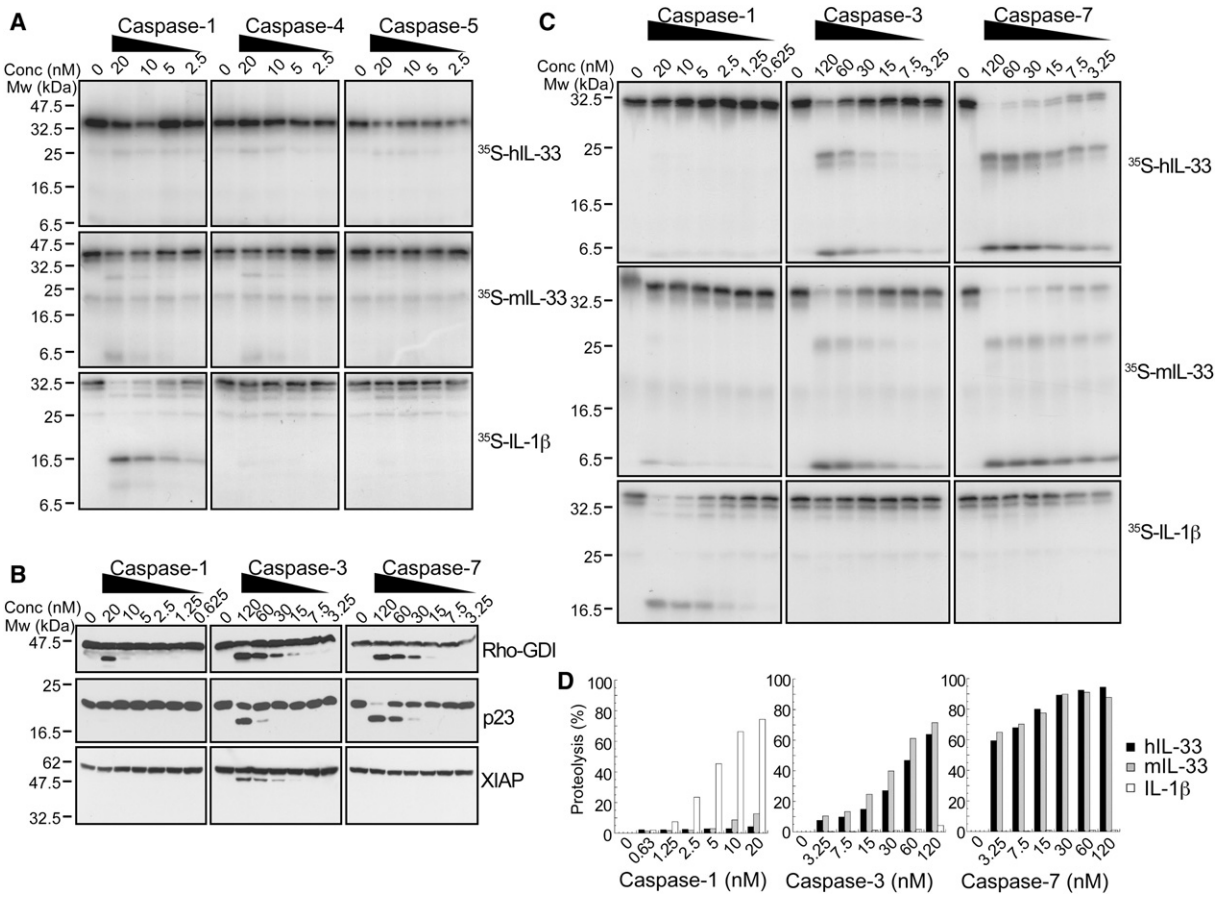


Figure 1. Processing of IL-33 by Apoptotic but Not Inflammatory Caspases

(A) ^{35}S-labeled hIL-33, mIL-33, and IL-1β were prepared by in vitro transcription and translation and were then incubated with the indicated concentrations of recombinant caspase-1, -4, and -5 for 2 hr at 37°C followed by analysis by SDS-PAGE and fluorography.

(B) Recombinant caspase-1, -3, and -7 were added to Jurkat cell-free extracts, at the indicated concentrations, followed by incubation at 37°C for 2 hr. Extracts were then analyzed by SDS-PAGE followed by immunoblotting for the indicated substrate proteins.

(C) ^{35}S-labeled hIL-33, mIL-33, and IL-1β, prepared by in vitro transcription and translation, were incubated with the indicated concentrations of recombinant caspase-1, -3, and -7 for 2 hr at 37°C followed by analysis by SDS-PAGE and fluorography.

(D) Densitometric analysis of the gels presented in (C). Scanned gels were analyzed with ImageJ software, and results are expressed as % proteolysis of the full-length forms of each protein relative to the untreated control.

Panels (A)–(D) represent data from at least three independent experiments.

is critical for IL-33 activity. Thus, IL-33 is preferentially processed by caspases activated during apoptosis rather than inflammation and this serves to reduce, rather than enhance, IL-33 activity in vivo.

RESULTS

IL-33 Is a Poor Substrate for Caspase-1

To explore whether IL-33 is a physiological substrate for caspases activated during inflammation, we incubated in vitro-transcribed and -translated human and mouse IL-33 in the presence of physiologically relevant amounts of caspase-1, -4, and -5 (Figure 1A). We used nonsaturating concentrations of caspase-1 that achieved robust proteolysis of the known caspase-1 substrate, IL-1β, and equimolar amounts of caspase-4 and -5. All caspases were active as indicated by hydrolysis of the synthetic peptide substrate WEHD-AMC (Figure S1 available online). However, although caspase-1 robustly cleaved IL-1β, IL-33 was not readily processed under the same conditions (Figure 1A). Caspase-4 and -5 also failed to process IL-33, suggesting that, in comparison with IL-1β, IL-33 is a poor substrate for the inflammatory caspases.

IL-33 Is a Substrate for Caspases Activated during Apoptosis

We next explored whether IL-33 could be cleaved by caspases that are activated during apoptosis rather than inflammation. Caspase-3 and -7 act as the major effector caspases within the cell-death machinery (Walsh et al., 2008); therefore, we used concentrations of caspase-3 and -7 that achieved robust, but incomplete, proteolysis of their known substrates, Rho GDP dissociation inhibitor (RhoGDI), cochaperone p23, and X-linked inhibitor of apoptosis protein (XIAP) (Figure 1B). These concentrations were chosen to avoid the use of saturating, nonphysiological amounts of the latter proteases.

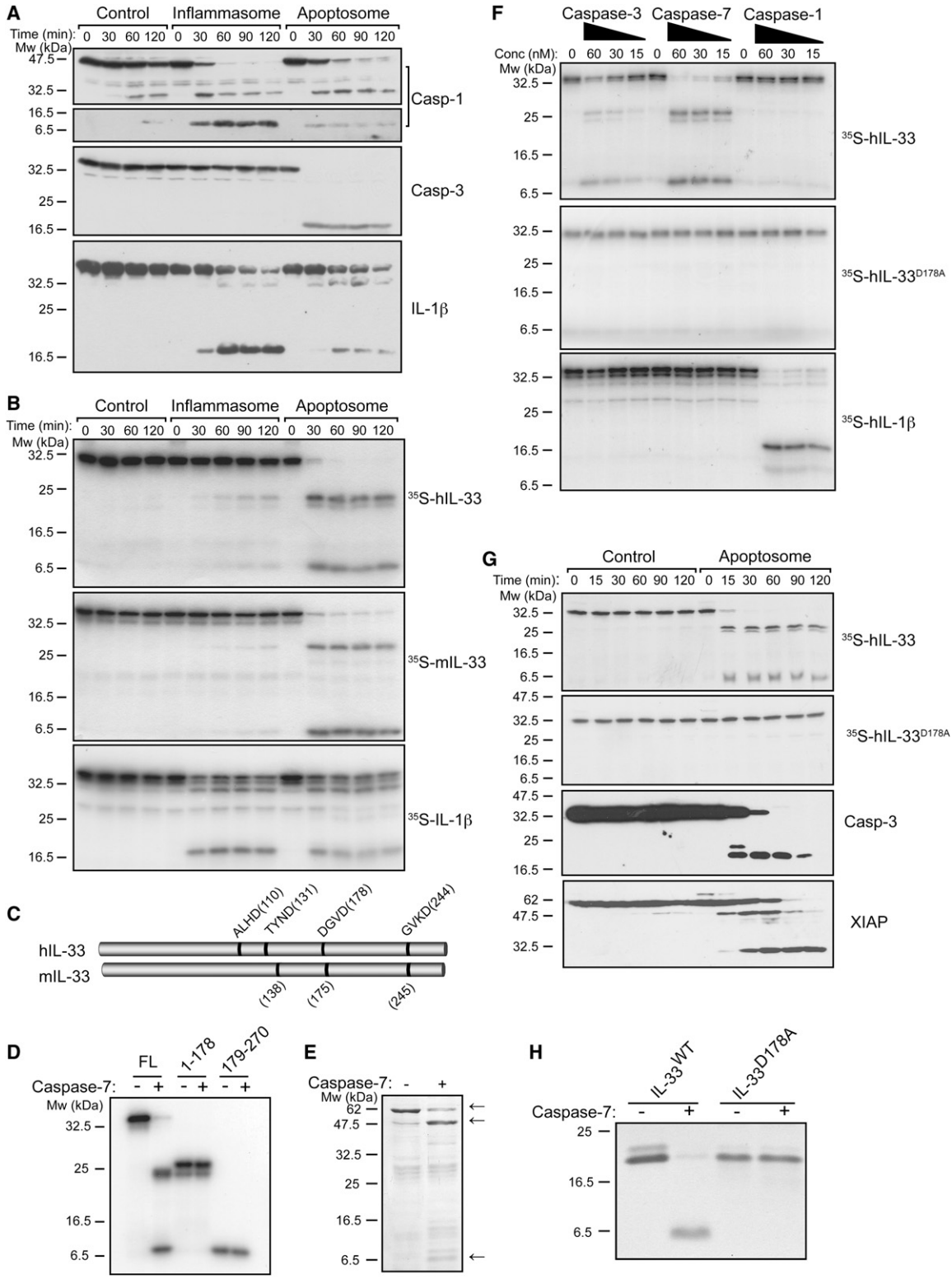


Figure 2. Proteolysis of IL-33 at Asp178 Occurs under Conditions of Apoptotic but Not Inflammatory Caspase Activation

(A) Cell-free extracts derived from THP-1 cells were incubated at 37°C to permit spontaneous activation of inflammatory caspases (Inflammasome) or in the presence of 50 μg/ml cytochrome c and 1 mM dATP to promote activation of apoptotic caspases (Apoptosome). As a control, caspase activation was suppressed through addition of 5 μM YVAD-CHO. Extracts were then immunoblotted for caspase-1, caspase-3, and IL-1β, as indicated. Representative blots are shown from at least three independent experiments.

As Figures 1C and 1D illustrate, caspase-3 and -7 readily processed human and murine IL-33, with caspase-7 being more efficient in this regard. Importantly, caspase-3 and -7 failed to cleave IL-1β under the same conditions (Figure 1C). Whereas robust IL-33 processing was observed at low concentrations (3–7 nM) of caspase-7, caspase-1 failed to cleave IL-33 even at several-fold-higher concentrations. Once again, caspase-1 readily processed IL-1β under conditions where it failed to process IL-33 to any substantial degree (Figures 1A and 1C). These data argue that IL-33 is preferentially cleaved by caspases that are activated during apoptosis as opposed to inflammation.

Proteolysis of IL-33 in Apoptotic Cell-Free Extracts

To explore IL-33 processing further, we used a cell-free system based upon cytosolic extracts derived from LPS-treated monocytic THP-1 cells, where caspase-1, -4, and -5 can be activated by incubating these extracts at 37°C (Yamin et al., 1996; Martinon et al., 2002). Upon incubation of THP-1 cell-free extracts at 37°C, caspase-1 was processed to its active form and maturation of endogenous IL-1β was readily detected (Figure 2A). As expected, caspase-3 was not activated under these conditions, as indicated by the failure of this protease to undergo proteolytic processing (Figure 2A). In sharp contrast to the robust processing of IL-1β seen under these conditions, processing of human or mouse IL-33 was barely detectable (Figure 2B), again suggesting that IL-33 is a poor substrate for caspase-1 and other caspases activated under inflammatory conditions.

With the same THP-1 cell-free system, caspase-3 and -7 can be activated by addition of cytochrome c and deoxyadenosine triphosphate (dATP) to the extracts, because the latter act as cofactors for assembly of the Apaf-1-caspase-9 apoptosome, which activates these caspases downstream (reviewed in Creagh et al., 2003). Under these conditions, caspase-1 activation was attenuated and IL-1β proteolysis was much less efficient, whereas caspase-3 was robustly activated (Figure 2A). In contrast to the lack of processing of IL-33 under conditions where caspase-1 was clearly activated, IL-33 was processed very efficiently upon activation of caspase-3 and -7 through addition of cytochrome c and dATP (Figure 2B), again arguing that IL-33 is preferentially cleaved by caspases activated during apoptosis but not inflammation.

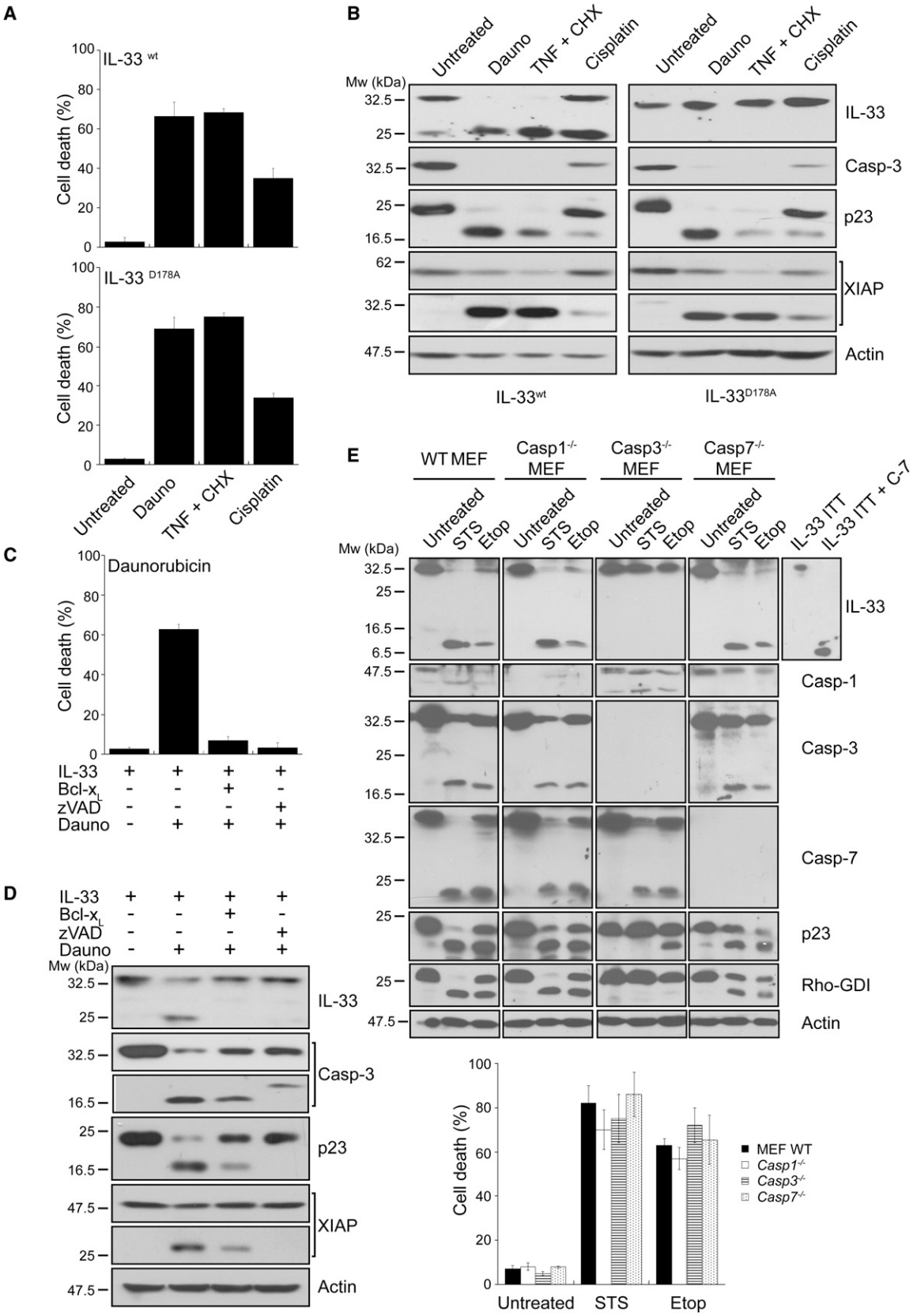
We also used a cell-free system based upon cytosolic extracts of Jurkat cells, which are essentially devoid of caspase-1 (Chow et al., 1999). Addition of cytochrome c and dATP to Jurkat extracts resulted in rapid activation of caspase-3 and -7 and proteolytic processing of known caspase substrates, such as XIAP and cochaperone p23 (Figure S2A). Proteolysis of human and murine IL-33 was again readily observed under these conditions (Figure S2B). Taken together with our earlier observations made with recombinant caspases (Figure 1), these results strongly suggest that IL-33 is a physiological substrate for caspases activated during apoptosis but is cleaved very inefficiently at physiologically relevant concentrations of caspase-1.

IL-33 Is Cleaved at a Single Conserved Site

It has been proposed that human IL-33 is cleaved by caspase-1 at Asp110 and that this represents the biologically active form of this cytokine (Schmitz et al., 2005). However, this site is not conserved between the human and murine forms of IL-33, making it highly unlikely that IL-33 is processed at this residue (Figure 2C). To identify the caspase-processing site within IL-33, we inspected the human and mouse IL-33 sequences for conserved tetrapeptide motifs containing Aspartate (Asp) residues that may qualify as caspase cleavage motifs. On the basis of the approximate molecular weights of the caspase-mediated cleavage products of IL-33 observed in our initial experiments (Figures 1C and 2B), a conserved caspase cleavage motif that was more likely to represent the site of caspase-mediated proteolysis was located at Asp178 within human IL-33 (^{175}DGVD^{178}) and Asp175 within murine IL-33 (^{172}DGVD^{175}) (Figure 2C). Notably, this site also conforms to a consensus caspase-3 and -7 DXXD cleavage motif, rather than the WI/V/LXD motif preferred by caspase-1.

We therefore expressed truncations of human IL-33 corresponding to the putative cleavage products generated through processing at Asp178. As can be seen from Figure 2D, these truncated IL-33 proteins displayed precisely the same SDS-PAGE mobilities as the caspase-7-cleaved form of IL-33. Furthermore, the truncated IL-33 mutants failed to be further processed by caspase-7 (Figure 2D), strongly suggesting that human IL-33 is processed at Asp178 and not Asp110, as previously claimed. We also expressed recombinant full-length GST-IL-33 in bacteria and cleaved this protein with caspase-7 (Figure 2E). The resulting fragments were then analyzed with MALDI-TOF mass spectrometry, and the peptide coverage of these fragments strongly indicated that the caspase cleavage site was located between amino acids 159 and 187 (Figure S3),

(B) ^{35}S-labeled hIL-33, mIL-33, and IL-1β were added to THP-1 cell-free extracts followed by treatment as described in (A). Reactions were sampled at the indicated times and were subsequently analyzed by SDS-PAGE and fluorography. Representative gels are shown from at least two independent experiments.
(C) Schematic representation of human and murine IL-33 depicting potential caspase cleavage motifs. Note that the proposed site of caspase-1-mediated proteolysis (ALHD^{110}; Schmitz et al., 2005) is not conserved between human and mouse IL-33.
(D) ^{35}S-labeled full-length (FL) hIL-33 and the indicated IL-33 deletion mutants were incubated in the presence of recombinant caspase-7 (40 nM) for 2 hr at 37°C followed by analysis by SDS-PAGE and fluorography. Representative gels are shown from at least two independent experiments.
(E) Recombinant GST-IL-33 was incubated for 2 hr at 37°C in the presence or absence of recombinant caspase-7 (600 nM), as indicated, followed by SDS-PAGE and Coomassie blue staining. Representative gels are shown from at least two independent experiments.
(F) ^{35}S-labeled wild-type hIL-33 and IL-33^{D178A} point mutant were incubated for 2 hr at 37°C with recombinant caspase-3, -7, and -1, as shown. Reactions were analyzed by SDS-PAGE and fluorography. Representative gels are shown from at least two independent experiments.
(G) ^{35}S-labeled wild-type hIL-33 and IL-33^{D178A} point mutant were added to Jurkat cell-free extracts followed by activation of apoptotic caspases by addition of cytochrome c and dATP. Reactions were sampled at the indicated times and were subsequently analyzed by SDS-PAGE and fluorography. Samples of the same reactions were also immunoblotted for caspase-3 and XIAP, as indicated. Representative gels and blots are shown from at least two independent experiments.
(H) Recombinant IL-33$^{112\text{-}270}$ and IL-33$^{112\text{-}270}$ D178A point mutant were incubated with recombinant caspase-7 (600 nM) for 4 hr at 37°C followed by analysis by SDS-PAGE and Coomassie blue staining. Representative gels are shown from at least three independent experiments.


A
IL-33 wt
IL-33 D178A
Cell death (%)
Untreated
Dauno
TNF + CHX
Cisplatin
B
Mw (kDa)
IL-33
Casp-3
p23
XIAP
Actin
IL-33wt
IL-33D178A
C
Daunorubicin
IL-33
Bcl-xL
zVAD
Dauno
D
E
WT MEF
Casp1-/- MEF
Casp3-/- MEF
Casp7-/- MEF
STS
Etop
IL-33 ITT
IL-33 ITT + C-7
Casp-1
Casp-7
Rho-GDI
MEF WT
Casp1-/-
Casp3-/-
Casp7-/-

which encompassed the conserved DGVD$^{175/178}$ motif discussed above. We then generated point mutations in human and murine IL-33 corresponding to the putative caspase cleavage site (Asp178 in human and Asp175 in mouse), and these mutants were completely resistant to processing by any of the caspases examined (Figure 2F and data not shown). Furthermore, this point mutant was also completely protected from proteolysis in apoptotic Jurkat cell-free extracts under conditions where wild-type IL-33 was completely cleaved (Figure 2G).

On the basis of the initial observations by Schmitz et al. (2005), all investigations carried out to date with IL-33 have used an artificially truncated form of this cytokine, IL-33$^{112\text{-}270}$, that is proposed to represent the caspase-cleaved form of this protein. However, our experiments indicate that this form of IL-33 would still contain the actual caspase cleavage site and therefore be susceptible to caspase-mediated proteolysis. To confirm this, we also generated the artificially truncated form of IL-33 (amino acids 112–270) as well as the D178A mutant form of this truncation. As Figure 2H shows, IL-33$^{112\text{-}270}$ was cleaved by caspase-7, whereas the IL-33$^{112\text{-}270}$ D178A mutant was completely resistant to proteolysis.

These data demonstrate that IL-33 is cleaved by caspase-3 and -7 within a conserved motif at Asp178 in the human form of this cytokine (Asp175 in the mouse). This has important implications, given that all previous studies on IL-33 have exclusively used a truncated form of this protein based on a predicted caspase cleavage site (at Asp110) that has failed to be verified by our investigations and is not conserved between human and mouse IL-33.

IL-33 Is Cleaved during Apoptosis

To confirm that IL-33 is cleaved during apoptosis in a cellular context, we transiently overexpressed FLAG-tagged IL-33 in human HeLa cells and induced these cells to die by exposure to a panel of proapoptotic stimuli, including Daunorubicin, tumor necrosis factor (TNF), and Cisplatin (Figure 3A). Robust processing of IL-33 was observed under conditions where apoptosis was initiated, but importantly, the IL-33^{D178A} point mutant was not cleaved under the same conditions (Figure 3B). Furthermore, inhibition of caspase activation or activity in HeLa cells, through overexpression of Bcl-xL or by inclusion of a polycaspase inhibitor (z-VAD-fmk) in the medium, also blocked apoptosis-associated proteolysis of IL-33 (Figures 3C and 3D). Thus, IL-33 is cleaved during apoptosis, and this occurs at the same site (Asp178) of caspase-mediated processing of IL-33 in vitro.

We also asked whether murine embryonic fibroblasts (MEFs) lacking *Casp1*, *Casp3*, or *Casp7* processed IL-33 during apoptosis. As Figure 3E shows, whereas IL-33 was readily processed upon induction of apoptosis in wild-type MEFs, *Casp1*$^{-/-}$ MEFs still retained the ability to process this cytokine, underscoring our earlier observations that caspase-1 is unlikely to cleave IL-33. In sharp contrast, processing of IL-33 was completely attenuated in *Casp3*$^{-/-}$ MEFs under the same conditions (Figure 3E). *Casp7*$^{-/-}$ MEFs exhibited processing of IL-33 similar to wild-type cells (Figure 3E), most likely because caspase-3 and -7 exhibit functional redundancy with respect to many caspase substrates, although this is unidirectional in many contexts because caspase-3 is a more abundant enzyme in certain cell types (Walsh et al., 2008).

Endogenous IL-33 is Preferentially Released during Necrosis

To ask whether endogenous IL-33 behaved similarly to the overexpressed cytokine, we initially explored conditions for induction of IL-33 protein expression in THP-1 cells, because previous studies have not addressed this issue. As shown in Figures 4A and 4B, THP-1 cells did not express IL-33 constitutively but were induced to do so upon stimulation with LPS and PMA, similar to IL-1β. However, in contrast to IL-1β, IL-33 remained completely cell associated and was not detected in medium from LPS- and PMA-stimulated cells (Figure 4C). Furthermore, the form of IL-33 that was detected under these conditions was the full-length form of this cytokine (Figure 4B).

We also immunoprecipitated endogenous IL-33 from LPS- and PMA-treated THP-1 cells to explore whether any of this cytokine could be found in a cleaved form, but failed to detect any cleaved IL-33 under these conditions (Figure 4D). However, upon induction of apoptosis in THP-1 cells via cytotoxic drugs, endogenous IL-33 was processed to a species that ran at an identical mobility to the caspase-3- and caspase-7-cleaved form of IL-33 (Figure 4E). Similar results were also obtained when apoptosis was induced by the physiological death-receptor ligands TNF, anti-Fas, or TRAIL (Figure S4). We also

Figure 3. IL-33 Is Cleaved during Apoptosis

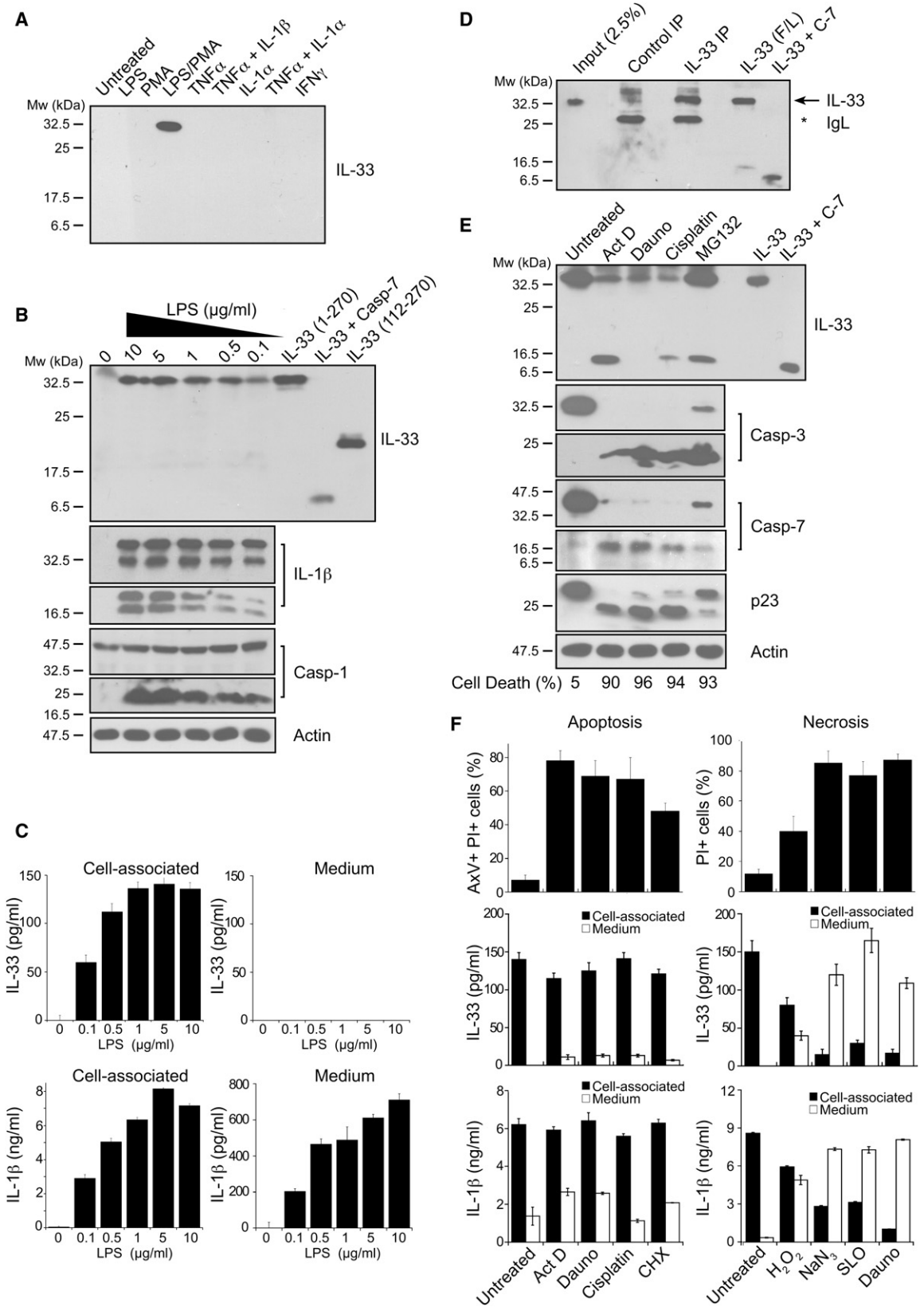
(A) HeLa cells were transfected with expression plasmids encoding either wild-type IL-33 (top panel) or IL-33^{D178A} point mutant (bottom panel). Twenty-four hours later, cells were then treated with Daunorubicin (Dauno; 5 μM), TNF (10 ng/ml), cycloheximide (CHX; 1 μM), and cisplatin (50 μM) and incubated for a further 8 hr before assessment of apoptosis. Results are representative of at least three independent experiments. Error bars represent the mean ± the standard error of the mean (SEM).

(B) Immunoblot analysis of IL-33, caspase-3, cochaperone p23, XIAP, and actin in cell lysates derived from HeLa cells transfected with either wild-type IL-33 (left panel) or the D178A point mutant (right panel), followed by incubation in the presence or absence of Daunorubicin (Dauno), TNF and cycloheximide, or Cisplatin at concentrations indicated in (A). Results are representative of at least three independent experiments.

(C) HeLa cells were transfected with an IL-33 expression plasmid for 24 hr followed by treatment for 8 hr with Daunorubicin (5 μM) to induce apoptosis. In parallel, HeLa cells were also treated with the polycaspase inhibitor Z-VAD-fmk (50 μM) or were transfected with a Bcl-xL expression plasmid as indicated. Results are representative of at least three independent experiments. Error bars represent the mean ± the SEM.

(D) Cell lysates were generated from the cells treated in (C) and were immunoblotted for the indicated proteins. Results are representative of at least three independent experiments.

(E) Murine embryonic fibroblasts from wild-type (WT), *Casp1*$^{-/-}$, *Casp3*$^{-/-}$, or *Casp7*$^{-/-}$ mice were transfected with an expression plasmid encoding hIL-33, followed by treatment for 24 hr with MG132 (0.2-0.4 μM) to facilitate IL-33 stabilization. Cells then either were left untreated or were treated for 24–30 hr with Staurosporine (40–80 μM) or Etoposide (0.5–1 mM) in order to achieve equivalent levels of cell death, followed by preparation of cell lysates and immunoblotting for the indicated proteins. The % of apoptotic cells in each condition was assessed by direct cell counts on a minimum of 300 cells per treatment with standard morphological criteria (bottom panel). Results are representative of at least three independent experiments. Error bars represent the mean ± the SEM.


A
Untreated
LPS
PMA
LPS/PMA
TNFα
TNFα + IL-1β
IL-1α
TNFα + IL-1α
IFNγ
Mw (kDa)
32.5
25
17.5
6.5
IL-33
B
LPS (μg/ml)
0
10
5
1
0.5
0.1
IL-33 (1-270)
IL-33 + Casp-7
IL-33 (112-270)
Mw (kDa)
32.5
25
17.5
6.5
IL-33
32.5
16.5
IL-1β
47.5
32.5
25
16.5
Casp-1
47.5
Actin
C
Cell-associated
Medium
IL-33 (pg/ml)
IL-1β (ng/ml)
IL-1β (pg/ml)
0 0.1 0.5 1 5 10
LPS (μg/ml)
D
Input (2.5%)
Control IP
IL-33 IP
IL-33 (F/L)
IL-33 + C-7
Mw (kDa)
32.5
25
16.5
6.5
IL-33
IgL
E
Untreated
Act D
Dauno
Cisplatin
MG132
IL-33
IL-33 + C-7
Mw (kDa)
32.5
25
16.5
6.5
IL-33
32.5
25
Casp-3
47.5
32.5
16.5
6.5
Casp-7
25
p23
47.5
Actin
Cell Death (%) 5 90 96 94 93
F
Apoptosis
Necrosis
AxV+ PI+ cells (%)
PI+ cells (%)
Cell-associated
Medium
IL-33 (pg/ml)
IL-1β (ng/ml)
Untreated
Act D
Dauno
Cisplatin
CHX
Untreated
H2O2
NaN3
SLO
Dauno

asked whether IL-33 was secreted during apoptosis, but again found that the majority of protein remained cell associated (Figure 4F, left panels). In contrast, upon induction of necrosis via hydrogen peroxide, sodium azide, streptolysin O, or a high concentration of daunorubicin, IL-33 was now readily detected in medium (Figure 4F, right panels).

We also performed similar experiments with primary bone marrow-derived mouse dendritic cells. Similar to what was observed in human THP-1 cells, IL-33 was upregulated upon LPS and PMA treatment of murine DCs but was not proteolytically processed or secreted under these conditions (Figures S5A and S5B). IL-1β was also induced upon LPS and PMA treatment of DCs but, in contrast to IL-33, was secreted under these conditions (Figure S5B). Upon triggering of apoptosis in these cells, IL-33 was once again processed to a fragment consistent with caspase-3- and caspase-7-dependent processing of this cytokine (Figure S5C).

Collectively, the above data indicate that IL-33 is processed in a caspase-dependent manner during apoptosis, but not in response to a proinflammatory stimulus (LPS) associated with caspase-1 activation. Furthermore, IL-33 appears to be preferentially released during necrosis, with relatively minor amounts being released from apoptotic cells.

IL-33 Does Not Require Proteolytic Processing for Activity

Certain members of the IL-1 family, such as IL-1β, undergo restricted proteolysis to convert their inactive precursors into the active cytokine (Mosley et al., 1987). However, other cytokines in this family, such IL-1α, display biological activity irrespective of whether they are proteolytically processed or not (Mosley et al., 1987). Because all previous studies on IL-33 have used an artificially truncated form of this cytokine (IL-33$^{112\text{-}270}$) that does not represent either the full-length or the bona fide caspase-cleaved form of IL-33, it is therefore not clear how proteolysis modulates the activity of this cytokine, given that the biological activity of full-length IL-33 has not been assessed.

To explore the impact of caspase-mediated proteolysis on the biological activity of IL-33, we generated recombinant full-length GST-IL-33 and incubated this protein with caspase-7 to generate cleaved IL-33 protein (see Figure 2E). Note that a GST-fusion protein was used due to the extreme insolubility of full-length untagged IL-33 when expressed in bacterial or yeast expression systems (data not shown). We then compared the ability of full-length GST-IL-33, versus the caspase-cleaved form of this protein, to promote NF-κB activation in an ST2-receptor-dependent manner. For this purpose, we used HEK293T cells transfected with the ST2 receptor along with a NF-κB-responsive promoter. As Figure 5A illustrates, whereas we detected NF-κB activation in response to the full-length IL-33 protein, the activity of the caspase-cleaved form of this protein was dramatically attenuated. Similar results were also observed with the artificially truncated form of IL-33 (amino acids 112–270), which also exhibited reduced activity upon caspase-mediated proteolysis (Figure 5A, bottom panel). These data suggest, in direct opposition to the prevailing view, that caspase-mediated proteolysis of IL-33 results in a decrease rather than an increase in the activity of this cytokine. Moreover, our data also suggest that full-length IL-33 is biologically active and does not require proteolytic processing for acquisition of ST2-dependent receptor activation.

We also compared the activity of full-length GST-IL-33 with the artificially truncated version of this protein (amino acids 112–270; Figure 5B) that is currently used by most laboratories as "mature" IL-33. As Figure 5C shows, IL-33$^{112\text{-}270}$ had comparable activity to full-length GST-IL-33 in the ST2-dependent NF-κB reporter assay. However, as we have shown above, this truncated form of IL-33 is not the form that would be produced through caspase-dependent proteolysis. Therefore, we also generated recombinant forms of IL-33 equivalent to the

Figure 4. Endogenous IL-33 Is Upregulated in Response to LPS and Is Selectively Released during Necrosis

(A) THP-1 cells either were left untreated or were treated with LPS (5 μg/ml), PMA (50 nM), TNFα (50 ng/ml), IL-1α (50 ng/ml), IL-1β (50 ng/ml), IFNγ (250 ng/ml), or combinations of these agents, as indicated. Twenty-four hours later, lysates were generated and immunoblotted for IL-33.

(B) THP-1 cells were treated with PMA (50 nM) together with the indicated concentrations of LPS. Twenty-four hours later, lysates were generated and immunoblotted for the indicated proteins. As controls, in vitro-transcribed and -translated full-length IL-33, caspase-7-cleaved full-length IL-33, and artificially truncated IL-33$^{112\text{-}270}$ were included to facilitate size comparison.

(C) THP-1 cells were treated as in (B); then, 24 hr later, cells and medium were harvested separately and cells were lysed in cell lysis buffer (see Experimental Procedures) to facilitate measurement of cell-associated cytokines. Cell-associated and -secreted IL-33 and IL-1β concentrations were then assessed by ELISA. Results are representative of at least three independent experiments. Error bars represent the mean ± the SEM.

(D) Immunoprecipitation of endogenous IL-33. THP-1 cells (10^8) were treated with LPS (5 μg/ml) and PMA (50 nM) for 24 hr, followed by treatment with the proteasome inhibitor MG132 (5 μM) for a further 24 hr to stabilize endogenous IL-33. Cells were lysed as in (C), then incubated overnight with Protein A/G agarose-immobilized control or IL-33 antibody. Immune complexes were then immunoblotted for IL-33 as indicated. The arrow indicates full-length IL-33, whereas the asterisk indicates antibody light chain used for the immunoprecipitation. As controls, in vitro-transcribed and -translated full-length IL-33 and caspase-7-cleaved full-length IL-33 were included to facilitate size comparison. Results are representative of at least two independent experiments.

(E) THP-1 cells were treated with LPS (5 μg/ml) and PMA (50 nM); then, 24 hr later, MG132 (5 μM) was added to stabilize endogenous IL-33. After a further 6 hr, cells were treated with the apoptosis-inducing agents Actinomycin D (Act D; 5 μM), Daunorubicin (Dauno; 10 μM), Cisplatin (500 μM), or MG132 (100 μM) and incubated for a further 36 hr before assessment of apoptosis. The % of apoptotic cells (counts were performed on a minimum of 300 cells per treatment) in each condition is indicated at the bottom of the figure. Cell lysates were immunoblotted for the indicated proteins. Results are representative of at least three independent experiments.

(F) Left panels: THP-1 cells were treated with LPS (5 μg/ml) and PMA (50 nM) for 24 hr. For induction of apoptosis, cells were then treated with Act D (5 μM), Daunorubicin (10 μM), Cisplatin (500 μM), or CHX (250 μM) for a further 12 hr before assessment of apoptosis by annexin V-FITC and propidium iodide (PI) staining and flow cytometry. Cells and medium were harvested separately and cells were lysed as in (C). IL-33 and IL-1β amounts in cell lysates and medium were then assessed by ELISA. Right panels: THP-1 cells were treated with LPS and PMA for 24 hr, as above. For induction of necrosis, cells were then treated for 1 hr with hydrogen peroxide (H_2O_2; 20 mM), sodium azide (NaN_2; 1 M), Streptolysin O (SLO; 10 μg/ml) or a high dose of Daunorubicin (100 μM) followed by assessment of necrosis via PI uptake in conjunction with flow cytometry. Cells and medium were harvested separately and cells were lysed as in (C). IL-33 and IL-1β in the cell lysates and media were assessed by ELISA. Results are representative of at least three independent experiments. Error bars represent the mean ± the SEM.

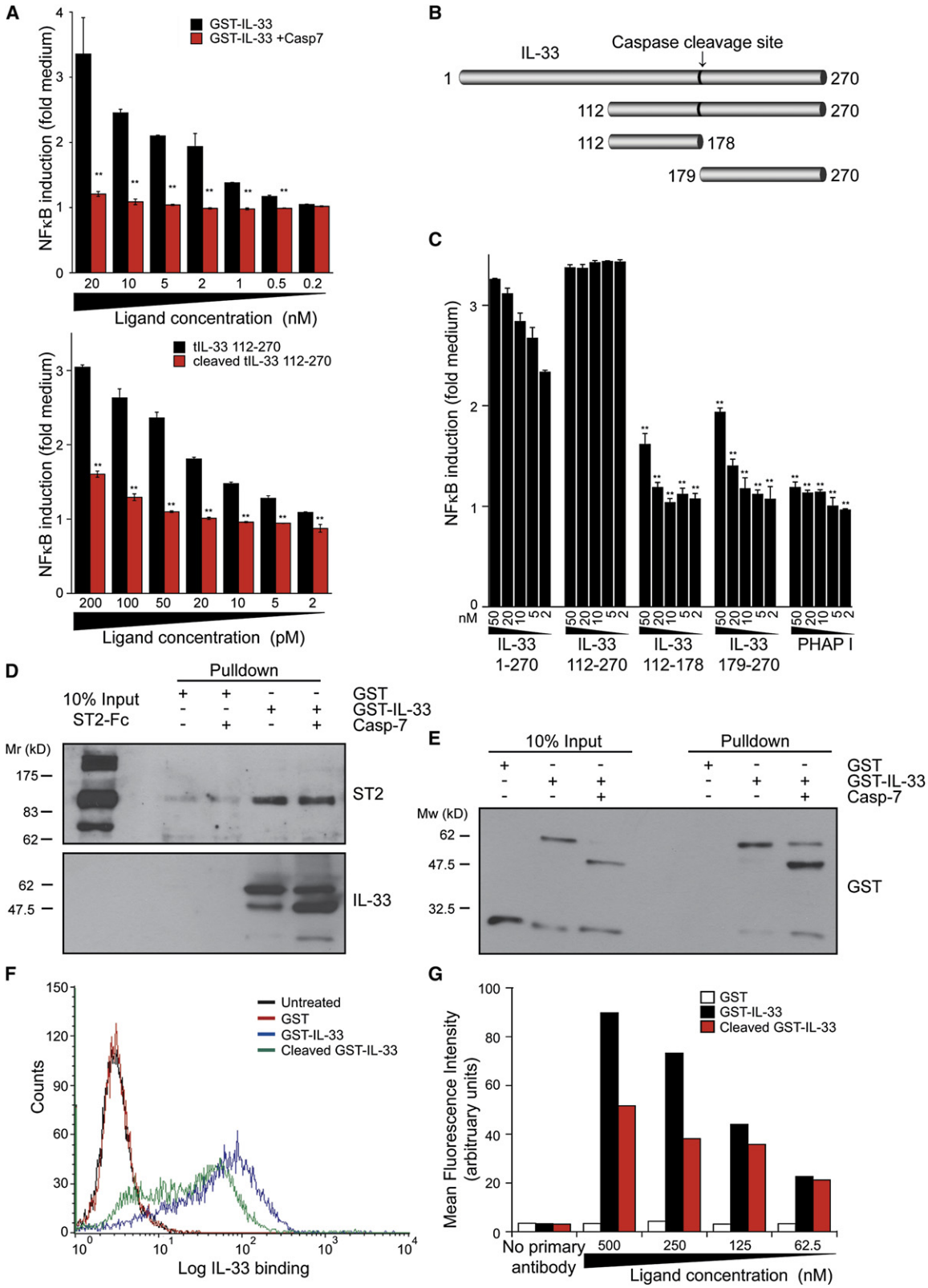


Figure 5. Caspase-Mediated Proteolysis of IL-33 Attenuates the Activity of This Cytokine In Vitro

(A) Upper panel: HEK293T cells were transfected with a ST2L receptor expression plasmid (200 ng per well of a 6-well plate) along with an NFκB luciferase reporter plasmid (10 ng). Twenty-four hours later, the indicated concentrations of full-length or caspase-7-cleaved GST-IL-33 were added for a further 8 hr.

caspase-generated cleavage products (IL-33$^{112-178}$ and IL-33$^{179-270}$; Figure 5B) to ask whether these fragments could promote ST2-dependent NF-κB activation. However, compared to either full-length GST-IL-33 or the artificially truncated IL-33$^{112-270}$, when expressed independently neither fragment was found to be capable of promoting ST2-dependent NF-κB activation (Figure 5C).

Collectively, these data suggest that IL-33 is active as a full-length molecule, or when artificially truncated after amino acid 111, and that caspase-mediated processing is not required for the production of mature IL-33. These observations are reminiscent of the pattern of activity reported for IL-1α because this cytokine displays biological activity both as a precursor and as an N-terminally-truncated protein (Mosley et al., 1987). Thus, the proposal that IL-33 is activated through proteolysis by caspase-1 (Schmitz et al., 2005), similar to IL-1β and IL-18, appears unfounded. Indeed, proteolytic processing of full-length IL-33 by caspases considerably undermined the activity of this cytokine (Figure 5A), possibly through destabilizing the protein and/or by promoting the separation of IL-33 into fragments that are incapable of promoting efficient ST2 receptor stimulation (Figure 5C).

Pro-IL-33 Can Bind to the ST2 Receptor

Because the preceding experiments indicated that pro-IL-33 possessed ST2-dependent biological activity, this suggested that full-length IL-33 was capable of interacting with the ST2 receptor. To confirm this, we performed in vitro pull-down assays where we incubated sepharose-immobilized full-length GST-IL-33, or caspase-cleaved GST-IL-33, with a soluble Fc-ST2 fusion protein to determine whether both forms of IL-33 bound to the ST2 receptor. As Figure 5D shows, both forms of GST-IL-33 specifically captured Fc-ST2 in the assay. We also carried out the reciprocal experiment where we immobilized Fc-ST2 on protein A/G agarose and assessed the binding of soluble full-length GST-IL-33 or the caspase-cleaved form of this protein (Figure 5E). Once again, we observed that both the cleaved and the full-length forms of GST-IL-33 were able to interact with the ST2 receptor.

We also carried out binding studies in ST2-transfected HEK293 cells to compare binding of GST, GST-IL-33, and caspase-7-cleaved GST-IL-33. Dose-dependent binding of full-length GST-IL-33, but not GST, to ST2-transfected cells was readily detected under these conditions. In agreement with the in vitro pull-down experiments (Figures 5D and 5E), the caspase-cleaved form of GST-IL-33 also bound to ST2-transfected cells, but with reduced efficiency (Figures 5F and 5G). Because caspase-processed IL-33 was still capable of interacting with the ST2 receptor, this suggests that the loss of biological activity observed (Figure 5A) was unrelated to loss of receptor binding per se but may be related to reduced binding efficiency and/or other factors. Furthermore, although receptor binding by the cleaved from of IL-33 was detected, it also remains possible that the cleaved ligand may not activate the ST2 receptor efficiently, and this may be responsible for the reduced biological activity observed.

IL-33 Stability Is Modulated through Caspase-Mediated Proteolysis

To explore the consequences of caspase-mediated cleavage of IL-33 further, we asked whether caspase-mediated proteolysis might destabilize this cytokine, possibly by opening the molecule up to attack by serum proteases. To test this, we used the serum protease α-chymotrypsin as a probe for IL-33 stability because many cytokines are rapidly inactivated through degradation in the peripheral circulation (Shechter et al., 2001). As Figure 6A shows, whereas IL-33 was relatively resistant to proteolysis by α-chymotrypsin, pretreatment of IL-33 with caspase-7 rendered this cytokine much more susceptible to degradation by this protease. Differential susceptibility of the caspase-cleaved form of IL-33, versus the uncleaved form, to α-chymotrypsin-mediated degradation was observed over a wide concentration range (Figures 6A and 6B). Similar results were also observed in response to proteinase K treatment (Figures 6C and 6D).

These data indicate that caspase-mediated proteolysis of IL-33 provokes structural changes that render this cytokine more susceptible to serum protease-mediated inactivation. This suggests that rather than completely abolishing the biological activity of IL-33 (by blocking ST2 receptor binding), caspases may be involved in reducing the half-life of IL-33, by increasing the sensitivity of this cytokine to attack by serum proteases.

Luciferase activity was assayed, in triplicate, in cell lysates and normalized against empty-vector-transfected cells. **$p < 0.01$ by Student's t test. Lower panel: the biological activity of His-tagged recombinant IL-33$^{112-270}$ or caspase-7-cleaved IL-33$^{112-270}$ was assessed as above. **$p < 0.01$ by Student's t test. Results are representative of at least three independent experiments. Error bars represent the mean ± the SEM.

(B) Schematic representation of IL-33 depicting the caspase cleavage site and the various His-tagged IL-33 deletion mutants generated for this study.

(C) Cells were transfected as in (A), followed by addition of the indicated molar amounts of GST-IL-33, the indicated deletion mutants of His-tagged IL-33, or the control protein, PHAP. Cell lysates were assayed for luciferase activity 8 hr after addition of recombinant proteins. **$p < 0.01$ by Student's t test. Results are representative of at least three independent experiments. Error bars represent the mean ± the SEM.

(D) Upper panel: capture of soluble ST2-Fc after incubation with sepharose-immobilized GST, GST treated with caspase-7, GST-IL-33, or GST-IL-33 treated with caspase-7, followed by probing for ST2. Lower panel: cleavage status of the IL-33 used for the pull-down assay was revealed by immunoblotting. Note that ST2-Fc was pulled down with the full-length and the cleaved form of IL-33 (upper panel). Results are representative of at least two independent experiments.

(E) Protein A/G immobilized ST2-Fc was used to assess binding of GST, GST-IL-33 full-length, or cleaved GST-IL-33. Note that both the full-length and the cleaved forms of IL-33 were captured by ST2, whereas the GST control was not. Results are representative of at least two independent experiments.

(F) Recombinant GST, GST-IL-33, or caspase-7-cleaved GST-IL-33 (all at 125 nM) were incubated for 1 hr with HEK293 cells stably expressing the ST2L receptor. Cells were then surface immunostained with anti-GST or anti-IL-33 antibodies where appropriate. Binding of IL-33 was detected by flow-cytometry analysis. Results are representative of at least three independent experiments.

(G) HEK293 cells expressing the ST2L receptor were incubated with the indicated concentrations of recombinant GST, GST-IL-33, or caspase-7-cleaved GST-IL-33 for 1 hr followed by immunostaining with GST or IL-33 antibodies where appropriate. Binding of IL-33 was detected by flow-cytometry analysis. Results are representative of at least three independent experiments.

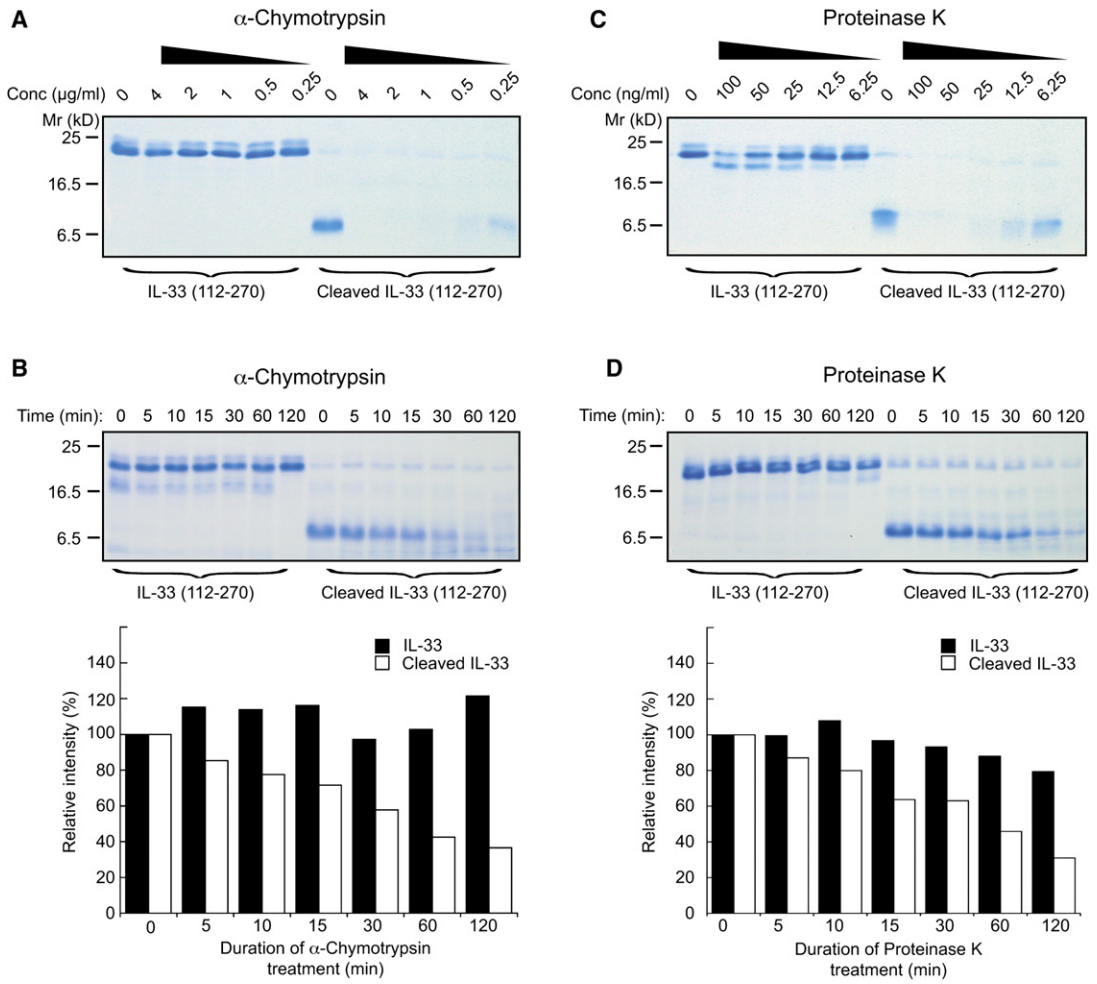


Figure 6. Caspase-Dependent Proteolysis of IL-33 Increases Susceptibility to Degradation by Serum Proteases

(A) Purified recombinant IL-33$^{112\text{-}270}$ or caspase-cleaved IL-33$^{112\text{-}270}$ was incubated for 2 hr at 37°C in the presence of the indicated concentrations of α-chymotrypsin, followed by analysis by SDS-PAGE and Coomassie blue staining.

(B) Purified recombinant IL-33$^{112\text{-}270}$ or caspase-cleaved IL-33$^{112\text{-}270}$ was incubated for the indicated times at 37°C with α-chymotrypsin (1 μg/ml) followed by analysis by SDS-PAGE and Coomassie blue staining. Histograms represent the relative intensities of each IL-33 species normalized to the 0 hr time point. Gels were quantitated with ImageJ software.

(C) Purified recombinant IL-33$^{112\text{-}270}$ or caspase-cleaved IL-33$^{112\text{-}270}$ was incubated for 2 hr at 37°C in the presence of the indicated concentrations of Proteinase K followed by analysis of cleavage reactions by SDS PAGE and Coomassie blue staining.

(D) Purified recombinant IL-33$^{112\text{-}270}$ or caspase-cleaved IL-33$^{112\text{-}270}$ was incubated at 37°C with Proteinase K (25 ng/ml) for the indicated times followed by analysis by SDS-PAGE and Coomassie blue staining. Histograms represent the relative intensities of each IL-33 species normalized to the 0 hr time point. Gels were quantitated with ImageJ software. Results from (A)–(D) are representative of at least three independent experiments.

The Caspase-Cleaved Form of IL-33 Exhibits Diminished Activity In Vivo

To ask whether the caspase-cleaved form of IL-33 was also less potent in vivo, we then compared the activity of both forms of IL-33 in a mouse model. Mice treated with daily injections of IL-33 (intraperitoneally [i.p.]) over a 6 day period exhibited dramatic increases in splenic weight and cellularity (Figure 7A and Figure S6A). Granulocyte numbers in the peritoneal space, the peripheral blood, and the spleen were highly elevated (Figures 7B and 7C and Figure S6B), with increases in eosinophil numbers particularly evident (Figure 7C and Figure S6B). In addition, serum IL-4 and IL-5 concentrations were dramatically elevated in response to IL-33, as previously reported (Figure 7D). Furthermore, IL-5 and IgA concentrations were also greatly elevated in the lungs of IL-33-treated mice (Figure 7D). Strikingly, all of these responses were significantly attenuated in mice treated with an identical regime of caspase-cleaved IL-33 (Figures 7A–7D and Figures S6A and S6B). Furthermore, whereas restimulation of splenocytes and mesenteric lymph node-derived lymphocytes from IL-33-treated mice resulted in robust IL-5 production, these responses were also blunted in mice treated with caspase-cleaved IL-33 (Figures 7E and 7F).

To rule out the possibility that residual caspase activity within caspase-7-treated IL-33 preparations was responsible for the reduced biological activity of this cytokine, we added the irreversible polycaspase inhibitor, zVAD-fmk, to both IL-33

preparations after caspase treatment. We confirmed complete neutralization of caspase activity after treatment with zVAD-fmk (Figure S7). Once again, caspase-cleaved IL-33 exhibited greatly reduced potency in vivo compared to the uncleaved protein (Figure S8), thereby ruling out the possibility that residual caspase activity contributed to the effects seen.

Mutation of Asp178 Attenuates IL-33 Activity In Vitro and In Vivo

Caspase-dependent proteolysis of IL-33 could lead to decreased biological activity by accelerating degradation by extracellular proteases or by destabilizing a region of the protein critical for proper receptor stimulation. To further explore the consequences of proteolysis of IL-33 at Asp178, we wondered whether mutation of this amino acid might affect the biological activity of this cytokine. Therefore, we compared the activity of wild-type IL-33, caspase-cleaved IL-33, and the IL-33^{D178A} mutant in vivo. As Figure S9 illustrates, wild-type IL-33 again displayed potent biological activity in vivo, which was greatly attenuated through caspase-mediated proteolysis, as before. Furthermore, mutation of the caspase cleavage site at D178 also dramatically reduced the biological potency of this cytokine, implying that this region is critical for activity. With ST2-transfected HEK293 cells, the IL-33^{D178A} mutant was also found to be inactive within the NF-κB reporter assay (Figure S9H).

Collectively, these data provide strong support for the idea that caspase-3- and caspase-7-mediated cleavage of IL-33 diminishes, rather than increases, the biological activity of this cytokine through destabilizing a region within IL-33 that is important for ST2 receptor stimulation.

DISCUSSION

Here we have shown that IL-33 is efficiently cleaved by apoptosis-associated caspases (caspase-3 and -7) but not inflammatory caspase-1, -4, or -5. Caspase-3 and -7 cleave IL-33 at a motif (DGVD^{178} in human and DGVD^{175} in mouse) that is fully conserved between the human and mouse forms of this protein. Proteolysis of IL-33 was not required for ST2 receptor binding or ST2 receptor-dependent NF-κB activation. Caspase-dependent proteolysis of IL-33 attenuated ST2-dependent NF-κB activation and increased susceptibility of IL-33 to degradation by serum proteases. Furthermore, mutation of IL-33 at Asp178 abolished the biological activity of this cytokine, suggesting that proteolysis at this site alters the conformation of a region within IL-33 that is critical for receptor signaling.

Because caspases are activated during apoptosis but not necrosis, an interesting implication of our experiments is that the half-life of IL-33 is reduced during apoptosis. Consistent with this, IL-33 failed to undergo proteolytic processing in necrotic cells (data not shown) but was readily cleaved during apoptosis. Similar to IL-1α and IL-1β, IL-33 does not possess a classical secretory sequence and is therefore unlikely to be released from cells via the classical ER-Golgi secretory pathway. Therefore, one possibility is that IL-33, similar to the nonclassical cytokine HMGB1 (Scaffidi et al., 2002), is released as a consequence of necrosis. Because caspases do not become activated during necrotic cell death (Kroemer and Martin, 2005), IL-33 is therefore likely to be released from necrotic cells as a full-length active molecule. Caspase-dependent proteolysis of IL-33 during apoptosis may therefore represent a means of reducing the proinflammatory activity of this cytokine. Interestingly, it has been demonstrated by several groups that apoptotic cells are much less proinflammatory than necrotic cells and can even exhibit anti-inflammatory effects that may dominate over necrotic cell-derived factors (Voll et al., 1997; Patel et al., 2007). Thus, the proteolysis of IL-33 during apoptosis may contribute to the damping down of the potentially proinflammatory effects of cell death. Furthermore, because apoptotic cells are typically engulfed by phagocytes prior to loss of plasma-membrane integrity, this further reduces the possibility of biologically active IL-33 being released from such cells. IL-33 may therefore represent an endogenous "danger signal" or "alarmin" that is more potent when released in the context of pathological cell death (necrosis) as opposed to apoptosis, which is more usually encountered in physiological settings.

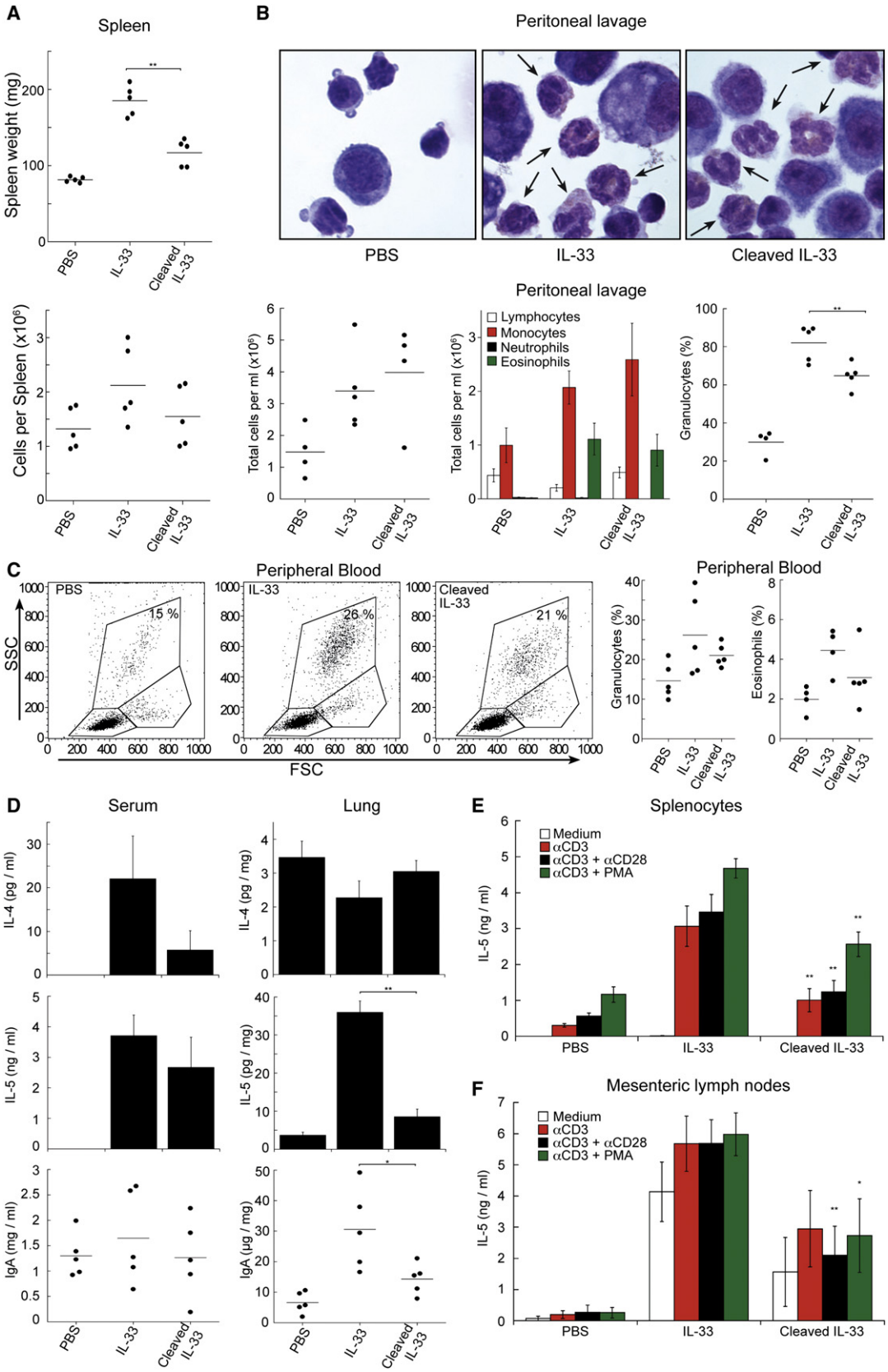
IL-33 is a nuclear protein that has been reported to possess activity as a regulator of transcription (Carriere et al., 2007). IL-1α also exhibits a nuclear expression pattern and is reported to have intracellular activities (Maier et al., 1994). Furthermore, pro-IL-1α is active as a full-length protein and is capable of binding to the IL-1 receptor (Mosley et al., 1987). It is also suspected that the major route of IL-1α release may be through necrosis. Thus, IL-33 and IL-1α share several features in common: both proteins are active as full-length molecules but also undergo proteolytic processing under certain circumstances, and both are released during necrosis.

In conclusion, here we have shown that IL-33 is active as a full-length cytokine, similar to IL-1α, and does not require proteolytic maturation by inflammatory caspases for production of the biologically active cytokine. However, IL-33 can be cleaved at physiological concentrations of caspase-3 and -7, which greatly attenuates the biological activity of this cytokine. Consistent with this, IL-33 was processed at this cleavage motif within apoptotic but not necrotic cells. Thus, contrary to the previous proposal that caspase-1 activates IL-33 (Schmitz et al., 2005), caspase-mediated proteolysis acts to suppress the proinflammatory properties of this cytokine. Direct inactivation of a cytokine represents a unique function for cell death-associated caspases and suggests that caspases activated during apoptosis may actively disable molecules with proinflammatory properties.

EXPERIMENTAL PROCEDURES

Reagents

Polyclonal antibodies were generated against hIL-33 by repeated immunization of rabbits with the hIL-33 peptide ^{58}CYFRRETTKRPSLKT^{72} (Sigma Genosys, UK). Additional IL-33 antibodies were purchased from Alexis (UK) and R&D systems (UK). Mouse IL-1β antibody was obtained from the National Institute for Biological Standards and Control (UK). Antibodies specific to caspase-3, caspase-7, RhoGDI, and XIAP were obtained from BD (UK). Anti-human caspase-1 antibodies were from Santa Cruz (UK). Anti-IL-1β antibodies were from R&D Systems (UK); anti-caspase-9 monoclonal antibodies were from Oncogene Research Products (UK). Anti-co-chaperone p23 antibodies were purchased from Affinity Bioreagents (UK). Anti-actin antibody was purchased from ICN (UK). Anti-GR-1-FITC antibody was purchased from ImmunoTools (Germany). The peptides z-YVAD-CHO, Ac-WEHD-AMC, Ac-DEVD-AFC, and zVAD-FMK were all purchased from Bachem (UK). Unless otherwise indicated, all other reagents were purchased from Sigma (Ireland) Ltd.


A
Spleen
Spleen weight (mg)
PBS
IL-33
Cleaved IL-33
B
Peritoneal lavage
PBS
IL-33
Cleaved IL-33
Cells per Spleen (x10⁶)
Peritoneal lavage
Total cells per ml (x10⁶)
Lymphocytes
Monocytes
Neutrophils
Eosinophils
Granulocytes (%)
C
Peripheral Blood
PBS
IL-33
Cleaved IL-33
15 %
26 %
21 %
SSC
FSC
Peripheral Blood
Granulocytes (%)
Eosinophils (%)
D
Serum
Lung
IL-4 (pg / ml)
IL-5 (ng / ml)
IgA (mg / ml)
IL-4 (pg / mg)
IL-5 (pg / mg)
IgA (µg / mg)
E
Splenocytes
Medium
αCD3
αCD3 + αCD28
αCD3 + PMA
IL-5 (ng / ml)
F
Mesenteric lymph nodes
Medium
αCD3
αCD3 + αCD28
αCD3 + PMA
IL-5 (ng / ml)

Primary Cell Culture

Bone marrow-derived DCs were generated from C57BL/6 mice as described (Lavelle et al., 2001). WT, *Casp1*$^{-/-}$, *Casp3*$^{-/-}$, and *Casp7*$^{-/-}$ MEFs were derived from C57BL/6 mice. WT and *Casp1*$^{-/-}$ MEFs were kindly provided by Richard Flavell (Yale University, CT, USA), *Casp3*$^{-/-}$ MEFs were kindly provided by Afshin Samali (NUI Galway, Ireland), and *Casp7*$^{-/-}$ MEFs were kindly provided by Peter Vandenabeele (Ghent University, Belgium).

Expression and Purification of Recombinant IL-33 and Caspases

Recombinant GST-IL-33 and various His-tagged forms of IL-33 were expressed and purified as described in the Supplemental Experimental Procedures. Recombinant polyhistidine-tagged caspase-1, -4, -5, -3, and -7 were also expressed and purified as described in the Supplemental Experimental Procedures.

Animals and In Vivo Treatment

C57BL/6 and Balb/c mice were obtained from Harlan (UK). Animal experiments and maintenance were approved and regulated by the Trinity College Dublin ethics committee and the Irish Department of Health.

Determination of Cytokine and IgA Levels

Cells were lysed in buffer containing 150 nM NaCl, 50 nM Tris (pH 8), 1% NP-40, and 0.1% SDS. Cytokines in cell lysates or medium were detected by ELISA with paired antibodies for IL-4, IL-5, IL-6 (BD PharMingen, UK), IL-33, and IL-1β (R&D Systems, UK). IgA levels were measured as described previously (Lavelle et al., 2001).

SUPPLEMENTAL DATA

Supplemental Data include nine figures and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/supplemental/S1074-7613(09)00269-6.

ACKNOWLEDGMENTS

We thank K. Yanagisawa for provision of ST2 expression plasmid and L. O'Neill and K. Mills for helpful discussions and for provision of reagents. We thank Science Foundation Ireland (PI1/B038) and The Health Research Board of Ireland (RP/2006/233) for their support of this work. S.J.M is a Science Foundation Ireland Fellow.

Received: March 13, 2008
Revised: February 23, 2009
Accepted: May 8, 2009
Published online: June 25, 2009

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Figure 7. Cleaved IL-33 Displays Diminished Biological Activity In Vivo

C57BL/6 mice (5 per treatment group) were injected (i.p.) for 6 consecutive days with PBS, IL-33$^{112\text{-}270}$ (1 μg per mouse per day), or caspase-cleaved IL-33$^{112\text{-}270}$ (1 μg per mouse per day). Note that the artificially truncated IL-33 was used here because of problems associated with purification of large quantities of full-length IL-33.

(A) Spleen weight and cellularity for each group of mice are shown. **$p < 0.01$ by Student's t test.

(B) Peritoneal lavage-derived cells were enumerated by hemocytometer, and cytospins were also made. Cytospins were stained with hematoxylin and eosin for assessment of cell morphology; arrows indicate granulocytes (top panels). Numbers of individual cell types were assessed by cytospin analysis and are expressed relative to the total number of cells found in the peritoneal lavages. Granulocyte numbers were also determined by forward-scatter (FSC) and side-scatter (SSC) analysis. **$p < 0.01$ by Student's t test. Measurements were taken in triplicate. Error bars represent the mean ± the SEM.

(C) Peripheral bloods were treated with flow-cytometry lysis solution to eliminate red blood cells followed by analysis by flow cytometry. Granulocyte numbers were scored on the basis of their high FSC and SSC properties, as shown. Eosinophil numbers were determined by counting H&E-stained cytospin preparations of peripheral bloods.

(D) IL-4, IL-5, and IgA concentrations were determined by ELISA in plasma samples or lung homogenates. Note that lung data are expressed per mg protein. *$p < 0.05$; **$p < 0.01$ by Student's t test. Measurements were taken in triplicate. Error bars represent the mean ± the SEM.

Splenocytes (E) and mesenteric lymph node cells (F) (10^6 cells/ml) were restimulated either with medium, 1 μg/ml anti-CD3, 1 μg/ml anti-CD3, and 1 μg/ml anti-CD28 or with 1 μg/ml anti-CD3 and 20 ng/ml PMA, as indicated. Supernatants were collected after 3 days and IL-5 concentrations were determined by ELISA. Measurements were taken in triplicate. Error bars represent the mean ± the SEM.

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