Legumain is activated in macrophages during pancreatitis

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ACINAR CELLS IN THE PANCREAS are responsible for producing proteases that are essential for digestion. Synthesized aszymogens, these enzymes are normally activated only upon secretion into the duodenum. Trypsinogen, one of the most abundant pancreaticzymogens, is cleaved byenterokinase to produce active trypsin in the lumen of the gut. If thesezymogens are prematurely activated within the pancreas, autodigestion and severe inflammation result in pancreatitis (12, 16, 34). Pancreatitis is painful and is one of the leading causes of hospitalization in the United States. Although acute pancreatitis is normally self-limiting, severe cases can result in acute respiratory distress syndrome, multiple organ failure, and death (9, 17a). Chronic inflammation associated with pancreatitis is implicated in the development of pancreatic cancer (20, 29). Approximately 4% of patients with non-hereditary chronic pancreatitis develop pancreatic ductal adenocarcinoma (PDAC) within 20 yr (21).

Cathepsin B is a lysosomal cyteine protease that is known to activate trypsinogen within acinar cells and is considered akey mediator during the initial stages of pancreatitis. Mice deficient in cathepsin B are protected fromcaerulein-induced pancreatitis, exhibiting reduced trypsin activation, lower levels of amylase and lipase in the serum, and fewer necrotic acini (14). In contrast to cathepsin B, cathepsin L irreversibly inactivates trypsinogen (38). While cathepsin L knockout mice exhibit increased trypsin activity during pancreatitis compared...
### A: Time course of legumain activation during caerulein-induced pancreatitis

<table>
<thead>
<tr>
<th>Condition</th>
<th>Time</th>
<th>Treatment</th>
<th>Legumain Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal PBS</td>
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<td>PBS, Caerulein</td>
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<tr>
<td>12h Pancreatitis</td>
<td>12h</td>
<td>Caerulein, LE28</td>
<td></td>
</tr>
<tr>
<td>18h Pancreatitis</td>
<td>18h</td>
<td>Caerulein, LI-1</td>
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<td>24h Pancreatitis</td>
<td>24h</td>
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1 hour = PBS
12 hours = DMSO Vehicle

### B: Legumain inhibitor trial I

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<tr>
<td>Pancreatitis 12h</td>
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<td>Caerulein, DMSO</td>
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<tr>
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<td>Caerulein, LI-1</td>
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*Injection of PBS/CAE at the same time point as DMSO Vehicle/LI-1

### C: Legumain inhibitor trial II

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<tr>
<td>Pancreatitis 52h</td>
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<td>Caerulein, LI-1</td>
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### D: Legumain knockout trial

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<td>Caerulein</td>
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<tr>
<td>Pancreatitis 8h</td>
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<td>Caerulein</td>
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Fig. 1. Experimental setup for pancreatitis models. A: schematic of legumain activation time course during pancreatitis, corresponding to Figure 2. B and C: schematic of legumain inhibitor trials, corresponding to Fig. 7. D: schematic of experiments with legumain knockout mice, corresponding to Fig. 10. CAE, caerulein.
with wild-type controls, the overall severity of disease is reduced (38). These results suggest that cathepsin L mediates additional factors that promote pancreatitis. In support of the studies in genetically modified mice, pharmacological inhibition of cysteine cathepsin activity provides therapeutic relief from the symptoms of pancreatitis (23, 33). Together, these studies indicate a causative role for cysteine cathepsins in pancreatitis.

Legumain, or asparaginyl endopeptidase, is a lysosomal cysteine protease related to cysteine cathepsins with known roles during inflammation (5, 7). Mice deficient in legumain exhibit impaired cleavage and activation of cathepsin B, L, and H (24, 31), and chemical inhibition of legumain activity disrupts cathepsin L processing in bone marrow-derived cells (8). We therefore hypothesized that legumain might function at the apex of a proteolytic cascade that leads to trypsinogen activation and subsequent pancreatic inflammation. In support of this possibility, a global proteomics analysis of patients with chronic pancreatitis revealed a fourfold increase in legumain expression compared with healthy tissue (2). This observation has not been further explored until now. Herein, we investigated the potential role of legumain in caerulein-induced murine pancreatitis and in human disease. Using mouse models, we demonstrate that legumain activity is strongly increased in the pancreas during acute and chronic pancreatitis and its expression is localized primarily to macrophages. Furthermore, legumain is increased in pancreas tissue from patients with pancreatitis, indicating that legumain is a novel biomarker for pancreatic inflammation.

**EXPERIMENTAL PROCEDURES**

*Pancreatitis models.* All animal experiments were approved by the Institutional Animal Care and Use Committee at Stanford University and the Animal Ethics Committees at Monash University and at the Regional Councils of Freiburg and Magdeburg, Germany. Eight-week-old female C57BL/6 mice were purchased from Jackson Lab-
oratories or bred at the Monash Animal Research Platform. To induce acute pancreatitis, caerulein [50 µg/kg in 100 µl sterile phosphate-buffered saline (PBS); American Peptide (now Bachem, Torrance, CA)] or PBS vehicle was intraperitoneally injected every hour for up to 12 h (refer to Fig. 1A). Six hours before endpoint, the legumain activity-based probe LE28 [20 nmol in 20% dimethylsulfoxide (DMSO)/PBS; synthesized in house according to Edgington et al. (7)] was injected intravenously. At the indicated time, mice were humanely killed by cervical dislocation. Pancreata were harvested and imaged ex vivo using an FMT system (Perkin Elmer, Waltham, MA) followed by histological and biochemical analysis using previously developed methods (6, 7). To induce chronic pancreatitis, caerulein (50 µg/kg in 100 µl sterile PBS) or PBS vehicle was injected hourly for up to 7 h on Monday, Wednesday, and Friday for 5 wk. Mice were humanely killed by cervical dislocation 3 or 8 h after the first injection, and pancreata were removed for analysis. Serum amylase and lipase activity was determined using the commercial kit AMYL2- and LIPC colorimetric assay purchased from Roche (Mannheim, Germany). Trypsin was measured by the method of Grady et al. (12) using BOC-Gln-Ala-Arg-7-amino-4-methyl-coumarin (30 µM; Bachem) at 37°C. Pancreatic myeloperoxidase (MPO) activity as an indicator of leukocyte infiltration was determined according to Hallowes et al. (14) and normalized to total protein content measured by Bradford assay.

Legumain inhibitor trials. Refer to Fig. 1, B and C, for diagram of the trials. To inhibit legumain activity in vivo, the LI-1 inhibitor was used [synthesized in house according to Lee et al. (18)]. The lowest intravenous dose of LI-1 that inhibited legumain in the kidney, liver, and pancreas was found to be 50 mg/kg, as assessed by LE28 labeling on tissue lysates harvested after 1 h. We later found that this dose could be reduced to 25 mg/kg if delivered intraperitoneally. For the 12-h inhibitor trial, LI-1 (50 mg/kg in 20% DMSO/PBS) was administered intravenously before the first and sixth caerulein injections. One hour after the 12th caerulein injection, mice were humanely killed by cervical dislocation. Pancreata were harvested for histology, and blood was collected for analysis of serum amylase activity using an Amylase Activity Assay Kit (Sigma, Sydney, Australia). For the 28- and 52-h trials, LI-1 (25 mg/kg) was administered intraperitoneally 1 h before the first caerulein injection and every 12 h until the end of the experiment. Pancreata were harvested at 28 and 52 h for histological and biochemical analyses.

Legumain knockout studies. C57BL/6 legumain knockout mice (25) and appropriate controls were bred at the animal facility of the University Freiburg. Experimental acute pancreatitis was induced by up to seven intraperitoneal injections of caerulein (50 µg/kg body wt; Bachem) at hourly intervals (see Fig. 1D). Control animals were treated with physiological saline (NaCl; 2.5 ml/kg body wt). Mice were humanely killed by cervical dislocation 3 or 8 h after the first injection, and pancreata were removed for analysis. Serum amylase and lipase activity was determined using the commercial kit AMYL2- and LIPC colorimetric assay purchased from Roche (Mannheim, Germany). Trypsin was measured by the method of Grady et al. (12) using BOC-Gln-Ala-Arg-7-amino-4-methyl-coumarin (30 µM; Bachem) at 37°C. Pancreatic myeloperoxidase (MPO) activity as an indicator of leukocyte infiltration was determined according to Hallowes et al. (14) and normalized to total protein content measured by Bradford assay.

Biochemical analysis of in vivo-labeled legumain. Pancreatic tissue was snap frozen and stored until the time of analysis. Tissue was then sonicated in muscle lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 4 mM dithiothreitol and PBS pH 7.4). Supernatants were cleared by centrifuging for 10 min at 4°C. Protein concentration was determined by BCA reaction and then sample was diluted in 4X sample buffer (40% glycerol, 200 mM Tris-Cl pH 6.8, 8% SDS, 0.04% bromophenol blue, and 5% β-mercaptoethanol). Fifty micrograms total protein were then analyzed on a 15% SDS-PAGE gel. To detect labeling, the gel was scanned on a Typhoon flatbed laser scanner. LE28 was detected in the Cy5 channel (630 nm/670 nm). Gels were quantified using ImageJ and SE is reported. Pancreata from the 18-h time point were also analyzed for total legumain expression.
using standard Western blotting protocols (sheep anti-mouse legumain, 1:1,000, and donkey anti-sheep-HRP, 1:5,000; R&D Systems, Minneapolis, MN).

**In vitro analysis of protease activity.** Pancreata, kidney, or acinar cells were sonicated in citrate buffer (50 mM trisodium citrate pH 5.5, 0.1% Triton X-100, and 4 mM dithiothreitol). Supernatants were cleared by centrifugation for 10 min at 4°C. Protein concentration was determined by BCA reaction. Fifty micrograms total protein were aliquotted to a volume of 20 µl with LE28 (1 µM) or BMV109 (100 nM). Samples were incubated at 37°C for 30 min followed by addition of 4× sample buffer. Fluorescent SDS-PAGE was performed as above. Total expression of legumain and cathepsin L was measured by Western blot [sheep anti-mouse legumain, 1:1,000 (R&D Systems); donkey anti-sheep-IR800, 1:10,000 (LI-COR); goat anti-mouse cathepsin L, 1:1,000 (R&D Systems); and donkey anti-goat-IR800, 1:10,000 (LI-COR)]. Hybridizations were overnight at 4°C for primaries and 1 h at room temperature (RT) for secondaries.

**Purification of pancreatic acini.** Pancreata were harvested from normal mice and immediately minced with scissors into 1- to 5-mm pieces. The pieces were then digested in 10 ml DMEM containing collagenase IV (1 mg/ml). The samples were then agitated in a shaker at 37°C for 1 h. Once most of the large pieces had digested, the mixture was strained through a 100-um filter. The flow-through was pelleted by centrifugation at 50 g for 2 min. The pellet containing enriched acini was washed with cold PBS and then centrifuged again at 50 g for 2 min. The acini pellet was then lysed by sonication in citrate buffer. One-hundred micrograms of total protein (as determined by BCA) were labeled with LE28 (1 µM) or BMV109 (100 nM), and protease activity was analyzed by fluorescent SDS-PAGE as above.

**Histological analysis.** Pancreata were fixed in 4% paraformaldehyde in PBS overnight, transferred to 70% ethanol, and embedded in paraffin. Five-micrometer sections were cut and stained with hematoxylin and eosin according to the standard methods. Slides were scanned using a Mirax Digital Slide Scanner (Zeiss). The extent of pancreatitis was scored in a blinded manner by a pathologist using the following system: acinar damage: normal = 0, vacuolization/rare degenerated cells = 1, common degenerated cells = 2, extensive acinar damage = 3; edema: normal = 0, increased intralobular space = 1, increased intralobular space with interlobular fibrosis = 2; inflammation: none = 0, slight, moderate = 1, severe = 2. Total damage scores were obtained by summing the three scores.

**Mouse immunohistochemistry.** Pancreata were fixed in 4% paraformaldehyde in PBS followed by 30% sucrose. Tissues were embedded in Optimal Cutting Temperature (OCT) Compound (TissueTek) and frozen on dry ice. Ten-micrometer sections were cut and air-dried. Slides were submerged in cold acetone for 10 min and dried at RT for 10 min. OCT was dissolved with PBS and sections were blocked for 30 min in blocking buffer (PBS at pH 7.4, 3% normal horse serum, and 0.05% Triton X-100). Alternatively, paraffin sections were dew-
axed and rehydrated according to standard protocols. Sections were incubated with the indicated primary antibody in blocking buffer at 4°C overnight followed by three washes with PBS [sheep anti-mouse legumain (1:100, R&D Systems); rat anti-mouse CD68 (1:500, clone FA-11, BioLegend); rat anti-mouse Gr-1 (1:100, clone RB6-8c5, BioLegend)]. Secondary antibodies (donkey anti-sheep/rat-AlexaFluor488, 1:500; Jackson ImmunoResearch) were added for 1 h at RT followed by DAPI staining (1 μg/ml) and three PBS washes. Sections were mounted with ProLong Gold (Life Technologies, Scoresby, Australia). Tissues were visualized using a Leica SP8 inverted confocal microscope.

Human immunohistochemistry. Formalin-fixed paraffin-embedded pancreata (normal and chronic pancreatitis) were provided by the Biospecimen Resource for Pancreas Research of the Mayo Clinic SPORE (Specialized Program or Research Excellence) in Pancreatic Cancer. All patients provided informed consent as approved by the Institutional Review Board (IRB) at the Mayo Clinic. For the tissue microarray, formalin-fixed paraffin-embedded tissues were obtained from archived materials in accordance with institutional guidelines and prior approval by the Mayo Clinic IRB. Sections were subjected to antigen retrieval in 10 mM sodium citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked by 3% H2O2 for 5 min [immunohistochemistry (IHC)] or 15 min [immunofluorescence (IF)]. Sections were washed with 0.5% Tween 20 (IHC) or 1 h (IF) at RT. Samples were stained with H&E incubated with goat anti-legumain antibody (1:5,000; R&D Systems) and prior approval by the Mayo Clinic IRB. Sections were incubated with the indicated primary antibody in blocking buffer at 4°C overnight followed by incubation with Streptavidin-HRP (Biocare Medical, Concord, MA) for 5 min (IHC) or 1 h (IF) at RT. Samples were then incubated with PNGase F (2 U; DAKO) and anti-legumain (1:2,500; R&D Systems) diluted in Antibody Diluent Background Reducing Solution (DAKO) at 4°C overnight. Slides were then washed three times in PBS with 0.025% Tween-20. Alexa Fluor 488 (mouse) and 647 (goat) secondary antibodies (Life Technologies, Scoresby, Australia) were then applied (1:500, 1 h at RT) followed by DAPI staining (125 μg/ml, 10 min at RT). Slides were mounted with LabVision PermaFluor (Thermo Scientific, Waltham, MA) and imaged with ScanScope FL scanners (both Aperio, Leica).

**Trypsinogen cleavage assay.** Recombinant trypsinogen (1 μg; Sigma) was diluted in legumain buffer (0.1 M NaOAc, 0.1 M NaCl at pH 4.5) in the presence and absence of recombinant legumain (0.2 μg; R&D Systems). After 3 h at 37°C, the pH was raised by addition of 0.4 μl 1 M NaOH, followed by labeling with a trypsin activity-based probe Cy5-PK-DPP (1 μM) (28). Reaction was quenched with 4× sample buffer and analyzed by fluorescent SDS-PAGE. Positive controls included legumain alone labeled with LE28 (1 μM) to verify its activity and active trypsin alone labeled with Cy5-PK-DPP to verify that trypsin activity could indeed be detected. Following scanning, the gel was stained with Coomassie to visualize the proteins.

**Immunoprecipitation assay.** The identity of proteins labeled by the activity-based probes LE28 and BMV109 was determined by immunoprecipitation with protease-specific antibodies as previously described (6, 8). Probe-labeled lysates (100 μg total protein) were divided into input and immunoprecipitation (IP) fractions. The IP samples were diluted into 500 μl IP buffer (PBS at pH 7.4, 1 mM EDTA, and 0.5% NP-40), and 5 μl of the indicated antibody were added on ice (goat anti-mouse legumain; goat anti-mouse calpains L1, goat anti-cathepsin B; R&D Systems). Protein A/G agarose beads (50 μl; Santa Cruz Biotechnology, Dallas, TX) were washed once in IP buffer and then added to the IP samples. Beads were rocked overnight at 4°C and then washed four times in IP buffer, followed by a final wash in 0.9% sodium chloride. Two times the sample buffer (20 μl) was added and beads were boiled to release the immunoprecipitated proteins. Input and IP samples were resolved by SDS-PAGE and the gel was scanned for Cy5 fluorescence using a Typhoon flatbed laser scanner.

**Deglycosylation assay.** LE28-labeled pancreas lysates (50 μg) were incubated with PNGase F (2 μl; New England BioLabs) for 4 h at 37°C. Reaction was quenched with sample buffer and proteins were resolved by SDS-PAGE. Mobility shift was detected by scanning the gel for Cy5 fluorescence to detect LE28 label.

**Antibody array.** Infamed pancreas samples treated with DMSO vehicle or LI-1 legumain inhibitor were harvested 24 h after the first caerulein injection and snap frozen. Two samples from each group were then analyzed using a Proteome Profiler Mouse XL Cytokine Array Kit (R&D Systems) exactly according to the manufacturer’s instructions. One hundred fifty micrograms of total protein were used. Arrays were detected using a Bio-Rad Chemidoc Touch system. Quantification was performed using MATLAB and ImageJ software. Mean fluorescence was determined for each protein using an exposure...
in which none of the signals were saturated. Low expressing proteins were validated using a longer exposure.

Statistical analysis. Data are represented as means ± SE. Results were compared using Student's t-test and P < 0.05 is considered to be significant.

RESULTS

Legumain activity is increased during acute pancreatitis. To determine whether legumain is activated during acute pancreatitis, we used a fluorescently quenched activity-based probe, LE28 (7). Legumain binds to LE28 in an activity-dependent manner, triggering the release of a QSY21 quenching group and subsequent emission of Cy5 near-infrared fluorescence. Hence, a signal can be detected only in the presence of active legumain. LE28 binds to legumain irreversibly, allowing for verification of its targets by gel electrophoresis and immunoprecipitation assays. This validation is not possible with commercially available substrate probes, which do not bind covalently and are not completely specific for legumain.

We administered LE28 to control mice and mice with caerulein-induced pancreatitis at 12-, 18-, and 24-h time points (Fig. 1A). At the end point, pancreata were removed and LE28 fluorescence was measured by ex vivo imaging. LE28 signal in the caerulein-treated pancreata was increased by ~3-fold at 12 and 18 h compared with controls and by 5.5-fold at 24 h, reflecting a time-dependent increase in legumain activity during pancreatitis (Fig. 2, A and C).

Biochemical analyses confirmed that the ex vivo LE28 signal was due to the specific binding of legumain (Fig. 2, B and D). Lysates prepared from pancreas tissues were resolved by SDS-PAGE, and the gel was scanned for Cy5 fluorescence to detect proteins labeled by LE28. A band at the expected size of fully activated legumain (36 kDa) was observed and its intensity increased during the course of pancreatitis. In the pancreatitis samples, but not the controls, a higher molecular weight species was also observed. Immunoprecipitation of probe-labeled lysates with a legumain-specific antibody revealed that both bands were legumain species (Fig. 2E). We initially hypothesized that the higher molecular weight species of legumain was a glycosylated form. However, after incubation with a deglycosylase, both bands shifted to a lower molecular weight, suggesting that this modification does not account for the size difference (Fig. 2F). Legumain is expressed as a zymogen and becomes activated at low pH after autocleavage of NH₂- and COOH-terminal pro-domains (3). The high-molecular weight species observed during pancreatitis is most likely the 42-kDa intermediate that has been cleaved only at the COOH terminus. We also examined total legumain expression by Western blotting and observed increased expression of the full-length 56-kDa form and the 42-kDa intermediate during pancreatitis (Fig. 2G). These findings provide further evidence that legumain is increased in the inflamed pancreas.

Legumain is predominantly activated in macrophages. To determine the cellular source of legumain in the pancreas, we analyzed LE28 labeling by confocal microscopy. In accordance with the ex vivo imaging, LE28 signal was dramatically increased in inflamed tissues compared with controls (Fig. 3A).

This pattern was recapitulated by immunofluorescence staining with a legumain-specific antibody (Fig. 4). There was a low level of legumain activity in acinar cells but high activity in resident macrophages of the normal pancreas and in infiltrating macrophages during inflammation, as demonstrated by colocalizing with CD68 (Fig. 3A). As expected, the legumain labeling exhibited a punctate pattern in the cytoplasm, reflecting a lysosomal distribution. Gr-1+ neutrophils, which are prominent immune infiltrates during pancreatitis, did not exhibit legumain activity (Fig. 3B). Collectively, these data suggest that legumain activity is increased during pancreatitis due to accumulation of macrophages.

Legumain activity is upregulated during chronic pancreatitis. To also study legumain in the context of chronic pancreatitis, we used a model in which caerulein was injected eight times per day, 3 days per week, for 5 wk. One day after the final round of caerulein injections, the LE28 legumain probe was administered and pancreata were imaged. As we observed in the acute model, probe signal was increased in inflamed tissues compared with controls (Fig. 5, A and C) and this corresponded to an approximately sixfold increase in legumain labeling as shown by biochemical analysis (Fig. 5, B and D). This result suggests that legumain upregulation is sustained during chronic inflammation.

Legumain is unlikely to initiate pancreatitis. By immuno-

histochemistry and activity-based profiling, legumain expres-

![Fig. 6. Legumain is not active in acinar cells and degrades trypsinogen. A: lysates of purified pancreatic acinar cells were labeled with LE28 or BMV109 to measure the activities of legumain or cathepsins, respectively. B: immunoprecipitation of BMV109-labeled acinar cell lysates with antibodies specific for cathepsin B and L. C: recombinant trypsinogen (TPG) was incubated in the presence and absence of recombinant legumain (Lgmn). After being resolved by SDS-PAGE, the gel was stained with Coomassie. Positive controls include legumain alone and active trypsin. D: the proteins above were labeled with Cy5-PK-DPP, an activity-based probe for trypsin, or LE28, an activity-based probe for legumain.](image-url)
Fig. 7. Legumain inhibition does not block pancreatitis. A: hematoxylin and eosin (H&E) staining and damage scores of healthy pancreata (PBS) and inflamed pancreata treated with vehicle (CAE + DMSO) or legumain inhibitor (CAE + LI-1) at the indicated time point. All caerulein-treated groups were statistically different from the PBS control (12h: *P < 0.02, n = 5; 12h + LI-1: **P = 0.0002, n = 5; 24h: ***P = 4e-8, n = 5; 52h: **P = 0.001, n = 5; 52h + LI-1: *P = 0.02, n = 5). The vehicle- and LI-1-treated groups were not significantly different at any time point. B and C: biochemical analysis of protease activity in the tissues shown in A. Lysates were labeled with LE28 (B) and BMV109 (C) to measure legumain and cathepsin activity, respectively. D: densitometry of bands shown in B. 24h DMSO vs. LI-1: ***P = 0.001, n = 5; 52h DMSO vs. LI-1: ****P = 0.00001, n = 5. E: densitometry of bands shown in C. Cathepsin activity in DMSO and LI-1-treated pancreata was not statistically significant (n = 5). F: serum amylase activity from healthy mice and those with pancreatitis (12h) treated with DMSO vehicle or LI-1. Amylase activity between DMSO and LI-1-treated pancreata was not statistically significant (NS, P = 0.07, n = 5).
Legumain inhibition does not influence inflammatory cytokine/chemokine production. Antibody arrays on pancreatitis tissues taken at the 28th time point after treatment with the LI-1 legumain inhibitor or DMSO vehicle control. This experiment was performed on pancreas from two mice per group, and each cytokine spotted in duplicate. 13/111 cytokines were measured as being significantly different between DMSO and LI-1-treated pancreata, all marginally increased (highlighted in yellow in Supplemental Table S1).

Legumain inhibition does not attenuate acute pancreatitis. Histological staining revealed that all caerulein-controls, with obvious signs of edema consistent with acute pancreatitis were macroscopically indistinguishable from the LI-1-treated tissues had extensive edema, immune infiltration, and necrosis, and total damage scores were significantly increased relative to the PBS control (Fig. 7A). No differences between DMSO and LI-1-treated tissues were observed. Analysis of serum amylase activity at the 12-h time point also revealed no statistically significant changes upon legumain inhibition (Fig. 7F). Under identical experimental conditions, cathepsin inhibition results in <25% reduction in serum amylase activity and improved histology scores (23). To further assess inflammation, we used an antibody array to measure levels of inflammatory cytokines and chemokines in 28-h pancreatitis tissues. Legumain inhibition had a minimal effect on the expression of 13 out of 111 cytokines tested, increasing by 7–37% compared with vehicle-treated controls (Fig. 8; Supplemental Table S1; Supplemental Material for this article is available online at the Journal website). Given the subtle nature of this effect, we did not investigate it further.

To confirm inhibition of legumain activity by LI-1, we biochemically analyzed pancreas lysates using the LE28 legumain probe. As expected, legumain activity was increased upon induction of pancreatitis and was still elevated at the 52-h point after treatment with the LI-1 legumain inhibitor or DMSO vehicle.

Fig. 8. Legumain inhibition does not influence inflammatory cytokine/chemokine production. Antibody arrays on pancreatitis tissues taken at the 28th time point after treatment with the LI-1 legumain inhibitor or DMSO vehicle control. This experiment was performed on pancreas from two mice per group, and each cytokine spotted in duplicate. 13/111 cytokines were measured as being significantly different between DMSO and LI-1-treated pancreata, all marginally increased (highlighted in yellow in Supplemental Table S1).

Fig. 9. Effects of legumain inhibition in vivo. A: legumain expression in inflamed pancreas from the 52h CAE groups in Fig. 7 with and without LI-1 legumain inhibitor treatment. B: legumain activity in the kidney (+/−LI-1) as measured by LE28 labeling. C: Legumain expression in the kidney (+/−LI-1) as measured by Western blot. D: cathepsin L expression in inflamed pancreas (+/−LI-1). E: cathepsin activity in the kidney (+/−LI-1) as measured by BMV109 labeling. F: cathepsin L expression in the kidney (+/−LI-1).
time point. LI-1 treatment specifically reduced legumain activity by 60–77\% (Fig. 7, B and D). As a control, we also confirmed that legumain activity was abolished in the kidney (Fig. 9B). Legumain inhibition resulted in an increased expression of legumain protein in the pancreas and kidney, which may represent a compensatory upregulation (Fig. 9, A and C).

To investigate effects of the legumain inhibitor on cathepsin activity, we labeled pancreas and kidney lysates with the BMV109 pan-cathepsin probe. In the pancreas, we detected cathepsin Z, B, S, and L activity, and their total levels were significantly increased in inflamed tissues compared with controls (Fig. 7, C and E). LI-1 treatment did not alter pancreatic

![Image](image_url)
cathepsin activity (Fig. 6, C and E) or expression (Fig. 9D). In the kidney, however, LI-1 treatment led to a reduction in cathepsin L activity (Fig. 9E). Western blotting indicated that the pro-form of cathepsin L was increased in these tissues (Fig. 9F). This effect was only observed in vivo in mice treated with LI-1 for 52 h but not 28 h (not shown).

Since we observed that the legumain inhibitor caused up-regulation of its expression (Fig. 9, A and C), there may have been some residual activity during the course of the pancreatitis experiment. To circumvent this potential problem, we also independently studied legumain-deficient mice (25) in the acute pancreatitis model (Fig. 1D). These mice were indistinguishable from wild-type controls, as assessed by pancreatic trypsin activity, serum amylase and lipase activity, lung myeloperoxidase activity, and pancreatic histology (Fig. 10, A–E). These results confirm our finding that legumain is not essential for the initiation of pancreatitis.

Legumain is a biomarker of human pancreatitis. To determine the translational relevance of legumain in caerulein-induced murine pancreatitis, we examined tissue from patients with chronic pancreatitis. In healthy pancreas, legumain expression was extremely low (Fig. 11, A and B). In contrast, legumain was strongly expressed in CD68+ macrophages from patients with chronic pancreatitis (Fig. 11, A and B). We also analyzed a tissue microarray of pancreas tissues from 20 patients with chronic pancreatitis. The number of legumain-expressing cells per field was consistently higher in inflamed areas compared with adjacent normal tissue (Fig. 11, B and C).

![Fig. 11. Legumain expression in human pancreatitis. H&E staining (A) and legumain staining (brown; B) of healthy human pancreas tissue (normal) and of tissue from 2 patients with chronic pancreatitis. C: coimmunofluorescent staining of legumain (red), CD68-expressing macrophages (green), and DAPI (blue). D: numbers of legumain-expressing cells per field in normal (blue) and pancreatitis (red) regions from a tissue microarray of 20 patients. E: average number of legumain-expressing cells per field in normal and pancreatitis regions (***P = 0.00009; n = 20).](image-url)
Legumain was highly upregulated in macrophages located in areas displaying acinar-to-ductal metaplasia, suggesting that this protease may influence reprogramming events that lead to inflammation-induced preneoplastic lesions. Thus legumain is a potential biomarker for macrophages associated with human pancreatitis.

DISCUSSION

In this study, we have demonstrated for the first time that the cysteine protease legumain is activated in the pancreas during caerulein-induced acute and chronic murine pancreatitis (Figs. 2 and 5). Importantly, we also demonstrated increased legumain expression in human disease, confirming translational significance (Fig. 11). We originally hypothesized that legumain might function to initiate the zymogen activation cascade. This was based on its demonstrated ability to cleave and activate cathepsin B and L, cysteine proteases that are known to play critical roles during the induction of pancreatitis in mice (14, 23, 24, 31, 33, 38). However, we found that legumain activity and expression within normal acinar cells were undetectable using a highly sensitive activity-based probe and Western blotting (Fig. 6). In contrast, cathepsin B and L were abundant and readily detectable in acini. The absence of legumain within acinar cells suggests that it is unlikely to be the protease responsible for activating cathepsins during pancreatitis. Further to this point, pancreatic cathepsin activity was unaffected by the legumain inhibitor in vivo (Fig. 7). As LI-1 did not completely block legumain activity, the low residual activity may have been sufficient for cathepsin activation.

We also examined the ability of legumain to activate trypsinogen (Fig. 6). While legumain robustly cleaved trypsinogen at several sites, the cleavage products did not exhibit trypsin activity. Alternatively, legumain may function to degrade or inactivate trypsin. Legumain-deficient mice did not have significantly altered levels of pancreatic trypsin activity. While it is unlikely that legumain is inactivating trypsin during pancreatitis, this degradation may be important in other physiological processes. Taken together, these data suggest that legumain is not responsible for initiating pancreatitis. This is further supported by the fact that legumain activity was more strongly activated at later time points after caerulein induction and remained high in chronic pancreatitis (Figs. 2 and 5). Its predominant localization within macrophages, which accumulate hours after the initiation of pancreatitis, suggests a role associated with the inflammatory response rather than the induction of acinar cell injury (Fig. 3).

We used a small-molecule legumain inhibitor, LI-1, to reduce legumain activity during the course of caerulein treatment (Fig. 7). Legumain-inhibited mice exhibited a similar degree of pancreatic inflammation as vehicle-treated controls, as assessed by serum amylase, gross and histological evaluation, and the degree of cathepsin upregulation. Similarly, legumain knockout mice were not protected from pancreatitis (Fig. 9). Studying protease activity is often complicated by their propensity for compensation. Inhibition or deletion of one protease can often lead to upregulation of other proteases, which may counteract the loss of function (1, 35). We have shown here that legumain inhibition leads to upregulation of total legumain protein expression (Fig. 9). This, combined with effects on other proteases, may also explain the lack of effects in the pancreatitis model. Additionally, legumain may function independently of its catalytic activity during pancreatitis, for example, through the binding of its RGD motif to integrins (4).

Macrophages have received increasing attention for their ability to drive the pathogenesis of pancreatitis (11, 17, 20, 32, 39). Depletion of macrophages using clodronate has a protective effect during caerulein-induced pancreatitis, resulting in lower levels of serum lipase and amylase and improved histological scores (30). The reprogramming of acinar cells into duct-like cells during pancreatitis-associated inflammation is also influenced by macrophages. Cytokines produced by macrophages induce NF-κB signaling in acinar cells, which is a key event that drives the transition from chronic pancreatitis to pancreatic intraepithelial neoplasia (PanIN) and PDAC (20). In human pancreatitis tissue, legumain expression was highest in regions of acinar-to-ductal metaplasia, suggesting that this protease may influence reprogramming events that can lead to preneoplastic lesions associated with inflammation. In breast, colon, and ovarian cancers, increased legumain expression strongly correlates with decreased overall survival rates (10, 13, 15, 27, 37). Legumain is abundantly expressed by tumor-associated macrophages (7, 22), and blocking legumain activity with a small-molecule inhibitor was recently shown to have therapeutic efficacy in a mouse model of breast metastasis (19).

Furthermore, legumain-deficient mice exhibited less accumulation of CD74+ antigen-presenting cells in a mouse model of stroke (16a). Whether this occurs during pancreatitis and contributes to tumorigenesis of pancreatic cancer is a subject of ongoing investigation.

In sum, we have demonstrated that legumain activity is increased in the pancreas during acute and chronic inflammation. Contrary to our initial hypothesis, legumain is not expressed by acinar cells, does not function to initiate pancreatitis, and does not appear to modulate cathepsin activity within the pancreas. Instead, we have established legumain as a novel biomarker for macrophages that infiltrate the pancreas during chronic inflammation, where legumain may have other functions during mouse and human disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Legumain in pancreatitis


REFERENCES


