On the Mechanism of Neutrophil Extracellular Traps in Acute Pancreatitis

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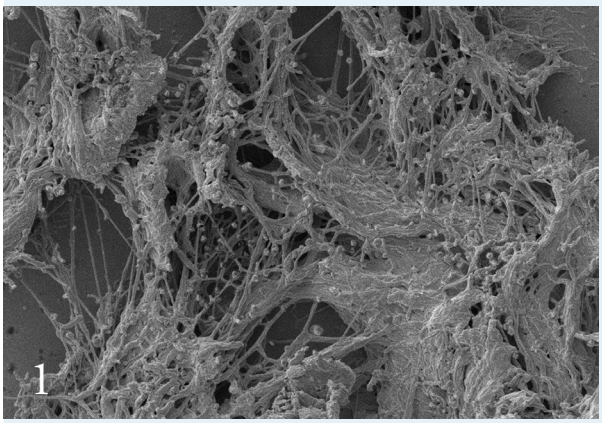
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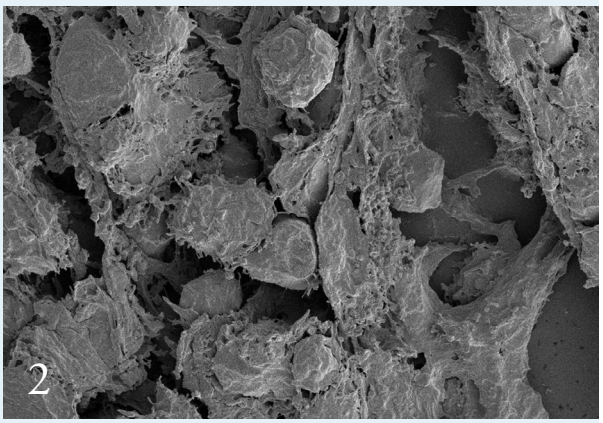
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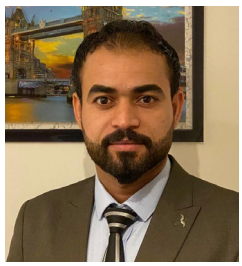

Raed Madhi is a biomedical scientist who obtained his MSc in immunogenetics from University of Thi-Qar. He worked as University teacher of immunology at Misan University in Iraq until 2015. Raed moved to Sweden and started his PhD in clinical medicine and experimental surgery at the faculty of medicine. The focus of his doctoral thesis is to investigate the mechanisms of neutrophil extracellular traps in severe acute pancreatitis.

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On the Mechanisms of Neutrophil Extracellular Traps in Acute Pancreatitis

On the Mechanisms of Neutrophil Extracellular Traps in Acute Pancreatitis

Raed Madhi

Faculty of Medicine
Section of Surgery

DOCTORAL DESSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden. To be defended at Surgical Clinic, Carl-Bertil Laurells gata 9, floor 3, room 3050, Malmö and will be available for public via Zoom on the 4th of November 2020 at 09:00 am.

Faculty opponent
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Abstract

Acute pancreatitis (AP) is a sudden inflammation that is characterized by protease activation and neutrophil recruitment, leading to acinar cells injury and tissue damage in the pancreatic tissue. Although pathphysiological studies indicate a strong connection betweeen neutrophil extracellar traps (NETs) and severe AP, very little is known about the mechanisms of NET formation in AP. The aim of this thesis was to investgate the role of PAD4, c-Abl kinase and platelet inositol hexakisphosphate kinase 1 (IP6K1) in regulating NET formation in AP.

Induction of AP was preformed by taurocholate infusion into pancreatic duct or by receiving intraperitoneal injection of L-arginine. Challenge with taurocholate caused clear-cut increase in pancreatic tissue damage as well as elevated inflitration of neutrophils, myeloperoxedase levels, chemokine and edema formation as well as acinar cell necrosis in the inflamed pancreas. Interesingly, targeting PAD4 by Cl-amidine or GSK484 substaintially attenuated formation of NETs as well as decreased infiltration of neutrophils in both inflamed pancreas and lung tissue. Moreover, inhibtion of c-Abl kinase activity by admenstration of GZD824 or ABL001 resulted in reduction in chemokine formation and neutrophil recruiment as well as attenuated NET formation in the inflamed pancreas. In vitro, TNF-α-induced reactive oxygen species (ROS) was markedly reduced by co-incubation of isolated neutropils with GZD824. In addtion, depletion of platelets resulted in a substaintial reduction in cytokine fromation, neutrophil inflitration and tissue damage as well as markedly decreased NET-MP complexes in the inflamed pancreas. These complexes were obseved to regulate amylase secretion and STAT3 signaling in isolated acinar cells. P-selectin provides a physical contact between platelets and neutrophils and immuno-targeting of P-selectin heavily reduced platelet-neutrophil aggregation (PNA) and NET formation in the pancreas as well as protected against development of severe AP. Disrupting platelet IP6K1 resulted in a substaintial decrease in tissue damage and neutrophil inflitration in the pancreas and lung, suggesting that IP6K1 controls both local and systemic inflammation. IP6K1 was also observed to support formation of NET-MP complexes in the inflamed pancreas. Adding exgenous polyphosphate restored thrombin-induced NET formation in IP6K1-deficient platelets and wild-type neutrophils mixtures.

Our novel findings uncover new pathways of NETs formation in the inflamed pancreas and suggest that targeting these pathways might be a useful stratigy to attenuate both local and systemic inflammation in severe AP.

Key words: Acute pancreatitis, chemokines, neutrophil recruitment, PAD4, c-Abl kinase, NET, NET-MPs complexes

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Lund University, Sweden 2020

Cover photo was used from paper 3. Photo 1; wild-type (WT) mice + taurocholate. Photo 2; IP6K1 knockout (IP6K1-/-) mice + taurocholate.

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قال الله تعالى:

﴿ قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ (32) ﴾

سورة البقرة

“They said, “Exalted are You; we have no knowledge except what you have taught us. Indeed, it is you who is the Knowing, the Wise”
Quran chapter Al- baqarah verse no. 32

To my family

Acknowledgment

The race of existence in the present world of competition is having will to come forward success. With this willing, I started my project. First, I would like to thank the God for his shower of blessing and his watching over me throughout the accomplishment of this research successfully.

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Table of Contents

Abbreviations

AP	Acute pancreatitis
SIRS	Systemic inflammatory response syndrome
CXCL2/MIP-2	Macrophage inflammatory protein-2
CXCL1/ KC	keratinocyte cytokine
MAC-1	Macrophage-1antigen
LFA-1	Lymphocyte function-associated antigen-1
NETs	Neutrophil extracellular taps
MPs	Microparticles
H3cit	Citrullinated histone 3
PAD4	Peptidyl arginine deiminase
c-Abl	C-Abelson
ROS	Reactive oxygen species
MPO	Myeloperoxidase
NE	Neutrophil elastase
PolyP	Platelet polyphosphate
IP6K1	Inositol hexakisphosphate kinase 1
RER	Rough endoplasmic reticulum
CCK	Cholecystokinin
CT	Computed tomography
MOF	Multi-organ failure
TLC	Taurolithocholic acid
IP3	Inositol 1,4,5-triphosphate
TAP	Trypsinogen activated peptide
RyR	Ryanodine receptor
NF-KB	Nuclear factor kappa-light-chain-enhancer of activation B cells
TNF-α	Tumor necrosis factor-α
IL	Interleukin
ARDS	Acute respiratory distress syndrome
ALI	Associated lung injury
MCP-1	Monocyte chemotactic protein 1
PSGL1	P-selectin glycoprotein ligand1
PECAM	Platelet endothelial cell adhesion molecule
L-selectin	Leukocytes selectin

P-selectin	Platelet selectin
E-selectin	Endothelial selectin
ICAM-1	Intercellular Adhesion Molecule 1
VCAM-1	Vascular cell adhesion molecule 1
MMP	Matrix metalloproteinase
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
MAPK	Mitogen-activated protein kinase
STAT3	Signal transducer and activator of transcription 3
ELISA	Enzyme linked immunosorbent assay
PE	Phycoerythrin
APC	Allophycocyanin
FITC	Fluorescein isothiocyanate
RPMI 1640	Memorial Institute medium 1640
FBS	Fetal bovine serum
QRT-PCR	Quantitative Reverse transcription polymerase chain reaction
PRP	Platelet-rich plasma
DAPI	Diamidino-2-phenylindole
ANOVA	Analysis of variance
i.p.	Intraperitoneal
i.v.	Intravenous
kDa	Kilo Dalton
DMSO	Dimetylsulfoxid
SEM	Standard error of the mean
PMA	Phorbol myristate acetate
PBS	Phosphate buffered saline
PNA	Platelet-neutrophil aggregates
TGFβ1	Transforming growth factor beta 1

List of Original Papers

I. **Madhi, R**., Rahman M., Taha D., Morgelin M., and Thorlacius H. Targeting Peptidylarginine Deiminase Reduces Neutrophil Extracellular Trap Formation and Tissue Injury in Severe Acute Pancreatitis. *J Cell Physiol* 2019:234: 11850-11860.[#]

II. **Madhi, R**., Rahman M., Morgelin M., and Thorlacius H. C-Abl Kinase Regulates Neutrophil Extracellular Traps Formation, Inflammation, and Tissue Damage in Severe Acute Pancreatitis. J Leukoc Biol 2019:106:455-466.[#]

III. **Madhi, R***., Rahman, M*., Taha, D., Linders, J., Merza, M., Wang, Y., Morgelin, M., Thorlacius, H. Platelet IP6K1 regulates neutrophil extracellular trap-microparticle complex formation in acute pancreatitis. *JCI Insight*, doi:10.1172/jci.insight.129270 (2019).[¤]

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Chapter 1

Thesis Introduction

1- *Introduction*

Acute pancreatitis (AP) is an inflammatory disease that encountered in many countries with increased incidences [1,2]. In spite of improvement in diagnostic, AP is still associated with high mortality rate that ranging from 20% to 30% [3, 4]. Since there is no specific therapy for AP, understanding the pathophysiological mechanism of this disease might be contributed in resolving the problem. The inflammatory reaction in pancreas results in pancreatic tissue auto-digestion, a local inflammation, and then developed into systemic inflammatory response syndrome (SIRS) [5]. In fact, these reactions are reported to cause various effects such as increase the permeability of endothelial plasma membrane and migration of leukocytes or enhancing bacterial translocation that can lead to multi-organ failure and death [6-8]. The hallmark of inflammatory response in AP is leukocytes sequestration into the pancreatic tissue [9]. It is well known that extravascular localization of leukocytes is orchestrated by secreted chemokines [9]. For instance, CXCL1 and CXCL2 have been observed to be potent stimulators for neutrophil accumulation [10]. Growing body of evidences have shown that leukocytes recruitment is a multiple steps process that initially roll on activated endothelial cells then adhere firmly and migrate into the extravascular space from the blood vessel [11, 12]. In addition, neutrophils infiltration are supported by specific cell adhesion molecules such as P-selectin [13], macrophage-1antigen (MAC-1) and Lymphocyte function-associated antigen-1 (LFA-1) [14].

It has been observed that activated neutrophils undergo to NETosis, a special kind of cell death program (different than necrosis and apoptosis), by which activated neutrophils release extracellular web-like structures (known as NETs, neutrophil extracellular traps). These structures consist of DNA, histones, and antimicrobial proteins [15]. NETs play an antimicrobial role by binding pathogens with extracellular traps during infection [16, 17]. However, convincing data have shown that NETs also contribute in pathogenesis of various inflammatory diseases [18] such as AP [19], sepsis [20] and inflammatory lung diseases [21]. Furthermore, it has observed that neutrophils can also shed off sphere-shaped intact vesicles that released from their membrane upon activation, called micro-particles (MPs) [22]. Indeed, MPs have shown to form a complex with NETs via interactions with histone phosphatidylserine. Moreover, it has found that NETs-MPs complexes can induce thrombin generation via intrinsic pathway of coagulation [23].

A recent study has demonstrated that histones modification have essential role in NETs formation via chromatin decondensation in which arginine on histones converted to citrullin [24]. It has been identified that citrullinated histone 3 (H3cit) is a predominant constitute of NETs [25-27]. In fact, citrullination process is orchestrated by peptidyl arginine deiminase (PAD4) that regulates NETs formation via hypercitrullination of the histone proteins [27, 28]. In addition, it was found that neutrophils from PAD4 gene deficient mice are incapable to form NETs.

Cellular stress and tissue injury can activate signaling pathways that regulate specific transcription factor, which control the gene expression of pro-inflammatory compounds. Indeed, intracellular kinases have reported to play a key role in many signaling cascades [29]. c-Abl kinase is a non-receptor tyrosine kinase belongs to Src family and expressed ubiquitously in mammalian cells [30]. It has

shown that c-Abl kinase regulates actin cytoskeleton dynamic that support cell adhesion and migration [31]. Several studies have observed that c-abl kinase has a potent role in neutrophil accumulation by mediating integrin β2-mediate neutrophil migration [32, 33]. Moreover, c-abl kinase has been implicated in causing of ROS generation from activated neutrophils that can damage the host tissue [34]. In fact, ROS formation is a potent contributor in NET formation. Furthermore, it has shown that ROS induce the release of MPO and neutrophil elastase (NE), essential components of NET formation, and that ROS generation facilitates histone citrullination which is the first step in formation of NETs via peptidyl arginine type 4 (PAD4) [35].

Platelets- neutrophil crosstalk has reported to be involved in various inflammatory conditions, such as reperfusion injury [36] and abdominal sepsis [37] as well as pulmonary infections [38] and acute myocardial disease [39]. Furthermore, previous studies have observed that activated platelets secrete pro-inflammatory compounds such as CCL5, CXCL4 and CD40L that involved in neutrophil migration [19, 40, 41]. Activated platelets have also reported to have a critical role in NET formation in infectious diseases, however, their role in NET formation in AP is not completely clear. Indeed, it has shown that activated platelets secrete polymer of phosphate unites, called platelet polyphosphate (PolyP) [42]. Serval studies have shown that PolyP play a critical pro-inflammatory role for instance; enhancing nuclear factor kappa-light-chain-enhancer of activation B cells (NF-kB) signaling as well as vascular permeability and activating complement system [43-45]. Indeed, PolyPs production has shown to be highly regulated by inositol hexakisphosphate kinase 1 (IP6K1) in platelets [42]. Recently, it has found that platelets IP6K1 has an important role in neutrophil activation and also that platelets IP6K1 regulates neutrophil-platelet aggregation in endotoxin-induced lung inflammation [46].

The present thesis has included two different experimental models of severe AP to identify the role of PAD4 and c-Abl kinase as well as platelet IP6K1 in NET formation in severe AP.

Chapter 2

Anatomy and Physiology of Pancreas

Contents

1. Anatomy of pancreas.
2. Physiology of pancreas.
3. Pancreatic enzymes.

1- ***Anatomy of pancreas***

The pancreas is an important glandular organ involve in both digestive and endocrine process. In normal human, it weighs approximately 70-150 grams and typically measuring 15-25cm in the length [47]. It is located in the retro-peritoneum, behind the stomach, particularly in the upper left part of the abdomen. The development of pancreatic tissue comes from precursory ventral and dorsal portions [47-49]. The head of pancreas, common bile duct, gallbladder and the liver are developed from the ventral part [49]. However, the dorsal portion gives the left parts of the gland, body and tail [50]. In fact, the head of pancreas lies in the second and third portions of the duodenum. But the body and tail are located in the posterior abdomen and extend into the hilum of spleen [51].

The main arterial blood supply of pancreas consists of two major arteries that supplying the abdominal organs, celiac and superior mesenteric artery [52]. The blood supply of pancreas by the celiac artery is through the superior pancreatic duodenal artery. However, the blood supply that provided by the superior mesenteric artery is through the inferior pancreatic duodenal artery [49]. The splenic vein and superior mesenteric vein represent the venous drainage of the pancreas which are entirely emptying into the portal vein [53].

2- ***Physiology of pancreas***

The pancreas is a dual function gland that works as an endocrine and exocrine gland. The endocrine part of pancreas is made up by many cell clusters called islets of Langerhans [54]. Generally, the cells in the islets are classified by their secretion into four types:α-alpha cells, β-beta cells, Δ-delta cells and ɣ-gamma cells which secrete glucagon, insulin, somatostatin and pancreatic polypeptide, respectively [55]. This part of gland occupies about 2% of pancreatic tissue and interfere with the acinar parenchyma [56]. However, the remaining part represents 98% of the pancreatic tissue which is made up from the pancreatic acinar cells. These cells are indeed organized in spherical masses to form what call acini. Acinar cells have truncated pyramid shape that arranged in groups around the center of ductal lumen. A group of organized acini assembles to compose lobules and a number of lobules together form pancreatic lobes [51]. In acinar cells, proteins are synthesized and assembled in the rough endoplasmic reticulum (RER) then transport to the Golgi apparatus and there undergo to some physiological processes such as post-translational modifications [57]. Moreover, high percentage of these proteins (more than 90%) is digested enzymes which are allocated to work outside the cell [58]. Normally, these enzymes are found in inactive form, pro-enzymes or zymogens, and packaged in vacuoles that carried toward the luminal plasma membrane [58]. After the fusion with the surface membrane, the zymogen granules set free their contents into acinar space [59]. In addition to their role in enzymes secretion, the exocrine pancreas also secretes ions and water into the duodenum of the gastrointestinal tract [60]. These two factors have an important role in transport the digestive enzymes from the point of their secretion in acinar cells to the intestine [51]. For instance, 1500-3000 mL/day fluid of iso-osmotic (pH > 8.0) is secreted by pancreas and carrying many enzymes and zymogens [61]. In fact, the alkalinity of this fluid is because the existing of NAHCO3 in high concentration, up to 150mM [62]. Indeed, the NAHCO3 plays a key role in neutralizing the acidity of gastric chyme that delivered from stomach to the intestine [63]. Giving the fact that neutral pH is necessary for the perfect function of digestive enzymes in the intestine

lumen as well as the epithelial function of the gastrointestinal surface [60].

3- *Pancreatic enzymes*

Pancreatic enzymes are variety of enzymes that synthesized, with exception of amylase and lipase, in an inactive form and converted to an active form upon reaching small intestine. When trypsinogen reaches the small intestine, it is cleaved from the N-terminal end by the intestine brush border enzymes such as enterokinase and releases trypsinogen activation peptide (TAP). Eventually, the active trypsin is formed and activates other pancreatic enzymes such as pro-carboxypeptidase, trypsinogen, chemotrypsinogen and elastase [64] (Figure 1). These enzymes can be divided into three major groups; 1) pancreatic amylase (Alpha amylase) is enzyme that catalyzes the hydrolysis of starch into sugar. In spite of it is secreted from salivary gland, the main source of amylase is pancreatic secretion [65].

Accordingly, the amylase has a diagnostic importance and can serves as biomarker of disordered or inflamed pancreas. It has been monitored that amylase is leaked out into blood circulation during pancreatic inflammation and increased levels of amylase can be used in the diagnosis of acute pancreatitis [66]. 2) proteases, which include several enzymes that synthesized in pancreas and secreted into small intestine such as trypsin and chymotrypsin, have an important role in proteins digestion [67]. Initially, protein digestion starts in the stomach by pepsin but the bulk of protein breakdown due to pancreatic proteases [68]. Upon activation, these enzymes can auto-digest the pancreatic tissue, however, they are packaging inside secretary vesicles in an inactive form which further contain trypsin inhibitor [67]. 3) pancreatic lipase is lipolytic enzyme that has an important role in fat digestion via hydrolysis the ester linkage of triglyceride [69]. In their natural form, dietary fat cannot be absorbed by the intestine; however, lipase can break it down into fatty acid that can be absorbed via intestinal surface [70]. Normally, pancreatic lipase concentration in serum is very low, nevertheless, during the disruption of pancreatic function in such conditions like pancreatitis, pancreatic lipase is leaked out into blood circulation and increased its concentration. Thus, detection of pancreatic lipase can be also used as an indicator in diagnosis of AP [71]. Moreover, pancreas produces other digestive enzymes which include carboxypeptidase, elastase, ribonuclease, and deoxyribionuclease [72].

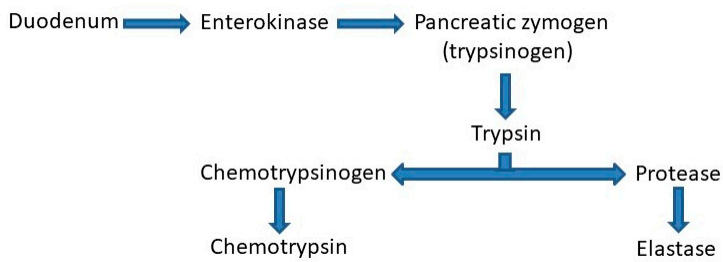

Figure 1. Diagram of Secretion of pancreatic enzymes. Trypsinogen is cleaved by enterokinase to form the active trypsin, then trypsin activates other zymogenes such as Chemotrypsin and Elastase.

Giving the fact that cholecystokinin (CCK) is the main gastrointestinal hormone that synthesized and secreted by duodenal mucosa upon food is reached to intestine. Indeed, CCK has an important role in stimulation of pancreatic enzyme secretion into pancreatic duct [73]. For example, CCK induces pancreatic secretion by either direct stimulation through CCK-A receptors on acinar cells or indirect mechanism via CCK-B receptors on nerves that followed by releasing of acetylcholine [74, 75]. However, acetylcholine, vasoactive intestinal polypeptide, and gastrin releasing peptide are less abundant stimulators of pancreatic acinar cells secretion [76]. In fact, the pancreatic enzyme secretion needs more knowledge to better understanding the pathogenesis of AP.

Chapter 3

Acute Pancreatitis

Contents

1. Definition of AP.
2. Pathophysiological mechanism of AP.
3. Trypsinogen activation in AP.
4. The role of calcium in AP.
5. Inflammation in AP.

1- Definition of AP

Acute pancreatitis (AP) is an inflammatory disease of pancreas that characterized by trypsin activation of acinar cells which is followed by inflammatory response with or without involvement of remote organs [77]. The increased incidence of AP has been encountered in many countries [3, 4]. The clinical manifestations of AP are variable and can range from mild, with self-limiting abdominal disturbance that may go away without treatment, to severe cases that can cause life-threatening complications [78]. Ultrasonographical or contrast enhanced computed tomography (CT) imaging shows that three things may detect within edematous of AP are homogenous interstitial edema with gland swelling, peripancreatic fat mild stranding and sometimes acute fluid collections. Normally, these circumstances associated with mild case may recover within couple of days [79]. In contrast, severe cases of AP can be developed and causing pancreatic necrosis and systemic inflammation that can present in 20-30% of patients and associated with up to 40% of mortality rate [80].

Because the mild and severe disease is almost with similar symptoms, it is difficult to recognize the severity of disease in early stage. Clinically, the severe pancreatitis progress becomes evident after the first 2-3 days. Generally, the progression of severe AP can be classified into two phases [81], early phase which is the first 7-14 days after dawn of AP. The common complications of this phase are systemic inflammatory response syndrome (SIRS) and multi-organ failure (MOF) [82]. 14-28 days represent the late phase after onset of AP and the disease complications can cause necrotizing pancreatitis, endothelial cells damage and secondary MOF [83, 84]. Periarterial edema or bleeding of pancreas that combines with endothelial damage can reduce perfusion through compression on pancreatic vessels. In case pancreatic perfusion reduction is continued, necrotizing pancreatitis would be developed [85]. The infectious risk of necrotic collections is low during the first week after onset of symptoms; however, it is highly later phase [86]. Pancreatic inflammation appears to be started by acinar cells injury which release signals that can affect surrounding tissue. Moreover, cellular permeability of blood vessels and intestine is increased as well as inflammatory cells are also recruited which can worsen the acinar cells damage. Subsequently, the inflammatory changes can extend from pancreas to lung, renal, stomach, colon and spleen [85]. Therefore, pancreatitis treatment at early stage is important to prevent necrosis and systemic inflammation.

2- *Pathophysiological mechanism of AP*

Giving the fact that there is no specific treatment available for AP, it is suggested that understanding the pathophysiological mechanism of this disease might contribute to the development of new strategy. In fact, many experimental studies have been performed in vivo and in vitro on pancreatic acinar cells to better understand the pathomechanism of AP. It has been shown that the progression of pancreatitis initiated in intra pancreatic acinar cells by elevating the concentration of calcium and premature activation of trypsinogen as well as the transcription factor activation such as NF-KB [87-89].

The taurolithocholic acid (TLC) has reported to be one of the most toxic bile acids to pancreatic acinar cells by causing Ca^{+2} signaling in acinar cells via inositol 1,4,5-triphosphate (IP3) [90]. Accordingly, the increased concentration of

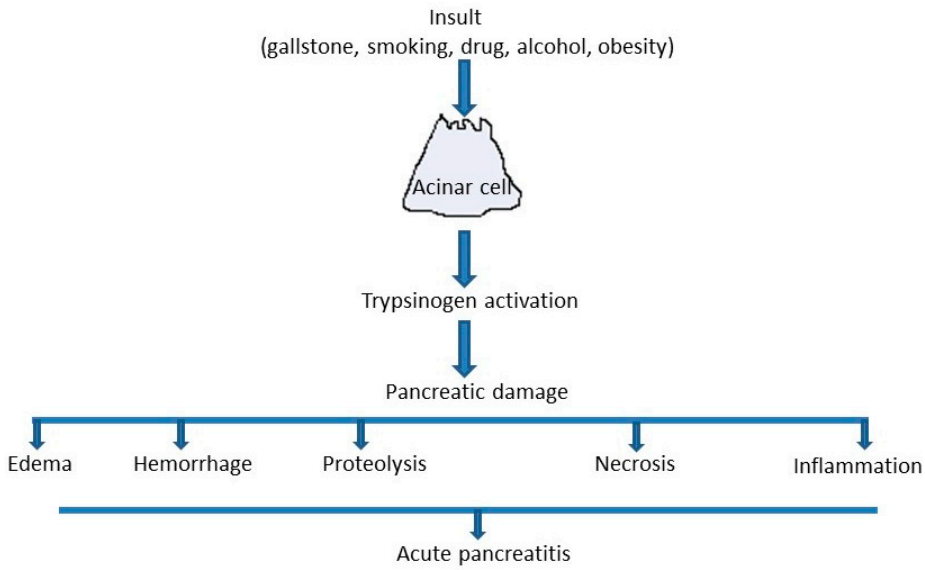

Figure 2. Basic schematic explanation of enzymatic activation of AP. Pancreatic activated enzymes can activate cytokines production and inflammatory cascade that led to tissue damage and acute pancreatitis.

Ca^{+2} can result in activation of pancreatic enzymes [91] or cell death [92].

3- *Activation of trypsinogen in AP*

Trypsinogen is precursor form of trypsin that synthesized in endoplasmic reticulum and moved to the Golgi apparatus where it is stored along with other pancreatic enzymes in the zymogen granules [93].Trypsinogen is small peptide with molecular weight (25 kDa) that presented in normal pancreatic juice. In normal physiological condition, this zymogen is activated to form trypsin in duodenum by enterokinase that secreted by the mucosa of duodenum. Enterokinase cleaves trypsinogen peptide bond at lysine residue, which is located after residue 15 [94, 95], and releases small fragment called trypsinogen activated peptide (TAP) [95, 96]. And this peptide (TAP) has been used as a marker for trypsin activation [13]. Indeed, activated trypsin can stimulate the activation of trypsinogen and other pancreatic enzymes [97]. Previous studies have observed immune-interaction against TAP inside the cytosolic vacuoles containing lysosomal cathepsin B [95, 98] . Suggesting that cathepsin B has an important role in pancreatic zymogens activation. In fact, cathepsin B was implicated in conversion of trypsinogen into active trypsin as proved by a previous study on lysosomal cathepsin B knockout mice [99]. The authors were shown that pancreatic trypsin activity in lysosomal cathepsin B knockout mice was attenuated by 80% compared with control mice. Moreover, lysosomal cathepsin B knockout mice resulted in a greatly reduction in pancreatic injury compared with control mice, as indicated by serum amylase and lipase levels as well as pancreatic acinar cells necrosis [99]. Acinar cells damage has been shown to be earliest morphological changed in the experimental model of AP. The mechanism by which acinar cells injured is not completely clear. Indeed, a previous study has observed that initial trypsinogen activation, 2 h after induction of AP, is independent of leukocytes,

however, later activation, 24h after induction of AP, has found to be dependent on leukocytes in the pancreas [100]. Accordingly, activated trypsin lead to pancreatic edema, necrotizing and inflammation (Figure 2).

4- ***The role of calcium in AP***

Under normal conditions, intracellular resting calcium (Ca^{+2}) concentration (10^{-7}M) is much lower than extracellular fluid (10^{-3}M) [101]. Calcium is one of important factors that might be involved in development of AP. Pancreatitis stimuli such as bile acids and caerulein have been reported to cause an increase in intracellular Ca^{+2} [90, 102]. It is generally accepted that the influx of Ca^{+2} in the apical side of acinar cells has a vital role in controlling secretary granules of acinar cells [103, 104]. After activation near the apical membrane, inositol 1,4,5-triphosphate receptors (IP3R) regulates rapid increase in the concentration of intracellular Ca^{+2} which is leading to release the zymogens into pancreatic duct [105]. The increase in intracellular Ca^{+2} concentrations is associated with vacuolization of acinar cells and trypsinogen activation during the early AP [106]. A previous study has shown that Ca^{+2} has an important role in trypsinogen activation as well as it was found that hypercalcemia results in edematous and necrotizing pancreas [91]. The role of Ca^{+2} in pancreatitis was further studied by disrupting the Ca^{+2} signaling in the acinar cells and a noted increase in intra-cellular Ca^{+2} was observed in experimental model of AP.

It is well known that gallstones are one of main cause of pancreatitis by obstructing the biliopancreatic duct that lead to reflux bile acid into pancreatic duct causing pancreatic tissue damage [107]. Indeed, bile acids cause an increase in the intracellular Ca^{+2} levels and prevent Ca^{+2} uptake to ER by inhibition of sarco-endoplasmic reticulum ATPase. Furthermore, bile acid is also implicated in releasing of Ca^{+2} from ER and apical store through different pathways such as open two channels of Ca^{+2}, the ryanodine receptor (RyR) and IP3R [90, 105]. A previous study was found that inhibition of RyR reduced intracellular Ca^{+2} oscillations as well as reduced pancreatic trypsin activity and protect against acinar cells injury in murine model [108]. Moreover, in vitro study has observed that transport-mediated bile acid uptake is involved in Ca^{+2} dependent cell death in pancreatic acinar cells [92]. Another study has found that pre-incubation of Na-taurocholate-induced trypsinogen activation acinar cells with an intracellular chelator 1,2-bis(2-aminophenoxy)-ethane-N,N:,N:N-tetra –acetic acid tetrakis/acetoxymethyl ester (BAPTA-AM) markedly attenuated Ca^{+2} dependent trypsinogen activation by 85% compared with trypsinogen activation in control acinar cells, which is indicated that Na-taurocholate-triggered trypsinogen activation is Ca^{+2} dependent [109].

Cell signaling proteins have a key role in development of pancreatitis. For example, NF-kB has been shown to be a critical protein in development of AP [110]. In fact, the activity of NF-kB has been shown to be dependent on Ca^{+2} influx [111-113]. It is generally held that NF-kB is involved in the inflammation by regulating transcription of different genes. These genes has indeed been reported to have a central role in development of AP [114]. In normal physiological condition, NF-kB can be initially controlled in the cytoplasm through binding to its inhibitory element, IkB [115].

Under pathophysiological conditions, IkB can be changed and phosphorylated and later degraded by the proteasomes. These changes on IkB can result in release of NF-kB and translocate into the nucleus, where it triggers region of different pro-inflammatory genes

[115]. Preclinical study on pancreatitis was revealed an increase in the activity of NF-kB and a decrease IkB expression in early phases of AP [111, 112, 116].

5- ***Inflammation in AP***

The initial phase of AP includes activation of premature pancreatic proteases which result in acinar cells disruption [117-119]. Local inflammatory cells are observed to be activated as well as various inflammatory chemokines are secreted during pancreatic injury [120]. The inflammatory response in pancreatic acinar cells initiates as a local inflammatory reaction that characterized by trypsinogen activation. However, in severe cases the inflammation can be extended to systemic inflammation causing SIRS [121]. A study by Gaiser et al, 2011 has observed that the activation of trypsinogen in pancreatic acinar cells is sufficient to induce AP. Moreover, this study was found that trypsinogen activation is implicated in leukocytes recruitment by various mechanisms [122]. However, another study was demonstrated that recruitment of leukocytes into inflamed site is independent process of trypsinogen activation. The authors suggested that intracellular trypsinogen activation results in death of pancreatic acinar cells in the initial phase of pancreatitis as well as that the progression of local and systemic inflammation does not require activation of trypsinogen [123]. Moreover, initial trypsinogen activation was reported to be independent on neutrophils but later the activation is dependent on neutrophils in pancreatic tissue [100]. Pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) have been reported to be critical mediators in pancreatitis [9, 124]. Indeed, these cytokines were shown to regulate production of interleukin-6 (IL-6) and interleukin-8 (IL-8) as well as initiate the systemic inflammation reaction such as acute respiratory distress syndrome (ARDS) [125, 126]. Furthermore, production of pro-inflammatory cytokines can enhance the inflammatory signaling pathways that together cause vascular endothelial cells activation in the body. Subsequently, this activation can increase the vasodilation of the capillary veins which supports recruitment of leukocytes into inflamed tissue as well as cause activation of coagulation cascades [127-130]. It is generally held that the inflammatory response in AP can be associated with different factors such as calcium overload, trypsin activation, production of oxygen free-radical species, production of cytokines, chemokines, recruitment of inflammatory cells, apoptosis and necrosis (Figure 2).

Chapter 4

Cytokines and Neutrophils in AP

Contents

1. Cytokines.
2. Chemokines.
3. Neutrophil recruitment.

1- *Cytokines*

Cytokines are a large family of small proteins with molecular weight (6 to 70 kDa) that have an important role in cell signaling. They have multiple effects on different target cells as well as activation of specific cell surface receptors. Based on their function, cytokines are divided into two groups: pro and anti-inflammatory cytokines. Accumulating evidences have been demonstrated that cytokines are up-regulated in both clinical and experimental pancreatitis [131, 132]. It is generally held that the expression of most cytokines is regulated by transcription factor such as NF-kB [133, 134]. A previous study has shown that NF-KB contributes in both local and systemic inflammatory response [135] and that inhibition of NF-KB led to a reduction in the severity of cerulein-induced pancreatitis that associated with lung injury [113]. In line with that, another study has also observed that transcription factors, such as NF-KB, can cause up-regulation of most cytokines expression [136]. During AP, it has found that cytokines are overexpressed in the inflamed pancreas. Furthermore, it has reported that acinar cells have ability to produce variety types of inflammatory mediators such as TNF-α, IL-6 and IL-1β. In addition, the acinar cells have also shown to have ability to produce anti-inflammatory mediator such as interleukin 10 (IL-10), which demonstrated to be expressed at the early stage of pancreatitis [137]. Produced cytokines can be leaked out into circulation and result in recruitment of pro-inflammatory cells into site of inflammation that can lead to SIRS [64]. Subsequently, the severity of pancreatitis can be associated with circulating cytokines levels. In fact, IL- 1β and TNF-α have been shown to be one of the major pro-inflammatory mediators that cause shock-like status as showed in animal experimental model in AP [138-140]. Consistently, previous studies have found that deficient IL-1 β receptors and TNF-α receptor in knockout mice or blockage of TNF-α by using antibodies directly against it, significantly attenuated pancreatic damage and systemic complications [141, 142]. Moreover, it was reported that IL-6 can be produced by variety cells such as monocytes/macrophage, fibroblast, endothelial cells and acinar cells in response to stimuli like TNF-α, IL-1β or endotoxin. Indeed, IL-6 is considered as primary inducer of the acute phase response in different inflammatory conditions [137, 143]. During AP, it is observed that the levels of IL-6 increased in patients serum samples in early course of AP [137, 144]. Clinically, the levels of IL-6 has speculated to be associated with the severity of AP. A study by Zhang et al, 2013 observed that IL-6 can be used as an important indicator of remote organ failure. The authors showed that AP associated lung injury (ALI) might be through the IL-6 trans-signaling dependent STAT3 pathway [137].

Interleukin (IL-10) is anti-inflammatory interleukin that has reported to be expressed by variety of cells specially by monocytes/macrophage and lymphocytes [145]. This interleukin appears to have an anti-inflammatory effects against different of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 and subsequently prevent tissue damage [146, 147]. Furthermore, IL-10 has shown to trigger synthesis of natural cytokines antagonists such as TNF-α receptor and IL-1 RA [141, 142]. Clinical study revealed that circulating levels of IL-10 has a correlation with the severity of AP and organic failure [145]. Consistently, it was shown that IL-10 has protective effect in several murine models of AP [148]. In fact, further studies are required to better understand the role of cytokines in AP.

2- *Chemokines*

Chemokines are a family of small cytokines with molecular weight (8-10 kDa) which have the ability to induce directed chemotaxis and trafficking of inflammatory cells to site of inflammation [149]. In fact, numerous of chemokines were identified as potent inflammatory mediators of leukocytes extravascular accumulation and they are involved in pathophysiological process of experimental pancreatitis [121, 150]. Based on the spacing of their first two cysteine residues, chemokines have classified into four groups: CXC, CX3C, C and CC [151]. IL-8 or GRO-α, CXC chemokines that contain two N-terminal cysteines which separated by one amino acid, were observed to have an important role in neutrophil chemotaxis [152]. Similarly, MIP-2 or CXCL2, which similar to IL-8 in human, has been reported to increase in experimental models after AP induction [100, 153]. Subsequently, treatment of mice with antileukinate, a specific MIP-2 blocker, or using an antibody against MIP-2 has found to attenuate pancreatitis associated with lung injury [154]. In addition, it has also shown that blocking MIP-2 signaling by using CXCR2 antagonist protected against AP [154]. Keratinocyte cytokine (KC) and MIP-2 are chemokines with approximately 63% of sequence identity and they have functional homologue of human IL-8. These chemokines have been reported to be secreted by macrophage and monocyte as well as other cells such as acinar cells [155]. Indeed, these chemokines have found to substantially attract neutrophils to site of inflammation [156] by their high affinity to CXCR2 receptor that expressed on neutrophils [157] and to mediate neutrophil recruitment in the pancreas [154]. Pancreatic acinar cells have shown to have the ability to produce monocyte chemotactic protein 1 (MCP-1), CC chemokine. Induction of acinar cell injury by using caerulein has been found to cause an increase in (MCP-1) production [158, 159]. Accordingly, chemokines have a critical role in development of AP via possibly directing inflammatory cells to the site of inflammation.

3-*Neutrophil recruitment*

Neutrophils are immune cells that consider the most plentiful type of granulocytes (40%-70%). They sever as first defense line of immune response and the main part of innate immune system [160]. Bone marrow stem cells are the main source of neutrophils formation. Indeed, neutrophils have a short live and characterized by their high mobility to inter the damaged tissue [161]. During inflammation of AP, it was reported that the inflamed organ can release chemoattractant into circulation which, in turn, induce neutrophil rolling and migration into inflamed pancreas [162]. For instance, experimental studies have shown that inflamed pancreas can release chemokine such as CXCL1 and CXCL2 that direct neutrophils into inflammatory site [154, 163]. In circulation, attracted neutrophils undergo to multiple cascade process to arrive in the inflamed site, which happens in four steps (Figure 3); 1) chemoattraction; upon inflammation, resident macrophages in the inflamed tissue produce cytokines such as IL-6, TNFα, IL-1β and chemokines that trigger endothelial cells near the site of inflammation to release cell adhesion molecules such as selectins [164]. 2) rolling and adhesion; circulating leukocytes express carbohydrate ligands, such as P-selectin glycoprotein ligand1 (PSGL1), that enables neutrophils to bind with selectins such as P-selectin on the inner surface of blood vessels. These interactions lead to slow down leukocytes movement and rolling along the inner wall of vessels. 3) tight adhesion;

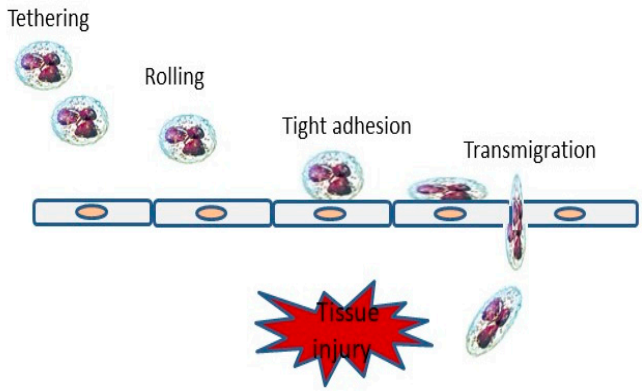

Figure 3. Recruitment cascade of leukocytes. A multi-step process of leukocytes recruitment, via integrins and selectins, into the inflamed site. This process includes tethering, rolling, tight adhesion and transmigration.

releasing chemokines activates leukocytes rolling and induce surface integrins to switch from low affinity adhesion to high affinity adhesion. This tight adhesion is caused by the tight binding of integrins to complementary receptors on endothelial cells. In spite of the force of ongoing blood flow, the adhesion causes leukocytes immobilization. 4) transmigration; in this step some of changes would occur on leukocyte phenotype in which cytoskeleton is reorganized in a way that allows leukocytes to leak out through the endothelial cells [165]. Leukocytes may pass the endothelial cells through gaps between endothelial cells and it is believed that this process could be mediated by platelet endothelial cell adhesion molecule (PECAM) proteins which are found on both leukocytes and endothelial cells surfaces [166].

Selectins are a family of three related glycoproteins: L-selectin (leukocytes selectin), P-selectin (platelet selectin) and E-selectin (endothelial selectin) [167]. L-selectin is expressed on leukocytes surface and has an important role in leukocytes rolling by binding to its ligand on the surface of endothelial cells [168]. E-selectin and P-selectin have been established to be expressed on the endothelial cell surface and bind with its oligosaccharide receptors on neutrophils [169, 170]. P-selectin is also expressed on platelet surface and has a critical role in platelet-neutrophil crosstalk by binding with PSGL-1 on neutrophils [13].

Experimental studies have found that the levels of P-selectin expression increased in early phase of AP in animal exposed to taurocholate and it suggested that this increased expression associated with prominent increase of NF-KB signaling and reactive oxygen species (ROS) [171, 172]. Furthermore, P-selectin mediates adhesion of leukocytes to the blood vessels in different inflammatory conditions. For example, P-selectin has been determined to mediate rolling and recruitment of neutrophils in severe pancreatitis [13]. Suggesting that target this protein might be a useful strategy to attenuate the inflammation in AP.

Other cell adhesion molecules, such as ICAM-1 (Intercellular Adhesion Molecule 1) and VCAM-1 (Vascular cell adhesion molecule 1), have been also implicated in adhesion of leukocytes. These integrins have reported to serve as a ligand for various integrins that expressed by neutrophils such as macrophage

antigen-1 (Mac-1) (CD11b/CD18) and lymphocyte function associated antigen-1 (LFA-1) (CD11a/CD18) [173, 174]. A previous study on murine model was found that ICAM-1 mediates leukocytes recruitment and tissue damage in AP [175]. In addition, LFA-1 and ICAM-1 were also shown to have an important role in intracellular leukocyte recruitment in AP [176].

Chapter 5

Neutrophil Extracellular Traps

Contents

1- *Introduction*

Neutrophils are essential immune cells of innate immune system and the first defense lines of host against various infectious pathogens such as bacteria, fungi and protozoa. After activation by the inflammatory stimuli, neutrophils migrate blood vessels and move forward to the inflamed tissues by following the chemotactic compounds. At inflammatory site, they attack and phagocytose invading pathogens by releasing proteolytic enzymes and antimicrobial proteins [177, 178]. In addition, it has been established that activated neutrophils release reactive oxygen species (ROS) as well as enzymes like elastase and matrix metalloproteinase-9 (MMP-9). Indeed, these products have suggested to be involved in neutrophil-mediated tissue damage in AP [13, 179]. Moreover, it was observed that activated neutrophils use another mechanism to kill and trap the pathogens by releasing web-like structures called neutrophil extracellular traps (NETs) [180, 181]. These structures are composed of DNA, nuclear and cytoplasmic proteins with embedded antimicrobial granular proteins [15, 182] which are extracellularly released in process called NETosis. It is another kind of cell death program neither apoptosis nor necrosis in which neutrophil chromatin undergoes to decondensation [183].

It has believed that NETs bind gram positive, negative bacteria and fungi so that provide the host cells with an important mechanism to trap and kill extracellular pathogens [15]. However, it has been found that NETs contribute in pathogenesis of various inflammatory diseases such as vascular disorder [184], inflammatory lung diseases [21], sepsis [20] and acute pancreatitis [19], as well as in autoimmune disease such as rheumatoid arthritis (RA) [185] and systemic lupus erythematosus (SLE) [186]. Suggesting that a direct expose to NETs components (DNA, histone, MPO, ROS and antimicrobial proteins) may result in host tissue damage [186-188]. Further support of the pathogenic role of NETs, it has observed that all core histones (H2A, H2B, H3, and H4) has a cytotoxic effect on pancreatic acinar cells [19].

2- *Mechanism of NETs formation*

Although mountain of studies have shown the formation of NETs but very little is known about the mechanism of NET formation. In fact, it was reported that neutrophils undergo to morphological modifications in the process of NETosis [189]. After activation, the cells become flat and the nucleus loses its lobules. In addition, chromatin is decondensed and the inner and outer nuclear membranes are separated from each other. After the nuclear membrane breaks down, the nucleoplasm and cytoplasm are merged into a homogenous mass. Then, the cells become spherical-like shape and contracted as well as the cytoplasmic membrane is broken and cell components are expelled into extracellular space and form web-like structures which are called NETs [181].

In fact, the process of NET formation has been reported to be a gradual process with several sequential steps.1) Signaling pathway of intracellular proteins such as p38, MAPK, MEK/ERK and Akt [190, 191]. 2) reactive oxygen species (ROS) generation have found to have essential role in NET formation by promoting the complex machinery that implicated in chromatin decondensation, nuclear membrane collapse and expel fiber structures of DNA connected with granular proteins [192, 193]. 3) Enzymes translocation, such as PAD4, neutrophil elastase (NE) and MPO, as well as histone modification [193]. PAD4 is a protein that catalyzes conversion of

arginine residues on H3, H4 and H2A to citrulline [194]. Overexpression of PAD4 leads to hypercitrullination that results in dissociation of histone from the nucleosome structure by losing the positive charge from the arginine residues and subsequently chromatin be decondensed [24]. Thus, PAD4 has a regulatory role in NET formation and considered the primary driving force in NETosis. In line with that, study by Wang group has shown that PAD4 gene-deficient mice neutrophils do not have the ability to form NETs [25]. Further support for this fact, previous studies have observed that PAD4 inhibitor, such as Cl-amidine and GSK484, resulted in a great inhibition in the formation of NETs in vivo and vitro [194, 195]. Subsequently, this enzyme considers as essential mediator for formation of NETs.

3- ***Platelets and NET formation***

Platelets are small (2-3µm) and enucleated fragments that derived from megakaryocytes. They have a crucial role in both hemostasis and thrombosis. Indeed, platelets shared the leukocytes by some features such as containing mitochondria, endoplasmic reticulum as well as actin and myosin filaments network. Moreover, platelets contain three types of store granules: α-granules, dense granules and lysosomes which release potent chemotactic factors and other pro-inflammatory mediators [196].

It is generally accepted that platelets under normal conditions do not adhere to each other, leukocytes or the wall of endothelial cells and this condition due to antithrombotic properties of vascular endothelium. During inflammation or vascular injury, activated platelets release pro-inflammatory mediators that stored in their granules such as platelet factor 4, CCL5, thromboxane A2, and adenosine diphosphate (ADP). These molecules trigger activation of other circulating platelets which results in platelet-platelet aggregation as well as leukocytes activation and recruitment [197-199]. Moreover, accumulating evidences have reported that platelets considered a main contributor in injury and inflammation [200] in different conditions such as acute lung injury (ALI) [201], rheumatoid arthritis [202] and pancreatitis [203]. A previous study was observed that activated platelets are capable to induce neutrophil-derived NET formation in transfusion-related acute lung injury [204]. Furthermore, it was also found that platelets trigger NETs formation in response to the challenge with bacterial and viral components [20, 205, 206]. However, the mechanism by which platelets induce NET formation in sterile inflammation is not completely understood. Upon activation, it was observed that platelets can activate neutrophils via binding between P-selectin on platelets and its receptor P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils surface [203]. The formation of platelet-neutrophil complexes are a frequent circumstance in a wide range of inflammatory conditions such as pulmonary infection [38], abdominal sepsis [37], acute myocardial disease [39] and reperfusion injury [36]. In this thesis, it was hypothesized that P-selectin mediated platelet-neutrophil aggregate could be involved in NETs formation in AP. Moreover, it has shown that platelet-secreted molecules might able to regulate neutrophils activation and NETs formation (Figure 4). Platelet polyphosphate (PolyP) is an inorganic molecule that consists of linear polymers of orthrophosphate (60 to 100 phosphate unit) linked by phosphoanhydride bonds. Upon platelets activation, PolyP are released from dense granules and mediated blood clotting cascade via extrinsic and intrinsic pathways [207]. In addition, a recent study has observed that polyphosphate play a substantial role in

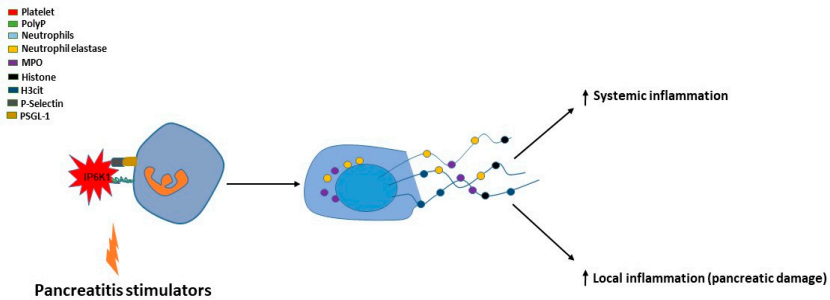

Figure 4. Basic illustration of platelet-induced NETs formation in AP. Activated platelets bind with neutrophil via P-selectin-PSGL-1 interaction and secrete IP6K1-regulated PloyP. This process lead to form neutrophil-derived NETs and increase both local and systemic inflammation in AP.

regulating of NETs formation [208]. Recently, it has been observed that hexakisphosphate kinase1 (IP6K1) regulates PolyP secretion via phosphorylation of inositol hexakisphosphate [42]. A previous study has demonstrated that IP6K1 gene-deficient mice displayed a significant reduction in platelet polyphosphate as well as resulted in slower platelet aggregation and lengthened plasma clotting time [42]. In addition to regulate PolyP, IP6K1 was also found to control the aggregation of neutrophil-platelet in endotoxin-induced lung inflammation, suggesting a pro-inflammatory role in systemic inflammation [46]. Therefore, it was interesting to investigate the role of IP6K1 mediated NETs formation in the pathophysiology of AP.

4- *Neutrophils microparticles*

Microparticles (MPs) are small vesicles (less than 1 μm) release from plasma membrane and contribute in various biological processes associated with inflammation, thrombosis, tumor cell growth, and intercellular signaling [209]. It is widely held that several types of cells such as leukocytes, platelets, endothelial cells and erythrocytes shed MPs upon activation or during apoptosis [32,210-212]. The mechanism of MPs generation has partly been understood; however, it has shown that increased cytosolic calcium leads to increase calpain activity [213]. A previous study on neutrophil MPs was shown that calpain enzyme is a potent mediator of degradation of talin, an essential protein of neutrophil cytoskeleton, and releasing of plasma membrane-derived vesicles [214]. Moreover, activated caspase regulates MPs formation through caspase 8 dependent pathway, for instance caspase 8 activates capase3 and the last one can activate calpain through cleavage of the calpain inhibitor, calpastatin (Figure 5) [215]. Furthermore, a recent study has observed that calpain and caspase have a critical role in MPs formation and that inhibition of these enzymes resulted in a significant reduction in PMA-stimulated neutrophil MPs generation [23].

The functional role of neutrophil microparticles as pro or anti-inflammatory is still under discussion. Generally, this criterion is based on the state of neutrophils and how neutrophil MPs are generated as well as it is also dependent on

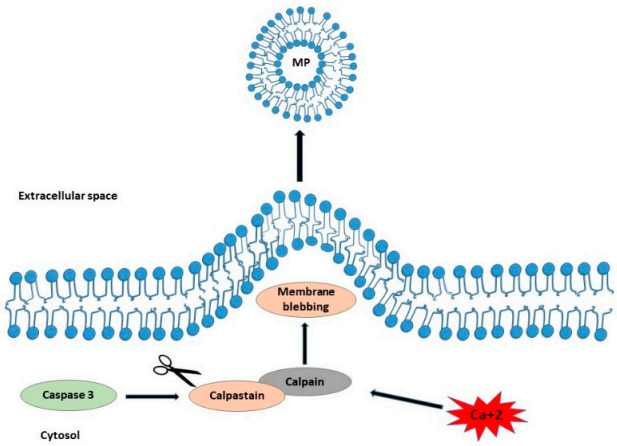

Figure 5. Basic illustration of MPs generation. Molecular mechanism leading to calpain activation that mediated MPs formation.

the cellular target. Indeed, previous studies have reported that MPs have cytoplasmic membrane and nuclear components from their parent cells [216] and retain the cell surface markers of parent cells [217]. For example, the expression of the cell surface adhesion molecules such as L-selectin and P-selectin glycoprotein 1 on the neutrophil MPs have shown to enhance the chemotaxis and migration of leukocytes [218]. Moreover, it was found that neutrophil MPs activate platelets and increase P-selectin expression through binding and activation of integrin αIIbβ [219]. It was also observed that neutrophil MPs increase the expression of endothelial cell tissue factor, which regulates the pro-coagulant response through Xa factor [220]. In addition, a recent study has reported that MPs, formed during neutrophil activation, are capable to bind with NETs and form NET-MP complexes. The authors found that these complexes are important for thrombin generation in sepsis [23]. In fact, neutrophil MPs were observed to increase in variety of inflammatory conditions. For example, it was reported that neutrophil MPs increased in patients with tubulointerstitial nephritis, IgA nephropathy, acute and chronic vasculitis as well as in patients with hemodialysis [221]. Subsequently, further studies are required to further understand the mechanism of neutrophil MPs in inflammatory disorders.

5- *c-Abl kinase and NETs formation*

c-Abelson (Abl) kinase is non-receptor tyrosine kinases that have been shown to be member of particular types of the Src family kinases and localized to both cytoplasm and nucleus [222]. In fact, the c-Abl family of tyrosine kinases are classified into two members, Abl and Arg (Abl-related gene), which are encoded by Abl 1 and Abl 2 genes in humans [223, 224]. Structurally, c-Abl kinase is composed of several domains: Src homology SH3, SH2 and tyrosine kinase domains which are located in N-terminal half that similar to Src family. In contrast, C-terminal half of c-Abl kinase consists of a DNA binding domain, an actin binding domain, and various short recognition motifs which are not considered as corresponding region in Src kinase [223-225]. It has been reported that the activity of c-Abl kinase is regulated by the SH3 and SH2 domains that found to be among the most

common domains of protein-protein interaction in human [226]. In the inactive condition, the inhibitory conformation of c-Abl kinase is provided by the SH3 and SH2 domains which combine with the distal domain of kinase and opposite of substrate binding site. These domains serve as a clamp that protects the kinase domain from conformational modification and led to low catalytic activity [227-229]. However, upon activation, c-Abl kinase molecule exposes to conformational change that results in relieved of SH3 and SH2 from their inhibitory role and trigger kinase activity through facilitate the interaction with the substrate. In addition, the major autophosphorylation site has shown to be Tyr-412 which is required to keep c-Abl in its active conformation [230]. In fact, several studies have used different inhibitors, such as imatinib [231], dasatinib [232], ABL001 [233] and GZD824 [234], to protect the c-Abl kinase domain from conformational change and inhibit its activity.

Up on activation by cellular stress and tissue injury, signaling pathways has been reported to play an important role in various biological processes by controlling gene expression of pro-inflammatory compounds [29]. c-Abl kinase serves as a control panel of cell signaling, for instance, it links diverse extracellular stimuli to signaling pathways that control the cell survive, growth, proliferation, invasion and migration [235]. It has also a critical role in cell spreading and dynamic formation or extension filopodia in response to extracellular signals [236]. It is well established that cell migration is multiple steps and a highly complicated process in which actin cytoskeleton regulation and alteration in cell shape have been reported to play an essential role in this process [237]. During the inflammatory response, it has demonstrated that c-Abl kinase contributes in the migration and recruitment of neutrophils by mediating actin polymerization and cytoskeleton rearrangement [32]. Subsequently, c-Abl kinase appears to be required for neutrophils phenotype change as well as their migration and recruitment. It is obvious that β2-integrin is expressed upon neutrophil activation. Indeed, β2-integrin has shown to play a critical role in neutrophil adhesion and migration. Previous report has shown that c-Abl kinase mediated neutrophil accumulation by regulating neutrophil β2-integrin expression [33].

Indeed, it was found that TNF α-activated c-Abl kinase phosphorylates and activates STAT3 and STAT5 transcription factors (Figure 6). Phosphorylated STAT3 triggers increase electrons influx into electron transport chain (ETC) in mitochondria. When the electrons fall into molecular oxygen results in generation of superoxide radical anion O^{-}_{2} that led to formation of ROS in the mitochondria of activated neutrophil [34]. Accumulating evidences have been shown that ROS generation is a potent stimulator of NETs formation from neutrophils [238]. In addition, ROS formation have reported to have a role in NE and MPO release from their granules as well as facilitate histone citrullination by peptidyl arginine deiminase 4 (PAD4) and finally expel NETs into extracellular space [35]. Therefore, it was of interest to investigate the role of c-Abl kinase in regulating NETs formation in AP.

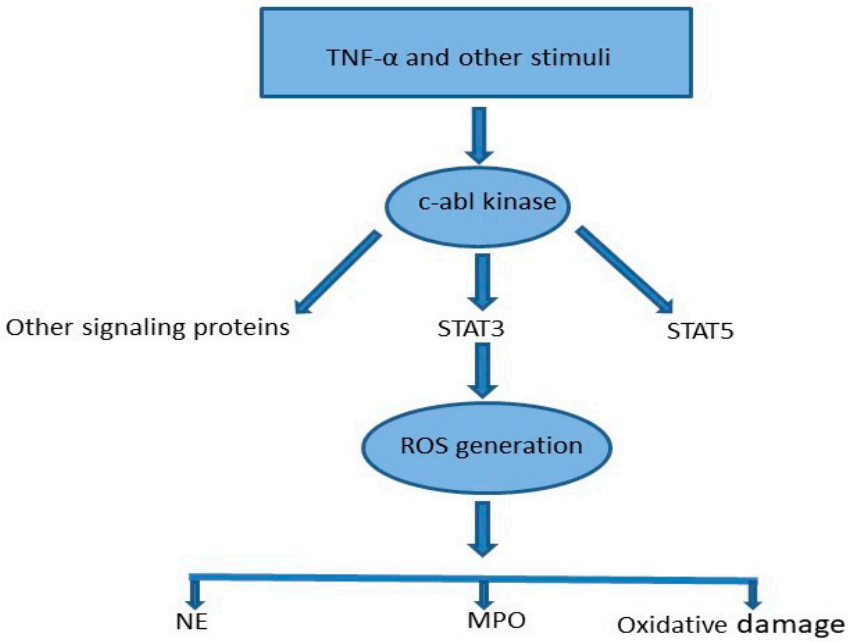

Figure 6. Mechanism of c-Abl kinase signaling cascade. Activated c-Abl kinase serves as a control panel that activates signaling proteins such as STAT3 and STAT5. This signaling cascade can results in oxidative damage via regulation of ROS generation, NE, MPO.

Chapter 6

Methodology

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1- ***Animals***

Animal experiments were done in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals that have been published by the US national institutes of health and approved by the ethical committee at Lund University, Sweden. Moreover, ARRIVE guidelines [239] and an editorial on the application of these guidelines to pharmacological studies [240] were consulted with the regard to all in vivo studies involving animals. Male wild-type C57BL/6 (20-26 g) were used in study I and II. In study III, male IP6K1 gene-deficient mice with C57BL/6 (21-25 g) background and wild-type C57BL/6 (21-25 g) control mice were used. They were maintained in climate-controlled room in a 12-hour light/dark cycle at 22°C with water accessible and fed a laboratory diet ad libitum. For anesthetize, mice were received intraperitoneal (i.p.) injection of 75 mg of ketamine hydrochloride and 25 mg of xylazine per kg body weight.

2- ***Induction of AP and experimental design***

Pancreatitis was induced in mice as described previously [109]. In brief, animals were anaesthetized and laparotomised and then the papilla of Vater was identified. The duodenum was immobilized by two 7,0 prollene sutures. Then the duodenal wall, opposite to the papilla of Vater was punctured by using 23 G needle was under microscopic observation. A polyethylene catheter connected to a micro-infusion pump (CMA/100, Carnegie Medicine, Stockholm, Sweden) was inserted 1 mm into the common bile duct. To prevent hepatic reflux, the common hepatic duct was identified at liver hilum and clamped by a clip at the liver hilum. 10 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. Animals were sacrificed 24 hours after pancreatitis induction. Then, blood samples were gathered from inferior vena cava for different assays. Pancreatic tissue was rapidly collected and separated into three pieces. One third was utilized for electron microscopy and one third was snap-frozen in liquid nitrogen for biochemical. One third was fixed in formalin for histological analysis. Lung tissue was also collected for measurement of MPO activity in the lung tissue. In separated experiments, the induction of AP was performed by administration of L-arginine (4 g/kg/dose) i.p. twice at an interval of 1h as described in detail previously [233]. Saline-received animals treated as negative controls. Blood was collected from the tail vein and inferior vena cava. All animals were sacrificed 72 h after the first administration of L-arginine.

3- ***Amylase*** **measurements**

Blood was collected from the tail vein and diluted in PBS (1:50). Levels of blood amylase were quantified in the diluted blood using a commercially available kit (Reflotron, Roche Diagnostics GmbH, Mannheim, Germany).

4- Myeloperoxidase assay

A piece of the pancreatic head and lung tissue were collected 24 hour after induction of pancreatitis and snap-frozen in liquid nitrogen in order to measure the activity of MPO. First, 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 (15300 g, 10 minutes) and the supernatant was stored at −20°C for

subsequent ELISA. To measure MPO activity, 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% hexadecyl-trimethylammonium bromide before MPO assay. Next, samples were thawed and sonicated for 90 seconds then incubated in a water bath at 60°C for 2 hours. After that samples were centrifuged for 5 minutes at 15300 g and then 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 per gram tissue in all studies that included in this thesis.

5- Enzyme liked immune sorbent assay

The levels of CXCL1, CXCL2, histone 3, histone 4, MMP-9 and IL-6 were measured in stored supernatants of homogenized pancreatic tissue or plasma by use of double-antibody ELISA kits (R&D Systems Europe, Abingdon, Oxon, UK and USCN, Life Science Inc., Burlington, NC, USA) according to manufacturer's instructions. Blood was obtained from the inferior vena cava and diluted (1:10) in acid citrate dextrose, then centrifuged at 15300 g for 10 minutes at 4°C and stored at -20°C until use. For detection of DNA-histone complexes plasma samples or in vitro samples were centrifuged at 15 300 g for 5 minutes at 4°C and the levels of DNA-histone complexes were determined by use of a Cell Death Detection Elisa Plus kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

6- *Histology and scoring system*

Pieces from the head of the pancreas were fixed in 4% formaldehyde overnight, dehydrated and embedded in paraffin. Tissue sections (6 µm) were stained by use of hematoxylin and eosin and then examined by light microscopy. A pre-existing scoring system were used in order to evaluate the severity of pancreatitis in a blinded manner. Quantifying edema, acinar cell necrosis, hemorrhage and neutrophil infiltration on a 0 (absent) to 4 (extensive) scales as previously described [242].

7- *Electron microscopy*

To identify NETs in paraffin-embedded pancreatic tissue, samples were examined by high resolution scanning electron microscopy. Samples on coverslips were subjected to deparaffinize and fixed in 2.5 % glutaraldehyde in 0.15 M sodium cacodylate, pH 7.4, (cacodylate buffer) for 30 minutes at room temperature. Samples then washed with cacodylate buffer and dehydrated with an ascending ethanol series from 50% (v/v) to absolute ethanol (10 minutes per step). The Samples were subjected to critical-point drying in CO2, with absolute ethanol as intermediate solvent, mounted on aluminum holders, and then sputtered with 30 nm palladium/gold. A Jeol/ FEI XL 30 FEG scanning electron microscope at the Core Facility for Integrated Microscopy at Panum Institute, University of Copenhagen, Denmark were used to examine samples. Location of individual target molecules in tissue NETs were analyzed at high resolution by ultra-thin sectioning and transmission immunoelectron microscopy. Samples on coverslips were embedded in Epon 812 and sectioned into 50-nm-thick ultrathin sections with a diamond knife in an ultramicrotome. Sections were incubated overnight (4°C) with primary antibodies against elastase and citrullinated H3 in study I and elastase, histone H4, Mac-1 and CD41 in study III (abcam, Cambridge, UK). Controls without primary antibodies were included. Grids were then incubated with species-specific gold-conjugated secondary antibodies (Electron Microscopy Sciences, Fort Washington, MD).

Finally, the sections were post-fixed in 2% glutaraldehyde and post-stained with 2% uranyl acetate and lead citrate. A Jeol/FEI CM100 transmission electron microscope operated at 80 kV accelerating voltage was used to examine the specimens.

8- Evaluation of MPs and performing pseudo-coloring

Cellular MPs are presented in different size (50 nm to 1000 nm in diameter) and shapes (globular, non-globular, oval, irregular, etc). MPs of original images were given a pseudo-color in order to highlight the tiny MPs (50-1000 nm) and simplify interpretation of the original images for the reader in analogy of using arrows or circles. Briefly, MPs were evaluated by using higher magnification with enhanced contrast of original images in order to have a clear viewing of MPs, so that made it easily to examine the MPs on NETs. Then, pseudo-coloring of MPs was performed by using pink color circular objects to denote MPs in the original images.

9- Flow cytometry

Mac-1 expression was determined on Ly6G positive cells in study II. Blood was collected from the inferior vena cava and diluted (1:10) in acid citrate dextrose. Fcγ III/II were blocked as mentioned above. Then, cells were incubated with a Phycoerythrin (PE) or allophycocyanin (APC)-conjugated anti-Ly6G (clone 1A8, BD Pharmingen, San Jose, CA) and a Fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 (clone M1/70, integrin αM china, rat IgG2b, BD Biosciences Pharmingen, San Jose, CA, USA) antibody at 4°C for 20 min. Flowcytometric analysis was performed according to standard setting on a Cytoflex flow cytometer (Beckman Coulter, Indianapolis, IN), and dead and fragmented cells were exclude by using the viable gate. In study III, flow cytometry was performed for the measurement of PNA and the percentage of platelets, blood was collected into syringes pre-filled with 1:10 acid citrate dextrose 24 h after challenged with taurocholate. To reduce non-specific labelling, blood samples were incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors for 10 minutes at room temperature. Next, samples were incubated with phycoerythrin- conjugated anti Ly6G (clone 1A8, BD Pharmingen, San Jose, USA) and fluorescein isothiocyanate-conjugated anti-CD41 (clone MWReg30, BD Pharmingen, San Jose, USA) in order to detect the percentage of neutrophil-platelet aggregates by considering neutrophils as cells positive for Ly6G and platelets as CD41+ cells. Then, samples were fixed with 2% formaldehyde solution and erythrocytes were lysed using ACK lysing buffer (Thermo Fisher Scientific, Somerset, NJ, USA). Cells were recovered following centrifugation. Flow-cytometric determination of neutrophil-platelet aggregates was performed by first gating all viable cells and then the percentage of neutrophils (Ly6G+) binding platelets (CD41+) was analyzed in this population. For comparison of leukocytes subtypes and platelets, blood was collected from wild-type and IP6K-gene deficient mice and incubated with an anti-CD16/CD32 antibody as described above. Samples were then incubated with PerCP-Cy5.5-conjugated anti-CD45 (clone 30-F11, Biolegend, San Diego, CA) antibody, PE-conjugated anti-Ly6G (clone 1A8, BD Pharmingen, San Jose, CA) antibody, APC-conjugated antiCD4 (clone GK 1.5, eBioscience, San Diego, CA) antibody and a FITC-conjugated anti-CD41 (clone eBioMWReg30, eBioscience, San Diego, CA) antibody at 4°C for 20 minutes. Flow-cytometric determination of leukocytes subtypes and platelets was performed and dead

and fragmented cells were exclude by using the viable gate.

10- ***Isolation of blood neutrophils***

To measure the activity of c-abl kinase in isolated neutrophils, mice were euthanized and blood was collected from the inferior vena cava and diluted (1:10) in acid citrate dextrose. Blood samples were compiled with Roswell Park Memorial Institute medium 1640 (RPMI 1640, Invitrogen, Stockholm, Sweden) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 2 mM EDTA (Sigma-Aldrich, Stockholm, Sweden). Then, by using a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden), neutrophils were extracted from other cells by subjecting to density gradient centrifugation using a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden). ACK lysing buffer (Thermo Fisher Scientific, Somerset, NJ) was used to lyse erythrocytes and neutrophils, next, were isolated and washed with RPMI 1640 and then resuspended at 4×10^6 cells/ml. Finally, the cells were homogenized to identify the activity of c-abl kinase.

11- ***In vitro ROS and NET formation***

Bone marrow neutrophils ($2x10^6$ cells/ml) were extracted from healthy C57BL/6 mice by density gradient centrifugation using a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden). FcϒIII/IIRs were blocked by incubating neutrophils with anti-CD16/CD32 to reduce non-specific labeling. Cells were stained by incubating with PE-conjugated anti-Ly6G antibodies. To measure ROS generation, cells were incubated with dihyrorhodamine 123 (Sigma) for 15 min at 37°C and then stimulated with 100 U/ml TNF-α (R&D Systems Europe Ltd., Abingdon, UK) for 1 hour at 37°C. In separate experiments, neutrophils were isolated and incubated with 100 U/ml TNF-α for 3 h at 37°C. Next, samples were subjected to centrifugation (400 g, 5 min) and supernatants were collected to detect DNA-histone complex levels as described above. In study II and III, NETs were detected by flow cytometry. First, the cells were fixed with 2% formaldehyde. Then, cells were stained by incubating with primary antibodies: Phycoerythrin (PE) conjugated anti-Ly6G (clone 1A8, BD Pharmingen), Fluorescein isothiocyanate (FITC) conjugated anti-MPO antibody (mouse: ab90812) and rabbit anti-H3cit (citrulline 2,8,17, ab5103; Abcam, Cambridge, MA) in PBS containing 5% donkey serum. Cells were washed tice and next, cells were incubated with rat anti-rabbit allophycocyanin (APC) conjugated secondary antibody (A-21038, Thermo Scientific, Rockford, IL). Flow cytometry analysis was done according to standard setting on a CytoFLEX flow cytometer (Becton Dickinson, Mountain View, CA, USA), and dead and fragmented cells were exclude by using the viable gate. To visualize NETs by immunofluorescence imaging, neutrophils were stimulated with TNF-α or PMA and permeabilized with 1% Triton X-100 for 10 min and then stained on glass coverslips as described above. Next, immunostaining was performed and then coverslips rinsed and mounted in fluoromount with DAPI (Thermo Fisher Scientific). Confocal microscopy was performed using LSM 800 confocal (Carl Zeiss, Jena, Germany) by a × 63 oil immersion objective (numeric aperture = 1.25). The pinhole was ~ 1 airy unit and the scanning frame was 1024×1024 pixels. Images were later processed using ZEN2012 software.

12- ***Generation of NETs and MP in Vitro***

Bone marrow neutrophils ($4 x10^6$ cells/ml) were isolated from healthy C57BL/6 mice and stimulated with 50 nM PMA for 3 h at 37°C in RPMI 1640. NETs without MPs has been

shown to be generated by co-incubated with caspase and calpain [23], accordingly, the cells were co-incubated with caspase (50 µM, Z-VAD-FMK, R&D Systems) and calpain (25 µM, PD150606, Sigma-Aldrich) inhibitors to stimulate NETs without MPs. After removal of supernatants, fresh media was added to isolate NETs. By extensive pipetting, the residual neutrophils and NETs were removed. Next, the mixture was subjected to centrifuge at 200 g for 5 minutes to discharge cellular components and supernatants that containing NETs were collected. Supernatants, that contained NET, were subjected to centrifugation at 19000 g (15 min) to collect NETs. To generate MPs, supernatants were centrifuged at high speed (21 000 g for 1h at 4°C). Finally, supernatants were discharged and the pellets contained MPs were re-suspended in phosphate buffer saline (PBS) and stored at -20°C until use.

13- ***Pancreatic acinar cells***

Pancreatic acinar cells were isolated from healthy C57BL/6 mice by collagenase digestion as previously described [243]. HEPES-ringer buffer containing collagenase from *Clostridium histolyticum* type 1 (2.5 ml, 1%, Sigma-Aldrich) was smoothly infused into the pancreatic duct. The animals were euthanized and pancreatic tissues were harvested. To have maximal exposure to collagenase, the pancreas was cut into pieces, gently shaken and incubated at 37°C for 15 minutes. In order to remove the collagenase and stop digestion, the solute was centrifuged and washed three times in cold HEPES-Ringer buffer, pH 7.4. Cold HEPES-Ringer buffer was then added to the acinar cells and the solute was passed through a 150 µm cell strainer (Partec, Canterbury, England). In order to check the viability of pancreatic acinar cells, trypan blue was used and the viability of cells was higher than 95%. Next, the cell suspension was divided in Eppendorf tubes and kept on ice until subsequent in vitro experiments. Acinar cells were incubated with NETs, NETs depleted of MPs (calpain/caspase treatment), DNase I treated NETs and neutrophil-derived MPs for 1 hour at 37°C. The mixture was centrifuged (1400 g, 5 minutes) and amylase secretion was determined in the supernatant as described earlier. The cell pellets were homogenized and used for western blot of STAT-3 phosphorylation, as described below.

14- ***Western blot***

Samples of pancreatic tissue and blood isolated neutrophils were obtained and homogenized in ice-cold RIPA buffer (RIPA Lysis and Extraction Buffer, ThermoFisher, USA) containing protease inhibitors (Halt Protease Inhibitor Cocktail; Pierce Biotechnology, Rockford, IL) for 20 min. Samples were then subjected to sonication and centrifugation (16000 g for 15 min, 4°C). Supernatants were collected and stored at -20°C. Pierce BCA Protein Assay Reagent (Pierce Biotechnology) was used in order to determine protein concentration of supernatants. Next, 8-16% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad) was utilized to separate proteins (20 µg per lane) and then proteins were transferred to polyvinylidene fluoride membranes (Novex, San Diego, CA, USA). Total protein gel image was taken before blotting by use Bio-Rad's stain-free gel chemistry. To block the membrane and avoid non-specific reaction, membranes were incubated in TBS/Tween 20 buffer containing 5% non-fat dry milk powder. Protein immunoblots were performed using anti-histone H3 in study I,II and III (citrulline 2,8,17, ab5103, Abcam, Cambaridge, MA, USA), anti-c-Abl kinase and anti-phosphorylated c-Abl kinase antibody in study II (Selleck Chemicals, Munich, Germany).

After wash, the membranes were incubated with secondary antibodies or anti-biotin conjugated with peroxidase. In study III, isolated acinar cells were exposed to NETs, NETs depleted of MPs (calpain/caspase treatment), DNase I-treated NETs and neutrophil-derived MPs to measure the activity of (Tyr 705) stat3 and stat3. Cell pellets were then homogenized and protein concentration of supernatants were determined as described above. Proteins separation and blocking were performed as described above. Protein immunoblots were performed using antibodies against phosphotyrosin (Tyr 705) stat3 and stat3 (Cell Signaling Technology, Beverly, MA). After wash, the membranes were incubated with secondary antibodies or anti-biotin conjugated with peroxidase. BioRad ChemiDoc™ MP imaging system and Image Lab™ software version 5.2.1 was used to normalized the interest band against the total protein in the corresponding lane.

15- ***Quantitative RT-PCR***

To determine the expression of IL-6 and TGFβ1, acinar cells were exposed to NETs, NETs depleted of MPs (calpain/caspase treatment), DNase I-treated NETs and neutrophil-derived MPs. Next, cell pellets were obtained and the expression of IL-6 mRNA and TGFB1 mRNA was examined by RT-qPCR. Briefly, RNA samples were isolated by using Direct-zol RNA extraction kit (Zymo Research, Irvine, CA, USA) kit according to manufacturer's recommendations. Nanodrop spectrophotometer at 260 nm absorbance was used to determine total RNA concentration and cDNA was synthesized in a final reaction volume of 10 µL according by using RevertAid First Strand cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. qRT-PCR was performed in a final volume of 25 µL by using SYBR Green dye (Takara Bio, USA) for relative expression of IL-6 mRNA and TGFβ1 mRNA. The PCR primers used were as follows; mouse IL-6 mRNA sense; 5'-GAGGATACCACTCCCAACAGACC-3', antisense; 5'-AAGTGCATCATCGTTGTTCATACA-3' ; and mouse TGFβ1 mRNA sense; 5′-TCCCGTGGCTTCTAGTGCTG-3′, antisense; 5′-ATTTTAATCTCTGCAAGCGCA-3′, and GAPDH sense; 5′-GTCCCAGCTTAGGTTCATAG-3′, GAPDH antisense; 5′-GATGGCAACAATCTCCACTTTG-3′. 2- ΔΔ CT method was performed to determine the expression of IL-6 mRNA and TGFB1 mRNA relative to house-keeping gene GAPDH.

16- ***Platelet-neutrophil interactions***

Bone marrow neutrophils were extracted from wild-type and IP6K1-gene deficient mice by subjected to density gradient centrifugation using a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden). Blood platelets were also isolated from wild type and IP6K1-gene deficient mice. Briefly, mice were scarified and blood was collected from the inferior vena cava with 1:10 acid citrate dextrose anticoagulant and immediately diluted with equal volumes of modified Tyrode solution (1 µg/ml prostaglandin E1 and 0.1 U/ml apyrase). The mixture then subjected to centrifuge at 200 g for 5 minutes. Platelet-rich plasma (PRP) was collected and subjected to centrifuge at 800g for 15 minutes. After wash twice with modified Tyrode solution, platelet pellets were immediately used for the experiment. To visualize NETs by immunofluorescence imaging, isolated neutrophils were co-incubated with isolated wild-type or IP6K1-gene deficient platelets with or without thrombin (0.2 U/ml, Sigma, USA) and with or without polyP (10 and 100 µM, Kerafast,

Boston, USA) over glass coverslips inside a 24-well plate for 3 hours at 37 °C. Neutrophils were then fixed with 2% formaldehyde and permeabilized with 1% Triton X-100 for 10 minutes. Next, cells were stained by incubating with primary antibodies: Fluorescein isothiocyanate (FITC) conjugated anti-MPO antibody (mouse: ab90812) and rabbit anti-H3cit (citrulline 2,8,17, ab5103; Abcam, Cambridge, MA) in PBS containing 5% donkey serum. After washing twice, cells were incubated with rat anti-rabbit secondary antibody conjugated with allophycocyanin (APC) (A-21038, Thermo Scientific, Rockford, IL). Next, cells were counterstained with Hoechst 33342 and then coverslips were rinsed and mounted in fluoromount (Thermo Fisher Scientific). Confocal microscopy was applied by using Zeiss LSM 800 (Carl Zeiss, Jena, Germany) by a × 63 oil immersion objective (numeric aperture = 1.25). The pinhole was ~ 1 airy unit and the scanning frame was 1024×1024 pixels. Images were then processed using ZEN2012 software.

17- *Confocal imaging of platelet-polyphosphate*

Blood platelets were isolated from wild-type and IP6K1-gene deficient mice as described above. To fix isolated platelets, the cells were later fixed in 4% paraformaldehyde in PBS for 30 minutes and then washed with PBS. Next, cells were dissolved in water and 1 mg/ml DAPI was added. After attaining, cells were mounted on the slides and confocal images were taken using Zeiss LSM 800 (Carl Zeiss, Jena, Germany) by a × 63 oil immersion objective (numeric aperture = 1.25). The fluorescence emission of DAPI shifts to higher wavelengths when binds with polyP (3, 4). Samples were excited at 405 nm and emission was observed at 530-570 nm. Images were processed using ZEN2012 software.

18- *Statistics*

Data are presented either as mean values ± standard errors of the means or box plot (25-75 percentiles) where horizontal line indicates median of the group; whiskers extend from the minimum to the maximum values. Statistical comparisons were done by using non-parametrical tests (Mann-Whitney or ANOVA on ranks followed by Dunnett's multiple comparisons). $P < .05$ was considered significant and n represents the number of animals or experiments.

Role of PAD4 in Regulating NET Formation in AP

Contents

1- Aim

To determine the role of PAD in NETs formation and tissue damage in AP.

2- Introduction

Neutrophils represent the main defense lines and first immune cells that arrive to site of inflammation [244]. In the circulation, neutrophils are directed into infected tissue by cytokines where they face the invading pathogens. Indeed, activated neutrophils engulf the pathogens into a phagosome in which they are destroyed [181]. Furthermore, activated neutrophil has been shown to use another mechanism by releasing extracellular web-like structures called NETs. NETs are composed of decondensed chromatin, histones and granular proteins [15]. Although their role in the innate immune system to trap and kill pathogens, the excessive NETs formation or disorder of NETs clearance have been negatively implicated in number of diseases [245, 246]. For example, it was observed that NETs contributed in development of inflammation in rheumatoid arthritis [247], systemic lupus erythematosus [248], AP [19], chronic obstructive pulmonary diseases [249], thrombosis [23] and cancer [250]. However, the molecular mechanism of NETs formation is still not completely understood. In fact, it is well known that histone modification has essential role in chromatin decondensation through a process called citrullination [24]. Having established that citrullinated histone 3 (H3cit) is a predominant component of NETs and considered as part of the neutrophil response to infection. Citrullination process is orchestrated by peptidyl arginine deiminases (PAD) which initiate NETs formation via hypercitrullination of the histone proteins, (**Figure 7**) [194]. Convincing data have demonstrated that PAD inhibitor Cl-amidine protected against histones citrullenation and NETs formation in different diseases such as; murine sepsis model [194], atherosclerosis [251] and colitis [252]. Moreover, a recent study has shown that a specific PAD4 inhibitor, GSK484, markedly abolished NETs formation in vitro [195].

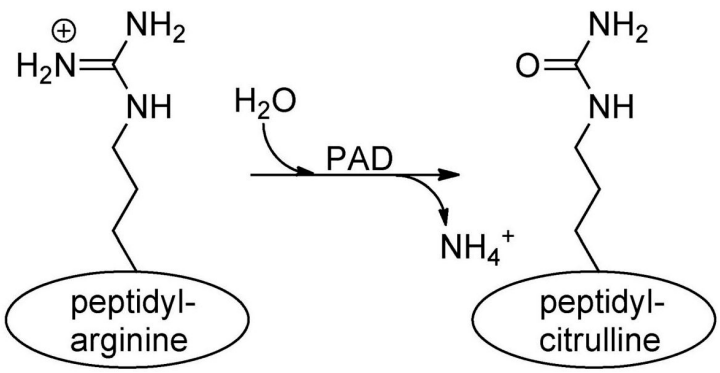


Figure 7. **Basic schematic illustration of histone citrullination process**. Arginine residue on histone proteins is converted into citrulline by peptidylarginine deiminase (PAD) enzymes [253].

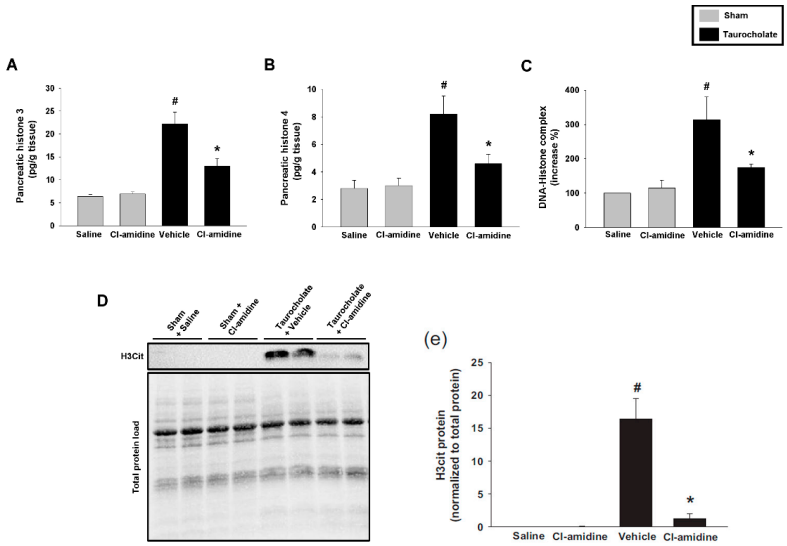


Figure 8. Quantifications of A) histone 3, B) histone 4 in the pancreas and C) extracellular DNA-histone complexes in the plasma. D) Pancreatic H3Cit was determined by western blot and E) aggregate data showing H3Cit protein normalized with stain-free total protein load. Sham mice (grey bars) were received only saline. Pancreatitis (black bars) was induced by retrograde infusion of (5%) sodium taurocholate into pancreatic duct. Animals were received i.p. injections of the Cl-amidine (50 mg/kg) or vehicle (DMSO) as described in Materials and Methods. 24 hours after pancreatitis, samples were collected. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus taurocholate without Cl-amidine.

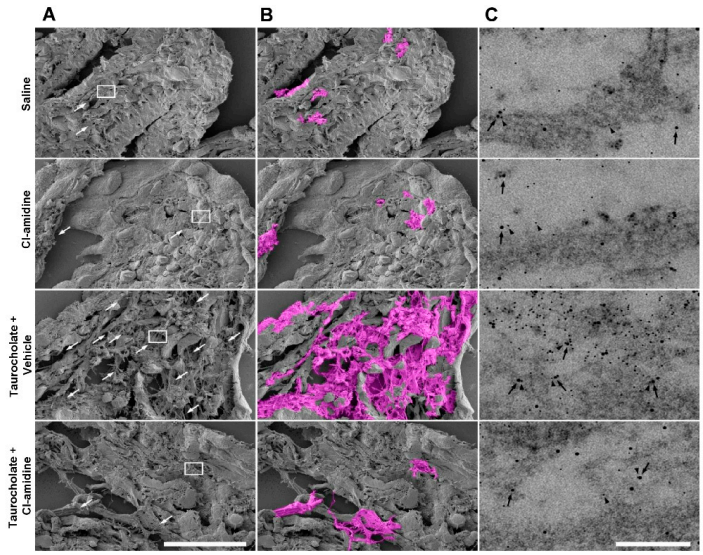


Figure 9. Role of PAD in NET formation in AP. A) Extracellular web-like structures were identified by scanning electron microscopy in the pancreas from mice challenged with taurocholate. Scale bar = 5 µm. B) NETs are indicated in pink color. C) Indicated area of interest from Figure 1A was identified by transmission electron microscopy and incubated with gold-labeled antibody against citrullinated histone 3 (large gold particles) and anti-elastase (small gold particles). Scale bar = 0.25 µm. Pancreatitis (black boxes) was induced by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (grey boxes) were infused with saline alone. Animals were received i.p. injections of the Cl-amidine (50 mg/kg) or vehicle (DMSO) as described in Materials and Methods. Samples were collected 24 hours after induction of pancreatitis.

Therefore, in this study we hypothesized that PAD might be involved in NETs formation, inflammation and tissue damage in severe AP.

3- Results and discussion

Histones and DNA-histone complex are widely accepted as major component of NETs structures. In the present study, we found that infusion of taurocholate into pancreatic duct increased the levels of pancreatic H3, H4 and citrullinated H3 by 3-fold, 4-fold and 410-fold, respectively (**Figure 8A, B, D and E**), as well as plasma levels of DNA-histone complex by more than 3-fold (**Figure 8C**) in animals challenged with taurocholate. Interestingly, it was found that animals pretreated with Cl-amidine had markedly decreased levels of H3, H4 and citrullinated H3 by 56%, 76% and 67% respectively, in the pancreas (**Figure 8A, B, D and E**), as well as the levels of DNA-histone complex by 91%, in plasma (**Figure 8C**). Subsequently, these results indicated that Cl-amidine effectively reduced NETs formation in the pancreas of mice exposed to taurocholate.

Moreover, this fact was confirmed by using electron microscopy and it was observed that challenged with taurocholate provoked web-like structures that contained neutrophil-derived elastase protein and citrullinated H3 in the inflamed pancreas (**Figure 9**). Indeed, administration of Cl-amidine greatly abolished formation of NETs in animals challenged with taurocholate (**Figure 9**), suggesting that PAD have a critical role in regulating of NETs formation in AP.

Next, we examined the role of PAD in regulating tissue damage in the taurocholate-induced pancreatitis model. In fact, it is generally accepted that amylase levels can be used as an indicator of acinar cell damage and tissue injury. We observed that challenge with taurocholate resulted in increase in the levels of blood amylase by 19-fold (**Figure 10**).

Notably, it was found that pretreated with Cl-amidine markedly reduced amylase levels by 43% (**Figure 10**). In addition, 24 hours after the challenge with taurocholate, the histological examinations showed clear-cut tissue damage characterized by intensive edema, hemorrhage and acinar cell necrosis as compared with sham animals (**Figure 11A-D**). However, pretreated with Cl-amidine protected against taurocholate-induced disruption of pancreatic architecture that typified by a markedly reduction of edema, acinar cell necrosis and hemorrhage by 60%, 62%, 59%, respectively, in taurocholate challenge animals (**Figure 11A-D**). Indicating that PAD activity controls a significant part of tissue injury in AP.

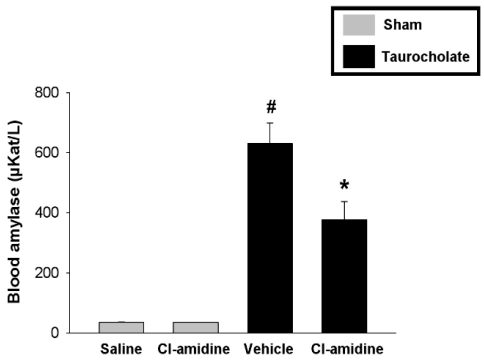


Figure 10. Levels of blood amylase measurements. Pancreatitis (black boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (grey boxes) were infused with saline alone. Animals were received i.p. injections of the Cl-amidine (50 mg/kg) or vehicle (DMSO) as described in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus taurocholate without Cl-amidine.

MPO is peroxidase enzyme that stored in azurophilic granules of neutrophils. It has a key role in production of hypochlorite during inflammatory condition by conversion of chloride and hydrogen peroxide [254]. In fact,

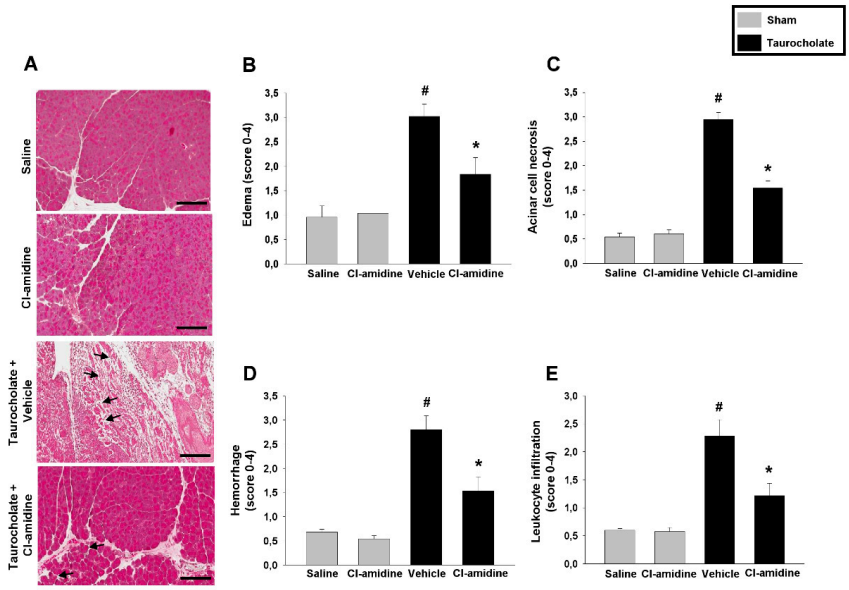


Figure 11. Role of PAD in taurocholate-provoked tissue damage in AP. Hematoxylin & eosin sections from the head of the pancreas of indicated groups. Scale bar = 100 µm. Histological scoring of B) edema (black arrows indicate the expansion of interlobar space), C) acinar cell necrosis, D) hemorrhage and E) leukocyte infiltration in the pancreas of sham (grey bars), (saline alone was infused into pancreatic duct), and taurocholate (5%)-challenged mice (black bars). Control mice (grey bars) were infused with saline alone. Animals were received i.p. injections of the Cl-amidine (50 mg/kg) or vehicle (DMSO) as described in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05

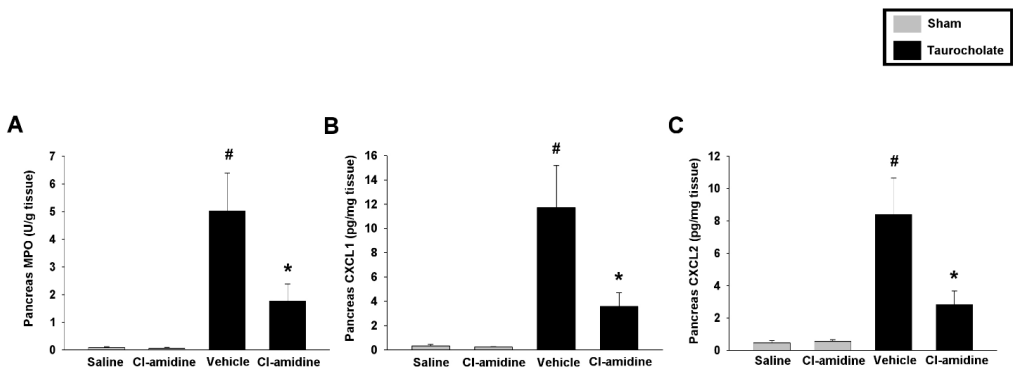


Figure 12. Role of PAD in taurocholate-provoked tissue inflammation in AP. Quantification measurement of levels of A) MPO, B) CXCL1 and C) CXCL2 in the pancreas. Pancreatitis (black boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (grey boxes) were infused with saline alone. Animals were received i.p. injections of the Cl-amidine (50 mg/kg) or vehicle (DMSO) as described in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus taurocholate without Cl-amidine.

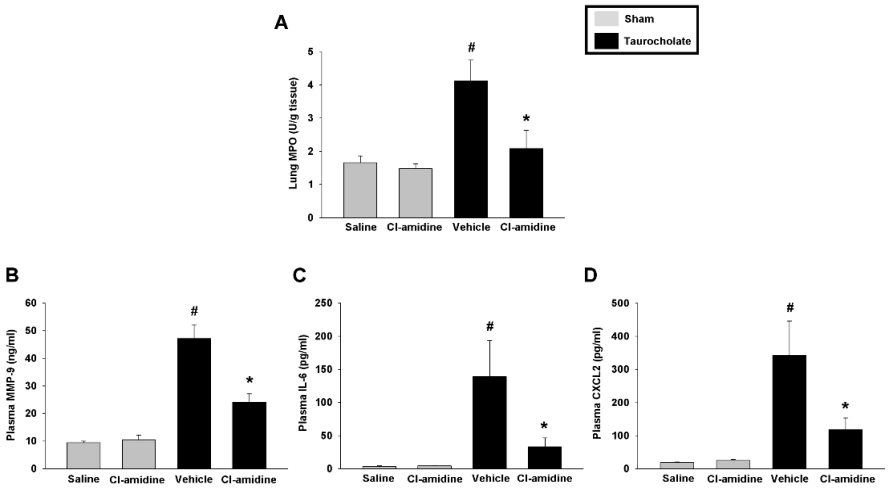


Figure 13. PAD control systemic inflammation in AP. Quantification measurement of A) MPO activity in the lung. Levels of B) MMP-9, C) IL-6 and D) CXCL1 in the plasma. Pancreatitis (black boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (grey boxes) were infused with saline alone. Animals were received i.p. injections of the Cl-amidine (50 mg/kg) or vehicle (DMSO) as described in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus taurocholate without Cl-amidine.

this enzyme has been used as an indicator of neutrophil recruitment in AP [255]. In the present study, we observed that taurocholate challenge caused clear cut increased in MPO levels and extravascular neutrophil infiltration in pancreatic tissue compared with sham group. Notably, administration of Cl-amidine significantly attenuated MPO activity and extravascular neutrophil infiltration by 67% (**Figure 12A**) and 63% (**Figure 11E**), respectively, in the inflamed pancreas. Suggesting that PAD regulates neutrophils infiltration and this fact might be explained the protection effect of Cl-amidine in severe AP. It is well known that CXC chemokines, such as CXCL1 and CXCL2, have an important role in pathogenesis of AP and neutrophils trafficking to site of inflammation [154, 256]. To evaluate the effect of PAD on chemokines secretion, pancreatic tissue was collected 24 h after challenge with taurocholate. We found low CXCL1 and CXCL2 concentrations in the pancreatic tissue of sham group and a significant increase in taurocholate-induced pancreatitis group (**Figure 12B and C**). Notably, administration of Cl-amidine markedly decreased the levels of CXCL1 and CXCL2 by 71%, and 70%, respectively, in the inflamed pancreas (**Figure 12B and C**). Indicating that PAD have a possible effect on CXC chemokines secretion and this notion can led us to explain the effect of Cl-amidine in the reduction of extravascular neutrophils recruitment in severe AP. Furthermore, infiltration of inflammatory cells into lung tissue has been shown to be as a part of systemic inflammatory response in AP [257]. In this context, activated neutrophils accumulation in lung tissue was examined as a part of systemic inflammatory response. We found that taurocholate-induced MPO greatly increased in lung tissue of taurocholate challenge animals compared with sham animals (**Figure 13A**). However, pretreated with Cl-amidine significantly reduced MPO levels in lung tissue. This observation was also provided by a notion that taurocholate-induced increase of plasma levels of MMP-9, IL-6 and CXCL2 which were

significantly attenuated by administration of Cl-amidine (**Figure 13B-D**). Taking together that PAD can regulate both local and systemic inflammation in severe AP.

Moreover, the results above were further supported by using another specific PAD4 inhibitor, GSK484. Indeed, it was found that administration of GSK484 markedly attenuated inflammation and NETs formation in AP. For example, we noticed a significant reduction in the levels of blood amylase from 539 ± 107 to 113 ± 24 μKat, corresponding to a 79% decrease, in mice pretreated with GSK484 and challenged with taurocholate (**Figure 14A**). In addition, we found that pretreated mice with GSK484 had significantly lower pancreatic histone 3 and histone 4 and plasma levels of DNA-histone complex (**Figure 14B-D**).

Moreover, it was observed that administration of GSK484 not only decreased the pancreatic levels of MPO (**Figure 14E**), CXCL1, IL-6 and MMP-9 (**Figure 15A-C**), but also markedly reduced the plasma levels of CXCL1, Il-6, and MMP-9 (**Figure 15D-F**), suggesting that PAD regulate both local and systemic inflammation in severe AP via possibly controls formation of NETs.

In conclusion, the present demonstrated that PAD4 has a potent stimulatory effect on NETs formation in severe AP. In addition, these findings were also found that PAD4 controls inflammation and tissue damage in severe AP. Therefore, the present study strongly suggests that targeting PAD4 could be a useful strategy to attenuate both local and systemic inflammation in severe AP.

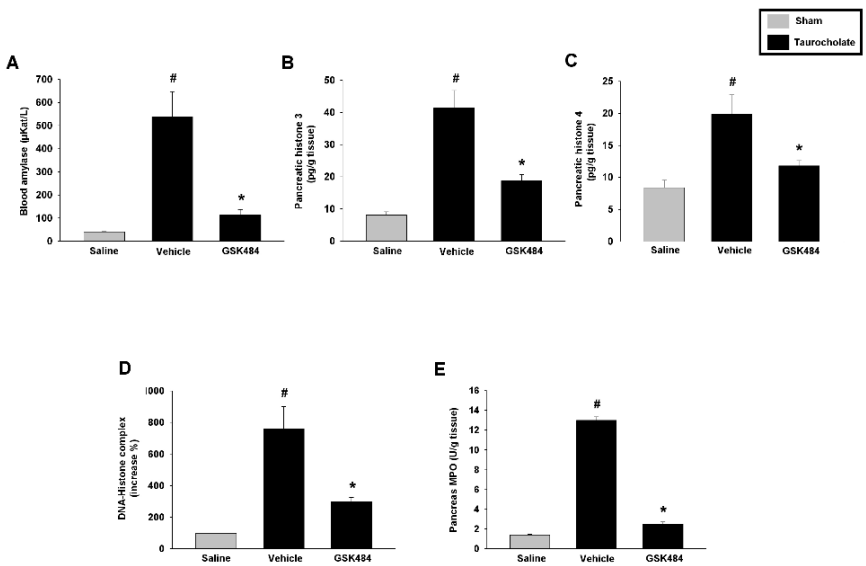


Figure 14. PAD4 regulates inflammation and NETs formation in AP. A) Levels of blood amylase. Levels of B) histone 3, C) histone 4 in pancreas D) levels of extracellular DNA-histone complexes in plasma and E) activity of MPO in the pancreas. Pancreatitis (black boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (grey boxes) were infused with saline alone. Animals were received i.p. injections of the GSK484 (4 mg/kg) or vehicle (alcohol) as described in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus taurocholate without GSK484.

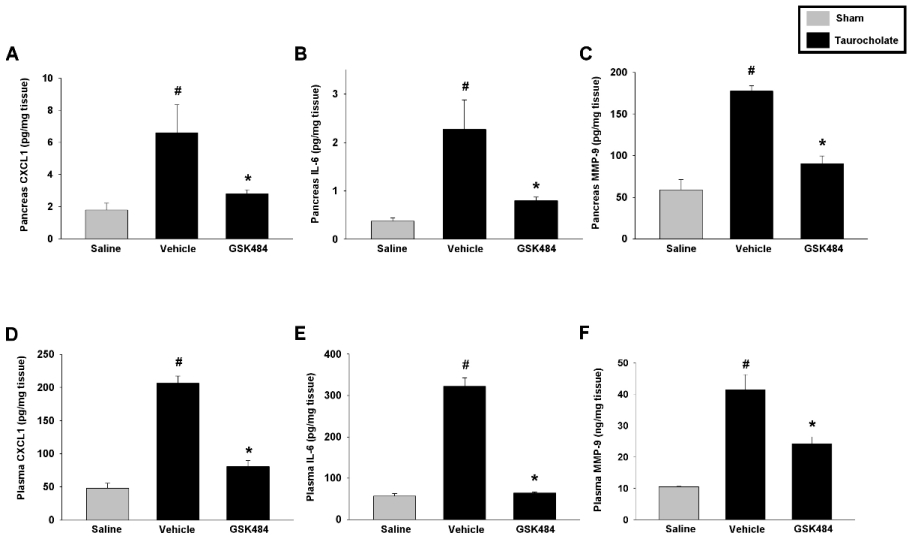


Figure 15. PAD4 controls both local and systemic inflammation in AP. Quantification measurement of levels of A) CXCL1, B) IL-6, and C) MMP-9 in the pancreas. Levels of D) CXCL1, E) IL-6, and F) MMP-9 in the plasma. Pancreatitis (black boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (grey boxes) were infused with saline alone. Animals were received i.p. injections of the GSK484 (4 mg/kg) or vehicle (alcohol) as described in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus taurocholate without GSK484.

Role of c-AbL-kinase Signaling in Regulating NET Formation in AP

Contents

1. Aim

To define the role of c-Abl kinase signaling in NETs formation and tissue damage in AP.

2. Introduction

c-Abl kinase is non-receptor tyrosin kinase that ubiquitously expressed in mammalian cells. c-Abl kinase was identified as a key regulator of myeloid cell transformation into leukemia [258]. Indeed, it has reported that c-Abl kinase has an important role in cell signaling that regulates actin dynamic and cytoskeleton rearrangement [259]. A previous study was shown that neutrophil accumulation to site of inflammation was orchestrated by c-Abl kinase via regulating β2-integrin-mediated neutrophil migration [32]. In addition to their essential role in leukemia and solid tumors, c-abl kinases have also reported to play an important role in inflammatory pathologies [260], endotoxin-induced lung damage and immunoglobulin-mediated renal injury [234, 261]. However, the potential role of c-Abl kinase in the induction of severe AP is unknown. Notably, it was reported that c-Abl regulates ROS generation in neutrophils [34] and convincing data have been demonstrated that ROS are involved in the expulsion of NETs from neutrophils [238]. In this context, we could link for the first time between the c-Abl kinase signaling and NETs formation and highlight the mechanism by which c-Abl kinase regulates NETs formation in severe AP.

3. Results and discussion

In this context, it was interest to evaluate the role of c-Abl kinase in AP. We found that taurocholate challenge increased c-Abl phosphorylation in circulating neutrophils and pancreas as compared with sham animals (**Figure 16A and B**). Importantly, it was found that administration of GZD824 markedly attenuated the c-Abl kinase phosphorylation in peripheral blood isolated neutrophils and pancreas of animals exposed to taurocholate (**Figure 16A and B**), suggesting that GZD824 inhibits c-Abl kinase activity. It was observed that pretreated GZD824 alone had no effect on the activation of c-Abl in neutrophils or in the pancreas in sham animals. Next, we asked whether neutrophils contribute in the c-Abl kinase activity in severe AP. To evaluate that, neutrophils were depleted by using a direct Ab against Ly6G on neutrophils which resulted in a significant reduction in the number of circulating neutrophils by more than 97% (**Figure 16C and D**). Notably, we found that depletion of neutrophil attenuated c-Abl phosphorylation and markedly decreased the levels of amylase by 85%, in animals with AP (**Figure 16E and F**), demonstrating that neutrophils have a significant contribution in regulating of c-Abl activity in the inflamed pancreas. It is generally held that NETs have an important role in innate immune system through trapping and killing the pathogens [262]. However, it has observed that neutrophil-derived NETs are involved in development of AP by stimulating trypsinogen activation and tissue damage in pancreas [19]. In the present study, we found that taurocholate challenge enhanced increase of NETs components such as pancreatic citrullinated histone 3 and plasma DNA-histone complexes by more than 58-fold and 8-fold in taurocholate challenged mice (**Figure 17**). Interestingly, inhibition of c-Abl kinase by GZD824 administration significantly attenuated the levels of citrullinated histone 3 in the pancreas and DNA-histone complexes in plasma by 62% and 77%, respectively (**Figure 17**). Indeed, it was interesting to ask whether c-Abl kinase directly regulates NETs formation in the neutrophils. To answer this question, isolated bone marrow neutrophils were stimulated with TNF-α and it was found that

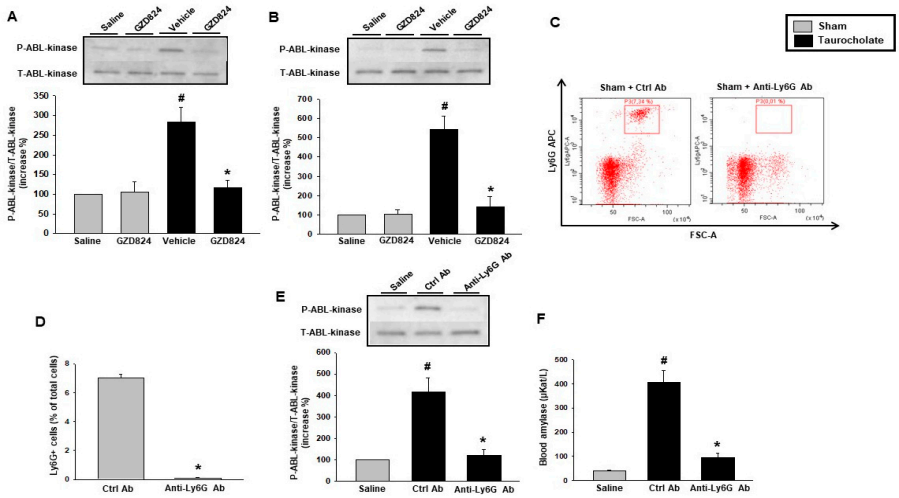


Figure 16. Phosphorylation of c-Abl kinase in AP. c-Abl kinase activity in A) peripheral blood isolated neutrophils and B) homogenate pancreatic tissue as evaluated by western blot and described in Materials and Methods. Pancreatitis (black bars) was triggered by retrograde infusion of taurocholate (5%) into pancreatic duct. Sham mice (grey bars) were received only saline. Animals were received i.v. injection of 5 mg/kg GZD824, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. Peripheral blood neutrophils (Ly6G+) were measured in animals received i.p. injection of anti Ly6G antibody (clone 1A8) or a control antibody prior to induction of pancreatitis. C) Representative dot plots of (Ly6G+) and D) aggregate data of dot plots. E) activity of c-Abl kinase and F) levels of blood amylase as described in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 4-5. #P < 0.05 versus control mice and *P < 0.05 versus vehicle + taurocholate.

TNF-α stimulation increased levels of neutrophil co-expression of MPO and citrullinated histone 3 as well as increased release of DNA-histone complexes (**Figure 18A and B**). As expected, inhibition of c-Abl kinase by co-incubating with GZD824 markedly decreased the levels of TNF-α-stimulated neutrophil co-expression of MPO and citrullinated histone 3 as well as DNA-histone complexes in isolated neutrophils (**Figure 18A and B**). Furthermore, these findings were confirmed by using confocal fluorescence microscopy. It was observed that stimulation of neutrophil with TNF-α provoked formation of DNA fibrillary co-localized with MPO and citrullinated H3 that more compatible

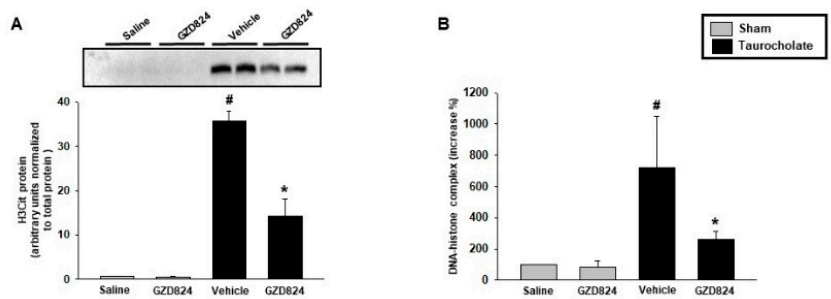


Figure 17. Role of c-Abl kinase in taurocholate-induced NET formation in AP. A) Pancreatic citrullinated histone 3 was determined by western blot and aggregate data showing H3Cit protein normalized with stain-free total protein load. B) Levels of extracellular DNA-histone complexes. Pancreatitis (black bars) was triggered by retrograde infusion of taurocholate (5%) into pancreatic duct. Sham mice (grey bars) were received only saline. Animals were received i.v. injection of 5 mg/kg GZD824, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus vehicle + taurocholate.

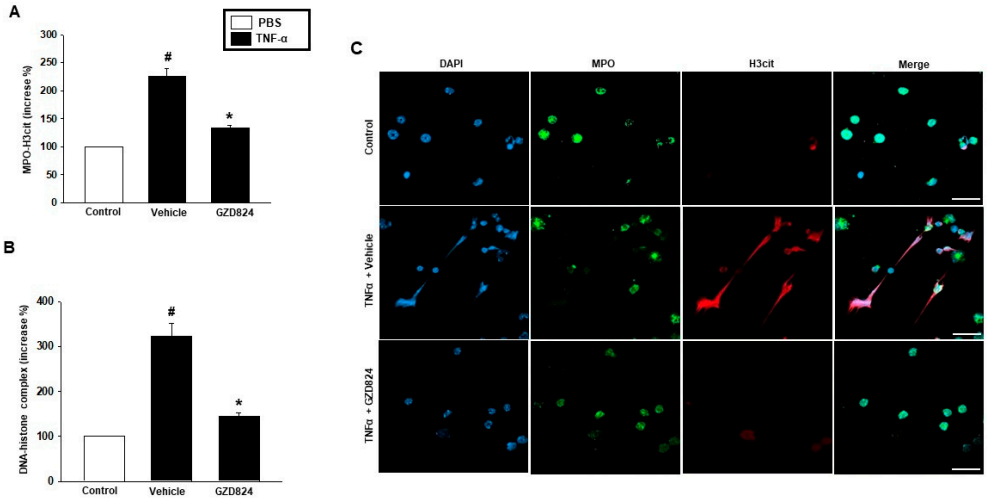


Figure 18. Role of c-Abl kinase in TNF-α-induced NETs-derived isolated neutrophils. A) Levels of citrullinated histone 3 and MPO in isolated neutrophils were quantified by FACS and B) DNA-histone complexes in the supernatant were determined by ELISA. Data represent means ± SEM and n = 5. #P < 0.05 versus control and *P < 0.05 versus vehicle + TNF-α. C) Neutrophils were immune-stained with antibodies to citrullinated histone 3 (H3Cit), myeloperoxidase (MPO), and DAPI nuclear stain. NETs were generated from isolated neutrophils by stimulation with TNF-α co-incubated with or without GZD824. Non-stimulated neutrophils served as a control. One representative experiment of four independent experiments. Scale bars = 10 µm.

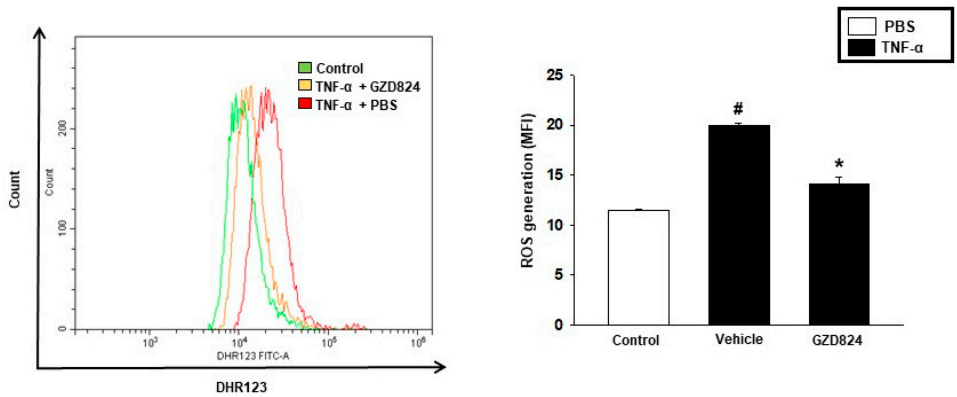


Figure 19. c-Abl kinase regulates ROS formation in isolated neutrophils. Measurement of ROS generation in isolated neutrophils by flow cytometry. Neutrophils were stimulated with TNF-α and pre-incubated with or without GZD824. Non-stimulated neutrophils served as a control. Representative histogram of ROS generation and aggregate data. Data represent means ± SEM and n = 5. #P < 0.05 versus control and *P < 0.05 versus vehicle + TNF-α.

with NETs morphology (**Figure 18 C**). Notably, we observed that pretreatment of isolated neutrophil with GZD824 greatly abolished TNF-α-induced formation of these web-like structures together with MPO and citrullinated histone 3 (**Figure 18C**), indicating that c-Abl kinase has a critical signaling role in regulating NETs formation.

A recent study has observed that ROS generation has a potent role in TNF-α-induced NET formation in neutrophils [263]. In this context, we tried to define the mechanism by which c-Abl kinase mediated formation of NETs-derived neutrophil. It was found that incubation of isolated neutrophil with TNF-α caused clear-cut generation of ROS (**Figure 19**). However, we observed that inhibition of c-Abl kinase significantly decreased TNF-α-induced ROS generation in isolated neutrophils (**Figure 19**). These findings suggested that c-Abl kinase-dependent ROS generation could be involved in NETs formation stimulated by TNF-α in isolated neutrophils. Taking together, our novel data shows for the first time that c-Abl kinase regulates NETs formation in AP.

In the present study, we used established pancreatic tissue damage scoring system to analyze H and E stained pancreatic tissue samples. We found that taurocholate challenge caused clear-cut destruction in pancreatic tissue structure as compared with sham group (**Figure 20**). Importantly, administration of GZD824 protected against taurocholate-induced tissue

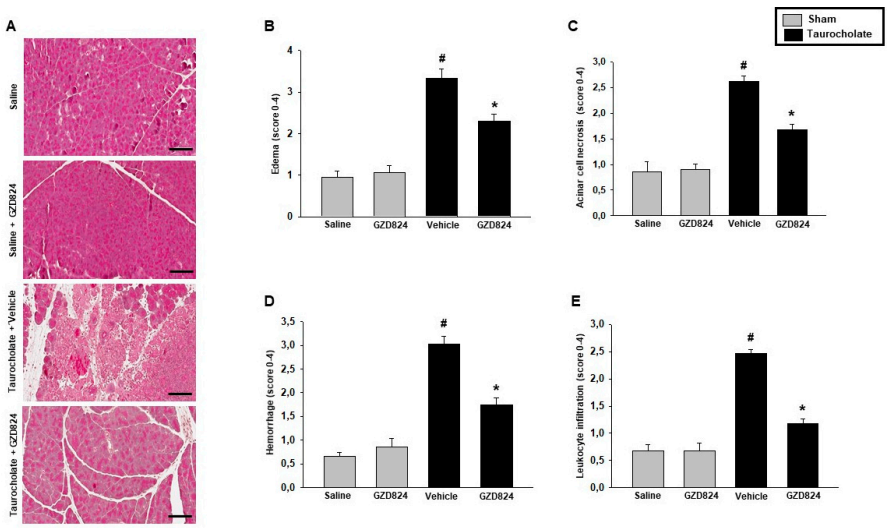


Figure 20. Role of c-Abl kinase in taurocholate-induced tissue damage in AP. A) Hematoxylin & eosin sections of the head of the pancreas from indicated groups. Scale bar = 100 µm. Histological scoring of B) edema, C) acinar cell necrosis, D) hemorrhage and E) leukocyte infiltration in the pancreas of sham (grey bars), (saline alone was infused into pancreatic duct), and taurocholate (5%)-challenged mice (black bars). Animals were received i.v. injection of 5 mg/kg GZD824, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus vehicle + taurocholate.

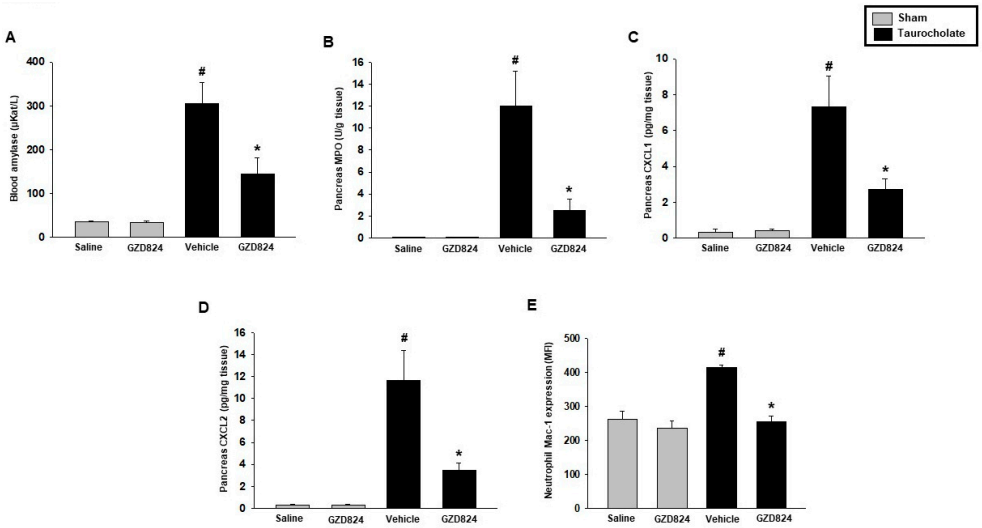


Figure 21. Role of c-Abl kinase in taurocholate-induced tissue injury and neutrophil accumulation in the pancreas. A) Quantitative measurements of blood amylase levels. Levels of B) MPO, C) CXCL1 and D) CXCL2 in the Pancreas. E) Mac-1 expression on peripheral blood neutrophils. Pancreatitis (black bars) was triggered by retrograde infusion of taurocholate (5%) into pancreatic duct. Sham mice (grey bars) were received only saline. Animals were received i.v. injection of 5 mg/kg GZD824, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus vehicle + taurocholate.

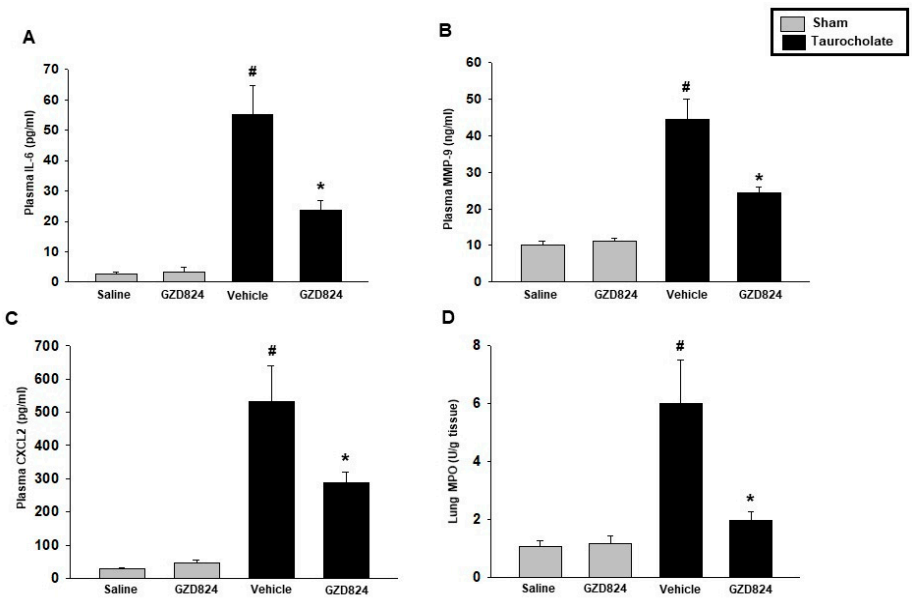


Figure 22. c-Abl kinase regulates taurocholate-induced systemic inflammation in AP. Levels of A) IL-6, (B) MMP-9, (C) CXCL2 in the plasma and D) MPO activity in the lung tissue. Pancreatitis (black bars) was triggered by retrograde infusion of taurocholate (5%) into pancreatic duct. Sham mice (grey bars) were received only saline. Animals were received i.v. injection of 5 mg/kg GZD824, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus vehicle + taurocholate.

damage and significantly reduced edema, acinar cell necrosis and hemorrhage by more than 43%, 52% and 55%, respectively, (**Figure 20B-D**). Furthermore, it is well known that blood amylase was used as an indicator of tissue damage in AP. We found that challenge with taurocholate increased blood levels of amylase by 9-fold in taurocholate-exposed mice (**Figure 21A**). Notably, administration of GZD824 greatly reduced the levels of blood amylase by 60%, in the inflamed pancreas (**Figure 21A**), suggesting that c-Abl kinase signaling controls a significant part of the tissue injury in severe AP.

Having established that leukocytes represent a hallmark in the pathophysiology of severe AP by mediating trypsin activation and tissue damage in the pancreas [13, 100]. Herein, we found that 24 hours after taurocholate challenge enhanced tissue accumulation of neutrophils in the pancreas as indicated by increased levels of MPO (**Figure 21B**) and number of extravascular leukocytes (**Figure 20E**). However, inhibition of c-Abl kinase significantly attenuated the activity of MPO and the number of extravascular leukocytes in the inflamed pancreas by 83% (**Figure 21B**) and 72% (**Figure 20E**), respectively. Thus, these findings strongly suggest that c-Abl kinase regulates neutrophils infiltration in the inflamed pancreas. Subsequently, this notion can led us to the fact that effect of GZD824 on activation and recruitment of neutrophils might illustrate the tissue protective effect of GZD824 in AP.

Previous studies have found that neutrophil trafficking to site of inflammation is orchestrated by secretion of CXC chemokines, such as CXCL1 and CXCL2 [10, 156]. Moreover, CXC chemokines have been reported to have a functional role in AP [154].

To evaluate the effect of GZD824 on chemokines secretion, pancreatic tissue samples were collected 24 hours after challenge with taurocholate. It was found that challenge with taurocholate markedly increased the pancreatic levels of each CXCL1 and CXCL2 as compared with sham animals (**Figure 21C and D**). Notably, administration of GZD824 significantly attenuated CXCL1 and CXCL2 levels in the inflamed pancreas (**Figure 21C and D**), demonstrated that inhibition of c-Abl kinase might control the CXCL1 and CXCL2 secretion in AP. Indeed, this fact could explain the inhibitory effect of GZD824 on neutrophil infiltration in the inflamed pancreas. Despite several studies have reported that Mac-1 has critical role in facilitating extravascular recruitment neutrophil in other tissues [14,264], the function of cell adhesion molecules in mediating neutrophil infiltration in the pancreas is not completely clear. In this study, it was interested to exam the effect of GZD824 on neutrophil Mac-1 expression in AP. In fact, we found that taurocholate challenge increased the neutrophil expression of Mac-1 in mice exposed to taurocholate (**Figure 21E**). Interestingly, pretreatment with GZD824 greatly decreased taurocholate-induced expression of Mac-1 on neutrophil surfaces in AP (**Figure 21E**). This fact could explain the inhibitory effect of GZD824 on neutrophils recruitment in the pancreas is possibly through two distinct pathways, CXC chemokines production and neutrophil Mac-1 expression.

Pulmonary infiltration of neutrophils is identified as a hallmark of systemic inflammation in AP [257]. In the present study, we found that levels of MPO in lung tissue were markedly higher in taurocholate-exposed mice than in sham group (**Figure 22D**). In line with that, taurocholate challenge was also observed to markedly increase the plasma levels of IL-6, MMP-9 and CXCL2 by 20-fold 5-fold and 18-fold, respectively, (**Figure 22A-C**). However, administration of GZD824 markedly reduced taurocholate-induced increase of IL-6, MMP-9

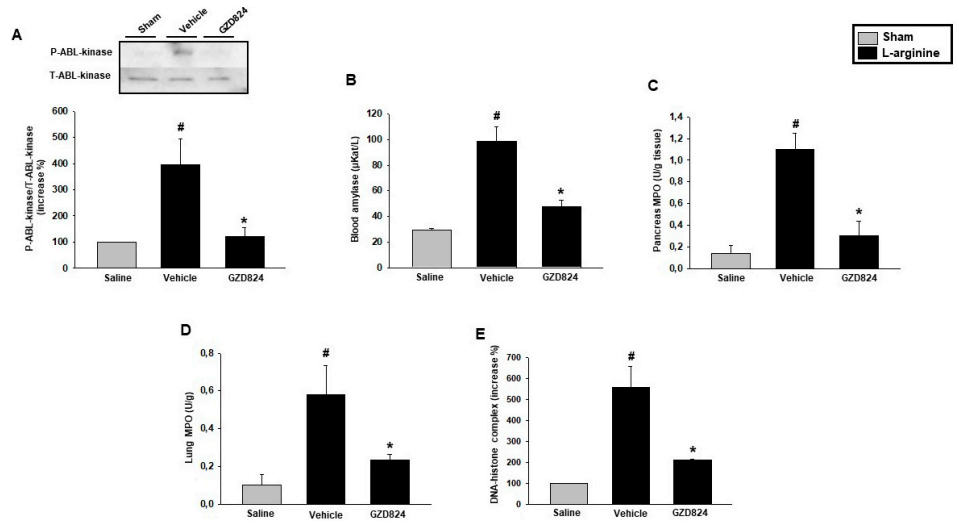


Figure 23. Role of c-Abl kinase in L-arginine-induced AP. A) Activity of c-Abl kinase in pancreatic tissue. Quantitative measurement of B) blood amylase levels, MPO activity in the C) pancreas and D) lung. E) Plasma levels of DNA-histone complexes. Pancreatitis (black bars) was triggered by i.p. injections of 4 g/kg/dose of L-arginine twice at an interval of one h. Sham mice (grey bars) were received only saline. Animals were received i.v. injection of 5 mg/kg GZD824, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. 72 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus vehicle + L-arginine.

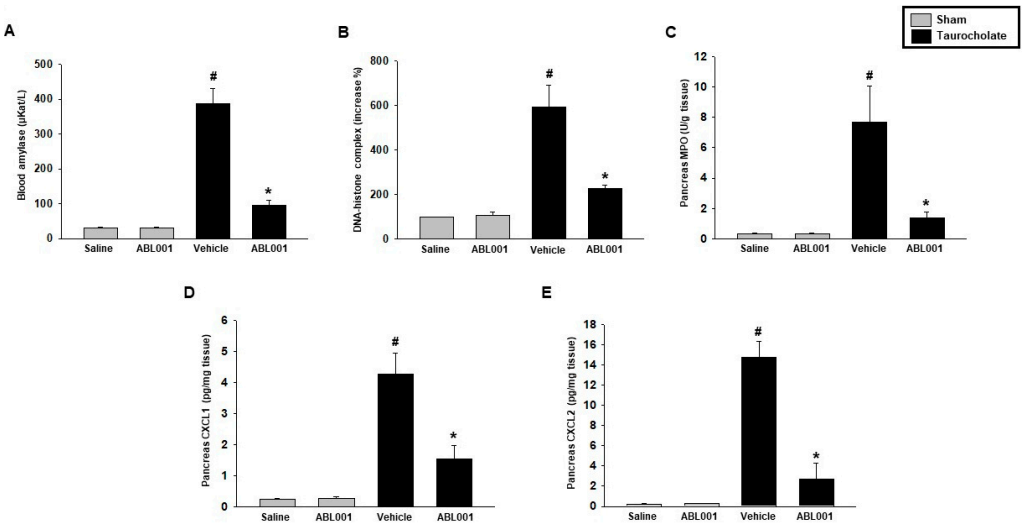


Figure 24. Role of c-Abl kinase in taurocholate-induced inflammation and tissue damage in AP. A) Levels of blood amylase. B) Levels of DNA-histone complexes in the plasma. Levels of C) MPO, D) CXCL1, and E) CXCL2 in the Pancreas. Pancreatitis (black bars) was triggered by retrograde infusion of taurocholate (5%) into pancreatic duct. Sham mice (grey bars) were received only saline. Animals were received i.v. injection of 5 mg/kg ABL001, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus taurocholate without ABL001.

and CXCL2 by 60%, 59% and 50% respectively, (**Figure 22A-C**). Taking together these findings indicating that c-Abl kinase controls both local and systemic inflammation in severe AP.

The role of c-Abl kinase in regulating of NETs formation and tissue damage in severe AP was also evaluated in an alternative experimental model of L-arginine. In present study, pancreatitis was provoked by i.p injection of C57BL/6 mice with L-arginine (4 g/kg) given at an interval of 1 hour. Notably, we found that L-arginine administration markedly increased phosphorylation of c-Abl kinase and blood amylase levels as well as elevated the activity of MPO in both pancreas and lung (**Figure 23A-D**). However, pretreated with GZD824 markedly attenuated the activity of c-Abl kinase and levels of blood amylase in mice challenged with L-arginine (**Figure 23A-B**). Moreover, administration of GZD824 also markedly reduced the activity of MPO in the pancreas and lung tissue in L-arginine-received mice (**Figure 23C and D**). In addition, challenge with L-arginine caused clear-cut increase in the plasma levels of DNA-histone complexes (**Figure 23E**). Importantly, it was observed that pretreatment with GZD824 significantly decreased plasma levels of DNA-histone complexes in mice challenge with L-arginine (**Figure 23E**). Taking together our novel results show that inhibition of c-Abl kinase attenuates NETs formation and tissue damage in severe AP in two different experimental models.

To validate the above findings and further detail the role of c-Abl kinase in AP, ABL001, an alternative inhibitor, was used. It was observed that pretreated with ABL001 greatly attenuated

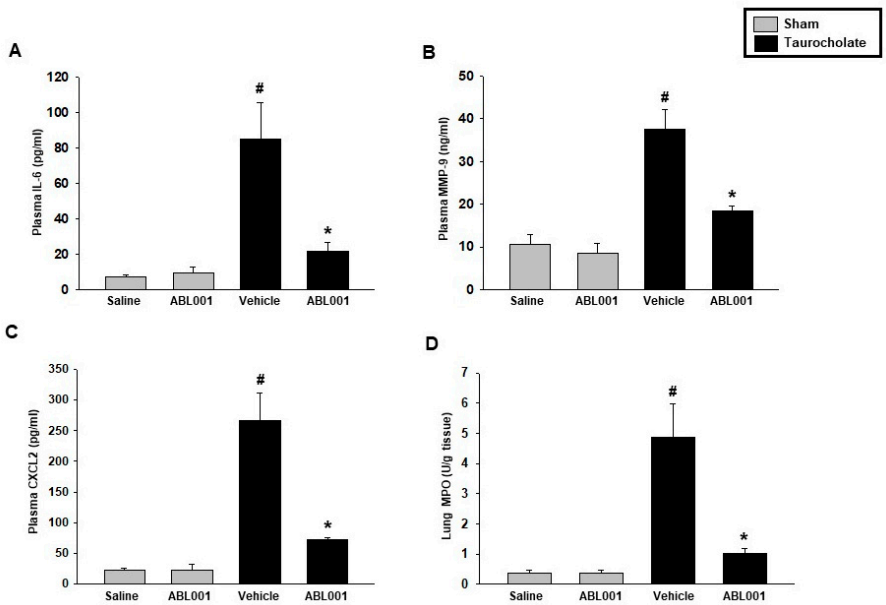


Figure 25. Role of c-Abl kinase in taurocholate-induced systemic inflammation in AP. Levels of A) IL-6, B) MMP-9, and C) CXCL2 in the plasma. D) Activity of MPO in lung tissue. Pancreatitis (black bars) was triggered by retrograde infusion of taurocholate (5%) into pancreatic duct. Sham mice (grey bars) were received only saline. Animals were received i.v. injection of 5 mg/kg ABL001, c-Abl kinase inhibitor, or vehicle (DMSO) before provoked pancreatitis. 24 hours after induction of pancreatitis, samples were collected. Data represent means ± SEM and n = 5. #P < 0.05 versus control mice and *P < 0.05 versus taurocholate without ABL001.

the levels of taurocholate-induced amylase by 82% (**Figure 24A**). In addition, administration of ABL001 significantly decreased plasma levels of DNA-histone complexes in taurocholate-exposed animals (**Figure 24B**). Furthermore, we observed that administration of ABL001 markedly reduced the levels of MPO, CXCL1 and CXCL2 by 85%, 70%, 83%, respectively, in the inflamed pancreas (**Figure 24C-E**). It was also observed that ABL001 regulates the systemic inflammation. For instance, pretreated with ABL001 significantly reduced levels of IL-6, MMP-9 and CXCL2 in the plasma as well as levels of MPO in the lung tissue (**Figure 25A-D**). Notably, administration of ABL001 alone had no effect on formation of NETs and inflammation in sham mice (**Figure 25A-D**).

In conclusion, our results found that the signaling pathways of c-Abl kinase regulate NETs formation in AP. Moreover, inhibition of c-Abl kinase attenuated neutrophil recruitment and tissue injury in the pancreas. In addition, these findings investigated that blocking the activity of c-Abl kinase results in decrease in systemic inflammation in mice with AP. Therefore, the present study not only shows the novel signaling mechanism regulating NET formation in AP but also suggests that blocking c-Abl kinase could be a useful therapeutic strategy to decrease both local and systemic inflammation in severe AP.

Role of Platelet IP6K-1 in Regulating NET-MP Formation in AP

Contents

1- Aim

To investigate the potential role and mechanism of platelets in NETs formation and tissue damage in AP.

2- Introduction

NETs have been found to have a substantial role in trypsin activation, neutrophil recruitment and tissue damage in AP [19]. Structurally, NETs consist of extracellular DNA that decorated with nuclear, cytoplasmic and granular proteins which have important biologic functions [15]. Moreover, activated neutrophils have also reported to shed off sphere-shaped intact vesicles that released from their membranes called microparticles (MPs) with a size less than 1μm [22]. Recently, it was observed that MPs form complexes with NETs via interactions with histone phosphatidylserine and this complex has found to be powerful inducers of thrombin generation by the intrinsic pathway of coagulation [23].

It is generally held that platelets have critical role in hemostasis and thrombosis, however, they are also reported to participate in inflammation via interaction with inflammatory cells or secretion of pre-stored pro-inflammatory mediators [266, 267]. Moreover, activated platelets have been found to support neutrophil migration into site of inflammation by secreting of CCL5, CXCL4 and CD40L [40, 41]. Several studies have shown that platelets have a key role in formation of NETs in infectious diseases but platelet-provoked NETs formation in AP is still elusive. Upon activation, it was found that platelets secrete polymer of phosphate unites (length 60 to 100 unites) that linked with each other by phosphoanhydride bonds [42]. Indeed, PolyPs has shown to have important pro-inflammatory effects for instance; enhancing NF-kB signaling and vascular permeability as well as activating complement system [43-45]. Convincing data have shown that production of PolyPs highly regulated by inositol hexakisphosphate kinase 1 (IP6K1) [42]. In fact, this enzyme has reported to have a critical role in neutrophil activation as well as regulates neutrophil-platelet aggregation in endotoxin-induced lung inflammation. Suggesting that IP6K1 has a pro-inflammatory role in systemic inflammation [46]. Herein and based on the considerations above, we hypothesized that platelet IP6K1 plays a role in mediating NETs formation as well as regulates subsequent organ inflammation and injury in severe AP.

3- Results and discussion

In this study it was interest to exam the role of platelets in NETs formation in AP. By scanning electron microscopy, it was observed that challenge with taurocholate induced extracellular fibrillar and web-like structures that compatible with NETs in the inflamed pancreas (**Figure 26A**). This fact was confirmed by transmission immune electrons which showed that neutrophil elastase and histone 4 were co-localized with the extracellular DNA in these extracellular web-like structures (**Figure 26B**). However, these structures were disappeared in healthy mice. Importantly, platelets depletion, by using an antibody directed against GP1bα (**Supplementary figure 1**), greatly attenuated NETs formation in the inflamed pancreas (**Figure 26A**). In line with a previous study, administration of DNase I greatly decreased formation of taurocholate-induced NET in inflamed pancreas (**Figure 26A**). Moreover, 24 h after challenge with taurocholate increased of H3 and H4 by 3-fold and 4-fold, respectively, (**Figure 26D-F**). Interestingly, we found that depletion of platelets significantly reduced the levels of DNA-histone complexes in plasma by 3-fold as well as enhanced pancreatic levels

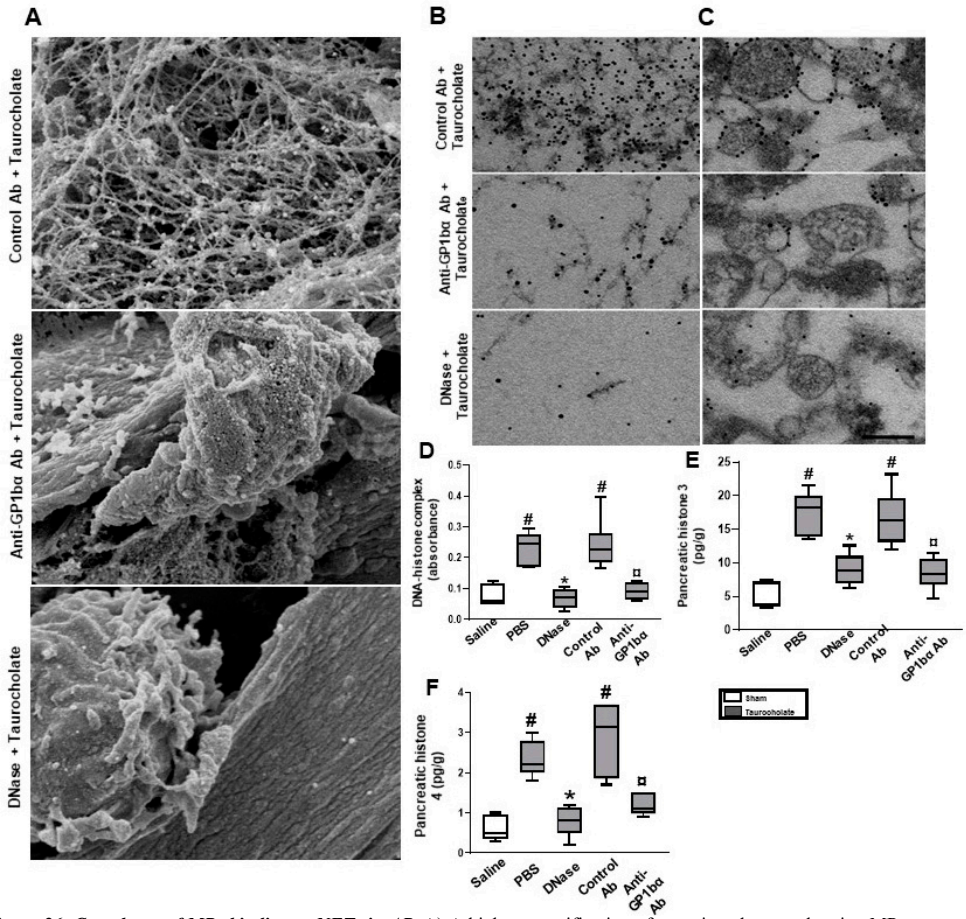


Figure 26. Complexes of MPs binding to NETs in AP. A) A higher magnification of scanning electron showing MPs connected with NETs in the inflamed pancreatic tissue. Scale bar = 2 µm. B) Indicated area of interest from Figure 1A was identified by transmission electron microscopy and incubated with gold-labeled anti-histone 4 (large gold particles) and anti-elastase (small gold particles) antibodies. Scale bar = 0.25 µm. C) Indicated area of interest from Figure 1A was identified by transmission electron microscopy and incubated with gold-labeled anti-Mac-1 (large gold particles) and anti-CD41 (small gold particles) antibodies. Scale bar = 0.25 µm. D) Quantification of extracellular DNA-histone complexes. Levels of E) histone 3 and F) histone 4 in the Pancreas. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (white boxes) were received only saline. Animals were received i.p. injections of the DNase I, a control antibody (Control Ab), anti-GP1b alpha antibody or vehicle (PBS) as showed in Materials and Methods. 24 hours after pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5-6. #P < 0.05 versus sham mice, *P < 0.05 versus PBS+taurocholate and ¤P < 0.05 versus Control Ab+taurocholate.

plasma levels of DNA-histone complexes by 86% as well as decreased the levels of H3 and H4 in the pancreas of mice exposed to taurocholate (**Figure 26D-F**). A recent study has observed MPs formed during neutrophil activation and can bind to NETs and form NET-MP complexes and these complexes have an important role in thrombin generation in sepsis [23]. Here, we applied scanning electron microscopy and it was observed that taurocholate challenge induced NETs in the pancreas with numerous round structures compatible with MPs. Indeed, MPs on NETs were examined first by using higher

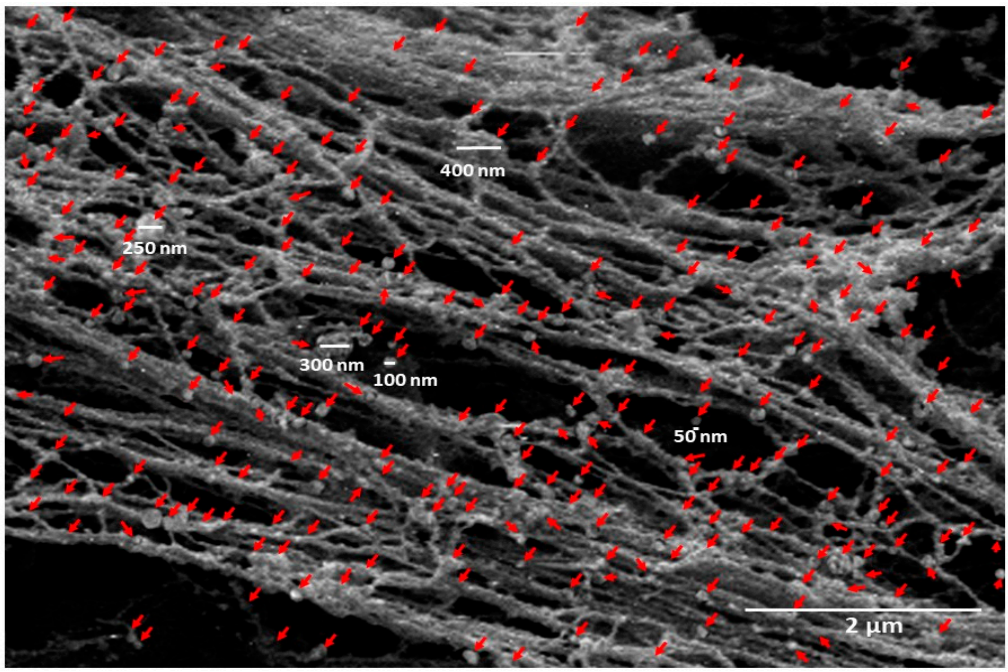


Figure 27. Evaluation of MPs on NETs. Contrast and brightness of one of original figures (figure1 PBS_Taurocholate) was adjusted for better visibility of MPs. Scale bar = 2 µm. The largest MP were about 400 nm and smallest MPs were in between 50-100 nm. All MPs are carefully evaluated in higher magnification with enhanced contrast. Clear MPs were denoted by red arrows.

magnification of original images with enhanced contrast to make it clear for analysis (**Figure 27**). Then, MPs denoted by pink color objects that applied on the original images in order to visualize the MPs and make them more readable, as described in materials and methods section. In fact, transmission electron microscopy manifested that these particles expressed CD41 or Mac-1. Indicating that these MPs originally from platelets and neutrophils, respectively (**Figure 26D**). Notably, treatment with DNase I greatly decreased NETs formation and consequently the density of MPs on NETs close to zero in the flamed pancreas.

Moreover, we observed that platelet depletion not only reduced NETs formation but also attenuated the density of MPs on remaining NETs. Furthermore, depletion of platelets resulted in a significant reduction in tissue damage (**Supplementary figure 1C-K**) and inflammation in taurocholate-challenged mice (**Supplementary figure 2A-F**).

In fact, it was interested to examine the role of MPs-attached NET complexes in acinar cell biology. In line with a recent study [23], we found that PMA provoked neutrophil-derived NETs containing numerous MPs. Scanning electron microscopy showed that co-incubation of neutrophils with caspase and calpain inhibitors resulted in NETs formation with greatly less MPs. Moreover, we observed that NETs depleted MPs had markedly lower effect to induce amylase secretion from acinar cells in vitro as compared with NET-MPs complexes (**Figure 28C**). However, neutrophil-derived MPs alone did not show any effect on amylase secretion from acinar cells in vitro (**Figure 28C**). Having established that signal transducer and activator of transcription-3 (STAT-3) has an important signaling role in acinar cells

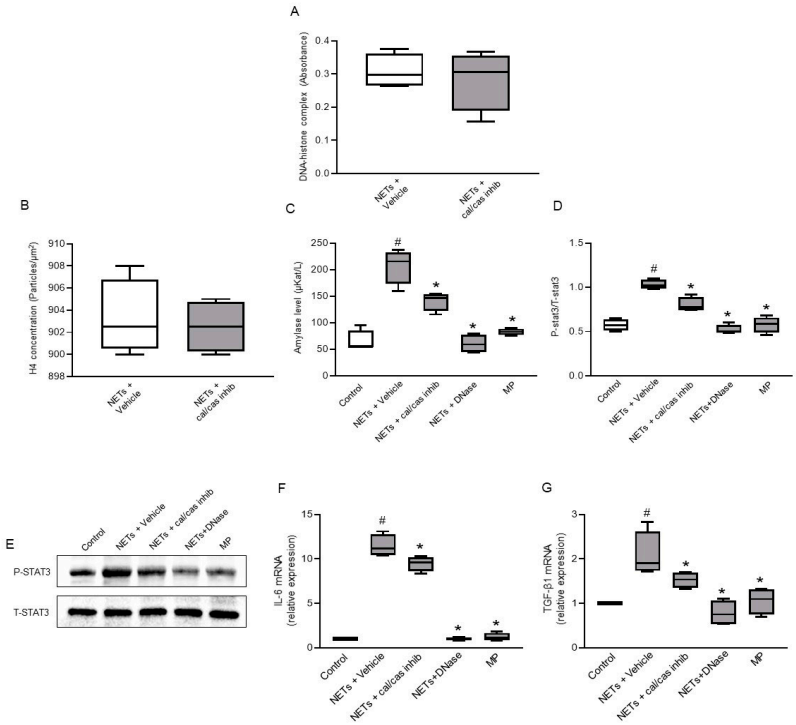


Figure 28. Role of NET-MP complexes in acinar cells damage. NETs were formed from PMA-stimulated bone marrow neutrophils in the presence of vehicle or caspase (50 µM, Z-VAD-FMK) and calpain (25 µM, PD150606) inhibitors. A) Levels of DNA-histone complex and B) histone 4 concentration on NETs with and without MPs. C) Levels of amylase secretion, D) Phosphorylated STAT-3 aggregate data normalized to total STAT-3 and E) Western blot showing phosphorylated STAT-3 and total STAT-3. Gene expression of F) IL-6 and G) TGFβ1. Acinar cells were triggered by NETs (grey boxes) in the presence of vehicle, a mixture of caspase and calpain inhibitors or DNase as well as by MPs of isolated neutrophil. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and $n = 4$. $\#P < 0.05$ *vs.* Control and $*P < 0.05$ *vs.* Vehicle+NETs.

biology [137]. In the present study, we found that stimulation of acinar cells with NETs significantly increased STAT-3 activity in acinar cells (**Figure 28D and E**), however, co-incubation with DNase I completely reduced NET-induced phosphorylation of STAT-3 cells (**Figure 28D and E**). Moreover, it was found that pretreatment with caspase and calpain inhibitors markedly reduced NET-provoked STAT-3 phosphorylation by 44% (**Figure 28D and E**). Importantly, PMA-induced NETs generation was not affected by co-incubation with calpain and caspase inhibitors as confirmed by quantification of levels of DNA-histone complex and NET content of histone 4 which showed that these inhibitors had no effect on their levels (**Figure 28A and B**). Next, we elucidated the pathway by which STAT-3 causes acinar cell damage. We next examined gene expression of STAT-3-targets IL-6 and TGFβ1 in acinar cells. It was observed that NET challenge markedly increased acinar cell mRNA levels of IL-6 and TGFβ1 (**Figure 28F and G**). Notably, pretreatment with DNase I or calpain and caspase inhibitors markedly attenuated NET-stimulated gene expression of IL-6 and TGFβ1 in acinar cells (**Figure 28F and G**). In line with the results above, MPs alone had no effect on mRNA levels of IL-6 and TGFβ1 in acinar cells (**Figure 28F and G**).

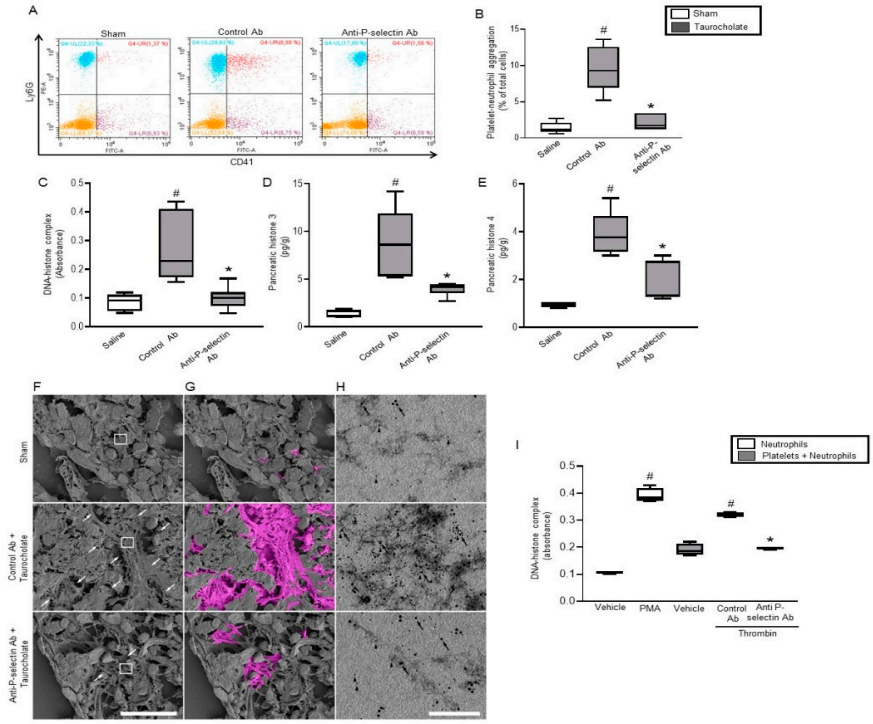


Figure 29. Platelet-neutrophil crosstalk in AP. A) Quantification of platelet-neutrophil aggregates as a percentage of neutrophils (Ly6G+) binding platelets (CD41+) in the blood, as showed in Materials and Methods. B) Aggregate data of platelet-neutrophil formation. C) Quantification of extracellular DNA-histone complexes. Levels of D) histone 3 and E) histone 4 in the pancreas. F) Extracellular web-like structures were identified by scanning electron microscopy in the pancreas from mice challenged with taurocholate. Scale bar = 25 µm. G) NETs are denoted in pink color. H) Indicated area of interest from Figure 1F was identified by transmission electron microscopy and incubated with gold-labeled anti-histone 4 (large gold particles) and anti-elastase (small gold particles) antibodies. Scale bar = 0.25 µm. I) Levels of DNA-histone complex. Mixtures of neutrophils and platelets were stimulated with thrombin and treated with a control or an anti-P-selectin antibody (Ab). PMA-induced neutrophils represent the positive control. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (white boxes) were infused with saline alone. Animals were received i.v. injections of a control or an anti-P-selectin antibody (Ab) as showed in Materials and Methods. 24 hours after pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 4-6. #P < 0.05 versus sham mice and *P < 0.05 versus Control Ab+taurocholate.

Taking to gather, these results indicate that NETs is a functional assembly scaffold for MPs in severe AP. Circulating platelet-leukocyte complexes have been shown to have a critical role in various inflammatory conditions, such as reperfusion injury [36], abdominal sepsis [37], pulmonary infections [38], and acute myocardial disease [39]. To our knowledge, the role of platelet-neutrophil aggregate-mediated NETs formation in AP has not been identified. In this study, we hypothesized that such complex formation may influence neutrophil activation and NET formation. Indeed, we have observed that challenge with taurocholate markedly enhanced formation of NPA in the circulation of mice with AP (**Figure 29A and B**). However, blockage of P-selectin significantly decreased taurocholate-induced formation of NPA (**Figure 29A and B**). To exam the role of P-selectin-mediated contact between platelets and neutrophils in NETs formation, isolated neutrophils co-incubated with thrombin-stimulated platelets and it was found that these mixtures increased levels of

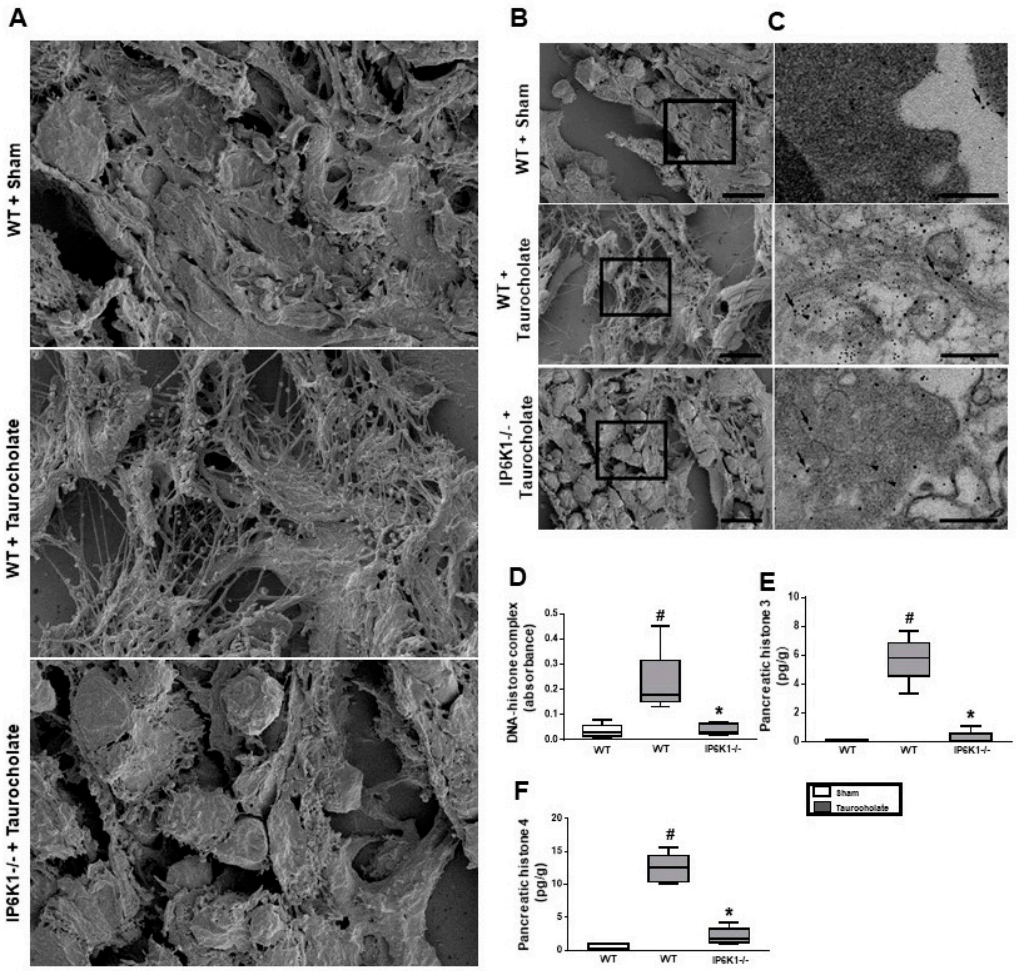


Figure 30. Role of IP6K1 in formation of NET in AP. A) A higher magnification of scanning electron showing MPs connected with NETs in the inflamed pancreatic tissue. Scale bar = 2 µm. B) Extracellular web-like structures were identified by scanning electron microscopy in the pancreas from mice challenged with taurocholate. Scale bar = 5 µm. C). Indicated area of interest from Figure 1A was identified by transmission electron microscopy and incubated with gold-labeled anti-Mac-1 (large gold particles) and anti-CD41 (small gold particles) antibodies. Scale bar = 0.25 µm. D) Quantification of extracellular DNA-histone complexes. Levels of E) histone 3 and F) histone 4 in the pancreas. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct of wild-type (WT) and IP6K1 knockout (IP6K1-/-) mice. Sham mice (white boxes) were infused with saline alone. 24 hours after pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5. #P < 0.05 versus Sham WT mice and *P < 0.05 versus taurochoalte+IP6K1-/- mice.

DNA-histone complexes (**Figure 29I**). Interestingly, it was observed that immunoneutralization of P-selectin significantly attenuated the levels of thrombin-induced formation of DNA-histone complexes in mixtures of platelets and neutrophils (**Figure 29I**). Thus, these findings indicate that P-selectin-mediated crosstalk between platelets and neutrophils is a critical for NETs formation. Furthermore, administration of P-selectin Ab abolished taurocholate-induced NETosis in the pancreas of taurocholate-challenged mice

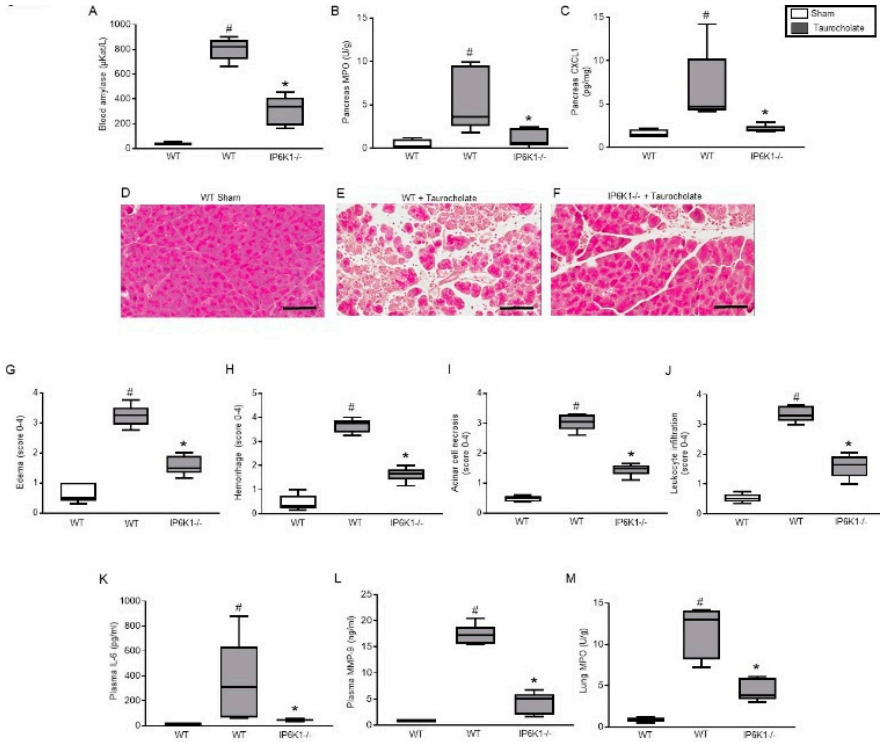


Figure 31. Role of IP6K1 in inflammation and tissue damage in AP. Levels of A) blood amylase, B) MPO and C) CXCL1in the pancreas. D-F) Representative hematoxylin & eosin sections of the head from the pancreas of indicated groups. Scale bar = 100 µm. Histological examinations of G) edema, H) hemorrhage, I) acinar cell necrosis and J) leukocyte infiltration. Levels of K) IL-6 and L) MMP-9 in the plasma as well as M) MPO activity in lung tissue. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct of wild-type (WT) and IP6K1 knockout (IP6K1 -/-) mice. Sham mice (white boxes) were infused with saline alone. 24 hours after pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5. #P < 0.05 versus Sham WT mice and *P < 0.05 versus taurochoalte+IP6K1-/- mice.

(**Figure 29C-E**). In addition, inhibition of P-selectin greatly attenuated the levels of DNA-histone complexes in the plasma and levels of H 3 and H 4 in the pancreas of animals exposed to taurocholate (**Figure 29C-E**) as well as reduced the inflammation (**Supplementary figure 3A-C and K-M**) and tissue damage (**Supplementary figure 3D-J**) in the pancreas of taurocholate-challenged mice. The above results suggested that physical contact between platelets and neutrophils are critical for platelet-mediated NETosis.

IP6K1 has been reported to be a critical enzyme in regulating homeostasis by controlling the production of PolyP from dense granule [45]. Recently, it has shown that IP6K1 has pro-inflammatory role in systemic inflammation via regulating of neutrophil-platelet aggregates and neutrophil recruitment in endotoxin-induced lung inflammation [46]. Therefore, we next sought IP6K1 role in platelet-dependent NETs formation and pathophysiology of AP. We first assessed the differences in the number of leukocyte subtypes and platelets between wild-type and IP6K1-deficienct animals and it was found no differences between them (**Supplementary figure 5A-E**). Then by using scanning electron microscopy, we observed that challenge with taurocholate caused clear-cut increased in NETs formation in the

inflamed pancreas as compared with sham group (**Figure 30A and B**). Interestingly, it was found that NET formation in the pancreas was markedly abolished in IP6K1 gene-deficient mice with AP **(Figure 30A and B)**. Indeed, taurocholate challenge markedly elevated the levels of extracellular DNA-histone complexes by more than 7-fold as well as pancreatic levels of H3 and H4 by 52-fold and 25-fold, respectively as compared with sham animals **(Figure 30C-E)**. Interestingly, we found that lacking IP6K1 significantly decreased the plasma levels of DNA- histone complexes by 81% and pancreatic levels of H3 and H4 by 97% and 87%, respectively, in mice exposed to taurocholate **(Figure 30C-E)**. Thus, our finding showing for the first time in the literature that IP6K1 is a key regulator of NET formation.

To evaluate the role of IP6K1 in severe AP, levels of blood amylase were initially measured as an indicator of tissue damage. We found that taurocholate challenge increased blood amylase levels that significantly attenuated in healthy mice **(Figure 31A)**. Notably, amylase levels were markedly reduced by 65% in IP6K1-deficient animals with AP **(Figure 31A)**. Furthermore, morphological examination showed that challenge with taurocholate caused

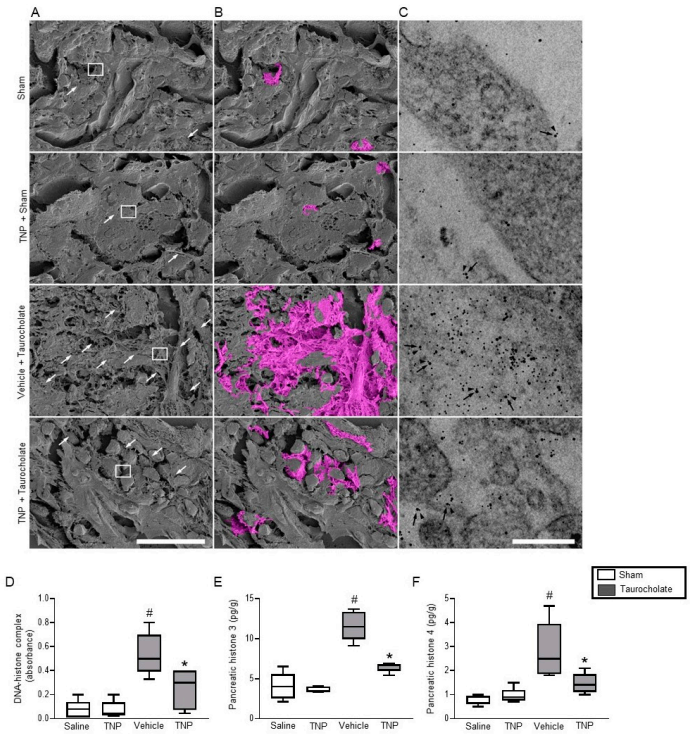


Figure 32. Role of TNP in NETs formation in AP. A) Extracellular web-like structures were identified by scanning electron microscopy in the pancreas from a mouse challenged with taurocholate. Scale bar = 25 µm. B) NETs denoted in pink color. C) Indicated area of interest from Figure 1A was identified by transmission electron microscopy and incubated with gold-labeled antibodies against histone 4 (large gold particles, arrows) and elastase (small gold particles, arrowheads). Scale bar = 0.25 µm. D) Quantification of plasma DNA-histone complexes. E) Histone 3 and F) histone 4 levels in the pancreas. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (white boxes) were infused with saline alone. Animals were received i.p. injections of the vehicle (PBS) or TNP as showed in Materials and Methods. 24 hours after pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5. #P < 0.05 versus PBS+Sham and *P < 0.05 versus Vehicle+taurocholate.

clear-cut destruction of the pancreatic tissue microarchitecture typified by increased edema, acinar cell necrosis, haemorrhage in pancreatic tissue as compared with sham group **(Figure 31D-I)**. Notably, disruption of IP6K1 protected against taurocholate-provoked destruction of pancreatic tissue architecture **(Figure 31D-I)**. For instance, knockout IP6K1 markedly reduced edema, acinar cell necrosis, haemorrhage by 64%, 61% and 63%, respectively, **(Figure 31E-I)**, thus these results suggest that IP6K1 regulate a major part of tissue damage in AP. Having established that neutrophil recruitment has a critical role in tissue damage associated with AP [100, 267]. It is generally held that MPO used as an indicator of neutrophil recruitment. In light of this observation, it was found that taurocholate challenge increased the levels of MPO by 12-fold as well as caused a massive infiltration of neutrophils number in the inflamed pancreas **(Figure 31B and J)**. It was noticed that knockout of IP6K1 significantly decreased taurocholate-provoked pancreatic activity of MPO and extravascular neutrophils recruitment by 86% and 62%, respectively, in pancreas of mice with severe AP **(Figure 31B and J)**. Suggesting that IP6K1 is a critical regulator of neutrophil recruitment. Moreover, the effect of disrupted IP6K1 on recruitment of neutrophil might help to explain the protective effect of tissue damage in AP. Secreted CXC chemokines were implicated in neutrophil chemoattractant to site of inflammation [268]. In the present study, we found that challenge with taurocholate provoked substantial increase of pancreatic levels of CXCL1 **(Figure 31C)**. Notably, taurocholate-increased levels of CXCL1 were significantly attenuated in taurocholate-exposed mice lacking IP6K1 **(Figure 31C)**, indicating that decreased expression of CXCL1 could help to explain the decreased neutrophil recruitment and tissue damage in the pancreas of IP6K1gene-deficient mice with AP.

It is known that the predominant effects of AP are systemic inflammatory response that including pulmonary neutrophilia [269, 270]. We herein observed that the levels of MPO were significantly increased in lung of animals with severe AP as compared with sham group (**Figure 31M**). Notably, taurocholate-induced increased of MPO activity was markedly decreased by 66% in the lung of IP6K1-deficient mice exposed to taurocholate (**Figure 31M**). Moreover, it was also observed that lacking IP6K1 significantly attenuated the plasma levels of IL-6 and MMP-9 (**Figure 31K and L**). Accordingly, these results indicating that disruption of IP6K1 not only markedly abolished NETs formation in the inflamed pancreas, but also attenuated both local and systemic inflammation in AP. It is herein strongly suggested that targeting IP6K1 might be a useful therapeutic approach in AP.

Further support to the above data, we next asked whether TNP, a specific inhibitor of IP6Ks [271], regulates taurocholate-induced inflammation and tissue damage. We addressed this issue by applying scanning electron microscopy and it was found that taurocholate challenge induced NETs formation in the inflamed pancreas which has observed to be markedly abolished by pretreatment with TNP (**Figure 32A-C**). We further confirmed that administration of TNP significantly decreased plasma levels of DNA-histone complex by 63% and pancreatic H3 and H4 levels by 68% and 67%, respectively, in taurocholate-exposed mice (**Figure 32D-F**). Moreover, it was also found that pretreated with TNP markedly reduced the levels of amylase, pancreatic MPO activity, CXCL1 and CXCL2 levels by 48%, 75%, 67% and 54%, respectively in taurocholate-challenged mice (**Figure 33A-D**). Notably, it was found that pretreatment with

TNP resulted in a significant reduction in plasma levels of IL-6 and MMP-9 as well as decreased MPO activity in the lung of AP mice (**Figure 33F-H**). Furthermore, administration of TNP was protected against taurocholate-induced tissue damage which characterized by markedly decreased edema, hemorrhage, acinar cell necrosis and leukocyte infiltration by 53%, 46%, 44% and 65%, respectively in the inflamed pancreas (**Figure 33A-D**). Therefore, these data further support the notion that IP6K1 regulates NETs formation, inflammation and tissue damage in AP.

Convinced data have shown that IP6K1 regulates homeostasis via controlling PloyP secretion [42]. Indeed, PloyP has shown to regulate blood clotting cascade via the extrinsic and intrinsic pathways [207]. Moreover, it was suggested that PolyP has important pro-inflammatory effects, such as activation of the kallikrein–kinin [45] and complement systems [272]. We therefore asked whether PolyP could be involved in formation of NETs. In fact, it was observed that lacking of IP6K1 markedly reduced the amount of polyphosphates in

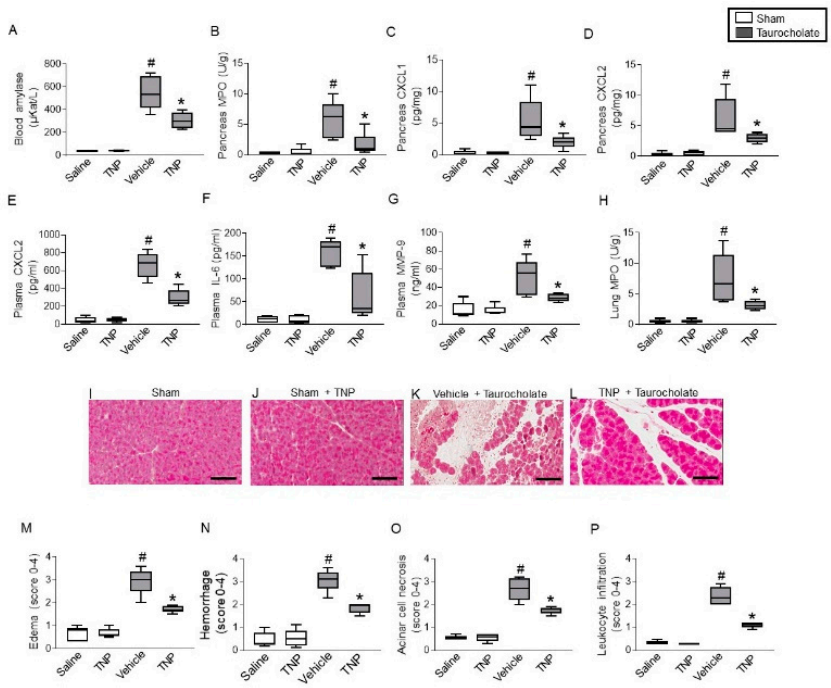


Figure 33. Role of TNP in inflammation and tissue damage in AP. Levels of A) blood amylase, B) MPO, C) CXCL1 and D) CXCL2 in the pancreas. Levels of E) CXCL2, F) IL-6 and G) MMP-9 in plasma as well as H) levels of MPO in lung tissue. I-L) Representative hematoxylin & eosin sections of the head from the pancreas of indicated groups. Scale bar = 100 μm. Histological examinations of M) edema, N) hemorrhage, O) acinar cell necrosis and P) leukocyte infiltration. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (white boxes) were infused with saline alone. Animals were received i.p. injection of the vehicle or TNP as showed in Materials and Methods. 24 hours after pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5. #$P < 0.05$ versus sham mice and *$P < 0.05$ versus Vehicle+taurocholate.

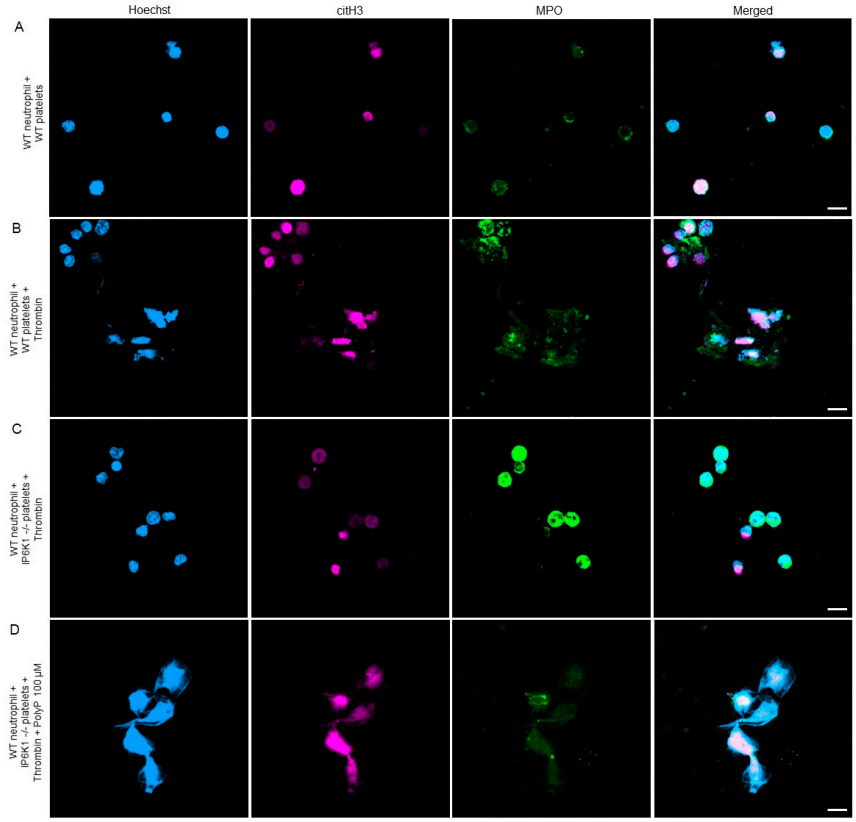


Figure 34. Role of PolyP in NETs formation. Confocal fluorescence microscopy showing NETs generated from isolated neutrophils by incubating with isolated wild-type platelets, A) with or B) without thrombin (0.2 U/ml), or C) incubating with isolated IP6K1-/- platelets with thrombin (0.2 U/ml) and D) with PolyP (100 µM) over coverslips at 37°C for 3 hours. Cells were fixed and permeabilized and then subjected to immune-stained with anti citrullinated histone 3 (citH3), myeloperoxidase (MPO) antibodies, and DNA was counterstained with Hoechst 33342. One representative experiment of four independent experiments. Scale bars = 10 µm.

isolated platelets (**Supplementary figure 4A and B**). To evaluate the effect of the PolyP on NETs formation, wild-type neutrophils co-incubated with wild-type or IP6K1 gene-deficient platelets. It was observed that the mixture of thrombin-stimulated wild-type platelets and neutrophils triggered NETs formation as observed by markedly increased in the levels of DNA-histone complexes (**Supplementary figure 4E**). Notably, we found that thrombin stimulation of IP6K1 gene-deficient platelets co-incubated with wild-type neutrophils resulted in 59% reduction in DNA-histone complexes (**Supplementary figure 4C**). Interestingly, we found that added exogenous polyphosphate to the mixture of thrombin stimulation of IP6K1-deficient platelets and wild-type neutrophils markedly enhanced the formation of DNA-histone complexes (**Supplementary figure 4C**). This notion was supported by confocal fluorescence microscopy. It was observed that thrombin stimulation of wild-type platelets and neutrophils resulted in extensive expulsion of DNA co-localizing with MPO and citrullinated histone 3 (**Figure 34B**). In contrast, disruption of IP6K1 protected against releasing of DNA co-localizing with MPO and citrullinated

histone 3 in mixture of thrombin stimulation of IP6K1-deficient platelets and wild-type neutrophils (**Figure 34C**). Indeed, it was interested to notice that addition of polyphosphate to the mixture of thrombin stimulation of IP6K1-deficient platelets and wild-type neutrophils rescued formation of NET (**Figure 34D**). Thus, our findings strongly indicate that platelet IP6K1 can regulate NETs formation via controlling the secretion of PolyP. Herein, we also suggested that PolyP could have a significant effect not only on AP but also other conditions, such as infectious and noninfectious, in which NETs play a critical pathophysiological role. Therefore, it indicates that IP6K1 might be a useful therapeutic target to antagonize the inflammation and coagulation in patients with AP. In separate experiments, isolated neutrophils from wild type and IP6K1-deficient were co-incubated with PMA. Importantly, it was found that PMA-induced NET formation was independent of IP6K1 in isolated neutrophils (**Supplementary figure 6A-C**).

Next, we examined whether IP6K1 also controls NET formation, neutrophil recruitment and tissue injury in another experimental model. In fact, L-arginine was used to induce pancreatitis. It was found that challenge with L-arginine causes clear-cut increased in NET formation in pancreatic tissue of mice with AP (**Supplementary figure 7A-C**). Moreover, L-arginine challenge also elevated levels ofcitrullinated histone 3 in the pancreas and DNA-histone complexes in the plasma (**Supplementary figure 7D and E**). Notably, pretreated with TNP greatly abolished NET formation in the inflamed pancreas as well as significantly decreased plasma levels of DNA-histone complexes and pancreatic levels of citrullinated histone 3 in animals challenged with L-arginine (**Supplementary figure 7D and E**). In addition, challenge with L-arginine provoked increase in blood amylase levels as well as pancreatic and plasma markers of inflammation (**Supplementary figure 8A-H**). However, we observed that administration of TNP decreased blood amylase as well as activity of MPO and pro-inflammatory compounds in the pancreas and plasma (**Supplementary figure 8A-H**). In addition, pretreated with TNP greatly reduced formation of edema, hemorrhage, acinar cell necrosis and leukocyte infiltration in the pancreas of animals challenged with L-arginine (**Supplementary figure 8I-O**).

In conclusion, our data found that platelets control NETs formation in the inflamed pancreas. Furthermore, the present study shows for the first time that NETs-MPs aggregates have a potential role in regulating amylase secretion and STAT-3 phosphorylation in isolated acinar cells. In addition, we could observe that IP6K1 controls NETs formation, inflammation and tissue damage in AP possibly via regulating secretion of PolyP from platelets. Therefore, our novel results provide a new function of IP6K1 in pancreatitis and suggest that targeting IP6K1 might be a useful strategy to decrease both local and systemic inflammation in AP.

Chapter 10

General Discussion and Future Perspectives

The purpose of preclinical experiments is to translate the findings and make them applicable into clinical trials. However, number of studies have failed to translate preclinical researches into clinical practice. And this could be due to the fact that animal models have slightly different in cellular and immunological system than human. Subsequently, animal models have limited ability to show complex process of inflammation because of high homogeneity of experimental animals. But, it is important to mention that animal models have essentially been used in the research purpose for long time because of practical and ethical concern in human experimentation.

In this thesis, we used animal models because we found that it is difficult to understand the pathophysiological mechanism of severe AP with only in vitro studies. In addition, we found that animal models are essential to study the role of different signaling pathways in a complex disease system.

Despite of great investigatory efforts in AP, there is no effective treatments of AP yet. Accordingly, understanding the pathophysiological mechanisms could help in resolving this problem. In this thesis, we identify three different pathways involved in NETs formation in severe AP. Suggesting that targeting any of these pathways might provide a therapeutic approach to reduce the pathologic inflammation in clinical AP.

A previous study has shown that leukocytes represent the hallmark of inflammatory response in severe AP [9]. In addition to engulf the pathogens [181] and release inflammatory mediators [13,179], activated neutrophils have been observed to release web-like structures called NETs [15]. It has been shown that NETs trap and kill the pathogens and provide a defense mechanism against invading pathogens [180,181]. In contrast, NETs have found to be involved in development of various inflammatory diseases such as vascular disorder [184], inflammatory lung diseases [21], sepsis [185] and acute pancreatitis [19], however, the mechanisms of NETosis in severe AP are still elusive. In this thesis, we investigated the role of three different pathways which might mediate NETs formation in AP. Our findings show that all of these pathways are required for NETs formation.

It is well established that citrullinated histone3 is main component of NETs and it is released outside neutrophils as a part of inflammatory response. Citrullination is process in which histone arginine converted into citrulline residues via PAD4 protein catalyzation [24]. Therefore in paper I, it was interested to investigate the role of PAD4 in severe AP as a first step in chromatin condensation of NETs. We found that challenge with taurocholate elevated NETs formation in both plasma and pancreatic tissue. For instance, we found substantial increased in plasma levels of DNA-histone complexes and pancreatic levels of H3 and H4 in mice exposed to taurocholate as compared with sham mice. Importantly, we found that administration of Cl-amidine significantly attenuated the levels of DNA-histone complexes in the plasma and levels of H3 and H4 in the inflamed pancreas. This fact was more confirmed by electron microscopy showing that taurocholate induced releasing of NETs into pancreatic tissue. Notably, administration of Cl-amidine greatly reduced NETs formation in the inflamed pancreas. These findings were in line with previous studies showing that Cl-amidine blocked formation of NETs in murine sepsis model [194], atherosclerosis [251] and colitis [252].

The role of PAD4 in regulating tissue damage was also examined. We found that targeting PAD4 protected against pancreatic tissue damage. For example, pretreatment with Cl-amidne greatly reduced blood levels of amylase

as well as edema, hemorrhage and acinar cell necrosis, suggesting that PAD4 has a critical role in pathology of AP. It is well known that neutrophils play an essential role in pathophysiology of severe AP [13, 100]. A recent study has found that NETs stimulate neutrophil rolling, adhesion and extravasation in the microvasculature of cremaster tissue. Indicating that NETs cause self-amplifying loop via increase neutrophilia [274]. In line with that we found that Cl-amidine-inhibited NETs formation resulted in decrease recruited neutrophils in the inflamed pancreas. Thus, these results suggested that PAD4 regulates neutrophils infiltration and this could explain the protective effect of Cl-amidine of tissue damage in severe AP. Moreover we found that inhibition of PAD4 reduced neutrophils infiltration in lung tissue, indicating that PAD4 regulates both local and systemic inflammation in severe AP.

Cellular stress and tissue injury have been shown to lead to signaling pathways that involved in various biological processes via controlling gene expression of pro-inflammatory compounds [29]. In paper II, we examined the signaling pathways of c-Abl kinase in regulating of NETs formation in severe AP. We found that inhibition c-Abl kinase activity reduced taurocholate-induced releasing of NETs formation in the inflamed pancreas. For instance, inhibition phosphorylation of c-Abl kinase significantly decreased the levels of DNA-histone complexes in the plasma and H3cit in the inflamed pancreas. Suggesting that c-Abl kinase has role in regulating NETs formation in severe AP. We next investigated the mechanism of c-Abl kinase mediated NETs formation in severe AP. A previous study has shown that c-Abl kinase plays an essential role in regulating of ROS generation in neutrophils [34]. It was interested to notice that a previous study has shown that ROS formation regulate release of NETs from neutrophils [238]. Herein, we found that TNF-α-exposed isolated neutrophil resulted in clear-cut generation of ROS. Importantly, we observed that co-incubation with GZD824 significantly reduced TNF-α-induced ROS generation in isolated neutrophils. Thus, our findings suggest that c-Abl kinase-dependent ROS generation could be involved in NETs formation in AP. The role of c-Abl kinase was also examined in regulating of neutrophil recruitment and tissue damage in AP. We found that inhibition of c-Abl kinase significantly reduced the levels of amylase as well as edema, hemorrhage and acinar cell necrosis, suggesting that c-Abl kinase signaling might be involved in pathology of severe AP. Moreover, inhibition the activity of c-Abl kinase greatly reduced the number of neutrophils and activity of MPO enzyme in the inflamed pancreas. Previous studies have shown that neutrophils extravasation are a hallmark in pathophysiology of AP via mediating trypsin activation and tissue damage [100, 268]. Thus, reduction of neutrophils in the inflamed pancreas could explain the tissue protective effect of GZD824 in severe AP. And these results were in line with the results of paper I showing that reduction of extravascular neutrophils protected against pancreatic tissue damage. In addition, we demonstrated that inhibition of c-Abl kinase also reduced neutrophilia in Lung tissue, indicating that c-Abl kinase mediated NETs formation regulates both local and systemic inflammation in severe AP. In addition to their role in providing the host with defense mechanisms against invading pathogens [15], excessive NETs formation have also shown to involve in tissue damage and organ failure in infectious diseases [20,23]. Indeed, it was interesting to note that previous study has observed that c-Abl kinase has a positive role in supporting bacterial and viral

pathogens to achieve entry, release and survival in the cells of mammalian host [275]. Therefore, a pharmacological inhibitor of c-Abl kinase activity could be a useful approach to antagonize the microbial pathogens and attenuate NETs formation that affect host organs tissue.

Several studies have reported that activated platelets support neutrophil migration and recruitment into site of inflammation by secreting of CCL5, CXCL4 and CD40L [40, 41]. Moreover, it has observed that platelets have essential role in regulation of NETs formation in infectious diseases but platelet-induce NETs expulsion in AP is not completely clear. In paper III, we investigated the potential role of platelet-mediated NETs formation in severe AP. Notably, we found that depletion of platelets greatly reduced NETs formation in taurocholate-challenged mice. For instance, administration of anti-GP1b alpha antibody significantly reduced the levels of DNA-histone complexes in the plasma as well as the levels of H3 and H4 in the inflamed pancreas. A previous study has found that that NET-derived histones have a critical role in epithelial cell damage [267] as well as activation of trypsinogen in pancreatic acinar cells [19]. Suggesting that reduced levels of pancreatic H3 and H4 might help to explain, at least in part, the beneficial effect of platelets depletion in AP. By applying scanning electron microscopy, we observed that taurocholate induced NETs formation contained round structures more compatible with MPs. We found that these MPs expressed CD41 or Mac-1, indicating that platelets and neutrophils are the origin of these MPs. Wang Y et al. 2018, have found that MPs bind with NETs possibly via interactions of histone phosphatidylserine. The authors have demonstrated that NETs-MPs complexes cause thrombin generation via intrinsic pathway of coagulation [23]. Therefore, it was interested to examine the role of these complexes in pancreatic acinar cell biology. We found that incubation of acinar cells with NETs-MPs complexes caused clear-cut increase in amylase secretion and activity of STAT3 as well as increased the gene expression of IL-6 and TGFβ1. Notably, we found that NETs-depleted MPs have greatly less effect on amylase secretion and STAT3 activity. However, MPs alone had no effect on acinar cell damage, indicating that MPs become powerful inducer for acinar cell damage when they form complexes with NETs. Targeting of platelets can provide a useful strategy that leads to reduce NETs formation and subsequently the inflammation and pancreatic tissue damage in severe AP. However, platelets have a crucial role in both hemostasis and thrombosis [196], and thus depletion of platelets would not be a good idea to translate into clinical practice. We therefore sought the mechanisms by which platelets stimulate NETs formation in sever AP. A previous study has reported that P-selectin on platelets is an adhesion molecule that provide a highly interaction between platelets and neutrophils [276]. We next assessed the role of P-selectin in platelet-neutrophil interaction mediated NETs formation. Immuno-neutralization of P-selectin substantially reduced NETs formation in the inflamed pancreas. Suggesting that P-selectin can provide a potential physical contact between platelet and neutrophil but P-selectin itself could not be able to induce NETs formation. That's mean platelet-neutrophil interaction can result in intracellular signaling might lead to induce NETs formation. Recent published data have been found that platelet IP6K1 has pro-inflammatory role in systemic inflammation in endotoxin-induced lung injury via regulating neutrophil-platelet aggregates in lung tissue [46]. Another study has reported that platelet IP6K1 has a potential role in controlling PolyP

secretion form platelets dense granules [42]. Moreover, it has been shown that PolyP has important pro-inflammatory effects, for instance regulates activation of the kallikrein-kinin [45] as well as controls blood clotting cascade via extrinsic and intrinsic pathways [207]. Thus, it was of our interest to investigate whether PolyP are involved in stimulation of NETs formation in severe AP. We found that thrombin stimulation of IP6K1 gene-deficient platelets co-incubated with wild-type neutrophils greatly reduced the levels of DNA-histone complexes. This notion was also confirmed by confocal fluorescence microscopy that thrombin stimulation of IP6K1 gene-deficient platelets co-incubated with wild-type neutrophils protected against releasing of DNA co-localizing with MPO and citrullinated histone 3. Moreover, IP6K1-deficienct animals showed greatly less NETs formation in the inflamed pancreas as compared with taurocholate-exposed wild type animals. Indicating that PolyP has a significant involvement in platelet-mediated NETs formation in severe AP. In paper III, induction of NETs formation might require both P-selectin-mediated physical contact between platelet and neutrophil as well as PolyP secretion. It is important to notice that a previous study has found that PolyP has an important role in activation of complement systems cascades [273]. We could show, in another submitted work not involved in this thesis, that complement component 3 has a critical role in NETs formation in severe AP. Thus, whether PolyP mediated complement systems activation is required for NETs formation this should be addressed in future studies.

Although extensive researches have done on AP, there is no available treatment for this disease. This could due to poor understanding of pathological mechanism of AP. That's mean new treatment strategies are required in order to prevent the early inflammatory response and subsequently inhibit multi organ failure. A previous study has observed that viscosity of purulent secretions of patients with cystic fibrosis increases greatly in the presence of large amounts of extracellular DNA, a major component of NETs. The authors found that using recombinant human DNase I resulted in disintegration of extracellular DNA in purulent sputum of patients with cystic fibrosis and led to reduce viscosity and transform the sputum from the gel-like structures to flowing liquid within minutes [277]. It is important to mention here that recombinant human DNase I have been used for patients with cystic fibrosis and systemic lupus erythematosus and appears to be safe. Therefore, targeting NETs formation could provide a promising strategy to inhibit both local and systemic inflammation in severe AP. However, whether targeting NETs formation can affect the other functions of neutrophil as well as whether it is possible to treat AP patients with DNase targeting NETs require adequate future clinical studies.

Chapter 11

Thesis Conclusions

- PAD4 is a critical regulator of NET formation in AP. Inhibition of PAD4 decreased not only NET generation but also attenuated neutrophil recruitment and tissue damage in AP.
- c-Abl kinase signaling is a potent stimulator of NET formation possibly via ROS generation. Inhibition of c-Abl kinase reduced chemokines secretion, neutrophil recruitment and tissue injury in AP.
- Platelets are a key regulator of NET generation and tissue damage in AP.
- P-selectin is a critical protein of platelet-neutrophil interaction and provides a physical contact between platelets and neutrophil. This physical contact mediates NET formation in the inflamed pancreas.
- NETs form aggregates with MPs in AP. NET-MP complexes are a critical stimuli for secretion of amylase and phosphorylation of STAT3 in isolated acinar cells.
- Platelet IP6K1 is a key regulator of NET formation and controls local and systemic inflammation in AP possibly via controlling PolyP secretion from platelets.

Populärvetenskaplig Sammanfattning

Akut pankreatit är en inflammatorisk sjukdom i bukspottkörteln. De vanligaste orsakerna är överkonsumtion av alkohol hos män och inkilad gallsten som stoppar sekretionen från bukspottkörteln. Inflammationen leder till aktivering av olika enzymsystem framförallt trypsin som i sin tur ökar inflammationen. Tryspinaktiveringen och inflammationen kan snabbt sprida sig runt bukspottkörteln lokalt och senare också till blodet där det uppstår en systemisk inflammation med risk för organsvikt och till och med död. De flesta fallen med akut pankreatit är milda eller moderata och endast ett fåtal (5-10%) är allvarliga med risk för organsvikt och död. Neutrofila granulocyter är en typ av vita blodkroppar som har visat sig spela en central betydelse för utvecklingen av akut pankreatit. Neutrofiler utsöndrar syreradikaler och enzymer som aktiverar trypsin och orsakar patologisk inflammation i bukspottkörteln. På senare tid har det visat sig att neutrofiler också kan bilda och utsöndra så kallade neutrofila extracellulära fällor (neutrophil extracellular traps, NETs). Det övergripande syftet med den aktuella avhandlingen var att studera hur NETs bildas vid akut pankreatit och dess betydelse frö inflammation och skada i bukspottkörteln.

Delarbete 1. Nyare forskning har visat att vissa specifika enzymer, peptidyl arginin deaminaser (PADs) kan vara viktig för bildningen av NETs. I detta delarbete var vi särskilt intresserade av att studera PAD4. För syftet användes två olika inhibitorer av PAD4, Cl-amidine och GSK484. Det kunde visas att stora mängder av NETs bildades i den inflammerade bukspottkörteln. Behandling med Cl-amidine och GSK484 minskade bildningen av NETs vid akut pankreatit. Dessutom minskade utsöndringen av amylas som är en indikator på vävnadsskada i pankreas. Med histologi kunde ses att inhibition av PAD4 reducerade blödning, celldöd, svullnad och inflammation i bukspottkörteln. Vidare kunde konstateras att blockering av PAD4 minskade antalet neutrofiler och bildningen av ämnen som attraherar neutrofiler (kemokiner) i den inflammerade bukspottkörteln. Behandling med Cl-amidine och GSK484 minskade också den systemiska inflammationen mätt som ökning av enzymer, IL-6 och kemokiner i plasma.

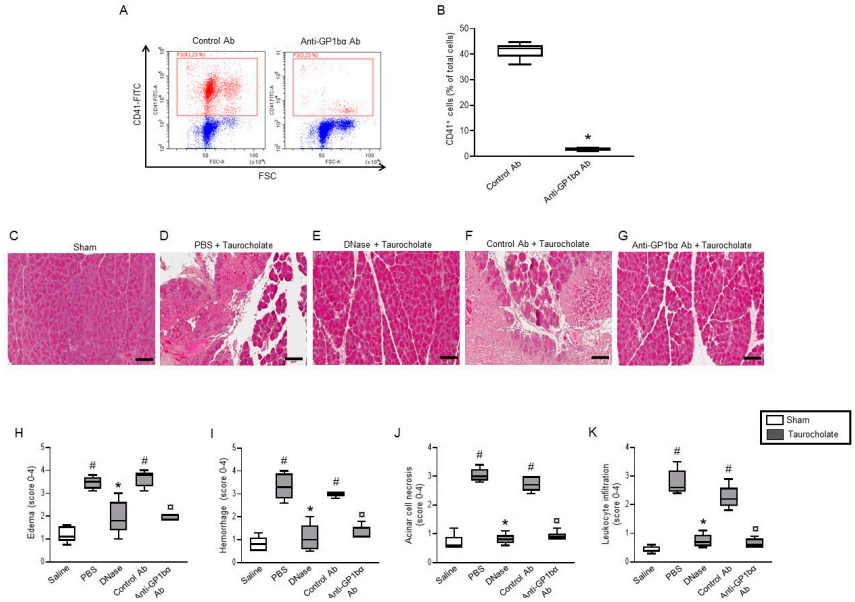
Delarbete 2. Detta arbete var fokuserat på intracellulära signalvägar för regleringen av NET syntes. C-Abl kinase är ett enzym som finns i alla celler och det har visat sig att c-Abl kinas kan reglera bildningen av syreradikaler i neutrofiler. Syreradikaler är viktigat för bildningen av NETs och målsättningen blev därför att studera betydelsen av c-Abl kinas i akut pankreatit med fokus på NET bildning. Först observerades att c-Abl kinas fosforylerades (aktiverades) och att GZD824 effektivt inhiberade forsforylering av c-Abl kinas i neutrofiler. I möss som gjordes neutropena (saknar neutrofiler) försvann forsforyleringen av c-Abl kinas i blodet som tecken på av neutrofiler var de dominerade cellerna i blodet där c-Abl kinas fosforylerades vid akut pankreatit. Inhibition av c-Abl kinas aktivering minskade bildningen av NETs i neutrofiler in vitro och i neutrofiler vid akut pankreatit. Dessutom kunde det konstateras att GZD824 minskade bildningen av syreradikaler i neutrofiler. Behandling med GZD484 minskade inte bara amylas i blodet utan också blödning, celldöd, svullnad och inflammation i bukspottkörteln. Vidare kunde konstateras att blockering av c-Abl kinas aktivering minskade antalet neutrofiler och bildningen av kemokiner

i den inflammerade bukspottkörteln. Mac-1 som är en adhesionmolekyl på neutrofiler uppregleras vid inflammation. Även Mac-1 ökningen minskade på neutrofiler i möss med akut pankreatit efter blockering av c-Abl kinas. Behandling med GZD484 minskade också den systemiska inflammationen mätt som ökning av enzymer, IL-6 och kemokiner i plasma. I detta arbete användes två olika modeller för akut pankreatit och effekterna av GZD484 var liknande i båda modeller vilket stärker av slutsatsen att c-Abl kinas spelar en viktig roll vid akut pankreatit via bildningen av NETs.

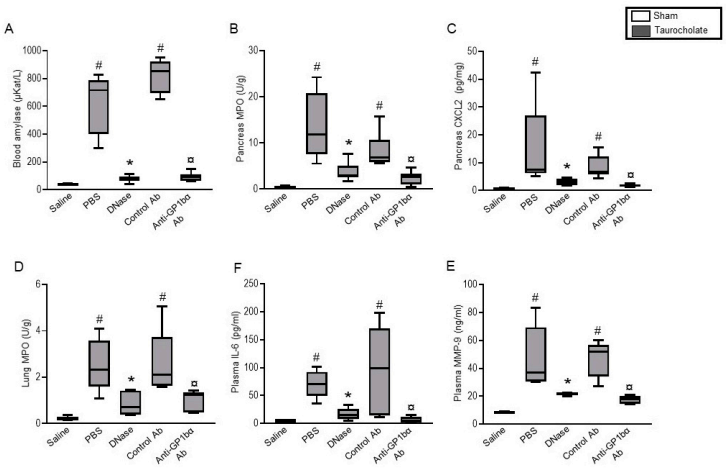
Delarbete 3. Trombocyter (blodplättar) har visat sig inte bara vara viktig för att stoppa blödning och blodets levring utan de har också visat sig spela stor betydelse för inflammatoriska reaktioner. Detta arbete fokuserade på betydelsen av trombocyter för aktivering av neutrofiler och bildning av NETs. Först kunde konstaterats att Net bildningen avsevärt minskade i den inflammerade bukspottkörteln i möss som saknade trombocyter. Dessutom kunde det konstateras att NETs var dekorerade med mikropartiklar (MP, små celldelar från både neutrofiler och trombocyter). Behandling med caspase och calpain inhibitorer kunde minska bildning av NETs med MP. Det visade sig av utsöndring av amylas från pankreas acinära celler ökade efter exponering med NETs. NET-inducerad amylas utsöndring var delvis beroende av MP på NETs medans MP per se inte kunde stimulera amylasutsöndringen från acinära celler. I separata experiment kunde det visas att kontakt mellan trombocyter och neutrofiler var avgörande för en effektiv bildning av NETs. Blockering av trombocyt-neutrofil interaktionen med en antikropp riktad mot P-selectin minskade trombocyt-beroende aktivering och bildning av NETs i neutrofiler. Inositol hexakifosfat kinase 1 (IP6K1) är ett enzym som bland annat reglerar bildningen av polyfosfater i trombocyter. Det observerades av möss som saknade IP6K1 bildade mycket mindre NETs i den inflammerade bukspottkörteln. Med histologi kunde ses att inhibition avsaknad av IP6K1 resulterade i minskad blödning, celldöd, svullnad och inflammation i bukspottkörteln. Dessutom minskade också den systemiska inflammationen mätt som ökning av enzymer och IL-6 i plasma och neutrofiler i lungorna hos möss utan IP6K1. Samma fynd som observerades med möss utan IP6K1 kunde upprepas med en substans som hämmar IP6K1 aktivitet. I separata experiment kunde det visas att IP6K1 reglerar polyfosfatutsöndringen från trombocyter och att polyfosfater är de ämnen som medierar trombocyt-beroende NET bildning i neutrofiler.

Sammantaget visar detta avhandlingsarbete att PAD4 och c-Abl kinas spelar en central roll för bildningen av NETs vid akut pankreatit och att hämning av dessa enzym skulle kunna vara relevanta måltavlor för att behandla akut pankreatit. Dessutom visar detta arbete att NETs bildar aggregat med MP som aktiverar acinära celler i pankreas och att IP6K1 medierar trombocyt-beroende bildning av NETs via utsöndring av polyfosfater.

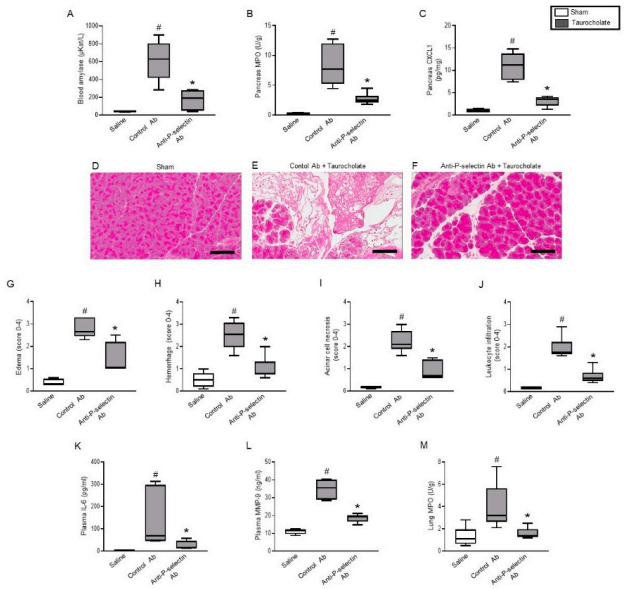
Supplementary Figures



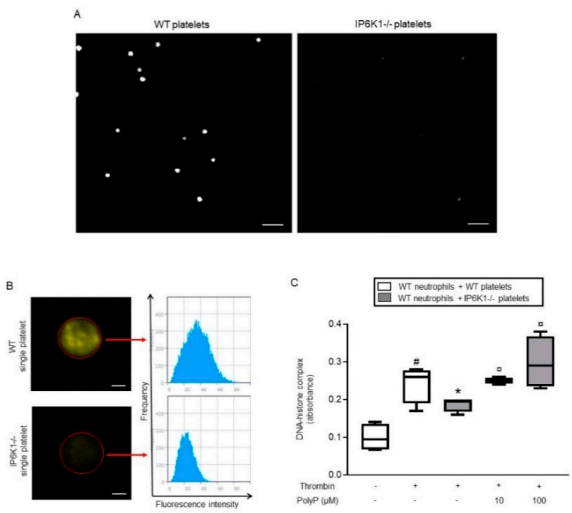
Supplementary figure 1. Role of Platelet in tissue damage in AP. Mice were received a control antibody (Ctrl Ab) and Ab against GP1b alpha for depleting platelets A) CD41+ platelets as showed by dot plots and B) aggregate data of CD41+ cells. C-G) Representative H & E sections of the pancreas from indicated groups. Scale bar = 100 µm. Histological examinations showing H) edema, I) hemorrhage, J) acinar cell necrosis and K) leukocyte infiltration. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (white boxes) were infused only saline. Animals were received i.p. injections of the DNase I, a control antibody or anti-GP1b alpha Ab or vehicle (PBS) as showed in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 4-6. #P < 0.05 versus sham mice, *P < 0.05 versus PBS+taurocholate and ¤P < 0.05 versus Control Ab+taurocholate.



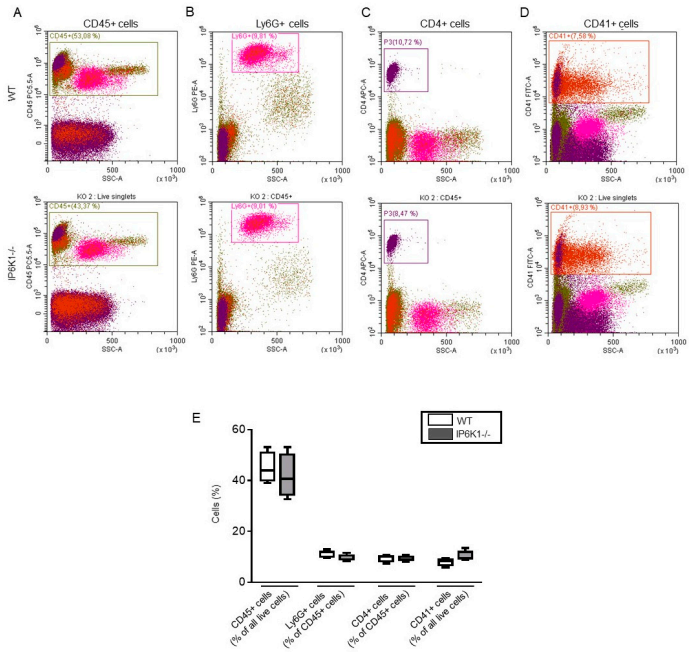
Supplementary figure 2. Role of Platelet in inflammation and tissue damage in AP. Levels of A) blood amylase, B) MPO and C) CXCL2 in the pancreas. D) Levels of MPO in lung tissue, E) IL-6 and F) MMP-9 levels in the plasma. Pancreatitis (grey boxes) was triggered by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (white boxes) were infused only saline. Animals were received i.p. injections of the DNase I, a control antibody, anti-GP1b alpha Ab or vehicle (PBS) as showed in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5-6. #P < 0.05 versus sham mice, *P < 0.05 versus PBS+taurocholate and ¤P < 0.05 versus Control Ab+taurocholate.



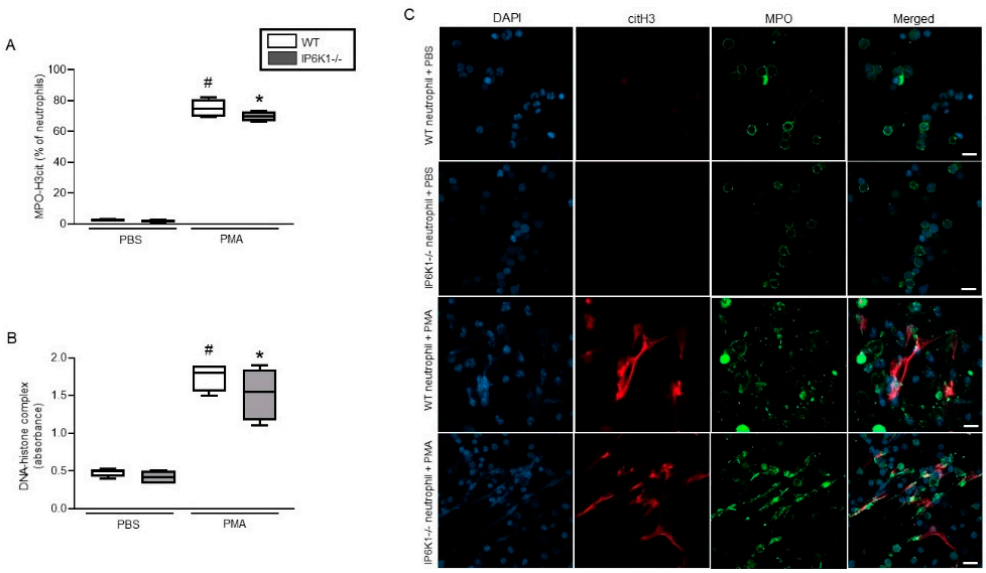
Supplementary figure 3. Role of NPA formation in inflammation and tissue damage in AP. Levels of A) blood amylase, B) MPO and C) CXCL1 levels in the pancreas. D-F) Representative H & E sections from the head of the pancreas of indicated groups. Scale bar = 100 μm. Histological examinations showing G) edema, H) hemorrhage, I) acinar cell necrosis and J) leukocyte infiltration. Levels of K) IL-6 and L) MMP-9 in the plasma as well as M) MPO activity in lung. Pancreatitis (grey boxes) was induced by retrograde infusion of sodium taurocholate (5%) into the pancreatic duct. Sham mice (white boxes) were infused only saline. Animals were received i.v. injections of a control antibody or an antibody against P-selectin as showed in Materials and Methods. 24 hours after induction of pancreatitis, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5-6. #P < 0.05 versus sham mice and *P < 0.05 versus Control Ab+taurocholate.



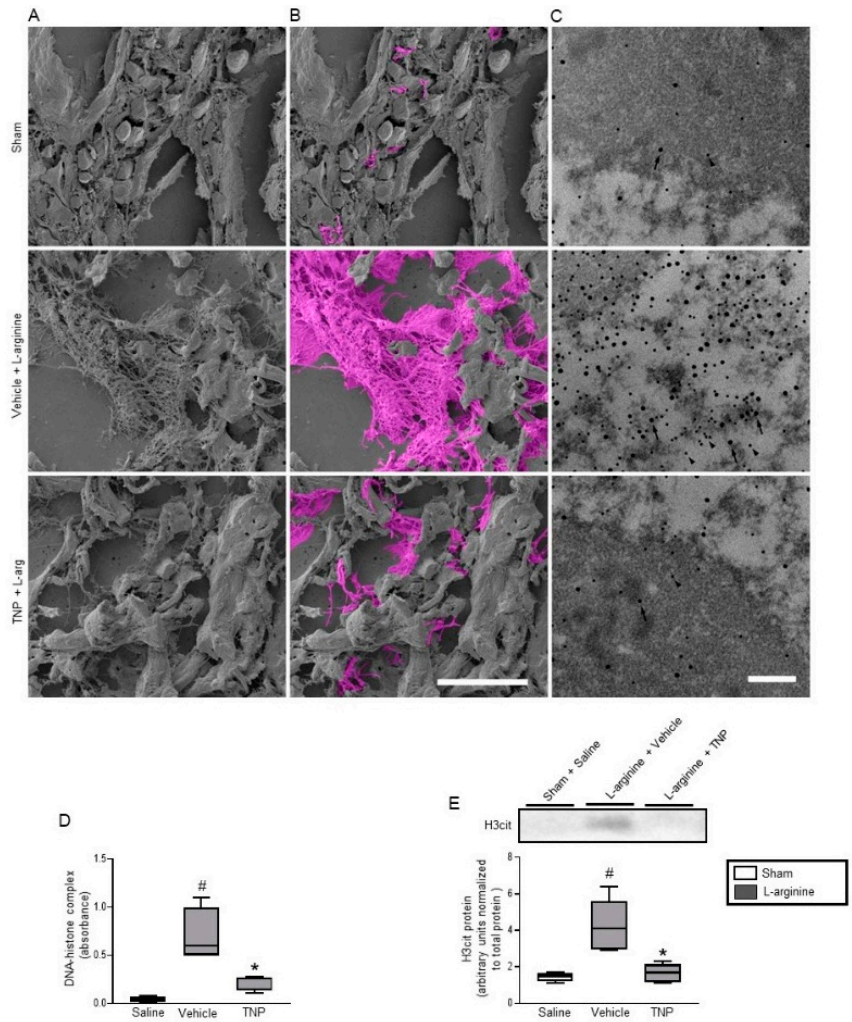
Supplementary figure 4. Role of Platelet IP6K1 in formation of DNA-histone complex in vitro. A) Cellular PolyP as showed by confocal fluorescence microscopy after staining with 4′,6-diamidino-2-phenylindole (DAPI). Scale bars = 10 μm. DAPI emits higher wavelengths light after binding with PolyP. B) DAPI fluorescence intensity was shown by a higher magnification imaging of single platelets and corresponding histograms showing. Scale bars = 0.2 μm. C) Bone marrow neutrophils were incubated with isolated platelets from wild-type or IP6K1-/- with or without thrombin (0.2 U/ml) and with or without PolyP (10 or 100 μM) for 3 hours at 37°C. Quantification measurement of DNA-histone complexes in the supernatant. Isolated neutrophils with non-stimulated wild-type platelets served as a control. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 4. #P < 0.05 versus WT neutrophils + WT platelets, *P < 0.05 versus WT neutrophils + WT platelets + thrombin and ¤P < 0.05 WT neutrophils + IP6K1-/- platelets + thrombin.



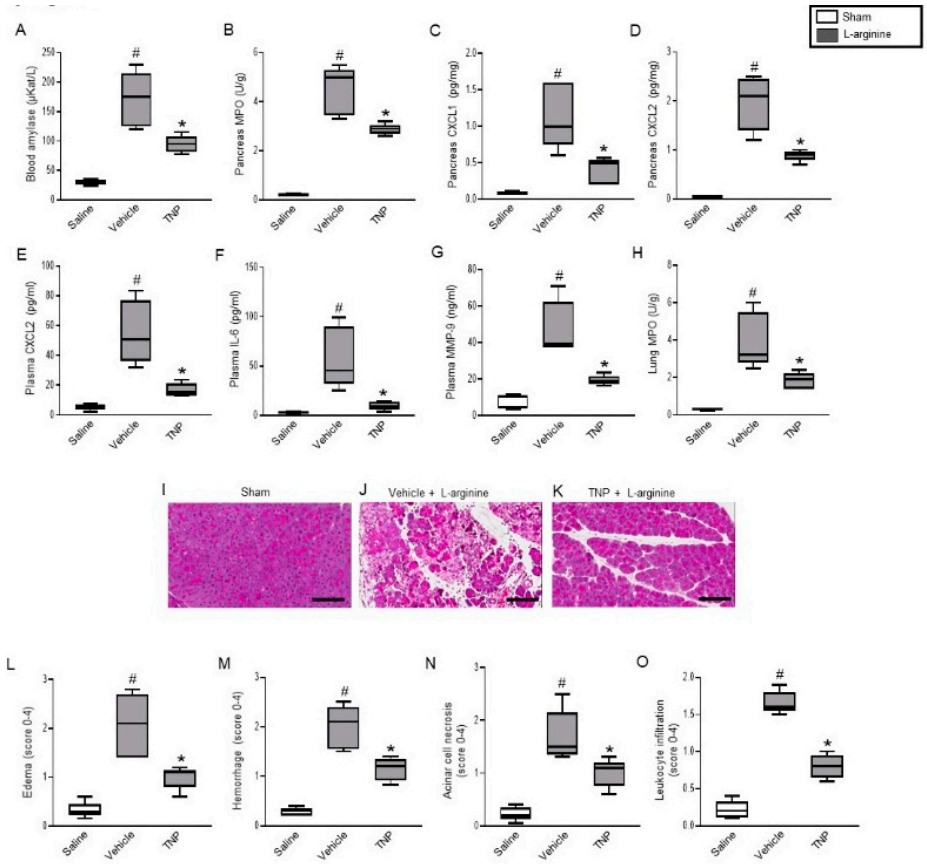
Supplementary figure 5. Comparison of wild-type and IP6K1-/- leukocyte subtypes and platelets using flow cytometry. Representative dot plots of A) CD45+ cells, B) Ly6G+ cells, C) CD4+ cells and D) CD41+ cells. E) Relative percentage of wild type and IP6K-/- leukocyte subtypes and platelets. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 4. No significant difference observed.



Supplementary figure 6. Generation of PMA-induced NET in vitro. NETs were stimulated by PMA (50 nM, 3h) from isolated wild-type and IP6K1-/- neutrophils. Non-stimulated neutrophils represented the control group. A) Quantification measurement of citrullinated histone 3 and MPO levels in the neutrophils were identified by FACS. B) Supernatant DNA-histone complexes was measured by ELISA as showed in Materials and Methods. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 4. C) Confocal fluorescence microscopy showing citrullinated histone 3 (H3Cit) and myeloperoxidase (MPO) in isolated neutrophils by co-incubated with with anti (H3Cit), (MPO) antibodies and Hoechst for nuclear staining. Images were taken using LSM 800 confocal microscope. One representative experiment of five independent experiments. Scale bars = 10 µm. #P < 0.05 versus PBS+WT and *P < 0.05 versus PBS+ IP6K-/-.



Supplementary figure 7. Role of TNP in L-arginine-induced NET formation in AP. A) Extracellular web-like structures were identified by scanning electron microscopy in the pancreas from mice challenged with taurocholate. Scale bar = 25 µm. B) NETs denoted in pink color. C) Indicated area of interest from Figure 1A was identified by transmission electron microscopy and incubated with gold-labeled anti-histone 4 (large gold particles, arrows) and anti-elastase (small gold particles, arrowheads) antibodies. Scale bar = 0.25 µm. D) Quantification of extracellular DNA-histone complexes. E) Pancreatic citrullinated histone 3 was determined by western blot and aggregate data showing H3Cit protein normalized with stain-free total protein load. Pancreatitis was induced by treatment with 4 g/kg/dose of L-arginine i.p. twice at an interval of one hour. Animals were received with i.p. injection of vehicle or TNP as showed in Materials and Methods prior on the first dose of L-arginine (grey boxes). Sham mice (white boxes) were received only saline. 72 hours after L-arginine challenge, samples were collected. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5. #P < 0.05 versus Saline+Sham and *P < 0.05 versus Vehicle+L-arginine.



Supplementary figure 8. Role of TNP in L-arginine-induced inflammation and tissue damage in AP. Levels of A) blood amylase, pancreatic B) MPO, C) CXCL1 and D) CXCL2 levels. Plasma levels of E) CXCL2, F) IL-6 and G) MMP-9 as well as H) lung levels of MPO. I-K) Representative haematoxylin & eosin sections of the head of the pancreas from indicated groups. Scale bar = 100 μm. Histological quantification of L) edema, M) haemorrhage, N) acinar cell necrosis and O) leukocyte infiltration. AP was induced by administration of 4 g/kg/dose of L-arginine i.p. twice at an interval of one h. Mice were treated with i.p. injections of vehicle or TNP as described in Materials and Methods before the first dose of L-arginine (grey boxes). Sham mice (white boxes) were infused with saline alone. Samples were collected 72 hours after induction of pancreatitis. Data represent median (25-75 percentile); whiskers extend from the minimum to the maximum values and n = 5. #P < 0.05 versus Saline+Sham and *P < 0.05 versus Vehicle+L-arginine.

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