Isoprenylation and NET formation in acute pancreatitis

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Abstract

Acute Pancreatitis (AP) is an inflammation disease that characterized by activation of protease and the innate immune system, leading to infiltration of neutrophils and tissue damage in the pancreas. The aim of this thesis was to determine the role of isoprenylation (farnesyltransferase and geranylgeranyltransferase) as well as NET formation in regulating recruitment of neutrophils and tissue damage in severe AP.

AP in mice was induced by retrograde infusion of Na-taurocholate into the pancreatic duct and intraperitoneal injection of L-arginine two times at hourly intervals. Induction of pancreatitis provoked a clear cut increase in tissue damage of the pancreas characterized by neutrophil infiltration, myeloperoxidase activity, chemokine levels, and acinar cell necrosis and edema formation in the pancreas. In paper I, farnesyltransferase mediates leukocyte sequestration and tissue injury in AP. Inhibition of farnesyltransferase attenuates infiltration of neutrophils in the pancreas and the lung and suggesting that farnesyltransferase controls both local and systemic inflammation in pancreatitis. Paper II demonstrates that geranylgeranyltransferase regulate severity in pancreatitis. Inhibition of geranylgeranyltransferase results in a reduction of neutrophil up-regulation Mac-1 and CXCL2 formation in the pancreas. Blocking geranylgeranyltransferase activity attenuated systemic inflammation and pulmonary neutrophils in animals with pancreatitis. In paper Paper III address the role of Ras-signalling in AP. Ras inhibition improves neutrophil infiltration, blood amylase, cytokine formation and and pancreatitis-associated systemic inflammation. Paper IV demonstrates that NETs are generated in the inflamed pancreas and play a critical role in the development of severe AP. Inhibition of NET decreased CXCL2 formation and neutrophil recruitment in the inflamed pancreas. Moreover, NETs regulates STAT3 activity and trypsin activation in acinar cells and histones might be important molecular mediators in these processes.

These findings identify a novel role of isoprenylation and NET formation in pancreatitis and suggest that targeting these mechanisms might be a useful way to ameliorate local and systemic inflammation in severe AP.

Key words: Acute pancreatitis, neutrophil recruitment, trypsinogen activation, NET

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List of Papers

The following papers will be included in this thesis and referred to in the text by their Roman numerals:

I. Mohammed Merza, Darbaz Awla, Rundk Hwaiz, Milladur Rahman, Stefan Appelros, Aree Abdulla, Sara Regner; Henrik Thorlacius. Farnesyltransferase regulates neutrophil recruitment and tissue damage in acute pancreatitis. *Pancreas*: 2014;43:427-435.

II. Mohammed Merza, Erik Wetterholm, Su Zhang, Sara Regner, Henrik Thorlacius. Inhibition of geranylgeranyltransferase attenuates neutrophil accumulation and tissue injury in severe acute pancreatitis. *Journal of Leukocyte Biology*: 2013; 94:493-502.

III. Changhui Yua, **Mohammed Merza**, Lingtao Luo, Henrik Thorlacius. Inhibition of Ras signalling reduces neutrophil infiltration and tissue damage in severe acute pancreatitis. *European Journal of Pharmacology:* 2015; 746:245–251.

IV. Hannes Hartman*, **Mohammed Merza***, Milladur Rahman, Rundk Hwaiz, Enming Zhang, Sara Regnér, Henrik Thorlacius. Neutrophil extracellular traps induce trypsin activation, inflammation and Tissue Damage in mice with severe acute pancreatitis. *Gastroenterology*: 2015 (Resubmitted).

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Abbreviations

Ach	acetylcholine
AP	acute pancreatitis
CCK	cholecystokinin
CCK-RF	cholecystokinin-releasing factor
CXCL2/MIP-2	macrophage inflammatory protein-2
DAMPs	damage-associated molecular patterns
ELISA	enzyme linked immunosorbent assay
EGF	epidermal growth factor
ERCP	endoscopic retrograde cholangio-pancreatography
ECM	extracellular matrix
GEF	guanine nucleotide exchange factor
GTP	guanosine triphosphate
ICAM-1	inter-cellular adhesion molecule-1
IP3	inositol triphosphate
GAP	guanine activating proteins
i.p.	intraperitoneal
i.v.	intravenous
JAMs	junctional adhesion molecules
JNK	c-Jun terminal kinase
kDa	kilo Dalton
LPS	lipopolysaccharide
LFA-1	lymphocyte function antigen-1
MMP	matrix metalloproteinase
MCP-1	monocyte chemotactic protein-1
MOF	multiple organ failure
Mac-1	macrophage-1 antigen
NET	neutrophil extracellular taps
NF-kB	nuclear factor kappa B
PECAM-1	platelet endothelial cell adhesion molecule-1
PMNL	polymorphonuclear leukocyte

PSGL-1	P-selectin glycoprotein ligand-1
Rac1	Ras-related C3 botulinum toxin substrate 1
Ras	rat sarcoma
RER	rough endoplasmic reticulum
Rho	Ras homologous
RT-PCR	reverse transcription-polymerase chain reaction
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
SPINK 1	serine protease inhibitor Kazal type 1
TAP	trypsinogen activation peptide
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule-1
ZG	zymogen granule

Introduction

Acute pancreatitis (AP) is a sudden swelling and inflammation of the pancreas. The inflammatory reaction within the pancreas, modulated by enzymatic cell damage, leading to pancreatic auto-digestion then results in systemic inflammatory response syndrome (SIRS) which is associated with a massive proinflammatory cytokine burst [1]. All these reactions will causes an increase in membrane permeability, migration of inflammatory cells or perhaps promoting bacterial translocation, as demonstrated in animal models, resulting in multiorgan failure and death [2,3,4]. However, the nature of the relationship between protease activation on one hand and inflammatory changes on the other is not clear owing to an incomplete understanding of the basic mechanisms regulating proteolysis and inflammation in the pancreas. The official mark of the inflammatory process in AP is leucocyte infiltration into the pancreas [5]. It is generally held that secreted chemokines are fundamental regulators of leukocyte activation and tissue navigation [6]. For example, CXCL2 known as macrophage inflammatory protein (MIP-2) is a potent neutrophil attractant and might play an important role in AP [7]. However, knowledge of the molecular mechanisms regulating leucocyte recruitment in the pancreas is limited. Several reports have shown that leucocytes initially roll on activated endothelium, followed by firm adhesion and transendothelial migration into the extravascular space [8,9,10]. Numerous studies have shown that Mac-1 is a dominating molecule in mediating tissue infiltration of neutrophils as well as in pancreas [11,12,13]. Since neutrophils accumulate in the lungs during acute respiratory disease syndrome (ARDS) and are also present in the lungs of taurocholate and L-arginine induced pancreatitis in mice models, it is likely that they play an important role in pancreatitis-associated acute lung injury (ALI) [14,15,16].

Neutrophil extracellular traps (NETs) emerge from activated neutrophils following stimulus by variety of proinflammatory stimuli such as lipopolysaccharides (LPS), interleukin-8 (IL-8), tumor necrosis factor (TNF) and various pathogens [17,18] and are extruded into the extracellular space, this series of events is also called (NETosis) [17]. NETs are composed of decondensed chromatin including DNA and histones and with antimicrobial factors such as neutrophil elastase and myeloperoxidase [19]. Before the discovery of NETs, increases in circulating free DNA (cf-DNA) in the blood had been reported in various diseases including cancers [20,21], sepsis [22,23,24] and cell-free DNA in pancreatitis [25,26] and several articles have suggested an association between NETs and cf-DNA [23,27,28].

Small GTP-binding proteins are intracellular regulators which must undergo post-translational modifications, including isoprenylation, for their activity. Isoprenylation is catalyzed by two enzymes: farnesyltransferase and geranylgeranyltransferase [29]. The Ras protein is a family of small guanosine triphosphate (GTPases) that can activate a variety of signal transduction cascades in the cytoplasm that eventually lead to activation of nuclear transcription factors [30]. However, a recent study reported that elevated Ras signaling is able to induce pancreatitis [31]. Rho-kinase, are known to act as molecular switches regulating numerous important cellular functions, such as cytoskeleton organization, cell adhesion, migration, reactive oxygen species formation and oncogenic transformation [32,33,34]. Rho-kinase inhibitor have been demonstrated to ameliorate and regulates trypsinogen activation in severe AP [35], protecting against tissue fibrosis [36] and abdominal sepsis [37]. In addition, one other small GTPase Rac1 (Ras-related C3 botulinum toxin substrate 1), a member of the Rho family of proteins, is a signal transducer involved in the control of several processes, including cell cycle progression, cell adhesion, cell migration and cytoskeletal reorganization [38]. Interestingly, a recent study demonstrated that Rac1 inhibition ameliorate the severity of pancreatitis and pancreatitis-associated lung injury [39].

Our recent studies have demonstrated the importance of previously unrecognized pathways leading to tissue inflammation in severe AP. In this thesis two different experimental models of severe AP in mice were used to determine the role of isoprenyl farnesyltransferase and geranylgeranyltransferase signals as well as role of NET formation in activation of innate immunity and tissue damage in severe AP.

Background

Anatomy and physiology of pancreas

The pancreas is a soft, elongated, flattened gland typically 15 cm long [40]. The adult pancreas weighs between 75 and 110 g and sits across the back of the abdomen, behind the stomach, specifically the upper left abdomen. Further, the pancreas develops from two portions, a ventral and dorsal [41,42]. The ventral portion is formed from the bud which gives rise to the liver, gallbladder, and common duct, and it gives the head of the pancreas [41]. The dorsal portion of the pancreas arise as a direct outpouching from the duodenum, it forms part of the body and tail of the gland [43]. The head of the pancreas is on the right side and located within the curvature of the duodenum [44]. However, the neck, body, and tail of the pancreas lie in the posterior abdomen, with the tail extending to the gastric surface of the spleen [45]. The arterial blood supply to the pancreas is from two major arteries supplying the abdominal organs such as (a) celiac and (b) superior mesenteric arteries [46]. The celiac artery is supplying the pancreas from the superior pancreatic duodenal artery. The superior mesenteric artery is supplying the pancreas from the inferior pancreatic duodenal artery [41]. The pancreas is a dual-function gland, having features of both endocrine and exocrine glands and the part of the pancreas with endocrine function is made up of many cell clusters called islets of Langerhans [47]. In general, there are four main cell types in the islets, they can be classified by their secretion into: α-alpha cells secrete glucagon that increase glucose in blood, β-beta cells secrete insulin that decrease glucose in blood, Δ-delta cells that secrete somatostatin and PP cells, or γ (gamma) cells, that secrete pancreatic polypeptide [48].

Regarding the pancreas endocrine gland, approximately 2% of the gross weight of the pancreas supports endocrine function. However, the remaining 98% is involved with exocrine function [49]. The exocrine portion of the gland is made up of pancreatic acinar cells arranged spherically in formations called acini. The pancreatic acinar cells (acinar from the Latin term meaning berry in a cluster) are a factory of protein synthesis [50]. Acinar cells are pyramidal in shape, a group of acini composed lobules and a number of lobules can make pancreatic lobes. Proteins which are synthesized by the acinar cells are assembled in the rough endoplasmic reticulum (RER), and transport to the golgi apparatus where some physiological processes like post-translation modifications occur [51]. However, more than 90% of the proteins synthesized by acinar cells consist of digestive enzymes which are destined to be

moved out of the cell [52]. Those proteins, which exist primarily as inactive pro-enzymes or zymogens, are packaged in considering vacuoles at the trans side of the golgi complex and carried towards the luminal plasma membrane [53]. At the plasma membrane, those zymogen granules fuse with the surface membrane and then release their contents into the acinar space [54]. Moreover, the exocrine pancreas is also responsible for secretion of ions and water into the duodenum of the gastrointestinal tract [55]. The flow of ions and water is necessary to transport the digestive enzymes from their origin in the pancreatic acinar cells to the intestine [56]. The pancreas secretes 1500-3000 mL of iso-osmotic alkaline (pH > 8.0) fluid per day containing many enzymes and zymogens [57]. In addition, this alkaline is due to a very high concentration of NaHCO3 (up to 150 mM) [58]. Further, at least one major function of the NaHCO3 is to neutralize the acidic pH of the gastric chyme (a liquid substance found in the stomach before passing through the pyloric valve and entering the duodenum) delivered to the intestine from the stomach [59]. A neutral pH in the intestinal lumen is necessary for optimal function of digestive enzymes as well as gastrointestinal surface epithelial function [55]. A clear alkaline secretion of the pancreas containing enzymes that aid in the digestion of proteins, carbohydrates and fats [58].

Pancreatic enzyme secretion

Pancreatic enzymes, except for amylase and lipase, are normally inactive until they reach small intestine. In the intestine brush border enzyme like enterokinase, cleaves a peptide from trypsinogen, forming the active enzyme trypsin and then trypsin activates the other enzymes such as chymotrypsin and elastase (Figure 1) [60]. There are three major groups of enzymes: 1) amylase (alpha-amylase) is the enzyme that digest carbohydrates and hydrolyses starch into sugar (a glucose-glucose disaccharide) [61]. The major source of amylase is pancreatic secretions, although amylase is also present in saliva [62]. When the pancreas is diseased or inflamed, amylase releases into the blood. Increased levels of amylase can help in the diagnosis and monitoring of acute pancreatitis. 2) Protease, the pancreas secretes several proteases, including trypsin and chymotrypsin, which is play very important role in the digestion of proteins [63]. Although the stomach starts the breakdown of proteins with another digestive enzyme known as pepsin, this process occurs in the intestine, with the help of proteases [61]. Normally, proteases could autodigest the pancreas if active, however, they are produced in an inactive form until released into the intestine, where they become active [63]. 3) Pancreatic lipase is another pancreatic enzyme, which play an important role in

digestion of fat [64]. Generally, dietary fats are composed of triglycerides, which cannot be absorbed by the intestine in their natural form. Lipase play important roles in the absorption of fats through break down of triglycerides into fatty acids, which can be easily absorbed by the intestinal surface [65]. Lipase levels also increased during pancreatitis [61,66]. In addition, the pancreas synthesis a host of other digestive enzymes, including ribonuclease, deoxyribonuclease, gelatinase, carboxypeptidase and elastases [67]. In the severe pancreatitis, enzymes of the exocrine pancreas damage the structure and tissue of the pancreas. Detection of elevated levels of amylase and lipase in the blood, are often used to diagnose AP.

Normally, after feeding, the presence of fat or amino acid, in the duodenum actively stimulates the release of trypsin-sensitive peptide terminal cholecystokinin releasing factor (CCK-RF) which in turn stimulates the release of CCK from duodenal wall [68]. Direct stimulation of pancreatic exocrine function related to the several hormones, which bind to receptors on the pancreatic acinar cells [69]. Indirect mechanisms depend on the activation of autonomic nervous reflexes [70]. It means that CCK stimulates pancreatic enzyme secretion both directly via CCK-A receptors on acinar cells and indirectly through CCK-B receptors on nerves, followed by acetylcholine release [70,71]. However, less abundant pancreatic acinar cell secretion stimulators have been addressed and including acetylcholine (Ach), vasoactive intestinal polypeptide, gastrin releasing peptide and substance P. Somatostatin acts as the main exocrine pancreatic secretion inhibitor [72]. Greater knowledge about pancreatic enzyme secretion and transport is necessary for better understanding of AP pathogenesis.

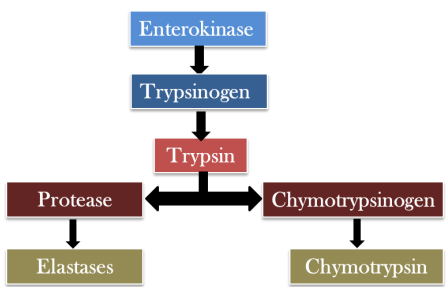

Figure 1: Pancreatic enzyme secretion. Enterokinase cleaves trypsinogen, forming the active enzyme trypsin, the trypsin then activates the other enzymes such as chymotrypsin and elastase.

Acute pancreatitis

Definition and classification

Pancreatitis is a disease characterized by inflammation of the pancreas. It is common about 40/100 000 per year accompanied by abdominal pain and elevated of serum/plasma or urine levels of pancreatic enzymes. The clinical manifestation have a great deal of variability and may present as mild, which is self-limiting abdominal disturbance or at the other extreme, may present with an acute abdominal and shock. Nausea and vomiting is common [73]. Sometimes, AP may also present without abdominal pain with symptoms of respiratory failure or confusion. Low grade to moderate fever is not uncommon in AP. Also tachycardia and hypotension and mild jaundice may be found [74]. AP is usually a mild edematous interstitial inflammation, which is a self-limiting disease and not associated with complications or organ dysfunction [75]. In contrast, a severe type of AP with a local necrotizing inflammation and systemic complications are present in 20%–30% of patients and associated with a mortality rate of up to 40% [76].

It is not possible to recognize severe disease early in the course, since patient with mild and severe disease initially appear with similar symptoms. The progress to severe pancreatitis is often clinically evident after the first 2-3 days. In general, symptoms of severe AP reveals its progresses into two phases [53]: 1) The early phase refers to the first 7 to 14 d after onset of AP where 1) systemic inflammatory response syndrome (SIRS) and 2) multi organic failure (MOF) are common complications [77]. In the late phase (14 to 28 d after onset of AP), the disease may be complicated by infection of pancreatic necrosis and causes secondary MOF. It has been mentioned that the mortality rate in this phase is about 0-20% [78,79]. Severe pancreatitis can cause serious complications and inflammation, local or systemic problems. The local complications include pancreatic necrosis, fluid collection, pancreatic pseudocyst, ascites, and infected pancreatic necrosis [80]. The systemic complications are usually seen in acute and severe pancreatitis [81]. These complications include pulmonary complications, followed by pulmonary edema and respiratory distress syndrome [75]. It has been shown that, inflammatory changes from the pancreas extended to the kidneys, stomach, colon and spleen [82,83].

The documented incidence of AP has ranged from 40 per 100,000 populations [84]. The cause of AP is evident after standard investigations in about 70%–80% of patients during or after the first attack. Gallstones are the most common cause of AP and it is about 45-60%, and the

second common cause is alcohol intake, ranged between 20%–25%, post-endoscopic retrograde cholangiopancreatography (ERCP) in 5%–7%, and miscellaneous in about 5% of cases [85]. Mortality in AP is usually due to SIRS and MOF in the first two-week period, while after two weeks it is usually due to sepsis and its complications [86].

Pathomechanism of AP

For investigation of pathomechanism and prevention as well as therapeutic possibilities of AP, several noninvasive and invasive experimental models are known. Although the pathomechanism is still not completely clarified yet, most of the research investigating the pathomechanism of AP has been done by *in vivo* and *in vitro* on pancreatic acinar cells. These studies have shown that the central intra pancreatic acinar cell proceeding in pancreatitis are elevation of intra acinar calcium concentration or premature activation of trypsinogen and the activation of the transcription factor like NF-κB which leads to acinar cell injury [87,88,89]. It has been demonstrated that one of the most toxic bile acids to acinar cells is the taurolithocholic acid (TLC). This bile acid causes Ca2+ signaling in pancreatic acinar cells through an inositol 1,4,5-trisphosphate (IP3) [90]. The elevated intracellular Ca2+ concentration can lead to pancreatic enzyme activation [91] or cell death [92] and consequences in severe acute necrotizing pancreatitis.

Trypsinogen activation in AP

The protein trypsinogen is synthesis in the endoplasmic reticulum (ER) and then transported to the golgi apparatus, where it is sorted together with other pancreatic enzyme into core particles [93]. Trypsinogen is a small around 25 kDa protein which is present in normal pancreatic fluid. It can be activated by the hydrolysis of up to ten amino acids at the N-terminus and the breakdown region is called trypsinogen activation peptide (TAP) [94]. Trypsin is capable of activating the trypsinogen by proteolytic cleavage, and, in turn, trypsin rapidly activates all the other pancreatic proenzymes (Figure 2) [94]. In acute experimental pancreatitis several reports suggested that premature activation of trypsinogen into active trypsin within the pancreas resulting in the release of trypsinogen activation peptide (TAP) [95,96,97]. Further, cellular studies of co-localization show that trypsin activation occurs within cytosolic vacuoles which contains both digestive enzymes and the lysosomal enzymes cathepsin B [98,99]. Some authors have demonstrated immunoreactivity against TAP in

vacuoles positive for lysosomal markers and cathepsin B [95,100]. This cathepsin B might play an important role in the activation of the pancreatic zymogens. The importance of cathepsin B in transforming trypsinogen into active trypsin is demonstrated in a well carried out study on mice knockout in lysosomal cathepsin B [98]. In this study, the trypsin activity in the pancreatic of mice deficient in lysosomal cathepsin B approximately more than 80% lower than in the control mice. Pancreatic tissue injury, as indicated by the serum activities of lipase and amylase, or by the extent of pancreatic acinar cell necrosis, approximately 50% lower in mice deficient in lysosomal cathepsin B compared with the control [98].

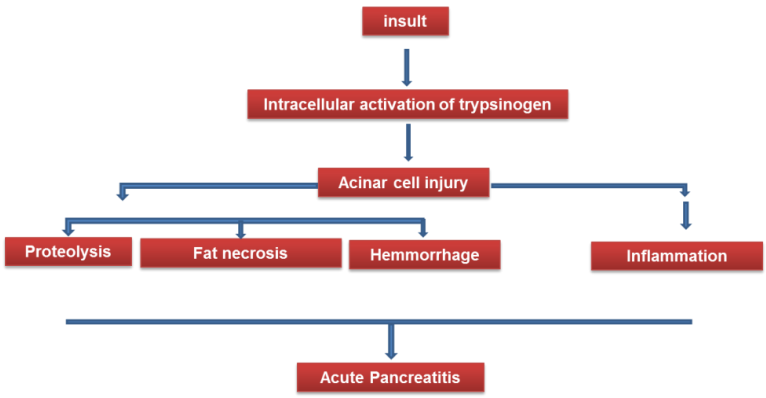

Figure 2: Schematic of enzymatic activation of AP. The enzymes can damage tissue and activate the complement system and the inflammatory cascade, producing cytokines. This process causes inflammation, edema and necrosis.

However, a recent study by Dawra *et al*, 2011 was carried out in an animal model of cerulein induced AP. The authors generated knock out mice lacking the trypsinogen isoform 7 gene T (−/−) and they found that deletion of trypsinogen isoform 7 gene reduced the total trypsinogen content without affecting the physiologic function. For example, T(−/−) mice lacked the pathologic activation of trypsinogen which occurs within the pancreatic acinar cells during the early stages of AP. This study also showed that the absence of trypsinogen activation in T(−/−) mice showed complete inhibition of acinar cell death *in vitro* and a 50% reduction in acinar cell damage during disease progression. This result strongly suggested that intracellular trypsin is an important factor in determining of pancreatic acinar cell death [101].

Role of calcium in AP

Calcium may also play an important role in early AP. Pancreatitis induced by caerulein hyperstimulation and bile acids has been shown to cause a rise in intracellular calcium and disruption of acinar cell calcium signaling [90,102]. This is associated with acinar cell vacuolization and the intracellular trypsinogen activation events that occur in early AP [103]. The role of calcium in the mechanistic activation of trypsinogen has been thoroughly studied in the past and recent years. It has been shown that hypercalcemia leads to edematous and necrotizing pancreatitis [91]. In addition, premature activation of serine protease is generally believed to be the crucial step in the escalation of acinar cell injury to AP [104]. The role of calcium Ca^{2+} in pancreatitis is further supported by the observation that disrupted Ca^{2+} signaling inside the acinar cells and a sustained rise in intra-cellular Ca^{2+} is seen in experimental models of AP. As mentioned before, a frequent cause of AP is gallstones, which are thought to cause disease by obstructing the pancreatic duct or obstructing a common bile pancreatic channel. The mechanism with reflux of bile acid into the pancreas could cause pancreatic injury and subsequent lung organ injury as well [105]. Previous study showed that transporter mediated bile acid uptake causes calcium dependent cell death in acinar cells *in vitro* [92]. Laukkarinen *et al*. 2007 in experiments using pre-incubation of mouse with 1, 2-bis(2-aminophenoxy)-ethane-N,N:,N:,N:',N:'-tetra-acetic acid tetrakis/acetoxymethhyl ester (BAPTA-AM) an intracellular chelator, reduced trypsinogen activation by 85% compared with the control acini, and indicated that Na-taurocholate induced trypsinogen activation is calcium dependent [106]. Bile acids and non-oxidative alcohol metabolites elicit calcium release from endoplasmic reticulum and apical store [107]. ER, membrane Ryanodine Receptors (RyR) and plasma membrane store operated calcium channels (SOCs) have been implicated as important sources of calcium response [108,109,110,111]. However, pharmacologic antagonism of RyR using, and pharmacologic inhibition of TRPC3, has been shown to reduce zymogen activation as well as pancreatic damage in AP [112]. Prolongation of the pathologic Ca^{2+} by inhibition of SERCA or by ATP depletion has been recognized as important mechanisms of pancreatic damage via ethanol metabolites and bile acids [113,114]. A recent study investigated the relationship between the formation of vacuoles and calcium influx developed in response to the inducers of pancreatitis by specific inhibitors of Ca^{2+}. They found that the formation of endocytic vacuoles is an important early step in the development of acinar cell damage triggered by the inducers of AP [115].

Another factor which has been studied in the severity of AP is nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB). NF-kB has been shown to play a critical role in the development of AP [116]. However, NF-kB is a nuclear transcription factor responsible for regulating the transcription of a wide variety of genes involved in immunity and inflammation. Many of these genes have been implicated as central players in the development and progression of AP [117]. In fact, NF-κB is initially sequestered in the cytoplasm bound to its inhibitory element which is called IκB [118]. It has been shown that, after stimulation, IκB is changed and phosphorylated then degraded by proteasomes. The degradation of IκB causes and releases NF-κB, allowing it to translocate into the nucleus and promote region of a number of proinflammatory genes [118]. In early phases of AP, induction of NF-κB binding activity and decreased IκB expression were observed in animal pancreatitis models [119,120,121], and NF-κB activity seems to be dependent on calcium influx (Figure 4) [120,121,122].

The role Ras (Rat sarcoma protein family) activity levels for pancreatic diseases have been implicated [31,123,124]. It has been shown that high levels of Ras activity leads to the generation of inflammation of the pancreas (Figure 3) [31]. In the recent study, carried out in animal models of taurocholate induced pancreatitis, the Rho signaling in regulating trypsinogen activation and tissue damage in severe AP are well addressed [35].

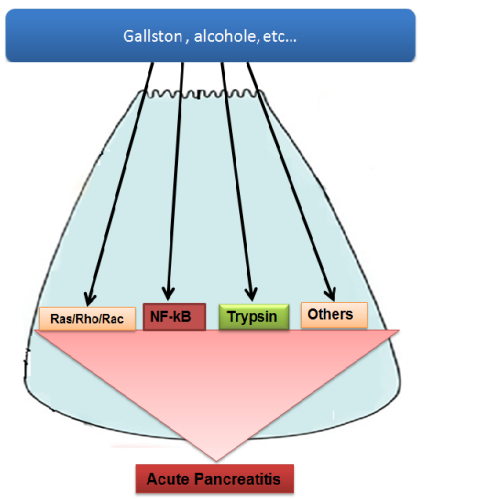

Figure 3: Cellular response cascades in AP. Induction of acute pancreatitis trypsin activity simultaneously activated together with several other mechanism when acinar cells are injured. Ras, Rho, Rac, NF-kB, trypsin and other factors initiate acute pancreatitis.

Inflammatory response in AP

The initial phase of AP involves triggering incident that cause premature activation of pancreatic proteases as a result of intracellular co-localization of the digestive and lysosomal enzymes and this leads to disruption of the pancreatic acinar cells [125,126,127]. Furthermore, pancreatic injury is followed by activation of local inflammatory cells and various inflammatory mediators cytokines [128]. The most common concepts are based on cellular experiments and in experimental animal models. Early in the course of AP, activation of trypsinogen occurs within the pancreatic acinar cells, which leads to a local inflammatory reaction, in severe cases, injury and inflammation in the pancreas can proceed to systemic inflammation causing SIRS [129]. Still the mutual relation between these two phases in AP is not well known. Gaiser *et al*, 2011 was demonstrated that the intra acinar activation of trypsinogen is sufficient to initiate AP. The authors demonstrated that trypsinogen activation and substrates like trypsin can trigger leukocyte recruitment through different mechanisms [130]. However, Dawra *et al*, 2011 reported that leukocyte recruitment into inflamed tissue is independent of the primary trypsinogen activation and both phases activate via two independent pathways initiated within the pancreatic acinar cells [131]. They suggested that intra-acinar trypsinogen activation leads to acinar death during early stages of pancreatitis and progression of local and systemic inflammation in AP does not require trypsinogen activation. Further, it has been demonstrated that initial trypsinogen activation is independent on neutrophils, whereas later activation is depend on neutrophils in the pancreas [132].

However, It has been reported that proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) are playing an important role in pancreatitis [5,133]. IL-1 and TNF- α are primary inducers of interleukin-6 (IL-6) and interlukin-8 (IL-8) production and are known to initiate systemic complication such as acute respiratory distress syndrome (ARDS) [134,135]. In addition, the production of cytokines enhances proinflammatory signals and this proinflammatory cytokines activate the vascular endothelium in the body. Thus, this activation accelerates leakage of the capillary veins and triggers migration of leukocytes into inflamed tissue and also cause promoting activation of coagulation cascades [136,137,138,139]. Currently, the inflammation response in AP is believed to be associated with some factors: trypsinogen activation, production of oxygen free-radical species, microcirculation disorder, calcium overload, rat sarcoma (RAS) and Ras

homologues (Rho), production of inflammatory mediators, cytokines, chemokines, adhesion molecules, apoptosis and necrosis (Figure 3).

Cytokines

Cytokine are small, with molecular weights ranging from 8 to 40,000 Dalton. On a functional basis, cytokines can be divided into two groups: the pro- and the anti-inflammatory cytokines. Several studies have shown that cytokines are upregulated in both human and experimental pancreatitis [140,141], indeed, there is evidence indicating that acinar cells itself produces inflammatory mediators, such as TNF-α, IL-1β, IL-6 and an anti-inflammatory cytokine interleukin 10 (IL-10) [142]. It has been shown that zymogen and cytokines released by the inflamed pancreas are absorbed into the circulation, leading to the SIRS [60]. In fact, disease severity can be correlated to the levels of these circulating cytokines. It has been shown that cytokines and NF-κB contribute to both local and systemic inflammatory response [143], however, inhibition of NF-κB [122,144], leukocyte depletion [132], genetic deletion of cytokine IL-1β and receptors to IL-1 type I, TNF-α type I [145] or intercellular adhesion molecule-1 (ICAM-1) have uniformly decreased the severity of pancreatitis associated lung injury [146]. It has been shown that intrapancreatic TNF-α and IL-1β one hour following induction of AP and levels increase rapidly over the following six hours [147,148]. Numerous investigators have employed specific antagonists to TNF-α or IL-1β in experimental models of AP [149,150]. Examples of this approach include the use of an anti-TNF-α antibody, soluble type 1 TNF receptor and IL-1ra. Blockade of these receptors revealed a significant reduction of intrapancreatic damage and systemic severity [149,150].

IL-6 is produced by a wide range of cells including monocytes or macrophages, endothelial cells, fibroblasts and acinar cells in response to potent proinflammatory stimuli such as endotoxin, IL-1 or TNF-α. IL-6 is the primary inducer of the acute-phase response in various inflammatory conditions [151,152]. Studies have shown that plasma levels of IL-6 are elevated in serum samples from patients early in the course of AP [151,153]. Moreover, IL-6 was found to be an excellent predictor of remote organ failure, and its main signaling pathway, STAT-3 has been implicated in AP. They indicated that IL-6 trans-signaling dependent STAT3 pathway is central to AP associated acute lung injury (ALI) [151].

IL-10 is a potent anti-inflammatory cytokine expressed by almost all cells but primarily released by activated monocytes/macrophages and lymphocytes [154]. This cytokine exerts its anti-inflammatory properties through inhibition of various proinflammatory cytokines. In

addition, IL-10 induces the synthesis of natural cytokine antagonists such as IL-1RA and TNF- receptors [149,150]. In human AP, circulating levels of IL-10 were found to correlate with the severity of the disease and with organic failure or death [155]. A number of experimental studies demonstrated a protective effect of IL-10 in several models of AP [156,157,158].

Chemokines

Chemokines are a family of cytokines, small (8-10 kDa), with activating and chemotactic effects on leukocytes, which provide a key stimulus for directing the migration of leukocytes into injured tissues [159]. Numerous chemokines have been identified as inflammatory mediators with potent leukocyte and monocyte activating properties and many of them have been shown to be involved in the pathophysiological process of experimental pancreatitis [129,160]. Basis of cysteine residue positioning, chemokines has been divided into four subfamilies: CXC, CX3C, C and CC [161]. CXC chemokines which contain a three amino acid ELR motif at the amino terminal end such as IL-8 or GRO-α are believed to act upon neutrophils [162]. MIP-2 or CXCL2, representing the human IL-8, was shown to be elevated after induction of AP in the rat and mice [35,132]. Treatment with neutralizing antibody against CXCL2 protects mice against AP associated lung injury. Furthermore, treatment of mice with antileukinate a specific blocker of CXCL2 reduces the associated lung injury [163]. However, a CC chemokine such as monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory proteins -1 (MIP-1) and regulated on activation normal T cell expressed and secreted (RANTES) have been believed to act principally upon monocytes and T cells, respectively [164]. However, It has been shown that pancreatic acinar cells produce the CC chemokine MCP-1 and that treatment with supermaximally stimulating dose of cerulein causes an upregulation of MCP-1 production [165,166]. Furthermore, an inhibition of MCP-1 protects against severity AP [167]. Cerulein-induced stimulation of chemokines production is regulating via NF-kB and Ca^{+2} [166].

Neutrophil recruitment

Trypsin and other pancreatic enzymes have proteolytic activity and can cleave components of the complement system, causes pancreas to produce the chemoattractants. However, levels of these chemoattractants rise in experimental pancreatitis and may cause priming of circulating

leukocyte and perhaps migration of activated neutrophils into the pancreas [168]. It is well known that leukocyte infiltration is a central feature of the inflammatory response, and numerous studies have reported that leukocytes are essential in severe AP [132,169,170]. Proinflammatory cytokines (IL-6, TNF-α, IL-8, IL-1β and others such as platelet-activating factor (PAF), free radicals) will result in a significant concentration of neutrophils activation [171,172]. Depletion of circulating leukocytes decreases both the magnitude of the local pancreatic damage and the systemic inflammatory response in AP [132]. For example, leukocyte-endothelium interactions were studied by use of intravital florescence microscopy of the pancreatic microcirculation [173,174,175]. Intraperitoneal injection of the anti Gr-1 antibody reduces leukocyte interactions with the vascular endothelium in the pancreas [132]. In general, the leukocyte adhesion and transmigration process occurs in four clearly defined steps: 1) leukocyte rolling mediated by weak adhesive interactions with the endothelium, 2) triggering, 3) strong, integrin-mediated adhesion, 4) leukocyte migration (Figure 4). The first step in this process is mediated by the selectins, a family of lectin-like adhesion molecules [176]. The selectins are surface glycoproteins, three selectins have been identified and are named for the principal cell types in which they occur: leukocyte selectin (L-selectin); endothelial selectin (E-selectin); and platelet selectin (P-selectin) [177]. L-selectin is widely expressed on the surface of leukocytes and binds to carbohydrate moieties on the surface of endothelial cells, is important for tethering and subsequent rolling on endothelial cells facilitating neutrophil migration into a site of inflammation [178]. Also E-selectin and P-selectin are expressed on the surface of endothelial cells following cytokine activation (e.g. IL-1, TNF-α) and bind to oligosaccharide receptors on neutrophils [179] [180]. P- selectin has been detected and expressed in one hour after induction of AP using the taurocholate model and suggested that this expression associated with prominent increase in reactive oxygen species (ROS) and NF-kB [181,182]. A recent study determined the role of P-selectin and showed that P-selectin mediated neutrophil rolling and recruitment in severe pancreatitis [183]. Furthermore, P-selectin expression was detected on lung three hour after AP, and the highest expression was detected 24 h after induction of AP [184]. It has been shown that P-selectin expression on the lung depends on the production of ROS, since xanthine oxidase inhibition prevents P-selectin expression and leukocyte recruitment [184]. Targeting P-selectin may be an effective strategy to ameliorate inflammation in AP. Clinically, a study demonstrated that soluble P-selectin and E-selectin are indicators for endothelial activation and serum levels correlate with the mortality rate in patient with AP [185]. In non-survival

patients, levels of soluble P-selectin were significantly higher than survival, while E-selectin levels were significantly higher in patient with other organ dysfunction [185].

ICAM-1 and vascular cell adhesion protein 1 (VCAM-1) are two important molecules involved in leukocyte adhesion [179]. They are ligands for leukocyte lymphocyte function associate antigen-1 (LFA -1) (CD11a/CD18), and macrophage antigen-1 or (Mac-1) (CD11b/CD18 on neutrophils [186,187]. Experimentally, cerulein induced AP model has been shown that ICAM-1 play a role in leukocyte recruitment and tissue damage in this disease [188]. Protective effects of P- and L-selectin inhibitors reduced the severity of AP in mice [189]. However, it has been shown that intracapillary leukocyte accumulation in AP depends on the expression of LFA-1 and ICAM-1 [190]. Furthermore, adhesion molecule platelet/endothelial cell adhesion molecule 1 (PECAM-1) has been shown that play critical role via localized at the level of the intercellular endothelial junctions and it is necessary to allow neutrophil migration [191,192,193]. Arriving at the interstitium, the activated neutrophil will cause considerable damage to a tissue, which has already suffered from hypoxia. All these processes are mainly related to the massive ROS production [194]. During AP, ROS are generated from hypoxic injured pancreatic tissue and activated neutrophils within the pancreas at the early stage of AP [193]. Several studies have demonstrated that ROS play a critical role in the pathogenesis of AP [195,196]. However, treatment with antioxidant and ROS scavengers' limited and reduces both local and systemic tissue damage in AP [197].

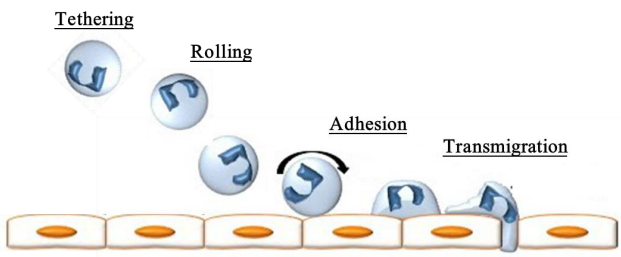

Figure 4: The leukocyte recruitment cascade. Neutrophil initially binds the blood vessel wall through selectins and undergoes a transient, rolling adhesion to the blood vessel. Integrins expressed by neutrophils change shape in response chemokines, causing the neutrophil to decelerate from rolling to a stable arrest. Finally, the combination of chemokines and integrin causes the neutrophil to transform from a spherical circulating cell into site of infection.

Overall, the neutrophilic granules, filled with proteases, collagenases, elastases, lipooxygenases, phospholipases and myeloperoxidases (MPO), will digest and disorganize neutrophil of extracellular trap (NET), which enables clearance of invading pathogens. This structure is composed of DNA in association with histones, as the most abundant protein in NET, as well as granular protein such as elastases and MPO. Leukocyte tethering, migration and activation can then occur at distant sites and lead to widespread organ damage.

Neutrophil extracellular traps

After recruitment to the inflammatory site, neutrophils attack invading pathogens by release of antimicrobial peptides and lytic enzymes as well as production of ROS followed by phagocytosis that enables of invading pathogens [198]. However, in 2004, a novel function was described antimicrobial mechanism of neutrophils is the formation of NET due to web like structure of the expelled DNA [199]. The main components of NETs are deoxyribonucleic acid (DNA), histones, neutrophil elastase and MPO [200]. Before the discovery of NETs, several studies reported on an increase in the concentration of circulation free DNA (cf-DNA) in the blood in various diseases [22,23,24]. However, a recent study of Gornic *et al*, 2011 carried out in an clinical pancreatitis, demonstrated that cf-DNA in plasma and serum on the first day after admission is significantly higher in patient who developed severe AP than in those in mild disease [25]. This study suggested that DNA quantification could be used as an early marker of severity AP. NETs effectively trapped and killed bacteria *in vitro* and were considered as a final step in a novel controlled cell death mechanism [17]. NE and MPO are stored in azuruphilic granules of neutrophils and play important role in regulating of NET, NE is a serine protease that depredate virulence pathogens and kill bacteria [201,202]. MPO catalyzes the oxidation and produces hydrogen peroxide [203]. NE and MPO deficient are susceptible to microorganism infections [204]. However, MPO plays an important role in neutrophil antimicrobial response and has been shown that neutrophils from deficient in MPO failed to form NET, suggested that MPO is necessary for making NETs in the inflammation disease [205,206].

Histones are considered to be involved in NET formation, undergoes a modification (citrulinated) that converts arginine residues to citrulline [207]. This citrullinate histone is catalyzed by peptidylarginine deiminase 4 (PAD4), and is localized in the nucleus of neutrophils. However, it has been shown that neutrophils from knock out PAD4 mice lost

their ability to hypercitrulinate histones and NET formation [208]. Upon i*n vitro* activation with phorbol myristate acetate (PMA), IL-8 or lipopolysaccharide (LPS), neutrophils release NETs has been shown to depend on ROS [17,199]. A recent study demonstrated that neutrophils from chronic granulomatous disease (CGD) patient caused by mutations in NADPH oxidase fail to produce NET upon PMA stimulation [17]. Furthermore, it has been found that inhibition of Raf, MEK-ERK signaling pathway upstream of NADPH oxidase blocked NET formation [209]. It is believed that chromatin decondensation and the association with antimicrobial proteins are two essential steps during NET formation [17]. The molecular mechanism linking ROS production to chromatin decondensation and binding to antimicrobial proteins is still unknown. A recent study show that ROS production leads to the release of NET and subsequently MPO from azuruphilic granules [204]. In addition, administration of recombinant human (rh) DNase, which effectively catalyzes hydrolysis of extracellular DNA, it has been used to examine the impact of NET formation *in vivo* [210]. A recent study examined the effect of rhDNase on inflammation and bacterial spread in a model of abdominal sepsis based on CLP [211]. This study demonstrated that treatment with rhDNase I decreased CLP-induced formation of CXC chemokines and found that NETs regulate pulmonary infiltration of neutrophils and tissue injury via formation of proinflammatory compounds in abdominal sepsis, suggested that NETs exert a proinflammatory role in septic lung injury.

Isoprenylation proteins

Isoprenylation is a post-translation modification involving the covalent 15-carbon farnesyl or a 20-carbon geranylgeranyl moiety to a carboxyl terminal cysteine residue [212]. Isoprenylation is required for proper membrane localization of the modified protein. In general, preneltransferase consist of three related enzymes 1) farnesyltransferase (FTase), 2) geranyltransferase I (GGTIase I), 3) GGTase II [213] and it is dependent on the guanine protein receptor proteins. Guanine nucleotide-binding proteins (G proteins) play an important role to transduce extracellular signals into intracellular changes [214]. G proteins include the heterotrimeric G proteins (known as large G proteins) that are activated by membrane G-protein-coupled receptors (GPCRs) and the monomeric small G proteins (also referred to as small GTPases) [215]. However, of the small G proteins, the RAS superfamily of GTPases is the most studied and comprises many small (20–40 kDa) monomeric G proteins [215]. It has

been reported that Ras superfamily is structurally divided into five major subfamilies: the RAS, RHO, RAB, RAN and ARF families [216]. These proteins control cellular signaling pathways which is responsible for adhesion, migration, growth, cytoskeletal integrity, differentiation and survival [217]. Despite their functional and structural diversity, they all possess GDP/GTP-binding and intrinsic GTPase activities that enable them to switch between biologically active (GTP-bound) and inactive (GDP-bound) conformations [217]. Switching between GDP/GTP is regulated by three major types of protein modulator agents [218]: 1) Guanine nucleotide Exchange Factors (GEFs) catalyze the exchange of GDP with GTP to promote Ras activation, 2) GTPase-Activating Proteins (GAPs) deactivate the Ras protein by stimulating hydrolysis of bound GTP to GDP, 3) deactivation can also be achieved by association with guanine nucleotide dissociation inhibitors (GDIs), which prevent membrane association, and GDP dissociation (Figure 5). All of these regulatory proteins serve to activate or inactivate Ras or Rho GTPase signaling pathways [219]. The interaction between the active GTP-bound GTPase and the effector molecule leads to activation of downstream signal transduction pathways effectors (Figure 5).

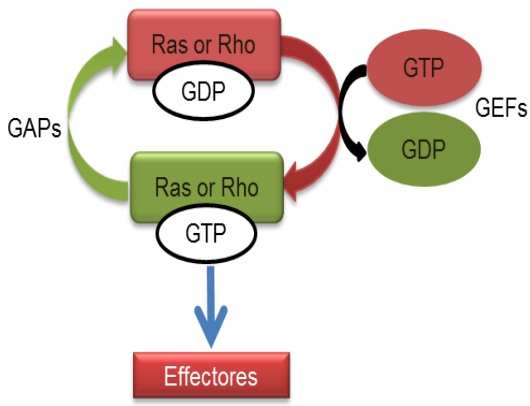

Figure 5. Mechanism of Ras/Rho regulation. GEFs catalyze the exchange of GDP with GTP to promote Ras/Rho activation. GAPs deactivate the Ras/Rho protein by stimulating hydrolysis of bound GTP to GDP. Deactivation association with GDIs, to prevent membrane association and GDP dissociation.

However, it is known that post-translational modifications, such as phosphorylation and methylation play important roles in regulating the function of proteins by localization or stability and altering activity [220]. The Ras superfamily of GTPases undergo numerous

modifications in their C-terminal region, and more specifically through their CAAX motif (where C is a Cysteine, A an aliphatic amino acid, and X any amino acid) before they can be appropriately localized and activated [221]. These modifications includes (1) prenylation of the cysteine by a prenyltransferase, (2) cleavage of the tripeptide AAX motif by Ras converting enzyme 1 (RCE1) and (3) methylation of the C-terminal cysteine by isoprenylcysteine carboxyl methyltransferase (ICMT). Following modifications, the protein binds to the plasma membrane of the cells and causes downstream effector proteins. These modifications cannot occur without covalent attachment of a non-sterol isoprenoid (either farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) to the cysteine residue of the CAAX motif by prenylation (farnesylation and geranylgeranylation, respectively) [222]. Moreover, FPP is believed to be intermediate products in the mevalonate pathway and can be converted to cholesterol or GGPP. In this context it is important to mention that farnesylation occurs when the CAAX sequence ends in any amino acid other than leucine and is catalysed by farnesyltransferase (FTase), whereas geranylgeranylation occurs when the CAAX sequence ends in leucine and is catalysed by geranylgeranyltransferase I (GGTase I) [215]. Farnesyltransferase inhibitors (FTIs) were originally designed to suppress the oncogenic property of all Ras proteins [223]. In a preclinical *in vivo* study in which FTI have been tested in mice with chemically induced lung tumor, the treatment with FTI inhibited tumor growth significantly [224]. Furthermore, it has been found that administration of an FTI to human cancer cell induced apoptosis through inhibition of PI3/Akt2 cascades [225]. The potential role of farnesyltransferase in regulating pulmonary recruitment and tissue damage in a sepsis model of inflammation has shown that farnesyltransferase regulates CXC chemokine formation in alveolar macrophages. Furthermore, inhibition of farnesyltransferase reduces neutrophil recruitment and attenuated acute lung injury in this study [226]. A recent study indicated that the elevation in protein farnesylation plays a role in derangements in immune function and mortality of septic mice and suggested that prevention of immune dysfunction by administration of FTI-277 improvement in survival of septic mice [227]. Numerous studies demonstrated the role of small GTPas and leukocyte infiltration in severe inflammations. For example, study reported that inhibition of Rho GTPase antagonized T-lymphocyte infiltration in the nervous system [228]. However, inhibition of Rho-kinase decreases tissue injury in numerous disease models, such as, AP, and abdominal sepsis [35,37]. It has been revealed that Rho kinase signaling regulates trypsinogen activation, CXCL2 chemokines, and neutrophil infiltration and tissue

damage in AP [35]. Protein geranylgeranylation modifies small G-proteins, including Rho A-C, Cdc42, and Rac1, which is critical for their function. Role of geranylgeranyltransferase in systemic activation and recruitment of neutrophil into the lung in a murine model of poly microbial sepsis has been studied [229,230]. Inhibition of geranylgeranyltransferase markedly decreased CXCL2 production and neutrophil recruitment in the lung, and attenuates acute lung injury in abdominal sepsis. Furthermore, Rac1 which is a member of the Rac subfamily of the family Rho family of GTPases has been shown to exert protective effects in models of injury in the liver, endotoxemia and AP [231,232,233]. The role of Rac1 signaling in regulating CXC chemokines formation, neutrophil infiltration, and lung injury in abdominal sepsis also has been studied [234]. In this thesis, based on the above, we hypothesized the role farnesyltransferase and geranylgeranyltransferase signaling may play an important role in AP (Paper I and II). The role of trans-farnesylthiosalicyclic acid (FTS), a specific Ras protein inhibitor has been tested. For example, intraperitoneal injection of FTS on Ras activation and development of paraneoplastic liver lesions in rats has been evaluated. It was found that FTS inhibits Ras activation and prevents paraneoplastic liver lesions in rats by inducing an apoptosis transformed hepatocyte through the activation of FAS/FAS ligand system [235]. A recent study, demonstrated that Ras inhibition decreased chemokine-mediated neutrophil migration *in vitro and in vivo* [236]. This study suggested that Ras signaling is a potent regulator of CXC chemokine formation and neutrophil infiltration in the lung and inhibition of Ras activity by FTS protected mice from acute lung injury.

Aims

1- To determine the role of farnesyltransferase in pancreatitis-induced leukocyte recruitment and tissue damage in AP.

2- To investigate the role of geranylgeranyltransferase regulating pathological inflammation and tissue damage in severe AP.

3- To define the impact of Ras signaling in the regulation of neutrophil infiltration and tissue injury in AP.

4- To define the potential role and mechanisms of action of NET formation in severe AP.

Methodology

Animals

All studies have been performed in mice. Male C57BL/6 mice 6-8 weeks (22-26 g) were used in these studies. Mice were allowed to acclimatize to facility environment for a minimum of one week before experimentation. They were either breaded from our own breading facilities or purchased from (Taconic Europa, Ry, Denmark) laboratories. Mice were maintained in an environmentally controlled room on a 12-hour light/dark cycle and fed water was available to animal ad libitum at all times. All experiments were done in accordance with legislation on the protection of animals and were approved by the Regional Ethical Committee at Lund University, Sweden. All experimental procedures were performed in a clean, well-ventilated laboratory space.

Experimental design

The Na-taurocholate infusion into the pancreatic duct causes a reproducible AP, with a pathophysiology similar to human disease, it reflects clinical pancreatitis in forms of etiology, complications and inflammation [106,237]. Here, we have used two different severe models of AP. The duodenum and the attached pancreatic head were exposed through a midline incision. The papilla of Vater was identified, the duodenum was immobilized by two 7-0 prolene sutures and a small puncture was made through the duodenal wall in parallel to the papilla of Vater with a 23G needle. A polyethylene catheter (internal diameter 0.28 mm) connected to a micro-infusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) was introduced one mm into the pancreatic duct. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp to prevent hepatic reflux. Overall 10 µl of either saline or 5% taurocholate (Sigma Chemical Company, St. Louis, Missouri, USA) was infused into the pancreatic duct at a rate of 2 µl/minute. Before suturing the abdominal wall, the bile duct clip was removed and the duodenal puncture closed (7-0 prolene). Animals were sacrificed 24 h after induction of AP and tissues collected for different assays. In separate experiments, AP was induced by i.p. administration of L-arginine (4 g/kg/dose, dissolved in saline), twice at an interval of one hour, as described in detail before [238]. Saline or Treatment was administered i.p. before the first dose of L-arginine. Saline animals served as

negative controls. Blood was sampled from the tail vein and inferior vena cava. Mice were killed, and samples were harvested, 72 h after the first administration of L-arginine.

Systemic leukocyte counts

A small volume of blood was taken from the tail vein and mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leukocytes were defined as monomorphonuclear leukocyte (MNL) and polymorphonuclear leukocyte (PMNL) cells in a Burker chamber.

Blood amylase

Amylase was quantified from blood with a commercially available assay (Reflotron®, Roche Diagnostics GmbH, Mannheim, Germany).

Myeloperoxidase activity

Frozen pancreatic and lung tissue were pre-weighed and homogenized in one ml mixture (4:1) of PBS and aprotinin 10,000 KIE/ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for one min. The homogenate was centrifuged (15339g, 10 minute) and the supernatant was stored at -20°C and the pellet was used for MPO assay as previously described [239]. In brief, the pellet was mixed with one ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 second, put in water bath 60°C for 2 h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units per gram tissue in all studies included in this thesis.

Cytokine and chemokine measurement

Pancreatic levels of CXCL2, IL-6, MMP-9, HMGB1, histone 3 and histone 4 were determined in stored supernatants from homogenized pancreatic tissue or blood plasma by use of double-antibody ELISA kits (R&D Systems Europe, Abingdon, Oxon, UK, Chondrex, Redmond, WA, USA and USCN, Life Science Inc., Burlington, NC, USA) according to manufacturers' instructions. Blood collected from the inferior vena cava was diluted (1:10) in

acid citrate dextrose, centrifuged (15300 g for 10 minutes at 4°C) and stored at -20°C until use.

Histology and scoring systems

Pancreas samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six micrometer sections were stained (haematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system including edema, acinar cell necrosis, hemorrhage and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scales as previously described [240].

Pull down and Western blot assay

Rac1 and Ras activities were determined by a pull-down assay by using Rac1 or Ras activation assay kit (Pierce Biotechnology, Rockford, Ill) as described previously[241] [242]. Briefly, 50 mg pancreas tissue was minced and homogenized in lysis buffer on ice. Samples were centrifuged at 15,000 g for 15 min, and 10 µl supernatant was removed to measure total protein content using the BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA), and the rest of the supernatant was used for the pull-down assay. Supernatants containing equal amount of proteins were then diluted with 2× SDS sample buffer and boiled for 5 min. Proteins were separated using SDS-PAGE (10% gel). After transfer to a nitrocellulose membrane (BioRad, Hercules, CA, USA), blots were blocked with 3% BSA at room temperature for one hour, followed by incubation with an anti-Rac1 or anti Ras antibodies (1:1000) at 4°C overnight. Binding of the antibody was visualized using peroxidase-conjugated anti-mouse antibody (1:100,000; Pierce Biotechnology) at room temperature for one hour and the ECL method (BioRad). Expression of internal control β-actin proteins and total Ras were detected by Western blot on aliquots taken before protein affinity purification. In another study (Tyr705) STAT3 and STAT3 activity were detected in acinar cells. Isolated acinar cells were incubated for three hour in suspensions containing 100 µg/ml of either histone 3 (Roche) or histone 4 (BioNordica). In separate experiments, acinar cells were co-incubated with 200 µl of NETs, DNase I-treated NETs or supernatant from non-stimulated neutrophils incubated with or without DNase I. Following incubation, the cells were centrifuged (56 g, 1 minute), washed twice and suspended in ice-cold lysing buffer (25

mM Tris HCl, pH 7.2, 150 mM NaCl, 5 mM MgCl2, 1% NP-40 and 5% glycerol) containing protease inhibitors (Halt Protease Inhibitor Cocktail - EDTA Free) for 20 minutes before homogenization and centrifugation (16,000 g,15 minutes at 4˚C). Protein concentration of the supernatant was determined by the Pierce BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA). An aliquot of 25 μg of protein was mixed with 3X protein loading buffer and boiled for 5 minutes before loading onto a 10-12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked in Tris-Buffered Saline/Tween 20 buffer containing 5% non-fat dry milk powder. Protein immunoblots were performed using specific antibodies to phosphotyrosine (Tyr705) STAT3 and STAT3 (Cell Signaling Technology, Beverly, MA, USA). The membranes were further incubated with peroxidase conjugated secondary antibodies, and protein bands were visualized using a commercial chemiluminescence detection kit (ECL Plus; Amersham Biosciences, Piscataway, NJ, USA) as described by the manufacturer.

Flow cytometry

Flow cytometry was performed for checking the activation of PMNL for Mac-1 expression on PMNLs. To block Fcg III/II receptors and reduce non-specific labelling, samples were incubated with an anti-CD16/CD32 for 5 min. Then samples were stained with a PE-conjugated anti-Gr-1 (clone RB6-8C5, eBioscience, San Diego, CA, USA) antibody and with a FITC-conjugated anti-Mac-1 (clone M1/70, Integrin αM chain, rat IgG2b) antibody at 4 °C for 30 min. Cells were recovered following centrifugation then analysed with FACS Calibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells. After gating the neutrophil population based on forward and side scatter characteristics, Mac-1 expression was determined on cells positive for Gr-1, which is a neutrophil marker.

Polymerase chain reaction (PCR)

The pancreas was harvested and frozen immediately in liquid nitrogen (Paper II). Total RNA was isolated from the frozen pancreas samples using TRI Reagent, following the manufacturer's technical bulletin (Sigma-Aldrich St. Louis, MO, USA), and the RNA concentrations were measured using a spectrophotometer (Model ND-1000; NanoDrop, Wilmington, DE, USA). Each cDNA was synthesized by reverse transcription from 5 μg total

RNA with the RevertAid First Strand cDNA synthesis kit and random hexamer primers (Fermentas Life Sciences, Burlington, Ontario, Canada), according to the manufacturer's protocol. After reverse transcription, the cDNA was diluted 10 times with Tris-EDTA buffer. Real-time PCR was performed with Brillian II SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and the Mx3000P detection system (Stratagene, La Jolla, CA, USA). The primer sequences of CXCL2 and β-actin were the following: CXCL2 (forward) 5′-GCT TCC TCG GGC ACT CCA GAC-3′, CXCL2 (reverse) 5-TTA GCC TTG CCT TTG TTC AGT AT-3′; β-actin (forward) 5′-ATG TTT GAG ACC TTC AAC ACC-3′, β-actin (reverse) 5′-TCT CCA GGG AGG AAG AGG AT-3′. PCR amplifications were performed in a total volume of 25 µl/well containing 12.5 µl SYBR Green PCR 2× Master Mix, 1 µl 0.10 µM each primer, 0.3751 µl reference dye, and 4 µl cDNA as a template and adjusted up to 25 µl with water. PCR reactions were started with 10 min denaturing of 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 minute and 1 minute of elongation at 72°C). Cycling time values for the specific target genes were related to that of β-actin in the same sample, and the β-actin gene is normalized. Real-time qRT-PCR analysis was then performed using comparative, quantitative software in the Mx3000P instrument.

Visualization and Quantification of Extracellular DNA

NETs are composed of extracellular DNA, neutrophil-derived granule proteins, and histones. The cell impermeable dye, Sytotox Green, was used to visualize extracellular DNA in the pancreas and co-localization of the neutrophil-derived granule protein MPO with DNA in the pancreas was used to visualize NET formation in the inflamed pancreas. Paraffin-embedded samples of the pancreas were sectioned (5 µm) and mounted on glass slides and blocked with 1% donkey serum and 3% bovine serum albumin. Specimens were subsequently incubated with a rabbit anti-MPO primary antibody (ab9535, Abcam, Cambridge, UK) overnight at 4°C in incubation buffer (1% bovine serum albumin, 0.3% triton X-100 and 0.01% sodium azide), followed by an anti-rabbit alexa-fluor 647 secondary antibody (ab150075, Abcam). Specimens were permeabilized by 0.3% triton X-during the time of primary antibody incubation. The permeabilized tissue sections were labeled with Sytox® Green nucleic acid stain just before the confocal microscopy. Confocal microscopy was performed using Meta 510 confocal microscope (Carl Zeiss, Jena, Germany) by a × 63 oil immersion objective (numeric aperture = 1.25). In separate experiments, extracellular DNA was labeled by i.v. injection of 100 µl of Sytox® Green nucleic acid stain (5 µM) was injected i.v. 10 minutes

before harvesting the samples. Pancreatic tissue samples were collected in iced tubes and loaded onto 35 mm µ-dish (Ibidi, GmbH, Martinsried, Germany) with a thin bottom for high end microscopy. Images were taken in bright field within 30 minutes of sample collection. Confocal microscopy was performed using Meta 510 confocal microscopy by a × 63 oil immersion objective (numeric aperture = 1.25). Sytox® Green was excited by 488 nm laser line and corresponding emission wavelength was collected by the filter of 500-530 nm. The pinhole was ~1 airy unit and the scanning frame was 512×512 pixels. Background fluorescence was adjusted by changing contrast to minimize variations in each image. Quantification of extracellular DNA in the pancreas was determined in dark field using ZEN2009 software and the area of fluorescence per high-power field was quantified.

Quantification of circulating cf-DNA

To quantify levels of circulating cf-DNA, blood were collected from the inferior vena cava and diluted (1:10) in acid citrate dextrose. The samples were centrifuged at 15300 g for 10 minutes at 4°C and a Quant-iT™ PicoGreen® dsDNA kit (Invitrogen GmbH, Darmstadt, Germany) was used to quantify cf-DNA according to the manufacturers' instructions. The fluorescence intensity reflected the amount of DNA and was measured at excitation and emission wavelengths of 480 nm and 530 nm, respectively.

Trypsin and chymotrypsin activation in acinar cells

Isolated acinar cells were stimulated with 100 nM caerulein (Sigma-Aldrich), NETs, DNase I-treated NETs, supernatant from non-stimulated neutrophils incubated with DNase I or HEPES-Ringer buffer pH 7.4 for 30 minutes at 37°C. In separate experiments, acinar cells were exposed to NETs co-incubated with PSA (20 µg/ml, Sigma-Aldrich) or 100 µg/ml of purified histone 2A (BioNordica, Stockholm, Sweden), histone 2B (BioNordica), histone 3 (Roche) or histone 4 (BioNordica). Next, the cells were centrifuged (56 g, 1 minute), washed twice and suspended in 250 mM sucrose, 5 mM 3-(N-morpholino) propanesulphonic acid buffer (pH 6.5). The cells were then homogenised with a potter-elvehjem type glass homogenizer. The homogenate was centrifuged (56 g, 5 minutes) and the supernatant was used for assay. Trypsin and chymotrypsin activity were measured fluorometrically as described previously using Boc-Glu-Ala-Arg-MCA and Suc-Ala-Pro-Phe-MCA, respectively, as substrates.4 Supernatant from acinar homogenates was added to a 96-well plate (50

µl/well) and mixed with 75 µl TRIS buffer (50 nM TRIS, 450 nM CaCl2 and 0.1% bovine serum albumin, pH 8.0). Addition of substrate initiated the reaction and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Levels of trypsin and chymotrypsin were determined using a standard curve generated from purified trypsin and chymotrypsin (Sigma-Aldrich) and the result was then normalized to the protein concentration and expressed as relative trypsin units (RTU)/pg and relative chymotrypsin units (RCU)/pg, respectively.

Isolation and preparation of neutrophils

Bone marrow neutrophils were extracted from the femur and tibia of healthy C57BL/6 mice using Ficoll-Paque research grade (Amersham Pharmacia Biotech, Uppsala, Sweden). In order to induce NET formation, $2x10^6$ neutrophils were seeded on a 6-well plate and exposed to either 50 nM PMA (Sigma-Aldrich) or PBS for 3 hours (37ºC). The cells were then carefully washed three times with PBS and reincubated (30 minutes, 37ºC) in a solution containing DNAse I (Pulmozyme, Roche) at a concentration of 20 µg/ml, or PBS. The cells were then resuspended and centrifuged (5 minutes, 50 g). NETs were taken from the supernatant and used for further in vitro experiments involving neutrophil Mac-1 expression, ROS formation, STAT3 phosphorylation and trypsin activation.

Isolation of pancreatic acinar cells

Pancreatic acinar cells were prepared by collagenase digestion as previously described4. Briefly, mice were sacrificed through cervical dislocation and pancreatic tissue was collected after collagenase (1%, Sigma-Aldrich) infusion into the pancreatic duct. In order to achieve maximal exposure to collagenase, the pancreas was cut into pieces and incubated at 37ºC for 15 minutes. The solute was then centrifuged and washed repeatedly in cold HEPES-Ringer buffer, pH 7.4 to stop digestion and remove the collagenase. Next, the acinar cells were suspended in HEPES-Ringer buffer and the solute was passed through a 150 µm cell strainer (Partec, Canterbury, England).

Human samples

The study was approved by the regional ethics committee at Lund University, Sweden (2009/413). Ten patients admitted to Skåne University Hospital (Malmö, Sweden) with AP

were included after oral and written informed consent. Blood samples were drawn at admission and 24 h after admission, placed in plasma separator tubes and centrifuged (2000 g, 25°C, 10 minutes) before plasma was frozen at -80°C. All patient samples were from patients fulfilling criteria for severe AP based on the Atlanta Classification. Blood samples from ten healthy controls were handled as patient samples. Plasma levels of cf-DNA were measured using a flourogenic assay for double stranded DNA (Quant-IT PicoGreen dsDNA kit, Invitrogen GmbH, Darmstadt, Germany). Plasma levels of DNA-histone complexes were quantified by use of a sandwich Elisa based on monoclonal antibodies directed against histones and DNA (Cell Death Detection Elisa plus, Roche Diagnostics). All analyses were performed according to the manufacturer's instructions.

Statistics

Data are presented as mean ± SEM. Statistical evaluations were performed by using non-parametrical tests (Mann-Whitney) and Kruskal-Wallis one-way, analysis of variance on ranks followed by multiple comparisons versus control group. $P < 0.05$ was considered significant and *n* represents the number of animals. SigmaStat® for Windows® version 3.5 software (Systat Software, Chicago, Illinois, USA) was employed.

Results and Discussions

I. Role of farnesyltransferase in regulation of AP.

Ras proteins are guanine nucleotide-binding proteins that play a major role in the regulation of proliferation of normal and transformed cells. In order for Ras to activate its downstream signaling effectors such as Raf-1/mitogen-activated protein kinase pathway and the Rac/Rho pathway it must be localized and bound to the plasma membrane [243]. Ras undergoes several post-translational modifications that facilitate its attachment to the surface of the plasma membrane. Inhibition of Ras farnesylation prevents the protein from binding to the plasma membrane and leads to block the conversion of Ras to its biologically active form and it is considered to be a potential therapeutic target [244]. Protein farnesyltransferase inhibitor (FTI) has been shown to affect the inflammatory response *in vivo* and *in vitro*. For example, pretreatment of FTI results in significant inhibition of NF-kB, TNF-α, IL-6, MCP-1, IL-1β and MIP-1α production in various disease [245,246]. Ras proteins are dependent on farnesylation in order to attach to the plasma membrane and thereby conduct their signaling. We hypothesized that Ras signaling is important in the pathophysiology of severe AP and that inhibition of farnesylation would ameliorate disease severity through decreased activity of Ras. First, results from Ras activation assay confirmed that Ras activity (total GTP-Ras levels) was significantly increased in pancreatic tissue from taurocholate challenged mice compared to saline infuse animals. However, pretreatment with FTI-277 inhibited the pancreatitis induced activation of Ras (Figure R1).

To determine the effect of farnesyltransferase inhibitor on another index of mice pancreatitis, we measured the blood amylase activity at 24 h after taurocholate infusion. Blood amylase levels are widely accepted as an indicator of acinar cell necrosis and subsequent pancreatic tissue injury in pancreatitis in mice. As expected, retrograde infusion of Na-taurocholate into the pancreatic duct resulted in AP and increased levels of blood amylase. In animals were FTI-277 was administrated just prior to taurocholate infusion; blood amylase levels were significantly attenuated. MPO has been proposed as a useful risk marker and diagnostic tool in AP [247]. It is stored in azurophilic granules of neutrophils and macrophages. MPO catalyzes the conversion of chloride and hydrogen peroxide to hypochlorite and is secreted during inflammatory condition [248].

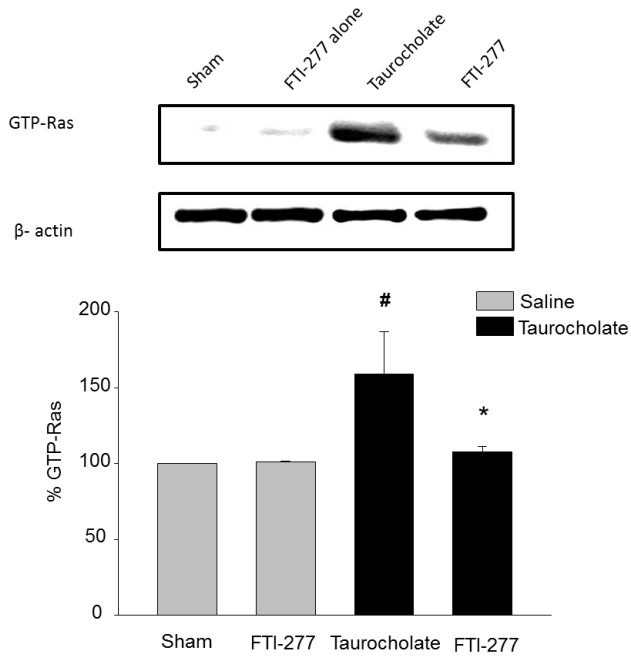

Figure R1. Ras activity in the pancreas. Ras activity was determined in sham (Saline) animals and taurocholate-exposed mice pretreated with saline or the FTI-277. Separate animals received FTI-277 alone. β-actin was used as an internal control for Ras. Data represent means ± SEM and $n = 4$. *$P < 0.05$ *vs*. Sham and #$P < 0.05$ *vs*. Vehicle + Taurocholate.

Next, we tested whether the farnesyltransferase inhibitor affects infiltration of neutrophils in the Na-taurocholate pancreatitis model. As shown in figure R2, 24 h after taurocholate challenge, a significant increase of MPO activities were noted in pancreatic tissue compared to tissue from sham animals. However, pretreatment with FTI-277 decreased the taurocholate induced increase of MPO activity. As part of a systemic inflammatory respond in severe AP, accumulation of activated neutrophils is seen in the lung. Taurocholate challenge provoked a significant lung injury, indicated by the increase in MPO levels in the lung tissue. When FTI-277 was given as pretreatment, a statistically significant decrease in pancreatitis related lung injury was noted (P<0.05). Considered together, these findings suggest that farnesyltransferase signaling regulates both local and systemic organ accumulation of neutrophils in AP. The effect might be subsequent to decrease in Ras activity since several reports suggest that Ras can mediate NF-kB activation [249,250,251]. NF-kB is a key

regulator of inflammatory molecule expression and it is activated early in experimental pancreatitis and correlated with the inflammatory response [252].

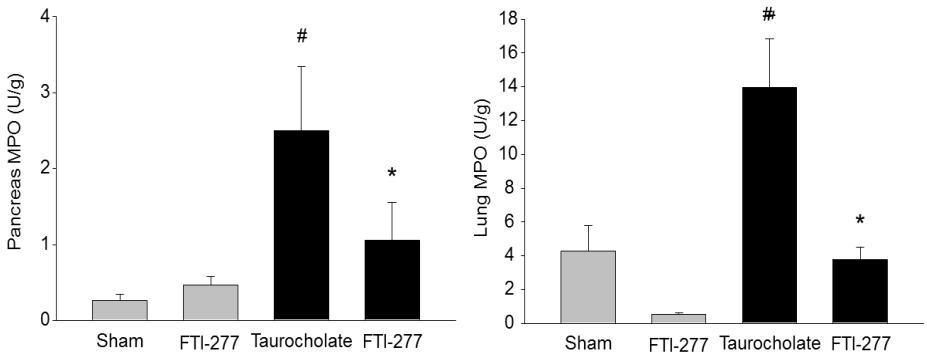

Figure R2. Farnesyltransferase controls taurocholate-induced neutrophil accumulation. MPO levels in the pancreas and lung in sham and taurocholate-exposed mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM and n = 8-10. #$P < 0.05$ *vs.* Sham and *$P < 0.05$ *vs.* Vehicle + Taurocholate.

To evaluate the effect of farnesyltransferase inhibition on taurocholate induced pancreatitis histological changes, pancreas tissue were harvested 24 h after taurocholate challenge. In sham-saline mice the histological features of the pancreas were typical of a normal architecture. The histological examination (at 24 h after infusion of Na-taurocholate) revealed tissue damage characterized by inflammatory cell infiltrates edema, hemorrhage and acinar cell necrosis. However, in taurocholate challenged mice, FTI-277 markedly attenuated the infiltration of inflammatory cells, specifically neutrophils, and improved acinar cell necrosis, edema and pancreas hemorrhage as compared with taurocholate challenged mice pretreated with vehicle (Figure R3).

These data indicate that the farnesyltransferase proteins affect critical steps in the intracellular signaling cascade regulating the process of inflammation in AP.

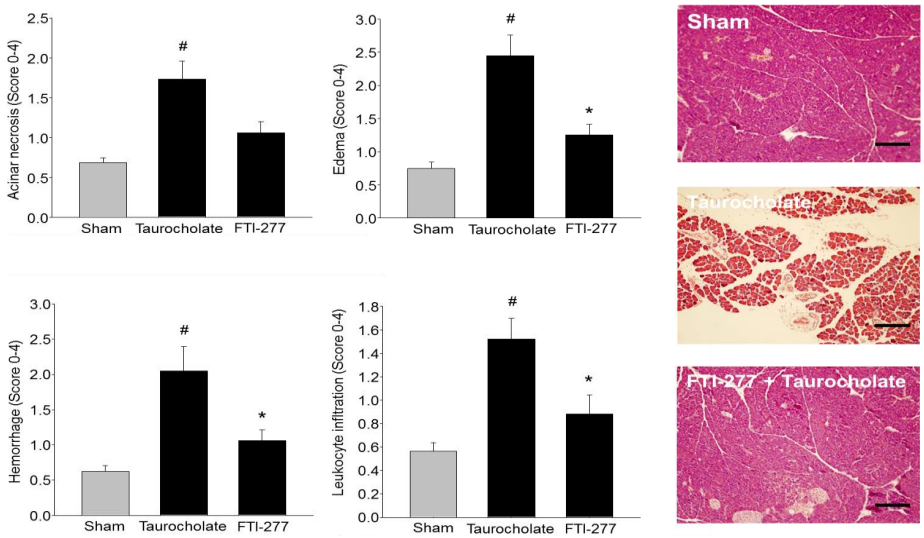

Figure R3. Farnesyltransferase regulates tissue damage in AP. Acinar cell necrosis, edema formation, hemorrhage and extravascular leukocytes in sham and taurocholate-exposed mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg), representative hematoxylin & eosin sections of the pancreas. Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM and n = 8-10. #P < 0.05 *vs.* Sham and *P < 0.05 *vs.* Vehicle + Taurocholate.

Chemokines are believed to play a key role in the pathogenesis of AP and neutrophils chemotaxis is known to be coordinating by macrophage inflammatory protein-2 (MIP-2) [253]. Herein we show that low MIP-2 concentration in the sham pancreas tissue and induction of Na-taurocholate significantly increased the MIP-2 levels concentration in the tissue (P<0.05). It has been explained that chemokines orchestrate neutrophil migration into tissues in two major ways: 1) binding of chemokine ligand to a neutrophil receptor leads to activation of cell surface integrins and allows strong adhesion to endothelium. 2) chemokines promote migration of adherent leukocytes across endothelium and through the extracellular matrix. However, FTI-277 had no significant effect on taurocholate-induced production of MIP-2. In this context, farnesyltransferase may be prerequisite involving in MIP-2 production. [163]. Mac-1 (β2 integrins) expressed on neutrophils and found to regulate and promote neutrophil recruitment. In the present study, we found that taurocholate challenged up regulate Mac-1 expression on neutrophil. Interestingly, administration of FTI-277 markedly reduced surface levels of Mac-1 on neutrophils, suggesting that farnesyltransferase signaling contributed to neutrophil expression of Mac-1 in AP. Moreover, this inhibitory

effect on Mac-1 expression may also help the inhibitory action of FTI-277 on neutrophil accumulation in severe AP observed.

In order to examine whether farnesyltransferase might also regulate tissue damage and neutrophil recruitment in an alternative experimental model, L-arginine was used to trigger AP. In this study, C57BL/6 mice were injected i.p with L-arginine in two dose of 4 g/kg each, 1 h a part. Blood amylase, MPO activity (pancreas and lung) tissues and histopathology were examined. We found that injection of L-arginine was followed by significant increase in blood amylase, pancreatic and lung MPO levels accompanied by marked histological changes. In addition, our results show that treatment of animal with FTI-277 reduces the severity of pancreatitis as evidenced by a significant attenuation of hyperamylesemia, pancreatic and lung MPO activity and histological evidence of diminished pancreatic injury (Paper I, Table 2). Taken together, our novel data shows that inhibition of farnesyltransferase ameliorates tissue damage in severe pancreatitis.

In conclusion, our findings demonstrate that farnesyltransferase signaling regulates tissue damage in severe AP. These results show that inhibition farnesyltransferase attenuates neutrophil expression of Mac-1 and infiltration in the pancreas and the lung, suggesting that farnesyltransferase controls both local and systemic inflammation in pancreatitis. Thus, these data not only delineate a novel signaling mechanism in AP but also indicate that targeting farnesyltransferase might be an effective way to ameliorate the pathological inflammation in severe AP.

However in order to clarify the role of other small GTPase in trypsinogen activation and tissue damage in AP, study II was performed as follow.

II. Role of geranylgeranyltransferase in regulation of AP.

Geranylgeranyltransferase is a prenyltransferase that mediates lipid medication of Rho small GTPase, including Rho, Rac and Cdc42. These proteins are involved in many critical cellular processes, including inflammation, proliferation, and migration [254,255,256]. GTP loading and isoprenylation are two important post-translational modifications of small GTPases, and are critical for their normal function. This study explores the role of geranylgeranyltransferase, using the pharmacological inhibitor GGTI-2133, for regulation tissue damage in severe AP. Here, we found that Na-taurocholate challenge induced high level of Rac1 activation compared with control saline group. This is in line with previous work results indicate that pharmacological Rac1 inhibition ameliorates the severity of

pancreatitis and pancreatitis-associated lung injury [39]. When mice where pretreated with GGTI- 2133 the taurocholate induced Rac1 activation was decreased (Figure R4). Indicating that activation of Rac1 in AP is dependent on geranylgeranyltransferase.

In order to identify the role of geranylgeranyltransferase in tissue injury of AP, pancreatic sections were stained with H&E and graded in a blinded manner using a scale from 0 to 4 for the degree of hemorrhage, neutrophil extravasation, acinar cell necrosis, and edema. Figure R5 shows the histological scoring for all groups of animals and Na-taurocholate challenge caused severe destruction of the pancreatic tissue characterized by edema, hemorrhage and necrosis. However, the structure of the pancreas was protected in GGTI-2133 treated mice challenged with Na-taurocholate. Importantly, all the analyzed parameters were significantly reduced when taurocholate infused animals were pretreated with GGTI-2133.

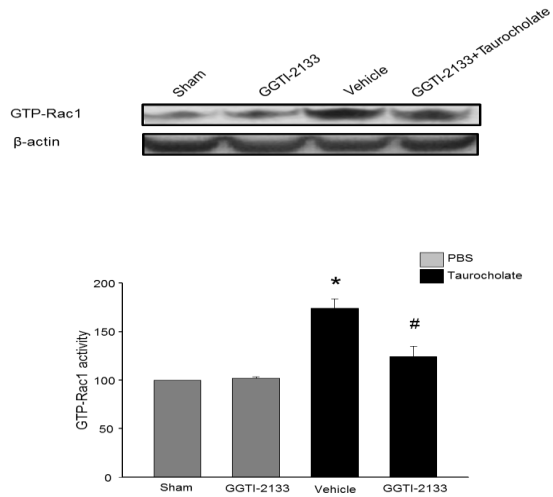

Figure R4. Geranylgeranyltransferase regulates Rac1 activity in the pancreas. Rac1 activity was determined in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). Separate animals received GGTI-2133 (20 mg/kg) alone. β-actin was used as an internal control for total Rac1. Data represent means ± SEM and $n = 5$. *$P < 0.05$ *vs.* Sham and #$P < 0.05$ *vs.* Vehicle + Taurocholate.

Blood levels of amylase were analyzed as an indicator of tissue damage in AP. It was found that Na-taurocholate challenge caused a clear cut increase blood amylase levels. Interestingly, inhibition of geranylgeranyltransferase reduced Na-taurocholate induced levels of amylase by 70% which was in accordance with the morphological change in the pancreas after Na-

taurocholate challenge. This study suggests that geranylgeranyltransferase activity regulates a major part of the tissue damage in severe AP.

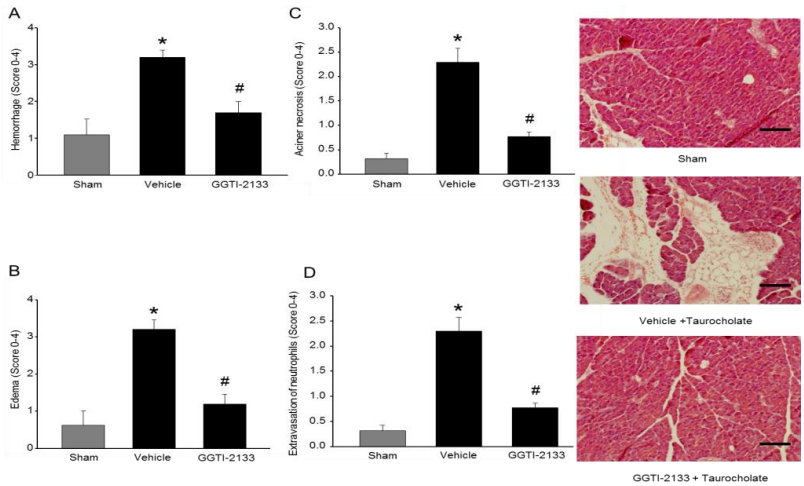

Figure R5. Geranylgeranyltransferase regulates tissue damage in AP. (A) Hemorrhage, (B) edema formation, (C) acinar necrosis, and (D) extravascular neutrophils in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI- 2133 and representative H&E sections of the pancreas. Samples were harvested 24 h after pancreatitis induction. Original bars represent100-m. Data represent means ± SEM, and n=5. *P < 0.05 *vs*. Sham; #P < 0.05 *vs.* Vehicle * Taurocholate.

Next, to study the role of geranylgeranyltransferase in neutrophil recruitment to the pancreas during AP, the pancreatic levels of MPO activities, an indicator of inflammatory cell infiltration, were measured. Our data indicated that taurocholate challenge caused a clear cut increase in the MPO levels in the pancreas. Interestingly, it was found that injection of the GGTI-2133 reduced taurocholate-provoked MPO levels in the pancreas by 50% (Figure R6) which was in line with morphological analysis of the pancreatic tissue. This result indicates that geranylgeranyltransferase regulates significant proportion of neutrophil infiltration in AP. It is known that neutrophil activation and tissue localization are orchestrated by secreted CXC chemokines [257]. CXCL2 is considered to be a major stimulus for neutrophil chemotaxis and has been shown to regulate tissue neutrophils in AP [163]. Plasma levels of CXCL2 increased significantly after taurocholate challenge.

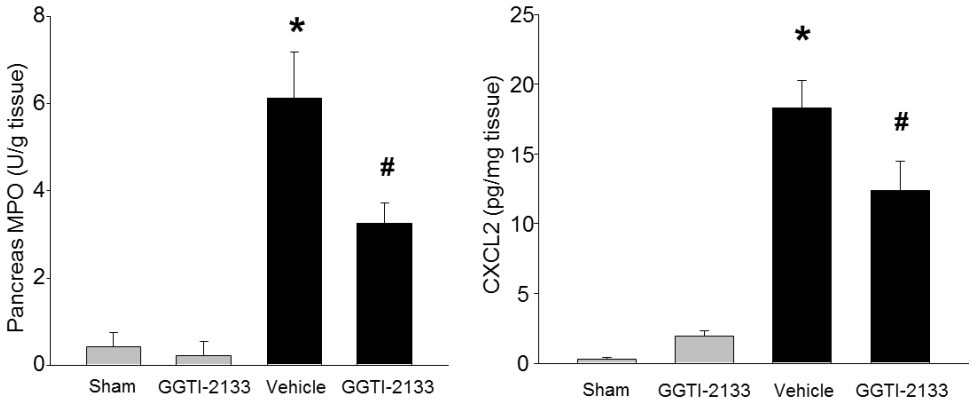

Figure R6. Geranylgeranyltransferase regulates tissue damage in AP. (Geranylgeranyltransferase controls taurocholate-induced inflammation. MPO levels in the pancreas and plasma levels of CXCL2. Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM, and n=5. *P < 0.05 *vs.* Sham; #P < 0.05 vs. Vehicle * Taurocholate.

In this experiment we noted that pretreatment with GGTI-2133 reduced taurocholate induced levels of CXCL2 substantially (Figure R6). Although the details of how adhesion molecules facilitates leukocyte accumulation in the pancreas is relatively unclear, recent studies have shown that Mac-1 is an important adhesion molecule which supports extravascular infiltration of neutrophils [258]. Herein we found that Mac-1 expression on neutrophils increased in response to taurocholate administration. Notably, administration of GGTI-2133 significantly decreased taurocholate-induced neutrophil expression of Mac-1, suggesting that geranylgeranyltransferase regulates neutrophil expression of Mac-1 on neutrophils in AP (Figure 7). Moreover, systemic complications of severe pancreatitis include pulmonary infiltration of inflammatory cells [259]. In the present study, it was found that pulmonary activity of MPO was clearly enhanced in response to taurocholate. Interestingly, GGTI-2133 reduced lung levels of MPO in taurocholate-exposed mice, indicating that geranylgeranyltransferase also controls systemic accumulation of neutrophils in the lung in severe AP. The notion that geranylgeranyltransferase controls systemic inflammation is also supported by our finding that GGTI-2133 markedly reduced the taurocholate-provoked increase in plasma levels of IL-6. IL-6 is an indicator of systemic inflammation, correlates with mortality of septic patients and appears to be a good marker of severity during bacterial infection [260].

In order to reduce the risk that effects of GGTI-2133 might be model-dependent, L-arginine-induced pancreatitis was performed. In a separate experiment, we found GGTI-2133 significantly reduced L-arginine-induced acinar cell necrosis, edema and hemorrhage in the pancreas, as shown in paper II. This data suggest that geranylgeranyltransferase play a key role in AP by affecting neutrophil infiltration in the pancreas.

Bacterial translocation and dissemination are characteristic features in severe pancreatitis. Experimental and clinical studies have greatly increased the knowledge of the pathophysiology of bacteria translocation during AP. However, earlier reports have shown that bacteria overgrowth in the small bowel is associated with bacterial translocation to extra intestinal site, including mesenteric lymph nodes (LN) and the pancreases during experimental AP. However, in our pancreatitis model, we observed that taurocholate challenge markedly increased bacterial counts in the blood and mesenteric LNs. Notably, inhibition of geranylgeranyltransferase greatly reduced bacterial counts in the blood and mesenteric LNs in pancreatitis mice (Figure R8). The mechanisms behind this beneficial effect of GGTI-2133 on bacterial spread are not known at present, although a recent study reported that inhibition of geranylgeranyltransferase improves T cell functions and bacterial clearance in abdominal sepsis [229].

The exact mechanisms of bacterial of bacterial translocation remain to be defined to provide new insights in prevention and treatment of infectious complications during AP.

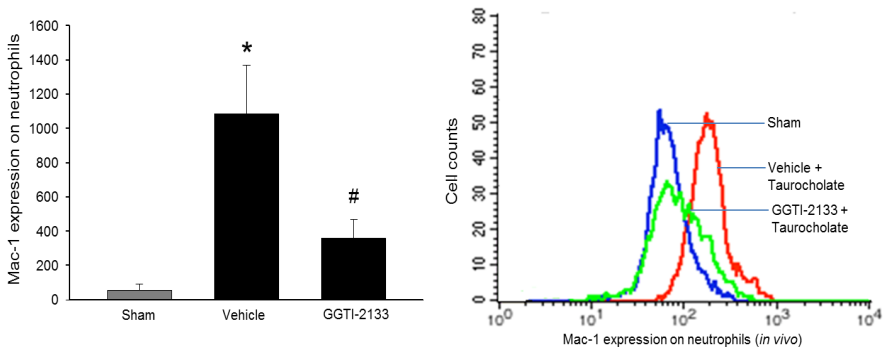

Figure R7. Mac-1 expression on circulating neutrophils (Gr-1+) in sham (PBS) mice or after taurocholate challenge in animals treated with vehicle or GGTI-2133 (20 mg/kg). Representative dot plot and histogram and data in aggregate. Data represents mean ± SEM and $n = 5$. *$P < 0.05$ *vs.* Sham and #$P < 0.05$ *vs.* Vehicle + Taurocholate.

In conclusion, this findings together provides the first evidence that geranylgeranyltransferase inhibitor substantially reduce the severity of AP in mice. Thus, the activation of geranylgeranyltransferase, in turn, results in a neutrophil up-regulation of Mac-1 and increased CXCL2 formation in the pancreas. Moreover, geranylgeranyltransferase inhibition decreased neutrophil infiltration and tissue damage in the pancreas. Finally, blocking geranylgeranyltransferase activity attenuated systemic inflammation and pulmonary neutrophils in animals with pancreatitis. Thus, this study not only delineates a novel signaling mechanism in AP but also suggests that interference with geranylgeranyltransferase might be a useful strategy to ameliorate local and systemic inflammation in severe AP.

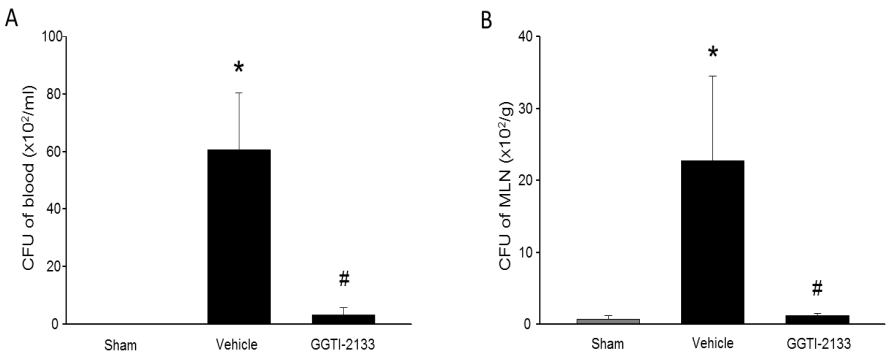

Figure R8. Geranylgeranyltransferase regulates bacterial clearance in AP. The number of bacterial colonies was quantified in the (A) blood and (B) mesenteric lymph nodes in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133. Data represent means ± SEM and $n = 5$. *$P < 0.05$ *vs*. Sham and #$P < 0.05$ *vs*. Vehicle + Taurocholate.

III. The role of Ras in AP

Ras protein is essential for regulation of signal transduction pathways that control cellular proliferation. It has been reported that cell surface receptors activate Ras proteins attached to the inner side of plasma membranes by the conversion of inactive Ras-GDP into active Ras-GTP [261]. Farnesylthiosalicylic acid (FTS) is a unique and potent competitive inhibitor of Ras. Structurally, FTS resembles the carboxy-terminal farnesylcysteine group and it acts as a functional Ras antagonist by interfering with Ras-plasma membrane interactions. Several studies revealed that FTS interacts with Ras anchorage domains leading to dislodgment from the membrane, which in turn, accelerates Ras degradation [262,263,264]. Experimental studies indicated that administration of FTS, which is potent Ras inhibitor, attenuates

systemic inflammation in several diseases [236,265,266,267]. However, the role of the Ras signaling in regulating CXC chemokine formation, neutrophil recruitment and tissue injury in severe acute AP is not known, In the present investigation we aimed to define the functional significance of Ras in controlling CXC chemokine formation, neutrophil Mac-1 expression and recruitment in experimental severe AP.

First, to evaluate the role of Ras signaling in this study on pancreatitis, blood amylase levels were determined. Na-taurocholate challenge enhanced blood amylase levels by 22-fold. Inhibition of Ras signaling decreases amylase levels with 80% indicating that Ras signaling is important in the pathophysiology in severe AP.

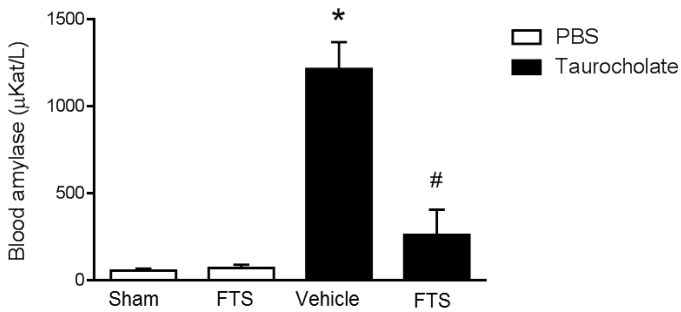

Figure R9. Blood amylase levels (µKat/L) in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the Ras inhibitor FTS (10 mg/kg). Separate animals received FTS (10 mg/kg) alone. Blood samples were obtained 24 h after pancreatitis induction. Data represent means ± S.E.M. and $n = 5$. *$P < 0.05$ *vs.* Sham and #$P < 0.05$ *vs*. Vehicle + Taurocholate.

Leukocyte accumulation represents a hall-mark in the pathophysiology of AP; in this study it is of interest to examine the effect of FTS on pancreas accumulation of neutrophils. Challenge with 5% of Na-taurocholate increased pancreas levels of MPO by more than 4-fold. Inhibition of Ras reduced the activity levels of MPO by 42% in taurocholate challenged animal. Plasma levels of CXCL1 and CXCL2 were low but detectable in sham animals, whereas taurocholate challenged caused a clear-cut increase in the plasma levels of CXCL1 and CXCL2 respectively. Administration of FTS reduced Na-taurocholate-provoked plasma levels of CXCL1 and CXCL2 by more than 80%. This result indicates that Ras signaling exert a regulatory function on the formation of CXC chemokines in AP. This Ras-dependent

generation of CXC chemokines could also help to explain the inhibitory effect of FTS on neutrophil recruitment in AP. Extravascular recruitment of neutrophils at sites of injury is a multistep process coordinated by specific adhesion molecules expressed on neutrophils, including P-selectin and Mac-1. We therefore asked whether Ras might regulate neutrophil activation and expression of Mac-1 in mice challenge with Na-taurocholate infusion into the pancreas. This study shows that Mac-1 expression on neutrophils did not change significantly after Na-taurocholate challenge. One explanation might be that those neutrophils with Mac-1 up-regulation accumulate in the inflamed tissues and that the harvested blood contains neutrophils with less Mac-1 expression.

Apart from local tissue injury, SIRS is the major patho-biological process responsible for the morbidity and mortality of severe AP. High mobility group box 1 (HMGB1) is a potent pro inflammatory cytokine and a late predictor of clinical outcome in severe AP [268]. In line with a previous study, we observe that AP caused a clear cut increase in the plasma levels of HMGB1. Notably, Ras inhibition reduced HMGB1 levels in the plasma by 79% in Na-taurocholate mice. This result indicates that FTS has a potent anti-inflammatory in severe AP. Moreover, challenge with Na-taurocholate enhanced plasma levels of IL-6 by 50-fold, pretreatment with FTS decreased plasma levels of IL-6 by more than 79% in animals exposed to taurocholate. This observation also supports the concept that inhibition of Ras attenuates the systemic inflammatory response in AP.

In conclusion, our novel findings demonstrate that inhibition of Ras improves neutrophil infiltration in severe AP. We found that of Ras signaling regulates blood amylase, cytokine information and causes systemic inflammation in severe AP. Moreover, we confirm that Ras inhibition improves histological changes in the pancreas and it may be a useful approach to protect from systemic inflammation and tissue injury in developing AP.

IV. Role of NET in AP

The aim of this study was to investigate whether NET signaling is activated in AP and weather it plays a role in trypsinogen activation, inflammation and tissue damage in severe AP. Neutrophils are the first line of defense and rapidly recruited in tissue during infection and inflammation [269]. One function of neutrophils, called ‘‘neutrophil extracellular traps’’ (NETs), and this distinctive phenomenon was first reported by Brinkmann in 2004 [199]. This structure is composed of DNA in association with histones, elastase and MPO [270]. In this

study, we have observed that NET signaling plays an important role in severe AP. We notice that AP is associated with increased NET activity and that pharmacological inhibition by DNase I restored the NET activity close to the base line. Visualization of NET was performed by intravascular injection of the cell impermeable dye sytox green. As shown in figure R10 no extracellular DNA was detected in the pancreas from sham mice. In pancreas from two separate animal models of pancreatitis Na-taurocholate induced and administration of L-arginine induce AP, widespread deposition of extracellular DNA was seen. DNase I which effectively catalyzes hydrolysis of extracellular DNA can be used to examine the impact of NET formation. Notably, administration of DNase I abolish taurocholate induced deposition of extracelluar DNA in the pancreas. Moreover depletion of neutrophils by use of Ly-6G ab, markedly reduced taurocholate induced pancreatitis deposition of extracellular DNA in the pancreas. To further confirm that the extracellular DNA was part of NETs formation in pancreas, MPO staining was added. Using confocal microscopy, we could confirm that induction of severe AP causes wide-spread DNA deposition in the inflamed pancreas, which co-localizes with MPO. This phenotype was abolished by depletion of neutrophils, indicating that NETs are the likely source of the extracellular DNA in the inflamed pancreas (Figure R10). We could confirm that taurocholate as well as L-arginine challenged in mice had signs of AP with elevated levels of amylase in blood, MPO in pancreas and lung and CXCL2 in blood plasma. The disturbed pancreatic microarchitectures were normalized in mice treated with DNase I injection before taurocholate challenge. Moreover, it was found that taurocholate challenge increased levels of cf-DNA/NET in plasma. Our result shows that cf-DNA/NET levels correlated with extent of pancreatic necrosis. In clinical samples cf-DNA levels correlated to severity of AP, indicating that it may be an early marker of severity. However, DNase I treatment after taurocholate challenge completely abolished cf-DNA/NET in plasma. This data indicate that degradation of NET by DNase I is possible *in vivo.*

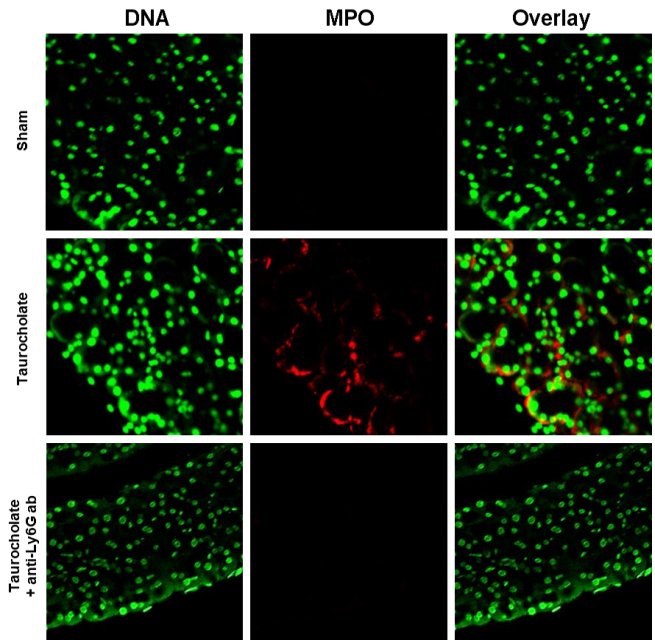

Figure R10. NET formation in AP. Pancreatic tissue was stained with Sytox® Green and an antibody against the neutrophil-derived granule protein MPO in the inflamed pancreas. Samples were collected 24 hours after induction of pancreatitis. Data represent means ± SEM and n = 4-7. #$P < 0.05$ *vs* control mice and ¤$P < 0.05$ *vs* Taurocholate without DNase I or anti-Ly6G.

Next, we wanted to elucidate the relevance of histones as the major protein components of NET structures. Herein we found that histone 3 and histone 4 levels are clear cut increased in pancreas after taurocholate challenge. Notably depletion of neutrophils as well as DNase I markedly decreased levels of histones in the pancreas and cf-DNA/NET in the plasma of mice exposed to taurocholate. This result indicates that neutrophils are the likely source of extracellular DNA in AP (Figure R11). In this context it is interesting to note that a recent study reported that NET-derived histones can directly cause epithelial and endothelial cell damage and death [271]. In light of this observation, our finding that NET degradation markedly decreased pancreatic levels of histone 3 and histone 4 might help to explain part of the beneficial effect of inhibiting generation of NETs in AP.

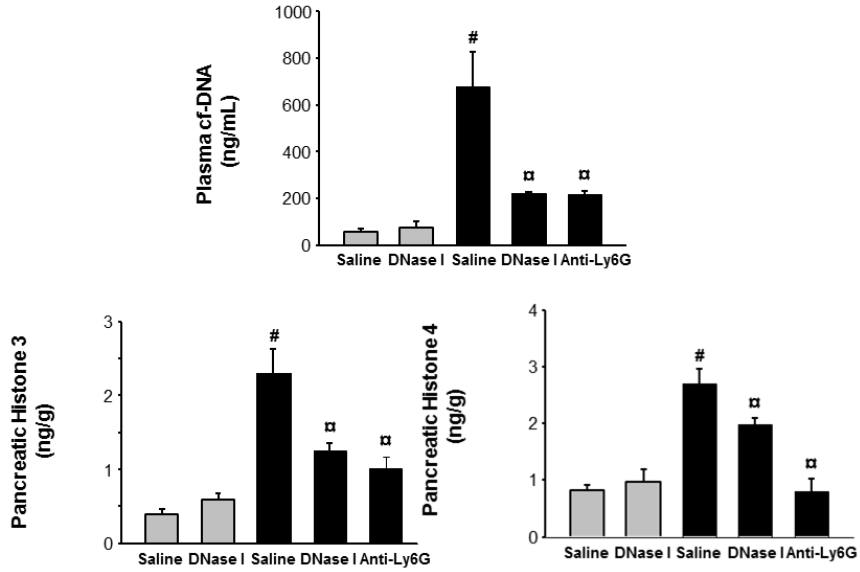

Figure R11. Quantification of extracellular DNA and Pancreatic levels of histone 3 and histone 4 in the pancreas by measuring the relative area of fluorescence per high-power field. Plasma levels of cf-DNA were determined as described in Materials and Methods. Pancreatitis (black bars) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.p. injections of the DNAse I, an antibody directed against Ly6G (anti-Ly6G) or vehicle (saline). Samples were collected 24 hours after induction of pancreatitis. Data represent means ± SEM and n = 4-7. #P < 0.05 *vs* control mice and ¤P < 0.05 *vs* taurocholate without DNAse I or anti-Ly6G.

As we mentioned previously, neutrophil infiltration is a key component in AP and neutrophil depletion or other inhibition of neutrophil recruitment and protected against tissue injury in pancreatitis [132]. Inhibition of NET significantly reduced taurocholate induced MPO levels in pancreas and plasma CXCL2 levels. This may help explain the attenuated accumulation of neutrophils in the inflamed pancreas. In addition, DNase I decreased taurocholate provoked Mac-1 expression on neutrophils and indicated that NETs play a role in expression of Mac-1 up-regulation on neutrophils. In separate experiment, we wanted to examine if NETs are able to activate neutrophils in a direct manner. Neutrophil-derived NETs were co-incubated with neutrophils and we observed that NETs not only increased expression of Mac-1 but also enhanced ROS production in isolated neutrophils. Moreover, co-incubation with DNAse I attenuated NET-provoked expression of Mac-1 and ROS formation in neutrophils. These findings also indicate that NETs can directly activate neutrophils. Considered together with the findings above on CXCL2 formation, it could be suggested that NETs regulate neutrophil

infiltration at two distinct levels, 1) indirectly via formation of CXCL2 in the pancreas and 2) directly via up-regulation of Mac-1 expression on neutrophils (Figure R12).

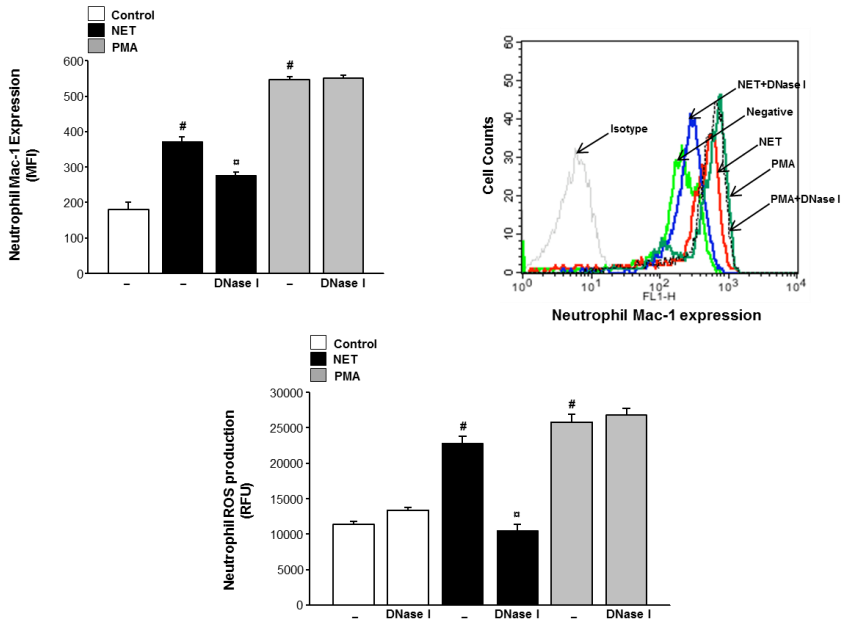

Figure R12. Mac-1 expression and ROS production in isolated neutrophils. Aggregate data and representative histogram of Mac1-expression on neutrophils. Quantification of ROS production in neutrophils by use of fluorescence microplate reader. Murine neutrophils were isolated from bone marrow and incubated with neutrophil-derived NETs (black bars) or PMA (grey bars). Unstimulated cells (white bars) served as controls. Neutrophils were co-incubated with DNAse I as indicated. Data represent means ± SEM and n = 5. #P < 0.05 versus control and ¤P < 0.05 versus NET without DNAse I.

Systemic complications of severe pancreatitis include pulmonary infiltration of inflammatory cells and pulmonary tissue damage. We observed that inhibition of NET formation decreased pulmonary tissue injury and recruitment of neutrophils in AP. We therefore examined plasma levels of IL-6 in pancreatitis animals and found that taurocholate challenge markedly increased the circulating levels of IL-6. However, levels of IL-6 significant decreased in animal treated with DNase I. In patients and animals with AP, serum levels of HMGB1 are significantly increased and positively correlate with the severity of the disease [272,273,274]. Inhibiting the release or cytokine activity of HMGB1 confers protection against experimental

AP [275]. Herein, our results show that taurocholate exposure significant increased HMGB1 levels compared with sham mice group. However, administration of DNase I reduced plasma levels of HMGB1. This data suggests that NET signaling controls both local and distant accumulation of neutrophils in AP. Furthermore, we also observed that pretreatment with DNase I inhibited pancreatitis induced neutrophil into the broncho-alveolar (BALF) compartment, suggesting that NET may regulate a substantial part of the tissue injury in AP. It is widely held that trypsinogen activation is a critical feature in the pathophysiology of AP [276,277]. Thus, we asked whether NETs might be involved in the activation of trypsin in acinar cells. Acinar cell were isolated and stimulated with 10 nM of caerulein, PMA derived NET, histone or NETs. We found that co-incubation with neutrophil-derived NETs enhanced trypsin activation in acinar cells to levels similar to caerulein, a well-known trypsin secretagogoue. Interestingly, co-incubation with DNase I abolished NET-induced activation of trypsin in acinar cells. Aiming to investigate more specifically how NETs induce trypsin activation histones were used in cellular experiments. Histones were found to activate trypsin and chymotrypsin in isolated acinar cells in a way similar to cerulein.

The signal transducer and activator of transcription 3 (STAT3) is a transcription factor that controls expressions of several genes involved in cell survival, proliferation and differentiation, and tissue inflammation. It has been shown that STAT3 knock out mice protected against caerulein-induced pancreatitis [278]. The importance of STAT3 in acinar cells has been tested [151]. Herein we investigate if induce STAT3 phosphorylation in acinar cells by use of western blot analysis. As shown in Figure R14 NETs, as well as histone 3 and 4, are potent stimulators of STAT3 phosphorylation. Co-incubation with DNase I decreased NET-induced STAT 3 phosphorylation. Altogether the results indicate that NET is a potent inducer of STAT3 activity, which is in line with the findings on trypsin activation.

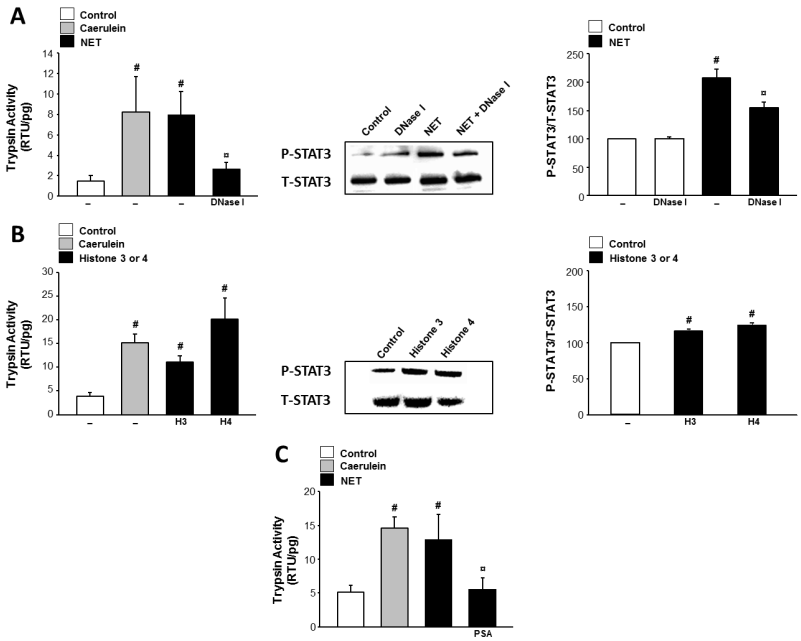

Figure R13. Trypsin activation and STAT3 phosphorylation. Total and phosphorylated STAT3 were determined by western blot and the ratio of phosphorylated STAT3 divided by total STAT3 was quantified. A) Acinar cells were co-incubated with caerulein (grey bars) or neutrophil-derived NETs (black bars) with or without DNAse I. STAT3 phosphorylation was analyzed after incubation with neutrophil derived NETs (black bars) with or without DNase I or saline (white bars) with or without DNase I. B) Acinar cells were co-incubated with caerulein (grey bars) or histone 3 and histone 4 (black bars). STAT3 phosphorylation was analyzed after incubation with histone 3 or histone 4 (black bars) or saline (white bars). C) Acinar cells were co-incubated with caerulein (grey bars) or neutrophil-derived NETs (black bars) with or without PSA. Data represent means ± SEM and $n = 5$. #$P < 0.05$ control cells and ¤$P < 0.05$ *vs* NET alone.

Polysialic acid (PSA) a long linear polymer composed entirely of negatively charged sialic acid (5′-N-acetylneuraminic acid) belongs to a class of functionally important anionic glycans and direct binds to histones [279]. It has been demonstrated to reduce both histone and NET mediated cytotoxicity [271]. In this study, we noticed that co-incubation with PSA abolish NET-provoked trypsinogen activation. In order to investigate the toxicity of histones in the pancreas, acinar cells were isolated and co-incubated with histones for 60 minutes, before the viability of acinar cells were assessed by trypan blue exclusion test. We found the viability of acinar cells to be reduced by 35% (Figure R13).

In conclusion, this study demonstrates for the first time that NETs are not only generated but also play a critical role in the development of severe AP. Inhibition of NET decreased CXCL2 formation and neutrophil recruitment in the inflamed pancreas. In addition, we show that

NETs regulate STAT3 activity and trypsin activation in acinar cells and histones might be important molecular mediators in these processes. Together, these findings identify a novel role of NETs in pancreatitis and suggest that targeting NETs might be a useful way to ameliorate local and systemic inflammation in severe AP.

Conclusions

1- Farnesyltransferase is a potent regulator of neutrophil infiltration in AP. Inhibition of farnesyltransferase attenuates pancreatitis associated neutrophil recruitment and tissue injury in AP.

2- Geranylgeranyltransferase is an important regulator of CXC chemokine production and neutrophil recruitment in AP. Inhibition of geranylgeranyltransferase attenuates systemic inflammation and the pancreas and lungs were protected from organ damage.

3- Ras signaling controls CXC chemokine formation, neutrophil recruitment and tissue injury in severe AP. Inhibition of Ras signaling attenuates local and systemic inflammation in severe AP.

4- Formation of NET plays a critical role in the development of severe AP. Inhibition of NET decreased CXCL2 formation and neutrophil recruitment in the inflamed pancreas. In addition, NETs regulate STAT3 activity and trypsin activation in acinar cells.

Populärvetenskaplig sammanfattning på svenska

Akut inflammation i bukspottskörteln, akut pankreatit är en vanlig sjukdom. Flertalet patienter som drabbas får en mild inflammation som ofta läker ut av sig själv. En del drabbas dock av komplikationer, en så kallad svår akut pankreatit. Vid denna form av pankreatit finns en betydande risk att dö och många patienter behöver vårdas länge på sjukhus. Det finns ingen specifik behandling av sjukdomen, delvis för att sjukdomsmekanismerna är oklara. Akut pankreatit kännetecknas av en aktivering av såväl bukspottskörtelenzymer, framför allt proteaser, som immunsystemet. Aktiverade proteaser kan orsaka nedbrytning av proteiner, proteolys och aktivering av immunsystemet leder till att vita blodkroppar, leukocyter, aktiveras. Vid akut pankreatit infiltrerar en typ av vita blodkroppar, neutrofila leukocyter, bukspottskörtelns vävnad. Vid svår pankreatit ses även infiltration av neutrofila leukocyter till lungorna. Dessutom ses vävnadsskada i såväl bukspottskörtel som lungor. För att kunna behandla sjudomen behövs en bättre förståelse av dess mekanismer och vad som styr dem.

En specifik mekanism som visats ha betydelse för sjukdomsförloppet vid andra sjukdomar kallas isoprenylering. Begreppet innebär att ett protein förändras så att det kan fästa vid ett membran, såsom cellväggen, vilket är en förutsättning för att proteinet ska kunna påverka fortsatt signalering i cellen. Isoprenylering styrs av enzymerna farnesyltransferas och geranylgeranyltransferas. En grupp av intracellulära proteiner, så kallade Guanin nucleotid-bindande proteiner, G-proteiner, är viktiga för signalöverföring i många celler. Genom att aktiveras eller inaktiveras reglerar de hur extracellulära stimuli överförs till intracellulära signaler. Två grupper av G-proteiner som är viktiga för inflammation och bildande av inflammatoriska mediatorer är Ras och Rac proteinerna. De är beroende av isoprenylering för att kunna fästa vid cellväggen och styra signalöverföring.

Neutrofila leukocyter är viktiga för sjukdomsföloppet vid akut pankreatit. Man har nyligen visat att neutrofiler vid aktivering kan ge upphov till extracellulära nätliknande strukturer (nuclerar extracellular traps, NETs). Dessa är uppbyggda av DNA och proteiner från aktiverade neutrofiler och deras betydelse för immunförsvaret är relativt outforskad.

Syftet med denna avhandling var att undersöka betydelsen av isoprenylering och NETs för neutrofilinfiltration och vävnadsskada vid pankreatit.

Dessa inflammatoriska mekanismer har studerats i musmodeller och cellmodeller. För att orsaka en svår akut pankreatit liknande den som ses vid svår gallstensutlöst pankreatit på människa infunderas gallsyran taurocholat i pankreasgången på möss. Försöken upprepas i en

alternativ pankreatitmodell där aminosyran L-arginin injiceras i bukhålan 2 gånger med en timmes mellanrum. Cellförsök görs med isolerade bukspottkörtelceller (acinära celler).

I arbete I hämmas farnesyltransferas genom att mössen förbehandlas med farnesyltransferashämmare (FTI)-277. Det intracellulära G-proteinet Ras undersöks specifikt då dess aktivering tros vara beroende av farnesyltransferas aktivering. Vid taurocholatutlöst pankreatit ses en tydlig Ras aktivering (mätt som RasGTPase) i bukspottkörteln. I möss som förbehandlats med FTI-277 minskar Ras aktivitet samt neutrofilinfiltration och vävnadsskada i bukspottkörteln. De neutrofila leukocyternas aktivering minskar när farnesyltransferas hämmas. Dessutom minskar neutrofilinfiltration i lungvävnaden.

I arbete II studeras effekter av geranylgeranyltransferas genom att mössen förbehandlas med geranylgeranyltransferas hämmare (GGTI)-2133. Utan förbehandling kan vi visa att den intracellulära signalvägen Rac-1 uppregleras vid taurocholatinducerad pankreatit. Förbehandling med GGTI-2133 leder till minskad Rac-1 aktivering, minskad aktivering samt infiltration av neutrofila leukocyter och minskad vävnadsskada i bukspottkörteln. Vidare ses tecken till minskad inflammation generellt i blodet med minskade nivåer av inflammatoriska mediatorer (cytokinen CXCL2) och minskad infiltration av neutrofilaleukocyter till lungorna.

I arbete III studeras effekter av G-proteinet Ras. När detta hämmas minskar den systemiska inflammationen och nivåer av inflammatoriska mediatorer i blodet. Även vävnadsskada i bukspottkörteln minskar när Ras hämmas.

I arbete IV kan vi för första gången visa att NETs bildas i bukspottskörteln vid akut pankreatit. Om de neutrofila leukocyterna slås ut innan pankreatit induceras ses inte NETs vilket stödjer teorin att NETs uppkommer från aktiverade neutrofila leukocyter. Genom att bryta ner NETs med enzymer som DNase kan vi konstatera att de är viktiga för många av sjukdomsprocesserna vid akut pankreatit. Bland annat minskar inflammatoriska mediatorer i blodet och neutrofilinfiltration samt vävnadsskada i bukspottkörteln. I cellulära försök visas att signalvägen STAT-3 aktiveras av NETs och att NETs kan aktivera proteaset trypsin.

Sammantaget har resultaten i denna avhandling visat att man genom att påverka de inflammatoriska signalvägarna kan lindra effekterna av akut pankreatit såväl i bukspottkörteln som i blod och lungor. Flera potentiellt viktiga mekanismer har undersökts och resultaten kan ligga till grund för framtida specifika behandlingar av akut pankreatit.

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Paper I

Farnesyltransferase Regulates Neutrophil Recruitment and Tissue Damage in Acute Pancreatitis

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Objectives: The signaling mechanisms controlling organ damage in the pancreas in severe acute pancreatitis (AP) remain elusive. Herein, we examined the role of farnesyltransferase signaling in AP.

Methods: Pancreatitis was provoked by the infusion of taurocholate into the pancreatic duct in C57BL/6 mice. Animals were treated with a farnesyltransferase inhibitor FTI-277 (25 mg/kg) before pancreatitis induction.

Results: FTI-277 decreased the blood amylase levels, pancreatic neutrophil infiltration, hemorrhage, and edema formation in the pancreas in mice challenged with taurocholate. Farnesyltransferase inhibition reduced the myeloperoxidase levels in the pancreas and lungs in response to taurocholate infusion. However, FTI-277 had no effect on the taurocholate-provoked formation of macrophage inflammatory protein-2 in the pancreas. Interestingly, farnesyltransferase inhibition abolished the neutrophil expression of macrophage-1 antigen in mice with pancreatitis. In addition, FTI-277 decreased the taurocholate-induced activation of the rat sarcoma protein in the pancreas. An important role of farnesyltransferase was confirmed in L-arginine–induced pancreatitis.

Conclusions: These results demonstrate that farnesyltransferase signaling plays a significant role in AP by regulating neutrophil infiltration and tissue injury via the neutrophil expression of macrophage-1 antigen. Thus, our findings not only elucidate novel signaling mechanisms in pancreatitis but also suggest that farnesyltransferase might constitute a target in the management of severe AP.

Key Words: amylase, chemokines, inflammation, leukocytes, pancreas

Abbreviations: AP - acute pancreatitis, IP - intraperitoneal, MIP-2 - macrophage inflammatory protein-2, MPO - myeloperoxidase, MNL - monomorphonuclear leukocytes, PBS - phosphate-buffered saline, PMNL - polymorphonuclear leukocytes

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Acute pancreatitis (AP) presents with a wide range of disease severity ranging from simple and transient pain to local and systemic complications.[1] Because of the limited understanding of the underlying pathophysiology, management of patients with severe AP poses a major challenge to clinicians and is largely limited to supportive therapies. Today, there is no effective method to predict the severity and outcome of AP. The literature suggests that trypsinogen activation, inflammation, and impaired microvascular perfusion are integrated components in the pathophysiology of pancreatitis.[2,3] Knowing that the activation of trypsinogen seems to be an early and transient process, inflammation in the pancreas persists longer and might be a more rational target for treatment.[4] Leukocyte accumulation is a hallmark of inflammation, and several reports have shown that leukocytes play a key role in the development of AP.[5,6] The extravasation process of leukocytes comprises multiple sequential steps mediated by specific adhesion molecules, such as P-selectin,[7,8] macrophage-1 antigen (Mac-1),[9] and LFA-1.[6,9] Tissue navigation of leukocytes is orchestrated by secreted chemokines.[10] CXC chemokines, such as macrophage inflammatory protein-2 (MIP-2), stimulate extravascular recruitment of neutrophils.[11] CXCR2 is the high affinity receptor on murine neutrophils for MIP-2 and KC,[12,13] and it has been shown that CXCR2 is critical in supporting neutrophil infiltration in the pancreas.[14] Although, the role of specific adhesion molecules and chemoattractants in leukocyte infiltration in the pancreas is relatively well known, the understanding of the signaling pathways coordinating proinflammatory actions in AP is limited.

Trauma and infection trigger multiple signaling cascades that converge on specific transcription factors controlling gene expression of proinflammatory substances. This signal transmission is predominately regulated by intracellular kinases phosphorylating downstream targets.[15] For example, small G proteins of the rat sarcoma (Ras) homologous (Rho) family and one of their effectors, Rho-kinase, are known to act as molecular switches regulating several functions, including cytoskeleton organization, vesicular trafficking, and cell migration.[16] Indeed, inhibition of Rho-kinase has been reported to attenuate pathologic inflammation in several disease models, including pancreatitis[17] and sepsis.[18] The Rho proteins must undergo posttranslational modifications, such as isoprenylation, to effectively localize at cell membranes and activate downstream effectors like Rho-kinase. Isoprenylation of Rho proteins is catalyzed by 2 enzymes, farnesyltransferase and geranylgeranyltransferase.[19] Interestingly, a previous study reported that the inhibition of farnesyltransferase with a specific inhibitor (FTI-277) antagonized the T-lymphocyte infiltration in the nervous system.[20] However, the potential role of farnesyltransferase in regulating neutrophil recruitment and tissue damage in the pancreas remains elusive.

Based on these considerations, we hypothesized that farnesyltransferase signaling might play a role in severe AP. We used 2 experimental models of severe AP in mice and interfered with farnesyltransferase activity by administration of FTI-277.

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The authors declare no conflict of interest.

EXPERIMENTAL PROCEDURES

Animals

All experiments were conducted using C57BL/6 male mice, weighting 20 to 25 g (6–8 weeks) purchased from Taconic (Denmark). The animals were maintained under a 12-hour light/dark cycle in a climate at 22°C and fed with water and standard food ad libitum. The study was approved by the Regional Ethical Committee for Animal Experimentation at Lund University, Sweden. Mice were anesthetized intraperitoneally (IP) with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kilogram body weight.

TAUROCHOLATE-INDUCED PANCREATITIS

Anaesthetized mice underwent midline laparotomy, and the second part of the duodenum and papilla of Vater were identified. Traction sutures were placed 1 cm from the papilla, and a small puncture was made through the duodenal wall with a 23-G needle in parallel to the papilla of Vater. A nonradiopaque polyethylene catheter connected to a micro infusion pump (CMA/100; Carnegie Medicine, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and 1 mm into the common bile duct. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp. Infusions of 10 μL of 5% sodium taurocholate (Sigma, St Louis, Mo) for 10 minutes were retrogradly infused into the pancreatic duct. After completion, the catheter was withdrawn, and the common hepatic duct clamp was removed. The duodenal puncture was closed with a purse-string suture. The traction sutures were removed, and the abdomen was closed in 2 layers. Animals were allowed to wake up and were given free access to food and water. FTI-277 (25 mg/kg, Sigma) or vehicle (phosphate-buffered saline [PBS]) was administered IP before bile duct cannulation. This dose and scheme of administration of the FTI-277 inhibitor were based on a previous investigation.[21] Animals exposed to taurocholate were pretreated with vehicle (n = 10) or FTI-277 (n = 8). One separate group of mice received 25 mg/kg of FTI-277 alone (n = 5). Sham mice undergoing laparotomy and sodium chloride infusion into the pancreatic duct were pretreated with vehicle (sham, n = 8). In separate experiments, mice that were exposed to taurocholate were treated with vehicle (n = 5) or FTI-277 (n = 5) 2 hours after the induction of pancreatitis. All animals were killed 24 hours after pancreatitis induction and assessed for all parameters included in this study.

L-Arginine–Induced Pancreatitis

In separate experiments (n = 8–10), AP was induced by the administration of L-arginine (4 g/kg per dose) IP twice at an interval of 1 hour as described in detail previously.[22] The vehicle and FTI-277 (25 mg/kg) were given IP before the first dose of L-arginine. Saline animals treated as negative controls.

RAS ACTIVITY

Active Ras was determined by a pull-down assay using Ras activation assay kit (Pierce Biotechnology, Rockford, Ill) according to a previously described method.[23] Briefly, 50 mg of pancreas tissue were minced and homogenized in lysis buffer on ice. Lysates were centrifuged at 15,000 g for 15 minutes at 4°C. From each supernatant, 10 μL were removed to measure protein content using Pierce BCA Protein Assay Reagent (Pierce Biotechnology), 20 μL were removed to determine β-actin in total lysate, and the rest of the volume was used for the pull-down assay. Lysates containing equal amount of proteins were then mixed with 80 μg of GST-Raf1-RBD. Samples were diluted in Laemmli sample buffer and boiled for 5 minutes. The proteins were separated using SDS-PAGE (10% gel). After the transfer to nitrocellulose membranes (Bio-Rad, Hercules, Calif), blots were blocked with 3% bovine serum albumin for 1 hour at room temperature, followed by incubation with Ras antibody (1:1000, 4°C) overnight. Binding of the antibody was visualized using horseradish peroxidase-coupled antimouse antibody (1:100000, 1 hour, room temperature) and enhanced chemiluminescence method (Amersham, Arlington Heights, Ill). The data represent 4 independent experiments. Quantification was performed by densitometry using the AlphaEaseFC Image software. The values obtained from the sham animals were set as 100.

Amylase Measurements

Blood amylase levels were determined in the blood collected from the tail vein by use of a commercially available assay (Reflotron; Roche Diagnostics GmbH, Mannheim, Germany).

Systemic Leukocyte Counts

Blood was collected from the tail vein for systemic leukocyte differential counts. The blood was mixed with Turks solution (0.2 mg gentian violet in 1 mL glacial acetic acid, 6.25% vol/vol) in a 1:20 dilution. Leucocytes were identified as monomorphonuclear and polymorphonuclear (PMNL) cells in a Bürker chamber.

Myeloperoxidase Activity

A piece of the pancreatic head and lung tissue were harvested for myeloperoxidase (MPO) measurements. All frozen pancreatic and lung tissues were preweighed and homogenized in a 1-mL mixture (4:1) of PBS and aprotinin 10,000 Kallikrein-inhibitor-unit per mL (Trasylol; Bayer HealthCare AG, Leverkusen, Germany) for 1 minute. The homogenate samples were centrifuged (15,339*g*, 10 minutes), and the supernatant was stored at −20°C, and the pellet was used for MPO assay as previously described.[24] All pellets were mixed with 1 mL of 0.5% hexadecyltrimethylammonium bromide. Next, the samples were frozen for 24 hours and then thawed, sonicated for 90 seconds, and put in a water bath with a temperature of 60°C for 2 hours, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units per gram tissue.

Tissue Histology

Pancreas samples from head were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six-millimeter sections were stained (hematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a preexisting scoring system including edema, acinar cell necrosis, hemorrhage, and neutrophil infiltrate on a scale of 0 (absent) to 4 (extensive) as previously described in detail.[25]

MIP-2 Levels

Macrophage inflammatory protein-2 levels in the pancreas were determined in stored supernatants from homogenized pancreatic tissues. Macrophage inflammatory protein-2 levels were assessed using double-antibody Quantikine enzyme–linked immunosorbent assay kits (R&D Systems Europe, Abingdon, United Kingdom) using recombinant murine MIP-2 as standard. The minimal detectable protein concentration is less than 0.5 pg/mL.

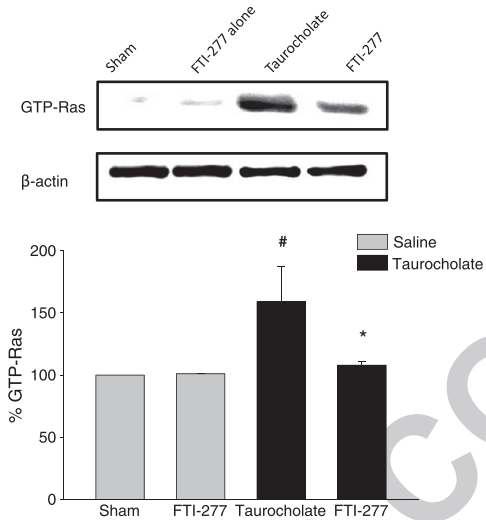

FIGURE 1. Ras activity in the pancreas. Ras activity was determined in sham (saline) animals and taurocholate-exposed mice pretreated with saline or the FTI-277 (25 mg/kg). Separate animals received FTI-277 (25 mg/kg) alone. β-Actin was used as an internal control. Data represent mean ± SEM and n = 4 ($^{\#}P < 0.05$ vs sham and $^{*}P < 0.05$ vs vehicle + taurocholate).

Flow Cytometry Assay

For analysis of surface expression of Mac-1 on circulating neutrophils, blood was collected from the inferior vena cava (1:10 acid citrate dextrose) 24 hours after taurocholate induction and incubated (10 minutes at room temperature) with an anti-CD16/CD32 antibody, blocking Fcγ III/II receptors to reduce nonspecific labeling and then incubated with phycoerythrin-conjugated anti–Gr-1 (clone RB6-8C5, rat IgG2b, eBioscience, Frankfurt, Germany) and fluorescein isothiocyanate–conjugated anti–Mac-1 (clone M1/70, integrin αM china, rat IgG2b κ, BD Biosciences Pharmingen, San Jose, Calif) antibodies. Cells were fixed, erythrocytes were lysed, and neutrophils were recovered after centrifugation. Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif) and analyzed with Cell-Quest Pro software (BD Bioscience). A viable gate was used to exclude dead and fragmented cells.

Trypsinogen Activation in Isolated Acinar Cells

Pancreatic acinar cells were prepared by collagenase digestion and gentle shearing as described previously.[26] Cells were suspended in HEPES-Ringer buffer (pH 7.4) saturated with

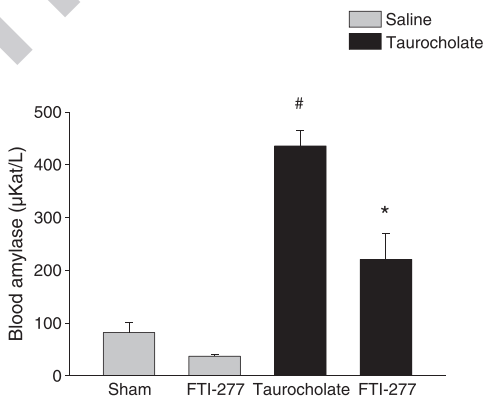

FIGURE 2. Blood amylase (μKat/L) in sham and taurocholate-exposed mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). One group of mice received 25 mg/kg of FTI-277 alone. Blood samples were obtained 24 hours after pancreatitis induction. Data represent mean ± SEM and n = 8–10 ($^{\#}P < 0.05$ vs sham and $^{*}P < 0.05$ vs vehicle + taurocholate).

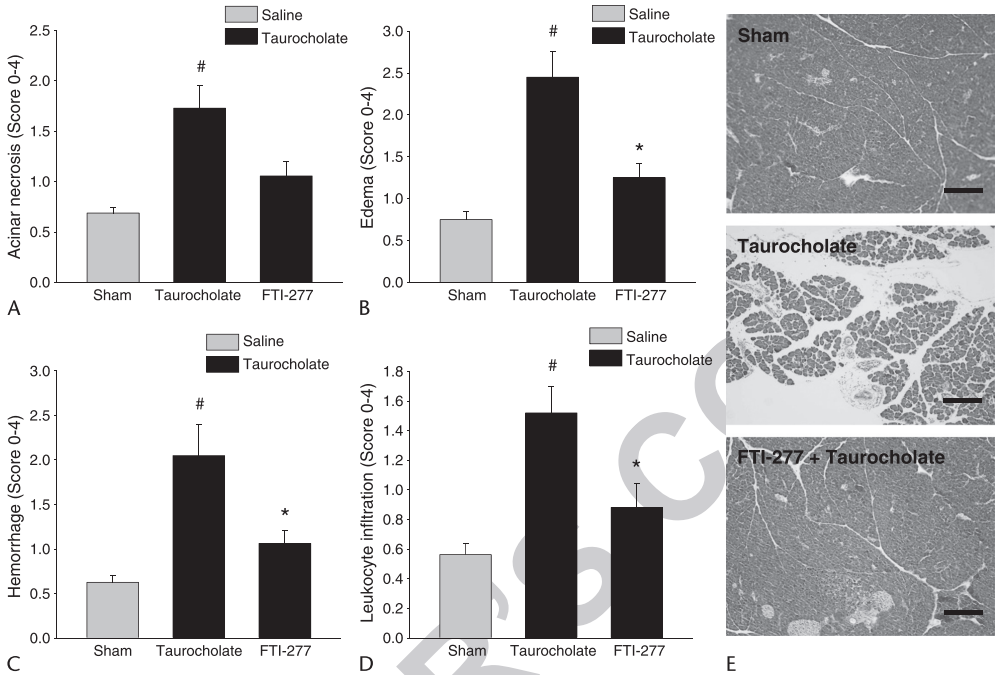

FIGURE 3. Farnesyltransferase regulates tissue damage in AP. A, Acinar cell necrosis, (B) edema formation, (C) hemorrhage, and (D) extravascular leukocytes in sham and taurocholate-exposed mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). E, Representative hematoxylin-eosin sections of the pancreas. Samples were harvested 24 hours after pancreatitis induction. Bars represent 100 μm. Data represent mean ± SEM and n = 8–10 (#$P < 0.05$ vs sham and *$P < 0.05$ vs vehicle + taurocholate).

oxygen and passed through a 150-μm cell strainer (Partec, England). Isolated acinar cells (1×10^7 cells per well) were preincubated with vehicle or FTI-277 (200 μM, 30 minutes) and stimulated with 100 nM cerulein (37°C, 30 minutes) in duplicate. The buffer was then discarded, and the cells were washed twice with a buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3-(morpholino) propanesulphonic acid, and 1 mM $MgSO_4$. The cells were next homogenized in a cold (4°C) 3-(morpholino) propanesulphonic acid buffer using a potter Elvejham-type glass homogenizer. The resulting homogenate was centrifuged ($56 \times g$, 5 minutes), and the supernatant was used for assay. Trypsin activity was measured flourometrically using BoC-Glu-Ala-Arg-MCA as substrate as described previously.[27] For this purpose, a 200-μL aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, and 0.1% bovine serum albumin, pH 8.0). The reaction was initiated by the addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin levels (pg/mL) were calculated using a standard curve generated by assaying purified trypsin. The viability of the pancreatic acinar cells was higher than 95% as determined by the trypan blue dye exclusion.

Statistics

Data are presented as mean values ± SEM. Statistical evaluations were performed by using nonparametrical tests (Mann-Whitney *U* test). $P < 0.05$ was considered significant, and n represents the number of animals.

RESULTS

Farnesyltransferase Regulates Ras Activation in the Pancreas

To investigate the activation of Ras in the pancreas by taurocholate and the effect of FTI-277 on the inhibition of Ras activity, pancreatic tissue from sham and pancreatitis mice

TABLE 1. Systemic Leukocyte Differential Counts

	PMNL	MNL	Total
Sham	0.9 ± 0.1	4.3 ± 0.5	5.2 ± 0.6
Vehicle + taurocholate	1.8 ± 0.2*	6.6 ± 0.3	8.4 ± 0.5
FTI-277 + taurocholate	1.1 ± 0.2†	5.9 ± 0.2	6.2 ± 0.4

Blood was collected from sham mice and taurocholate-treated animals pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). Cells were identified as monomorphonuclear leukocytes (MNL) and PMNL. Data represent mean ± SEM, 10^6 cells/mL, and n = 8–10.

*$P < 0.05$ versus sham.

†$P < 0.05$ versus vehicle + taurocholate.

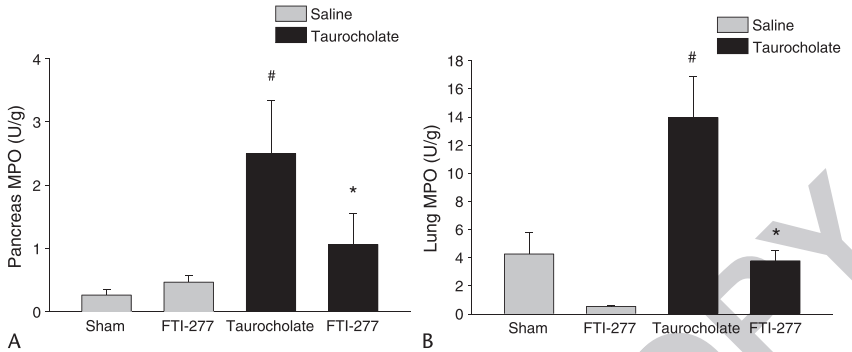

FIGURE 4. Farnesyltransferase controls taurocholate-induced neutrophil accumulation. MPO levels in the (A) pancreas and (B) lung in sham and taurocholate-exposed mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). One group of mice received 25 mg/kg of FTI-277 alone. Samples were harvested 24 hours after pancreatitis induction. Data represent mean ± SEM and n = 8–10 ($^{\#}P < 0.05$ vs sham and $^{*}P < 0.05$ vs vehicle + taurocholate).

were harvested for Ras activation assay. It was found that taurocholate increased the active form (Guanosine-5'-triphosphate binding form) of Ras (Fig. 1). The administration of FTI-277 decreased the taurocholate-evoked activation of Ras in the pancreas (Fig. 1).

Farnesyltransferase Controls Tissue Damage in Pancreatitis

To examine the role of farnesyltransferase in severe AP, serum amylase levels were first examined as an indicator of tissue damage. We observed that retrograde infusion of taurocholate in the pancreatic duct increased the blood amylase levels by 5-fold (Fig. 2). Treatment with the farnesyltransferase inhibitor FTI-277 decreased the taurocholate-induced blood amylase levels from 411.2 ± 29.3 μKat/L to 220.4 ± 18.3 μKat/L, corresponding to a 46% reduction (Fig. 2). Administration of FTI-277 alone had no effect on taurocholate-induced amylase levels in the blood (Fig. 2). Examination of tissue morphology revealed that sham mice had normal pancreatic microarchitecture (Fig. 3), whereas taurocholate infusion lead to significant destruction of the pancreatic tissue structure typified by acinar cell necrosis, edema formation, and neutrophil accumulation (Fig. 3). We found that the inhibition of farnesyltransferase protected against taurocholate-provoked tissue destruction (Fig. 3). For example, the administration of FTI-277 reduced the taurocholate-evoked edema by 49% in the pancreas (Fig. 3B). Moreover, FTI-277 decreased the number of extravascular leukocytes by 43% in pancreatitis mice (Fig. 3D). Challenge with taurocholate increased the number of circulating PMNLs, suggesting an on-going systemic activation (Table 1). Inhibition of farnesyltransferase significantly decreased the number of systemic PMNLs in taurocholate-treated animals (Table 1).

Farnesyltransferase Regulates Neutrophil Infiltration in Pancreatitis

Tissue levels of MPO were used as an indicator of neutrophil infiltration. We found that challenge with taurocholate increased the pancreatic MPO activity by 11-fold (Fig. 4A). Farnesyltransferase inhibition reduced the taurocholate-provoked pancreatic levels of MPO by 64% (Fig. 4A). As part of a systemic inflammatory response in severe AP, activated neutrophils accumulate in the pulmonary microvasculature. Indeed, it was observed that challenge with taurocholate markedly enhanced the

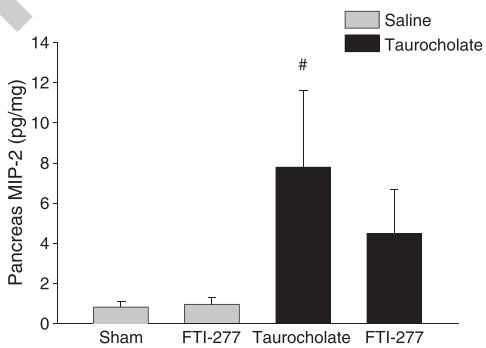

FIGURE 5. Chemokine formation in the pancreas. Pancreatic levels of MIP-2 were determined in sham and taurocholate-exposed mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). One group of mice received 25 mg/kg of FTI-277 alone. Samples were harvested 24 hours after pancreatitis induction. Data represent mean ± SEM and n = 8–10 ($^{\#}P < 0.05$ vs sham and $^{*}P < 0.05$ vs vehicle + taurocholate).

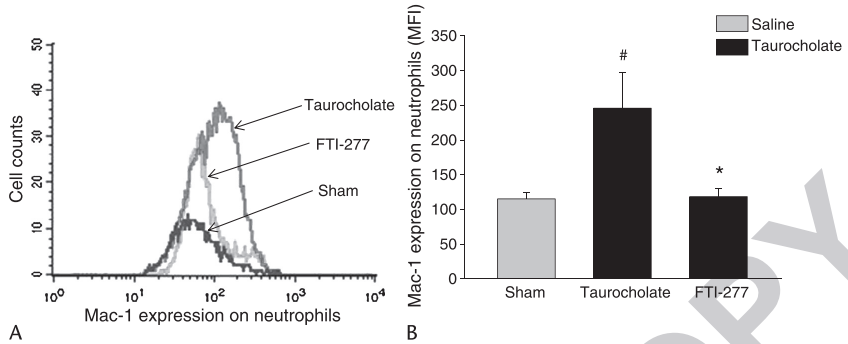

FIGURE 6. Farnesyltransferase regulates Mac-1 expression on neutrophils. Mac-1 expression on neutrophils was determined in sham and taurocholate-exposed in mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). Samples were harvested 24 hours after induction of pancreatitis. Histograms are representative of 5 samples. Data represent mean ± SEM and n = 5 ($^{\#}P < 0.05$ vs sham and $^{*}P < 0.05$ vs vehicle + taurocholate).

MPO activity in the lung (Fig. 4B). Farnesyltransferase inhibition reduced MPO levels in the lung by more than 71% in mice challenged with taurocholate (Fig. 4B). Administration of FTI-277 alone had no effect on MPO levels in the pancreas and lung (Figs. 4A, B). In addition, we observed that challenge with taurocholate increased the MIP-2 levels in the pancreas (Fig. 4). The inhibition of farnesyltransferase had no significant effect on MIP-2 levels in the inflamed pancreas in animals challenged with taurocholate (Fig. 5). The administration of FTI-277 alone had no effect on MIP-2 levels in the pancreas (Fig. 5). Moreover, we noted that Mac-1 expression was increased on the surface of neutrophils in mice with pancreatitis (Fig. 6A, B). The inhibition of farnesyltransferase signaling markedly reduced the neutrophil expression of Mac-1 in pancreatitis (Fig. 6A, B). The administration of FTI-277 2 hours after the induction of pancreatitis had no effect on taurocholate-induced increases in MPO in the pancreas and lung (not shown). In addition, pancreatic levels of MIP-2 and Mac-1 expression on neutrophils were intact in mice treated with FTI-277 2 hours after the taurocholate administration (not shown).

Trypsinogen Activation in Acinar Cells In Vitro

We next asked whether farnesyltransferase may regulate trypsinogen activation in pancreatic acinar cells in vitro. For this purpose, we isolated acinar cells from the pancreas of mice and incubated the cells with cerulein. It was found that cerulein stimulation increased the trypsinogen activation by more than 8-fold compared with unstimulated cells (Fig. 7). However, preincubation of the acinar cells with FTI-277 had no effect on the secretagogue-induced activation of trypsinogen (Fig. 7).

Farnesyltransferase Regulates Tissue Injury in L-Arginine–Induced AP

To examine whether farnesyltransferase might also regulate tissue damage and neutrophil recruitment in an alternative experimental model, L-arginine was used to trigger AP. The administration of 4 g/kg L-arginine caused extensive tissue damage and clear-cut infiltration of neutrophils in the pancreas (Table 2 and Fig. 8). The inhibition of farnesyltransferase markedly decreased the L-arginine–provoked acinar cell necrosis, edema, and hemorrhage in the pancreas (Table 2) and protected against L-arginine–provoked damage of the tissue microarchitecture in the pancreas (Fig. 8). Finally, the administration of FTI-277 significantly decreased the L-arginine–evoked MPO levels and the number of extravascular neutrophils in the pancreas (Table 2) as well as MPO levels in the lung (Table 2).

DISCUSSION

Signaling cascades controlling proinflammatory pathways in pancreatitis are incompletely understood. Our present study demonstrates for the first time that farnesyltransferase is an important regulator of the pathophysiology in severe AP. These results demonstrate that farnesyltransferase is involved in the surface up-regulation of Mac-1 on neutrophils. We found that the inhibition of farnesyltransferase activity not only decreases neutrophil infiltration in the pancreas but also attenuates tissue damage and blood amylase levels in AP. In addition, our findings show that farnesyltransferase inhibition abolishes accumulation of

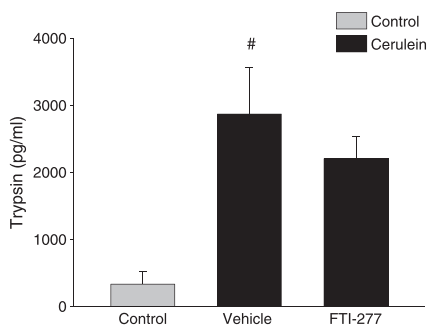

FIGURE 7. Acinar cell activation of trypsinogen was measured in negative control cells and cerulein-exposed acinar cell homogenate pretreated with PBS or FTI-277 (200 μM). Trypsinogen activation was quantified by measuring enzymatic activity of trypsin fluorometrically by using BoC-Gln-Ala-Arg-MCA as the substrate as described in detail in the Excremental Procedure. Trypsin levels were calculated using a standard curve generated by assaying purified trypsin. Data represent mean ± SEM and n = 4 ($^{\#}P < 0.05$ vs control).

TABLE 2. L-arginine–Induced Pancreatitis

	Saline	Vehicle + L-Arginine	FTI-277 + L-Arginine
Blood amylase (μ Kat/L)	77.0 ± 9.1	127.7 ± 45.1*	83.4 ± 29.4†
MPO in the pancreas (U/g)	0.4 ± 0.06	4.0 ± 1.2*	0.8 ± 0.1†
MPO in the lung (U/g)	0.2 ± 0.07	3.1 ± 0.6*	1.2 ± 0.4†
Acinar cell necrosis (score 0–4)	0.5 ± 0.1	2.3 ± 0.3*	0.8 ± 0.2†
Neutrophil infiltration (score 0–4)	0.5 ± 0.2	1.8 ± 0.1*	1.1 ± 0.1†
Edema (score 0–4)	0.6 ± 0.2	2.7 ± 0.3*	1.2 ± 0.3†
Hemorrhage (score 0–4)	0.7 ± 0.1	2.1 ± 0.4*	1.4 ± 0.2

Blood amylase, MPO levels in the pancreas and lung, as well as histology scoring in saline and L-arginine–exposed mice pretreated with vehicle or the farnesyltransferase inhibitor FTI-277 (25 mg/kg). Samples were harvested 72 hours after pancreatitis induction. Data represent mean ± SEM and n = 8–10.

*$P < 0.05$ versus saline.

†$P < 0.05$ versus vehicle + L-arginine.

neutrophils in the lung, suggesting that farnesyltransferase controls both local and systemic inflammation in severe AP.

Protein isoprenylation is mainly recognized for its role in oncogenesis by regulating functional activity of small G proteins, such as Ras and Rho.[28] Nonetheless, accumulating data point to a potential role of isoprenylation also in inflammatory processes. For example, farnesyltransferase activity has been reported to control proinflammatory actions in experimental models of multiple sclerosis[29] and sepsis.[21] Herein, we could demonstrate that farnesyltransferase inhibition with a specific farnesyltransferase inhibitor (FTI-277) not only decreased Ras activity but also significantly reduced tissue injury in severe AP. For example, the administration of FTI-277 decreased the taurocholate-provoked increase in blood amylase by 46% and edema formation by 49%, suggesting that farnesyltransferase activity regulates a significant part of the tissue damage in severe AP. These data constitute the first evidence in the literature that the farnesyltransferase signaling pathway is involved in the pathophysiology of AP. In this context, it is interesting to note that statins, which are mainly used to regulate cholesterol levels

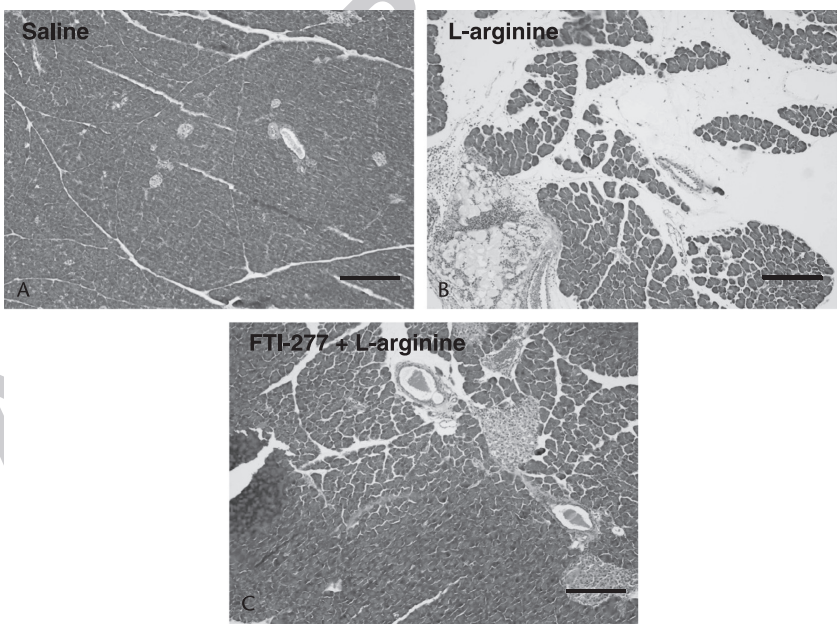

FIGURE 8. Representative hematoxylin-eosin sections of the pancreas. A, Saline animals served as negative controls. AP was induced by the administration of 4 g/kg per dose of L-arginine IP twice at an interval of 1 hour. Mice were treated with (B) vehicle or (C) the farnesyltransferase inhibitor FTI-277 (25 mg/kg) IP before the first dose of L-arginine. Samples were harvested 72 hours after the first dose of L-arginine. Bars represent 100 μm.

in patients with cardiovascular diseases, have been reported to attenuate experimental pancreatitis.[30] Statins do not only reduce mevalonate, the precursor for cholesterol formation, but also farnesyl pyrophosphate, which is used as substrate for farnesyltransferase in the prenylation of small G proteins.[19] Thus, our present findings may therefore also help to explain the reported anti-inflammatory effects of statins in pancreatitis. In this context, it should be mentioned that the statin-induced depletion of mevalonic acid not only decreases farnesyl pyrophosphates but also reduces the levels of geranylgeranyl pyrophosphates, which might contribute to the proinflammatory effects in AP.

It is widely held that neutrophil infiltration is a prominent feature in pancreatitis.[5,6,31] For example, neutrophil depletion has repeatedly been reported to ameliorate tissue damage in AP.[31] In the present study, we found that taurocholate challenge markedly enhanced the MPO activity and the number of extravascular neutrophils in the pancreas. The administration of FTI-277 significantly reduced the MPO levels (64%) and the number of extravascular neutrophils (43%) in the pancreas, indicating that farnesyltransferase activity is an important regulator of neutrophil recruitment in the inflamed pancreas. Considering the critical role of neutrophils in the pathophysiology of pancreatitis,[5,6] it might be forwarded that the inhibitory effect of FTI-277 on neutrophil responses might explain the protective effect of FTI-277 on tissue injury in AP. In addition, systemic complications of severe AP include pulmonary accumulation of neutrophils.[32] We found that lung levels of MPO were clearly increased in response to taurocholate challenge. Interestingly, FTI-277 decreased the pulmonary MPO activity, suggesting that farnesyltransferase also regulates systemic activation and infiltration of neutrophils in the lung in severe AP. Numerous studies have shown that specific adhesion molecules control the extravasation process of leukocytes.[6,24,33,34] Although the detailed role of adhesion molecules in facilitating leukocyte accumulation in the pancreas is relatively unclear, several reports have documented that Mac-1 is a dominating molecule in mediating tissue infiltration of neutrophils.[9,35] Herein, it was observed that Mac-1 expression on neutrophils are increased in response to taurocholate induction infusion. Notably, the treatment with FTI-277 greatly decreased the taurocholate-induced neutrophil expression of Mac-1, suggesting that farnesyltransferase regulates Mac-1 expression on neutrophils in AP. We next asked whether this inhibitory effect of FTI-277 might be attributed to the formation of CXC chemokine, such as MIP-2, which is a particularly potent activator of neutrophils.[11] It was found that taurocholate provoked a significant increase in MIP-2 levels in the pancreas. However, inhibition of farnesyltransferase had no significant effect on taurocholate-induced production of MIP-2 in the pancreas.

Trypsinogen activation is generally considered to be a central feature in the pathophysiology of AP. A recent study showed that Rho-kinase signaling regulates trypsinogen activation in acinar cells.[17] In general, isoprenylation mediated by farnesyltransferase and geranylgeranyltransferase is necessary for the function of Rho proteins. It was therefore of interest to examine whether farnesyltransferase might be involved in the activation of trypsin. However, we observed that the inhibition of farnesyltransferase activity had no effect on the secretagogue-induced activation of trypsin in isolated acinar cells in vitro. In this context, it is important to note that Rho-kinase activity is controlled by RhoA, RhoB, and RhoC, which are mainly prenylated by geranylgeranyltransferase,[36,37] although farnesyltransferase has been shown to prenylate RhoB.[38] Considered together, it could be speculated that gerangeryltransferase might be relatively more important for regulating Rho-kinase–dependent trypsin activation in acinar cells. The precise role of gerangeryltransferase in AP needs to be addressed in future studies. Nonetheless, these findings suggest that the protective effects of FTI-277 are the downstream of trypsin activation in pancreatitis. This notion is in line with the concept in the present study suggesting that a dominant role of farnesyltransferase in AP is related to the inhibition of Mac-1 expression and neutrophil accumulation in the pancreas and lung.

In conclusion, our findings demonstrate that farnesyltransferase signaling regulates tissue damage in severe AP. These results show that the inhibition of farnesyltransferase attenuates the neutrophil expression of Mac-1 and infiltration in the pancreas and the lung, suggesting that farnesyltransferase controls both local and systemic inflammation in pancreatitis. Thus, these data not only delineate a novel signaling mechanism in AP but also indicate that targeting farnesyltransferase might be an effective way to ameliorate the pathologic inflammation in severe AP.

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Paper II

JLB

Article

Inhibition of geranylgeranyltransferase attenuates neutrophil accumulation and tissue injury in severe acute pancreatitis

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ABSTRACT

Leukocyte infiltration and acinar cell necrosis are hallmarks of severe AP, but the signaling pathways regulating inflammation and organ injury in the pancreas remain elusive. In the present study, we investigated the role of geranylgeranyltransferase in AP. Male C57BL/6 mice were treated with a geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg) prior to induction of pancreatitis by infusion of taurocholate into the pancreatic duct. Pretreatment with GGTI-2133 reduced plasma amylase levels, pancreatic neutrophil recruitment, hemorrhage, and edema formation in taurocholate-evoked pancreatitis. Moreover, administration of GGTI-2133 decreased the taurocholate-induced increase of MPO activity in the pancreas and lung. Treatment with GGTI-2133 markedly reduced levels of CXCL2 in the pancreas and IL-6 in the plasma in response to taurocholate challenge. Notably, geranylgeranyltransferase inhibition abolished neutrophil expression of Mac-1 in mice with pancreatitis. Finally, inhibition of geranylgeranyltransferase had no direct effect on secretagogue-induced activation of trypsinogen in pancreatic acinar cells in vitro. A significant role of geranylgeranyltransferase was confirmed in an alternate model of AP induced by L-arginine challenge. Our findings show that geranylgeranyltransferase regulates neutrophil accumulation and tissue damage via expression of Mac-1 on neutrophils and CXCL2 formation in AP. Thus, these results reveal new signaling mechanisms in pancreatitis and indicate that targeting geranylgeranyltransferase might be an effective way to ameliorate severe AP. *J. Leukoc. Biol.* 94: 493–502; 2013.

Introduction

Severe AP is associated with high morbidity and mortality [1]. Management of patients with severe AP is a significant challenge to clinicians and is largely limited to supportive therapies as a result of the limited understanding of the underlying pathophysiology. It is generally considered that activation of trypsinogen, leukocyte recruitment, and impaired microvascular perfusion are integrated components in the pathophysiology of severe AP [2–5]. Leukocyte infiltration is a central feature of the inflammatory response, and numerous studies have reported that leukocytes are essential in severe AP [6, 7]. Leukocyte extravasation involves multiple sequential steps supported by specific adhesion molecules, including P-selectin [8, 9] and LFA-1 [7]. Extravascular direction of leukocytes is coordinated by secreted chemokines [10, 11]. CXC chemokines, including CXCL1 and CXCL2, are potent stimulators of neutrophil accumulation [12]. CXCR2 is the high-affinity receptor on neutrophils for CXCL1 and CXCL2 [13] and has been demonstrated to mediate neutrophil recruitment in the pancreas [14]. Thus, the role of specific adhesion molecules and chemoattractants as regulators of leukocyte accumulation in pancreatitis is relatively well known; however, the knowledge about the signaling mechanism orchestrating pathological inflammation in pancreatitis is limited.

Cellular stress and microbial invasion activate signaling pathways converging on specific transcription factors, which in turn, regulate gene expression of proinflammatory compounds. Intracellular kinases phosphorylating downstream targets play a dominant role in many signaling cascades [15]. Small GTP-binding proteins of the Rho family, including RhoA, Cdc42, and Rac1, as well as one of their effectors, Rho-kinase, operate as molecular switches, controlling numerous cell functions, such as cytoskeleton reorganization, vesicular trafficking, and chemotaxis [16]. Inhibition of Rho-kinase decreases tissue injury in numerous disease models, such as AP [17], endotoxemia [18], and abdominal sepsis [19]. To activate downstream effectors, such as Rho-kinase, small GTP-binding proteins must undergo post-translational modifications, including isoprenylation, which is catalyzed by two enzymes: farnesyltransferase and geranylgeranyltransferase [20]. Interest-

Abbreviations: AP=acute pancreatitis, Boc-Glu/Gln-Ala-Arg=t-butyloxycarbonyl-L-glutamine/L-glutaminyl-L-alanyl-L-arginine, CFU=colonic forming unit, Gr-1=granulocyte receptor-1, Mac-1=membrane-activated antigen-1, MLN=mesentry lymph node, MNL=monomorphonuclear leukocyte, PMNL=polymorphonuclear leukocyte, qPCR=quantitative polymerase chain reaction, Rac1=Ras-related C3 botulinum toxin substrate 1, Rho=Ras homologous

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ingly, a recent study reported that inhibition of geranylgeranyltransferase with a specific inhibitor decreased T-lymphocyte infiltration in the CNS [21]. However, the potential role of geranylgeranyltransferase in controlling neutrophil accumulation and tissue injury in pancreatitis is not known.

Based on the above considerations, we hypothesized that geranylgeranyltransferase signaling might play a role in severe AP. Two different experimental models of severe AP in mice were used, and inhibition of geranylgeranyltransferase activity was achieved by use of GGTI-2133.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (20–25 g) were housed on a 12–12 h light–dark cycle and fed a laboratory diet and water *ad libitum*. All experimental procedures were approved by the Ethical Committee at Lund University. Mice were anesthetized i.p. with 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/kg body weight.

Bile salt ductal perfusion

Anesthetized animals underwent midline laparotomy, and the second part of duodenum and papilla of vater was identified. Traction sutures were placed 1 cm from the papilla, and a small puncture was made through the duodenal wall with a 23-G needle in parallel to the papilla of vater. A polyethylene catheter connected to a microinfusion pump (CMA/100; Carnegie Medicin, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and 1 mm into the common bile duct. The common hepatic duct was identified and clamped at the liver hilum. Sodium taurocholate (10 μl of 5%; Sigma-Aldrich, St. Louis, MO, USA) was infused into the pancreatic duct for 10 min. After completion, the catheter and the common hepatic duct clamp were removed. The duodenal puncture was closed with a purse-string suture. The traction sutures were removed, and the abdomen was closed in two layers. Mice were allowed to wake up and were given free access to food and water. Sham mice underwent laparotomy and PBS infusion into the pancreatic duct and were pretreated i.p. with vehicle (PBS; n=5). Vehicle (PBS) or the GGTI-2133 inhibitor (4-{[*N*-(imidazol-4-yl)methyleneamino]-2-(1-naphthyl) benzoyl} leucine; Sigma-Aldrich, Stockholm, Sweden) was given 20 mg/kg i.p. prior to bile duct cannulation and induction of AP. One group of mice received 20 mg/kg GGTI-2133 alone without bile duct cannulation. This dose and scheme of administration of the GGTI-2133 inhibitor were based on a previous investigation [22]. All mice were killed 24 h after pancreatitis induction and assessed for all parameters included in this study.

L-Arginine-induced, acute necrotizing pancreatitis

In separate experiments, AP was induced by i.p. administration of L-arginine (4 g/kg/dose, dissolved in saline), twice at an interval of 1 h, as described in detail before [23]. Saline (n=4) or GGTI-2133 (20 mg/kg; n=4) was administered i.p. before the first dose of L-arginine. Saline animals served as negative controls. Blood was sampled from the tail vein and inferior vena cava. Mice were killed, and samples were harvested, 72 h after the first administration of L-arginine.

Amylase measurements

Blood amylase levels were determined in blood collected from the tail vein by use of a commercially available assay (Reflotron; Roche Diagnostics GmbH, Mannheim, Germany).

Leukocyte differential counts

Blood was collected from the tail vein for systemic leukocyte differential counts. The blood was mixed with Turks solution (Merck, Damnstadt, Germany) in a 1:20 dilution. Leukocytes were identified as MNL and PMNL cells in a Burker chamber.

Measurement of MPO activity

A piece of the pancreatic head and lung tissue was harvested for MPO measurements. All frozen pancreatic and lung tissues were preweighed and homogenized in 1 ml mixture (4:1) of PBS and aprotinin 10,000 kallikrein inactivator units/ml (Trasylol; Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenate samples were centrifuged (15,339 *g*, 10 min), and the supernatant was stored at −20°C, and the pellet was used for MPO assay, as described previously [24]. All pellets were mixed with 1 ml 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 s, and incubated in a water bath at 60°C for 2 h, after which, the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of hydrogen peroxide (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units/g tissue.

Morphologic evaluation of pancreas

Tissue pieces from the head of the pancreas tissue were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Sections (6 μm) were stained (H&E) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system, including edema, acinar cell necrosis, hemorrhage, and neutrophil infiltrate on a 0 (absent)–4 (extensive) scale, as described previously [25].

ELISA

Pancreatic levels of CXCL2 and plasma levels of IL-6 were determined by use of a double-antibody Quantikine ELISA kit (R&D Systems Europe, Abingdon, UK) using murine rCXCL2 and rIL-6 as standards. The minimal detectable protein concentration is <0.5 pg/ml.

CXC chemokine mRNA expression in qPCR

The pancreas was harvested and frozen immediately in liquid nitrogen. Total RNA was isolated from the frozen pancreas samples using TRI Reagent, following the manufacturer's technical bulletin (Sigma-Aldrich St. Louis, MO, USA), and the RNA concentrations were measured using a spectrophotometer (Model ND-1000; NanoDrop, Wilmington, DE, USA).

Each cDNA was synthesized by reverse transcription from 5 μg total RNA with the RevertAid First Strand cDNA synthesis kit and random hexamer primers (Fermentas Life Sciences, Burlington, Ontario, Canada), according to the manufacturer's protocol. After reverse transcription, the cDNA was diluted 10 times with Tris-EDTA buffer.

Real-time PCR was performed with Brillian II SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and the Mx3000P detection system (Stratagene, La Jolla, CA, USA). The primer sequences of CXCL2 and β-actin were the following: CXCL2 (forward) 5′-GCT TCC TCG GGC ACT CCA GAC-3′, CXCL2 (reverse) 5-TTA GCC TTG CCT TTG TTC AGT AT-3′; β-actin (forward) 5′-ATG TTT GAG ACC TTC AAC ACC-3′, β-actin (reverse) 5′-TCT CCA GGG AGG AAG AGG AT-3′.

PCR amplifications were performed in a total volume of 25 μl/well containing 12.5 μl SYBR Green PCR 2× Master Mix, 1 μl 0.10 μM each primer, 0.3751 μl reference dye, and 4 μl cDNA as a template and adjusted up to 25 μl with water. PCR reactions were started with 10 min denaturing of 95°C, followed by a total of 40 cycles (95°C for 30 s and 55°C for 1 min and 1 min of elongation at 72°C). Cycling time values for the specific target genes were related to that of β-actin in the same sample, and the β-actin gene is normalized. Real-time qRT-PCR analysis was then performed using comparative, quantitative software in the Mx3000P instrument.

Mac-1 expression on neutrophils

For analysis of surface expression of Mac-1 on circulating neutrophils, blood was collected (1:10 acid citrate dextrose), 24 h after taurocholate

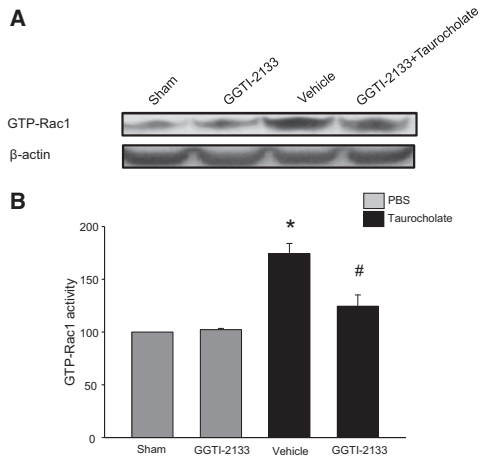

Figure 1. Geranylgeranyltransferase regulates Rac1 activity in the pancreas. Rac1 activity was determined in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). Separate animals received GGTI-2133 (20 mg/kg) alone. β-Actin was used as an internal control for total Rac1. Data represent means ± SEM, and $n = 5$. *$P <$ 0.05 versus sham; #$P < 0.05$ versus vehicle + taurocholate.

induction, and incubated (10 min at room temperature) with an anti-CD16/CD32 antibody blocking FcγIII/IIR to reduce nonspecific labeling and then incubated with PE-conjugated anti-Gr-1 (Clone RB6-8C5, rat IgG2b; eBioscience, Frankfurt, Germany) and FITC-conjugated anti-Mac-1 (Clone M1/70, integrin αM China, rat IgG2bκ; BD Biosciences PharMingen, San Jose, CA, USA) antibodies. Cells were fixed, erythrocytes were lysed, and neutrophils were recovered following centrifugation. Flow cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with CellQuest Pro software (BD Biosciences PharMingen).

Rac1 activity

Rac1 activity was determined by using a Rac1 activation assay kit, as described previously [26]. Briefly, 50 mg pancreas tissue was minced and homogenized in lysis buffer on ice. Samples were centrifuged at 15,000 *g* for 15 min, and 10 μl supernatant was removed to measure protein content using the BCA Protein Assay Reagent (Pierce Biotechnology, Rockford, IL, USA), and the rest of the supernatant was used for the pull-down assay. Supernatants containing equal amount of proteins were then diluted with 2× SDS sample buffer and boiled for 5 min. Proteins were separated using SDS-PAGE (10% gel). After transfer to a nitrocellulose membrane (BioRad, Hercules, CA, USA), blots were blocked with 3% BSA at room temperature for 1 h, followed by incubation with an anti-Rac1 antibody (1:1000) at 4°C overnight. Binding of the antibody was visualized using peroxidase-conjugated anti-mouse antibody (1:100,000; Pierce Biotechnology) at room temperature for 1 h and the ECL method (BioRad). Expression of internal control β-actin proteins was detected by Western blot on aliquots taken before protein affinity purification.

Bacterial quantification

Blood samples (inferior vena cava) and mesenteric LNs were harvested for bacterial quantification. Mesenteric LN was weighed and processed immediately for cultures of bacteria. Samples were cultured on blood agar and evaluated after 24 h of incubation (37°C). Bacterial counts in the blood and mesenteric LNs are given as CFU/ml and CFU/gram, respectively.

Acinar cell isolation

Pancreatic acini cells were prepared by collagenase digestion and gentle shearing, as described previously [27]. Cells were suspended in HEPES-Ringer buffer (pH 7.4) saturated with oxygen and passed through a 150-μm cell strainer (Partec, Görlitz, Germany). Isolated acinar cells (1×10^7 cells/well) were preincubated immediately with PBS or GGTI-2133 (100 μM, 30 min) and stimulated with 100 nM cerulein (37°C, 30 min) in duplicates. The buffer was then discarded, and the cells were washed twice with buffer (pH 6.5) containing 250 mM sucrose, 5 mM MOPS, and 1 mM magnesium sulfate. The cells were next homogenized in cold (4°C) MOPS buffer using a Potter-Elvehjem-type glass homogenizer. The resulting homogenate was centrifuged (56 *g*, 5 min), and the supernatant was used for assay. Trypsin activity was measured flourometrically using Boc-Glu-Ala-Arg-amino-4-methylcoumarin as substrate, as described previously [28]. For this purpose, a 200-μl aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, and 0.1% BSA, pH 8.0). The reaction was initiated by the addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin levels (pg/ml) were calculated using a standard curve generated by assaying purified trypsin. Viability of the pancreatic acinar cells was >95%, as determined by trypan blue dye exclusion.

Statistical analysis

Data are presented as mean values ± SEM. Statistical evaluations were performed by using nonparametrical tests (Mann-Whitney). $P < 0.05$ was considered significant, and n represents the number of animals.

RESULTS

Geranylgeranyltransferase-dependent activation of Rac1 in pancreatitis

To investigate the activation of Rac1 in the pancreas and the effect of GGTI-2133 on Rac1 activity, pancreas from sham and

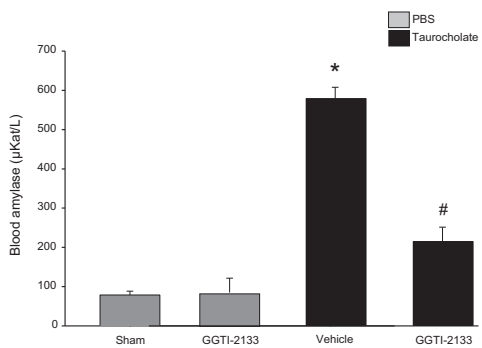

Figure 2. Blood amylase (μKat/L) in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). Separate animals received GGTI-2133 (20 mg/kg) alone. Blood samples were obtained 24 h after pancreatitis induction. Data represent means ± SEM, and $n =$ 5. *$P <$ 0.05 versus sham; #$P < 0.05$ versus vehicle + taurocholate.

pancreatitis mice was harvested for Rac1 activation assay. Taurocholate challenge increased the active form (GTP-binding form) of Rac1 (**Fig. 1**). Administration of GGTI-2133 significantly decreased taurocholate-induced activation of Rac1 (Fig. 1). Pretreatment with GGTI-2133 alone had no effect on Rac1 activity in the pancreas (Fig. 1).

Geranylgeranyltransferase controls tissue damage in pancreatitis

To examine the role of geranylgeranyltransferase in severe AP, blood amylase levels were first examined as an indicator of tissue damage. We observed that retrograde infusion of taurocholate in the pancreatic duct increased blood amylase levels by sevenfold (**Fig. 2**). Pretreatment with the geranylgeranyltransferase inhibitor GGTI-2133 decreased taurocholate-induced blood amylase levels from 579.6 ± 28.7 μKat/L to 215.0 ± 36.5 μKat/L, corresponding to a 70% reduction (Fig. 2). Administration of GGTI-2133 alone had no effect on taurocholate-induced amylase levels in the blood (Fig. 2). Examination of tissue morphology revealed that sham mice had normal pancreatic microarchitecture (**Fig. 3**), whereas taurocholate challenge led to significant destruction of the pancreatic tissue structure typified by acinar cell necrosis, edema formation, and neutrophil accumulation (Fig. 3). We found that inhibition of geranylgeranyltransferase protected against taurocholate-provoked tissue destruction (Fig. 3). Challenge with taurocholate decreased the number of circulating PMNLs and MNLs, suggesting ongoing systemic activation (**Table 1**). Inhibition of geranylgeranyltransferase significantly inhibited the taurocholate-induced decrease in systemic leukocytes (Table 1).

Geranylgeranyltransferase regulates neutrophil infiltration in pancreatitis

Tissue levels of MPO were used as an indicator of neutrophil infiltration. We found that challenge with taurocholate increased pancreatic MPO activity by 15-fold (**Fig. 4A**). Geranylgeranyltransferase inhibition reduced taurocholate-pro-

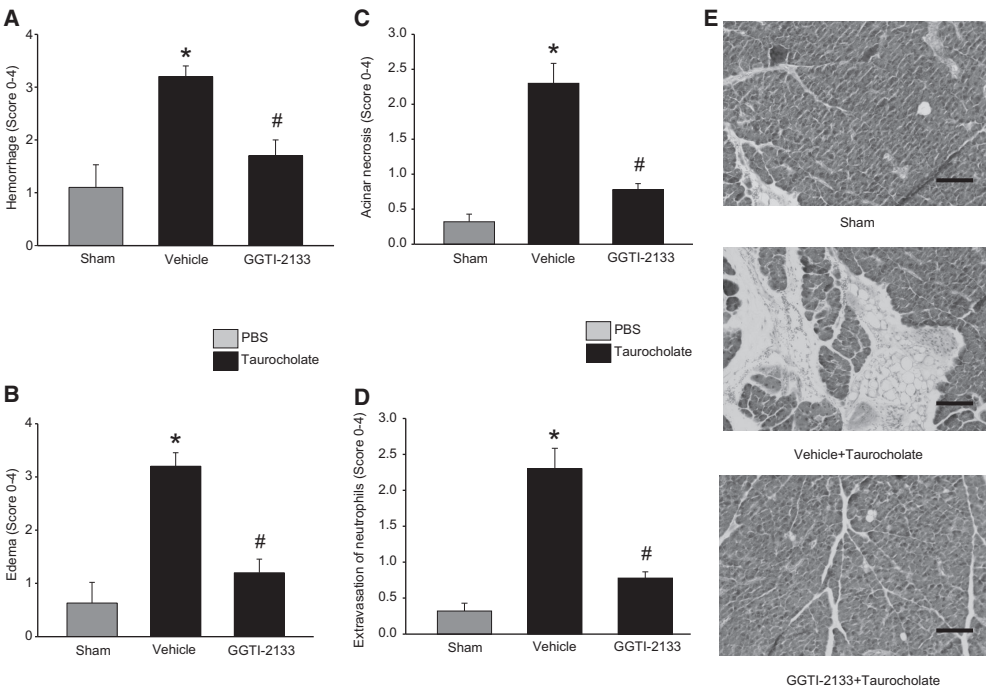

Figure 3. Geranylgeranyltransferase regulates tissue damage in AP. (A) Hemorrhage, (B) edema formation, (C) acinar necrosis, and (D) extravascular neutrophils in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). (E) Representative H&E sections of the pancreas. Samples were harvested 24 h after pancreatitis induction. Original bars represent 100 μm. Data represent means ± SEM, and $n = 5$. *$P < 0.05$ versus sham; #$P < 0.05$ versus vehicle + taurocholate.

TABLE 1. Systemic Leukocyte Differential Counts

	PMNL	MNL	Total
PBS	1.6 ± 0.1	10.3 ± 0.3	11.9 ± 0.4
Vehicle + taurocholate	0.4 ± 0.1[a]	4.7 ± 0.1[a]	5.1 ± 0.2[a]
GGTI-2133 + taurocholate	0.9 ± 0.2[b]	6.2 ± 0.2[b]	7.1 ± 0.4[b]

Blood was collected from sham mice and taurocholate-challenged animals pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). Cells were identified as MNL and PMNL. Data represent mean ± SEM, 10^6 cells/ml, and $n = 5$. [a]$P < 0.05$ versus PBS; [b]$P < 0.05$ versus vehicle + taurocholate.

voked pancreatic levels of MPO by 50% (Fig. 4A). As part of a systemic inflammatory response in severe AP, activated neutrophils accumulate in the pulmonary microvasculature. Indeed, it was observed that challenge with taurocholate markedly enhanced the MPO activity in the lung (Fig. 4B). Geranylgeranyltransferase inhibition reduced MPO levels in the lung by >74% in mice challenged with taurocholate (Fig. 4B). In addition, we observed that challenge with taurocholate increased gene expression and protein levels of CXCL2 in the pancreas (Fig. 4C and D). Administration of GGTI-2133 significantly decreased CXCL2 mRNA and protein levels in the inflamed pancreas in animals challenged with taurocholate (Fig. 4C and D). Moreover, taurocholate challenge markedly increased plasma levels of IL-6, and administration of GGTI-2133 normalized plasma levels of IL-6 in mice with pancreatitis (Fig. 4E). We noted that Mac-1 expression was increased on the surface of neutrophils in mice with pancreatitis (**Fig. 5A** and **B**). Inhibition of geranylgeranyltransferase signaling markedly reduced neutrophil expression of Mac-1 in AP (Fig. 5A and B).

Geranylgeranyltransferase inhibits bacterial spread in pancreatitis

Challenge with taurocholate markedly increased the number of bacteria in the blood (**Fig. 6A**) and mesenteric LNs (Fig. 6B). Pretreatment with GGTI-2133 significantly decreased the number of bacteria in the blood and mesenteric LNs of septic animals (Fig. 6B).

Trypsinogen activation in acinar cells in vitro

We next asked whether geranylgeranyltransferase may regulate trypsinogen activation in pancreatic acinar cells in vitro. For this purpose, we isolated acinar cells from the pancreas of mice and incubated the cells with cerulein. It was found that cerulein stimulation increased trypsinogen activation by more than ninefold compared with unstimulated cells (**Fig. 7**). However, preincubation of the acinar cells with GGTI-2133 had no effect on secretagogue-induced activation of trypsinogen (Fig. 7).

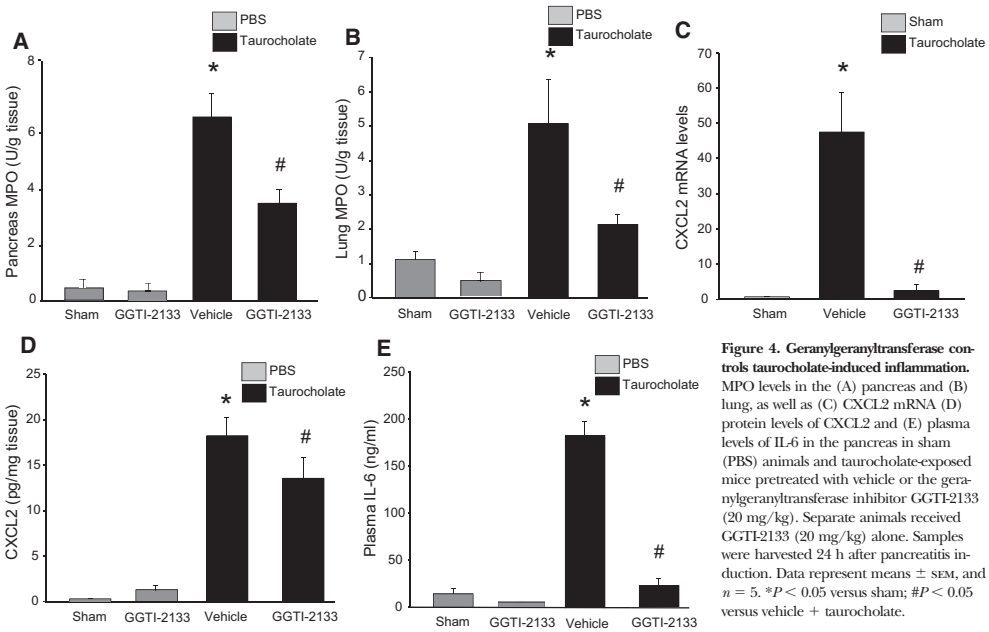

Figure 4. Geranylgeranyltransferase controls taurocholate-induced inflammation. MPO levels in the (A) pancreas and (B) lung, as well as (C) CXCL2 mRNA (D) protein levels of CXCL2 and (E) plasma levels of IL-6 in the pancreas in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). Separate animals received GGTI-2133 (20 mg/kg) alone. Samples were harvested 24 h after pancreatitis induction. Data represent means ± SEM, and $n = 5$. *$P < 0.05$ versus sham; #$P < 0.05$ versus vehicle + taurocholate.

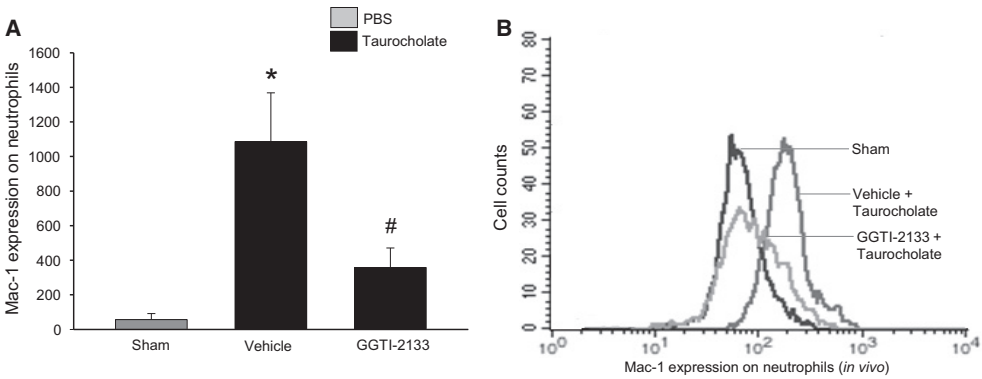

Figure 5. Mac-1 expression on circulating neutrophils (Gr-1$^+$) in sham (PBS) mice or after taurocholate challenge in animals treated with vehicle or GGTI-2133 (20 mg/kg). Representative dot plot and histogram (A) and data in aggregate (B). Data represent means ± SEM, and $n = 5$. *$P <$ 0.05 versus sham; #$P < 0.05$ versus vehicle + PBS.

Geranylgeranyltransferase regulates tissue injury in L-arginine-induced AP

To examine whether geranylgeranyltransferase might also regulate tissue damage and neutrophil recruitment in an alternative experimental model, L-arginine was used to trigger AP. Administration of 4 g/kg/dose L-arginine caused extensive tissue damage and clear-cut infiltration of neutrophils in the pancreas (**Table 2** and **Fig. 8**). Inhibition of geranylgeranyltransferase markedly decreased L-arginine-provoked acinar cell necrosis, edema, and hemorrhage in the pancreas (Table 2) and protected against L-arginine-provoked damage of the tissue microarchitecture in the pancreas (Fig. 8). Finally, administration of GGTI-2133 significantly decreased L-arginine-evoked MPO levels and the number of extravascular neutrophils in the pancreas (Table 2), as well as MPO levels in the lung (Table 2).

DISCUSSION

Our present study demonstrates for the first time that geranylgeranyltransferase is an important regulator of the pathophysiology in severe AP. These results demonstrate that geranylgeranyltransferase is involved in the surface up-regulation of Mac-1 on neutrophils and CXC chemokine formation in the pancreas. Moreover, we found that inhibition of geranylgeranyltransferase activity not only decreases neutrophil infiltration

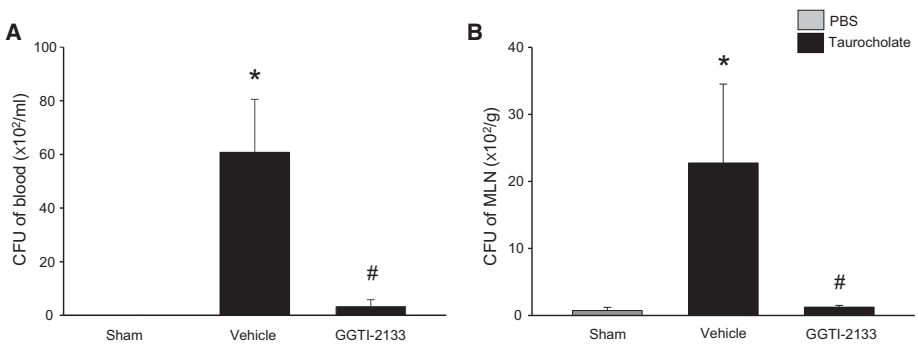

Figure 6. Geranylgeranyltransferase regulates bacterial clearance in AP. The number of bacterial colonies was quantified in the (A) blood and (B) mesenteric LNs (MLN) in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). Data represent means ± SEM, and $n = 5$. *$P <$ 0.05 versus sham; #$P < 0.05$ versus vehicle + taurocholate.

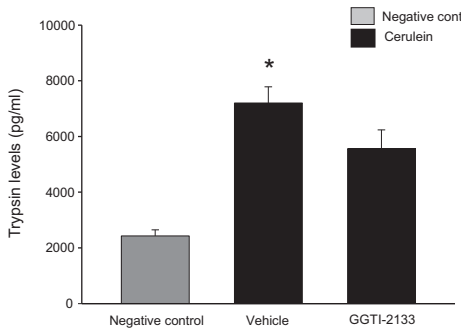

Figure 7. Acinar cell activation of trypsinogen was measured in negative control cells and in cells exposed to cerulein with PBS or GGTI-2133 (100 μM). Activation of trypsinogen activation was quantified by measuring enzymatic activity of trypsin fluorometrically by using Boc-Gln-Ala-Arg-4-methylcoumaryl-7-amide as the substrate, as described in detail in Materials and Methods. Trypsin levels were calculated using a standard curve, generated by assaying purified trypsin. Data represent means ± SEM, and $n = 5$. $^*P < 0.05$ versus negative control.

in the pancreas but also attenuates acinar cell necrosis and serum amylase levels in AP. In addition, our findings show that geranylgeranyltransferase inhibition abolishes IL-6 formation and accumulation of neutrophils in the lung, suggesting that geranylgeranyltransferase controls local and systemic inflammation in severe AP.

Isoprenylation processes are mainly recognized for their role in oncogenesis by regulating functional activity of the small G protein, such as Rac1, Cdc42, and RhoA proteins [29]. Nonetheless, emerging data point to a potential role of isoprenylation, also in inflammatory processes. For example, geranylgeranyltransferase activity has been reported to control proinflammatory actions in experimental models of multiple sclerosis [21] and asthma [30]. In the present study, it was demonstrated that geranylgeranyltransferase inhibition with a specific geranylgeranyltransferase inhibitor (GGTI-2133) not only reduced Rac1 activity in the pancreas but also markedly decreased tissue injury in severe AP. For example, administration of GGTI-2133 decreased a taurocholate-provoked increase in blood amylase by 70%, suggesting that geranylgeranyltransferase activity regulates a major part of the tissue damage in severe AP. These data constitute the first evidence in the literature that geranylgeranyltransferase is involved in the pathophysiology of AP. However, we also observed that administration of GGTI-2133 after taurocholate challenge had no significant effect on inflammatory parameters or tissue injury in the pancreas, suggesting that targeting geranylgeranyltransferase may have a limited influence on the treatment of patients with ongoing pancreatitis. Nonetheless, it can be speculated that high-risk patients undergoing endoscopic retrograde cholangiopancreatography might benefit from prophylactic inhibition of geranylgeranyltransferase. In this context, it is interesting to note that statins, which are mainly used to regulate cholesterol levels in patients with cardiovascular diseases, have been reported to attenuate experimental pancreatitis [31]. Statins not only reduce mevalonate, the precursor for cholesterol formation, but also pyrophosphates, which are used as substrates for franesyltransferase and geranylgeranyltransferase in the isoprenylation of small G proteins [32]. Thus, our present findings may therefore also help to explain the reported anti-inflammatory effects of statins in pancreatitis.

Numerous evidence suggests that neutrophil infiltration is a key component in AP [6, 7, 33]. For example, neutrophil depletion or inhibition of neutrophil recruitment has repeatably been shown to protect against tissue injury in pancreatitis [7, 17, 34]. Herein, it was observed that taurocholate challenge increased MPO levels and the number of extravascular neutrophils in the inflamed pancreas. Treatment with GGTI-2133 markedly decreased the activity of MPO and the number of extravascular neutrophils in the pancreas, suggesting that geranylgeranyltransferase is a prominent regulator of neutrophil recruitment in the pancreas. Considering the critical role of neutrophils in the pathophysiology of pancreatitis [6, 7, 35], it might be forwarded that the inhibitory effect of GGTI-2133 on neutrophil activation and infiltration could help to explain the tissue-protective effect of GGTI-2133 in the inflamed pancreas. Moreover, systemic complications of severe pancreatitis include pulmonary infiltration of inflammatory cells [36]. In the present study, it was found that pulmonary activity of MPO was clearly enhanced in response to taurocholate. Interestingly, GGTI-2133 reduced lung levels of MPO in taurocholate-exposed mice, indicating that geranylgeranyltransferase also controls systemic accumulation of neutrophils in the lung in severe AP. The notion that geranylgeranyltransferase controls systemic inflammation is also supported by our finding that GGTI-2133 markedly reduced the taurocholate-provoked in-

TABLE 2. L-Arginine-Induced Pancreatitis Model

	PBS	Vehicle + L-arginine	GGTI-2133 + L-arginine
Blood amylase (μKat/L)	82 ± 28	532 ± 48[a]	262 ± 35[b]
MPO in the pancreas (U/g)	0.03 ± 0.0	1.7 ± 0.7[a]	0.1 ± 0.04[b]
MPO in the lung (U/g)	0.07 ± 0.03	2.2 ± 0.7[a]	0.2 ± 0.1[b]
Acinar cell necrosis (Score 0–4)	0.07 ± 0.03	2.2 ± 0.7[a]	0.2 ± 0.1[b]
Neutrophil infiltration (Score 0–4)	0.33 ± 0.05	1.98 ± 0.09[a]	0.88 ± 0.12[b]
Edema (Score 0–4)	0.41 ± 0.05	1.9 ± 0.18[a]	0.67 ± 0.09[a]
Hemorrhage (Score 0–4)	0.75 ± 0.45	2.75 ± 0.4[a]	1.25 ± 0.12[b]

Blood amylase and MPO levels in the pancreas and lung and histology score in the pancreas in saline- and L-arginine-exposed mice pretreated with vehicle or the gera-nylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg). Samples were harvested 72 h after pancreatitis induction. Data represent means ± SEM, and $n = 4$. [a]$P < 0.05$ versus PBS; [b]$P < 0.05$ versus vehicle + L-arginine.

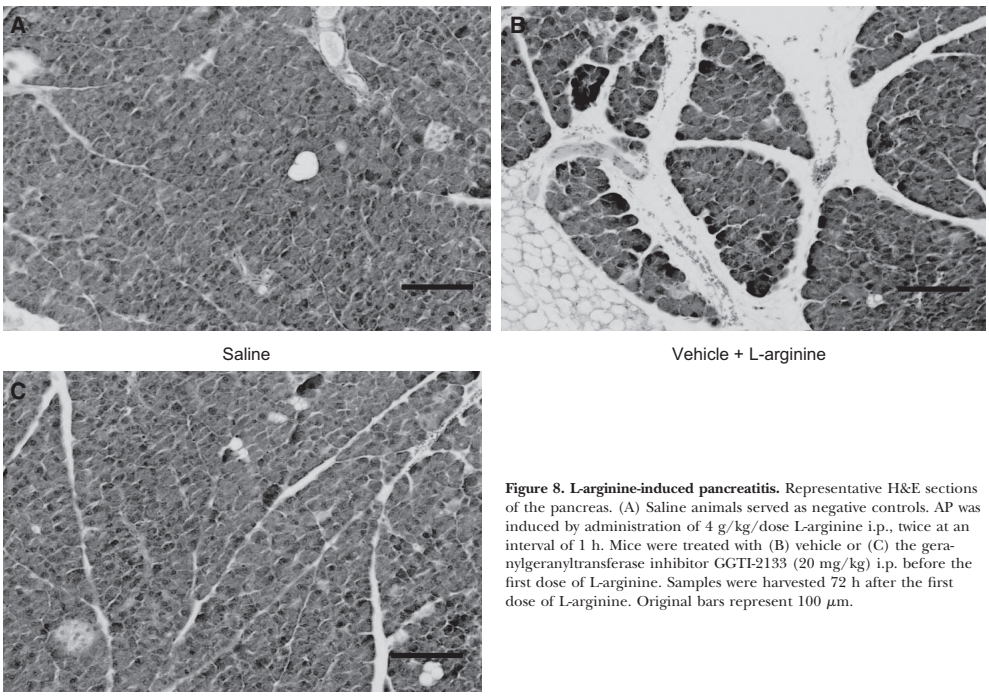

Figure 8. L-arginine-induced pancreatitis. Representative H&E sections of the pancreas. (A) Saline animals served as negative controls. AP was induced by administration of 4 g/kg/dose L-arginine i.p., twice at an interval of 1 h. Mice were treated with (B) vehicle or (C) the geranylgeranyltransferase inhibitor GGTI-2133 (20 mg/kg) i.p. before the first dose of L-arginine. Samples were harvested 72 h after the first dose of L-arginine. Original bars represent 100 μm.

crease in plasma levels of IL-6, which is an indicator of systemic inflammation and correlates with mortality of septic patients [37]. Although the detailed role of adhesion molecules in facilitating leukocyte accumulation in the pancreas is relatively unclear, recent studies have shown that Mac-1 is an important adhesion molecule in supporting extravascular infiltration of neutrophils [38]. We found that Mac-1 expression on neutrophils increased in response to taurocholate administration. Notably, administration of GGTI-2133 significantly decreased taurocholate-induced neutrophil expression of Mac-1, suggesting that geranylgeranyltransferase regulates neutrophil expression of Mac-1 in AP. Neutrophil trafficking to sites of inflammation is coordinated by secreted CXC chemokines, such as CXCL1 and CXCL2 [12], and a functional role of CXC chemokines has been proposed in pancreatitis [6, 7, 17]. It was found that taurocholate provoked a significant increase in CXCL2 levels in the pancreas. Administration of GGTI-2133 markedly reduced pancreatic levels of CXCL2 in AP, suggesting that geranylgeranyltransferase regulates CXCL2 formation in the inflamed pancreas. This finding might also help to explain the inhibitory effect of GGTI-2133 on neutrophil recruitment in AP. Considered together with the findings on Mac-1 expression above, it could be suggested that geranylgeranyltransferase regulates neutrophil infiltration in the pancreas at two distinct levels, i.e., formation of CXCL2 in the pancreas and Mac-1 expression on neutrophils.

Bacterial translocation and dissemination are characteristic features in severe pancreatitis [39]. Herein, we observed that taurocholate challenge markedly increased bacterial counts in the blood and mesenteric LNs. Notably, inhibition of geranylgeranyltransferase greatly reduced bacterial counts in the blood and mesenteric LNs in pancreatitis mice. The mechanisms behind this beneficial effect of GGTI-2133 on bacterial spread are not known at present, although a recent study reported that inhibition of geranylgeranyltransferase improves T cell functions and bacterial clearance in abdominal sepsis [40]. Whether similar mechanisms are involved in AP is an issue for future studies.

It is widely held that activation of trypsinogen is a critical feature in the pathophysiology of AP. Thus, we asked whether geranylgeranyltransferase might be involved in the activation of trypsin in acinar cells. However, it was found that GGTI-2133 had no impact on secretagogoue-induced activation of trypsin in isolated acinar cells, suggesting that geranylgeranyltransferase activity is not directly involved in the activation of trypsin in acinar cells. In this context, it is interesting to note

that we reported recently that Rho-kinase activity controls activation of trypsinogen in acinar cells [17]. Rho-kinase activation is dependent on isoprenylation, which is controlled by geranylgeranyltransferase and farnesyltransferase [41, 42]. Considered together, it could be speculated that farnesyltransferase might be relatively more important or that geranylgeranyltransferase or farnesyltransferase activity is sufficient for mediating Rho-kinase-dependent trypsin activation in AP. The precise importance of farnesyltransferase in regulating trypsinogen activation needs to be addressed in future studies on AP. Nonetheless, these findings suggest that the beneficial effects exerted by GGTI-2133 are downstream of trypsin activation in pancreatitis. This notion is in line with the concept in the present study, suggesting that a dominant role of geranylgeranyltransferase in AP is related to inhibition of Mac-1 expression and neutrophil accumulation in the pancreas and lung.

Taken together, these results show that geranylgeranyltransferase signaling controls tissue injury in severe AP. Our findings demonstrate that inhibition of geranylgeranyltransferase decreases neutrophil up-regulation of Mac-1 and CXCL2 formation in the pancreas. Moreover, geranylgeranyltransferase inhibition decreased neutrophil infiltration and tissue damage in the pancreas. Finally, blocking geranylgeranyltransferase activity attenuated systemic inflammation and pulmonary neutrophils in animals with pancreatitis. Thus, this study not only delineates a novel signaling mechanism in AP but also suggests that interference with geranylgeranyltransferase might be a useful strategy to ameliorate local and systemic inflammation in severe AP.

AUTHORSHIP

M.M. and E.W. performed experiments and contributed to the writing. S.Z. performed experiments, designed the study, and contributed to the writing. S.R. designed the study and contributed to the writing. H.T. conceived of and designed the study and contributed to the writing.

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DISCLOSURES

The authors state no conflict of interest.

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Paper III

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Immunopharmacology and inflammation

Inhibition of Ras signalling reduces neutrophil infiltration and tissue damage in severe acute pancreatitis

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ABSTRACT

Neutrophil recruitment is known to be a rate-limiting step in mediating tissue injury in severe acute pancreatitis (AP). However, the signalling mechanisms controlling inflammation and organ damage in AP remain elusive. Herein, we examined the role of Ras signalling in AP. Male C57BL/6 mice were treated with a Ras inhibitor (farnesylthiosalicylic acid, FTS) before infusion of taurocholate into the pancreatic duct. Pancreatic and lung tissues as well as blood were collected 24 h after pancreatitis induction. Pretreatment with FTS decreased serum amylase levels by 82% and significantly attenuated acinar cell necrosis, tissue haemorrhage and oedema formation in taurocholate-induced pancreatitis. Inhibition of Ras signalling reduced myeloperoxidase (MPO) levels in the inflamed pancreas by 42%. In addition, administration of FTS decreased pancreatic levels of CXC chemokines as well as circulating levels of interleukin-6 and high-mobility group box 1 in animals exposed to taurocholate. Moreover, treatment with FTS reduced taurocholate-induced MPO levels in the lung. Inhibition of Ras signalling had no effect on neutrophil expression of Mac-1 in mice with pancreatitis. Moreover, FTS had no direct impact on trypsin activation in isolated pancreatic acinar cells. These results indicate that Ras signalling controls CXC chemokine formation, neutrophil recruitment and tissue injury in severe AP. Thus, our findings highlight a new signalling mechanism regulating neutrophil recruitment in the pancreas and suggest that inhibition of Ras signalling might be a useful strategy to attenuate local and systemic inflammation in severe AP.

1. Introduction

One tenth of all patients with acute pancreatitis (AP) develop a severe form associated with a high mortality rate (Mann et al., 1994). The most feared complication in AP is lung failure. Although the exact mechanisms remain elusive, it has been reported that interleukin-6 (IL-6) and high-mobility group box 1 (HMGB1) play a role in promoting lung damage in severe AP (Luan et al., 2013; Zhang et al., 2013). However, treatment of patients with severe AP is mainly limited to supporting vital functions. The lack of more specific therapies is partly related to an incomplete knowledge of the underlying pathophysiology. Trypsin activation, leucocyte recruitment and microvascular perfusion failure are well-accepted components in the induction of severe AP (Regner et al., 2008; van Acker et al., 2006; Zhang et al., 2009). Numerous studies have shown that neutrophil accumulation is a rate-limiting step in AP. For example, blocking specific adhesion molecules necessary for neutrophil trafficking, such as P-selectin, intercellular adhesion molecule-1 (ICAM-1) and lymphocyte function-associated antigen-1 (LFA-1) reduces tissue damage in the inflamed pancreas (Awla et al., 2011a; Hartman et al., 2012; Werner et al., 1998). Moreover, tissue accumulation of neutrophils is also coordinated by secreted chemokines, including CXCL1 and CXCL2 (Bacon and Oppenheim, 1998; Li et al., 2004). CXCR2 is the high affinity receptor on neutrophils for CXCL1 and CXCL2 (Huber et al., 1991) and targeting CXCR2 has been found to protect against tissue damage in AP (Bhatia and Hegde, 2007). Considered together, the role of adhesion molecules and chemoattractants in regulating neutrophil infiltration in the inflamed pancreas is relatively well described, however, the knowledge about signalling mechanisms orchestrating pathological inflammation in pancreatitis is limited.

We have recently shown that Rho-kinase regulates trypsin activation and neutrophil recruitment in severe AP (Awla et al., 2011b). Rho-kinase is one of many effector molecules acting downstream of the Ras supefamily, including more than 50 different small GTPases (Gutkind and Vitale-Cross, 1996). These small GTPases act as molecular switches regulating important

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cellular functions, including vesicle transport, cytoskeleton organization, adhesion, migration and survival (Alblas et al., 2001; Itoh et al., 1999; Slotta et al., 2006). GTPases are attached to the inner side of plasma membranes where they convert inactive Ras-guanosine-diphosphate (GDP) into active Ras-guanosine-triphosphate (GTP) (Ehrhardt et al., 2002; Hancock, 2003). Farnesylthiosalisylic acid (FTS) is a synthetic derivative of carboxylic acid, resembling the farnesyl cysteine group common to all small GTPases (Nevo et al., 2011). FTS interferes with the binding of activated Ras proteins to their escort proteins and therefore inhibits Ras–plasma membrane interactions. Although Ras signalling has mainly been associated with cell growth and oncogenic transformation, recent reports suggest that Ras is also involved in the pathogenesis of arthritis, nephritis, autoimmune diseases and infections (Clarke et al., 2003; Kafri et al., 2005; Katzav et al., 2001). However, the potential effect of FTS on inflammation and tissue injury in AP has not been investigated.

Based on the above, the aim of the present study was to define the functional significance of Ras signalling in regulating trypsin activation, CXC chemokine production, neutrophil accumulation and tissue damage in severe AP.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (20–25 g) were housed on a 12–12 h light dark cycle and fed a laboratory diet and water ad libitum. All experiments were approved by the local ethical committee at Lund University. Mice were anesthetized intraperitoneally (i.p.) with 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight.

2.2. Acute pancreatitis

Through a midline laparotomy in anaesthetized animals were the second part of duodenum and papilla of vater identified. Traction sutures were placed 1 cm from the papilla and a small puncture was made through the duodenal wall (23 G needle) in parallel to the papilla of vater. A polyethylene catheter connected to a microinfusion pump (CMA/100, Carnegie Medical, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and 1 mm into the common bile duct. The common hepatic duct was temporarily clamped at the liver hilum. 10 μl of 5% sodium taurocholate (Sigma, St. Louis, MO, USA) was infused into the pancreatic duct for 10 min. Then, the catheter and the common hepatic duct clamp were removed. The duodenal puncture was closed with a purse-string suture. Traction sutures were removed and the abdomen was closed. Using this protocol we observed a total mortality rate of 5% within 24 h of pancreatitis induction. Sham mice underwent laparotomy and phosphate buffered saline (PBS) infusion into the pancreatic duct and were pretreated i.p. with vehicle (PBS, $n=5$). Vehicle (PBS) or the Ras inhibitor, FTS (10 mg/kg, Cayman Chemical, Boston, USA) was given i.p. prior to bile duct cannulation and induction of AP. One group of mice received 10 mg/kg of FTS alone without bile duct cannulation. This dose and scheme of administration of FTS was based on a previous investigation (Zhang et al., 2014). All animals were killed 24 h after induction of pancreatitis.

2.3. Amylase measurements

Blood amylase levels were determined in blood collected from the tail vein by use of a commercially available assay (Reflotron®, Roche Diagnostics GmbH, Mannheim, Germany).

2.4. Myeloperoxidase (MPO) activity

A piece of the pancreatic head and lung tissue were harvested for MPO analyses. All frozen tissues were pre-weighed and homogenized in 1 ml mixture (4:1) of PBS and aprotinin 10,000 KIE per ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenates were centrifuged (15,339g, 10 min) and the supernatant was stored at −20 °C. The pellet was used for MPO assay as previously described (Laschke et al., 2007). All pellets were mixed with 1 ml of 0.5% hexadecyltrimethylammonium bromide and the samples were frozen for 24 h. Thawed samples were sonicated for 90 s, incubated in a water bath at 60 °C for 2 h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25 °C). Values are expressed as MPO units per g tissue.

2.5. Histology

Tissue pieces from the head of the pancreas were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. 6 μm sections were stained (haematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system, including oedema, acinar cell necrosis, haemorrhage and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scales as previously described (Schmidt et al., 1992).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Pancreatic levels of CXCL1 and CXCL2 as well as plasma levels of IL-6 and HMGB1 were determined by use of a double-antibody Quantikine enzyme linked immunosorbent assay kits (R & D Systems Europe, Abingdon, UK) using recombinant murine CXCL1, CXCL2, IL-6 and HMGB1 as standards. The minimal detectable protein concentration is less than 0.5 pg/ml.

2.7. Mac-1 expression on neutrophils

Blood was collected in acid citrate dextrose and incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce non-specific labelling. Neutrophils were stained with APC-conjugated anti-Mouse Ly-6C (Clone: AL-21, BD Biosciences Pharmingen, San Jose, CA, USA), phycoerythrin-conjugated anti-mouse Ly-6G (clone 1A8, BD Biosciences) and fluorescein isothiocyanatee-conjugated anti-mouse CD11b (clone M1/70, BD Biosciences) antibodies. Cells were fixed and erythrocytes were lysed. Neutrophils were recovered following centrifugation and defined as $Ly\text{-}6G^+/Ly\text{-}6C^+$ cells. Flow-cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with Cell-Quest Pro software (BD Bioscience).

2.8. Acinar cell isolation

Acinar cells from the pancreas were prepared by collagenase digestion and gentle shearing as described previously (Saluja et al., 1999). Cells were suspended in HEPES–Ringer buffer (pH 7.4) and passed through a 150 μm cell strainer (Partec, Görlitz, Germany). Isolated acinar cells (1×10^7 cells per well) were immediately preincubated with PBS or FTS (10 or 100 μM, 30 min) and stimulated with cerulein (100 nM, 30 min) in duplicates. The buffer was then discarded and the cells were washed twice with buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3-(morpholino) propanesulphonic acid and 1 mM MgSO4. The cells were next

homogenized in cold (4 °C) 5 mM 3-(morpholino) propanesulphonic acid buffer using a potter Elvehjem-type glass homogenizer. The resulting homogenate was centrifuged (56g, 5 min), and the supernatant was used for assay. Trypsin activity was measured flourometrically using Boc–Glu–Ala–Arg–AMC as substrate as described previously (Kawabata et al., 1988). For this purpose, a 200 μl aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$ and 0.1% bovine serum albumin, pH 8.0). The reaction was initiated by the addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin levels (pg/ml) were calculated using a standard curve generated by assaying purified trypsin. Viability of the pancreatic acinar cells was higher than 95% as determined by trypan blue dye exclusion.

2.9. Statistical analysis

Data are presented as mean values ± S.E.M. Statistical evaluations were performed by using non-parametrical tests (Mann–Whitney). $P < 0.05$ was considered significant and n represents the number of animals.

3. Results

3.1. Ras signalling regulates tissue injury in AP

To study the role of Ras signalling in severe AP, blood amylase levels were initially determined as an indicator of tissue injury. Taurocholate challenge enhanced blood amylase levels by 22-fold (Fig. 1). Administration of the Ras inhibitor FTS reduced taurocholate-provoked blood amylase levels from 1216 ± 154.6 μKat/L down to 260.9 ± 146.6 μKat/L, corresponding to a 82% decrease (Fig. 1). Normal pancreatic microarchitecture was found in sham mice (Fig. 2), whereas taurocholate challenge caused destruction of the pancreatic tissue structure, characterized by acinar cell necrosis, haemorrhage, oedema formation and neutrophil accumulation (Fig. 2). We found that inhibition of Ras signalling decreased taurocholate-induced tissue damage (Fig. 2). For example, FTS reduced oedema and necrosis by 50% and 49%, respectively, in taurocholate-exposed animals (Fig. 2).

3.2. Ras signalling regulates neutrophil recruitment in AP

MPO activity was used as used as an indicator of neutrophil infiltration in the inflamed pancreas. Taurocholate challenge enhanced pancreatic levels of MPO by 4-fold (Fig. 3A).

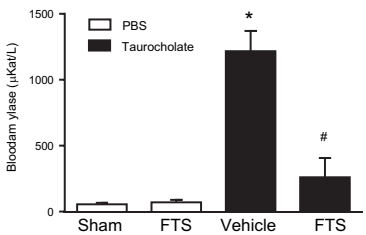

Fig. 1. Blood amylase levels (μKat/L) in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the Ras inhibitor FTS (10 mg/kg). Separate animals received FTS (10 mg/kg) alone. Blood samples were obtained 24 h after pancreatitis induction. Data represent means ± S.E.M. and $n=5$. $^{*}P < 0.05$ vs. Sham and $^{\#}P < 0.05$ vs. Vehicle + Taurocholate.

Administration of FTS decreased taurocholate-induced pancreatic activity of MPO by 42% (Fig. 3A). Moreover, it was found that challenge with taurocholate increased the levels of CXCL1 and CXCL2 in the pancreas by 3-fold and 8-fold, respectively (Fig. 3B and C). Administration of FTS decreased CXCL1 and CXCL2 levels in the inflamed pancreas by more than 80% (Fig. 3B and C).

3.3. Ras signalling regulates systemic inflammation in AP

As part systemic inflammation in severe AP, activated neutrophils accumulate in the lung. Indeed, we found that taurocholate challenge increased MPO levels in the lung by 3-fold (Fig. 4A). Inhibition of Ras signalling decreased MPO levels in the lung by 72% in mice exposed to taurocholate (Fig. 4A). Moreover, challenge with taurocholate enhanced plasma levels of IL-6 by 50-fold and HMGB1 by 3-fold (Fig. 4B and C). Pretreatment with FTS decreased plasma levels of IL-6 and HMGB1 by more than 79% in animals exposed to taurocholate (Fig. 4B and C). However, inhibition of Ras activity had no effect on taurocholate-induced levels of Mac-1 on neutrophils (Fig. 4D).

3.4. Trypsin activation in acinar cells

Acinar cells were isolated from the pancreas of mice and incubated with cerulein to study the role of Ras signalling in trypsin activation. We found that cerulein stimulation enhanced trypsin activation by 3-fold compared to unstimulated cells (Fig. 5). However, preincubation of the acinar cells with FTS had no effect on secretagogue-induced trypsin activation (Fig. 5).

4. Discussion

This study shows for the first time that Ras signalling regulates pathological inflammation in severe AP. Our findings indicate that Ras is involved CXC chemokine formation and neutrophil recruitment in the inflamed pancreas. We found that inhibition of Ras signalling decreased pancreatic tissue damage and serum amylase levels in AP. Moreover, Ras inhibition reduced accumulation of neutrophils in the lung as well as systemic formation of IL-6 and HMGB1 in animals with AP. Thus, targeting Ras signalling may be an effective way to control both local and systemic inflammation in severe AP.

It is widely held that Ras signalling plays a central role in oncogenesis by regulating the functional activity of ERK 1/2, Akt and RalA proteins in tumour cells (Schubbert et al., 2007). Interestingly, accumulating data suggest that Ras signalling also exert important functions in inflammatory conditions, such as arthritis, nephritis and autoimmune diseases (Clarke et al., 2003; Kafri et al., 2005; Katzav et al., 2001). Ras proteins are ubiquitously expressed in mammalian cells and activation of cell surface receptors promotes localization of Ras proteins to the inner side of plasma membranes by the conversion of inactive Ras-GDP into active Ras-GTP (Ehrhardt et al., 2002; Hancock, 2003). FTS is a unique and potent competitive inhibitor of Ras. FTS interacts with Ras anchorage domains leading to dislodgment from the membrane and accelerated degradation of Ras (Kloog and Cox, 2000; Kloog et al., 1999; Marom et al., 1995). In the present study, we observed for the first time that inhibition of Ras signalling by use of FTS decreases amylase levels in plasma and tissue injury in the pancreas of mice exposed to taurocholate, indicating that Ras constitute an important signalling pathway in the pathophysiology of local tissue damage in severe AP. This notion is also indirectly supported by a previous study demonstrating that targeting farnesyltransferase, an upstream regulator of Ras activation, had the capacity to attenuate taurocholate-induced tissue damage in

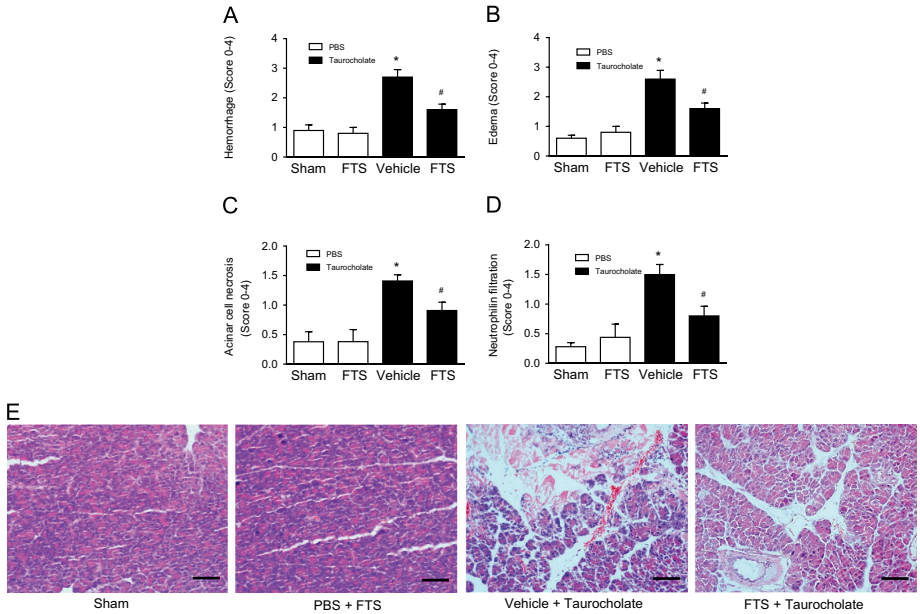

Fig. 2. Ras regulates tissue damage in AP. (A) Haemorrhage (B) oedema formation (C) acinar cell necrosis and (D) extravascular neutrophils in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the Ras inhibitor FTS (10 mg/kg). Separate animals received FTS (10 mg/kg) alone. (E) Representative haematoxylin and eosin sections of the pancreas. Samples were harvested 24 h after pancreatitis induction. Bars represent 100 μm. Data represent means ± S.E.M and $n=5$. $^{*}P<0.05$ vs. Sham and $^{\#}P<0.05$ vs. Vehicle + Taurocholate.

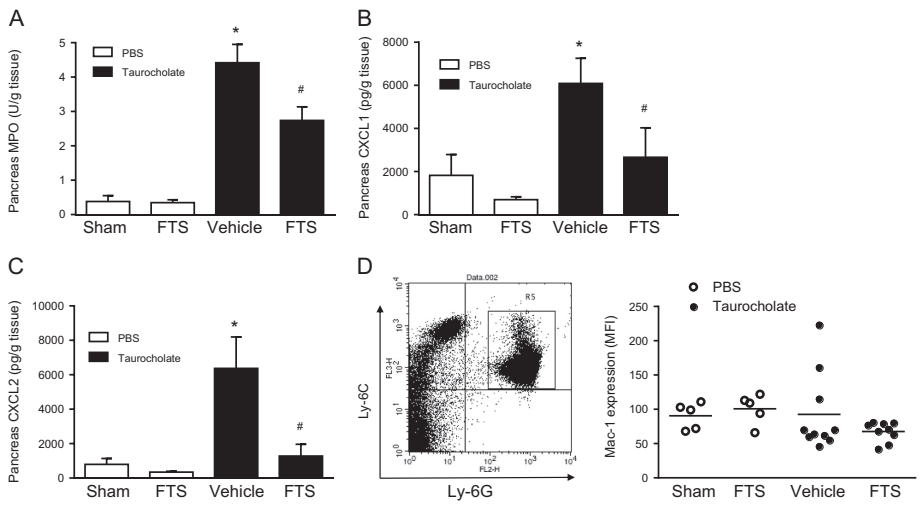

Fig. 3. Ras regulates taurocholate-induced neutrophil recruitment. Levels of (A) MPO, (B) CXCL1 and (C) CXCL2 in the pancreas as well as (D) Mac-1 expression on circulating neutrophils (Ly-6G^{+}/Ly-6C^{+} cells) in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the Ras inhibitor FTS (10 mg/kg). Separate animals received FTS (10 mg/kg) alone. Samples were harvested 24 h after pancreatitis induction. Data represent means ± S.E.M. and $n=5$. $^{*}P<0.05$ vs. Sham and $^{\#}P<0.05$ vs. Vehicle + Taurocholate.

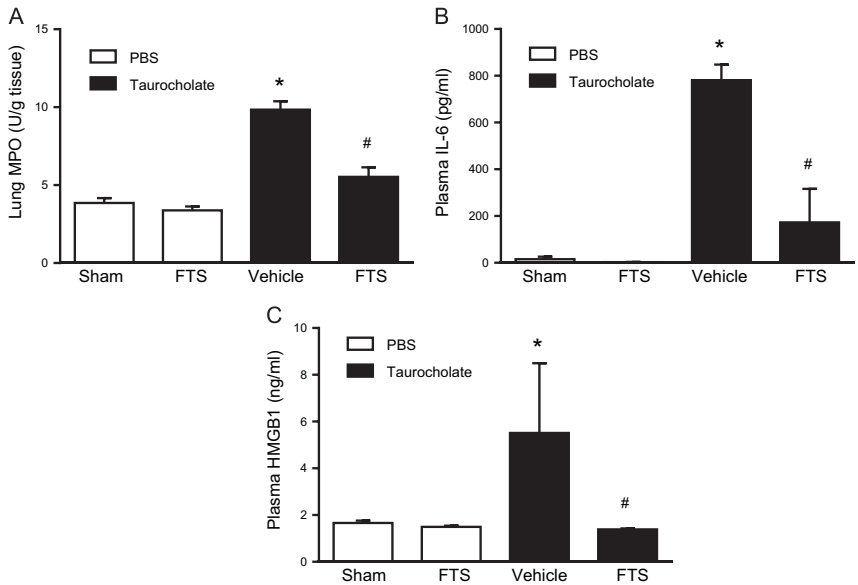

Fig. 4. Ras regulates taurocholate-induced systemic inflammation. MPO levels in the (A) lung as well as plasma levels of (B) IL-6 and (C) HMGB1 in sham (PBS) animals and taurocholate-exposed mice pretreated with vehicle or the Ras inhibitor FTS (10 mg/kg). Separate animals received FTS (10 mg/kg) alone. Samples were harvested 24 h after pancreatitis induction. Data represent means ± S.E.M and $n=5$. $^{*}P<0.05$ vs. Sham and $^{\#}P<0.05$ vs. Vehicle+Taurocholate.

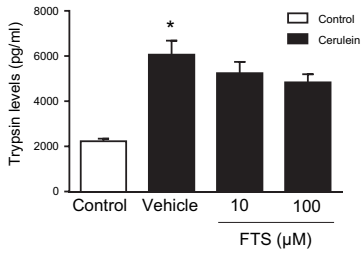

Fig. 5. Acinar cell activation of trypsinogen was measured in negative control cells and in cells exposed to cerulein with PBS or FTS (10 and 100 μM). Activation of trypsinogen activation was quantified by measuring enzymatic activity of trypsin fluorometrically by using Boc–Gln–Ala–Arg–MCA as the substrate as described in detail in Section 2. Trypsin levels were calculated using a standard curve generated by assaying purified trypsin. Data represent means ± S.E.M. and $n=5$. $^{*}P<0.05$ vs. negative control.

AP (Merza et al., 2014). Considered together, our findings add AP to the growing list of inflammatory diseases in which Ras inhibition appears to be of beneficial value. Moreover, systemic complications of severe AP include neutrophil accumulation in the lung (Pezzilli et al., 2009). Herein, it was observed that taurocholate challenge significantly enhanced pulmonary MPO activity. Notably, administration of FTS markedly decreased lung levels of MPO in animals exposed to taurocholate, indicating that Ras signalling also regulates systemic activation and recruitment of neutrophils in the lung in severe AP. It was recently reported that IL-6 constitute an important link between local inflammation in the pancreas and systemic inflammation and lung damage (Zhang et al., 2013). In the present study, we found that taurocholate greatly increased plasma levels of IL-6 and that treatment with FTS decreased IL-6 levels in the plasma by more than 79%. Whether this Ras-dependent formation of IL-6 plays a mechanistic role in lung injury and mortality in severe AP is a topic for further studies. HMGB1 is another marker of systemic inflammation and has been shown to promote lung injury in AP (Luan et al., 2013). Herein, we found that FTS significantly decreased plasma levels of HMGB1 in animals with pancreatitis. Taken together, these findings suggest that Ras not only regulates local inflammation but also systemic inflammatory responses in severe AP.

Convincing data have documented a key role of neutrophils in the induction of AP. For example, depletion of neutrophils markedly decreases tissue damage in AP (Abdulla et al., 2011). One recent study identified neutrophil-derived matrix metalloproteinase-9 as a potent stimulus for trypsin activation in acinar cells and tissue damage in AP (Awla et al., 2012). In the present study, we found that infusion of taurocholate into the pancreatic duct enhanced the activity of MPO and the number of extravascular neutrophils in the pancreas. Pretreatment with FTS substantially reduced the levels of MPO and the number of extravascular neutrophils in the inflamed pancreas, indicating that Ras signalling is an important regulator of neutrophil accumulation in AP, which helps to explain the tissue protective effects of FTS in the inflamed pancreas. Tissue localization of neutrophils is orchestrated by secreted CXC chemokines, such as CXCL1 and CXCL2 (Bacon and Oppenheim, 1998; Li et al., 2004). Indeed, functional inhibition of CXC chemokines has been shown to ameliorate tissue damage in AP (Bhatia and Hegde, 2007). We therefore analyzed pancreatic levels of CXC chemokines and found that challenge with taurocholate markedly enhanced CXCL1 and CXCL2 levels in the pancreas. Treatment with FTS attenuated CXCL1 and CXCL2 levels in the inflamed pancreas, indicating that Ras

signalling exert a regulatory function on the formation of CXC chemokines in AP. This Ras-dependent generation of CXC chemokines could also help to explain the inhibitory effect of FTS on neutrophil recruitment in AP. Adhesion to the inflamed endothelium is a precondition for neutrophil extravasation at sites of inflammation. Mac-1 is one of the important adhesion molecules mediating firm adhesion of neutrophils to activated endothelial cells (Asaduzzaman et al., 2008). Herein, it was observed that Mac-1 expression on neutrophils did not change significantly after taurocholate challenge. One reason might be that those neutrophils with Mac-1 up-regulation accumulate in the inflamed tissues and that the harvested blood contains neutrophils with less Mac-1 expression.

Trypsin activation is a key component in the induction of AP. In the present study, we examined if Ras signalling is involved in the activation of trypsin in isolated acinar cells. However, we observed that FTS had no effect on secretagogoue-evoked trypsin activation in acinar cells. Thus, Ras signalling seems not directly involved in trypsin activation in acinar cells. This conclusion is in line with a previous report showing that the inhibition of farnesyltransferase, an upstream regulator of Ras activation, had no impact on secretagogoue-provoked trypsin activation in acinar cells (Merza et al., 2014). These results suggest that the role of Ras signalling is more related to pro-inflammatory processes than proteolytic components in the pathophysiology of severe AP.

5. Conclusions

In conclusion, our findings suggest that Ras signalling regulates tissue damage in severe AP. These findings show that blocking Ras activity reduces CXC chemokine formation and neutrophil accumulation in the pancreas. In addition, Ras inhibition decreased systemic inflammation and lung recruitment of neutrophils in animals with pancreatitis. Thus, this work not only highlights a novel signalling mechanism in AP but also indicate that targeting Ras signalling could be effective in order to attenuate local and systemic inflammation in severe AP.

Authorship

Changhui Yu – performed experiments and contributed to the writing.

Mohammed Merza – performed experiments and contributed to the writing.

Lingtau Luo – performed experiments and contributed to the writing.

Henrik Thorlacius – conceived and designed the study and contributed to the writing.

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Paper IV

Neutrophil Extracellular Traps Induce Trypsin Activation, Inflammation and Tissue Damage in Mice with Severe Acute Pancreatitis

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Short title: Neutrophil extracellular traps and pancreatitis

Abbreviations: (AP) acute pancreatitis; (cf-DNA) cell free-deoxyribonucleic acid; (ELISA) enzyme-linked immunosorbent assay; (HMGB1) high-mobility groups protein 1; (IL-6) interleukin-6; (i.p.) intraperitoneal; (i.v.) intravenous; (Mac-1) macrophage-1 antigen; (MIP-2/CXCL2) macrophage inflammatory protein-2; (MMP-9) matrix metalloproteinase-9; (MPO) myeloperoxidase; (NETs) neutrophil extracellular traps; (PBS) phosphate buffered saline; 12-phorbol 13-myristate acetate (PMA); (PSA) polysialic acid; (ROS) reactive oxygen species; (RTU) relative trypsin units; (STAT3) signal transducer and activator of transcription 3

Conflict of interest: The authors state no conflict of interest

*Authors contributed equally to this work

Authorship: Hannes Hartman, Mohammed Merza, Milladur Rahman, Rundk Hwaiz, Erik Rehnström and Enming Zhang performed experiments, analyzed data and contributed to the writing. Sara Regnér and Henrik Thorlacius conceived and designed the study and contributed to the writing

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ABSTRACT

BACKGROUND & AIMS: Neutrophils are involved in development of acute pancreatitis (AP), but it is not clear how neutrophil-induced tissue damage is regulated. In addition to secreting antimicrobial compounds, activated neutrophils eliminate invading microorganisms by expelling nuclear DNA and histones to form extracellular web-like structures called neutrophil extracellular traps (NETs). However, NETs have been reported contribute to organ dysfunction in patients with infectious diseases. We investigated whether NETs contribute to development of AP in mice.

METHODS: AP was induced in C57BL/6 mice by infusion of taurocholate into the pancreatic duct or by intraperitoneal administration of L-arginine. Pancreata were collected and extracellular DNA was detected by Sytox green staining; levels of CXC chemokines, histones, and cytokines were also measured. Cell-free DNA was quantified in plasma samples. Signal transducer and activator of transcription 3 (STAT3) phosphorylation and trypsin activation were analyzed in isolated acinar cells. NET was depleted by administration of DNase I to mice. Plasma was obtained from healthy individuals (controls) and patients with severe AP.

RESULTS: Infusion of taurocholate induced formation of NETs in pancreatic tissues of mice and increased levels of cell-free DNA in plasma. Neutrophil depletion prevented taurocholate-induced deposition of NETs in the pancreas. Administration of DNase I to mice reduced neutrophil infiltration and tissue damage in the inflamed pancreas and lung, and decreased levels of blood amylase, CXCL2, interleukin-6, and high-mobility groups protein 1. In mice given taurocholate, DNase I administration also reduced expression of integrin α M (MAC1) on circulating neutrophils. Similar results were in mice with L-arginine–induced AP. Addition of NETs and histones to acinar cells induced formation of trypsin and activation of STAT3; these processes were blocked by polysialic acid. Patients with severe AP had increased plasma levels of NET components compared to controls.

CONCLUSIONS: NETs form in the pancreata of mice during development of AP and their levels increase in plasma of patients with AP. NETs regulate organ inflammation and injury in mice with AP, and might be targeted to reduce pancreatic tissue damage and inflammation in patients.

KEYWORDS: Chemokines, Histones, Inflammation, Leukocytes

INTRODUCTION

The clinical presentation of acute pancreatitis (AP) ranges from reversible and mild symptoms to a severe and life-threatening condition with systemic complications, such as lung damage and compromised gaseous exchange.[1, 2] In fact, severe AP with systemic inflammation and lung damage is associated with a mortality rate as high as 25%.[3] Due to limited understanding of the underlying pathophysiology, management of patients with severe AP poses a significant challenge to clinicians and is largely restricted to supportive therapies. It is generally considered that trypsin activation, leukocyte recruitment and impaired microvascular perfusion constitute integrated components in the pathophysiology of AP.[4-6] Indeed, convincing data have shown that neutrophil recruitment is a rate-limiting step in the development of AP. For example, depletion of neutrophils or inhibition of neutrophil infiltration by targeting specific adhesion molecules, including P-selectin, lymphocyte function antigen-1 (CD11a/CD18) and intercellular adhesion molecule-1 (CD54), ameliorates tissue damage in AP.[7-11] Extravascular accumulation of neutrophils in the pancreas is coordinated by secreted CXC chemokines, such as macrophage inflammatory protein-2 (CXCL2) and blocking CXC chemokine function decreases tissue injury in the inflamed pancreas,[12] further supporting a role of neutrophils in AP. It has been reported that neutrophil-derived reactive oxygen species (ROS) and enzymes, such as elastase and matrix metalloproteinase-9 (MMP-9) might contribute to neutrophil-mediated tissue damage in AP.[13, 14]

On top of those proposed mechanisms thus far, new aspects of neutrophil biology might help to explain how neutrophils cause tissue injury in the inflamed pancreas. Recent findings have demonstrated that activated neutrophils, beside secretion of antimicrobial compounds and phagocytic killing, can eliminate invading microorganisms by expelling nuclear DNA forming extracellular web-like structures decorated with nuclear histones as well as granular and cytoplasmic proteins.[15, 16] These DNA structures are referred to as neutrophil extracellular traps

(NETs).[15, 17, 18] On one hand, NETs have been reported to exhibit antimicrobial functions by trapping extracellular invading pathogens in the blood and tissues during infections.[17-20] On the other hand, NETs and their associated histones have been demonstrated to cause epithelial and endothelial cell damage.[21] In addition, intravascular deposition of NETs has been suggested to impair microvascular perfusion in endotoxemic liver injury.[19] However, the potential role of NETs in the activation of trypsin, neutrophil recruitment and tissue damage in AP is not known.

Based on the above considerations, we hypothesized herein that NET formation is not only increased but also might play a functional role in the pathophysiology of severe AP in mice. For this purpose, we used two different models of AP induced by taurocholate[22] or L-arginine.[23]

Materials and Methods

For a detailed version of this section, please see Supplementary Materials and Methods.

Animals

All experimental procedures were performed in line with recommendations of the Guide for the Care and Use of Laboratory Animals, published by the US national institutes of health and approved by the ethical committee at Lund University, Sweden. Male C57BL/6 mice (20-25 g) were housed under standardized conditions at 22°C in a 12-hour light dark cycle, fed a laboratory diet with water accessible *ad libitum*. Mice were anesthetized by intraperitoneal (i.p.) injection of 75 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kg body weight in 200 µl saline. Analgesia was obtained by subcutaneous injection of buprenophin hydrochloride (0.75 mg/kg, Schering-Plough, New Jersey, USA).

Induction of AP and Experimental Design

Induction of pancreatitis was performed as described previously[22]. Briefly, anaesthetized animals underwent midline laparotomy and the papilla of Vater was identified. A puncture through the duodenal wall, opposite to the papilla of Vater was made under microscopic observation using a 23 G needle. A polyethylene catheter connected to a microinfusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) was inserted one mm into the common bile duct. The common hepatic duct was identified and clamped with a microvessel clip at the liver hilum to prevent hepatic reflux. Ten µl of 5% sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA) or 0.9% sodium chloride was infused into the pancreatic duct at a rate of 2 µl/min. Methylene blue was used to enable visual control of pancreatic infusion. The catheter and the bile duct clip were removed and the duodenal puncture was closed before suturing of the abdominal wall. To

explore the effect of NET depletion, mice were divided into five groups: 1) sham; 2) sham + DNase I; 3) taurocholate + vehicle 4) taurocholate + DNase I and 5) taurocholate + anti-Ly6G antibody. DNase I (1000 U/mg, Pulmozyme, 5 mg/kg, Roche, Grenzach-Wyhlen, Germany) or phosphate buffered saline (PBS) was administered 10 minutes prior to induction of AP and 23 hours after taurocholate challenge. Using this protocol caused elevated plasma levels of DNase I throughout the experimental period of 24 hours (Supplementary Figure 1). Anti-Ly6G antibody (1A8, rat IgG2a, BioXcell, West Lebanon, NH, USA) was administrated i.p. 24 hours before induction of pancreatitis to deplete animals of neutrophils. Extracellular DNA was labelled by an intravenous (i.v.) injection of Sytox® Green nucleic acid stain (5 μM, Life Technologies Europe, Netherlands) 10 minutes before sacrificing the animals. Animals were killed 24 hours after pancreatitis induction and tail vein blood was obtained for systemic leukocyte differential counts and determination of blood amylase levels. Moreover, blood samples were collected from the inferior vena cava for measurement of cell-free (cf)-DNA, plasma CXCL2, interleukin-6 (IL-6), high-mobility groups 1 (HMGB1) protein and MMP-9. Pancreatic tissue was rapidly removed and separated in three for later analyses. One third was used for confocal microscopy and one third was snap-frozen in liquid nitrogen for biochemical quantification of pancreatic myeloperoxidase (MPO), CXCL2, histone 3 and histone 4 levels. One third was fixed in formalin for histological analysis. Lung tissue was harvested for pulmonary MPO measurement and morphological analyses. In separate experiments, pancreatitis was induced by two i.p. injections of L-arginine (4 g/kg) given at an interval of 1 hour and sacrificed 72 hours later[23]. L-arginine exposed animals were treated with DNase I or vehicle as above and saline-treated mice served as negative controls.

NET Generation In Vitro

Bone marrow neutrophils were isolated from the femur and tibia of healthy C57BL/6 mice using Ficoll-Paque research grade (Amersham Pharmacia Biotech, Uppsala, Sweden). Freshly

isolated bone marrow neutrophils (2 x 10^6 cells) were seeded on each well of a 6-well plate and incubated in RPMI 1640 medium containing 50 nM 12-phorbol 13-myristate acetate (PMA, Sigma) or PBS for 3 hours at 37°C as previously described.[24] The cells were then carefully washed three times in PBS and re-incubated for 30 minutes in PBS or PBS containing DNase I (pulmozyme, Roche) at a concentration of 20 µg/ml. Finally, cells were resuspended and centrifuged for 5 minutes (50 g) and the supernatant was used to assay activity of NETs on Mac-1 expression, ROS formation, STAT3 phosphorylation and trypsin activation.

Statistics

Data are presented as mean values ± SEM. Statistical evaluations were performed using non-parametrical tests (Mann-Whitney or ANOVA on ranks followed by student-Newman-Keuls test for pairwise comparisons). $P < .05$ was considered significant and n represents the number of animals.

Results

NET Formation in AP

NETs are composed of extracellular DNA, neutrophil-derived granule proteins and histones.[15, 21] The cell impermeable dye, Sytotox Green, was used to visualize extracellular DNA in the pancreas. We found that the neutrophil-derived granule protein MPO co-localized with extracellular DNA in the pancreas after infusion of taurocholate into the pancreatic duct, demonstrating that NETs are formed in the inflamed pancreas (Figure 1A). Moreover, depletion of neutrophils by use of an antibody directed against Ly6G (99.5% reduction in circulating neutrophils, Supplementary Figure 2) markedly reduced taurocholate-induced formation of NETs in the pancreas (Figure 1A). Quantification of extracellular DNA was subsequently performed in mice exposed to taurocholate and in mice depleted of neutrophils or treated with DNase I prior to taurocholate challenge. We found markedly increased deposition of extracellular DNA in the inflamed pancreas 6 hours (Supplementary Figure 3) and 24 hours (Figure 1B) after challenge with taurocholate. Administration of DNase I, which effectively catalyses hydrolysis of extracellular DNA, can be used to examine the impact of NET formation.[24, 25] Notably, treatment with DNase I decreased taurocholate-induced deposition of extracellular DNA by 98% in the pancreas (Figure 1A). To further support the notion that induction of pancreatitis is associated with formation of NETs, we determined levels of cf-DNA. It was observed that taurocholate challenge increased cf-DNA in plasma by more than 11-fold (Figure 1B). Treatment with DNase I decreased plasma levels of cf-DNA by 68% (Figure 1B). We next quantified tissue levels of histones in the pancreas. Pancreatic levels of histone 3 and histone 4 were low in sham animals (Figure 1C). Notably, infusion of taurocholate increased the levels of histone 3 and histone 4 by 5-fold and 3-fold, respectively, in the inflamed pancreas (Figure 1C). Treatment with DNase I significantly decreased histone 3 and histone 4 levels in the pancreas in mice exposed to taurocholate (Figure 1C). Administration of DNase I alone had no effect on NET formation in the pancreas (Figure 1). Moreover, we observed that treatment with DNase I had no effect on the

number of circulating neutrophils, i.e. circulating numbers of neutrophils were 1.9 x 10^6 per ml in vehicle-treated and 2.1 x 10^6 per ml in DNase I-treated animals. In addition, depletion of neutrophils markedly reduced taurocholate-induced deposition of extracellular DNA and histone levels in the pancreas as well as decreased plasma levels of cf-DNA (Figure 1).

NETs Regulate Tissue Damage in AP

Taurocholate challenge caused severe destruction of the pancreatic tissue structure, typified by extensive acinar cell necrosis, edema, infiltration of leukocytes, and hemorrhage (Figure 2A-E). Inhibition of NET formation significantly reduced taurocholate-provoked edema formation, leukocyte infiltration, and hemorrhage in the pancreas (Figure 2A-E). For example, NET inhibition attenuated taurocholate-induced edema formation by 53% in the pancreas (Figure 2C). Moreover, we found that severe AP induced by taurocholate was associated with significant lung tissue damage (Supplementary Figure 4). NET inhibition protected against taurocholate-induced lung damage (Supplementary Figure 4). Blood amylase levels are a useful indicator of tissue damage in the pancreas. Retrograde infusion of taurocholate in the pancreatic duct increased blood amylase levels by 10-fold, i.e. from 51.3 μKat/L to 532.8 μKat/L (Figure 3A). Administration of DNase I decreased taurocholate-induced blood amylase levels down to 308.5 μKat/L, corresponding to a 42% reduction (Figure 3A). Administration of DNase I alone had no effect on blood levels of amylase or tissue morphology in the pancreas or lung (Figure 2 and 3A, Supplementary Figure 4).

NETs Regulate Local and Pulmonary Recruitment of Neutrophils in AP

Tissue levels of MPO were used as an indicator of neutrophil infiltration. We found that challenge with taurocholate increased pancreatic MPO activity by 30-fold (Figure 3B). Treatment with DNase I reduced taurocholate-provoked pancreatic levels of MPO by 69% (Figure 3B). As part of a systemic inflammatory response in severe AP, activated neutrophils accumulate in the

lung. Indeed, it was found that challenge with taurocholate increased the MPO activity and the number of neutrophils in the alveolar space in the lung (Figure 3B, Supplementary Figure 4D). Administration of DNase I reduced MPO levels in the lung by 46% and bronchoalveolar lavage fluid neutrophils by 86% in mice exposed to taurocholate (Figure 3B). Moreover, taurocholate challenge increased CXCL2 levels in the pancreas by more than 40-fold (Figure 3C). Inhibition of NET formation attenuated tissue levels of CXCL2 by 75% in the inflamed pancreas (Figure 3C). In addition, we observed that Mac-1 was up-regulated on circulating neutrophils in animals with AP (Figure 3C). Inhibition of NET formation significantly decreased taurocholate-induced Mac-1 expression on neutrophils (Figure 3C). To determine if NETs can directly activate neutrophils, we isolated bone marrow neutrophils and stimulated them with NET isolated from PMA-activated neutrophils. It was found that NETs were a potent stimulator of Mac-1 expression and ROS formation in isolated neutrophils (Supplementary Figure 4). Co-incubation of neutrophils with DNase I significantly attenuated NET-induced Mac-1 up-regulation and ROS formation in neutrophils (Supplementary Figure 5). DNase I alone had no effect on neutrophil recruitment in the pancreas and lung as well as the levels of Mac-1 expression or reactive oxygen spices generation in isolated neutrophils (Supplementary Figure 5).

NET Activity Regulates Systemic Inflammation

Systemic inflammation in patients with severe AP is characterized by increased levels of pro-inflammatory compounds in the circulation. It was found that plasma levels of IL-6 and HMGB1 were low but detectable in sham animals (Figure 4). In contrast, taurocholate challenge increased plasma levels of HMGB1 by 7-fold, i.e. from 0.44 ± 0.11 ng/ml to 3.17 ± 0.19 ng/ml (Figure 4). Administration of DNase I reduced plasma levels of HMGB1 to 0.50 ± 0.09 ng/ml, corresponding to an 84% decrease (Figure 4). Moreover, it was found that the plasma levels of IL-6 were greatly increased in pancreatitis animals compared to sham mice (Figure 4). Interestingly, treatment with DNase I attenuated plasma levels of IL-6 by 64% in pancreatitis

animals (Figure 4). Plasma levels of CXCL2 were markedly elevated in pancreatitis mice (Figure 4). Injection of DNase I reduced taurocholate-induced plasma levels of CXCL2 by 45% (Figure 4). A recent study showed that neutrophil-derived MMP-9 might play an important role in AP.[13] We found that challenge with taurocholate increased plasma levels of MMP-9 by nearly 8-fold (Figure 4). Inhibition of NET formation reduced circulating levels of MMP-9 by 93% in animals exposed to taurocholate (Figure 4). Administration of DNase I alone had no effect on plasma levels of HMGB1, IL-6, CXCL2, or MMP-9 (Figure 4).

NETs Regulate Trypsin Activation in Acinar Cells

We next asked whether NETs might regulate trypsin activation in pancreatic acinar cells *in vitro*. For this purpose, we isolated acinar cells from the pancreas of mice. As a positive control, it was found that stimulation with caerulein, a well-known acinar cell secretagogue, increased trypsin activation by more than 5-fold compared to unstimulated acinar cells (Figure 5A). Notably, co-incubation of acinar cells with NETs derived from PMA-stimulated neutrophils enhanced trypsin activation by 5-fold (Figure 5A). This NET-induced trypsin activation was abolished by co-incubation of acinar cells with DNase I (Figure 5A). Signal transducer and activator of transcription 3 (STAT3) is an important signaling molecule in acinar cells.[26] It was found that NET stimulation markedly increased STAT-3 activity in acinar cells (Figure 5A) and that co-incubation with DNase I decreased NET-induced phosphorylation of STAT-3 by 48% (Figure 5A). To elucidate the relevance of histones as the major protein components of NETs structures[27], we next examined whether pure histones can activate acinar cells. We found that histone 2A, 2B, 3 and 4 increased trypsin activation by more than 3-fold in acinar cells, which is similar to the effect observed with caerulein (Figure 5B, Supplementary Figure 6A). Histones could also increase chymotrypsin activation in acinar cells (Supplementary Figure 6B). Moreover, we found that co-incubation with histone 3 and histone 4 also increased STAT3 phosphorylation in acinar cells (Figure 5B). Polysialic acid (PSA) is a highly negatively charged

glycan that directly binds to histones[28] and has been demonstrated to block NET- and histone-induced cell cytotoxicity.[21] Herein, we found that co-incubation with polysialic acid abolished NET-provoked trypsin activation in acinar cells (Figure 5C). Polysialic acid had no effect on caerulein-induced trypsin activation in acinar cells (Supplementary Figure 7). Knowing that histones exert toxic effects on epithelial and endothelial cells[21], we next examined the potential toxic effect of histones on acinar cell. Indeed, we observed that co-incubation with all core histone types reduced the number of viable acinar cells, suggesting that histones exert cytotoxic effects on acinar cells (Supplementary Figure 8).

L-Arginine-induced AP

Challenge with L-arginine caused wide-spread deposition of extracellular DNA deposition in the pancreas (Figure 6A and B) and increased the levels of cf-DNA in plasma (Figure 6C). It was found that administration of DNase I significantly decreased extracellular DNA deposition in the pancreas (Figure 6A and B) and plasma levels of cf-DNA (Figure 6C) in mice exposed to L-arginine. Moreover, treatment with DNase I significantly decreased L-arginine-induced amylase release (Figure 6D), MPO activity in the pancreas and lung (Figure 6E) as well as pancreatic tissue damage (Figure 6F).

NET formation in patients with AP

Our findings indicated that NET formation is important in AP and may therefore represent a potential therapeutic target. Therefore, we next examined the clinical relevance of these experimental findings in patients with severe AP. Serum was drawn at admission and 24 hours after admission from individuals with severe AP. Notably, we found that both levels of cf-DNA (Figure 7A) and DNA-histone complexes (Figure 7B) were significantly elevated in serum from patients with severe AP compared to healthy controls.

Discussion

Patients with severe AP pose a major challenge to clinicians due to the high mortality rate and lack of specific treatment. It is well-known that neutrophils play a key role in the development of inflammation in the pancreas although the mechanisms remain elusive. This study demonstrates for the first time that neutrophil-derived NETs constitute a central component in the pathophysiology in severe AP. Moreover, we also found that patients with severe AP showed increased plasma levels of NET components. Thus, therapeutic strategies directed against NET formation or function may provide a clinical benefit for patients with severe AP.

NETs are normally formed in response to infections to participate in the anti-bacterial defense of the host. However, accumulating reports have shown that NETs are formed also in sterile models of inflammation, such as thrombosis and endotoxemia.[19, 23] Herein, we provide evidence showing for the first time that induction of severe AP causes wide-spread DNA deposition in the inflamed pancreas, which co-localized with the neutrophil-derived granule protein MPO. This phenotype was abolished by depletion of neutrophils, indicating that NETs are the likely source of the extracellular DNA in the inflamed pancreas. Moreover, it was found that taurocholate challenge increased cf-DNA in plasma as well as histone 3 and histone 4 levels in the pancreas. Notably, depletion of neutrophils markedly decreased DNA deposition and levels of histones in the pancreas as well as cf-DNA levels in the plasma of animals exposed to taurocholate, suggesting that neutrophils are the likely source of extracellular DNA in AP. Numerous studies have shown that pharmacological inhibition by use of DNase I is an effective way to block formation of NETs both in vitro and in vivo.[21, 29] Indeed, we found that NET formation in the inflamed pancreas is susceptible to treatment with DNase I and therefore useful to explore the role of NETs in severe AP. Herein, administration of DNase I reduced the taurocholate-induced increase in serum amylase by 42% and pancreatic edema by 53%, suggesting that NETs regulate a significant part of the tissue damage in pancreatitis. Thus, NET formation plays an important role in the pathophysiology behind severe AP and targeting NET

induction or function might be a useful strategy in order to ameliorate tissue damage in the inflamed pancreas. This notion is also supported by our patient data showing that individuals with AP have increased plasma levels of cf-DNA and DNA-histone complexes, constituting components of NET.[15, 21] In this context, it is interesting to note that a recent study reported that NET-derived histones can directly cause epithelial and endothelial cell damage and death.[21] In light of this observation, our finding that NET degradation markedly decreased pancreatic levels of histone 3 and histone 4 might help to explain part of the beneficial effect of inhibiting generation of NETs in AP. Although the function of NETs in the host defense against microbial infection is somewhat controversial, this study adds AP to the list of non-infective diseases, such as transfusion-induced lung injury, endotoxemia, vasculitis and arthritis, in which excessive NET formation appears to be deleterious.[19, 30-32]

Numerous studies suggest that neutrophil infiltration is a key component in AP.[7,8, 33] For example, neutrophil depletion or inhibition of neutrophil recruitment by targeting specific adhesion molecules have repeatedly been shown to protect against tissue injury in pancreatitis.[9-11] In the present study, we found that taurocholate challenge increased MPO levels and the number of extravascular neutrophils in the inflamed pancreas. Administration of DNase I markedly decreased the activity of MPO and the number of extravascular neutrophils in the pancreas, suggesting that NETs are prominent regulators of neutrophil recruitment in the pancreas. In light of the important role of neutrophils in the pathophysiology of pancreatitis,[7, 8, 33] it might be forwarded that the tissue protective effect of inhibiting formation of NETs is related, at least in part, to decreased infiltration of neutrophils in the inflamed pancreas. Specific chemokines secreted from resident tissue cells co-ordinate tissue navigation of neutrophils and CXCL2 has been shown to play a significant role in AP.[12] It was therefore of interest to examine local formation of CXCL2 in the pancreas in the present study. It was found that taurocholate provoked a clear-cut increase in CXCL2 production in the pancreas. Notably, inhibition of NET formation decreased taurocholate-induced expression of CXCL2 by 75%, which may help

explain the attenuated accumulation of neutrophils in the inflamed pancreas. Although the detailed role of adhesion molecules in mediating leukocyte recruitment in the pancreas is relatively unclear, recent investigations have demonstrated that Mac-1 is an important adhesion molecule in facilitating extravascular accumulation of neutrophils at sites of inflammation. We observed that Mac-1 expression was markedly elevated on circulating neutrophils in AP and that DNase I treatment decreased pancreatitis-associated expression of Mac-1 on neutrophils, indicating that NETs play a role in Mac-1 up-regulation on neutrophils in AP. Considering that CXCL2 is a potent stimulator of neutrophil up-regulation of Mac-1[34, 35], it may be forwarded that the reduced levels of plasma CXCL2 in pancreatitis animals treated with DNase I might help to explain how NETs promoted neutrophil expression of Mac-1. Next, we wanted to examine if NETs can also activate neutrophils in a direct manner. Neutrophil-derived NETs were co-incubated with neutrophils and we observed that NETs not only increased expression of Mac-1 but also enhanced ROS production in isolated neutrophils. Moreover, co-incubation with DNase I attenuated NET-provoked expression of Mac-1 and ROS formation in neutrophils. These findings also indicate that NETs can directly activate neutrophils. Considered together with the findings above on CXCL2 formation, it could be suggested that NETs regulate neutrophil infiltration at two distinct levels, *i.e.* indirectly via formation of CXCL2 in the pancreas and directly via up-regulation of Mac-1 expression on neutrophils. Systemic complications of severe pancreatitis include pulmonary infiltration of inflammatory cells and tissue damage in AP.[12, 26] We observed that inhibition of NET formation decreased pulmonary tissue injury and recruitment of neutrophils in AP. In this context, it is interesting to note that a recent study reported that IL-6 seems to be an important link between local inflammation in the pancreas on one hand and systemic inflammation and lung damage on the other hand.[26] We therefore examined plasma levels of IL-6 in pancreatitis animals and found that taurocholate challenge markedly increased the circulating levels of IL-6. Notably, taurocholate-induced plasma levels of IL-6 were decreased by more than 64% in DNase I-treated animals, suggesting that NETs control systemic

levels of IL-6 in severe AP. Whether this NET-regulated formation of IL-6 plays a mechanistic role in pancreatitis-associated lung damage and mortality remains to be studied in the future.

It is widely held that trypsinogen activation is a critical feature in the pathophysiology of AP.[4, 5, 36] Thus, we asked whether NETs might be involved in the activation of trypsin in acinar cells. We found that co-incubation with neutrophil-derived NETs enhanced trypsin activation in acinar cells to levels similar to caerulein, a well-known trypsin secretagogoue. Interestingly, co-incubation with DNase I abolished NET-induced activation of trypsin in acinar cells. STAT3 is an important signaling molecule in acinar cells.[25] Herein, NETs were found to be a potent inducer of STAT3 activity, which is in line with the findings on trypsin activation. It should be mentioned that a recent study reported that neutrophil-derived MMP-9 is a potent inducer of trypsin activation in acinar cells.[14] Herein, we observed that inhibition of NET generation decreased plasma levels of MMP-9, which might also explain the pathological role of NETs in AP. We next asked whether histones, which are the dominating NET-associated proteins[26], could be involved in trypsin secretion from acinar cells. Indeed, we found that both histone 3 and histone 4 provoked both an increase in STAT3 phosphorylation and trypsin activation in acinar cells. Notably, we found that PSA, which binds to and blocks the function of histones[21, 28], abolished NET-induced trypsin activation in acinar cells, indicating that the trypsin activating potential of NETs is in great part mediated by histones. Although histones constitutes close to 70% of all proteins in NETs[27], these structures also contain other proteins, such as enzymes and anti-microbial peptides, which might also be involved in the activation of trypsin in acinar cells. Nonetheless, a pathophysiological role of histones in AP is also corroborated by a recent study showing that inhibition of histone 3 decreases neutrophil recruitment and tissue injury in L-arginine-induced AP.[37] In addition, NETs are known to exert cytotoxic effects[21] and we observed that all core histones (H2A, H2B, H3 and H4) exerted a direct cytotoxic effect on acinar cells but whether these effects completely explain the role of histones in AP or whether there are also specific receptors for histones on acinar cells will be important to address in future studies.

In conclusion, this study demonstrates for the first time that NETs are not only generated but also play a critical role in the development of severe AP. Inhibition of NET decreased CXCL2 formation and neutrophil recruitment in the inflamed pancreas. In addition, we show that NETs regulate STAT3 activity and trypsin activation in acinar cells and that histones might be important molecular mediators in these processes. Together, these findings identify a novel role of NETs in pancreatitis and suggest that targeting NETs might be a useful way to ameliorate local and systemic inflammation in severe AP.

Figure legends

Figure 1. NET formation in AP. A) Pancreatic tissue was stained with Sytox® Green and an antibody against the neutrophil-derived granule protein MPO in the inflamed pancreas. B) Quantification of extracellular DNA in the pancreas by measuring the relative area of fluorescence per high-power field. Plasma levels of cf-DNA were determined as described in Materials and Methods. C) Pancreatic levels of histone 3 and histone 4 were determined by ELISA. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.p. injections of the DNase I, an antibody directed against Ly6G (anti-Ly6G) or vehicle (saline) as described in Materials and Methods. Samples were collected 24 hours after induction of pancreatitis. Data represent means ± SEM and n = 4-7. $^{\#}P < 0.05$ versus control mice and $^{¤}P < 0.05$ versus taurocholate without DNase I or anti-Ly6G.

Figure 2. A) Representative hematoxylin & eosin sections of the head of the pancreas from indicated groups. Scale bar = 100 µm. Histological quantification of B) acinar cell necrosis C) edema D) hemorrhage and E) leukocyte infiltration is shown from samples collected 24 hours after induction of pancreatitis. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.p. injections of the DNase I or vehicle (saline) as described in Materials and Methods. Data represent means ± SEM and n = 4-10. $^{\#}P < 0.05$ versus control mice and $^{¤}P < 0.05$ versus taurocholate without DNase I.

Figure 3. Quantitative measurements of A) blood amylase levels B) pancreatic MPO, lung MPO and the number of neutrophils in the bronchoalveolar lavage fluid. C) Pancreatic levels of CXCL2 and Mac-1 expression on circulating neutrophils. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were

infused with saline alone. Animals were treated with i.p. injections of the DNase I or vehicle (saline) as described in Materials and Methods. Samples were collected 24 hours after induction of pancreatitis. Data represent means ± SEM and n = 4-6. $^{\#}P < 0.05$ versus control mice and $^{¤}P < 0.05$ versus taurocholate without DNase I.

Figure 4. Plasma levels of A) HMGB1 B) IL-6 C) CXCL2 and D) MMP-9. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.p. injections of the DNase I or vehicle (saline) as described in Materials and Methods. Samples were collected 24 hours after induction of pancreatitis. Data represent means ± SEM and n = 4-5. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus taurocholate without DNase I.

Figure 5. Trypsin activation and STAT3 phosphorylation. Trypsinogen activation was determined by measuring enzymatic activity of trypsin fluorometrically, using Boc-Glu-Ala-Arg-MCA as the substrate as described in detail in the Supplemental Material section. Trypsin levels were calculated using a standard curve generated by assaying purified trypsin and normalized to protein concentrations. Total and phosphorylated STAT3 were determined by western blot and the ratio of phosphorylated STAT3 divided by total STAT3 was quantified. A) Acinar cells were co-incubated with caerulein (grey bars) or neutrophil-derived NETs (black bars) with or without DNase I. STAT3 phosphorylation was analyzed after incubation with neutrophil derived NETs (black bars) with or without DNase I or saline (white bars) with or without DNase I. B) Acinar cells were co-incubated with caerulein (grey bars) or histone 3 and histone 4 (black bars). STAT3 phosphorylation was analyzed after incubation with histone 3 or histone 4 (black bars) or saline (white bars). C) Acinar cells were co-incubated with caerulein (grey bars) or neutrophil-derived NETs (black bars) with or without PSA. Data represent means ± SEM and n = 5. $^{\#}P < 0.05$ control cells and $^{¤}P < 0.05$ versus NET alone.

Figure 6. NET formation in L-arginine-induced AP. A) Intravascular administration of Sytox Green was used to visualize extracellular DNA in the inflamed pancreas. B) Quantification of extracellular DNA in the pancreas by measuring the relative area of fluorescence per high-power field. C) Plasma levels of cf-DNA, D) Blood amylase levels, E) pancreatic and lung MPO activity. F) Histological quantification of acinar cell necrosis, leukocyte infiltration, hemorrhage and edema were determined as described in Materials and Methods. Pancreatitis (black bars) was induced by i.p. challenge with L-arginine. Control mice (grey bars) were treated i.p. with saline alone. Animals were treated with i.p. injections of the DNase I or vehicle (saline) as described in Materials and Methods. Samples were collected 72 hours after induction of pancreatitis. Data represent means ± SEM and $n = 6$. $^{\#}P < 0.05$ versus control mice and $^{¤}P < 0.05$ versus L-arginine without DNase I.

Figure 7. NET components in patients with AP. Plasma was drawn at admission and 24 hours after admission from patients with severe AP. Plasma levels of A) cf-DNA and B) DNA-histone complexes. Healthy individuals served as controls. Data represent means ± SEM and $n = 10$. $^{\#}P < 0.05$ versus healthy controls.

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Supplementary Materials and Methods

Visualization and Quantification of NETs and Extracellular DNA

NETs are composed of extracellular DNA, neutrophil-derived granule proteins, and histones. The cell impermeable dye, Sytotox Green, was used to visualize extracellular DNA in the pancreas and co-localization of the neutrophil-derived granule protein MPO with DNA in the pancreas was used to visualize NET formation in the inflamed pancreas. Paraffin-embedded samples of the pancreas were sectioned (5 μm) and mounted on glass slides and blocked with 1% donkey serum and 3% bovine serum albumin. Specimens were subsequently incubated with a rabbit anti-MPO primary antibody (ab9535, Abcam, Cambridge, UK) overnight at 4°C in incubation buffer (1% bovine serum albumin, 0.3% triton X-100 and 0.01% sodium azide), followed by an anti-rabbit alexa-fluor 647 secondary antibody (ab150075, Abcam). Specimens were permeabilized by 0.3% triton X-during the time of primary antibody incubation. The permeabilized tissue sections were labeled with Sytox® Green nucleic acid stain just before the confocal microscopy. Confocal microscopy was performed using Meta 510 confocal microscope (Carl Zeiss, Jena, Germany) by a × 63 oil immersion objective (numeric aperture = 1.25). In separate experiments, extracellular DNA was labeled by i.v. injection of 100 μl of Sytox® Green nucleic acid stain (5 μM) was injected i.v. 10 minutes before harvesting the samples. Pancreatic tissue samples were collected in iced tubes and loaded onto 35 mm μ-dish (Ibidi, GmbH, Martinsried, Germany) with a thin bottom for high end microscopy. Images were taken in bright field within 30 minutes of sample collection. Confocal microscopy was performed using Meta 510 confocal microscopy by a × 63 oil immersion objective (numeric aperture = 1.25). Sytox® Green was excited by 488 nm laser line and corresponding emission wavelength was collected by the filter of 500-530 nm. The pinhole was ~1 airy unit and the scanning frame was 512×512 pixels. Background fluorescence was adjusted by changing contrast to minimize variations in each

image. Quantification of extracellular DNA in the pancreas was determined in dark field using ZEN2009 software and the area of fluorescence per high-power field was quantified.

Quantification of Circulating cf-DNA

To quantify levels of circulating cf-DNA, blood were collected from the inferior vena cava and diluted (1:10) in acid citrate dextrose. The samples were centrifuged at 15300 g for 10 minutes at 4°C and a Quant-iT™ PicoGreen® dsDNA kit (Invitrogen GmbH, Darmstadt, Germany) was used to quantify cf-DNA according to the manufacturers' instructions. The fluorescence intensity reflected the amount of DNA and was measured at excitation and emission wavelengths of 480 nm and 530 nm, respectively.

Amylase Measurements

Blood amylase levels were determined in blood collected from the tail vein by use of a commercially available assay (Reflotron®, Roche Diagnostics GmbH, Mannheim, Germany).

Systemic Leukocyte Counts

Tail vein blood was collected and mixed with Turks solution (1:20, Merck, Damnstadt, Germany) for leukocyte differential counts. Leukocytes were identified as mononuclear leukocytes (MNLs) or polymorphonuclear leukocytes (PMNLs) in a Burker chamber.

MPO Assay

A piece of the pancreatic head and lung tissue were snap-frozen in liquid nitrogen for biochemical quantification of MPO. Before analysis, tissues were pre-weighed and homogenized for 1 minute in a 1 ml mixture (4:1) of PBS and aprotinin containing 10 000 Kallikrein Inhibitor Units per ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany). The homogenates were centrifuged (16000 g, 10 minutes) and the supernatant was stored at −20°C for subsequent ELISA. Pellets were resuspended in 0.02 M PB pH 7.4, centrifuged and frozen overnight in 0.05 M PB, pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide before MPO assay. Briefly, samples were thawed, sonicated for 90 seconds, incubated in a water bath at 60°C for 2 hours and centrifuged for 5 minutes at 18800 g after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of hydrogen peroxide (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units/g tissue.

Histological Analysis

Tissue pieces from the head of the pancreas and lung were fixed in 4% formaldehyde overnight, dehydrated and embedded in paraffin. Sections (6 µm) were stained with hematoxylin and eosin and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system, quantifying edema, acinar cell necrosis, hemorrhage and neutrophil infiltration on a 0 (absent) to 4 (extensive) scales as previously described.[1] Lung injury was quantified in a blinded manner by adoption of a preexisting scoring system, including extent of alveolar collapse, thickness of alveolar septa, alveolar fibrin deposition and neutrophil infiltration graded on a 0 (absent) to 4 (extensive) scales.[2, 3]

ELISA

Pancreatic levels of CXCL2, histone 3 and histone 4 were determined in stored supernatants from homogenized pancreatic tissue by use of double-antibody ELISA kits (R&D Systems Europe, Abingdon, Oxon, UK and USCN, Life Science Inc., Burlington, NC, USA) according to manufacturers' instructions. Blood collected from the inferior vena cava was diluted (1:10) in acid citrate dextrose, centrifuged (15300 g for 10 minutes at 4°C) and stored at -20°C until use. Commercially available ELISA kits were used to quantify plasma levels of CXCL2 (R&D Systems Europe), IL-6 (R&D Systems Europe), MMP-9 (R&D Systems Europe) and HMGB1 (Chondrex, Redmond, WA, USA) according to manufacturers' instructions. DNAse I levels in plasma were measured by use of ORG 590 DNase Activity Immunometric Enzyme Immunoassay for the Quantitative Determination of DNase Activity (Orgentec, Mainz, Germany) according to the manufacturer's instruction.

Neutrophil Expression of Mac-1

Blood from the inferior vena cava was diluted (1:10) in acid citrate dextrose and incubated (10 minutes, room temperature) with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce non-specific labeling. Next, samples were incubated with phycoerythrin (PE)-conjugated anti-Gr-1 (clone RB6-8C5, rat IgG2b, eBioscience, Frankfurt, Germany) and fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 (clone M1/70, integrin αM, rat IgG2b, BD Biosciences Pharmingen, San Jose, CA, USA) antibodies. The labeled cells were fixed and erythrocytes were lysed. Neutrophils were recovered following centrifugation and data were acquired in a flow cytometer.

Mac-1 Expression and ROS Formation in Isolated Neutrophils

Bone marrow neutrophils were incubated with NETs (100 µl), DNase I-treated NETs (100 µl), PMA (100 nM) or DNase I-treated PMA (100 nM) for 15 minutes at 37°C. Cells were then fixed with 1% paraformaldehyde and stained with antibodies as described above. Flow-cytometric analysis was performed according to standard settings on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analyzed with Cell-Quest Pro software (BD Bioscience). Generation of ROS by neutrophils was detected by incubating with dihydrorhodamine 123 (Sigma-Aldrich). Briefly, isolated neutrophils (2.5×10^5 cells) were washed twice with HBSS (0.1% bovine serum albumin, without Ca^{2+} and Mg^{2+}) and incubated with 1 µM dihydrorhodamine 123 in a 96-well plate for 1 hour at 37°C. Then, neutrophils were incubated with NETs (100 µl), DNase I-treated NETs (100 µl), PMA (100 nM) or DNase I-treated PMA (100 nM). Fluorescence emission at 536 nm was monitored after 30 minutes in response to excitation at 488 nm by a fluorescence microplate reader (Spectramax Gemini, Molecular Devices, Sunnyvale, CA, USA).

Isolation of Pancreatic Acinar Cells

Pancreatic acinar cells were prepared by collagenase digestion as previously described.[5] HEPES-ringer buffer containing collagenase from Clostridium histolyticum type 1 (2.5 ml, 1%, Sigma-Aldrich) was gently infused into the pancreatic duct of male C57BL/6 mice. The animals were sacrificed through cervical dislocation and pancreatic tissue was collected. In order to achieve maximal exposure to collagenase, the pancreas was cut into pieces, gently shaken and incubated at 37°C for 15 minutes. The solute was then centrifuged and washed three times in

cold HEPES-Ringer buffer, pH 7.4 to stop digestion and remove the collagenase. Next, the acinar cells were suspended in cold HEPES-Ringer buffer and the solute was passed through a 150 µm cell strainer (Partec, Canterbury, England). The cell suspension was then divided in Eppendorf tubes and kept on ice until subsequent in vitro experiments involving protease activity.

Trypsin and Chymotrypsin Activation in Acinar Cells

Isolated acinar cells were stimulated with 100 nM caerulein (Sigma-Aldrich), NETs, DNase I-treated NETs, supernatant from non-stimulated neutrophils incubated with DNAse I or HEPES-Ringer buffer pH 7.4 for 30 minutes at 37°C. In separate experiments, acinar cells were exposed to NETs co-incubated with PSA (20 µg/ml, Sigma-Aldrich) or 100 µg/ml of purified histone 2A (BioNordica, Stockholm, Sweden), histone 2B (BioNordica**)**, histone 3 (Roche) or histone 4 (BioNordica). Next, the cells were centrifuged (56 g, 1 minute), washed twice and suspended in 250 mM sucrose, 5 mM 3-(N-morpholino) propanesulphonic acid buffer (pH 6.5). The cells were then homogenised with a potter-elvehjem type glass homogenizer. The homogenate was centrifuged (56 g, 5 minutes) and the supernatant was used for assay. Trypsin and chymotrypsin activity were measured fluorometrically as described previously using Boc-Glu-Ala-Arg-MCA and Suc-Ala-Pro-Phe-MCA, respectively, as substrates.[4] Supernatant from acinar homogenates was added to a 96-well plate (50 µl/well) and mixed with 75 µl TRIS buffer (50 nM TRIS, 450 nM $CaCl_2$ and 0.1% bovine serum albumin, pH 8.0). Addition of substrate initiated the reaction and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Levels of trypsin and chymotrypsin were determined using a standard curve generated from purified trypsin and chymotrypsin (Sigma-Aldrich) and the result was then normalized to the protein

concentration and expressed as relative trypsin units (RTU)/pg and relative chymotrypsin units (RCU)/pg, respectively.

Western Blot

NETs and acinar cells were isolated as described in materials and methods. Isolated acinar cells were incubated for 3 hours in suspensions containing 100 µg/ml of either histone 3 (Roche) or histone 4 (BioNordica). In separate experiments, acinar cells were co-incubated with 200 µl of NETs, DNase I-treated NETs or supernatant from non-stimulated neutrophils incubated with or without DNase I. Following incubation, the cells were centrifuged (56 g, 1 minute), washed twice and suspended in ice-cold lysing buffer (25 mM Tris HCl, pH 7.2, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40 and 5% glycerol) containing protease inhibitors (Halt Protease Inhibitor Cocktail, Pierce Biotechnology, Rockford, IL, USA) for 20 minutes before homogenization and centrifugation (16 000 g, 15 minutes at 4˚C). Protein concentration of the supernatant was determined by the Pierce BCA Protein Assay Reagent (Pierce Biotechnology). An aliquot of 25 µg of protein was mixed with 3X protein loading buffer and boiled for 5 minutes before loading onto a 10-12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blocked in Tris-Buffered Saline/Tween 20 buffer containing 5% non-fat dry milk powder. Protein immunoblots were performed using specific antibodies to phosphotyrosine (Tyr705) STAT3 and STAT3 (Cell Signaling Technology, Beverly, MA, USA). The membranes were further incubated with peroxidase conjugated secondary antibodies, and protein bands were visualized using a commercial chemiluminescence detection kit (ECL Plus; Amersham Biosciences, Piscataway, NJ, USA) as described by the manufacturer.

Histone Cytotoxicity

Isolated acinar cells were incubated in HEPES-Ringer buffer, pH 7.4 (60 minutes, 37°C) or HEPES-Ringer buffer, pH 7.4 containing 50 µg/ml of histone 2A, histone 2B, histone 3 or histone 4. Cells were then centrifuged (1 minute, 56 g) and resuspended twice in PBS. Cell suspensions were mixed with a solution of trypan blue (0.4% in PBS) in a 1:1 dilution. Histone induced cytotoxicity was assessed after 60 minutes by trypan blue exclusion test of cell viability.

Human Samples

The study was approved by the regional ethics committee at Lund University, Sweden (2009/413). Ten patients admitted to Scania University Hospital (Malmö, Sweden) with AP were included after oral and written informed consent. Blood samples were drawn at admission and 24 h after admission, placed in plasma separator tubes and centrifuged (2000 *g*, 25°C, 10 minutes) before plasma was frozen at -80°C. All patient samples were from patients fulfilling criteria for severe AP based on the Atlanta Classification.[6] Blood samples from ten healthy controls were handled as patient samples. Plasma levels of cf-DNA were measured using a flourogenic assay for double stranded DNA (Quant-IT PicoGreen dsDNA kit, Invitrogen GmbH, Darmstadt, Germany). Plasma levels of DNA-histone complexes were quantified by use of a sandwich Elisa based on monoclonal antibodies directed against histones and DNA (Cell Death Detection Elisa plus, Roche Diagnostics). All analyses were performed according to the manufacturer's instructions.

Supplementary References

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Figure 1

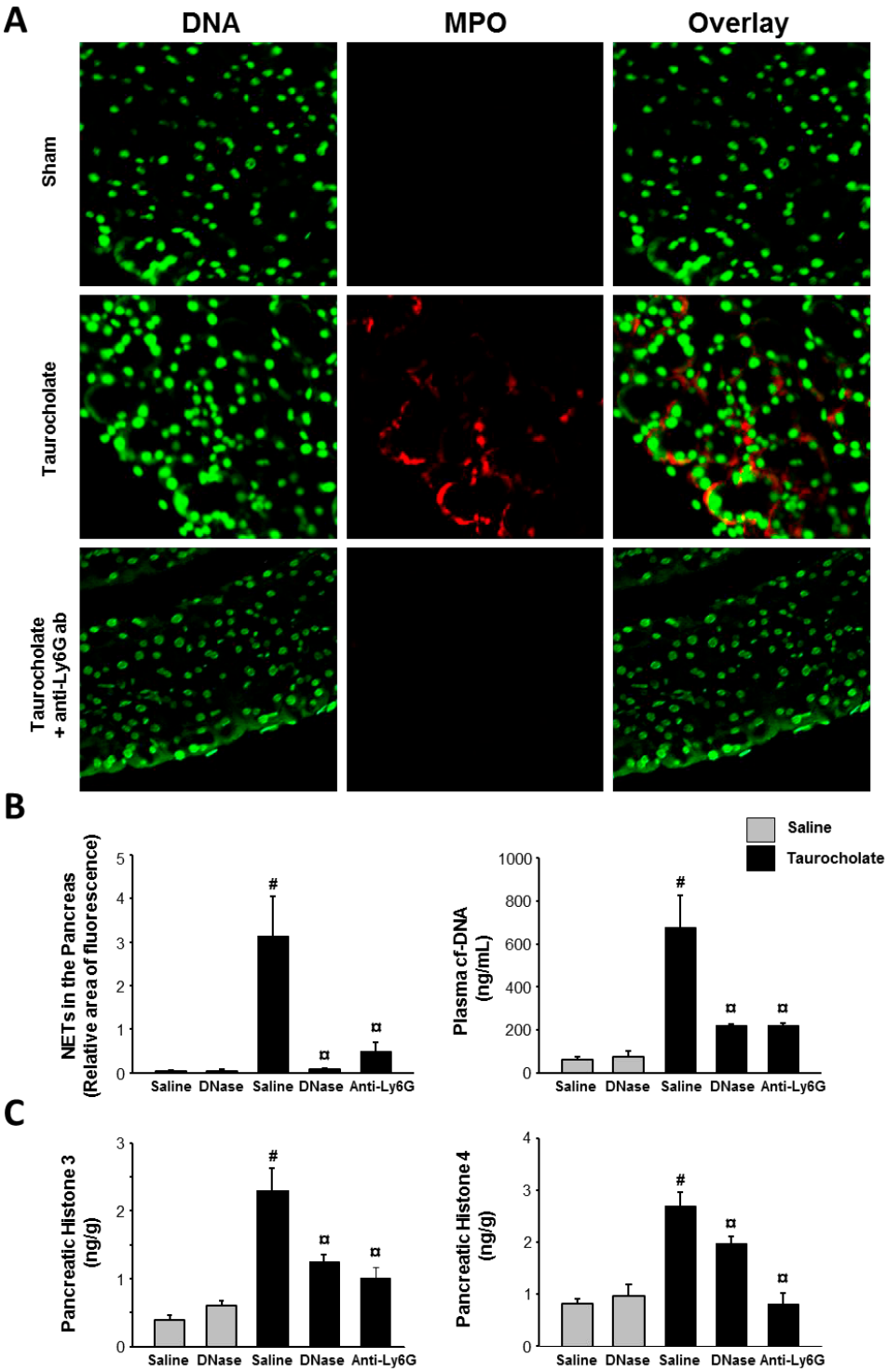

A
DNA
MPO
Overlay
Sham
Taurocholate
Taurocholate + anti-Ly6G ab
B
NETs in the Pancreas (Relative area of fluorescence)
Plasma cf-DNA (ng/mL)
Saline
Taurocholate
Saline DNase Saline DNase Anti-Ly6G
C
Pancreatic Histone 3 (ng/g)
Pancreatic Histone 4 (ng/g)

Figure 2

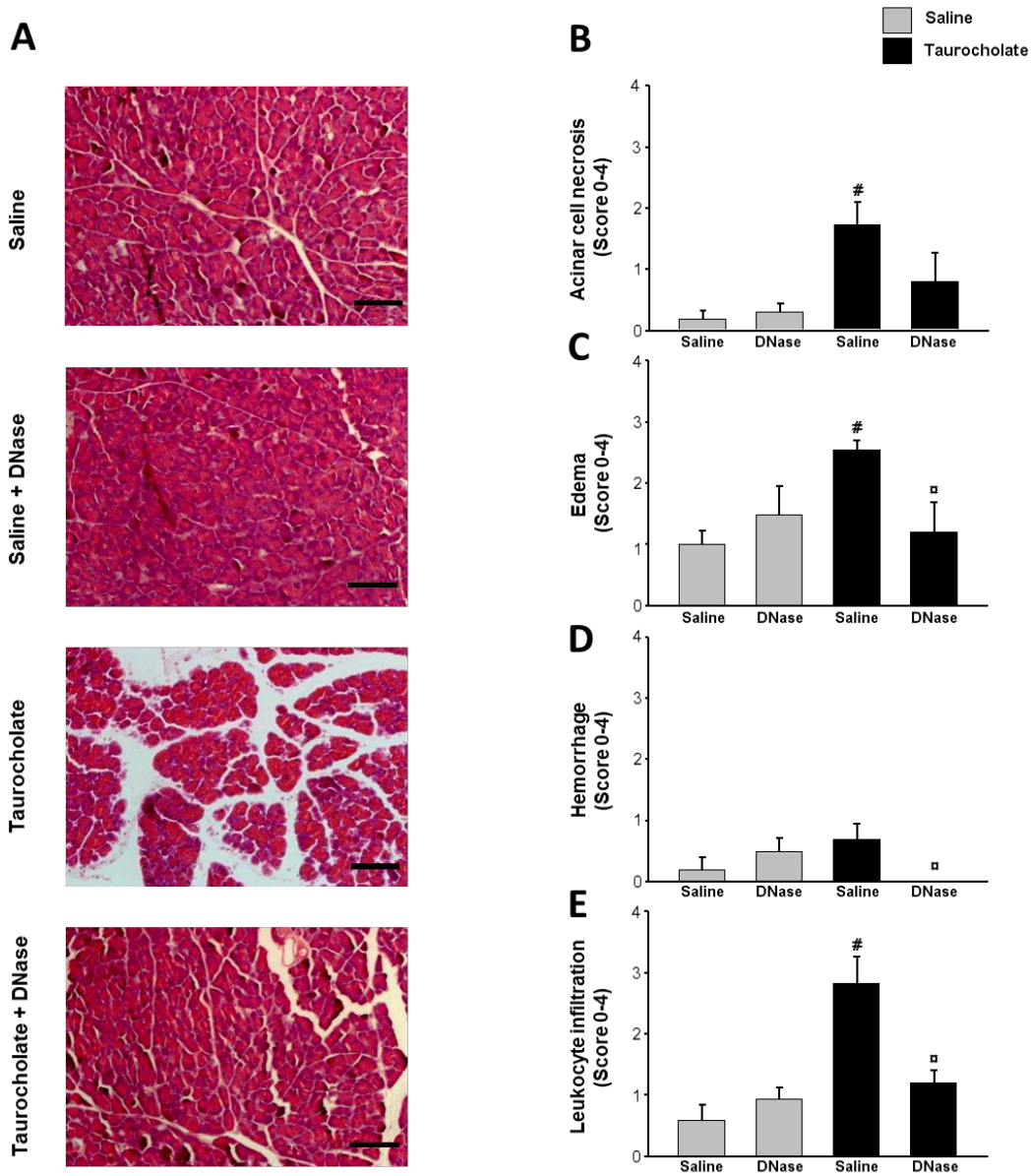

A
Saline
Saline + DNase
Taurocholate
Taurocholate + DNase
B
Saline
Taurocholate
Acinar cell necrosis (Score 0-4)
C
Edema (Score 0-4)
D
Hemorrhage (Score 0-4)
E
Leukocyte infiltration (Score 0-4)
Saline
DNase
Saline
DNase
#
¤

Figure 3

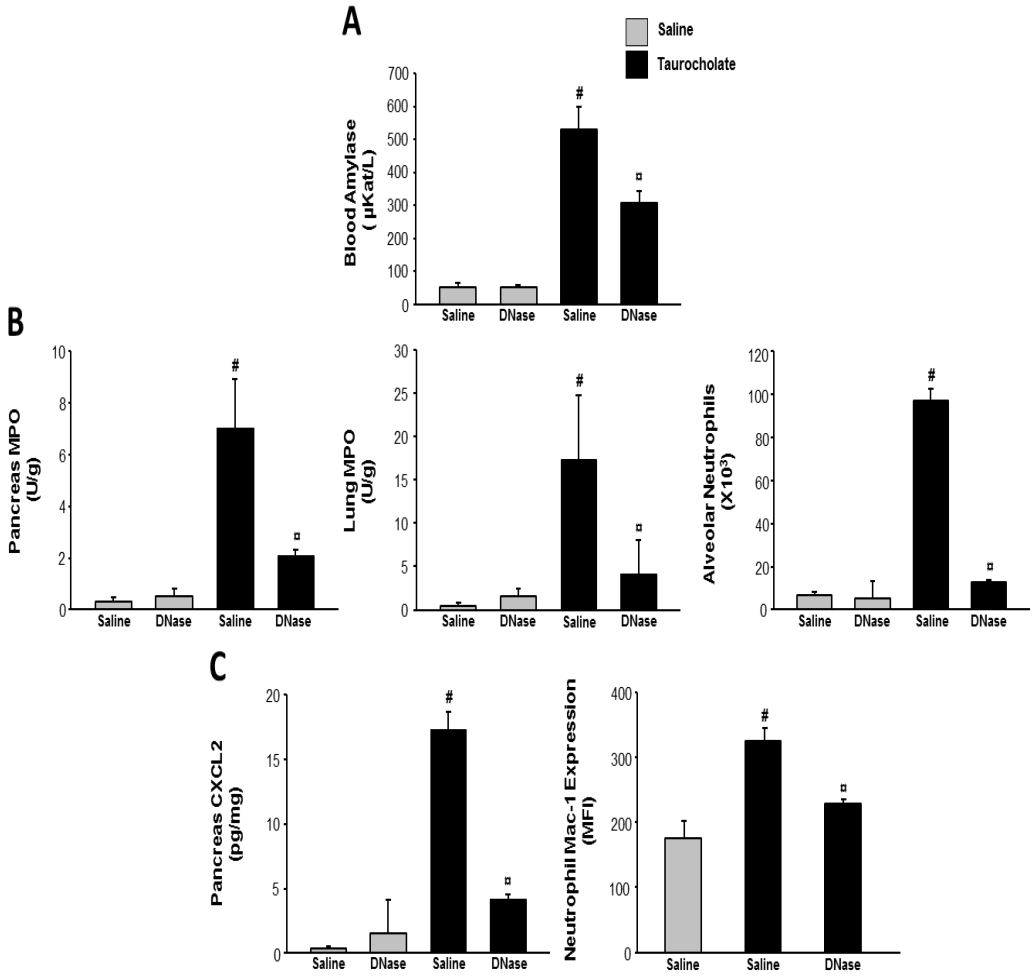

A
Saline
Taurocholate
Blood Amylase (µKat/L)
700
600
500
400
300
200
100
0
#
¤
Saline
DNase
Saline
DNase
B
Pancreas MPO (U/g)
10
8
6
4
2
0
#
¤
Saline
DNase
Saline
DNase
Lung MPO (U/g)
30
25
20
15
10
5
0
#
¤
Saline
DNase
Saline
DNase
Alveolar Neutrophils (X10³)
120
100
80
60
40
20
0
#
¤
Saline
DNase
Saline
DNase
C
Pancreas CXCL2 (pg/mg)
20
15
10
5
0
#
¤
Saline
DNase
Saline
DNase
Neutrophil Mac-1 Expression (MFI)
400
300
200
100
0
#
¤
Saline
Saline
DNase

Figure 4

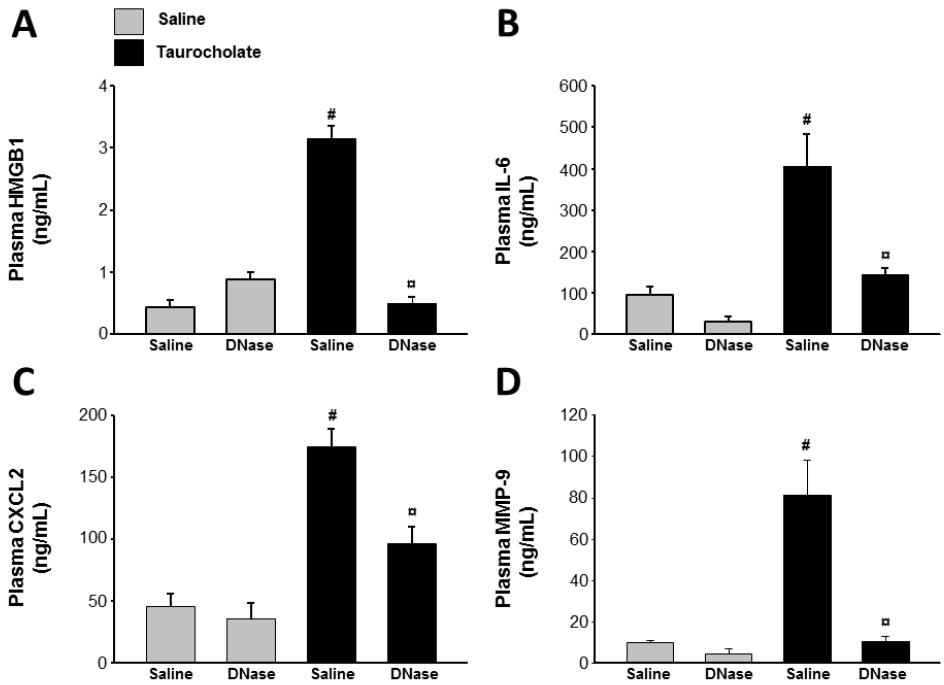

A
Saline
Taurocholate
Plasma HMGB1 (ng/mL)
0
1
2
3
4
#
¤
Saline
DNase
Saline
DNase
B
Plasma IL-6 (ng/mL)
0
100
200
300
400
500
600
#
¤
Saline
DNase
Saline
DNase
C
Plasma CXCL2 (ng/mL)
0
50
100
150
200
#
¤
Saline
DNase
Saline
DNase
D
Plasma MMP-9 (ng/mL)
0
20
40
60
80
100
120
#
¤
Saline
DNase
Saline
DNase

Figure 5

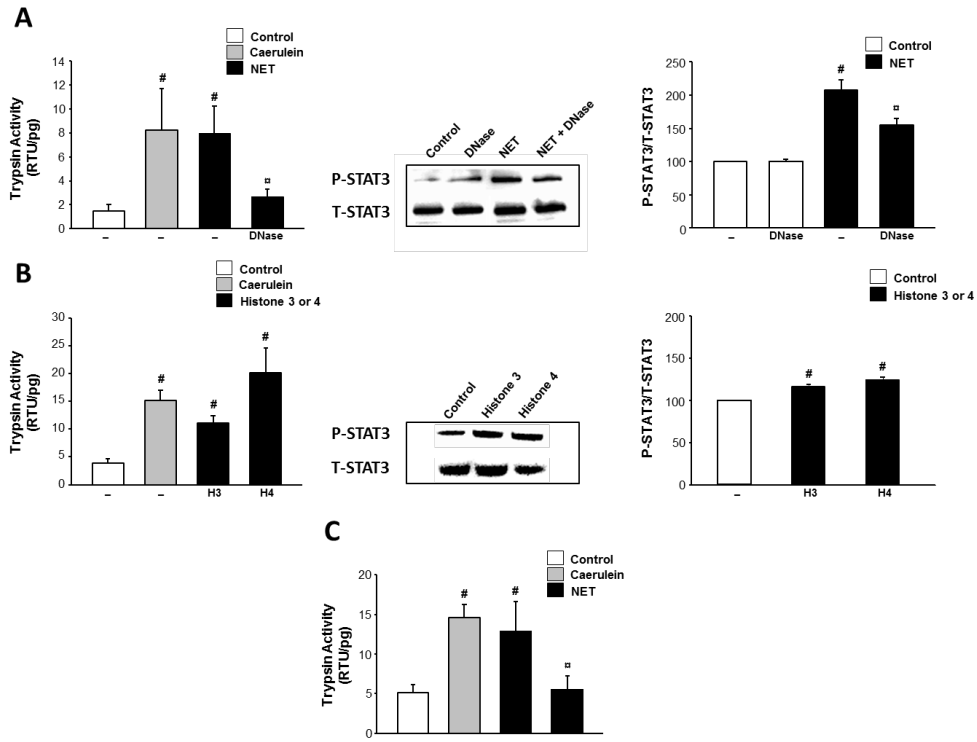

A
Control
Caerulein
NET
Trypsin Activity (RTU/pg)
DNase
Control
DNase
NET
NET + DNase
P-STAT3
T-STAT3
Control
NET
P-STAT3/T-STAT3
DNase
B
Control
Caerulein
Histone 3 or 4
Trypsin Activity (RTU/pg)
H3
H4
Control
Histone 3
Histone 4
P-STAT3
T-STAT3
Control
Histone 3 or 4
P-STAT3/T-STAT3
C
Control
Caerulein
NET
Trypsin Activity (RTU/pg)

Figure 6

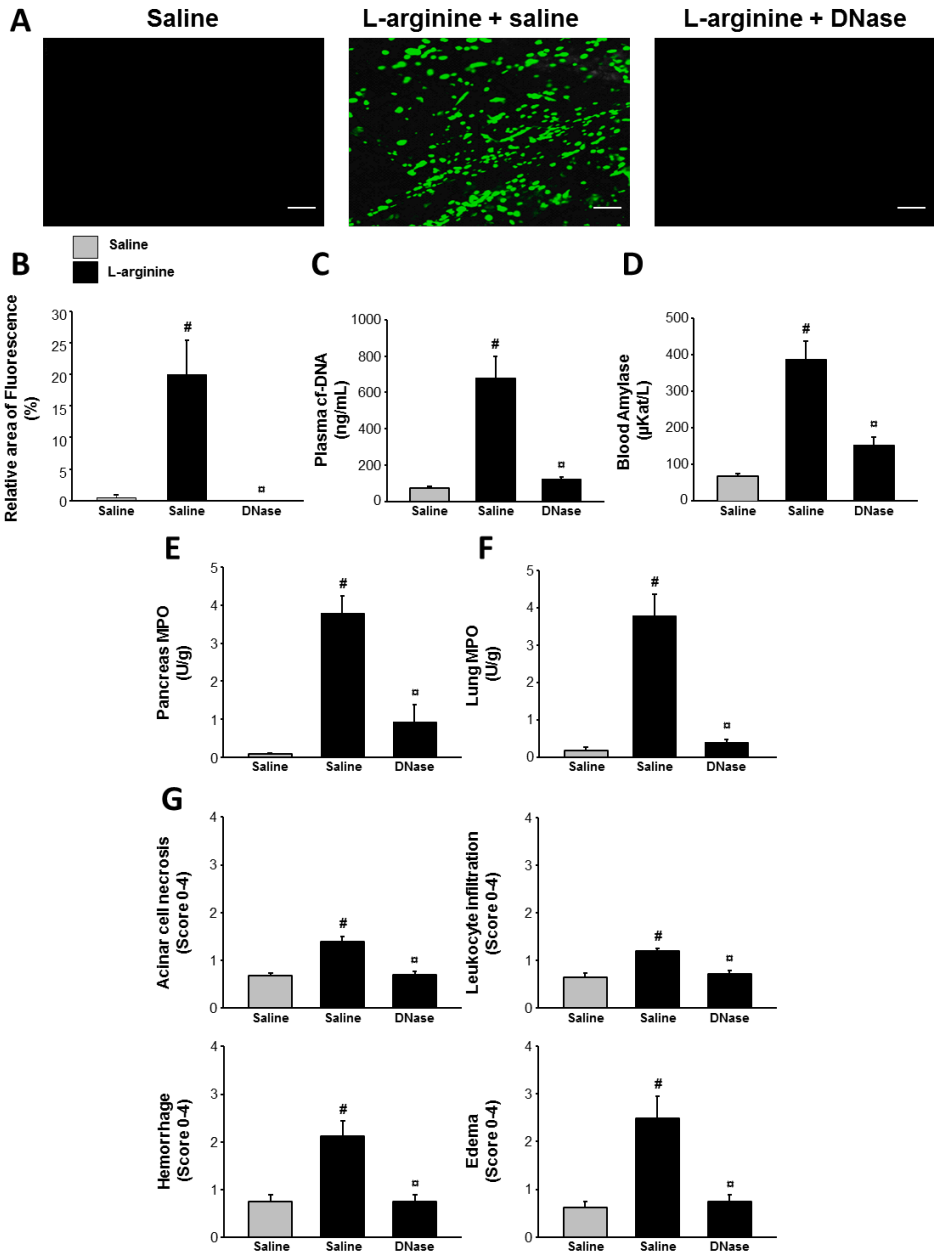
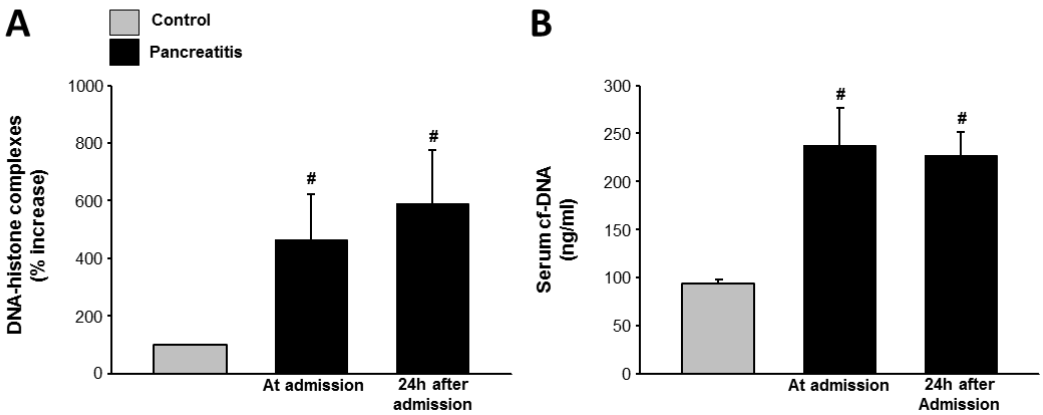
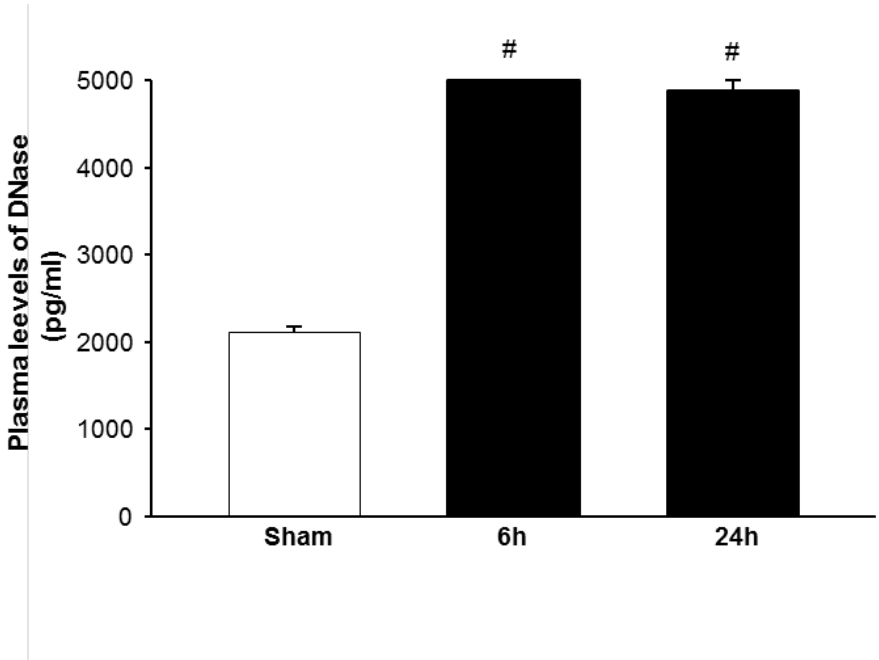

A
Saline
L-arginine + saline
L-arginine + DNase
B
Saline
L-arginine
Relative area of Fluorescence (%)
C
Plasma cf-DNA (ng/mL)
D
Blood Amylase (µKat/L)
E
Pancreas MPO (U/g)
F
Lung MPO (U/g)
G
Acinar cell necrosis (Score 0-4)
Leukocyte infiltration (Score 0-4)
Hemorrhage (Score 0-4)
Edema (Score 0-4)
Saline
Saline
DNase
#
¤

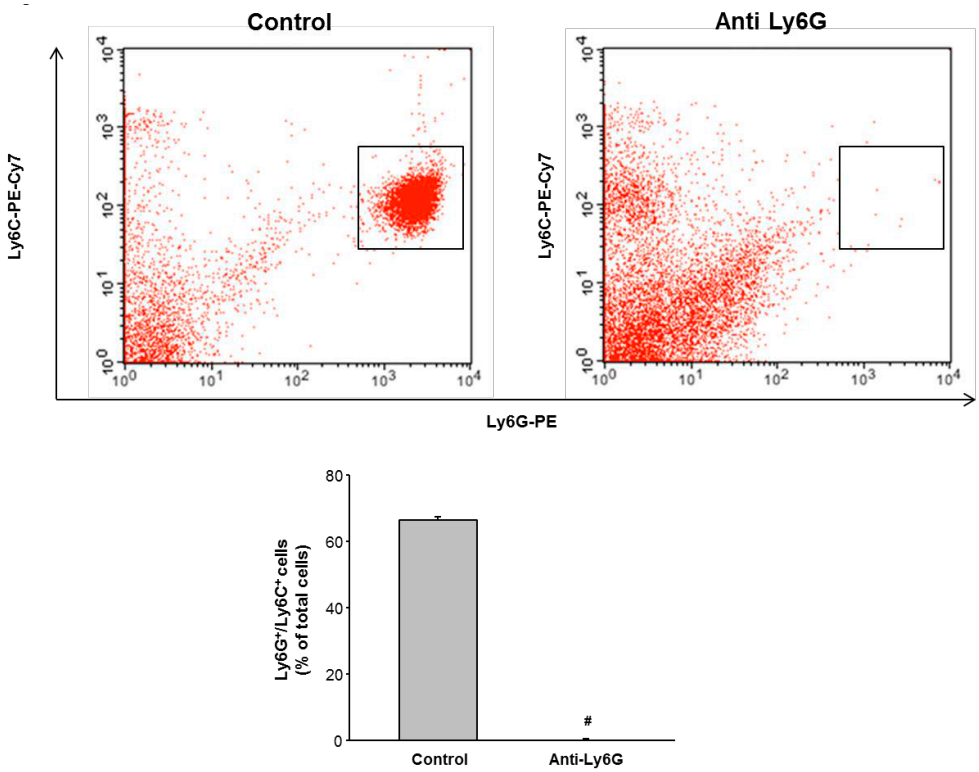
Figure 7


A
Control
Pancreatitis
DNA-histone complexes (% increase)
1000
800
600
400
200
0
#
#
At admission
24h after admission
B
Serum cf-DNA (ng/ml)
300
250
200
150
100
50
0
#
#
At admission
24h after Admission

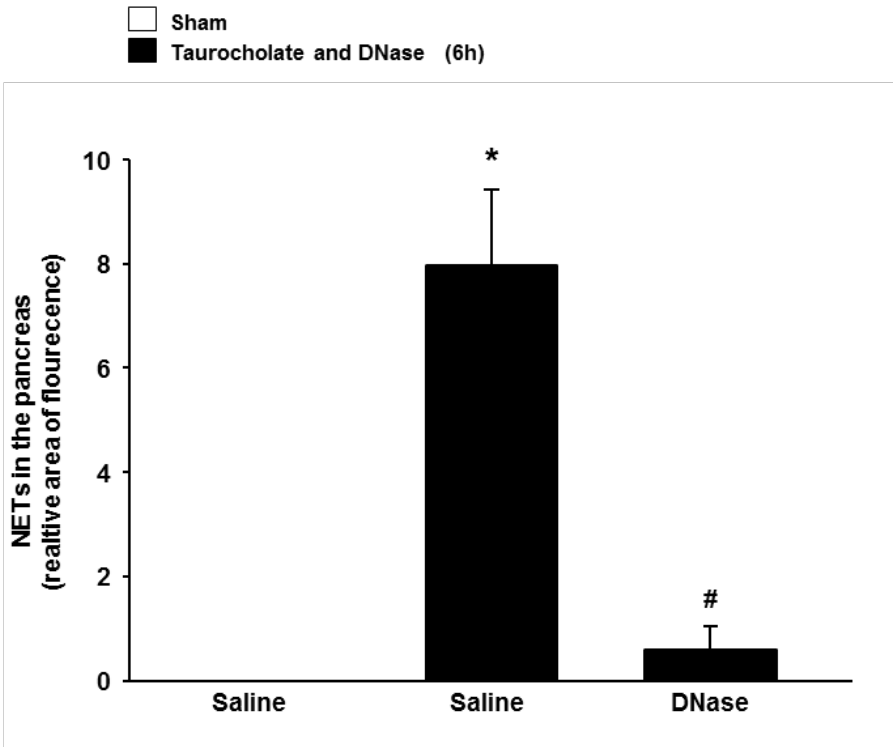
Suppl. Figure 1



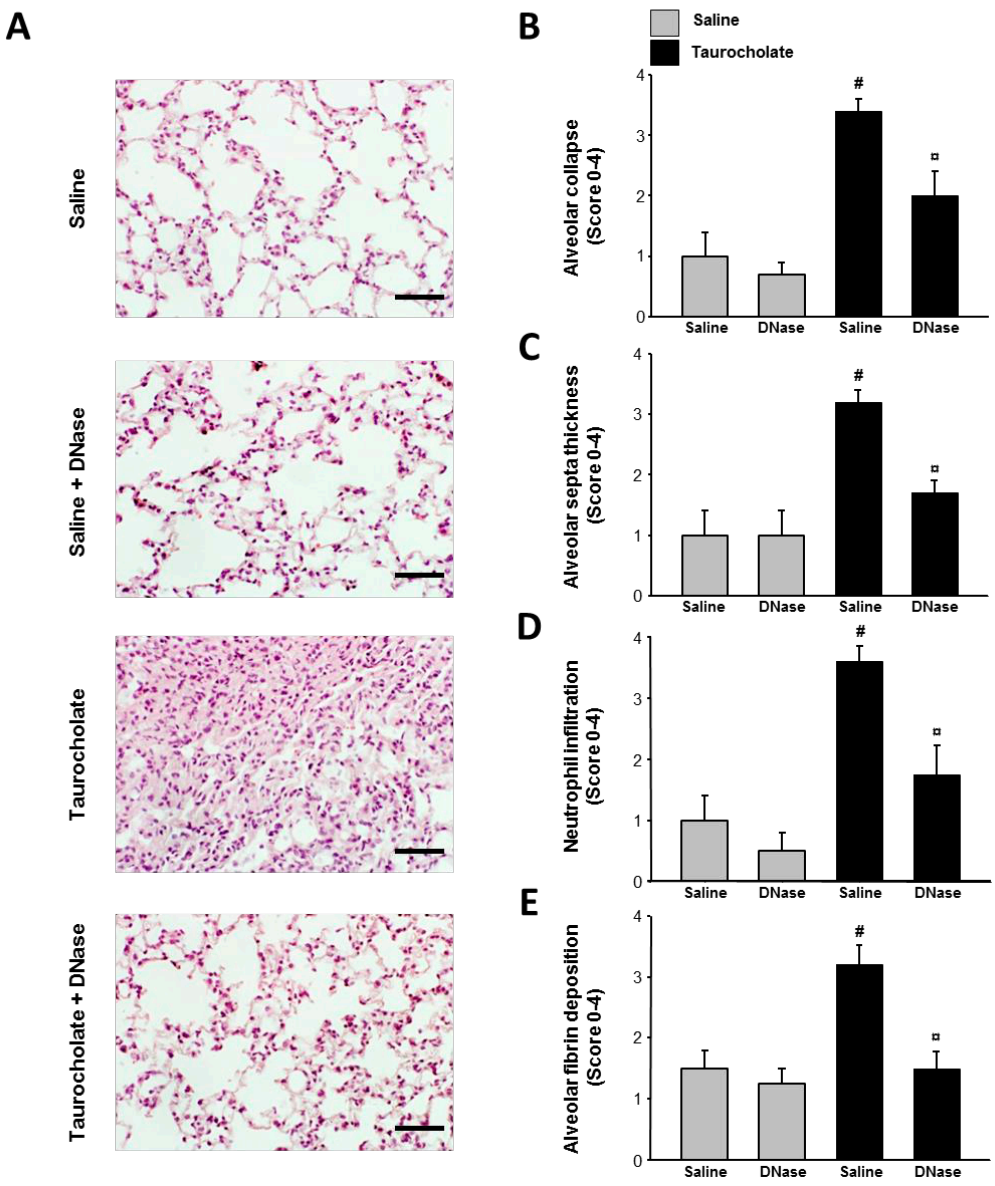
Suppl. Figure 2



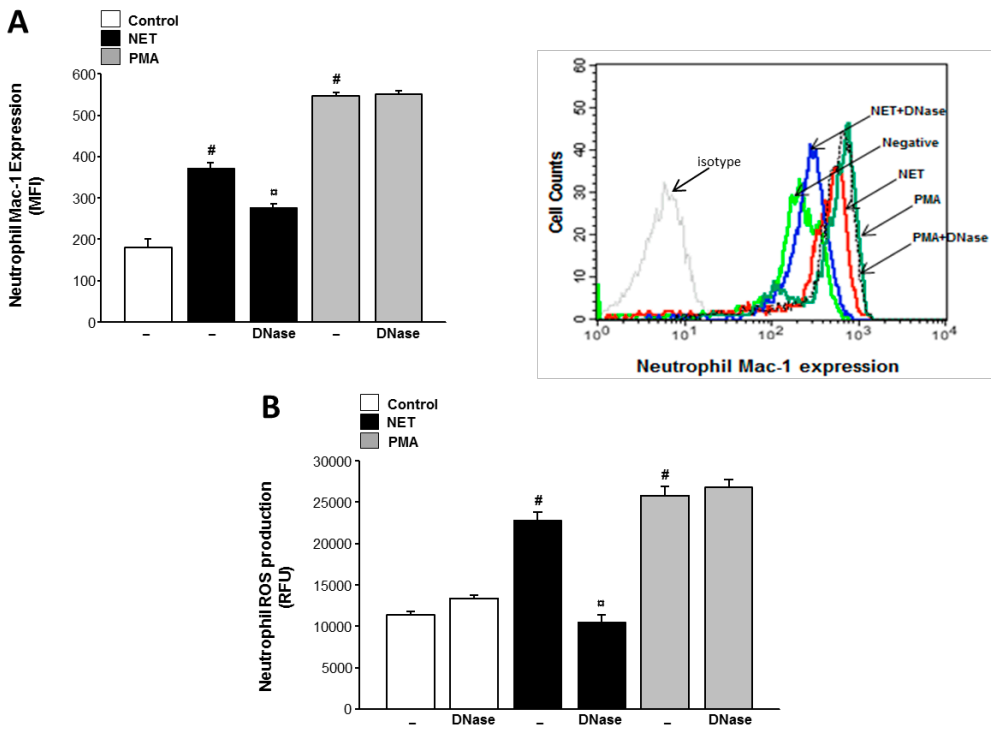
Suppl. Figure 3



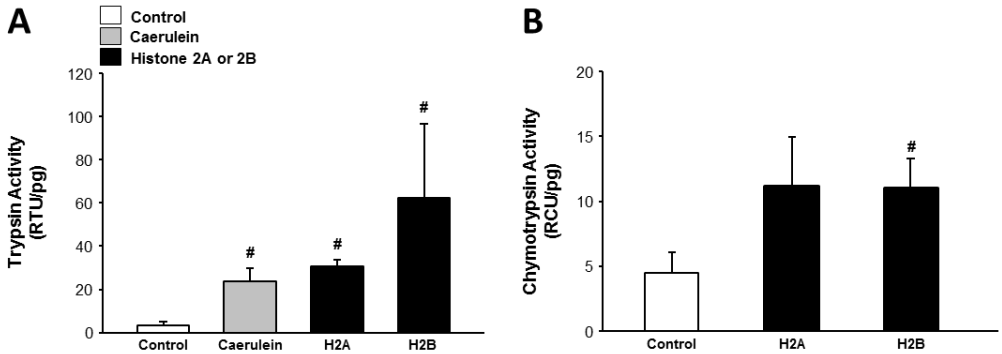
Suppl. Figure 4



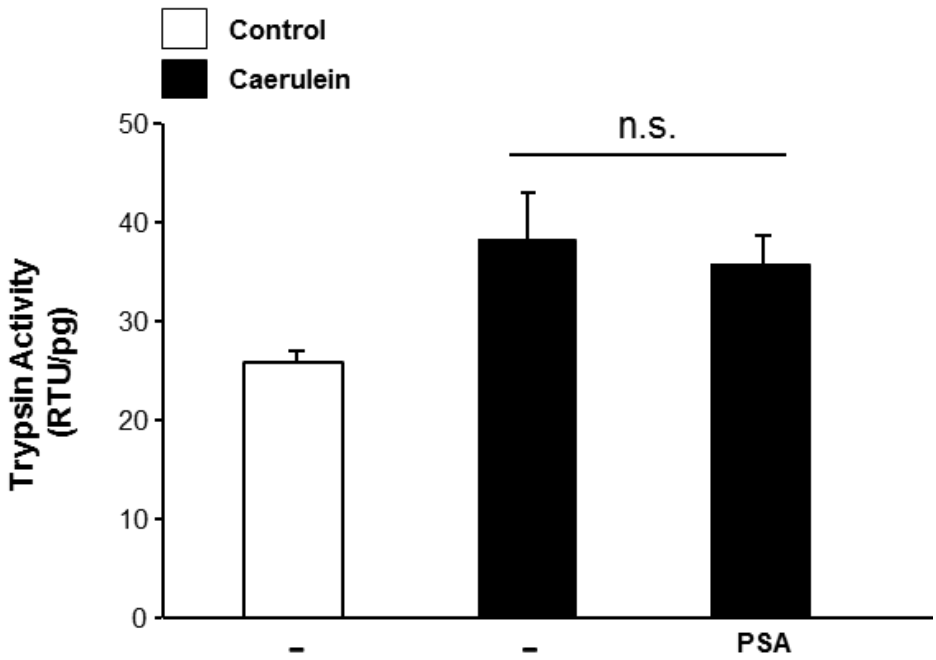
Suppl. Figure 5



Suppl. Figure 6



Suppl. Figure 7



Supp. Figure 8