# Supplementary Materials and Methods

### *Acini Preparation*

Briefly, cells were maintained and stimulated in Dulbecco's modified Eagle medium containing 10 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethansulfonic acid (HEPES), 2% of bovine serum albumin (BSA) and 1% Penstrep. Stimulation of acinar cells was performed with 0.001 mmol/L CCK over 30 minutes; afterwards, cells were centrifuged for 30 seconds at 500 rpm and resuspended in fresh media to wash out residual CCK.

## Reagents and Antibodies

Macrophage colony stimulating factor (MCSF) was purchased from Miltenyi Biotec. (Auburn, CA). Collagenase of *Clostridium histolyticum* (EC.3.4.24.3) from Serva (lot no. 14007, Heidelberg, Germany) was used for acinar cell isolation. LPS from Escherichia coli O26:B6 was obtained from Sigma (St. Louis, MO). Purified enzymes of pancreatic porcine amylase and bovine trypsinogen and caerulein, cholecystokinine (CCK), CA074me, E-64d, and cytochalasin-B from Drechslera dematioideum were obtained from Sigma (St. Louis, MO). Bafilomycin-A1 was obtained from InvivoGen (San Diego, CA).

Protease activity was measured using the following fluorogenic substrates: trypsin R110-(CBZ-Ile-Pro-Arg)$_2$ from Invitrogen (Carlsbad, CA), cathepsin B AMC-Arg$_2$, cathepsin L R110-(CBZ-Phe-Arg) from Invitrogen, and chymotrypsin Suc-Ala-Ala-Pro-Phe-AMC from Bachem (Bubendorf, Switzerland).

The following antibodies were used: anti-Ly6g (ab25377) from Abcam (Cambridge, UK), anti-CD68 (ABIN181836) from antibodies-online, GmbH (Aachen, Germany), anti-CD206 (OASA05048) from Aviva Systems Biology (San Diego, CA), anti-NF-$\kappa$B p65 (#8242) from Cell Signaling Technology (Danvers, MA), anti-cathepsin B (AF965) from R&D Systems (Minneapolis, MN), anti-amylase (sc-46657) from Santa Cruz Biotechnology (Dallas, TX), anti-glyceraldehyde-3-phosphate dehydrogenase (clone 6C5) from Meridian (Memphis, TN), anti-chymotrypsin (sc-80750) from Santa Cruz Biotechnology, anti-F4/80 (MCA497R) from AbD Serotec (Raleigh, NC), anti-human CD68 (clone PG-M1) from Dako (Glostrup, Denmark), anti-trypsin (AB1823) from Chemicon International (Temecula, CA), anti-caspase-1(#2225) from Cell Signaling Technology, anti-human CD206 (MAB25341) from R&D Systems, and anti-syncollin (ab178415) from Abcam.

## Induction of Pancreatitis in Mice

A mild form of pancreatitis was induced in C57Bl/6 mice by hourly intraperitoneal injections of caerulein (50 $\mu$g/kg body weight) up to 8 hours.

A second model of severe acute pancreatitis was induced in C57Bl/6 and CTSB-/-mice by partial duct ligation of the pancreatic duct with a single additional supramaximal stimulation with caerulein 2 days after ligation, as previously reported.[1] Animals were sacrificed 3, 7, and 14 days after partial duct ligation. Adoptive transfer experiments were performed 2 days after duct ligation by injection of 2 million cells intravenously; 24 hours after the adoptive transfer animals were sacrificed. Cells for adoptive transfer were isolated from bone marrow as described above and maintained in 10-cm dishes for 5–7 days.

## Tissue Handling

Pancreas was snap frozen in liquid nitrogen and stored at -80°C for enzyme measurement. For histology, tissue was fixed in 4.5% formalin for paraffin embedding or embedded in TissueTec (OCT, Sakura, Los Angeles, CA) for cryo sections. Collected blood samples were centrifuged and serum was stored at -80°C.

## Isolation of BMDM

Femur and tibia of C57Bl/6, CTSB-/-, and CTSL-/- mice were prepared under sterile conditions. Bone marrow was flushed out of the bones with sterile phosphate-buffered saline (PBS) and passed through a cell strainer (70 $\mu$mol/L). Cells were washed with sterile PBS, counted, and maintained in 6-well plates or chamber slides for immunofluorescence staining in a concentration of 2.5 million cells/well with RPMI medium (1% Penstrep and 5% fetal calf serum [FCS]). Six hours after isolation from bone marrow medium, non-attaching cells were removed and cells resuspended in fresh medium containing 20 $\mu$g/ml macrophage colony-stimulating factor (M-CSF). Cells were used 5–7 days after isolation for experiments.

Before stimulation, cells were washed with sterile PBS and medium was replaced by fresh medium. Cells were co-incubated with freshly prepared acinar cells as described before, or with 10 $\mu$g/ml bovine trypsinogen over 6 hours, untreated cells served as controls. For different experimental settings, 50 $\mu$mol/L nafamostate, 100 nmol/L bafilomycin A1, 50 $\mu$mol/L CA074me, or 10 $\mu$g/ml cytochalasin B were added. Stimulation with 1 $\mu$g/ml LPS served as positive control for cytokine secretion or NF$\kappa$Bp65 translocation into the nucleus.

Supernatant was harvested, centrifuged at 10,000 rpm, and frozen at -80°C for cytokine measurement. Cells were washed 3 times with PBS to remove residual acinar cells, cellular waste or remaining bovine trypsinogen and subsequently scraped from the plate in the presence of 100 $\mu$l PBS. Cell suspension was lysed by ultrasound sonification and stored at -80°C for measurement of protease activity. Samples for Western blotting were lysed in buffer containing 25 mmol/L HEPES, 75 mmol/L NaCl, 0.5% Triton X-100, 5% glycerin, 1 mmol/L EDTA in the presence of 1 mmol/L PMSF (phenylmethylsulfonyl fluoride), 5 mmol/L $Na_4P_2O_7$, 10 mmol/L NaF (sodium fluoride), and 1 $\mu$g/ml aprotinin.

## In Vivo Imaging of Proteases in Macrophages

Serum amylase and different protease activities were measured as previously reported using substrates

R110-Ile-Pro-Arg for trypsin, Suc-Ala-Ala-Pro-Phe-AMC for chymotrypsin, R110-Phe-Arg for cathepsin L, and AMC-$Arg_2$for cathepsin B. In vivo imaging of active proteases within macrophages was performed in $\mu$-dish from Ibidi (Martinsried, Germany). Cells were maintained in $\mu$-dishes for 5–7 days in the presence of 20 $\mu$g/ml MCSF. Co-incubation with freshly prepared acinar cells or bovine trypsinogen was performed over 6 hours; afterwards, cells were washed 3 times with PBS before they were loaded with fluorogen substrates, R110-Ile-Pro-Arg, and/or Suc-Ala-Ala-Pro-Phe-AMC in a concentration of 10 $\mu$mol/L for 30 minutes. Cells were washed again carefully and maintained in PBS for microscopy.

## FACS Analysis of BMDM

Cells were washed by centrifugation, re-suspended in PBS, and strained through a 40-$\mu$m strainer. Cells were re-centrifuged and re-suspended in PBS and stained with Zombie-NIR (1:100, BioLegend, San Diego, CA) as a marker for necrotic cells in the dark for 30 minutes at room temperature. After that, cells were washed by centrifugation with FACS buffer (1% FCS in PBS) and then incubated with antibodies for 30 minutes at 4°C. BD Horizon V450-conjugated rat anti-mouse/human CD11b (1:100, BD Biosciences, San Jose, CA) and PE-conjugated rat anti-mouse CD69 (1:100, BioLegend) were used. Cells were washed by centrifugation at +4°C and re-suspended in 200 $\mu$l FACS buffer to be ready for FACS analysis (Becton Dickinson, Franklin Lakes, NJ). The percentage of activated cells was analyzed by FlowJo (https://www.flowjo.com/). CD11b was used as a marker for macrophages, whereas CD69 is an activation marker that is only expressed on activated immune cells.

## Isolation of Zymogen Fraction

Subcellular fractionation of healthy whole pancreas from wild type (WT) mice was performed by sucrose gradient centrifugation in a buffer containing 240 mmol/L sucrose, 5 mmol/L MOPS (3-(N-morpholino)propanesulfonic acid), and 1 mmol/L $MgSO_4$, as previously described.[2] Zymogen-enriched fraction was stained with membrane bound red fluorochrome Vybrant CM-Dil cell-labeling solution from Invitrogen.

## Cytokine Measurement in Supernatant

The cytokines IL6 and TNF$\alpha$ and the chemokine MCP-1 in medium were measured by Cytometric Bead Array (CBA) mouse inflammation Kit from Becton Dickinson and Company (BD Bioscience). IL1$\beta$ in supernatant was determined by Quantikine ELISA from R&D Systems.

## Histology, Immunohistochemistry, and Immunofluorescence

For Masson Goldner staining, a kit from Merck (Darmstadt, Germany) was used. Immunohistochemical staining of F4/80 and cathepsin B as well as CD68 in human chronic pancreatitis and necrosis samples was performed on paraffin sections. For antigen retrieval target, retrieval solution from Dako was used. Antibodies were used in a dilution of 1:100 in 20% fetal calf serum (FCS) from PAN Biotech (Aidenbach, Germany).

Immunofluorescence stainings were performed from cryo-embedded tissue as previously reported.[3] Anti-CD68 was used as marker for M1 macrophages, anti-CD206 for M2 macrophages, and anti-Ly6g for neutrophils. Quantification of immunologic infiltrate was performed by cell count/field of view. A minimum of 5 pictures/animal was quantified.

Immunofluorescence stainings of BMDM was performed in chamber slides. Cells were maintained as previously described in the presence of 20 $\mu$g/ml MCSF. Co-incubations with bovine trypsinogen or freshly prepared acinar cells were performed under sterile conditions. Cells were washed carefully after co-incubation with PBS before they were fixed in ice-cold acetone for 30 minutes; 20% FCS was used for blocking as well as for antibody dilution. Incubation with antibodies was performed over night at 4°C; antibodies were used in a dilution of 1:100. Secondary antibody incubation was performed for 1 hour at room temperature. Nuclei were stained by DAPI (1:10,000), slides were mounted with DACO mounting medium for fluorescence slides.

Area quantification of macrophages was performed by the software CellSens Dimensions 1.7.1 from Olympus cooperation (Tokyo, Japan). Quantification of secretory tissue and fibrosis in Masson-Goldner staining was performed by color deconvolution technique using the software ImageJ (Supplement 1).

## Microarray-based Transcriptome Analysis

Microarray-based transcriptome analysis was performed as previously described.[4] Shortly, total RNA was extracted from cells using TRIzol reagent, followed by further column purification and quality control. Individual RNA samples ($n = 3$) were subjected to transcriptome analysis using Affymetrix GeneChip Mouse Gene 2.0 ST Arrays and GeneChip WT PLUS Reagent Kit (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions. Microarray data analysis was performed using the Rosetta Resolver software system (Rosetta Bio Software, Seattle, WA). Significantly different mRNA levels were defined using the following criteria: one-way ANOVA with Benjamini and Hochberg FDR ($P \leq .05$), signal correction statistics (Ratio Builder software) $P \leq .05$), and an expression value ratio between the different conditions $\geq$ 1.5-fold.

In-silico pathway and functional analysis of differentially expressed genes was carried out using the commercial systems biology-oriented package Ingenuity Pathway Analysis (Ingenuity Systems, Inc. Redwood City, CA).

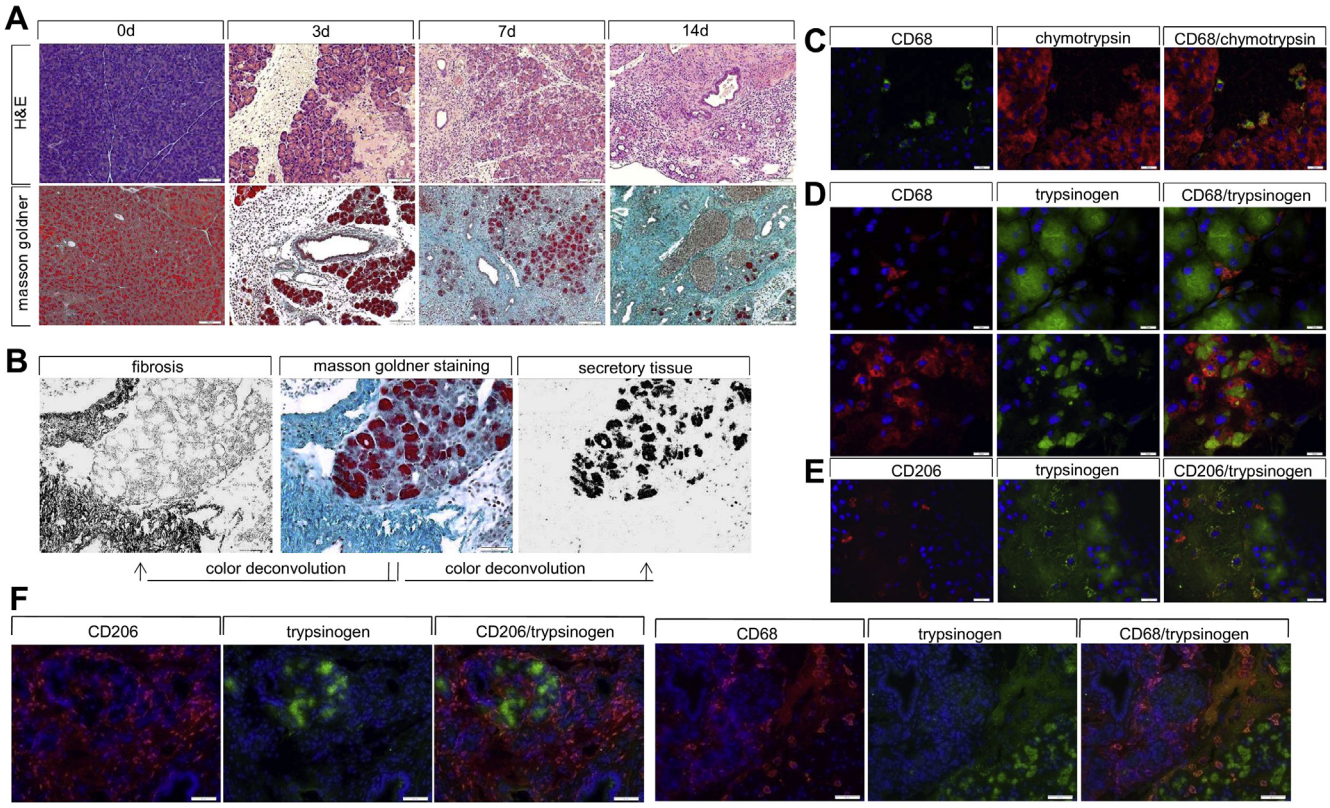
## Western Blot

Protein concentrations of samples were determined by Bradford assay; 20 $\mu$g of total protein was loaded on polyacrylamide gels and transferred onto nitrocellulose for immunoblotting. Anti-amylase, anti-trypsin, and anti-GAPDH antibodies were used in a dilution of 1:1000.

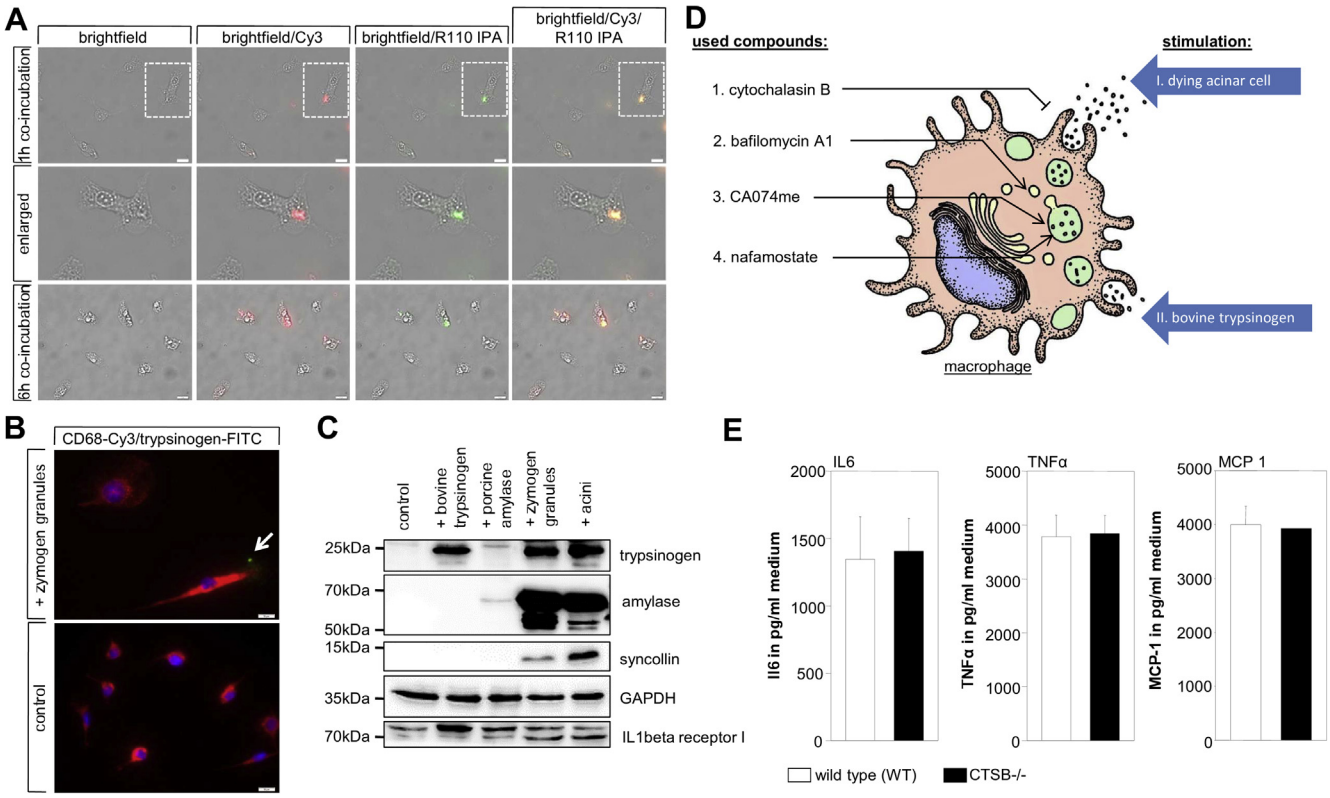
## References


1. **Sendler M, Beyer G**, Mahajan UM, et al. Complement component 5 mediates development of fibrosis, via activation of stellate cells, in 2 mouse models of chronic pancreatitis. Gastroenterology 2015;149:765–776.e10.
2. Sendler M, Maertin S, John D, et al. Cathepsin B activity initiates apoptosis via digestive protease activation in pancreatic acinar cells and experimental pancreatitis. J Biol Chem 2016;291:14717–14731.
3. Lerch MM, Lutz MP, Weidenbach H, et al. Dissociation and reassembly of adherens junctions during experimental acute pancreatitis. Gastroenterology 1997; 113:1355–1366.
4. Lietzow J, Golchert J, Homuth G, et al. 3,5-T2 alters murine genes relevant for xenobiotic, steroid, and thyroid hormone metabolism. J Mol Endocrinol 2016;56: 311–323.

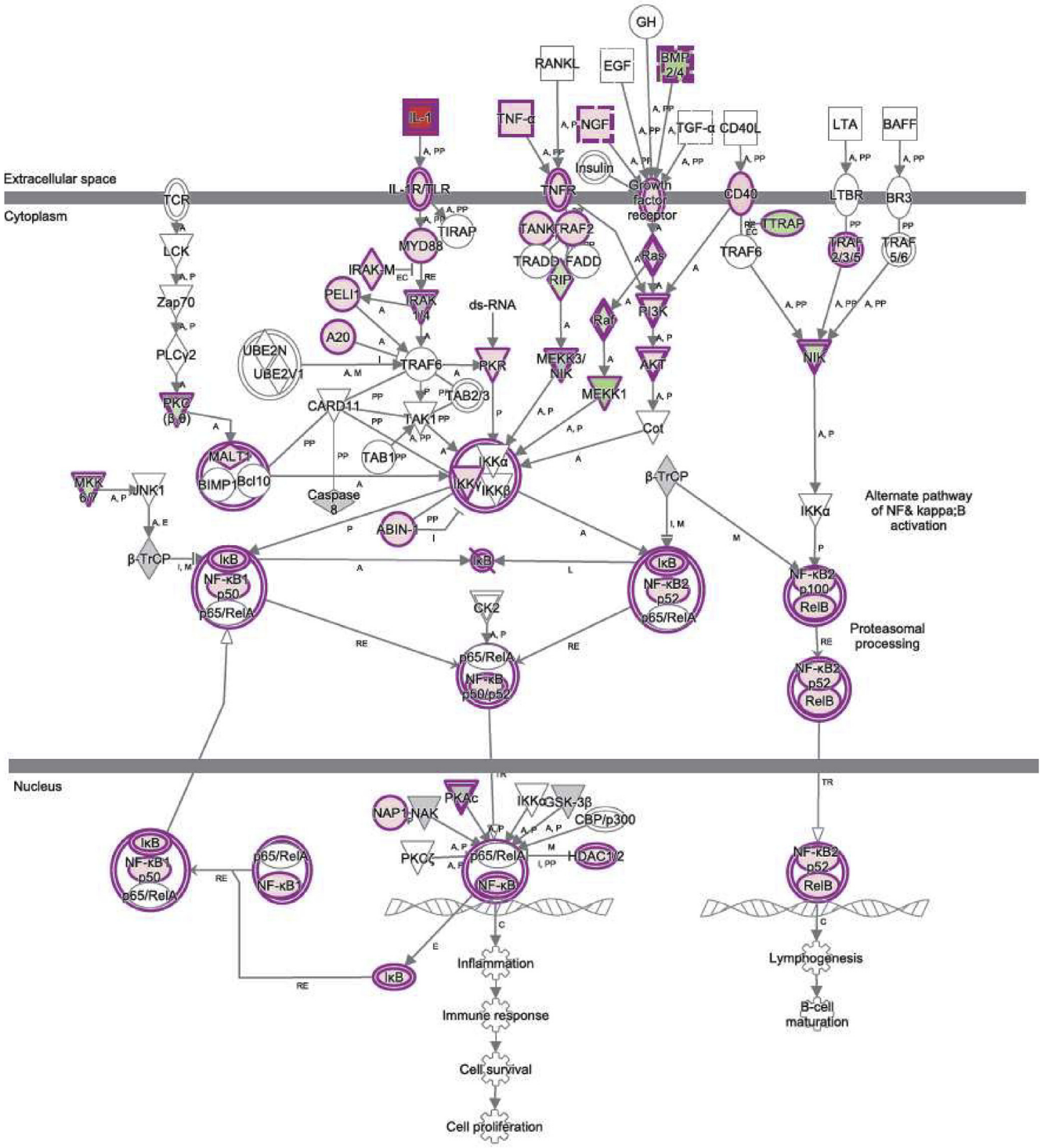

**Author names in bold designate shared co-first authorship.**



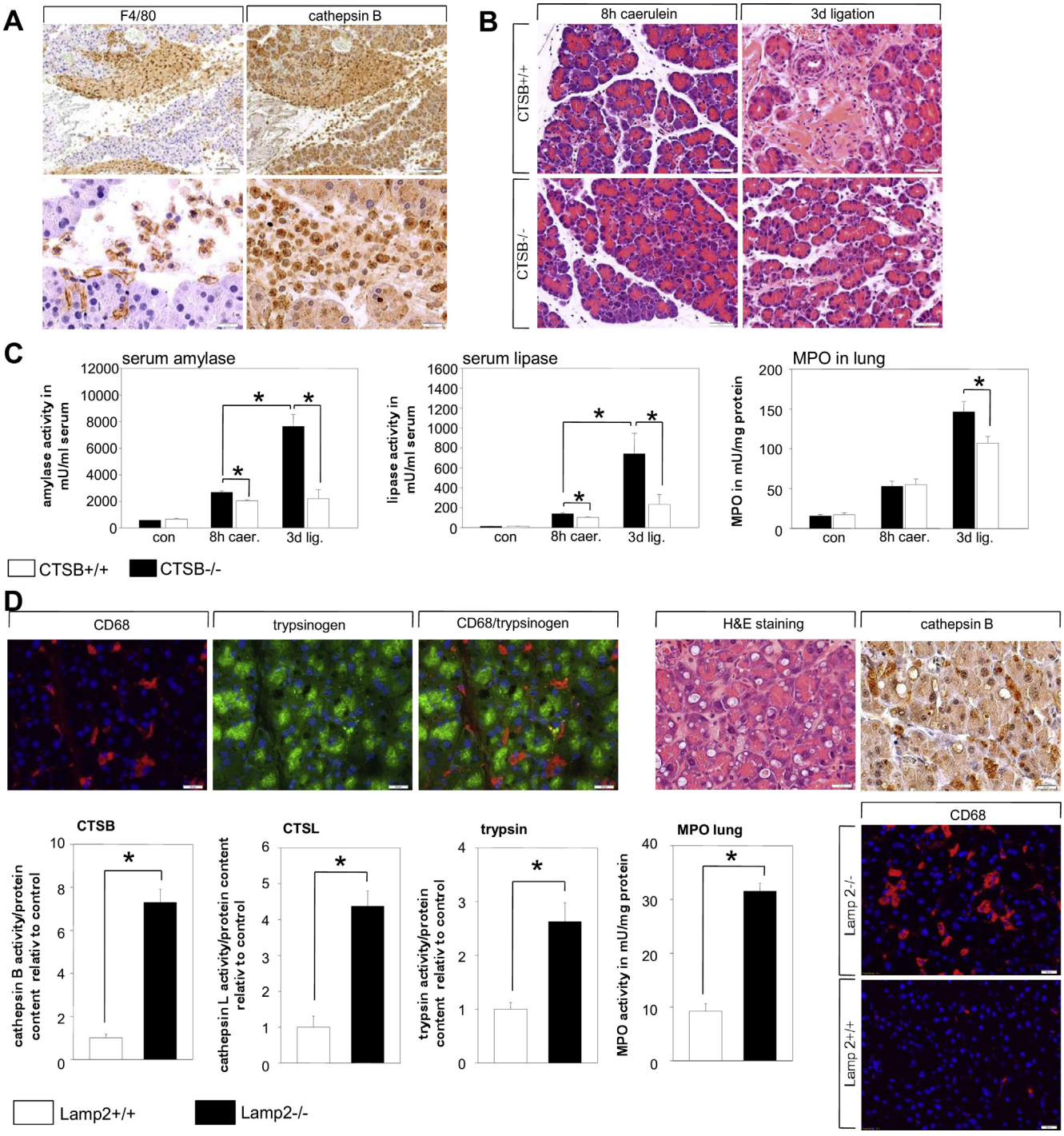

**Supplementary Figure 1.** H&E and Masson-Goldner staining of the pancreas from partial duct ligated C57Bl/6 animals. H&E stainings illustrate necrosis development 3 and 7 days after operation during the severe acute phase of the disease. Masson-Goldner staining revealed development of fibrosis over time, with a maximum at later time points such as day 14 (*A*). These stainings reflected the necrosis fibrosis sequence in this model. Quantification of fibrosis or healthy exocrine tissue was performed by color deconvolution with ImageJ (*B*), whereas red areas were regarded as exocrine tissue and green areas as fibrotic tissue. Quantification was calculated in percentage of image taken. Staining of CD68-positive macrophages (Cy3) and trypsinogen (FITC) in pancreas showed intact acini and CD68-positive macrophages without co-localization in necrosis-free areas, but a distinct intracellular localization of trypsinogen in macrophages in necrotic areas (*C*). Here large amounts of trypsinogen were not associated with DAPI staining and represent extracellular zymogens within necrosis. Co-staining of trypsinogen (FITC) with CD206 (Cy3), a marker for M2 macrophages, in contrast to CD68-positive M1 macrophages (*D*), showed no co-localization (*D*). Another pancreatic enzyme, chymotrypsin (Cy3), was also co-localized within CD68-positive macrophages (FITC) (*E*). Fourteen days after duct ligation we observed an increase of CD206-positive M2 macrophages. These cells do not co-localize with pancreatic trypsinogen (*F*), but also during this late phase of disease some necrotic areas could be detected where CD68-positive macrophages showed distinct intracellular granules positive for trypsinogen (*F*).



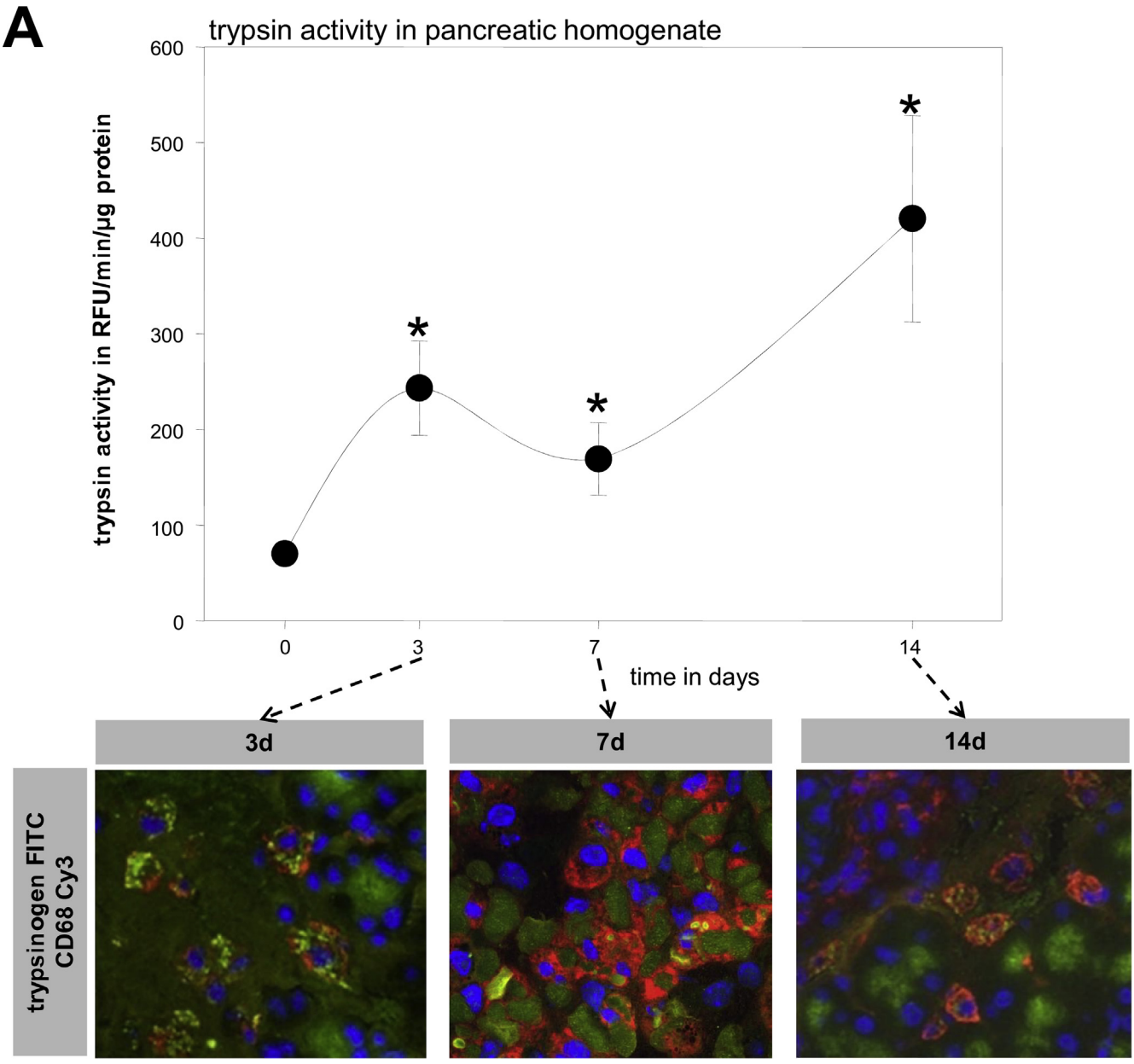

**Supplementary Figure 2.** Co-incubation of BMDM with isolated and fluorescent-labeled zymogen granules revealed an uptake of zymogens by macrophages within 1 hour (*A*). Loading cells with fluorescent substrate for active trypsin (R110 IPA) demonstrated trypsin activity in phagocytosed zymogen granules. Fluorescent staining of CD68 and trypsinogen showed intracellular localization of trypsinogen in macrophages after co-incubation with isolated zymogen granules (*B*). Western blot analysis of BMDM confirmed uptake of trypsinogen if macrophages were co-incubated with bovine trypsinogen, zymogen granules, or acini, but not in control cells or cells that were co-incubated with porcine amylase (*C*). Amylase could only be detected in cells co-incubated with zymogen granules, acini, or purified protein. Syncollin, a zymogen marker, could only be detected in cells co-incubated with zymogen granules or acini. GAPDH served as loading control. The IL1$\beta$ receptor was expressed independently of co-incubation on macrophages (*C*). Schematic illustration of experimental set-up for the co-incubation experiments of isolated macrophages (*D*). BMDM were fed with (I) freshly prepared acinar cell that were stimulated with 0.001 mmol/L CCK before co-incubation, or (II) with 10 $\mu$g/ml of bovine trypsinogen. Macrophages phagocytose both dying acinar cells as well as bovine trypsinogen. To investigate the mechanism of macrophage-mediated intracellular protease activation we use different compounds: (1) Cytochalasin B inhibits actin cytoskeleton reorganization and thus prevents phagocytosis; (2) Bafilomycin A1 inhibits V-ATPases and therefore prevents acidification of the lysosomal and phagosomal compartment, which leads to reduced cathepsin B activity; (3) CA074me is a cell-permeable inhibitor of cathepsin B, the major activator of trypsinogen; (4) Nafamostate is a serine protease inhibitor that directly inhibits active trypsin. Stimulation of BMDM from WT and CTSB-/- mice with LPS did not show differences in cytokine secretion (*E*).



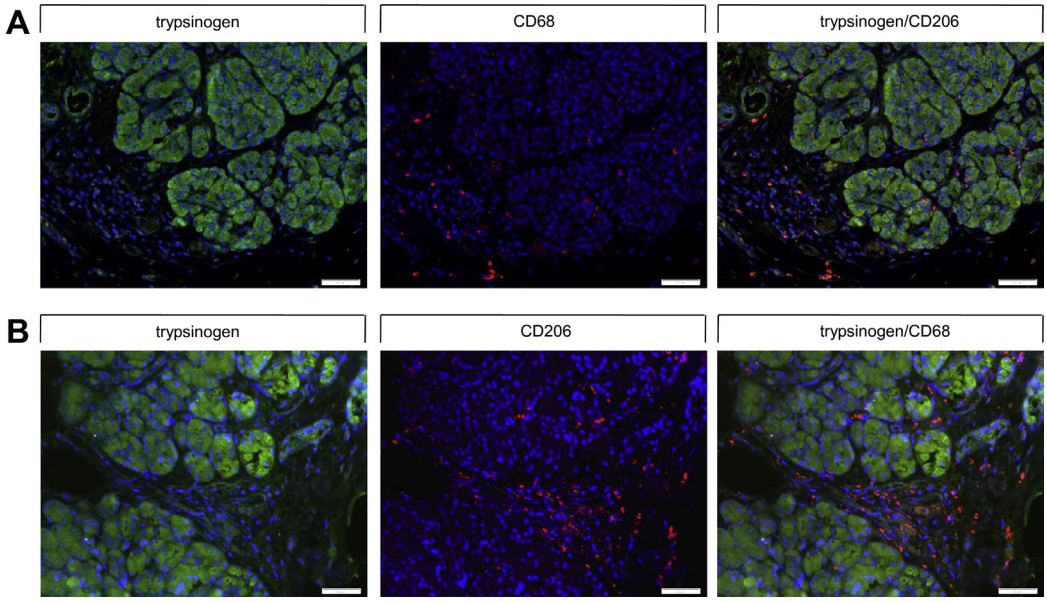

**Supplementary Figure 3.** Transcritome analysis of macrophages co-incubated with acini compared with unstimulated controls suggested a pronounced induction of the NF$\kappa$B pathway. One pathway to induce NF$\kappa$B translocation into the nucleus is the IL1$\beta$ signaling. IPA analysis suggested a critical role for IL1$\beta$-mediating NF$\kappa$B activation and translocation into the nucleus.



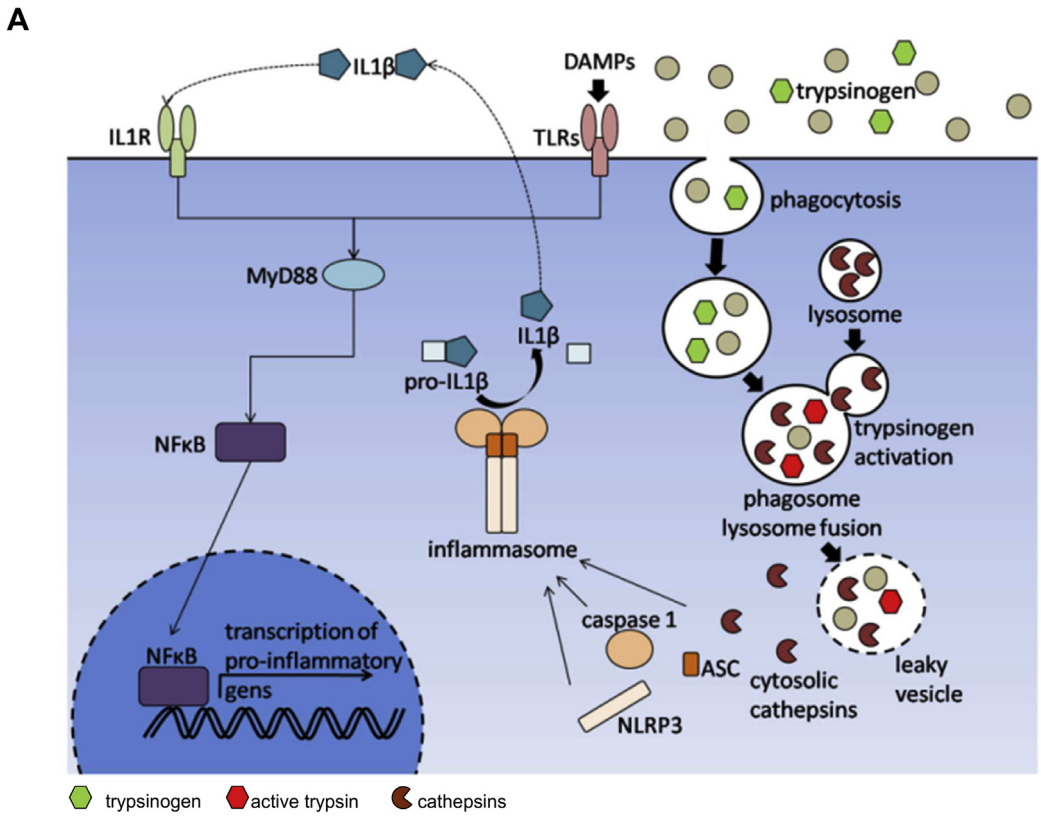

**Supplementary Figure 4.** Macrophages are the dominant infiltrating immune cells during severe necrotizing pancreatitis in mice, demonstrated by F4/80 staining of the pancreas 3 days after duct ligation (*A*). Staining of cathepsin B illustrates that these infiltrating immune cells carry large amounts of cathepsin B (*A*). Comparison of caerulein-induced pancreatitis with duct ligation pancreatitis in CTSB-/- animals showed slightly decreased pancreatic damage in the model of caerulein pancreatitis and a significant reduction of severity in a severe model of necrotizing pancreatitis (*B-C*). Serum amylase and lipase were significantly reduced upon duct ligation pancreatitis in CTSB-/- mice. In contrast to caerulein-induced pancreatitis, MPO levels in the lung were decreased in CTSB-/- 3 days after duct ligation (*C*).This illustrates the crucial role of CTSB mediating the systemic immune response in a severe model of pancreatitis. LAMP2-deficient mice develop chronic pancreatitis spontaneously. In this genetic model of chronic pancreatitis, CD68-positive phagocytosing macrophages co-localized with trypsinogen within the pancreas (*D*). Staining of CTSB shows CTSB-positive infiltrating cells within the pancreas. Enzyme measurement of CTSB and CTSL demonstrated a significant increase of lysosomal enzymes within the pancreas and trypsinogen activation follows this pattern. LAMP2-deficient mice also showed an increased systemic immune response, illustrated by increased levels of lung MPO. This form of chronic pancreatitis is associated with a strong infiltration of CD68-positive M1 macrophages.




**Supplementary Figure 5.** Trypsin activity was measured over the time course of 14 days after induction of partial duct ligation pancreatitis in pancreatic tissue homogenate. Trypsinogen activation was significantly elevated at all time points (3, 7, and 14 days) compared with untreated control mice (0 days). In addition to the trypsin activity, we observed a co-localization of trypsinogen within CD68-positive macrophages at all time points after induction of pancreatitis.




**Supplementary Figure 6.** Staining of alternatively activated macrophages (M2) by CD206 in human chronic pancreatitis resection specimen and trypsinogen staining excluded co-localization of these proteins. Phagocytosis of zymogens resulted in the differentiation to na M1 phenotype, and thus the surface marker CD206 that is only expressed on M2 macrophages was lost. However, not all M1 macrophages phagocytosed zymogens but expressed CD68, suggesting alternative pathways of activation to be in place (*B*).




**Supplementary Figure 7.** NF$\kappa$B activation in phagocytosing macrophages depended in part on intracellular protease activation and an autocrine loop via IL1$\beta$ receptor signaling. Zymogens that were engulfed by macrophages undergo lysosomal degradation and during this process trypsinogen was co-localized with macrophage-derived cathepsin B in a phagolysosomal compartment. Cathepsin B-mediated activation of trypsinogen led to phagolysosomal leakiness, which resulted in the cytosolic redistribution of cathepsins. Inflammasome activation was induced by cytosolic cathepsin and resulted in the maturation of IL1$\beta$ through the proteolytic processing of pro-IL1$\beta$ by caspase-1. IL1$\beta$ is a crucial activator of the NF$\kappa$B pathway, as suggested by transcriptome analysis (Supplementary Figure 3).