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TLR9 and the NLRP3 Inflammasome Link Acinar Cell Death With Inflammation in Acute Pancreatitis

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Abstract

BACKGROUND & AIMS—Acute pancreatitis is characterized by early activation of intracellular proteases followed by acinar cell death and inflammation. Activation of damage-associated molecular pattern (DAMP) receptors and a cytosolic complex termed the inflammasome initiates forms of inflammation. In this study, we examined whether DAMP-receptors and the inflammasome provide the link between cell death and the initiation of inflammation in pancreatitis.

METHODS—Acute pancreatitis was induced by caerulein stimulation in wild-type mice and mice deficient in components of the inflammasome (ASC, NLRP3, caspase-1), Toll-like receptor 9 (TLR9), or the purinergic receptor P2X₇. Resident and infiltrating immune cell populations and pro-IL-1 β expression were characterized in control and caerulein-treated adult murine pancreas. TLR9 expression was quantified in pancreatic cell populations. Additionally, wild-type mice were pretreated with a TLR9 antagonist prior to induction of acute pancreatitis by caerulein or retrograde bile duct infusion of tauro lithocholic acid 3-sulfate (TLCS).

RESULTS—Caspase-1, ASC, and NLRP3 were required for inflammation in acute pancreatitis. Genetic deletion of *Tlr9* reduced pancreatic edema, inflammation, and pro-IL-1 β expression in pancreatitis. TLR9 was expressed in resident immune cells of the pancreas, which are predominantly macrophages. Pretreatment with the TLR9 antagonist IRS954 reduced pancreatic edema, inflammatory infiltrate, and apoptosis. Pretreatment with IRS954 reduced pancreatic necrosis and lung inflammation in TLCS-induced acute pancreatitis.

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CONCLUSIONS—Components of the inflammasome, specifically ASC, caspase-1, and NLRP3, are required for the development of inflammation in acute pancreatitis. TLR9 and P2X₇ are important DAMP receptors upstream of inflammasome activation, and their antagonism could provide a new therapeutic strategy for treating acute pancreatitis.

Keywords

Pancreatitis; TLR9; Caspase-1; ASC; Inflammasome

Acute pancreatitis is characterized by a sequence of events that begins with intracellular activation of stored zymogens within the acinar cell. This can lead to acinar cell death and subsequent pancreatic inflammation.¹ This model has received support from experimental systems such as caerulein-induced pancreatitis in which each of these pathologic responses can be demonstrated.² Clinical and experimental studies have documented that the extent of the subsequent inflammatory response is directly related to disease severity and survival.³ An important role for inflammation in the pathogenesis of pancreatitis is supported by systems which show a requirement for inflammatory components in the development of pancreatitis. For example, neutrophils contribute to injury through the generation of reactive oxygen species.⁴ Other inflammatory components which are required for maximal pancreatitis include the IL-1 receptor, caspase -1, and TNF- α .⁵

Collectively, these studies suggest that a complex cytokine and cellular inflammatory response mediates injury in acute pancreatitis. However, whether acinar cell death could promote an inflammatory response has not been explored. A central feature of necrotic cell death is the release of intracellular contents. Some intracellular molecules on release into the extracellular compartment induce an immune response, and are known as damage associated molecular patterns (DAMPs).⁶ Over twenty DAMPs have been identified; they bind to a wide range of DAMP-receptors including members of the TOLL, and purinergic receptor families. Activation of DAMP-receptors results in up-regulation of pro-cytokines such as pro-IL-1 β , and activation of cellular proteases required for their cleavage to functional cytokines.⁷ Recently caspase-1 has been identified as a central component of a cytosolic complex termed the inflammasome which is required for the initiation of many types of sterile inflammatory responses. We demonstrate that the inflammasome is activated during acute pancreatitis, and components of the inflammasome are required for maximal pancreatitis. Furthermore, we identify the DAMP-receptors TLR9 and P2X₇ as upstream of the inflammasome and vital for the development of pancreatic injury.

Materials and Methods

Animals

C57BL/6 male mice five to eight weeks of age were purchased from the National Cancer Institute. *ASC*^{-/-}, *Casp1*^{-/-}, *Nlrp3*^{-/-}, *Tlr9*^{-/-} and *P2X7*^{-/-} mice have been described.⁸ All experiments and animal handling were performed in accordance with Yale University Institutional Animal Care guidelines.

Induction of Pancreatitis and Immunohistochemical Assessment of Pancreatic Severity

Mice were fasted for 12 hours and administered eight hourly intraperitoneal injections of caerulein (50 μ g/kg body wt).⁴ Controls received saline. Mice were euthanized one hour after the last intraperitoneal injection for subsequent analysis.

Taurolithocholic acid 3-sulfate (TLCS)-induced pancreatitis was performed in C57BL/6 wild-type male mice as previously described.⁹ Briefly, mice were anesthetized and administered 3mM TLCS by retrograde infusion into the pancreatic duct. Sham surgical

controls were performed involving manipulation of the duodenum and cannulation of the distal biliary pancreatic duct without TLCS infusion. Mice were euthanized 24 hours later.

Pancreatic and lung tissues were fixed, and neutrophil quantitation was performed after immunolabelling with GR-1 monoclonal antibody (BD Biosciences, San Jose, CA) by scoring for positive cells in five high power fields (40x). Pancreatic sections were stained with hematoxylin and eosin, and assessed at 20x magnification over five to ten separate fields for severity of pancreatitis by scoring for edema, inflammatory infiltrate, acinar cell apoptosis, and necrosis. Lung sections stained with hematoxylin and eosin were assessed at 20x magnification over ten separate fields for severity of lung injury by scoring for edema and inflammatory infiltrate. Apoptosis was further assessed by TUNEL staining of paraffin-embedded sections and quantification of TUNEL-positive cells by light microscopy in five high power fields (40x). Analysis was performed in a blinded manner.

Treatment with antagonists of TLR9 and P2X₇

The TLR9 antagonist IRS954 (Dynavax Technologies)⁸ was injected subcutaneously at a dose of 150 µg per mouse one hour prior to the first intraperitoneal injection of caerulein or normal saline, TLCS infusion, or sham surgery in wild-type C57BL/6 male mice. The P2X₇ antagonist A-438079 (Tocris Biosciences, Ellisville, MO) was injected subcutaneously [300 umoles/ kg] one hour prior to the aforementioned interventions in wild-type C57BL/6 male mice.¹⁰

Preparation of Pancreatic Cells

Pancreatic cells were isolated by a modification of the technique of Leach et al.¹¹

Immunostaining for Flow Cytometry

Single cell suspensions of pancreatic cells were immunolabeled with fluorochrome conjugated antibodies at 1:200 dilution. Pancreatic cuboidal ductal and microvascular endothelial cells were cell sorted based on immunophenotypes of CD34⁺ CD45⁻ CD133⁺ and CD34⁺ CD45⁻ CD133⁻, respectively.¹² Cells of hematopoietic origin within the pancreas were assessed by immunostaining with CD45.1 MAb from BD Biosciences (San Jose, CA). Antibodies used in this study from BD Biosciences (San Jose, CA) included anti: GR1, CD3epsilon, CD4, CD8, CD11c, CD19, CD34, and NK1.1. Anti-F4/80 and CD133, were obtained from ebiosciences (San Diego, CA).

Flow Cytometry and Cell Sorting

Flow cytometry was performed on FACS Calibur (BD Biosciences, San Jose, CA), data acquisition on CellQuest software, and data analysis on WinMDI version 2.9 software. Cells were sorted on FACS aria (BD Biosciences, San Jose, CA).

Quantitative Polymerase Chain Reaction for Expression of TLR9 and pro-IL-1β

RNA was extracted from pancreatic tissue, acinar cells, and cell sorted pancreatic populations with RNeasy Plus Mini Kit from Qiagen (Valencia, CA). Reverse transcription was performed with AffinityScript reverse transcriptase from Stratagene (Cedar Creek, TX). Quantitative real-time PCR was performed for mRNA expression for *pro-IL-1β* and *Tlr9* using commercial primer probe sets from Applied Biosystems (Foster City, CA) and the Applied Biosystems 7500 real-time PCR System. Expression of GAPDH was used to standardize the samples. Results were expressed as a ratio of untreated splenocytes and saline-treated pancreas for *Tlr9* and *pro-IL-1β*, respectively.

Amylase Measurement

Amylase was measured using a Phadebas Kit (Amersham Pharmacia, Rochester, NY).

Pancreatic Trypsin Activity Assay

Pancreata from mice were harvested, flash frozen, and stored at -80°C . Pancreatic trypsin activity was assessed by fluorometric determination. Total pancreatic trypsinogen content was assessed by incubating pancreatic homogenates with 10 U/L enterokinase (Sigma, St. Louis, MO) in trypsin assay buffer with 10mM CaCl_2 for 15 minutes at 37°C prior to fluorometric determination of trypsin activity.

Acinar Cell Zymogen Activation

Mice were fasted for 12 hours, pancreata were harvested, minced, and digested in 50 U/mL type IV collagenase from Worthington (Freehold, NJ) for 60 minutes at 37°C . The digest was filtered through a 300 μm mesh (Sefar American, Depew, NY), washed extensively, plated in 24-well polystyrene tissue culture plates, incubated at 37°C under constant oxygen, and the media exchanged in one hour.¹³ Acini were stimulated with caerulein (100 pM and 100 nM) concentrations for 60 minutes. Chymotrypsin activity was assessed using a fluorogenic substrate from Calbiochem (San Diego, CA). The cumulative fluorescence using was measured over 10 minutes using a HTS 7000 fluorescent plate reader (380-nm excitation and 440-nm emission wavelengths) from Perkin Elmer (Shelton, CT). Chymotrypsin activity is expressed as percentage of the activity induced by supraphysiologic caerulein (100 nM), a concentration known to induce maximal intracellular zymogen activation.

Detection of Circulating DNA in Acute Pancreatitis

Blood was collected from mice after one injection of caerulein. Blood plasma was isolated by centrifugation to remove cells and microparticles. DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with on-column RNase digestion. Quantitative real-time PCR (QPCR) was performed for β -actin DNA using commercial primer probe sets from Applied Biosystems (Foster City, CA) and the Applied Biosystems 7500 real-time PCR System. QPCR for the mitochondrial gene *ATP6* was similarly performed using previously published custom primer probe sets from Applied Biosystems.¹⁴ Results were expressed as fold detection relative to saline treated mice for a fixed volume of blood plasma (100 μL) analyzed. Serum amylase level was assessed concurrently and similarly normalized.

Treatment of Thioglycollate-elicited Peritoneal Macrophages with Pancreatic Homogenate and DNA

Wild-type mice were administered 3% thioglycollate broth (Sigma Aldrich, St. Louis, MO) by intraperitoneal injection and macrophages harvested 3-4 days thereafter. Pancreata were harvested from euthanized mice, disrupted by dounce homogenization, and immediately co-incubated with macrophages for 2 hours in the presence or absence of 5 μM IRS954 or 25 μM A-438079. DNA was also isolated from fresh pancreata with Dnazol reagent (Invitrogen, Carlsbad, CA) and added to macrophages at 100 μg per mL for 2 hours in the presence or absence of 5 μM IRS954.

Enzyme-linked Immunosorbent Assay (ELISA) for IL-1 β Release

IL-1 β release into the culture supernatant of thioglycollate-elicited peritoneal macrophages was assessed by ELISA using mouse IL-1 β , rat anti-mouse IL-1 β monoclonal antibody, and biotinylated goat anti-mouse IL-1 β polyclonal antibody (R&D Systems, Minneapolis, MN). Data are expressed as pg/mL IL-1 β .

NF-κB Immunostaining

Thioglycollate-elicited peritoneal macrophages were plated at 2 million cells per 10mm cover glasses, treated with pancreatic DNA or pancreatic homogenate, and then fixed and immunostained with anti-NF-κB p65 subunit rabbit polyclonal IgG (Delta Biolabs, Gilroy, CA) and HRP-conjugated anti-rabbit IgG secondary antibody (Cell Signaling, Danvers, MA) in Ultra V Block (Ultravision, Fremont, CA). Slides were then stained with DAB substrate kit (Vectorlabs, Burlingame, CA), and counterstained with Gill's hematoxylin. NF-κB activation was quantitated by counting immunostained nuclei in ten 40x fields with an Olympus inverted microscope (Olympus, Center Valley, PA).

Statistical Analysis

Data represent mean of at least three independent experiments, and error bars represent standard deviation and standard error of the mean. Unpaired 2-tailed Student's t-test was used to compare groups. A P value of less than 0.05 was considered significant.

Results

Deletion of caspase-1 (*Casp1*^{-/-}) reduces the severity of caerulein-induced acute pancreatitis

Acute pancreatitis was induced by caerulein administration in wild-type and *Casp1*^{-/-} mice. In wild-type mice, this led to pancreatic edema, leukocyte infiltrate, and acinar cell apoptosis. These features were absent in pancreata of wild-type or *Casp1*^{-/-} animals treated with normal saline (Figures 1A and 1C). Compared to wild-type animals treated with caerulein (n=5), *Casp1*^{-/-} mice treated with caerulein (n=5) showed significantly reduced pancreatic edema, leukocyte infiltrate, and acinar cell apoptosis, (Figures 1A and 1C). Gr1 immunolabeling at one hour after the last caerulein injection revealed significantly fewer neutrophils per high power field in *Casp1*^{-/-} mice versus wild-type animals (Figures 1B and 1D).

To ensure that the lower degree of pancreatitis in the *Casp1*^{-/-} mice was not due to reduced pancreatic trypsinogen content, or an inability of caerulein to activate chymotrypsin, both responses were examined. Supplemental figure 1B shows that pancreatic trypsinogen is not reduced in *Casp1*^{-/-} mice. Furthermore supraphysiologic caerulein treatment (100 nM) induced similar chymotrypsin activation in both wild-type (n=4) and *Casp1*^{-/-} (n=4) mice (supplemental figure 1C).

Genetic deletion of ASC, Nlrp3, P2X₇, or TOLL-like receptor 9 reduced the severity of caerulein-induced pancreatitis

Having demonstrated reduced edema, inflammation and apoptosis in *Casp1*^{-/-} mice, we next examined the requirement for cytosolic inflammasome associated proteins (ASC and NLRP3), as well as membrane proteins in related pathways (P2X₇ and TLR9). In comparison to wild-type (n=5), mice genetically deficient in ASC (n=5), NLRP3 (n=5), P2X₇ (n=6) or TLR9 (n=5) treated with caerulein had significantly less pancreatic edema and leukocyte infiltrate (figures 2A and 2B). In addition, in comparison to wild-type mice, there was significantly less acinar cell apoptosis in *Nlrp3*^{-/-} and *P2X7*^{-/-} animals. Relative to a wild-type caerulein treatment group, genetic deficiency of TLR3 (n=5) did not significantly alter the severity of caerulein-induced acute pancreatitis as assessed by pancreatic edema, leukocyte infiltrate, and acinar cell apoptosis. Of note, late pancreatic trypsin activation in caerulein-induced pancreatitis was significantly reduced with genetic deficiency of *caspase-1* (n=4) and *P2X7* (n=4) (supplementary figure 1A).

Tlr9 mRNA is strongly expressed in bone-marrow derived cells in the pancreas and promotes pancreatic neutrophil infiltrate and pro-IL-1 β expression in caerulein-induced injury

To further explore the molecules that link cell death to inflammation, we investigated details of the TLR9 response, a receptor for DNA that can function as a DAMP. One consequence of TLR9 activation is the up-regulation of pro-IL-1 β ; its level would be expected to be lower in *Tlr9*^{-/-} mice. *Tlr9* mRNA expression was quantified by real-time PCR from wild-type adult mouse pancreatic subpopulations (n=5) with expression reported relative to whole spleen (Figure 3A). *Pro-IL-1 β* mRNA expression in whole pancreas was increased in wild-type mice treated with caerulein one hour after the last caerulein injection. This was significantly reduced in whole pancreas of *Tlr9*^{-/-} mice (n=5) treated with caerulein (pancreas *pro-IL-1 β* mRNA from *Tlr9*^{-/-} was 24.2 ± 0.5 fold greater than whole spleen, versus 37.1 ± 3.0 fold for wild-type, $P < 0.05$). These findings demonstrate that *Tlr9* deletion results in less *pro-IL-1 β* mRNA in this pancreatitis model.

To determine the cell distribution of TLR9, pancreatic endothelial (CD34⁺ C133⁻ CD45⁻) and small duct cell (CD34⁻ CD133⁺ CD45⁻) populations were purified by immunolabeling single cell suspensions and cell sorting (Figure 3B). Pancreatic endothelial cells and small duct cells constituted 3.3 ± 0.04 and 6.8 ± 3.8 percent of total pancreatic cells, respectively. The bone marrow derived cell population was purified by CD45 staining and further characterized by FACS staining for F4/80 and ER-MP23. F4/80 positivity identified 93.3 ± 2.0 percent of pancreatic macrophages, including ER-MP23⁺ CD45⁺ macrophages (Figure 3C and 3D).

Tlr9 mRNA was expressed in pancreatic endothelial cells at levels significantly higher than whole spleen (Figure 3E). *Tlr9* mRNA was also expressed in pancreatic small duct cells at low levels that were not significantly different from whole spleen (Figure 3E). Resident CD45⁺ cells in the pancreas expressed *Tlr9* 29-fold higher relative to spleen cells and at much higher compared to all other groups ($P < 0.05$), with CD45⁺ cells constituting 5.3 ± 0.01 percent of total pancreatic cells (Figure 3E). These data show that TLR9 is expressed on a number of pancreatic cell populations, but bone marrow derived CD45⁺ cells have by far the highest expression level.

Macrophages are the predominant CD45⁺ resident bone marrow derived cell in the adult murine pancreas

CD45⁺ pancreatic and spleen cells were isolated and immunolabeled to identify resident neutrophils (GR1⁺ve), macrophages (F4/80⁺ve), NK/NKT cells (NK1.1⁺ve), and thymocytes (CD3⁺ve). Representative histograms for antigen expression in bone marrow derived CD45⁺ cells and pancreatic CD45⁺ cells are shown (Figure 4A). F4/80⁺ macrophages represented the predominant CD45⁺ cell population within the adult murine pancreas, constituting 71.6 ± 2.5 percent of all CD45⁺ pancreatic cells (Figure 4B).

Genetic deletion of Tlr9 reduces the neutrophil infiltrate in caerulein-induced acute pancreatitis

Acute pancreatitis in wild-type and *Tlr9*^{-/-} mice was induced by repeated caerulein administration and single cell suspensions of pancreata prepared one hour after the last injection. Resident and infiltrating immune cells were analyzed by flow cytometry one hour after the last caerulein injection. Representative histograms for antigen expression in CD45⁺ cells are shown (Figure 4C). GR1⁺ neutrophils were less than 5% of the CD45⁺ cells in control pancreas, but constituted greater than 50% of the CD45⁺ cells after caerulein treatment in wild-type animals (n=5). In the absence of TLR9 the increase in neutrophils in response to supraphysiologic doses of caerulein was significantly less (Figure 4D).

Pretreatment with TLR9 and of P2X₇ antagonists reduces the severity of caerulein-induced acute pancreatitis

Having demonstrated a requirement for TLR9 using a genetic deletion approach, we wished to confirm this finding using a pharmacological inhibitor. Wild-type mice were given a subcutaneous injection of the TLR9 antagonist IRS954 (n=5), or saline (n=5) one hour prior to inducing pancreatitis by caerulein. Pancreatic edema, leukocyte infiltrate, and acinar cell apoptosis one hour after the last caerulein injection were significantly reduced with IRS954 pretreatment (Figures 5A and 5D). Pretreatment with IRS954 alone followed did not result in histologic pancreatic injury. Gr1 immunolabeling at one hour after the last caerulein injection revealed significantly fewer infiltrating neutrophils (per 20 x mag field) in mice pretreated with IRS954 versus saline (5B and 5C). TUNEL staining at one hour after the last caerulein injection revealed significantly fewer apoptotic acinar cells in wild-type mice pretreated with IRS954 versus saline (Figure 5E). Similarly, TUNEL staining in mice treated with caerulein was markedly reduced in *Casp1*^{-/-} animals compared to wild-type animals (Figure 5E). Serum amylase elevation at one hour after the last caerulein injection was reduced by IRS954 pretreatment (Figure 6E). Pancreatic *pro-IL-1 β* elevation at one hour after the last caerulein injection was reduced by IRS954 pretreatment (Figure 5F). To determine whether TLR9 antagonism might have therapeutic value, IRS954 or vehicle was given after inducing pancreatitis (n=6 in each group). Similar to the responses observed with pretreatment, the delayed administration of the inhibitor also resulted in significantly less pancreatic edema, pancreatic leukocyte infiltrate and pancreatic apoptosis (Supplementary figure 2A and 2B).

We next tested if antagonism of P2X₇ the second DAMP-receptor we have identified, provided protection. As can be seen from supplemental figure 3, administration of the P2X₇ antagonist A-439079 (n=6) versus saline (n=6) prior to serial treatment with caerulein resulted in significant less pancreatic edema, and significantly less pancreatic leukocyte infiltrate.

Pretreatment with TLR9 antagonist reduces the severity of TLCS-induced acute pancreatitis

We next investigated if TLR9 inhibition could reduce the severity of organ injury in a more severe model of acute pancreatitis, TLCS infusion. Wild-type mice were given a subcutaneous injection of the TLR9 antagonist IRS954 (n=6), or saline (n=6) one hour prior to inducing pancreatitis by TLCS infusion. Pancreatic necrosis 24 hours after TLCS infusion was significantly reduced with IRS954 pretreatment (Figures 6A and 6C). Pretreatment with IRS954 alone followed by sham surgery did not result in histologic pancreatic injury relative to vehicle treated animals subject to sham surgery. Lung inflammatory cell infiltrate was also significantly reduced with IRS954 pretreatment (Figures 6B and 6D). Serum amylase elevation was reduced by IRS954 pretreatment (Figure 6E).

Pancreatic injury releases pro-inflammatory DAMPs

Having shown that TLR9 and P2X₇ are required for full pancreatic injury and inflammation in acute pancreatitis, we next investigated if pancreatic injury could release DAMPs with pro-inflammatory properties *in vivo* and *in vitro*. Self DNA is a ligand for TLR9. We therefore quantitated circulating genomic DNA released in acute pancreatic injury by QPCR for serum β -actin DNA. A single supramaximal dose of caerulein resulted in significantly marked elevations in serum β -actin (>10⁶ fold increase, P<0.05) concordant with significantly increased serum amylase which confirmed pancreatic injury (Figure 7A). Circulating mitochondrial DNA was similarly quantitated by QPCR for serum *ATP6* DNA with no significant difference noted between saline and caerulein treated animals (Figure 7A). To test if pancreatic DAMPs could induce P2X₇ and inflammasome dependent pro-

inflammatory responses in immune cells, IL-1 β release was determined in peritoneal macrophages co-incubated with pancreatic homogenate. Pancreatic homogenate markedly stimulated IL-1 β release in peritoneal macrophages and P2X₇ was required for maximal IL-1 β release (Figure 7B). NF- κ B activation is required for TLR-mediated pro-inflammatory signaling and for full pancreatic injury. Pancreatic homogenate and DNA both induced significant NF- κ B activation in isolated peritoneal macrophages (Figure 7C). This required TLR9 but not P2X₇ as treatment with IRS954 reduced NF- κ B activation to baseline levels (Figure 7C). Representative immunostaining for NF- κ B activation in isolated peritoneal macrophages is shown in Figure 7D.

Discussion

Clinical pancreatitis is characterized by a rapid inflammatory response which can lead to a systemic inflammatory syndrome, and multi-organ failure.¹ The mechanisms that couple the initial acinar cell death to the subsequent inflammatory response in pancreatitis have not been fully defined.

Release of potential DAMPs has been documented in pancreatic injury as genomic DNA and high-mobility group protein box-1 (HMGB1) were found to be released into the circulation in a cohort of patients with AP.¹⁵ Moreover, DAMPs mediate inflammatory injury in the pancreas as pharmacologic blockade of the DAMP HMGB1 decreases pancreatic necrosis and inflammation in an experimental model of severe acute pancreatitis.¹⁶

To address whether other innate immune components are significant contributors to this process, we initially confirmed that caspase 1, a cytosolic enzyme central to the activation of the pro-inflammatory cytokines IL-1 β , is required for pancreatitis.¹⁷ In the absence of caspase 1 there is a marked inability of the initial activation of acinar cell proteases to induce inflammation. This was not due to decreased pancreatic trypsinogen content, or an inability of caerulein to induce early acinar cell trypsin *in vivo* or chymotrypsin *in vitro* in caspase-1 deficient mice (Supplementary figure 1A-C). An important consequence of the lower level of inflammation in caspase-1 deficient mice is a reduction in overall acinar cell injury (Figure 1B and 5E). Such a reduction in overall organ injury after inhibition of the inflammatory response provides a therapeutic impetus for identifying the pathways initiating inflammation.

Having confirmed a non-redundant role for caspase-1 we attempted to define a role for two cytosolic molecules that are required for caspase-1 activation by some stimuli. NLRP3 is a member of the NLR (NOD-like receptors) family of molecules, is expressed in the cytosol and functions to sense a range of signals including uric acid, and ATP which are important in generating an inflammatory response.¹⁸ To identify the initiating and other inflammasome activating molecules in pancreatitis, we tested the requirement for NLRP3 and ASC. In the absence of ASC or NLRP3, caerulein induced edema and inflammation were substantially reduced (Figure 2A and 2B). The identification of the NLRP3-ASC limb of the inflammasome pathway has important implications for understanding the inflammasome activating DAMPs, as NLRP3 is known to be required for inflammasome activation by uric acid and ATP. Recently uric acid was shown to be an important mediator of sterile inflammation in the liver and the peritoneum, and may also have a role in pancreatitis.¹⁹ ATP-induced inflammasome activation is mediated via the plasma membrane purinergic receptor P2X₇. Based on the NLRP3 result, we tested a role for P2X₇ by inducing pancreatitis in P2X₇^{-/-} mice, and confirmed a requirement for P2X₇ for the induction of pancreatic edema and inflammation (Figure 2A and 2B).

Activation of the inflammasome results in caspase-1-dependent cleavage of pro-IL-1 β by into active IL-1 β and its subsequent release from the cell.²⁰ Most cells do not have significant amounts of preformed pro-IL-1 β and its up-regulation requires activation of specific regulators, especially TOLL receptors. The first characterized ligands for TOLL receptors were pathogen derived molecules, but some TOLL receptors can also be activated by self molecules, demonstrating that signals from non-self pathogens and signals from damaged-self share some of the same molecular machinery.⁶ Among the TOLL receptors, TLR9 is a leading candidate for detecting self molecules, and can detect self DNA, both nuclear as well as un-methylated mitochondrial DNA.²¹ Circulating genomic DNA is markedly increased early in the setting of acute pancreatic injury though serum level of mitochondrial DNA appear to be unaltered (Figure 7A). This is consistent with literature suggesting that mitochondrial DAMPs are largely restricted to the site of tissue injury.²² Our data clearly demonstrates that signaling through TLR9 has a significant, but not exclusive role, in up-regulation of pro-IL-1 β in pancreatitis (Figure 3A). Further, although ductal epithelial and endothelial cells express TLR9 mRNA, the highest levels are found in CD45+ bone marrow derived cells. Within the CD45+ population, in the healthy pancreas there is a high representation of F4/80 +ve, ER-MP23 +ve macrophage cells (Figure 3C). The onset of pancreatitis results in a rapid recruitment and increase in the percentage of GR1+ve neutrophils, and this is dependent on TLR9 (Figure 4D).

Our central finding, in addition to the requirement for inflammasome components, is a requirement for the two DAMP-receptors TLR9 and P2X₇ for maximal pancreatitis (Figure 2). This clearly places pancreatitis within the group of diseases with a DAMP and inflammasome-mediated mechanism. One value of identifying novel pathways is that it can stimulate new therapeutic approaches. In relation to our work, the potential of TLR9 antagonists to limit pancreatic injury was tested. The TLR9 antagonist IRS954 significantly reduced edema, inflammation and acinar cell apoptosis as confirmed by light microscopy and TUNEL staining (Figure 5). There was also a reduction in neutrophil infiltrate. It is notable that we were not able to demonstrate a significant reduction in apoptosis in TLR9 deficient mice as compared with the effect using IRS954 (Figure 2B). This may be due to the number of animals studied, or due to the fact that IRS954 also antagonizes TLR7, which may be important. Additionally, IRS954 post-treatment significantly reduces pancreatic edema and infiltrate in this model (Supplementary figure 2A-B). Moreover, IRS954 significantly reduces pancreatic necrosis and lung inflammation in the TLCS model of severe pancreatitis. To further test the requirement of the identified DAMP-receptors, we demonstrated the ability of a P2X₇ receptor antagonist to decrease pancreatic edema and leukocyte infiltrate in acute pancreatitis (Supplementary figure 3A-B). These findings have direct therapeutic implications for pancreatitis as DAMP-receptor antagonists are under development for a range of inflammatory diseases.²³

IL-6 responses are correlated with disease severity in human pancreatitis.²⁴ IL-6 can be synergistically induced by HMGB1, TLR9 ligands, or IL-1 β .²⁵ This is consistent with our finding of a role for TLR9 and the Nlrp3 inflammasome in pathologic sterile inflammatory injury in experimental acute pancreatitis (Figure 2).

Our demonstration of a requirement for the TLR9 inflammasome pathway helps to explain earlier findings in the literature including a requirement for IL-1 receptor activation for maximal pancreatitis in experimental models. IL-1 β is required for full distant organ injury in experimental models of severe acute pancreatitis.²⁶ This is consistent with our findings that the TLR antagonist IRS954 can decrease both pancreatic IL-1 β expression (Figure 5F) and lung inflammation (Figure 6D) in experimental acute pancreatitis.

Collectively our data place a DAMP-receptor mediated pathway of P2X₇, TLR9 and inflammasome activation in the center of converting signals from damaged acinar cells to an inflammatory response. This allows for an integration of the available information on inflammatory mediators in pancreatitis (Figure 7E), and has identified new therapeutic avenues such as TLR9 and P2X₇ antagonism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard abbreviations used

ASC	apoptosis-associated speck-like protein containing a CARD
NLRP 3	NACHT, LRR, and pyrin domain- containing protein 3

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Figure 1.

Deletion of caspase-1 (*Casp1*^{-/-}) reduces the severity of acute pancreatitis. Acute pancreatitis in wild-type and *Casp1*^{-/-} mice was induced by caerulein. Representative histology (A) and histology scores (C) are shown. Gr1 immunohistochemistry (B) and GR1 positive cell counts in pancreatic tissue section (D) are shown. Asterisks denote significant differences ($P < 0.05$) in bracketed groups. NS, not significant.

Figure 2.

Deletion of inflammasome component *ASC* (*ASC*^{-/-}), *Nlrp3* (*Nlrp3*^{-/-}), *P2X7* receptor (*P2X7*^{-/-}), or TOLL-like receptor 9 (*Tlr9*^{-/-}) reduces the severity of acute pancreatitis. Acute pancreatitis in wild-type, *ASC*^{-/-}, *P2X7*^{-/-}, *Tlr9*^{-/-}, and *Tlr3*^{-/-} mice was induced by caerulein. Representative histology (A) is shown. Histology scores for pancreatic edema, inflammatory infiltrate, and apoptosis (B) are shown. Asterisks denote significant differences (P < 0.05) in bracketed groups.

Figure 3.

Genetic deletion of *Tlr9* (*Tlr9*^{-/-}) reduces pro-inflammatory cytokine production in caerulein-induced acute pancreatitis. *Tlr9* mRNA is strongly and predominantly expressed in bone marrow-derived cells. Acute pancreatitis in wild-type and *Tlr9*^{-/-} mice was induced by caerulein. One hour after the last injection of caerulein, pancreata were harvested and pancreatic *pro-IL-1 β* mRNA was quantified by real-time PCR (A). Pancreatic cuboidal ductal cells (CD34⁺ CD133⁺ CD45⁻) and endothelial cells (CD34⁺ CD133⁻ CD45⁻) were isolated by flow cytometry (B). CD45⁺ bone marrow derived cells within the pancreas were assessed by flow cytometry for cell surface expression of tissue macrophage markers F4/80 and ER-MP23 (C and D). *Tlr9* mRNA was quantified by real-time PCR from wild-type pancreatic subpopulations and expressed relative to whole spleen (E). Open bars: wild-type mice treated with saline. Solid bars: wild-type mice treated with caerulein. Gray bars: report results from *Tlr9*^{-/-} mice treated with saline. Diagonal striped bars: *Tlr9*^{-/-} mice treated with caerulein. Asterisks denote significant differences (P<0.05) in bracketed groups. # denotes significant difference (P<0.05) compared to all other bars shown.

Figure 4.

Macrophages are the predominant immune cell in the pancreas. The early inflammatory infiltrate in caerulein-induced acute pancreatitis is neutrophil predominant and genetic deletion of *Tlr9* reduces this neutrophil infiltration. Pancreas and spleen were isolated from adult wild-type mice, single cell suspensions prepared, and flow cytometric analysis for cell surface proteins performed. Representative histograms for Gr1, F4/80, NK1.1, and CD3 cell surface expression in CD45⁺ bone marrow derived cells in the pancreas and spleen are shown (A). Resident immune cell populations are reported as percentage of total cells analyzed and as percentage of total CD45⁺ cells analyzed (B). Acute pancreatitis in wild-type and *Tlr9*^{-/-} mice was induced by caerulein. Representative histograms (C) are shown for Gr1, F4/80, NK1.1, and CD3 cell surface expression in CD45⁺ pancreatic cells from wild-type and *Tlr9*^{-/-} mice treated with caerulein. Resident immune cell populations are reported as percentage of total CD45⁺ cells analyzed (D). # denotes the predominant resident immune cell population within the pancreas. Asterisks denotes significant differences ($P < 0.05$) between marked groups.

Figure 5.

Pretreatment with the TLR9 antagonist IRS954 reduces the severity of caerulein-induced acute pancreatitis. Representative histology (A) and histology scores for pancreatic edema, inflammatory infiltrate, and apoptosis (D) are shown. Gr1 immunohistochemistry (B) and Gr1 positive cell counts in pancreatic tissue section (C) are shown. TUNEL positivity (E) was scored in the groups above as well as in *Casp1*^{-/-} mice subjected to caerulein pancreatitis. Pancreatic *pro-IL-1 β* transcription is shown (F).

Figure 6.

Pretreatment with the TLR9 antagonist reduces the severity of acute pancreatitis. Representative pancreatic head histology after hematoxylin and eosin staining (A) and histology scores for inflammatory infiltrate and necrosis (C) are shown. Representative lung histology after hematoxylin and eosin staining (B) and histology scores for lung edema and inflammatory infiltrate (D) are shown. Serum amylase is shown for both the TLCS and caerulein induced pancreatitis models (E). Asterisks denote significant differences ($P<0.05$) in bracketed groups. NS, not significant.

Figure 7.

Pancreatic injury releases DAMPs. Circulating genomic DNA and mitochondrial DNA was quantitated in acute pancreatic injury by QPCR of serum for β -actin and ATP6 DNA (A). IL-1 β release into supernatant was quantitated in peritoneal macrophages stimulated with pancreatic homogenate in the presence or absence of P2X₇ antagonist A-438079 (B). NF- κ B activation was quantitated by cell counts for immunochemically detected nuclear translocation in macrophages treated with pancreatic DNA or homogenate in the presence of TLR9 antagonist IRS954 or P2X₇ antagonist A-438079, respectively (C). Representative imaging of nuclear localized NF- κ B (D). Schematic representation of TLR9 and P2X₇ mediated innate immune responses in acute pancreatic injury (E). Pancreatic acinar cell injury and necrosis results in release of DAMPs, including nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) as well as intracellular ATP. Resident macrophages within the pancreas sense these DAMPs through (i) TLR9 receptors with respective induction of NF- κ B translocation and *pro-IL1 β* transcription and through (ii) P2X₇ with proteolytic maturation of IL-1 β through the Nlrp3 inflammasome. IL-1 β dependent pathways induce further pancreatic injury.