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ARTICLE

c-Abl kinase regulates neutrophil extracellular trap formation, inflammation, and tissue damage in severe acute pancreatitis

Raed Madhi[1] | **Milladur Rahman**[1] | **Matthias Mörgelin**[2] | **Henrik Thorlacius**[1]

[1]Department of Clinical Science, Malmö, Section for Surgery, Skåne University Hospital, Lund University, Malmö, Sweden

[2]Colzyx, Lund, Sweden

Correspondence
Henrik Thorlacius, MD, PhD, Department of Clinical Science, Malmö, Section for Surgery, Skåne University Hospital, Lund University, 205 02 Malmö, Sweden.
Email: henrik.thorlacius@med.lu.se

Abstract

Neutrophil extracellular traps (NETs) are involved in acute pancreatitis (AP) but mechanisms controlling NET expulsion in AP are incompletely understood. Herein, we examined the role of c-Abelson (c-Abl) kinase in NET formation and tissue damage in severe AP. AP was induced by taurocholate infusion into pancreatic duct or intraperitoneal administration of L-arginine in mice. Pancreatic, lung, and blood samples were collected and levels of phosphorylated c-Abl kinase, citrullinated histone 3, DNA-histone complexes, myeloperoxidase, amylase, cytokines, and CXC chemokines were quantified. Citrullinated histone 3, reactive oxygen species (ROS), and NET formation were determined in bone marrow neutrophils. Taurocholate challenge increased phosphorylation of c-Abl kinase and levels of citrullinated histone 3 in the pancreas as well as DNA-histone complexes in the plasma. Administration of the c-Abl kinase inhibitor GZD824 not only abolished activation of c-Abl kinase but also decreased levels of citrullinated histone 3 in the pancreas and DNA-histone complexes in the plasma of animals with AP. Moreover, GZD824 decreased plasma levels of amylase, IL-6, and MMP-9 as well as edema, acinar cell necrosis, hemorrhage, CXC chemokine formation, and neutrophil infiltration in the inflamed pancreas. A beneficial effect of c-Abl kinase inhibition was confirmed in L-arginine-induced pancreatitis. In vitro, inhibition of c-Abl kinase reduced TNF-α-induced formation of ROS, histone 3 citrullination, and NETs in isolated bone marrow neutrophils. Our findings demonstrate that c-Abl kinase regulates NET formation in the inflamed pancreas. In addition, inhibition of c-Abl kinase reduced pancreatic tissue inflammation, and damage in AP. Thus, targeting c-Abl kinase might be a useful way to protect the pancreas in severe AP.

KEYWORDS
adhesion, chemokines, histones, leukocyte, pancreas

1 | INTRODUCTION

Severe acute pancreatitis (AP) is associated with a high risk of severe morbidity and mortality.[1] Management of patients suffering from severe AP poses a significant challenge to clinicians and is mainly restricted to supportive therapies, which is at least partly related to a limited understanding of the disease-promoting pathophysiology. It is generally accepted that activation of trypsinogen, leukocyte accumulation, and impaired microvascular perfusion are integrated components in the etiology of severe AP.[2–5] Neutrophil recruitment is a key feature of inflammatory reactions and a rate-limiting step in trypsinogen activation and tissue damage in severe AP.[6,7] Neutrophil extravasation involves multiple sequential steps supported by specific adhesion molecules, such as P-selectin[8,9] and LFA-1.[7] Extravascular tissue localization of neutrophils is coordinated by secreted chemokines.[10–12] CXC chemokines, including CXCL1 and CXCL2, are potent stimulators neutrophil accumulation.[11] CXCR2 is the high affinity receptor on neutrophils for CXCL1 and CXCL2,[13] and has been demonstrated to mediate neutrophil recruitment in the pancreas.[14] A recent study showed that neutrophil extracellular traps (NETs) play a significant role in trypsin activation, neutrophil recruitment and tissue injury in AP.[15] NETs are composed of neutrophil-derived DNA forming extracellular web-like structures decorated with nuclear histones as well as granular and cytoplasmic proteins.[16,17] Another study

Abbreviations: AP, acute pancreatitis; c-Abl, c-Abelson; Mac-1, Macrophage-1 antigen; MMP-9, matrix metallopeptidase-9; MPO, myeloperoxidase; NET, neutrophil extracellular traps; ROS, reactive oxygen species.

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reported that NETs contribute to secretory obstruction in the inflamed pancreas.[18] Although NETs appear to be important in the pathophysiology of AP, the signaling mechanisms regulating NET formation in severe AP remain elusive.

Cellular stress and injury activate signaling pathways converging on specific transcription factors, which in turn control gene expression of pro-inflammatory compounds. Intracellular kinases phosphorylating downstream targets play a dominant role in many signaling cascades.[19] c-Abelson (c-Abl) kinase is a nonreceptor tyrosine-protein kinase ubiquitously expressed in mammalian cells and was initially identified as a potent driver of myeloid cell transformation into leukemia.[20] Subsequent data demonstrated that c-Abl kinase exert a central role in regulating actin cytoskeleton dynamics forming the basis for cell adhesion and migration.[21] Studies have shown that c-Abl kinase play a key role in neutrophil accumulation by regulating β_2-integrin-mediated neutrophil migration.[22,23] Moreover, inhibition of c-Abl kinase activity has been reported to reduce endotoxin-induced neutrophilia and tissue damage in the lung.[24] In addition, it has been showed that c-Abl kinase is involved in vascular leakage formation in sepsis[25] and in IgG-mediated glomerular injury.[26] Thus, accumulating evidence suggest that c-Abl plays an important function in versatile inflammatory conditions but the potential role of c-Abl kinase in the induction of severe AP is unknown. In this context, it is interesting to note that c-Abl kinase has been suggested to regulate ROS generation in neutrophils[27] and ROS has been shown to be involved in the expulsion of NETs from neutrophils.[28] Thus, accumulating data suggest that c-Abl kinase plays an important role in neutrophils functions and in different models of inflammation and the role of c-Abl kinase in NET formation and tissue damage in AP warrants further studies.

Based on the considerations above, we hypothesized that c-Abl kinase signaling might play a role in regulating tissue inflammation and injury in severe AP with special focus on formation of NETs.

2 | METHODS

2.1 | Animals

All experiments were performed in accordance with legislation on the protection of animals and approved by the Regional Ethics Committee for animal experimentation at Lund University, Sweden. Male C57BL/6 mice weighing 20–26 g (8-9 weeks old) were housed in an animal facility with 12-12 h light-dark cycle at 22°C. Animals were fed standard laboratory diet, and given water ad libitum. Mice were anesthetized by intraperitoneal administration with 75 mg ketamine hydrochloride (Hoffmann-La Roche, Basle, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) per kilogram body weight.

2.2 | Taurocholate-induced pancreatitis

Anesthetized mice underwent a midline incision and the second part of duodenum and papilla of Vater were identified. The duodenum was immobilized by traction sutures placed 1 cm from the papilla and a small puncture was made through the duodenal wall (23 G needle) in parallel to the papilla of Vater. A polyethylene catheter connected to a micro-infusion pump (CMA/100, Carnegie Medical, Stockholm, Sweden) was inserted through the punctured hole in the duodenum and 1 mm into the common bile duct. The common hepatic duct was temporarily clamped at the liver hilum to prevent hepatic reflux. Then 10 μl of 5% sodium taurocholate (Sigma, St. Louis, MO) or 0.9% sodium chloride was infused into the pancreatic duct for 10 min. After that, the catheter and the common hepatic duct clamp were removed. The duodenal puncture was closed with a purse-string suture. Traction sutures were removed and the abdomen was closed. Sham mice underwent laparotomy and saline was infused into the pancreatic duct. Vehicle (DMSO) or c-ABL kinase specific inhibitors GZD824 or ABL001 (5 mg/kg, Selleck Chemicals, Houston, TX, USA) were administered i.v. 1 h prior to intraductal infusion with saline or taurocholate. In separate animals, a neutrophil deleting Ab directed against Ly6G (20 mg/kg, clone 1A8, rat IgG, BioXcell, West Lebanon, NH) or a control Ab (20 mg/kg, rat IgG, BioXcell) was administered before induction of pancreatitis. All animals were killed 24 h after induction of pancreatitis.

2.3 | L-Arginine-induced pancreatitis

In separate experiments, AP was induced by administration of L-arginine (4 g/kg/dose) i.p. twice at an interval of 1 h as described in detail previously.[29] Vehicle or GZD824 (5 mg/kg) were given i.v. before the first dose of L-arginine. Saline-treated animals served as negative controls. All animals were sacrificed 72 h after induction of pancreatitis.

2.4 | Amylase measurements

Levels of blood amylase were quantified in blood collected from the tail vein using a commercially available kit (Reflotron, Roche Diagnostics GmbH, Mannheim, Germany).

2.5 | Myeloperoxidase activity

Pieces of the pancreatic head and lung were harvested for quantification of myeloperoxidase (MPO) activity as previously described.[30] Briefly, all frozen pancreatic and lung samples were pre-weighed and homogenized in 1 ml mixture (4:1) of PBS and aprotinin (10,000 kallikrein inactivator units per ml, Trasylol, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. Homogenates were centrifuged (15,300 × *g*, 10 min at 4°C), and supernatants were stored at −20°C. Pellets were mixed with 1 ml of 0.5% hexadecyltrimethylammonium bromide. Next, samples were frozen for 24 h and then thawed, sonicated for 90 s, and put in a water bath (60°C for 2 h) after which MPO activity of the supernatant was quantified 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.

2.6 | Tissue histology

Samples of the pancreatic head 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. Severity of pancreatitis was evaluated in a blinded manner by use of a preexisting scoring system including edema, acinar cell necrosis, hemorrhage, and neutrophil infiltrate on a 0 (absent) to 4 (extensive) scale as previously described in detail.[31]

2.7 | DNA-histone complexes

Blood was collected from the inferior vena cava and diluted (1:10) in acid citrate dextrose. Samples were centrifuged at 15,300 × *g* for 5 min at 4°C and a Cell Death Detection ELISA Plus kit (Roche Diagnostics, Mannheim, Germany) was used to quantify DNA-histone complex according to the manufacturers' instructions.

2.8 | Enzyme-linked immunosorbent assay

Pancreatic levels of CXCL1 and CXCL2 were analyzed in stored supernatants from homogenized pancreatic tissue by use of double-Ab, ELISA kits (R&D Systems Europe, Abingdon, Oxon, UK) according to the manufacturers' instructions. Blood collected from the inferior vena cava was diluted (1:10) in acid citrate dextrose, centrifuged (15,300 × *g* for 5 min at 4°C), and stored at −20°C until use. Commercially available ELISA kits were used to quantify plasma levels of IL-6, matrix metallopeptidase-9 (MMP-9), and CXCL2 (R&D Systems Europe) according to the manufacturers' instructions.

2.9 | Flow cytometry

Blood was collected from the inferior vena cava and diluted (1:10) in acid citrate dextrose. Cells were incubated with an anti-CD16/CD32 for 5 min to block Fc_{γ}III/IIRs and reduce nonspecific labeling. Then, cells were incubated with a Phycoerythrin (PE) or allophycocyanin (APC)-conjugated anti-Ly6G (clone 1A8, BD Pharmingen, San Jose, CA) and a Fluorescein isothiocyanate (FITC)-conjugated anti-**Mϕ**-1 Ag (Mac-1; clone M1/70, integrin α_M china, rat IgG2b, BD Biosciences Pharmingen, San Jose, CA) Ab at 4°C for 20 min. Cells were fixed with 2% formaldehyde, erythrocytes were lysed and neutrophils were recovered following centrifugation. Flow-cytometric analysis was performed according to standard setting on a Cytoflex flow cytometer (Becton Dickinson, Mountain View, CA, USA), and viable gate was used to exclude dead and fragmented cells. Neutrophils were defined as $Ly6G^+$ cells. After gating the neutrophil population based on forward- and side-scatter characteristics, Mac-1 expression was determined on Ly6G positive cells.

2.10 | Isolation of blood neutrophils

Mice were euthanized and blood was collected from the inferior vena cava and diluted (1:10) in acid citrate dextrose. Blood samples were added to Roswell Park Memorial Institute medium 1640 (RPMI 1640, Invitrogen, Stockholm, Sweden) supplemented with 10% FBS (Invitrogen) and 2 mM EDTA (Sigma–Aldrich, Stockholm, Sweden). Neutrophils were separated from mononuclear cells by density gradient centrifugation using a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden). Erythrocytes were lysed by using ACK lysing buffer (Thermo Fisher Scientific, Somerset, NJ) and then neutrophils were recovered following centrifugation and cells were resuspended at 4×10^6 cells/ml. Next, the cells were homogenized and the activity of c-Abl kinase determined in isolated neutrophils as described below.

2.11 | Western blot

Pancreatic and blood isolated neutrophils samples were collected and homogenized in ice-cold RIPA buffer (RIPA Lysis and Extraction Buffer, ThermoFisher, USA) containing protease inhibitors (Halt Protease Inhibitor Cocktail; Pierce Biotechnology, Rockford, IL) for 20 min and then sonicated and centrifuged (16,000 × *g* for 15 min, 4°C). Supernatants were collected and stored at −20°C. Protein concentration of supernatants was determined by use of Pierce BCA Protein Assay Reagent (Pierce Biotechnology). Proteins (20 μg per lane) were separated by 8–16% Mini-PROTEAN® TGX Stain-Free™ Gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes (Novex, San Diego, CA, USA). Before blotting, total protein gel image was taken using Bio-Rad's stain-free gel chemistry. Membranes were blocked in TBS/Tween 20 buffer containing 5% nonfat dry milk powder. Protein immunoblots were performed using anti-histone H3 (citrulline 2,8,17, ab5103, Abcam, Cambaridge, MA), anti-c-Abl kinase, and anti-phosphorylated c-Abl kinase Ab (Selleck Chemicals, Munich, Germany). Membranes were then incubated with peroxidase conjugated secondary Abs or anti-biotin. Protein bands were developed and analyzed using the BioRad ChemiDoc™ MP imaging system and Image Lab™ software version 5.2.1. The Image Lab™ software (version 5.2.1) was used to normalize the band signal against the total protein in the respective lane.

2.12 | ROS and NET formation

Bone marrow neutrophils (2×10^6 cells/ml) were freshly isolated from healthy C57BL/6 mice by density gradient centrifugation using a Ficoll-Paque gradient (GE Healthcare, Uppsala, Sweden). Neutrophils were incubated with anti-CD16/CD32 (to block Fc_{γ}III/IIRs and reduce nonspecific labeling) and PE-conjugated anti-Ly6G Abs. To detect ROS generation, cells were incubated with dihyrorhodamine 123 (Sigma) for 15 min at 37°C and then stimulated with 100 U/ml TNF-α (R&D Systems Europe Ltd., Abingdon, UK) for 1 h at 37°C. In separate experiments, isolated neutrophils were incubated with 100 U/ml TNF-α for 3 h at 37°C. Samples were centrifuged (400 × *g*, 5 min) and supernatants were collected to measure DNA-histone complex as described above. For detection of NETs by flow cytometry, neutrophils were fixed with 2% formaldehyde. After washing 2 times with PBS containing 2% FBS, cells were incubated with primary Abs: Phycoerythrin (PE) conjugated anti-Ly6G (clone 1A8, BD Pharmingen), Fluorescein isothiocyanate (FITC) conjugated anti-MPO Ab (mouse: ab90812), and rabbit anti-H3cit (citrulline 2,8,17, ab5103; Abcam, Cambridge, MA) in PBS containing 5% donkey serum. After washing 2 times, cells were incubated with rat anti-rabbit allophycocyanin (APC) conjugated secondary Ab (A-21038, Thermo Scientific, Rockford, IL). Flow cytometry analysis was performed according to standard setting on a CytoFLEX flow cytometer (Becton Dickinson, Mountain View, CA), and viable gate was used to exclude dead and fragmented cells. For immunofluorescence imaging of NETs, neutrophils were stimulated with TNF-α and permeabilized with 1% Triton X-100 for 10 min and then stained

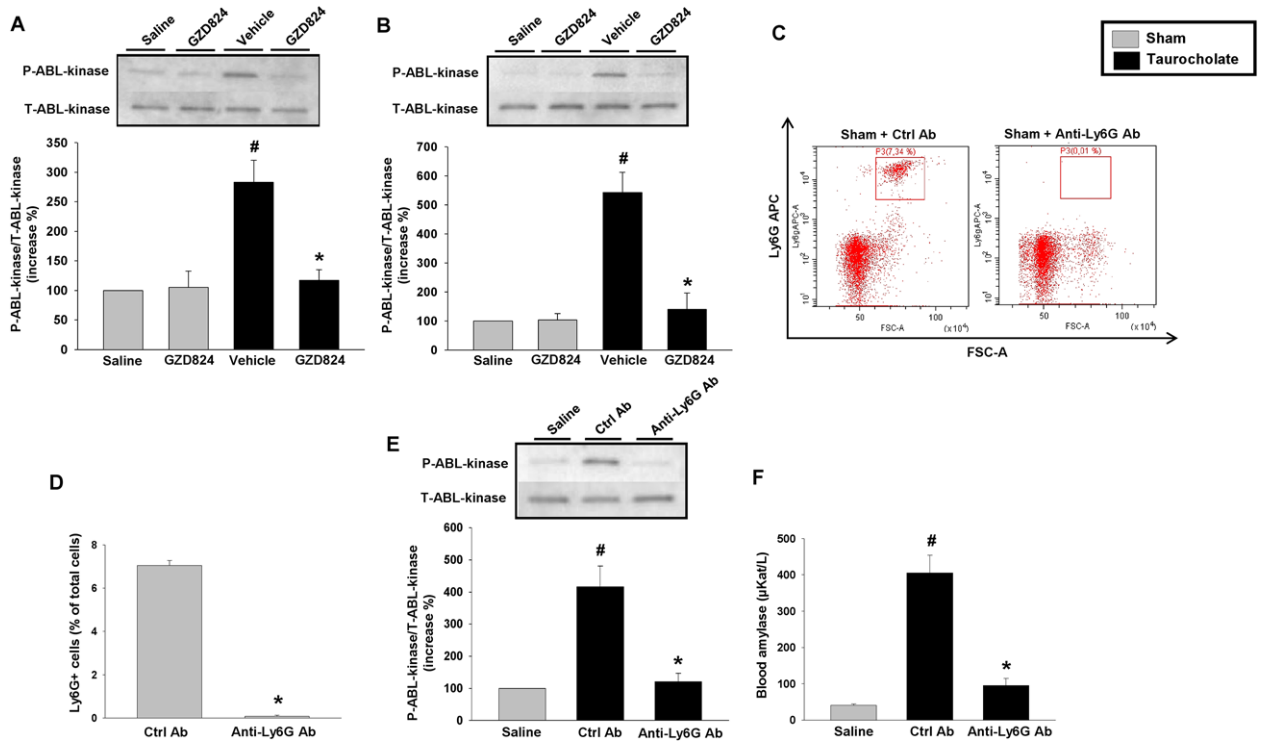

FIGURE 1 **c-Abl kinase activity in AP.** Phosphorylation of c-Abl kinase in (**A**) isolated neutrophils (peripheral blood) and (**B**) pancreatic lysates were examined by western blot as described in Methods section. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.v. injections of the c-Abl kinase inhibitor GZD824 (5 mg/kg) or vehicle (DMSO) prior to induction of pancreatitis. Circulating neutrophils (Ly6G+) were quantified in animals treated i.p. with an Ab directed against Ly6G (clone 1A8) or a control Ab before induction of pancreatitis. (**C**) Representative dot plots of 4 other independent experiments and (**D**) aggregate data. (**E**) c-Abl kinase phosphorylation in the pancreas and (**F**) blood amylase levels as described in Methods section. Samples were collected 24 h after induction of pancreatitis. Data represent mean ± SEM and $n = 4–5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + taurocholate

on glass coverslips as described above. After immunostaining, coverslips were rinsed and mounted in fluoromount with DAPI (Thermo Fisher Scientific). Confocal microscopy was performed using LSM 800 confocal (Carl Zeiss, Jena, Germany) by a ×63 oil immersion objective (numeric aperture = 1.25). The pinhole was ~1 airy unit and the scanning frame was 1024 × 1024 pixels. Images were later processed using ZEN2012 software.

2.13 | Statistical analysis

Data are presented as mean values ± SEM. Statistical evaluations were performed using Kruskal–Wallis 1-way analysis of variance on the ranks followed by multiple comparisons versus the control group (Dunnett's method). $P < 0.05$ was considered significant, and n represents the number of animals in each group.

3 | RESULTS

3.1 | c-Abl kinase activation in AP

To investigate activation of c-Abl kinase in AP, western blot was used to determine phosphorylation of c-Abl kinase. Taurocholate challenge markedly increased c-Abl phosphorylation in circulating neutrophils (Fig. 1A) and in the pancreas (Fig. 1B) compared to sham-treated animals (Fig. 1A-B). Moreover, administration of GZD824 abolished phosphorylation of c-Abl kinase in neutrophils and in the pancreas of animals with AP, suggesting that GZD824 is a potent inhibitor of c-Abl kinase function (Fig. 1A and B). Administration of GZD824 alone had no effect on c-Abl kinase activation in neutrophils or in the pancreas of sham animals (Fig. 1A and B). We next wanted to examine the role of contribution of neutrophils to the c-Abl kinase activity in the inflamed pancreas. For this purpose, we used an Ab against Ly6G on neutrophils, which reduced the number of circulating neutrophils by more than 97% (Fig. 1C and D). It was found that neutrophil depletion decreased the taurocholate-induced increase in c-Abl phosphorylation by 50% in the pancreas (Fig. 1E). Moreover, we observed that depletion of neutrophils significantly reduced blood amylase levels in mice with AP (Fig. 1F).

3.2 | c-Abl-kinase regulates NETs formation in AP

Citrullinated histone 3 was used as a specific indicator of NETs formation. It was found that taurocholate challenge enhanced levels of citrullinated histone 3 in the inflamed pancreas (Fig. 2A). In addition, we observed that plasma levels of DNA-histone complexes increased by more than 8-fold in animals with severe AP (Fig. 2B). Notably, administration of GZD824 reduced levels of citrullinated histone 3 in the pancreas and DNA-histone complexes in the plasma

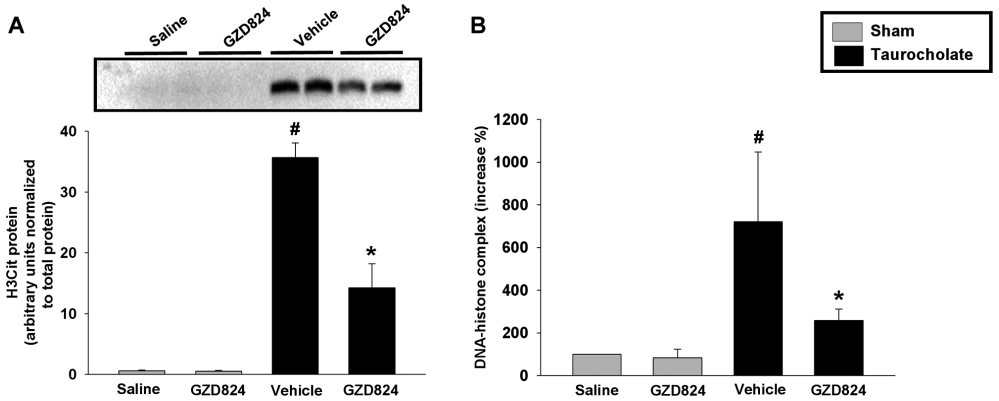

FIGURE 2 **NET formation in AP.** (**A**) Western blot of pancreatic citrullinated histone 3 and aggregate data showing H3Cit protein normalized with stain-free total protein load. (**B**) Plasma levels of DNA-histone complexes. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.v. injections of the c-Abl kinase inhibitor GZD824 (5 mg/kg) or vehicle (DMSO) prior to induction of pancreatitis. Samples were collected 24 h after induction of pancreatitis. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + taurocholate

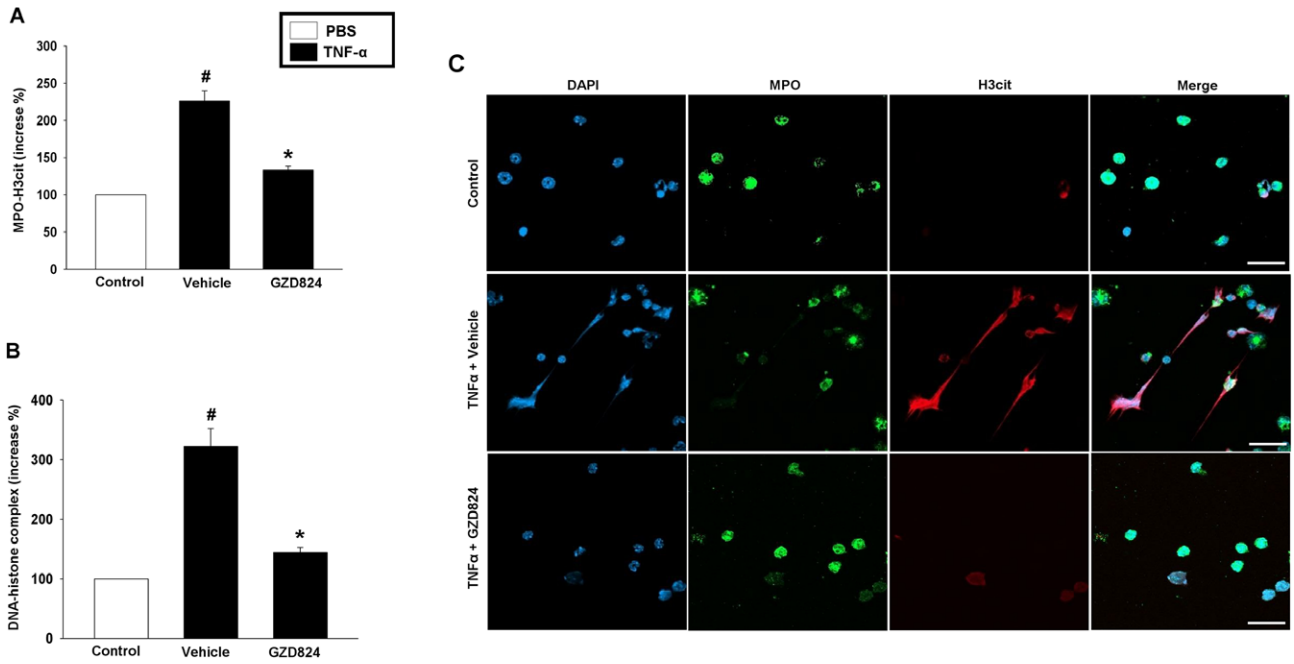

FIGURE 3 **NET formation in vitro**. NETs were generated from isolated neutrophils by stimulation with TNF-α co-incubated with or without GZD824. Non-stimulated neutrophils served as a control. (**A**) Levels of citrullinated histone 3 and MPO in isolated neutrophils detected by FACS and (**B**) DNA-histone complexes in the supernatant determined by ELISA. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control and $^{*}P < 0.05$ versus vehicle + TNF-α. (C) Neutrophils were immune-stained with Abs to citrullinated histone 3 (H3Cit), myeloperoxidase (MPO), and DAPI nuclear stain. One representative experiment of 4 independent experiments. Scale bars = 10 μm

by 62% (Fig. 2A) and 77% (Fig. 2B), respectively. To further study the role of c-Abl kinase in regulating NET formation, we used isolated bone marrow neutrophils in vitro. Stimulation of neutrophils with TNF-α markedly increased neutrophil co-expression of neutrophil-derived granule protein MPO and citrullinated histone 3 (Fig. 3A) as well as DNA-histone complexes (Fig. 3B). Notably, inhibition of c-Abl kinase decreased TNF-α-induced co-expression of MPO and citrullinated histone 3 (Fig. 3A) levels as well as DNA-histone complexes (Fig. 3B) in isolated neutrophils. Moreover, by use of confocal fluorescence microscopy, it was observed that TNF-α stimulation triggered formation of DNA fibrillar and web-like structures that colocalized with MPO and citrullinated histone 3 (Fig. 3C). Indeed, co-incubation of neutrophils with GZD824 inhibited TNF-α-induced formation of these DNA structures with MPO and citrullinated histone 3 (Fig. 3C), demonstrating that c-Abl kinase regulates NET formation. Administration of GZD824 alone had no effect on NET formation in healthy mice (Fig. 2). Knowing that ROS plays a key role in NET formation,[28] it was of interest to study the role of c-Abl kinase in TNF-α-induced formation of ROS. TNF-α stimulation triggered clear-cut formation of ROS in isolated neutrophils (Fig. 4). Co-incubation of neutrophils with

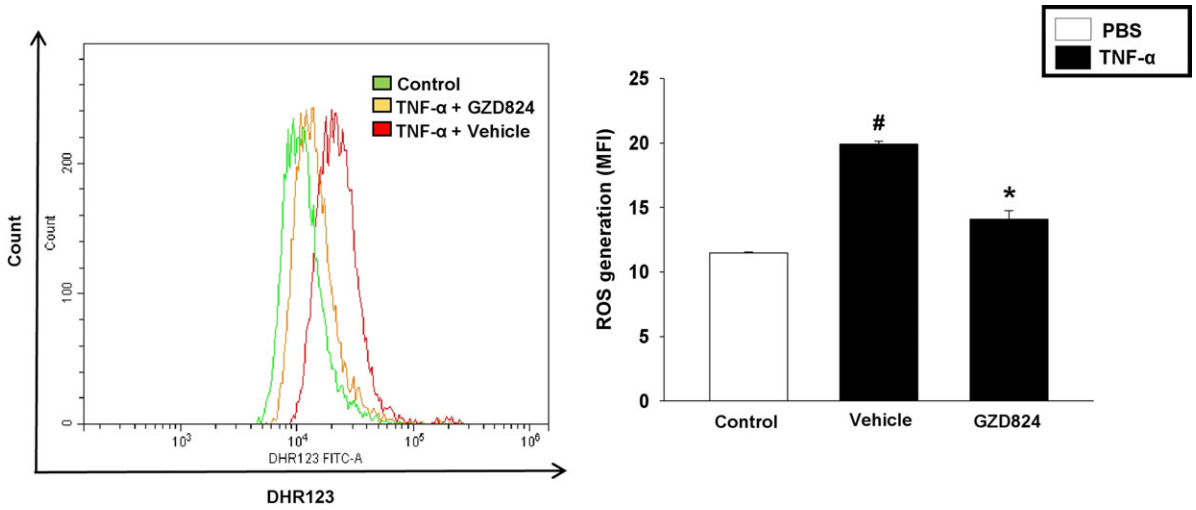

FIGURE 4 **ROS formation in neutrophils**. Quantification of ROS formation in isolated neutrophils by flow cytometry. TNF-α stimulation of neutrophils with or without GZD824. Nonstimulated neutrophils served as a control. Representative histogram of ROS generation and aggregate data. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control and $^{*}P < 0.05$ versus vehicle + TNF-α

GZD824 markedly decreased TNF-α-provoked generation of ROS in neutrophils (Fig. 4).

3.3 | c-Abl kinase regulates tissue damage in the pancreas

Examination of tissue morphology showed normal pancreatic microarchitecture in sham animals (Fig. 5A–E), whereas taurocholate challenge caused massive destruction of the pancreatic tissue structure characterized by edema formation (Fig. 5B), acinar cell necrosis (Fig. 5C), hemorrhage (Fig. 5D), and neutrophil accumulation (Fig. 5E). We found that administration of GZD824 protected against taurocholate-induced tissue damage (Fig. 5A–E). For example, inhibition of c-Abl attenuated edema formation and acinar cell necrosis by 43% and 52%, respectively, in the inflamed pancreas (Fig. 5B and C). Furthermore, treatment with GZD824 reduced extracellular leukocytes by 72% in the pancreas of animals with AP (Fig. 5E). In addition, it was found that taurocholate challenge elevated blood amylase levels by 9-fold (Fig. 6A). Pretreatment with GZD824 significantly decreased taurocholate-induced blood amylase by 60% (Fig. 6A). Administration of GZD824 alone had no effect on taurocholate-induced tissue damage or amylase levels in the blood (Figs. 5 and 6A).

3.4 | c-Abl kinase controls neutrophil infiltration in the pancreas

Tissue levels of MPO were used as an indicator of neutrophil infiltration. We found that challenge with taurocholate increased pancreatic MPO activity by 24-fold (Fig. 6B). Inhibition of c-Abl kinase reduced taurocholate-induced pancreatic MPO activity by 83% (Fig. 6B). Moreover, taurocholate challenge markedly increased CXCL1 and CXCL2 levels in the pancreas (Fig. 6C and D). Inhibition of c-Abl kinase decreased CXCL1 by 66% and CXCL2 by 70% in the inflamed pancreas (Fig. 6C and D). Moreover, we noted that Mac-1 expression was increased on the surface of neutrophils in mice with pancreatitis (Fig. 6E). Administration of GZD824 markedly reduced neutrophil expression of Mac-1 in AP (Fig. 6E).

3.5 | c-Abl kinase regulates systemic inflammation in AP

Challenge with taurocholate increased plasma levels of IL-6 by 20-fold, MMP-9 by 5-fold, and CXCL2 by 18-fold (Fig. 7A–C). Inhibition of c-Abl kinase markedly reduced taurocholate-induced plasma levels of IL-6, MMP-9, and CXCL2 by more than 60%, 59%, and 50%, respectively (Fig. 7A–C). As part of a systemic inflammatory response in severe AP, activated neutrophils accumulate in the lung microvasculature. Indeed, it was observed that challenge with taurocholate markedly enhanced the MPO activity in the lung (Fig. 7D). Administration of GZD824 attenuated pulmonary MPO activity by 80% in mice with severe AP (Fig. 7D). Administration of GZD824 alone had no effect on taurocholate-induced systemic inflammation in animals with AP (Fig. 7A–D).

3.6 | c-Abl kinase regulates tissue injury in L-arginine-induced AP

In order to examine whether c-Abl might also regulate tissue damage and neutrophil recruitment in an alternative experimental model, L-arginine was used to trigger AP. Administration of L-arginine caused c-Abl phosphorylation, elevation of blood amylase as well as increased levels of MPO in the pancreas and lung (Fig. 8A–D). Moreover, L-arginine challenge increased plasma levels of DNA-histone complexes (Fig. 8E). Administration of GZD824 abolished phosphorylation of c-Abl kinase in the pancreas of animals exposed to L-arginine (Fig. 8A), suggesting that GZD824 is a potent inhibitor of c-Abl function in L-arginine-induced AP. In addition, inhibition of c-Abl kinase significantly reduced L-arginine-induced increases in blood amylase, pancreas, and lung MPO activity as well as plasma levels of DNA-histone complexes (Fig. 8B–E)

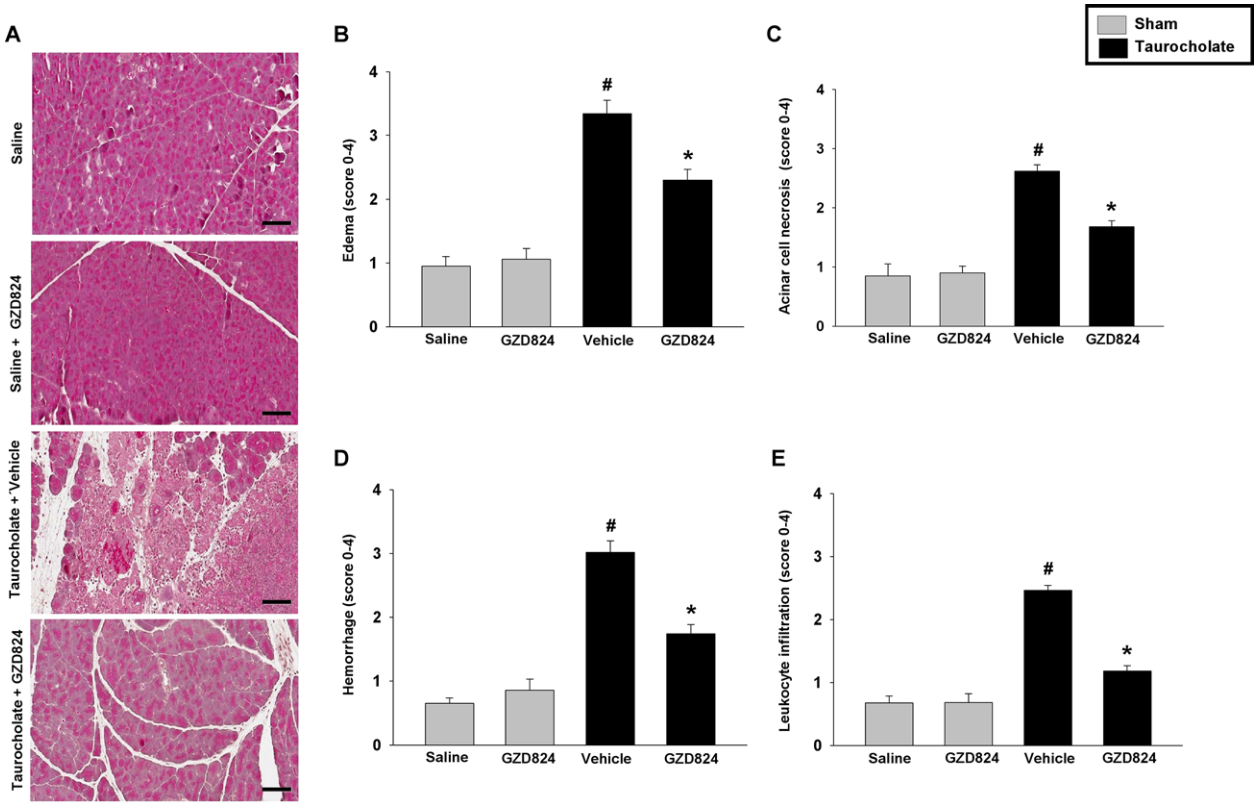

FIGURE 5 **Tissue damage in the inflamed pancreas.** (**A**) Representative hematoxylin and eosin sections of the head of the pancreas from indicated groups. Scale bar = 100 μm. Histological quantification of (**B**) edema, (**C**) acinar cell necrosis, (**D**) hemorrhage, and (**E**) leukocyte infiltration. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.v. injections of the c-Abl kinase inhibitor GZD824 (5 mg/kg) or vehicle (DMSO) prior to induction of pancreatitis. Samples were collected 24 h after induction of pancreatitis. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + taurocholate

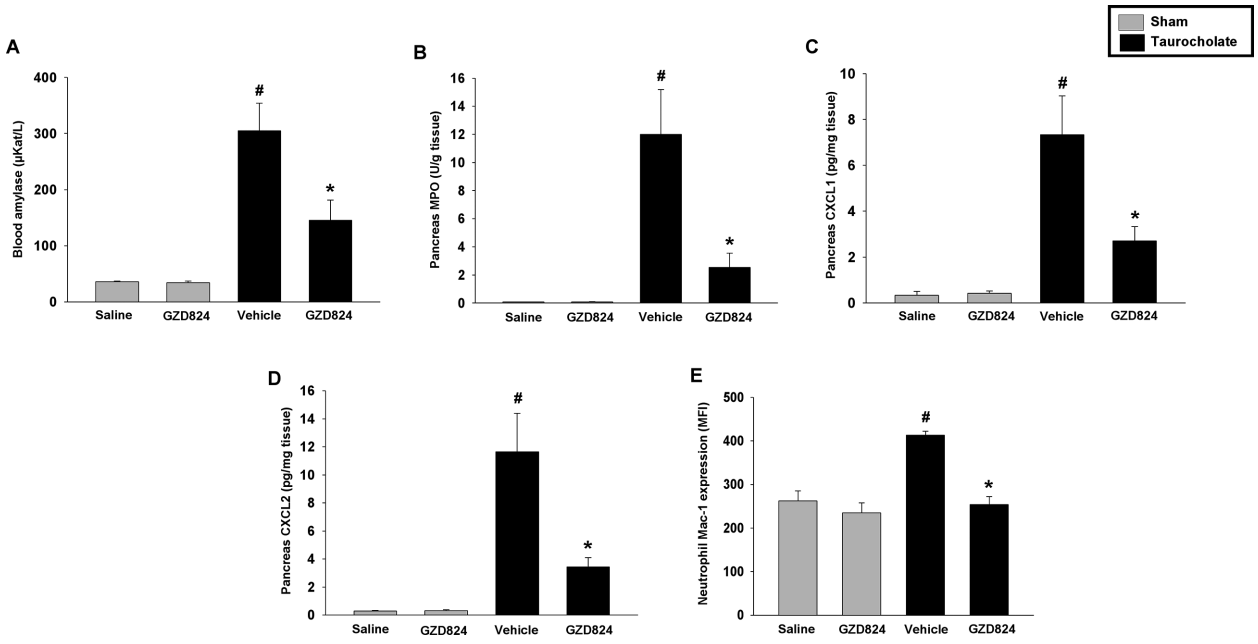

FIGURE 6 **(A) Blood levels of amylase.** Pancreatic levels of (**B**) MPO, (**C**) CXCL1, and (**D**) CXCL2. (**E**) Mac-1 expression on circulating neutrophils. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.v. injections of the c-Abl kinase inhibitor GZD824 (5 mg/kg) or vehicle (DMSO) prior to induction of pancreatitis. Samples were collected 24 h after induction of pancreatitis. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + taurocholate

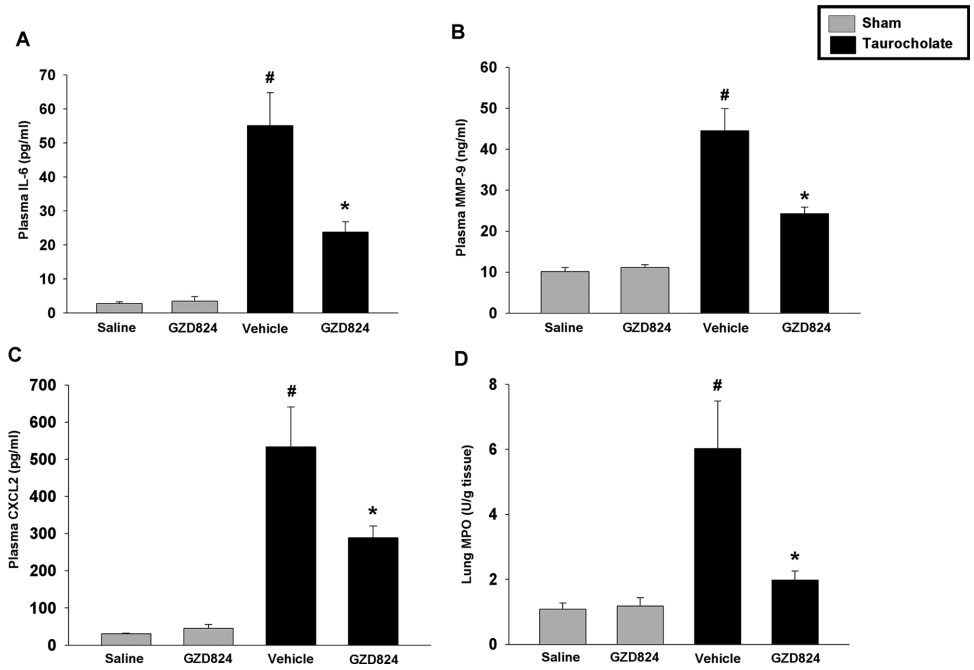

FIGURE 7 **Systemic inflammation in AP**. Plasma levels of (**A**) IL-6, (**B**) MMP-9, (**C**) CXCL2, and (**D**) pulmonary levels of MPO. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.v. injections of the c-Abl kinase inhibitor GZD824 (5 mg/kg) or vehicle (DMSO) prior to induction of pancreatitis. Samples were collected 24 h after induction of pancreatitis. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + taurocholate

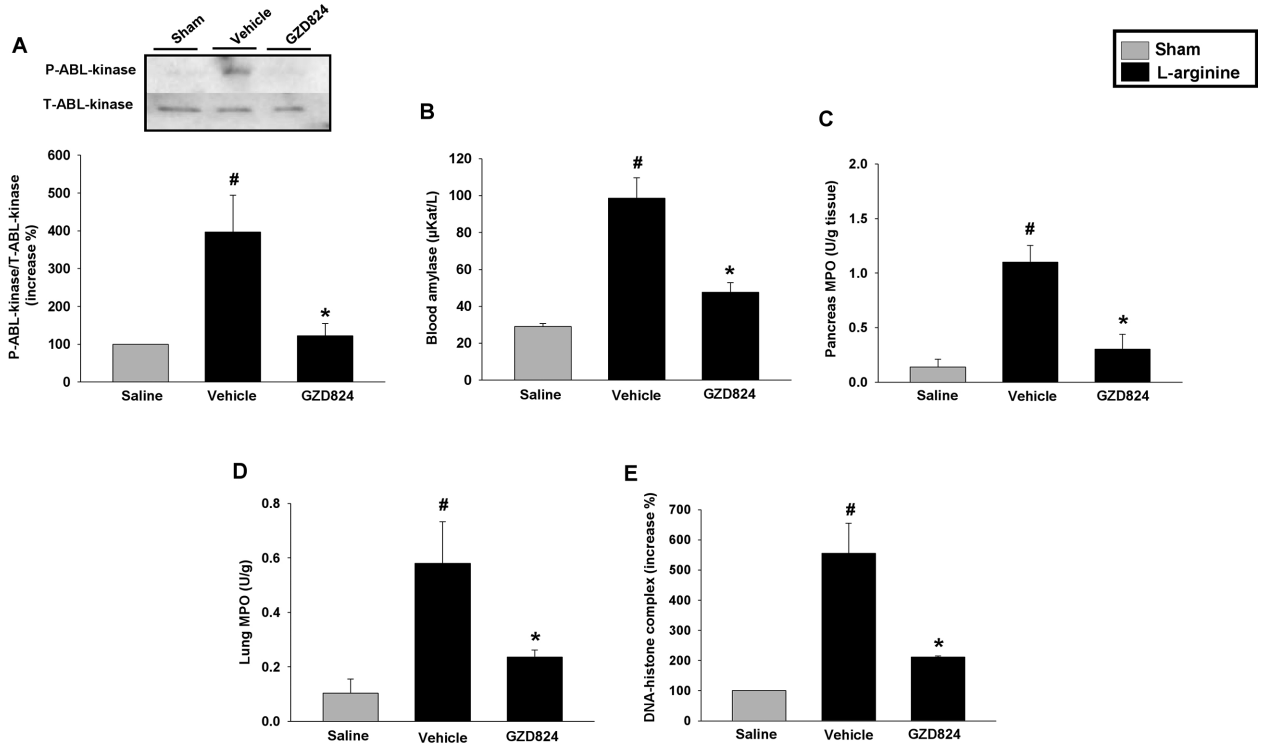

FIGURE 8 **c-Abl kinase regulates L-arginine-induced AP**. (**A**) Phosphorylation of c-Abl kinase in the pancreas, (**B**) blood amylase levels, (**C**) MPO activity in the (**C**) pancreas and (**D**) lung as well as (**E**) DNA-histone complexes were determined 72 h after induction of pancreatitis. AP was induced by administration of 4 g/kg/dose of L-arginine i.p. twice at an interval of 1 h. Mice were treated with vehicle or the c-Abl kinase inhibitor GZD824 (5 mg/kg) i.v. before the first dose of L-arginine. Control mice were injected with saline alone. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + L-arginine

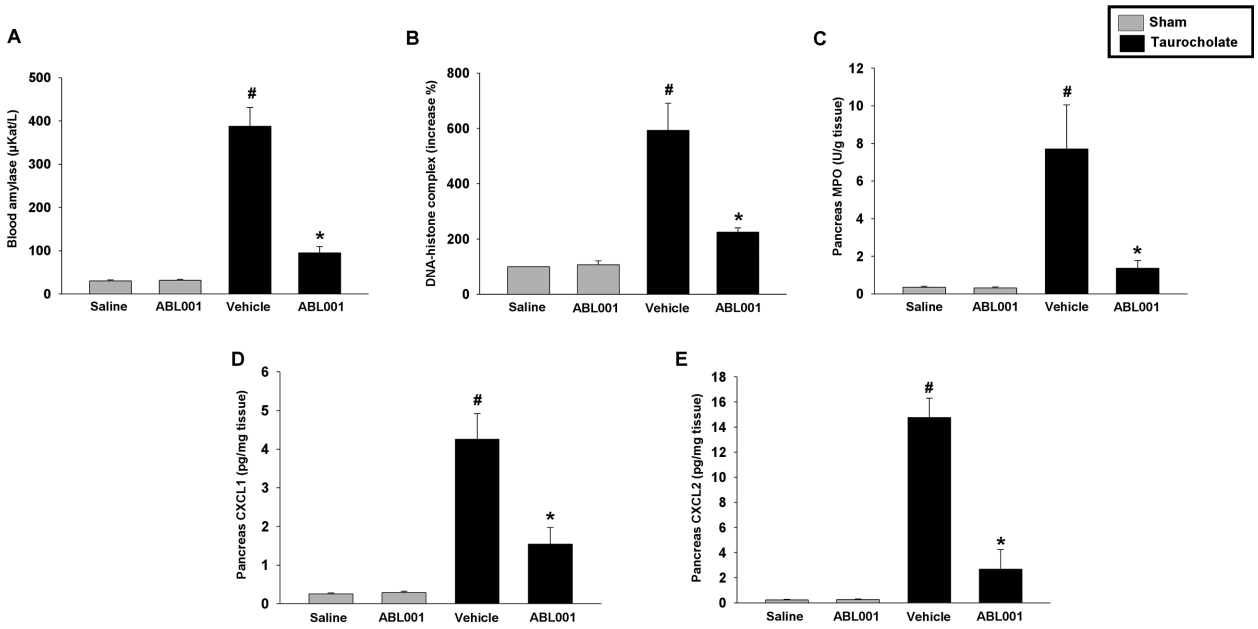

FIGURE 9 **(A) Quantitative measurements of blood amylase levels.** (**B**) Plasma levels of DNA-histone complexes. Pancreatic levels of (**C**) MPO, (**D**) CXCL1, and (**E**) CXCL2. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.v. injections of the ABL001 (5 mg/kg) or vehicle (DMSO) as described in Methods section. Samples were collected 24 hours after induction of pancreatitis. Data represent means ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + taurocholate

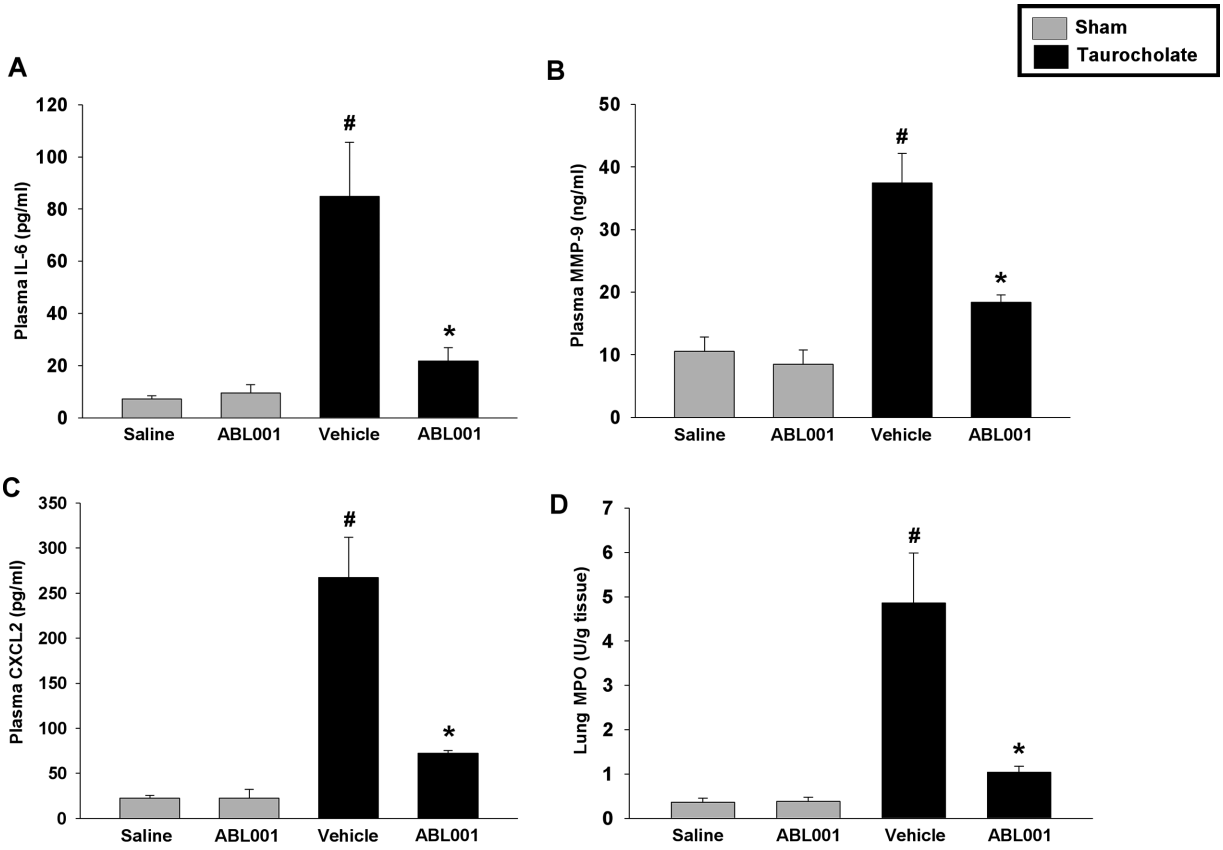

FIGURE 10 **Plasma levels of (A) IL-6, (B) MMP-9, and (C) CXCL2.** (**D**) Pulmonary levels of MPO. Pancreatitis (black bars) was induced by infusion of sodium taurocholate (5%) into the pancreatic duct. Control mice (grey bars) were infused with saline alone. Animals were treated with i.v. injections of the ABL001 (5 mg/kg) or vehicle (DMSO) as described in Methods section. Samples were collected 24 h after induction of pancreatitis. Data represent mean ± SEM and $n = 5$. $^{\#}P < 0.05$ versus control mice and $^{*}P < 0.05$ versus vehicle + taurocholate

3.7 | ABL001 reduces tissue injury, NET formation, and inflammation in AP

To validate the role of c-Abl kinase in AP, an alternative c-Abl kinase inhibitor, ABL001, was used. Administration of ABL001 decreased taurocholate-induced blood amylase levels by 82% (Fig. 9A). Moreover, pretreatment with ABL001 significantly decreased DNA-histone complex levels in the plasma of animals with AP (Fig. 9B). Administration of ABL001 significantly reduced pancreatic levels of MPO, CXCL1, and CXCL2 by more than 85%, 70% and 83%, respectively, in response to taurocholate challenge (Fig. 9C–E). Treatment with ABL001 attenuated taurocholate-induced increases of plasma levels of IL-6 (Fig. 10A), MMP-9 (Fig. 10B), and CXCL1 (Fig. 10C) as well as pulmonary activity of MPO (Fig. 10D). Administration of ABL001 alone had no effect on NET formation and inflammation in sham mice (Figs. 9 and 10).

4 | DISCUSSION

This study demonstrates for the first time that c-Abl kinase plays a key role in the pathophysiology of severe AP. Our findings show that c-Abl kinase is an important regulator of NET formation and AP. Moreover, we observed that inhibition of c-Abl kinase activity not only decreases NET formation in the inflamed pancreas but also attenuates neutrophil recruitment, pancreatic tissue damage, and systemic inflammation in severe AP. Thus, targeting c-Abl kinase activity might be a useful therapeutic approach in patients suffering from AP.

Current evidence supports that c-Abl kinase, as an important signaling molecule, is involved in cytoskeleton reorganization in many cellular processes. It is widely accepted that c-Abl kinase is a critical signaling molecule regulating cellular actin dynamics and cytoskeletal rearrangement.[21,32] Although c-Abl kinase is best known for it´s role in human leukemias,[20,33] accumulating data suggest that c-Abl exerts more pluripotent functions in different inflammatory conditions, such as endotoxemic lung damage, Ig-mediated renal injury, and nephrotoxicity.[25,26,34] In the present study, we could demonstrate that targeting c-Abl kinase with a specific inhibitor (GZD824) reduced c-Abl phosphorylation in both circulating neutrophils and in the pancreas of animals with AP. In order to define the contribution of neutrophils to the c-Abl kinase activity in the inflamed pancreas, animals were depleted of neutrophils. It was found that administration of an Ab against Ly6G reduced neutrophils counts by more than 97% and decreased the taurocholate-evoked increase of c-Abl kinase phosphorylation in the pancreas by 50%, suggesting that neutrophils contribute to a significant proportion of the c-Abl kinase activity in the inflamed pancreas. Importantly, we observed that treatment with GZD824 not only decreased activation of c-Abl kinase but also significantly decreased tissue damage in severe AP. For example, administration of GZD824 reduced taurocholate-provoked increase in blood amylase by 60% as well as acinar cell necrosis and edema formation by more than 50% and 43%, respectively, suggesting that c-Abl kinase activity regulates a significant part of the tissue damage in severe AP. Importantly, we found that GDZ824 also decreased c-Abl kinase activity and tissue damage in an alternative model of AP, i.e., L-arginine-induced pancreatitis. These data constitute the first evidence in the literature that c-Abl kinase signaling is involved in the development of AP.

Recent findings have shown that neutrophil-derived NETs play an important role in the pathophysiology of AP by triggering activation of trypsinogen and tissue inflammation in the pancreas.[15] Herein, we found that inhibition of c-Abl kinase by use of GZD824 decreased taurocholate-induced increases of histone 3 citrullination in the pancreas and DNA-histone complexes in the plasma, suggesting that c-Abl kinase regulates NET formation in severe AP. We then asked whether c-Abl kinase directly regulates NET formation. It was found that TNF-α stimulation of isolated neutrophils caused a marked increase in neutrophil co-expression of MPO and citrullinated histone 3 as well as generation of DNA-histone complexes. Inhibition of c-Abl kinase reduced TNF-α-induced co-expression of MPO and citrullinated histone 3 levels as well as DNA-histone complexes in isolated neutrophils, suggesting that c-Abl kinase regulates NET formation. This notion was supported by our findings showing co-incubation of neutrophils with GZD824 inhibited TNF-α-induced formation of extracellular DNA fibrillar structures containing MPO and citrullinated histone 3. Thus, this is the first time in the literature that c-Abl kinase has been shown to regulate NET formation and AP. Notably, convincing data have shown that induction of ROS is pivotal in TNF-α-induced NET formation.[35] Herein, we found that inhibition of c-Abl kinase markedly reduced TNF-α-triggered formation of ROS in neutrophils. Thus, c-Abl kinase-dependent generation of ROS might be involved in TNF-α-induced formation of NETs in neutrophils. Although neutrophil-derived NETs are recognized for their important role in the innate immune system by trapping microbes facilitating microbiological clearance,[36,37] several studies have shown that excessive formation of NETs is known to cause tissue injury and organ failure in infectious conditions.[38,39] In this context, it is interesting to note that accumulating reports demonstrate that Abl kinase function is subverted by numerous bacterial and viral pathogens to achieve entry, motility, release, and survival in mammalian host cells.[40] Thus, pharmacological targeting of c-Abl kinase function might be a useful strategy to treat infectious diseases in which excessive NET formation harm host organs tissue while simultaneously antagonizing microbial pathogens.

It is generally held that neutrophil infiltration is a key component in mediating tissue damage in severe AP.[6,8,41,42] For example, neutrophil depletion or inhibition of neutrophil accumulation have repeatable been shown to protect against tissue injury in pancreatitis.[6–8,42,43] Moreover, neutrophils are known to regulate trypsin activation in acinar cells in the pancreas.[6,44] In the present study, we found that taurocholate challenge elevated MPO activity and the number of extravascular neutrophils in the inflamed pancreas. Inhibition of c-Abl kinase greatly reduced MPO levels and the number of extravascular neutrophils in the pancreas, indicating that c-Abl kinase is an important regulator of neutrophil infiltration in the inflamed pancreas. Knowing the key role of neutrophils in the development of pancreatitis[6–8,42–44] it might be suggested that the inhibitory effect of GZD824 on neutrophil activation and recruitment could help to explaining the tissue protective effect of GZD824 in AP. Neutrophil trafficking to sites of inflammation is orchestrated by secreted CXC chemokines, including, CXCL1 and CXCL2[11,12,45,46] and a functional role of CXC chemokines

has been documented in AP.[14] We observed that taurocholate caused a clear-cut increase in CXCL1 and CXCL2 levels in the pancreas. Treatment with GZD824 greatly decreased pancreatic levels of CXCL1 and CXCL2 in the inflamed pancreas, indicating that c-Abl kinase controls CXC chemokine generation in AP, which helps explaining the inhibitory effect of GZD824 on neutrophil infiltration in the inflamed pancreas. The detailed function of specific adhesion molecules in mediating neutrophil recruitment in the pancreas is relatively unclear, although convincing studies have shown that Mac-1 is a key molecule in facilitating extravascular accumulation of neutrophils in other tissues.[47,48] Herein, it was observed that taurocholate challenge increased neutrophil expression of Mac-1. Notably, treatment with GZD824 substantially attenuated expression of Mac-1 on neutrophils increased in response to taurocholate challenge, indicating that c-Abl kinase controls neutrophil expression of Mac-1 in AP. Taken together with the findings on formation of CXC chemokines above, it might be forwarded that c-Abl kinase controls recruitment of neutrophils in the pancreas at 2 distinct levels, i.e., generation of CXC chemokines in the pancreas and expression of Mac-1 on neutrophils. A hallmark of systemic complications in severe AP consists of pulmonary infiltration of neutrophils.[49] Herein, we observed that pulmonary levels of MPO were markedly increased in response to taurocholate challenge. Interestingly, administration of GZD824 decreased pulmonary activity of MPO in taurocholate-exposed animals, suggesting that c-Abl kinase also controls systemic infiltration of neutrophils in the lung in severe AP. The notion that c-Abl kinase regulates systemic inflammation is also in line with our observation that GZD824 attenuated the taurocholate-induced elevation of plasma levels of IL-6, which is an indicator of systemic inflammation and correlates with mortality of septic patients.[50]

In conclusion, our findings demonstrate that c-Abl kinase signaling regulates NET formation in the inflamed pancreas. These results suggest that inhibition c-Abl kinase reduces neutrophil expression of Mac-1 and pancreatic formation of CXC chemokines in severe AP. In addition, c-Abl kinase inhibition reduced neutrophil recruitment and tissue injury in the pancreas. Thus, c-Abl kinase regulates tissue damage in the inflamed pancreas via both NET formation and neutrophil recruitment. Finally, blocking c-Abl kinase activity decreased systemic inflammation and pulmonary neutrophilia in mice with pancreatitis. Thus, this study not only delineates a novel signaling mechanism regulating NET formation in AP but also suggests that blocking c-Abl kinase might be a useful strategy to ameliorate local and systemic inflammation in severe AP.

AUTHORSHIP

R.M., M.R., and M.M. performed experiments, interpreted results, and contributed to the writing. H.T. conceived and designed the study and contributed to the writing. All authors approved the final manuscript.

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DISCLOSURE

No conflicts of interest, financial or otherwise, are declared by the authors.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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