Supporting Information

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SI Results

MS Analysis of IL-33 Processing by Neutrophil Serine Proteases. Recombinant IL-33, expressed in *E. coli* as a GST fusion, was purified on glutathione-Sepharose beads and digested on beads with either elastase or cathepsin G, and cleavage products were separated from full-length protein by 1D SDS/PAGE. To identify the new N terminus of these cleavage products, a quantitative proteomic mapping was performed: gel bands corresponding respectively to the full-length protein (control) and to the cleaved IL-33 were excised, further in-gel digested with a specific enzyme (trypsin or Glu-C), and the resulting smaller peptides were identified and quantified by MS. Peptides originating from the C-terminal part (IL-1 domain) were detected both in the full-length and cleaved proteins, and peptides originating from the N-terminal part of the protein (amino acids 1–90) were specifically detected in full-length IL-33. Only one peptide was specifically detected in the cleavage product (Fig. S1 *B*, *D*, and *F*, red spot on the intensity plots), and this peptide was, therefore, assigned as the new N terminus of the protein processed by elastase or cathepsin G. The sequences of these peptides were deduced from fragmentation MS/MS spectra acquired during the analysis (Fig. S1 *A*, *C*, and *E*).

Specific Tryptic or Glu-C Fragments from the Proteomic Mapping. Table S1 summarizes the results by listing the specific tryptic or Glu-C fragments expected from the proteomic mapping, encompassing the cleavage sites identified for the two neutrophil proteases. The number of MS/MS spectra acquired for each species reflects the abundance of the peptide. Short peptides corresponding to the new N terminus were specifically detected, with many MS/MS sequencing events only in the processed forms of IL-33.

SI Materials and Methods

Plasmid Construction and Protein Production. IL-33 deletion mutants were amplified by PCR using the human (NM_033439) and mouse (NM_133775) IL-33/NF-HEV cDNAs (1, 2) as templates. The PCR fragments, thus obtained, were cloned into plasmid pcDNA3.1 (Invitrogen). The human IL-33_{F94G}, IL-33_{I98G}, IL-33_{V101G}, and IL-33_{L108G} mutants were generated by PCR and cloned into the same expression vector. All primer sequences are available upon request. Wild-type and mutant IL-33 proteins were synthesized in vitro in RRL using the TNT-T7 kit (Promega). Human IL-$33_{1–270}$ was also synthesized using WGEs (Promega) and HES (Pierce). For expression in *Escherichia coli*, cDNAs encoding mature forms IL-$33_{95–270}$, IL-$33_{99–270}$, and IL-$33_{109–270}$ were subcloned into expression vector pET-15b (Novagen). Recombinant proteins were produced in *E. coli* BL21pLysS (Novagen) and purified using Ni-NTA agarose column (Qiagen). His tag was removed by cleavage with thrombin, and proteins were further purified by gel filtration (FPLC; GE Healthcare). Endotoxin levels were <0.01 EU/μg of protein, as determined by the *Limulus* amebocyte lysate QCL-1000 method (Lonza).

Protein Cleavage Assays with Neutrophil Proteases and Isolated Neutrophils. In vitro-translated proteins (2-5 μL lysate) were incubated with neutrophil elastase (0.3 U; Calbiochem), cathepsin G (1 mU; Calbiochem), or PR3 (70 μU; Calbiochem) in 15 μL of assay buffer (2–5 μL of RRL lysate plus 10 μL of PBS) for 30 min to 1 h at 37 °C. Endogenous native IL-33 protein isolated from human endothelial cell freeze-thaw extracts (3) was used in some experiments. Cleavage assays with activated neutrophils (6×10^4–10^6 neutrophils; 15 min to 2 h at 37 °C) were performed using human neutrophils from healthy blood donors (Etablissement Français du sang; Contract 21/PVNT/TOU/IPBS01/2009-0052), isolated using Polymorphprep cell separation media (Axis-Shield) and activated with PMA (25 mM; 2 h), or mouse neutrophils isolated by Percoll density gradient from femur and tibia bone marrow and activated by stimulation with cytochalasin B (5 μg/mL; 15 min) and fMLP (40 μM; 3 h). In some experiments, neutrophils or neutrophil supernatants were incubated with serine protease inhibitor AEBSF (1–8 mM; Calbiochem) or Cathepsin G Inhibitor I and Elastase Inhibitor IV (50 μM; Calbiochem). Cleavage products were analyzed by SDS/PAGE and Western blot.

Western Blot Analysis. Proteins were fractionated by SDS/PAGE, electroblotted, and detected with mAb to human IL-33-Cter (305B; 1/1,000; Alexis Biochemicals), rabbit antiserum to human IL-33-Nter [IL-$33_{1–15}$; 1/400 (1, 2)] or goat antiserum to mouse IL-33-Cter (AF326; 1/500; R&D Systems), followed by HRP-conjugated goat anti-mouse, goat anti-rabbit, or donkey anti-goat polyclonal antibodies (1/10,000; Promega), and finally an enhanced chemiluminescence kit (GE Healthcare). Quantitative IR Western blots were performed to quantify the four IL-33 forms used in cellular bioassays, using the IL-33-Cter mAb 305B, goat anti-mouse IgG IRDye800 secondary antibody (610-132-121; 1/10,000; Rockland Immunochemicals), and Odyssey IR Imager (LI-COR Biosciences). A standard curve was performed using purified recombinant IL-$33_{95–270}$ protein.

IL-33 Activity Assays. In vitro-translated full-length IL-33 or mature forms (5 μL lysate/well; 24-h treatment) were used to stimulate IL-33-responsive MC/9 mast cells (ATCC; 10^5–2×10^5 cells/well in 96-well plates) (3) and KU812 basophil-like chronic myelogenous leukemia cells (ATCC; 5×10^5 cells/well in 96-well plates) (4). Cytokine levels in supernatants were determined using DuoSet IL-6 and IL-5 ELISAs (R&D Systems).

Animals. BALB/c and C57BL/6 wild-type mice were purchased from Charles River Laboratories. Female BALB/c mice received daily i.p. injections of 4 μg of recombinant human IL-$33_{95–270}$, IL-$33_{99–270}$, IL-$33_{109–270}$, or saline for 7 d. Blood and histologic analyses were performed on day 8. Acute lung injury was induced in female C57BL/6 wild-type or *IL-33*$^{-/-}$ mice (8–10 wk old) by i.v. injection of OA (0.8 μL/g body weight; Sigma) in a 15% solution with 0.1% BSA. Lung histology and BAL fluid were analyzed 2 h after OA injection. All mice were bred under specific pathogen-free conditions and handled according to institutional guidelines under protocols approved by the IPBS and "Région Midi-Pyrénées" animal care committees.

Histology. Histological evaluation was performed on formalin-fixed mouse tissues. Five-micron paraffin-embedded tissue sections (jejunum) or cryosections (lung) were prepared and stained with hematoxylin and eosin for morphological evaluation. Periodic acid Schiff and alcian blue staining was used to detect the presence of mucus in jejunum tissue sections.

Analysis of Blood, Spleen, Lung, BAL, and Serum Samples. Peripheral blood was obtained by cardiac puncture and stored in EDTA-containing tubes at 4 °C until analysis with an automated hematological analyzer (ABX Micros 60). Spleens and lungs were collected, and the weights determined. Two hours after OA injection, mice were killed, and the lungs were lavaged in situ with 300 μL of PBS. The resultant BAL fluid was analyzed for protein content (Nanodrop 1000 Spectrophotometer; Thermo Fischer), leukocyte May–Grunwald–Giemsa staining of cytospins, and

presence of endogenous IL-33 forms (Western blot). IL-5 cytokine levels in serum were determined using a Cytometric Bead Array Kit (BD Biosciences).

Mapping of Cleavage Sites by MS. To map cleavage sites after processing by neutrophil proteases, recombinant IL-33 was expressed in *E. coli* Rosetta 2 (Novagen) as a GST fusion protein (pGEX-2T vector; GE Healthcare), purified on glutathione-Sepharose beads and digested on beads with either purified cathepsin G (0.25 mU) or neutrophil elastase (0.12 U). Beads were eluted with Laemli buffer, and the resulting fragments, as well as control nondigested GST-IL33, were analyzed by 1D SDS/PAGE. For the cathepsin G experiment, two cleavage products were detected specifically after digestion with the protease and excised from the gel. In the case of elastase digestion, one major cleavage product was detected, and the band was cut from the gel. To identify the corresponding cleavage sites, these three processed fragments were further in-gel digested with specific enzymes into small peptides that were then extracted from the gel and analyzed by MS. Mapping was performed either with trypsin or with endoproteinase Glu-C, to extend the protein sequence coverage and generate MS-detectable peptides in the cleavage region. To confidently assign the N terminus of each processed fragment, a comparative mapping was performed between each cleavage product and the full-length protein excised from the control gel lane. Before digestion, gel bands were washed by cycles of incubation in 100 mM ammonium bicarbonate/acetonitrile (1:1), and cysteine residues were reduced and alkylated in-gel with iodoacetamide. Proteins were then digested by 0.2 μg of modified sequencing grade trypsin (Promega) or 0.4 μg of Glu-C (Sigma) in 50 mM ammonium bicarbonate (5 h at 37 °C). The resulting peptides were extracted from the gel by 10% formic acid/acetonitrile (1:1), dried in a speed-vac (miVac sample concentrator, Genevac), and analyzed by nano-LC-MS/MS using an Ultimate3000 system (Dionex) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific). Peptides were separated on C-18 column (75 μm i.d. × 15 cm; PepMap C18; Dionex) using a 60-min gradient of acetonitrile at 300 nL/min flow rate. The LTQ-Orbitrap Velos (Velos) was operated in data-dependent acquisition mode with the XCalibur software. Survey scan MS were acquired in the Orbitrap on the 300–2,000 *m/z* range with the resolution set to a value of 60,000. The 20 most intense ions per survey scan were selected for MS/MS fragmentation and the resulting fragments were analyzed in the linear trap (LTQ). Dynamic exclusion was used within 60 s to prevent repetitive selection of the same peptide. The Mascot Daemon software (Matrix Science) was used to perform searches against a database containing all *E. coli* entries from Uniprot and the GST-IL33 fusion protein sequence (63,080 total sequences). Carbamidomethylation of cysteines was set as a fixed modification, and oxidation of methionine and protein N-terminal acetylation were set as variable modifications. Specificity of digestion was set for cleavage after K or R for trypsin and after E for Glu-C. Two missed cleavage sites were allowed, as well as semispecific cleavages to enable identification of the N-terminal peptide from processed fragments. The mass tolerances in MS and MS/MS were set to 5 ppm and 0.8 Da, respectively. Only MS/MS peptide sequence matches with Mascot score higher than 20 were considered for identification of the peptides. Quantification of peptides from cleavage products and full-length IL33 was performed using the MFPaQ software.

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2. Carriere V, et al. (2007) IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. *Proc Natl Acad Sci USA* 104:282–287.
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4. Tare N, et al. (2010) KU812 cells provide a novel in vitro model of the human IL-33/ST2L axis: functional responses and identification of signaling pathways. *Exp Cell Res* 316:2527–2537.

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Fig. S1. MS analysis of IL-33 processing by neutrophil elastase and cathepsin G. (*A*, *C*, and *E*) Typical annotated MS/MS spectra identifying N-terminal peptides from IL-33 cleavage products after treatment of purified recombinant IL-33-GST fusion protein with cathepsin G (*A* and *C*) or elastase (*E*). (*B*, *D*, and F) Intensity plots of peptides from cleavage products versus full-length IL-33. Peptides derived from trypsin- or Glu-C-specific enzymatic cleavage of cleavage products or full-length protein were analyzed by MS and quantified based on their extracted ion chromatogram (XIC) area. Specific, highly intense peptides, observed only in the processed fragments and characteristic of the cleavage sites are indicated in red: (*i*) fragment 1 obtained after cathepsin G processing of IL-33, proteomic mapping with trypsin (*B*); (*ii*) fragment 2 obtained after cathepsin G processing of IL-33, proteomic mapping with Glu-C (*D*); and (*iii*) major fragment obtained after elastase processing of IL-33, proteomic mapping with Glu-C (*F*).

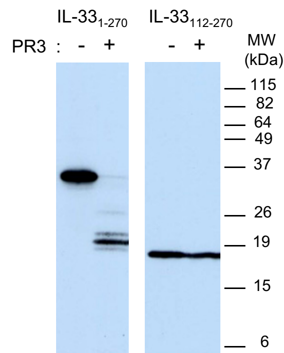

Fig. S2. IL-33 is a substrate for neutrophil proteinase 3. In vitro-translated IL-33$_{1–270}$ and IL-33$_{112–270}$ proteins were incubated with purified neutrophil proteinase 3 (PR3) (4.66 μU/μL; 30 min at 37 °C). Proteins were separated by SDS/PAGE and revealed by Western blot with anti-IL-33-Cter mAb 305B. Blots are representative of at least two independent experiments.

Table S1. List of the specific tryptic or Glu-C fragments expected from the proteomic mapping and containing respectively the two cleavage sites identified for cathepsin G and the main cleavage site identified for elastase

Protease	IL-33 fragment	IL-33 peptide	IL-33 peptide sequence	No. of MS/MS
Cathepsin G, site 1	Full-length IL-33	Peptide 79–103	HLVLAACQQQSTVECFAFGISGVQK	80
		Peptide 95–103	AFGISGVQK	0
	IL-33 cleaved by cathepsin G Fragment 1	Peptide 79–103	HLVLAACQQQSTVECFAFGISGVQK	2
		Peptide 95–103	AFGISGVQK	51
Cathepsin G, site 2	Full-length IL-33	Peptide 93–121	CFAFGISGVQKYTRALHDSSITGISPITE	0
		Peptide 109–121	HDSSITGISPITE	0
	IL-33 cleaved by cathepsin G Fragment 2	Peptide 93–121	CFAFGISGVQKYTRALHDSSITGISPITE	0
		Peptide 109–121	HDSSITGISPITE	34
Elastase	Full-length IL-33	Peptide 93–121	CFAFGISGVQKYTRALHDSSITGISPITE	0
		Peptide 99–121	SGVQKYTRALHDSSITGISPITE	0
	IL-33 cleaved by elastase	Peptide 93–121	CFAFGISGVQKYTRALHDSSITGISPITE	0
		Peptide 99–121	SGVQKYTRALHDSSITGISPITE	11

Although the long peptide encompassing the cleavage site and expected from the tryptic or Glu-C mapping was not always detected for the full-length protein (probably due to low ionization efficiency or poor extraction from the gel), the short peptide corresponding to the N terminus of the processed forms of IL-33 was identified with many MS/MS spectra, specifically during analysis of the cleavage products.