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Mast Cell Chymase Degrades the Alarmins Heat Shock Protein 70, Biglycan, HMGB1, and Interleukin-33 (IL-33) and Limits Danger-induced Inflammation*

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Background: Mast cell chymase may be both pro-inflammatory and anti-inflammatory during infection and tissue damage.
Results: Human and mouse chymases modulate extracellular levels of the alarmins Hsp70, biglycan, HMGB1, and IL-33.
Conclusion: Mast cell chymase degrades alarmins and may limit inflammation.
Significance: Identifying the physiological chymase substrates is crucial for understanding the role of chymase in immune responses and could aid in drug development.

During infection and tissue damage, virulence factors and alarmins are pro-inflammatory and induce activation of various immune cells including macrophages and mast cells (MCs). Activated MCs instantly release preformed inflammatory mediators, including several proteases. The chymase mouse mast cell protease (MCPT)-4 is thought to be pro-inflammatory, whereas human chymase also degrades pro-inflammatory cytokines, suggesting that chymase instead limits inflammation. Here we explored the contribution of MCPT4 and human chymase to the control of danger-induced inflammation. We found that protein extracts from wild type (WT), carboxypeptidase A3-, and MCPT6-deficient mice and MCs and recombinant human chymase efficiently degrade the *Trichinella spiralis* virulence factor heat shock protein 70 (Hsp70) as well as endogenous Hsp70. MC-(W^{sash})-, serglycin-, NDST2-, and MCPT4-deficient extracts lacked this capacity, indicating that chymase is responsible for the degradation. Chymase, but not MC tryptase, also degraded other alarmins, *i.e.* biglycan, HMGB1, and IL-33, a degradation that was efficiently blocked by the chymase inhibitor chymostatin. IL-7, IL-22, GM-CSF, and CCL2 were resistant to chymase degradation. MCPT4-deficient conditions *ex vivo* and *in vivo* showed no reduction in added Hsp70 and only minor reduction of IL-33. Peritoneal challenge with Hsp70 resulted in increased neutrophil recruitment and TNF-α levels in the MCPT4-deficient mice, whereas IL-6 and CCL2 levels were similar to the levels found in WT mice. The rapid and MC chymase-specific degradation of virulence factors and alarmins may depend on the presence of accessible extended recognition cleavage sites in target substrates and suggests a protective and regulatory role of MC chymase during danger-induced inflammation.

Mast cells (MCs)[3] are thought to play a role in surveillance and maintenance of the tissue and to regulate important functions in innate and adaptive immunity. By lining the body surfaces, MCs provide a rapid, almost instant, response to infections and injury. In this context MCs have been shown to protect us against bacterial and parasitic attacks, poisonous bites from snakes and lizards, as well as poisonous stings from the honeybee and scorpions (1–4). MCs have also been suggested to play a role in tissue homeostasis, by regulation of intestinal epithelial barrier function and by degradation/inactivation of endogenous proteins such as fibronectin, endothelin, neurotensin, and vasointestinal peptide (4–8). Upon activation MCs release a number of preformed mediators by degranulation, rapidly synthesize arachidonic acids, and start synthesis and secretion of many cytokines (8, 9). Of the preformed mediators, including histamine, proteoglycans (heparin), and proteases, the MC-specific proteases may play negative or positive roles in disease. For example they have been associated with a destructive role in arthritis, but a protective role in asthma (10–12). The MC-specific proteases are normally divided into three groups, the chymases, the tryptases, and the carboxypeptidase A3 (CPA3). In humans, MCs can be classified by their protease content, where mucosal MCs express only tryptase (MC_T) and

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[3] The abbreviations used are: MC, mast cell; MCPT, mast cell protease; Hsp, heat shock protein(s); CPA, carboxypeptidase A; TLR, Toll-like receptor; HMGB1, high mobility group protein B1; PCMC, peritoneal cell-derived mast cell; STP, soluble *T. spiralis* proteins; DAMP, danger-associated molecular pattern; HC, human chymase; rHC, recombinant human chymase; rHT, recombinant human tryptase; rh, recombinant human; rm, recombinant mouse.

connective tissue MCs express tryptase, chymase, and carboxypeptidase A3 (MC_{TC}). In the mouse, mucosal MCs express the chymases, mast cell protease (MCPT) 1 and MCPT2, whereas connective tissue type MCs expresses the chymase MCPT4 and the elastase MCPT5, the tryptases MCPT6 and MCPT7, and CPA3.

In vivo the connective tissue type MC proteases show various degrees of dependence on the serglycin proteoglycan heparin for granular storage and after degranulation. At least chymase, which performs its function in a macromolecular complex with heparin, also depends on heparin for enhanced enzymatic activity. In addition, the highly negatively charged glycosaminoglycan chains may attract positively charged proteins that thus become potential substrates for the chymase (13–16). The fine substrate specificity of the MC chymases has been extensively investigated by phage display, and the human chymase and MCPT4 prefer to cleave protein substrates that contain aromatic amino acids in the P1 position and acidic amino acids in the P2′ position (17, 18).

Infectious agents carry a set of factors called pathogen-associated molecular patterns (PAMPs) that innate cells recognize and respond to via pattern recognition receptors, *e.g.* Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NODs), RIG-1, and MDA-5. Engagement of the pattern recognition receptors on innate cells, such as MCs and macrophages, may cause release of inflammatory mediators, *e.g.* proteases, leukotrienes, and cytokines, which promote the recruitment of leukocyte effector cells and the development of an adaptive immune response (19). However, stress and injury may also cause release of several potent endogenous factors, *i.e.* danger-associated molecular patterns (DAMPs, from here referred to as alarmins) from damaged and necrotic cells that may induce a sterile type of inflammation via pattern recognition receptors. Alarmins released during injury include *e.g.* the high mobility group protein B1 (HMGB1) and cytokines of the IL-1 family, such as IL-1α, IL-18, and IL-33 (20–25). In addition to necrotic and activated cells, the extracellular matrix can also release alarmins, *e.g.* biglycan (26). Similar to the PAMPs, alarmins act on pattern recognition receptors expressed on innate immune cells to induce inflammation. For example IL-33, which binds to the T1/ST2 receptor, acts directly on MCs to release the pro-inflammatory cytokines IL-6 and TNF-α (27). Also the heat shock proteins (Hsp) have been described as prototypic alarmins (28–30), but recently they have been ascribed a dampening rather than an activating role during the immune response (30, 31).

The body has developed ways to limit the pro-inflammatory activities of PAMPs and alarmins because exaggerated inflammation can lead to tissue destruction and death. In this study we explored a possible role of the MC chymase/MCPT4 in limiting the response to infection and danger-associated inflammation. We show that MC chymase, but not MC tryptase, specifically degrades the virulence factor Hsp70 of *Trichinella spiralis* and several alarmins such as endogenous Hsp70, biglycan, HMGB1, and IL-33, and thus may contribute to the control of inflammation. The identification of alarmins as likely *in vivo* substrates for chymase provides additional evidence for a protective role of the MC proteases during infection and tissue damage and strongly implies that MCs are key cells in the regulation of inflammation.

EXPERIMENTAL PROCEDURES

Mouse Strains—All mouse strains used were on the C57BL/6 background. The following mouse strains were used for *in vivo* and *in vitro* studies: wild type (WT); the serglycin-deficient mouse strain ($SG^{-/-}$) (32, 33); and the mouse mast cell protease 4-deficient mouse strain ($Mcpt4^{-/-}$) (6, 34, 35). The following mouse strains were used for *in vitro* studies only: the mast cell-deficient W^{sash} mice; the heparin-deficient mouse strain ($NDST2^{-/-}$) (14); the two carboxypeptidase A-deficient mouse strains, $Cpa3^{-/-}$ (which also lack MCPT5) and $Cpa3^{inact}$ (which carry an inactivating mutation of the catalytic site and maintain MCPT5 expression) (36, 37); and the mouse mast cell protease 6-deficient mouse strain ($Mcpt6^{-/-}$) (10). The mice were bred and maintained at the Uppsala University animal care unit and used for experiments under permission from the local ethical board.

Peritoneal Cell-derived Mast Cells—Peritoneal cell-derived mast cells (PCMCs) were obtained, from the above described mouse strains, according to the protocol of Malbec *et al.* (38). In this protocol purified PCMCs were cultured in DMEM plus GlutaMAX (Gibco, Invitrogen, Paisley, UK) supplemented with 10% supernatant of stem cell factor-transfected Chinese hamster ovary cells (a gift from Dr. Marc Daeron, Pasteur Institute, Paris, France), 10% FBS, 60 μg/ml streptomycin, 50 μg/ml penicillin, 100 μM minimum essential medium nonessential amino acids, and 50 μM 2-mercaptoethanol. Medium was changed every 4th to 5th day. Unless otherwise stated, 4–5-week-old PCMC-cultures at a density of 10^6 cells/ml were used for all experiments.

Recombinant Proteins and Antibodies—Recombinant human (rh) tryptase (rHT) (G706A Promega), rh heat shock protein 70 (Hsp70) (HSP0603, ATGEN), rh HMGB1 (HMG0801, ATGEN), and rh biglycan (2667-CM, R&D Biosystems), as well as recombinant mouse (rm) interleukin (IL)-33 (12340333, ImmunoTools AS) rm IL-7 (12340073, ImmunoTools AS), rh IL-22 (11340222, ImmunoTools AS), rh GM-CSF (11343122, ImmunoTools AS),rh CCL2/MCP1 (11343380, ImmunoTools AS), rh IL-6 (11340060, ImmunoTools AS), and rm IL-6 (12340063, ImmunoTools AS or 216-16, PeproTech, EU) were used in the degradation assays. Anti-rabbit mMCP4 sera, a serum generated after immunization of rabbits with a 13-amino acid surface-exposed peptide from mMCP-4 (C-AAKKAKETPSVNV) coupled to KLH, as well as primary antibody toward Hsp70 (4872, Cell Signaling) and secondary antibodies (LI-COR Biosciences), were used according to the concentrations suggested by the suppliers. A rabbit polyclonal IgG (H-196 from Santa Cruz Biotechnology) was used as primary antibody to detect actin levels.

Purification of Soluble T. spiralis Proteins—*T. spiralis* (strain ISS03, Istituto Superiore di Santa, Rome, Italy) was maintained in BALB/c mice, and larvae were recovered by pepsin-acid digestion of the whole carcass. Soluble *T. spiralis* proteins (STP) were prepared from the infective larvae according to Yera *et al.* with some minor modifications (39). In brief, larvae were washed three times in phosphate-buffered saline, resuspended

in Tris-EDTA buffer (0.25 M sucrose, 1 mM EDTA, 40 mM Tris, and 1% Triton X-200), and homogenized at 4 °C for 1 h followed by three cycles of thawing and freezing in liquid nitrogen and sonication (six times for 1 min each time). The crude extract was centrifuged at 100,000 × *g* for 1 h, and the supernatant was filtered (0.22-μm pore size) and stored at −80 °C. Protein content was estimated by Bradford assay. The purified soluble protein was kept in −80 °C until used in various degradation assays.

Purification of Proteases from Ear Tissue and Peritoneal Cell-derived Mast Cells—Tissues and PCMCs were snap-frozen in liquid nitrogen and kept at −20 °C until processed. Frozen tissues were homogenized in liquid nitrogen using a mortar and pestle followed by lysis with low salt lysis buffer (PBS/0.5 M NaCl/1% Triton), whereas the PCMCs were directly subjected to the low salt lysis buffer. After occasional shaking for 30 min at room temperature, lysed tissues and PCMCs were centrifuged at 3000 × *g*. The low salt supernatant was saved and kept at −20 °C. The tissue and cell pellets were resuspended in a high salt lysis buffer (PBS/2 M NaCl/1% Triton) and extracted for another 30 min at room temperature with occasional shaking. After centrifugation at 3000 × *g*, the 2 M NaCl supernatants were saved and kept at −20 °C until used in different degradation assays.

Production and Purification of Recombinant Human Chymase—The construction of the expression plasmids for the human chymase (HC) WT enzyme has previously been described in detail (18). The vector constructs encoding the HC were transfected into the human embryonic kidney cell line (HEK 293 EBNA) at ~80% confluency, using Lipofectamine (Invitrogen) as described previously (40). Selection of transfected cells was initiated by the addition of 1.5 μg/ml puromycin to the cell culture medium (DMEM supplemented with 5% FCS, 50 μg/ml gentamicin, and 5 μg/ml heparin). The level of puromycin was decreased to 0.5 μg/ml after approximately 7 days of selection.

Conditioned medium was collected and centrifuged to remove cell debris followed by the addition of 300 μl of nickel-nitrilotriacetic acid agarose beads (Qiagen GmbH, Hilden, Germany) per liter of conditioned medium. After 1 h of incubation with gentle agitation at 4 °C, the beads were pelleted by centrifugation and transferred to 1.5-ml reaction tubes (Trefflab, Degersheim, Switzerland). The collected nickel-nitrilotriacetic acid beads were then transferred to a 2-ml column and washed five times with washing buffer (1 M NaCl, 0.2% Tween in PBS). Bound protein was then eluted with elution buffer (100 mM imidazole, 0.2% Triton X-100 in PBS). Protein purity and concentration were estimated by separation on 12.5% SDS-PAGE gels. Protein samples were mixed with sample buffer, and β-mercaptoethanol was added to a final concentration of 5%. To visualize the protein bands, the gel was stained with colloidal Coomassie Brilliant Blue according to previously described procedures (41).

Activation of Recombinant Human Chymase—Approximately 30 μg of the recombinant human chymase (rHC) was diluted 1:2 in double-distilled H_2O and digested for 5 h at 37 °C with EKMax™ enterokinase (Invitrogen), using one unit per 10 μg of recombinant protease. Enzymatic activity was measured toward the chromogenic substrate L-2130 (Suc-Leu-Leu-Val-Tyr-pNA, Bachem, Bubendorf, Switzerland, where Suc indicates succinyl and pNA indicates *p*-nitroanilide). Measurements were performed in 96-well microtiter plates with a substrate concentration of 0.18 mM in 200 μl of PBS. Hydrolysis of L-2130 was monitored spectrophotometrically at 405 nm in a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA).

Protease Inhibitors—Degradation assays both *in vitro* and *in vivo* were done with or without the addition of the inhibitor chymostatin (27250, Sigma), which inhibits mast cell chymase and other chymotrypsin-like proteases, and the potato tuber inhibitor (C0279, Sigma), which inhibits CPA3. Cells and crude extracts/elutes were preincubated with the inhibitors for 10 min before starting the experiments.

Hsp70 and Alarmin Degradation Assays—To study the degradation of soluble *Trichinella* antigens, 20 μg of purified STP was mixed with the high salt (2 M) crude extracts from ears of WT mice and from different knock-out mouse tissues as well as from WT and knock-out PCMCs. Initially, the reactions were left to proceed for a wide range of time points varying between 2 h and 12 h, and the degradation of STP was studied by separation on SDS-PAGE followed by Coomassie Brilliant Blue staining. Extended degradation times with WT extracts resulted in almost complete degradation of the STP, which is why we decided to focus our analysis on the immediate to early degradation period (up to 2 h). The cleavage reactions were also analyzed on two-dimensional gels. The protein bands from the STP that rapidly disappeared, *i.e.* within <2 h, when adding the WT extract as compared with the *Mcpt4*$^{-/-}$ extracts were cut out from the control gel and identified by mass spectrometry. Hsp70 was identified as one of these bands in the mass spectrometry analysis. To study the role of chymase in the degradation of Hsp70, 1 μg of recombinant human Hsp70 (rhHsp70) was mixed with crude and fractionated 2 M extracts. Chymase-specific degradation of Hsp70 was analyzed at different time points, 0, 5, 10, 15, 20, and 30 min. Finally, we examined whether rHC and mouse chymase (MCPT4) could degrade 1 μg of the alarmins, *i.e.* HMBG1, biglycan, and IL-33. We also analyzed the degradation of rmIL-7, rhIL-22, rhGM-GSF, and rhMCP-1/CCL2. From the reaction tube, samples were taken at 30 and 60 min, and degradation was analyzed on silver-stained SDS-PAGE.

Silver Staining—The SDS-PAGE gels were fixed in a 1:1 solution of 10% acetic acid and 40% EtOH for 30 min, washed in water to restore pH to 5.5, and then incubated with dithiothreitol (5 μg/ml H_2O) for 30 min and in 0.1% $AgNO_3$ for 30 min. After this gels were washed in H_2O for 30 s and developed in a formaldehyde solution (250 μl of 37% formaldehyde in 500 ml of 3% Na_2CO_3) until most protein bands showed clearly. The reaction was stopped by the addition of citric acid, and the gels were washed in H_2O for 10 min before a photo was taken (using a Nikon D90/AF-S NIKKOR 18–105-mm 1:3.5–5.6G).

Cell Culture Experiments—PCMCs from three separate experiments, each with PCMCs derived from three individual mice of each genotype, WT and *Mcpt4*$^{-/-}$, were cultivated in stem cell factor-conditioned medium (38). The PCMCs, at 1 × 10^6 cells/ml, were induced to degranulate by the addition of the

calcium ionophore A23187 in the presence or absence of 1 μg/ml rhHsp70 or 20 ng/ml IL-33 and with or without the inhibitor chymostatin (at 50 μM). The assays were done in Hanks' balanced salt solution buffer supplemented with 2% FBS. All stimulations were done in triplicates, and supernatants were collected at 1, 2, 4, and 6 h after stimulation. The viability of the PCMCs was checked with trypan blue exclusion in an automated cell counter (Countess™, Invitrogen) and was ≥95% both before and after the various assays, indicating that none of the treatments induced apoptosis or necrosis in the PCMCs.

β-Hexosaminidase Assay—To measure the level of Hsp70-induced MC degranulation, mature PCMCs (1×10^6 cells/ml) of each genotype (WT and Mcpt4$^{-/-}$) in triplicates were incubated at 37 °C in 5% CO_2 in Hanks' balanced salt solution supplemented with 2% FBS for 0.5–2 h in the presence of either 2 μM calcium ionophore A23187 (as a positive control) or 1 μg/ml Hsp70 alone or in a combination of 1 μg/ml Hsp70 and A23187 with or without the chymase inhibitor chymostatin. Samples were taken at each time point, and cells were centrifuged at 300 × *g* for 10 min. Supernatants were incubated with 1 mM *p*-nitro-phenyl-N-acetyl-β-D-glucosaminide (Sigma) in 0.05 M citrate buffer (pH 4.5) at 37 °C for 1 h. As a control for total β-hexosaminidase content, cells were lysed with 1% Triton X-100 and incubated as above. All reactions were quenched by the addition of 100 μl of 0.05 M Na_2CO_3 (pH 10.0), and the absorbance was read at 405 nm.

In Vivo Experiments—In ≥2 separate experiments, groups of WT and *Mcpt4*$^{-/-}$ mice were injected intraperitoneally with either 1 μg of Hsp70 in PBS ($n = 6$–7 per genotype), 0.5 μg of IL-33 ($n = 4$ per genotype), 0.5 μg of IL-33 + 1 μg of Hsp70 ($n = 4$ per genotype), or PBS ($n = 3$ or 4 per genotype). The animals were euthanized after 16 h, and the peritoneum was washed with sterile PBS. In one additional experiment, mice received 1 μg of Hsp70 in PBS intraperitoneally, and the peritoneal lavage fluid was collected at 3 h (WT, $n = 4$ and *Mcpt4*$^{-/-}$, $n = 2$) and at 6 h (WT, $n = 4$ and *Mcpt4*$^{-/-}$, $n = 3$). The peritoneal lavage fluid was used for analysis of Hsp70, IL-33, IL-6, MCP1/CCL2, and TNF-α content with Western blot and/or ELISA. The peritoneal lavage cells were collected on cytospin slides (5 min at 600 rpm) and stained with May-Grunewald/Giemsa for differential counting of leukocytes.

T. spiralis (strain ISS03, Istituto Superiore di Sanità, Rome, Italy) was maintained in BALB/c mice, and larvae were recovered by pepsin-acid digestion. To analyze *in vivo* the intestinal Hsp70 levels, experimental infections were carried out in 8–10-week-old WT and *Mcpt4*$^{-/-}$ mice, by oral gavage with 500 *T. spiralis* larvae suspended in PBS with 0.1% agar. Infected mice and uninfected control mice were killed at 12 days after infection, and the jejunum was collected. From infected and control animals, jejunum protein extracts were purified in radioimmune precipitation assay buffer supplemented with protease inhibitors and subjected to Western blot with anti-Hsp70 antiserum.

Detection of Hsp70, IL-33, IL-6, CCL2, and TNF-α—The levels of Hsp70, IL-33, IL-6, CCL2, and TNF-α were analyzed in both the *in vitro* and the *in vivo* experiments with commercial ELISA kits from R&D systems (DYC1663 for Hsp70 and DY3626 for IL-33) and PeproTech (900-K50 for IL-6, 900-K126 for CCL2 and 900-K54 for TNF-α). In other experiments, Hsp70 was detected by Western blot analysis using a polyclonal rabbit anti-Hsp70 antiserum from Cell Signaling (4872) and visualized with a donkey anti-rabbit IRDye 800 CW secondary antibody (LI-COR Biosciences). Membranes were scanned using the Odyssey scanner (LI-COR Biosciences).

Statistical Analysis—Statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Statistical differences between groups were calculated using the Student's *t* test (unpaired, two-tailed) or the Mann Whitney *U* test, with *p* values ≤0.05 considered significant. Results are presented as mean values, with *p* values indicated: $p > 0.05$, *, $p \leq 0.05$, **, $p < 0.001$, ***, $p < 0.0001$ *versus* WT cells and WT mice, unless stated otherwise in the figure legends.

RESULTS

The Chymase Mouse Mast Cell Protease 4 Degrades T. spiralis Virulence Factors via a Heparin/Serglycin-dependent Mechanism—To identify physiological and potentially important substrates of the connective tissue MC proteases during infection with *T. spiralis,* we analyzed the degradation of STP after the addition of ear tissue extract on SDS-PAGE. Low salt (0.5 M NaCl) and high salt (2.0 M NaCl) ear tissue extracts from wild type (WT) or MC-deficient W^{sash} mice were incubated together with STP, proteolysis of the STP was allowed to proceed for 1 h up to 12 h, and samples were taken at different time points during this incubation. Incubation with the low salt ear tissue extracts did not result in any substantial degradation (data not shown), whereas in the presence of the high salt WT extracts, marked degradation of STP was already observed at 4 h. In contrast, extracts from the W^{sash} mice failed to process STP, suggesting that MCs contribute substantially to the observed proteolysis (Fig. 1*A*). The degradation of STP in WT high salt extracts, but not in *Ndst2*$^{-/-}$, *SG*$^{-/-}$, and *Mcpt4*$^{-/-}$ extracts (Fig. 1*B*), suggested that heparin-dependent MC proteases, possibly MCPT4, caused most of the observed degradation.

To determine which MC protease(s) was responsible for the proteolysis of STP, the degradation of STP by high salt extracts from the *Mcpt4*$^{-/-}$, *Mcpt6*$^{-/-}$, and *Cpa3*^{inact} mouse strains was analyzed. Ear tissue extracts lacking MCPT6 retained full capacity similar to WT extracts in the degradation of STP at 4 h (Fig. 1*C*). In contrast, the MCPT4- and CPA3-deficient extracts did not degrade STP to the same extent as WT extracts (Fig. 1, *B* and *C*), suggesting that both MCPT4 and CPA3 had multiple targets in the STP. Importantly, although some chymase, tryptase, and CPA activity remained in extracts from the *SG*$^{-/-}$ and *Ndst2*$^{-/-}$ mice, knock-out targeting of respective proteases completely abolished the specific enzymatic activity toward the chromogenic substrates (Fig. 1*D*). To identify which STP was degraded by chymase/MCPT4, prominent protein bands of the STP that already showed significant reduction in WT extracts at 2 h but remained after degradation in MCPT4-deficient extracts were cut out from the SDS-PAGE and analyzed by mass spectrometry (Table 1). Of these potential substrates, the heat shock protein Hsp70 (ABR58855) and the small heat shock

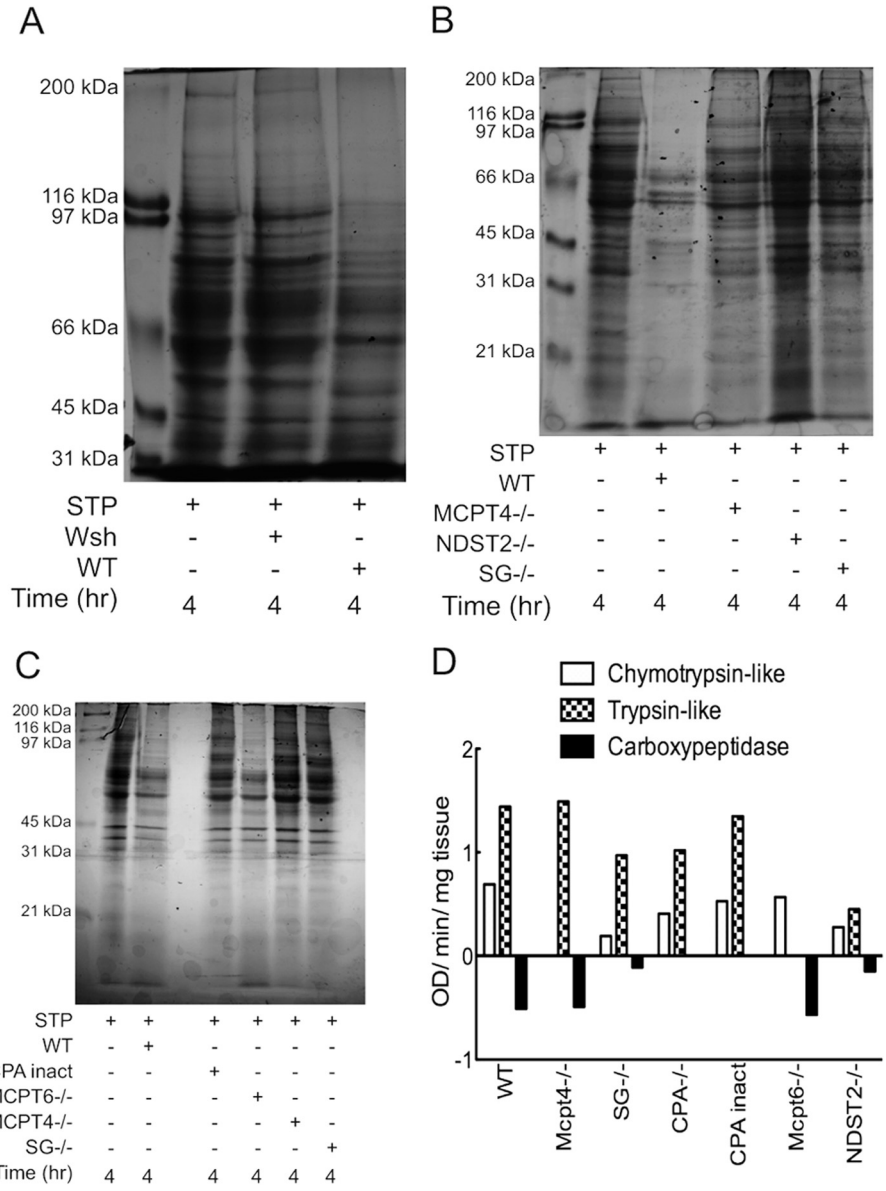

FIGURE 1. **Mast cell chymase MCPT4 degrades soluble *T. spiralis* antigens, via a serglycin- and heparin-dependent mechanism.** Degradation of STP by mouse ear tissue protein extracts (crude, 2 M NaCl) was analyzed on SDS-PAGE (Coomassie Brilliant Blue-stained). *Panels A–C* show representative gel pictures out of ≥3 experiments. The content in each reaction is indicated below the individual gel pictures, and the molecular standard marker is indicated. *A*, STP degradation by WT and mast cell-deficient W^{sash} ear tissue extracts (*Wsh*) at 4 h. *B*, STP degradation by WT, *Mcpt*4$^{-/-}$, SG$^{-/-}$, and heparin-deficient NDST2$^{-/-}$ ear tissue extracts at 4 h. *C*, STP degradation by WT, CPA^{inact}, *Mcpt*6$^{-/-}$, *Mcpt*4$^{-/-}$, and SG$^{-/-}$ ear tissue extracts at 4 h. *D*, proteolytic activity against chromogenic substrates in mouse ear tissue extracts from the respective genotypes used in *panels A–C*. *OD*, optical density.

TABLE 1

Identification of virulence factors as chymase substrates

STP were mixed with 2 M WT and MCPT4-deficient ear tissue extracts, and degradation was allowed for up to 2 h. Mass spectrometry analyses were performed on protein bands and spots in one-dimensional SDS-PAGE control gels not subjected to degradation.

Molecular size (kDa)	Condition after treatment with 2 M WT extracts	Mass spectrometry analysis	Accession number
70	Faint spot	Heat shock protein 70[a]	ABR58855
48	Faint spot	Serine protease[a]	AYO28974
42	Almost missing	Actin muscle protein	EFV54220.1
33	Completely missing	NADH-oxidoreductase chain 1 protein	Q9B8A1
32	Fainter band and slightly smaller	Actin-5C isoform 2	XP_976050
23	Completely missing	Small heat shock protein	ABJ55914

[a] Indicates results from both one- and two-dimensional SDS-PAGE gel electrophoresis with corresponding bands and spots given for mass spectrometry analysis.

protein (ABJ55914), the serine protease (AYO28974), and the NADH-oxidoreductase chain 1 protein (Q9B8A1) have been identified as potential virulence factors of *T. spiralis* (42–45).

Chymase but Not Tryptase Rapidly Degrades the Alarmins Hsp70, Biglycan, HMGB1, and IL-33—The rapid and potentially MC chymase-specific degradation of *T. spiralis* heat

shock protein 70 (Hsp70) and the fact that endogenous Hsp70 is classified as a DAMP/alarmin (46) led us to speculate that alarmins could be important physiologic substrates for chymase. When degradation of rhHsp70 was assessed with WT and $Mcpt4^{-/-}$ ear tissue extracts, only MCPT4-containing extracts could efficiently degrade human Hsp70 (Fig. 2*A*). In WT extracts degradation was already observed at 5 min, and degradation was almost complete at 20 min (Fig. 2*B*). The inhibitor chymostatin effectively blocked the degradation, whereas inhibition of CPA3 did not block the degradation of rhHsp70 in WT extracts (Fig. 2, *B* and *C*). Furthermore, rHC also efficiently degraded rhHsp70 and Hsp70 in STP in a time-dependent manner, with substantial degradation of rhHsp70 already observed at 20 min, and at 1 h most of the *T. spiralis* Hsp70 protein was degraded (Fig. 2, *D* and *E*).

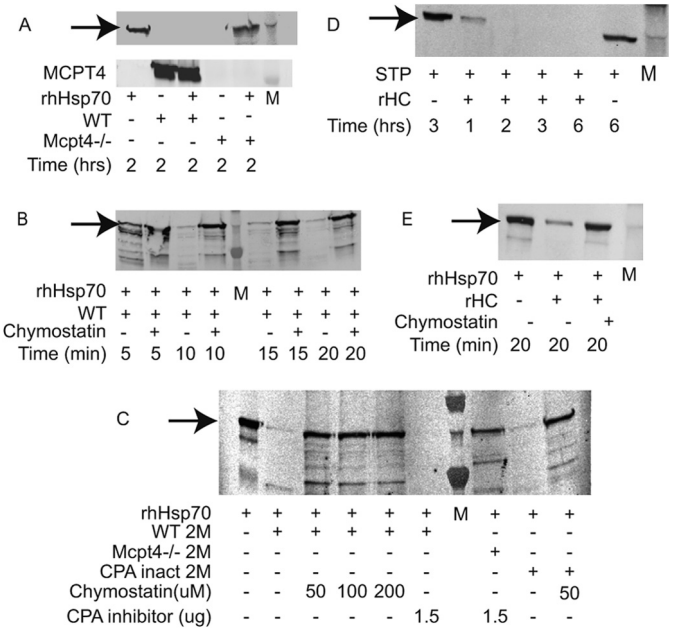

FIGURE 2. **MCPT4 and human chymase can degrade Hsp70.** Degradation of Hsp70 by MCPT4 and human chymase was analyzed by Western blots. The content in each reaction is indicated below the individual gel pictures, and detection of Hsp70 and MCPT4 with antisera is indicated to the *left* of each photo. *Arrows* indicate where a positive signal for Hsp70 should appear. *M*, molecular standard marker. *A*, MCPT4-specific degradation of rhHsp70 at 2 h. *B*, the rapid degradation of rhHsp70 by MCPT4 can be blocked with chymostatin. *C*, degradation of rhHsp70, with or without inhibitors, in WT, $Mcpt4^{-/-}$, and CPA^{inact} ear tissue extracts. *D* and *E*, degradation by rHC of Hsp70 in STP (*D*) and of rhHsp70 (*E*).

Chymase Released from Peritoneal Cell-derived Mast Cells Can Degrade rhHsp70—To assess the MCPT4-specific degradation of Hsp70 *ex vivo*, rhHsp70 was added to cultures of peritoneal MCs. A significant and time-dependent decrease of rhHsp70 was evident in WT PCMC-cultures, whereas degradation was completely absent in MCPT4-deficient PCMCs (Fig. 4*A*). No rhHsp70 degradation was observed in WT PCMCs in

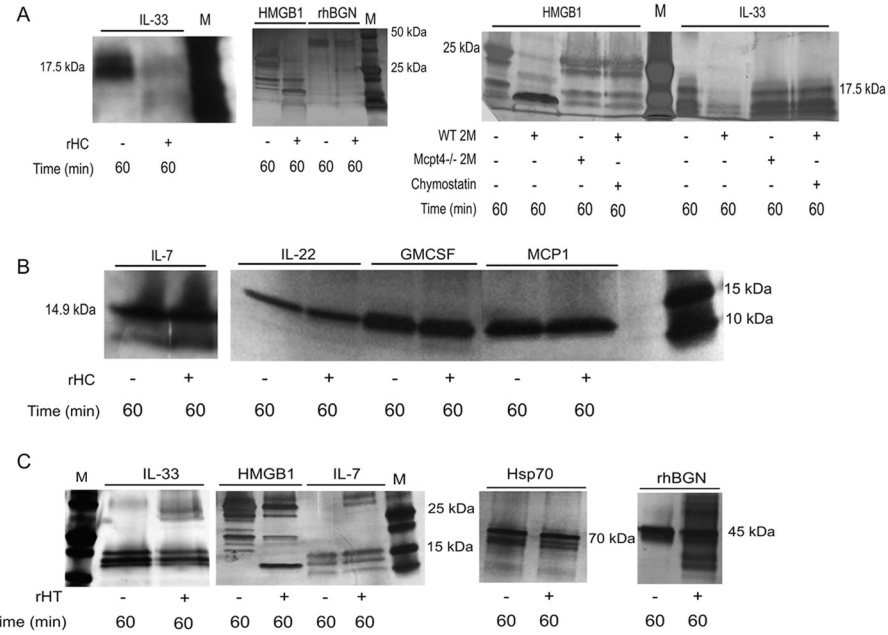

FIGURE 3. **MCPT4 and human chymase can degrade IL-33, HMGB1, and biglycan.** Alarmins can be degraded by recombinant human chymase and mouse chymase MCPT4. The content in each reaction is indicated below the individual gel pictures (silver-stained SDS-PAGE). Note that MCPT4-induced degradation of HMGB1 and IL-33 can be blocked by chymostatin. *M*, molecular standard marker. *A*, rHC/MCPT4-induced degradation of HMGB1 (25 kDa), IL-33 (17.5 kDa), and biglycan (45 kDa) at 1 h. *B*, IL-7, IL-22, GM-CSF, and CCL2/MCP1 were included as examples of proteins that are not cleaved by chymase at 1 h. *C*, degradation of IL-33, HMGB1, IL-7, Hsp70, and biglycan by rHT at 1 h. Note that the doublet above 25 kDa in the IL-33 and IL-7 gel pictures and below 45 kDa in the biglycan gel picture is the added recombinant human tryptase.

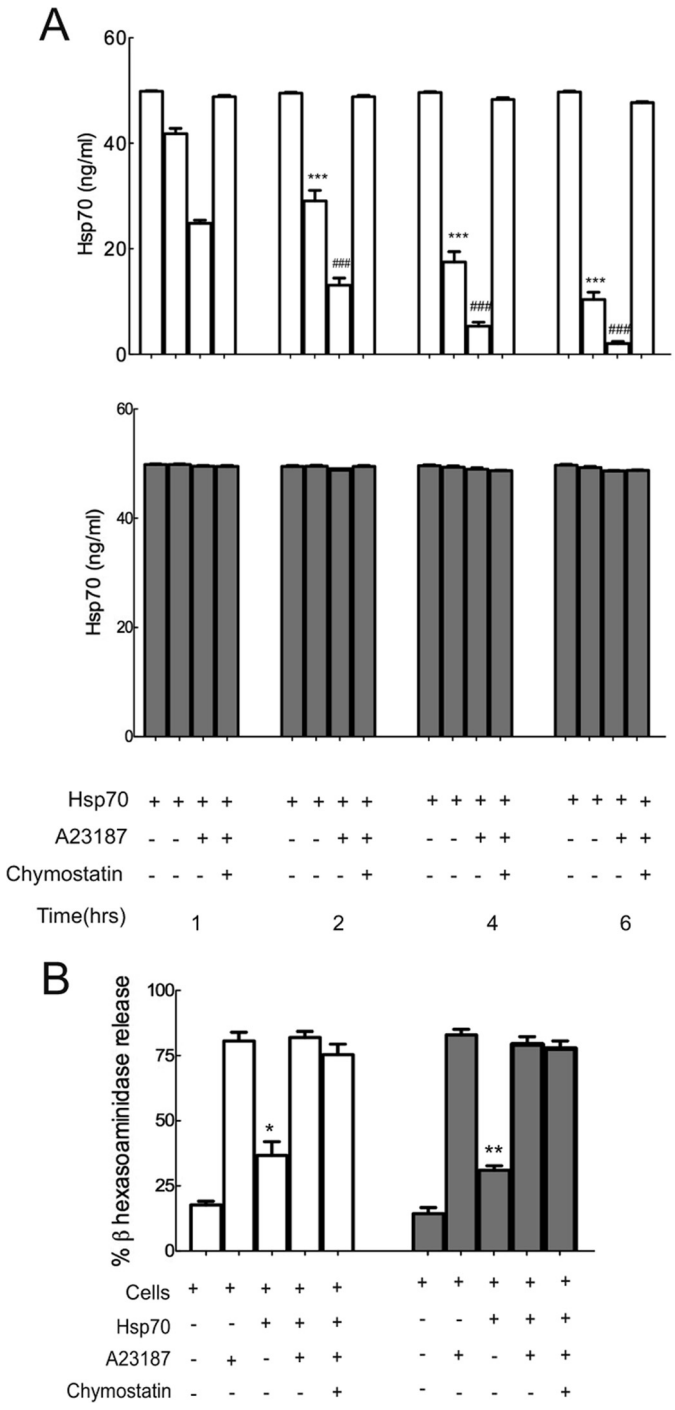

FIGURE 4. **Hsp70 degradation *ex vivo* in peritoneal cell-derived mast cells.** rhHsp70 was added to WT and MCPT4-deficient PCMC cultures, and the chymase-specific degradation of rhHsp70 was analyzed with ELISA. The content in each reaction is indicated below the panels. Panels show WT and *Mcpt4*$^{-/-}$ PCMCs with *white* and *gray bars*, respectively. *A*, MCPT4-specific degradation of rhHsp70 in PCMCs at 1, 2, 4, and 6 h. Note that degradation of Hsp70 is absent in *Mcpt4*$^{-/-}$ PCMC cultures and that degradation is enhanced in WT PCMCs upon the addition of a calcium ionophore and the degranulating agent A23187, and completely blocked by chymostatin. ***, $p < 0.0001$ *versus* 1-h Hsp70 stimulated cells, and ###, $p < 0.0001$ *versus* WT Hsp70 stimulated cells. *B*, Hsp70 induces moderate MC degranulation in WT and MCPT4-deficient PCMCs at 1 h. Background β-hexosaminidase release is also shown. *, $p < 0.05$ **, $p < 0.01$ *versus* unstimulated WT and *Mcpt4*$^{-/-}$ cells, respectively.

the presence of the inhibitor chymostatin, confirming a role of MCPT4/chymase in Hsp70 degradation. Furthermore, the degradation of rhHsp70 was more prominent in WT PCMCs when added together with a degranulating agent, *i.e.* the calcium ionophore A21837 (Fig. 4*A*), indicating that MCPT4 released from the PCMCs is the major mast cell protease involved in this degradation process. In addition, WT PCMCs degraded rhHsp70 without stimulation of A23187, suggesting that chymase may also be released by piecemeal secretion. Alternatively, Hsp70 induce MC degranulation. In support of the alternative explanation, the addition of rhHsp70 caused a significantly increased but moderate β-hexosaminidase release in both MCPT4-deficient and WT PCMCs (Fig. 4*B*), explaining the degradation of rhHsp70 in WT PCMC cultures not stimulated with A23187.

Chymase/MCPT4-dependent Degradation of Hsp70 in Vivo—To study the role of chymase in degradation of Hsp70 *in vivo*, rhHsp70 was injected intraperitoneally in WT and *Mcpt4*$^{-/-}$ mice. After 3, 6, and 16 h, the peritoneal lavage fluid levels of Hsp70 were analyzed by Western blot and ELISA, and Hsp70 already showed complete degradation at 3 h in WT mice. In contrast, injected Hsp70 persisted for at least 16 h in *Mcpt4*$^{-/-}$ mice (Fig. 5, *A* and *B*). Interestingly, endogenously released mouse Hsp70 was also detected in PBS-injected *Mcpt4*$^{-/-}$ mice, suggesting that MCPT4/chymase is one of the proteases involved in the degradation of endogenous danger-released Hsp70 (Fig. 5*A*). The peritoneal lavage level of MC tryptase, MCPT6, was similar between the Hsp70-challenged mouse strains (Fig. 5*C*), indicating that tryptase has a less prominent role in the Hsp70 degradation as compared with MCPT4.

Finally, to investigate whether chymase can regulate Hsp70 during *e.g.* parasitic infection, we analyzed the levels of Hsp70 at day 12 in the jejunum of *T. spiralis*-infected WT and *Mcpt4*$^{-/-}$ mice. Infected MCPT4-deficient mice showed high Hsp70 levels in the intestine, whereas uninfected control mice and infected WT mice had Hsp70 levels below the detection level (Fig. 5*D*).

Chymase/MCPT4-dependent Degradation of IL-33 in Vivo—Because chymase/MCPT4 rapidly degraded several DAMPs *in vitro*, we also decided to study the degradation of IL-33 *ex vivo* and *in vivo*. When assessing MCPT4-specific degradation of rmIL-33 *ex vivo* in PCMC cultures, degradation of IL-33 was observed in WT PCMCs, but only after the addition of the calcium ionophore A21387 (Fig. 6*A*). In addition, stimulation with A21387 also caused some release of PCMC-derived endogenous IL-33 that was rapidly degraded in WT PCMCs. A minor IL-33 degradation was observed in degranulated MCPT4-deficient PCMCs (Fig. 6*A*), suggesting that other MC proteases may also be involved in the degradation of IL-33. To investigate whether MC degranulation occurs in IL-33-challenged PCMCs, we analyzed the MCPT4 level in the cell culture supernatants. Although IL-33 did not induce release of MCPT4, A23187 induced a substantial release (Fig. 6*B*).

Peritoneal injection of rmIL-33 in WT mice caused moderate degradation, a degradation that was significantly reduced in *Mcpt4*$^{-/-}$ mice (Fig. 6*C*). Furthermore, to assess whether Hsp70 could enhance IL-33 degradation *in vivo*, we injected both proteins and studied IL-33 degradation by ELISA. As compared with rmIL-33 alone, the addition of Hsp70 enhanced deg-

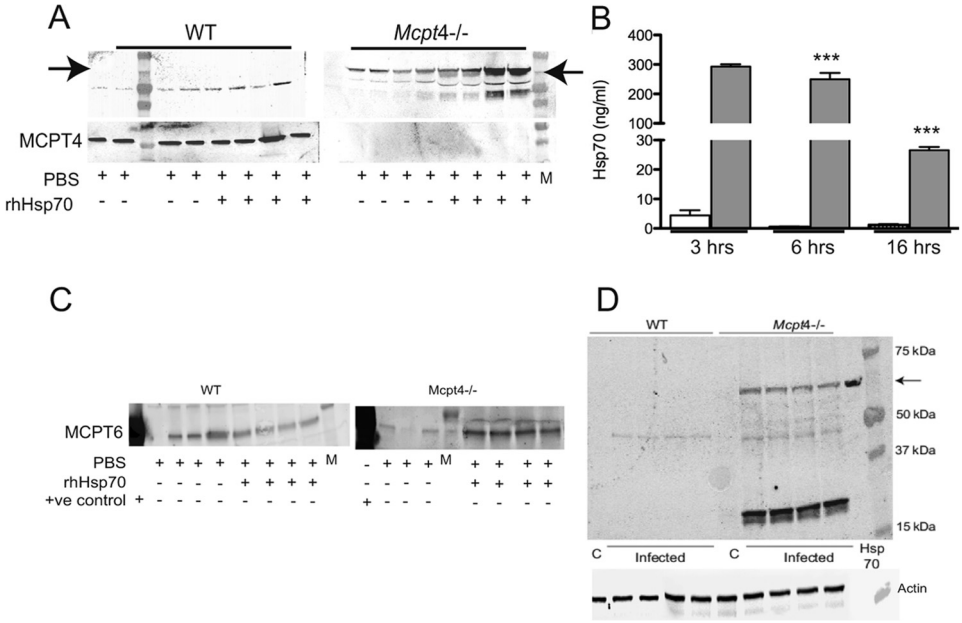

FIGURE 5. **Hsp70 degradation *in vivo* in WT and *Mcpt4*$^{-/-}$ mice.** *A* and *B*, to study chymase-dependent degradation of Hsp70 *in vivo*, rhHsp70 was injected into the peritoneum of WT and *Mcpt4*$^{-/-}$ mice, and the levels of Hsp70 in peritoneal lavage fluid were analyzed. Hsp70 levels in peritoneal lavage fluid of Hsp70-challenged WT and *Mcpt4*$^{-/-}$ mice were detected by Western blot at 16 h ($n = 4$ per genotype) (*A*) and by ELISA at 3, 6, and 16 h ($n = 2$–6 per genotype) (*B*). ***, $p < 0.0001$ *versus* Hsp70-challenged WT mice. *Panel B* shows WT mice and *Mcpt4*$^{-/-}$ mice with *white* and *gray bars*, respectively. The *arrow* in *A* indicates where a positive signal for Hsp70 should appear. *C*, peritoneal lavage fluid levels of MCPT6, detected by Western blot, indicate similar MC activation in Hsp70-challenged WT and *Mcpt4*$^{-/-}$ mice. *M* stands for size marker, and *+ve control* stands for positive control; the lane contains chymase-positive PCMC protein extracts. *D*, to study degradation of Hsp70 *in vivo* during *e.g.* parasitic infection, WT and *Mcpt4*$^{-/-}$ mice were infected with *T. spiralis.* At day 12 after infection, jejunum was excised, and protein extracts were prepared and subjected to Western blot. Infected WT and *Mcpt4*$^{-/-}$ mice, $n = 4$ per genotype; uninfected control mice, $n = 1$ per genotype. Results shown are from 4 out of 6 infected mice and from 1 out of 3 uninfected mice. The *arrow* indicates where a positive signal for Hsp70 should appear, and the molecular standard marker is indicated. In the lane marked *Hsp 70*, recombinant human Hsp70 is shown as a positive control. The protein level of actin was used as a loading control. *C* stands for control.

radation of IL-33 in both WT and *Mcpt4*$^{-/-}$ mice, although the degradation remained significantly reduced in the *Mcpt4*$^{-/-}$ mice as compared with WT mice, indicating that other proteases are also involved in IL-33 degradation *in vivo* (Fig. 6*C*). To investigate whether challenge with IL-33 and IL-33/Hsp70 causes MC degranulation *in vivo*, we analyzed the MCPT4 and MCPT6 levels in the peritoneal lavage fluid of WT and *Mcpt4*$^{-/-}$ mice, and the MCPT6 levels were found to be similar in challenged WT and *Mcpt4*$^{-/-}$ mice (Fig. 6*D*).

Chymase/MCPT4 May Limit Alarmin-induced Inflammation in Vivo—Next we assessed the potential role of chymase in limiting inflammation induced by the alarmins Hsp70 and IL-33. The counting of leukocytes in the peritoneum of challenged mice showed that Hsp70 induced a significantly increased neutrophil recruitment and neutrophil elastase activity in the Mcpt4$^{-/-}$ mouse strain as compared with WT mice, whereas ratios of MCs, macrophages, and lymphocytes were not significantly different between WT and *Mcpt4*$^{-/-}$ mice (Fig. 7*A* and data not shown). Furthermore, although the levels of IL-6 and CCL2 were not significantly altered in Hsp70-challenged *Mcpt4*$^{-/-}$ mice (Fig. 7, *B* and *C*), both Hsp70 and IL-33 induced a significantly higher TNF-α response in *Mcpt4*$^{-/-}$ mice as compared with WT mice (Fig. 7, *D* and *E*), suggesting that chymase is involved in the regulation of TNF-α levels during infection and danger-induced inflammation.

Taken together, the rapid and specific degradation of the *T. spiralis* virulence factor Hsp70 as well as several alarmins suggests that MC chymase may provide a protective role during danger-induced inflammation by regulation of leukocyte recruitment and modulation of alarmin-induced cytokine responses.

DISCUSSION

MCs are known for their role in the protection against infections caused by parasite and bacteria. However, the contribution by the various MC-specific proteases to this protection has remained largely unknown. Two important exceptions are the chymase MCPT1, which has been shown to be of importance for the expulsion of *T. spiralis* from the intestine (47) and the tryptase MCPT6, which has been shown to be important for the eosinophil recruitment into the muscle tissue and around encysted *T. spiralis* larvae (48). *In vitro* studies have identified several potential substrates for the MC-specific proteases. However, only a few of these have been verified as true *in vivo* substrates for the MC-specific proteases. The recently developed protease-specific knock-out mouse strains have become important tools in identifying such potential *in vivo* substrates, and examples of substrates identified using these knock-out mice are fibronectin, endothelin, and vasointestinal peptide. Previous work also showed that chymase and CPA3 could cooperate in the degradation of potential substrates (4, 6, 7, 34). Our results also suggest that chymase may be required for the initiation of the degradation process.

Here we show that virulence factors of *T. spiralis* as well as endogenous proteins that may act as alarmins can be true physiologic substrates of chymase/MCPT4. Interestingly, the heparin-dependent chymase/MCPT4 was found to be the major alarmin-degrading activity in MC-containing connective tis-

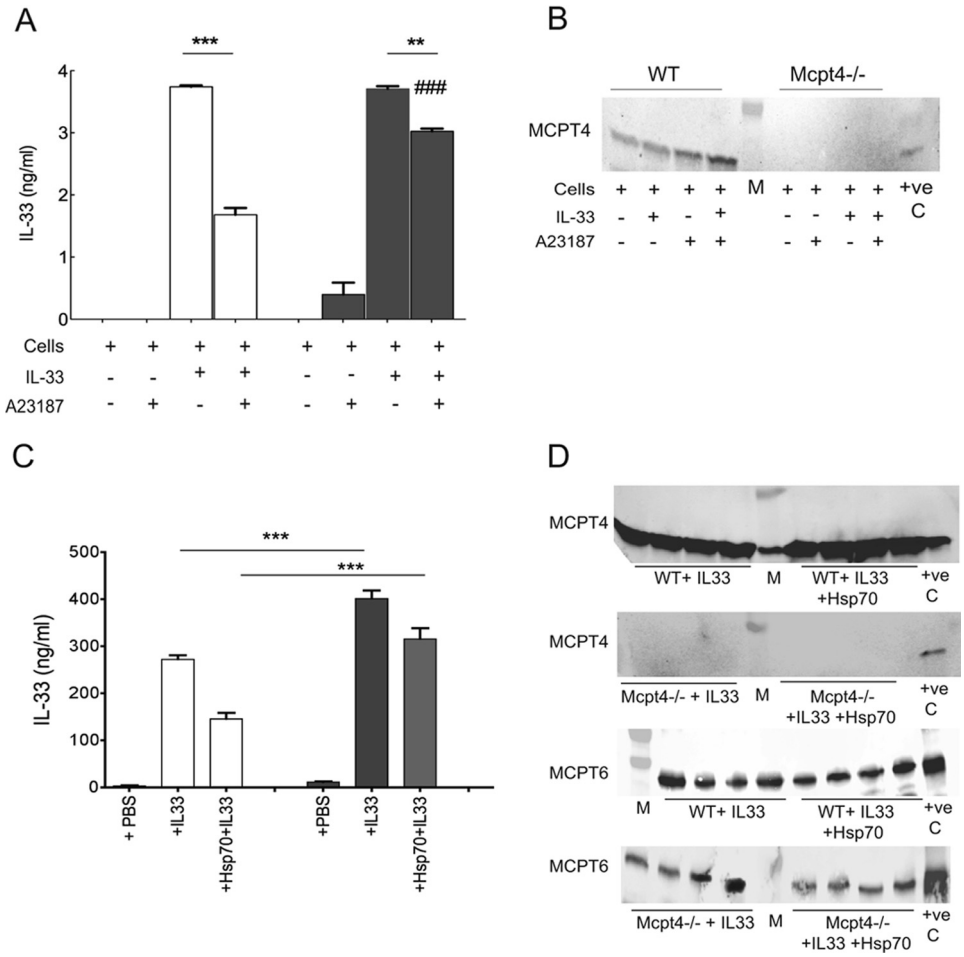

FIGURE 6. **IL-33 degradation *ex vivo* and *in vivo* by MCPT4.** rmIL-33 was added to WT and MCPT4-deficient PCMC cultures or injected intraperitoneally, alone or in combination with rhHsp70, to study degradation of IL-33. MCPT4-specific degradation of rmIL-33 was analyzed with ELISA. The content in each reaction is indicated below the panels. *Panels A* and *C* show WT and *Mcpt4*$^{-/-}$ PCMCs with *white* and *gray bars*, respectively. *A*, MCPT4-specific degradation of rmIL-33 in PCMCs at 6 h. Note that only minor degradation of IL-33 occurs in *Mcpt4*$^{-/-}$ PCMC cultures and that degradation is enhanced in WT PCMCs upon the addition of a calcium ionophore, the degranulating agent A23187. **, $p < 0.001$, ***, $p < 0.0001$ *versus* IL-33 stimulated cells, and ###, $p < 0.0001$ *versus* WT A23187+IL-33 stimulated cells. *B*, the level of MCPT4 in PCMC cell culture supernatants was detected by Western blot. The content in each lane is indicated below the panel. *C*, the level of IL-33 in peritoneal lavage of IL-33- and IL-33 + Hsp70-challenged WT and *Mcpt4*$^{-/-}$ mice after 16 h detected by ELISA ($n = 4$ per genotype). PBS-challenged control mice, $n = 4$ per genotype. ***, $p < 0.0001$ *versus* alarmin-challenged WT mice. *D*, the levels of MCPT4 and MCPT6 in peritoneal lavage fluid in IL-33- and IL-33 + Hsp70-challenged WT and *Mcpt4*$^{-/-}$ mice after 16 h were detected by Western blot. The content in each lane is indicated below the panel. In *C* and *D*, *M* stands for size marker, and +*ve C* stands for positive control; the lane contains chymase-positive PCMC protein extracts.

sues, where tryptase had little effect. Using a biochemical approach, we were able to identify Hsp70 via degradation of STP and recombinant protein as a potential *in vivo* substrate for chymase. Interestingly, both human and mouse MC chymases seem to prefer to cut proteins with an extended recognition site that contains aromatic amino acids in the P1 position and acidic amino acids in the P2′ position (Fig. 8*A*) (18). Comparison of the amino acid sequences of *T. spiralis*, mouse, and human Hsp70 showed extensive amino acid similarity in the N-terminal region, and several extended recognition sites for chymase were identified (Fig. 8*B*). Furthermore, comparison of the amino acid sequences in human and mouse IL-33, HMGB1, and biglycan revealed that each alarmin contains several extended recognition sites for chymase (Fig. 8*C* and data not shown). In contrast, although the resistant proteins IL-7, IL-22, GM-CSF, and CCL2 contained numerous aromatic amino acids, they displayed no extended recognition sites (data not shown). Thus, although chymase will act to degrade many peptides *in vitro*, potential *in vivo* substrates may also require extended recognition sites that are accessible on the surface and not buried in the tertiary structure of the protein.

Interestingly, besides being a virulence factor, Hsp70 has also been suggested to act as a DAMP/alarmin. Thus, to begin delineating the physiological relevance of chymase/MCPT4-dependent alarmin degradation, recombinant pure proteins were used to show that Hsp70 as well as biglycan, HMGB1, and IL-33 are degraded rapidly after exposure to chymase/MCPT4 (Figs. 2 and 3). Biglycan is normally associated with collagen in the extracellular matrix, but upon MC-induced tissue remodeling, the chymase/MCPT4 can activate the collagenase MMP-9 (35), and biglycan could be released to induce inflammation. Once released biglycan may act over TLR2 and TLR4 as a pro-inflammatory danger signal (26, 49). To control biglycan signaling during inflammation, chymase, but not MC tryptase, may directly act to degrade it.

HMGB1 was originally described as a ubiquitous nuclear protein, but the discovery that HMGB1 was secreted during danger-induced inflammation suggested a critical role in host

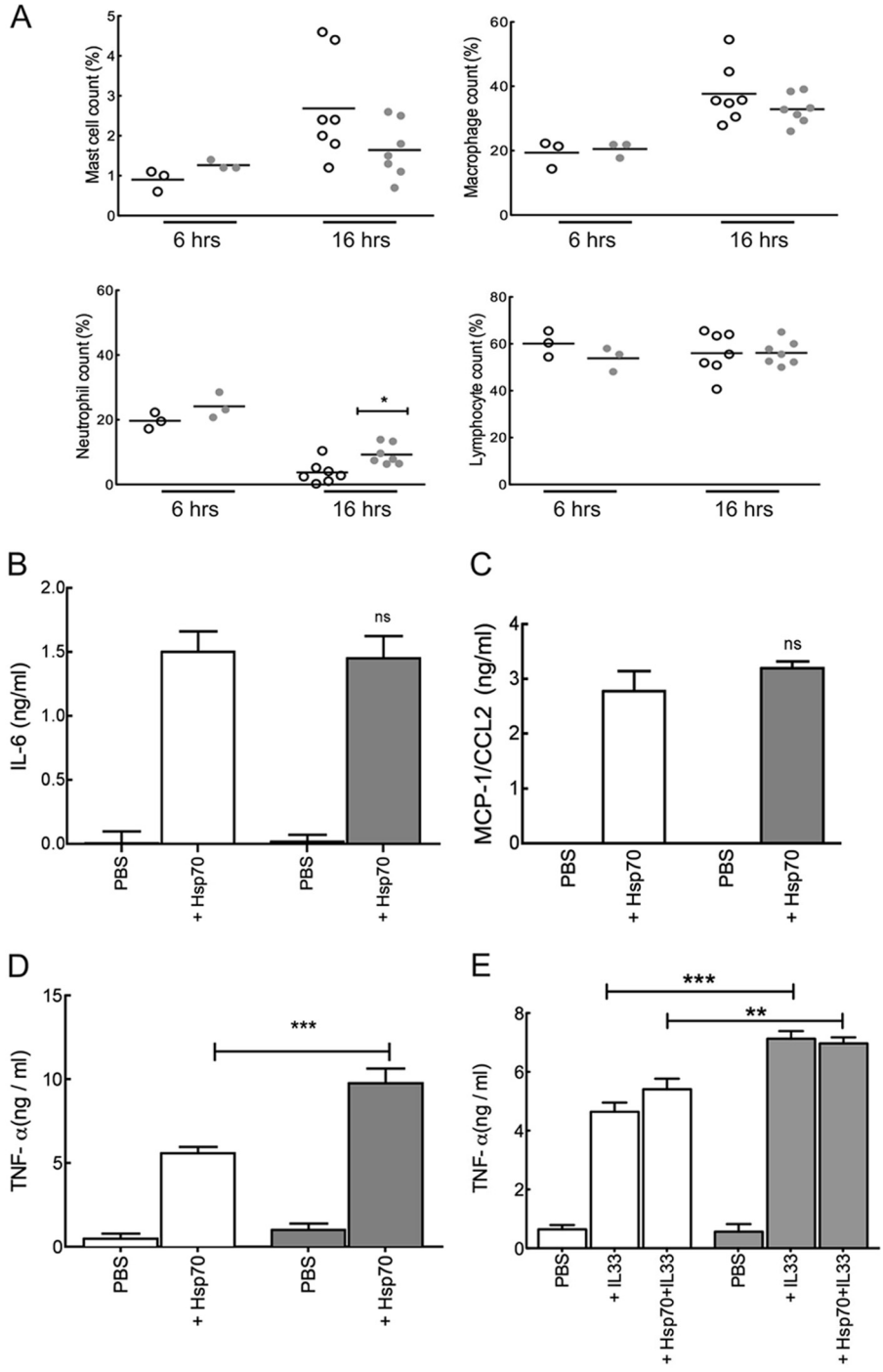

FIGURE 7. **Hsp70 and IL-33 induced inflammation *in vivo* in WT and *Mcpt4*$^{-/-}$ mice.** To study alarmin-induced inflammation, recombinant human Hsp70 and/or recombinant mouse IL-33 was injected into the peritoneum of the WT and *Mcpt4*$^{-/-}$ mice. *A*, leukocyte recruitment at 6 h in WT ($n = 3$) and *Mcpt4*$^{-/-}$ ($n = 3$) and at 16 h in WT ($n = 7$) and *Mcpt4*$^{-/-}$ ($n = 7$) mice challenged with Hsp70. Note the enhanced ratio of neutrophils in the Hsp70-challenged *Mcpt4*$^{-/-}$ mice. *B–D*, IL-6 levels (*B*), MCP-1/CCL2 levels (*C*), and TNF-α levels (*D*) at 16 h in Hsp70-challenged WT ($n = 6$) and *Mcpt4*$^{-/-}$ ($n = 6$) mice. PBS-challenged control mice, $n = 3$ per genotype. *E*, TNF-α levels at 16 h in IL-33- and IL-33 + Hsp70-challenged WT ($n = 4$) and *Mcpt4*$^{-/-}$ ($n = 4$) mice. PBS-challenged control mice, $n = 4$ per genotype. *Panels A* (at 16 h) and *B–E* show results from one representative experiment out of ≥2 experiments. Panels show WT and *Mcpt4*$^{-/-}$ mice with *white* and *gray circles* and *bars*, respectively. *, $p < 0.05$, **, $p < 0.001$, ***, $p < 0.0001$ *versus* challenged WT mice. *ns*, not significant.

inflammatory responses (23). HMGB1 has been suggested to bind *e.g.* TLR4 and induces a strong pro-inflammatory response in leukocytes with release of cytokines and proliferation as well as maturation of lymphocytes (21, 27, 50–55). Our data suggest that MC chymase, and to a much lesser extent MC tryptase, can directly regulate the levels of extracellular HMGB1.

IL-33, a chromatin-associated nuclear factor, functions as a cytokine when released from necrotic cells. IL-33 activates cells over the T1/ST2 receptor, and this receptor is highly expressed on Th2 cells and innate cells, such as MCs. Stimulation with IL-33 induces secretion of a Th2 cytokine profile from these cells, which may promote the pathogenesis of asthma and ana-

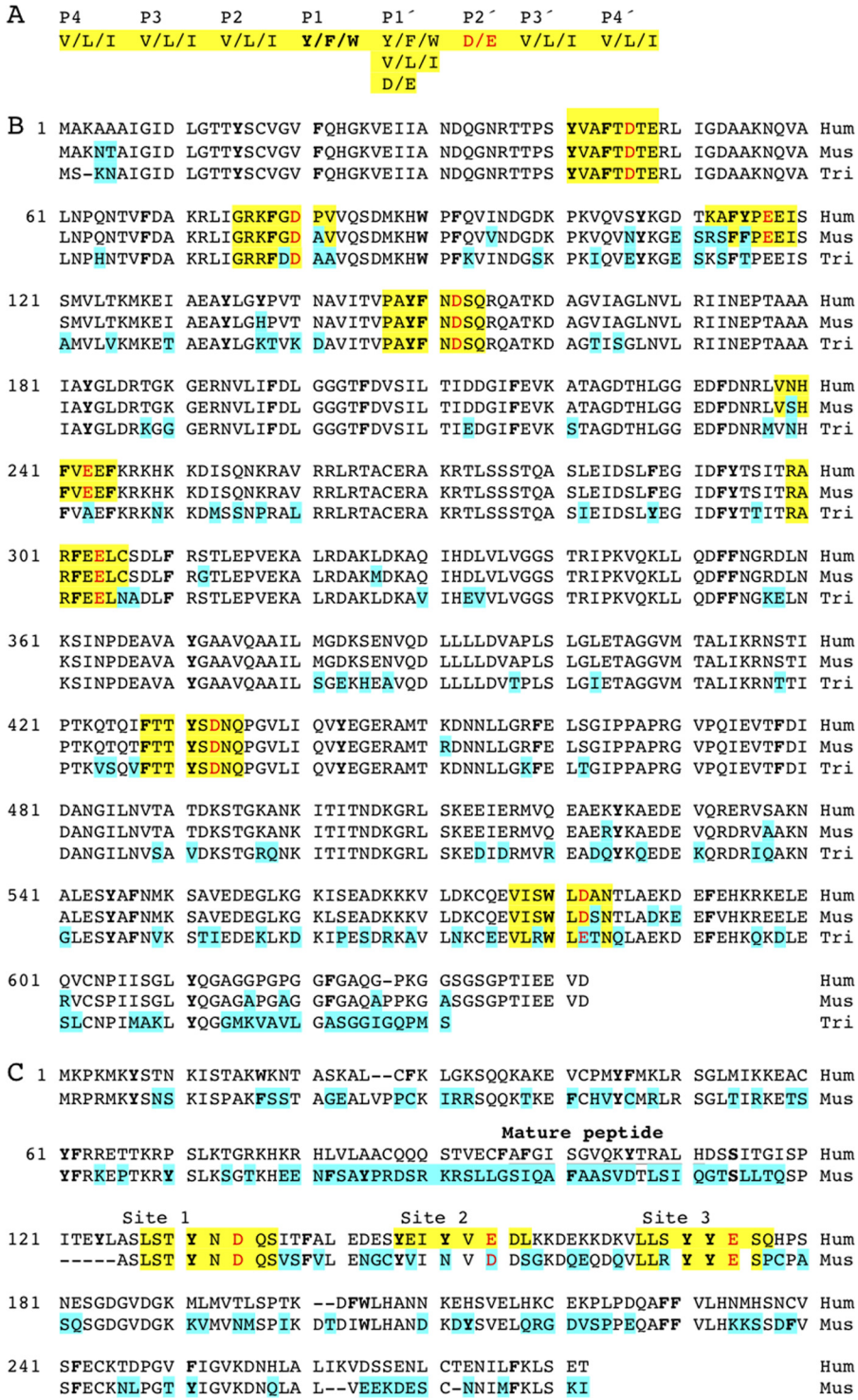

FIGURE 8. **Alignment and comparison of human, mouse, and *T. spiralis* Hsp70 and human and mouse IL-33.** *A*, an extended cleavage site for human chymase and MCPT4 (8 amino acids, *yellow box*). Human chymase/MCPT4 seems to prefer small and hydrophobic residues in P4 to P2 and to require an acidic residue in P2′ (*red*) to make the cut after the aromatic amino acid (*bold*) in P1 position (18). *B*, comparison of human Hsp70 (*Hum*) from ATGen (HSP0603, without the His tag) (64, 65), mouse Hsp70 (*Mus*) (GenBank™ AAC84168.1), and *T. spiralis* Hsp70 (*Tri*), residues 1–630 (of the 1003 amino acids of HspA, GenBank EFV56367). *C*, comparison of human and mouse IL-33 (National Center for Biotechnology Information (NCBI) Ref. Seq: human IL-33 (NP_254274.1) and mouse IL-33 (NP_001158196.1)). The suggested caspase cleavage site for the IL-1 family members, the serine residue in position 112, is indicated in *bold*. The activation cleavage sites for *N*-elastase/cathepsin G at residues 95–109 are *underlined* (56). *Yellow boxes* (8 amino acids) indicate the potential extended cleavage sites for chymase in Hsp70 and IL-33. Note that both Hsp70 and IL-33 contain additional aromatic amino acids (*bold*) in positions that lack the acidic residue in P2′. *Blue boxes* indicate nonconserved amino acids.

phylaxis (27). IL-33 is normally released in the proform as a 251-amino acid protein, and the neutrophil proteases elastase and cathepsin G have been shown to perform activating cleavages around residues 95–107, resulting in a 10-fold increased activity of the remaining ~18-kDa IL-33 peptide (56). Our data show that rHC/MCPT4, but not MC tryptase, could degrade

the 18-kDa peptide further. When chymase cuts in the three potential cleavage sites identified, although sites 1 and 3 may be hidden in the tertiary structure, it removes up to 6 kDa of the N terminus that may interact with the ST2 receptor (Fig. 8*C*). However, whether any activity of the residual ~12-kDa IL-33 peptide remains after chymase cleavage needs to be determined. Interestingly, caspase 1 has been shown to cleave and inactivate IL-33 at residue Asp^{178} (25), which is 15 amino acids downstream of the potential chymase cleavage site 3. Although MCs may respond to IL-33 with degranulation (57) or without (Ref. 27 and our results) degranulation, other factors released from pathogens and injured tissue, *e.g.* Hsp70, may provoke MC degranulation and hence the release of chymase, which thereby affects the local levels of bioactive IL-33.

Assessment of the degradation of Hsp70 and IL-33 *ex vivo* in a cell culture-based assay with WT and MCPT4-deficient PCMCs, and *in vivo* by intraperitoneal injections in WT and *Mcpt4*$^{-/-}$ mice, showed that MCPT4-deficient MCs and mice largely failed to process Hsp70 and IL-33, whereas WT MCs and mice degraded Hsp70 and IL-33 efficiently. In further support for a role of chymase in Hsp70 degradation *in vivo*, the base line of endogenous Hsp70 in PBS-injected *Mcpt4*$^{-/-}$ mice was found to be higher than in WT mice (Fig. 5*A*). The decline in *Mcpt4*$^{-/-}$ mice of injected Hsp70 levels could depend on absorption and/or receptor binding, or could indicate that other proteases than MCPT4 can degrade Hsp70.

Our results show that MCPT4/chymase is one of the major MC proteases involved in the degradation of Hsp70 and IL-33, and we provide evidence for a possible link between MCs and the regulation of inflammation during infection and tissue injury. The inflammatory properties and action of Hsp70 have been debated, but it seems that Hsp70 can act over several receptors, *e.g.* TLR2, CD40, LOX-1, and CD91 (58–61). These receptors are expressed by MCs, and because Hsp70 causes moderate MC degranulation (Fig. 4) and *Mcpt4*$^{-/-}$ mice fail to degrade injected rhHsp70 and Hsp70 during a *T. spiralis* infection (Fig. 5), our results suggest a physiological interaction between chymase/MCPT4 and Hsp70, and hence a possible role of the chymase in moderating inflammation during infection- and injury-induced inflammation. The increased neutrophil recruitment and TNF-α levels observed after Hsp70 challenge in the *Mcpt4*$^{-/-}$ mice also support such a conclusion (Fig. 7). Furthermore, in support of a requirement of extended recognition sites for effective chymase degradation, TNF-α displayed three extended recognition sites (data not shown). This finding supports a recent study where chymase was shown to degrade TNF-α and to affect neutrophil recruitment during microbial infection (62).

In humans, chymases seem to play a significant role in IL-6 degradation (63). In contrast, mouse IL-6 that contains four extended cleavage sites, although poorly conserved, was not significantly regulated by MCPT4 in Hsp70-challenged mice and in an *in vitro* degradation assay (Fig. 7*B* and data not shown). This suggests that in mice the IL-6 levels may be regulated primarily by other proteases than MCPT4.

In conclusion, the rapid and specific degradation of Hsp70 and IL-33 *in vivo* suggests that alarmins are physiological substrates for chymase during danger-induced inflammation. Once activated the MCs, through chymase, can act on several alarmins and thereby modulate inflammation. This suggests that MC chymase has multiple substrates, both early during the pro-inflammatory response and later during the down-regulation of an immune response. The intricate interplay between pro-inflammatory and anti-inflammatory properties of MCs is still largely unknown, but our data further point at the important and complex role of the MC proteases during infection and injury.

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