Signaling and Adhesive Mechanisms in Acute Pancreatitis

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Abstract

Abstract:

Acute pancreatitis (AP) is an inflammatory disease with variable severity ranging from mild interstitial edematous to severe necrotizing disease. The overall mortality rate of AP is 8-9%. Specific treatment of AP is lacking which is partly related to an incomplete understanding of the basic pathophysiology behind the disease. It is widely held that premature intra-cellular trypsinogen activation and leukocyte recruitment play key roles in the pathophysiology of the AP. However, the signaling and adhesive mechanisms remain elusive. The aim of this thesis was to investigate the signaling and adhesive mechanisms in AP.

In this thesis, biliary pancreatitis was induced by retrograde infusion of taurocholate into the pancreatic duct in different mice strains to elucidate the role of toll-like receptor 2 (TLR2), TLR4, lymphocyte function antigen-1 (LFA-1), matrix metalloproteinases (MMPs) and Rho-kinase as well as nuclear factor of activated T-cell (NFAT) signaling in AP.

We found that TLR4 but not TLR2 plays a role in AP. LFA-1 adhesive mechanisms play a role in tissue damage and leukocyte recruitment but not trypsinogen activation. Furthermore, neutrophil-derived MMP-9 mediates tissue damage and neutrophil-dependent trypsinogen activation. Rho-kinase signaling regulates trypsinogen activation, leukocyte recruitment and tissue damage in AP. Moreover, NFATc3 is activated and translocated to the acinar cell nucleus and regulates trypsinogen activation, leukocyte recruitment and tissue damage in AP.

Taken together, the results of this thesis demonstrate that signaling and adhesive mechanisms are of particular importance in the pathophysiology of AP and could be used as useful targets in the management of patients with AP.

Key words: Acute pancreatitis, trypsinogen activation, leukocyte recruitment and adhesion

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To my daughter (Kani)

What we do for ourselves dies with us.
What we do for others and the world remains and is immortal.
(Albert Pine)

Table of Contents

Abbreviations

Ach	acetylcholine
AP	acute pancreatitis
α-1PI	α-1 protease inhibitor
BTP	bis(trifluoromethyl)pyrazole
CCK	cholecystokinin
CCK-RF	cholecystokinin-releasing factor
CXCL2/MIP-2	macrophage inflammatory protein-2
CsA	cyclosporin A
DAMPs	damage-associated molecular patterns
ELISA	enzyme linked immunosorbent assay
EGF	epidermal growth factor
ERCP	endoscopic retrograde cholangio-pancreatography
ECM	extracellular matrix
ICAM-1	inter-cellular adhesion molecule-1
IP3	inositol triphosphate
IL-2	interleukin-2
i.p.	intraperitoneal
JAMs	junctional adhesion molecules
JNK	c-Jun terminal kinase
kDa	kilo Dalton
LPS	lipopolysaccharide
LRRs	leucine-rich repeats
LAD-1	leukocyte adhesion deficiency-1
LFA-1	lymphocyte function antigen-1
MMP	matrix metalloproteinase
MCP-1	monocyte chemotactic protein-1
MOF	multiple organ failure
Mac-1	macrophage-1 antigen
NFAT	nuclear factor of activated T-cell
NFAT-luc	NFAT-luciferase
NF-kB	nuclear factor kappa B
PRRs	pattern recognition molecular receptors
PAMPs	pathogen-associated molecular patterns
PECAM-1	platelet endothelial cell adhesion molecule-1
PKA	protein kinase A
PMNL	polymorphonuclear leukocyte
PSGL-1	P-selectin glycoprotein ligand-1
RER	rough endoplasmic reticulum
RT-PCR	reverse transcription-polymerase chain reaction
ROS	reactive oxygen species
SIRS	systemic inflammatory response syndrome
SPINK 1	serine protease inhibitor Kazal type 1
TAP	trypsinogen activation peptide
TLR	toll-like receptor
TIR	toll/IL-1 receptor
TNF-α	tumor necrosis factor-α
TIMPs	tissue inhibitors of metalloproteinases
VCAM-1	vascular cell adhesion molecule-1
ZG	zymogen granule

List of original papers

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

I. **Awla D**, Abdulla A, Regnér S and Thorlacius H. TLR4 but not TLR2 regulates inflammation and tissue damage in acute pancreatitis induced by retrograde infusion of taurocholate. *Inflamm. Res.* 2011 [Epub ahead of print]. *

II. **Awla D**, Abdulla A, Zhang S, Roller J, Menger MD, Regnér S, Thorlacius H. Lymphocyte function antigen-1 regulates neutrophil recruitment and tissue damage in acute pancreatitis. *Br. J. Pharmacol.* 2011; 163(2):413-423. **

III. **Awla D**, Abdulla A, Ingvar S, Jeppsson B, Regnér S and Henrik Thorlacius. Neutrophil-derived matrix metalloproteinase-9 activates trypsinogen in acinar cells in acute pancreatitis. *J. Leukoc. Biol.* 2011; in press. #

IV. **Awla D**, Hartman H, Abdulla A, Zhang S, Rahman M, Regnér S, Thorlacius H. Rho-kinase signalling regulates Trypsinogen activation and tissue damage in severe acute pancreatitis. *Br. J. Pharmacol.* 2011; 162(3):648-658. **

V. **Awla D**, Zetterqvist A.V., Abdulla A., Camello C., Berglund L.M., Spegél P., Pozo M.J., Camello P.J., Regnér S., Gomez M.F., and Thorlacius H. NFATc3 regulates trypsinogen activation, neutrophil recruitment and tissue damage in a mouse model of acute pancreatitis. Sumitted to *Gastroenterology* 2011.

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\# will be featured under the Frontline Science Section of *Journal of Leukocyte Biology* as "Leading Edge Research" in the Table of Contents.

The papers where the author has contributed during his PhD but which are not included in this thesis

1. **Awla, D,** Abdulla A, Regnér S, Thorlacius H. PAR-2 exerts no role in acute pancreatitis induced by sodium taurocholate. Manuscript.

2. Abdulla A, **Awla D**, Hartman H, Rahman M, Jeppsson B, Regnér S, Thorlacius H. Role of platelets in experimental acute pancreatitis. *Br. J. Surg.* 2011; 98(1):93-103.

3. Abdulla A, **Awla D**, Jeppsson B, Regnér S, Thorlacius H. CD40L is not involved in acute experimental pancreatitis. *Eur. J. Pharmacol.;* 2011. [Epub ahead of print].

4. Abdulla A, **Awla D**, Thorlacius H, Regnér S. Role of neutrophils in the activation of trypsinogen in severe acute pancreatitis. *J. Leukoc. Biol.;* 2011 [Epub ahead of print].

5. Hartman H, Abdulla A, **Awla D**, Lindkvist B, Jeppsson B, Thorlacius H and Regnér S. P-selectin mediates neutrophil rolling and recruitment in acute pancreatitis. *Br. J. Surg* 2011*;* in press.

Introduction

Acute Pancreatitis (AP) is a spectrum of clinical presentations ranging from mild edematous and self limiting pancreatitis to severe necrotizing and sometimes a fatal condition [1]. AP is a relatively common disease with the annual incidence ranging from 5-55/100000 [2]; however, the incidence, causative etiology and severity vary from one population to another based on the incidence of gall bladder stones and average alcohol consumption. In 1992, during the Atlanta classification symposium, they aimed to unify various AP definitions. According to this classification, AP was broadly and clinically divided into interstitial pancreatitis; characterized by the lack of parenchymal necrosis and necrotizing pancreatitis with local and systemic complications [3]. There is a general desire to revise Atlanta classification to exclude some criterias such as intestinal bleeding and include more and it has been revisited more than once [4].

In almost 75% of AP cases the disease will take the mild form represented by transient abdominal pain which disappears within some days [5]. In the rest 25% of cases, AP will progress into a severe disease with local and systemic complications and potentially devastating consequences and mortality as high as 30% [6]. Approximately half of AP-related deaths occur within the first two weeks during the course of the disease and are generally attributed to organ failure. The rest half of deaths occur weeks to months after first interval and mortality in this case is related to organ failure associated with infected necrosis or complications of the sterile pancreatic necrosis [7].

There is no clear-cut definition for the clinical recognition of AP cases, but there is a general widely-applied criteria all over the world, this criteria requires at least two of the following three features for the diagnosis of AP: 1) abdominal pain strongly suggestive of AP, 2) serum amylase and/or lipase at least three folds higher than the upper normal limit and 3) AP characteristic findings on trans-abdominal ultrasonography or contrast-enhanced C-T scan. The lack of a specific and highly-dependent marker for predicting severity in AP accounts for insufficient management of severe cases and recognition between mild and severely affected patients. Treatment of AP is mainly hampered to supportive care in form of fluid resuscitation and symptomatic management and specific drug against AP is lacking, which is in part due to incomplete understanding of the basic pathophysiology leading to the disease. Even though supportive treatment may improve prognosis in severely affected patients; specific therapy against AP continues to be the subject of intense investigative interest.

Early recognition, fluid resuscitation and supportive care in an advanced unit by a multidisciplinary team including gastroenterologists, interventional radiologists, intensivists, and surgeons may improve the outcome in potentially severe patients with AP, but still a number of these patients will end up in complications. The complicated AP may affect pancreas locally or involve distant organs and produce a systemic disease. The local complications of AP include pancreatic necrosis, infected necrosis, pus collection inside the pancreas, pancreatic pseudocyst development and etc. [8]. However systemic complications adopt involvement of remote organs such as lung and kidney, producing a systemic inflammatory response syndrome (SIRS). An exaggerated SIRS leads to distant organ damage and multiple organ failure (MOF) [9]. The agreement has been established on the concept that repeated attacks of AP may lead to chronic pancreatitis [7, 10]. Chronic pancreatitis *per se* carries a profound risk for progression to pancreatic cancer [11], which is nowadays ranked as

number four among cancer-related deaths [12].

In most cases, AP happens when a stone obstructs the distal biliopancreatic ductal system. However, the mechanism through which obstruction of biliopancreatic ductal opening triggers AP has been the subject of controversy for more than a century. It has been hypothesized that pancreatic exocrine secretions, such as trypsinogen, cause auto digestion of the pancreatic tissue and induce AP. According to another theory, the biliopancreatic obstruction causes regurgitation of the bile salts into the pancreas and thereby stimulates the initiation of AP [13, 14]. To date the evidence that pancreatic secretions without bile salt involvement causing AP is scant. The second most common cause of AP is heavy alcohol consumption, however, animal experiments revealed that ethanol consumption alone is not sufficient to produce AP; it should be combined with other contributing factors such as reduction of pancreatic blood flow and microcirculation [15]. Other less common causes of AP apart from bile salts and alcohol have been well-described, including: idiopathic, trauma, drugs, infections such as viral infections, steroids, hyperlipidemia, hypertriglyceridemia, hypercalcemia, hypothermia, scorpion and snake bite, autoimmune pancreatitis and patients undergoing endoscopic retrograde cholangio-pancreatography (ERCP) [16-30].

The exact pathophysiology of AP is vague, that is why the specific treatment is lacking. However, trypsinogen activation, leukocyte recruitment and disturbed microcirculation are considered to play a key role in the pathophysiology of AP [31-35]. Early in the course of AP, premature activation of trypsinogen occurs within the acinar cells, which leads to a local inflammatory reaction. During the later phases of the disease leukocytes infiltrate into the site of inflammation and if this inflammation is marked, it can lead to SIRS [36]. The interrelation between these two phases in AP is unclear. Some believe that trypsinogen activation and substrates like trypsin can trigger leukocyte recruitment through different mechanisms [32, 35]. While others showed that infiltrated leukocytes into the inflamed pancreas aggravate primary trypsinogen activation resulting in a continuous and exaggerated activation of the primary phase of trypsinogen activation [33, 34]. It has been shown also that leukocyte recruitment is independent on the primary trypsinogen activation and both phases activate through two independent pathways initiated within acinar cells [37]. Furthermore, some researchers believe that intra-cellular trypsinogen activation could be protective in AP [38-41] and some others believe that leukocytes infiltrating into the pancreas during AP exert a protective step in AP especially during the early phases of the disease [42]. The explanation of the pathophysiology and discrepancies are illustrated in figure 1.

Background

Anatomy and Physiology of the pancreas

During embryologic organogenesis, pancreas develops from out-pouching of the primitive foregut endoderm in the region of the duodenum [43, 44]. Development of the exocrine pancreas necessitates involvement of both endoderm and mesoderm whereas endocrine pancreatic development requires only endodermal tissue [45]. Congenital anomalies of the pancreas are quite rare but can happen during the developmental stages such as pancreas divisum [46]; the affected cases may suffer from recurrent attacks of AP [47]. Other rare pancreatic congenital anomalies are solitary and multiple pancreatic cysts, heterotopic pancreatic tissue, annular pancreas and an

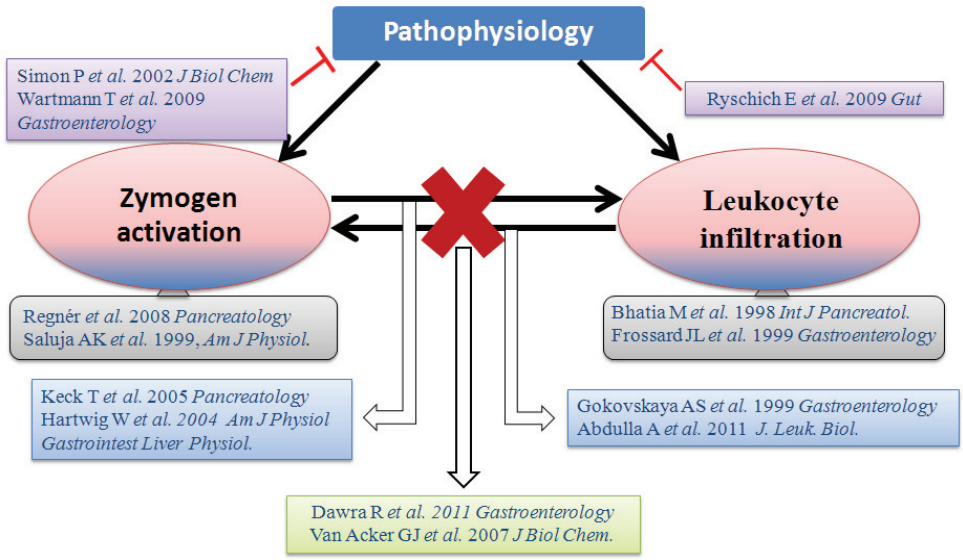

Figure 1: Schematic explanation of the pathophysiology of AP.

anomalous junction of the bile and pancreatic ducts [48].

The pancreas was first identified by the Greek anatomist and surgeon Herophilus (335–280 BC). A few hundred years later, Rufus of Ephesus, another Greek anatomist, gave the pancreas its name. The term "pancreas" is a Greek term, “pan” means all or whole and “creas” stands for flesh, presumably because of its fleshy consistency. The normal human pancreas is around 120 g in weight, 15 cm long and located in the retroperitoneum. Pancreas anatomically divided into head (embedded in the second part of the duodenum) and tail of the pancreas (extends from the head to the hilum of the spleen). Microscopically, pancreatic tissue composed of 3 functionally integrated but structurally distinct components: exocrine pancreatic tissue comprises 80% of the pancreas, 18% includes ducts, nerves, vessels and connective tissue and the rest 2% is endocrine portion of the pancreas [49].

The endocrine glands of the pancreas, islets of Langerhans, are diffusely distributed in the pancreas and around 1 million in each human pancreas, include 4 types of cells: B cells; secret insulin, PP cells; contain pancreatic polypeptide, A cells; release glucagon and D cells; contain glucagon. Each islet is surrounded by larger acinar cells with condensed granules compared to centrally located acini [50]. The endocrine pancreatic tissue will not be further addressed in this thesis.

The exocrine pancreatic tissue is composed of pancreatic acinar cells which are quite large but smaller than Langerhans cells. They are pyramidal in shape, arranged in spherical masses termed acini, a group of acini compose lobules and a number of lobules make pancreatic lobes. The ductal drainage system originates as small ductules collecting acini secretions, tending to enlarge into ducts and eventually draining into the main pancreatic duct. The small ductules are lined by small centro-acinar cells which secret water and bicarbonate. The larger ducts are lined by columnar epithelium, goblet cells which secret mucus and argentaffin cells which have peptides necessary for pancreatic secretion. The acinar cells are condensed with apically located eosinophilic granules known as

zymogen granules (ZGs), filled with enzymes formed by and assembled in the extensive rough endoplasmic reticulum (RER). After feeding and upon stimulation by cholecystokinin (CCK), the apical ZGs release enzymes into ductules and then they appear as empty holes. The Romanian biologist George Palade, the most influential cell biologist ever, awarded Noble price in 1974 in Medicine and Physiology for his description of intra-cellular pathway of synthesizing and release of proteins in the pancreatic acinar cells [51].

The exocrine pancreatic tissue secretes each day approximately 2.5 L of a clear, colorless and alkaline secretion. This secretion contains inorganic components in form of Na^+, K^+, Cl^- and HCO_3^- and enzymatic secretions of exocrine pancreatic tissue which are: amylase; degrades starch, lipase; hydrolyzes fatty acids, trypsin and chymotrypsin; they are potent proteolytics which break down DNA and RNA and thereby degrade meal protein and nucleases. Under basal conditions pancreatic secretion is minimal. After feeding, the fat and aminoacid contents of the meal inside the duodenal lumen enhance release of cholecystokinin-releasing factor (CCK-RF), this CCK-RF stimulates release of CCK which has capability of stimulating acinar cells to release secretions accumulated in their apical ZGs into ductules and thus transferred into the duodenal lumen. Activated trypsin can deactivate CCK-RF and control further stimulation thereby prevents excessive secretion. Less abundant pancreatic acinar cell secretion stimulators have been addressed including acetylcholine (Ach), vasoactive intestinal polypeptide, gastrin releasing peptide and substance P. Somatostatin acts as the main exocrine pancreatic secretion inhibitor [49, 52-56].

Trypsinogen activation

Trypsinogen is a small around 25 kDa protein which is present in normal pancreatic juice. Under normal conditions, the exocrine pancreatic tissue secretes trypsinogen in the pro-enzyme form. Inactive trypsinogen together with other pancreatic secretions is then transferred into the duodenum through the pancreatic duct. Inside the duodenal lumen, trypsinogen is activated into active trypsin and end-product trypsinogen activation peptide (TAP) by intestinal enterokinase. Trypsin is a 24 kDa proteolytic enzyme. It has serine in its structure and can bind lysine and arginine residues of other proteins and destroy their peptide bonds. The trypsin can further activate trypsinogen and a number of proteases (Pro-carboxypeptidase B, chymotrypsin-ogen, pro-elastase, pro-colipase, pro-phospholipase A2, pro-carboxypeptidase A) into their active forms [57-62], see illustration in figure 2.

The site and mechanism of trypsinogen activation in AP have been enduring mysteries. Initially, investigators believed that the disease begins within the peri-ductal area thus pancreatic secretion leakage from the ducts is responsible for the early events during AP [63]. Later, when pathologists found pancreatic fat necrosis at the time of autopsy in the victims of AP, led the researchers to hypothesize that the peri-pancreatic fat necrosis under the effect of pancreatic lipase secretions from acinar cells are the initial events leading to AP. Subsequent controlled studies performed in animal models reported that during AP premature activation of pancreatic zymogens occurs either in the interstitium, intra-ductal or inside the pancreatic acinar cells. However the third possibility and “co-localization theory” or “crinophagy” is more culminated and received a more general agreement. It has been shown that intra-ductal trypsinogen activation is not sufficient to trigger development of AP [64]. Interstitial trypsinogen is not excluded as in studies using enterokinase; the course of the disease has shifted from mild to severe [64, 65], but further studies

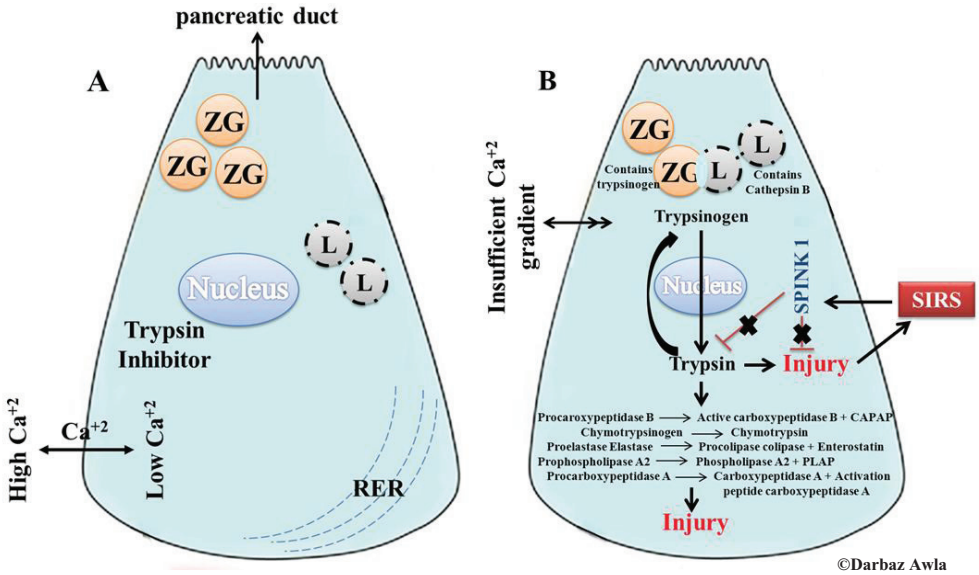

Figure 2: Cellular events in a normal acinar cell (A) and in AP (B), according to the "co-localization" theory.

are mandatory to substantiate this possibility and its influence in the progression to AP. Trypsinogen can be activated by enterokinase, trypsin and cathepsin B.

The exact mechanism how intra-cellular trypsinogen activation occurs is obscure but several possibilities have been proposed to explain this premature trypsinogen activation in AP which include: 1) auto-activation of trypsinogen to trypsin which is more evidenced *in vitro* since it is optimal in pH 5 and *in vivo* is deactivated by natural protease inhibitors [66, 67], 2) lysosomal hydrolase cathepsin B cleaves trypsinogen into trypsin when ZGs converge with lysosomes containing cathepsin B, in a process called co-localization. This cathepsin B is capable of activating trypsinogen, however, cathepsin B and trypsinogen have been observed within the same subcellular compartment [68, 69] and when Halangk and his co-workers induced AP in cathepsin B-deficient mice there was only 80% reduction in trypsin activity compared to wild-type mice [69]. Furthermore, even recently it has been demonstrated that cathepsin B has substantial ability to activate trypsinogen at neutral pH; its ability for such activation is optimal at pH around 5, putting in consideration that pH at the co-localization surrounding is neutral. So by these observations the window will stay open for discussion and there might be other possibilities and pathways explaining trypsinogen activation apart from cathepsin B, 3) failure of circulating and intra-cellular trypsin inhibitors such as serine protease inhibitor Kazal type 1 (SPINK1). It has been shown that a defect in the gene controlling SPINK 1 is associated with increased risk of repeated attacks of AP [70-73], but the role of trypsin inhibitors present in blood such as α-1 protease inhibitor (α-1PI) and α-2 macroglobulin is vague, 4) proteolytic activation occurs after leakage of lysosomal enzymes into

the cytoplasm [74], 5) establishment of a shunt between intra-cellular space and membrane-bound compartments which enables the zymogens to pass through these compartments [75], 6) uptake and processing of secreted zymogens by endocytic pathways [76] and 7) the zymogen becomes more susceptible to proteolysis by oxidation and decondensation reactions [77]. The first three mechanisms have received more attention. The role of Ca^{2+} in the mechanistic activation of trypsinogen has been thoroughly studied in the recent years [78] and we will discuss it in the consequent text of this thesis.

The question of whether trypsinogen activation and trypsin are prerequisites for AP has been the subject of controversy and remained an unsolved issue in pancreatology to date [79]. In a study Hartwig *et al.*; showed that exogenous trypsin can up-regulate both soluble and membrane bound inter-cellular adhesion molecule-1 (ICAM-1) on pancreatic endothelial cells and thus contribute to leukocyte migration to the pancreas in AP [35]. Also a couple of published studies showed that co-localization of zymogens can trigger further trypsinogen activation and subsequent leukocyte infiltration [80-82], still these studies implicated the use of chemical trypsin inhibitors or more recently, using adenoviral mediated gene expression techniques [83-85]. A recent published study used trypsin-7 knock-out mice, which in this study is considered as the cationic trypsin and the main trypsin isoform in mice and human, with the deletion of this major trypsin isoform there was partial but significant reduction in tissue damage in AP but inflammation and leukocyte recruitment remained intact both locally and systemically [86]. Once trypsin has been activated however, its inhibition does not influence the course of AP because other proteases such as elastase, lipase, chymotrypsin and phospholipase A_2 which are activated by trypsin; cause the subsequent cell damage [87]. There is mounting evidence that although trypsinogen activation to trypsin initiates the events, trypsin *per se* exerts least direct harmful effects to the pancreas and it has been reported that on molecular basis the proteases activated by trypsin are more potent in damaging acinar cells when compared to trypsin [88].

It is not easy to measure the direct trypsin activity which is why most studies (including the studies in this thesis), use indirect indicators of trypsin activity such as α-1PI-complex and TAP (analyzed in the studies included in this thesis) to track trypsinogen activation and trypsin activity. It has been shown that urinary TAP concentration is a more sensitive marker than serum amylase and lipase concentrations for predicting the severity in AP [89], as up to 20% of patients with AP may have normal serum pancreatic enzyme concentrations [90], thus urinary TAP represents a good alternative tool for clinicians. However, TAP requires a laborious expensive enzyme linked immunosorbent (ELISA) method performed by skilled laboratory personnel, which takes several hours and not available in every hospital.

The role Calcium in AP

There are substantial direct and indirect evidences that Ca^{2+} is involved in the pathophysiology of AP. The endocrine situations resulting in hypercalcemia are predisposed to AP, thus establishing a link between AP and hypercalcemia. The AP developing in patients on extracorporeal blood circulation maintenance during major cardiac operations seems to be due hypercalcemia [91]. The role of Ca^{2+} in AP is further supported by the observation that disrupted Ca^{2+} signaling inside the acinar cells and a sustained rise in intra-cellular Ca^{2+} in experimental models of AP has been evidenced in numerous studies [78, 92-94].

Nowadays it is well-described that Ca^{2+} controls secretion of proteases regardless

whether it is a normal protease secretion or pathological secretion of zymogen proteases. That's why it is of particular importance to differentiate between transient physiological rises in cytosolic Ca^{2+} and sustained rise and export from extracellular spaces.

The intra-cytosolic resting Ca^{2+} concentration is 10^{-7} M, which is much lower compared to extracellular fluid (10^{-3} M) and intra-cellular stores (10^{-4} M) [95]. These Ca^{2+} concentration discrepancies enable the cell to increase Ca^{2+} levels in its different regions.

In normal resting pancreatic acinar cells, by the effect of neurotransmitter Ach and hormone CCK, Ca^{2+} controls secretion of acinar enzymes, encoded within apically located ZGs. This has been shown by using physiological doses of Ach and CCK which aid in short-lasting Ca^{2+} oscillations between the ZGs and intra-cellular cytosolic space (calcium spikes) [92, 94]. However, high concentrations of Ach, CCK and various stimuli causing AP result in a global, toxic and abnormal rise in cytosolic Ca^{2+} resulting in the crucial step of intra-cellular protease activation and initiating the events leading to AP [7, 93, 96]. Recently has been reported that the pathological rise is not necessarily sustained but sufficient enough to result in intra-cellular trypsinogen activation [97].

Apart from the direct detergent effect, it has been reported that transporter-mediated bile acid uptake causes Ca^{2+}-dependent cell death in rat isolated pancreatic acinar cells [98]. Bile acids exert their primary effect by releasing Ca^{2+} from both the RER and acid stores in the apical granular region through activation of 1,4,5 inositol triphosphate (IP3) and ryanodine receptors (intra-cellular calcium channels) [98, 99], resulting in either apoptosis or necrosis. The intra-cellular ATP level plays a crucial role in determining which type of cell death occurs [100].

Inflammation

Inflammation is a characteristic feature of AP. The inflammatory events that occur subsequent to acinar cell stimulation and intra-cellular trypsinogen activation are believed to determine the severity of AP. Accumulating data in the literature have demonstrated the cardinal role of inflammatory cells in tissue damage and systemic manifestations in AP [33, 34, 101]. In contrast to trypsinogen activation, pancreatic inflammatory cell accumulation has been regarded to occur late and not early during the course of AP [102]. Although we could not detect significant rise in inflammatory cell infiltration neither in cerulein- nor in taurocholate-induced AP before 6 h post-pancreatitis induction [103, 104], Mayerle and his colleges reported inflammatory cell infiltration as early as 1 h after the start of secretagogue-induced AP [105]. Generally the process of inflammation in the pancreas includes changes in the microvascular system such as vasodilation, plasma leakage and leukocyte recruitment. Microvascular endothelium cell linings play a crucial role in mediating leukocyte recruitment.

Leukocyte recruitment

Leukocyte (also spelled as Leucocyte) is a Greek word that means white. It stands for white blood cells. Leukocytes comprise different types including: neutrophils (polymorphonuclear leukocytes; PMNLs), band cells (slightly immature neutrophils), T-lymphocytes (T-cells), B-lymphocytes (B-cells), monocytes, eosinophils and basophils. Upon activation, leukocytes release proteolytic enzymes (for example elastases) and are capable to degrade various tissues. Once leukocytes migrated into inflamed tissue can release both pro-inflammatory and inflammatory cytokines and further induce formation of free radicals (for example reactive oxygen species (ROS)) of importance for

aggravation, tissue destruction and different phases of AP [106].

Among inflammatory cells, PMNLs represent the first responders concerning migration into the site of inflammation. Leukocyte recruitment from the circulation to the inflamed tissue is a complex and well-described multi-step cascade including at least 4 steps which have been thoroughly studied during the past two decades and a paradigm formulated that can be found in most classical immunology and pathology textbooks (figure 3). These inter-dependent steps are: leukocyte tethering, rolling, adhesion and transmigration. There are emerging concepts that add additional steps in this cascade, including intraluminal crawling, to be responsible for bridging adhesion to crawling. All of these steps are characterized by the close interaction between leukocytes in one hand and microvascular endothelial cells on the other hand and are strongly governed by specific adhesion molecules expressed on the surface of both cell types. These include the major adhesion molecule families of selectins, integrins and the immunoglobulin superfamily [107, 108].

Leukocyte rolling

Locally produced chemokines under various stimuli and conditions activate leukocytes inside the microvasculature. Leukocytes, then initiate the multistep recruitment cascade by tethering (initial attachment) and rolling. Leukocytes are considered to be rolling when they slow down their velocity approximately 50 times compared to the base-line. Many pro-inflammatory mediators play a cardinal role in initiating the leukocyte rolling step such as histamine, thrombin and oxygen free radicals [109, 110]. The rolling of leukocytes is mediated by selectins which are a family of calcium-dependent type I transmembrane glycoproteins which constitute three closely related members namely E-selectin (ELAM-1, CD62E) and P-selectin (PADGEM, CD62P) on venular endothelium and L-selectin (LAM-1, CD62L) on many leukocyte subsets [111, 112]. All selectins have a unique structure with an extracellular region composed of N-terminal lectin domain, an epidermal growth factor (EGF)-linked domain, 2-9 short consensus repeat units homologous to domains found in complement binding proteins, a single transmembrane region and a cytoplasmic region [110]. Although leukocyte adhesion is preceded by leukocyte rolling in most cases, it has been shown that leukocyte rolling is not always a prerequisite for subsequent adhesion and leukocyte recruitment, as for example, in liver sinusoids, leukocytes adhere without preceding rolling due to sinusoidal narrow lumens [113]. The same observations have been reported in the lung [114]. We have shown that p-selectin is of particular importance in AP (accepted data for publication).

Leukocyte adhesion

After successful rolling of leukocytes, next step in leukocyte recruitment is firm adhesion to the activated microvascular endothelial lining. The effect of chemokines released by inflamed tissue endothelial cells starts activation. The interaction between endothelial selectins and leukocytic P-selectin glycoprotein ligand-1 (PSGL-1) on the one hand and chemokine receptors on the activated leukocytes and secreted chemokines on the other initiate up-regulation of a group of heterodimeric adhesion molecules and mediate firm adhesion of leukocytes to the activated microvascular endothelium [107].

The adhesion molecules referred to as β2-integrins as they share a β-subunit (CD18) but have different α-subunits (CD11a-d). Integrins such as lymphocyte function antigen-1 (LFA-1, CD11a), macrophage-1 antigen (Mac-1, CD11b), p150,95 (CD11c) and less abundant αdβ2 (CD11d) [107] are expressed on the leukocyte surface. Upon activation they

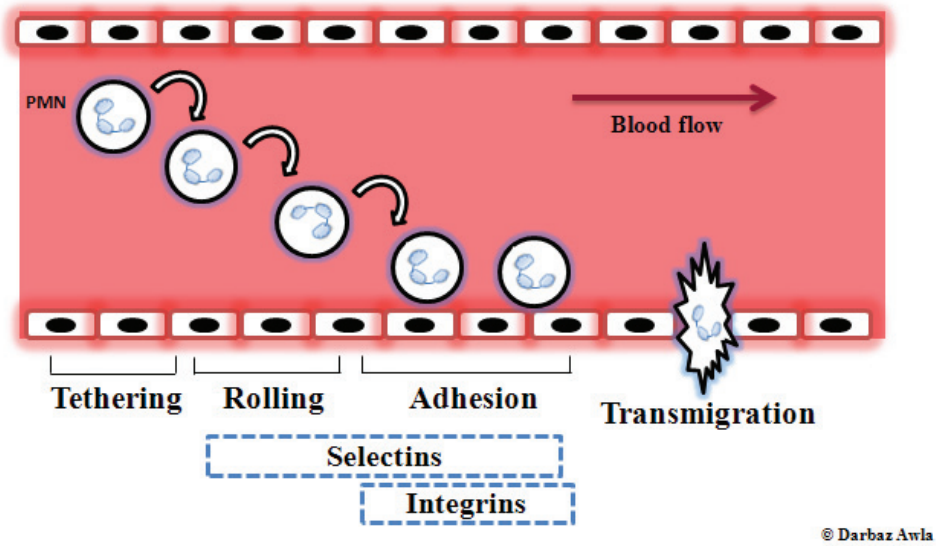

Figure 3: The leukocyte recruitment cascade. Leukocyte recruitment is a multi-step process, which comprises leukocyte tethering/rolling, firm adhesion and transendothelial migration.

will bind to their ligands on the microvascular endothelial cell lining and thus enhancing capture of rolling leukocytes. These ligands are transmembrane glycoproteins and members of the immunoglobulin superfamily namely ICAMs (ICAM-1 - ICAM-5), vascular cell adhesion molecule-1 (VCAM-1) and junctional adhesion molecules (JAMs) [107]. ICAM-1 is constitutively expressed on venular endothelium, it is up-regulated upon stimulation by pro-inflammatory cytokines [115] as well as trypsin [35] and binds to LFA-1. Interestingly, it has been shown that ICAM-1 may play a role in leukocyte recruitment and tissue damage in cerulein-induced AP [101].

LFA-1

LFA-1 is constitutively expressed on leukocytes. Once leukocytes become activated the already existing LFA-1 changes its avidity from low avidity (inactive) state to high avidity (active) state rather than increase in its surface expression. The relevance of the effect of LFA-1 in clinical practice is forwarded by the functional deficiency of LFA-1 in a rare autosomal recessive disorder known as Leukocyte Adhesion Deficiency-1 syndrome (LAD-1). In this situation failure of leukocytes to change their expression avidity to high avidity phase results in failure of adherence of rolling leukocytes and as a compensatory mechanism, patients have more leukocytes in the circulation. Accumulating body in the literature have documented the role of LFA-1 in leukocyte recruitment and organ damage under various inflammatory conditions like septic liver injury [116], ischemia-reperfusion colonic injury [117], cholestatic liver damage [118], viral hepatitis [119] as well as in alcoholic liver disease [120] and graft-versus host disease [121]. However, the potential role of LFA-1 in leukocyte recruitment and subsequent tissue damage in AP remains elusive.

Based on the above considerations we have studied the role of LFA-1 in leukocyte recruitment and tissue damage in AP which is included in this thesis.

Transmigration:

The process of leukocyte recruitment ends with the passage of adhered leukocytes into the inflamed tissue. Different paracellular pathways through which leukocytes end up in the inflamed tissue have been described, including passing the endothelial cells either by transcytotic migration or through a pre-existing holes in endothelial cells [122]. However the well-described paradigm implicates intrinsic capability of leukocytes to adapt their shapes with help from pseudopod-like extensions. This transfer necessitates the use of many endothelial cell adhesion molecules where PECAM-1 has been shown to play a great role [123].

Chemokines

The activation and navigation of leukocytes to the site of inflammation is orchestrated by at least 40 recognized members of small (~8-14 kDa) and structurally related members of the cytokine family with chemotactic activities called chemokines [124]. They exert their effects by interaction with seven transmembrane G protein-coupled receptors. According to the new classification system, which depends on the structural arrangement of the N-terminal cysteine residues, chemokines are divided into two main groups, CC and CXC chemokines. If there was an amino acid between the first two cysteine residues they are classified under CXC chemokines, however if the 2 terminal cysteine residues are adjacent without any amino acid separating them they are classified as CC chemokines. Two other less abundant groups of chemokines have been described which are C or SCYc and CX3C or SCYd. The C chemokine doesn't possess the cysteines one and three of the typical chemokine structure [125]. The fourth group, CX3C, is the only membrane-bound chemokine and has three aminoacids between the first two cysteines [126]. The CC chemokines such as monocyte chemotactic protein-1 (MCP-1) mainly activate monocytes while CXC chemokines such as macrophage inflammatory protein-2 (CXCL2/MIP-2) tend to activate neutrophils to a higher extent.

CXCL2/MIP-2

CXCL2 is the murine homologue of human interleukin-8 (IL-8). The 27 amino acid mature mouse CXCL2 is formed by cleavage from around 100 amino acid residue precursor proteins encoded in the mouse CXCL2 cDNA. Mouse CXCL2 exhibits a great similarity to the mouse CXCL1. A recent study has reported that pancreatic acinar cells can serve as a source of CXCL2 during the course of AP [127] and numerous studies forwarded the positive and effective role of CXCL2 in the initiation of inflammation and tissue damage in AP [127-130]. In the studies included in this thesis we have selected CXCL2 as the representative member of the chemokine family, as a previous study has shown that CXCL2 seems to play an important role in AP [128].

Signaling pathways

During the last decade, the abundant role of intra-cellular pathways mediated by small GTP-binding proteins has been highlighted. Their importance in different pathological conditions such as cancers, atherosclerosis and inflammatory conditions such as sepsis has been studied [131]. However, not much is known about the impact of different intra-cellular pathways in AP.

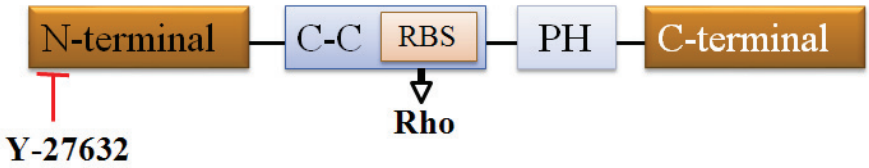

Figure 4: Structure of ROCK and inhibitory site of Y-27632.

Rho-kinase

Rho GTPase family of proteins is considered as the most important member of the Rho-kinase group, includes Rac, Rho and Cdc42. Under basal conditions the proteins of the Rho GTPase family exist in an inactive GDP-bound form whereas upon activation they undergo phosphorylation and become bound to GTP (GTP-bound) [132].

Rho-kinases (ROCKs) are considered as the most abundantly studied and recognizable member of the Rho family. Two isoforms of the Rho-kinase have been described with a great similarity, ROCK-I and ROCK-II. Rho-kinase is exclusively expressed in almost all human, rat and mouse tissues; however, ROCK-I is abundant in the liver, testis and kidney whereas ROCK-II is mainly expressed in the brain and striated muscles. The structural composition of ROCKs includes an N-terminal catalytic kinase domain, central coiled-coil domain (C-C) which has a Rho-binding site (RBD), a C-terminal pleckstrin homology (PH) domain and a C-terminal cysteine-rich domain (CRD) (figure 4).

ROCK-I and ROCK-II are very similar in the N-terminal domain [133] and to a lesser extent in the C-terminal coiled-coil and pleckstrin domains [133]. C-terminal coiled-coil acts as a Rho-binding site after activation while ROCK inhibitors such as fasudil and Y-27632 mediate their inhibitory effect by binding to the N-terminal domain. Y-27632 is highly potent, cell-permeable, selective inhibitor of Rho-associated protein kinase. It inhibits both ROCK-I and ROCK-II with similar potency. Inhibitory effect is achieved by competing with ATP for binding to the N-terminal catalytic site [134].

Since this specific intra-cellular pathway is closely related with angiotensin-II, platelet derived growth factor and thrombin, the role of Rho pathway has been first studied in cardiovascular diseases [135]. Among others, it was shown that inhibition of Rho-kinase significantly augmented the outcome of obstructive ischemic stroke [136, 137]. More recently fasudil has been passed the clinical trial and now is commercially available as an effective drug in pulmonary hypertension to prevent cardiovascular complications. Growing body in the literature has showed an effective role of Rho-kinase in inflammatory processes with primary focus on chemokine expression and leukocyte-endothelial interaction in colonic ischemia reperfusion and lipopolysaccharide (LPS)-induced platelet capture to the endothelium [138-141]. It has been demonstrated that Rho-kinase signaling regulates cytoskeleton organization [142], cell adhesion and migration [138], ROS formation [143], oncogenic transformation [144] and tissue fibrosis [145]. It has been shown also that interference with Rho-kinase signaling may ameliorate the course or decrease the severity in obstructive cholestasis [146], cerebral ischemia [147],

intestinal ischemia [148] and pulmonary hyperten- sion [149]. However the potential role of Rho-kinase signaling in AP is not known. Thus, based on the above considerations, we hypothesized that Rho-kinase signaling blockade could be an interesting target to treat patients with AP.

Toll-like receptors (TLRs)

The immune system is composed of two closely related systems, referred to as the innate and adaptive immune systems. The action of adaptive immune system is standing on the response to specific antigens and establishes an immunological memory. However, the innate immune system is responsible for an immediate first line host defense against different microbial pathogens. The hall-marks of the innate immune system are known as pattern recognition molecular receptors (PRRs) that act as sentinels of the cell and function in recognition of danger signals from molecular structures which are broadly shared by pathogens known as pathogen-associated molecular patterns (PAMPs) [150]. A growing body in the literature indicates the role of PRRs in recognition of endogenous substances released from dying host cells known as damage-associated molecular patterns (DAMPs) [151] such as heat shock proteins, which may play a role in AP [152].

TLRs were the first PRRs identified; they recognize various PAMPs and DAMPs [153-156]. Toll word is the German slang which means fantastic. The Toll gene of *Drosophilia* was discovered and found to be involved in embryonic development in 1985 by the German biologist Christiane Nüsslein-Volhard, who later awarded Nobel Prize in Physiology and Medicine in 1995 for this discovery [157, 158]. After that, Jules Hoffmann in 1996 discovered that Toll gene-mutant fruit flies infected with bacteria or fungi died because they could not mount an effective defense. Two years later in 1998, Bruce Beutler discovered that mutant-mice in a gene similar to the Toll gene of the fruit fly are resistant to LPS. This TLR was turned out to be the elusive LPS receptor and nowadays is known as TLR4 [159]. The discoveries of Hoffmann and Beutler triggered an explosion of research in innate immunity and they awarded Nobel Prize in Physiology and Medicine in October 3, 2011.

Nowadays the TLR family expanded to include 11 mammalian and 13 murine members. TLRs are type I transmembrane glycoproteins with molecular weight ranging from 90-115 kDa, they are structurally characterized by extracellular leucine-rich repeats (LPRs) and Toll/IL-1 receptor (TIR) signaling domains [160, 161]. TLRs exhibit different expression patterns and localization as for example; some of the TLRs including TLR2 and TLR4 are expressed on the surface of the cells while other members of the TLR family (TLR3, TLR7, TLR8 and TLR9) are expressed within intra-cellular vesicles and aid in recognition of nucleic acids [162], TLR11 is expressed on both the cell surface and in intra-cellular compartments [163]. TLR family members are expressed in innate immune cells such as PMNLs, adaptive immunity cells such as T- and B-lymphocytes, which is why they implicate a wide-range impact on both adaptive and innate immune systems, and other cells such as epithelial and endothelial cells [164]. This wide-range distribution makes them an attractive target in several disease modalities.

Once PAMPs and DAMPs have been recognized by TLRs they initiate a series of signaling cascades that compromise a defensive mechanism by the host tissue [165]. Up to date we can summarize functions of TLRs in 5 points: 1) first line of host defense as in the respiratory system, 2) induce secretion of chemokines and expression of adhesion molecules such as E-selectin and ICAM-1 making them

important molecules in the mediation of leukocyte rolling and adhesion, 3) increase phagocytic activity, 4) regulate the differentiation and maturation of immune cells and 5) tissue regeneration after injury as in the liver [166, 167].

TLR4 is mainly present on myeloid cells (monocytes, macrophages and myeloid dendritic cells), mast cells, NK cells, T- and B- lymphocytes, endothelial and epithelial cells, keratinocytes, fibroblasts and even tumor cells. TLR4 has been shown to be the receptor for LPS, paclitaxel, endogenous heat shock proteins, fibronectin and heparin sulfate [168].

TLR2 is demonstrated to recognize various danger signals from viruses, bacteria, parasites and fungi. It can make complexes with TLR1, TLR2 or non-TLR molecules such as CD36 to discriminate the molecular structure of various ligands from the above mentioned microbes [153].

It has been demonstrated that trypsin and trypsin substrates such as elastase can activate TLR4 and produce a SIRS-like reaction in mice [169]. Furthermore, TLR4-deficient mice are associated with better survival when challenged with i.p. LPS-free pancreatic elastase compared to wild-type mice [159].

The literature concerning the role of TLR2 and TLR4 in AP is rather complex and partly contradictory. There is a study showing that TLR4 plays a protective role in AP [170]; however this is contradictory [171]. Moreover, a recent published study reported that TLR4 polymorphisms are not associated with occurrence or severity of AP [172].

Interestingly, a study has reported that polymorphism in the TLR2 gene in human is associated with increased susceptibility and severity of AP [173]. However, the potential functional role of TLR2 in AP remains elusive.

Nuclear Factor of Activated T-cells (NFAT)

NFAT was originally described as an inducible factor with the capability to bind the distal antigen receptor response elements of the human interleukin-2 (IL-2) promoter [174, 175]. Since their discovery more than twenty years ago [175], an increasing body in the literature has shown that NFAT transcription factors are operative not only in T-cells but also control critical processes in many vertebrate developmental systems [176-179]. The NFAT family consists of five recognized members (NFATc1-c4 and NFAT5), of which all are related to the Rel/nuclear factor kappa B (NF-kB) [180]. Adding to the complexity of this system, NFAT family members may have more than one isoform. For example, there are three protein isoforms of NFATc1: A, B, and C, which are driven from two different promoters and may have different biologic activities [181]. NFATc1 - c4 are completely dependent on calcium/calcineurin for their activation. Although NFAT5 is similar to other NFAT isoforms in composition and function, it does not require calcium/calcineurin for activation.

The NFATs consist of a regulatory N-terminal domain and a Rel homology C-terminus domain [181]. The regulatory domain is a unique characteristic of the NFAT and composed of around 300 amino acid regions encoded by a single exon [174, 181]. Under basal conditions the regulatory domain is heavily phosphorylated and in an inactive form in the cytoplasm. When there is increased intracellular calcium such as in AP, the serine/threonine phosphatase calcineurin is activated and binds to NFAT removing the calcium and thus translocating NFAT into the nucleus where it is activated by binding to importins and through the nuclear core complexes [182-184]. A number of associated proteins have been described to phosphorylate NFAT in various cell types

including c-Jun terminal kinase 2 (JNK2), casein kinase 1, protein kinase A (PKA) and glycogen synthase kinase-3 (GSK-3) [178, 185]. Inside the nucleus, active NFAT will bind with a number of partner proteins to maintain significant DNA-binding and transcriptional activity and thus enhance transcription of pro-inflammatory proteins such as IL-2 and tumor necrosis factor-α (TNFα) [186].

What has been mentioned above provides us with the notion that calcium is of particular important in activation of NFAT. In immune cells transient intra-cellular calcium elevation is not sufficient to cause nuclear accumulation of the NFAT, instead a sustained rise in calcium is mandatory [187]. However in some cells outside the immune system, for example in hippocampal neurons, NFATc4 is activated by increased extracellular K^+ causing depolarization by spontaneous synaptic activity [188]. NFAT has low DNA-binding affinity *per se* but association with the partner proteins enhances its DNA binding [185]. Due to engagement of NFAT in development, almost all cells express at least one NFAT isoform [176, 189]. In the pancreas, we have shown that both NFATc1 and NFATc3 are expressed in pancreatic acinar cells and tissue.

Both fungal metabolites cyclosporin A (CsA) and FK506 (tacrolimus) have been used to inhibit NFAT. They exert their inhibitory effect through forming complexes with cyclophilin and FK506-binding proteins, respectively [190]. These immunosuppressive drugs which are used in the clinical practice to prevent graft-versus host disease and autoimmune diseases [191], they block calcineurin making them not selective for NFAT. A novel NFAT blocker which is used in the study included in this thesis is a bis(trifluoromethyl)pyrazole (BTP) derivative is called A-285222. A-285222 exerts its inhibitory effect without interference with calcineurin; instead it blocks dephosphorylation of NFAT and keeps it in an inactive form in the cytosol without cytokine transcriptional activity [192-194].

Matrix Metalloproteinases (MMPs)

MMPs, also known as matrixins, constitute 23 members of structurally related zinc- and calcium-dependent endopeptidase family that function in the breakdown of extracellular matrix (ECM) and belong to the metzincin superfamily [195-197]. They can also process a large number of non-ECM proteins such as growth factors, cytokines, chemokines, cell receptors, serine proteinase inhibitors and other MMPs [198]. The MMP family is composed of 23 members in humans and 24 in mouse of structurally similar to a great extent. In human 24 genes control MMPs, as MMP-1 is encoded by 2 genes on chromosome 1. The first MMP identified in tadpole tails in 1962 and it was functioning in degradation of fibrillar collagen during metamorphosis [199].

Under resting conditions, MMP mRNA is expressed in a low level; however transcription is enhanced by various stimuli such as cytokines and growth factors [200, 201]. The synthesized inactive MMPs are found either within the ECM or anchored to the surface of the cells [200]. Under normal conditions MMPs activity is controlled by a family of endogenous MMP inhibitors referred to as tissue inhibitors of metalloproteinases (TIMPs). TIMPs are constituted of 4 members and are capable in regulating the action of all MMPs [202-204]. Normally MMPs function in tissue remodeling, embryogenesis, angiogenesis, cell adhesion and proliferation and wound healing [205]. Dysregulation of MMP activity is associated with diseases such as multiple sclerosis, periodontal diseases and cancer [206, 207] and inflammatory diseases such as arthritis and chronic obstructive pulmonary disease [208, 209].

There are different classifications of MMP family as for example according to their domain structure [210], however the most widely used is based on substrate-specificity, in which they are classified into 4 classes: the collagenases (MMP-1, -8 and -13), the gelatinases (MMP-2 and MMP-9), the stromelysins (MMP-3, -10 and -11), matrilysin (MMP-7), metallo-elastase (MMP-12), enamelysin (MMP-20), endometase (MMP-26) and eiplysin (MMP-28) [211, 212]. They are also classified based on cellular and tissue localization and membrane binding and regulation. In this context it should be mentioned that some studies reported that different MMPs are localized intra-cellular and process intra-cellular substrates such as troponin 1 [213], αB-crystallin [214] and lens βB1 crystallin [215].

Exploiting the advantage that MMPs have Zinc ion and various substrate-binding sites, a number of MMP inhibitor generations have been designed and used in the past time. The first generation, including batimastat (BB-94) which is used in our study included in this thesis, is composed of small-molecules with high similarity to the naturally-occurring MMP substrates. These are able to inhibit all MMPs at low concentrations by binding to the hydroxamic acid zinc-binding group to chelate the catalytic zinc ion and thus inactivate the protein [216]. The second and third generations of MMP inhibitors were no more based on MMP substrate; instead they were designed with peptidomimetic and non-peptidomimetic structures and hold a higher selectivity against specific MMPs [217].

MMP-9

MMP-9 (also referred to as gelatinase B, 92 kDa type IV collagenase and type V collagenase). MMP-9 is expressed in 92 kDa pro-enzyme form and upon stimulation changed to smaller 83 kDa active form. MMP-9 can degrade ECM with high specificity for denaturated collagen (gelatin), it can also cleave a number of non-ECM substances and either increase or decrease their activity (depending on the site of cleavage) such as IL-1β, IL-8, connective tissue activating peptide III, platelet factor-4, substance P and etc. [218, 219]. MMP-9 was first discovered in 1974 in neutrophils [220], but nowadays evidence has been established that MMP-9 is produced by various other cell types including monocytes, macrophages, astrocytes, fibroblasts, osteoclasts, chondrocytes, keratinocytes, endothelial and epithelial cells. However, its constitutive presence is only in neutrophil granules and rapidly secreted after stimulation [221], whereas MMP-9 is inducible in other cell types [222]. Neutrophil-derived MMP-9 forms a covalent complex with neutrophil gelatinase B-associated lipocalin and thus can be distinguished from MMP-9 of other sources than neutrophils [223]. MMP-9 is mainly inhibited by TIMP-1 [224]. MMP-9 is capable of processing chemokines and cytokines, cleaving IL-1β and transforming growth factor β [225]. A recent study published by Vaisar *et al.* reported that MMP-9 sheds β2-integrin (CD18) from macrophages [226].

Due to lack of selective inhibitors, mechanistic studies involving the specific roles of MMP-9 in diseases is restricted on using MMP-9 gene deficient mice. By using these mice, it has been reported that MMP-9 plays a crucial role in cell migration such as recruitment of inflammatory cells to the site of inflammation [227] and migration of embryo to the site of endometrial implantation, that's why MMP-9 deficient mice are associated with low fertility rate [228]. Lack of MMP-9 *in vivo* also results in impaired osteoclast migration and defective angiogenesis [218, 229].

Aims

1) To better understand the basic mechanisms behind the pathophysiology of AP.
2) To investigate the role of TLR2 and TLR4 in severe AP.
3) To study the impact of LFA-1 in leukocyte recruitment and its consequences in the course of severe AP.
4) To analyze the role of MMP-9 in neutrophil-dependent trypsinogen activation in severe AP.
5) To define the role of Rho-kinase signaling in severe AP.
6) To examine the role of NFAT and specifically NFATc3 in trypsinogen activation and leukocyte recruitment in severe AP.

Materials and Methods

Animals

All studies have been performed in mice. The male mice 6-8 weeks (22-26 g) of the following strains have been used in the studies included in this thesis: C57BL/6 (I, II, III and IV), Balb/c (V), TLR2- and TLR4-deficient (I), LFA-1-deficient (II), MMP-9-deficient (III), NFAT-luciferase (NFAT-luc), NFATc3-heterozygous and NFATc3-deficient (V). They were either breaded from our own breading facilities or purchased from Jax laboratories. Animals were maintained on a 12-hour light/dark cycle and fed water *ad libitum*. The accommodation period was 1 week before experimentation. All experiments were done in accordance with legislation on the protection of animals and were approved by the Regional Ethical Committee at Lund University, Sweden.

Experimental design

Studies investigating the mechanism of AP demand animal models because clinical material is generally not available during the early phase of the disease. Furthermore there is no clear-cut marker to identify those patients with a severe form and differentiate them from mild pancreatitis cases early in the disease [7]. A number of pancreatitis models have been used for studying AP (bile salt-pancreatitis, cerulein, closed duodenal loop, L-arginine and etc.). Among experimental AP models bile salt-induced pancreatitis is preferred over other models because it is a reproducible model, represents a model of severe AP and, the most important, it reflects clinical pancreatitis in forms of etiology, complications and inflammation [230]. The primary idea of biliary pancreatitis was raised by Opie at the beginning of 20th century when he reported two separate cases of AP [13]. In these two cases he proposed 2 different potential mechanisms for AP. Based on the fact that the pressure is 3-4 times higher inside the pancreatic duct compared to the common bile duct under normal conditions, he discussed two different mechanisms as the proposal. In the first theory, the common channel theory, Opie suggested that the gall bladder stone or sludge could obstruct the distal common biliopancreatic duct making a common channel behind it, through which the bile might retrogradely reflux into the pancreatic duct. In the second theory, duct obstruction theory, he proposed that the stone obstructs the pancreatic duct and prevents the outflow of pancreatic juice and subsequently pancreatic ductal hypertension. In this context it should be mentioned that until now the confirmation that pancreatic ductal hypertension without bile reflux resulting in pancreatitis is deficient and limited exclusively to studies using the American opossum [14]. Based on the first theory, Opie was also the first to induce pancreatitis by injecting bile into the pancreatic duct in dogs. However, failure to reproduce bile-pancreatitis because of the frequent presence of an accessory pancreatic duct in dogs pushed the researchers to use retrograde bile salt pancreatitis method in rats, which was first performed during the seventies. Lately, for the aim of reducing experimental costs and after development of genetically modified techniques especially in mice, the researchers turned to perform bile salt pancreatitis in mice. Even though it had been partly used before, Boston pancreatitis group in 2007 thoroughly studied and used the taurocholate pancreatitis induction [231]. The taurocholate-induced pancreatitis is used in the studies included in this thesis. Briefly the duodenum and the attached pancreatic head were exposed through a midline incision. The papilla of Vater was identified, the duodenum was immobilized by two 7-0 prolene sutures and a small puncture was made through the duodenal wall in parallel to the papilla of Vater with a 23G needle. A polyethylene catheter (internal diameter 0.28 mm) connected to a

micro-infusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) was introduced 1 mm into the pancreatic duct.

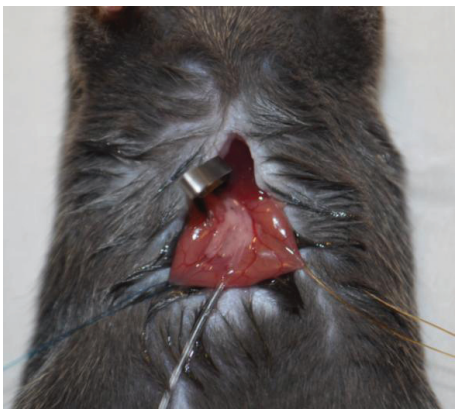

Figure 5: Bile salt pancreatitis induction by retrograde infusion of sodium taurocholate.

The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp to prevent hepatic reflux. Overall 10 µl of either saline or 5% taurocholate (Sigma Chemical Company, St. Louis, Missouri, USA) was infused into the pancreatic duct at a rate of 2 µl/min (figure 5). Before suturing the abdominal wall, the bile duct clip was removed and the duodenal puncture closed (7-0 prolene). Animals were sacrificed 24 h after induction of AP and tissues collected for different assays.

Antibodies and Drugs

Anesthesia was performed by intraperitoneal (i.p.) injection of a mixture of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium). Analgesia was obtained by subcutaneous injection of buprenorfin hydrochloride 0.1 mg/kg (Schering-Plough Corporation, New Jersey, USA). During the experiments, the animals were kept on a heating pad (37ºC).

Immunoneutralization of LFA-1 was obtained by i.p. injection of purified anti-mouse LFA-1 antibody (5 µg/g) prior to induction of pancreatitis (clone M17/4, rat IgG2a, eBioscience, San Diego, CA, USA). The control group received a control antibody (5 µg/g, rat IgG2a, eBioscience, San Dieogo, CA, USA).

MMP inhibition was achieved by i.p. injection of Batimastat (BB-94) (Calbiochem®, Darmstadt, Germany) (40 mg/kg body weight/dose), administered 48 h, 24 h and just before AP induction.

As described in the fourth paper Rho-kinase signaling was inhibited by i.p. injection of Y-27632 [(R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexaneca-rboxamide] (Calbiochem, San Diego, USA) (0.5 -5 mg/kg), either 30 min before or 2 h after bile duct cannulation.

In the last paper, to study the role of NFAT in AP, we used i.p. injections of a novel NFAT blocker, the derivative of BTP, A-285222 (0.15 mg/kg body weight, administered twice daily for 1 week and in the morning of the AP induction). A-285222 was kindly provided by Abbott Laboratories.

Systemic leukocyte counts

A small volume of blood was taken from the tail vein (I, II and IV) and mixed with Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leukocytes were identified as MNLs and PMNLs in a Burker chamber.

Blood amylase

Amylase was quantified either from blood (I, III, IV and V) or serum (II) with a commercially available assay (Reflotron®, Roche Diagnostics GmbH, Mannheim, Germany).

MPO assay

Frozen pancreatic and lung tissue were pre-weighed and homogenized in one ml mixture (4:1) of PBS and aprotinin 10 000 KIE/ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for one min. The homogenate was centrifuged (15339g, 10 min) and the supernatant was stored at -20°C and the pellet was used for MPO assay as previously described [232]. In brief, the pellet was mixed with one ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 sec, put in water bath 60°C for two h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units per gram tissue in all studies included in this thesis.

CXCL2 levels

Levels of CXCL2 were determined in serum (I, II and III) and pancreatic tissue homogenate (I, II, III, IV and V) by using double-antibody Quantikine enzyme linked immunosorbent assay (ELISA) kits (R & D Systems Europe, Abingdon, UK) using recombinant murine CXCL2 as standard. The minimal detectable protein concentration is less than 0.5 pg/ml.

Histology

Pancreas samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six micrometer sections were stained (haematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated (I, II, III, IV and V) in a blinded manner by use of a pre-existing scoring system including edema, acinar cell necrosis, hemorrhage and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scales as previously described [233].

TAP levels

Trypsinogen is activated to trypsin in a reaction where TAP is cleaved off and thus can be used as a marker of trypsinogen activation [89]. The RIA was performed as described previously [234]. A 0.1 M Tris HCL buffer (pH 7.5) containing 0.15 M NaCl, 0.005 M EDTA and 2 g/l bovine serum albumin (Sigma-aldrich) was used as assay buffer. Samples of 100 µl diluted in assay buffer were incubated (16 h, 4°C) with 200 µl of I^{125}Tyr-TAP (=20 000 counts per min) in assay buffer and 200 µl of antiserum diluted 1/750 in assay buffer. Parallel incubations with the synthetic activation peptides TAP diluted in assay buffer in a series of concentrations from 0.078 to 20 nM, were used as standards in the assays. Free and bound radioactivities were separated by means of a second step antibody precipitation. For this, 100 µl of a cellulose coupled anti-mouse IgG suspension (Sc-Cel® IDA, Boldon, England) was added to the samples. After 30 min of incubation, 1 ml of water was added and tubes were centrifuged (704 g, 5 min, room temperature). The supernatant was decanted and radioactivity of the precipitate was counted in a γ-spectrophotometer (I, II, III, IV and V).

Reverse-Transcription Polymerase Chain Reaction

Total RNA was extracted from the blood samples of the knockout mice and wild type mice using RNeasy Mini-kit (Qiagen Gmbh, Hilden, Germany) and treated with RNease-free DNease (Amersham Pharmacia Biotech AB, Sollentuna, Sweden) to remove potential genomic DNA contaminants according to manufacturer's handbook. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically.

Reverse-transcription polymerase chain reaction (RT-PCR) was performed with Superscript One-Step RT-PCR system (GIBCO BRL Life Technologies, Grand Islands, NY). The RT-PCR profile was 1 cycle of cDNA synthesis at 50°C for 30 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 10 min. After RT-PCR, aliquots of the RT-PCR products were separated on 2% agarose gel containing ethidium bromide and photographed. The primers sequences were as follows: CD11a (f) 5'-AGA TCG AGT CCG GAC CCA CAG-3'; CD11a (r) 5'-GGC AGT GAT AGA GGC CTC CCG-3'. β-actin (f) 5'-ATG TTT GAG ACC TTC AAC ACC-3' β -actin (r) 5'-TCT CCA GGG AGG AAG AGG AT-3'. β-actin served as a house keeping gene to control for the loading amount of cDNA.

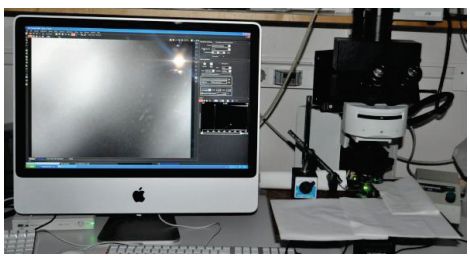

Figure 6: Intravital fluorescence microscopy.

Intravital microscopy

The pancreatic microcirculation was examined by using intravital fluorescence microscopy (II, III). A 5-min equilibration time was allowed before analysis of leukocyte rolling and adhesion was performed in postcapillary venules in the pancreas. Contrast enhancement by intravenous injection of fluorescein isothiocyanate-labelled dextran 150,000 (0.05 ml, 5 mg/ml, Sigma Chemical Co.) and in vivo labelling of leukocytes with rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma Chemical Co.) enabled analysis of leukocyte-endothelium interactions in the microvascular bed. For observations of the microcirculation, we used a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) and recorded videos on a computer for later off-line analysis of leukocyte-endothelium interactions. Several postcapillary venules were evaluated in each animal and leukocyte rolling was measured by counting the number of cells rolling along the endothelial lining during 20 sec and is expressed as cells per minute. Leukocyte adhesion was measured by counting the number of cells that adhered and remained stationary for more than 30 sec during the observation time and is expressed as cells per mm^2. Certain animals received an anti-P-selectin antibody (40 μg, intravenous, clone RB40.34, BD Biosciences Pharmingen) immediately before capturing microphotographs of the postcapillary venules in the pancreas in order to abolish leukocyte rolling and thereby enable visualization of the remaining leukocytes that were firmly adherent to the endothelium.

Trypsin assay

Pancreatic acinar cells were prepared by collagenase digestion and gentle shearing as described previously [235]. Cells were suspended in HEPES-Ringer buffer (pH 7.4) saturated with O_2 and passed through a 150 μm cell strainer (Partec, England). Isolated acinar cells (10^7 cells per well) were preincubated with vehicle, cerulein (100 nM), activated recombinant MMP-9 (R & D Systems) or activated neutrophils or secretions of activated neutrophils from wild-type or MMP-9-deficient mice (37°C, 1 h). The buffer was then discarded and the cells were washed twice with a buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3 (morpholino) propanesulphonic acid (MOPS) and 1 mM $MgSO_4$. The cells were next homogenized in cold (4°C) MOPS buffer using a potter Elvejham-type glass homogenizer. The resulting homogenate was centrifuged (56x *g*, 5 min), and the supernatant was used for assay. Trypsin

activity was measured flourometrically using Boc-Glu-Ala-Arg-MCA as the substrate as described previously [236]. For this purpose, a 200 µl aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM CaCl2 and 0.1% bovine serum albumin (BSA), pH 8.0). The reaction was initiated by the addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin levels (pg/µl) were calculated using a standard curve generated by assaying purified trypsin (IV) or normalized to protein concentration and expressed as relative trypsin units (RTU/pg) (III and IV). Viability of the pancreatic acinar cells was higher than 95% as determined by trypan blue dye exclusion.

Flow cytometry

Flow cytometry was performed in (II) for expression of LFA-1 on leukocytes, (III) for checking the activation of PMNL for Mac-1 expression and (IV) for expression of Mac-1 and CXCR2 on PMNLs. To block Fcg III/II receptors and reduce non-specific labelling, samples were incubated with an anti-CD16/CD32 for 5 min. Then samples were stained with a PE-conjugated anti-Gr-1 (clone RB6-8C5, eBioscience, San Diego, CA, USA) antibody and with a FITC-conjugated anti-Mac-1 (clone M1/70, Integrin α_M chain, rat IgG_{2b}) antibody at 4 °C for 30 min. Cells were recovered following centrifugation then analysed with FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells. After gating the neutrophil population based on forward and side scatter characteristics, Mac-1 expression was determined on cells positive for Gr-1, which is a neutrophil marker.

Luciferase reporter assay

Luciferase activity was measured in pancreas, aorta, lung, and spleen in NFAT-luc mice. Assays were performed as previously described [237] and optical density measured using a Tecan Infinite M200 instrument (Tecan Nordic AB, Mölndal, Sweden) and normalized to protein concentration and expressed as relative luciferase units (RLU) per µg protein (V).

gas chromatography/mass spectrometry (GC/MS)

To measure the concentration of A-285222 in plasma, blood from healthy controls and taurocholate infused mice was collected from the portal vein at the time of euthanasia. Plasma was pooled from 5-6 mice for each experimental condition. Pooled plasma from 5 mice treated with saline was used as a negative control. All samples were run in duplicates in a randomized order. A known concentration (2.5 µM) of the analogous compound A-216491 was added as an internal standard to all plasma samples. Samples (300 µl) were extracted twice with ethyl acetate (400 µl), followed by evaporation. The dried residues were finally redissolved in chloroform (30 µl) for GC/MS analysis. Identification was based on mass spectra and retention indexes, calculated from the injection of a homologous series of n-alkanes. The concentration of A-285222 in plasma was determined using a calibration curve calculated from analyses of plasma from untreated mice, spiked with known concentrations of A-285222 and A-216491.

NFAT isoform expression

Pancreas from young (18-22 days) and adult (5-6 weeks) mice were removed and placed in a physiological saline solution with trypsin inhibitor. To obtain lobules, pancreas was cut in small pieces of 10 mg and washed in PS. Acinar cells were obtained as explained above. For RNA isolation, tissue or cells was placed in RNA Later (Ambion, Applied Biosystems, Madrid, Spain) immediately after

harvesting and RNA was later isolated using Trizol (Invitrogen Life Technologies, Paisley, UK) as previously described (31). Total RNA was further purified using RNeasy Mini Kit (Qiagen, Valencia, CA) with simultaneous DNase treatment (Qiagen, Valencia, CA). For cDNA synthesis, 2 µg of total RNA was used for reverse transcription with the Sensiscript Reverse Transcription Kit (Qiagen, Valencia, CA) with oligo-dT primers according to the manufacturer's instructions. Primer sequences and cycling conditions for NFAT family members were performed as previously described (31), with the following modification of annealing temperatures for mouse samples: NFATc1: 46.5ºC, NFATc2: 50.0ºC, NFATc3: 54.0ºC and NFATc4: 44.0ºC. GAPDH primers used were forward: 5'-TCACCATCTTCCAGGAGCGA-3' and reverse: 5'-CACAATGCCGAAGTGGTCGT-3'. The HotStarTaq Master Mix Kit (Qiagen, Valencia, CA) was used for the PCR reaction and products were analyzed on a 1.5% agarose gel. cDNA from mouse thymus and spleen were used as positive controls.

Confocal Immunofluorescence

Pancreatic acinar cells were seeded on coverslips and stimulated with or without the agonists CCK (10 pM or 10 nM) or ACh (10 µM) for various times as indicated in the text, or with or without the calcium ionophore ionomycin (10 µM, 10 min); each stimuli in the presence or absence of the calcineurin inhibitor CsA (1µM, 30 min). Treatments were done in PS at RT. For measurements of NFATc3 nuclear accumulation, experiments were performed as previously described (31). Briefly, cells were fixed with 4% paraformaldehyde in PBS (15 min, RT), permeabilized with 0.2% TritonX-100 in PBS (15 min, RT) and blocked with 2% BSA in PBS for 2 h. Primary antibody, rabbit polyclonal anti-NFATc3 (1:250, Santa Cruz Biotechnology, Inc) and FITC-labeled secondary antibody, goat anti-rabbit IgG (1:250, Santa Cruz Biotechnology, Inc) were used. Prior staining with the nucleic acid dye PI (1 µg/ml, Molecular Probes, Invitrogen Life Technologies, Paisley, UK) for nuclear identification, cells were treated with ribonuclease A type X-A (Sigma Chemical Company) (30 min, 37ºC) to remove cytosolic RNA and increase the specificity of the PI for nuclear DNA. Cells were examined at x60 magnification using a Bio-Rad MRC 1024ES laser scanning confocal microscope (Bio-Rad Lab, Life Sciences Division, CA, USA). Specificity of immune staining was confirmed by the absence of staining when primary or secondary antibodies were omitted. FITC and PI were excited at 488 nm and the emitted light was collected through 515/30-nm and 605/32-nm bandpass filters, respectively. Multiple fields for each coverslip were acquired and images analyzed using Image J software (NIH, Bethesda, MD, USA). Red fluorescent PI images were used to generate a mask to define the nuclear area. Mean fluorescence intensity of NFATc3 (green) in the nuclear area of each cell was determined and normalized to the mean nuclear NFATc3 fluorescence of the matched control. The number of cells examined and animals used for each experimental condition is indicated in the figure legend.

Statistics

Data are presented either as median (ranges) (I) or mean ± SEM in (II, III, IV and V) papers. Statistical evaluations were performed by using non-parametrical tests (Mann-Whitney) (I, II, III and V) paper and Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons versus control group (Dunnet's method) (IV). $P < 0.05$ was considered significant and n represents the number of animals. SigmaStat® for Windows® version 3.5 software (Systat Software, Chicago, Illinois, USA) was employed.

Results and discussion

Role of TLR2 and TLR4 in AP

We live in a dangerous environment full of pathogenic micro-organisms such as bacteria, fungi, viruses and parasites, they threaten us continuously. Despite external threats, we are vulnerable to endogenous threats rising from major programmed cell death or under various errors in the intrinsic mechanisms regulating the nature of life. We should not worry about our body equilibrium because we have been equipped with complex-regulated systems of the innate and adaptive immunity. The innate immune system is the first line protection, however if the causative agent could pass through this mechanism, we have an adaptive immune to combat the invader and it produces an immunological memory with a more powerful defence upon next attack by the same invader. Although, these two systems are associated with a high accuracy but they also pose a risk. If the activation threshold is too low or if the endogenous stimulus is too strong, a pathological inflammation may follow as in AP. Premature intra-cellular trypsinogen activation followed by microvascular inflammatory cell infiltration represents a hall-mark in the pathophysiology of AP [33, 36]. At the start of this thesis we developed a reproducible experimental model of AP by injecting taurocholate into the pancreatic duct which resembles the most clinically occurring cases of AP [238]. We performed pilot studies to evaluate extent of severity and the degree of inflammation by measuring MPO activity in the pancreas and extent of trypsinogen activation by measuring levels of TAP. We found that taurocholate enhanced leukocyte activation and extravasation into the pancreatic tissue. Furthermore we found that the MPO levels peaked 24 h after pancreatitis induction. So we selected 24 h as the optimum time-point during the course of AP (I, II, III, IV, and V).

Numerous studies have reported that endogenous substances such as heat shock proteins can trigger the innate immune system; these endogenous substances have been elucidated to play a role in AP [152, 239, 240]. In the first study (I), we found that taurocholate challenge enhanced a profound inflammation in the pancreas and there was a protective response by the innate immune system. This response was demonstrated by lower levels of inflammation and tissue damage in TLR4-deficient mice compared to their control littermates in all the studied parameters including pancreatic MPO activity, chemokines, blood amylase levels and microstructure evaluation revealing the extent of tissue damage and degree of inflammation. The literature regarding the role of TLR4 in AP was partly contradictory as a study by Sharif *et al.* reported that TLR4 plays an important role in cerulein-induced AP [170] and this is in line with our study, however a similar study using the same pancreatitis model showed that TLR4 gene-deficient mice were not protected neither from local nor from distant organ damage provoked by pancreatitis. Furthermore; they reported that TLR4-deficient mice showed less damage after co-enhancing systemic inflammation with LPS [171]. Considering this controversy in the literature it was of value to elucidate the role of TLR4 in taurocholate-induced AP. We found also that TLR4-deficient mice were not different from their control littermates in the aspect of trypsinogen activation; this finding provides us with the notion that trypsinogen activation is upstream of TLR4 and trypsinogen activation is independent of TLR4-mediated tissue

damage and leukocyte recruitment in the pancreas.

Next, we found that TLR2 exhibited similar phenotype compared to their wild-type control littermates regarding leukocyte recruitment, tissue damage and trypsinogen activation. This notion provides us with the fact that TLR2 does not play a role in taurocholate-induced AP. In this context it should be mentioned that it has been shown that TLR2 gene polymorphism in human is associated with increased incidence and the degree of severity in AP [173]. However, the mice with taurocholate-pancreatitis were neither protected nor associated with the alteration in the course of severity.

Taken together, we can summarize the results we have observed in the paper (I) in that innate immune system plays a role in AP and its cells such as neutrophils could be of particular importance to examine in AP. Furthermore, targeting TLR4 but not TLR2 could be of great importance to alter the severity of AP, thus TLR4 could be of value as a therapeutic target in AP and finally that trypsinogen activation is independent on TLR4-mediated inflammation in the pancreas during AP.

Role of neutrophils and LFA-1 in AP

During the last decades, several studies with the main focus on inflammation, considered the cardinal role of leukocytes in the pathophysiology of AP [34, 241]. The role of leukocytes may be mediated by their content of inflammatory mediators, capacity to produce numerous elastatses upon activation and mediate tissue aggravation by producing ROS following their recruitment [242]. Even though, the role of leukocytes in the pathophysiology of AP has been addressed clearly, the mechanisms how leukocytes migrate to the pancreas in case of AP have been subjected to intense investigative interest for decades and the specific role of adhesive mechanisms mediating leukocyte-endothelium interaction and subsequent migration remains elusive. Surprising data published by Ryschich *et al.* reported that leukocytes implicate a protective role in AP by trapping into the capillaries and thereby prevent hemorrhage, thus LFA-1 deficient mice are associated with severe course of pancreatitis with high mortality [42]. According to these hypotheses, we started the next study (II) to re-evaluate the role of leukocytes in AP and determine the mechanisms mediating leukocyte-endothelium interactions and more specifically study the role of adhesive molecule LFA-1 in mediating leukocyte adhesion and consequent leukocyte migration in taurocholate-induced AP.

The findings in the second paper provide us with the notion that LFA-1 plays a key role in mediating leukocyte recruitment by regulating firm leukocyte adhesion to the endothelium. Furthermore, the findings of the second paper not only explain the mechanism of leukocyte adhesion in AP but also confirm the role of leukocytes in mediating tissue damage in AP. However trypsinogen activation was independent of LFA-1 mediated leukocyte recruitment in AP.

Interestingly we found that mice lacking LFA-1 exhibited less MPO activity, thus LFA-1 deficient mice exhibit significantly lower levels of pancreatic tissue accumulation of leukocytes. This lower accumulation of leukocytes in the pancreas accounts for the less tissue injury and necrosis in the pancreas. This observation is in line with previous studies, which demonstrated the significant role of leukocytes in AP [33, 34 and 243]. Upon activation, leukocytes change their surface expression of LFA-1 from low avidity to high avidity and then the high avidity

LFA-1 expressing leukocytes combine to their receptor ICAM-1 on endothelium. By using intravital microscopy, we were able to directly visualize the leukocyte-endothelium interactions in the postcapillary venules and address the role of LFA-1 in leukocyte adhesion to the activated endothelium. We observed that taurocholate greatly enhanced the number of leukocytes adhered to the activated endothelium. However, this increase in leukocyte adhesion was greatly reduced in mice lacking LFA-1 or in mice treated with the anti-LFA-1 antibody. Besides that leukocyte adhesion and subsequent recruitment is debilitated in LFA-1 deficient mice, we also found that these mice were significantly protected from pancreatitis-induced tissue damage; however the trypsinogen activation in AP was upstream of LFA-1-mediated leukocyte recruitment. Thus these notions assured us with the fact that leukocytes are of great importance during the course of AP and furthermore; illustrated the mechanisms behind leukocyte adhesion.

Role of MMP-9 in AP

Intra-cellular trypsinogen activation is an early feature of AP; however, trypsinogen activation persists during the later courses of AP even when the stimulating agent is no more pending. It was believed that leukocytes are responsible for this late trypsinogen activation [34] and in a published study neutrophil depletion in mice [33] resulted in a significant reduction in the levels of taurocholate-induced trypsinogen activation in the pancreas. This confirmed earlier evidence that leukocytes play a cardinal role in tissue damage in AP, however no significant role in the levels of trypsinogen activation (II). These findings made us to hypothesize that there are substances released from activated neutrophils floating in the plasma and responsible for this neutrophil-mediated trypsinogen activation. To elucidate this issue, we selected MMPs particularly MMP-9 as possible candidates to examine. For this purpose, first we used a first generation MMP inhibitor with similar inhibiting activity toward all MMPs.

The data of the third paper demonstrate that MMPs mediate neutrophil-dependent trypsinogen activation, formation of CXC chemokines, neutrophil recruitment and tissue damage in AP. Furthermore, the findings of the third paper elucidate that plasma levels of MMP-9 but not MMP-2 are elevated in AP. AP-induced trypsinogen activation, leukocyte recruitment and tissue injury in the pancreas as well as pulmonary accumulation of neutrophils were greatly reduced in mice lacking MMP-9. Moreover, we found that MMP-9 is a potent activator of trypsinogen in acinar cells and that neutrophil-derived MMP-9 is capable of activating trypsinogen in pancreatic acinar cells. Thus, our novel data demonstrates that MMP-9 is an important regulator of neutrophil-dependent trypsinogen activation and may be a useful therapeutic target in AP.

The MMPs are known to control degradation and formation of the ECM, but accumulating data in the literature also implicate MMPs in several features of inflammatory reactions, such as leukocyte migration and cytokine formation [244-247]. Herein, first we used a broad spectrum, first generation MMP inhibitor (BB-94) which significantly reduced tissue damage, leukocyte recruitment and inflammation in the pancreas. This is in line with previous studies demonstrating the role of MMPs in AP [248-255]. Interestingly, we found that the taurocholate-induced pancreatic TAP levels were significantly decreased in BB-94 treated mice by 61%. Furthermore, we found that not MMP-2 but rather MMP-9 is increased in the plasma of mice

challenged with taurocholate. It has been reported that neutrophils contain a substantial amount of MMP-9 in tertiary (secretory) granules, which are the first granules to be mobilized and degranulate upon chemotactic activation of neutrophils [256, 257]. After we confirmed the role of MMPs in AP, next, MMP-9 was selected for investigation of its role in AP and particularly in trypsinogen activation. For this purpose, we used MMP-9 deficient mice challenged with taurocholate. Interestingly, we found that MMP-9 gene deficient mice were protected from taurocholate-induced tissue injury and leukocyte recruitment in AP. The pancreatic and pulmonary levels of MPO were reduced by more than 64% and 65% respectively. The blood amylase levels were significantly decreased in MMP-9 deficient mice compared to wild-type mice after challenge with taurocholate. Furthermore, the pancreatic microarchitecture was significantly improved in MMP-9 deficient mice. Direct visualizing of pancreatic microcirculation by intravital fluorescence microscopy revealed that MMP-9 deficient mice exhibited lower numbers of adherent leukocytes to the endothelium after taurocholate challenge compared to wild-type mice, however, the rolling remained intact and was not different from wild-type mice.

Co-incubation of isolated pancreatic acinar cells with both neutrophils and secretions isolated from activated neutrophils from wild-type mice greatly increased trypsinogen activation *in vitro*; however, neither neutrophils isolated from MMP-9 deficient mice nor their secretions were capable of stimulating trypsinogen activation in pancreatic acinar cells. Furthermore, activated recombinant MMP-9 significantly enhanced trypsinogen activation in isolated pancreatic acinar cells. These novel findings provide us with the knowledge that MMP-9 is a strong stimulator for intra-acinar trypsinogen activation and neutrophil-derived MMP-9 is responsible for neutrophil-mediated trypsinogen activation. In this context, it should be mentioned that researchers have reported that trypsin can activate MMP-9 [258, 259]. Considered together with our observation that MMP-9 triggers trypsinogen activation, it may be proposed that there might be self-amplifying loops involving trypsin and MMP-9 activation in AP.

Taken together, our findings of the fourth paper provide us with the knowledge that MMP-9 mediates trypsinogen activation, leukocyte recruitment and tissue damage in AP. Furthermore, we found that neutrophil-derived MMP-9 is a potent activator of trypsinogen activation in acinar cells in AP. Thus MMP-9 could be used as a useful target in the management of AP.

Role of Rho-kinase signaling in AP

During the last two decades numerous studies have demonstrated the importance of small GTP-binding proteins such as Rho GTPase proteins and one of their effectors Rho-kinase seems to be an important component in regulating intra-cellular signaling pathways [135, 260]. Rho-kinase influences several components of inflammation including ROS formation [143] and leukocyte-platelet-endothelium interactions [261, 262] making it an important aspect in early phases of the inflammatory processes. Based on these considerations the aim of our fourth study was to determine the role of Rho-kinase signaling in trypsinogen activation, inflammation and tissue damage. Our data demonstrated that inhibition of Rho-kinase signaling by Y-27632 protects against

taurocholate-induced tissue damage and trypsinogen activation in a dose-dependent manner. These novel findings suggest that interference with Rho-kinase signaling may be a useful target to protect patients with AP.

Administration of Y-27632 at 30 min prior to bile duct canulation greatly decreased taurocholate-enhanced tissue damage and inflammation. For example administration of 5 mg/kg Y-27632 significantly reduced the AP-induced blood amylase by 83% and pancreatic acinar necrosis by more than 90% concluding that Rho-kinase regulates a profound part of tissue damage in AP. In this context it should be mentioned that a previous study by Kusama *et al.* reported that inhibition of ROCKs enhances cerulein-induced amylase release from isolated acinar cells, however, the outcome and course of pancreatitis is more likely to be dependent on the model at which AP is induced [263]. As they are investigating the role of Y-27632 in cerulein-induced amylase release and our study demonstrates the significant effects of Y-27632 in taurocholate-induced AP. More recently, in a study published by Du *et al.,* it has been demonstrated that RhoA mediates endothelial hyperpermeability in patients with AP complicated by lung injury, furthermore, this RhoA-mediated injury to the endothelial cells can be reversed by pre-treatment with Y-27632. Nonetheless, our study adds AP to the list of conditions which may be ameliorated by interference with Rho-kinase signaling including ischemia-reperfusion [264, 265], endotoxemia [141], septic lung injury [266], tissue fibrosis [145] and obstructive cholestasis [146].

Leukocyte recruitment represents a hall-mark in the pathophysiology of AP [33, 34]. Herein, we observed that taurocholate challenge enhanced tissue accumulation of neutrophils in the pancreas demonstrated by increased MPO levels and the number of extravascular leukocytes in histological sections. Furthermore, pre-treatment with Y-27632 significantly decreased MPO level by 73% and extravascular leukocytes by 88% suggesting an important role of Rho-kinase signaling in mediating leukocyte trafficking in AP. It has been reported that Mac-1 can mediate leukocyte adhesion in various tissues [267-269]. The exact mechanism how Y-27632 affects accumulation of leukocytes in the pancreas during AP is not clear but since it has been reported that Y-27632 regulates cell adhesion [138], we hypothesized that Y-27632 may regulate leukocyte adhesion to the activated endothelium in AP. For this purpose, we investigated the effect of Y-27632 on the adhesion molecule Mac-1. Interestingly, we found that AP increased Mac-1 expression on neutrophils and this increase was significantly reduced in animals treated with Y-27632. It should be mentioned that this reduction in Mac-1 does not exclude the role of other adhesion molecules such as LFA-1, which could be regulated by Y-27632 as well. The inhibitory effect of Y-27632 on adhesive molecules may help to explain the effect of Y-27632 on systemic manifestations of AP. The pre-treatment with Y-27632 significantly reduced the taurocholate-induced accumulation of leukocytes in the lung as demonstrated by decrease MPO level. This increase in pulmonary level was significantly reduced in taurocholate challenged mice pre-treated with Y-27632.

Tissue accumulation of leukocytes is coordinated by secreted chemokines, which initiate the leukocyte activation and later attract leukocytes to the site of inflammation. In the fourth study; we observed that taurocholate-induced tissue levels of CXCL2 were significantly

reduced in Y-27632 mice. This effect of Y-27632 may also explain the effective role of Rho-kinase inhibition on Mac-1 expression and accumulation of leukocytes in pancreas and lung but still there is a possibility that Y-27632 directly affects Mac-1 expression on neutrophils. CXC chemokine receptor 2 (CXCR2) serves as a receptor for CXCL2. In the fourth study, we observed that despite that Y-27632 reduced CXCL2 levels in the tissue, the expression of CXCR2 on leukocytes remained. In this context it should be mentioned that CXCR2 expression was significantly reduced in taurocholate-challenged mice and this is in line with other models of systemic inflammation such as sepsis [270] and trauma [271]. The reason behind this discrepancy, i.e. CXCL2 level increase and its receptor CXCR2 decrease is not fully understood but it may be that those neutrophils expressing CXCR2 have already recruited to the inflamed pancreas during AP and there is a subsequent relative accumulation of those neutrophils which are not expressing the receptor.

The second effect of Y-27632 in AP is seen in the aspect of trypsinogen activation. TAP is cleaved when trypsinogen is activated to trypsin and thus TAP can be used as a marker for trypsinogen activation. Taurocholate challenge significantly enhanced tissue levels of TAP. Interestingly, we found that pre-treatment with Y-27632 significantly reduced trypsinogen activation and thus tissue levels of TAP. There is some evidence that leukocytes can enhance trypsinogen activation in pancreatic acinar cells [34] and we raised the question whether the reduced trypsinogen activation in our study is due to direct effects of Rho-kinase signaling inhibition or if it is an indirect consequence of the above mentioned effects of Y-27632 on inflammation and leukocyte migration leading to reduced trypsinogen activation. To clear out this effect of Y-27632 we stimulated pancreatic acinar cells *in vitro* and performed a trypsin assay. We found that Y-27632 completely blocked intra-acinar trypsinogen activation indicating that trypsinogen activation is the main target of the Rho-kinase inhibitor in AP.

Moreover, we observed that administration of Y-27632 2 h after taurocholate challenge had no effect on the studied parameters. This notion supported even more the concept that trypsinogen activation is the target in AP. This means also that Y-27632 has limited effect in patients with on-going pancreatitis.

Taken together, the findings of the fourth paper, report that inhibition of Rho-kinase signaling abolished trypsinogen activation, tissue damage and leukocyte recruitment. However, this effect is only evidence when administered before pancreatitis induction or more clearly as a prophylactic treatment but has no or a limited effect when used as a treatment in AP. However, it may be a useful approach to protect patients undergoing ERCP from developing AP.

Role of NFAT in AP

In the fifth study, we have observed that NFAT signaling plays an important role in AP. We noticed that AP is associated with increased NFAT transcriptional activity and that pharmacological inhibition of NFAT restored the NFAT transcriptional activity close to the base-line. We found also that taurocholate-mediated trypsinogen activation, blood amylase and CXCL2 levels, disturbed microarchitecture and leukocyte recruitment were normalized in NFATc3-deficient mice. Furthermore; we reported that pharmacological inhibition of NFAT diminished stimulator-

enhanced trypsinogen activation in acinar cells and that acinar cells isolated from NFATc3-deficient mice exhibited significant reduction in trypsinogen activation almost to the base-line. Taken together, our novel findings in the fifth paper report for the first time that NFAT plays an effective role in the pathophysiology of AP and that targeting NFATc3 could be a useful approach for treating patients with AP.

NFAT was originally described as an inducible factor with the capability to bind the distal antigen receptor response elements of the human IL-2 promoter [174, 175] and it was generally considered to control tissue development including muscle and bone formation, vasculogenesis and axonal outgrowth [272-276]. However, a growing body of literature also implicates the role of NFAT in numerous aspects of inflammation such as atherosclerosis and autoimmune diseases [277, 278].

In the fifth study, we hypothesized that NFAT transcriptional activity is increased in AP. For this purpose, we used the taurocholate model of AP. We found that taurocholate challenge greatly increased NFAT transcriptional activity in the pancreas, lung, aorta and spleen. Furthermore, this AP-enhanced transcriptional activity significantly decreased in animals treated with the novel NFAT blocker A-285222 before challenge with taurocholate. Next, we examined whether the novel NFAT blocker, A-285222, is capable to reduce the severity of taurocholate-induced AP. Interestingly, we found that A-285222 significantly reduced taurocholate-induced serum amylase by 59%. Also treatment with A-285222 greatly reduced taurocholate-provoked levels of pancreatic and lung MPO and CXCL2 in the pancreas. Furthermore, we found that administration of A-285222 significantly reduced taurocholate-induced levels of TAP, which is used as a marker for trypsinogen activation. In this context it should be mentioned that a previous *in vivo* study has reported that FK506, a calcineurin inhibitor and a commercially available drug to prevent graft versus host disease after organ transplantation, decreases protease activation and tissue damage in AP [279]. Considering that calcineurin regulates NFAT activity [174], our findings help explain the protective effects exerted by FK506 in AP. Collectively, our data suggest a pathological role for the calcium/calcineurin-NFAT signaling axis in the pathophysiology of AP similar to that proposed for the development of cardiac hypertrophy, diabetes-induced vascular inflammation and arteriosclerosis [278, 280, 281].

Leukocyte accumulation represents a hall-mark in the pathophysiology of AP, in this study; it is interesting to observe that taurocholate-induced leukocyte infiltration was greatly reduced in NFAT blocker treated group, such as for example treatment with A-285222 reduced both MPO activity and tissue neutrophils by 79% and 71%, respectively. In AP, lung is involved as a part of the systemic manifestations of the disease. We observed that inhibition of NFAT transcriptional activity significantly reduced pulmonary accumulation of neutrophils, suggesting that NFAT signaling controls both local and distant accumulation of neutrophils in AP. It is interesting to observe that NFAT significantly reduced taurocholate provoked levels of CXCL2 which plays a cardinal role in the activation and tissue navigation of neutrophils to the site of inflammation.

Having established that NFAT signaling plays a role in the pathophysiology of AP, next, we examined the specific isoforms of NFAT in AP. Various tissues express different NFAT isoform and they perform unique functions [282, 283]. We found that both NFATc1 and NFATc3 were readily detected both in extracts of pancreatic lobules and of isolated acini and NFATc4 was not detected. Interestingly, the very modest expression of NFATc2 was expressed only in adult (5-6 weeks) but not young (18-22 days) mice. In a study by Gurda *et al.*, they have shown that NFATc2 is also expressed in pancreatic acinar cells [284], these discrepancies may be related to that significant plasticity in the expression levels of NFAT proteins occur during development and/or growth stimulatory conditions [285, 286].

Next, we examined the role of NFATc3 in AP. We found that mice lacking NFATc3 completely were protected from taurocholate-induced tissue damage and leukocyte recruitment. Levels of pancreatic and lung MPO were significantly decreased in NFATc3 knock-out mice compared to NFATc3-competent mice. We found also that levels of CXCL2 were significantly reduced in NFATc3-deficient mice. Furthermore, we observed that trypsinogen activation was abolished in NFATc3-deficient mice as evidenced by lower levels of pancreatic TAP in NFATc3-deficient mice. Moreover, we found that heterozygous NFATc3 mice exhibited an intermediate phenotype to the NFATc3-deficient and NFATc3-competent mice. Next, by the use of confocal microscopy, we observed that NFATc3 is translocated to the nucleus after secretagogue stimulation. With the aid of RT-PCR, we were able to demonstrate that NFATc3 is accumulated in the nucleus upon agonist stimulation and this accumulation was abolished when co-incubated with the calcineurin inhibitor CsA.

Taken together, we can conclude that NFAT signaling and particularly NFATc3 may be a useful target to treat patients with AP.

Conclusions

1) TLR4 but not TLR2 regulates pathological inflammation in AP.
2) LFA-1 is a critical mediator of leukocyte recruitment and tissue damage in AP; however, trypsinogen activation is independent of LFA-1 in AP.
3) Neutrophil-derived MMP-9 regulates trypsinogen activation in acinar cells.
4) Rho-kinase signalling regulates trypsinogen activation, leukocyte recruitment and tissue damage in AP.
5) NFATc3 controls trypsinogen activation, leukocyte recruitment and tissue damage in AP.

Sammanfattning på svenska

Akut bukspottkörtelinflammation, akut pankreatit (AP), är en sjukdom med varierande kliniska presentationer; från en mild och ödematös, till en svår och nekrotiserande form. Ungefär 75% av patienter med AP utvecklar en mild, självbegränsande sjukdom, men hos resten av patienterna progredierar sjukdomen till en svår form och dödligheten är så hög som 30% i denna grupp av patienter. I dag finns det ingen specifik behandling av AP, men stödjande behandling och vätskerehydrering ges och tros förbättra prognosen. Avsaknaden av en specifik terapi mot AP beror delvis på en ofullständig förståelse av de basala sjukdomsmekanismerna som leder till sjukdomen. Även om den exakta patofysiologin är oklar anses prematur aktivering av bukspottkörtelenzymet trypsinogen redan i bukspottkörtelns (pankreas) acinära celler, och inflammation med rekrytering av vita blodkroppar (leukocyter) representera viktiga mekanismer vid AP. Vid initieringen av AP aktiveras trypsinogen till trypsin av det lysosomala enzymet cathepsin B. Aktivt trypsin aktiverar andra proenzymer och initierar de inflammatoriska signalvägarna vid AP. CXC kemokiner, och framför allt, CXCL2 spelar en avgörande roll för att aktivera leukocyter och att styra deras infiltration och rörelse mot inflammationshärden. Leukocytrekryteringen möjliggörs även av specifika molekyler som gör att leukocyterna saktar ner (rullar) och fastnar (adhererar) på kärlväggens endotel för att slutligen migrera igenom kärlväggen till den inflammerade vävnaden. Vilka exakta molekyler som styr denna process vid AP har hittills varit okänt. Syftet med studierna i denna avhandling är att bidra med kunskap för att bättre kunna förstå patofysiologin vid AP och att möjliggöra insikter som kan leda till en effektiv terapi vid AP. För detta syfte hypotetiserade vi att signalerings och adhesionsmekanismer är speciellt viktiga för sjukdomsförloppet.

För att utforska dessa hypoteser utfördes 5 studier med en reproducerbar, kliniskt relevant modell för AP där gallsalt (Taurocholat, 5%, 10 µl på 10 minuter) injiceras i pankreasgången. För att bestämma sjukdomens svårighetsgrad 24 timmar efter induktionen togs blodprov och vävnad från pankreas och lunga, som analyserades avseende bukspottkörtelenzymet amylas, mängd av aktiverade leukocyter (myeloperoxidas, MPO), CXCL2 nivåer, histologisk pankreasskada och leukocytinfiltration och trypsinogenets aktiveringspeptid (TAP). Ytterligare vävnad sparades och analyserades för andra parametrar vilka beskrivs i detalj i material och metodavsnittet.

I den första studien undersöktes betydelsen av två medlemmar i Toll familjen, nämligen Toll like receptor (TLR) 2 och 4, vilka spelar en avgörande roll vid initiering av det ospecifika immunförsvaret. För detta användes TLR2 och TLR4 genmodifierade möss. Vi fann att frånvaro av TLR4 genen skyddade mössen från AP utlöst inflammation i pankreas, jämfört med icke genmodifierade möss. Möss utan uttryck av TLR2 genen skilde sig däremot inte från kontrollmöss som injicerats med taurocholat. Aktivering av trypsinogen skilde sig inte mellan någon av grupperna (TLR2 och TLR4 modifierade möss samt

kontroll möss). Således kan modulering av TLR4 men inte TLR2 vara ett användbart sätt att behandla patienter med AP.

I den andra studien analyseras betydelsen av adhesionsmolekylen lymfocyt function antigen-1 (LFA-1) för leukocytrekrytering och vävnadsskada vid AP. För att klargöra detta använde vi både genmodifierade möss utan LFA-1 uttryck och antikroppar riktade mot LFA-1. Vi fann att utslagning av LFA-1 genom genmodifiering eller genom antikroppsbehandling orsakade mindre leukocytrekrytering till pankreas och även mindre vävnadsskada. Genom att studera leukocyterna i pankreas venoler med intravital mikroskopi på sövda möss med AP, klargjordes att frånvaro av LFA-1 funktion orsakade mindre adhesion till kärlväggen. Leukocyternas rullning var däremot oförändrad. Dessutom fann vi att trypsinogen aktivering var oberoende av LFA-1 funktion vid A. Sammanfattningsvis visar resultaten av den andra studien att LFA-1 är den dominerande adhesionsmolekylen som medierar leukocytrekrytering vid AP och att trypsinogenaktivering inte var beroende av LFA-1 funktionen vid AP.

I det tredje arbetet undersöks betydelsen av matrix metalloproteinaser (MMPs), och speciellt MMP-9, för patofysiologin och neutrofil medierad trypsinogen aktivering vid AP. För att undersöka denna mekanism användes bredspektrum MMP hämmaren batimastat (BB-94) och (take away en) genmodifierade möss med avsaknad av MMP-9 uttryck. Vi fann att förbehandling med BB-94 signifikant minskade vävnadsskada, leukocytrekrytering och TAP bildning vid AP. Dessutom var möss utan MMP-9 uttryck skyddade från taurocholat inducerad vävnadsskada och leukocytrekrytering. Experiment gjorda på pankreas acinära celler visade att MMP-9 som utsöndrats från neutrophila leukocyter kan aktivera trypsinogen. Sammanfattningsvis visar resultaten i det tredje arbetet att MMP-9, som utsöndrats från neutrofila leukocyter, kan orsaka trypsinogen aktivering och vävnadsskada vid AP.

I det fjärde arbetet hypotetiserade vi att intra-cellulär Rho-kinas signalering spelar en nyckelroll för patofysiologin vid AP. För att undersöka detta användes en selektiv Rho-kinas hämmare (Y-27632), 30 minuter innan induktion av AP. Detta ledde till signifikant minskad vävnadsskada, leukocytrekrytering och trypsinogenaktivering på ett dosberoende sätt. Dessutom minskades trypsinfrisättning från stimulerade acinära celler när Rho-kinas hämmades. Om Y-27632 tillfördes 2 timmar efter taurocholatinfusion sågs emellertid ingen effekt på de studerade parametrarna. Således kan inhibering av Rho-kinas signalering vara användbart som förebyggande behandling för patienter som genomgår ERCP och riskerar att drabbas av ERCP utlöst AP, men inte som behandling för patienter med pågående AP.

I det femte arbetet studeras effekten av intra-cellulär NFAT signalering vid sjukdomsutvecklingen vid AP. NFAT är framför allt viktig då den styr gentranskription och därmed vilka processer som ska sättas igång i cellen. NFATs betydelse för AP är aldrig studerad tidigare. Genom att använda NFAT-luciferas möss kunde NFAT aktivitet mätas och vi fann att den var förhöjd både lokalt i pankreas och systemiskt i andra organ efter taurocholatinfusion. Denna ökning reverseras vid farmakologisk

NFAT blockad. Vi fann även att farmakologisk hämning av NFAT signifikant minskade vävnadsskada, CXCL2 nivåer, leukocytrekrytering och trypsinogen aktivering vid AP. Dessa resultat upprepades då AP inducerades på genmodifierade möss, med avsaknad av NFATc3 uttryck. Cellförsök med acinära celler visade att blockad av NFAT minskade trypsinogen aktivering och att NFAT c3 förflyttas till cellkärnan vid aktivering. Vi konkluderar att NFAT signalering är en viktig mekanism i patofysiologin vid AP och att påverkan på NFAT signalering kan vara sätt att behandla patienter med AP i framtiden.

I sin helhet visar våra nya resultat att signallerings och adhesionsmekanismer kan vara användbara mål för att utveckla terapeutiska strategier för behandling av AP.

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Paper I

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Inflammation Research

ORIGINAL RESEARCH PAPER

TLR4 but not TLR2 regulates inflammation and tissue damage in acute pancreatitis induced by retrograde infusion of taurocholate

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Abstract
Objective Neutrophil infiltration is a key regulator in the pathophysiology of acute pancreatitis (AP), although the impact of Toll-like receptors (TLRs) in AP remains elusive. The aim of this study was to define the role of TLR2 and TLR4 in leukocyte recruitment and tissue damage in severe AP.
Experimental design AP was induced by retrograde infusion of sodium taurocholate into the pancreatic duct in wild-type, TLR2- and TLR4-deficient mice. Samples were collected 24 h after induction of AP.
Results Taurocholate challenge caused a clear-cut pancreatic damage characterized by increased acinar cell necrosis, neutrophil infiltration, focal hemorrhage and edema formation, as well as increased levels of blood amylase and CXCL2 (macrophage inflammatory protein-2) in the pancreas and serum. Moreover, challenge with taurocholate increased activation of trypsinogen in the pancreas. Notably, *TLR2* gene-deficient mice exhibited a similar phenotype to wild-type mice after challenge with taurocholate. In contrast, tissue damage, pancreatic and lung myeloperoxidase (MPO) activity, serum and pancreatic levels of CXCL2 as well as blood amylase were significantly reduced in TLR4-deficient mice exposed to taurocholate. However, taurocholate-induced activation of trypsinogen was intact in TLR4-deficient mice.
Conclusion Our data suggest a role for TLR4 but not TLR2 in the pathogenesis of severe AP in mice.

Keywords Toll-like receptors · Amylase · Chemokines · Inflammation · Neutrophils · Pancreatitis

Abbreviations

AP	Acute pancreatitis
MPO	Myeloperoxidase
MNL	Monomorphonuclear leukocytes
PBS	Phosphate-buffered saline
PMNL	Polymorphonuclear leukocytes
RIA	Radioimmunoassay
TAP	Trypsinogen activation peptide
TLR	Toll-like receptor
HPF	High-power field

Introduction

The clinical presentation of acute pancreatitis (AP) ranges from a mild edematous and local condition to a severe necrotizing and systemic disease [1]. The treatment of AP is not specific and is mainly restricted to supportive care, due to an incomplete understanding of the basic mechanisms regulating the development of pancreatitis. AP is characterized by activation of proteases and the innate immune system, leading to massive infiltration of neutrophils and tissue damage in the pancreas [2]. Activation and tissue localization of neutrophils are orchestrated by secreted CXC chemokines [3]. CXCL2 (macrophage inflammatory

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protein-2) is considered to be a major stimulus for neutrophil chemotaxis and has been demonstrated to regulate tissue neutrophilia in AP [4]. The innate immune system is controlled by specific families of pattern-recognizing proteins with the capacity to bind certain pathogen- and host-derived molecules [2, 5, 6]. The Toll-like receptor (TLR) family constitutes 13 different receptors which are particularly effective in recognizing molecules broadly shared by pathogens but distinguishable from host molecules [7, 8]. For example, TLR4 is the primary signaling receptor for endotoxins from Gram-negative bacteria, whereas TLR2 recognizes bacterial lipoproteins and lipoteichoic acid from Gram-positive bacteria [9]. Not only bacteria-derived compounds may activate TLR2 and TLR4 but also endogenous substances, such as heat shock proteins, which are potent ligands of these TLRs and may play a role in AP [10]. TLR2 and TLR4 signaling can regulate several components in the tissue response to injury, including inflammatory cell recruitment, microvascular leakage and cellular apoptosis [11, 12]. There is some data in the literature suggesting that TLR4 may play a role in AP [13], although this is controversial [14]. Interestingly, a recent study reported that polymorphism in the *TLR2* gene is associated with increased susceptibility and severity of AP [15]. However, there is no information on the potential functional role of TLR2 in the pathophysiology of AP.

Based on the above considerations, the aim of this study was to determine the role of TLR2 and TLR4 in activation of innate immunity and tissue damage in AP.

Materials and methods

Animals

All experiments were done in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for animal experimentation at Lund University, Sweden. Wild-type C57BL/6 and BALB/c mice were used. Breeder pairs of TLR2-deficient (B6.129-Tlr2^{tm1Kir}/J) and TLR4-deficient (C.C3-Tlr4^{LPS-d}/J) mice were purchased from Jaxmice (Sacramento, CA, USA). All animals (20–26 g, 6–8 weeks) were maintained in a climate-controlled room at 22°C and exposed to a 12:12-h light–dark cycle. Animals were fed standard laboratory diet and given water ad libitum. Mice were anesthetized by intraperitoneal (i.p.) administration of 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight in 200 µl saline.

Experimental protocol

Through a small (1–2 cm) upper midline incision, the second part of the duodenum and papilla of Vater were identified (Electronic Supplementary Material Fig. 1). Traction sutures (7–0 prolene) were placed 1 cm from the papilla. Under microscopic observation, parallel to the papilla of Vater at the duodeno-pancreatic junction on the posterior surface of the duodenum, a small puncture was made through the duodenal wall with a 23G needle. A non-radiopaque polyethylene catheter (inner diameter 0.28 mm) connected to a micro infusion pump (CMA/100, Carnegie Medicine, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and 1 mm into the pancreatic duct. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp. Infusion of 10 µl of either 5% sodium taurocholate (Sigma-Aldrich, St. Louis, MO, USA) or 0.9% sodium chloride for 5 min at a rate of 2 µl/min was performed, and after completion the catheter was withdrawn and the common hepatic duct clamp was removed. The duodenal puncture was closed with a 7–0 prolene suture, the traction sutures were removed and the abdomen was closed in two layers. Animals were allowed to wake up and were given free access to food and water. Control mice received saline into the pancreatic duct. All animals were killed 24 h after pancreatitis induction and assessed for all parameters included in this study in a blinded manner. Blood was collected from the tail vein for systemic leukocyte differential counts and determination of blood amylase levels. Blood samples were also collected from the inferior vena cava for measurement of serum CXCL2. Pancreatic tissue was removed and kept in two pieces; one piece was snap frozen in liquid nitrogen for biochemical analysis of myeloperoxidase (MPO), CXCL2 and trypsinogen activation peptide (TAP), and the other piece was fixed in formalin for later histological analysis. Lung tissue was harvested for MPO measurements.

Systemic leukocyte counts

Tail vein blood was mixed with Turk's solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leukocytes were identified as monomorphonuclear (MNLs) and polymorphonuclear (PMNLs) cells in a Burker chamber.

Blood amylase

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

MPO assay

Frozen pancreatic and lung tissue were pre-weighed and homogenized in 1-ml mixture (4:1) with phosphate-buffered saline (PBS) and aprotinin 10,000 kIE/ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenate was centrifuged (15,339*g*, 10 min); the supernatant was stored at −20°C and the pellet was used for MPO assay as previously described [16]. In brief, the pellet was mixed with 1 ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 s, and put in a water bath at 60°C for 2 h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units per gram tissue.

CXCL2 levels

Tissue levels of CXCL2 were determined in serum and pancreatic tissue by using double-antibody Quantikine enzyme-linked immunosorbent assay kits (R&D Systems Europe, Abingdon, UK) using recombinant murine CXCL2 as standard. The minimal detectable protein concentration is <0.5 pg/ml.

Histology

Pancreas samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin-embedded. Six-micrometer sections were stained (hematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system including edema, acinar cell necrosis, hemorrhage and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scale as previously described [17] and detailed in Table 1.

TAP levels

Trypsinogen is activated to trypsin in a reaction where TAP is cleaved off and thus can be used as marker of trypsinogen activation [18]. The radioimmunoassay (RIA) was performed as described previously [19]. A 0.1-M Tris HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.005 M EDTA and 2 g/L bovine serum albumin (Sigma-Aldrich) was used as assay buffer. Samples of 100 µl diluted in assay buffer were incubated (16 h, 4°C) with 200 µl of I^{125}Tyr-TAP (=20,000 counts per min) in assay buffer and 200 µl of antiserum diluted 1/750 in assay buffer. Parallel incubations with synthetic TAP diluted in assay buffer in a series of concentrations from 0.078 to 20 nM were used as standards in the assays. Free and bound radioactivities were separated by means of a second-step antibody precipitation.

Table 1 Histology scoring system

Score	Edema	Acinar necrosis	Hemorrhage	Leukocyte infiltration
0	Absent	Absent	Absent	0–1 intralobular or perivascular leukocytes/HPF
0.5	Focal expansion of interlobar septae	Focal occurrence of 1–4 necrotic cells/HPF	1 focus	2–5 intralobular or perivascular leukocytes/HPF
1	Diffuse expansion of interlobar septae	Diffuse occurrence of 1–4 necrotic cells/HPF	2 foci	6–10 intralobular or perivascular leukocytes/HPF
1.5	Same as 1 + focal expansion of interlobular septae	Same as 1 + focal occurrence of 5–10 necrotic cells/HPF	3 foci	11–15 intralobular or perivascular leukocytes/HPF
2	Same as 1 + diffuse expansion of interlobular septae	Diffuse occurrence of 5–10 necrotic cells/HPF	4 foci	16–20 intralobular or perivascular leukocytes/HPF
2.5	Same as 2 + focal expansion of interacinar septae	Same as 2 + focal occurrence of 11–16 necrotic cells/HPF	5 foci	21–25 intralobular or perivascular leukocytes/HPF
3	Same as 2 + diffuse expansion of interacinar septae	Diffuse occurrence of 11–16 necrotic cells/HPF (foci of confluent necrosis)	6 foci	26–30 intralobular or perivascular leukocytes/HPF
3.5	Same as 3 + focal expansion of intercellular spaces	Same as 3 + focal occurrence of >16 necrotic cells/HPF	7 foci	>30 leukocytes/HPF or focal microabscesses
4	Same as 3 + diffuse expansion of intercellular spaces	>16 necrotic cells/HPF (extensive confluent necrosis)	≥8 foci	>35 leukocytes/HPF or confluent microabscesses

Histological scoring system as described by Schmit et al. (1992)

HPF high-power field

For this, 100 µl of a cellulose coupled anti-mouse IgG suspension (Sac-Cel®, IDS, Boldon, England) was added to the samples. After 30 min of incubation, 1 ml of water was added and the tubes were centrifuged (704*g*, 5 min, room temperature). The supernatant was decanted and the radioactivity of the precipitate was counted in a gamma spectrophotometer (1 min).

Statistics

Data are presented as medians (ranges). Statistical evaluations were performed by using non-parametrical tests (Mann–Whitney). $P < 0.05$ was considered significant and *n* represents the number of animals.

Results

Role of TLR4 and TLR2 in AP

First, we determined the role of TLR4 in taurocholate-induced AP. As shown in Table 2, we found that taurocholate increased blood amylase more than 12-fold as well as causing a clear-cut infiltration of leukocytes in the pancreas and lung (Table 2). Moreover, taurocholate-induced pancreatic injury elevated CXCL2 levels in the pancreas and serum and caused significant acinar cell necrosis, edema and hemorrhage (Table 2). TAP, a marker of trypsinogen activation, was also increased after taurocholate challenge (Table 2). It was found that taurocholate-induced blood amylase levels as well as pancreatic and pulmonary activity of MPO were markedly reduced in animals lacking TLR4 (Table 2). In addition, taurocholate-induced formation of CXCL2 in the pancreas and serum was reduced by 75% and 94%, respectively, in *TLR4* gene-deficient mice (Table 2). As shown in Table 2, acinar cell necrosis, edema and hemorrhage induced by challenge with taurocholate were significantly decreased in TLR4-deficient mice, suggesting a role of TLR4 in AP. We next examined the role of TLR2 in taurocholate-induced AP. We observed that levels of blood amylase, pancreatic and pulmonary MPO, pancreatic and serum CXCL2 as well as acinar cell necrosis, edema and hemorrhage were not significantly different in mice lacking TLR2 compared to wild-type controls (Table 3). TAP is a marker of trypsinogen activation [18]. We found that challenge with taurocholate markedly increased formation of TAP in the pancreas and that generation of TAP was intact in both TLR2- and TLR4-deficient animals (Tables 2 and 3). Moreover, there were no significant differences in the numbers of mononuclear and polymorphonuclear leukocytes in the blood of wild-type, *TLR2* and *TLR4* gene-deficient animals receiving taurocholate (data not shown).

Discussion

Accumulating data in the literature suggest that pattern-recognizing receptors play a significant role in acute inflammatory diseases [20, 21]. In the present study, we observed that pancreatitis-associated amylase release, neutrophil recruitment and tissue damage were greatly decreased in animals lacking TLR4 whereas animals lacking TLR2 were no different from wild-type animals. Thus, our data suggest that TLR4 but not TLR2 signaling

Table 2 Role of TLR4 in taurocholate-induced pancreatitis

	BALB/c + saline ($n = 5$)	BALB/c + taurocholate ($n = 5$)	TLR4 −/− + taurocholate ($n = 4$)
Blood amylase (µKat/L)	48 (37–59)	560 (490–690)#	138 (44–172)*
MPO in the pancreas (U/g)	0.8 (0.3–1.1)	2.5 (2.1–3.2)#	0.8 (0.2–1.8)*
MPO in the lung (U/g)	0.8 (0.2–1.2)	1.7 (1.4–5.1)#	0.6 (0.3–0.9)*
CXCL2 in the pancreas (pg/mg)	0.1 (0.04–1)	7.5 (5.1–12.7)#	2.2 (0.1–3)*
CXCL2 in the serum (pg/mL)	1.3 (0.2–2.8)	53.6 (7.5–190)#	3.2 (1.1–7.2)*
Acinar cell necrosis (score 0–4)	0.5 (0–0.5)	3.5 (3.5–4)#	1 (0.5–1.5)*
Neutrophil infiltration (score 0–4)	0.3 (0–0.5)	3.5 (3.5–4)#	1 (0.5–1.5)*
Edema (score 0–4)	0.5 (0.5–1)	3.3 (3–4)#	1.5 (1.5–2)*
Hemorrhage (score 0–4)	0.5 (0.5–1)	3.5 (3–3.5)#	1.5 (1–1.5)*
Pancreatic TAP (µg/g)	105 (11–210)	362 (209–776)#	245 (62–351)

Acute pancreatitis was induced by retrograde infusion of 10 µl sodium taurocholate (5%) into the pancreatic duct in BALB/c and *TLR4* gene-deficient (TLR4 −/−) mice. Control animals (BALB/c + saline) underwent infusion of 10 µl saline into the pancreatic duct. Myeloperoxidase (*MPO*), CXCL2 in pancreas and serum, acinar cell necrosis, edema and hemorrhage, as well as serum amylase, MPO in the lung and TAP in the pancreas were determined 24 h after infusion. Values represent medians (ranges). # $P < 0.05$ versus BALB/c + saline and * $P < 0.05$ versus BALB/c + taurocholate

Table 3 Role of TLR2 in taurocholate-induced pancreatitis

	C57BL/6 + saline ($n = 5$)	C57BL/6 + taurocholate ($n = 5$)	TLR2 −/− + taurocholate ($n = 5$)
Blood amylase (μKat/L)	57 (48–133)	614 (563–901)[#]	655 (155–750)
MPO in the pancreas (U/g)	0.4 (0.1–0.7)	1.7 (1.5–2.7)[#]	1.6 (1–2.2)
MPO in the lung (U/g)	0.6 (0.2–1)	2.4 (1.1–2.6)[#]	1.4 (1.2–3.2)
CXCL2 in the pancreas (pg/mg)	0.9 (0.5–1.6)	7.2 (4.4–8.4)[#]	4.9 (3.7–8.8)
CXCL2 in the serum (pg/mL)	2.5 (1.5–10)	54.6 (23.6–97.3)[#]	53.8 (23.6–69.6)
Acinar cell necrosis (score 0–4)	0.3 (0.3–0.8)	3.5 (3–3.8)[#]	3 (2–3.5)
Neutrophil infiltration (score 0–4)	0.5 (0.5–1)	3.5 (3–4)[#]	3 (2.5–3.5)
Edema (score 0–4)	0.5 (0–1.5)	3 (2.5–3.5)[#]	3 (2.5–3.5)
Hemorrhage (score 0–4)	1.5 (0.5–2)	3.5 (3–4)[#]	3 (2.5–3.5)
Pancreatic TAP (μg/g)	93 (54.6–270)	377 (350–462)[#]	252 (69–941)

Acute pancreatitis was induced by retrograde infusion of 10 μl sodium taurocholate (5%) into the pancreatic duct in C57BL/6 and *TLR2* gene-deficient (TLR2 −/−) mice. Control mice (C57BL/6 + saline) underwent infusion of 10 μl saline into the pancreatic duct. Myeloperoxidase (*MPO*), CXCL2 in pancreas and serum, acinar cell necrosis, edema and hemorrhage, as well as serum amylase, MPO in the lung and TAP in the pancreas were determined 24 h after infusion. Values represent medians (ranges). [#] $P < 0.05$ versus C57BL/6 + saline

constitutes a key component in severe AP induced by retrograde injection of taurocholate.

AP is characterized by enhanced proteolytic activation triggering overwhelming tissue injury, which in turn may provoke systemic inflammation and remote organ damage [22]. Numerous investigations have identified pattern-recognizing receptors, such as TLRs, as key molecules regulating pathological inflammation in both infectious and non-infectious diseases [23–25]. In this context, it is interesting to note that a recent investigation by Takagi et al. [15] found that polymorphism in the *TLR2* gene is associated with enhanced risk and severity of AP. However, the outcome of AP may be species-specific, as the study by Takagi et al. [15] was carried out in humans, and we are investigating the role of TLR2 in mice. Thus, we considered it important to examine the potential functional role of TLR2 in AP in mice. In the present study, we found that the taurocholate-induced increases in blood amylase, acinar cell injury and tissue edema in the pancreas were similar in TLR2-deficient and wild-type mice. These findings suggest that TLR2 does not play an important role in severe AP induced by retrograde injection of taurocholate. In contrast, it was observed that the taurocholate-provoked elevation of blood amylase, acinar cell damage and tissue edema in the pancreas were markedly attenuated in *TLR4* gene-deficient mice, suggesting that TLR4 function regulates cell and tissue damage in AP. The literature on the role of TLR4 in pancreatitis is complex and partly contradictory. For example, some studies have reported a role of TLR4 in AP induced by cerulein and L-arginine [13, 26]. Others have shown that TLR4 had no effect on the severity of pancreatitis and pancreatitis-associated lung damage in cerulein-induced AP [14]. In addition, Sawa et al. [27] reported that TLR4 deficiency had no effect on pancreatic damage, but reduced liver and kidney injury in closed duodenal loop-induced AP. These discrepancies may be related to differences in species used, severity of pancreatitis and methods to induce pancreatitis, but our data demonstrate that TLR4 plays a role, at least, in the development of severe AP after initial pancreatic injury. Convincing data in the literature shows that neutrophil-mediated tissue injury is a key feature in AP. Thus, several studies have reported that depletion of neutrophils decreases tissue injury in AP [22, 28]. Here, we observed that taurocholate-induced increases in MPO activity in the pancreas and lung as well as the neutrophil chemoattractant CXCL2 in the pancreas and serum were significantly decreased in *TLR4* but not *TLR2* gene-targeted mice, which parallel the changes in tissue damage discussed above and are in line with a critical role of neutrophils in AP. Moreover, we found that pancreatitis-associated trypsinogen activation, i.e., increases in TAP levels in the pancreas, were not reduced in *TLR2* or *TLR4* gene-deficient animals. Thus, these findings suggest that TLR4-mediated inflammation and tissue damage in AP is an event downstream of proteolytic activation in acinar cells.

Taken together, our data suggest that TLR4 but not TLR2 regulates chemokine formation, neutrophil recruitment and tissue damage in severe AP. Thus, we suggest that targeting the function or signaling pathways of TLR4 but not TLR2 may provide a useful therapeutic option against pathological inflammation in AP.

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Paper II

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RESEARCH PAPER

Lymphocyte function antigen-1 regulates neutrophil recruitment and tissue damage in acute pancreatitis

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BACKGROUND AND PURPOSE
Leucocyte infiltration is a rate-limiting step in the pathophysiology of acute pancreatitis (AP) although the adhesive mechanisms supporting leucocyte-endothelium interactions in the pancreas remain elusive. The aim of this study was to define the role of lymphocyte function antigen-1 (LFA-1) in regulating neutrophil-endothelium interactions and tissue damage in severe AP.

EXPERIMENTAL APPROACH
Pancreatitis was induced by retrograde infusion of sodium taurocholate into the pancreatic duct in mice. LFA-1 gene-targeted mice and an antibody directed against LFA-1 were used to define the role of LFA-1.

KEY RESULTS
Taurocholate challenge caused a clear-cut increase in serum amylase, neutrophil infiltration, CXCL2 (macrophage inflammatory protein-2) formation, trypsinogen activation and tissue damage in the pancreas. Inhibition of LFA-1 function markedly reduced taurocholate-induced amylase levels, accumulation of neutrophils, production of CXC chemokines and tissue damage in the pancreas. Notably, intravital microscopy revealed that inhibition of LFA-1 abolished taurocholate-induced leucocyte adhesion in postcapillary venules of the pancreas. In addition, pulmonary infiltration of neutrophils was attenuated by inhibition of LFA-1 in mice challenged with taurocholate. However, interference with LFA-1 had no effect on taurocholate-induced activation of trypsinogen in the pancreas.

CONCLUSIONS AND IMPLICATIONS
Our novel data suggest that LFA-1 plays a key role in regulating neutrophil recruitment, CXCL2 formation and tissue injury in the pancreas. Moreover, these results suggest that LFA-1-mediated inflammation is a downstream component of trypsinogen activation in the pathophysiology of AP. Thus, we conclude that targeting LFA-1 may be a useful approach to protect against pathological inflammation in the pancreas.

Abbreviations
AP, acute pancreatitis; MNL, monomorphonuclear leucocytes; MPO, myeloperoxidase; PMNL, polymorphonuclear leucocytes; RIA, radioimmunoassay; ROS, reactive oxygen species; TAP, trypsinogen activation peptide

Introduction

The clinical presentation of acute pancreatitis (AP) ranges from a mild and local condition to a severe and systemic disease (Van Laethem *et al.*, 1998). Despite significant investigative efforts specific therapy is not yet available and treatment of patients with AP is largely limited to supportive care, which is related to an incomplete understating of the basic

pathophysiology. It is widely held that protease activation, inflammation and impaired microvascular perfusion are involved in the pathophysiology of pancreatitis (Wang *et al.*, 2009; Zhang *et al.*, 2009) although their interrelationships are not well understood. For example, it is not known whether activation of trypsinogen into trypsin in the pancreas is dependent on neutrophil infiltration in the pancreas or not.

Infiltration of neutrophils represents a hallmark in AP (Gukovskaya *et al.*, 2002). Secreted chemokines coordinate leucocyte migration to sites of tissue damage. For example, CXCL2 (macrophage inflammatory protein-2) is a potent neutrophil attractant and one previous study reported that CXCL2 plays an important role in AP (Pastor *et al.*, 2003). Moreover, CXCR2 is the main receptor of CXCL2 and it has been shown that inhibition of CXCR2 protects against AP (Bhatia and Hegde, 2007). In general, leucocyte extravasation is a multistep process including initial rolling along activated endothelial cells followed by firm adhesion and transmigration (Månsson *et al.*, 2000; Riaz *et al.*, 2002). The interactions between leucocytes and endothelium are mediated by specific adhesion molecules of the selectin and integrin families (Butcher, 1991). Numerous studies have shown that leucocyte rolling is supported by P-, E- and L-selectins (Thorlacius *et al.*, 1994; Ridger *et al.*, 2005). Stationary adhesion of leucocytes to the microvascular endothelium is mainly mediated by a group of heterodimeric molecules referred to as β_2-integrins, such as lymphocyte function antigen-1 (LFA-1; CD11a/CD18), membrane-activated complex-1 (Mac-1; CD11b/CD18) and p150,95 (CD11c/CD18). The literature is rather complex and partly contradictory with respect to the function of individual β_2-integrins in leucocyte adhesion, and the relative importance of specific β_2-integrins appears to vary depending on the type of inflammatory stimulus and experimental model (Argenbright *et al.*, 1991; Issekutz and Issekutz, 1992; Rutter *et al.*, 1994; Issekutz, 1995). Notably, by use of LFA-1 gene-targeted mice, we and others have demonstrated that LFA-1 plays an important role in supporting firm leucocyte adhesion in striated muscles (Thorlacius *et al.*, 2000; Dunne *et al.*, 2002), peritoneum (Schmits *et al.*, 1996; Lu *et al.*, 1997), skin (Schramm *et al.*, 2002), colon (Riaz *et al.*, 2002) and liver (Li *et al.*, 2004; Dold *et al.*, 2008). Nonetheless, the importance of β_2-integrins for leucocyte recruitment and tissue damage in the pancreas is not known.

Based on these considerations, the aim of this study was to examine the role of LFA-1 in regulating recruitment of neutrophils and tissue damage in the pancreas. In addition, we wanted to determine the impact of inhibiting pancreatic infiltration of neutrophils on protease activation in AP. For this purpose, we used a mouse model of AP based on retrograde infusion of taurocholate into the pancreatic duct.

Methods

Animals

All experiments were done in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for animal experimentation at Lund University, Sweden. Fifty C57BL/6 wild-type and 10 LFA-1 gene-targeted male mice weighing 20–26 g (6–8 weeks) were maintained in a climate-controlled room at 22°C and exposed to a 12:12-h light-dark cycle. Animals were fed standard laboratory diet and given water *ad libitum*. Mice were anaesthetized by i.p. administration of 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight in 200 μL saline.

Taurocholate-induced AP

Through a small (1–2 cm) upper midline incision, the second part of duodenum and papilla of Vater were identified. Traction sutures (7–0 prolene) were placed one cm from the papilla. Parallel to the papilla of Vater a small puncture was made through the duodenal wall with a 23 G needle. A non-radiopaque polyethylene catheter (ID 0.28 mm) connected to a micro infusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and one mm into the common bile duct. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp. Infusion of 10 μL of either 5% sodium taurocholate (Sigma-aldrich, USA) or 0.9% sodium chloride (n = 5) for 5 min was performed and after completion, the catheter was withdrawn and the common hepatic duct clamp was removed. The duodenal puncture was closed with a purse-string suture (7–0 monofilament). The traction sutures were removed and the abdomen was closed in two layers. Animals were allowed to wake up and were given free access to food and water. Sham operated animals underwent the same procedure without any infusion into the pancreas (n = 5). Control (5 $\mu g \cdot g^{-1}$, rat IgG2a, eBioscience, San Diego, CA, USA) antibody (n = 5) or purified anti-mouse LFA-1 antibody (5 $\mu g \cdot g^{-1}$, clone M17/4, rat IgG2a, n = 5, eBioscience, San Diego, CA, USA) was administered i.p. prior to bile duct cannulation. This dose and scheme of administration of the anti-mouse LFA-1 antibody was based on a previous investigation (Asaduzzaman *et al.*, 2008). In addition, LFA-1 gene-deficient (n = 5) and wild-type (n = 5) mice were also challenged with 10 μL of 5% sodium taurocholate. All animals were killed 24 h after pancreatitis induction and assessed for all parameters included in this study. Blood was collected from the tail vein for systemic leucocyte differential counts. Blood samples were also collected from the inferior vena cava for determination of serum amylase levels and measurements of serum CXCL2. Pancreatic tissue was removed and kept in two pieces; one piece was snap frozen in liquid nitrogen for biochemical analysis of myeloperoxidase (MPO), CXCL2 and trypsinogen activation peptide (TAP) and the other piece was fixed in formalin for later histological analysis. Lung tissue was also harvested for MPO measurements.

Systemic leucocyte counts

Tail vein blood was mixed with Turks solution (0.2 mg gentian violet in 1 mL glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leucocytes were identified as monomorphonuclear and polymorphonuclear cells in a Burker chamber.

Serum amylase

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

MPO assay

Frozen pancreatic and lung tissue were pre-weighed and homogenized in 1-mL mixture (4:1) of PBS and aprotinin 10 000 KIE·mL^{-1} (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenate was centrifuged (153 39× *g*, 10 min) and the supernatant was stored at –20°C and the pellet was used for MPO assay as previously described (Laschke *et al*., 2007). In brief, the pellet was mixed with 1 mL of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 s, put in a water bath 60°C for 2 h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalysed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units·g^{-1} tissue.

Flow cytometry

Blood was collected (1:10 acid citrate dextrose) from wild-type and LFA-1 gene-targeted mice. To block Fcg III/II receptors and reduce non-specific labelling samples were incubated with an anti-CD16/CD32 for 5 min. Then samples were stained with a PE-conjugated anti-Gr-1 (clone RB6-8C5, eBioscience, San Diego, CA, USA) antibody and with a FITC-conjugated anti-LFA-1 (clone 2D7, BD Biosciences Pharmingen, San Jose, CA) antibody at 4°C for 30 min. Erythrocytes were lysed and Cells were fixed. Cells were recovered following centrifugation before being analysed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells. After gating the neutrophil population based on forward and side scatter characteristics, LFA-1 expression was determined on cells positive for Gr-1, which is a neutrophil marker.

CXCL2 levels

Tissue levels of CXCL2 were determined in serum and pancreatic tissue by using double-antibody Quantikine enzyme linked immunosorbent assay kits (R & D Systems Europe, Abingdon, UK) using recombinant murine CXCL2 as standard. The minimal detectable protein concentration is less than 0.5 pg·mL^{-1}.

Histology

Pancreas samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six micrometre sections were stained (haematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system including oedema, acinar cell necrosis, haemorrhage and neutrophil infiltrate on a 0 (absent) to four (extensive) scale as previously described in detail (Schmidt *et al*., 1992).

TAP levels

Trypsinogen is activated to trypsin in a reaction where TAP is cleaved off and thus can be used as marker of trypsinogen activation (Chen *et al*., 2003). The RIA was performed as described previously (Lindkvist *et al*., 2008). A 0.1 M Tris HCL buffer (pH 7.5) containing 0.15 M NaCl, 0.005 M EDTA and 2 g·L^{-1} bovine serum albumin (Sigma, St Louis, USA) was used as assay buffer. Samples of 100 μL diluted in assay buffer were incubated (16 h, 4°C) with 200 μL of I 125Tyr-TAP (=20 000 counts·min^{-1}) in assay buffer and 200 μL of antiserum diluted 1/750 in assay buffer. Parallel incubations with the synthetic activation peptides TAP1 diluted in assay buffer in a series of concentrations from 0.078 to 20 nM, were used as standards in the assays. Free and bound radioactivities were separated by means of a second step antibody precipitation; 100 μL of a cellulose coupled anti-mouse IgG suspension (Sc-Cel® IDA, Boldon, England) was added to the samples. After 30 min of incubation 1 mL of water was added and tubes were centrifuged (704× *g*, 5 min, room temperature). The supernatant was decanted and radioactivity of the precipitate was counted in a γ-spectrophotometer (1 min).

Reverse-transcription polymerase chain reaction

Total RNA was extracted from the blood samples of the knockout mice and wild-type mice using RNeasy Mini-kit (Qiagen Gmbh, Hilden, Germany) and treated with RNease-free DNease (Amersham Pharmacia Biotech AB, Sollentuna, Sweden) to remove potential genomic DNA contaminants according to manufacturer's handbook. RNA concentrations were determined by measuring the absorbance at 260 nm spectrophotometrically. Reverse-transcription polymerase chain reaction (RT-PCR) was performed with Superscript One-Step RT-PCR system (Gibco BRL Life Technologies, Grand Islands, NY). The RT-PCR profile was one cycle of cDNA synthesis at 50°C for 30 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 10 min. After RT-PCR, aliquots of the RT-PCR products were separated on 2% agarose gel containing ethidium bromide and photographed. The primers sequences were as follows: CD11a (f) 5′-AGA TCG AGT CCG GAC CCA CAG-3′, CD11a (r) 5′-GGC AGT GAT AGA GGC CTC CCG-3′, β-actin (f) 5′-ATG TTT GAG ACC TTC AAC ACC-3′, β-actin (r) 5′-TCT CCA GGG AGG AAG AGG AT-3′. β-Actin served as a house keeping gene to control for the loading amount of cDNA.

Intravital microscopy

A 5-min equilibration time was allowed before analysis of leucocyte rolling and adhesion was performed in postcapillary venules (19–51 μm) in the pancreas. Contrast enhancement by i.v. injection of fluorescein isothiocyanate-labelled dextran 150 000 (0.05 mL, 5 mg·mL^{-1}, Sigma Chemical Co.) and *in vivo* labelling of leucocytes with rhodamine 6 G (0.1 mL, 0.5 mg·mL^{-1}, Sigma Chemical Co.) enabled analysis of leucocyte–endothelium interactions in the microvascular bed. For observations of the microcirculation, we used a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) and recorded videos on a computer for later off-line analysis of leucocyte-endothelium

interactions. Twenty-five C57BL/6 wild-type and five LFA-1 gene-targeted male mice were used, two to six postcapillary venules were evaluated in each animal and leucocyte rolling was measured by counting the number of cells rolling along the endothelial lining for 20 s and is expressed as cells·min^{-1}. Leucocyte adhesion was measured by counting the number of cells that adhered and remained stationary for more than 30 s during the observation time and is expressed as cells·mm^{-2}. Certain animals received an anti-P-selectin antibody (40 μg, i.v., clone RB40.34, BD Biosciences Pharmingen) immediately before capturing microphotographs of the postcapillary venules in the pancreas in order to abolish leucocyte rolling and thereby enable visualization of the remaining leucocytes that were firmly adherent to the endothelium.

Statistics

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

Results

Role of LFA-1 in taurocholate-induced tissue damage in the pancreas

First, we examined LFA-1 expression at the mRNA and protein level in the LFA-1 gene-targeted mice used herein and found that these animals completely lacked LFA-1 (Figure S1). Retrograde infusion of sodium taurocholate into the pancreatic duct enhanced serum amylase levels by nearly 16-fold (Figure 1). Taurocholate-induced serum levels of amylase were reduced by more than 70% in LFA-1-deficient animals and in mice treated with an antibody against LFA-1 (Figure 1). Tissue damage was evaluated by quantification of acinar cell necrosis, oedema formation and haemorrhage in the pancreas. Taurocholate-induced acinar cell necrosis, oedema formation and interstitial haemorrhage were markedly attenuated in LFA-1 gene-targeted animals and in mice treated with the anti-LFA-1 antibody (Figure 2). Moreover, morphological examination of the pancreas revealed normal microstructure in control animals, whereas taurocholate challenge caused severe destruction of the pancreatic tissue structure characterized by extensive cell necrosis, oedema and massive infiltration of neutrophils (Figure 3). However, the structure of the pancreas was protected in LFA-1-deficent and in anti-LFA-1 antibody-treated mice challenged with taurocholate (Figure 3).

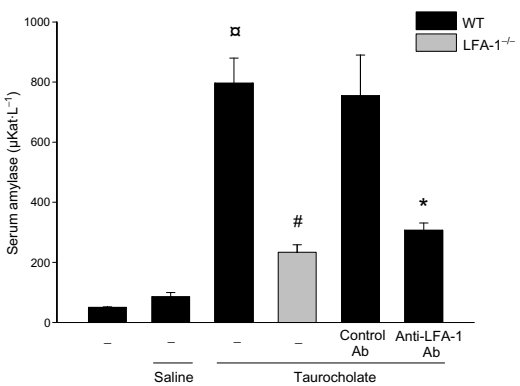

Figure 1

Serum amylase (μKat·L^{-1}) in wild-type (WT) and lymphocyte function antigen-1 (LFA-1)-deficient mice. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice received saline alone. Certain mice received a control antibody (Ab) or an Ab against LFA-1 prior to pancreatitis induction. Blood samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 5$. ¤$P < 0.05$ versus saline control, #$P < 0.05$ versus WT and *$P < 0.05$ versus control Ab.

Role of LFA-1 in taurocholate-induced neutrophil recruitment in the pancreas

Levels of MPO, a neutrophil indicator, in the pancreas peaked 24 h after taurocholate challenge (21-fold increase). Pancreatic MPO activity was reduced by more than 80% in LFA-1 gene-targeted mice and in animals receiving the anti-LFA-1 antibody (Figure 4A). Similarly, histological quantification of taurocholate-provoked neutrophil infiltration revealed markedly reduced numbers of pancreatic neutrophils in LFA-1-deficient and in anti-LFA-1 antibody-treated mice (Figure 2C). Systemic inflammation such as pulmonary infiltration of neutrophils is a central feature of severe AP. Challenge with taurocholate provoked a clear-cut increase in MPO activity in the lung (Figure 4B). Taurocholate-induced pulmonary levels of MPO were markedly reduced in LFA-1-deficient animals and in mice treated with an antibody against LFA-1 (Figure 4B). We used intravital microscopy of the pancreatic microcirculation in order to study the role of LFA-1 in leucocyte-endothelium interactions in AP. Taurocholate challenge triggered a clear-cut increase in leucocyte-endothelium interactions in the pancreas (Figure 5 and Video S1A,B). It was found that taurocholate challenge increased leucocyte rolling and adhesion by threefold and sevenfold respectively, in postcapillary venules of the pancreas (Figure 5C,D). Notably administration of taurocholate did not enhance leucocyte interactions or trapping in the pancreatic capillaries (Video S2). Inhibition of LFA-1 function did not reduce taurocholate-induced leucocyte rolling (Figure 5C and Video S1B). In contrast, it was observed that taurocholate-induced leucocyte adhesion was decreased by 61% in animals treated with the anti-LFA-1 antibody (Figure 5D). Moreover, the number of firmly adherent leucocytes in taurocholate-treated mice deficient in LFA-1 was reduced by 75% (Figure 5D and Video S1B). We found also that the number of circulating mononuclear leucocytes and neutrophils increased in severe AP, indicating systemic activation in this model (Table 1).

Role of LFA-1 in taurocholate-induced chemokine formation in the pancreas and serum

At baseline levels of CXCL2 in the pancreas were 0.2 ng·pg^{-1}. Administration of taurocholate caused a 13-fold and eightfold increase in the levels of CXCL2 in the pancreas

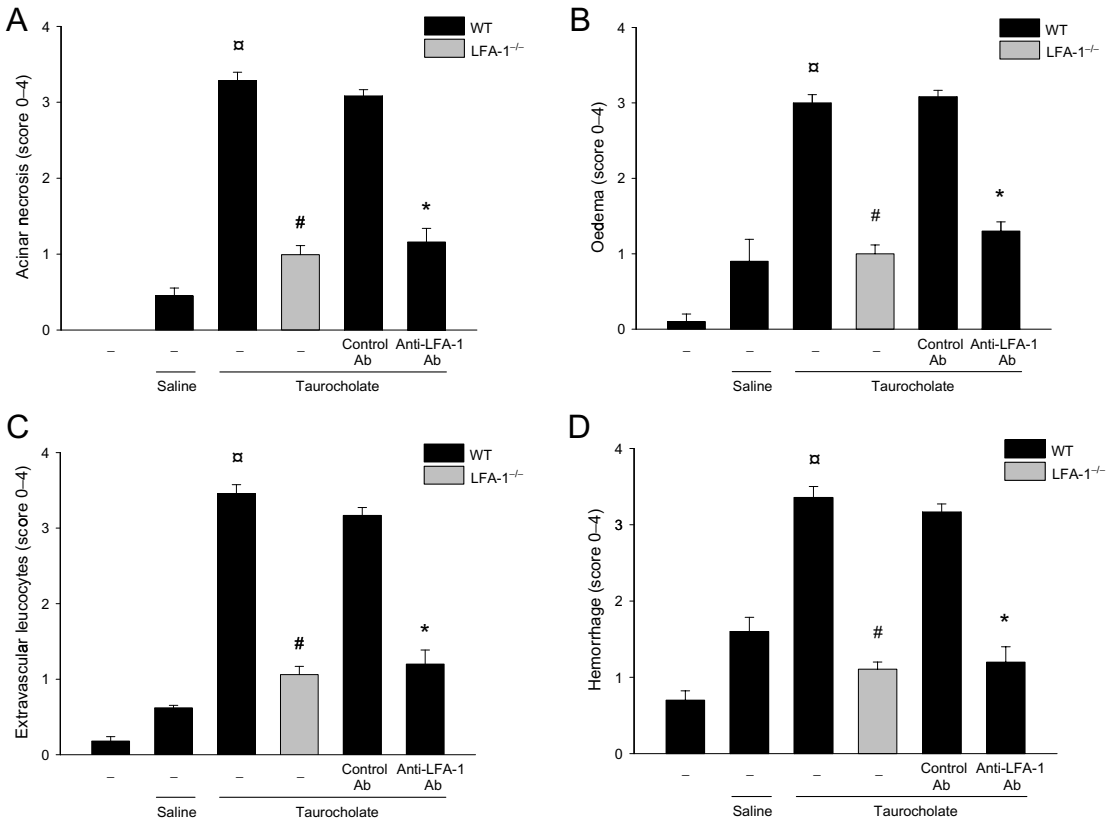

Figure 2

Taurocholate-induced tissue damage in the pancreas. (A) Acinar cell necrosis (B) oedema formation (C) infiltration of neutrophils in the pancreas and (D) haemorrhage. Control mice received saline alone. Certain mice received a control antibody (Ab) or an Ab against LFA-1 prior to pancreatitis induction. Blood samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 5$. $^{\circ}P < 0.05$ versus saline control, $\#P < 0.05$ versus WT and $*P < 0.05$ versus control Ab.

(Figure 6A) and serum (Figure 6B) respectively. It was observed that immunoneutralization of LFA-1 decreased taurocholate-induced production of CXCL2 in the pancreas by 64% (Figure 6A). Also, we found that taurocholate-provoked formation in the serum was reduced by 63% in LFA-1-deficient animals (Figure 6B).

Role of LFA-1 in taurocholate-induced protease activation in the pancreas

Trypsinogen activation into trypsin was determined by measuring pancreatic levels of TAP. Taurocholate administration significantly enhanced trypsinogen activation reflected by a more than twofold increase in TAP levels in the pancreas (Figure 7). However, it was observed that taurocholate-induced activation of trypsinogen was not changed in LFA-1 gene-targeted animals ($P > 0.05$ vs. wild-type, Figure 7) or in mice treated with the anti-LFA-1 antibody ($P > 0.05$ vs. control antibody, Figure 7).

Discussion and conclusions

This study documents an important role of LFA-1 in AP. Our findings show that LFA-1 is a key regulator of neutrophil infiltration into the pancreas by regulating firm adhesion in postcapillary venules. Interference with LFA-1 not only decreased adhesion and recruitment of neutrophils but also protected against tissue damage in AP. However, these data show that trypsinogen activation into trypsin is not apparently dependent on LFA-1 in AP, suggesting that LFA-1-mediated inflammation is a downstream component of protease activation in the pathophysiology of AP. Taken together; these novel results indicate that targeting LFA-1

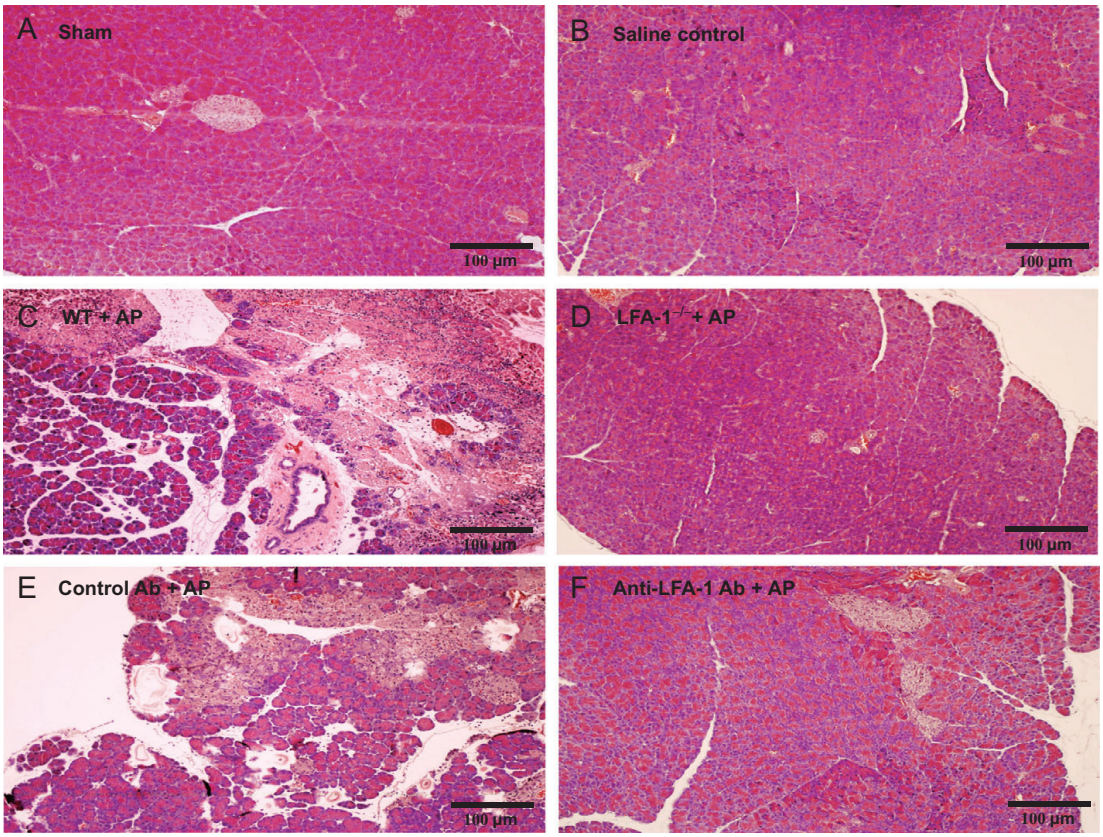

Figure 3

Representative haematoxylin & eosin sections of the pancreas from wild-type (WT) of (A) sham (B) saline infused to the pancreas (C) pancreatitis (D) lymphocyte function antigen-1 (LFA-1)-deficient mice with pancreatitis (E) control antibody (Ab) with pancreatitis and (F) anti-LFA-1 Ab with pancreatitis. Pancreatitis was induced by infusion sodium taurocholate into the pancreatic duct. Control mice received saline alone. Certain mice received a control Ab or an Ab against LFA-1 prior to pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Bars represent 100 μm.

may be an effective approach to ameliorate pathological inflammation in AP.

It is well recognized that leucocyte recruitment is a fundamental feature in inflammatory diseases. Numerous mechanisms of neutrophil-mediated tissue injury have been forwarded. For example, neutrophils are potent producers of reactive oxygen species (ROS), such as hydroxyl radicals and superoxide, which can exert harmful effects on tissue and endothelial cells in the pancreas (Mossman, 2003). Indeed, several studies have demonstrated that depletion of neutrophils protects against tissue injury in AP (Frossard *et al.*, 1999; Gukovskaya *et al.*, 2002). LFA-1 has been shown to mediate neutrophil adhesion and tissue recruitment (Ding *et al.*, 1999; Thorlacius *et al.*, 2000) but the role of LFA-1 in AP is not known. Our data show that LFA-1-deficient mice exhibited significantly reduced acinar cell necrosis, tissue oedema and haemorrhage as well as serum amylase, indicating that LFA-1 plays an important role in mediating organ damage in AP. This notion was confirmed by our findings that immunoneutralization of LFA-1 markedly decreased taurocholate-induced pancreatic tissue destruction and serum amylase levels. Thus, these data suggest for the first time that LFA-1 is a key regulator of pancreatic injury in AP. This adds AP to the list of conditions in which LFA-1 has turned out to be a significant target; these include septic and cholestatic liver injury (Li *et al.*, 2004; Dold *et al.*, 2008), alcoholic liver disease (Ohki *et al.*, 1998), viral hepatitis (Matsumoto *et al.*, 2002), endotoxaemia (Li *et al.*, 2004), graft-versus-host disease (Kimura *et al.*, 1996; Sato *et al.*, 2006) and colonic ischaemia-reperfusion (Wan *et al.*, 2003). In this context, it should be mentioned that a previous study reported that depletion of neutrophils increases pancreatic haemorrhage in response to taurocholate challenge (Ryschich *et al.*, 2009), suggesting that leucocytes protect against haemorrhage in AP. This is in

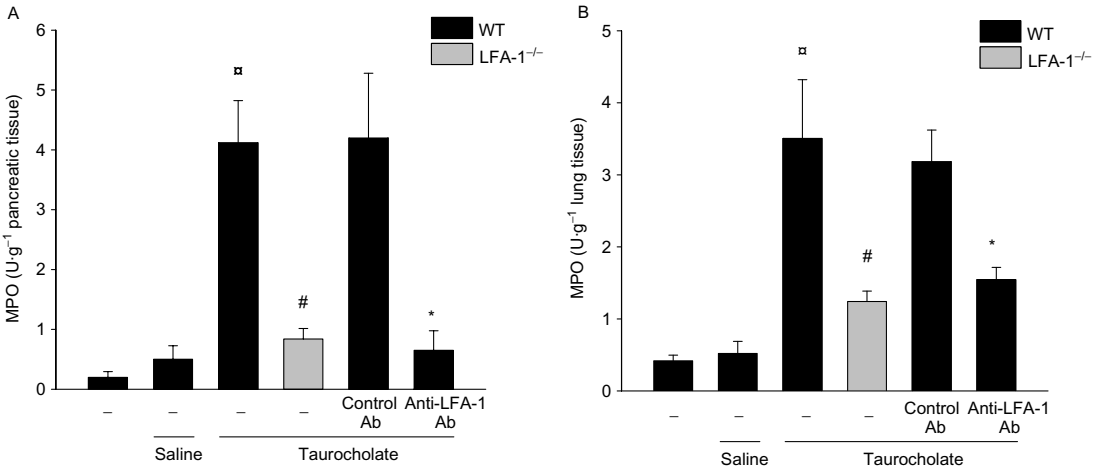

Figure 4

Myeloperoxidase (MPO) levels (U·g^{-1} tissue) for (A) pancreas and (B) lung in wild-type (WT) and lymphocyte function antigen-1 (LFA-1)-deficient mice. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice received saline alone. Certain mice received a control antibody (Ab) or an Ab against LFA-1 prior to pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 5$. ¤$P < 0.05$ versus saline control, #$P < 0.05$ versus WT and *$P < 0.05$ versus control Ab.

Table 1

Systemic leucocyte differential counts

	PMNL	MNL	Total
WT: sham	0.8 ± 0.1	4.5 ± 0.1	5.3 ± 0.2
WT: saline	0.8 ± 0.1	4.6 ± 0.1	5.4 ± 0.2
WT: taurocholate	1.8 ± 0.2¤	6.7 ± 0.6¤	8.5 ± 0.8¤
LFA-1-deficient: taurocholate	3.9 ± 0.4	11.8 ± 0.3	15.7 ± 0.7
WT + control Ab: taurocholate	1.9 ± 0.1	5.9 ± 0.1	7.8 ± 0.2
WT + anti-LFA-1 Ab: taurocholate	1.3 ± 0.3	6.4 ± 0.1	7.7 ± 0.4

Blood samples were collected from wild-type (WT) and lymphocyte function antigen-1 (LFA-1) gene-targeted mice. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice received saline alone. Certain mice received a control antibody (Ab) or an Ab against LFA-1 prior to pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Cells were identified as monomorphonuclear leucocytes (MNL) and polymorphonuclear leucocytes (PMNL). Data represent means ± SEM, 10^6 cells·mL^{-1} and $n = 5$. ¤$P < 0.05$ versus saline control.

contrast to previous studies showing that neutrophil depletion reduces tissue damage in AP (Weiss, 1989; Gukovskaya *et al.*, 2002) and we have also recently depleted mice of neutrophils and found no signs of increased haemorrhage but instead a clear-cut decrease in taurocholate-induced haemorrhage in the pancreas, suggesting that neutrophils do not protect against tissue haemorrhage in AP (data not shown). In fact, this notion is also supported by our present findings showing that inhibition of neutrophil accumulation in the pancreas by targeting LFA-1 function also reduced taurocholate-induced haemorrhage in the pancreas.

The extravasation of leucocytes is a multistep process supported by a sequential engagement of adhesive receptors, such as selectins and integrins (Butcher, 1991). Although the function of these receptors has been extensively studied in certain organs, the role of specific adhesion molecules in pancreatic infiltration of leucocytes is virtually unknown. Two previous studies have reported that LFA-1 expression is increased on the surface of circulating neutrophils in pancreatitis (Sun *et al.*, 2006; 2007). We have extended these observations and demonstrated herein that genetic deficiency or functional inhibition of LFA-1 greatly reduces pancreatic infiltration of neutrophils, suggesting that LFA-1 mediate tissue accumulation of neutrophils in AP. This finding is also supported by a previous study showing that chemoattractant-induced leucocyte recruitment in the pancreas is mediated by LFA-1 (Ryschich *et al.*, 2009). By use of intravital microscopy, we could document a direct and dominating role of LFA-1 in supporting firm adhesion of leucocytes in the postcapillary venules of the microcirculation in AP. Systemic depletion of neutrophils abolished leucocyte-endothelium interactions in the pancreas, suggesting that neutrophils constitute the main leucocyte subtype interacting with the microvascular endothelium in AP (not shown). Although our findings show that LFA-1 is the predominant adhesion molecule supporting pancreatic adhesion and infiltration of neutrophils, these data do not exclude the possibility that other β_2-integrins may also be important in AP. For example, Hentzen *et al.* (2000) have shown that Mac-1 and LFA-1 cooperate for optimal

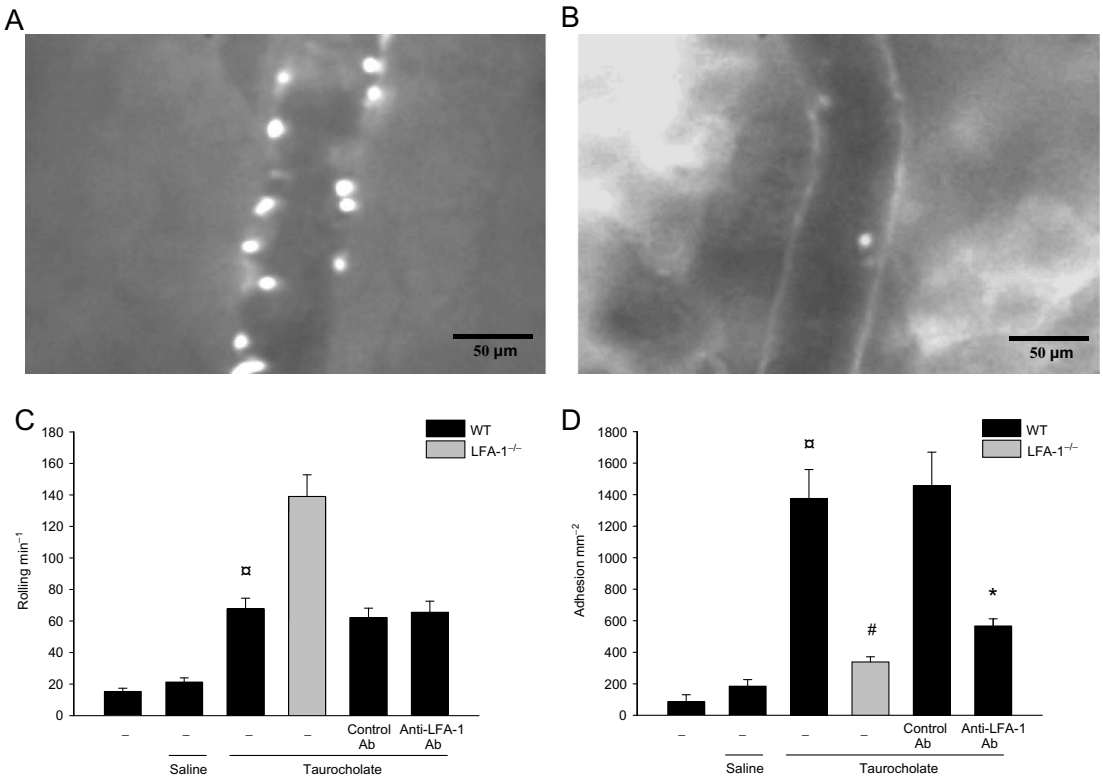

Figure 5

Leucocyte-endothelium interactions in the pancreas. Intravital photos of postcapillary venules in the pancreas after pancreatitis in (A) wild-type (WT) and (B) lymphocyte function antigen-1 (LFA-1)-deficient animals after i.v. administration of an anti-P-selectin antibody (40 µg). The anti-P-selectin antibody was given to abolish leucocyte rolling and leave the remaining firmly adherent leucocytes visible. Quantification of (C) leucocyte rolling (cells·min^{-1}) and (D) adhesion (cells·mm^{-2}) in WT and lymphocyte function antigen-1 (LFA-1)-deficient mice. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice received saline alone. Certain mice received a control antibody (Ab) or an Ab against LFA-1 prior to pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 5$. $^{\circ}P < 0.05$ versus saline control, $\#P < 0.05$ versus WT and $*P < 0.05$ versus control Ab.

recruitment of inflammatory cells, that is, LFA-1 initiates first stable contact and Mac-1 establishes a more sustainable adhesion onto the endothelium of inflamed organs. In this context, it is interesting to note that one previous study has reported that inhibition of LFA-1 decreases neutrophil formation of ROS in AP (Inoue *et al.*, 1996). Thus, considered collectively, these data suggest that LFA-1 may be of importance at several steps in the pathophysiology of AP, including both tissue leucocyte recruitment and ROS-mediated organ damage. Activation and extravascular navigation of neutrophils are orchestrated by secreted CXC chemokines, such as CXCL2 (Bacon and Oppenheim, 1998). In the present study, we found that both pancreatic and systemic levels of CXCL2 were markedly enhanced after taurocholate challenge. Interestingly, taurocholate-induced formation of CXCL2 was significantly decreased in LFA-1-deficient animals. Similarly, inhibition of LFA-1 function also attenuated tissue formation of CXCL2 in AP. These observations are somewhat surprising considering that CXC chemokines are largely secreted by cells resident in the tissue of the pancreas (Bradley *et al.*, 1999). Nonetheless, our findings indicate that LFA-1 exerts an early feature in the pathophysiology of pancreatitis upstream of CXC chemokine formation. Thus, our data suggest that LFA-1-mediated functions regulate subsequent formation of CXCL2 in AP. The link between LFA-1 function and CXCL2 production is speculative but may be related to pro-inflammatory compounds secreted from activated leucocytes, which in turn may activate tissue-resident cells in the pancreas. For example, LFA-1-depedent formation of ROS may be involved as ROS have been shown to have the capacity to stimulate chemokine formation (Riaz *et al.*, 2003; Kina *et al.*, 2009).

Inflammation and trypsinogen activation are recognized as central components in the pathophysiology of AP. However, the relationship between neutrophil recruitment on

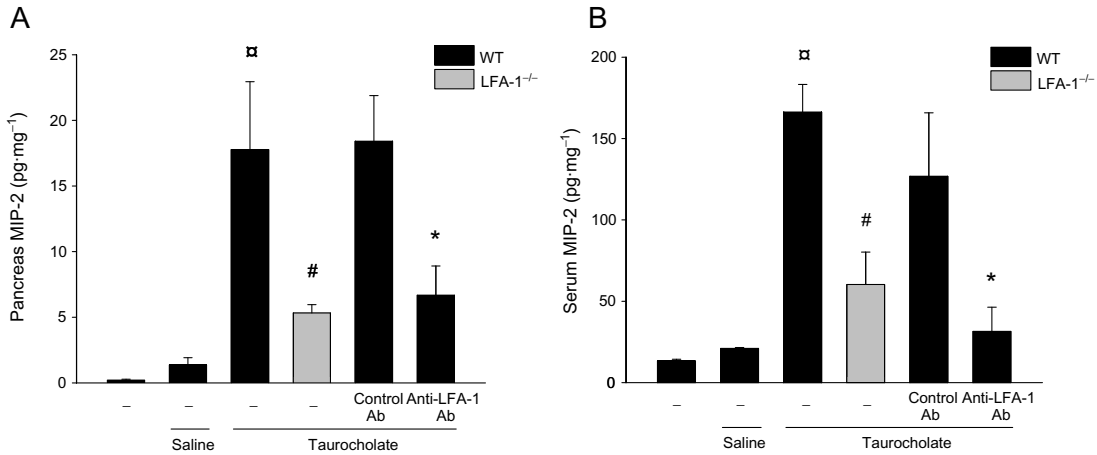

Figure 6

CXCL2 in the (A) pancreas and (B) serum in wild-type (WT) and lymphocyte function antigen-1 (LFA-1)-deficient mice. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice received saline alone. Certain mice received a control (Ab) or an Ab against LFA-1 prior to pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and n = 5. $^{\circ}P < 0.05$ versus saline control, #$P < 0.05$ versus WT and *$P < 0.05$ versus control Ab.

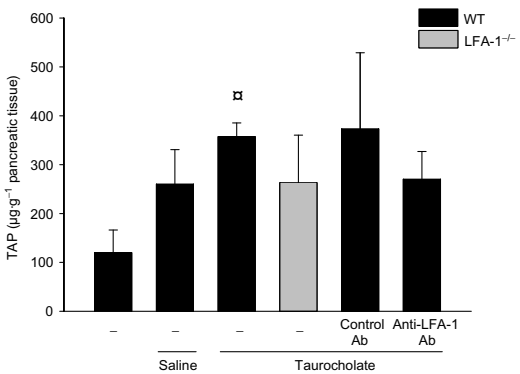

Figure 7

Trypsinogen activation peptide (TAP) levels (µg·g^{-1} pancreatic tissue) in wild-type (WT) and lymphocyte function antigen-1 (LFA-1)-deficient mice. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice received saline alone. Certain mice received a control antibody (Ab) or an Ab against LFA-1 prior to pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and n = 5. $^{\circ}P < 0.05$ versus saline control.

one hand and protease activation on the other hand in the pancreas is not known. Since we found that LFA-1 was an important regulator of pancreatic infiltration of neutrophils, we next asked whether LFA-1 controls activation of trypsinogen into trypsin, which is associated with the formation of TAP. Notably, a previous investigation has shown that levels of TAP correlate with disease severity in the early phases of AP (Frossard, 2001). Indeed, we observed that taurocholate challenge markedly increased the levels of TAP in the pancreas. However, taurocholate-induced levels of TAP were not altered in LFA-1 gene-targeted animals or in mice treated with a blocking antibody directed against LFA-1. These findings indicate that trypsinogen activation is independent of LFA-1-mediated neutrophil accumulation in the pancreas. Thus, pancreatic infiltration of neutrophils seems not to be a precondition for protease activation in AP. Whether neutrophils may exert intravascular functions, such as secretion of pro-inflammatory compounds, which may trigger intrapancreatic activation of trypsinogen cannot be excluded by our present findings. It should be noted that trypsin is a potent activator of proteinase-activated receptor-2 (PAR2), which is a 7-transmembrane G-protein-coupled receptor expressed by pancreatic acinar and ductal cells (Nguyen *et al.*, 1999). A recent study reported that taurocholate-triggered calcium transients, kinase activation and acinar cell injury is markedly reduced in isolated pancreatic acini from PAR2 gene-deficient mice, suggesting that PAR2 activation may support acinar cell damage in AP (Laukkarinen *et al.*, 2008). Whether trypsin-mediated activation of PAR2 may explain LFA-1-independent effects, such as trypsinogen activation, in the present study is a matter of future studies. Nonetheless, considering that activation of trypsinogen seems to be an early process, neutrophil recruitment and inflammation in the pancreas persists longer and targeting LFA-1 might be a more favourable strategy for specific therapeutic interventions (Regnér *et al.*, 2008).

In conclusion, our novel data demonstrate not only that neutrophil adhesion and infiltration in AP are mediated by LFA-1 but also that LFA-1-dependant recruitment of

neutrophils regulates tissue damage in the pancreas. In addition, these findings also indicate that trypsinogen activation is independent of LFA-1-mediated neutrophil accumulation in the pancreas. Taken together, we conclude that LFA-1 may be a useful target to antagonize pathological inflammation in AP.

Acknowledgements

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Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (A) mRNA and (B) protein expression of CD11a mRNA in peripheral neutrophils from wild-type (WT) and lymphocyte function antigen-1-deficient (LFA-1$^{-/-}$) mice. β-Actin serves as a housekeeping gene. The results presented are from one experiment, which is representative of four others.
Video S1 Intravital fluorescence clip of a postcapillary venule in a) wild-type and b) lymphocyte function antigen-l-deficient mouse. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. The video was recorded 24 h after induction of pancreatitis.
Video S2 Intravital fluorescence clip of capillaries in a wild-type mouse. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. The video was recorded 24 h after induction of pancreatitis.

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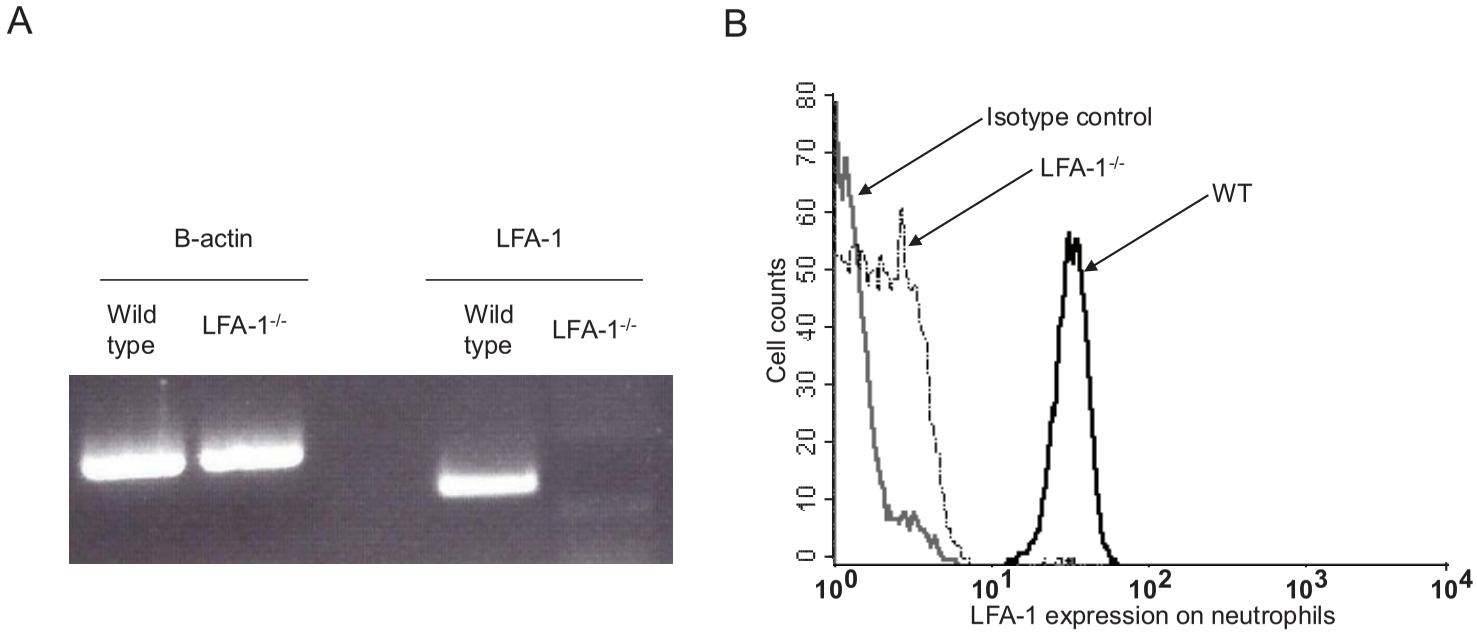

Supplementary Figure 1

Paper III

Neutrophil-derived matrix metalloproteinase-9 is a potent activator of trypsinogen in acinar cells in acute pancreatitis

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Running title: MMP-9 and acute pancreatitis

Key words: Metalloproteinases, amylase, chemokines, inflammation, neutrophils, pancreas

Summary sentence: Neutrophil-derived MMP-9 regulates trypsinogen activation, pathological inflammation and tissue damage in acute pancreatitis.

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Abbreviations
(AP) acute pancreatitis; (HPF) high power field; (KO) knock-out; (CXCL2/MIP-2); macrophage inflammatory protein-2; (Mac-1) macrophage-1 antigen; (MMP) matrix metalloproteinase; (MNL) monomorphonuclear leukocytes; (MPO) myeloperoxidase; (PBS) phosphate-buffered saline; (PMNL) polymorphonuclear leukocytes; (RIA) radioimmunoassay; (TAP) trypsinogen activation peptide; (TIMP) tissue inhibitor of metalloproteinases; (WT) wild-type

Abstract

Matrix metalloproteinases (MMPs) are generally considered to regulate degradation and remodeling of the extracellular matrix. Convincing data also implicate a role for MMPs in inflammatory conditions, such as acute pancreatitis (AP), although the mechanisms are not known. The aim of this study was to define the role of MMPs in regulating activation of trypsinogen and tissue damage in AP. AP was induced by infusion of taurocholate into the pancreatic duct in mice. A broad-spectrum MMP inhibitor (BB-94) and MMP-9 gene-deficient mice were used. Neutrophil secretions and recombinant MMP-9 were used to stimulate trypsinogen activation in isolated acinar cells. Taurocholate challenge increased serum amylase, neutrophil infiltration, macrophage inflammatory protein-2 (CXCL2) formation, trypsinogen activation and tissue damage in the pancreas. Treatment with the broad spectrum inhibitor of MMPs BB-94 markedly reduced activation of trypsinogen, levels of CXCL2, infiltration of neutrophils and tissue damage in AP. Taurocholate challenge increased serum levels of MMP-9 but not MMP-2. Taurocholate-induced amylase levels, neutrophil accumulation, production of CXCL2, trypsinogen activation and tissue damage in the pancreas were abolished in MMP-9-deficient mice. Moreover, secretions from activated neutrophils isolated from wild-type but not from MMP-9-deficient animals stimulated trypsinogen activation in acinar cells. Notably, recombinant MMP-9 greatly enhanced activation of trypsinogen in acinar cells. These findings demonstrate that neutrophil-derived MMP-9 is a potent activator of trypsinogen in acinar cells and regulates pathological inflammation and tissue damage in AP.

Based on the findings, this manuscript will be featured by *JLB* under the Frontline Science Section of *JLB* as "Leading Edge Research" in the Table of Contents.

Introduction

The clinical presentation of acute pancreatitis (AP) ranges from a mild and self-limiting inflammation to a severe necrotizing disease [1, 2]. Severe pancreatitis poses a major challenge to clinicians and the mortality is close to 30% in this group of patients [3]. Specific treatment for patients with AP is lacking and management is restricted to supportive care, which is related to our incomplete understanding of the pathophysiology in AP. It is widely held that trypsinogen activation and neutrophil infiltration in the pancreas represent key components in the progression of AP [4-7]. The interrelationship between proteolytic activation and inflammation in AP is complex and not very well understood. On one hand, several studies have shown that inhibition of neutrophil infiltration into the pancreas does not attenuate trypsinogen activation in AP [8, 9]. On the other hand, a recent study showed that systemic depletion of neutrophils markedly decrease activation of trypsinogen in AP [10]. Thus, combining these findings, it appears that compound(s) released from circulating neutrophils may be involved in the regulation of trypsinogen activation in the pancreas. However, the identity of such neutrophil-derived substances remains elusive.

Matrix metalloproteinases (MMPs) comprise a super family of more than 25 structurally and functionally related endopeptidases [11] with capacity to cleave the majority of matrix proteins as well as many non-matrix targets, such as chemokines, cytokines, adhesion molecules and surface receptors [12]. In this context, it is interesting to note that previous investigations have reported that certain MMPs, in particular members of the gelatinase subfamily (MMP-2 and MMP-9), are increased in the plasma in different experimental models of pancreatitis [13-16]. In addition, one recent study showed that MMP-9 is elevated in

	vehicle + saline (n=5)	vehicle + taurocholate (n=5)	BB-94 + taurocholate (n=5)
Blood amylase (μKat/L)	46.2 ± 2	571.6 ± 35.7#	135 ± 34.4*
MPO in the pancreas (U/g)	0.07 ± 0.03	1.8 ± 0.1#	0.34 ± 0.2*
MPO in the lung (U/g)	0.7 ± 0.06	2.6 ± 0.6#	1.1 ± 0.14*
CXCL2 in the pancreas (pg/mg)	0.16 ± 0.1	5.4 ± 0.9#	1 ± 0.2*
CXCL2 in the serum (pg/mL)	3.6 ± 1	55.1 ± 15#	12.6 ± 3.6*

Table 1: Role of MMPs in taurocholate-induced pancreatitis. Acute pancreatitis was induced by retrograde infusion of 10 μl sodium taurocholate (5%) into the pancreatic duct in vehicle + saline, vehicle + taurocholate and BB-94 + taurocholate. Control mice (vehicle + saline) underwent infusion of 10 μl saline into the pancreatic duct. Blood amylase, myeloperoxidase (MPO) in the pancreas and lung, as well as levels of macrophage inflammatory protein-2 (CXCL2) in pancreas and serum were determined 24 h after pancreatitis induction. Values represent Mean ± SEM. $^{\#}P < 0.05$ versus vehicle + saline. $^{*}P < 0.05$ versus vehicle + taurocholate.

the serum of patients with pancreatitis and was forwarded as a potential prognostic marker in AP [17]. Accumulating data in the literature have demonstrated that treatment with broad-spectrum inhibitors of MMPs attenuates leukocyte recruitment to the pancreas and distant organs in AP [14, 15, 18-20]. However, the role of MMPs in regulating trypsinogen activation in AP has not been examined. Interestingly, neutrophils contain abundant quantities of MMP-9 in secretory granules [21], which made us hypothesize that neutrophil-derived MMP-9 might be involved in trypsinogen activation.

Based on the above considerations, we hypothesized herein that MMPs and in particular MMP-9 might be involved in the regulation of trypsinogen activation in AP. For this purpose, we used a broad-spectrum inhibitor of MMPs and MMP-9 gene-deficient mice in an experimental model of AP based on retrograde infusion of taurocholate in mice.

Materials and Methods

Animals

All experiments were done in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for animal experimentation at Lund University, Sweden. Wild-type C57BL/6 and MMP-9-deficient mice (B6.FVB(Cg)-Mmp9^{tm1Tvu}/J) were purchased (Jaxmice, Sacramento, USA) and used at age 6–8 weeks (20-26 g). All animals were maintained in a climate-controlled room at 22°C and exposed to a 12:12-h light-dark cycle. Animals were fed standard laboratory diet and given water *ad libitum*. Mice were anesthetized by intraperitoneal (i.p.) administration of 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight in 200 μl saline.

Experimental protocol

AP was induced by retrograde infusion of bile salt (taurocholate) into the pancreas as previously described [22, 23]. Briefly the duodenum and the attached pancreatic head were exposed through a midline incision. The papilla of Vater was identified, the duodenum was immobilized by two 7-0 prolene sutures and a small puncture was made through the duodenal wall in parallel to the papilla of Vater with a 23G needle. A polyethylene catheter (internal diameter 0.28 mm) connected to a micro-infusion pump

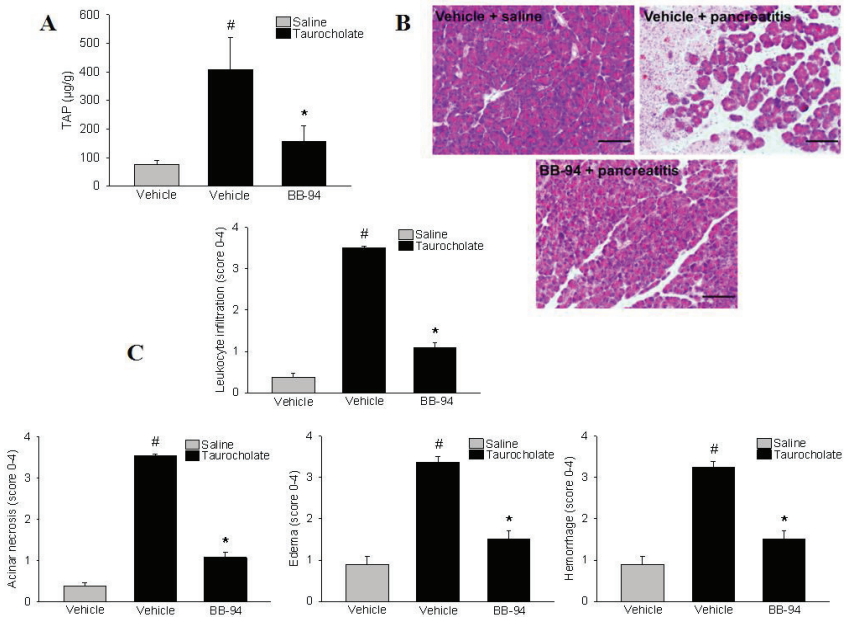

Figure 1. MMPs regulate trypsinogen activation and tissue damage in AP. A) Quantitative measurements of TAP levels (µg/g) in the pancreas, B) representative hematoxylin & eosin sections of the pancreas and C) quantitative analysis of leukocyte infiltrate, acinar cell necrosis, edema and hemorrhage in the pancreas. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline into the pancreas and i.p. vehicle injection. MMPs were inhibited by i.p. injections of BB-94 once daily for 2 days and just before pancreatitis induction. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus vehicle + saline, $^{*}P < 0.05$ *vs.* vehicle + taurocholate. Bars represent 100 µm.

(CMA/100, Carnegie Medicin, Stockholm, Sweden) was introduced 1 mm into the pancreatic duct. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp to prevent hepatic reflux. Overall 10 µl of either saline or 5% taurocholate (Sigma Chemical Company, St. Louis, Missouri, USA) was infused into the pancreatic duct at a rate of 2 µl/min. Before suturing the abdominal wall, the bile duct clip was removed and the duodenal puncture closed (7-0 prolene). Animals were sacrificed 24 h after induction of AP. Blood was obtained from the vena porta and plasma was frozen at -20˚C. The stomach, duodenum and the pancreatic head were rapidly removed in one piece. The pancreatic head was carefully separated from the duodenum to avoid any contamination by mucosal enterokinase. Two thirds of the pancreatic sample and lung was divided in two pieces and flash frozen in liquid nitrogen while the rest was fixed in formaldehyde. For *in vivo* experiments using the MMP inhibitor, animals were randomized into three groups: 1) a positive taurocholate-infused group pre-treated with vehicle (saline, $n = 5$), 2) a negative control also operated but infused with saline only ($n = 5$) and 3) a taurocholate-infused group treated with the MMP inhibitor BB-94 (Calbiochem®, Darmstadt, Germany) (40 mg/kg body weight/dose) ($n = 5$), administered i.p., 48 h, 24 h and just before AP induction. For experiments using the MMP-9 gene deficient mice, animals were randomized

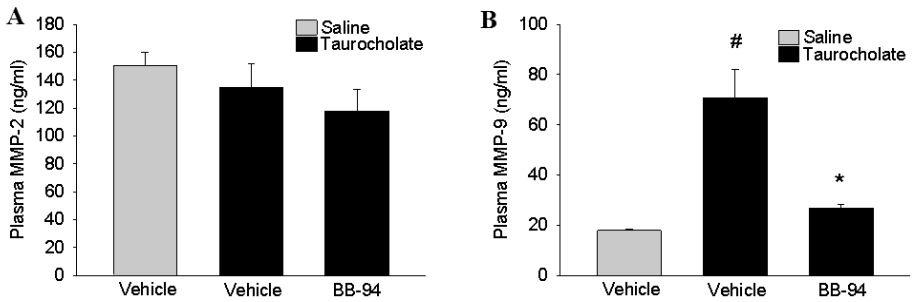

Figure 2. MMP-9 levels are increased in AP. Plasma levels (ng/ml) of A) MMP-2 and B) MMP-9 were determined 24 h after pancreatitis induction. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline into the pancreatic duct and i.p. vehicle injection. MMPs were inhibited by i.p. injections of BB-94 once daily for 2 days and just before pancreatitis induction. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus vehicle + saline, $^{*}P < 0.05$ versus vehicle + taurocholate.

into three groups: 1) wild-type mice infused with taurocholate ($n = 5$), 2) wild-type mice operated but infused with saline only ($n = 5$) and 3) MMP-9 gene-deficient mice infused with taurocholate ($n = 5$). Blood was collected from the tail vein for determination of blood amylase levels 24 h after induction of pancreatitis.

Blood amylase

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

MPO assay

Frozen pancreatic and lung tissue were pre-weighed and homogenized in one ml mixture (4:1) with PBS and aprotinin 10 000 KIE/ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for one min. The homogenate was centrifuged (15300 g, 10 min) and the supernatant was stored at -20°C and the pellet was used for MPO assay as previously described [24]. In brief, the pellet was mixed with one ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 sec, put in water bath 60°C for two h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units per gram tissue.

ELISA

Plasma levels of MMP-2 and MMP-9, serum and pancreatic homogenate levels of CXCL2 were analyzed by using commercially available ELISA kits (R & D Systems). The MMP-2 and MMP-9 ELISA kits recognize pro-, active, and tissue inhibitor of metalloproteinases (TIMP)-complexed forms of MMP-2 and MMP-9. Total MMP-2 and MMP-9 were analyzed in heparinized plasma according to manufacturer's protocols and centrifuged for 20 min at 2000 g immediately after collection. An additional centrifugation at 10000 g for 10 min at 4°C was employed for complete removal of platelets and stored at -20°C for further use. Plasma samples were then diluted 10 times with a sterile buffer (10% fetal calf serum in PBS, pH 7.4) to overcome the matrix effects and analyzed as per the protocols provided.

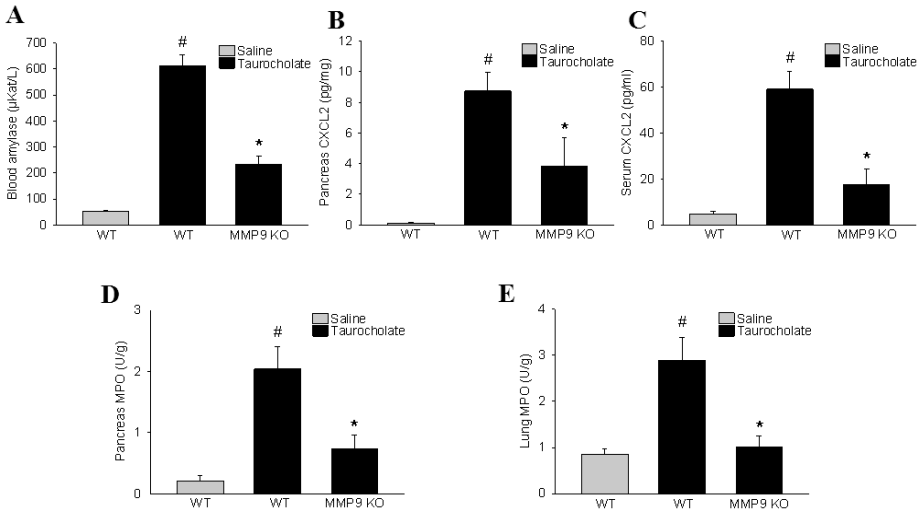

Figure 3. Quantitative measurements of A) pancreatic CXCL2 (pg/mg) B) serum CXCL2 (pg/ml) as well as MPO levels (U/g) in the C) pancreas and D) lung MPO in wild-type (WT) and MMP-9 knock-out (MMP-9 KO) mice. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline into the pancreas. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus WT + saline, $^{*}P < 0.05$ versus WT + taurocholate.

Histology

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

TAP levels

Trypsinogen is activated to trypsin in a reaction where TAP is cleaved off and thus can be used as marker of trypsinogen activation [26]. The RIA was performed as described previously [27]. A 0.1 M Tris HCl buffer (PH 7.5) containing 0.15 M NaCl, 0.005 M EDTA and 2 g/l bovine serum albumin (Sigma-Aldrich) was used as assay buffer. Samples of 100 µl diluted in assay buffer were incubated (16 h, 4°C) with 200 µl of I^{125}Tyr-TAP (=20 000 counts per min) in assay buffer and 200 µl of antiserum diluted 1/750 in assay buffer. Parallel incubations with the synthetic activation peptides TAP diluted in assay buffer in a series of concentrations from 0.078 to 20 nM, were used as standards in the assays. Free and bound radioactivities were separated by means of a second step antibody precipitation. For this, 100 µl of a cellulose coupled anti-mouse IgG suspension (Sc-Cel® IDA, Boldon, England) was added to the samples. After 30 min of incubation 1 ml of water was added and tubes were centrifuged (704 g, 5 min, room temperature). The supernatant was decanted and radioactivity of the precipitate was counted in a γ-spectrophotometer.

Intravital microscopy

Analysis of leukocyte rolling and adhesion was performed in postcapillary venules in

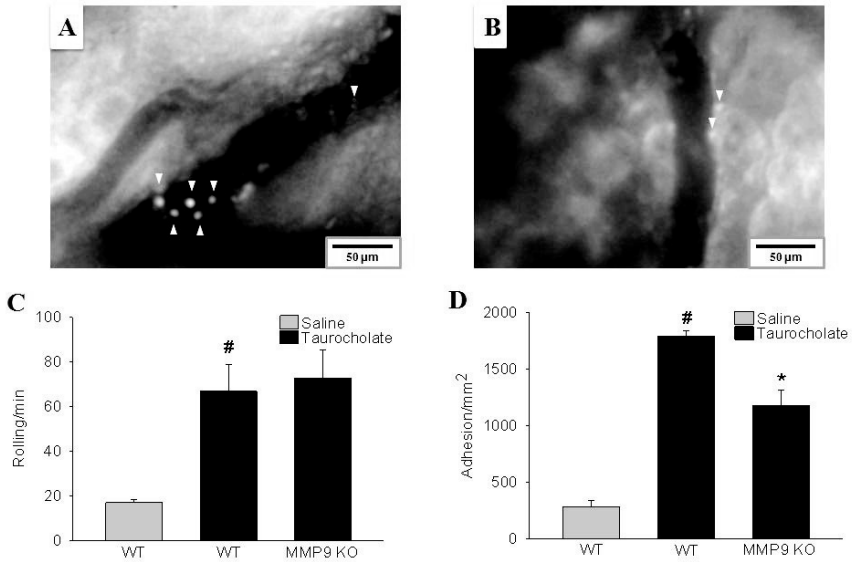

Figure 4. Leukocyte-endothelium interactions in the pancreas. Intravital micrographs of postcapillary venules in the pancreas 24 h after pancreatitis induction in A) wild-type and B) MMP-9-deficient animals after intravenous administration of an anti-P-selectin antibody (40µg). The anti-P-selectin antibody was given to abolish leukocyte rolling and leave the remaining firmly adherent leukocytes visible. Quantification of leukocyte C) rolling (cells/min) and D) adhesion (cells/mm^2) in wild-type (WT) and MMP-deficient (MMP-9 KO) mice. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline into the pancreas. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus WT + saline and $^{*}P < 0.05$ versus WT + taurocholate.

the pancreas in 10 C57BL/6 wild-type and 5 MMP-9-deficient male mice. Contrast enhancement by intravenous injection of fluorescence isothiocyanate-labelled dextran 150 000 (0.05 ml, 5 mg/ ml, Sigma Chemical Co.) and *in vivo* labeling of leukocytes with rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma Chemical Co.) enabled analysis of leukocyte-endothelium interactions in the microvascular bed. For observations of the microcirculation, we used a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) and recorded videos on a computer for later off-line analysis of leukocyte-endothelium interactions. In each animal, 2-6 postcapillary venules were evaluated and a 5-min equilibration time was allowed before recording. Leukocyte rolling was measured by counting the number of cells rolling along the endothelial lining during 20 sec and is expressed as cells per minute. Leukocyte adhesion was measured by counting the number of cells that adhered and remained stationary for more than 30 sec during the observation time and is expressed as cells per mm^2. Certain animals received an anti-P-selectin antibody (40 µg, intravenous, clone RB40.34, BD Biosciences Pharmingen) immediately before capturing microphotographs of the postcapillary venules in the pancreas in order to abolish leukocyte rolling and thereby enable visualization of the remaining leukocytes that were firmly adherent to the endothelium.

Trypsinogen activation in isolated acinar cells

Bone marrow neutrophils were freshly extracted from healthy C57BL/6 and MMP9-deficient mice by using Ficoll-

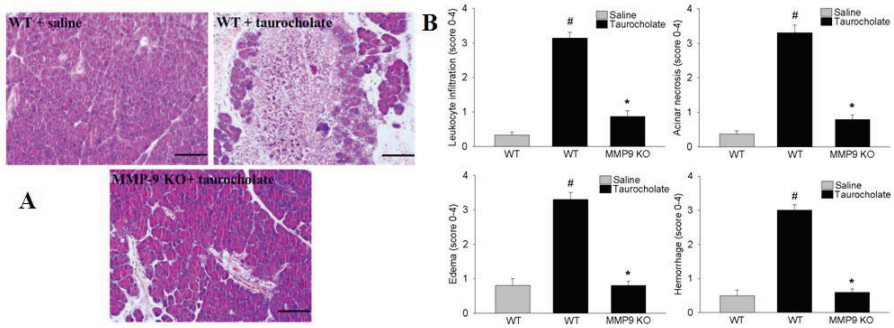

Figure 5. MMP-9 regulates pancreatic tissue damage in AP. A) Representative hematoxylin & eosin sections of the pancreas and B) quantitative analysis of leukocyte infiltrate, acinar cell necrosis, edema and hemorrhage in the pancreas in wild-type and MMP-9-deficient animals. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline into the pancreas. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus WT + saline and $^{*}P < 0.05$ versus WT + taurocholate. Samples were obtained 24 h after induction of pancreatitis. Bars represent 100 μm.

Paque research grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was >80%, as assessed by Turk's stain in a hematocytometer [28]. Neutrophil activation was achieved after 30 min of incubation (10^7 cells/ml) with CXCL2 in 37°C. Neutrophil secretion products were decanted after centrifugation (15,300 g, 5 min, 4°C). Pancreatic acinar cells were prepared by collagenase digestion and gentle shearing as described previously [29]. Cells were suspended in HEPES-Ringer buffer (pH 7.4) saturated with O_2 and passed through a 150 μm cell strainer (Partec, England). Isolated acinar cells (10^7 cells per well) were preincubated with vehicle, cerulein (100 nM), activated recombinant MMP-9 (R & D Systems) or activated neutrophils or secretions of activated neutrophils from wild-type or MMP-9-deficient mice (37°C, 1 hr). The buffer was then discarded and the cells were washed twice with a buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3 (morpholino) propanesulphonic acid (MOPS) and 1 mM $MgSO_4$. The cells were next homogenized in cold (4°C) MOPS buffer using a potter Elvejham-type glass homogenizer. The resulting homogenate was centrifuged (56x *g*, 5 min), and the supernatant was used for assay. Trypsin activity was measured flourometrically using Boc-Glu-Ala-Arg-MCA as the substrate as described previously [30]. For this purpose, a 200 μl aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM CaCl2 and 0.1% bovine serum albumin (BSA), pH 8.0). The reaction was initiated by the addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin levels (pg/μl) were calculated using a standard curve generated by assaying purified trypsin, normalized to protein concentration and expressed as relative trypsin units (RTU/pg). Viability of the pancreatic acinar cells was higher than 95% as determined by trypan blue dye exclusion.

Flow cytometry

Isolated neutrophils incubated with CXCL2 analysed for macrophage-1 antigen (Mac-1). To block Fcg III/II receptors and reduce non-specific labelling, samples were incubated with an

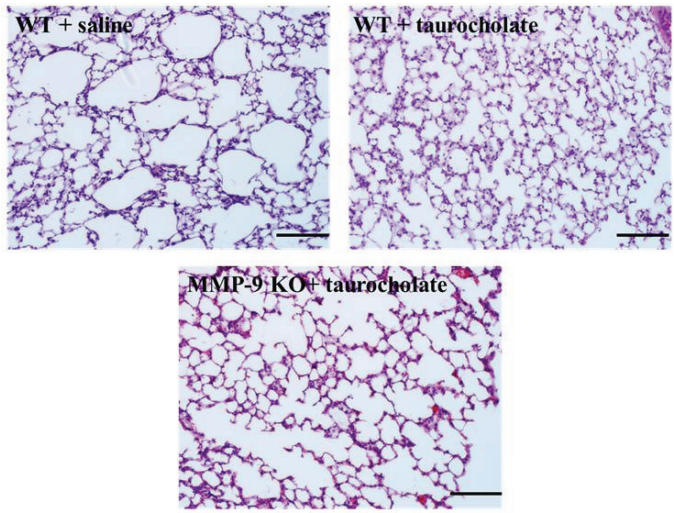

Figure 6. MMP-9 regulates lung damage in AP. Representative hematoxylin & eosin sections of the lung from wild type (WT) and MMP-9 knock-out (MMP-9 KO) + taurocholate. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline into the pancreas. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus saline control, $^{*}P < 0.05$ versus WT + taurocholate. Bars represent 100 μm.

anti-CD16/CD32 for 5 min. Then samples were stained with a PE-conjugated anti-Gr-1 (clone RB6-8C5, eBioscience, San Diego, CA, USA) antibody and with a FITC-conjugated anti-Mac-1 (clone M1/70, Integrin α_M chain, rat IgG_{2b}) antibody at 4 °C for 30 min. Cells were recovered following centrifugation then analysed with FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells. After gating the neutrophil population based on forward and side scatter characteristics, Mac-1 expression was determined on cells positive for Gr-1, which is a neutrophil marker.

Statistics

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

Results

MMPs mediate trypsinogen activation and tissue damage in AP

In order to examine the role of MMPs in AP, we used a broad-spectrum MMP inhibitor BB-94. Taurocholate challenge caused a clear-cut increase in blood amylase, pancreatic and lung MPO activity, as well as pancreatic and serum CXCL2 levels (Table 1). Administration of BB-94 decreased the taurocholate-induced increase in amylase by 76% (Table 1). Moreover, MMP inhibition reduced MPO activity in the pancreas and lung by 80% and 58%, respectively, in animals challenged with taurocholate. Taurocholate-induced levels of CXCL2 in the pancreas and serum were significantly decreased by BB-94 (Table 1). TAP is a

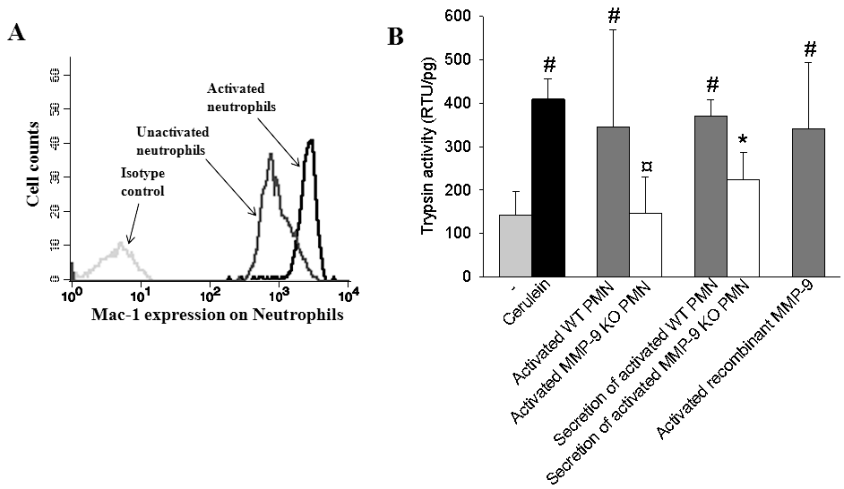

Figure 7. MMP-9 regulates trypsinogen activation in acinar cells. A) Neutrophils (PMN) were activated with CXCL2 and activation was confirmed by quantifying Mac-1 expression on neutrophils by flow cytometry. B) Acinar cell activation of trypsinogen was measured in (negative) control cells, cerulein-exposed acinar cell homogenate, activated neutrophils or secretions from wild-type mice, activated neutrophils or secretions from MMP-9-deficient mice as well as activated recombinant MMP-9. Trypsinogen activation was determined by measuring enzymatic activity of trypsin fluorometrically by using Boc-Glu-Ala-Arg-MCA as the substrate as described in detail in Methods. Trypsin levels (pg/µl) were calculated using a standard curve generated by assaying purified trypsin, normalized to protein concentration and expressed as relative trypsin units (RTU/pg). Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus negative control, $^{¤}P < 0.05$ versus activated wild-type PMN, $^{*}P < 0.05$ versus secretion of activated wild-type PMN.

cleavage product from trypsinogen and TAP level is a useful marker of trypsinogen activation [26, 31]. Interestingly, inhibition of MMPs reduced pancreatic TAP formation by 61% in mice challenged with taurocholate (Figure 1A). Morphologic examination revealed normal tissue structure in pancreatic tissue in controls (Figure 1B), whereas taurocholate challenge caused severe destruction of the pancreatic microarchitecture characterized by extensive acinar cell necrosis, hemorrhage, edema and massive infiltration of neutrophils (Figure 1B). MMP inhibition protected against taurocholate-induced destruction of the tissue architecture (Figure 1B). Quantification of histological changes revealed that treatment with BB-94 decreased taurocholate-induced acinar cell necrosis by 69%, edema by 55%, neutrophil infiltration by 69% and hemorrhage by 54% in the pancreas (Figure 1C).

MMP-9 regulates trypsinogen activation and tissue damage in AP

Taurocholate markedly increased plasma levels of MMP-9 but had no effect of MMP-2 levels in the plasma (Figure 2). Interestingly, administration of BB-94 significantly decreased the taurocholate-induced elevation of MMP-9 levels in the plasma (Figure 2b).Taurocholate-induced amylase release was abolished in MMP-9 gene-deficient animals (Figure 3). Furthermore, CXCL2 levels in the pancreas and serum were greatly decreased in mice lacking MMP-9 and challenged with taurocholate (Figure 3). In addition, the taurocholate-evoked enhancement of MPO activity in the pancreas and lung was reduced by more than 64% and 65% respectively in MMP-9-deficient animals (Figure 3). In order to study the role of MMP-9 in neutrophil recruitment, we used intravital fluorescence microscopy of the pancreatic microcirculation. We observed

that taurocholate challenge increased both leukocyte rolling and firm adhesion in postcapillary venules in the pancreas (Figure 4). Taurocholate-induced leukocyte rolling was intact but the number of firmly adherent leukocytes was significantly decreased in MMP-9 gene-deficient animals (Figure 4c + d). Moreover, histological examination revealed that the severe destruction of the pancreatic tissue structure characterized by extensive cell necrosis, edema, hemorrhage and massive infiltration of neutrophils evoked by taurocholate was markedly decreased in mice lacking MMP-9 (Figure 5). Administration of taurocholate also caused neutrophil infiltration and reduced alveolar spaces in the lung (Figure 6). These taurocholate-induced changes in the lung were reduced in MMP-9-deficient animals (Figure 6).

MMP-9 activates trypsinogen in acinar cells

To examine whether neutrophils and MMP-9 can stimulate trypsinogen activation, we performed *in vitro* experiments with isolated pancreatic acinar cells. Acinar cells were incubated with neutrophils and secretions from neutrophils activated with CXCL2. In order to assure activation of isolated neutrophils by CXCL2 we analyzed Mac-1 expression and found that CXCL2 markedly up-regulated Mac-1 expression on isolated neutrophils (Figure 7a). As expected, cerulein challenge caused a clear-cut activation of trypsinogen in acinar cells (Figure 7b). Co-incubation of activated wild-type neutrophils significantly increased trypsin activation in acinar cells (Figure 7b). In contrast, co-incubation of neutrophils from MMP-9 gene-deficient mice had no effect on trypsin activation in acinar cells (Figure 7b). Moreover, stimulation of acinar cells with secretions from CXCL2-activated wild-type neutrophils enhanced trypsin activity by 62% (Figure 7b). Notably, trypsinogen activation was significantly lower when co-incubated with CXCL2-provoked secretions from MMP-9-deficient neutrophils (Figure 7b). Finally, it was found that incubation of acinar cells with activated recombinant MMP-9 increased trypsin activation by 59% in acinar cells (Figure 7b).

Discussion

Our data demonstrate that MMPs regulate trypsinogen activation, formation of CXC chemokines, neutrophil recruitment and tissue damage in AP. Moreover, these results show that plasma levels of MMP-9 but not MMP-2 are elevated in AP. Taurocholate-induced activation of trypsinogen, inflammation and tissue injury in the pancreas as well as pulmonary accumulation of neutrophils were markedly attenuated in mice lacking MMP-9. Furthermore, it was found that neutrophil-derived MMP-9 regulates trypsinogen activation and that MMP-9 is a potent activator of trypsinogen in acinar cells. Thus, our novel data demonstrates that MMP-9 is an important regulator of neutrophil-dependent trypsinogen activation and may be a useful therapeutic target in AP.

The MMP family of proteases is generally considered to control degradation and formation of the extracellular matrix, but convincing data in the literature also implicate MMPs in several features of inflammatory reactions, such as leukocyte migration and cytokine formation [32-36]. Herein, we found that administration of BB-94, a broad-spectrum inhibitor of MMPs, markedly reduced tissue injury in AP. Thus, MMP inhibition significantly decreased the taurocholate-induced enhancement of serum amylase, suggesting that MMPs indeed regulate a significant part of the tissue damage in pancreatitis. Moreover, administration of BB-94 markedly decreased taurocholate-induced MPO activity and tissue infiltration of neutrophils in the pancreas, suggesting that

MMPs orchestrate neutrophil recruitment in AP. This notion is in line with previous studies on experimental pancreatitis [14-16, 18-20, 37]. The relationship between proteolytic activity on one hand and the inflammatory response on the other hand in AP is complex and not clearly delineated. A recent study showed that neutrophils play an important role in the activation of trypsinogen in AP [10]. However, infiltration of neutrophils in the pancreas *per se* does not seem to be a critical feature in this neutrophil-dependent activation of trypsinogen [8, 9]. Based on these findings, we hypothesized that mediators released from activated neutrophils, such as MMPs, may be responsible for neutrophil-mediated trypsinogen activation. Thus, we asked whether MMPs might regulate trypsinogen activation in the pancreas. Interestingly, we found that BB-94 greatly reduced TAP levels in pancreatitis animals, suggesting for the first time that MMPs may be involved in the conversion of trypsinogen to active trypsin in AP.

Neutrophils are known to contain large amounts of MMP-9 in tertiary (secretory) granules, which are the first granules to be mobilized and degranulate upon chemotactic activation of neutrophils [21, 38]. Having established that MMPs play an important role in AP, we next studied plasma levels of MMP-2 and MMP-9 in AP. We found that the plasma levels of MMP-9, but not MMP-2 were greatly enhanced in AP. This observation is consistent with previous experimental studies and one clinical study showing increased plasma levels of MMP-9 in ongoing pancreatitis [17]. In this context, it was interesting to note that administration of BB-94 abolished taurocholate-induced formation of MMP-9 in the plasma, indicating a pro-inflammatory self-perpetuating cycle in which MMP formation triggers further MMP formation. In this context, it should be mentioned that previous studies have shown that trypsin has the capacity to activate MMP-9 [39, 40]. Considered together with our observation that MMP-9 triggers trypsin activation, it may be proposed that there might be self-amplifying loops involving trypsin and MMP-9 activation in AP. In order to further define the role of MMP-9 in AP, we used MMP-9 gene-deficient mice. In these animals lacking MMP-9, we observed that taurocholate-provoked increases in serum amylase and tissue injury in the pancreas were significantly decreased, suggesting that MMP-9 is involved in the pathophysiology of AP. In addition, we found that taurocholate-induced neutrophil recruitment in the pancreas was significantly reduced in MMP-9-deficient animals. To study the role of MMP-9 in controlling leukocyte responses, we examined leukocyte-endothelium interactions in the pancreatic microcirculation. It was found that taurocholate-induced firm adhesion, but not rolling, in post capillary venules was significantly decreased in MMP-9 gene-deficient mice. Knowing that CXC chemokines, such as CXCL2, are potent activators of neutrophils triggering firm adhesion to endothelial cells, it was of interest to study the role of MMP-9 in the production of CXCL2 in AP. Herein, we found that taurocholate-induced formation of CXCL2 in the pancreas and serum was markedly reduced in animals lacking MMP-9, suggesting that MMP-9 regulates CXCL-2 formation and may help to explain the role of MMP-9 in controlling adhesion and tissue accumulation in pancreatitis described above. In this context, it is interesting to note that a previous study reported that MMP-9 has the capability to cleave the CXC chemokine IL-8 and enhance the neutrophilic chemotactic activity of IL-8 by ten times [41]. Taken together with our findings it may be forwarded that MMP-9 may control neutrophil chemotaxis in AP by two distinct mechanisms, i.e. formation and potency of CXC chemokines. At any

rate, our data show for the first time that MMP-9 is an important regulator of neutrophil recruitment and tissue damage in AP. Considering our finding that MMPs are required for trypsinogen activation in AP, we next wanted to define the role of MMP-9 in regulating trypsin activity in isolated acinar cells. First we confirmed that activated neutrophils and neutrophil secretions had the capacity to increase trypsin activity as demonstrated previously [10]. Interestingly, we found that activated neutrophils and neutrophil secretions derived from MMP-9 gene-deficient animals lacked capacity to trigger activation of trypsinogen in acinar cells. Notably, we also observed that activated recombinant MMP-9 caused a clear-cut increase in trypsin activity in acinar cells. These results suggest for the first time that neutrophil-derived MMP-9 is critical in the activation process of trypsinogen. Moreover, our novel data unifies the observations that on one hand neutrophils regulate trypsinogen activation [10, 42] and on the other hand that trypsinogen activation is independent of actual infiltration of neutrophils into the pancreas [8, 9]. Thus, our work has identified a critical link between neutrophils and trypsinogen activation in AP by showing that neutrophil-derived MMP-9 is a potent activator of trypsinogen.

To conclude, our novel data demonstrate that MMPs regulate trypsinogen activation and tissue damage in AP. Moreover, we found that plasma levels of MMP-9 are increased in AP and that mice lacking MMP-9 are protected against pathological inflammation and tissue injury in AP. Finally, our results show that MMP-9 is a potent activator of trypsinogen and critical in mediating neutrophil-dependent activation of trypsinogen. Thus, our results suggest that MMP-9 is a critical link between neutrophils and proteolytic activation in the pancreas and that MMP-9 may be an effective target in the treatment of AP.

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RESEARCH PAPER

Rho-kinase signalling regulates trypsinogen activation and tissue damage in severe acute pancreatitis

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BACKGROUND AND PURPOSE
Severe acute pancreatitis (SAP) is characterized by trypsinogen activation, infiltration of leucocytes and tissue necrosis but the intracellular signalling mechanisms regulating organ injury in the pancreas remain elusive. Rho-kinase is a potent regulator of specific cellular processes effecting several pro-inflammatory activities. Herein, we examined the role of Rho-kinase signalling in acute pancreatitis.

EXPERIMENTAL APPROACH
Pancreatitis was induced by infusion of taurocholate into the pancreatic duct in C57BL/6 mice. Animals were treated with a Rho-kinase inhibitor Y-27632 (0.5–5 mg·kg^{-1}) before induction of pancreatitis.

KEY RESULTS
Taurocholate infusion caused a clear-cut increase in blood amylase, pancreatic neutrophil infiltration, acinar cell necrosis and oedema formation in the pancreas. Levels of pancreatic myeloperoxidase (MPO), macrophage inflammatory protein-2 (MIP-2), trypsinogen activation peptide (TAP) and lung MPO were significantly increased, indicating local and systemic disease. Inhibition of Rho-kinase activity dose-dependently protected against pancreatitis. For example, 5 mg·kg^{-1} Y-27632 reduced acinar cell necrosis, leucocyte infiltration and pancreatic oedema by 90%, 89% and 58%, respectively, as well as tissue levels of MPO by 75% and MIP-2 by 84%. Moreover, Rho-kinase inhibition decreased lung MPO by 75% and blood amylase by 83%. Pancreatitis-induced TAP levels were reduced by 61% in Y-27632-treated mice. Inhibition of Rho-kinase abolished secretagogue-induced activation of trypsinogen in pancreatic acinar cells *in vitro*.

CONCLUSIONS AND IMPLICATIONS
Our novel data suggest that Rho-kinase signalling plays an important role in acute pancreatitis by regulating trypsinogen activation and subsequent CXC chemokine formation, neutrophil infiltration and tissue injury. Thus, these results indicate that Rho-kinase may constitute a novel target in the management of SAP.

Abbreviations
i.p., intraperitoneal; MIP-2, macrophage inflammatory protein-2; MNL, monomorphonuclear leucocytes; MPO, myeloperoxidase; MOPS, 3-(morpholino) propanesulphonic acid; PBS, phosphate-buffered saline; PMNL, polymorphonuclear leucocytes; RIA, radioimmunoassay; SAP, severe acute pancreatitis; TAP, trypsinogen activating peptide

Introduction

The clinical course of acute pancreatitis includes a wide spectrum of presentations from simple and transient pain to development of local and systemic complications (Andersson *et al*., 2007). At present, there is no useful method to predict the severity and outcome of acute pancreatitis. Despite substantial investigative efforts, there is still no specific therapy

available against acute pancreatitis and treatment is mainly limited to supportive care, which is partly related to an incomplete understanding of the underlying pathophysiology. In general, trypsinogen activation, inflammation and impaired microvascular perfusion have been implicated in the pathophysiology of pancreatitis (Wang *et al.*, 2009; Zhang *et al.*, 2009). Considering that trypsinogen activation seems to be an early and temporary process, inflammation in the pancreas persists longer and might be a more favourable target for specific therapeutic interventions (Regner *et al.*, 2008). Tissue accumulation of leucocytes constitutes a hallmark of inflammation and numerous studies have documented a critical role of leucocyte recruitment in the pathophysiology of acute pancreatitis (Glasbrenner and Adler, 1993; Bhatia *et al.*, 2000; Granger and Remick, 2005; Ryschich *et al.*, 2009). Activation and tissue navigation of leucocytes are coordinated by secreted chemokines (Bacon and Oppenheim, 1998). The chemokine family is subdivided into two main groups (CC and CXC) based on structural properties. In the mouse, the CXC chemokine family includes macrophage inflammatory protein-2 (MIP-2), which is known to be a murine homologue of human growth-related oncogenic chemokines (Tekamp-Olson *et al.*, 1990). MIP-2 is considered to predominately attract neutrophils and has been implicated as an important mediator of several severe conditions, such as endotoxaemia-induced lung and liver injury (Li *et al.*, 2004; Mangalmurti *et al.*, 2009), glomerulonephritis (Feng *et al.*, 1995), bacterial meningitis (Klein *et al.*, 2006) and hepatic ischaemia-reperfusion (Monson *et al.*, 2007). Indeed, one previous study has shown that MIP-2 may also be an important regulator of neutrophil infiltration in the pancreas (Pastor *et al.*, 2003). Although, the role of specific chemoattractants in leucocyte infiltration in the pancreas is relatively well described, the understanding of the signalling pathways orchestrating pro-inflammatory actions in the pancreas is limited.

Extracellular stress signals, such as ischaemia and infection, initiate intracellular signalling cascades that converge on specific transcription factors regulating gene expression of pro-inflammatory mediators. This signal transmission is largely regulated by intracellular kinases phosphorylating down-stream targets (Itoh *et al.*, 1999). For example, small (~21 kDa) guanosine triphosphatases of the Ras-homologus (Rho) family and one of their effectors, Rho-kinase, are known to act as molecular switches regulating numerous important cellular functions, such as cytoskeleton organization, cell adhesion, migration, reactive oxygen species formation and oncogenic transformation (Itoh *et al.*, 1999; Alblas *et al.*, 2001; Slotta *et al.*, 2006). Notably, Rho-kinase inhibitors have been demonstrated to ameliorate reperfusion and endotoxaemic injury in the liver (Slotta *et al.*, 2008) as well as protecting against tissue fibrosis (Kitamura *et al.*, 2007), obstructive cholestasis (Laschke *et al.*, 2008), cerebral and intestinal ischaemia (Shin *et al.*, 2007; Santen *et al.*, 2010) and pulmonary hypertension (Oka *et al.*, 2008). However, the role of the Rho-kinase signalling in regulating trypsinogen activation, leucocyte recruitment and tissue injury in acute pancreatitis is not known.

Based on the above, we hypothesized that Rho-kinase signalling may play an important role in acute pancreatitis. We used a new experimental model of severe acute pancreatitis (SAP) in mice and interfered with Rho-kinase activity by administration of Y-27632, a specific Rho-kinase inhibitor.

Methods

Animals

All experiments were done in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for animal experimentation at Lund University, Sweden. Male C57BL/6 mice weighing 20–26 g (6–8 weeks) were maintained in a climate-controlled room at 22°C and exposed to a 12:12 h light-dark cycle. Animals were fed standard laboratory diet and given water *ad libitum*. Mice were anaesthetized by i.p. administration of 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) 100 g^{-1} body weight in 200 μL saline.

Experimental model of taurocholate-induced pancreatitis

The second part of duodenum and papilla of Vater was identified through a small (1–2 cm) upper midline incision. Traction sutures (7-0 prolene) were placed 1 cm from the papilla. A small puncture was made through the duodenal wall in parallel to the papilla of Vater with a 23G needle. A non-radiopaque polyethylene catheter (ID 0.28 mm) connected to a microinfusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) was inserted through the punctured hole in the duodenum, via the papilla of Vater and 1 mm into the common bile duct. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp. 10 μL of either 5% sodium taurocholate (Sigma-Aldrich, USA) or sodium chloride (0.9%) was infused for 5 min. Then the catheter was withdrawn and the common hepatic duct clamp was removed. The duodenal puncture closed by a purse-string suture (7-0 monofilament). The traction sutures were removed and abdomen was closed in two layers. Animals were allowed to wake up and given free access to food and water. Sham-operated animals underwent the same procedure without any infusion into the pancreas. Vehicle or the Rho-kinase inhibitor, Y-27632 [(R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide; Calbiochem, San Diego, USA], was given (0.5–5 mg·kg^{-1}) i.p. 30 min prior to bile duct cannulation.

In separate experiments animals were treated with 5 mg·kg^{-1} Y-27632 2 h after taurocholate challenge. Animals were killed 24 h after the induction of pancreatitis. One group of mice received 5 mg·kg^{-1} Y-27632 alone without bile duct cannulation. Blood was collected from the tail vein for systemic leucocyte differential counts and determination of blood amylase levels. Blood samples were also collected from the inferior vena cava for flow cytometric studies of neutrophils. Pancreatic tissue was removed and kept in two pieces; one piece was snap-frozen in liquid nitrogen for biochemical analysis of myeloperoxidase (MPO), trypsinogen activating peptide (TAP) and MIP-2 and the other piece was fixed in formalin for later histological analysis.

Systemic leucocyte counts

Tail vein blood was mixed with Turks solution (0.2 mg gentian violet in 1 mL glacial acetic acid, 6.25% v/v) in a 1:20 dilution. Leucocytes were identified as monomorphonuclear (MNLs) and polymorphonuclear (PMNLs) cells in a Burker chamber.

Blood amylase

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

MPO assay

Frozen pancreatic tissue was preweighed and homogenized in 1 mL mixture (4:1) with phosphate-buffered saline and aprotinin 10 000 KIE mL^{-1} (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. The homogenate was centrifuged (15339× *g*, 10 min) and the supernatant was stored at –20°C and the pellet was used for MPO assay as previously described (Laschke *et al*., 2007). In brief, the pellet was mixed with 1 mL of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 s, put in a water bath set at 60°C for 2 h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units g^{-1} tissue.

Flow cytometry

For analysis of Mac-1 and CXCR2 expression on circulating neutrophils, blood was collected into syringes prefilled with 1:10 acid citrate dextrose at 24 h post taurocholate challenge. Immediately after collection, blood samples were incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce non-specific labelling for 10 min at room temperature and then incubated with FITC-conjugated anti-Mac-1 (clone M1/70, Integrin α_M chain, rat IgG_{2b}), APC-conjugated anti-Gr-1 (clone RB6-8C5, Rat IgG_{2b}) and PerCP Cy5.5-conjugated anti-mouse CD182 (CXCR2) (clone TG11/CXCR2, rat IgG2a, Biolegend, San Diego, CA, USA) antibodies. Cells were fixed with 1% formaldehyde solution, erythrocytes were lysed using red blood cell lysing buffer (Sigma Chemical Co., St. Louis, MO, USA) and neutrophils were recovered following centrifugation. Flow-cytometric definition of neutrophils was based on $Gr\text{-}1^+$ cells in the neutrophil population of cells based on forward and side scatter characteristics on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells.

MIP-2 levels

Tissue levels of MIP-2 were determined in stored supernatant from homogenized pancreatic tissue by using double-antibody Quantikine enzyme linked immunosorbent assay kits (R & D Systems Europe, Abingdon, UK) using recombinant murine MIP-2 as standard. The minimal detectable protein concentration is less than 0.5 $pg{\cdot}mL^{-1}$.

Histology

Pancreas samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six micrometer sections were stained (haematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a preexisting scoring system including oedema, acinar cell necrosis and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scale, as previously described in detail (Schmidt *et al*., 1992).

Radioimmunoassay

RIA was performed as described previously (Lindkvist *et al*., 2008). A 0.1 M Tris HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.005 M EDTA and 2 $g{\cdot}L^{-1}$ bovine serum albumin (Sigma, St Louis, USA) was used as assay buffer. Samples of 100 μL diluted in assay buffer were incubated (16 h, 4°C) with 200 μL of [^{125} I]-Tyr-TAP (=20 000 counts min^{-1}) in assay buffer and 200 μL of antiserum diluted 1/750 in assay buffer. Parallel incubations with the synthetic activation peptide TAP diluted in assay buffer in a series of concentrations from 0.078 to 20 nM, were used as standards in the assays. Free and bound radioactivities were separated by means of a second step antibody precipitation; 100 μL of a cellulose coupled anti-mouse IgG suspension (Sc-Cel® IDA, Boldon, England) was added to the samples. After 30 min of incubation, 1 mL of water was added and tubes were centrifuged (704× *g*, 5 min, room temperature). The supernatant was decanted and radioactivity of the precipitate was counted in a γ-spectrophotometer (1 min).

Tryspsinogen activation in isolated acinar cells

Pancreatic acini cells were prepared by collagenase digestion and gentle shearing as described previously (Saluja *et al*., 1999). Cells were suspended in HEPES-Ringer buffer (pH 7.4) saturated with O_2 and passed through a 150 μm cell strainer (Partec, England). Isolated acinar cells (1×10^7 cells per well) were preincubated with vehicle or Y-27632 (100 nM, 10 min) and stimulated with 100 nM cerulein (37°C, 30 min). The buffer was then discarded and the cells were washed twice with buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3-(morpholino) propanesulphonic acid (MOPS) and 1 mM $MgSO_4$. The cells were next homogenized in cold (4°C) MOPS buffer using a potter Elvejham-type glass homogenizer. The resulting homogenate was centrifuged (56× *g*, 5 min), and the supernatant was used for assay. Trypsin activity was measured flourometrically using Boc-Glu-Ala-Arg-MCA as the substrate as described previously (Kawabata *et al*., 1988). For this purpose, a 200 μL aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$ and 0.1% BSA, pH 8.0). The reaction was initiated by the addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin levels ($pg{\cdot}mL^{-1}$) were calculated using a standard curve generated by assaying purified trypsin. Viability of the pancreatic acinar cells was higher than 95% as determined by trypan blue dye exclusion.

Statistics

Data are presented as mean values ± SEM. Statistical evaluations were performed using Kruskal-Wallis one-way analysis of variance on ranks followed by multiple comparisons versus

control group (Dunnett's method). $P < 0.05$ was considered significant, and n represents the number of animals.

Results

Rho-kinase activity regulates tissue damage in pancreatitis

To study the role of Rho-kinase, we first examined blood amylase levels as an indicator of tissue damage in SAP. It was found that retrograde infusion of sodium taurocholate into the pancreatic duct enhanced blood amylase levels by nearly 17-fold (Figure 1, $P < 0.05$ vs. sham, $n = 5–7$). Administration of the Rho-kinase inhibitor Y-27632 reduced taurocholate-provoked levels of blood amylase from 834.4 ± 117.3 μKat·L^{-1} down to 141.2 ± 28.5 μKat·L^{-1}, corresponding to an 83% reduction (Figure 1, $P < 0.05$ vs. vehicle + taurocholate, $n = 5–7$). Morphological examination revealed that pancreas tissue from control animals had a normal microstructure (Figure 2, $n = 5–7$), whereas taurocholate challenge caused severe destruction of the pancreatic tissue structure characterized by extensive acinar cell necrosis, oedema and massive infiltration of neutrophils (Figure 2, $n = 5–7$). It was observed that Rho-kinase inhibition protected against taurocholate-induced destruction of the tissue structure (Figure 2, $n = 5–7$). For example, inhibition of Rho-kinase activity decreased taurocholate-induced acinar cell necrosis by 90% and oedema by 58% in the pancreas (Figure 3A and B, $P < 0.05$ vs. vehicle + taurocholate, $n = 5–7$). Indeed, the number of circulating MNL and neutrophils increased in SAP, indicating systemic activation in this model (Table 1). Rho-kinase inhibition reversed systemic changes in leucocyte differential counts towards baseline levels in controls (Table 1). Notably, administration of 5 mg·kg^{-1} Y-27632 ($n = 6$) after induction of pancreatitis had no effect on taurocholate-induced acinar cell necrosis, oedema or infiltration of neutrophils in pancreas (not shown).

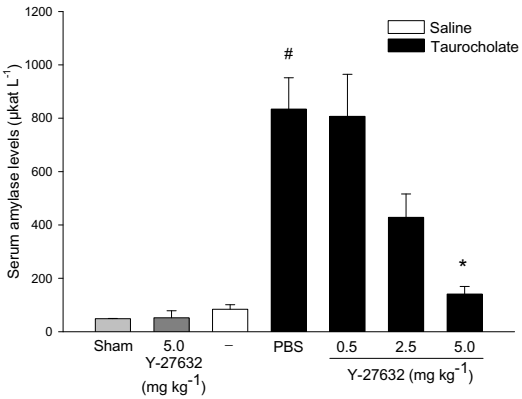

Figure 1

Blood amylase (μKat·L^{-1}) in sham and control animals infused with saline alone into the pancreatic duct. Animals were treated with PBS or the Rho-kinase inhibitor Y-27632 (0.5–5.0 mg·kg^{-1}) before infusion with sodium taurocholate into the pancreatic duct. One group of mice was given 5 mg·kg^{-1} Y-27632 alone without bile duct cannulation. Blood samples were obtained after 24 h. Data represent means ± SEM and $n = 5–7$. $^{\#}P < 0.05$ versus sham and $^{*}P < 0.05$ versus PBS + taurocholate.

Rho-kinase activity controls neutrophil recruitment in pancreatitis

Pancreatic levels of MPO were used as a marker of inflammatory cell infiltration. Peak levels of MPO were observed 24 h after taurocholate challenge (not shown) and this time-point was used for subsequent studies of neutrophil infiltration in the pancreas. It was found that challenge with taurocholate enhanced pancreatic levels of MPO by seven-fold (Figure 4A, $P < 0.05$ vs. sham, $n = 5–7$). Inhibition of Rho-kinase signalling decreased taurocholate-induced MPO levels in the pancreas by 73% (Figure 4A, $P < 0.05$ vs. vehicle + taurocholate, $n = 5–7$). Moreover, histological analysis of pancreatic tissue showed that taurocholate challenge provoked a clear-cut enhancement in extravascular neutrophils (Figure 4B, $P < 0.05$ vs. sham, $n = 5–7$). Notably, administration of 5 mg·kg^{-1}

Table 1

Systemic leucocyte differential counts

	PMNL	MNL	Total
Sham	0.8 ± 0.1	4.6 ± 0.2	5.4 ± 0.2
Y-27632 5 mg·kg^{-1}	0.9 ± 0.2	4.3 ± 0.2	5.2 ± 0.4
Saline control	0.9 ± 0.1	4.6 ± 0.1	5.5 ± 0.2
PBS + pancreatitis	1.7 ± 0.2$^{\#}$	6.9 ± 0.6$^{\#}$	7.6 ± 0.7$^{\#}$
Y-27632 0.5 mg·kg^{-1} + pancreatitis	1.7 ± 0.3	6.1 ± 0.1	7.8 ± 0.4
Y-27632 2.5 mg·kg^{-1} + pancreatitis	1 ± 0.1*	4.9 ± 0.1*	5.9 ± 0.2*
Y-27632 5 mg·kg^{-1} + pancreatitis	0.9 ± 0.1*	5.3 ± 0.1*	6.2 ± 0.2*

Blood was collected from sham, saline control, taurocholate-treated animals receiving PBS or the Rho-kinase inhibitor Y-27632 (0.5–5 mg·kg^{-1}). One group of mice received 5 mg·kg^{-1} Y-27632 alone without bile duct cannulation. Cells were identified as monomorphonuclear leucocytes (MNL) and polymorphonuclear leucocytes (PMNL). Data represents mean ± SEM, 10^6 cells mL^{-1} and $n = 5–7$. $^{\#}P < 0.05$ versus sham and $^{*}P < 0.05$ versus PBS + taurocholate.

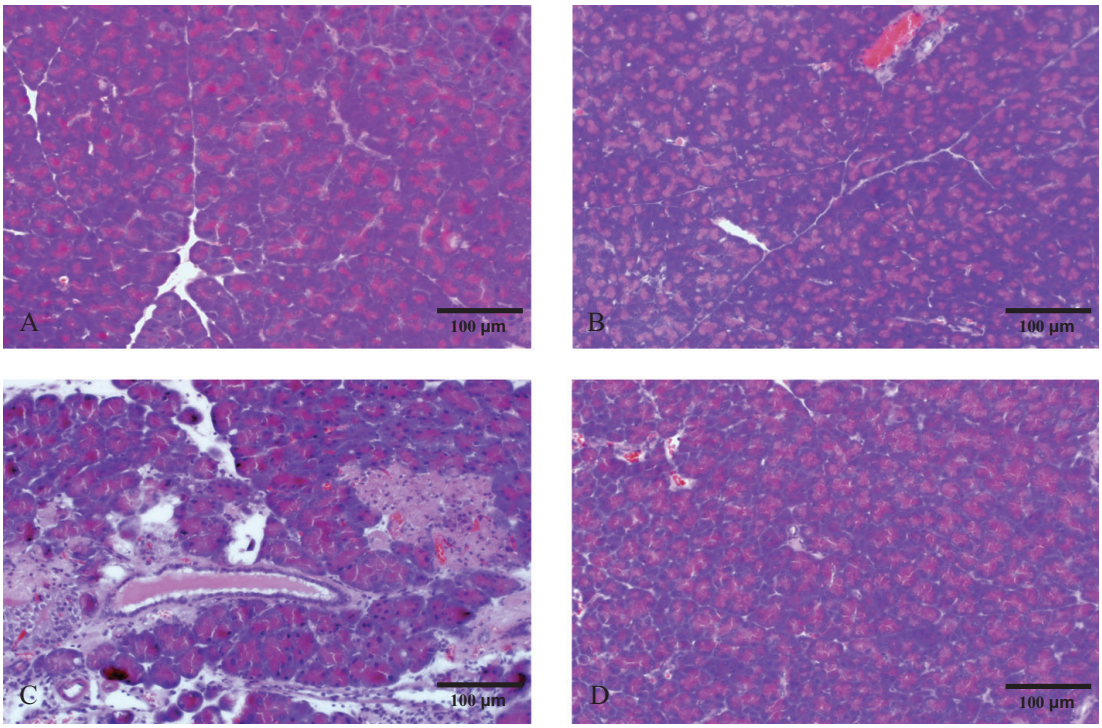

Figure 2

Representative haematoxylin and eosin stained sections of the pancreas. (A) Sham animals and (B) control animals infused with saline alone into the pancreatic duct. Taurocholate-exposed mice were pretreated with (C) PBS or (D) 5 mg·kg^{-1} of the Rho-kinase inhibitor Y-27632. Samples were harvested 24 h later. Bars represent 100 µm.

Y-27632 reduced taurocholate-provoked infiltration of neutrophils in the pancreas by 88% (Figure 4B, $P < 0.05$ vs. vehicle + taurocholate, $n = 5–7$). Neutrophil chemotaxis is known to be coordinated by MIP-2 (Pastor *et al.*, 2003). Herein, we observed that MIP-2 levels were low but detectable in normal pancreas and that challenge with taurocholate markedly increased MIP-2 levels in the pancreas up to 22.1 ± 5.3 pg·mg^{-1} tissue (Figure 4C). Notably, Rho-kinase inhibition greatly decreased MIP-2 levels in the inflamed pancreas (Figure 4C). In addition, we noted that Mac-1 expression was increased on the surface of circulating neutrophils in mice with pancreatitis (Figure 5A), indicating systemic activation in this experimental model. Inhibition of Rho-kinase signalling markedly reduced neutrophil expression of Mac-1 in pancreatitis (Figure 5A). In contrast, the expression of chemokine receptor CXCR2 on neutrophils decreased after taurocholate challenge and administration of Y-27632 had no effect on CXCR2 expression on neutrophils in animals with pancreatitis (Figure 5B). As part of the systemic response to SAP, activated neutrophils accumulate in the lung. Indeed, we observed that MPO levels in the lung were significantly increased in animals with pancreatitis. Pretreatment with Y-27632 decreased pulmonary levels of MPO by 75% in mice challenged with taurocholate (Figure 5C). In contrast, treatment with 5 mg·kg^{-1} Y-27632 ($n = 6$) had no effect on MPO levels in the pancreas or the lung when given after taurocholate challenge (not shown).

Rho-kinase activity regulates trypsinogen activation in pancreatitis

TAP is a cleavage product from trypsinogen and TAP is a useful marker of trypsinogen activation (Gudgeon *et al.*, 1990; Hartwig *et al.*, 1999). Herein, it was found that taurocholate challenge increased TAP levels in the pancreas by 3-fold (Figure 6, $P < 0.05$ vs. sham, $n = 5–7$), suggesting that trypsinogen is indeed activated in this model of pancreatitis. Interestingly, we observed that administration of the Rho-kinase inhibitor significantly reduced taurocholate-induced TAP levels from 357.2 ± 28.2 down to 146.8 ± 56.8 µg·g^{-1} tissue, corresponding to a 61% reduction in trypsinogen activation (Figure 6, $P < 0.05$ vs. vehicle + taurocholate, $n = 5–7$).

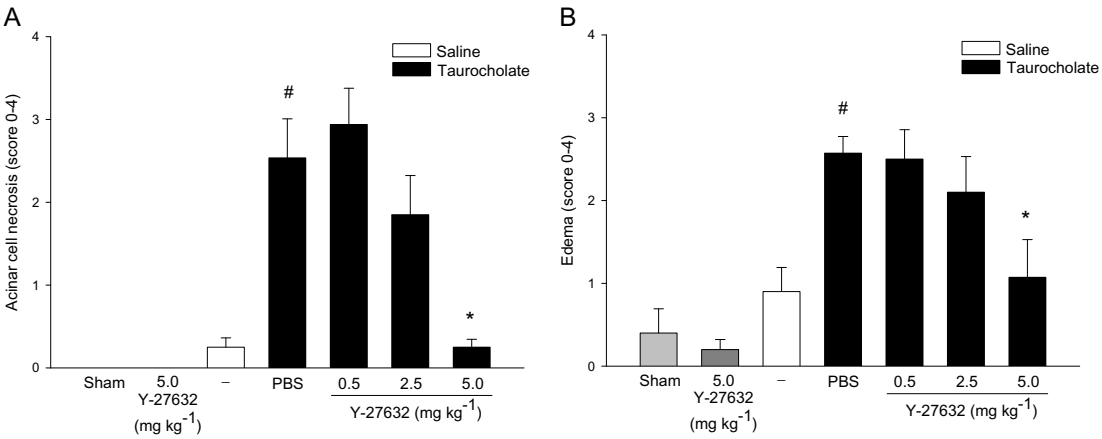

Figure 3

Rho-kinase regulates taurocholate-induced tissue damage in the pancreas. (A) Acinar cell necrosis and (B) oedema formation in sham, control (saline alone into the pancreatic duct) and taurocholate-exposed mice pretreated with PBS or the Rho-kinase inhibitor Y-27632 (0.5–5 mg·kg^{-1}). One group of mice was given 5 mg·kg^{-1} Y-27632 alone without bile duct cannulation. Samples were obtained after 24 h. Data represent means ± SEM and n = 5–7. $^{\#}P < 0.05$ versus sham and $^{*}P < 0.05$ versus PBS + taurocholate.

Rho-kinase activity regulates activation of trypsinogen in acinar cells in vitro

We next determined whether Rho-kinase regulates trypsinogen activation in pancreatic acinar cells *in vitro*. For this purpose, we isolated acinar cells from the pancreas of mice and incubated the cells with cerulein as described previously (Saluja *et al*., 1999). It was found that cerulein stimulation increased trypsinogen activation by two-fold compared with unstimulated cells (Figure 7, $P < 0.05$ vs. control, $n = 5$). Notably, preincubation of the acinar cells with Y-27632 decreased secretagogue-induced activation of trypsinogen by 69% (Figure 7, $P < 0.05$ vs. vehicle + cerulein, $n = 5$).

Discussion and conclusions

Signalling mechanisms regulating pathophysiological processes in acute pancreatitis are incompletely understood. The present study reveals that Rho-kinase signalling plays an integral part in the pathophysiology of SAP. In fact, inhibition of Rho-kinase activity markedly reduced acinar cell necrosis and blood amylase levels in acute pancreatitis. Our findings show that inhibition of Rho-kinase activity abolishes trypsinogen activation in the pancreas, which helps to explain the attenuated inflammatory response and tissue damage in SAP. These novel findings indicate that targeting Rho-kinase activity may be a useful approach to protect against SAP.

Rho-kinase activity is generally considered to regulate cytoskeletal dynamics, including cell contraction and vesicular trafficking, but there is increasing evidence also implicating Rho-kinase signalling in numerous features of inflammatory reactions, such as leucocyte migration, phagocytosis and cytokine formation (Riento and Ridley, 2003; Bokoch, 2005). In the present study, we observed that administration of Y-27632, a specific Rho-kinase inhibitor, markedly decreased tissue damage in SAP. For example, inhibition of Rho-kinase signalling reduced the taurocholate-induced increase in blood amylase by 83% and acinar cell necrosis by more than 90%, indicating that Rho-kinase indeed controls a substantial part of the tissue injury in pancreatitis. It should be mentioned that a previous study reported that Y-27632 increased secretion of amylase from acinar cells in cerulein-induced pancreatitis (Kusama *et al*., 2003). That observation may seem to be in contrast to our present findings but the reduction of blood amylase in our present work is more likely to be related to the protective effect of Y-27632 against taurocholate-induced cell injury in the pancreas. Nonetheless, our present data constitutes the first evidence in the literature that inhibition of the Rho-kinase signalling pathway protects against SAP. Thus, our data add SAP to the growing list of conditions, which may be ameliorated by interference with Rho-kinase signalling, including ischaemia-reperfusion (Bao *et al*., 2004; Shiotani *et al*., 2004), endotoxaemia (Thorlacius *et al*., 2006), septic lung injury (Tasaka *et al*., 2005), tissue fibrosis (Bourgier *et al*., 2005) and obstructive cholestasis (Laschke *et al*., 2009). In this context, it is interesting to note that the present findings show that inhibition of Rho-kinase attenuates TAP levels in animals with pancreatitis, suggesting that Rho-kinase is involved in the conversion of trypsinogen to active trypsin. This caused us to ask whether Rho-kinase may regulate trypsinogen activation in acinar cells in the pancreas. Interestingly, we found that Y-27632 abolished secretagogue-induced activation of trypsinogen *in vitro*, suggesting that Rho-kinase indeed regulates trypsinogen activation in acinar cells. Considering that trypsin formation is an early and important component in the pathophysiology of AP (Mithöfer *et al*., 1998), it is

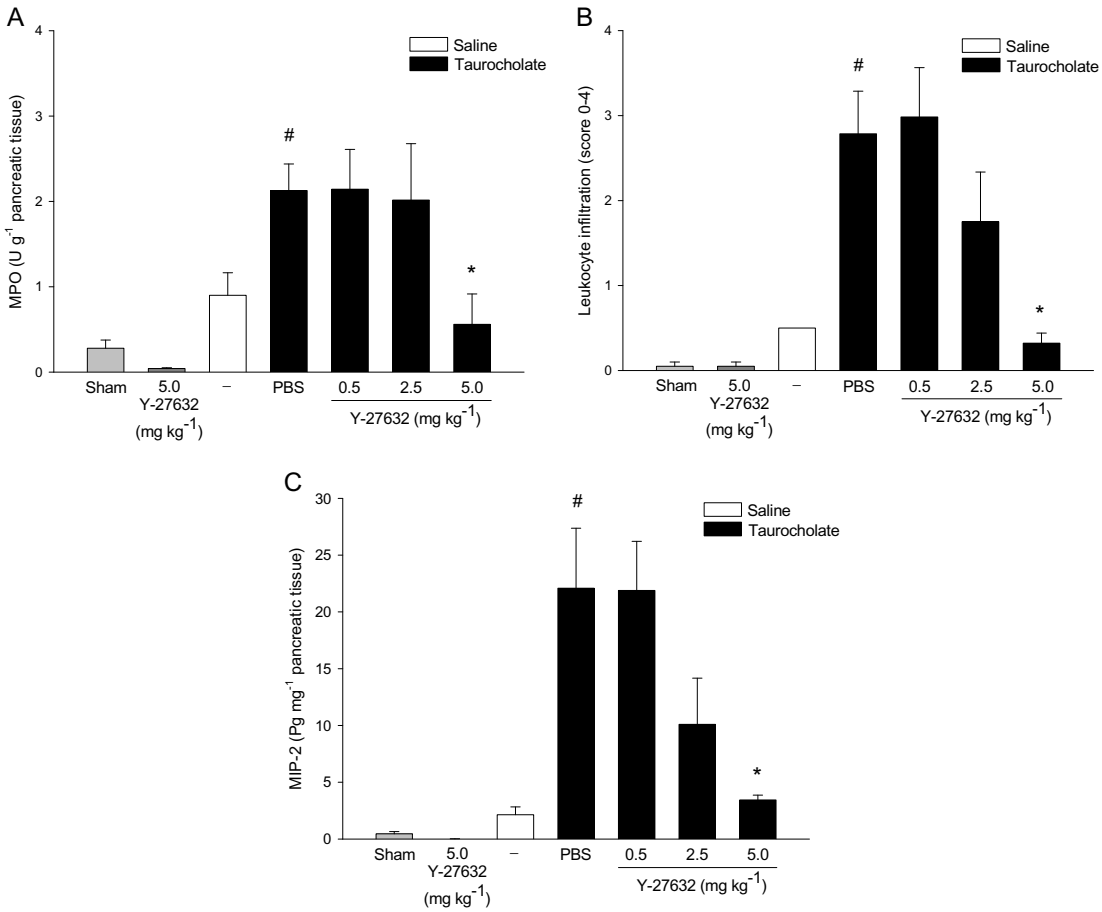

Figure 4

Rho-kinase regulates taurocholate-induced neutrophil accumulation in the pancreas. (A) MPO levels, (B) extravascular neutrophils, (C) MIP-2 levels in the pancreas in sham, control (saline alone into the pancreatic duct) and taurocholate-exposed mice pretreated with PBS or the Rho-kinase inhibitor Y-27632 (0.5–5 mg·kg^{-1}). One group of mice was given 5 mg·kg^{-1} Y-27632 alone without bile duct cannulation. Samples were obtained after 24 h. Data represent means ± SEM and n = 5–7. $^{\#}P < 0.05$ versus sham and $^{*}P < 0.05$ versus PBS + taurocholate.

tempting to speculate that trypsinogen activation is a main target of the Rho-kinase inhibitor in AP. However, the relationship between trypsin activity on one hand and the inflammatory response on the other hand in SAP is not clearly delineated. It may be that both develop in parallel and potentiate each other or there may be a sequential relationship with one preceding the other. Nonetheless, we also observed that administration of Y-27632 after taurocholate challenge had no significant effect on inflammatory parameters or tissue damage in the pancreas, supporting the notion above that trypsinogen activation in acinar cells rather than secondary chemokine formation and neutrophil activation may be the main protective mechanism exerted by the Rho-kinase inhibitor. In this context, it should be noted that targeting Rho-kinase activity may have a limited influence on the treatment of patients with on-going pancreatitis considering that Rho-kinase-regulated activation of trypsinogen is an early feature in AP and that delayed treatment with the Rho-kinase inhibitor did not ameliorate tissue damage in the inflamed pancreas. However, it is possible that high-risk patients undergoing endoscopic retrograde cholangiopancreatography may benefit from prophylactic administration of Rho-kinase inhibitors.

Activation and extravascular accumulation of leucocytes are key components in the inflammatory response following injury and infection, but in certain instances, leucocytes may cause organ damage, including graft rejection and sepsis (Carlos and Harlan, 1994). In fact, numerous studies have

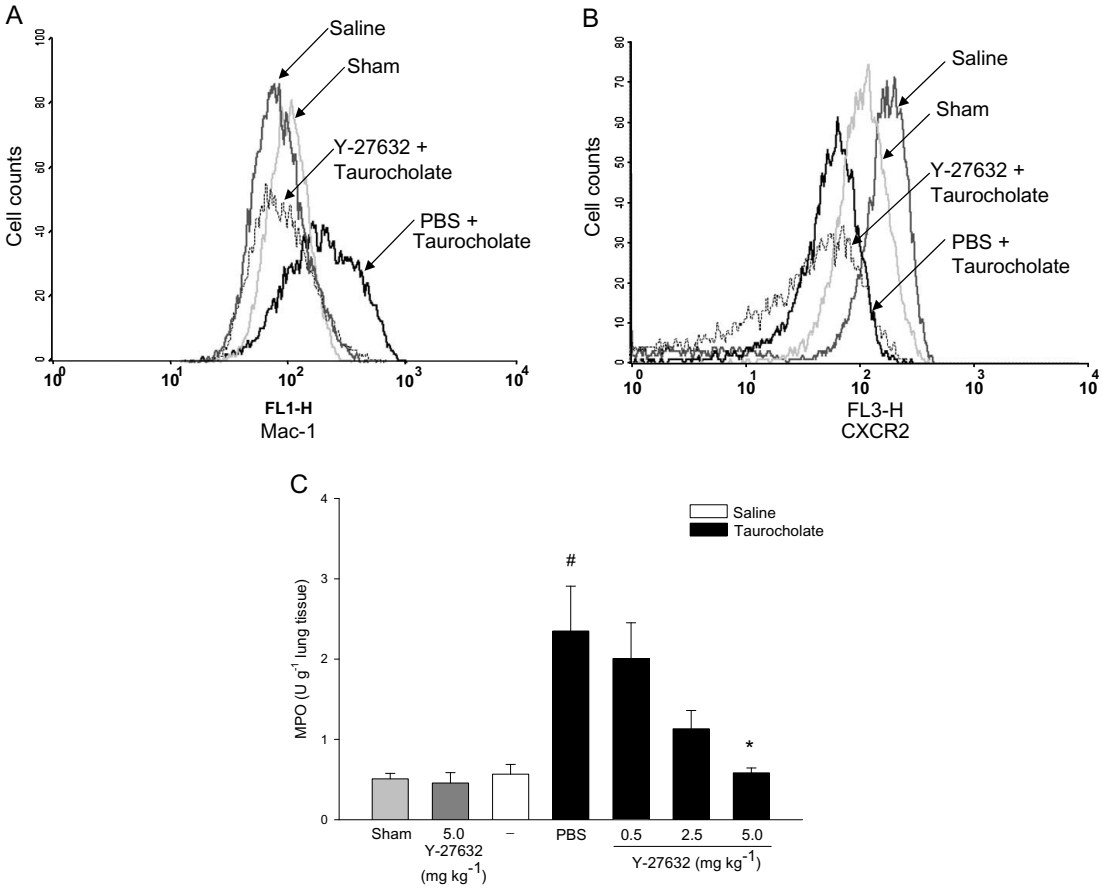

Figure 5

A representative sample of (A) Mac-1, (B) CXCR2 expression on neutrophils and (C) lung MPO levels in sham, control (saline alone into the pancreatic duct) and taurocholate-exposed mice pretreated with PBS or the Rho-kinase inhibitor Y-27632 (0.5–5 mg·kg^{-1}). One group of mice was given 5 mg·kg^{-1} Y-27632 alone without bile duct cannulation. Samples were obtained after 24 h. Data represent means ± SEM and n = 5–7. $^{\#}P < 0.05$ versus sham and $^{*}P < 0.05$ versus PBS + taurocholate.

documented that leucocyte recruitment constitutes a rate-limiting step in pancreatitis by demonstrating markedly attenuated tissue destruction in neutrophil-depleted animals (Kyriakides *et al.*, 2001). Herein, we observed that taurocholate challenge increased MPO activity and the number of extravascular neutrophils in the pancreas. Administration of Y-27632 greatly decreased both MPO levels (73%) and extravascular neutrophils (88%) in the pancreas, suggesting that Rho-kinase activity is a potent regulator of neutrophil trafficking in pancreatitis. Specific adhesion molecules regulate the recruitment process of leucocytes to extravascular sites of inflammation (Kelly *et al.*, 2007). Although the detailed role of specific adhesion molecules in supporting leucocyte recruitment in the pancreas is relatively unclear, numerous studies have shown that Mac-1 is a dominating molecule in mediating tissue infiltration of neutrophils (Asaduzzaman *et al.*, 2008; Lee *et al.*, 2009; Rahman *et al.*, 2009). In the present study, we found that taurocholate challenge upregulated Mac-1 expression on neutrophils. Interestingly, administration of Y-27632 markedly reduced surface levels of Mac-1 on neutrophils, indicating that Rho-kinase signalling contributes to neutrophil expression of Mac-1 in pancreatitis. Moreover, this inhibitory effect on Mac-1 expression may also help explain the inhibitory action of Y-27632 on neutrophil accumulation in SAP observed herein. In addition, systemic complications of SAP include pulmonary accumulation of neutrophils (Sharif *et al.*, 2009). Indeed, we observed that lung MPO activity was clearly increased in taurocholate-treated animals. Notably, inhibition of Rho-kinase function clearly attenuated pulmonary MPO levels, indicating that

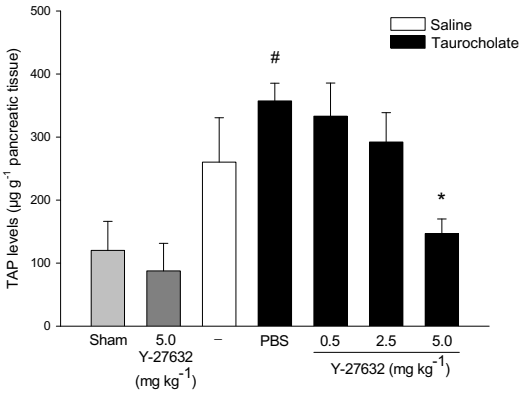

Figure 6

Rho-kinase regulates taurocholate-induced activation of trypsinogen. Levels of TAP in the pancreas in sham, control (saline alone into the pancreatic duct) and taurocholate-exposed mice pretreated with PBS or the Rho-kinase inhibitor Y-27632 (0.5–5 mg·kg^{-1}). One group of mice was given 5 mg·kg^{-1} Y-27632 alone without bile duct cannulation. Samples were obtained after 24 h. Data represent means ± SEM and n = 5–7. $^{\#}P < 0.05$ versus sham and $^{*}P < 0.05$ versus PBS + taurocholate.

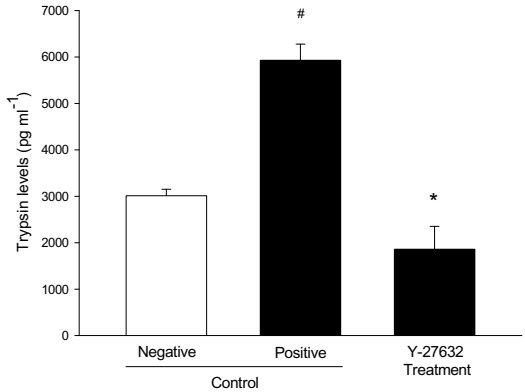

Figure 7

Rho-kinase regulates activation of trypsinogen in acinar cells. Acinar cell activation of trypsinogen was measured in (negative) control cells and cerulein-exposed acinar cell homogenate pretreated with vehicle (positive control) or the Rho-kinase inhibitor Y-27632 (100 nM). Trypsinogen activation was determined by measuring enzymatic activity of trypsin fluorometrically by using Boc-Gln-Ala-Arg-MCA as the substrate as described in detail in Methods. Trypsin levels (pg·mL^{-1}) were calculated using a standard curve generated by assaying purified trypsin. Data represent means ± SEM and n = 5. $^{\#}P < 0.05$ versus control and $^{*}P < 0.05$ versus positive control.

Y-27632 protects against systemic activation and infiltration of neutrophils in the lung. Considered together, these findings suggest that Rho-kinase signalling regulates both local and distant organ accumulation of neutrophils in acute pancreatitis.

It is generally held that secreted chemokines are fundamental regulators of leucocyte activation and tissue navigation. CXC chemokines, such as MIP-2, are particularly potent activators of neutrophils. It was, therefore, of great interest to examine local formation of MIP-2 in the pancreas in this study. We observed that taurocholate caused a clear-cut increase in MIP-2 formation in the pancreas. It is interesting to note that inhibition of Rho-kinase activity reduced taurocholate-induced expression of MIP-2 by 84%. Indeed, this marked attenuation of MIP-2 formation may account for the inhibitory effect of Y-27632 on neutrophil expression of Mac-1 as well as on the infiltration of neutrophils in the pancreas and lung. However, these findings do not exclude the possibility that Rho-kinase signalling also directly regulates Mac-1 expression and migratory function in neutrophils. For example, a previous study has reported that Rho-kinase can coordinate chemoattractant-induced leucocyte migration *in vitro* (Satoh *et al*., 2001). Moreover, it is valuable to note that these findings do not exclude a potential role of other protein kinases; p38 mitogen-activated protein kinase signalling has also been shown to play a role in pancreatitis (Chen *et al*., 2007). In general, MIP-2 effects are mediated through binding to the CXC chemokine receptor 2 (CXCR2), which is the high affinity receptor on murine neutrophils for MIP-2 (Cacalano *et al*., 1994; Jones *et al*., 1997). Herein, we observed that taurocholate challenge decreased CXCR2 expression on neutrophils, which is in line with other models of systemic inflammation such as sepsis (Rios-Santos *et al*., 2007) and trauma (Quaid *et al*., 1999). The reason behind the discrepancy between decreased expression of CXCR2 on the one hand and increased tissue migration of neutrophils on the other is not known but may be related to a relative accumulation of neutrophils with low levels of CXCR2 in the circulation after vascular extravasation of neutrophils with higher expression of CXCR2. Alternatively, there may be a time-dependent component, that is, neutrophils exit the circulation prior to the downregulation of CXCR2 in the remaining population of neutrophils in the blood. Nonetheless, we observed that administration of Y-27632 had no effect on CXCR2 expression on neutrophils in pancreatitis, suggesting that pancreatitis-associated CXCR2 shedding from the surface of neutrophils is not related to Rho-kinase activity.

Taken together, our novel data show that inhibition of Rho-kinase signalling ameliorates tissue damage in pancreatitis. Indeed, our findings show that Rho-kinase regulates trypsinogen activation in pancreatitis and that interference with Rho-kinase activity decreased MIP-2 formation, neutrophil activation (Mac-1 expression) and recruitment in the pancreas. Thus, our results not only elucidate important signalling mechanisms in pancreatitis but also suggest that targeting Rho-kinase activity may be a useful approach to protect against pathological tissue damage in SAP.

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Conflict of interest

The authors state no conflict of interest.

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Paper V

NFATc3 regulates trypsinogen activation, neutrophil recruitment and tissue damage in acute pancreatitis in mice

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Running title: NFAT and acute pancreatitis
Key words: NFAT**,** amylase, inflammation, leukocytes and pancreas
Summary sentence: NFATc3 plays an important role in trypsinogen activation, leukocyte recruitment and tissue damage in acute pancreatitis

Abbreviations:
(AP) acute pancreatitis; (NFAT) nuclear factor of activated T-cells; (TAP) trypsinogen activation peptide; (i.p.) intraperitoneal; (MPO) myeloperoxidase; (MIP-2/CXCL2) macrophage inflammatory protein-2; (RT) room temperature; (CCK) cholecystokinin; (CsA) cyclosporin A; (NFAT-luc) NFAT-luciferase; (GC/MS) gas chromatography/mass spectrometry; (BSA) bovine serum albumin; (PI) propidium iodide; (RLU) relative luciferase units; (PS) physiological solution; (RTU) relative trypsin units; (Ach) acetylcholine

Conflict of interest:
The authors state no conflict of interest

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Abstract:

Acute pancreatitis (AP) is an inflammatory disease with a wide clinical spectrum. Elevated cytosolic calcium, trypsinogen activation and inflammation are key components in the pathophysiology of AP but the molecular mechanisms leading to the initiation and propagation of these events are still unclear. Here, we explore the potential involvement of the calcium/calcineurin-dependent transcription factor nuclear factor of activated T-cells (NFAT) in AP. Retrograde infusion of sodium taurocholate into the pancreatic duct in transgenic NFAT-luciferase (NFAT-luc) reporter mice resulted in elevated blood amylase, pancreas and lung myeloperoxidase (MPO), pancreas macrophage inflammatory protein-2 (CXCL2), trypsinogen activation and severe tissue destruction, as indicated by acinar cell necrosis, edema, leukocyte infiltration and hemorrhage in the pancreas. In these mice, NFAT-dependent transcriptional activity was significantly increased in pancreas, as well as in aorta, lung and spleen. Treatment with the NFAT inhibitor A-285222 completely blocked taurocholate-induced NFAT activation in all organs and led to restored levels of inflammatory mediators as well as preserved tissue architecture. Similarly, NFATc3-deficient mice were protected from taurocholate-induced trypsinogen activation, inflammation and destruction of pancreatic tissue. Confocal immunofluorescence experiments revealed that NFATc3 is expressed in acinar cells and readily activated by stimuli leading to increased intracellular calcium (i.e. cholecystokinin "CCK", acetylcholine "Ach", ionomycin) and that this is prevented by the calcineurin blocker cyclosporine A (CsA) or by A-285222. Secretagogue-induced activation of trypsinogen in acinar cells *ex vivo* was also NFAT-dependent, since pharmacological inhibition with A-285222 or lack of NFATc3 protein abolished the response. Our data suggests that NFATc3 is a critical regulator of trypsinogen activation, neutrophil recruitment and pancreatic tissue damage and may be a potential therapeutic target in AP.

Introduction

Acute pancreatitis (AP) includes a wide spectrum of clinical presentations ranging from a mild condition with transient pain to a severe disease with local and systemic complications (1, 2). At the moment, there is no valid method to predict the severity and outcome of AP. In spite of significant research efforts, specific treatment of patients with AP is lacking and management is largely limited to supportive care, which is in part due to insufficient understanding of the basic pathophysiology (3). It is widely held that trypsinogen activation, inflammation and disturbed microvascular perfusion are important components in AP (4-6). Activation of trypsinogen appears to be an early and transient phenomenon, whereas the inflammatory process is longer in duration and might be a more useful target for specific therapeutic interventions in AP (7, 8). A prominent feature of the inflammatory response is the extravascular accumulation of leukocytes at sites of tissue injury. Indeed, several reports have shown that leukocyte recruitment is a critical feature in the pathophysiology of AP (9-12). Leukocyte activation and tissue navigation are coordinated by secreted chemokines, such as CC and CXC chemokines. In the mouse, the CXC chemokine family includes macrophage inflammatory protein-2 (MIP-2/CXCL2) and cytokine-induced neutrophil chemoattractant (KC/CXCL1), which predominately attract neutrophils (13). CXC chemokine receptor-2 (CXCR2) is the high affinity receptor on murine neutrophils for CXCL1 and CXCL2 and has been shown to regulate neutrophil accumulation and tissue injury in AP (14). Moreover, recent studies

have demonstrated that adhesion molecules, such as lymphocyte function antigen-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) mediate neutrophil-dependent tissue damage in AP (10, 11). Although, the role of specific chemoattractants and adhesion molecules orchestrating neutrophil infiltration in the pancreas is relatively well described, the understanding of the signaling pathways underlying pro-inflammatory actions in AP is limited.

Extracellular stress signals, such as ischemia and infection, initiate intracellular signaling cascades that converge on specific transcription factors regulating gene expression of pro-inflammatory mediators. A dysregulated increase in cytosolic calcium is a major determinant of protease activation in the pancreas (15, 16). One key target of calcium in eukaryotic cells is calcineurin, a unique calcium/calmodulin activated serine/threonine protein phosphatase (also known as protein phosphatase 2B, PP2B), which plays a central role in a number of cellular processes and calcium-dependent signal transduction pathways (17, 18). Calcineurin is potently inhibited by the immunosuppressant drugs FK506 and CsA, which are widely used for preventing transplant rejection (19). Interestingly, a recent study reported that FK506 inhibits protease activation in pancreatic acinar cells and protects against mild pancreatitis *in vivo* (20). However, calcineurin inhibition, due to its ability to engage a broad range of substrates and binding partners (i.e. transcriptional regulators, receptor ion channels, cytoskeleton, cell cycle/apoptosis proteins and critical scaffolders) that confer substrate specificity (17, 21, 22), is associated with serious side-effects and may not be suitable to use in patients with AP (23, 24). Instead, we hypothesized herein that inhibition of downstream targets of calcineurin signaling may be a more useful approach to inhibit protease activation and pathological inflammation in AP. One important downstream target of calcineurin is the family of four nuclear factors of activated T cells (NFATc1-c4) transcription factors, which are heavily phosphorylated and cytosolic under basal conditions, but able to translocate to the nucleus upon stimulation and dephosphorylation by calcineurin (18). NFAT activation initiates a cascade of transcriptional events involved in physiological and pathological processes (25, 26). NFAT was originally described as a transcriptional activator of cytokine and immunoregulatory genes in T cells (27), but is now known to play a role in several cell types outside the immune system (25). However, the potential role of NFAT in the pathophysiology of AP remains elusive.

Based on the considerations above, the aim of this study was to investigate whether NFAT is activated in AP *in vivo* and whether it plays a role in trypsinogen activation, inflammation and tissue damage in AP. More specifically, we explored the role of NFATc3 in the regulation of trypsinogen activation, neutrophil recruitment and pancreatic tissue damage in AP.

Results

NFAT-dependent transcriptional activity in AP

In order to examine whether AP engages the NFAT signaling pathway, we measured local and systemic NFAT-dependent transcriptional activity in response to retrograde infusion of sodium taurocholate in the pancreatic duct in transgenic NFAT-luc reporter mice. This procedure caused a significant increase in luciferase activity in the pancreas as well as in the aorta, lung and spleen 24 h after taurocholate infusion when compared to control mice in which saline infusion was performed instead (Figure 1). Our results indicate that both local and

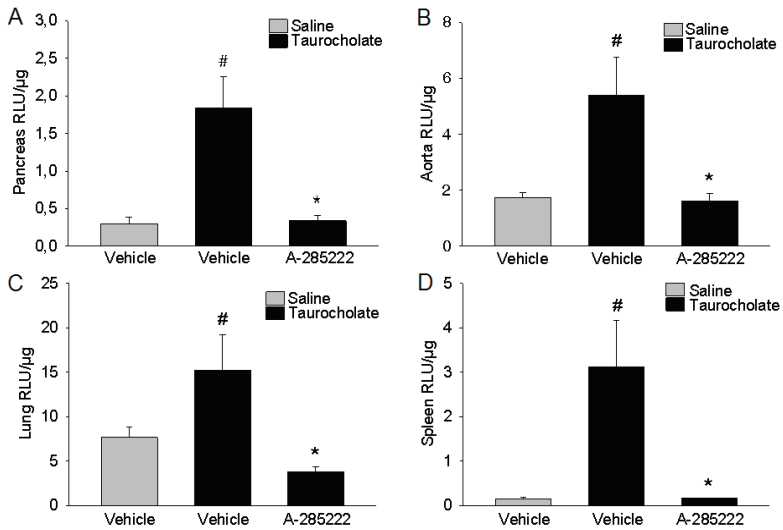

Figure 1. Luciferase levels (RLU/µg protein) in A) pancreas B) aorta C) lung and D) spleen in NFAT-luc mice. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline alone. Animals were pre-treated with i.p. injections of the NFAT blocker A-285222 or vehicle twice daily for 1 week before pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 6$. $^{\#}P < 0.05$ versus saline control, $^{*}P < 0.05$ versus vehicle + taurocholate.

systemic NFAT-dependent transcriptional activity take place in response to AP (Figure 1). Treatment with the NFAT inhibitor A-285222 completely blocked the taurocholate-induced NFAT activation in all studied organs, demonstrating that A-285222 is an effective inhibitor of NFAT transcriptional activity *in vivo* (Figure 1). A-285222 (0.15 mg/kg body weight) was administered i.p. twice a day for 7 days before surgery and once in the morning prior to surgery. Using this protocol, we found that plasma levels of A-285222 ranged between 10 and 98 nM 24 h after taurocholate injection.

NFAT regulates tissue damage in AP

Blood levels of amylase, an established indicator of pancreatic injury, were significantly enhanced after challenge with taurocholate when compared to saline controls (Figure 2A). Treatment with the NFAT blocker A-285222 significantly decreased taurocholate-provoked amylase levels by 59% (Figure 2A). MPO levels can be used as a marker of neutrophil infiltration. We found that taurocholate infusion resulted in increased pancreatic and lung MPO levels and that these increases were markedly reduced by treatment with A-285222 (Figure 2B and C). Neutrophil trafficking is known to be coordinated by CXCL2 (28). Herein, it was found that CXCL2 levels were low in normal pancreas but significantly increased in response to taurocholate challenge (Figure 2D). Administration of A-285222 greatly reduced CXCL2 levels in the inflamed pancreas (Figure 2D). Trypsinogen activation was determined by measuring pancreatic levels of trypsinogen activation peptide (TAP). Taurocholate injection significantly increased TAP levels in the pancreas (Figure 2E). Administration of A-285222 markedly decreased the taurocholate-induced increase

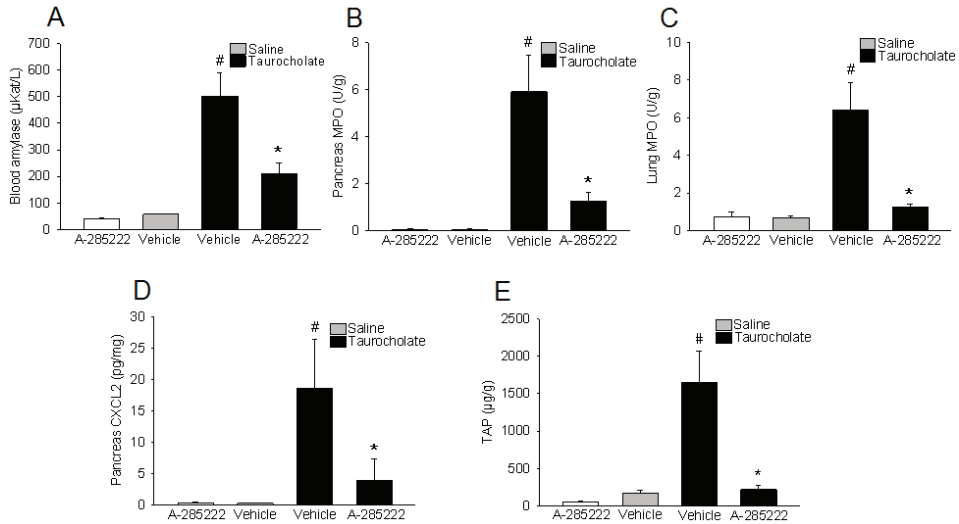

Figure 2. Quantitative measurements of A) blood amylase levels (µKat/L) B) pancreatic MPO (U/g tissue) C) lung MPO (U/g tissue) D) pancreatic CXCL2 (pg/mg tissue) and E) pancreatic TAP (µg/g tissue) in NFAT-luc mice. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline alone. Animals were pre-treated with i.p. injections of A-285222 or vehicle twice daily for 1 week before pancreatitis induction. One group (white bar) received A-285222 without bile duct cannulation. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 6$. $^{\#}P < 0.05$ versus saline control, $^{*}P < 0.05$ versus vehicle + taurocholate.

in TAP levels in the pancreas (Figure 2E). Blood amylase, pancreas and lung MPO, pancreas CXCL2 and TAP levels in control mice treated with A-285222 alone were not different from saline infused control mice (Figure 2). Morphological examination revealed normal microarchitecture in the pancreas from control animals, whereas challenge with taurocholate caused severe destruction of the pancreatic tissue structure characterized by extensive acinar cell necrosis, edema, massive infiltration of leukocytes and hemorrhage (Figure 3A-D). Treatment with A-285222 protected against taurocholate-induced destruction of the tissue architecture, significantly reducing taurocholate-induced acinar cell necrosis, edema, leukocyte infiltration and hemorrhage in the pancreas (Figure 3E-H). Tissue architecture was not affected in mice treated with A-285222 alone when compared to saline infused control mice (Figure 3A-H).

NFATc3 regulates tissue damage in AP

Differences in expression and unique functions have been attributed to each of the NFAT isoforms in various tissues (29, 30), therefore, we next asked which NFAT isoforms are expressed in the pancreas. Using RT-PCR, we found that NFATc1 and NFATc3 were readily detected both in extracts of pancreatic lobules and of isolated acini, whereas NFATc2 was expressed weakly, if at all, and NFATc4 was not detected (Figure 4). As expected, all NFAT isoforms were expressed in the thymus (Figure 4). Since we and others have described significant plasticity in the expression levels of NFAT proteins during

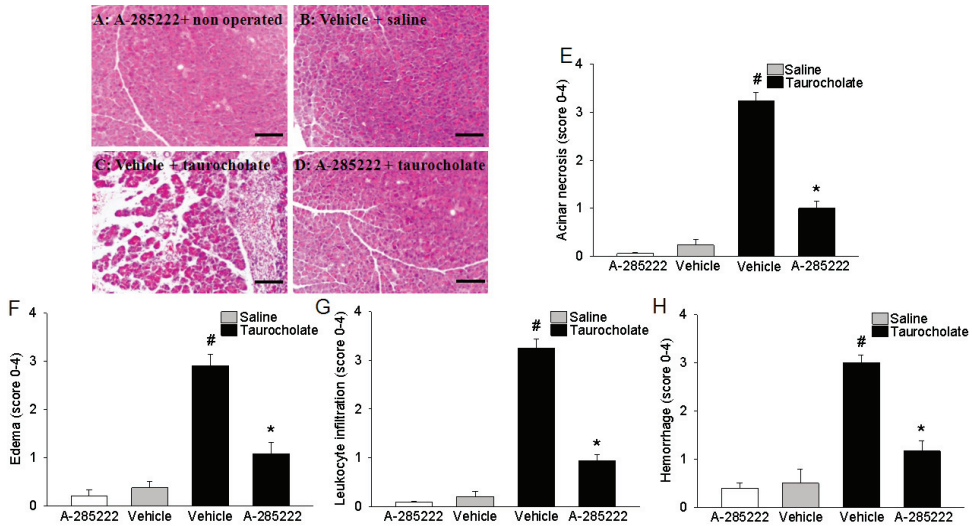

Figure 2. Quantitative measurements of A) blood amylase levels (μKat/L) B) pancreatic MPO (U/g tissue) C) lung MPO (U/g tissue) D) pancreatic CXCL2 (pg/mg tissue) and E) pancreatic TAP (μg/g tissue) in NFAT-luc mice. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline alone. Animals were pre-treated with i.p. injections of A-285222 or vehicle twice daily for 1 week before pancreatitis induction. One group (white bar) received A-285222 without bile duct cannulation. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 6$. $^{\#}P < 0.05$ versus saline control, $^{*}P < 0.05$ versus vehicle + taurocholate.

development and/or growth stimulatory conditions (31, 32), we examined the pattern of NFAT isoform expression in young (18-22 days) and adult (5-6 weeks) mice. Interestingly, the very modest expression of NFATc2 was only detected in adult mice and not in the young ones (Figure 4B). We then examined the role of NFATc3 in AP. For this purpose, we used NFATc3 heterozygous ($NFATc3^{+/-}$), homozygous knockout mice ($NFATc3^{-/-}$) and control littermates ($NFATc3^{+/+}$). Twenty four hours after infusion of taurocholate, blood amylase and pancreas and lung MPO levels were significantly increased in control NFATc3-competent mice, whereas the response was greatly reduced in NFATc3-deficient mice (Figure 5A-C). Moreover, induction of CXCL2 levels observed in $NFATc3^{+/+}$ mice with AP was blunted in $NFATc3^{-/-}$ mice (Figure 5D). Notably, the taurocholate-induced elevation of TAP levels in the pancreas were abolished in $NFATc3^{-/-}$ animals (Figure 5E). It was also observed that mice lacking NFATc3 were protected against morphological tissue damage evoked by taurocholate (Figure 6A-E). Quantification of histological parameters showed that taurocholate-provoked acinar necrosis, edema, leukocyte infiltration and hemorrhage were greatly attenuated in $NFATc3^{-/-}$ mice (Figure 6F-I). Heterozygous $NFATc3^{+/-}$ animals exhibited an intermediate phenotype, with significantly reduced taurocholate-induced acinar necrosis, edema, leukocyte infiltration and hemorrhage when compared to NFATc3 competent mice (Figures 6A-E); as well as trends towards reduced blood amylase, pancreas MPO, CXCL2 and TAP levels (Figure 5).

NFATc3 regulates trypsinogen activation in acinar cells

We next asked whether NFATc3 may regulate trypsinogen activation in pancreatic

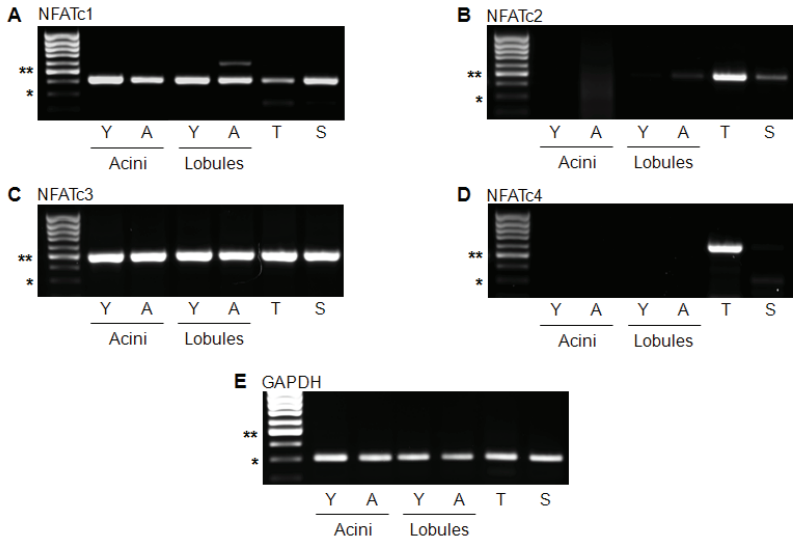

Figure 4. Expression of NFATc1-c4 isoforms and the housekeeping gene GAPDH was determined using RT-PCR in isolated pancreatic acini and lobules from young (Y, 18-22 days) and adult (A, 5-6 weeks) mice. Thymus (T) and spleen (S) were used as controls. The expected sizes of the products are: NFATc1 416 bp, NFATc2 452 bp, NFATc3 499 bp, NFATc4 649 bp and GAPDH 293 bp. * and ** indicate 300 and 500 bp markers, respectively.

acinar cells. For this purpose, we isolated acinar cells from the pancreas of mice and incubated them with the CCK related peptide cerulein. Cerulein stimulation significantly enhanced trypsinogen activation compared to control unstimulated cells (Figure 7). Preincubation of the acinar cells with A-285222 markedly decreased secretagogue-induced activation of trypsinogen (Figure 7). Furthermore, we found that cerulein-induced trypsinogen activation was abolished in acinar cells isolated from mice lacking NFATc3 (Figure 7).

Nuclear accumulation of NFATc3 in acinar cells

Under similar experimental conditions as the ones used to induce trypsinogen activity in Figure 7, incubation of pancreatic acinar cells with a supramaximal dose of CCK (10 nM), which is equivalent to the dose used for cerulein, resulted in significantly increased NFATc3 nuclear accumulation (Figure 8A and B). Confocal images showing NFATc3 in green and nuclei stained with propidium iodide (PI) in red, demonstrated predominantly cytosolic distribution of endogenous NFATc3 under basal non-stimulated conditions, with low but detectable nuclear staining in some cells (Figure 8A). In response to CCK stimulation, NFATc3 nuclear accumulation was significantly increased (indicated by the lack of black holes, "NFATc3-empty" nuclei or the white nuclei in the merged panel in Figure 8A). Summarized data from confocal experiments demonstrated that the response was quite rapid with a peak at 7 min, but still elevated NFATc3 nuclear accumulation after 50 min of CCK stimulation (Figure 8B). To further explore the activation properties of NFATc3 in acinar cells, we tested a lower more physiological dose of CCK (10 pM) and the muscarinic agonist Ach (10 µM). Both agonists resulted in a significant and transient elevation of NFATc3 nuclear accumulation (supplemental Figure 1A and B). All agonist-induced responses were

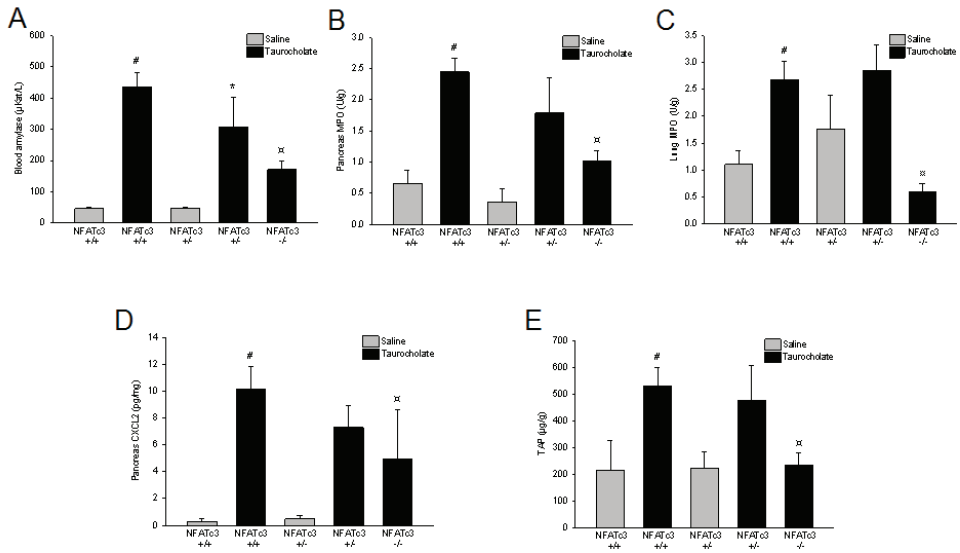

Figure 5. Quantitative measurements of A) blood amylase levels (µKat/L) B) pancreatic MPO (U/g) C) lung MPO (U/g) D) pancreatic CXCL2 (pg/mg) and E) pancreatic TAP (µg/g) in NFATc3 $^{+/+}$, NFATc3 $^{+/-}$ and NFATc3 $^{-/-}$ mice. Pancreatitis (black bar) was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice (grey bar) received saline alone. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM and $n = 8$. $^{\#}P < 0.05$ versus saline control, $^{¤}$ and $^{*}P < 0.05$ versus taurocholate.

prevented by pre-incubation for 30 min with CsA (10 µM, supplemental figure 1C). Incubation for 10 min with the calcium ionophore ionomycin (10 µM) also resulted in NFATc3 nuclear accumulation (supplemental Figure 1C). CsA alone had no effect on NFATc3 nuclear accumulation.

Discussion

This study documents for the first time that NFAT signaling is a key feature in the pathophysiology of AP. It was observed that AP is associated with increased NFAT transcriptional activity in the pancreas as well as in the lung, aorta and spleen and that pharmacological inhibition of NFAT signaling reduces AP-induced activation of trypsinogen, neutrophil recruitment and tissue damage. We found that trypsinogen activation, CXCL2 expression as well as neutrophil infiltration in the pancreas and lung were markedly reduced in NFATc3-deficient mice. In addition, secretagogue-induced activation of trypsinogen in isolated acinar cells was associated with increased nuclear accumulation of NFATc3. Agonist-provoked trypsin activity was decreased by inhibition of NFAT signaling and abolished in acinar cells from NFATc3-deficient mice. Thus, these findings show that NFAT signaling plays an important role in AP and that targeting NFATc3 may be a useful strategy to ameliorate pathological inflammation in the pancreas.

NFAT activity is generally considered to control key aspects of tissue development, including vasculogenesis, axonal outgrowth, muscle and bone formation as well as maturation of the gastrointestinal tract and immune system (33-37). However, a growing body of literature also implicates a role of NFAT signaling in inflammatory

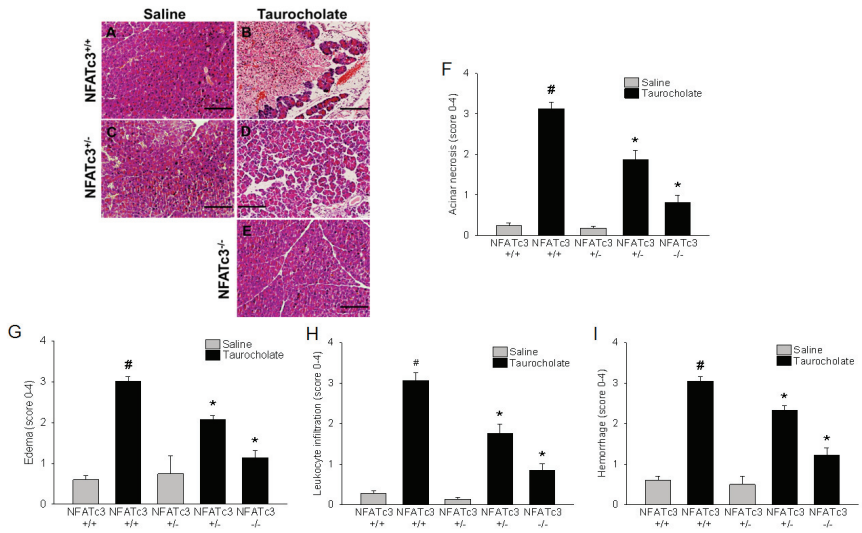

Figure 6. Representative haematoxylin & eosin sections of the pancreas from NFATc3$^{+/+}$, NFATc3$^{+/-}$ and NFATc3$^{-/-}$ mice of A) NFATc3$^{+/+}$ + saline B) NFATc3$^{+/+}$ + taurocholate C) NFATc3$^{+/-}$ + saline D) NFATc3$^{+/-}$ + taurocholate and E) NFATc3$^{-/-}$ + taurocholate and histological quantification of F) acinar cell necrosis G) edema H) leukocyte infiltration and I) hemorrhage. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct. Control mice received saline alone. Samples were obtained 24 h after induction of pancreatitis. Bars represent 100 µm. Data represent means ± SEM and $n = 8$. $^{\#}P < 0.05$ versus saline control, $^{*}P < 0.05$ versus vehicle + taurocholate.

processes, such as arteriosclerosis and autoimmune diseases (38, 39). In the present study, it was hypothesized that AP, which is an acute inflammatory disease, is associated with increased NFAT-dependent transcriptional activity. We found that NFAT was not only activated in the pancreas but also in the aorta, spleen and lung, suggesting that AP is associated with both local and systemic engagement of NFAT signaling. The role of NFAT was investigated by the use of a novel inhibitor, A-285222, which prevents nuclear accumulation of all NFAT isoforms (40). Indeed, it was observed that *in vivo* administration of this NFAT inhibitor completely blocked AP-induced NFAT-dependent transcriptional activity not only in the pancreas but also in the aorta, spleen and lung, suggesting that A-285222 is an effective inhibitor of NFAT activation. Previous experiments in cynomolgus monkeys showed that the inhibitory effect of A-285222 on T-cell cytokine production was similar to that of CsA, when administered at concentrations that did not yield any toxic side effects (41). In this study, animals were treated with 7.5 mg/kg of A-285222 twice daily during 14 days, a regime that resulted in plasma levels between 1 and 2 µg/ml (2.4 and 4.8 µM). In a recent study using mice, we found that lower doses (0.15 mg/kg) were sufficient to achieve NFAT-signaling inhibition in the vasculature and that the drug was well tolerated for a 4-week study period (42). In the current study, we treated the animals with 0.15 mg/kg twice daily for a week, which resulted in plasma levels in the nanomolar range and no side effects. Interestingly, at this low plasma level A-285222 significantly reduced tissue damage in AP, as evidence by the reduction in taurocholate-induced serum amylase

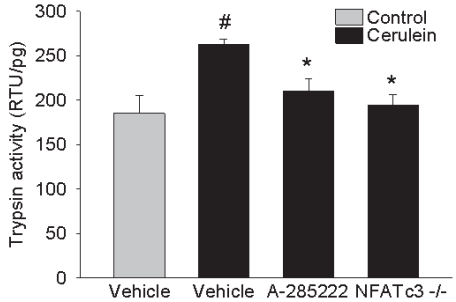

Figure 7. Activation of trypsinogen was measured in acinar cells from wild type and NFATc3-deficient mice. Cerulein was used to activate trypsinogen in acinar cells. The cells were pre-treated with vehicle or the NFAT blocker A-285222 (1 μM). Trypsinogen activation was determined by measuring enzymatic activity of trypsin fluorometrically by using Boc-Glu-Ala-Arg-MCA as the substrate as described in detail in Methods. Trypsin levels (RTU/pg) were calculated using a standard curve generated by assaying purified trypsin and normalized to protein concentration. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control + vehicle and $^{*}P < 0.05$ versus cerulein + vehicle.

and acinar cell necrosis by more than 41% and 69% respectively. This indicates that NFAT may regulate a substantial part of the tissue injury in pancreatitis. It is widely held that premature and local activation of proteolytic enzymes, such as trypsinogen, constitutes an early component in the pathophysiology of AP (5, 12). Activation of trypsinogen increases the formation of TAP, which has been shown to reflect disease severity in early phases of AP (43). In this context, it is worth to note that inhibition of NFAT activity attenuates TAP levels in the pancreas, indicating that NFAT signaling is involved in the activation of trypsinogen in AP. Interestingly, a recent study reported that FK506, a calcineurin inhibitor, ameliorates protease activation and tissue damage in AP (20). Considering that NFAT activity is regulated by calcineurin (27), our findings may help explain the protective effects exerted by FK506 in AP. Collectively, our data suggest a pathological role for the calcium/calcineurin-NFAT signaling axis in the development of AP similar to that proposed for the development of cardiac hypertrophy, diabetes-induced vascular inflammation and arteriosclerosis (39, 44, 45). Nonetheless, our present results constitute the first evidence that the NFAT signaling pathway plays an important role in protease activation and tissue injury in AP. Leukocyte accumulation at extravascular sites is a hallmark of inflammatory processes. Although the primary purpose of leukocyte recruitment is to protect against infection and promote wound healing, under certain circumstances leukocytes may cause tissue injury, such as graft rejection, cholestasis and sepsis (46-49). In fact, several studies have documented that neutrophil depletion markedly protects against tissue damage in pancreatitis, suggesting that neutrophil infiltration is a rate-limiting step in AP (9-12, 50). In the present study, we found that taurocholate challenge markedly increased MPO levels and the number of extravascular neutrophils in the pancreas. Treatment with A-285222 significantly reduced both MPO activity (79%) and tissue neutrophils (71%) in the pancreas, indicating that NFAT signaling coordinates neutrophil infiltration in AP. Moreover, systemic complications of severe AP include neutrophil accumulation in the lung (51). Indeed, we found that administration of A-285222 significantly reduced the taurocholate-induced increase in MPO activity in the lung, suggesting that NFAT activity also regulates systemic pulmonary recruitment of neutrophils. Considered together, our results demonstrate that NFAT signaling controls both local and distant organ neutrophilia in AP. Specific chemokines secreted from resident tissue cells co-ordinate tissue navigation of leukocytes to sites of inflammation (28). CXC chemokines, such as CXCL2, are particularly potent activators of neutrophils and CXCL2 has been shown to play a significant role in AP (52). It was therefore of interest to examine local formation of

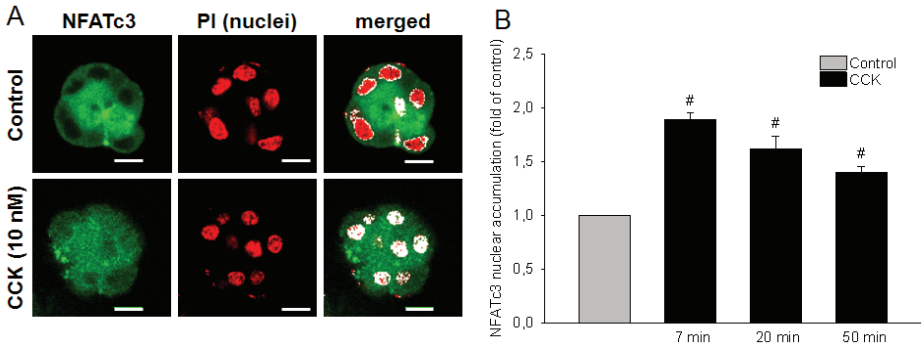

Figure 8. A) Confocal immunofluorescence images showing predominantly cytosolic distribution of endogenous NFATc3 under basal non-stimulated conditions (control, upper panels) and increased NFATc3 nuclear accumulation in response to 10 nM CCK stimulation for 7 min (lower panels). NFATc3 staining is shown in green, PI-stained nuclei in red, co- localization of the signals is shown in white in the merged images. Scale bars represent 10 µm. B) Summarized data from confocal experiments as in A, showing NFATc3 nuclear accumulation in response to 7, 20 and 50 min stimulation with 10 nM CCK. Results are expressed as fold of control; $^{\#}P < 0.05$ versus control (331, 106 and 101 cells were examined from 8, 4 and 4 mice, respectively for each time-point).

CXCL2 in the pancreas in this study. We observed that taurocholate caused a clear-cut increase in CXCL2 formation in the pancreas. Notably, inhibition of NFAT activity reduced taurocholate-induced expression of CXCL2 by 78%, which may help explain the inhibitory effect of A-285222 on neutrophil accumulation in AP as observed herein. Considering the critical role of neutrophils in pancreatitis (9-12, 50), our findings suggest that NFAT-regulated trafficking of neutrophils is a key component in the protective effects exerted by A-285222 in AP.

The NFAT family consists of four Ca^{2+}-dependent isoforms (NFATc1-c4), which are phosphorylated and located in the cell cytosol under basal conditions. Upon dephosphorylation by calcineurin, activated NFATs are translocated to the nucleus where they trigger the transcription of numerous pro-inflammatory genes (53). Having established that NFAT activity constitutes an important signaling pathway in AP, we next examined the pattern of NFAT isoform expression in the pancreas. Both NFATc1 and NFATc3 were detected in pancreatic lobules and isolated acini. NFATc1-deficient mice are embryonic lethal due to a defect in cardiac development (54, 55). Hence, as an alternative approach to the pharmacological inhibition of NFAT with A-285222, we used NFATc3-deficient mice to further establish the role of NFAT signaling in AP. In these mice lacking NFATc3, taurocholate-induced increases in serum amylase and tissue damage in the pancreas were markedly attenuated. Moreover, they exhibited significantly decreased infiltration of neutrophils in the pancreas and lungs as well as reduced CXCL2 formation in the pancreas in response to taurocholate when compared to NFATc3 competent mice. Consistently, heterozygous NFATc3 mice had an intermediate phenotype between knockout and wild-type animals when inflammatory and tissue injury parameters were examined. These findings suggest for the first time that NFATc3 is an important regulator of neutrophil recruitment and tissue damage in AP. In this context, it is interesting to note that another isoform, NFATc2, appears instead to be a negative regulator of

lymphocyte and eosinophil accumulation in allergic inflammation (56, 57). Considered together with our data, it may be concluded that specific NFAT isoforms may exert opposing effects on leukocyte infiltration in different models of inflammation. Moreover, we found that taurocholate-induced formation of TAP in the pancreas was completely inhibited in NFATc3 gene-deficient mice, indicating that NFATc3 is required for trypsinogen activation in AP. To define the role of NFAT signaling in regulating trypsin activity in acinar cells, we isolated pancreatic acini and stimulated them with the secretagogue cerulein. We observed that cerulein-induced trypsin activity in acinar cells was abolished by administration of the NFAT inhibitor A-285222. In addition, secretagogue-provoked trypsin activity was blunted in acinar cells isolated from NFATc3 gene-deficient mice. These results suggest that NFATc3-mediated cell signaling controls trypsinogen activation in the pancreas. This notion was also supported by our findings showing that secretagogue stimulation of isolated acinar cells resulted in a rapid and clear-cut increase in NFATc3 nuclear accumulation. CsA, a calcineurin inhibitor, significantly decreased secretagogue-induced NFATc3 nuclear accumulation in acinar cells. This is in line with a previous study showing that inhibition of calcineurin ameliorates protease activation and tissue damage in the pancreas (20), and suggests that calcineurin-dependent activation of NFATc3 may constitute a new and central signaling pathway in the regulation of trypsinogen activation, neutrophil recruitment and tissue injury in AP, which may open new opportunities to treat patients with AP. The NFAT signaling pathway has been well recognized in adaptive immunity, but little evidence exists to suggest a role for NFAT in innate immunity. Interestingly a recent study reported that toll-like receptor signaling in bone marrow-derived macrophages appears to be dependent on NFATc3 and NFATc4 activity (58). Our findings showing that NFATc3 is required for the induction of AP, a process dominated by increased activation of innate immunity, further strengthens the evolving concept that NFAT signaling may constitute a significant component in innate inflammatory processes.

In conclusion, our data show that NFAT-dependent transcriptional activity is increased in AP and that inhibition of NFAT signaling ameliorates tissue damage in AP. Indeed, interference with NFAT activity reduced CXCL2 formation and neutrophil recruitment in the pancreas. Moreover, we demonstrate that NFAT signaling regulates trypsinogen activation in acinar cells and that in particular NFATc3 is an important molecular mediator in these processes. Thus, our results not only elucidate critical signaling mechanisms in pancreatitis but also suggest that targeting NFATc3 activity may be a useful strategy to protect against pathological tissue damage in AP.

Materials and Methods

Animals

All experiments were done in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for animal experimentation at Lund University, Sweden and University of Extremadura, Spain. Male mice of the following strains were bred and used at age 6-8 weeks (20-26 g): FVBN 9x-NFAT-luciferase reporter mice (NFAT-luc) and wild-type littermates, as well as BALB/c NFATc3 knockout ($NFATc3^{-/-}$), heterozygous ($NFATc3^{+/-}$) and wild-type littermates ($NFATc3^{+/+}$) (59). NFAT-luc mice are phenotypically normal and express nine copies of an NFAT binding site from the interleukin-4 promoter, positioned 5' to a

minimal promoter from the α-myosin heavy chain gene (-164 to +16) and inserted upstream of a luciferase reporter gene (42). For isolation of pancreatic acinar cells for RT-PCR and confocal immunofluorescence experiments, male Swiss ICR (CD-1, Harlan Laboratory Models, Barcelona, Spain) mice were used. Animals were maintained in a climate-controlled room at 22°C and exposed to a 12:12-h light-dark cycle. Animals were fed standard laboratory diet and given water *ad libitum.* Mice were anesthetized by intraperitoneal (i.p.) administration of 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight in 200 µl saline. Analgesia was obtained by subcutaneous injection of buprenorfin hydrochloride 0.1 mg/kg (Schering-Plough Corporation, New Jersey, USA).

AP induction and experimental design

AP was induced by retrograde infusion of bile salt (taurocholate) into the pancreas as previously described (60, 61). Briefly, the duodenum and the attached pancreatic head were exposed through a midline incision. The papilla of Vater was identified, the duodenum was immobilized by two 7-0 prolene sutures and a small puncture was made through the duodenal wall in parallel to the papilla of Vater with a 23G needle. A polyethylene catheter (internal diameter 0.28 mm) connected to a micro-infusion pump (CMA/100, Carnegie Medicin, Stockholm, Sweden) was introduced 1 mm into the pancreatic duct. The common hepatic duct was identified at the liver hilum and clamped with a neurobulldog clamp to prevent hepatic reflux. Overall 10 µl of either saline or 5% taurocholate (Sigma Chemical Company, St. Louis, Missouri, USA) was infused into the pancreatic duct at a rate of 2 µl/min. Before suturing the abdominal wall, the bile duct clip was removed and the duodenal puncture wound closed (7-0 prolene). Animals were sacrificed 24 h after induction of AP. Blood was obtained from the vena porta and plasma was frozen at -20˚C. The stomach, duodenum and the pancreatic head were rapidly removed in one piece. The pancreatic head was carefully separated from the duodenum to avoid any contamination by mucosal enterokinase. Two thirds of the pancreatic sample was divided in two pieces and flash frozen in liquid nitrogen while the rest was fixed in formaldehyde. A piece of lung, aorta and spleen were also frozen for luciferase measurements. For *in vivo* experiments using NFAT-luc mice, animals were randomized into three groups: 1) a positive taurocholate-infused group pre-treated with vehicle (saline), 2) a negative control also operated but infused with saline only and 3) a taurocholate-infused group treated with the NFAT blocker A-285222 (0.15 mg/kg body weight, administered i.p., twice daily for 1 week and in the morning of the AP induction). To test the effects of pharmacological treatment on control healthy mice, an additional non-operated group received A-285222 as group 3 above. For experiments using BALB/C NFATc3 knockout mice, animals were operated and infused either with taurocholate or saline as controls. A-285222 was kindly provided by Abbott Laboratories and has been previously described (40).

Luciferase reporter assay

Luciferase activity was measured in pancreas, aorta, lung, and spleen in NFAT-luc mice treated as specified in the text. Assays were performed as previously described (31, 62) and optical density measured using a Tecan Infinite M200 instrument (Tecan Nordic AB, Mölndal,

Sweden) and normalized to protein concentration and expressed as relative luciferase units (RLU) per µg protein.

Blood amylase

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

MPO assay

Frozen pancreatic and lung tissue were pre-weighed and homogenized in one ml mixture (4:1) with PBS and aprotinin 10 000 KIE/ml (Trasylol®, Bayer HealthCare AG, Leverkusen, Germany) for one min. The homogenate was centrifuged (15339x*g*, 10 min) and the supernatant was stored at -20°C and the pellet was used for MPO assay as previously described (63). In brief, the pellet was mixed with one ml of 0.5% hexadecyltrimethylammonium bromide. Next, the sample was frozen for 24 h and then thawed, sonicated for 90 sec, put in water bath 60°C for two h, after which the MPO activity of the supernatant was measured. The enzyme activity was determined spectrophotometrically as the MPO-catalyzed change in absorbance in the redox reaction of H_2O_2 (450 nm, with a reference filter 540 nm, 25°C). Values are expressed as MPO units per gram tissue.

CXCL2 levels

Tissue levels of CXCL2 were determined in serum and pancreatic tissue by using double-antibody Quantikine enzyme linked immunosorbent assay kits (R & D Systems Europe, Abingdon, UK) using recombinant murine CXCL2 as standard. The minimal detectable protein concentration is less than 0.5 pg/ml.

Histology

Pancreas samples were fixed in 4% formaldehyde phosphate buffer overnight and then dehydrated and paraffin embedded. Six micrometer sections were stained (hematoxylin and eosin) and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner by use of a pre-existing scoring system including evaluation of acinar cell necrosis, leukocyte infiltrate, edema and hemorrhage on a 0 (absent) to 4 (extensive) scale as previously described in detail (64).

TAP levels

Trypsinogen is activated to trypsin in a reaction where TAP is cleaved off and thus can be used as a marker of trypsinogen activation (43)**.** Radioimmunosorbent assay was performed as described previously (65). A 0.1 M Tris HCl buffer (PH 7.5) containing 0.15 M NaCl, 0.005 M EDTA and 2 g/l bovine serum albumin (BSA) (Sigma Chemical Company, St. Louis, Missouri, USA) was used as assay buffer. Samples of 100 µl diluted in assay buffer were incubated (16 h, 4°C) with 200 µl of [I^{125}]-Tyr-TAP (=20 000 counts per min) in assay buffer and 200 µl of antiserum diluted 1/750 in assay buffer. Parallel incubations with the synthetic activation peptides TAP diluted in assay buffer in a series of concentrations from 0.078 to 20 nM, were used as standards in the assays. Free and bound radioactivities were separated by means of a second step antibody precipitation. 100 µl of a cellulose coupled anti-mouse IgG suspension (Sc-Cel® IDA, Boldon, England) was added to the samples. After 30 min of incubation one ml of water was added and tubes were centrifuged (704xg, 5 min, room temperature "RT"). The supernatant was decanted and radioactivity of the precipitate was counted in a γ-spectrophotometer.

Preparation of pancreatic acinar cells

Pancreatic acini were prepared by collagenase digestion and gentle shearing as previously described (66), either from Swiss ICR (CD-1) mice for RT-PCR and confocal imaging experiments or from BALB/c wild type for the trypsinogen activation assay. In brief, animal killing was performed by cervical dislocation. Cells were suspended in a physiological solution (PS) containing (mM): 140 NaCl, 4.7 KCl, 2 $CaCl_2$, 1.1 Mg Cl_2, 10 glucose, 10 N-2-hydroxyethylpiperazine-N-2-sulfonic acid (HEPES) and 0.1% BSA (pH 7.4) saturated with O_2. During isolation of the cells used for confocal imaging and PCR procedure 0.01% trypsin inhibitor (soybean) was added to this solution and then incubated with collagenase solution one mg/ml (Sigma Chemical Company, St. Louis, Missouri, USA) at 37°C under gentle agitation (60 cycles/min, 15 min). After stopping the digestion by washing with collagenase free PS, the pancreas was agitated vigorously to release a mixture of single cells and acini to the medium. The supernatant was pipetted with a fire-polished plastic tip to further disintegrate cell clumps and washed twice by mild centrifugation to remove cellular debris (70xg, 1 min, 4ºC) then passed through a 150 µm cell strainer (Partec®, Münster, Germany). Viability of the pancreatic acinar cells was higher than 95% as determined by trypan blue dye exclusion.

Trypsinogen activation in isolated acinar cells

Isolated acinar cells (1 x 10^7 cells per well) were preincubated in PS with A-285222 (1 µM) or vehicle (20 min, 37°C), after which they were stimulated with 100 nM cerulein (1 h, 37°C). The buffer was then discarded and cells washed twice with a buffer (pH 6.5) containing 250 mM sucrose, 5 mM 3-(morpholino) propanesulphonic acid (MOPS) and 1 mM $MgSO_4$. Cells were then homogenized in cold (4°C) MOPS buffer using a potter Elvejham glass homogenizer and centrifuged (56xg, 5 min). Trypsin activity was measured fluorometrically using Boc-Glu-Ala-Arg-MCA as the substrate as described previously (67). For this purpose 200 µl aliquot of the acinar cell homogenate was added to a cuvette containing assay buffer (50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$ and 0.1% BSA, pH 8.0). The reaction was initiated by the addition of substrate, and the fluorescence emitted at 440 nm in response to excitation at 380 nm was monitored. Trypsin levels (pg/µg) were calculated using a standard curve generated by assaying purified trypsin, normalized to protein concentration and expressed as relative trypsin units (RTU) per pg protein.

gas chromatography/mass spectrometry (GC/MS)

To measure the concentration of A-285222 in plasma, blood from healthy controls and taurocholate infused mice was collected from the portal vein at the time of euthanasia. Plasma was pooled from 5-6 mice for each experimental condition. Pooled plasma from 5 mice treated with saline was used as a negative control. All samples were run in duplicates in a randomized order. A known concentration (2.5 µM) of the analogous compound A-216491 was added as an internal standard to all plasma samples. Samples (300 µl) were extracted twice with ethyl acetate (400 µl), followed by evaporation. The dried residues were finally redissolved in chloroform (30 µl) for GC/MS analysis. Identification was based on mass spectra and retention indexes, calculated from the injection of a homologous series of n-alkanes. The concentration of A-285222 in plasma was

determined using a calibration curve calculated from analyses of plasma from untreated mice, spiked with known concentrations of A-285222 and A-216491.

NFAT isoform expression

Pancreas from young (18-22 days) and adult (5-6 weeks) mice were removed and placed in PS solution with trypsin inhibitor. To obtain lobules, pancreas was cut in small pieces of 10 mg and washed in PS. Acinar cells were obtained as explained above. For RNA isolation, tissue or cells was placed in RNA Later (Ambion, Applied Biosystems, Madrid, Spain) immediately after harvesting and RNA was later isolated using Trizol (Invitrogen Life Technologies, Paisley, UK) as previously described (31). Total RNA was further purified using RNeasy Mini Kit (Qiagen, Valencia, CA) with simultaneous DNase treatment (Qiagen, Valencia, CA). For cDNA synthesis, 2 µg of total RNA was used for reverse transcription with the Sensiscript Reverse Transcription Kit (Qiagen, Valencia, CA) with oligo-dT primers according to the manufacturer's instructions. Primer sequences and cycling conditions for NFAT family members were performed as previously described (31), with the following modification of annealing temperatures for mouse samples: NFATc1: 46.5ºC, NFATc2: 50.0ºC, NFATc3: 54.0ºC and NFATc4: 44.0ºC. GAPDH primers used were forward: 5'-TCACCATCTTCCAGGAGCGA-3' and reverse: 5'-CACAATGCCGAAGTGGTCGT-3'. The HotStarTaq Master Mix Kit (Qiagen, Valencia, CA) was used for the PCR reaction and products were analyzed on a 1.5% agarose gel. cDNA from mouse thymus and spleen were used as positive controls.

Confocal Immunofluorescence

Pancreatic acinar cells were seeded on coverslips and stimulated with or without the agonists CCK (10 pM or 10 nM) or ACh (10 µM) for various times as indicated in the text, or with or without the calcium ionophore ionomycin (10 µM, 10 min); each stimuli in the presence or absence of the calcineurin inhibitor CsA (1µM, 30 min). Treatments were done in PS at RT. For measurements of NFATc3 nuclear accumulation, experiments were performed as previously described (31). Briefly, cells were fixed with 4% paraformaldehyde in PBS (15 min, RT), permeabilized with 0.2% TritonX-100 in PBS (15 min, RT) and blocked with 2% BSA in PBS for 2 h. Primary antibody, rabbit polyclonal anti-NFATc3 (1:250, Santa Cruz Biotechnology, Inc) and FITC-labeled secondary antibody, goat anti-rabbit IgG (1:250, Santa Cruz Biotechnology, Inc) were used. Prior staining with the nucleic acid dye PI (1 µg/ml, Molecular Probes, Invitrogen Life Technologies, Paisley, UK) for nuclear identification, cells were treated with ribonuclease A type X-A (Sigma Chemical Company) (30 min, 37ºC) to remove cytosolic RNA and increase the specificity of the PI for nuclear DNA. Cells were examined at x60 magnification using a Bio-Rad MRC 1024ES laser scanning confocal microscope (Bio-Rad Lab, Life Sciences Division, CA, USA). Specificity of immune staining was confirmed by the absence of staining when primary or secondary antibodies were omitted. FITC and PI were excited at 488 nm and the emitted light was collected through 515/30-nm and 605/32-nm bandpass filters, respectively. Multiple fields for each coverslip were acquired and images analyzed using Image J software (NIH, Bethesda, MD, USA). Red fluorescent PI images were used to generate a mask to define the nuclear area. Mean fluorescence intensity of NFATc3 (green) in the nuclear area of each cell was determined and normalized to the mean nuclear NFATc3

fluorescence of the matched control. The number of cells examined and animals used for each experimental condition is indicated in the figure legend.

Statistics

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

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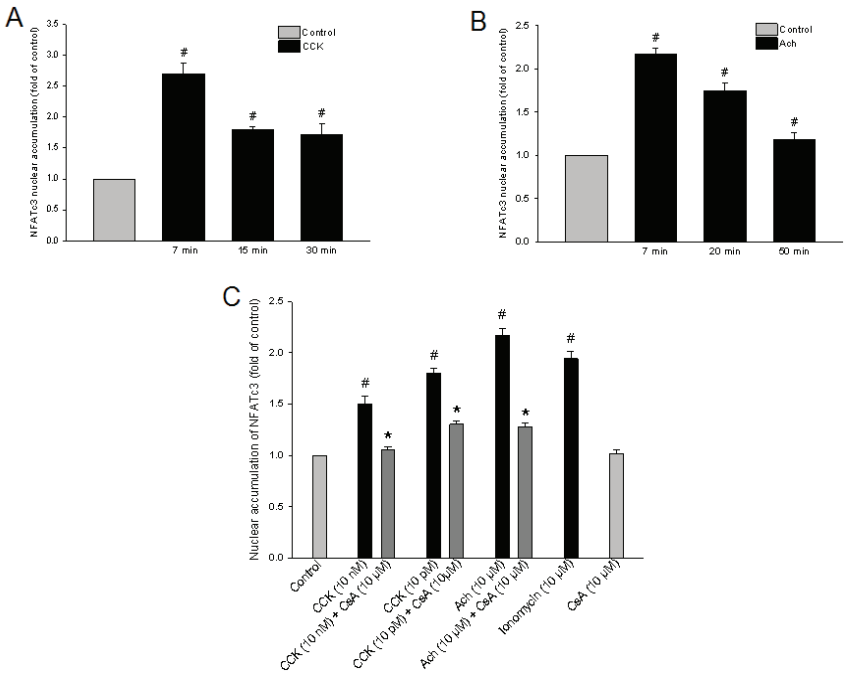

Supplemental Figure 1. Summarized data from confocal immunofluorescence experiments showing NFATc3 nuclear accumulation after stimulation with: A) 10 nM CCK for 7, 15 or 30 min (121, 687 and 103 cells were examined from 4, 12 and 4 mice, respectively for each time-point); and B) 10 µM Ach for 7, 20 and 50 min (426, 185 and 142 cells were examined from 12, 4 and 4 mice, respectively for each time point). C) Agonist-induced NFATc3 nuclear accumulation was prevented by pre-incubation for 30 min with CsA (10 µM). Incubation for 10 min with ionomycin (10 µM) resulted in increased NFATc3 nuclear accumulation, while CsA alone had no effect. $^{\#}P < 0.05$ versus control and $^{*}P < 0.05$ for "agonist + CsA"-treated cells versus corresponding agonist alone. At least 120 cells were examined and 4-12 mice were used in each group. Data is expressed as fold of controls in all graphs.

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1985	Anders Lindhagen
1985	Stefan Arvidsson
1985	Kent Jonsson
1985	Hans Hedlund
1985	Mans Bohe
1986	Henry Svensson
1987	Heitti Teder
1987	Hans Högstrom
1988	Per Uden
1988	Erik Svartholm
1988	Per-Anders Abrahamsson
1989	Toste Länne
1990	Bengt Hjelmqvist
1990	Nils H Persson
1990	Henrik Åkesson
1990	Thomas Mätzsch
1990	Magnus Delshammar
1990	Anders Törnqvist
1990	Magnus Erlansson
1990	Jan Brunkwall
1991	Johan Ottosson

1991	Lars Salemark
1991	Claes Forssell
1991	Agneta Montgomery
1991	Jan Berglund
1991	Hans Olof Håkansson
1992	Henrik Bengtsson
1992	Thomas Troeng
1992	Anita Ringberg
1992	Peter Björk
1992	Michael Hartmann
1992	Henrik Weibull
1992	Erney Mattsson
1992	Thorvaldur Jonsson
1992	Magnus Bergenfeldt
1993	Anders Lundell
1993	Baimeng Zhang
1994	Staffan Weiber
1994	Stefan Matthiasson
1994	Björn Sonesson
1995	Jan Stewenius
1995	Björn Arnljots
1995	Jan Holst
1995	Leif Israelsson
1995	Per Jönsson
1996	Norman Jensen
1996	Jens Peter Game
1996	Hans Bohe
1997	Wayne Hawthorne
1997	Öyvind Östraat
1997	Yilei Mao
1998	Diya Adawi
1998	Liselotte Frost-Arner
1998	Martin Malina
1998	Thomas Björk
1998	Mats Hedberg
1998	Håkan Brorson
1998	Magnus Becker
1999	Zhonquan Qi
1999	Stefan Appelros
1999	Göran Ahlgren
1999	Håkan Weiber
2000	Ingvar Syk
2001	Xiao Wei Zhang
2001	Christer Svedman
2001	Mats Bläckberg
2001	Peter Månsson
2001	Tor Svensjö
2001	Ursula Mirastschijski
2001	Torbjörn Söderstrom
2002	Thomas Sandgren
2002	Max Nyström
2002	Rene Schramm
2002	Ervin Tóth
2002	Daniel Klintman
2002	Åke Mellstrom
2002	Amjid Riaz
2002	Matthias Corbascio
2003	Nina Kvorning
2003	Gudmundur Danielsson
2003	Fritz Berndsen
2003	Salathiel Mzezewa
2004	Marianne Starck
2004	Li Xiang
2004	Karl MaIm
2004	Claes Jansen
2004	Peter Danielsson
2004	Lisa Rydén
2005	Ann-Cathrin Moberg
2005	Anders Holmström
2005	Helene MaIm
2005	Carolin Freccero
2005	Cecilia Österholm Corbascio
2005	Elzanaty Saad
2005	Björn Lindkvist
2006	Louis Banka Johnson
2006	Henrik Dyhre
2006	Erik Almqvist
2006	Yusheng Wang
2008	Peter Mangell
2008	Martin Persson
2008	Sara Regnér
2008	Stefan Santén
2008	Asaduzzaman Muhammad
2008	Matthias Laschkse
2009	Martin Almquist
2009	Farokh Collander Farzaneh
2010	Dorthe Johansen
2010	Björn Schönmeyr
2010	Fredrik Jörgren
2010	Patrik Velander
2010	Andrada Mihăescu
2011	Salma Butt
2011	Emma Hansson