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Suppression of IL-33 Bioactivity through Proteolysis by Apoptotic Caspases

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Summary

IL-33 is a member of the IL-1 family and is involved in polarization of T cells towards a T_H2 phenotype. It is widely believed that IL-33 is activated via caspase-1-dependent proteolysis, similar to the pro-inflammatory cytokines IL-1β and IL-18, but this remains unproven. In opposition to this view, here we show that IL-33 is processed by caspases activated during apoptosis (caspases -3 and -7) but is 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 NFκB activation. 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 pro-inflammatory 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). Caspases -1, -4, -5 are activated in response to pathogen products such as lipopolysaccharide (LPS) that signal via members of the Toll-like receptor 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-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 novel IL-1 family cytokine, IL-33/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_H2 cells and negative regulation of IL-1R and TLR4 signalling (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 T_H1 responses and attenuation of T_H2-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 enhanced production of T_H2 cytokines from *in vitro* polarized T_H2 cells and suppressed T_H1 cytokine production (Schmitz et al., 2005). Administration of the same truncated form of IL-33 *in vivo* induced expression of IL-4, IL-5 and IL-13 and also led to eosinophilia, splenomegaly and increased levels of serum 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 have been reported to cleave IL-33 *in vitro* and this has been 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, caspase-4 and caspase-5 in the maturation of IL-33. Surprisingly, we find little evidence that IL-33 is a physiological substrate for the inflammatory caspases. Rather, we show that this cytokine is efficiently processed by caspases-3 and -7, proteases that are selectively activated during apoptosis. Furthermore, caspase-mediated proteolysis of IL-33 increased 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 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 physiologically-relevant amounts of caspases -1, -4 and -5 (Figure 1A). We used non-saturating concentrations of caspase-1 that achieved robust proteolysis of the known caspase-1 substrate, IL-1β, and equimolar amounts of caspases –4 and –5. All caspases were active as indicated by hydrolysis of the synthetic peptide substrate WEHD-AMC (Figure S1). However, while caspase-1 robustly cleaved IL-1β, IL-33 was not readily processed under the same conditions (Figure 1A). Caspases-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), thus we used concentrations of caspases -3 and -7 that achieved robust, but incomplete, proteolysis of their known substrates, RhoGDI2, co-chaperone p23 and XIAP (Figure 1B). These concentrations were chosen to avoid using saturating, non-physiological, amounts of the latter proteases.

As Figures 1C and 1D illustrate, caspases-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 caspases-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.

Using the same THP-1 cell-free system, caspases -3 and -7 can be activated by addition of cytochrome c and dATP to the extracts, as the latter act as co-factors 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 caspases-3/-7 and proteolytic processing of known caspase substrates, such as XIAP and co-chaperone 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 using 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 site that is conserved between the human and murine forms of this cytokine

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 Asp residues that may qualify as caspase cleavage motifs. Based upon the approximate molecular weights of the caspase-mediated cleavage products of IL-33 observed in our initial experiments (Figure 1C and Figure 2B), a conserved caspase cleavage motif was located at Asp178 within human IL-33 (^{175}DGVD^{178}) and Asp175 within murine IL-33 (^{172}DGVD^{175}) that was more likely to represent the site of caspase-mediated proteolysis (Figure 2C). Notably, this site also conforms to a consensus caspase -3/-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 analysed using 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), 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).

Based upon the initial observations of Schmitz et al. (2005), all investigations carried out to date with IL-33 have used an artificially-truncated form of this cytokine, IL-33$^{112-270}$, that was 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$^{112-270}$ was cleaved by caspase-7 whereas the IL-33$^{112-270}$ 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, as 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 pro-apoptotic stimuli, including Daunorubicin, 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 poly-caspase inhibitor (z-VAD-fmk) in the medium, also blocked apoptosis-associated proteolysis of IL-33 (Figures 3C and D). 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 either *CASP-1*, *CASP-3*, or *CASP-7* processed IL-33 during apoptosis. As Figure 3E shows, whereas IL-33 was readily processed upon induction of apoptosis in wild type MEFs, *CASP-1*$^{-/-}$ 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 *CASP-3*$^{-/-}$ MEFs under the same conditions (Figure 3E). *CASP-7*$^{-/-}$ MEFs exhibited processing of IL-33 similar to wild type cells (Figure 3E), most likely because caspase-3 and caspase-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 induced by LPS/PMA treatment but only 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, as previous studies have not addressed this issue. As shown in Figure 4A and 4B, THP-1 cells did not express IL-33 constitutively, but were induced to do so upon stimulation with LPS/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/PMA-stimulated cells (Figure 4B and 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/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 using cytotoxic drugs, endogenous IL-33 was processed to a species that ran at an identical mobility to the caspase-3/7-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 asked whether IL-33 was secreted during apoptosis, but again found that the majority of protein remained cell-associated (Figure 4F, upper panels). In contrast, upon induction of necrosis using hydrogen peroxide, sodium azide, streptolysin O, or a high concentration of daunorubicin, IL-33 was now readily detected in medium (Figure 4F, lower panels).

We also performed similar experiments using primary bone marrow-derived mouse dendritic cells. Similar to what was observed in human THP-1 cells, IL-33 was upregulated upon LPS/PMA-treatment of murine DCs but was not proteolytically processed or secreted under these conditions (Figure 4G and 4H). IL-1β was also induced upon LPS/PMA-treatment of DCs but, in contrast to IL-33, was secreted under these conditions (Figure 4H). Upon triggering of apoptosis in these cells, IL-33 was once again processed to a fragment consistent with caspase-3/-7-dependent processing of this cytokine (Figure 4I).

Collectively, the above data indicate that IL-33 is processed in a caspase-dependent manner during apoptosis, but not in response to a pro-inflammatory stimulus (LPS) associated with caspase-1 activation.

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, as 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 NFκB activation in an ST2-receptor-dependent manner. For this purpose, we used HEK293T cells transfected with the ST2 receptor along with a NFκB-responsive promoter. As Figure 5A illustrates, whereas we detected NFκB 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 using 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 NFκB 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 caspase-generated cleavage products (IL-$33^{112\text{-}178}$ and IL-$33^{179\text{-}270}$; Figure 5B) to ask whether these fragments could promote ST2-dependent NFκB activation. However, compared to either full length GST-IL-33 or the artificially-truncated IL-$33^{112\text{-}270}$, when expressed independently neither fragment was found to be capable of promoting ST2-dependent NFκB 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 as this cytokine displays biological activity both as a precursor as well 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* pulldown 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 as well as 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 pulldown experiments (Figure 5D and 5E), the caspase-cleaved form of GST-IL-33 also bound to ST2-transfected cells, but with reduced efficiency (Figure 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 as 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, pre-treatment 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.

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 (i.p.) over a 6 day period exhibited dramatic increases in splenic weight and cellularity (Figure 7A). Granulocyte numbers in the peritoneal space, the peripheral blood and the spleen were highly elevated (Figure 7B-D), with increases in eosinophil numbers particularly evident (Figure 7C and 7D). In addition, serum IL-4 and IL-5 levels were dramatically elevated in response to IL-33, as previously reported (Figure 7E). Furthermore, IL-5 and IgA levels were also greatly elevated in the lungs of IL-33-treated mice (Figure 7E). Strikingly, all of these responses were significantly attenuated in mice treated with an identical regime of caspase-cleaved IL-33 (Figure 7A-E). 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 7F and 7G).

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 poly-caspase inhibitor, zVAD-fmk, to both IL-33 preparations after caspase treatment. We confirmed complete neutralization of caspase activity after treatment with zVAD-fmk (Figure S5). Once again, caspase-cleaved IL-33 exhibited greatly reduced potency in vivo compared to the uncleaved protein (Figure S6), 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 due to accelerated degradation by extracellular proteases, or through 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 S7 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.

Using ST2-transfeced HEK293 cells, the IL-33^{D178A} mutant was also found to be inactive within the NFκB reporter assay (Figure S7H).

Collectively, these data provide strong support for the idea that caspase-3/-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 (caspases -3 and -7) but not inflammatory caspases –1, -4 or –5. Caspases –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 NFκB activation. Caspase-dependent proteolysis of IL-33 attenuated ST2-dependent NFκB 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 signalling.

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 non-classical 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 pro-inflammatory activity of this cytokine. Interestingly, it has been demonstrated by several groups that apoptotic cells are much less pro-inflammatory 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 pro-inflammatory 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 as 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/-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 pro-inflammatory properties of this cytokine. Direct inactivation of a cytokine represents a novel function for cell death-associated caspases and suggests that caspases activated during apoptosis may actively disable molecules with pro-inflammatory 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 anti-caspase-1 antibody was a kind gift from P. Vandenabeele. Mouse IL-1β antibody was obtained from the National Institute for Biological Standards and Control (UK). Antibodies specific to caspase-3, caspase-7 and XIAP were obtained from BD (UK). Anti-human caspase-1 antibodies were from Santa Cruz (UK). Antibodies specific to caspase-4 and caspase-5 were purchased from MBL (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 zVAD-FMK were all purchased from Bachem (UK). Unless otherwise indicated, all other reagents were purchased from Sigma (Ireland) Ltd.

Primary cell culture

Bone marrow-derived DCs were generated from C57BL/6 mice as described (Lavelle et al., 2001). WT, *CASP-1*$^{-/-}$, *CASP-3*$^{-/-}$, and *CASP-7*$^{-/-}$ MEFs were derived from C57BL/6 mice.

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 procedures. Recombinant poly-histidine-tagged caspases –1, -4, -5, -3, and –7 were also expressed and purified as described in the supplemental procedures.

Animals and *in vivo* treatment

C57BL/6 and Balb/c mice were obtained from Harlan U.K. 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

Cytokines 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

The Supplemental Data include Supplemental Experimental Procedures and seven figures and can be found with this article online at http://www.immunity.com/supplemental/XXXXXX.

ACKNOWLEDGEMENTS

We thank Dr. Ken Yanagisawa for provision of ST2 expression plasmid and Profs. Luke O'Neill and Kingston 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. The authors have no conflicting financial interests.

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Figure Legends

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/translation and were then incubated with the indicated concentrations of recombinant caspase-1, -4 and -5 for 2 h at 37°C followed by analysis by SDS-PAGE and fluorography.

(B) Recombinant caspases –1, -3 and -7 were added to Jurkat cell-free extracts, at the indicated concentrations, followed by incubation at 37°C for 2 h. Extracts were then analysed 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/translation, were incubated with the indicated concentrations of recombinant caspase-1, -3 and -7 for 2 h at 37°C followed by analysis by SDS-PAGE/fluorography.

(D) Densitometric analysis of the gels presented in C. Scanned gels were analysed using ImageJ software (http://rsb.info.nih.gov/ij/) and results are expressed as % proteolysis of the full-length forms of each protein relative to the untreated control.

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.

(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 analysed by SDS-PAGE/fluorography.

(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 h at 37°C followed by analysis by SDS-PAGE/fluorography.

(E) Recombinant GST-IL-33 was incubated for 2 h at 37°C in the presence or absence of recombinant caspase-7 (600 nM), as indicated, followed by SDS-PAGE/Coomassie blue staining.

(F) ^{35}S-labeled wild-type hIL-33 and IL-33^{D178A} point mutant were incubated for 2 h at 37°C with recombinant caspase-3, -7 and –1, as shown. Reactions were analysed by SDS-PAGE/fluorography.

(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 analysed by SDS-PAGE/fluorography. Samples of the same reactions were also immunoblotted for caspase-3 and XIAP, as indicated.

(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 h at 37°C followed by analysis by SDS-PAGE/Coomassie blue staining.

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). 24 h 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 h before assessment of apoptosis.

(B) Western blot analysis of cell lysates derived from HeLa cells transfected either with 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/cycloheximide, or Cisplatin at concentrations indicated in A.

(C) HeLa cells were transfected with an IL-33 expression plasmid for 24 h followed by treatment for 8 h with Daunorubicin (5 μM) to induce apoptosis. In parallel, HeLa cells were also treated with the poly-caspase inhibitor Z-VAD-fmk (50 μM), or were transfected with a Bcl-xL expression plasmid as indicated.

(D) Cell lysates were generated from the cells treated in C and were immunoblotted for the indicated proteins.

(E) Murine embryonic fibroblasts from wild type (WT), *CASP-1* null, *CASP-3* null, or *CASP-7* null mice were transfected with an expression plasmid encoding hIL-33, followed by treatment for 24h with MG132 (0.2-0.4 μM) to facilitate IL-33 stabilization. Cells were then either left untreated, or were treated for 24-30 h 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 using standard morphological criteria (bottom panel).

Figure 4. Endogenous IL-33 is upregulated in response to LPS and is selectively released during necrosis

(A) THP-1 cells were either 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. 24 h later, lysates were generated and immunoblotted for IL-33. Note that LPS/PMA induced strong upregulation of endogenous IL-33.

(B) THP-1 cells were treated with PMA (50 nM) together with the indicated concentrations of LPS. 24 h later, lysates were generated and immunoblotted for the indicated proteins. As controls, in vitro transcribed/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. Note that cleaved IL-33 was not detected in LPS-stimulated cells.

(C) THP-1 cells were treated as in (B) then 24 h later, cells and medium were harvested separately and cells were lysed in buffer containing 150 nM NaCl, 50 nM Tris pH 8, 1 % NP-40, 0.1 % SDS to facilitate measurment of cell-associated cytokines. Cell-associated and secreted IL-33 and IL-1β levels were then assessed by ELISA. Note that whereas IL-1β was secreted into the medium under these conditions, IL-33 was not.

(D) Immunoprecipitation of endogenous IL-33. THP-1 cells (10^8) were treated with LPS (5 μg/ml) and PMA (50 nM) for 24 h, followed by treatment with the proteasome inhibitor MG132 (5 μM) for a further 24 h 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 while the asterix indicates antibody light chain used for the immunoprecipitation. As controls, in vitro transcribed/translated full length IL-33 and caspase-7-cleaved full length IL-33 were included to facilitate size comparison.

(E) THP-1 cells were treated with LPS (5 μg/ml) and PMA (50 nM) then 24 h later, MG132 (5 μM) was added to stabilize endogenous IL-33. After a further 6 h, 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 h 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.

(F) Upper panels, THP-1 cells were treated with LPS (5 μg/ml) and PMA (50 nM) for 24 h. To induce apoptosis, cells were then treated with Act D (5 μM), Daunorubicin (10 μM), Cisplatin (500 μM) or CHX (250 μM) for a further 12 h before assessment of apoptosis by annexin V-FITC/ 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 □□□□□□ in cell lysates and medium were then assessed by ELISA. Lower panels, THP-1 cells were treated with LPS/PMA for 24 h, as above. To induce necrosis, cells were then treated for 1 h

with Hydrogen Peroxide (H_2O_2; 20 mM), Sodium Azide (NaN_2; 1 M), Steptolysin O (SLO; 10 µg/ml) or a high dose of Daunorubicin (100 µM) followed by assessment of necrosis using PI uptake in conjunction with flow cytometry. Cells and medium were harvested separately and cells lysed as in (C). IL-33 and IL-1β in the cell lysates and media were assessed by ELISA.

(G) Primary bone-marrow-derived mouse DCs were treated with 50 nM PMA, either alone, or together with the indicated concentrations of LPS. 24 h later, cell lysates were immunoblotted for the indicated proteins. As controls, in vitro transcribed/translated full length IL-33 and caspase-7-cleaved full length IL-33 were included to facilitate size comparison.

(H) Primary bone-marrow-derived mouse DCs were treated with LPS/PMA as in (G) then 24 h later, cells and medium were harvested separately and IL-33 and IL-1β levels were measured by ELISA.

(I) Primary bone-marrow-derived mouse DCs were treated with LPS (5 µg/ml) and PMA (50 nM) for 24 h, followed by treatment for a further 12 h with MG132 (0.2 µM) to stabilize IL-33 levels. Cells were then treated for 30 h with the apoptosis-inducing agents Staurosporine (STS; 40 µM), TPCK (500 µM) or MG132 (100 µM) before assessment of cell death by direct counts on a minimum of 300 cells per treatment. Lysates were generated and immunoblotted for the indicated proteins.

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). 24 h later, the indicated concentrations of full length or caspase-7-cleaved GST-IL-33 were added for a further 8 h. Luciferase activity was assayed, in triplicate, in cell lysates and normalised against empty vector transfected cells. **p<0.01 by student's t-test. Lower panel, the biological activity of His-tagged recombinant IL-$33^{112\text{-}270}$, or caspase-7-cleaved IL-$33^{112\text{-}270}$ was assessed as above. **p<0.01 by student's t-test.

(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, or the indicated deletion mutants of His-tagged IL-33, or the control protein, PHAP. Cell lysates were assayed for luciferase activity 8 h after addition of recombinant proteins. **p<0.01 by student's t-test.
(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 pulldown assay was revealed by immunoblotting. Note that ST2-Fc was pulled down with the full length as well as the cleaved form of IL-33 (upper panel).
(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 full-length as well as the cleaved forms of IL-33 were captured by ST2 whereas the GST control was not.
(F) Recombinant GST, GST-IL-33 or caspase-7 cleaved GST-IL-33 (all at 125nM) were incubated for 1 h 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 using flow cytometry analysis.
(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 h followed by immunostaining with anti-GST or anti-IL-33 antibodies where appropriate. Binding of IL-33 was detected using flow cytometry analysis.

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-$33^{112\text{-}270}$, were incubated for 2 h at 37^{o}C in the presence of the indicated concentrations of α-chymotrypsin, followed by analysis by SDS-PAGE/Coomassie blue staining.

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

(C) Purified recombinant IL-33$^{112\text{-}270}$ and caspase-cleaved IL-33$^{112\text{-}270}$ were incubated for 2 h at 37^{o}C in the presence of the indicated concentrations of □□□□□□□□□□, followed by analysis of cleavage reactions by SDS-PAGE/Coomassie blue staining.

(D) Purified recombinant IL-33$^{112\text{-}270}$, or caspase-cleaved IL-33$^{112\text{-}270}$, were incubated at 37^{o}C with □□□□□□□□□□ (25 □g/ml) for the indicated times followed by analysis by SDS-PAGE/Coomassie blue staining. Histograms represent the relative intensities of each IL-33 species normalized to the 0 h time point. Gels were quantitated using Image-J software.

Figure 7. Cleaved IL-33 displays diminished biological activity in vivo

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

(A) Spleen size, weight and cellularity for each group of mice are shown. Photographs show representative spleens for two mice per group. **p<0.01 by student's t-test.

(B) Peritoneal lavage-derived cells were enumerated by haemocytometer 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 FSC/SSC analysis. **p<0.01 by student's t-test.

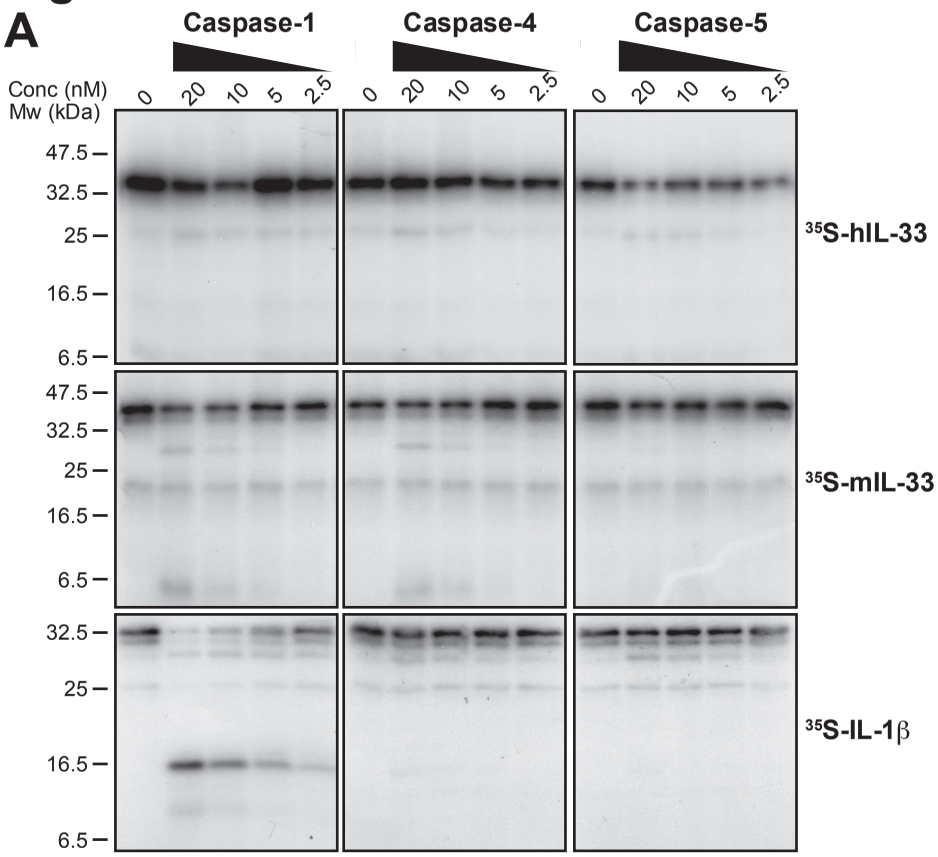
(C) Peripheral bloods were treated with FACS lysis solution to eliminate RBCs followed by analysis by flow cytometry. Granulocyte numbers were scored based on their high FSC/SSC properties, as shown. Eosinophil numbers were determined by counting H&E-stained cytospin preparations of peripheral bloods.

(D) Spleen-derived granulocytes were enumerated as described in (C) and neutrophil and eosinophil numbers were scored on H&E-stained cytospin preparations. *p<0.05, **p<0.01 by student's t-test.

(E) IL-4, IL-5 and IgA levels 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.

Splenocytes (F) and mesenteric lymph node cells (G) (10^6 cells/ml) were restimulated either with medium, 1 µg/ml anti-CD3, 1µg/ml anti-CD3 and 1 µg/ml anti-CD28, or 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.

Figure 1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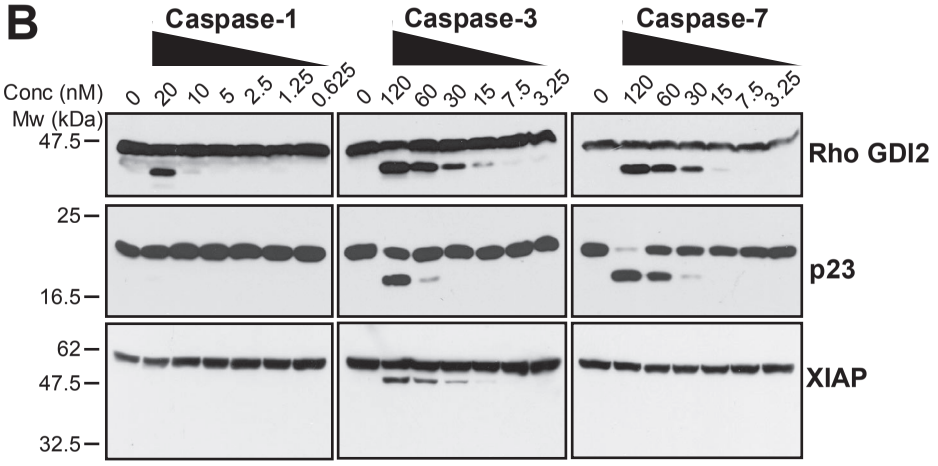
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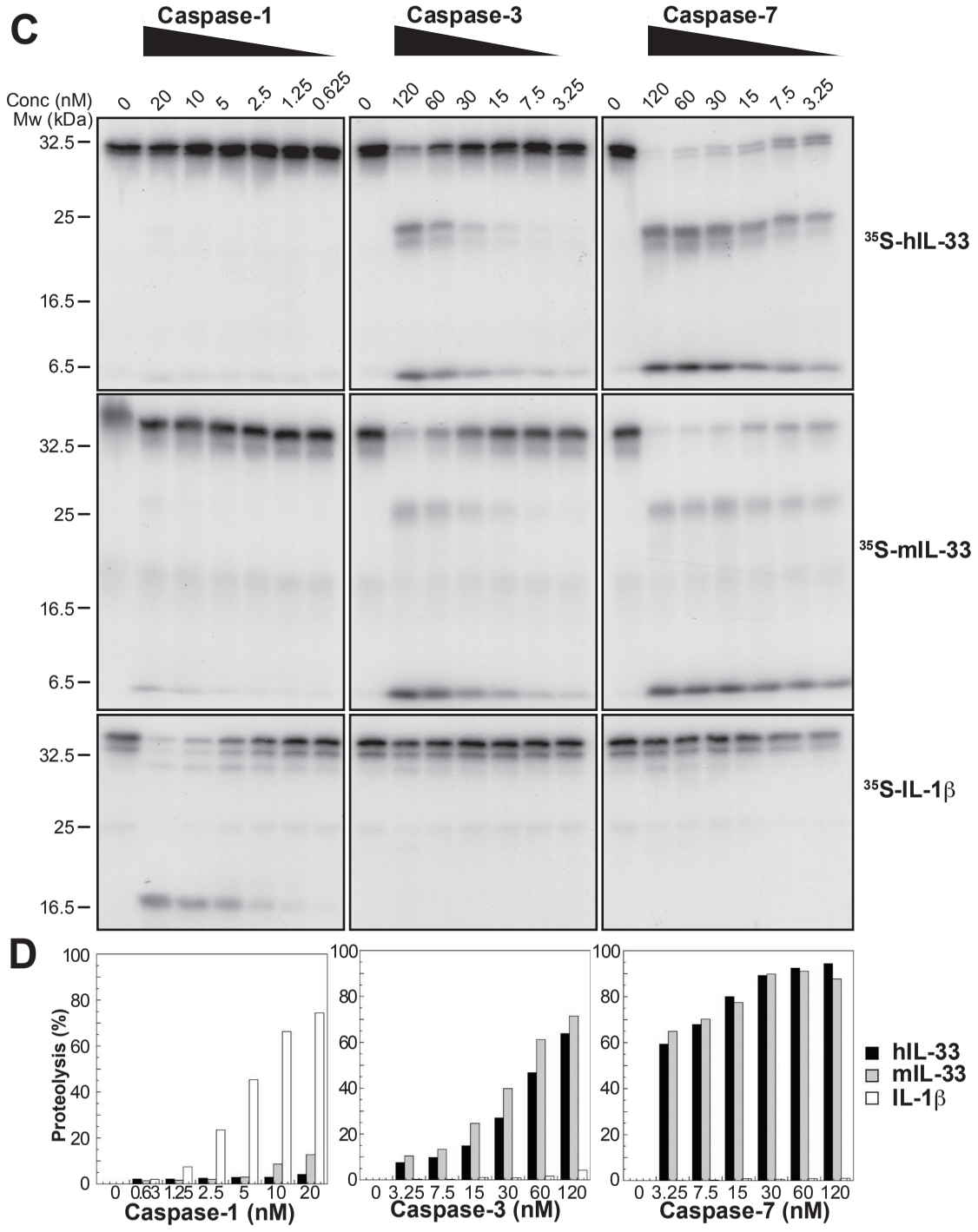
XIAP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Caspase-7
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35S-mIL-33
35S-IL-1β
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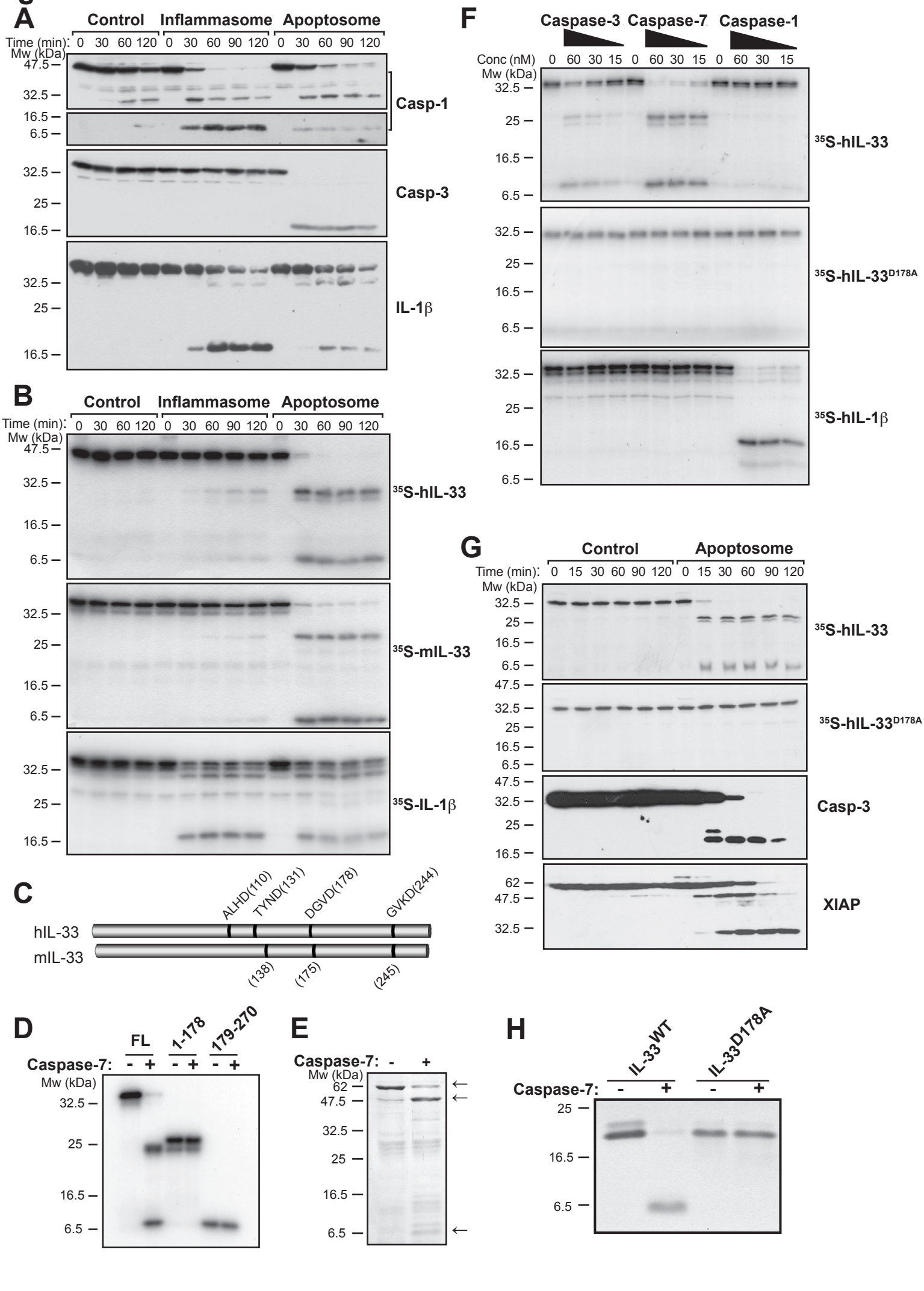
Caspase-3 (nM)
Caspase-7 (nM)
hIL-33
mIL-33
IL-1β

Figure 2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Apoptosome
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Casp-1
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Control
Inflammasome
Apoptosome
Time (min): 0 30 60 120 0 30 60 90 120 0 30 60 90 120
Mw (kDa)
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Caspase-1
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Control
Apoptosome
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Mw (kDa)
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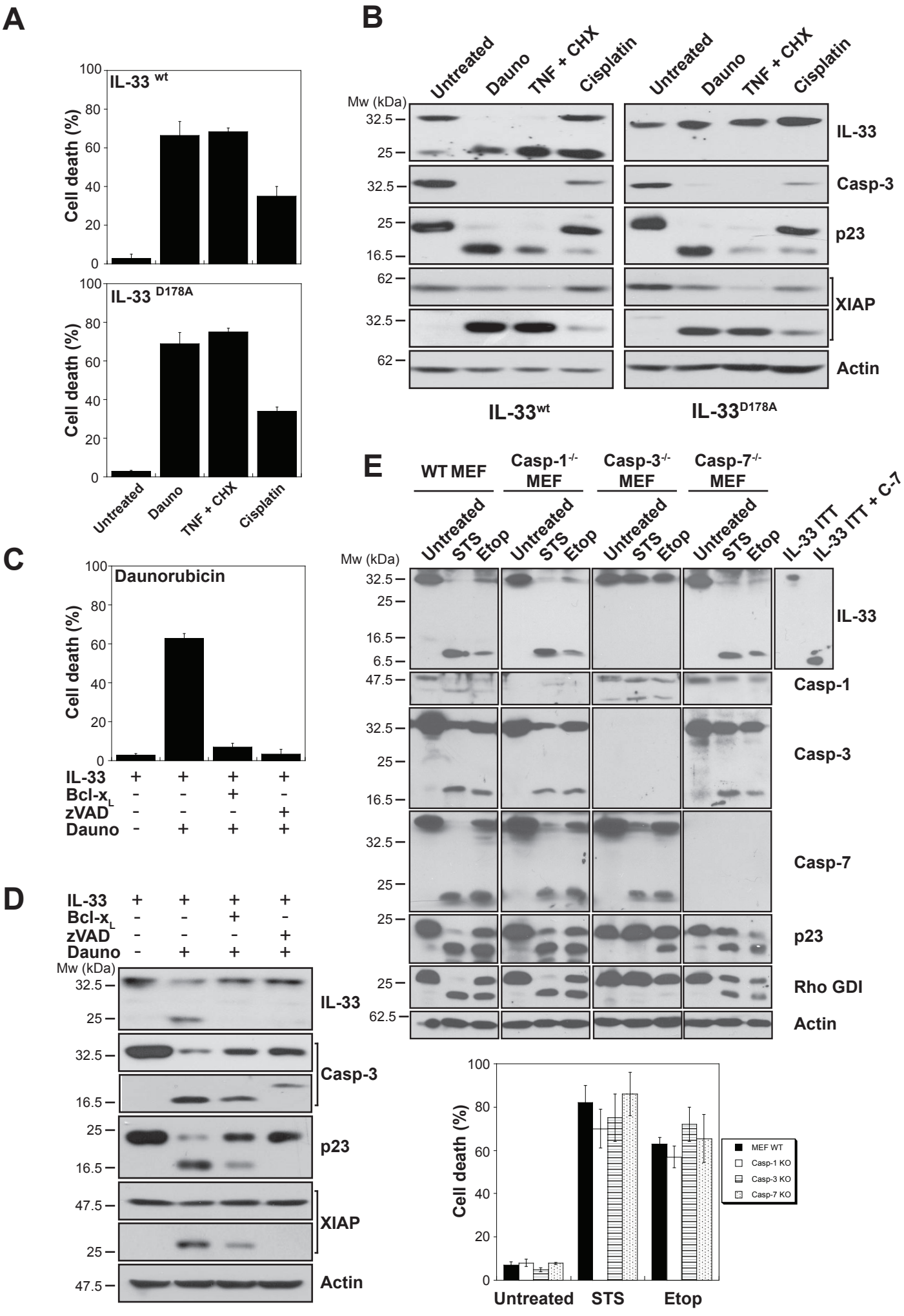
Figure 3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Dauno
TNF + CHX
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Mw (kDa)
IL-33
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XIAP
Actin
IL-33wt
IL-33D178A
C
Daunorubicin
IL-33
Bcl-xL
zVAD
Dauno
D
E
WT MEF
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Casp-3-/- MEF
Casp-7-/- MEF
STS
Etop
IL-33 ITT
IL-33 ITT + C-7
Casp-1
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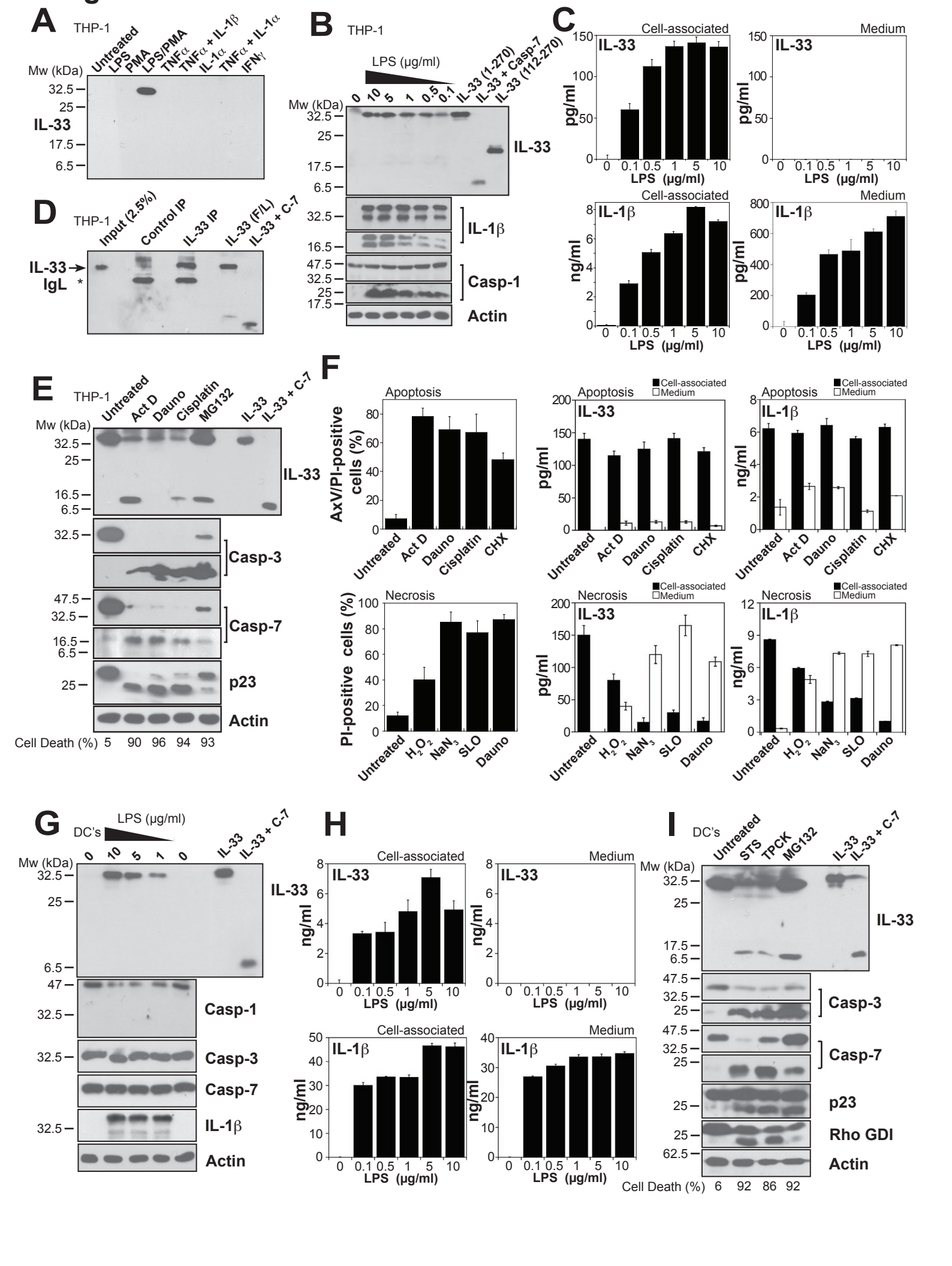
Casp-7 KO

Figure 4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THP-1
Untreated
LPS
PMA
LPS/PMA
TNFα
TNFα + IL-1β
IL-1α
TNFα + IL-1α
IFNγ
Mw (kDa)
32.5
25
IL-33
17.5
6.5
B
THP-1
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
17.5
Casp-1
Actin
C
Cell-associated
Medium
IL-33
pg/ml
150
100
50
0
0 0.1 0.5 1 5 10
LPS (μg/ml)
IL-1β
ng/ml
8
6
4
2
800
600
400
200
D
THP-1
Input (2.5%)
Control IP
IL-33 IP
IL-33 (F/L)
IL-33 + C-7
IL-33
IgL
*
E
THP-1
Untreated
Act D
Dauno
Cisplatin
MG132
IL-33
IL-33 + C-7
Mw (kDa)
32.5
25
16.5
6.5
IL-33
Casp-3
47.5
32.5
Casp-7
p23
Actin
Cell Death (%) 5 90 96 94 93
F
Apoptosis
AxV/PI-positive cells (%)
80
60
40
20
0
Untreated
Act D
Dauno
Cisplatin
CHX
Cell-associated
Medium
IL-33
pg/ml
200
150
100
50
IL-1β
ng/ml
8
6
4
2
Necrosis
PI-positive cells (%)
100
80
60
40
20
0
Untreated
H2O2
NaN3
SLO
Dauno
12
9
6
3
G
DC's
LPS (μg/ml)
0 10 5 1 0
IL-33
IL-33 + C-7
Mw (kDa)
32.5
25
6.5
IL-33
47
32.5
Casp-1
Casp-3
Casp-7
IL-1β
Actin
H
Cell-associated
Medium
IL-33
ng/ml
8
6
4
2
0
0 0.1 0.5 1 5 10
LPS (μg/ml)
IL-1β
50
40
30
20
10
I
DC's
Untreated
STS
TPCK
MG132
IL-33
IL-33 + C-7
Mw (kDa)
32.5
25
17.5
6.5
IL-33
47.5
32.5
25
Casp-3
Casp-7
p23
Rho GDI
62.5
Actin
Cell Death (%) 6 92 86 92

Figure 5

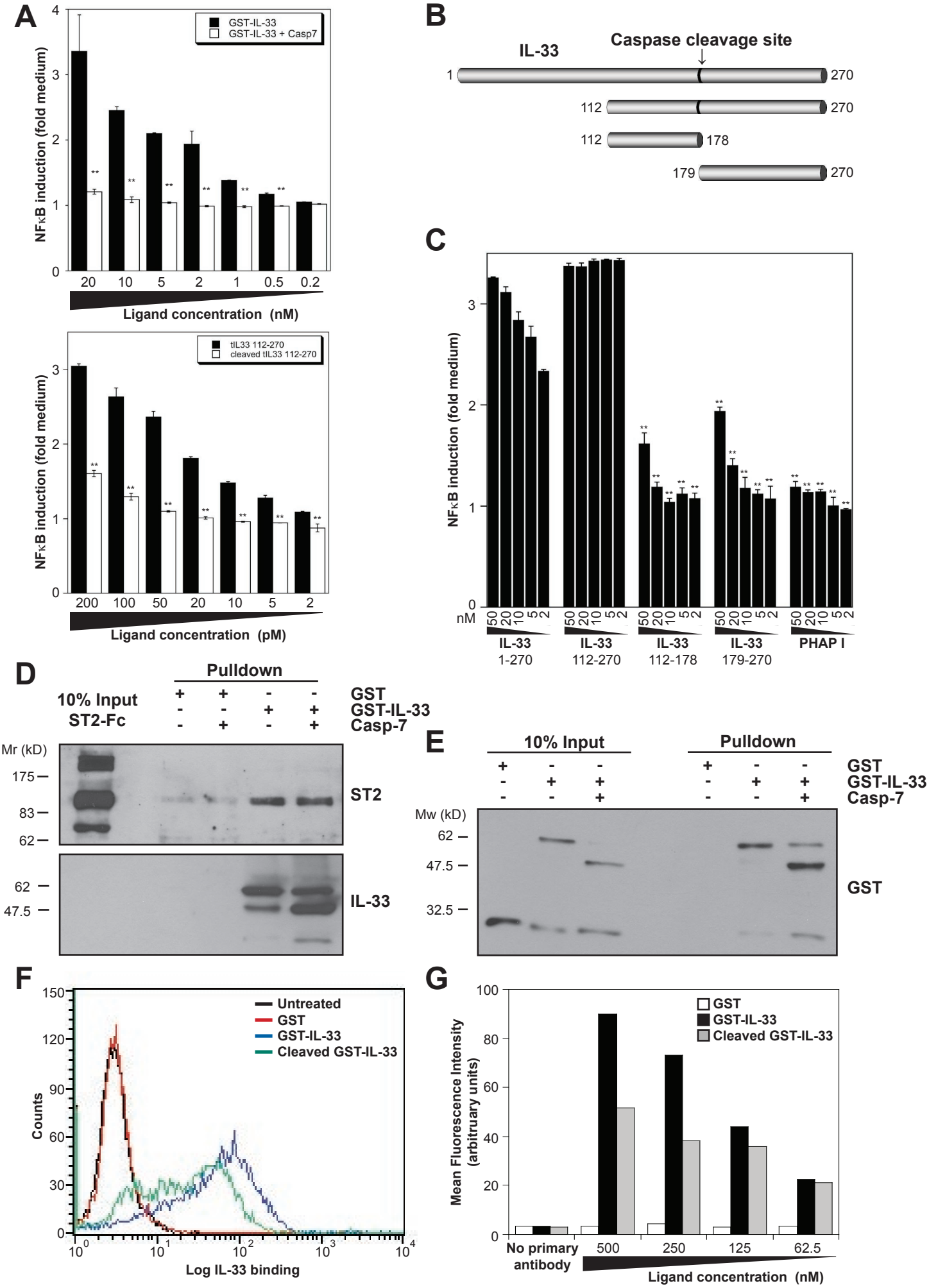

A
GST-IL-33
GST-IL-33 + Casp7
NFκB induction (fold medium)
20 10 5 2 1 0.5 0.2
Ligand concentration (nM)
tIL33 112-270
cleaved tIL33 112-270
200 100 50 20 10 5 2
Ligand concentration (pM)
B
IL-33
Caspase cleavage site
1 270
112 270
112 178
179 270
C
NFκB induction (fold medium)
nM
IL-33 1-270
IL-33 112-270
IL-33 112-178
IL-33 179-270
PHAP I
D
Pulldown
10% Input
ST2-Fc
GST
GST-IL-33
Casp-7
Mr (kD)
175
83
62
ST2
62
47.5
IL-33
E
10% Input
Pulldown
GST
GST-IL-33
Casp-7
Mw (kD)
62
47.5
32.5
GST
F
Untreated
GST
GST-IL-33
Cleaved GST-IL-33
Counts
Log IL-33 binding
G
GST
GST-IL-33
Cleaved GST-IL-33
Mean Fluorescence Intensity (arbitruary units)
No primary antibody
500 250 125 62.5
Ligand concentration (nM)

Figure 6

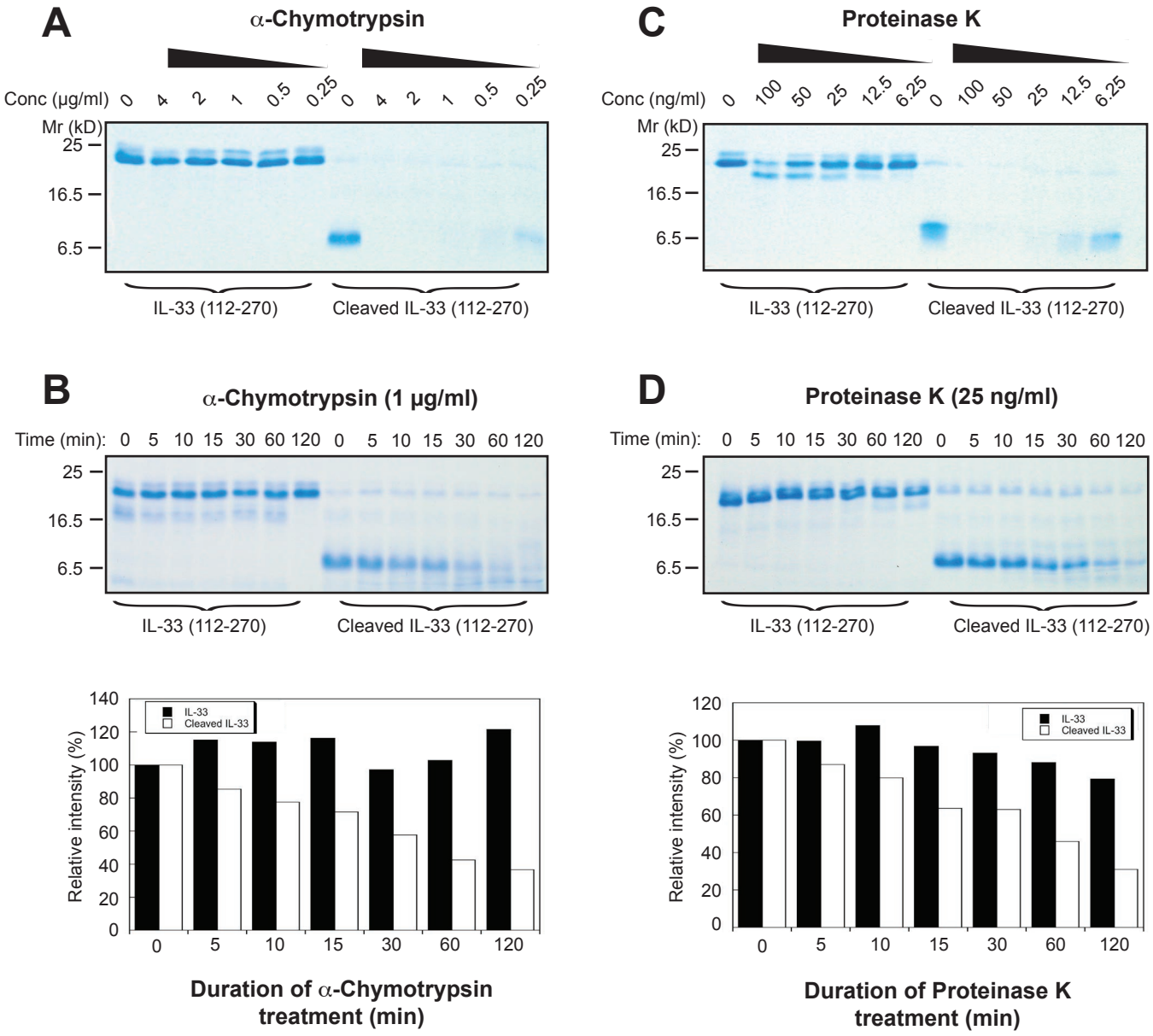
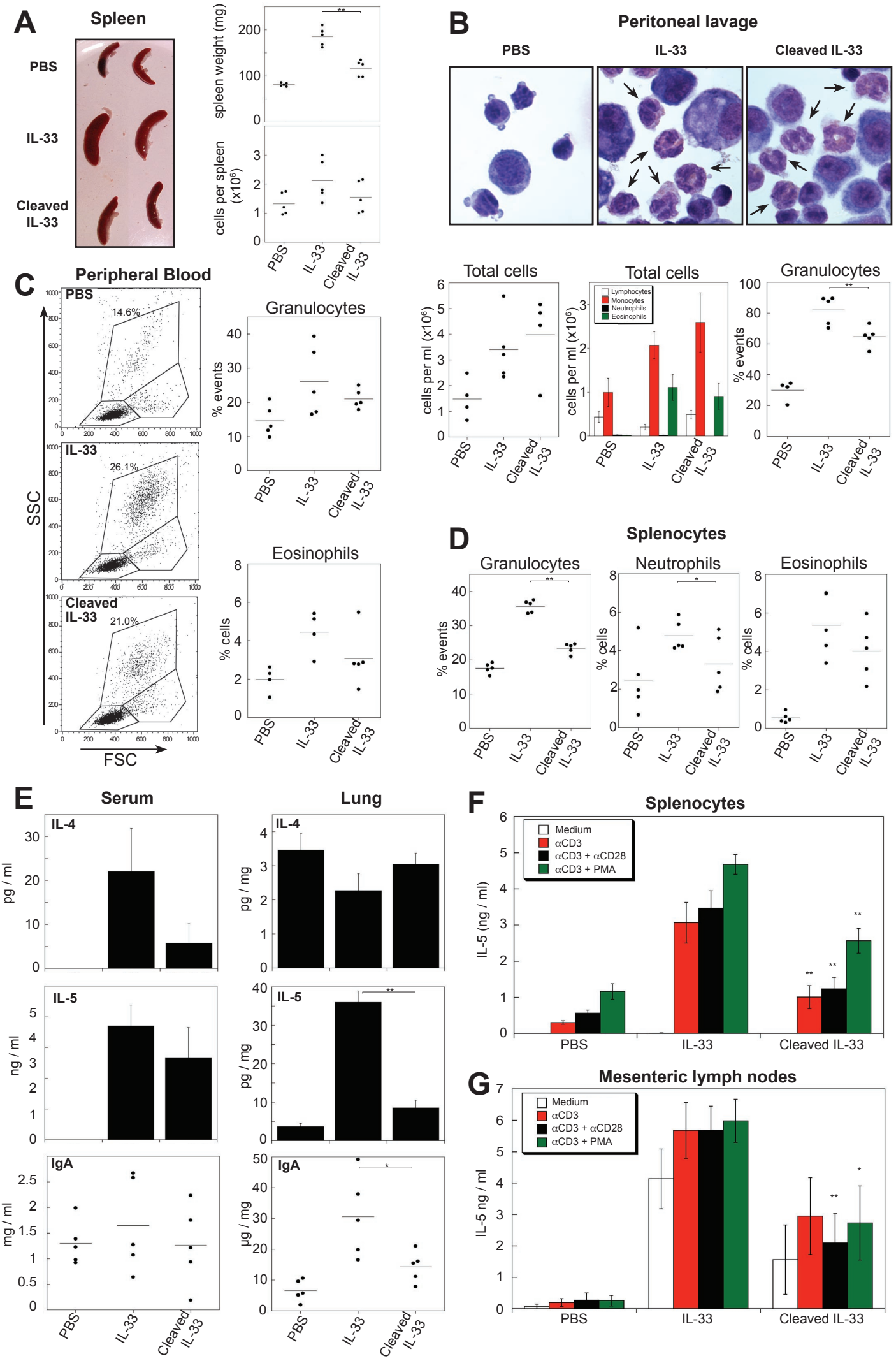

A
α-Chymotrypsin
Conc (μg/ml) 0 4 2 1 0.5 0.25 0 4 2 1 0.5 0.25
Mr (kD)
25
16.5
6.5
IL-33 (112-270)
Cleaved IL-33 (112-270)
C
Proteinase K
Conc (ng/ml) 0 100 50 25 12.5 6.25 0 100 50 25 12.5 6.25
Mr (kD)
25
16.5
6.5
IL-33 (112-270)
Cleaved IL-33 (112-270)
B
α-Chymotrypsin (1 μg/ml)
Time (min): 0 5 10 15 30 60 120 0 5 10 15 30 60 120
25
16.5
6.5
IL-33 (112-270)
Cleaved IL-33 (112-270)
D
Proteinase K (25 ng/ml)
Time (min): 0 5 10 15 30 60 120 0 5 10 15 30 60 120
25
16.5
6.5
IL-33 (112-270)
Cleaved IL-33 (112-270)
IL-33
Cleaved IL-33
Relative intensity (%)
0 20 40 60 80 100 120 140
0 5 10 15 30 60 120
Duration of α-Chymotrypsin treatment (min)
IL-33
Cleaved IL-33
Relative intensity (%)
0 20 40 60 80 100 120
0 5 10 15 30 60 120
Duration of Proteinase K treatment (min)

Figure 7


A
Spleen
PBS
IL-33
Cleaved IL-33
spleen weight (mg)
cells per spleen (x10^6)
B
Peritoneal lavage
PBS
IL-33
Cleaved IL-33
C
Peripheral Blood
PBS
14.6%
IL-33
26.1%
Cleaved IL-33
21.0%
SSC
FSC
Granulocytes
% events
Eosinophils
% cells
Total cells
cells per ml (x10^6)
Lymphocytes
Monocytes
Neutrophils
Eosinophils
D
Splenocytes
Granulocytes
Neutrophils
Eosinophils
E
Serum
Lung
IL-4
IL-5
IgA
pg / ml
ng / ml
mg / ml
pg / mg
µg / mg
F
Splenocytes
Medium
αCD3
αCD3 + αCD28
αCD3 + PMA
IL-5 (ng / ml)
G
Mesenteric lymph nodes
IL-5 ng / ml

Supplemental Data

Suppression of IL-33 Bioactivity through Proteolysis by Apoptotic Caspases

Alexander U. Lüthi, Sean P. Cullen, Edel A. McNeela, Patrick J. Duriez, Inna S. Afonina, Clare Sheridan, Gabriela Brumatti, Rebecca C. Taylor, Kristof Kersse, Peter Vandenabeele, Ed C. Lavelle, and Seamus J. Martin

SUPPLEMENTAL FIGURES and METHODS

Supplemental Figure S1

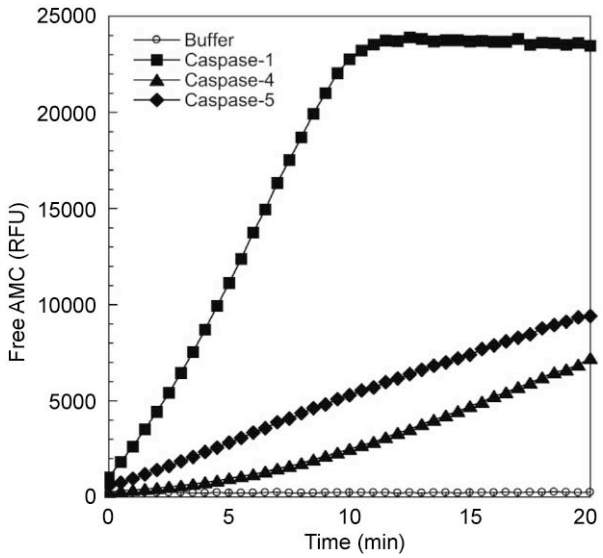
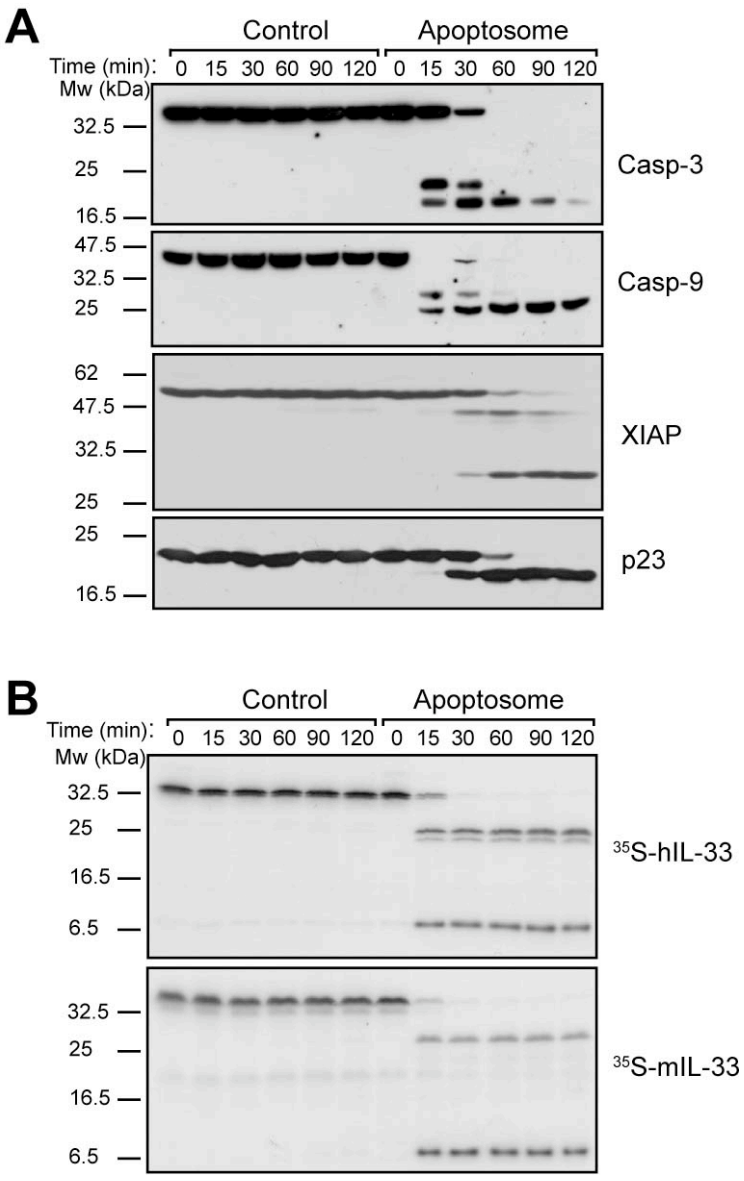


Figure S1. Hydrolysis of a synthetic caspase substrate peptide by recombinant caspases –1, -4 and -5

Hydrolysis of the synthetic caspase substrate, WEHD-AMC, by recombinant caspase-1, -4 and –5 (20nM each). Note that recombinant inflammatory caspases cleave WEHD-AMC with different efficiencies. Active site titrations with zVAD-fmk confirmed that the molar amounts of each caspase were identical (data not shown).

Supplemental Figure S2



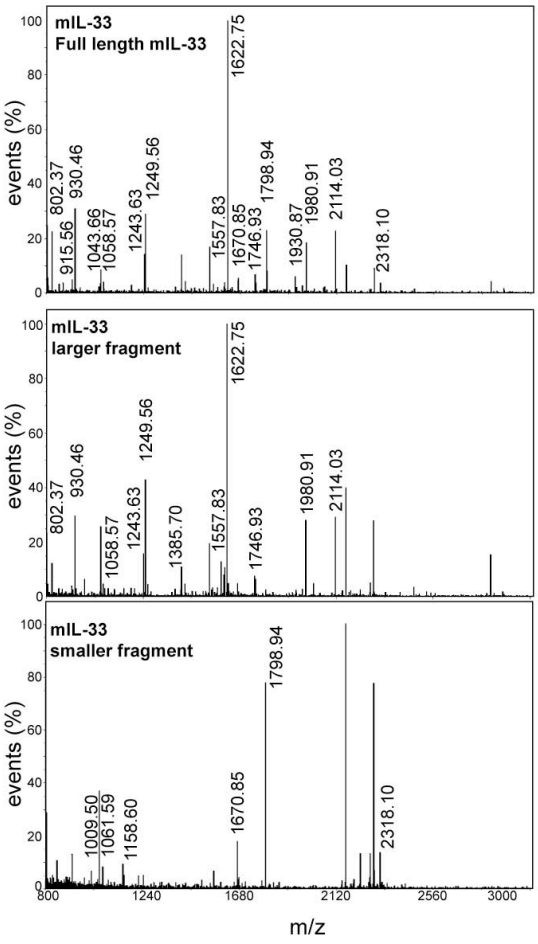
Supplemental Figure S2. Proteolysis of IL-33 in apoptotic Jurkat cell-free extracts

(A) Cell-free extracts derived from Jurkat cells were incubated at 37°C in the presence (Apoptosome) or absence (Control) of cytochrome c/dATP, as indicated, followed by immunoblotting for caspase-3, caspase-9, XIAP or p23.

(B) ^{35}S-labeled hIL-33 or mIL-33 were added to Jurkat cell-free extracts which were treated as described in Figure 2B. Reactions were sampled at the indicated times and were subsequently analysed by SDS-PAGE followed by fluorography.

Supplemental Figure S3

A



Full lenght mIL-33

```
MRPRMKYSNS KISPAKFSST AGEALVPPCK IRRSQQKTKE  40
FCHVYCMRLR SGLTIRKETS YFRKEPTKRY SLKSGTKHEE  80
NFSAYPRDSR KRSLLGSIQA FAASVDTLSI QGTSLLTQSP 120
ASLSTYNDQS VSFVLENGCY VINVDDSGKD QEQDQVLLRY 160
YESPCPASQS GDGVDGKKVM VNMSPIKDTD IWLHANDKDY 200
SVELQRGDVS PPEQAFFVLH KKSSDFVSFE CKNLPGTYIG 240
VKDNQLALVE EKDESCNNIM FKLSKI                266
```

Large fragment

```
MRPRMKYSNS KISPAKFSST AGEALVPPCK IRRSQQKTKE  40
FCHVYCMRLR SGLTIRKETS YFRKEPTKRY SLKSGTKHEE  80
NFSAYPRDSR KRSLLGSIQA FAASVDTLSI QGTSLLTQSP 120
ASLSTYNDQS VSFVLENGCY VINVDDSGKD QEQDQVLLRY 160
YESPCPASQS GDGVDGKKVM VNMSPIKDTD IWLHANDKDY 200
SVELQRGDVS PPEQAFFVLH KKSSDFVSFE CKNLPGTYIG 240
VKDNQLALVE EKDESCNNIM FKLSKI                266
```

Small fragment

```
MRPRMKYSNS KISPAKFSST AGEALVPPCK IRRSQQKTKE  40
FCHVYCMRLR SGLTIRKETS YFRKEPTKRY SLKSGTKHEE  80
NFSAYPRDSR KRSLLGSIQA FAASVDTLSI QGTSLLTQSP 120
ASLSTYNDQS VSFVLENGCY VINVDDSGKD QEQDQVLLRY 160
YESPCPASQS GDGVDGKKVM VNMSPIKDTD IWLHANDKDY 200
SVELQRGDVS PPEQAFFVLH KKSSDFVSFE CKNLPGTYIG 240
VKDNQLALVE EKDESCNNIM FKLSKI
```

Figure S3. Mass spectrometry analysis of caspase cleaved IL-33
Recombinant GST-IL-33 was cleaved by caspase-7, as depicted in Figure 2E, followed by analysis of the cleavage products by MALDI-TOF mass spectrometry. Mass spectrograms for each IL-33 species (i.e. full length, large and small fragments) are shown, along with the corresponding peptide coverage of each. The peptide coverage (highlighted in bold) of each IL-33 species indicates that the site of caspase-7-mediated proteolysis lies between residues 159 and 187.

Supplemental Figure S4

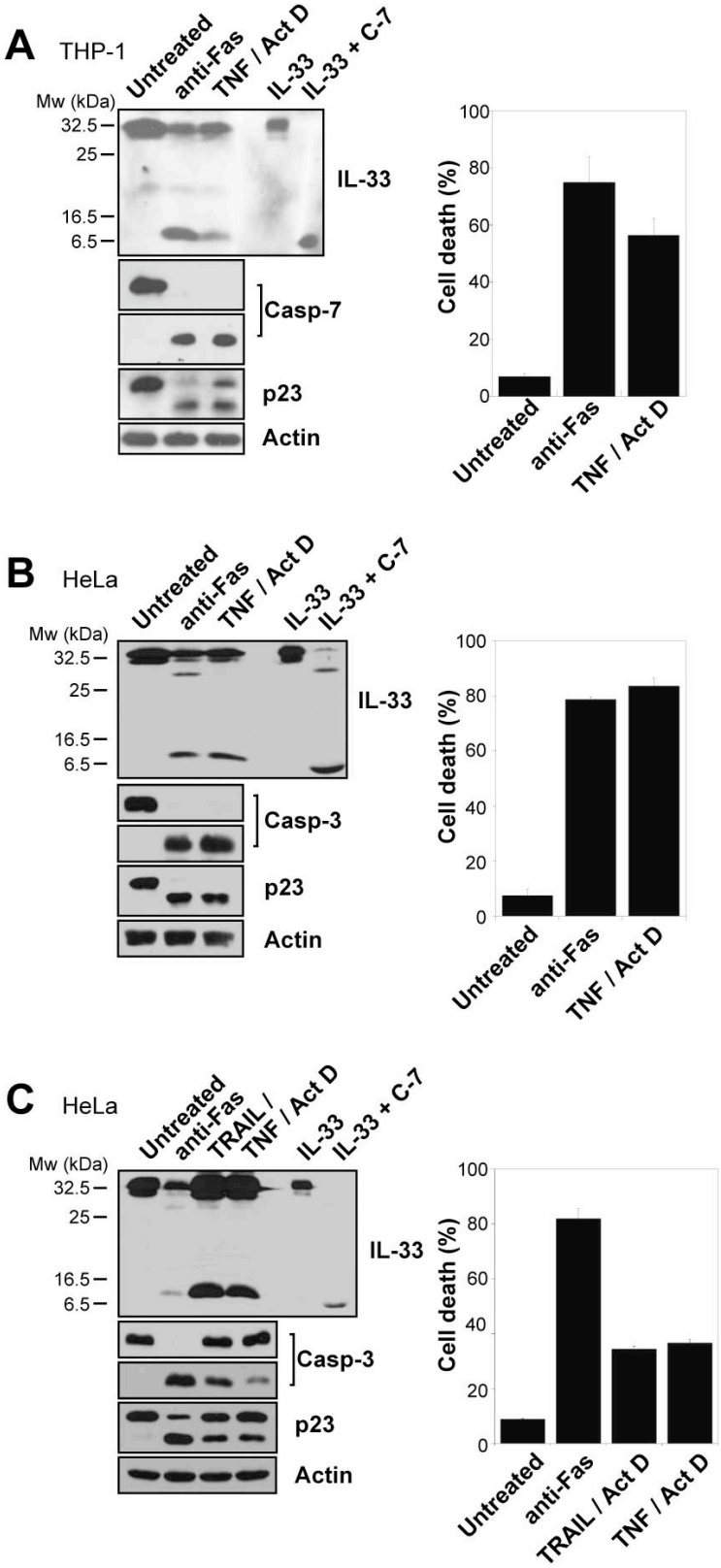


Figure S4. Proteolytic processing of IL-33 during apoptosis induced by death receptor ligands

(A) THP-1 cells were treated with LPS (5 µg/ml) and PMA (50 nM) for 20 h to induce expression of endogenous IL-33, MG132 (2 µM) was then added to stabilize IL-33. After a further 6 h, cells were treated with anti-Fas CH11 IgM antibody (2.5 µg/ml), or TNF (500 ng/ml) plus act D (300 nM), to induce apoptosis. 30 h after addition of pro-apoptotic stimuli, apoptosis was quantitated by direct cell counts using standard morphological criteria (counts were performed on triplicate fields of a minimum of 100 cells per treatment). Cell lysates were then generated and immunoblotted for the indicated proteins. As controls, in vitro transcribed/translated full length IL-33 and caspase-7-cleaved full length IL-33 were included to facilitate size comparison.

(B) HeLa cells were transfected with 1 µg of expression plasmid encoding human FLAG epitope-tagged IL-33. 24 h later, MG132 (1 µM) was added to stabilize IL-33, followed by treatment with with anti-Fas CH11 IgM antibody (0.5 µg/ml), or TNF (200 ng/ml) plus act D (300 nM), for 12 h to induce apoptosis. Apoptosis was quantitated by direct cell counts using standard morphological criteria (counts were performed on triplicate fields of a minimum of 100 cells per treatment). Cell lysates were then generated and immunoblotted for the indicated proteins. As controls, in vitro transcribed/translated full length IL-33 and caspase-7-cleaved full length IL-33 were included to facilitate size comparison.

(C) HeLa cells were transfected with 1 µg of expression plasmid encoding human FLAG epitope-tagged IL-33. 24 h later, MG132 (1 µM) was added to stabilize IL-33, followed by treatment with with anti-Fas CH11 IgM antibody (0.5 µg/ml), TRAIL (100 ng/ml) plus act D (300 nM), or TNF (200 ng/ml) plus act D (300 nM), for 12 h to induce apoptosis. Apoptosis was quantitated by direct cell counts using standard morphological criteria (counts were performed on triplicate fields of a minimum of 100 cells per treatment). Cell lysates were then generated and immunoblotted for the indicated proteins. As controls, in vitro transcribed/translated full length IL-33 and caspase-7-cleaved full length IL-33 were included to facilitate size comparison.

Supplemental Figure S5

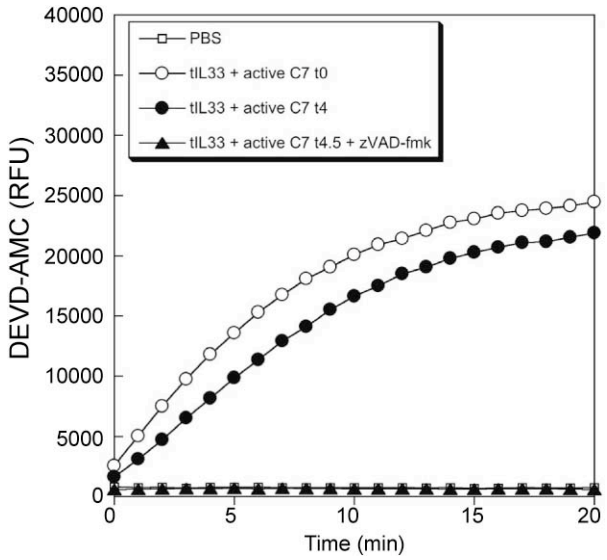


Figure S5. Elimination of residual caspase activity in caspase-treated IL-33 preparations after treatment with an irreversible caspase inhibitor.
Purified recombinant IL-33 was incubated with 300 nM caspase-7 at 37°C for 4 hours then 30 minutes with 2 μM zVAD-fmk on ice. IL-33 pre- (open circles), post- caspase (closed circles) and post zVAD-fmk (filled triangles) addition was assayed for DEVDase activity.

Supplemental Figure S6

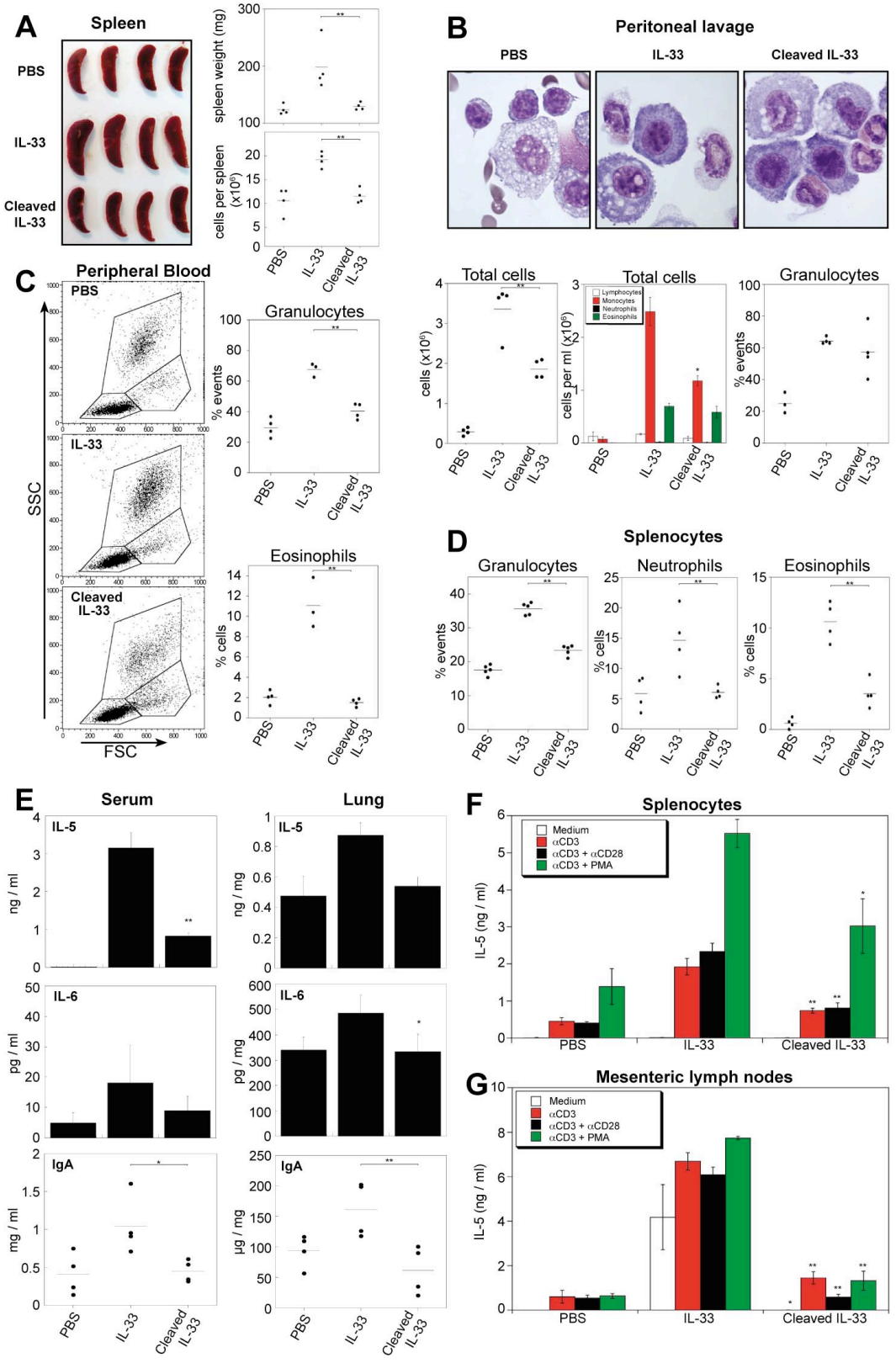


Figure S6. Attenuation of IL-33 bioactivity through caspase-dependent proteolysis.

To ensure that residual caspase activity in caspase-treated IL-33 preparations did not contribute to the reduced biological effects seen, recombinant IL-33 was treated for 4 h with either heat-inactivated caspase-7 (300 nM), or active caspase-7 (300 nM), followed by addition of 2 μM of the irreversible caspase inhibitor, zVAD-fmk, to both IL-33 preparations to eliminate any residual caspase activity. Quenching of caspase activity was confirmed by assessing hydrolysis of the synthetic caspase substrate, DEVD-AMC, as shown in Figure S4. BALB/c mice (n=4 per group) were then injected (i.p.) with either PBS, or zVAD-fmk-treated IL-33 (0.8 μg per day per mouse), or zVAD-fmk-treated cleaved IL-33 (0.8 μg per mouse), for 5 consecutive days.

(A) Spleen size, weight and cellularity for each group of mice are shown.

(B) Peritoneal lavage-derived cells were enumerated from cytospins as described in Fig 6B. Percentages of individual cell types were multiplied by the total number of cells (bottom middle panel) and granulocyte numbers were also determined by FSC/SSC analysis.

(C) Peripheral bloods were analysed as described in Fig 6C.

(D) Spleen-derived granulocytes were enumerated as described in Fig 6C and neutrophil and eosinophil numbers were scored on H&E-stained cytospin preparations.

(E) IL-5, IL-6 and IgA levels were determined by ELISA in plasma samples or lung homogenates, as indicated.

Splenocytes (F) and mesenteric lymph node cells (G) (10^6 cells/ml) were restimulated either with medium, 1 μg/ml anti-CD3, 1μg/ml anti-CD3 and 1 μg/ml anti-CD28, or 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.

Supplemental Figure S7

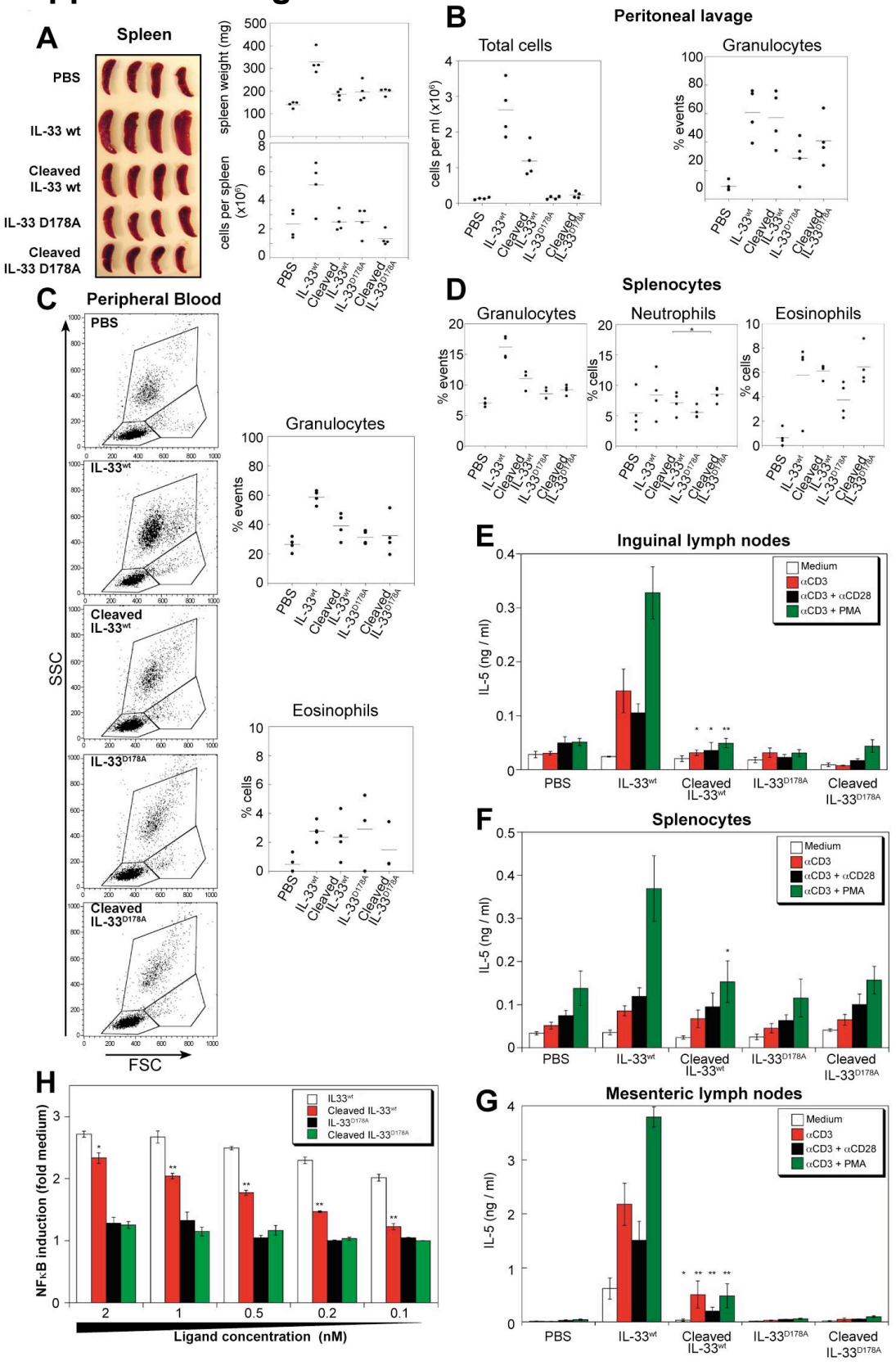


Figure S7. Mutation of the caspase cleavage site Asp178 is sufficient to abolish IL-33 biological activity in vivo.

BALB/c mice (4 per treatment group) were injected (i.p.) either with PBS, IL-$33^{112\text{-}270}$, caspase-cleaved IL-$33^{112\text{-}270}$, IL-$33^{112\text{-}270}$ D178A mutant, or caspase-7-treated IL-$33^{112\text{-}270}$ D178A mutant. All IL-33 treatment groups received 0.8 µg per mouse per day for 5 consecutive days.

(A) Spleen size, weight and cellularity for each group of mice are shown.

(B) Peritoneal lavage-derived cells were enumerated by haemocytometer counting. Granulocyte numbers were determined by FSC/SSC analysis.

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

(D) Spleen-derived granulocytes were enumerated by flow cytometry as described in (C), and neutrophil and eosinophil numbers were scored on H&E-stained cytospin preparations.

Inguinal lymph node-derived lymphocyte (E), splenocytes (F), and mesenteric lymph node-derived lymphocytes (G), were restimulated either with medium, 1 µg/ml anti-CD3, 1µg/ml anti-CD3 and 1 µg/ml anti-CD28, or 1 µg/ml anti-CD3 and 20 ng/ml PMA, as indicated. Supernatants were collected after 3 days and IL-5 concentration was determined by ELISA.

(H) The indicated amounts of untreated or caspase-treated $IL33^{wt}$ or $IL33^{D178A}$ mutant protein was added to the HEK ST2-NFκB reporter cell line as described in Fig 5A.

SUPPLEMENTAL METHODS

Cell-free reactions

Cell-free extracts were generated from exponentially growing healthy THP-1 cells as described previously (Murphy et al., 2003; Slee et al., 1999). Briefly, THP-1 cells were treated for 5 hours with 1 µg/ml LPS then harvested by centrifugation at 800 x g into a Dounce-type homogenizer. Three volumes of ice-cold cell extract buffer were added CEB (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 100 µM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin), and the cells were allowed to swell for 15–20 min on ice. Cells were then lysed by homogenization with 10–15 strokes of a B-type pestle. Lysates were clarified by centrifugation at 15,000 x g for 30 min to remove nuclei, mitochondria, and other cellular debris. Extracts were then aliquoted and frozen at -70ºC prior to use. For *in vitro* activation of caspases involved in inflammation, THP-1 cell-free extracts were diluted to 80% in CEB and incubated at 37ºC for 2 hours. To provoke apoptosome-dependent caspase activation, bovine heart cytochrome c and dATP were added to reactions to final concentrations of 50 µg/ml and 1 mM, respectively.

Expression and purification of recombinant caspases

Caspase-1.p30, Caspase-4.p30, Caspase-5.p30, Caspase-3 and Caspase-7 were produced by PCR-mediated amplification of the relevant coding sequences from the respective full-length cDNA, followed by subcloning of the resulting PCR products in-frame with the His coding region of pET15b (Novagen, UK) for Caspase-1, pet23b (Novagen, UK) for Caspase-3 and -7, or pGEX4T1 (Amersham, UK) for Caspase-4 and –5. Plasmids encoding His-tagged and were transformed into Escherichia coli DH5α, GST-tagged fusion proteins were transformed into BL21pLysS and bacteria were induced to express the recombinant proteins in the presence of 0.1 to 0.6 mM IPTG (Melford, UK). Recombinant caspases were subsequently purified using Ni2+ beads (Qiagen, UK) or Glutathione Sepharose 4B (Amersham, UK) according to standard procedures.

Expression and purification of recombinant IL-33

GST-IL-33 was generated by inserting the human IL-33 coding sequence into the pGEX4T2 bacterial expression vector. GST-IL-33 was expressed by addition of 100 µM IPTG to exponentially growing cultures of DH5α strain *E. coli* followed by incubation for 1 h at room temperature. Bacteria were lysed by sonication and GST-IL-33 was captured using Glutathione Sepharose 4B, followed by elution into PBS, pH 7.2, in the presence of 20 mM reduced gluthathione. Protein was then extensively dialysed against PBS prior to use. His-tagged IL-33 deletion mutants were generated through inserting the relevant coding sequences into pET45b (Novagen, UK), followed by expression in BL21pLysS strain *E. coli* and were purified using Ni2+ beads (Qiagen, UK) according to standard procedures. Proteins were then eluted from the affinity capture matrix and were extensively

dialysed against PBS, pH 7.2, followed by depletion of LPS by several rounds of incubation to agarose-immobilized polymyxin B (Sigma). For caspase-mediated processing, recombinant IL-33 was incubated with recombinant caspase-7 (300nM) for 4 h at 37ºC, or with an identical concentration of heat-inactivated caspase-7 as a control.

Transient transfection and reporter gene assays

HeLa cells were seeded at a density of 2 x 10^5 cells per well of a 6-well tissue culture plate and were transiently transfected 24 h later with GeneJuice (Merck, Ireland), according to the manufacturer's instructions. HEK293T cells were seeded at a density of 4 x 10^5 cells per well of a 6-well tissue culture plate 24 h prior to transfection. Cells were transfected with plasmids according to the standard calcium phosphate precipitation method, and DNA complexes were allowed to remain on cells for 14 h before replacing with fresh medium. For the luciferase reporter assay the cells were lysed in 200 µl RLS (100 mM HEPES, pH 8, 2 mM $MgCl_2$, 2% Triton X-100) of which 20 µl were assayed with 50 µl LAR (20mM N-Glycylglycine, 1mM $MgCl_2$, 100 µM EDTA, 27.8 µg/ml ATP, 21.3 µg/ml Coenzyme A and 160 µg/ml beetle luciferin (Promega, UK). Luminescence was measured in a Spectrafluor Plus (TECAN, UK).

Pulldown and immunprecipitation assays

GST-IL-33 (1 µg) was immobilised on 30 µl Glutathione Sepharose 4B (Amersham Biosciences, UK) by rotation at 4ºC for 30 minutes, followed by addition of 1 µg of recombinant ST2.Fc (Alexis, UK) and further incubation for 4 h in 1 ml of reaction buffer (50 mM Tris, pH 7.6, 120 mM NaCl, 0.1% CHAPS). The reciprocal pulldown experiments were performed under essentially the same conditions.

For immunoprecipitation of endogenous IL-33, THP-1 cells (10^8) were treated with LPS (5 µg/ml) and PMA (50 nM) for 24 h, followed by addition of MG132 (5 µM) and incubation for a further 6 h to stabilize endogenous IL-33. Cells were then lysed in buffer containing 150 mM NaCl, 50 mM Tris pH 8, 1 % NP-40, 0.1 % SDS. The clarified supernatant was used for immunprecipitation by adding 3 µg of control or anti-IL-33 antibody and 30 µl A/G agarose (Santa Cruz). After overnight incubation, the beads were washed extensively, and samples were electrophoresed on 13 % SDS-PAGE followed by immunoblotting.

Caspase activity assays

For the assessment of caspase activity, recombinant caspases -1, -3, -4 and –5 and -7 were diluted to a final volume of 50 µl in CEB containing 50 µM Ac-WEHD-AMC (for the inflammatory caspases) or Ac-DEVD-AMC (for apoptotic caspases). Samples were then measured over 20 minutes in an automated fluorimeter (Spectrafluor Plus, TECAN, UK) at wavelengths of 360 nm (excitation) and 465 nm (emission). Caspases were active site titrated by incubation with a range of concentrations (0, 6.25, 12.5, 25, 50, 100 nM) of the poly-caspase inhibitor zVAD-fmk for 30 min at 37ºC, followed by measurement of

residual caspase activity by monitoring the hydrolysis of WEHD-AMC or Ac-DEVD-AMC as described above.

Coupled *in vitro* transcription/translation reactions

In vitro transcription/translation reactions were carried out using purified plasmid templates added to a rabbit reticulocyte lysate system (Promega, UK) with ^{35}S-Methionine (Amersham, UK) as described previously (Slee et al., 1999).

Analysis of peripheral blood, spleen and peritoneal lavage samples

Blood was collected from the tail vein followed by addition of 100 µM EDTA as an anticoagulant. Red blood cells were lysed in 20 volumes of 150 mM NH_4CL, 10mM $NaHCO_3$ and 100 µM EDTA. Cytospins were prepared and stained with hematoxylin/eosin and scored for lymphocytes, monocytes, neutrophils and eosinophils. For flow cytometry, cells were fixed in FACS lysis buffer (BD, UK) according to the manufacturer's instructions and analysed on a FACScalibur (BD, UK).

IL-33-ST2 cell binding assay

HEK293 cells stably over-expressing the ST2L receptor were incubated with GST, GST-IL-33 or caspase-7-cleaved GST-IL-33 at concentrations indicated in the figure legends for 1 h followed by immunostaining with anti-GST or anti-IL-33 antibodies. Binding of IL-33 was detected using flow cytometry analysis.

SUPPLEMENTAL REFERENCES

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