Published in final edited form as:

Pancreatology. 2005 ; 5(1): . doi:10.1159/000084489.

Degradation and Inactivation of Plasma Tumor Necrosis Factor-Alpha by Pancreatic Proteases in Experimental Acute Pancreatitis

G. Alsfasser, B. Antoniu, S.P. Thayer, A.L. Warshaw, and C. Fernández-del Castillo

Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, Mass., USA

Abstract

Background—Release of TNF is thought to play an important role in mediating systemic effects in acute pancreatitis (AP). We have been unable to find an elevation of plasma TNF in AP and hypothesize that it is susceptible to catabolism by circulating pancreatic proteases.

Methods—(1) AP was induced in Sprague-Dawley rats by cerulein hyperstimulation preceded by intraductal infusion of saline (mild) or glycodeoxycholic acid (severe). Healthy and sham-operated animals served as controls. Severity of pancreatitis was confirmed by histology. Plasma TNF levels were measured at various time points after induction of AP with competitive ELISA. (2) Recombinant rat TNF (rrTNF) was incubated with trypsin, elastase, chymotrypsin and pepsin. Western Blot was performed to visualize TNF degradation. (3) RrTNF was incubated in a concentration and time-dependant manner with proteases and TNF bioactivity was evaluated with a cytotoxicity assay.

Results—(1) Plasma TNF levels in severe pancreatitis were significantly lower than in sham-operated controls after 0.5 and 6 h. (2) Incubation with proteases showed degradation in the presence of trypsin, elastase and chymotrypsin and no effect of pepsin. (3) There was a concentration dependent inactivation of rrTNF in the presence of pancreatic proteases and a complete time-dependent inactivation in the presence of trypsin.

Conclusion—Plasma TNF does not rise in experimental AP, and levels are significantly lower in severe pancreatitis compared to sham-operated controls. Our study demonstrates degradation and inactivation of TNF by pancreatic proteases, suggesting that it is unlikely it plays an important role in the development of distant organ failure.

Keywords

Acute pancreatitis; Tumor necrosis factor-alpha; Cytokine; Trypsin digestion; Cytokine; Pancreatic proteases, bioassay

Introduction

Distant organ failure is the most important determinant of severity in acute pancreatitis [1, 2], yet the mechanisms involved in the development of extra-pancreatic organ injury remain poorly understood. Inflammatory cytokines have been implicated in the pathogenesis of pancreatitis as well as the development and progression of multisystem organ failure [3-5].

Several studies have shown that IL-6, IL-10, IL-1 [6-8] and in particular tumor necrosis factor- (TNF) [3, 4] may play a significant role in this disease. The latter is not surprising, given the well-established role of TNF in the course of sepsis. However, the importance of TNF in acute pancreatitis is still controversial. While some studies have shown that acinar cells are capable of TNF production [9], that TNF mRNA levels in tissue are increased in pancreatitis [10], and even that anti-TNF therapy decreased mortality in experimental pancreatitis [11], other authors have reported worsening of pancreatitis with anti-TNF therapy [12] or no TNF secretion at all during experimental pancreatitis [13]. In the clinical setting, TNF is not elevated in the overwhelming majority of patients [6, 14] and has no prognostic value [6].

To further elucidate the role of this cytokine in acute pancreatitis we sought to measure TNF release in experimental pancreatitis with graded severity, and investigated the effects of pancreatic enzymes on TNF in vitro.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 300 ± 50 g were obtained from Charles River Laboratories (Wilmington, Mass., USA). Care was provided in accordance with the procedures outlined in the Guide of Care and Use of Laboratory Animals (DHHS Publication No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, Bethesda, Md., USA). The study was approved by the subcommittee on animal research at our institution. Rats were housed individually in hanging cages at standard conditions (12-hour light/dark cycle and $21 \pm 1^\circ\text{C}$) and fed regular rat chow. The animals were fasted overnight before the experiment with free access to water.

All reagents were obtained from Sigma (St. Louis, Mo., USA) unless otherwise specified.

Experimental Design

Fifty-nine animals were divided into 5 different groups: Healthy controls ($n = 3$), LPS controls (i.e. positive control) ($n = 2$), sham-operated controls ($n = 18$), mild ($n = 18$) and severe ($n = 18$) pancreatitis. Healthy animals were anesthetized and blood was collected by cardiac puncture. Blood of the remaining experimental groups was collected in the same manner 0.5, 1, 2, 6, 12 and 24 h after induction of pancreatitis from 3 animals per group per time point. Autopsy was performed after blood collection and signs of pancreatitis were evaluated. Myeloperoxidase (MPO) in lungs was measured to verify the severity of pancreatitis.

Anesthesia and Catheter Placement

Surgical anesthesia was induced by intraperitoneal pentobarbital (Nembutal® 50 mg/kg BM, Abbott Laboratories, North Chicago, Ill., USA) and intramuscular ketamine (Ketalar® 10 mg/kg BM, Monarch Pharmaceuticals Inc, Bristol, Tenn., USA). The right internal jugular vein was cannulated using a soft polyethylene catheter (Intramedic, ID 0.023', Clay Adams, Parsippany, N.J., USA) for infusion. This catheter was tunneled subcutaneously to the neck and brought out via a flow-through tether, which permitted free movement.

Induction of Pancreatitis

Experimental pancreatitis was induced as previously described [15]. Briefly, the biliopancreatic duct was cannulated with a 24-gauge angiocath (Becton Dickinson, Sandy, Utah, USA) and bile and pancreatic juice were drained by gravity in a 60°C reverse Trendelenburg position for 5 min. During the last 2 min of this procedure, the main duct was

clamped below the liver to allow complete emptying of the biliary and pancreatic ductal system and to facilitate the subsequent intraductal infusion. For induction of mild pancreatitis saline solution, and for severe pancreatitis freshly prepared 10 mmol/l glycodeoxycholic acid (GDOC) glycylglycine-NaOH-buffered solution (pH 8.0 at room temperature) was then infused in a pressure- (30 mm Hg) and volume- (0.12 ml/100 g) controlled manner over 10 min. After completion of the intraductal infusion, all animals received continuous secretory hyperstimulation for 6 h with intravenous cerulein (5 µg/kg/h) mixed in saline at 8 ml/kg/h as baseline hydration. Sodium bicarbonate (0.2 ml/100 g) and ketamine (0.2 ml) were added to this infusion. Sham-operated controls underwent a median laparotomy and received saline infusion at 8 ml/kg/h for 6 h.

Blood Collection

Blood was drawn, collected in citrate (citrate to blood ratio 1:9), placed on ice and immediately spun at 4°C, 2,500 g for 15 min. Plasma was aliquoted and frozen at -80°C.

Quantification of TNFα

TNF was measured using a competitive ELISA-Kit for murine TNF (Neogen Corporation, Lexington, Ky., USA) according to the instructions of the manufacturer.

LPS controls were carried out according to the method of Murakami et al. [16]. Briefly, 5 mg/kg LPS were administered intravenously as a bolus. After 90 min rats were anesthetized with pentobarbital and blood was drawn by cardiac puncture.

Histological Analysis

In all groups necrosis, edema and leukocyte infiltration of pancreata were evaluated by a previously described scoring system [17].

MPO Measurements in Lungs

Lungs were harvested, the blood vessels were flushed with normal saline and the tissue was immediately snap frozen. Frozen tissue was homogenized followed by freeze-thaw cycles and sonication. MPO was measured as described by others [18, 19].

Digestion of Cytokines

Recombinant rat TNF (rrTNF) from Pharmingen, San Diego, Calif., USA was used for the following experiments:

Digestion for Western Blotting—10 ng of rrTNF were digested with 1 µg/ml of trypsin, elastase or chymotrypsin in digestion buffer consisting of 2 mM CaCl₂, 1 mM MgCl₂, 50 mM TRIS at pH 7.4 for 30 min at 37°C. Pepsin, which does not digest TNF, was used as control.

20 µl of this cytokine (1 µg/ml) were mixed with 20 µl of each enzyme (2 µg/ml in 2 × digestion buffer). Reaction was stopped by adding 10 µl of 5 × SDS-PAGE sample buffer ± β-mercapto-ethanol and boiling of samples for 3 min. 20 µl of this mixture were placed on the gel for Western Blotting as described below.

Digestion for Bioassay—In pilot studies we determined the dose required to induce cell death in 50% of cells (EC₅₀) to be 0.3 ng for rrTNF, and this concentration was then used in the remaining experiments.

0.3 ng rrTNF was incubated with 0.1, 0.3, 1, 3, 10, 30 and 100 µg/ml trypsin, elastase or chymotrypsin for 30 min at 37°C. Additionally, 0.3 ng rrTNF was incubated with 0.1 µg/ml trypsin in a time-dependent manner at 37°C. Pepsin was again used as a control.

In detail, 1.2 ng/ml rrTNF dissolved in 2× digestion buffer, was incubated with 0.4, 1.2, 4, 12, 40, 120 and 400 µg/ml trypsin, elastase, chymotrypsin and pepsin in 200-µl aliquots at 37°C. The reaction was stopped after 30 min by adding 100 µl of RPMI-1,640 supplemented with 10% FBS and 2 mM glutamine. Then 3 × 90 µl of this mixture were transferred to three wells of a 96-well plate. 30 µl of 4× Sytox® Green and Actinomycin D, mixed in supplemented RPMI, were added to each well. 100 µl of this mixture was transferred to the cells to start the bioassay as described below. For the time-dependent incubation of rrTNF with trypsin, aliquots were taken and the reaction was stopped at 30 min, 1, 2, 3 and 4 h.

Western Blot

After digestion, samples were subject to 15% SDS-polyacrylamide gel electrophoresis using MiniProtean II Dual Slab Cell apparatus (Bio-Rad, Richmond, Calif., USA). Transfer to a PVDF membrane was performed using Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). The membrane was blocked with 3% BSA overnight at 4°C, washed in TBS/0.05% Tween-buffer, and incubated with 1.0 µg/ml mouse anti-rat-TNF monoclonal antibody MAB 510 (Jackson Immuno Research, West Grove, Okla., USA) for 2 h at room temperature. It was then washed three times and incubated with Biotin-SP-conjugated Affini-Pure rabbit anti-mouse IgG (Jackson Immuno Research) (dilution 1:100,000) for 1 h at room temperature. After washing, the membrane was incubated with Streptavidin-HRP (DAKO, Carpinteria, Calif., USA) for 45 min, washed again and ECL reagent applied (Amersham Biosciences, Piscataway, N.J., USA) followed by autoradiography.

Bioactivity Assay

For evaluation of bioactivity, the Vybrant™ Tumor Necrosis Factor Assay Kit (V-23100) (Molecular Probes, Eugene, Oreg., USA) was used. This cytotoxicity assay evaluates TNF bioactivity using SYTOX® Green stain to detect TNF induced cell death in mouse fibroblast-derived WEHI 13var cells in the presence of actinomycin D. Briefly, WEHI-13var cells were seeded at a density of 4×10^5 cells/ml in an untreated black 96-well plate (Fisher Scientific, Pittsburgh, Pa., USA) and incubated overnight in a humidified chamber with 5% CO₂ in RPMI-1640 supplemented with 10% FBS and 2 mM glutamine. On the day of the experiment rrTNF was digested with graded concentration of trypsin, elastase, chymotrypsin and pepsin as described in detail above. The assay mixture was transferred to the cells and incubated for 7 h. TNF-induced cell death results in binding of the fluorescent dye SYTOX® Green to DNA which in turn increases the fluorescent signal, measured at 485/530nm with a Wallac Victor 1,420 plate reader (PerkinElmer Life Sciences, Boston, Mass., USA). Cells without addition of rrTNF and cells lysed with 0.1% Triton X served as negative and positive control for determination of percentage of dead cells and TNF bioactivity. The percentage of dead cells did not exceed 20%.

These experiments were repeated at least four times to ensure reproducibility. Toxic effects of the proteases were ruled out by adding every concentration to the cells without TNF and comparing them to controls.

Statistical Analysis

Data are presented as mean ± SEM. Differences between groups were compared using Student t test. Data were analyzed with Graph-Pad Prism version 3.03 for Windows (GraphPad Software, San Diego, Calif., USA, www.graphpad.com).

Results

Pancreatitis and TNF Levels in Plasma

Induction of severe pancreatitis resulted in hemorrhage and edema within the pancreas as early as 30 min, and fat necrosis and development of ascites reaching a maximum at six hours. This was paralleled by elevated MPO levels in lungs with mean MPO levels (mU/mg protein) at 0.5 h of 4.5 ± 0.2 and 0.9 ± 0.3 and at 6 h of 6.9 ± 2.1 and 3.8 ± 0.3 in severe pancreatitis and sham-operated controls, respectively. In pancreata of sham operated controls no pathology was found. Induction of mild pancreatitis produced a markedly edematous pancreas. In pancreata of animals with severe pancreatitis confluent necrosis and increased leukocyte infiltration could be demonstrated (data not shown). TNF levels in healthy animals were 4.8 ± 2.4 ng/ml. At 0.5 h, levels were 10.53 ± 1.34 ng/ml, 7.26 ± 1.75 ng/ml and 3.95 ± 1.56 in sham-operated controls, mild and severe pancreatitis, respectively. The levels in severe pancreatitis were significantly lower than in sham-operated controls ($p = 0.03$). This difference persisted up to 6 h (4.01 ± 0.18 ng/ml vs. 7.54 ± 0.84 ng/ml, $p = 0.01$). The positive control (plasma of LPS-treated animal) demonstrated a TNF concentration of 105 ng/ml (fig. 1).

Western Blot

Western Blot demonstrated marked degradation of rrTNF in the presence of both trypsin and chymotrypsin and, to a lesser extent, in the presence of elastase. As expected, Pepsin did not degrade TNF (fig. 2).

Determination of TNF α Bioactivity

A concentration-dependent inactivation of rrTNF in the presence of trypsin, elastase and chymotrypsin was demonstrated by bioassay. EC_{50} of each enzyme was 10.89, 2.74 and 1.5 μ g/ml, respectively. Pepsin did not inactivate rrTNF (fig. 3). Time-dependent incubation of rrTNF with trypsin showed continuous decrease of bioactivity whereas activity of undigested rrTNF remained constant over time at about 50% cell killing. Activity was compared to lysis with Triton X, which causes 100% cell killing. At 0.5 h after incubation with trypsin, the bioactivity of rrTNF was decreased to $51 \pm 8\%$ (equals cell killing in $31 \pm 4\%$ of cells). After 1 and 2 h, bioactivity of digested TNF was decreased to $20 \pm 3\%$ ($11 \pm 2\%$ cell killing) and $5 \pm 2\%$ ($2 \pm 1\%$ cell killing), respectively. After 3 h the bioactivity of digested TNF was 0% (fig. 4). These results as well demonstrate that adding RPMI cell culture media indeed stops the reaction. If the reaction had not been stopped, we would not have been able to see any bioactivity, because the bioactivity assay itself requires an additional 7 h for completion.

Discussion

TNF was first described in 1975 by Carswell et al. [20] as a substance that mediates endotoxin-induced necrosis in various tumors. Later it was identified as a protein that usually forms a trimer which is dissociated by SDS-PAGE into a monomeric form with a molecular weight of 17,000–18,000 [21, 22]. The important role of TNF in the course of many diseases, like sepsis, rheumatoid arthritis, congestive heart failure, and others is well-established [23]. Not surprisingly, the possible role of TNF in pancreatitis has been subject to extensive research in this field. In 1992, Exley et al. [14] described its presence in 30% (11/38) of patients with acute pancreatitis, and suggested this cytokine could be a prognostic marker of severe pancreatitis. Subsequent clinical studies have shown that very few patients with acute pancreatitis have detectable levels of TNF in serum, and that it has no prognostic significance [6]. Others had not been able to detect any TNF in serum of patients with post-ERCP pancreatitis [24, 25]. Although some researchers attribute this lack

of detection on account of a short half-life or wrong timing of blood collection [26], others have considered TNF to be an inappropriate target for anticytokine therapy in acute pancreatitis [5].

In the experimental setting, many studies have also investigated the role of TNF in the pathogenesis of pancreatitis and distant organ injury. Data to support that TNF is involved in this disease include several studies showing up-regulation of TNF mRNA in pancreas, liver and lung in experimental acute pancreatitis [10]. Elevated TNF levels in serum were detected by Western blotting in rabbit pancreatitis induced by intraductal bile infusion [27]. However, exact quantification is impossible with this method. Others report elevated TNF levels, measured by ELISA, in rat taurocholate pancreatitis compared to controls [28]. The group of Gaber and co-workers published a study showing improvement in biochemical markers of pancreatitis, but not in histological score, using anti-TNF antibodies [29], and a subsequent study where this form of treatment improved survival in rats with pancreatitis induced by retrograde pancreatic ductal infusion of bile [11]. These authors as well report a higher plasma level of TNF in untreated animals at 30 min after induction of pancreatitis, however, the plasma levels were measured using a bioassay and no information about the percentage of dead cells is given, which influences the accuracy of the method. The improvement of survival with anti-TNF therapy has not been confirmed by others, and in fact another study showed worsening of pancreatitis with anti-TNF therapy [12].

In our own and in other's experience there has been failure to demonstrate detectable levels of TNF in serum of rats with pancreatitis or in patients with pancreatitis [6, 13, 26]. While this has also been attributed to interference of the soluble TNF receptor with the detection of TNF [30], the findings of the present study indicate that circulating TNF does not increase following the induction of pancreatitis, and furthermore, that TNF levels were significantly lower in rats with severe pancreatitis compared to sham operated controls. In our study we used a competitive ELISA to measure total TNF and therefore rule out interference with the soluble receptor. Folch et al. [30] recommend the use of a competitive ELISA for the measurement of this cytokine, which detects free and receptor bound TNF. They validated the use of this particular assay for rat TNF. In our study we used the same assay and found detectable levels in healthy rats at a similar level than others [Closa et al., unpubl. data]. This seemingly paradoxical finding of decreased TNF in severe pancreatitis can probably be explained by our subsequent findings showing TNF degradation by proteases. Incubating TNF with pancreatic proteases showed marked degradation in the presence of chymotrypsin and trypsin and to a lesser extent with elastase. Others, as well, have shown that TNF is susceptible to enzymatic digestion. Van Kessel et al. [31] reported degradation of TNF by enzymes released from stimulated human neutrophils. Aggarwal et al. [22] characterized TNF in detail and used trypsin and chymotrypsin to digest TNF for further characterization of its structure. Unfortunately, there are no details about any concentrations of proteases mentioned. In our study TNF formed a band of about 19 kDa on a 15% SDS-PAGE followed by Western blotting, which is in accordance to reports of the molecular weight of TNF to be 17.9 kDa. Bands at higher molecular weight can appear, if repeated freeze-thaw cycles were present, because of aggregation of the protein (source: R&D Systems, Inc, Minneapolis, Minn., USA).

After demonstrating a proteolytic degradation we investigated whether these digestion products are in fact biologically active. Therefore we evaluated the activity of the remaining TNF after incubation with proteases. We used a recently described bioassay with high sensitivity [32] and used graded concentrations including physiologic concentrations of pancreatic proteases. These concentrations were based upon circulating TAP levels in our model of acute pancreatitis published earlier [33, 34] and levels of proteases in acute pancreatitis described by others [35-37]. A concentration-dependent inactivation of TNF

was seen in the presence of all pancreatic proteases. Of interest is the fact that after incubation with elastase, inactivation could be demonstrated even in absence of marked degradation. The mechanism of this effect still remains unclear and warrants further studies. The time-dependent incubation of TNF with trypsin revealed a 50% reduction of TNF bioactivity after 30 min, and after two hours no demonstrable bioactivity was noted while the bioactivity of TNF without trypsin remained constant. This observation indicates that the activity of TNF was not compromised by long exposure to a temperature of 37°C. This is in accordance with Haranaka et al. [21] who demonstrated stable TNF at 37°C for 40 h. The same authors also mention inactivation of mouse TNF by various enzymes including trypsin, but no details about the incubation are given [21]. Another group [31] reported decrease of TNF bioactivity in the presence of neutrophil elastase as well as other proteases, but the concentrations used were unphysiologic (100 µg/ml). Of note is that so far these findings have not been linked to protease digestion of TNF in acute pancreatitis.

Under normal conditions, circulating proteases are rapidly cleared from the blood after conjugation with plasma protease inhibitors [37]. However, antiprotease levels may be inadequate to inactivate proteases completely during the early phase of acute pancreatitis [38]. Many studies indicate that pancreatic proteases in acute pancreatitis are not fully inhibited [39-41] and therefore remain capable of digestive activity.

In summary, we were unable to demonstrate elevation of plasma TNF during experimental acute pancreatitis, and, in fact, plasma levels were higher in sham-operated controls compared to severe pancreatitis. Our study demonstrated marked degradation of TNF in the presence of trypsin, chymotrypsin and to a lesser extent with elastase. Bioactivity of TNF was decreased in the presence of pancreatic proteases in a concentration and time-dependent manner, even in absence of marked degradation. Whatever the local effect of TNF in acute pancreatitis, we suggest that its enzymatic degradation and inactivation limit any substantial role in systemic pathophysiology.

References

1. Perez A, Whang EE, Brooks DC, Moore FD Jr, Hughes MD, Sica GT, Zinner MJ, Ashley SW, Banks PA. Is severity of necrotizing pancreatitis increased in extended necrosis and infected necrosis? *Pancreas*. 2002; 25:229–233. [PubMed: 12370532]
2. Tenner S, Sica G, Hughes M, Noordhoek E, Feng S, Zinner M, Banks PA. Relationship of necrosis to organ failure in severe acute pancreatitis. *Gastroenterology*. 1997; 113:899–903. [PubMed: 9287982]
3. Norman J. The role of cytokines in the pathogenesis of acute pancreatitis. *Am J Surg*. 1998; 175:76–83. [PubMed: 9445247]
4. Bhatia M, Brady M, Shokuhi S, Christmas S, Neoptolemos JP, Slavin J. Inflammatory mediators in acute pancreatitis. *J Pathol*. 2000; 190:117–125. [PubMed: 10657008]
5. Kingsnorth A. Role of cytokines and their inhibitors in acute pancreatitis. *Gut*. 1997; 40:1–4. [PubMed: 9155566]
6. De Beaux AC, Goldie AS, Ross JA, Carter DC, Fearon KC. Serum concentrations of inflammatory mediators related to organ failure in patients with acute pancreatitis. *Br J Surg*. 1996; 83:349–353. [PubMed: 8665189]
7. Zou WG, Wang DS, Lang MF, Jin DY, Xu DH, Zheng ZC, Wu ZH, Liu XY. Human interleukin 10 gene therapy decreases the severity and mortality of lethal pancreatitis in rats. *J Surg Res*. 2002; 103:121–126. [PubMed: 11855927]
8. Chen CC, Wang SS, Lu RH, Chang FY, Lee SD. Serum interleukin 10 and interleukin 11 in patients with acute pancreatitis. *Gut*. 1999; 45:895–899. [PubMed: 10562589]
9. Gukovskaya AS, Gukovsky I, Zaninovic V, Song M, Sandoval D, Gukovsky S, Pandolfi SJ. Pancreatic acinar cells produce, release, and respond to tumor necrosis factor- α : Role in regulating cell death and pancreatitis. *J Clin Invest*. 1997; 100:1853–1862. [PubMed: 9312187]

10. Norman JG, Fink GW, Denham W, Yang J, Carter G, Sexton C, Falkner J, Gower WR, Franz MG. Tissue-specific cytokine production during experimental acute pancreatitis: A probable mechanism for distant organ dysfunction. *Dig Dis Sci.* 1997; 42:1783–1788. [PubMed: 9286248]
11. Hughes CB, Grewal HP, Gaber LW, Kotb M, El din AB, Mann L, Gaber AO. Anti-TNF α therapy improves survival and ameliorates the pathophysiologic sequelae in acute pancreatitis in the rat. *Am J Surg.* 1996; 171:274–280. [PubMed: 8619467]
12. Guice KS, Oldham KT, Remick DG, Kunkel SL, Ward PA. Anti-tumor necrosis factor antibody augments edema formation in caerulein-induced acute pancreatitis. *J Surg Res.* 1991; 51:495–499. [PubMed: 1943086]
13. Rongione AJ, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. Interleukin 10 reduces the severity of acute pancreatitis in rats. *Gastroenterology.* 1997; 112:960–967. [PubMed: 9041259]
14. Exley AR, Leese T, Holliday MP, Swann RA, Cohen J. Endotoxaemia and serum tumour necrosis factor as prognostic markers in severe acute pancreatitis. *Gut.* 1992; 33:1126–1128. [PubMed: 1398241]
15. Schmidt J, Rattner DW, Lewandrowski K, Compton CC, Mandavilli U, Knoefel WT, Warshaw AL. A better model of acute pancreatitis for evaluating therapy. *Ann Surg.* 1992; 215:44–56. [PubMed: 1731649]
16. Murakami K, Okajima K, Uchiba M, John M, Nakagaki T, Okabe H, Takatsuki K. Activated protein C prevents LPS-induced pulmonary vascular injury by inhibiting cytokine production. *Am J Physiol.* 1997; 272:L197–L202. [PubMed: 9124369]
17. Schmidt J, Lewandrowski K, Fernandez-del Castillo C, Mandavilli U, Compton CC, Warshaw AL, Rattner DW. Histopathologic correlates of serum amylase activity in acute experimental pancreatitis. *Dig Dis Sci.* 1992; 37:1426–1433. [PubMed: 1380425]
18. Trush MA, Egner PA, Kensler TW. Myeloperoxidase as a biomarker of skin irritation and inflammation. *Food Chem Toxicol.* 1994; 32:143–147. [PubMed: 8132173]
19. Schierwagen C, Bylund-Fellenius AC, Lundberg C. Improved method for quantification of tissue PMN accumulation measured by myeloperoxidase activity. *J Pharmacol Methods.* 1990; 23:179–186. [PubMed: 2158602]
20. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. *Proc Natl Acad Sci USA.* 1975; 72:3666–3670. [PubMed: 1103152]
21. Haranaka K, Carswell EA, Williamson BD, Prendergast JS, Satomi N, Old LJ. Purification, characterization, and antitumor activity of nonrecombinant mouse tumor necrosis factor. *Proc Natl Acad Sci USA.* 1986; 83:3949–3953. [PubMed: 3520561]
22. Aggarwal BB, Kohr WJ, Hass PE, Moffat B, Spencer SA, Henzel WJ, Bringman TS, Nedwin GE, Goeddel DV, Harkins RN. Human tumor necrosis factor: Production, purification, and characterization. *J Biol Chem.* 1985; 260:2345–2354. [PubMed: 3871770]
23. Raza A. Anti-TNF therapies in rheumatoid arthritis, Crohn's disease, sepsis, and myelodysplastic syndromes. *Microsc Res Tech.* 2000; 50:229–235. [PubMed: 10891888]
24. Messmann H, Vogt W, Falk W, Vogl D, Zirngibl H, Leser HG, Scholmerich J. Interleukins and their antagonists but not TNF and its receptors are released in post-ERP pancreatitis. *Eur J Gastroenterol Hepatol.* 1998; 10:611–617. [PubMed: 9855088]
25. Messmann H, Vogt W, Holstege A, Lock G, Heinisch A, von Furstenberg A, Leser HG, Zirngibl H, Scholmerich J. Post-ERP pancreatitis as a model for cytokine induced acute phase response in acute pancreatitis. *Gut.* 1997; 40:80–85. [PubMed: 9155580]
26. Dinarello CA, Gelfand JA, Wolff SM. Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. *JAMA.* 1993; 269:1829–1835. [PubMed: 8459516]
27. Osman MO, Gesser B, Mortensen JT, Matsushima K, Jensen SL, Larsen CG. Profiles of pro-inflammatory cytokines in the serum of rabbits after experimentally induced acute pancreatitis. *Cytokine.* 2002; 17:53–59. [PubMed: 11886171]
28. Folch E, Prats N, Hotter G, Lopez S, Gelpi E, Rosello-Catafau J, Closa D. P-selectin expression and Kupffer cell activation in rat acute pancreatitis. *Dig Dis Sci.* 2000; 45:1535–1544. [PubMed: 11007102]

29. Grewal HP, Mohey el Din A, Gaber L, Kotb M, Gaber AO. Amelioration of the physiologic and biochemical changes of acute pancreatitis using an anti-TNF-alpha polyclonal antibody. *Am J Surg.* 1994; 167:214–218. [PubMed: 8311136]
30. Folch E, Serrano A, Sabater L, Gelpi E, Rosello-Catafau J, Closa D. Soluble receptors released during acute pancreatitis interfere with the detection of tumor necrosis factor-alpha. *Crit Care Med.* 2001; 29:1023–1026. [PubMed: 11378616]
31. Van Kessel KP, Van Strijp JA, Verhoef J. Inactivation of recombinant human tumor necrosis factor-alpha by proteolytic enzymes released from stimulated human neutrophils. *J Immunol.* 1991; 147:3862–3868. [PubMed: 1940372]
32. Jones LJ, Singer VL. Fluorescence microplate-based assay for tumor necrosis factor activity using SYTOX Green stain. *Anal Biochem.* 2001; 293:8–15. [PubMed: 11373072]
33. Hartwig W, Jimenez RE, Werner J, Lewandrowski KB, Warshaw AL, Fernandez-del Castillo C. Interstitial trypsinogen release and its relevance to the transformation of mild into necrotizing pancreatitis in rats. *Gastroenterology.* 1999; 117:717–725. [PubMed: 10464149]
34. Fernandez-del Castillo C, Schmidt J, Rattner DW, Lewandrowski K, Compton CC, Jehanli A, Patel G, Hermon-Taylor J, Warshaw AL. Generation and possible significance of trypsinogen activation peptides in experimental acute pancreatitis in the rat. *Pancreas.* 1992; 7:263–270. [PubMed: 1375747]
35. Geokas MC, Brodrick JW, Johnson JH, Largman C. Pancreatic elastase in human serum. Determination by radioimmunoassay. *J Biol Chem.* 1977; 252:61–67. [PubMed: 833129]
36. Geokas MC, Largman C, Brodrick JW, Johnson JH, Fassett M. Immunoreactive forms of human pancreatic chymotrypsin in normal plasma. *J Biol Chem.* 1979; 254:2775–2781. [PubMed: 429317]
37. Rinderknecht, H. Pancreatic secretory enzymes. In: Vay Liang, WG.; Dimagno, EP.; Gardner, JD.; Lebenthal, E.; Reber, HA.; Scheele, GA., editors. *The Pancreas: Biology, Pathobiology, and Disease.* Raven Press; New York: 1993. p. 219-251.
38. Larvin M, Switala SF, McMahon MJ. Altered protease-antiprotease relationships in plasma during severe acute pancreatitis: a role for r1-protease inhibitor? *Br J Surg.* 1988; 75:1253.
39. Leese T, Holliday M, Heath D, Hall AW, Bell PR. Multicentre clinical trial of low volume fresh frozen plasma therapy in acute pancreatitis. *Br J Surg.* 1987; 74:907–911. [PubMed: 2444309]
40. Goodman AJ, Bird NC, Johnson AG. Antiprotease capacity in acute pancreatitis. *Br J Surg.* 1986; 73:796–798. [PubMed: 3768647]
41. Ohlsson K, Ganrot PO, Laurell CB. In vivo interaction between trypsin and some plasma proteins in relation to tolerance to intravenous infusion of trypsin in dog. *Acta Chir Scand.* 1971; 137:113–121. [PubMed: 5149486]

Fig. 1.

Mean plasma levels of TNF α in healthy controls, sham operated controls, mild and severe pancreatitis at different time points after induction of pancreatitis. Significant differences noted between sham operated controls and severe pancreatitis at 0.5 and 6 h (* p = 0.03, ** p = 0.01, Student's t test).

Fig. 2. Western Blot of rrTNF after incubation with 1 µg/ml of different enzymes at 37°C for 30 min. This picture demonstrates digestion of rrTNF by trypsin, chymotrypsin and, to a lesser extent, elastase. 1: rrTNF only, 2: rrTNF + trypsin, 3: trypsin only, 4: rrTNF + elastase, 5: elastase only, 6: rrTNF + chymotrypsin, 7: chymotrypsin only, 8: rrTNF + pepsin, 9: pepsin only.

Fig. 3. Bioactivity of TNF after incubation for 30 min at 37°C with different concentrations (0.1, 0.3, 1, 3, 10, 30 and 100 $\mu\text{g/ml}$) of trypsin, elastase, chymotrypsin and pepsin. Data presented as mean $\pm$ SEM.

Fig. 4. Bioactivity of TNF expressed as '% cell killing'. Cell lysis with Triton X equals killing of all cells. TNF was incubated with 1 μ g/ml trypsin at 37°C and bioactivity was assessed after 0.5, 1, 2, 3 and 4 h. Data presented as mean $\pm$ SEM.