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Role of neutrophils in the activation of trypsinogen in severe acute pancreatitis

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ABSTRACT

The relationship between inflammation and proteolytic activation in pancreatitis is an unresolved issue in pancreatology. The purpose of this study was to define the influence of neutrophils on trypsinogen activation in severe AP. Pancreatitis was induced by infusion of taurocholate into the pancreatic duct in C57BL/6 mice. For neutrophil depletion, an anti-Gr-1 antibody was administered before pancreatitis induction. Administration of the anti-Gr-1 antibody reduced circulating neutrophils by 97%. Pancreatic TAP and serum amylase levels increased 2 h and 24 h after induction of pancreatitis. Neutrophil depletion reduced pancreatic TAP and serum amylase levels at 24 h but not at 2 h after pancreatitis induction. Pancreatic MPO and infiltration of neutrophils, as well as MIP-2 levels, were increased 24 h after taurocholate infusion. Two hours after taurocholate administration, no significant pancreatic infiltration of neutrophils was observed. Injection of the anti-Gr-1 antibody abolished MPO activity, neutrophil accumulation, and MIP-2 levels, as well as acinar cell necrosis, hemorrhage, and edema in the pancreas at 24 h. Moreover, taurocholate-provoked tissue damage and MPO activity in the lung were normalized by neutrophil depletion. Intravital fluorescence microscopy revealed a 97% reduction of leukocytes in the pancreatic microcirculation after administration of the anti-Gr-1 antibody. Our data demonstrate that initial trypsinogen activation is independent of neutrophils, whereas later activation is dependent on neutrophils in the pancreas. Neutrophils are critical in mediating pancreatic and lung tissue damage in severe AP. *J. Leukoc. Biol.* 90: 975–982; 2011.

Introduction

AP is a common condition with a large spectrum of disease severity and clinical outcome. In general, patients with AP are usually identified as having a mild or a severe form of the disease. On one hand, mild forms of AP are usually reversible and uncomplicated. On the other hand, severe forms of AP are frequently complicated and associated with a high rate of mortality [1, 2]. Despite substantial investigative efforts, there is still no specific treatment of AP available. One reason for this is related to our incomplete understanding of the pathophysiology of AP. It is widely held that neutrophil accumulation and protease activation constitute key components in the development of AP [3–6].

Tissue infiltration of neutrophils in the pancreas is a multistep process, coordinated by specific adhesion molecules, such as P-selectin [7, 8] and LFA-1 [9], and by secretion of CXC chemokines, including MIP-2 [10]. Numerous studies have demonstrated that neutrophils play an important role in mediating tissue damage. For example, it has been shown that systemic depletion of neutrophils protects against pancreatitis induced by caerulein [5, 6], which represents a mild form of experimental AP. It is important to note that in line with clinical observations, there are significant differences between mild and severe experimental models of AP, including distant organ complications and levels of proteolytic activation in the pancreas [11–13]. Consequently, the relative contribution of neutrophils may also be different in mild and severe models of AP. Thus, one purpose of the present study was to define the role of neutrophils in a severe form of AP based on infusion of taurocholate into the pancreas.

Beside neutrophil infiltration, proteolytic activation in the pancreas is considered to be a critical feature in the pathophysiology of AP [11, 14]. Activation of trypsinogen into trypsin is the most central proteolytic feature in AP [15]. Exaggerated trypsin activation causes local complications and systemic inflammation, which constitute hallmarks of severe pancreatitis [15, 16]. Although neutrophil accumulation and trypsin activation are considered to be important in AP, their inter-relationships have remained an unresolved issue in pancreatology. For example, it is not known whether trypsinogen activation is dependent on neutrophil infiltration or vice versa in severe pancreatitis.

Abbreviations: AP=acute pancreatitis, MCA=methylcoumarinamide, MMP-9=matrix metallopeptidase-9, PMNL=PMN leukocyte, TAP=trypsinogen activation peptide, UMAS=Malmö University Hospital

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Based on the considerations above, the purpose of this study was to determine the role of neutrophils in a severe model of pancreatitis and in particular, the role of neutrophils in regulating trypsinogen activation in the pancreas. For this purpose, we studied taurocholate-induced pancreatitis in mice, with or without depletion of neutrophils.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice (Taconic Europe, Ry, Denmark), weighing 20–26 g (6–8 weeks old), were kept at standardized conditions at 22°C and exposed to a 12-h light-dark cycle. Animals were fed standard laboratory diet (R3, Lactamin AB, Kimstad, Sweden) and given water ad libitum. Mice were anesthetized by i.p. administration of 7.5 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium)/100 g body weight, diluted in 200 μl saline. Analgesia was obtained by s.c. injection of buprenorfin hydrochloride (0.75 mg/kg, Schering-Plough, Kenilworth, NJ, USA). 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 (Malmö, Sweden).

Model of taurocholate-induced pancreatitis

Duodenum and the attached pancreatic head were exposed through a small (1–2 cm), upper-midline incision. Stay sutures (7-0 prolene) were placed 1 cm from the papilla of Vater before a small puncture was made through the duodenal wall with a 23-G needle. A polyethylene catheter (inner diameter, 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 1–2 mm into the pancreatic duct. The common hepatic duct was identified and clamped with a small vessel clamp to prevent hepatic reflux before 10 μl 5% sodium taurocholate (Sigma-Aldrich, Sollentuna, Sweden), or 0.9% sodium chloride (n=6) was infused into the pancreatic duct (2 μl/min). Methylene blue was added to the infusion to gain visual control of the distribution in the pancreas. Next, the catheter was withdrawn, and the common hepatic duct clamp was removed. The duodenal puncture was closed with a simple suture (7-0 monofilament), and stay sutures were removed before the laparotomy was sutured in two layers. Animals were allowed to wake up and given free access to food and water. In this model, no significant effect on body weight or other clinical signs were observed up to 24 h after administration of taurocholate. Blood was collected from the tail vein for systemic leukocyte differential counts and determination of serum amylase levels and from the inferior vena cava, for analysis of systemic differential cell count. Animals were killed, and tissue from the pancreatic head and lung was harvested, 2 h and 24 h after induction of pancreatitis. One piece of each tissue was snap-frozen in liquid nitrogen for analysis of MPO, MIP-2, and TAP. The other pieces were fixed in formalin for later histological analysis. Sham-operated animals (n=6) were subjected to laparotomy and had a catheter inserted into the pancreatic duct with infusion of 0.9% sodium chloride. An antibody directed against Gr-1 (RB6-8C5, rat IgG2b, 3.0 mg/kg, n=6, Emfret Analytics GmbH and Co. KG, Eibelstadt, Wurzburg, Germany), which is known to effectively deplete mice of neutrophils [17, 18], or a control antibody (rat IgG2b, 3.0 mg/kg, n=6, Emfret Analytics GmbH and Co. KG) was administered i.p. 2 h before induction of pancreatitis.

Intravital fluorescence microscopy

Intravital fluorescence microscopy was performed in separate animals, 24 h after induction of pancreatitis. The abdominal incision was reopened, and the microcirculation was observed using a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany). An equilibration time of 5 min was allowed before videos were recorded digitally for later analysis. FITC-labeled dextran 150,000 (0.05 ml, 5 mg/ml, Sigma-Aldrich) and rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma-Aldrich) were injected into the jugular vein, enabling visualization of leukocyte-endothelium interactions. In each animal, two to five postcapillary venules were analyzed. Leukocyte rolling was defined as the number of leukocytes rolling along a defined part of the endothelium during 20 s and is expressed as cells/min. Leukocyte 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.

Serum amylase and systemic leukocyte counts

At the end of the experiments, blood was sampled from the tail vein. Serum amylase was analyzed with a commercially available assay (Reflotron, Roche Diagnostics GmbH, Mannheim, Germany). A Burker chamber was used for quantification of leukocytes. Blood was diluted 1:20 in Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% vol/vol) for quantification of PMNL. In separate experiments, the number of monocytes, basophils, lymphocytes, erythrocytes, and platelets, as well as neutrophils, was determined 24 h after i.v. injection of 3 mg/kg of the anti-Gr-1 antibody (RB6-8C5) and a control antibody (n=4; Emfret Analytics GmbH and Co. KG). Differential blood cell counts were quantified by use of a multiparameter automated hematology analyzer (CELL-DYN Ruby, Abbott Scandinavia AB, Diagnostics Division, Sweden), which is based on flow cytometric techniques to analyze cell populations.

TAP radioimmunoassay

Upon trypsinogen activation, trypsin and the activation peptide TAP are formed in equimolar amounts, and TAP can thus be used as a marker of trypsinogen activation [19]. Using synthetically made rat TAP (fpledddk, Eurodiagnostica, Malmö, Sweden) as a standard (0.078–20 nM) and as a tracer (I^{125}TAP, Department of Radiophysics, Scania University Hospital, Malmö, Sweden), the RIA was performed as described previously [20]. Frozen pancreatic tissue was homogenized in a 1-ml mixture of 80% PBS and 20% Trasylol 10,000 kallikrein inactivating units/ml (aprotinin, Bayer HealthCare AG, Leverkusen, Germany) for 1 min. Homogenate (100 μl), diluted 1:2 in assay buffer [0.15 M NaCl, 5 mM EDTA, and 2 g/l BSA (Sigma-Aldrich)], and tracer (200 μl) were incubated at 4°C overnight, together with rabbit antisera. Next, samples were incubated with 100 ml of a cellulose-coupled anti-mouse IgG suspension (Sac-Cel®, IDS, Boldon, UK) for 30 min before centrifugation (700 g, 5 min, 20°C) to separate free and bound radioactivities. After decantation of the supernatant, the radioactivity of the precipitate was counted in a γ-spectrophotometer.

MPO assay

Frozen pancreatic and lung tissue was preweighed and homogenized as described above. The homogenate was centrifuged (15,300 g, 10 min, 4°C), and the supernatant was stored at –20°C. The pellet was resuspended in 1 ml 0.5% hexadecyl trimethyl ammonium bromide (pH 7.4), centrifuged as above, and frozen overnight. Next, samples were 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 spectrophotometrically, as described previously [9, 21]. Values are expressed as MPO units/g tissue.

MIP-2 levels

MIP-2 levels were analyzed in stored supernatant from homogenized pancreatic tissue using double-antibody Quantikine ELISA kits (R&D Systems Europe, Abingdon, UK). The minimal detectable protein concentration is <0.5 pg/ml.

Histology

Paraffin-embedded samples from pancreas or lung were cut in sections of 6 μm, stained with H&E, and examined by light microscopy. The severity of pancreatitis was evaluated in a blinded manner in 10 high-power fields/

animal. Edema, acinar cell necrosis, hemorrhage, and neutrophil infiltrate were quantified using 0 (absent)–4 (extensive) scales, as described previously in detail [22].

Isolation of neutrophils and acinar cells

Bone marrow neutrophils were freshly extracted from healthy mice by using Ficoll-Paque research grade (Amersham Pharmacia Biotech, Uppsala, Sweden). The purity of the isolated neutrophils was >70%, as assessed by Turk stain in a hematocytometer [23]. Neutrophil activation was achieved after 20 min of incubation (10^7 cells/ml) with PMA (2 μg/ml; Sigma-Aldrich) in 37°C [24]. Neutrophil secretion products were decanted after centrifugation (15,300 g, 5 min, 4°C). Acinar cells were isolated from the pancreas of healthy mice by collagenase digestion and gentle shearing, as described previously [25]. Cells were suspended in HEPES-Ringer buffer (pH 7.4), saturated with O_2, and passed through a 150-μm cell strainer (Partec, Canterbury, UK). Isolated acinar cells (10^7 cells/well) were stimulated with 100 nM caerulein (37°C, 30 min) or preincubated with activated neutrophils or supernatant from activated neutrophils. The suspending buffer was harvested, and the cells were washed twice with buffer (250 mM sucrose, 5 mM MOPS, and 1 mM $MgSO_4$, pH 6.5) before homogenization in cold (4°C) MOPS buffer using a Potter-Elvejham-type glass homogenizer. The homogenate was centrifuged (50 g, 5 min), and the supernatant was taken for assay. Trypsin activity was measured fluorometrically, as described previously [26]. 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 addition of Boc-Glu-Ala-Arg-MCA as the substrate, and the fluorescence—emitted at 440 nm in response to excitation at 380 nm—was monitored. Trypsin levels (pg/ml) were calculated using a standard curve, generated by assaying purified trypsin. Viability of the pancreatic acinar cells was >95%, as determined by trypan blue dye exclusion.

Statistics

Data are presented as mean ± SEM. Statistical evaluations were performed using Mann Whitney rank sum test unless stated otherwise. $P < 0.05$ was considered significant, and n represents the number of animals.

RESULTS

Taurocholate-induced tissue injury in the pancreas

Injection of the anti-Gr-1 antibody reduced systemic levels of neutrophils by >97%, which is in accordance with earlier studies [27, 28]. In separate experiments, it was observed that administration of the anti-Gr-1 antibody had no effect on the circulating number of monocytes, basophils, lymphocytes, erythrocytes, and platelets (Supplemental Table 1). Taurocholate infusion into the pancreatic duct increased serum levels of amylase by eightfold after 2 h and 23-fold after 24 h (**Fig. 1A**). Neutrophil depletion did not affect amylase levels after 2 h but abolished the taurocholate-induced increase in serum amylase at 24 h (Fig. 1A). Morphological examination revealed that neutrophil depletion protected against taurocholate-induced destruction of the pancreatic microarchitecture (Fig. 1B). Acinar cell necrosis, hemorrhage, and edema were quantified in the pancreas with a histological scoring system 24 h after induction of pancreatitis. Taurocholate challenge triggered a clear-cut increase in acinar cell necrosis, hemorrhage, and edema formation (**Table 1**). Neutrophil depletion markedly reduced taurocholate-provoked changes in acinar cell necrosis, hemorrhage, and edema (Table 1).

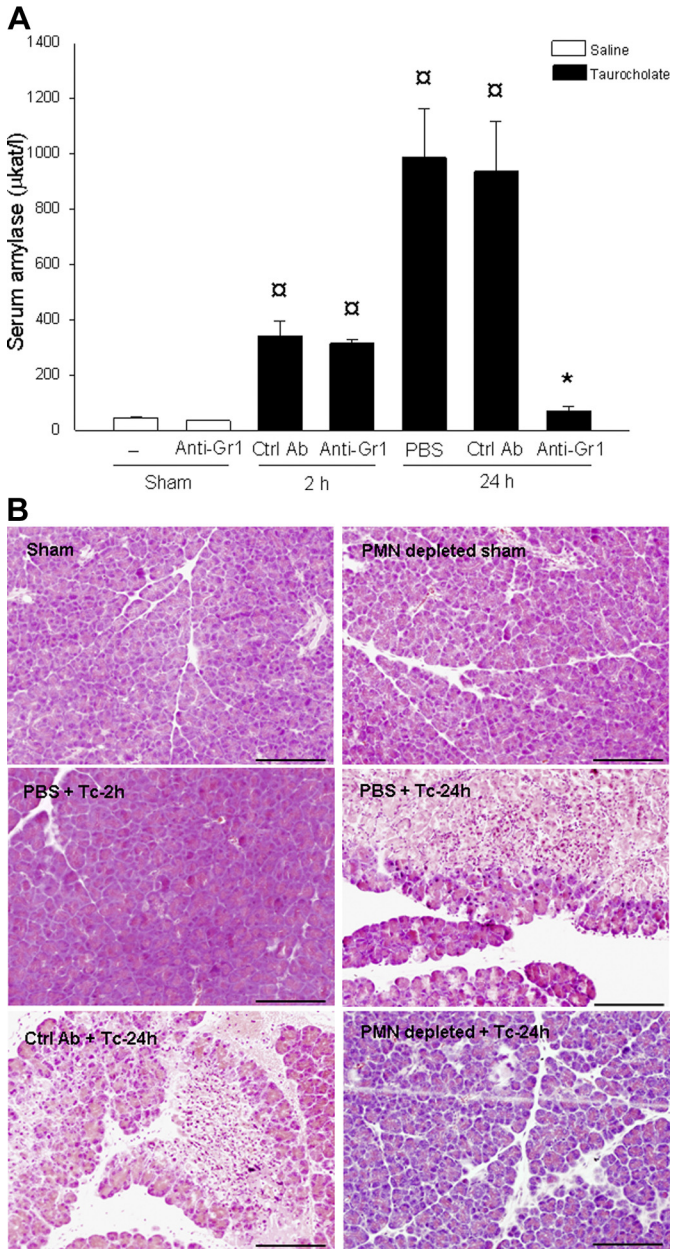

Figure 1. Neutrophils are critical in severe AP. (A) Serum amylase (μkat/l) and (B) representative H&E sections of the pancreas (bars represent 100 μm). Animals were pretreated with PBS, a control antibody (Ctrl Ab), and a neutrophil-depleting antibody (Anti-Gr1) prior to pancreatitis induction. Pancreatitis was induced by infusion of sodium taurocholate (Tc) into the pancreatic duct (black bars). Sham mice (white bars) received saline alone into the pancreatic duct, and certain mice received a neutrophil-depleting antibody before saline injection. Samples were obtained 2 h and 24 h after induction of pancreatitis. Data represent means ± SEM, and $n = 6$. $^{\circ}P < 0.05$ versus Sham; $^{*}P < 0.05$ versus control antibody.

Taurocholate-induced neutrophil infiltration in the pancreas

MPO activity, an indicator of neutrophils, was measured in the pancreas. Pancreatic levels of MPO were not increased

TABLE 1. Neutrophils Mediate Pancreatic and Remote Lung Injury

	Sham-operated		Taurocholate-induced pancreatitis		
	—	Anti-Gr-1	PBS	Control antibody	Anti-Gr-1
MPO in the pancreas (U/g, n=6)	0.3 ± 0.05	0.3 ± 0.05	5.4 ± 1.4[a]	5.6 ± 0.9[b]	0.3 ± 0.07[b]
MPO in the lung (U/g, n=6)	0.9 ± 0.05	0.3 ± 0.16	4.8 ± 1.0[a]	4.3 ± 1.1[a]	0.9 ± 0.2[b]
Acinar cell necrosis (Score 0–4, n=6)	0.1 ± 0.03	0.3 ± 0.07	2.5 ± 0.2[a]	2.9 ± 0.2[a]	1.3 ± 0.2[b]
Neutrophil infiltration (Score 0–4, n=6)	0.3 ± 0.04	0.4 ± 0.1	2.5 ± 0.3[a]	2.8 ± 0.2[a]	0.7 ± 0.2[b]
Edema (Score 0–4, n=6)	0.5 ± 0.13	0.8 ± 0.2	2.8 ± 0.3[a]	2.8 ± 0.2[a]	1.5 ± 0.2[b]
Hemorrhage (Score 0–4, n=6)	0.3 ± 0.2	0.6 ± 0.1	2.9 ± 0.3[a]	2.9 ± 0.2[a]	1.3 ± 0.2[b]

Neutrophils mediate pancreatic and remote lung injury. Neutrophil depletion was obtained in certain mice by injection of an antibody against Gr-1 (Anti-Gr-1) prior to pancreatitis induction. Acute pancreatitis induced by retrograde infusion of 10 μl sodium taurocholate (5%) into the pancreatic duct. Control mice received saline alone, and certain mice received a control antibody or an antibody against Gr-1 prior to pancreatitis induction. Samples were obtained 24 h after induction of pancreatitis. Data represent means ± SEM, and $n = 6$. [a]$P < 0.05$ versus Sham; [b]$P < 0.05$ versus control antibody.

(0.24±0.04 U/g, n=6) 2 h after administration of taurocholate and were not changed by depleting neutrophils (0.16±0.02 U/g, n=6). In contrast, MPO activity was increased by 24-fold, 24 h after taurocholate challenge. Injection of the anti-Gr-1 antibody reduced taurocholate-induced MPO levels by 95% at 24 h (Table 1). Similarly, histological evaluation of the pancreas revealed no infiltration of neutrophils 2 h after taurocholate administration but widespread accumulation of neutrophils 24 h after induction of pancreatitis (Fig. 1B). Administration of the anti-Gr-1 antibody markedly inhibited taurocholate-provoked neutrophil infiltration in the pancreas (Table 1). Leukocyte-endothelium interactions were studied by use of intravital fluorescence microscopy of the pancreatic microcirculation. Injection of the anti-Gr-1 antibody abolished leukocyte interactions with the vascular endothelium in the pancreas (**Fig. 2A**), indicating that neutrophils are the dominating subtype of leukocytes recruited in this experimental model of AP. MIP-2 is an important regulator of neutrophil trafficking and has been shown to play a significant role in AP [10, 29]. We found that pancreatic levels of MIP-2 were slightly enhanced 2 h after taurocholate challenge (Fig. 2B). Notably, this minor increase in MIP-2 formation in the pancreas was not affected by depletion of neutrophils (Fig. 2B). In contrast, pancreatic levels of MIP-2 were markedly increased 24 h after taurocholate challenge, and this major enhancement of MIP-2 formation was nearly abolished in mice depleted of neutrophils (Fig. 2B).

Taurocholate-induced activation of trypsinogen

TAP is used as an indicator of trypsinogen activation [30, 31]. Taurocholate infusion into the pancreatic duct caused a fivefold elevation of pancreatic TAP levels after 2 h and 24 h (**Fig. 3**). Depletion of neutrophils had no effect on TAP formation in the pancreas 2 h after taurocholate administration (Fig. 3). In contrast, it was found that neutrophil depletion abolished the taurocholate-induced increase in TAP levels in the pancreas at 24 h (Fig. 3), indicating that late, but not early, activation of trypsinogen is dependent on neutrophils. To examine whether neutrophils can stimulate trypsinogen activation, we performed in vitro experiments with isolated pancreatic acinar cells, which were incubated with activated neutrophils or secretions from activated neutrophils. We observed that coincubation of activated neutrophils significantly increased trypsin activation in acinar cells (**Fig. 4**). Moreover, stimulation of acinar cells with secretions from PMA-activated neutrophils significantly enhanced trypsin activity by 72% (Fig. 4).

Taurocholate-induced neutrophil recruitment and tissue injury in the lung

Neutrophil accumulation and tissue damage in the lung are features of the systemic response to severe AP. It was found that MPO activity (Table 1) and tissue damage (not shown) in the lung were increased significantly in mice challenged with taurocholate. Administration of the anti-Gr-1 antibody abolished pulmonary activity of MPO in mice challenged with taurocholate (Table 1). Moreover, we observed that depletion of neutrophils protected the lung against taurocholate-induced tissue injury (not shown).

DISCUSSION

Our data demonstrate that neutrophils play important roles in severe AP. Thus, we found that neutrophils are critical in mediating local tissue damage in the pancreas, as well as remote organ injury in the lung in severe pancreatitis. Of particular interest, our findings reveal the detailed interrelationship between neutrophils and trypsinogen activation in the pancreas. Thus, we observed that late but not early activation of trypsinogen in AP is dependent on neutrophils.

Neutrophil infiltration is a prominent feature in severe pancreatitis [9, 21, 32]. The present study shows that neutrophils are critical in the pathophysiology of severe AP. Neutrophil depletion with the anti-Gr-1 antibody not only reduced the number of circulating neutrophils but also abolished leukocyte-endothelium interactions in the microcirculation, MPO levels, and neutrophil accumulation in the

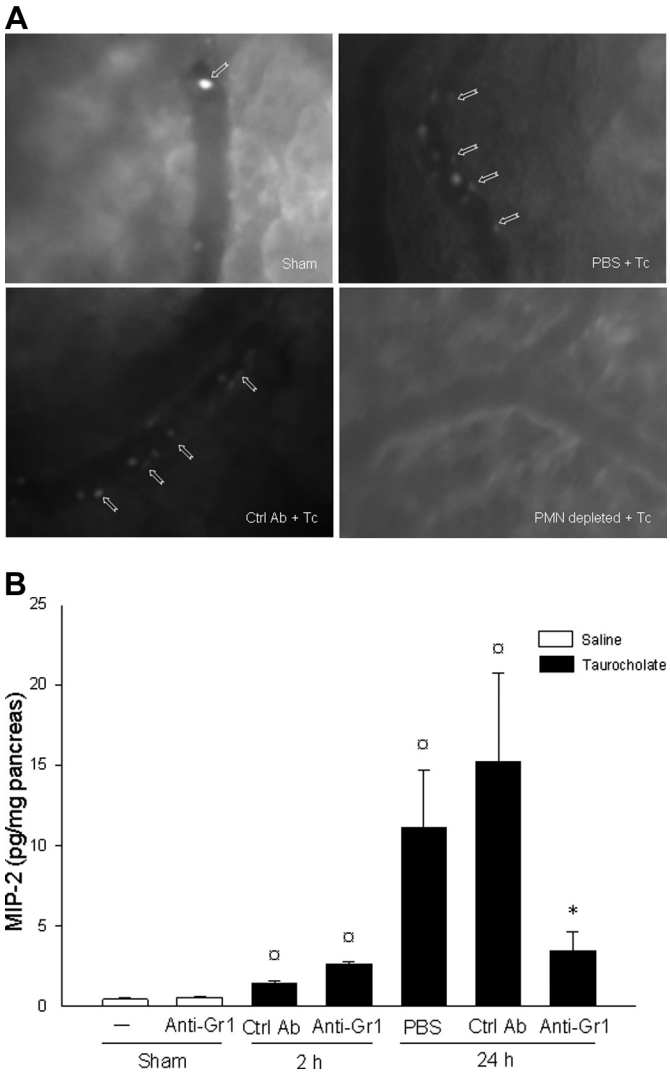

Figure 2. (A) Representative microphotos from intravital fluorescence microscopy and (B) MIP-2 levels (pg/mg) in the pancreas. Animals were pretreated with PBS, a control antibody, and a neutrophil-depleting antibody prior to pancreatitis induction. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct (black bars). Sham mice (white bars) received saline alone into the pancreatic duct, and certain mice received a neutrophil-depleting antibody before saline injection. Samples were obtained 2 h and 24 h after induction of pancreatitis. Data represent means ± SEM, and $n = 6$. $^{\circ}P < 0.05$ versus Sham; $^{*}P < 0.05$ versus control antibody.

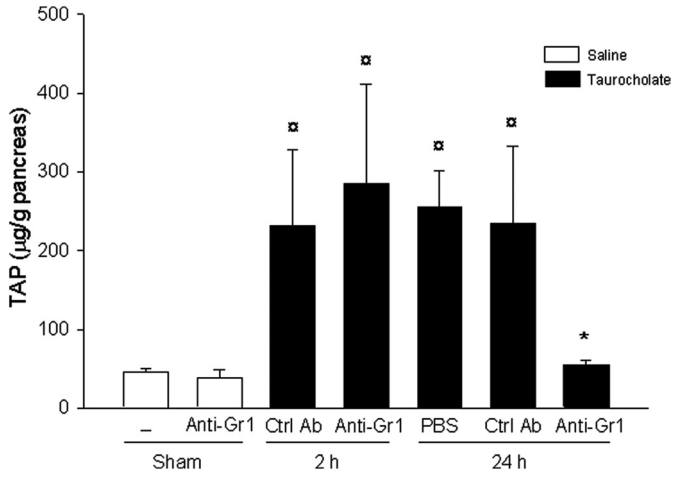

Figure 3. Role of neutrophils in trypsinogen activation. Levels of TAP in the pancreas. Animals were pretreated with PBS, a control antibody, and a neutrophil-depleting antibody prior to pancreatitis induction. Pancreatitis was induced by infusion of sodium taurocholate into the pancreatic duct (black bars). Sham mice (white bars) received saline alone into the pancreatic duct, and certain mice received a neutrophil-depleting antibody before saline injection. Samples were obtained 2 h and 24 h after induction of pancreatitis. Data represent means ± SEM, and $n = 6$. $^{\circ}P < 0.05$ versus Sham; $^{*}P < 0.05$ versus control antibody.

pancreas. It is interesting to note that neutrophil depletion abolished leukocytes in the pancreatic microvasculature, suggesting that neutrophils constitute the dominating leukocyte subtype involved in AP, at least up to 24 h after taurocholate challenge. Moreover, we observed that depletion of neutrophils markedly reduced serum amylase levels and tissue damage in the pancreas and lung, indicating that neutrophils are of great importance for local and systemic inflammation in AP. In this context, it should be mentioned that administration of the anti-Gr-1 antibody has been reported to recognize eosinophils and subsets of monocytes [33, 34]. However, herein, we did not observe any effect on major leukocyte subtypes, including monocytes, lymphocytes, and basophils, which are in line with other studies [28, 35, 36]. Nonetheless, as a result of the possibility that this anti-Gr-1 antibody may still affect non-neutrophils, it

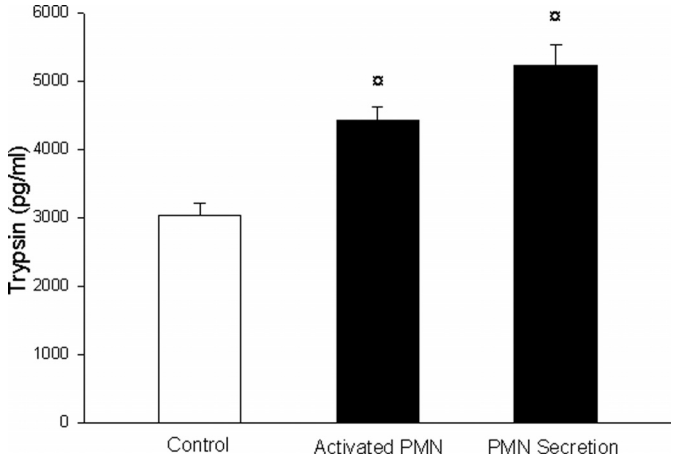

Figure 4. Neutrophils and trypsinogen activation. Acinar cell activation of trypsinogen was measured in negative control cells and acinar cells coincubated with activated neutrophils or neutrophil-derived secretions. Trypsinogen activation was determined by measuring enzymatic activity of trypsin fluorometrically by using Boc-Glu-Ala-Arg-MCA as the substrate, as described in detail in Materials and Methods. Trypsin levels (pg/ml) were calculated using a standard curve generated by assaying purified trypsin. Data represent means ± SEM, and $n = 5$. $^{\circ}P < 0.05$ versus control.

cannot be excluded that circulating cells other than neutrophils may also contribute to the tissue damage in this model of AP. A recent study reported that neutrophil depletion enhances taurocholate-provoked hemorrhage in the pancreas [37], suggesting that neutrophils may protect against hemorrhage in severe AP. This is clearly in contrast to our present findings, showing that neutrophil depletion markedly decreased taurocholate-induced hemorrhage in the pancreas. The reason for these discrepant results is not known at present. Nonetheless, our data are in line with previous studies demonstrating that depletion of neutrophils reduces tissue injury in caerulein-induced AP [5, 38]. In addition, numerous investigations have shown that targeting specific adhesion molecules reducing neutrophil recruitment in the pancreas decreases hemorrhage in different models of AP [8, 9]. Activation and tissue navigation of neutrophils are coordinated by secreted chemokines, such as MIP-2 [39, 40]. Herein, we found that taurocholate challenge enhanced pancreatic levels of MIP-2. It was interesting to note that MIP-2 levels were only decreased late (24 h) but not early (2 h) in the development of AP in neutrophil-depleted mice. These findings are somewhat surprising, knowing that MIP-2 is mainly generated by tissue resident cells in the pancreas [29]. Nonetheless, our results suggest that neutrophils play a role in the pathophysiology of pancreatitis upstream of MIP-2 formation. The link between neutrophils and pancreatic production of MIP-2 is not known but may be related to proinflammatory compounds secreted from activated leukocytes, which in turn, may activate tissue-resident cells in the pancreas. For example, neutrophils can generate large quantities of ROS, which have been shown to induce chemokine synthesis [41, 42].

Trypsinogen activation is widely held as a central feature in the pathophysiology of AP [11]. One previous study reported that neutrophils influence intrapancreatic activation of trypsin in a mild form, i.e., caerulein-provoked pancreatitis [5]. However, the importance of neutrophils for protease activation in severe pancreatitis has remained an unresolved issue. Herein, we asked whether neutrophils might control activation of trypsinogen into trypsin in taurocholate-induced pancreatitis. Trypsinogen activation is associated with the formation of TAP, and a previous study has reported that TAP levels correlate with disease severity in AP [43]. We found that taurocholate challenge markedly increased the levels of TAP in the pancreas at 2 h and remained elevated up to 24 h after pancreatitis induction. Interestingly, it was observed that the early elevation of TAP levels was insensitive to neutrophil depletion. In contrast, we found that depletion of neutrophils markedly reduced TAP levels 24 h after taurocholate challenge, suggesting that late activation of trypsin is dependent on neutrophils and that early trypsinogen activation is independent of neutrophils. In separate experiments, we asked whether activated neutrophils, as well as neutrophil secretions, had the capacity to stimulate trypsin activation in isolated acinar cells in vitro. We observed that activated neutrophils and neutrophil secretions were potent stimulators of trypsin activation in acinar cells, which further supports the conclusion that late formation of trypsin is regulated by neutrophils in vivo. The molecular mechanism behind neutrophil-mediated activation of trypsinogen remains elusive. One potential candidate may be MMP-9, which is an abundant protease in neutrophils and has been implicated in the pathophysiology of viral-induced and biliary models of pancreatitis [44, 45]. Notably, MMP-9 is known to cleave various substrates, such as gelatin [46], chemokines [47], and insulin [48], and whether MMP-9 may control trypsinogen activation in severe AP is a topic of future research. Nonetheless, this neutrophil-dependent activation of trypsin observed herein may also help to explain the reduced MIP-2 levels in the pancreas of neutrophil-depleted animals, knowing that trypsin can promote secretion of MIP-2 from acinar cells via binding to proteinase-activated receptor-2 [49]. Likewise, the lack of effect of neutrophil depletion on trypsinogen activa-

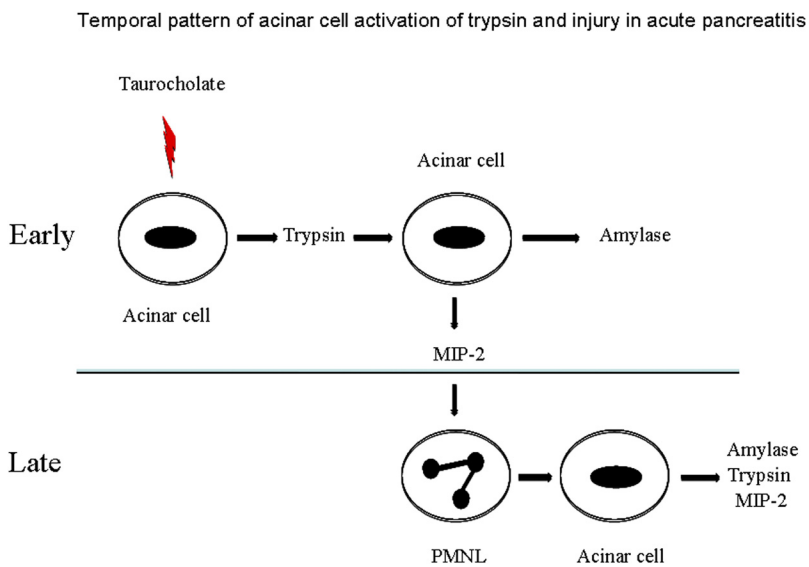

Figure 5. The schematic diagram summarizes the proposed hypothesis that neutrophils regulate late but not early activation of trypsinogen in acinar cells in severe AP. In the early phase, trypsinogen is activated and causes acinar cell injury (amylase release), independently of neutrophils. The initial injury triggers formation of MIP-2, which in turn, stimulates neutrophil chemotaxis into the pancreas. Once in the pancreas, neutrophils cause further acinar cell injury (amylase release), MIP-2 formation, and activation of trypsinogen in the late phase of severe AP.

tion at 2 h after pancreatitis induction helps to explain the intact formation of MIP-2 in animals depleted of neutrophils, 2 h after taurocholate challenge. In this context, it should be noted that neutrophil depletion, which had no effect on early activation of trypsin, reduced tissue damage in AP. These findings suggest that early trypsin formation may not be an essential, therapeutic target in AP and that interference with neutrophil functions may be a more useful strategy in the management of AP.

In conclusion, our data show that neutrophils are critical in mediating acinar cell necrosis and tissue damage in severe pancreatitis. Moreover, these findings suggest that proteolyic activation in the pancreas is a dynamic process characterized by an early neutrophil-independent and a late neutrophil-dependent phase (**Fig. 5**). These findings delineate complex mechanisms regulating tissue damage in AP and may pave the way for new strategies to interfere with pathological inflammation in the pancreas.

AUTHORSHIP

A.A. and D.A. performed experiments, analyzed data, and contributed to the writing. H.T. and S.R. conceived of and designed the study and contributed to the writing.

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DISCLOSURE

The authors declare no competing financial interests.

REFERENCES

1. Mann, D. V., Hershman, M. J., Hittinger, R., Glazer, G. (1994) Multicentre audit of death from acute pancreatitis. *Br. J. Surg.* **81,** 890–893.
2. Appelros, S., Lindgren, S., Borgstrom, A. (2001) Short and long term outcome of severe acute pancreatitis. *Eur. J. Surg.* **167,** 281–286.
3. Kyriakides, C., Jasleen, J., Wang, Y., Moore Jr., F. D., Ashley, S. W., Hechtman, H. B. (2001) Neutrophils, not complement, mediate the mortality of experimental hemorrhagic pancreatitis. *Pancreas* **22,** 40–46.
4. Sandoval, D., Gukovskaya, A., Reavey, P., Gukovsky, S., Sisk, A., Braquet, P., Pandol, S. J., Poucell-Hatton, S. (1996) The role of neutrophils and platelet-activating factor in mediating experimental pancreatitis. *Gastroenterology* **111,** 1081–1091.
5. Gukovskaya, A. S., Vaquero, E., Zaninovic, V., Gorelick, F. S., Lusis, A. J., Brennan, M. L., Holland, S., Pandol, S. J. (2002) Neutrophils and NADPH oxidase mediate intrapancreatic trypsin activation in murine experimental acute pancreatitis. *Gastroenterology* **122,** 974–984.
6. Pastor, C. M., Vonlaufen, A., Georgi, F., Hadengue, A., Morel, P., Frossard, J. L. (2006) Neutrophil depletion—but not prevention of Kupffer cell activation—decreases the severity of cerulein-induced acute pancreatitis. *World J. Gastroenterol.* **12,** 1219–1224.
7. Hackert, T., Buchler, M. W., Werner, J. (2010) Targeting P-selectin in acute pancreatitis. *Expert Opin. Ther. Targets* **14,** 899–910.
8. Hackert, T., Sperber, R., Hartwig, W., Fritz, S., Schneider, L., Gebhard, M. M., Werner, J. (2009) P-selectin inhibition reduces severity of acute experimental pancreatitis. *Pancreatology* **9,** 369–374.
9. Awla, D., Abdulla, A., Zhang, S., Roller, J., Menger, M., Regner, S., Thorlacius, H. (2011) Lymphocyte function antigen-1 regulates neutrophil recruitment and tissue damage in acute pancreatitis. *Br. J. Pharmacol.* **163,** 413–423.
10. Bhatia, M., Hegde, A. (2007) Treatment with antileukinate, a CXCR2 chemokine receptor antagonist, protects mice against acute pancreatitis and associated lung injury. *Regul. Pept.* **138,** 40–48.
11. Regnér, S., Manjer, J., Appelros, S., Hjalmarsson, C., Sadic, J., Borgstrom, A. (2008) Protease activation, pancreatic leakage, and inflammation in acute pancreatitis: differences between mild and severe cases and changes over the first three days. *Pancreatology* **8,** 600–607.
12. Chen, H. M., Sunamura, M., Shibuya, K., Yamauchi, J. I., Sakai, Y., Fukuyama, S., Mikami, Y., Takeda, K., Matsuno, S. (2001) Early microcirculatory derangement in mild and severe pancreatitis models in mice. *Surg. Today* **31,** 634–642.
13. Medveczky, P., Szmola, R., Sahin-Toth, M. (2009) Proteolytic activation of human pancreatitis-associated protein is required for peptidoglycan binding and bacterial aggregation. *Biochem. J.* **420,** 335–343.
14. Ohlsson, K., Balldin, G., Bohe, M., Borgstrom, A., Genell, S., Lasson, A. (1988) Pancreatic proteases and antiproteases in pancreatic disease; biochemical, pathophysiological and clinical aspects. *Int. J. Pancreatol.* **3** (Suppl. 1), S67–S78.
15. Bhatia, M., Wong, F. L., Cao, Y., Lau, H. Y., Huang, J., Puneet, P., Chevali, L. (2005) Pathophysiology of acute pancreatitis. *Pancreatology* **5,** 132–144.
16. Halangk, W., Lerch, M. M., Brandt-Nedelev, B., Roth, W., Ruthenbuerger, M., Reinheckel, T., Domschke, W., Lippert, H., Peters, C., Deussing, J. (2000) Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J. Clin. Invest.* **106,** 773–781.
17. Chen, L., Watanabe, T., Watanabe, H., Sendo, F. (2001) Neutrophil depletion exacerbates experimental Chagas' disease in BALB/c, but protects C57BL/6 mice through modulating the Th1/Th2 dichotomy in different directions. *Eur. J. Immunol.* **31,** 265–275.
18. Chen, L., Zhang, Z., Sendo, F. (2000) Neutrophils play a critical role in the pathogenesis of experimental cerebral malaria. *Clin. Exp. Immunol.* **120,** 125–133.
19. Chen, J. M., Kukor, Z., Le Marechal, C., Toth, M., Tsakiris, L., Raguenes, O., Ferec, C., Sahin-Toth, M. (2003) Evolution of trypsinogen activation peptides. *Mol. Biol. Evol.* **20,** 1767–1777.
20. Lindkvist, B., Wierup, N., Sundler, F., Borgstrom, A. (2008) Long-term nicotine exposure causes increased concentrations of trypsinogens and amylase in pancreatic extracts in the rat. *Pancreas* **37,** 288–294.
21. Abdulla, A., Awla, D., Hartman, H., Rahman, M., Jeppsson, B., Regner, S., Thorlacius, H. (2011) Role of platelets in experimental acute pancreatitis. *Br. J. Surg.* **98,** 93–103.
22. Schmidt, J., Rattner, D. W., Lewandrowski, K., Compton, C. C., Mandavilli, U., Knoefel, W. T., Warshaw, A. L. (1992) A better model of acute pancreatitis for evaluating therapy. *Ann. Surg.* **215,** 44–56.
23. Liu, Q., Wang, Y., Thorlacius, H. (2000) Dexamethasone inhibits tumor necrosis factor-α-induced expression of macrophage inflammatory protein-2 and adhesion of neutrophils to endothelial cells. *Biochem. Biophys. Res. Commun.* **271,** 364–367.
24. Lieberman, M. M., Sachanandani, D. M., Pinney, C. A. (1996) Comparative study of neutrophil activation by chemiluminescence and flow cytometry. *Clin. Diagn. Lab. Immunol.* **3,** 654–662.
25. Bruzzone, R., Halban, P. A., Gjinovci, A., Trimble, E. R. (1985) A new, rapid, method for preparation of dispersed pancreatic acini. *Biochem. J.* **226,** 621–624.
26. Kawabata, S., Miura, T., Morita, T., Kato, H., Fujikawa, K., Iwanaga, S., Takada, K., Kimura, T., Sakakibara, S. (1988) Highly sensitive peptide-4-methylcoumaryl-7-amide substrates for blood-clotting proteases and trypsin. *Eur. J. Biochem.* **172,** 17–25.
27. Sitia, G., Isogawa, M., Kakimi, K., Wieland, S. F., Chisari, F. V., Guidotti, L. G. (2002) Depletion of neutrophils blocks the recruitment of antigen-nonspecific cells into the liver without affecting the antiviral activity of hepatitis B virus-specific cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* **99,** 13717–13722.
28. Soehnlein, O., Zernecke, A., Eriksson, E. E., Rothfuchs, A. G., Pham, C. T., Herwald, H., Bidzhekov, K., Rottenberg, M. E., Weber, C., Lindbom, L. (2008) Neutrophil secretion products pave the way for inflammatory monocytes. *Blood* **112,** 1461–1471.
29. Orlichenko, L. S., Behari, J., Yeh, T. H., Liu, S., Stolz, D. B., Saluja, A. K., Singh, V. P. (2010) Transcriptional regulation of CXC-ELR chemokines KC and MIP-2 in mouse pancreatic acini. *Am. J. Physiol. Gastrointest. Liver Physiol.* **299,** G867–G876.
30. Awla, D., Hartman, H., Abdulla, A., Zhang, S., Rahman, M., Regner, S., Thorlacius, H. (2011) Rho-kinase signaling regulates trypsinogen activation and tissue damage in severe acute pancreatitis. *Br. J. Pharmacol.* **162,** 648–658.

31. Pezzilli, R., Venturi, M., Morselli-Labate, A. M., Ceciliato, R., Lamparelli, M. G., Rossi, A., Moneta, D., Piscitelli, L., Corinaldesi, R. (2004) Serum trypsinogen activation peptide in the assessment of the diagnosis and severity of acute pancreatic damage: a pilot study using a new determination technique. *Pancreas* **29,** 298–305.
32. Inagaki, H., Nakao, A., Kurokawa, T., Nonami, T., Harada, A., Takagi, H. (1997) Neutrophil behavior in pancreas and liver and the role of nitric oxide in rat acute pancreatitis. *Pancreas* **15,** 304–309.
33. Su, S. B., Grajewski, R. S., Luger, D., Agarwal, R. K., Silver, P. B., Tang, J., Tuo, J., Chan, C. C., Caspi, R. R. (2007) Altered chemokine profile associated with exacerbated autoimmune pathology under conditions of genetic interferon-γ deficiency. *Invest. Ophthalmol. Vis. Sci.* **48,** 4616–4625.
34. Dunay, I. R., Fuchs, A., Sibley, L. D. (2010) Inflammatory monocytes but not neutrophils are necessary to control infection with *Toxoplasma gondii* in mice. *Infect. Immun.* **78,** 1564–1570.
35. Nagendra, S., Schlueter, A. J. (2004) Absence of cross-reactivity between murine Ly-6C and Ly-6G. *Cytometry A* **58,** 195–200.
36. Leendertse, M., Willems, R. J., Giebelen, I. A., Roelofs, J. J., Bonten, M. J., van der Poll, T. (2009) Neutrophils are essential for rapid clearance of *Enterococcus faecium* in mice. *Infect. Immun.* **77,** 485–491.
37. Ryschich, E., Kerkadze, V., Deduchovas, O., Salnikova, O., Parseliunas, A., Marten, A., Hartwig, W., Sperandio, M., Schmidt, J. (2009) Intracapillary leukocyte accumulation as a novel antihemorrhagic mechanism in acute pancreatitis in mice. *Gut* **58,** 1508–1516.
38. Guice, K. S., Oldham, K. T., Caty, M. G., Johnson, K. J., Ward, P. A. (1989) Neutrophil-dependent, oxygen-radical mediated lung injury associated with acute pancreatitis. *Ann. Surg.* **210,** 740–747.
39. Pastor, C. M., Rubbia-Brandt, L., Hadengue, A., Jordan, M., Morel, P., Frossard, J. L. (2003) Role of macrophage inflammatory peptide-2 in cerulein-induced acute pancreatitis and pancreatitis-associated lung injury. *Lab. Invest.* **83,** 471–478.
40. Bacon, K. B., Oppenheim, J. J. (1998) Chemokines in disease models and pathogenesis. *Cytokine Growth Factor Rev.* **9,** 167–173.
41. Riaz, A. A., Schramm, R., Sato, T., Menger, M. D., Jeppsson, B., Thorlacius, H. (2003) Oxygen radical-dependent expression of CXC chemokines regulate ischemia/reperfusion-induced leukocyte adhesion in the mouse colon. *Free Radic. Biol. Med.* **35,** 782–789.
42. Kina, S., Nakasone, T., Takemoto, H., Matayoshi, A., Makishi, S., Sunagawa, N., Liang, F., Phonaphonh, T., Sunakawa, H. (2009) Regulation of chemokine production via oxidative pathway in HeLa cells. *Mediators Inflamm.* **2009,** 183760.
43. Frossard, J. L. (2001) Trypsin activation peptide (TAP) in acute pancreatitis: from pathophysiology to clinical usefulness. *JOP* **2,** 69–77.
44. De Palma, A. M., Thibaut, H. J., Li, S., Van Aelst, I., Dillen, C., Swinnen, M., Verbeken, E., Neyts, J., Opdenakker, G. (2009) Inflammatory rather than infectious insults play a role in exocrine tissue damage in a mouse model for coxsackievirus B4-induced pancreatitis. *J. Pathol.* **217,** 633–641.
45. Mikami, Y., Dobschütz, E. V., Sommer, O., Wellner, U., Unno, M., Hopt, U., Keck, T. (2009) Matrix metalloproteinase-9 derived from polymorphonuclear neutrophils increases gut barrier dysfunction and bacterial translocation in rat severe acute pancreatitis. *Surgery* **145,** 147–156.
46. Van den Steen, P. E., Proost, P., Grillet, B., Brand, D. D., Kang, A. H., Van Damme, J., Opdenakker, G. (2002) Cleavage of denatured natural collagen type II by neutrophil gelatinase B reveals enzyme specificity, post-translational modifications in the substrate, and the formation of remnant epitopes in rheumatoid arthritis. *FASEB J.* **16,** 379–389.
47. Van den Steen, P. E., Proost, P., Wuyts, A., Van Damme, J., Opdenakker, G. (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-α and leaves RANTES and MCP-2 intact. *Blood* **96,** 2673–2681.
48. Descamps, F. J., Van den Steen, P. E., Martens, E., Ballaux, F., Geboes, K., Opdenakker, G. (2003) Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin. *FASEB J.* **17,** 887–889.
49. Hirota, M., Ohmuraya, M., Baba, H. (2006) The role of trypsin, trypsin inhibitor, and trypsin receptor in the onset and aggravation of pancreatitis. *J. Gastroenterol.* **41,** 832–836.

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