

Active Caspase-3 Is Stored within Secretory Compartments of Viable Mast Cells

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Caspase-3 is a main executioner of apoptotic cell death. The general notion is that, in viable cells, caspase-3 is found as a cytosolic inactive proenzyme and that caspase-3 activation is largely confined to processes associated with cell death. In this study, we challenge this notion by showing that enzymatically active caspase-3 is stored in viable mast cells. The enzymatically active caspase-3 was undetectable in the cytosol of viable cells, but was recovered in subcellular fractions containing secretory granule-localized proteases. Moreover, active caspase-3 was rapidly released into the cytosolic compartment after permeabilization of the secretory granules. Using a cell-permeable substrate for caspase-3, the presence of active caspase-3-like activity in granule-like compartments close to the plasma membrane was demonstrated. Moreover, it was shown that mast cell activation caused release of the caspase-3 to the cell exterior. During the course of mast cell differentiation from bone marrow cells, procaspase-3 was present in cells of all stages of maturation. In contrast, active caspase-3 was undetectable in bone marrow precursor cells, but increased progressively during the process of mast cell maturation, its accumulation coinciding with that of a mast cell-specific secretory granule marker, mouse mast cell protease 6. Together, the current study suggests that active caspase-3 can be stored within secretory compartments of viable mast cells. *The Journal of Immunology*, 2013, 191: 1445–1452.

Mast cells are main effector cells of the immune system, being implicated in a variety of pathological contexts, including allergic disorders, cancer, atherosclerosis, arthritis, and host defense (1). A hallmark characteristic of mast cells is their large content of lysosome-like secretory granules, filled with numerous preformed bioactive compounds such as histamine, serotonin, cytokines, serglycin proteoglycans, lysosomal enzymes, and different mast cell-specific proteases, the latter comprising tryptases, chymases, and carboxypeptidase A3 (CPA3) (2). When mast cells are activated, a process that can be accomplished in many ways, the cells degranulate and thereby release the contents of their secretory granules to the cell exterior (3).

The caspases comprise a family of ubiquitously expressed cysteine proteases (4). The caspase family can be subdivided into the proinflammatory members (caspase-1, -4, -5, -12) and into caspases with a role in apoptosis (5, 6). Among the proapoptotic caspases, caspase-2, -8, -9, and -10 are regarded as initiator caspases, that is, their main function is to act early in the pathways leading to apoptotic cell death (6, 7). Caspase-3, -6, and -7, in contrast, are

classified as executioner caspases, with a main function in the late events of apoptotic cell death (6, 7).

Caspase-3, like all other caspases, is synthesized as a proenzyme lacking an endoplasmic reticulum localization signal peptide, that is, it is translated in the cytosol and is localized in this compartment as an inactive proenzyme under latent conditions. Upon the induction of apoptosis, procaspase-3 is proteolytically activated, and activated caspase-3 then executes many of the hallmark events of apoptosis (4, 8). Hence, procaspase-3 activation is generally considered as an event associated with apoptotic cell death. Consequently, procaspase-3 is the predominant form present in living cells, whereas active caspase-3 is normally not found. In this study, we present evidence that contests this general notion, by showing that enzymatically active caspase-3 is stored within granule-like compartments of live mast cells and that mast cells can release enzymatically active caspase-3. Moreover, we show that storage of activated caspase-3 accompanies the differentiation of precursor cells into mature mast cells.

Materials and Methods

Reagents

H-Leu-Leu-OMe-HBr (L-leucyl-L-leucine methyl ester [LLME]) was from Bachem (Bubendorf, Switzerland). Digitonin was from Sigma-Aldrich (Steinheim, Germany). Rabbit anti-procaspase-3 and anti-activated caspase-3 were from Abcam (Cambridge, U.K.), and goat anti- β -actin was from Santa Cruz Biotechnology (Santa Cruz, CA). EasyColl was from Biochrom (Berlin, Germany).

Mice

Mice (8–18 wk old) were all on C57BL/6J genetic background, and all animal experiments were approved by the local ethical committee.

Bone marrow-derived mast cells

Bone marrow-derived mast cells (BMMCs) were obtained by culturing bone marrow cells in the presence of IL-3, as previously described (9).

Activation of mast cells

Cells were sensitized with IgE anti-trinitrophenyl at 1 μ g/ml overnight at 37°C. After being washed, cells were resuspended in Tyrode's buffer (130

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Abbreviations used in this article: BMMC, bone marrow-derived mast cell; CPA3, carboxypeptidase A3; LDH, lactate dehydrogenase; LLME, L-leucyl-L-leucine methyl ester; mMCP-6, mouse mast cell protease 6; PCMC, peritoneal cell-derived mast cell; SCF, stem cell factor.

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mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA [pH 7.4]) and stimulated with TNP-OVA (0.4 µg/ml). Alternatively, 1 µM A23187 was used. At different time points of incubation, media and cell fractions were frozen and stored at -20°C until analysis. Control experiments showed that A23187 was not toxic for the mast cell populations studied (data not shown). β-Hexosaminidase activity was assayed, as described (9).

Preparation of granule-enriched fractions

Granule-enriched fractions were obtained from BMMCs by soft disruption in a bath sonicator and density centrifugation in Percoll, as previously described (10).

Enzymatic assays

Caspase-3-like activity was measured using the Z-DEVD-R110-based EnzChek caspase-3 assay kit 2 procedure (Molecular Probes/Invitrogen, Carlsbad, CA), and fluorescence intensity was monitored at 496 nm (excitation) and 520 nm (emission). Cysteine cathepsin-like activity was measured using 20 µM Z-Phe-Arg-AMC (Bachem). AMC release was monitored at 390 nm (excitation) and 460 nm (emission). Lactate dehydrogenase (LDH) was determined by a decrease in absorbance at 340 nm, resulting from the oxidation of 0.2 mM NADH. Trypsin-like activity was measured, as previously described (11). Histone deacetylase activity was measured using the HDAC Assay Kit, Fluorometric Detection (Millipore, Billerica, MA), following instructions from the manufacturer. For all measurements, samples were transferred in duplicates into individual wells of 96-well flat-bottom plates, and readings were performed at room temperature using a microplate reader (Infinite M200; TECAN).

Immunoblot analysis

Samples corresponding to equal numbers of cells were solubilized in SDS-PAGE sample buffer containing 5% DTT, as previously described (12). Membranes were scanned using an Odyssey Infrared Imager (Li-cor).

Digitonin extracts and cytosolic extract preparation

BMMCs (10⁶ cells) were collected by centrifugation (1200 rpm, 8 min, 4°C) in 1.5-ml Eppendorf tubes and then resuspended in 300 µl different concentrations of digitonin in ice-cold digitonin extraction buffer (250 mM sucrose, 20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA). After 10-min incubation on ice, the supernatant was quickly removed by centrifugation (13,000 rpm; 2 min, 4°C) and frozen (-20°C). Cytosolic extracts were obtained at the same conditions by using 10 µg/ml digitonin in ice-cold digitonin extraction buffer.

Caspase-3-like activity in live cells

Aliquots of 100 µl from 0.5 × 10⁶ cells/ml suspension were transferred into individual wells of a 96-well flat-bottom plate, in the presence and/or absence of 20 µM Z-DEVD-FMK (Sigma-Aldrich, Steinheim, Germany). After 1-h incubation at 37°C in 5% CO₂, 5 µM PhiPhiLux-G₂D₂ (OncoImmunin, Gaithersburg, MD) was added, followed by incubation for 2 h. Thirty and 10 min before the end of the incubation time, 20 µM calcein-AM (Invitrogen, Carlsbad, CA) and 0.3 µg DAPI were added. Cells were then transferred into 1.5-ml tubes and washed twice with 300 µl PBS. Pellets were resuspended in 100 µl PBS, transferred to microscopy glasses, fixed with 4% paraformaldehyde for 15 min, and covered with fluoromount-G mounting medium (Southern Biotech, Birmingham, AL) and cover glass. The entire assay was performed in the dark. Samples were analyzed using a laser-scanning microscope equipped with ZEN 2009 software (LSM 710; Carl Zeiss, Berlin, Germany).

Total RNA isolation and quantitative PCR

Total RNA was isolated and analyzed for levels of caspase-3 mRNA, as described (9), using the following primers for caspase-3: 5'-TCAG-AGGCGACTACTGCCGGA-3' as forward primer and 5'-CCACCGGT-ATCTTCTGGCAAGCC-3' as reverse primer. Hypoxanthine guanine phosphoribosyl transferase was used as housekeeping gene using the primers specified previously (9).

Statistical analysis

Data shown are means ± SEM. Statistical analyses were performed by using GraphPad Prism 4.0c (GraphPad Software) and paired Student *t* test for two-tailed distributions (**p* < 0.05, ***p* < 0.01, ****p* < 0.001).

Results

Active caspase-3 is present in mast cells and is released by mast cell activation

In a previous study, we showed that mast cells are sensitive to cell death induced by secretory granule-permeabilizing agents, and that mast cell apoptosis in response to such agents is accompanied by caspase-3 activation (13). Unexpectedly, we noted signs of the presence of active caspase-3 even in the absence of cell death-inducing agent (13). Possibly, the activated caspase-3 could have been present in an apoptotic subpopulation of the primary mast cell population used for those studies. However, we in this study decided to investigate the alternative possibility, that is, that enzymatically active caspase-3 may in fact be present in viable mast cells. For this purpose, we developed mast cells by differentiation of bone marrow cells into mature mast cells, that is, BMMCs (9). In addition, we derived mast cells by expansion of the peritoneal mast cell population, that is, peritoneal cell-derived mast cells (PCMCs) (14).

To assay for caspase-3 activity, we used Z-DEVD-R110. As shown in Fig. 1A, BMMCs indeed possessed caspase-3-like activity in the absence of cytotoxic agent, and this activity was completely abrogated by addition of a caspase-3 inhibitor (Ac-DEVD-CHO). Moreover, immunoblot analysis using an Ab detecting active caspase-3 (but not the proenzyme) revealed the presence of processed caspase-3 in BMMCs (Fig. 1B). Notably, two bands were recognized by the anti-caspase-3 Ab, migrating at positions corresponding to ~22.5 and ~19.5 kDa. These bands are thus in agreement with the partly processed, enzymatically active caspase-3 forms recently reported by Pederzoli et al. (15). Both of these bands were confirmed to correspond to caspase-3, as judged by abrogated signals in the presence of a blocking peptide (Fig. 1B).

Because active caspase-3 is unlikely to be present in the cytosol of viable cells, we considered the possibility that it may be sequestered within secretory granules. To address this possibility, we added agents that cause mast cell degranulation and assessed whether this resulted in release of active caspase-3. As seen in Fig. 1C, release of caspase-3-like activity into the medium was indeed induced by the calcium ionophore A23187, an agent that is known to produce robust mast cell degranulation. However, caspase-3 activity was also found in the cell supernatant in the absence of mast cell-activating stimuli, suggesting that active caspase-3 is released spontaneously by mast cells. In the presence of a caspase-3 inhibitor (Ac-DEVD-CHO), the secreted caspase-3-like activity was almost completely abrogated (Fig. 1C), whereas a general inhibitor of serine proteases (Pefabloc SC) had no effect. The addition of calcium ionophore did not cause any increase in the levels of caspase-3 mRNA, indicating that the increase in extracellular caspase-3 activity was not a consequence of increased caspase-3 mRNA expression (Fig. 1D). Moreover, the release of caspase-3 activity into the medium was accompanied by a decrease in the contents of intracellular caspase-3, as judged by both activity measurements (Fig. 1A) and immunoblot analysis (Fig. 1B). In contrast to the response toward calcium ionophore, we did not detect any increase in caspase-3 release in response to IgE receptor cross-linking (Fig. 1A). As a positive control to verify that the used stimulants induce mast cell degranulation, the stimulation by both calcium ionophore and IgE receptor cross-linking was shown to cause release of β-hexosaminidase, a granular marker (Fig. 1E).

To extend these findings, we assessed whether enzymatically active caspase-3 was also found in PCMCs, a cell population that represents terminally differentiated mast cells (14). Indeed, processed caspase-3 was detected by immunoblot analysis of cell extracts from PCMCs (Fig. 2A). Moreover, activation of PCMCs

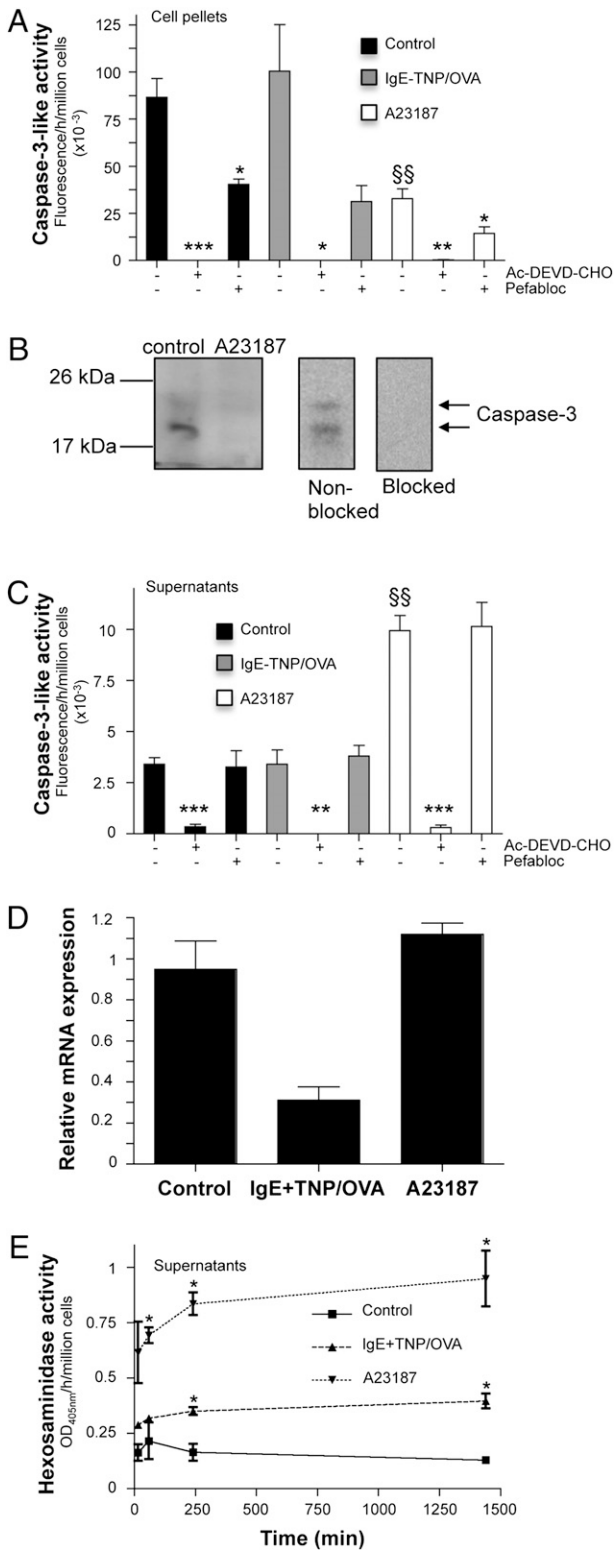


FIGURE 1. Active caspase-3 is present in mast cells and is released by mast cell degranulation. BMMCs (4×10^6 cells) were either nontreated (control) or induced to degranulate using calcium ionophore (A23187) or IgE receptor cross-linking (IgE-TNP/OVA). (A) After centrifugation, cell pellets were solubilized and cell extracts were analyzed for caspase-3-like activity, in the absence or presence of either a caspase-3 inhibitor (Ac-DEVD-CHO) or a serine protease inhibitor (Pefabloc SC), as indicated. (B) Immunoblot analysis for active caspase-3 using an Ab that detects active caspase-3, but not the proenzyme, showing the presence of processed caspase-3 in nonactivated BMMCs, and depletion of cell-associated caspase-3 after mast cell degranulation using A23187. The specificity of the caspase-3 Ab was verified by inclusion of soluble

with calcium ionophore resulted in increased release of caspase-3 activity into the medium (Fig. 2B), accompanied by a depletion of cellular caspase-3 (Fig. 2A). In contrast, IgE receptor cross-linking did not cause a significant depletion of cell-associated caspase-3 (Fig. 2A) or any significant increase in the release of caspase-3 into the medium (Fig. 2B). Activation of the PCMCs by calcium ionophore or by IgE receptor cross-linking did not cause any significant effects on caspase-3 mRNA expression (Fig. 2C). Approximately 25 and 8% of total caspase-3-like activity was released by BMMCs and PCMCs, respectively, in response to calcium ionophore stimulation, whereas ~60% of the total trypsin activity was released.

Accumulation of active caspase-3 accompanies the differentiation of bone marrow precursor cells into mature mast cells

To investigate whether the presence of active caspase-3 is linked to mast cell phenotype, we studied the expression of caspase-3 during the course of differentiation of bone marrow precursor cells into mature BMMCs. As expected, based on the ubiquitous expression of caspase-3, caspase-3 mRNA was found in cells of all stages of maturation (Fig. 3A). Notably though, the level of caspase-3 mRNA increased progressively as the cells matured into mature mast cell phenotype. An increase in caspase-3 mRNA expression in maturing mast cells was apparent using three different protocols for inducing mast cell differentiation, each producing a mast cell population of distinct phenotype (9, 16, 17). To produce mast cells with a phenotype that resembles that of connective tissue-type mast cells, cells were cultured in either WEHI-3B-conditioned medium (contains IL-3) or IL-3 plus stem cell factor (SCF; produces BMMCs of more mature connective tissue-type mast cell-like phenotype). To produce mast cells with a phenotype that resembles that of mucosal-type mast cells, cells were cultured in IL-3 plus SCF plus IL-9 plus TGF- β (Fig. 3A).

At the protein level, procaspase-3 was found in approximately equal amounts in cells of all stages of maturation (Fig. 3B, upper panel). In contrast, active caspase-3 was undetectable in the bone marrow cell population (day 0) and at early stages of mast cell maturation, but was clearly detected in cells of later stages of mast cell maturation (Fig. 3B, middle panel). At all stages of cell culture, cell viability exceeded 95%, with no increase in the percentage of dead cells in late-stage cultures. Hence, the accumulation of caspase-3 in late-stage cultures is not a reflection of an increased population of nonviable, apoptotic cells. Notably, the accumulation of active caspase-3 coincided with the accumulation of mouse mast cell protease 6 (mMCP-6), the latter being a mast cell-specific protease localized to the secretory granules (18) (Fig. 3B, lower panel). The levels of caspase-3 protein were similar in mature mast

peptide corresponding to the epitope used for immunization (peptide blocking; right panels). (C) After 4 h of incubation of control cells or cells treated with either calcium ionophore (A23187) or IgE receptor cross-linking (IgE-TNP/OVA), samples (50 μ l) were collected from the medium and analyzed for caspase-3-like activity, in the absence or presence of either a caspase-3 inhibitor (Ac-DEVD-CHO) or a serine protease inhibitor (Pefabloc SC), as indicated. (D) Effect of mast cell activation by either IgE receptor cross-linking or calcium ionophore stimulation on expression of caspase-3 mRNA as measured by quantitative PCR analysis. (E) Control experiment showing robust β -hexosaminidase release in response to both A23187 and IgE receptor cross-linking. $n = 3$ independent experiments; duplicate measurements. (A, C) $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with its noninhibited sample (i.e., A23187 + Ac-DEVD-CHO compared with A23187 alone); $§§p < 0.01$ compared with nonstimulated control samples. (E) $*p < 0.05$ compared with nonstimulated control cells.

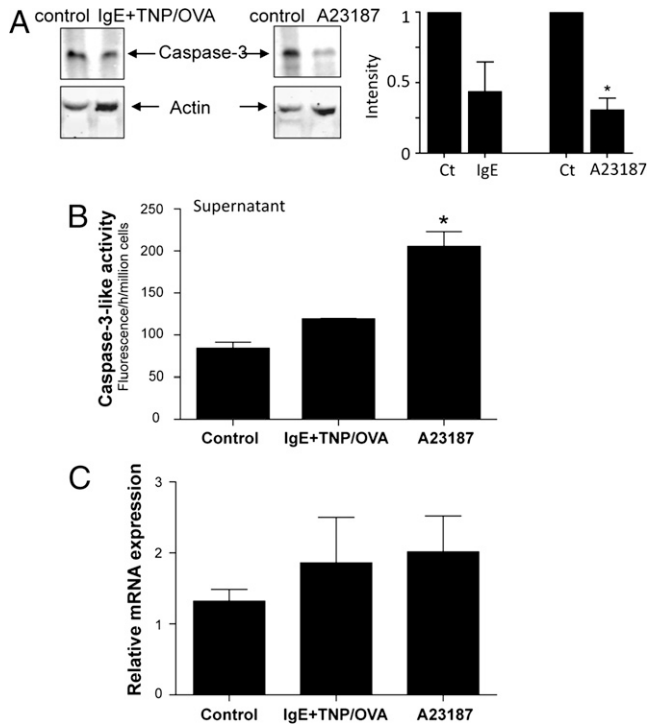


FIGURE 2. Enzymatically active caspase-3 is found in PCMCs. PCMCs (4×10^6 cells) were nontreated (control; Ct) or induced to degranulate using calcium ionophore (A23187) or IgE receptor cross-linking, as indicated. **(A)** Immunoblot analysis for active caspase-3 showing the presence of active caspase-3 in nonactivated PCMCs, and depletion of cell-associated active caspase-3 after mast cell degranulation using A23187, but not IgE receptor cross-linking. Quantification of the band intensities is shown in the *right panel* relative to control (set to 1). **(B)** Samples (50 μ l) from the conditioned media were collected after 4 h of culture in the absence or presence of mast cell-activating stimulus, as indicated, and were assayed for caspase-3 activity. **(C)** Effect of IgE receptor cross-linking or calcium ionophore (A23187) stimulation on expression of caspase-3 mRNA in PCMCs, as measured by quantitative PCR analysis. (A) $n = 3$ independent experiments; (B) $n = 4$ independent experiments, duplicate measurements; (C) $n = 3$ independent experiments, duplicate measurements. * $p < 0.05$.

cells developed with either of the protocols used (Fig. 3C). Together, these data indicate that high caspase-3 mRNA expression and accumulation of high levels of active caspase-3 protein accompany mast cell maturation.

Active caspase-3 is found within granule-like compartments in mast cells

To address the subcellular localization of active caspase-3 in mast cells, we first analyzed for its presence in cytosolic extracts. A titration experiment showed that digitonin concentrations $>2 \mu\text{g/ml}$ were sufficient to recover the cytosol marker LDH from the mast cells along with a general release of cellular protein (Fig. 4A). However, no detectable active caspase-3 was recovered at digitonin concentrations up to $\sim 20 \mu\text{g/ml}$, thus arguing against its presence within the cytosolic compartment. In contrast, solubilization of active caspase-3 was seen when increasing the detergent concentrations $>20 \mu\text{g/ml}$ (Fig. 4A). At these conditions, we also observed solubilization of CPA3, CPA3 being a mast cell-specific granule protease (19) (Fig. 4B), as well as solubilization of enzymatic activity toward Z-Phe-Arg-AMC, a substrate commonly used for detection of lysosomal cysteine cathepsin activity (Fig. 4A). These findings suggest that active caspase-3 is localized in

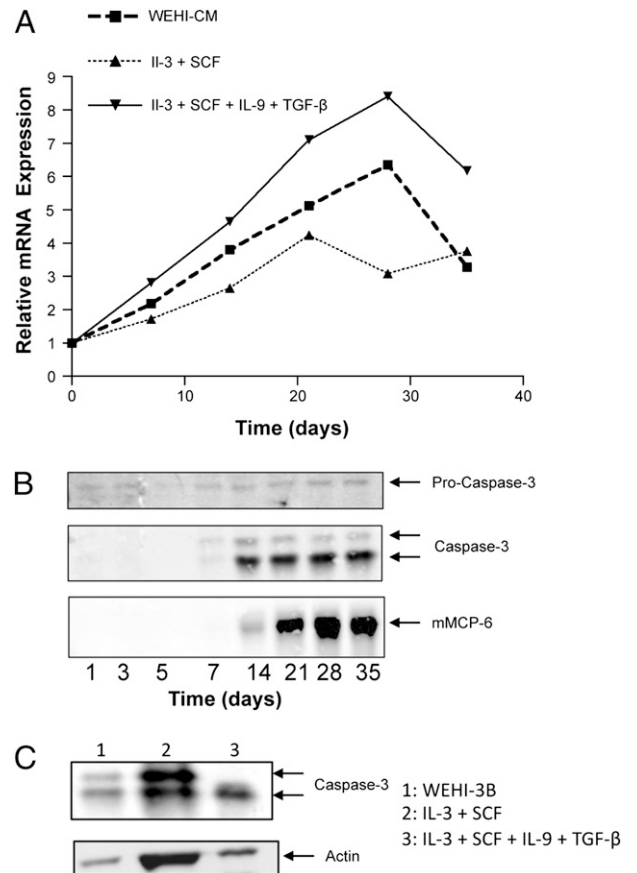


FIGURE 3. Expression of active caspase-3 protein and of caspase-3 mRNA is linked to mast cell maturation. Bone marrow cells were isolated and were differentiated into mast cell phenotype. **(A)** Caspase-3 mRNA levels in bone marrow cells at different stages of mast cell maturation. As indicated, mast cell maturation was induced using either conditioned medium from WEHI-3B cells (WEHI-CM; contains IL-3 and produces mast cells of connective tissue-like subtype), by a combination of IL-3 and SCF (alternative protocol resulting in mast cells of more mature connective tissue-like subtype), or by a combination of IL-3 + SCF + TGF- β + IL-9 (produces mast cells of mucosal-like subtype). **(B)** At the time points indicated, samples (corresponding to 0.3×10^6 cells) were analyzed by immunoblot for procaspase-3 (*upper panel*) and for active caspase-3 (*middle panel*) protein. For comparison, cells were assessed for levels of mMCP-6, a mast cell-specific protease localized to secretory granules. Note that the appearance of active caspase-3 protein coincides with the appearance of mMCP-6, the latter a sign that the cells have acquired mature mast cell phenotype. **(C)** Immunoblot analysis showing the levels of active caspase-3 in mature (day 28) mast cells developed using different protocols, as indicated. The results are representative of two independent experiments.

mast cell compartments that have characteristics similar to those of the secretory granules/lysosomes.

To further analyze the subcellular localization of active caspase-3 in mast cells, we used a cell-permeable substrate (PhiPhiLux) to detect caspase-3 activity in live cells. As seen in Fig. 5A (*upper panels*) showing a representative cell; additional stained cells are shown in Supplemental Fig. 1), staining of mast cells with PhiPhiLux revealed the presence of caspase-3-positive granule-like compartments, showing a predominant localization close to the cell membrane. Importantly, the active caspase-3-positive cells also stained positively for calcein-AM, the latter a marker for cellular viability (Fig. 5A). A Z stack showing the three-dimensional distribution of active caspase-3 in mast cells is displayed in Supplemental Fig. 2. The staining with PhiPhiLux was

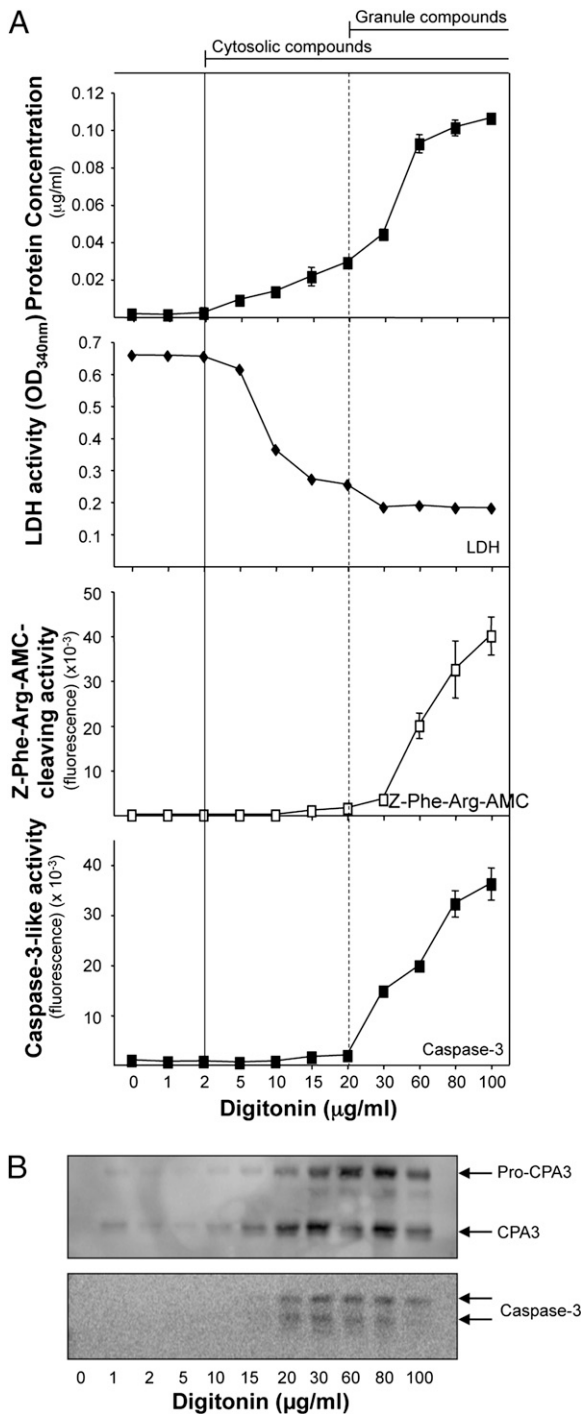


FIGURE 4. Active caspase-3 is absent in the cytosol of mast cells, but is recovered under conditions that cause secretory granule solubilization. **(A)** Mast cells (BMMCs; 1×10^6 cells) were subjected to digitonin at the concentrations indicated. After 10-min incubation on ice, cells were pelleted by centrifugation and the remaining supernatant was analyzed for total protein (upper panel), LDH activity (cytosol marker), activity toward Z-Phe-Arg-AMC (substrate for lysosomal cysteine cathepsins), and caspase-3-like activity, as indicated. **(B)** Cell extracts obtained by solubilization with digitonin at the indicated concentrations were analyzed by immunoblot for the presence of pro- and active CPA3 (secretory granule marker) and for active caspase-3. Note that active caspase-3 is solubilized under similar conditions as those required for solubilization of CPA3.

completely abrogated after addition of a caspase-3 inhibitor (Fig. 5B, right panel). After activation of mast cells with calcium ionophore, the intracellular caspase-3-positive granule-like com-

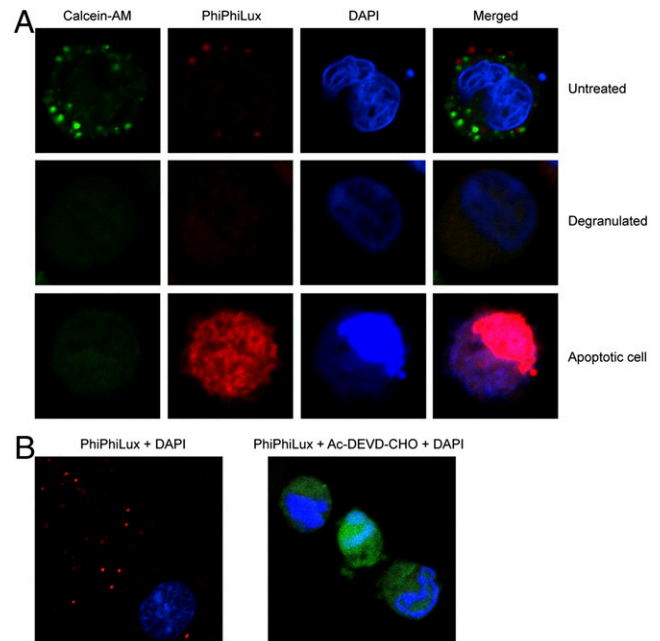


FIGURE 5. Active caspase-3 is localized in granule-like secretory vesicles of mast cells. **(A)** Mast cells (BMMCs) were stained with calcein-AM (viability marker), PhiPhiLux (cell-permeable fluorescent caspase-3 substrate), and DAPI (nuclear staining), followed by confocal microscopy analysis. Staining was performed on either nontreated cells (upper panels) or cells that had undergone calcium ionophore-induced degranulation (middle panels). Note the presence of caspase-3-positive compartments close to the plasma membrane in nontreated cells and the depletion of these after degranulation. The lower panels depict a representative apoptotic cell (occasional apoptotic cells are observed in cultures of BMMCs), showing negativity for calcein-AM and intense caspase-3 activity throughout the entire cytoplasm. **(B)** The left panel shows a lower magnification of degranulated mast cells, where released caspase-3-positive compartments are seen in the extracellular space. The right panel depicts the abrogated PhiPhiLux staining after addition of caspase-3 inhibitor. Original magnification $\times 400$.

partments disappeared (Fig. 5A, middle panels), along with the recovery of corresponding caspase-3-positive entities in the extracellular space (Fig. 5B, left panel). For a comparison, a cell with typical signs of apoptosis is depicted in Fig. 5A (lower panels), showing strong cytoplasmic positivity for active caspase-3. Together, these data suggest the presence of preformed active caspase-3 in granule-like, releasable compartments found close to the cell membrane of mast cells.

To provide further insight into the subcellular location of active caspase-3 in mast cells, subcellular fractionation was performed using methodology previously established for purification of secretory granules from rat peritoneal mast cells (20). The granule-enriched fraction obtained contained undetectable levels of LDH activity, indicating that there was no significant contamination of cytosolic proteins in the granule fraction (Fig. 6A). Note that LDH activity results in NADH consumption and thus reduced absorbance from initial levels (indicated by a dashed line). The left bar (cells) represents LDH activity in sonicated cells, that is, the total activity of the cell preparation used for purification of granules. Moreover, there were negligible levels of histone deacetylase activity in the granule fraction, suggesting that the contamination by nuclear compounds was minimal (data not shown). In contrast, the granule fraction contained substantial levels of trypsin-like activity (Fig. 6B). As most of the trypsin-like activity in mast cells is due to granule-located tryptase, this suggests that the purified

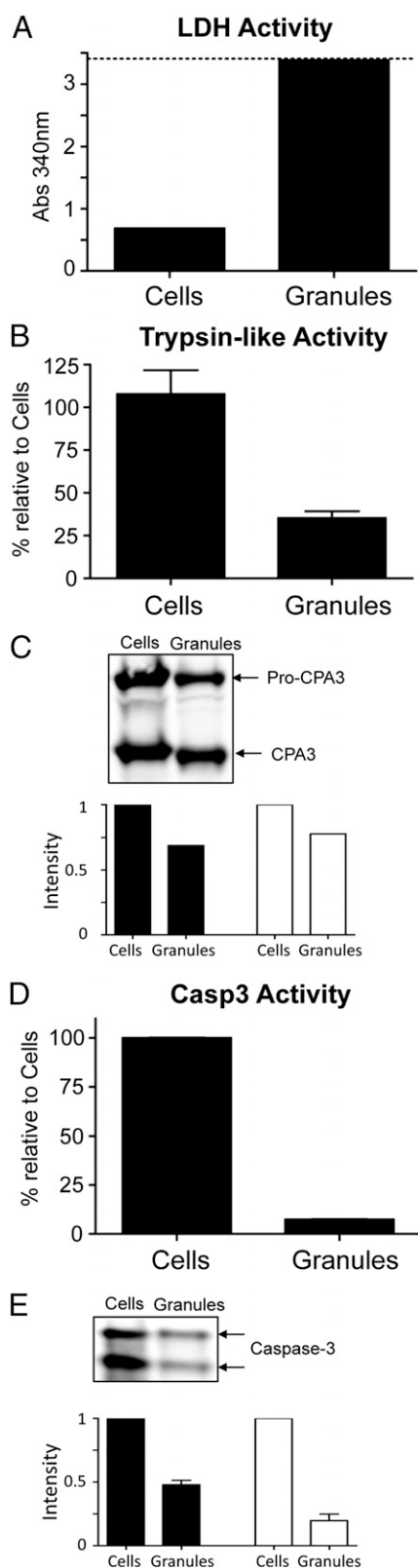


FIGURE 6. Active caspase-3 is recovered in granule-enriched fractions. Secretory granules were purified from BMMCs. **(A)** LDH activity (60 min) in sonicated cells used as starting material (cells) and in purified granules. Note that LDH activity results in decreased absorbance from initial level indicated by a dashed line, that is, LDH activity is undetectable in purified granules. **(B)** Trypsin-like activity (30 min) in starting material (Cells) and in purified granules. **(C)** Immunoblot analysis showing CPA3 levels in starting material (Cells) and in purified granules. The *lower panel* shows quantification of band intensities relative to control intensity (set to 1). **(D)**

fraction indeed is enriched with secretory granules. In further support for this, the granule fraction contained substantial levels of the granule-specific protein CPA3 as shown by immunoblot analysis (Fig. 6C). As seen in Fig. 6D, it was also evident that caspase-3 activity was detected in the granule-enriched fraction, and that caspase-3 protein was readily detected by immunoblot analysis (Fig. 6E). Hence, these data support that active caspase-3 is located in secretory granules of mast cells.

Secretory granule permeabilization causes release of active caspase-3 into the cytosol

The findings described above suggest that preformed active caspase-3 is present in granule-like compartments of mast cells. If so, disruption of the mast cell granule membrane would have the potential to result in release of active caspase-3 into the cytosol. To evaluate this possibility, we subjected mast cells to LLME, a lysosomotropic agent that has mast cell granule-permeabilizing properties (13, 21), and analyzed cytosolic extracts for the presence of active caspase-3. As shown in Fig. 7, LLME induced a robust and rapid release of active caspase-3-like activity (Fig. 7A, *lower panel*) and protein (Fig. 7B, *upper panel*) as well as release of Z-Phe-Arg-AMC-cleaving activity (Fig. 7A, *upper panel*) into the cytosol. Moreover, LLME induced a release of mast cell granule marker proteins (CPA3 and mMCP-6) into the cytosolic fraction (Fig. 7B, *middle and lower panels*, respectively), thus confirming its granule-permeabilizing properties. Together, these findings suggest that permeabilization of mast cell granules results in a rapid release of preformed active caspase-3 into the cytosolic compartment.

Discussion

The general notion is that activation of procaspase-3 to enzymatically active protease is an event mainly associated with cell death, and thus that caspase-3 is predominantly present as inactive proenzyme in viable cells. In this study, we present data challenging this notion by showing that enzymatically active caspase-3 is present in viable mast cells.

The presence of active caspase-3 in live mast cells was supported by the presence of caspase-3-like enzymatic activity and protein bands corresponding to processed caspase-3 in the absence of cell death-inducing agents. Importantly, active caspase-3 was found both in mast cells derived by *in vitro* differentiation of bone marrow precursors to mature mast cells (BMMCs) and in mast cells expanded from *in vivo*-differentiated mast cells (PCMCs), indicating that expression of active caspase-3 protein is a general property of mast cells. In strong support of the latter notion, we show that the appearance of active caspase-3 is strongly associated with maturation of bone marrow precursor cells into mature mast cells, whereas procaspase-3 was present in cells of all stages of differentiation. It was also intriguing to note that the mRNA levels for caspase-3 showed an increase as cells gained a mature mast cell phenotype, suggesting that high levels of caspase-3 mRNA expression are a phenotypic characteristic of mature mast cells.

An important issue to resolve was the intracellular location of active caspase-3. Normally, if procaspase-3 is activated within the cytosolic compartment, this will lead to apoptotic cell death. However, because our data suggested that active caspase-3 was in

Caspase-3-like activity in starting material (Cells) and in purified granules (16 h). **(E)** Immunoblot analysis showing levels of active caspase-3 in starting material (Cells) and in purified granules. The *lower panel* shows quantification of band intensities. Experiments were performed in duplicates from two independent granule-enriched purifications.

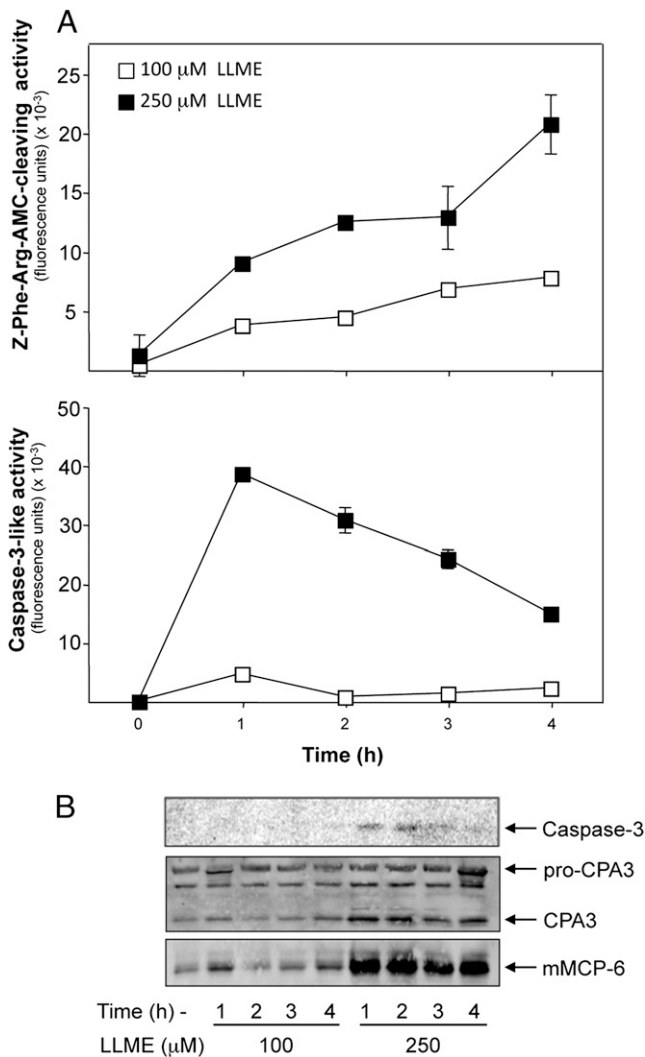


FIGURE 7. Secretory granule permeabilization causes release of active caspase-3 into the cytosolic compartment. **(A)** Mast cells (BMMCs; 1×10^6 cells) were incubated in the presence of 100 μ M or 250 μ M LLME. At the indicated time points, cytosolic extracts were prepared and were analyzed for levels of activity toward Z-Phe-Arg-AMC and for caspase-3-like activity. **(B)** Cytosolic extracts of untreated or LLME-treated mast cells were analyzed by immunoblot for the presence of active caspase-3 protein and for granule marker proteases (CPA3 and mMCP-6). Note absence of active caspase-3 in the cytosol of untreated cells and its appearance in the cytosol after secretory granule permeabilization.

fact present in live mast cells, we considered the possibility that the active protease may be present in a compartment separated from the cytosol. A dominating morphological feature of mast cells is their large content of lysosome-like secretory granules, and we decided to investigate whether the active caspase-3 may be sequestered within these organelles and thus being topologically separated from the cytosolic compartment. To address this possibility, we used several approaches. First, we subjected the mast cells to agents that cause mast cell degranulation, whereby the contents of their secretory granules are released to the exterior of the cells. In agreement with our hypothesis, mast cell degranulation using calcium ionophore caused a depletion of intracellular caspase-3 along with the appearance of caspase-3 activity in the cell medium. Another approach to this issue was to investigate whether permeabilization of the granule membrane would lead to release of active caspase-3 into the cytosolic compartment. Indeed, secretory granule permeabilization caused release of active caspase-3 into the

cytosol. In further support for a localization of active caspase-3 in secretory granules, we detected caspase-3 activity in rounded, granule-like compartments close to the cell membrane, and we also showed that mast cell activation caused the release of such caspase-3-positive entities to the extracellular space. Importantly, caspase-3-positive cells were also positive for calcein-AM, a dye that marks cellular viability, adding further support to the notion that caspase-3 is present in viable cells. Finally, by using subcellular fractionation, we show that active caspase-3 is recovered in a granule-enriched fraction. Taken together, we thus provide strong support for the presence of enzymatically active caspase-3 in secretory granules of mast cells.

The present findings have several implications for our understanding of caspase-3 function. First, our findings implicate caspase-3 in extracellular events. In this context, it is tempting to speculate that caspase-3 released from mast cells may have cytotoxic activity on target cells. In line with such a notion, limited evidence suggests that mast cells can exert cytotoxic activity on tumor cells (22, 23). However, it has been suggested that the cytotoxic activity of mast cells is mainly attributed to TNF (22, 23). Alternatively, extracellular caspase-3 may have functions that are not necessary associated with cell death. For example, there is limited evidence indicating that caspase-3 may have a role in cytokine processing (24, 25). It is thus conceivable that active caspase-3 released from mast cells may have a role in the regulation of inflammatory processes, for example, those orchestrated by mast cells (26). Another intriguing question is how caspase-3, a protein that is devoid of an endoplasmic reticulum localization signal peptide, is transported into the secretory granule compartment of mast cells. In a recent report, a role of autophagy in mast cell degranulation processes was suggested (27), and it was also shown that LC3-II, an autophagy-associated compound that is normally not present within organelles of the secretory pathway, was localized in mast cell granules (27). Based on these findings, it could thus be speculated that autophagy may have a bearing on mast cell granules and that, possibly, cytosolic compounds may enter the secretory granule compartment through autophagy. However, we have not seen any effect of autophagy inhibitors on the levels of active caspase-3 in mast cell secretory granules (G. Garcia-Faroldi, F.R. Melo, M. Grujic, and G. Pejler, unpublished data).

In some analogy with the current study, a recent report indicated that neutrophils contain a partly processed, enzymatically active caspase-3 variant (15). However, differently to our data, the neutrophil product was localized to the plasma membrane fraction and there was no evidence that the partly processed caspase-3 was released upon degranulation. It is also worth noting that we did not detect active caspase-3 in the bone marrow cell population, despite that this cell population is abundant in neutrophils (28). In another study, evidence was presented suggesting that caspase-3 can be transiently activated in CD8⁺ T cells without signs of cell death (29), and it has also been suggested that temporal activation of caspase-3 can have a role in monocyte-to-macrophage differentiation (30).

Interestingly, whereas calcium ionophore stimulation resulted in robust release of caspase-3 accompanied by a reduction of intracellular caspase-3, IgE receptor cross-linking failed to induce significant caspase-3 release. Hence, caspase-3 appears to be present in secretory vesicles distinct from those that are released by IgE-mediated mechanisms. Clearly, this may be in line with recent studies that have identified granule subtypes of distinct contents in mast cells (31).

Disclosures

The authors have no financial conflicts of interest.

References

- Kalesnikoff, J., and S. J. Galli. 2008. New developments in mast cell biology. *Nat. Immunol.* 9: 1215–1223.
- Lundequist, A., and G. Pejler. 2011. Biological implications of preformed mast cell mediators. *Cell. Mol. Life Sci.* 68: 965–975.
- Rivera, J., N. A. Fierro, A. Olivera, and R. Suzuki. 2008. New insights on mast cell activation via the high affinity receptor for IgE. *Adv. Immunol.* 98: 85–120.
- Earnshaw, W. C., L. M. Martins, and S. H. Kaufmann. 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* 68: 383–424.
- Taylor, R. C., S. P. Cullen, and S. J. Martin. 2008. Apoptosis: controlled demolition at the cellular level. *Nat. Rev. Mol. Cell Biol.* 9: 231–241.
- Pop, C., and G. S. Salvesen. 2009. Human caspases: activation, specificity, and regulation. *J. Biol. Chem.* 284: 21777–21781.
- Elmore, S. 2007. Apoptosis: a review of programmed cell death. *Toxicol. Pathol.* 35: 495–516.
- D'Amelio, M., V. Cavallucci, and F. Cecconi. 2010. Neuronal caspase-3 signaling: not only cell death. *Cell Death Differ.* 17: 1104–1114.
- Rönnberg, E., and G. Pejler. 2012. Serglycin: the master of the mast cell. *Methods Mol. Biol.* 836: 201–217.
- García-Faroldi, G., C. E. Rodríguez, J. L. Urdiales, J. M. Pérez-Pomares, J. C. Dávila, G. Pejler, F. Sánchez-Jiménez, and I. Fajardo. 2010. Polyamines are present in mast cell secretory granules and are important for granule homeostasis. *PLoS One* 5: e15071.
- Åbrink, M., M. Grujic, and G. Pejler. 2004. Serglycin is essential for maturation of mast cell secretory granule. *J. Biol. Chem.* 279: 40897–40905.
- Braga, T., M. Grujic, A. Lukinius, L. Hellman, M. Åbrink, and G. Pejler. 2007. Serglycin proteoglycan is required for secretory granule integrity in mucosal mast cells. *Biochem. J.* 403: 49–57.
- Melo, F. R., I. Waern, E. Rönnberg, M. Åbrink, D. M. Lee, S. M. Schlenner, T. B. Feyerabend, H. R. Rodewald, B. Turk, S. Wernersson, and G. Pejler. 2011. A role for serglycin proteoglycan in mast cell apoptosis induced by a secretory granule-mediated pathway. *J. Biol. Chem.* 286: 5423–5433.
- Malbec, O., K. Roget, C. Schiffer, B. Iannascoli, A. R. Dumas, M. Arock, and M. Daéron. 2007. Peritoneal cell-derived mast cells: an in vitro model of mature serosal-type mouse mast cells. *J. Immunol.* 178: 6465–6475.
- Pederzoli, M., C. Kantari, V. Gausson, S. Moriceau, and V. Witko-Sarsat. 2005. Proteinase-3 induces procaspase-3 activation in the absence of apoptosis: potential role of this compartmentalized activation of membrane-associated procaspase-3 in neutrophils. *J. Immunol.* 174: 6381–6390.
- Miller, H. R., S. H. Wright, P. A. Knight, and E. M. Thornton. 1999. A novel function for transforming growth factor-beta1: upregulation of the expression and the IgE-independent extracellular release of a mucosal mast cell granule-specific beta-chymase, mouse mast cell protease-1. *Blood* 93: 3473–3486.
- Gurish, M. F., N. Ghildyal, H. P. McNeil, K. F. Austen, S. Gillis, and R. L. Stevens. 1992. Differential expression of secretory granule proteases in mouse mast cells exposed to interleukin 3 and c-kit ligand. *J. Exp. Med.* 175: 1003–1012.
- Pejler, G., E. Rönnberg, I. Waern, and S. Wernersson. 2010. Mast cell proteases: multifaceted regulators of inflammatory disease. *Blood* 115: 4981–4990.
- Pejler, G., S. D. Knight, F. Henningsson, and S. Wernersson. 2009. Novel insights into the biological function of mast cell carboxypeptidase A. *Trends Immunol.* 30: 401–408.
- Krüger, P. G., D. Lagunoff, and H. Wan. 1980. Isolation of rat mast cell granules with intact membranes. *Exp. Cell Res.* 129: 83–93.
- Melo, F. R., A. Lundequist, G. Calounova, S. Wernersson, and G. Pejler. 2011. Lysosomal membrane permeabilization induces cell death in human mast cells. *Scand. J. Immunol.* 74: 354–362.
- Benyon, R. C., E. Y. Bissonnette, and A. D. Befus. 1991. Tumor necrosis factor-alpha dependent cytotoxicity of human skin mast cells is enhanced by anti-IgE antibodies. *J. Immunol.* 147: 2253–2258.
- Déry, R. E., T. J. Lin, A. D. Befus, C. D. Milne, R. Moqbel, G. Ménard, and E. Y. Bissonnette. 2000. Redundancy or cell-type-specific regulation? Tumor necrosis factor in alveolar macrophages and mast cells. *Immunology* 99: 427–434.
- Zhang, Y., D. M. Center, D. M. Wu, W. W. Cruikshank, J. Yuan, D. W. Andrews, and H. Kornfeld. 1998. Processing and activation of pro-interleukin-16 by caspase-3. *J. Biol. Chem.* 273: 1144–1149.
- Akita, K., T. Ohtsuki, Y. Nukada, T. Tanimoto, M. Namba, T. Okura, R. Takakura-Yamamoto, K. Torigoe, Y. Gu, M. S. Su, et al. 1997. Involvement of caspase-1 and caspase-3 in the production and processing of mature human interleukin 18 in monocytic THP.1 cells. *J. Biol. Chem.* 272: 26595–26603.
- Rodewald, H. R., and T. B. Feyerabend. 2012. Widespread immunological functions of mast cells: fact or fiction? *Immunity* 37: 13–24.
- Ushio, H., T. Ueno, Y. Kojima, M. Komatsu, S. Tanaka, A. Yamamoto, Y. Ichimura, J. Ezaki, K. Nishida, S. Komazawa-Sakon, et al. 2011. Crucial role for autophagy in degranulation of mast cells. *J. Allergy Clin. Immunol.* 127: 1267–1276.e6.
- Day, R. B., and D. C. Link. 2012. Regulation of neutrophil trafficking from the bone marrow. *Cell. Mol. Life Sci.* 69: 1415–1423.
- McComb, S., R. Mulligan, and S. Sad. 2010. Caspase-3 is transiently activated without cell death during early antigen driven expansion of CD8(+) T cells in vivo. *PLoS One* 5: e15328.
- Chan, G., M. T. Nogalski, and A. D. Yurochko. 2012. Human cytomegalovirus stimulates monocyte-to-macrophage differentiation via the temporal regulation of caspase 3. *J. Virol.* 86: 10714–10723.
- Puri, N., and P. A. Roche. 2008. Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. *Proc. Natl. Acad. Sci. USA* 105: 2580–2585.