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the inflammatory response in critical illness

van Westerloo, David Johannes
The inflammatory response in critical illness.
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the inflammatory response in critical illness

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the inflammatory response in critical illness

voor mijn drie lieve ouders: Lieneke, Gerard en Enno

stellingen

1. Try to stay healthy before you get sick.
   This thesis.

2. The cholinergic anti inflammatory pathway is an essential regulator of the im mune response during sepsis and pancreatitis
   This thesis.

3. The vagus nerve, and nicotinic acetylcholine receptors are both promising new therapeutic targets in the treatment of inflammatory diseases.
   This thesis.

4. PPAR-gamma ligands may be of therapeutic use in the treatment of chronic pan creatitis
   This thesis.

5. The brain boggles the mind
   James Watson

6. I shall never be ashamed of citing a bad author if the line is good.
   Seneca (and this thesis)

7. It is better, of course, to know useless things than to know nothing.
   Seneca

8. The great tragedy of Science - the slaying of a beautiful hypothesis by an ugly fact.
   Thomas Huxley

9. The best way to have a good idea, is to have lots of ideas.
   Linus Pauling.

10. Allways prefer the sultan solution.
    The sultans

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chapter 1

introduction, a preface to the thesis

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Introduction

In this thesis we describe investigations in three areas of translational research of the immune response during critical illness. This thesis consists of three parts. First, studies are presented that investigate the inflammatory response to secondary injury and infections in a previously injured critically ill host. Second, the role of the "cholinergic anti inflammatory pathway" was investigated in several animal models of critical illness. Finally, an animal model of chronic pancreatitis was used to investigate the pathogenesis of, and new treatment options for, chronic pancreatitis.

Part I: The inflammatory response to infection during critical illness

In the first part of this thesis we focus on a problem that clinicians are aware of but which has not been investigated in great detail: many patients hospitalized with a critical illness are prone to develop secondary infectious complications. Examples of this are patients with trauma, burns or pancreatitis who are at risk for secondary infection in general or patients with sterile lung injury who are at risk for the development of pneumonia in particular. In the first part of this thesis we evaluated whether the presence of a previous injury, pancreatitis or sterile lung injury, influences the inflammatory response to a secondary infection. In several studies an experimental model of acute pancreatitis is used therefore we start with a detailed description of the current concepts of pancreatitis, its severity assessment and pathogenesis (*Chapter 2*).

in *Chapter 3* a clinical study is presented that adds to the understanding of the pathophysiology of acute pancreatitis and suggests a potential new molecular target for its prophylaxis or treatment. In *Chapter 3* we evaluate whether prophylactic administration of Semapimod, a small molecule inhibiting mitogen activated protein (MAP) kinase pathways, is effective in the prevention of post ERCP pancreatitis. Since inflammatory activation is an early event during the pathophysiology of acute pancreatitis and MAP kinase pathways are critical intracellular pathways in the inflammatory response we postulated that inhibition of MAP kinase pathways lower the incidence of pancreatitis after an ERCP. For this, 260 patients undergoing an ERCP in our hospital were included in a randomized double blind trial comparing semapimod with placebo.

In *Chapter 4* we illustrate the interaction of one disease with another. In this study we evaluated whether patients with end stage renal disease requiring hemodialysis and peritoneal dialysis are at risk for the development of pancreatitis (*Chapter 4*). This study builds on a case report of two patients with end stage renal failure who developed acute pancreatitis during continuous ambulatory peritoneal dialysis which raised the question whether

patients with end stage renal failure undergoing dialysis have an increased risk for acute pancreatitis. In the remainder of the first part of this thesis we focus on the interaction of sterile inflammation with subsequent infection and investigate the immunopathology of infection in the already injured host.

Many severe infections do not develop in previously healthy patients but in patients with a pre-existing disease such as a trauma, vascular occlusive disease, pancreatitis or sterile lung injury. This is an area where animal models are underdeveloped. Indeed, most preclinical studies on pneumonia and sepsis are performed in previously healthy animals whereas animal models in which sepsis is preceded by a primary injury such as pancreatitis reflect the clinical scenario of many patients more accurately. Hence, current models fail to recognize that infectious complications frequently develop in already compromised patients. In this thesis we designed several animal models, described as “two-hit” or “two-event” models, to study the inflammatory response during infection preceded by a primary injury. We specifically chose to develop two distinct two event models, one in which infection is preceded by sterile inflammation in a different organ (pancreatitis followed by pneumonia or sepsis) and one in which infection is preceded by inflammation in the same organ (acid aspiration lung injury followed by pneumonia). Using these models we evaluated the effects of local and systemic inflammation on the inflammatory response to subsequent infection.

In cases of severe acute pancreatitis, the outcome is determined by the development of severe systemic inflammation as well as secondary infectious complications in the pancreas and in extra pancreatic organs[1]. The majority of such infections occurs in the inflamed pancreas and surrounding tissues but sepsis and nosocomial pneumonia are feared complications of this critical illness[2]. To study the interaction between pancreatitis and subsequent infection we first implemented a pancreatitis model. Of several pancreatitis models described in the literature we chose to use the cerulein hyperstimulation model since this model is most suitable for the use in mice, well defined and mimics the early events in human pancreatitis. To evaluate the effects of pre existent pancreatitis on subsequent extra pancreatic infection we designed “two hit” experiments in which pancreatitis was followed by pneumonia (C*hapter 5*) or septic peritonitis (*Chapter 6*). In *Chapter 5* pancreatitis was followed by *Pseudomonas aeruginosa* pneumonia, since this pathogen is frequently associated with nosocomial pneumonia in the intensive care setting. The effects of pancreatitis on the immune response in the lung as well as the effect of pneumonia on the course of pancreatitis were investigated. In *Chapter 6* we addressed not only the interaction between pancreatitis and septic peritonitis (caused by the injection of *Escherichia coli*), but also evaluated the role of Toll like receptor (TLR) 4 in pancreatitis and in the host response to pancreatitis and subsequent septic peritonitis. This is of interest since TLRs have been identified as crucial mediators of the innate immune response to infection. TLRs are pattern recogni-

tion receptors among which TLR4 is the signaling receptor for lipopolysaccharide (LPS), the proinflammatory constituent of the Gram-negative cell wall. TLR4 deficient macrophages are unresponsive to LPS and mice with a genetic or functional TLR4 deficiency are protected from the toxic effects of LPS[3-5]. The adequate "sensing" of LPS by the host has been implicated as an important early event in the innate immune response to Gram-negative bacteria[6]. The main objective of *Chapter 6* was to examine the innate immune response and the role of TLR4 during abdominal sepsis in the setting of pre-existing pancreatitis. For this we first studied the role of TLR4 in *E. coli* induced sepsis in previously healthy mice. Arguing that, as discussed above, in the clinical setting Gram-negative sepsis frequently is a complication of acute pancreatitis, we thereafter examined the role of TLR4 in abdominal sepsis in mice with pre-existing pancreatitis.

Since all experimental studies described above involve the interaction of a primary disease with an infection located in another organ we next studied the effects of a primary sterile inflammatory stimulus on an infection in the same body compartment. In this study, reported in *Chapter 7*, we used an animal model mimicking aspiration pneumonitis caused by the aspiration of gastric acid in the lung. Aspiration of gastric contents occurs in various hospitalized patients, in particular those with a reduced consciousness[7, 8]. The associated lung injury, commonly referred to as aspiration pneumonitis, is primarily caused by gastric acid. Aspiration pneumonitis predisposes the host to development of acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and pneumonia[9]. Although the exact underlying mechanisms for this association is unclear, it has been suggested that acid aspiration primes the lung for an enhanced inflammatory response to a subsequent challenge[10]. In *Chapter 7*, we induced pneumonia by intranasal inoculation with *Klebsiella pneumoniae*, a common nosocomial respiratory pathogen[2], in mice with or without preceding acid aspiration, and compared inflammatory responses and bacterial outgrowth in the lungs. In addition, arguing that tumor necrosis factor (TNF)-α has been implicated as an important mediator in various inflammatory lung diseases including aspiration pneumonitis, ALI/ARDS and pneumonia[11-14], we also evaluated the role of this pluripotent proinflammatory cytokine in lung inflammation during *Klebsiella* pneumonia in mice with or without preexisting aspiration pneumonitis.

Part II: The cholinergic anti inflammatory pathway as a regulator of the inflammatory response to critical illness

Recently, the vagus nerve and nicotinic acetylcholine (Ach) receptors have been identified as crucial mediators of the inflammatory response. In vitro studies have shown that immune cells are susceptible to Ach, the principle parasympathetic neurotransmitter. When macrophages are exposed to Ach these cells are effectively deactivated[15]. This Ach-induced

deactivation is characterized by a dose-dependent reduction in the release of a series of proinflammatory cytokines[15]. Ach acts through two types of receptors: muscarinic and nicotinic. In addition to the brain and "wire-innervated" peripheral structures, these Ach receptor subtypes are also expressed by immune cells[16-20]. Evidence indicates that the anti-inflammatory effects of Ach are mediated by nicotinic Ach receptors, and in particular by the α7 subunit of the nicotinic Ach receptor[20]. In vitro studies have shown that Ach and nicotine inhibit endotoxin-induced proinflammatory cytokine release by macrophages; this Ach effect can be prevented by nicotine receptor antagonists, and macrophages deficient for the α7 subunit of the nicotinic Ach receptor can not be inhibited with regard to cytokine release by Ach or nicotine[15, 20]. In vivo studies in endotoxemia and other models of inflammation have shown that macrophages are directly influenced by vagus nerve derived Ach, suggesting that the vagus nerve provides a hard wired anti inflammatory pathway called the "cholinergic anti inflammatory pathway"[16]. In these studies, electrical stimulation of the efferent vagus nerve inhibited TNF release induced by injection of endotoxin into rats and mice and prevents shock; however, electrical stimulation of the vagus nerve in mice deficient for the α7 subunit of the nicotinic Ach receptor did not result in a reduced cytokine release upon endotoxin administration[15].

A comprehensive review on neuro immune interactions and the cholinergic anti inflammatory pathway is reported in *Chapter 8*. Knowledge of the role of the anti-inflammatory cholinergic pathway during actual infection and pancreatitis in vivo is not available. Therefore, in *Chapter 9* we determined whether this anti-inflammatory pathway regulates host responses during experimental abdominal sepsis induced by intraperitoneal injection with *Escherichia coli.* We studied the host response to infection in mice in which this pathway was disrupted by vagotomy, and in animals in which the peripheral part of this pathway, nicotinic acetylcholine receptors on macrophages, was stimulated by pretreatment with nicotine. In *Chapter 10* we determined whether this anti-inflammatory pathway regulates host responses during experimental pancreatitis. We studied the inflammatory response and the severity of experimental pancreatitis in mice in which this pathway was disrupted by vagotomy. In a separate study, pancreatitis was induced in mice in which the peripheral part of this pathway, nicotinic Ach α 7 receptors on immune competent cells, was stimulated with a selective agonist, 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21) [21, 22]. In other mice the reciprocal approach was undertaken and nicotinic receptors were blocked by using mecamylamine [23]. In *Chapter 11* we report on the effects of acute vagotomy and show that timing of vagotomy in experiments is crucial to be able to interpret the results adequately. Sepsis and endotoxemia are associated with concurrent activation of inflammation and the hemostatic mechanism, which both contribute to organ dysfunction and death. Electrical vagus nerve stimulation has been found to inhibit tumor necrosis factor-α release during endotoxemia, sepsis and pancreatitis in rodents. In *Chapter 12* we investigated the effect

of electrical vagus nerve stimulation on coagulation and fibrinolysis during endotoxemia. In *Chapter 13* we investigated the capacity of the selective α7 cholinergic receptor agonist GTS-21 to inhibit LPS-induced inflammatory responses in mice during endotoxemia. In this paper we specifically investigated the mechanisms by which chemical α7 cholinergic receptor stimulation influences the inflammatory response.

Part II: Anti inflammatory strategies for the prevention of acute and the treatment of chronic pancreatitis

Chronic pancreatitis is characterized by progressive destruction of parenchymal tissue ultimately leading to exocrine and endocrine function loss. Clinical symptoms include abdominal pain, steatorrhea and diabetes mellitus. The incidence of chronic pancreatitis varies from region to region, from 7-15 per 100.000 per year, and is rising[24]. Knowledge of the pathophysiology of chronic pancreatitis is limited. Chronic pancreatitis is considered to result from chronic repetitive inflammation within the pancreas due to alcohol abuse or recurrent bouts of even minor events of pancreatic inflammation, resulting in recurrent repair of pancreatic damage and ultimately in activation of a profibrotic cascade. This hypothesis is usually referred to as the necrosis fibrosis hypothesis. Fibrosis formation in the pancreas is initiated by differentiation and activation of pancreatic stellate cells (PSC) which produce collagen as a result[25]. In this part of the thesis we report several studies investigating the pathophysiology of chronic pancreatitis. Also, we tried to identify potential new treatment targets for the treatment of chronic pancreatitis. Chronic pancreatitis is a disease which is especially hard to study since the organ is inaccessible for sampling in humans. Therefore, we used a chronic pancreatitis model with repetitive cerulein hyperstimulation since this model adheres to the necrosis fibrosis hypothesis of chronic pancreatitis pathophysiology. This model consists of 6 hourly cerulein injections (episodes of acute pancreatitis), three times a week for six weeks followed by one week of recovery.

In *Chapter 14* we investigated the therapeutic effects of troglitazone, a ligand for Peroxisome Proliferator Activated Receptor (PPAR)-γ, in this model. PPAR-γ is a member of the nuclear receptor family of transcription factors[26]. Considerable evidence indicates that PPAR-γ agonists inhibit inflammatory responses during inflammatory diseases[26-29]. Furthermore, PPAR-γ decreases TGF-β1 production and may therefore inhibit PSC activation and fibrosis formation[30, 31]. Taken together, PPAR-γ ligands may have anti-inflammatory and antifibrotic properties which both may exert a beneficial effect on the development and course of chronic pancreatitis[28, 32]. Treatment with troglitazone was given either during the whole duration of cerulein administration or in a therapeutic setting where troglitazone was added only during the last three weeks of the model.

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chapter 2

new insights in the pathophysiology and severity assessment of acute pancreatitis

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Introduction

Acute pancreatitis is an acute sterile inflammation of the pancreas. The diagnosis is made on the basis of a distinct clinical syndrome consisting of acute onset abdominal pain, radiating to the back, frequently accompanied by nausea and/or vomiting, combined with a more than threefold increase of serum amylase or lipase above the upper limit of normal. In the western world the most common causes of acute pancreatitis are alcohol abuse and gallstones[1]. The disease is characterized by the premature activation of digestive enzymes in the pancreas, followed by a massive immunological response resulting in autodigestion of the gland, local and subsequent systemic inflammation. The incidence of the disease varies between 5-20 per 100.000 persons per year and in the end, with 10-20% of patients developing severe pancreatitis of which up to 30% may die as a result of the development of secondary complications such as pancreatitis associated lung injury, infectious complications or multiple organ failure[1]. This review will focus on recent developments in the understanding of the pathophysiology and immunopathology of acute pancreatitis and its complications and discuss the importance of early severity prediction including the merits of various prognostic markers.

Pathophysiology

The pathophysiology of acute pancreatitis can be divided into several phases. An intra-acinar phase, a local inflammatory phase, a systemic inflammatory phase and, in a subset of patients, a fourth phase with the development of extra pancreatic or infectious complications. In the majority of cases the inflammatory process is contained within the pancreas itself and resolves in a matter of days. However, in up to 20% of cases, the disease takes a more serious turn and complications develop. These complications can be local (e.g., infection of pancreatic necrosis, pseudocyst-formation), or systemic (e.g., sepsis, acute respiratory distress syndrome)[1]. The development of extra pancreatic involvement is associated with a poorer prognosis.

Experimental models of acute pancreatitis

This review will focus on the pathophysiology and immunopathology of acute pancreatitis and on the lessons learned from experimental studies and their implications for staging and clinical management of the disease. Human studies of acute pancreatitis face several problems. First, patients usually present for medical care between 12 and 24 hours after onset of symptoms. At this point of the disease the intra acinar phase has already fully developed and the essential primary events can no longer be assessed. Second, the pancreas is not readily accessible for sampling, which significantly limits the possibilities to study

pathophysiological events on a (sub)cellular level. Therefore, most of the developments in the understanding of acute pancreatitis are derived from animal models. Many animal models of pancreatitis have been developed in rodents, rabbits, dogs, pigs and opossums. The most widely used animal model for edematous pancreatitis is the cerulein model (hyperstimulation), in which pancreatitis is induced in rodents by 6-12 hourly intraperitoneal injections of the Cholecystokinine (CCK) analogue cerulein. This model is characterized by the development of edema, neutrophil sequestration in the pancreas, mild necrosis and systemic inflammation as well as mild pancreatitis associated lung injury. Severe necrotising pancreatitis can be induced by the retrograde injection of taurocholate in the pancreatic duct in rats, or by the administration of a choline deficient ethionine (CDE) supplemented diet to young female mice. Finally, a so far underused human model is post endoscopic retrograde cholangio pancreatography (ERCP) pancreatitis. The risk of pancreatitis after ERCP can be as high as 15% in selected patients. Post-ERCP pancreatitis in humans is a feasible model to study the pathophysiological events and potential inhibitors in this setting especially because the initial noxious event is well-defined in time and patients can be followed during the essential first hours of the progression of the disease.

Intra-pancreatic digestive enzyme activation

The first and key event in the pathophysiology of acute pancreatitis is the premature activation of digestive enzymes in the pancreas[2] (Figure 1). Although the understanding of this phenomenon is expanding rapidly, the mechanism leading to it is still largely unknown. In the normal pancreas several protective mechanisms protect the organ from autodigestion. First, all proteolytic and lipolytic pancreatic enzymes are stored as zymogens, pro enzymes, and will become active only after activation by enzymes in the duodenum. However, within the acinary cell, lysosomal hydrolases have the capacity to activate trypsinogen and other zymogens as well. Therefore one of the most important lines of defense is the physical separation in granules of these zymogens and activating hydrolases. At present, two theories have been postulated as to the site and mechanisms of early enzyme activation in acute pancreatitis, the co-localization and the trypsinogen auto activation theory.

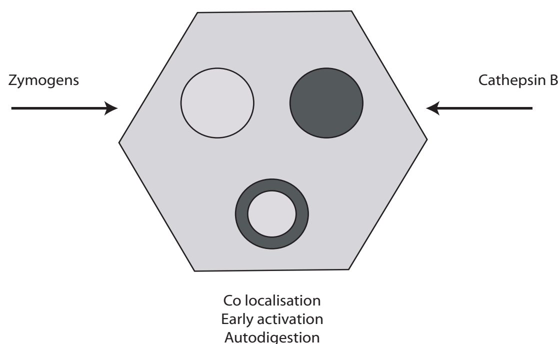

Figure 1: Co localisation. Co-localisation of zymogens and lysosomal hydrolases leads to premature enzyme activation and autodigestion of the pancreas.

Co localization theory

The leading theory is that in acute pancreatitis, this separation is lost and that zymogens and hydrolases co-localize leading to intra acinar enzyme activation[3] (figure 1). A direct proof for this so called colocalization theory is still lacking. However, studies have indicated that colocalization of lysosomal hydrolases with digestive enzyme zymogens occurs before zymogen activation in experimental pancreatitis and that the colocalization of these two types of enzymes occurs within the compartment in which zymogen activation occurs. In vitro data indicates that the lysosomal hydrolase Cathepsin B might play a pivotal role in the early activation of trypsinogen. When pancreatic acini are exposed to a supramaximally stimulating dose of cerulein the activation of trypsinogen is prevented by inhibitors of the lysosomal hydrolase cathepsin B(CTSB)[4]. Recent in vivo evidence indicates that a redistribution of lysosomal cathepsin B into a zymogen-containing subcellular compartment triggers trypsinogen activation[5]. In this study CTSB knockout mice were, protected from cerulein induced acute pancreatitis. In another study inhibitors of cathepsin B prevented the activation of trypsinogen and dramatically reduced the severity of experimental pancreatitis in two different animal models[6]. Therefore, there is growing evidence that cathepsin B might play a pivotal role in the intrapancreatic activation of trypsinogen in vivo and pharmacological inhibition of cathepsin B might be a valuable option to prevent pancreatitis or reduce its severity. However, more evidence from human studies is needed to confirm that colocalization actually occurs in humans and that it is not applicable to rodent pancreatitis alone.

Trypsinogen auto activation theory

One of the most interesting aspects of trypsinogen is that it is capable of auto activating as well as inhibiting itself. Under normal circumstances only a fraction of human trypsinogen activates to trypsin. However, in the presence of secretory blockade combined with an enhanced sensitivity to trypsinogen activation this process may become uncontrolled and lead to autodigestion of the gland. Two lines of defense regulate the auto activation properties of trypsinogen. The first failsafe mechanism to eliminate prematurely activated trypsinogen is depended on the action of a small peptide called pancreatic secretory trypsin inhibitor (PSTI) also referred to as serine protease inhibitor Kazal type 1 (SPINK1)[7]. SPINK1 inhibits trypsin by blocking its active site. Due to its limited availability (ratio to trypsin 1:5), SPINK1 is only capable of inhibiting 20% of all potential trypsin activity. However, SPINK1 is an important defense mechanism against prematurely activated trypsinogen. In recent years it has become clear that the frequency of SPINK1 mutations (N34S, P55S) in patients with idiopathic chronic pancreatitis is increased up to 25%.[8] However, since SPINK1 mutations are common in the general population (2%) there is no clear-cut direct causal relation between the presence of a SPINK1 mutation and chronic pancreatitis; in fact there are far more ‘healthy’ carriers than chronic pancreatitis patients with a SPINK1 mu-

tation. SPINK1 mutations are therefore considered disease modifying genes. A second line of defense is formed by trypsin itself. To keep its own activity in check trypsin hydrolyzes the chain connecting the two globular domains of the trypsin molecule at position R122H. Mutations of the gene encoding for this R122H domain result in an inactivation resistant trypsin. Basically, both mutations in the SPINK gene and in the gene encoding for the R122H probably lower the threshold for the development of acute and chronic pancreatitis. So this theory postulates that, whereas in the normal pancreas low levels of trypsinogen are activated constantly activated to trypsin, under influence of environmental and genetic factors the normal protective mechanisms are bypassed and pancreatitis may develop.

Pancreatic inflammation

As a result of intrapancreatic enzyme activation an auto digestive process is initiated which is followed by a massive inflammatory response (Figure 2 and 3).This second, inflammatory phase in the pathophysiology of acute pancreatitis is characterized by the involvement of several soluble inflammatory mediators, cytokines and chemokines[9]. All of these mediators are involved in the inflammatory cascade subsequent to acinar cell damage but do not play a causal role in the disease. To illustrate this, treatment of isolated acinar cells directly with proinflammatory cytokines, such as interleukin-1 beta (IL-1β) or tumor necrosis factor alfa (TNF-α) does not result in co-localization of zymogen granules and lysosomes or the activation or release of enzymes. Moreover, perfusion of the isolated human pancreas with IL-1β and TNF-α does not induce acute pancreatitis[9].

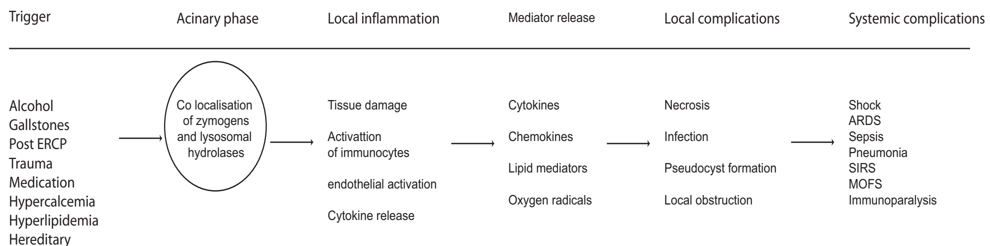

Figure 2: Phases in acute pancreatitis pathofysiology

Cytokines, including TNF-α, are primarily produced by immune competent cells but by pancreatic acinar cells as well[10]. Many experimental anti-cytokine therapies have been administered following induction of experimental pancreatitis, and some have proved to be effective. Usually, the cytokine network is discussed as consisting of proinflammatory cytokines, anti-inflammatory cytokines and soluble inhibitors of proinflammatory cytokines. At the end of this section we will review the mechanisms by which the cytokine network is activated during the first phase of the inflammatory response and the role of these mediators in acute pancreatitis as well as the involvement of neutrophils in pancreatitis.

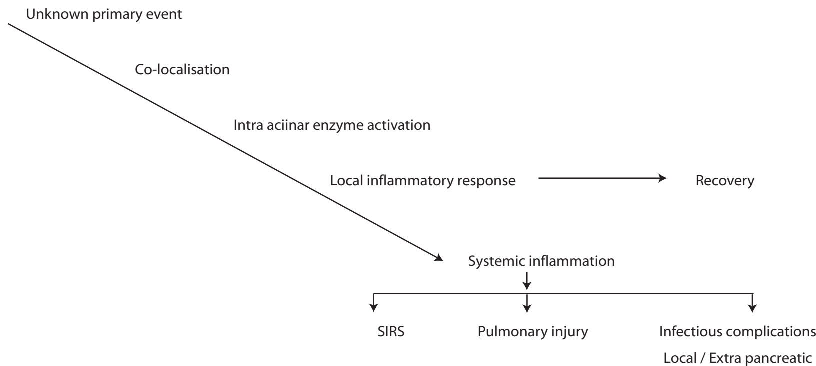

Figure 3: Schematic overview of disease progression in acute pancreatitis

Transcription factors

Experimental evidence indicates that the inflammatory process is initiated by the early activation of the transcription factor nuclear factor κ-B (NF-κB). NF-κB is a transcription factor that, upon degradation and activation, translocates to the nucleus where it activates a plethora of stress related genes such as genes encoding cytokines, chemokines, growth hormones, adhesion molecules and acute phase proteins. To illustrate the importance of NF-κB, neutralization of NF-κB, either by the antioxidant N-acetyl cysteine (NAC) or NF-κB antibody ameliorates experimental CCK induced pancreatitis[11, 12]. Furthermore, NF-κB levels are highest in areas most damaged by pancreatitis and the activation of NF-κB alone induces an inflammatory response in the pancreas[13]. In a recent study, NF-κB was directly activated within the pancreas using adenoviral-mediated transfer of an active subunit, RelA/p65 (Adp65), delivered by intraductal injection[13]. This led to the activation of NF-κB, the expression of NF-κB target genes, and an inflammatory response causing widespread damage to pancreatic acinar cells. The activation and degradation of NF-κB is in turn mediated by several intracellular messenger pathways such as the mitogen activated protein-kinase (MAPK) pathway. Inhibition of this specific pathway, for example by administration of the small molecule CNI-1493, a synthetic guanylhydrazone which inhibits the phosphorylation of p38 MAP kinase, attenuated the production of L-1β and TNF-α mRNA in pancreatic as well as pulmonary tissue associated with decreased severity of pancreatitis and pancreatitis associated lung injury[14, 15].

Pro inflammatory cytokines and platelet activating factor

From animal models, as well as clinical studies, we know that IL-1β, IL-6, TNF-α and the lipid mediator platelet activating factor (PAF) all play an important pro inflammatory role in the disease[16-19].

TNF-α

TNF-α is released in response to a variety of stimuli by monocytes and macrophages. Injection of high doses of TNF-α into experimental animals causes a syndrome that is indistinguishable from septic shock. TNF-α is one of the most important cytokines in the pathogenesis of sepsis and is associated with a plethora of inflammatory diseases, such as rheumatoid arthritis and Crohn's disease[20]. TNF-α is a pivotal and major component of the inflammatory cascade and induces the upregulation of various other cytokines such as IL-1β and IL-6. TNF-α has been detected in high concentrations in serum of patients with acute pancreatitis, however, its release is very phasic and TNF-α is rapidly degraded by the liver. A better alternative to obtain insight in the TNF-α load is to measure soluble TNF receptors (sTNFR), which persist in the circulation and can be detected in the plasma of patients with acute pancreatitis[21]. Increased levels of circulating sTNFR predicted organ failure in patients with pancreatitis even when TNF levels were not detectable[18]. In animal models, anti-TNF-α antibody, as well as inhibition of TNF-α release ameliorate acute pancreatitis[22]. Timing of anti-TNF therapy seems to be essential. In a study which used the CDE model blockade of TNF by the administration of a soluble TNF receptor attenuated the severity of pancreatitis, decreased levels of inflammatory cytokines, and improved survival. Delaying antagonism until pancreatitis was manifest and circulating cytokines were elevated but not yet maximal was more protective than prophylactic TNF antagonism (survival benefit was 20% in the prophylactic and 40% in the delayed therapeutic group)[16].

IL-1β

IL-1β is another potent pro-inflammatory cytokine which is derived predominantly from macrophages. IL-1β activates neutrophils, upregulates adhesion molecules and induces a shock like state in animals when injected at high doses. The antagonist for IL-1β, IL-1 receptor antagonist (IL-1ra) is also produced within the pancreas during acute pancreatitis and levels of IL-1β and IL-1ra correlate with the severity of acute pancreatitis[23]. Administration of recombinant IL-1ra, as well as targeted disruption of the IL-1 receptor gene (IL-1 receptor knock out mice) decreased the severity of cerulein induced pancreatitis[24]. If in the same model interleukin-1 converting enzyme (ICE) (which cleaves pro-IL-1β to IL-1β) was inhibited the extent of acinar necrosis and parenchymal destruction was significantly reduced[25].

IL-6

IL-6 is an important cytokine that is upregulated in a large variety of inflammatory conditions, such as sepsis, burns, infections, surgery and pancreatitis[26]. IL-6 is produced by a wide range of cells in response to stimulation by endotoxin, IL-1β, and TNF-α[27]. A major role of IL-6 is the stimulation of the synthesis of acute phase proteins, such as C-Reactive

Protein (CRP). IL-6 levels are raised in patients with acute pancreatitis and correlate with disease severity[28].

PAF

PAF has multiple actions of which platelet activation is the least important in the context of acute pancreatitis. More important is the ability of PAF to activate neutrophils, first locally in the pancreas and, after leakage from the pancreas to the circulation, systemically. Furthermore, PAF enhances the permeability of the capillary endothelium, thus promoting tissue edema and endothelial leakage. Therefore, antagonism of PAF has long been regarded as a potential treatment option in acute pancreatitis. Indeed, in animal models of pancreatitis, PAF ameliorated local pancreatic damage as well as pancreatitis associated systemic inflammation, as judged by lower serum cytokine levels (TNF, IL-1, IL-6), and less lung injury[29]. Unfortunately, after initial hopeful data, a recent large multi center trial evaluating the effect of lexiplafant, a potent PAF antagonist, has been stopped due to lack of clinical efficacy[30, 31].

Anti inflammatory cytokines

IL-10

IL-10 is a prominent anti-inflammatory cytokine. Plasma levels are elevated in animal models of endotoxemia and inhibits the release of pro-inflammatory cytokines (i.e. IL-1β, IL-6, and TNF-α) from monocytes/macrophages[32]. In acute pancreatitis, levels are markedly raised within the first 24 h of an attack followed by a steady decline over the following days. Experimental evidence shows that IL-10 plays a very important anti inflammatory role in pancreatitis. In IL-10 knockout mice pancreatitis is more severe and after the administration of recombinant IL-10 or in IL-10 transgenic mice local and systemic inflammation is largely ameliorated[33]. With regard to human pancreatitis, there is data that suggests that prophylactic administration of IL-10 reduces the incidence of post ERCP pancreatitis[34].

Chemokines and neutrophils

Finally, the overwhelming production of chemokines and cytokines leads to activation and influx of neutrophils. Acute pancreatitis is characterized by an strong influx of neutrophils. This is of pathogenetic significance since neutrophil depletion ameliorates the severity of experimental acute pancreatitis[35]. Leukocyte migration is a multi step process involving neutrophil rolling, firm adhesion and transmigration through the endothelial layer (reviewed in:[36]). The attraction of leukocytes, their adhesion to the endothelial wall and extravasation into tissues is essential for inflammation and the host response to inflammation. One of the most important adhesion molecules involved in rolling and firm adhesion is intercellular adhesion molecule-1 (ICAM-1). Pancreatitis severity and associated lung injury is reduced

in mice deficient in ICAM-1 by approximately 50%, an extent which is comparable to that seen in mice depleted of neutrophils. Combining ICAM-1 deficiency and neutrophil depletion does not provide additional protection[37]. Chemokines, a group of small chemotactic proteins, provide the signals that convert the low-affinity, selectin-mediated interaction of leukocyte adhesion into the higher-affinity, integrin-mediated interaction that leads to extravasation of leukocytes. Chemokines can be produced by virtually all cells given the appropriate stimulus and therefore the dramatic increase in the secretion of chemokines during inflammation results in the selective recruitment of leukocytes into inflamed tissue. CXC chemokines are a subset of chemokines that can be further divided in two different groups on the basis of the presence or absence of a 3 amino acid motif termed “ELR”. ELR-positive CXC chemokines are chemotactic for neutrophils and include, among others, IL-8, epithelial neutrophil-activating protein 78 (ENA-78) and growth-related oncogene-alpha (GRO-α) in humans and KC and macrophage inflammatory protein (MIP)-2, in mice. IL-8, ENA-78 and GRO-α, are detected in high concentrations in the blood of patients during an attack of acute pancreatitis and are prognostic for clinical outcome[38]. Anti-IL-8 treatment decreases the severity of acute pancreatitis in animal models[39]. Recently, Gukovskaya et al have shown that neutrophils do not only play a role in the immunological response to tissue damage but are themselves capable of activating digestive enzymes in the pancreas[40]. This is very interesting since it indicates that neutrophil migration to the pancreas might lead to a positive feedback loop which sustains the continuous activation of pancreatic enzymes, pancreatic damage and subsequent inflammation. Whereas neutrophils may be needed to clear cellular debris and provide a first line of protection against infection of pancreatic necrosis they may in fact do more harm than good. Therefore, inhibition of neutrophil influx, either by interfering with the transmigration process or by inhibition of CXC chemokines, might be a valuable therapeutic approach in the treatment of acute pancreatitis. This is confirmed by data obtained in various animal models, e.g. hyperstimulation pancreatitis in ICAM knock out mice and rabbits treated with anti IL-8, in which inhibition of neutrophil migration is associated with a better outcome[41, 42].

Systemic inflammation

In most patients, the inflammatory process is self-controlled; the inflammation subsides with pancreatic rest and supportive measures. However, in a subset of patients the inflammatory process is not controlled. As a result of the release of activated pancreatic enzymes, cytokines, chemokines and other mediators, and migration of activated neutrophils and monocytes from the pancreas, a systemic inflammatory syndrome (SIRS) may develop. All of these mediators and activated cells trigger a generalized capillary endothelial leakage leading to hypovolemia, hypotension and fluid sequestration in the lungs. The most frequently affected extrapancreatic organ is the lung, in which pancreatitis may lead to pancreatitis

associated lung injury, a syndrome quite indistinguishable from the acute respiratory distress syndrome (ARDS)[43]. Moreover, hypovolemia and hypoxemia lead to reduced oxygen delivery to vital organs such as the kidney (renal failure) and the gut (intestinal ischemia). Intestinal ischemia is especially important since it reduces the mucosal barrier function of the gut against translocation of bacteria making the host very susceptible to subsequent infectious complications. Ultimately pancreatitis associated SIRS may lead to multiorgan failure (MOF) and death.

Infectious complications

The most prevalent and serious complications of pancreatitis are, however, infectious in nature[44]. Infectious complications, primarily bacterial contamination of pancreatic necrosis, peripancreatic fat tissue or abcess formation in the pancreas but also extrapancreatic such as nosocomial pneumonia, are important contributors to pancreatitis related morbidity[44, 45]. Pancreatic infections develop in 40-70% of patients with necrotising pancreatitis with a mortality rate up to 50%[44]. The vast majority of these infections are caused by Gram negative organisms derived from the gut[44]. Pancreatic infection is caused by bacterial translocation from the gut lumen to the blood stream and secondary to the pancreas. Three principle causes have been implicated in this process: reduced motility with disruption of intestinal flora, mucosal barrier damage caused by hypoperfusion and immune system failure[46]. Many studies have been undertaken to evaluate the effect of prophylactic broad spectrum antibiotics or selective gut decontamination in pancreatitis patients at high risk for infection[47]. The outcome of most of these studies indicates that this may in fact be beneficial, although there is some concern and evidence for secondary complications such as fungal superinfections. However, the problem is to identify which patients are at high risk for infectious complications and might benefit the most from prophylactic antibiotics. The diagnostic dillema is to distinguish between sterile and infected pancreatic necrosis. Diagnosis of pancreatic infection can be made by the clinical picture, rising CRP levels and high Ranson scores (>4), but more reliably by CT scan and CT guided fine needle aspiration (FNA) and culture[48]. If pancreatic infection is proven surgery is indicated because further conservative treatment will lead to almost 100% mortality in patients with signs of sepsis. Therefore, the treatment is primarily surgical. Surgical methods for the treatment of necrosis and infection vary and the best method has yet to be identified; yet even in the most experienced hands the mortality is still between 15 and 80%[48, 49].

It has already been mentioned that decreased immune function enhances the risk of bacterial translocation and pancreatic infection. Recent studies have further suggested that in severely ill patients antibacterial host defense is significantly impaired[50]. This state is called immunoparalysis and patients with this syndrome are far more susceptible to infections. Prelimininary data from our group show that concurrent pancreatitis renders mice more

susceptible to pneumonia caused by Pseudomonas aeruginosa, a frequently encountered bacterial pathogen in nosocomial pneumonia, which in turn aggravates the severity of pancreatitis[51]. The same phenomenon can be seen in mice with concurrent pancreatitis and abdominal sepsis[52]. Therefore in the case of pancreatitis and secondary infectious complications there might be evidence for a pathological vicious circle in which bacterial host defense is impaired and further inflammation due to infection leads to enhancement of the severity of local damage in the pancreas.

Identification of high risk patients

One of the most important clinical issues in acute pancreatitis is to find prognostic markers that identify patients who will develop mild self limiting disease and patients who will progress to a more complicated outcome[53]. Finding a reliable marker would have major implications for the medical management of pancreatitis patients. Patients evaluated as being at high risk should then receive intensive observation and care, and possibly in the future experimental immune modifying drugs as well as prophylactic antibiotics.

Clinical scoring systems

Initial clinical assessment can be used to grade pancreatitis severity. Upon presentation in the emergency room the history should include a duration of symptoms, considering that an onset of symptoms shorter than 12 hours before seeking medical care is a prognostic indicator for an adverse outcome. Another fast prognostic indicator is calculation of the body mass index (BMI). Patients with a BMI over 30 kg/m2 have a mortality of 35%, as compared to 5% in patients with a lower BMI[54]. However, the overall prognostic power of clinical assessment is very limited. Several studies have shown that only in very specialized centers clinical assessment has a sensitivity to predict severe disease of more than 60% and a specificity of 80%, whereas the initial clinical assessment of inexperienced physicians only has a sensitivity of 40% and a specificity of 60%[55, 56]. To overcome these issues, several specific clinical scoring systems have been developed. All of these scoring systems have their merits but also their draw backs. The best known and the most widely used prognostic scoring system is the Ranson score. Ranson's original paper, published in 1974, described an 11 point scoring system that predicts severe pancreatitis if 3 points are present[57]. However, a major drawback of this system is that it cannot appropriately be used in patients with biliary pancreatitis. To overcome this a modified Ranson score has been developed (Table 1). However, the problem with the Ranson score remains which system should be used in an individual patient, making the results of the score difficult to interpret in large trials. The Ranson derived 8 point Glasgow score is more appropriate for the use in individual patients since it is not cause dependent, easy to perform and equally good as the Ranson score (Table 2). The major drawback of the Ranson and Glasgow scoring systems is that a delay of 48 hours is necessary to make a good assessment. Overall, the Ranson and

Glasgow scores have a sensitivity of 70% and 55 % and a specificity 67% and 91% respectively for severity assessment.

On admission		After 48 hours	
Age	>70	Hematocrit fall	>10%
WBC count	>18 x 10^9/l	Calcium	<2 mmol/l
Blood glucose	> 12 mmol/l	Base deficit	>5 meg/l
LDH	> 400 IU/l	BUN increase	>0.5 mmol/l
AST	>240 IU/l	Fluid sequestration	>6 litres
		Arterial pO2	<60 mm Hg

Table 1: Modified Ranson's criteria for the assessment of acute pancreatitis severity. A severe attack is predicted by the presence of three or more positive factors in patients with gallstone pancreatitis.

Contrast enhanced CT scan

Contrast CT has been utilized to assess severity in acute pancreatitis. Most studies have used the score originally described by Balthazar, modified for contrast enhanced CT scanning (Table 3). Unfortunately, it takes three to five days before necrosis can be appreciated on a CT scan and before that time CT scans have a very low sensitivity and specificity in predicting severe pancreatitis[58]. Actually, the value of contrast enhanced CT scan is quite comparable to that of the clinical scoring systems. We believe that the value of CT scans as a prognostic indicator is limited, although CT scans do help in patients who have progressed to severe disease and especially in those suspected of pancreatic infection.

Serum markers

New serum markers of disease severity have recently emerged and their potential for providing additional information on the severity of the disease is currently being evaluated.

CRP

The most readily available serum marker is CRP, widely available, cheap and currently underused in the clinical setting. CRP levels over 120 mg/l detected 95% of necrotising pancreatitis cases confirmed by laparotomy in a clinical study. Unfortunately, the delay of this test is at least 24-48 hours, and therefore it is more comprehensive to use it to monitor disease progression than for providing early prognostic indication.

IL-6

As mentioned before IL-6 is one of the most potent inducers of the acute phase response. Since CRP is a quite reliable indicator of severity and CRP is induced by IL-6 it is conceivable that IL-6 might be a reliable and earlier indicator of severity. Indeed, IL-6 is an excel-

lent indicator of severity, as good as CRP but with a delay that is approximately 24 hours shorter. However, the test is expensive and the lack of an automated assay has kept its use from the clinic so far.

TAP

Recently a new test has been introduced, urinary trypsinogen activation peptide[59]. The hypothesis behind the test is that necrotising pancreatitis is associated with a massive activation of pancreatic zymogens such as trypsinogen whereas edematous pancreatitis is associated with a much less severe zymogen activation. To quantify zymogen activation, and thereby early pancreatitis severity, Trypsinogen Activation Peptide (TAP) is measured. TAP is a small peptide which is released in equimolar concentrations during the activation of trypsinogen to trypsin. TAP is inert and is excreted in the urine within two hours. TAP levels in the urine over 2 nmol/l on admission predicts a severe attack with a sensitivity of 85% and a specificity of 90%[59]. The measurement of urinary TAP concentrations represents the best means of early and rapid (the test takes 4 hours) prediction of pancreatitis severity. However it is only used in highly specialized centers as of now, because the test is relatively unknown and highly expensive although currently commercially available (Biotrin, Ireland).

Conclusion

The key event in the pathophysiology of acute pancreatitis is the co-localization of digestive zymogens with activating hydrolases within the acinar cell. The mechanism leading to this co-localization is as of yet unidentified. The result is acinar cell damage which is followed by a primary local activation of acinar and immune competent cells, leading to activation of intracellular stress pathways, cytokine and chemokine production and the migration of neutrophils to the inflamed pancreas. Experimental studies have indicated that inhibition of several of these processes leads to diminished inflammation and a better outcome. However, whether these promising results can be duplicated in human disease remains to be evaluated.

An important subset of patients will develop extrapancreatic complications. In this group of patients morbidity and mortality is high due to serious systemic inflammation and the development of infectious complications. In such cases, administration of prophylactic antibiotics or selective gut decontamination seems beneficial.

Risk assessment for a stratified patient management by means of prognostic testing should be performed in every patient. Current clinical guidelines do advise to use a clinical (Ranson, Glasgow), a radiological (contrast CT, in selected patients) and a serum indicator (CRP, urinary TAP) of severity in all patients with acute pancreatitis. Patients identified as high

risk should receive intensive clinical observation and care, maximal supportive treatment, experimental drugs if feasible, or prophylactic antibiotics selective gut decontamination. At present, different immunomodulatory interventions are being evaluated and it is conceivable that such treatment will become standard in the care of patients suspected of developing severe pancreatitis.

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chapter 3

prophylaxis of post-ERCP pancreatitis: a randomized, placebo controlled trial using intravenous infusion of semapimod, a mitogen activated protein kinases inhibitor

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Introduction

Endoscopic Retrograde Cholangio-Pancreatography (ERCP) is a commonly performed procedure for the management of a wide range of biliary and pancreatic disorders. An asymptomatic increase in serum pancreatic enzyme levels without clinical features of pancreatitis is observed after ERCP in up to 70% of the cases [1]. Moreover, ERCP can induce acute pancreatitis, which may cause significant morbidity, prolonged hospital stay and even death[2]. The estimated incidence of ERCP-induced pancreatitis is 7%[3]. The incidence varies greatly with the indication for the procedure and is <5% in case of a small bile duct stone, 15% for therapeutic procedures involving the pancreatic duct, and >20% after sphincter of Oddi manometry [4].

The pathophysiology of post ERCP pancreatitis is incompletely understood. Several factors may act independently or together to induce post ERCP pancreatitis, such as: mechanical injury, hydrostatic injury, chemical injury, enzymatic (intestinal) injury, infection, and thermal injury [5-8]. Although most cases of clinically overt post ERCP acute pancreatitis are mild (40%) or moderate (50%) in severity, significant morbidity and prolonged hospital stay are generally the rule [9, 10]. In recent years prophylactic administration of a wide range of agents such as gabexate messilate, prednisone, nifedipine, allopurinol, Interleukin (IL) 10, somatostatin and low molecular weight heparin has been tried to lower the incidence of post ERCP pancreatitis[11-17]. Although some of these agents showed promising results (such as IL 10, diclofenac, allopurinol and gabexate messilate), these are either yet unconfirmed or subsequent studies have provided conflicting results. Therefore, considerable controversy remains about the usefulness of the administration of preprocedural drugs for the prevention post-ERCP pancreatitis.

Although the exact pathophysiology of post ERCP pancreatitis is not fully understood, it is believed that local activation of the inflammatory response is essential in its development. In this study we evaluated the effect of a single preprocedural intravenous injection of semapimod, a novel anti inflammatory drug, on the incidence of post ERCP pancreatitis. Semapimod is a synthetic guanylhydrazone which inhibits the phosphorylation of p38 Mitogen Activated Protein kinase (MAPK) as well as Jun N-terminal kinase (JNK) and MEK. Recently c-RAF was identified as the direct molecular target of Semapimod[18]

Methods

Patients

A prospective double blinded placebo controlled randomized clinical trial was conducted in a single center. From 2001 to 2005 266 consecutive patients who underwent a therapeutic

ERCP in our hospital were included in the study. Exclusion criteria were 1) age under 18 years, 2) pregnancy, 3) severe abdominal pain prior to ERCP (as determined by a visual analogue abdominal pain scale (VAS, 0-100, score > 50), 4) known kidney failure (defined as serum creatinin >180 µmol/l, 5) stent exchange for malignant disease, 6) previous sphincterotomy, 7) refusal to participate, 8) mental disability. Upon arrival at our department the patient's charts and laboratory data were reviewed for possible enrolment and a pre-ERCP VAS score was obtained. Eligible patients were informed of the study and asked to participate. After consent was granted, blood was drawn. One hour prior to ERCP patients received a single IV infusion of semapimod (50 milligrams dissolved in 100 cc of 5% glucose) or placebo (100 cc of 5% glucose)[19]. The study was reviewed and approved by the medical ethics committee of the Academic Medical Center in Amsterdam.

Randomization

Upon study entry patients were assigned the next available randomization number in sequence. The study drug administered (placebo or semapimod) corresponded to the assigned randomization number, following prior random allocation in fixed blocks of size 6. The randomization scheme was developed and blinded labels applied by Eminent Services Corporation, Frederick, MD, USA. Concealed vials, showing the randomization number only, were kept in the pharmacy and delivered one hour before each ERCP. None of the participants of the study was aware of the randomization scheme and of the codes of the medications received (placebo vs. semapimod). Randomization codes were revealed after the end of the study and the closure of the database.

ERCP procedure

ERCP's were carried out by gastroenterology fellows under constant supervision of an experienced endoscopist who each had a life-time experience of at least 750 ERCP procedures. ERCP was performed with a standard therapeutic duodenoscope (Olympus, Tokyo, Japan). Local anaesthesia was applied to the pharynx by means of lidocaine spray 10% (AstraZeneca, London, United Kingdom). Procedures were performed under conscious sedation induced via intravenous administration of 5 to 10 mg midazolam (Roche, Basel, Switserland) and 50 to 100 microgram fentanyl (Hameln pharmaceuticals, Hameln, Germany). Vital signs were monitored throughout the procedure. Telebrix 30 M (Guerbet, Roissy CdG Cedex, France) was used as contrast agent and injected manually under fluoroscopic guidance. During ERCP various data regarding the procedure were noted and logged in the study database including performance of a (precut)sphincterotomy, number of contrast injections and CBD/PD cannulation , procedure difficulty (graded on a 0-10 scale by the endoscopist) and duration. Patients coming from other hospitals were observed for another 2-4 hours after ERCP and subsequently transferred to the referring hospital; patients coming from our own department were either observed at our hospital wards or, in some cases, sent

home after several hours of observation.

Follow up

Follow up data and blood specimens were actively obtained from referring hospitals. Patients discharged to their home were asked to report to the nearest lab for blood sampling every day during the next two days. In the first 48 hours after ERCP three blood samples were taken from each patient for the estimation of plasma amylase, lipase and plasma cytokines. Also, during these days patients were asked to complete a VAS pain score for abdominal pain. Forms were returned by regular mail and patients were contacted by telephone if these forms had not been received during the first week after the procedure. Moreover, one month after the procedure all patients, as well as their physicians, were contacted by telephone and follow up data concerning overall outcome, occurrence of adverse events and duration of hospital stay were collected.

Assays

Blood samples were collected in EDTA coated vacutainer tubes pre ERCP as well as 4, 24 and 48 hours thereafter. Samples were immediately spun; plasma was removed and stored at -20^0 Celsius until analysis. Blood samples collected in outside hospitals were shipped to the Academic Medical Center in frozen condition. All assays were performed in the same laboratory in large batches. Amylase (normal range 40-220 U/ml), Lipase (normal range 20-60 U/ml) were determined with a commercially available kit (Sigma, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Exclusion from analysis

Patients who participated in the study were excluded from analysis when the study drug (placebo or semapimod) was not injected. Also, patients were excluded from analysis if ERCP did not result in cannulation of the papilla or when the amount of follow up data that was missing precluded the assessment of the primary endpoint (non-informative drop-out)

Primary and secondary outcomes

Post ERCP pancreatitis was the primary endpoint of this study and was defined by a more than threefold increase above the upper limit of normal in plasma amylase (>660 U/ml) or lipase (>180 U/ml), combined with an increase in VAS abdominal pain score relative to pre ERCP of at least 30 points (on a 1-100 scale) lasting for at least 24 hours. Post ERCP hyperamylasemia, the secondary endpoint, was defined as a plasma amylase level more than twofold above the upper limit of normal at 24 and/or 48 hours after ERCP[20]. Furthermore, kinetics of amylase levels were compared between the two groups during the first 48 hours after ERCP as well. Severity of post ERCP pancreatitis was defined as follows:

1) mild: symptoms persisting < 4 days combined with a need for intervention or hospitalization for <4 days combined with a normal appearance of the pancreas on CT/US if performed,
2) moderate, symptoms persisting 4-10 days combined with a need for intervention or hospitalization for 4-10 days
3) severe, need for intervention or hospitalization > 10 days, or ICU admission (in part) attributable to acute pancreatitis or death.

Sample size and power calculation

The sample size for the study was based on a preliminary estimate that a two group χ^2 test with a 0.05 one-sided significance level will have 82% power to detect a clinically relevant decrease in proportions of patients with post-ERCP pancreatitis from 0.15 in the placebo group to 0.05 in the semapimod group (odds ratio of 0.298). The calculated sample size per group was 120.

Statistical analysis

After the exclusion of patients following randomization the categorical demographic characteristics and medical history data in both groups at baseline were analyzed using a two-tailed Pearson χ^2 test or, in case of low frequencies, the Fisher's exact test. Continuous baseline data were analyzed using a two-tailed Student's T test or a two-tailed Mann-Whitney U-test in case of deviations from normality. Likewise, differences in ERCP findings and procedures between the groups were analyzed using the two-tailed Pearson χ^2 test, the Fisher's exact test, Student's T test or Mann-Whitney U-test, whichever appropriate. One-tailed Pearson χ^2 tests were performed to test for reduced incidences of pancreatitis and hyperamylasemia following semapimod injection compared with placebo injection. Two-tailed repeated measures analyses based on linear mixed modeling were performed to study the differential impact (over time) of semapimod and placebo injections on amylase and lipase during the first 24 hours following ERCP. In addition, two-tailed Student's T tests or Mann-Whitney U tests were performed to study whether the groups (still) differed by amylase, lipase at 48 hours after ERCP. In all instances, a p-value below 0.05 indicated statistical significance. Statistical analyses were performed with SPSS 12.0.2 for Windows and Compare2, version 1.25 [Abramson JH. Compare2 manual, revised edition. June 2004].

Results

In total, 266 patients were included in the study. ERCP failed in 14 patients, 5 additional patients were excluded from analysis for various reasons, while follow-up was incomplete for another 5 patients (Table 1). Data for 242 patients were available for further analysis. The exclusion of the 24 patients did not influence the comparability of the placebo and semapimod groups at baseline with respect to age, gender, previous ERCP history and indications

for ERCP (Tables 2 and 3). Moreover, findings at ERCP as well as procedures performed during ERCP were comparable between the two groups (Table 4).

Total number of inclusions	**266**
Exclusions	**19**
Incomplete ERCP (e.g. papilla not reached because of BII stomach resection, duodenal stenosis)	14
Patient refused infusion after having signed informed consent	2
Infusion not performed	2
Repeat ERCP < 48 hours	1
Incomplete follow up	5
Non excluded patients	**242**

Table 1. Exclusions . Included patients received placebo or Semapimod intravenously one hour pre ERCP. Twenty four patients were excluded from analysis because of failure of ERCP, failure of infusion or incomplete follow up. Reasons for exclusion of analysis are shown.

	Placebo	**Semapimod**	**test statistic**	**p value**
Number of patients	121	121		
Male/Female	51/70	62/59	$\chi2 = 2.01$	0.156
Mean age (sd)	61.7 (15.3)	63.3 (14.7)	t = -0.78	0.435
Age > 60	78	88	$\chi2 = 1.92$	0.166
Caucasian	113	112	$\chi2 = 0.06$	0.801

Table 2. Patients' demographic data. One hour pre ERCP patients received placebo (N=121) or Semapimod (N=121) intravenously. Demographic data of included patients in both groups are shown.

	Placebo	Semapimod	test statistic	p value
Previous ERCP's				
Patients with prior ERCP's (N)	42	53	$\chi2 = 2.10$	0.148
Previous ERCP's (mean ± SD)	0.45 ± 0.7	0.64 ± 0.9	Z = -1.57	0.116
Patients with previous attack of acute pancreatitis (N)	3	1		0.622
ERCP indications			7.0	0.756
Suspected CBD stones	18			
Removal of CBD stones	37			
Suspected pancreatic malignancy	30			
Biliary dilatation	8			
Recurrent pancreatitis	6			
Post cholecystectomy lesion	6			
Suspected cholangiocarcinoma	5			
Active cholangitis	1			
Suspected SOD	0			
Primary sclerosing cholangitis	1			
Other	9			

Table 3. Previous ERCP history and indications for ERCP. One hour pre ERCP patients received placebo (N=121) or Semapimod (N=121) intravenously. Previous ERCP data and indications for ERCP of included patients in both groups are shown.

Overall, post ERCP pancreatitis developed in 29 patients (12%). In the placebo group pancreatitis was observed in 18 patients whereas only 11 patients from the semapimod group developed pancreatitis (Table 5). However, this 40% difference did not reach statistical significance (14.9 vs 9.1%; p = 0.117)). No difference was observed in the severity of post ERCP pancreatitis (Table 5). Post ERCP hyperamylasemia (>=440) was observed in 34 patients from the placebo group versus 21 patients in the semapimod group (Table 5, 29.8% *v* 18.4%; p = 0.031). During the first 24 hours after the ERCP procedure amylase levels were lower (mean difference: -303, 95% CI: -18 to -589, F=4.38, p=0.037) in the semapimod group compared with the placebo group (Table 6 and Figure 1)..

No serious adverse events related to study drug medication were noted. More patients in the semapimod group suffered from vomiting and paraesthesias. No differences were observed for other mild adverse events (Table 7).

	Placebo	Semapimod	test statistic	p value
ERCP findings				0.107
Normal	7 (6%)	9 (7%)		
Incomplete/Failed	3 (2%)	0 (0%)		
Biliary pathology	**75 (61%)**	**65 (33%)**		
CBD stones	53	52		
Malignancy	14	10		
PSC	2	1		
Post cholecystectomy lesion	6	1		
Pancreatic pathology	31 (26%)	34 (28%)		
Malignancy	27	30		
PD stones	3	0		
Recurrent pancreatitis	1	2		
Pancreas divisum	0	1		
Pseudocyst	0	1		
Papillar pathology	**5 (4%)**	**13 (11%)**		
SOD	0	1		
Papillar adenoma or stenosis	2	6		
Malignancy	1	4		
Juxtapapillar diverticulum	2	2		
ERCP procedures				
Biliary sphincterotomy	61	70	$\chi2 = 1.35$	0.246
Precut papillotomy	50	36	$\chi2 = 3.54$	0.060
Guidewire used for cannulation	18	16	$\chi2 = 0.14$	0.711
Balloon dilatation of sphincter	5	4		1.000
Stone removal performed	38	37	$\chi2 = 0.15$	0.928
Stent placement	50	56		
Brushing performed	22	21	$\chi2 = 0.01$	0.914
Balloon dilatation of stricture	12	13	$\chi2 = 0.05$	0.833
Pancreatic sphincterotomy	2	2		

Tissue sampling performed	23	23		
ERCP duration (in minutes)	41 ± 23.2	37 ± 20.0	t = 1.50	0.134
ERCP difficulty (0-10)	5.4 ± 2.7	4.9 ± 2.5	t = 1.75	0.081
Number contrast injections CBD	2.9 ± 2.0	3.0 ± 2.1	t = -0.35	0.726
Number contrast injections PD	2.6 ± .2.8	2.0 ± 2.4	t = 1.67	0.095

Table 4. ERCP findings and procedures. One hour pre ERCP patients received placebo (N=121) or Semapimod (N=121) intravenously. Findings and procedures during ERCP of included patients in both groups are shown.

	Placebo	Semapimod	test statistic	p value
Post ERCP pancreatitis	18	11	$\chi2 = 1.92$	0.11
Severe	4	2		
Moderate	4	3		
Mild	10	6		
Mean hospital stay in patients with pancreatitis	5.0 ± 5.8 (0-20)	5.4 ± 8.1 (0-27)	t = 0.18	0.86
Mortality attributable to acute pancreatitis	0			
Hyperamylasemia	34 (29.8%)	21 (18.4%)	$\chi2 = 4.03$	0.031

Table 5. Frequency and severity of pancreatitis, related duration of hospital stay, related mortality, and frequency of hyperamylasemia. One hour pre ERCP patients received placebo (N=121) or Semapimod (N=121) intravenously. Occurrence and severity of post ERCP pancreatitis (as defined in the methods section) as well as hyperamylasemia (as defined in the methods section) of included patients in both groups are shown.

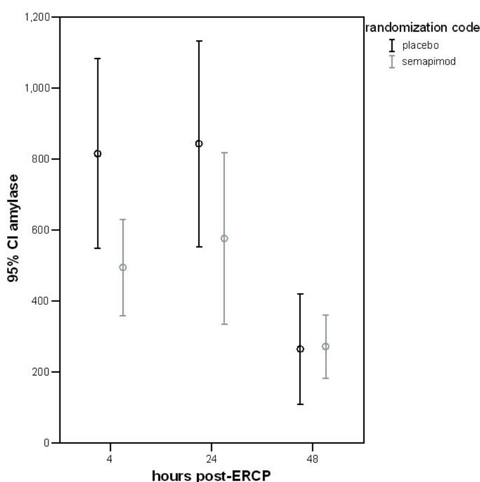

Figure 1. Amylase levels post ERCP. One hour pre ERCP patients received placebo (N=121) or Semapimod (N=121) intravenously. Amylase levels during the first 48 hours after ERCP of included patients in both groups are shown.

	Placebo (N=121)	Semapimod (N=121)	test statistic	p value
Amylase				
Baseline	174 ± 265	141 ± 227		
after 4 hours	816 ± 1478	495 ± 741	} F=4.38, p=0.037	
after 24 hours	843 ± 1566	577 ± 1300		
after 48 hours	265 ± 714	271 ± 414		
Lipase				
Baseline	90 ± 222	76 ±292		
after 4 hours	658 ± 1010	487 ± 844		
after 24 hours	482 ± 857	387 ± 785		
after 48 hours	153 ± 303	135 ± 385		

Table 6. Levels of plasma hydrolases post ERCP. One hour pre ERCP patients received placebo (N=121) or Semapimod (N=121) intravenously. Amylase and lipase levels at baseline and during the first 48 hours after ERCP of included patients in both groups are shown.

	Placebo	Semapimod	test statistic	p value
Serious adverse events				
Number of patients with serious adverse events (e.g. death or readmission during 30 day follow up	17	16	$\chi 2 = 0.04$	0.851
Serious adverse events related to study drug infusion	0	0		1.0
Mild and moderate adverse events (number of patients)				
Nausea	19	**19**	$\chi 2 = 0$	1.0
Vomiting	2	10		0.034
Pain at infusion site	3	3		1.0
Paraesthesias nos	1	8		0.036
Rash	0	4		0.122
Dizziness	3	8		0.216
Headache	5	3		0.722
Hypotension	1	0		1.0
Fever	13	9	$\chi 2 = 0.80$	0.503

Table 7. Adverse events. One hour pre ERCP patients received placebo (N=121) or Semapimod (N=121) intravenously. Serious (30 day follow up) and mild/moderate adverse events (48 hour follow up) after ERCP of patients in both groups are shown.

Discussion

We tested the efficacy of prophylactic treatment with semapimod, an inhibitor of MAPK pathways, to prevent post ERCP pancreatitis and hyperamylasemia in patients undergoing a therapeutic ERCP. In this double blinded, placebo controlled clinical trial we show that prophylactic administration of semapimod is safe and reduces post ERCP plasma amylase levels during the first 24 hours after ERCP. The incidence of post ERCP pancreatitis was not significantly decreased (P=0.11), but did show a trend in favor of semapimod.

Post ERCP pancreatitis and hyperamylasemia are frequent complications of therapeutic ERCP's. An elevation of pancreatic enzymes after ERCP can be found in up to 70% of patients undergoing an ERCP. Clinical pancreatitis, defined as the occurrence of abdominal pain with hyperamylasemia and /or hyperlipasemia (most often defined by an elevation more than 3 times upper limit of normal) occurs less commonly. Incidence rates vary from 2% in unselected patients up to 40% in high-risk patients [21]. In this study, we found an overall pancreatitis rate of 12% which is in line with previously described rates in patients undergoing a therapeutic ERCP[22, 23].

The pathophysiology of post ERCP pancreatitis is incompletely understood. Several factors may act independently or together to induce post ERCP pancreatitis, such as: mechanical injury, hydrostatic injury, chemical injury, enzymatic (intestinal) injury, infection and thermal injury [24-27]. Current concepts dictate that the co-localisation of digestive enzymes and lysosomal hydrolases in acinar cells leads to a premature activation of the digestive enzymes. This process takes place in the early phase of acute pancreatitis. Cell injury induced by premature intra-acinar trypsinogen activation to trypsin subsequently leads to activation of immunecompetent cells and the release of cytokines and chemokines[28-31].

Pharmacological prevention focused on the reduction of active intra-pancreatic enzymes, preventing the premature activation of digestive enzymes and blocking of the inflammatory cascade. Prophylactic administration of a wide range of agents such as gabexate messilate, prednisone, nifedipine, allopurinol, Interleukin (IL) 10, somatostatin and low molecular weight heparin has been tried to lower the incidence of post ERCP pancreatitis[32-38]. Although some of these agents showed promising results (such as IL 10, diclofenac, allopurinol and gabexate messilate) these are either unconfirmed or subsequent studies have provided conflicting results. In this article we undertook a novel anti inflammatory approach by administering semapimod, a small molecule that is a synthetic guanylhydrazone, which is known to inhibit MEK, JNK and p38 MAPK phosphorylation [39-41]. Although it is widely accepted that MAPK's constitute major inflammatory signaling pathways in various inflammatory diseases including experimental pancreatitis[42, 43], the importance of these kinases in

human acute pancreatitis has not been evaluated so far. Inhibition of MAPK phosphorylation by semapimod inhibited inflammatory responses in preclinical animal models such as endotoxic shock [44, 45], the acute respiratory distress syndrome [46] as well as in the treatment of mild to severe Crohn's disease in humans [47].

In this article we used a novel definition of post ERCP pancreatitis. Pancreatitis is usually defined as a clinical syndrome of abdominal pain combined with a more than threefold elevation of plasma amylase and/or lipase. However, since post ERCP hyperamylasemia is very frequent and many patients experience some form of abdominal pain after ERCP due to the procedure itself and insufflations of air, we felt that a more stringent definition of post ERCP pancreatitis should be used. In this study, we defined pancreatitis as a more than threefold elevation of plasma amylase and/or lipase over the upper limit of normal at 24 or 48 hours post ERCP. In addition, this elevation of enzymes had to be combined with an increase of post ERCP VAS scores for abdominal pain (compared to preprocedural VAS scores) of at least 30 points (on a 100 point scale) persisting for at least 24 hours. Using this definition we attempted to more accurately identify true pancreatitis cases by distinguishing them from patients with temporary abdominal discomfort due to air insufflations and concurrent hyperamylasemia and/or hyperlipasaemia.

A number of risk factors for post-ERCP pancreatitis have been identified over the years that can be divided into operator-, patient- and procedure-related factors. All patient and procedural risk factors for the development of post ERCP pancreatitis were comparable between placebo and semapimod treated patients. A total of 242 patients were analyzed in this study. This number was based upon a sample size analysis which assumed an incidence of post-ERCP pancreatitis of 15% in the placebo group and 5 % in the semapimod treated group (α=0.05 one-sided, power=0.82) under the assumption that the effect of semapimod was considered clinically relevant when post ERCP pancreatitis incidence was decreased by two-thirds. Indeed, in the placebo group 15% of patients developed post ERCP pancreatitis versus only 9% of semapimod treated patients. This decrease of the incidence of post ERCP pancreatitis by 40% fell short of reaching statistical significance. Post ERCP hyperamylasemia is widely regarded as a precursor for the development of post ERCP pancreatitis and frequently used as a secondary end point is studies evaluating the efficacy of prophylactic pharmacological agents for post ERCP pancreatitis[48-54]. In this study we show that semapimod indeed lowers the incidence of post ERCP hyperamylasemia and, furthermore, that it decreases the levels of post ERCP amylase during the first 24 hours after ERCP in general. These latter data suggest that semapimod may exert a protective effect on the *development/ pathogenesis* of post ERCP pancreatitis.

In accordance with the findings of other studies, most cases of post-ERCP pancreatitis n this

study were mild. Mortality from post-ERCP pancreatitis is rare. The main consequence of this complication is prolonged hospital stay. Hence it is important to consider the reduction in hospital stay for pancreatitis using prophylactic semapimod versus the cost of the therapy. However, because semapimod is still an investigational drug, the costs of the compound are not yet available to perform such an analysis at this point.

In conclusion, a single dose of intravenous semapimod one hour pre ERCP is safe and exerts a biological effect demonstrated by a statistically significant reduction of the incidence of hyperamylasemia and levels of post ERCP amylase, and a non-significant trend towards a protective effect for the development of post ERCP pancreatitis.

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chapter 4

acute pancreatitis in peritoneal dialysis and haemodialysis: risk, clinical course, outcome, and possible aetiology

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Introduction

In 1985 a case report was published on two patients with end stage renal failure who developed acute pancreatitis during continuous ambulatory peritoneal dialysis[1]. This raised the question whether patients with end stage renal failure undergoing dialysis have an increased risk for acute pancreatitis.[2] Subsequent reports seemed to confirm this assumption.[3-5] Some studies reported a significantly higher incidence of acute pancreatitis in patients on peritoneal dialysis compared with patients on haemodialysis.[3] Others, however, did not confirm this observation.[4] We have reviewed all the clinical data of patients undergoing long term peritoneal dialysis and/or haemodialysis in our dialysis centre over a nine year period. We assessed whether the incidence of acute pancreatitis in these patients is increased compared with the general population and calculated risk estimates. Furthermore, we evaluated its clinical course and outcome and attempted to identify possible aetiological factors.

Patients and methods

We retrospectively reviewed all medical data of patients undergoing chronic dialysis at the dialysis centre of the Kennemer Gasthuis, lokatie Elizabeth Gasthuis, Haarlem, The Netherlands, from January 1989 to March 1998. Chronic dialysis was defined as a total dialysis period longer than six weeks. Medical data that were reviewed included medical charts, laboratory reports, and reports from radiological investigations. An extensive database was created in which demographic data, data relating to dialysis (total dialysis time, type of dialysis, periods of dialysis, number of transplantations, cause of renal failure), and data relating to periods of pancreatitis (number of attacks, presenting symptoms, alcohol consumption, concomitant medication, history of peritonitis, amylase/lipase/calcium/triglyceride concentrations, peritoneal fluid cell counts and cultures, peritoneal fluid concentrations of amylase and lipase, findings on imaging investigations, total length of hospital stay, complications, and clinical course and outcome) were scored.

Acute pancreatitis was defined as a period with acute abdominal pain and discomfort with a more than threefold transient increase in serum amylase and/or lipase. To obtain "normal" values of amylase and lipase activities in plasma and peritoneal fluid we randomly selected nine patients on long term haemodialysis and nine patients on long term peritoneal dialysis who had no history of acute pancreatitis. At the time of blood sampling none of these patients had symptoms or signs compatible with acute pancreatitis. In the random selection of patients on long term peritoneal dialysis without acute pancreatitis we also determined triglyceride and calcium concentrations. In order to identify possible aetiological risk factors for acute pancreatitis in peritoneal dialysis, we compared these "normal" values with concentrations in patients on peritoneal dialysis during their attack of acute pancreatitis.

Statistiscs

The incidence of acute pancreatitis in haemodialysis or peritoneal dialysis during the study period was calculated by dividing the number of cases by the total number of patients at risk. We calculated risk estimates such as the number of attacks of acute pancreatitis per person year and the number of patients per person year. The standardised ratio (incidence in haemodialysis or peritoneal dialysis/incidence in general population) was calculated together with its 95% confidence interval (CI). The incidence of acute pancreatitis in The Netherlands from 1985 to 1995 was 15 per 100 000 person years.[6] This incidence rate was used to calculate the standardised ratios. Additionally, to test the firmness of these outcomes, "worst case" calculations were performed using the lowest and highest reported incidence of acute pancreatitis in northern Europe. The estimated incidence of acute pancreatitis in the general population for northern European countries is reported to be in the range from 5 to 73 per 100 000 person years.[7-11] For comparison of continuous variables, appropriate t tests or non-parametric tests were used. For categorical data, the Chi square test for trend was used. Differences between groups were considered significant if the p value was less than 0.05 for a two tailed test.

Results

Haemodialysis

There were 269 patients (167 men and 102 women) undergoing long term haemodialysis. Their mean age was 64 years (range 22-87). The total duration of haemodialysis in these 269 patients was 614 person years (median duration 19 months, range 2-202). During this period one patient developed an attack of acute pancreatitis. The incidence of acute pancreatitis over the study period (9.3 years) was 0.4% (1/269). There were 0.0016 (1/614) attacks (and patients) of (with) acute pancreatitis per person year. The standardised ratio between the incidence of acute pancreatitis in haemodialysis and the general population was 11 and not significantly different from 1 (95% CI 0.275 to 60.5). Using the lowest reported incidence in northern European countries (5 per 100 000 person years) the standardised ratio was 54 (95% CI 0.825 to 181), still showing no significantly increased risk.
The patient on haemodialysis who had an attack of pancreatitis finally underwent surgery because of an acute cholecystitis. During surgery a stone was found in the gall bladder neck. Peroperative cholangiography did not show stones in the common bile duct. As increases in amylase, lipase, and cholestatic liver tests were present before surgery and already dropping, pancreatitis in this patient was probably owing to a common bile duct stone or stones that had passed spontaneously. The clinical outcome was uneventful. The overall mortality rate of acute pancreatitis in patients on haemodialysis was 0%.

Peritoneal dialysis

There were 128 patients (84 men and 44 women) undergoing long term peritoneal dialysis. Their median age was 59 years (range 20-81). The total duration of peritoneal dialysis in these 128 patients was 241 person years (median duration 17 months, range 2-92). During this period seven patients had nine attacks of acute pancreatitis. The incidence of acute pancreatitis in peritoneal dialysis during the study period (9 years and 3 months) was 5.4% (7/128). There were 0.037 (9/241) attacks of acute pancreatitis per person year and 0.029 (7/241) patients per person year. The standardised ratio between the incidence of acute pancreatitis in peritoneal dialysis and the incidence in The Netherlands was 249, and highly and significantly different from 1 (95% CI 114 to 473). Using the highest reported incidence in northern European countries (73 per 100 000 patient years) the standardised ratio is 51 (95% CI 23 to 97), still showing a significantly increased risk. The incidence of acute pancreatitis between long term haemodialysis and peritoneal dialysis was also significantly different ($p<0.001$).

Presentation and patient history

Six patients had one attack of acute pancreatitis. One patient had three attacks. In between these attacks this patient had no abdominal complaints and serum amylase and lipase concentrations returned to normal. Median time on peritoneal dialysis until the attack of acute pancreatitis was 16 months with a wide range of 3-48 months. Four of seven patients had been maintained only on peritoneal dialysis and had never undergone haemodialysis. Three of seven patients had earlier episodes of haemodialysis (durations of 1, 20, and 41 months). Two of seven patients had a history of renal transplantation with rejection. None of the patients used medication known to be causally related to acute pancreatitis.[12] None of the patients had a history of alcohol abuse. Only two of seven patients had a history of peritonitis.

Laboratory data

Table 1 lists the (peak) serum amylase, (peak) serum lipase, amylase, and lipase concentrations in peritoneal fluid for each individual patient together with the results of both imaging techniques and a selection of outcome parameters. Table 2 lists the mean/median outcomes of (peak) serum amylase, (peak) serum lipase, amylase, and lipase concentrations in peritoneal fluid, triglyceride concentration, and calcium concentration in patients on long term peritoneal dialysis who suffered from an attack of acute pancreatitis and the random selection of patients on peritoneal dialysis who did not. Serum triglyceride and calcium concentrations did not differ significantly between both groups. In all of the randomly selected patients on peritoneal dialysis without acute pancreatitis, peritoneal lipase and amylase concentrations were below 10 U/l and 30 U/l, respectively. Peritoneal lipase concentrations rose above 10 U/l during seven of the total of nine attacks of acute pancreatitis. Peritoneal amylase concentrations rose above 30 U/l during five attacks. Five patients had negative peritoneal

fluid culture. Four of these patients had normal peritoneal fluid cell counts while one patient had a cell count of 1100 cells/mm. In two patients peritoneal fluid cultures were positive (Gram positive cocci and Gram negative rods with yeast). These patients had peritoneal fluid cell counts of 27 and 2700 cells/mm, respectively.

	Amylase at admision (U/l)	Peak amylase	Lipase at admission (U/l)	Peak lipase (U/l)	Amylase in PD fluid (U/l)	Lipase in PD fluid (U/l)	US	CT	Necrosis	Pseudocyst	Death
1	100	104	13100	13100	30	361	+	+	+	+	+
2	192	406	849	2144	30	15	+	-	-	-	-
3	340	451	1978	3818	30	1	+	+	-	-	-
4	251	659	3070	4510	130	144	NV	+	-	-	-
4	323	1070	2310	14000	33	46	NA	-	-	-	-
4	589	1070	5640	6730	616	1336	NA	-	-	-	-
5	1150	1150	7780	7780	62	561	NV	+	-	+	-
6	1339	1339	8620	8620	1229	21950	-	+	+	-	-
7	482	482	5700	5700	NA	NA		+	-	+	-

Table 1: Laboratory parameters and imaging results and a selection of outcome parameters listed for each patient who developed acute pancreatitis while on peritoneal dialysis (PD). NV= Not visualized. NA+ not available, US= ultrasound, CT= computed tomography. Patient 4 experienced 3 seperate attacks of acute pancreatitis.

	PD without pancreatitis	**PD with pancreatitis**
Serum amylase (U/l)*	81 (28), 47-131	530 (433), 100-1339
Peak serum amylase (U/l)*	-	748 (421), 104-1339
Serum lipase (U/l)*	202 (139), 81-460	5450 (3919), 849-13100
Peak serum lipase (U/l)*	-	7378 (4028), 2144-14000
Amylase peritoneal fluid (U/l)#	<30	48, 30-1229
Lipase peritoneal fluid (U/l)#	<10	253, 1-21950
Serum triglyceride (mmol/l) •	3.6 (2.0), 1.5-5.4	2.6, 1.1-9.3
Serum calcium (mmol/l) •	2.60 (0.14), 2.40-2.84	2.42 (0.28), 2.20-3.06

Table 2: Laboratory outcomes; comparison between patients on peritoneal dialysis (PD) with and without pancreatitis. Mean (SD), range. #Median, range. •p>0.05.

Imaging investigations

Ultrasound (US) showed a normal pancreas in two patients, pancreatitis in three patients, and could not visualise the pancreas in the remaining two patients (table 1). Computed

tomography (CT) showed signs of pancreatitis in six patients. The patient with negative CT findings showed signs of pancreatitis during US. Most probably, this is explained by the fact that the CT scan was performed a few days after the US examination at which time the swelling of the pancreas had already disappeared. In the patient who had three attacks of pancreatitis, CT showed oedema of the pancreatic tail area only during the first attack. CT was normal during the subsequent two attacks. Endoscopic retrograde cholangiopancreatography (ERCP) was performed in three patients. In one patient choledocholithiasis was found and the calculi were removed; in one patient it was normal, and in one patient cannulation failed.

	HC	HT	CH	AL	TH	ME	HP	CP	Total
1	+	+	-	-	-	-	+	+	4
2	-	-	-	-	-	-	-	-	0
3	-	+	-	-	-	-	-	-	1
4	-	NA	-	-	-	-	-	-	1
4	+	-	-	-	-	-	-	-	1
4	+	-	-	-	-	-	-	-	1
5	-	+	-	-	+	-	-	-	2
6	-	-	+	-	-	-	+	-	2
7	-	+	-	-	+	+	-	+	4
Total	3	4	1	-	2	2	2	2	

Table 3: Presence of known risk factors and factors that have been postulated to be associated with acute pancreatitis in peritoneal dialysis (PD). +, yes; , - no; NA, not available; HC, hypercalcaemia; HT, hypertriglyceridaemia; CH, choledocholithiasis; AL, alcohol; TH, transplantation history; ME, medication; HP, history of peritonitis; CP, concomitant peritonitis.

Clinical course, outcome, and possible aetiology

The median number of days spent in the hospital was 37 (range 16-113). Five patients had oedematous pancreatitis while in two patients necrotising pancreatitis developed. Three patients developed pseudocysts. One patient died 113 days after hospital admission. This patient developed a large pseudocyst that was drained transgastrically by means of endoscopy. Because of a persisting bleed from the puncture aperture she underwent a laparotomy at which time areas with extensive pancreatic necrosis were found. Debridement and a cystgastrostomy were performed. This patient finally died because of respiratory insufficiency and multiple organ failure. All the other patients recovered uneventfully. The overall mortality rate of acute pancreatitis in peritoneal dialysis calculated per patient was 14% (1/7) and counted per attack 11% (1/9). Table 3 lists known risk factors and factors that have been postulated to be associated with acute pancreatitis in peritoneal dialysis (see

Discussion). From this table it becomes clear that there is no single aetiological factor that can be identified in our patients.

Discussion

At postmortem examinations pancreatic abnormalities are reported in up to 60% of patients who were maintained on long term haemodialysis.[13-15] Histological changes were found to be diffusely present throughout the pancreas and included duct ectasia, periductal fibrosis, ductular proliferation, acinar ductular metaplasia, inspissated secretions, atrophy, thickening and fibrosis of arterioles, haemosiderin deposits, calcifications, amyloidosis, hyalinisation, cystic changes, necrosis of peripancreatic fat, and abscess formation. This high prevalence of pancreatic pathology after long term haemodialysis does not seem to match the incidence of clinically observed pancreatic disease (for example, acute/chronic pancreatitis and exocrine pancreatic insufficiency). However, the true incidence and prevalence of exocrine pancreatic insufficiency in long term haemodialysis or peritoneal dialysis is unknown. Autopsy data regarding patients who were maintained on long term peritoneal dialysis are not available.

In patients with renal insufficiency concentrations of gastrointestinal hormones such as cholecystokinin, serum gastric inhibitory polypeptide, and glucagon are significantly increased in relation to the degree of renal impairment.[16] During long term haemodialysis these increased hormone concentrations do not return to normal. This increase in hormone concentrations causes hypersecretion of pancreatic enzymes, predominantly trypsin.[17] The continued stimulation of the exocrine pancreas by cholecystokinin may account for the morphological changes that are observed in these patients. This may eventually lead to impaired pancreatic function. Therefore, in the differential diagnosis of the wasting syndrome that is sometimes seen in patients on chronic dialysis, exocrine pancreatic insufficiency with energy losses owing to steatorrhoea should be considered.[18]

In the absence of acute pancreatitis, plasma concentrations of amylase and lipase are frequently increased in uraemic patients and patients on chronic dialysis, both haemodialysis and peritoneal dialysis.[9] However, serum amylase activity greater than a threefold increase, together with acute onset abdominal pain is suggestive of acute pancreatitis. If there is doubt, determination of amylase isoenzymes, serum lipase concentrations, and lipase and amylase concentrations in the peritoneal fluid are useful to help distinguish a pancreatic from a non-pancreatic aetiology.[20] Whenever a patient on long term peritoneal dialysis presents with acute onset abdominal pain, even in the presence of increased concentrations of amylase and/or lipase, (concomitant) bacterial peritonitis should be excluded by peritoneal fluid cell counts and cultures. If dialysate amylase concentrations are at least 100 IU/l, acute pancreatitis is highly probable. In our patients, taking into consideration the clinical picture,

the results of laboratory investigations (together with resolution to normal concentrations after the attacks), and the outcome of the imaging investigations, the diagnosis acute pancreatitis seems without doubt.

The 10 year incidence of acute pancreatitis in patients with end stage renal disease (without further specification as to whether or not and what type of renal replacement therapy was started) is reported to be around 2.5%.[3, 21] In our series the 10 year incidence (recalculated from 9.25 years) was 0.4% for haemodialysis and 5.8% for peritoneal dialysis. The incidence per 100 person years is reported to be in the range from 0.63 to 1.41 in long term haemodialysis and from 0.46 to 4.3 in long term peritoneal dialysis.[3, 5, 21] We found incidence rates of 0.16 and 4.3, respectively. The remarkably lower incidence rate of acute pancreatitis in peritoneal dialysis (0.46 per 100 person years) reported by Pannekeet et al may be owing to recall bias. In this study, cases of acute pancreatitis during peritoneal dialysis were identified by means of interviews with nephrologists in dialysis centres. It is not unlikely that physicians who were interviewed were more likely to recall the more severe cases. This seems also to be reflected by the extremely high mortality rate of 58% in this series. Other groups also observed a high mortality rate but, as stated by the authors, less severe cases of acute pancreatitis may easily have been missed because symptoms of acute pancreatitis were often similar to those of dialysis associated peritonitis.[2] We observed a mortality rate of 11% of acute pancreatitis in long term peritoneal dialysis that does not differ from the reported overall (general population) mortality rate of acute pancreatitis in controlled clinical trials. In agreement with previous studies we noted that acute pancreatitis occurred more frequently in peritoneal dialysis than in haemodialysis.[2, 3, 21] In fact, in our series we observed no increased risk for acute pancreatitis for patients on haemodialysis compared with the general population. The 95% confidence interval, however, is very broad. One additional patient on haemodialysis with acute pancreatitis would yield a rate ratio of 21.7 (95% CI 2.63 to 78.4), indicating a significantly higher risk of acute pancreatitis in haemodialysis.

Of great interest and significant importance is the pathophysiological mechanism that may be responsible for the highly increased risk of acute pancreatitis in long term peritoneal dialysis. At first our results suggested that it is associated with the act of peritoneal dialysis and not with renal insufficiency as such. However, as stated above, the sample size may have been too small to detect a statistically significant difference between the general population and patients on haemodialysis. Others did find a higher incidence of acute pancreatitis in haemodialysis compared with the general population. An increased incidence of acute pancreatitis after renal transplantation is also reported but may be related to the surgery and the use of immunosuppressive agents. The risk of developing pancreatitis in long term peritoneal dialysis may be explained by a summation of separate risk factors: general population risk factors, risk factors related to renal insufficiency (uraemia, secondary hyperparathyroi-

dism with hypercalcaemia, hypertriglyceridaemia, use of drugs), and as yet unidentified risk factors related to the act of peritoneal dialysis. With respect to the latter one could speculate that chronic infusion of a large amount of fluid with a non-physiological composition under non-physiological high intra-abdominal pressure renders the pancreas more susceptible to parenchymal damage. Impairment of microvascularisation and hypoxaemia may induce premature activation of proteolytic enzymes, thereby provoking acute pancreatitis. The increase in intra-abdominal pressure during long term peritoneal dialysis is considerable and is known to cause complications such as herniation, prolapse of the uterus or rectum, and rarely hydrothorax. The composition of the dialysate may also (indirectly) influence factors known to be related to acute pancreatitis such as induction or aggravation of hyperglycaemia because of high glucose concentrations leading to hyperlipidaemia. Moreover, increased concentrations of pancreatic stimulating hormones together with the high prevalence of morphological changes may render the pancreas more susceptible to stimuli that potentially provoke acute pancreatitis.

More than one "known" or proposed risk factor was present in four of seven patients (table 3), the most prevalent one being hypertriglyceridaemia in four of seven patients. However, serum triglyceride concentrations below 5.64 mmol/l are less likely to be associated with pancreatitis and this level was exceeded in one patient only (9.3 mmol/l).[22] Overall there was no statistically significant difference between serum triglyceride concentrations in patients on long term peritoneal dialysis with and without acute pancreatitis. The cumulative effect of increased triglyceride concentrations as well as increased calcium concentrations (possibly masked by low albumin concentrations) is not known. The cumulative duration of chronic dialysis did not seem to be a determining factor in our study. This is in agreement with Padilla et al who also observed a wide range in the time interval between the start of treatment and the onset of acute pancreatitis for both haemodialysis and peritoneal dialysis.[21] Others suggested that a history of recurrent or concomitant episode(s) of peritonitis might be a risk factor for acute pancreatitis by leakage of infected peritoneal fluid into the lesser sac or haematogeneous spread to the peripancreatic region. Based on our results we cannot refute or confirm this hypothesis.
From the results of our series and previous studies it becomes clear that there is no single aetiological factor that accounts for the increased incidence of acute pancreatitis in peritoneal dialysis. Its pathophysiological mechanism seems multifactorial. There seems to be a stepwise risk increase for acute pancreatitis from the general population to patients with renal insufficiency (who may be maintained on haemodialysis) and finally to patients on long term peritoneal dialysis. The latter step, according to the results of this study, adds greatly to the overall risk. The causal mechanism by which long term peritoneal dialysis increases the risk of acute pancreatitis remains as yet unknown.

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chapter 5

acute pancreatitis in mice impairs bacterial clearance from the lungs, while concurrent pneumonia prolongs the course of pancreatitis

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Introduction

In cases of severe acute pancreatitis, the outcome is determined by the development of severe systemic inflammation as well as secondary infectious complications[(1)]. Although such infections most often occur in the inflamed pancreas and surrounding tissues, nosocomial pneumonia is a feared complication of critical illness[(2)]. *Pseudomonas (P.) aeruginosa* is a common pathogen in nosocomial pneumonia[(2,3)]; of note, this bacterium is also frequently involved in secondary infections in patients with pancreatitis[(4)]. In the present investigation we sought to determine whether pancreatitis influences bacterial clearance and pulmonary inflammatory responses, and, vice versa, whether concurrent pneumonia influences the course and severity of pancreatitis. For this, we induced pneumonia by intranasal inoculation with *P. aeruginosa* in mice after induction of pancreatitis[(5,6)], and compared bacterial clearance and inflammatory responses in lungs, as well as the course of acute pancreatitis.

Methods

Animals

Female C57BL/6 mice (Harlan, Horst, the Netherlands), 10-12 weeks old, were used in all experiments. The Institutional Animal Care and Use Committee approved the protocol.

Experimental groups and design

Pancreatitis was induced by 12 hourly intraperitoneal (i.p.) injections of cerulein (50 µg/kg in 200 µl saline; Research Plus, Manasquan, NJ) [(7)]. Sham mice received sterile saline. Mice (8 mice per group) were killed 12 hours after the first injection. In a separate study, one hour after the last i.p. injection of cerulein (pancreatitis) or saline (sham), pneumonia was induced by intranasal (i.n.) administration of *P. aeruginosa* strain PA 103 (50 µl containing 5.5x 10^5 Colony Forming Units, CFU, per mouse) [(8,9)]. Control mice were i.n. inoculated with 50 µl saline. Mice were killed 24 hours postinfection. Hence, 4 groups were studied (10 mice per group): sham/control, pancreatitis/control, sham/pneumonia, and pancreatitis/pneumonia (Figure 1).

Tissue handling

Mice were anesthetized with Hypnorm (Janssen, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the *vena cava inferior* Pancreatic and lung edema was estimated by expressing relative organ weights [(10)]. Parts of pancreas and one whole lung were fixed in formalin. Remaining parts of pancreas and lungs were either frozen in liquid nitrogen or removed for enumeration of bacteria as previously described [(8,9)]. In brief, whole lungs were harvested and homogenized at 4^0C in 5 volumes

of sterile 0.9% NaCl in a tissue homogenizer. After each homogenization, the homogenizer was carefully cleaned and disinfected with 70% alcohol. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates and blood, and 50 µl volumes were plated onto sheep-blood agar plates and incubated at 37^{0}C and 5% CO_2. CFU were counted after 24 hours.

Histologic examination

Hematoxylin and eosin stained specimens (2 specimens per organ per mouse) were scored as follows by a pathologist (SF) unaware of their origin. *Lungs*: Interstitial (0-3 points) and perivascular inflammatory infiltrates (0-3), pleural inflammation (0-3) and percentage of infected lung tissue (0-10% = 1, 10-20% = 2, 20-50% =3, >50% = 4). *Pancreas*: Pancreatitis severity was scored as described [(11)]. Briefly, necrosis was scored on a 0-3 scale (0=normal, 1= periductal necrosis < (5%), 2= focal necrosis (5-20%), 3= diffuse necrosis (>50%)), inflammatory cell infiltration was scored on a 0-3 scale (0= normal, 1= inflammatory infiltration in ducts, 2= infiltration in the parenchyma (<50%), 3= in the parenchyma (>50%), and edema was scored on a 0-3 scale (0= normal, 1= focal increase between lobules, 2= diffuse increase between lobules, 3= acini disrupted and separated). A total severity score was calculated by adding all scores.

Assays

Amylase was determined with a commercially available kit (Sigma, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany). Pancreas and lung homogenates were prepared as described [(8,9,12)]. Myeloperoxidase (MPO) content was measured in pancreas and lung homogenates, as described elsewhere and is expressed as percentage of values measured in sham/control mice [(13,12)]. In brief, tissue was homogenized in potassium phosphate buffer and pelleted at 4500 g for 20 min at 4°C. Pelleted cells were lysed in potassium phosphate buffer (pH 6.0) supplemented with 10 mM EDTA and 0.5% hexadecyltrimethyl ammoniumbromide. MPO activity was determined by measuring the H_2O_2-dependent oxidation of 3,3’ 5,5’ tetramethylbenzidine. Reaction was stopped with glacial acetic acid, followed by optical density reading at 655 nm. Cytokine and chemokine levels were measured by ELISA according to the manufacturers recommendations, standard curves were performed in standard buffer: tumor necrosis factor (TNF)-α (R&D systems, Minneapolis, MN), interleukin (IL)-6 (R&D), IL-10 (R&D), macrophage inflammatory protein (MIP)-2 (R&D) and KC (R&D).

Statistical analysis

All data are expressed as means ± SEM. Comparisons between groups where conducted using the Man Whitney U test or the Kruskal Wallis test followed by Dunn’s post test where appropriate. Significance was set at $P < 0.05$.

Results

Severity of pancreas and lung damage in cerulein induced pancreatitis

Twelve hourly injections of cerulein elicited pancreatitis, as indicated by profound increases in the plasma concentrations of amylase, the occurrence of pancreas edema and histopathologic examination demonstrating marked edema, infiltration of neutrophils and focal areas of necrosis (Figure 1, Table 1). In addition, plasma IL-6 was elevated in cerulein treated mice (Table 1). Cerulein induced pancreatitis was associated with mild pulmonary inflammation as determined by higher pulmonary MPO activity (reflecting the number of neutrophils), higher lung concentrations of the chemokine MIP-2 in lung and plasma and edema (P = 0.07 versus sham, Table 1) as compared to sham mice, (Table 1). Lung pathology (Figure 1, Table 1) and pulmonary cytokines (TNF-α, IL-6, IL-1β, IL-10; data not shown) were comparable between pancreatitis and sham mice.

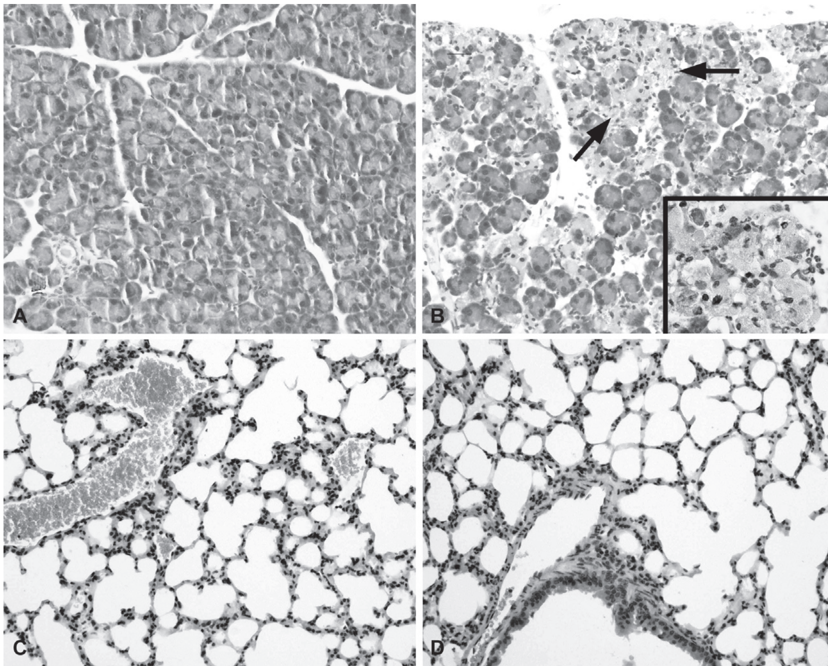

Figure 1: Effects of 12 hourly cerulein injections on pancreas and lung histology. Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 i.p. saline injections. Pancreas and lungs were harvested one hour after the 12th injection. Representative pancreas (A, B) and lung (C, D) histology slides are shown from a total of 8 sham mice (A, C) and 8 pancreatitis mice (B, D). H&E staining , magnification x 10, insert x 40. Arrows indicate areas of necrotic cells.

Bacterial clearance is impaired during pancreatitis and subsequent *P. aeruginosa* pneumonia

In a separate study, we induced pancreatitis and then inoculated mice i.n. with live *P. aeruginosa* immediately thereafter (control mice received saline). Mice were sacrificed 24 hours postinfection. Mice not inoculated with *P. aeruginosa* (sham/control and pancreatitis/control mice) did not display bacteria in lungs, pancreas or blood. Remarkably, mice with pancreatitis and pneumonia had significantly more *P. aeruginosa* CFU in their lungs and pancreas than sham/pneumonia mice (P < 0.05 for the difference between groups (Figure 2). In addition, all pancreatitis/pneumonia mice had positive blood cultures at 24 hours postinfection, versus only 50 percent of sham/pneumonia mice (Figure 2).

	Sham	Pancreatitis
Plasma	6 ± 2.9	69.5 ± 3.9*
Amylase (U/ml)	42 ± 6	329 ± 12*
IL-6 (pg/ml)	22 ± 4	112 ± 8*
MIP-2 (pg/ml)		
Pancreas		
Relative weight	8.4 ± 0.4	11.1 ± 0.36*
Histology score	0.8 ± 0.4	6.8 ± 0.4*
MPO (% of control)	100	188 ± 17*
Lung		
Relative weight	6.7 ± 0.2	7.3 ± 0.1
Histology score	1.7 ± 0.3	2.2 ± 0.4
MPO (% of control)	100	163 ± 1*
MIP-2 (pg/ml)	48 ± 6	262 ± 12*

Table 1: Effects of 12 hourly cerulein injections on pancreas and lungs. Pancreatitis was induced by 12 i.p. injections of cerulein (50 µg/kg); sham mice received saline i.p. Mice were sacrificed one hour after the 12th injection. Data are means ± SEM of 8 mice per group. * P < 0.05 versus sham.

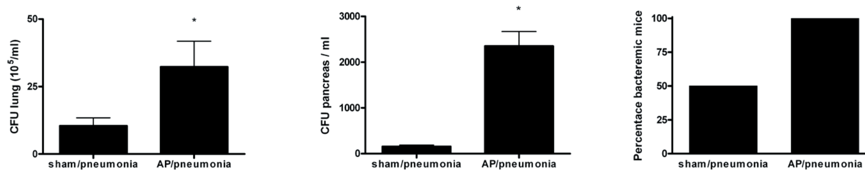

Figure 2: Pancreatitis impairs the clearance of P. aeruginosa from the lungs, and facilitates dissemination of the infection.
Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 i.p. saline injections. Directly thereafter, mice were i.n. inoculated with P.aeruginosa (pneumonia) or saline (control), and sacrificed 24 hours later. No bacteria were recovered from lungs, pancreas or blood of sham/control and pancreatitis/control mice (data not shown). Data are CFU's (means +/- SEM) of 10 mice per group in lung homogenates (left panel), and pancreas homogenates (middle panel), and the percentage of mice with positive blood cultures (right panel) *P < 0.05 vs sham/pneumonia.

	sham control	pancreatitis control	sham pneumonia	pancreatitis pneumonia
Plasma				
Amylase (U/ml)	6.7 ± 0.4	5.8 ± 1.4	4.9 ± 0.5	14.0 ± 1.8*†‡
Pancreas				
Relative weight	7.3 ± 0.3	7.8 ± 0.4	7.8 ± 0.4	10.4 ± 0.5*†‡
Histology score	0.1 ± 0.1	4.9 ± 0.5*	0.1 ± 0.1	6.9 ± 0.5*†‡
MPO (% of control)	100	155 ± 7*	90 ± 8	400 ± 22*†‡
Lung				
Relative weight	6.4 ± 0.1	6.6 ± 0.1	8.1 ± 0.3*†	9.8 ± 0.4*†‡
Histology score	2.5 ± 0.2	3.0 ± 0.4	8.3 ± 0.8*†	10.1 ± 0.4*†‡
MPO (% of control)	100	160 ± 11*	1480 ± 460*†	2569 ± 320*†‡

Table 2: Pancreas and lung damage during pancreatitis and/or pneumonia. Pancreatitis was induced by 12 i.p. injections of cerulein (50 µg/kg); sham mice received saline i.p. One hour after the 12th i.p. injection mice were intranasally inoculated with *P.aeruginosa* (pneumonia) or sterile saline (control) and sacrificed 24 hrs thereafter. Data are means ± SEM of 10 mice per group * $P < 0.05$ versus sham/control, †$P < 0.05$ vs pancreatitis/control, ‡ $P < 0.05$ versus sham/pneumonia.

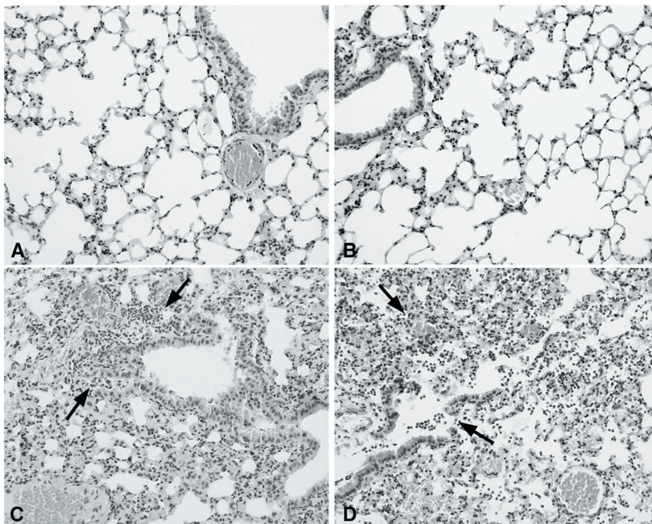

Figure 3: Lung histopathology during pancreatitis and/or pneumonia. Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 i.p. saline injections. Directly thereafter, mice were i.n. inoculated with *P.aeruginosa* (pneumonia) or saline (control), and sacrificed 24 hours later. Representative lung histology slides from a total of (10 mice are shown of sham/control (A), pancreatitis/control (B), sham/pneumonia (C) and pancreatitis/pneumonia (D) mice. H&E staining , magnification x 10. Corresponding lung histology scores are shown in Table 2. Arrows indicate areas of peribronchial inflammation with destruction of normal architecture.

	sham control	pancreatitis control	sham pneumonia	pancreatitis pneumonia
Plasma				
IL-6 (pg/ml)	40 ± 14	42 ± 18	42 ± 18	1452 ± 182*†
MIP-2	26 ± 3	42 ± 3	2132 ± 318*†	2453 ± 298*†
KC	23 ± 3	51 ± 6	872 ± 96*†	901 ± 83*†
Lung				
TNF-α	93 ± 9	82 ± 10	314 ± 33*†	312 ± 29*†
IL-6 (pg/ml)	116 ± 3	494 ± 293	5738 ± 971*†	6084 ± 386*†
MIP-2 (pg/ml)	37 ± 5	37 ± 8	11895 ± 998*†	13146 ± 1148*†
KC (pg/ml)	22 ± 3	26 ± 4	3202 ± 280*†	3628 ± 312*†

Table 3: Cytokine and chemokine levels. Pancreatitis was induced by 12 i.p. injections of cerulein (50 µg/kg); sham mice received saline i.p. One hour after the 12th i.p. injection mice were intranasally inoculated with *P.aeruginosa* (pneumonia) or sterile saline (control) and sacrificed 24 hrs thereafter. Cytokine and chemokine levels in lung homogenates are shown and therefore represent intra- and extracellular cytokine/chemokine levels. Data are means ± SEM of 10 mice per group * P < 0.05 versus sham/control, †P < 0.05 vs pancreatitis/control, ‡ P < 0.05 versus sham/pneumonia.

Pancreatitis increases pulmonary histological damage, MPO and edema during pneumonia

Pancreatitis/control and sham/control mice demonstrated normal lung histology (Figure 3). Both pneumonia groups showed interstitial inflammation, edema, perivascular infiltrates as well as extensive pleural inflammation (Figure 3). The extent of pulmonary injury as assessed by histology was larger in pancreatitis/pneumonia than in sham/pneumonia mice (P < 0.05, Table 2). In addition, pancreatitis/pneumonia mice displayed more edema than sham/pneumonia mice (P < 0.05; Table 2). Pancreatitis/pneumonia and sham/pneumonia mice displayed elevated levels of MPO activity in their lungs but MPO levels were higher in pancreatitis/pneumonia as compared to sham/pneumonia (P < 0.05, Table 2). The concentrations of pro-inflammatory cytokines TNF-α and IL-6, and the chemokine MIP-2 in plasma and lungs did not differ between pancreatitis/pneumonia and sham/pneumonia groups (Table 3).

***P. aeruginosa* pneumonia prolongs the course of pancreatitis**

Cerulein induced pancreatitis induces a severe but self limited disorder with maximal changes 6-12 hours after the cerulein and complete resolvement of the injury over the next couple of days[14,5]. Within 24 hours after the last cerulein injection, some regeneration of pancreas and lung injury did occur (Table 1 and Table 2, pancreatitis vs pancreatitis/

control). Pancreatitis/pneumonia mice displayed more pancreatic inflammation and damage as assessed by histological analysis than pancreatitis/control mice (Table 2, $P < 0.05$). In addition, pancreatitis/pneumonia mice showed higher MPO levels as well as edema than pancreatitis/control mice (Table 2, $P < 0.05$). While in pancreatitis/control mice plasma amylase had returned to normal values, in pancreatitis/pneumonia mice plasma amylase levels remained significantly higher than in pancreatitis/control mice ($P < 0.05$; Table 2), suggesting that in pancreatitis/pneumonia mice the course of pancreatic injury was prolonged.

Discussion

Nosocomial pneumonia is a feared complication in the critically ill patient[2]. Acute pancreatitis is frequently complicated by both local and distant infections and the outcome is largely determined by the presence or absence of such infectious complications[15,1]. The current study aimed to establish (1) whether experimentally induced pancreatitis influences the course of respiratory tract infection with *P. aeruginosa*, and (2) whether pneumonia influences the course of pancreatitis. We here demonstrate that pancreatitis impairs the clearance of *P. aeruginosa* from the lungs, which was accompanied by enhanced dissemination of the infection and exaggerated pulmonary injury. In turn, pneumonia resulted in a prolonged pancreatitis, which was associated with infection of the inflamed pancreas by *P. aeruginosa*.

We first evaluated the extent of pulmonary inflammation induced by pancreatitis alone. After 12 hourly cerulein injections, histopathological examination of the lungs did not reveal visible abnormalities, and relative pancreas weights and pulmonary cytokine levels remained unaltered. Other investigators did observe modest histological alteration in the lungs in this model of acute pancreatitis. Differences in mouse strain and gender and in the time of cerulein injections (day or, in our study, night) may have played a role herein. We did find an increase in pulmonary MPO and MIP-2 levels, as well as in pulmonary edema, indicating that cerulein induced acute pancreatitis results in modest lung inflammation, which is in line with previous reports[5,16].

Our first objective was to evaluate the influence of pancreatitis on bacterial clearnce. We showed that cerulein-induced pancreatitis impairs the bacterial clearance during subsequent *P. aeruginosa* pneumonia, as evidenced by more than three-fold higher bacterial counts in the lungs of, and a higher percentage of positive blood cultures in pancreatitis/pneumonia mice when compared to sham/pneumonia mice.

The proinflammatory shift in the lungs induced by pancreatitis may have impaired the clearance of *P. aeruginosa* from the respiratory tract, as has been demonstrated previously by our and other laboratories in experimental murine pneumonia caused by *P. aeruginosa*[8,9,17]. Of note, these investigations studied the pulmonary response to pancreatitis in the absence of a local inflammatory stimulus in the respiratory tract. To the best of our knowledge, this study is the first to examine the bacterial clearance in the lungs to a respiratory pathogen, administered via the airways, in the context of co-existing pancreatitis.

The higher bacterial loads in pancreatitis/pneumonia mice were accompanied by an increase in pulmonary edema, histological abnormalities and pulmonary MPO, MIP-2 and KC levels. Higher bacterial loads, providing a more potent pulmonary proinflammatory stimulus, together with a modest proinflammatory milieu at the onset of pneumonia, may explain the more profound inflammatory reaction in the pulmonary compartment in pancreatitis/pneumonia mice. In line with these data, it has recently been reported that intraperitoneal endotoxin potentiates the pulmonary injury of cerulein induced pancreatitis which was mediated, in part, by an increase in the activation of hepatic nuclear factor-κB[18]. Our findings expand these data; in contrast to this latter study we studied the effect of live bacteria instilled locally in the lungs (rather than intraperitoneally injected endotoxin), thereby more closely resembling the clinical situation and allowing us to investigate the effects of pancreatitis on pulmonary bacterial clearance.

Our second objective was to evaluate the influence of concurrent pneumonia on the regeneration of acute pancreatitis. We show that pancreatitis severity declines in the first 24 hours after the last cerulein injection but that concurrent pneumonia prolongs the course of pancreatitis, as indicated by more severe histological damage in the pancreas, higher relative pancreas weights and an increased neutrophil content. In addition, plasma amylase, which normally rapidly returns to baseline values after discontinuation of hyperstimulation with cerulein[19,14,5] (and Tables 1 and 2), remained elevated in pancreatitis/pneumonia mice. The mechanism by which concurrent pneumonia adversely influences pancreatitis may well lie in the fact that during pancreatitis more mice became bacteremic during pneumonia, leading to localization of bacteria in necrotic areas in the pancreas and local proliferation. Indeed, whereas in sham/pneumonia mice only few bacteria were harvested from the pancreas, in pancreatitis/pneumonia mice abundant bacteria were isolated. These bacteria elicit an inflammatory response, adding to the already ongoing pancreatic inflammation. Pancreas tissue of pancreatitis/pneumonia mice contained higher MPO levels than pancreas tissue of sham/pneumonia mice. This is of special interest since neutrophils not only play a role in the immunological response to tissue damage but are themselves capable of activating digestive enzymes in the pancreas [20], enhanced neutrophil migration due to pancreatic

localisation of *P.aeruginosa* might add to a positive feedback loop which prolongs pancreatic injury.

In conclusion, we have demonstrated that experimental pancreatitis leads to a reduced bacterial clearance in the pulmonary compartment during *P. aeruginosa* pneumonia. Vice versa, pneumonia prolonged the course of pancreatitis which was associated with a localization of bacteria in the inflamed pancreas and an enhanced influx of neutrophils. These data provide an interesting concept that adds to the understanding of the fact that patients with pancreatitis and complicating infections have a poor prognosis.

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chapter 6

toll-like receptor 4 deficiency and acute pancreatitis act similarly in reducing host defense during murine Escherichia coli peritonitis

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Introduction

Sepsis is a clinical syndrome that results from a systemic host response to an infection[1-3]. After pneumonia, peritonitis is the second most frequent cause of sepsis[4]. The most commonly encountered pathogens in abdominal sepsis are enteric Gram-negative bacteria, among which *Escherichia coli* (*E. coli*) can be found in up to 60% of cases[5]. Importantly, sepsis frequently complicates pre-existing conditions such as trauma, surgery, burn injury or severe inflammatory syndromes such as pancreatitis[2, 6, 7]. In contrast, the vast majority of preclinical investigations on the pathophysiology of sepsis studied the response to severe infection in the previously healthy host. These studies do not necessarily provide adequate insight into the immune response to sepsis in patients with pre-existing diseases. Indeed, abundant clinical and experimental evidence exists that such patients display profound changes in their capacity to react to inflammatory and infectious stimuli[1, 2, 8-10].

Acute pancreatitis is one of the most prevalent and serious diseases associated with the development of secondary Gram-negative infection and sepsis[6, 11, 12]. The mortality of severe pancreatitis is as high as 40%, with Gram-negative abdominal sepsis as the most common cause[13]. In these cases *E. coli* is the most frequently isolated causative organism[6, 14, 15].

The innate immune system recognizes invading pathogens by means of a repertoire of Toll-like receptors (TLRs)[16]. TLRs are pattern recognition receptors among which TLR4 is the signaling receptor for lipopolysaccharide (LPS), the proinflammatory constituent of the Gram-negative cell wall. TLR4 deficient macrophages are unresponsive to LPS and mice with a genetic or functional TLR4 deficiency are protected from the toxic effects of LPS[17-19]. The adequate "sensing" of LPS by the host has been implicated as an important early event in the innate immune response to Gram-negative bacteria[20]. As a consequence, TLR4 deficient mice have been reported to have a reduced defense against a number of infections caused by Gram-negative bacteria including *Salmonella typhimurium*, *Klebsiella pneumoniae* and *E. coli*[21-26].

The main objective of the present study was to examine the innate immune response and the role of TLR4 during abdominal sepsis in the setting of pre-existing pancreatitis. For this we first studied the role of TLR4 in *E. coli* sepsis in previously healthy mice. Arguing that in the clinical setting Gram-negative sepsis frequently is a complication of another illness and that in particular acute pancreatitis can be complicated by Gram-negative infection of the peritoneal cavity, we thereafter examined the role of TLR4 in abdominal sepsis in mice with pre-existing pancreatitis.

Materials and methods

Animals

Female C3H/HeN (wild type, WT) mice and C3H/HeJ (TLR4 mutant) mice (Harlan, Horst, the Netherlands), 8-10 weeks old, were used in all experiments. C3H/HeJ mice have been demonstrated to have a mis-sense mutation in the third exon of TLR4, yielding a non functional TLR4[17, 18]. The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Experimental groups and animal models

Experimental groups and study designs are shown in Figure 1.

E. coli or saline i.p.

Study 1

4 hrs

20 hrs

WT and TLR4 mutant mice were injected with *E. coli* and sacrificed 4 or 20 hours thereafter

Groups: WT abdominal sepsis and TLR4 mutant abdominal sepsis

Cerulein or saline i.p (12 hourly injections)

Study 2

WT and TLR4 mutant mice were injected with cerulein (pancreatitis) or saline (sham)
and sacrificed one hour after the 12th cerulein injection

Groups: WT sham, TLR4 mutant sham, WT pancreatitis and TLR4 mutant pancreatitis

Cerulein or saline i.p (12 hourly injections)

E. coli or saline i.p.

Study 3

4 hrs

20 hrs

WT and TLR4 mutant mice were injected with cerulein (pancreatitis) or saline (sham),
one hour after the 12th cerulein injection mice were injected with *E. coli* and sacrificed 4 or 20 hours thereafter

Groups:
WT sham/abdominal sepsis (I),
TLR4 mutant sham/abdominal sepsis (II),
WT pancraetitis/abdominal sepsis (III)
TLR4 mutant pancreatitis/abdominal sepsis (IV)

Figure 1. Study design and experimental groups.

In study 1, the role of TLR4 in host defense against *E. coli* abdominal sepsis was established in previously healthy mice. Abdominal sepsis was induced as described previously[27-29]. WT and TLR4 mutant mice were injected intraperitoneally (i.p.) with 5 x 10^4 CFU *E. coli* O18:K1 in 200µl sterile saline. Mice were sacrificed 4 or 20 hours postinfection. Hence, in study 1, two experimental groups were studied: WT abdominal sepsis and TLR4 mutant

abdominal sepsis.

In study 2, the role of TLR4 in acute experimental pancreatitis was established. Acute pancreatitis was induced by 12 i.p. injections (one hour intervals) of 50 μg/kg cerulein (Research Plus, Manasquan, NJ), mice were sacrificed one hour thereafter. This model is widely established and results in acute pancreatitis in a highly reproducible way[9]. WT and TLR4 mutant mice received either 12 hourly injections of cerulein (pancreatitis) or saline (sham). Hence, in study 2, four experimental groups were studied: WT sham, TLR4 mutant sham, WT pancreatitis and TLR4 mutant pancreatitis.

In study 3, the role of TLR4 in the immune response to *E. coli* abdominal sepsis was evaluated in mice with pre-existing acute pancreatitis. For this WT and TLR4 mutant mice received 12 hourly cerulein (pancreatitis) or saline (sham) injections before i.p. injection of *E. coli* as described above. Mice were killed 4 or 20 hours after infection. Hence, in study 3, four experimental groups were studied: WT sham/abdominal sepsis (I), TLR4 mutant sham/abdominal sepsis (II), WT pancreatitis/abdominal sepsis (III) and TLR4 mutant pancreatitis/abdominal sepsis (IV).

Tissue handling

Before they were killed, mice were anesthetized with i.p. injection of 0.07 ml/g FFM (Fentanyl 0.315 mg/ml, Fluanisone 10 mg/ml (both Janssen, Beersen, Belgium), and Midazolam 5 mg/ml (Roche, Mijdrecht, The Netherlands). Blood was collected from the *vena cava inferior* in heparin coated vacutainer tubes and immediately placed on ice. Tubes were spinned for 10 min at 3600 rpm, after which the plasma was removed and stored at -20^0C. In study 2, one longitudinal dissected part of the pancreas was immediately removed and frozen in liquid nitrogen to prevent degradation and stored at -70^0C until further assays. Other parts of the pancreas were stored for histological analysis. In other studies, mice inhaled isoflurane (Forene, Abott, Queensborough, Kent, UK) and 5 ml of sterile saline was injected in the peritoneal cavity, the abdomen was massaged for 10 seconds and peritoneal lavage fluid (PLF) was aspirated and put on ice. Subsequently, mice received anesthesia with FFM and blood was collected as described above.

Histology

Pancreatitis severity was scored as previousy described[9, 30]. Parts of the pancreas were fixed in 4% formalin and embedded in paraffin. 4 μm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist who was blinded for groups. Briefly, necrosis,inflammation and edema were scored on a 0-3 scale, and a total severity score was calculated (maximum= 9 points).

Ex vivo macrophage stimulation

In a separate study, WT mice received 12 hourly i.p. cerulein or saline injections; one hour after the 12th injection peritoneal lavage was performed. Isolation and stimulation of peritoneal macrophages were performed as previously described[28]. In brief, peritoneal macrophages were washed, counted and resuspended in RPMI 1640 containing 1mM pyruvate, 2mM L-glutamine, penicillin (10,000 U/ml) and streptomycin (1000 µg/ml) in a final concentration of $1x10^5$ cells/ml. Cells were then cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn, The Netherlands) for 2h and washed. Adherent cells were stimulated with heat-killed *E. coli* (O18:K1, equivalent to 1 x 10^5 CFU/ml), or RPMI 1640 for 20h. Stimulations were carried out in the presence of 10% FCS (Gibco, Detroit, MI). Supernatants were collected and stored at –70°C until assayed.

Assays

Pancreatic edema was estimated by expressing relative organ weights as described[9, 31]. Amylase was determined with a commercially available kit (Sigma, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany). Results are expressed in international units per ml. Myeloperoxidase (MPO) content was measured in pancreas homogenates, as described elsewhere[32, 33]. Tumor necrosis factor (TNF)-α , Interleukin (IL)-6, IL-10 and KC were measured in PLF by ELISA (R&D Systems, Minneapolis, MN).

Cell counts and differentials

Cell counts were determined in PLF using an automated counter (Beckham Coulter, Model Coulter ZF, Mijdrecht, The Netherlands). Subsequently PLF was centrifuged at 1400 x g for 10 min; the pellet was suspended in PBS until a final concentration of 10^5 cells/ml and differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzerland).

Statistical analysis

For variables that were normally distributed (Kolmogorov-Smirnov test, P-value > 0.05), values are expressed as mean ± SE. Otherwise, values are expressed as median and interquartile range (IQR). For all variables, non-parametric tests were used to examine differences between groups (per time point). For two groups, a Mann-Whitney U test was used. For more than two groups, a Kruskall-Wallis test was used followed by Mann-Whitney U tests where appropriate. CFU data were log10 transformed before analysis. Throughout, a P-value < 0.05 was considered statistically significant. All statistical analyses were carried out using SPSS 12.02.

Results

TLR4 deficiency results in an increased bacterial load during abdominal sepsis in previously healthy mice.

To evaluate the role of TLR4 during primary abdominal sepsis healthy WT and TLR4 mutant mice were i.p. injected with *E. coli*. Whereas at 4 h postinfection the number of *E. coli* CFU was similar in PLF and blood from TLR4 mutant and WT mice, at 20 h TLR4 mutant mice displayed > ten times more *E. coli* CFU in PLF and > 1000 times more *E. coli* CFU in blood as compared to WT mice (Figure 2, P<0.05), indicating that TLR4 is important for an adequate antibacterial host response. Considering that cytokines and chemokines are important for the orchestration of the innate immune response to bacterial infection, we went on to investigate which of these mediators were influenced by the absence of functional TLR4. At 4 h post infection, TLR4 mutant mice had lower PLF concentrations of TNF-α, IL-6 and KC as compared to WT mice (Table I; all P<0.05). Remarkably, at 20 hours postinfection TLR4 mutant mice had higher IL-6, TNF-α, and KC PLF concentrations compared to WT mice (Table I; all P<0.05), whereas the PLF levels of the anti-inflammatory cytokine IL-10 were similar in both mouse strains at all time points (Table I). Given that neutrophil influx to the site of infection is important for an adequate innate immune response, we next determined leukocyte counts and differentials in PLF. In particular at 20 hours postinfection TLR4 mutant mice showed a remarkably reduced number of leukocytes in PLF as compared to WT mice, which was caused by a strongly impaired recruitment of neutrophils (Table II; P<0.05). Hence, this first study indicated that TLR4 deficiency results in an increased bacterial load during *E. coli* abdominal sepsis in previously healthy mice, which is related to an impairment of early cytokine release as well as migration of leukocytes to the site of i

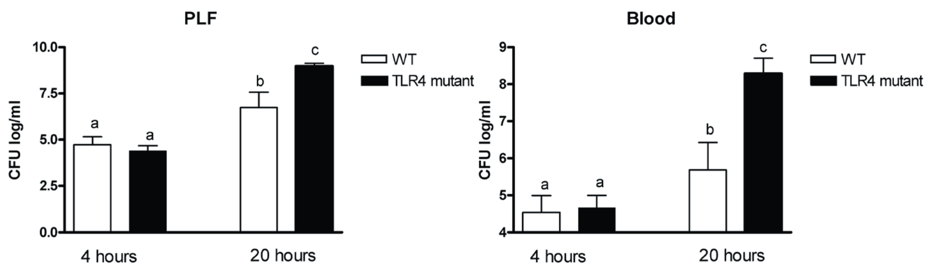

Figure 2. TLR4 deficiency results in an increased bacterial load during abdominal sepsis in previously healthy mice. WT and TLR4 mutant mice received an i.p. injection of 5 x 10^4 *E. coli* and were sacrificed 4 or 20 hours thereafter. Open bars represent WT mice and closed bars indicate TLR4 mutant mice. CFU derived from PLF and blood are shown. Data are means ± SE of 8 mice per group at each time point. a, b = Groups not sharing the same letter are significantly different from each other (P<0.05).

	4 hours		20 hours	
	WT	TLR4 mutant	WT	TLR4 mutant
TNF-α (pg/ml)	344 ± 6 [a]	112 ± 8 [b]	48 ± 4 [a]	112 ± 9 [b]
IL-6 (pg/ml)	227 ± 17 [a]	119 ± 8 [b]	614 ± 196 [a]	2898 ± 98 [b]
KC (pg/ml)	392 (146) [a]	148 (81) [b]	163 (32) [a]	998 (287) [b]
IL-10 (pg/ml)	98 (26) [a]	92 (21) [a]	354 (132) [a]	342 (101) [a]

Table I: Cytokine and chemokine levels in peritoneal lavage fluid of WT and TLR4 mutant mice with primary ***E. coli*** **peritonitis.** WT and TLR4 mutant mice received an i.p. injection of 5 x 10^4 *E. coli* in WT and TLR4 mutant mice and were sacrificed 4 or 20 hours thereafter. Data are mean ± SE or median (IQR), (n = 8 mice per group at each time point). a, b = Groups within one time point not sharing the same letter are significantly different from each other (P<0.05).

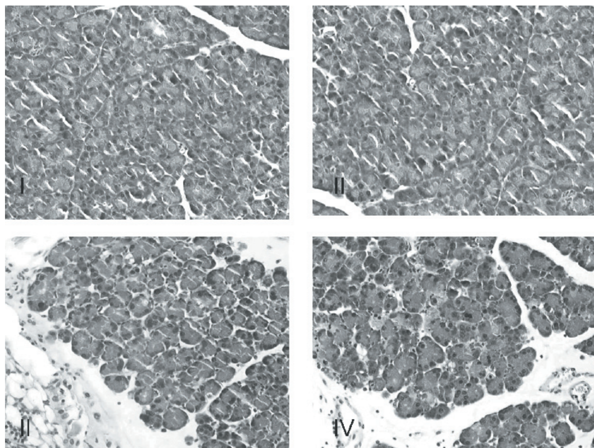

Figure 3. TLR4 does not contribute to the severity of cerulein-induced pancreatitis. Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 saline injections. Pancreas was harvested one hour after the 12th injection. Representative pancreas histology slides from a total of 8 mice per group are shown of sham (upper panels) and pancreatitis (lower panels) mice. Left panels (I and III) represent WT and right panels (II and IV) TLR4 mutant mice. Magnification X 10.

Pancreatitis severity is not influenced by the presence of TLR4.

Considering that the second objective of our study was to assess the role of TLR4 in the defense against *E. coli* sepsis in a host that is already afflicted by a disease (i.e. acute pancreatitis) that predisposes him to infectious complications, it was important to ensure that pancreatitis severity developed independently of TLR4. TLR4 mutant and WT mice received either 12 hourly i.p. injections of cerulein or saline. Induction of pancreatitis elicited profound increases in the plasma concentrations of amylase in WT and TLR4 mutant mice as compared to saline injected mice (Table III). Furthermore, relative pancreas weights, neutrophil recruitment to the pancreas and markers of histological damage were increased relative to saline injected mice but no differences were observed between pancreatitis WT and pancreatitis TLR4 mutant mice (Figure 3, Table III). Together these data clearly established that TLR4 is not involved in the pathogenesis of cerulein-induced acute pancreatitis.

	4 hours		20 hours	
	WT	TLR4 mutant	WT	TLR4 mutant
Cell count (10^5/ml)	5.1 (0.5) [a]	4.7 (0.7) [a]	62.3 (12.0) [a]	13.3 (3.1) [b]
Neutrophils	2.9 (0.6) [a]	2.0 (0.5) [a]	52.4 (8.1) [a]	8.1 (2.1) [b]
Macrophages	2.0 (0.2) [a]	2.6 (0.7) [a]	9.2 (2.1) [a]	5.1 (1.7) [b]

Table II: Leukocyte counts and differentials in peritoneal lavage fluid of WT and TLR4 mutant mice with primary *E. coli* peritonitis. WT and TLR4 mutant mice received an i.p. injection of 5 x 10^4 *E. coli* in WT and TLR4 mutant mice and were sacrificed 4 or 20 hours. Data are mean ± SE or median (IQR), (n = 8 mice per group at each time point). a, b = Groups within one time point not sharing the same letter are significantly different from each other (P<0.05).

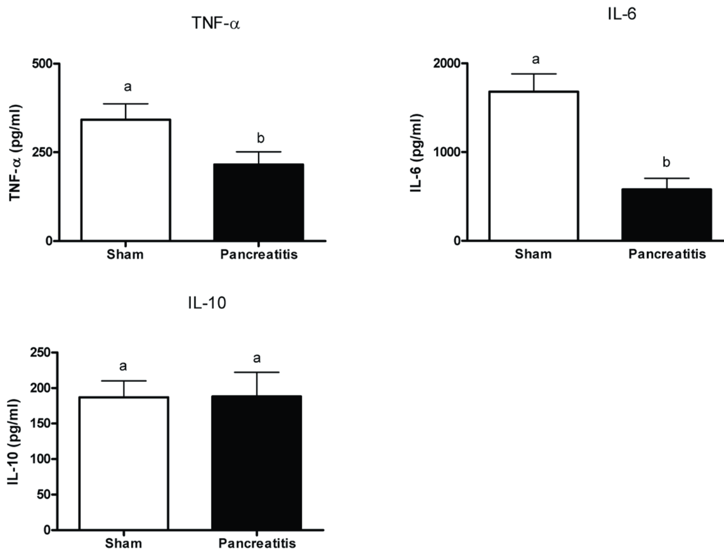

Figure 4. Pancreatitis is associated with reduced *E. coli* induced cytokine release by peritoneal macrophages. Pancreatitis was induced in wildtype mice by 12 hourly i.p. injections of cerulein; sham mice received 12 saline injections. Peritoneal macrophages were isolated one hour after the last injection and stimulated with heat killed ***E. coli*** for 20 hours. Subsequently, TNF-α, IL-6 and IL-10 levels were measured in the supernatant. Data are means ± SE of 8 mice per group. a, b = Groups not sharing the same letter are significantly different from each other (P<0.05).

Pancreatitis results in a diminished capacity of macrophages to release proinflammatory cytokines.

Since a reduced capacity of macrophages to produce proinflammatory cytokines is a hallmark of so called immunoparalysis[8], we next wished to establish the influence of experimentally induced pancreatitis on the release of cytokines by “ex vivo” stimulated peritoneal macrophages. Macrophages isolated from pancreatitis mice produced significantly less TNF-α and IL-6, but not IL-10, than macrophages isolated from sham treated mice (both

P<0.05; Figure 4).

Acute pancreatitis impairs cytokine release and increases the bacterial load during subsequent abdominal sepsis.

Having shown that pancreatitis induces a decrease in cytokine production by peritoneal macrophages, we evaluated whether pancreatitis impairs host defense during subsequent abdominal sepsis. In line with the fact that pancreatitis was associated with a reduced capacity of macrophages to produce proinflammatory cytokines upon "ex vivo" stimulation, pancreatitis/abdominal sepsis WT mice had lower PLF levels of TNF-α, IL-6 and KC 4 hours postinfection as compared to sham/abdominal WT sepsis mice, whereas the levels of IL-10 were comparable in both groups (P<0.05; Group I versus III, Table IV). At 20 hours postinfection, pancreatitis/abdominal sepsis WT mice had significantly more *E. coli* CFU in PLF and blood as compared to sham/abdominal sepsis WT mice (Figure 5, Group I versus III, both P<0.05). Notably, at this time point PLF levels of TNF-α, IL-6 and KC were higher in pancreatitis/abdominal sepsis WT mice than in sham/abdominal sepsis WT mice (all P<0.05). These findings were unrelated to the number of neutrophils that migrated to the peritoneal cavity, since neutrophil numbers were similar in these two WT groups at each time point (Table V).

	Sham		**Pancreatitis**	
	WT	TLR4 mutant	WT	TLR4 mutant
Plasma amylase (U/ml)	4.0 (0.0) [a]	4.5 (1.0) [a]	152.0 (54.2) [b]	119.0 (25.0) [b]
Histology score	1.0 (0.3) [a]	0.9 (0.3) [a]	7.0 (3.0) [b]	7.0 (1.5) [b]
Relative pancreas weight	6.2 (0.8) [a]	6.1 (0.8) [a]	9.6 (0.7) [b]	10.2 (2.8) [b]
MPO (% of WT sham)	100 (0.0) [a]	103 (30) [a]	199 (19) [b]	161 (21) [b]

Table III: TLR4 does not contribute to the severity of cerulein-induced pancreatitis. Pancreatitis was induced by 12 hourly i.p. injections of cerulein in WT and TLR4 mutant mice. Sham mice received 12 saline injections. Pancreas and plasma were harvested one hour after the 12th injection. Data are median (IQR), (n = 8 mice per group). MPO = myeloperoxidase. a, b = Groups not sharing the same letter are significantly different from each other (P<0.05).

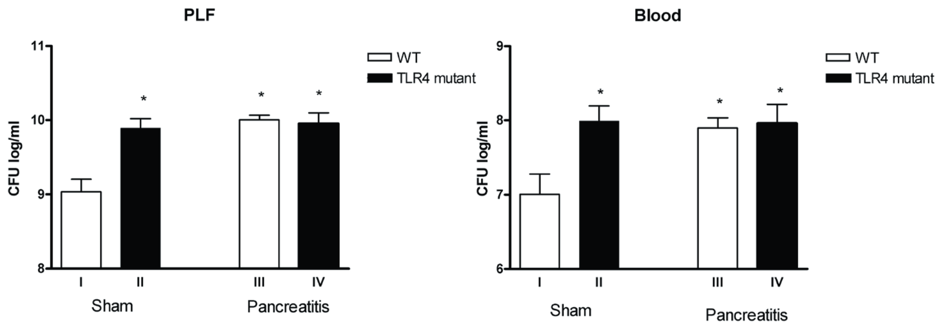

Figure 5. TLR4 deficiency results in an increased bacterial load during abdominal sepsis only in previously healthy mice. Pancreatitis was induced by 12 hourly i.p. injections of cerulein, sham mice received 12 saline injections. Abdominal sepsis was induced by i.p. injection of 5 x 104 E coli in WT sham/abdominal sepsis and TLR4 mutant sham/abdominal sepsis as well as WT pancreatitis/abdominal sepsis and TLR4 mutant pancreatitis/abdominal sepsis mice. Open bars represent WT mice and closed bars indicate TLR4 mutant mice. Mice were sacrificed 20 hours post infection. CFU derived form PLF and blood are shown. Data are means ± SE of 8 mice per group. a, b = Groups

TLR4 deficiency does not influence the bacterial load in abdominal sepsis complicating pancreatitis.

Concurrently with the two experimental WT groups described above, TLR4 mutant mice were subjected to the same protocol. In line with the results shown in study 1, sham/abdominal TLR4 mutant mice displayed significantly more *E. coli* CFU in PLF and blood 20 h postinfection, lower initial cytokine and chemokine release as well as significantly less influx of neutrophils, 4 as well as 20 hours postinfection, as compared to WT mice (Figure 5, Table IV and Table V, Group I versus II, P<0.05). In contrast, during pancreatitis/abdominal sepsis these differences between WT and TLR4 mutant mice were not reproduced. The number of *E. coli* CFU in PLF and blood was comparable between WT and TLR4 mutant pancreatitis/abdominal sepsis mice (Figure 5, Group III versus IV P<0.05). Furthermore, no difference in initial cytokine and chemokine release, or in neutrophil recruitment, was shown between WT and TLR4 mutant pancreatitis/abdominal sepsis mice (Table IV and Table V, Group III versus IV, P>0.05).

		Sham/abdominal sepsis		Pancreatitis/abdominal sepsis	
		I	II	III	IV
4 hours	TNF-α	210 ± 18 [a]	41± 3 [b]	32 ± 6 [b]	41 ± 3 [b]
	IL-6	316± 30 [a]	158 ± 10 [b]	236 ± 30 [a b]	219 ± 26 [a b]
	KC	388 (136) [a]	178 (78) [b]	100 (102) [b]	152 (113) [b]
	IL-10	103 (27) [a]	99 (39) [a]	85 (18) [a]	116 (17) [a]
20 hours	TNF-α	50 ± 5 [a]	88 ± 13 [b]	112 ± 12 [b]	120 ± 16 [b]
	IL-6	818 ± 72 [a]	3846 ± 621 [b]	3034 ± 194 [b]	3453 ± 185 [b]
	KC	173 (142) [a]	482 (1019) [b]	412 (762) [b]	1923 (1717) [b]
	IL-10	470 (200) [a]	414 (92) [a]	378 (92) [a]	392 (99) [a]

Table IV: Cytokine and chemokine levels in peritoneal lavage fluid during *E. coli* **peritonitis with or without pre-existing pancreatitis. Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 saline injections.** Thereafter, abdominal sepsis was induced by i.p. injection of 5 x 10^4 *E coli*.WT and TLR4 mutant mice were sacrificed 4 or 20 hours after infection. Please refer to figure 1 for a description of the groups. Note the difference between groups I and II which is not reproduced in groups III and IV. Data are mean ± SE or median (IQR), (n = 8 mice per group at each time point). a, b = Groups not sharing the same letter are significantly different from each other (P<0.05).

		Sham/ abdominal sepsis		Pancreatitis/ abdominal sepsis	
		I	II	III	IV
4h	Cell count (10^5/ml)	10.0 (1.0) [a]	9.0 (0.5) [a]	12.5 (3.5) [a]	12.8 (3.8) [a]
	Neutrophils	7.4 (0.7) [a]	4.9 (0.3) [b]	8.6 (2.4) [a]	8.9 (2.6) [a]
	Macrophages	2.6 (0.3) [a]	4.1 (0.2) [b]	3.9 (1.1) [b]	4.1 (1.4) [b]
20h	Cell count (10^5/ml)	249.5 (83.0) [a]	59.0 (13.5) [b]	300.9 (73.0) [a]	262.7 (66.7) [a]
	Neutrophils	229.5 (76.4) [a]	46.0 (10.5) [b]	300.9 (73.0) [a]	262.7 (66.7) [a]

Table V: Leukocyte counts and differentials in peritoneal lavage fluid of WT and TLR4 mutant mice with *E. coli* **peritonitis preceded by the induction of pancreatitis or sham.** Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 saline injections. Thereafter, abdominal sepsis was induced by i.p. injection of 5 x 10^4 *E coli*.WT and TLR4 mutant mice were sacrificed 4 or 20 hours after infection. Please refer to figure 1 for a description of the groups. Note the difference between groups I and II which is not reproduced in groups III and IV. Data are mean ± SE, (n = 8 mice per group at each time point). a, b = Groups not sharing the same letter are significantly different from each other (P<0.05).

Discussion

Abdominal sepsis is a common life threatening condition in the critically ill patient. TLR4 is the signaling component of the LPS receptor complex and is therefore thought to be essential in the innate immune response to Gram-negative infection. The present study sought to determine the role of TLR4 in host defense against abdominal sepsis induced by i.p. administration of *E. coli*, the most frequently isolated pathogen in this condition. Considering that abdominal sepsis often occurs in an already injured host, we compared the growth of *E. coli* and the accompanying innate immune response in WT and TLR4 mutant that were previously healthy or had been subjected to experimentally induced acute pancreatitis, a disease that regularly is complicated by intraabdominal and systemic Gram-negative infection. The main finding of our study is that whereas the presence of TLR4 reduces the bacterial load and the dissemination of *E. coli* in previously healthy mice, it does not play a role of importance in host defense against *E. coli* in mice with pre-existing pancreatitis. Hence, our data suggest that the pathogenetic significance of TLR4 in the immune response to Gram-negative infection at least in parts depends on the presence or absence of a pre-existing disease.

We show that mice lacking functional TLR4 release less TNF-α, IL-6 and KC early during the infection and have an impaired influx of neutrophils to the site of infection which ultimately results in an increased bacterial load. Our present experiments in previously healthy WT and TLR4 mutant mice are in line with an earlier report showing that the clearance of i.p. administered *E. coli* O18:K1 (which was also used here) is impaired in TLR4 mutant mice[21]. The importance of an adequate early TNF-α release during abdominal sepsis is further stressed by this report showing that pretreatment with TNF-α/IL-1β in this model restored bacterial killing and could rescue TLR4 mutant mice from lethality[21]. Furthermore, a recent investigation from our group showed that in mice lacking LPS binding protein, which facilitates the presentation of LPS to the LPS receptor complex, early TNF-α release, neutrophil recruitment and bacterial clearance are impaired in a similar fashion as described here in TLR4 mutant mice[28]. Whereas the absence of functional TLR4 clearly diminished early cytokine release during *E. coli* peritonitis in previously healthy mice, at 20h after infection cytokine concentrations were markedly higher in TLR4 mutant than in WT mice. These findings are in line with our earlier report on LPS binding protein deficient mice, which also displayed elevated TNF-α concentrations later on during *E. coli* infection when compared with WT mice[28].

Subsequently, we investigated whether the induction of pancreatitis influences the host response to abdominal sepsis. We established that peritoneal macrophages isolated from animals during pancreatitis display a reduced release of pro inflammatory cytokines in

response to heat killed *E. coli*. In vivo, pancreatitis resulted in a diminished early release of TNF-α, IL-6 and of the chemokine KC and an increased bacterial load in WT mice relative to WT mice without pancreatitis. Likely, the diminished early cytokine release of peritoneal macrophages resulted in an impairment of bacterial clearance in pancreatitis/abdominal sepsis mice[21, 28]. Previous reports have indicated that severe sterile injury renders the host more susceptible to secondary infection due to subsequent deactivation of the innate and adaptive immune system, which is usually referred to as `immunoparalysis”[1-3, 34]. It is known that systemic inflammatory responses caused by LPS or other related TLR stimuli in uninjured hosts can cause hyporesponsiveness to restimulation. In this study, we add a novel observation and show that pancreatitis induces hyporesponsiveness of peritoneal macrophages to *E. coli* ex vivo, as well as impairment of early cytokine release and bacterial clearance in vivo. This finding adds to the understanding why pancreatitis patients are especially vulnerable to develop secondary Gram-negative infections[6].

Interestingly, at 20h postinfection cytokine levels were higher in infected mice with pre-existing pancreatitis than in mice without pancreatitis. This finding indicates that the macrophage hyporesponsiveness caused by cerulein-induced pancreatitis is transient (consistent with the fact that pancreatitis produced by repeated cerulein injections is self-limiting[35, 36]) and/or can be overcome by higher bacterial loads (providing a stronger stimulus to release cytokines).

Having established that TLR4 as well as pancreatitis increase the bacterial load during subsequent abdominal sepsis in similar ways, we investigated the role of TLR4 in mice that were subjected to pancreatitis before induction of abdominal sepsis. We first established whether pancreatitis develops independently of TLR4. Indeed, all parameters of pancreatitis severity were equally increased in WT and TLR4 mutant mice after 12 hourly cerulein injections. This is in line with a recent study which evaluated the role of TLR4 in pancreatitis which was published while our study was in progress[37]. Surprisingly, in pancreatitis/abdominal sepsis mice no additional changes in bacterial load, neutrophil influx or cytokine levels were apparent in TLR4 mutant mice as compared to WT mice. This implicates that, whereas TLR4 deficiency as well as pre existent pancreatitis reduces initial cytokine release and increase the bacterial load during abdominal sepsis, in mice pre-injured by pancreatitis WT and TLR4 mutant mice show a comparable phenotype. Notably, the early cytokine response to *E. coli* was blunted in a similar way in previously healthy TLR4 mutant mice (without pancreatitis) and in WT mice with acute pancreatitis; in both cases the release of TNF-α, IL-6 and KC was diminished 4 hours after i.p. injection with bacteria. As discussed above, this attenuated early local inflammatory reaction likely at least in part was responsible for the increased bacterial load later on. Whereas in healthy mice TLR4 apparently is important for the recognition of *E. coli* by the host and thereby for the induction of an

inflammatory response, in mice with acute pancreatitis the responsiveness of immunocompetent cells, in particular macrophages, was reduced. These data suggest that acute pancreatitis results in a state of immunoparalysis (as illustrated by a reduced release of proinflammatory cytokines by peritoneal macrophages *ex vivo* and by a blunted early cytokine release during *E. coli* peritonitis *in vivo*) during which the role of TLR4 to subsequent challenges is minimized, explaining why an additional worsening of defense mechanisms was not observed in TLR4 mutant pancreatitis/peritonitis mice. A shortcoming of this study is the lack of survival data, which is related to the nature of the model used in this report. The peritonitis model used here is acute in onset and aggressive in nature. Furthermore, there is a small window in this model. Therefore the model is not optimal for the study of changes in mortality. However, since the *E. coli* model is timely, well defined, and uses a unibacterial inoculate (enabling us to evaluate bacterial outgrowth and dissemination) this model is better equipped to study the mechanisms of host defense during abdominal sepsis. Since this was the primary goal of our study we chose to use this model, whereas survival might have better been evaluated using the cecal ligation and puncture model.

Whereas most preclinical studies on sepsis are performed in previously healthy animals, animal models in which sepsis is preceded by a primary injury such as pancreatitis reflect the actual clinical setting more accurately. The discrepancy between the role of TLR4 during primary *E. coli* sepsis and *E. coli* sepsis complicating pancreatitis as shown in this study underlines the need to study the regulation of the innate immune response and potential new targets in the treatment of sepsis not only in healthy animals but also in experimental settings that make use of clinically relevant models of pre-existing critical illness.

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chapter 7

aspiration pneumonitis primes the host for an eaggerated inflammatory response during pneumonia

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Introduction

Aspiration of gastric contents occurs in various hospitalized patients, in particular those with a reduced consciousness[1, 2]. The associated lung injury, commonly referred to as aspiration pneumonitis, is primarily caused by gastric acid. Aspiration pneumonitis predisposes the host to development of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[3]. Although the exact underlying mechanisms for this association is unclear, it has been suggested that acid aspiration primes the lung for an enhanced inflammatory response to a subsequent challenge[4].
Nosocomial pneumonia is the leading cause of death from hospital-acquired infections, with an associated crude mortality rate of approximately 30 percent[5, 6]. Interestingly, risk factors for aspiration pneumonitis and nosocomial pneumonia are partly overlapping[1, 2, 7], and recent studies have suggested that aspiration pneumonitis may render the host more susceptible to bacterial pneumonia[8, 9]. In the present study we sought to further examine the influence of aspiration pneumonitis on the host response to subsequent pneumonia. For this, we induced pneumonia by intranasal (i.n.) inoculation with *Klebsiella (K.). pneumoniae,* a common nosocomial respiratory pathogen[7], in mice with or without preceding acid aspiration, and compared inflammatory responses and bacterial outgrowth in the lungs. In addition, arguing that tumor necrosis factor (TNF)-α has been implicated as an important mediator in various inflammatory lung diseases including aspiration pneumonitis, ALI/ARDS and pneumonia[10-13], we also evaluated the role of this pluripotent proinflammatory cytokine in lung inflammation during *Klebsiella* pneumonia in mice with or without preexisting aspiration pneumonitis.

Methods

Animals

Female C57BL/6 mice (Harlan, Horst, the Netherlands), 10-12 weeks old, were used in all experiments. The Institutional Animal Care and Use Committee of the Academic Medical Center approved the protocol.

Experimental groups & Design

Acid aspiration was done in essence as described[14, 15]. Mice were anesthetized by intraperitoneal injection of 0.07 ml/g FFM (Fentanyl 0.315 mg/ml, Fluanisone 10 mg/ml, both Janssen, Beersen, Belgium; Midazolam 5 mg/ml, Roche, Mijdrecht, The Netherlands). The trachea was exposed and mice were subjected to intratreacheal (i.t.) injection of 50 µl

of 0.1 N hydrochloric acid (HCl, pH 1.5) (Sigma, St Louis, MO) or an equivalent volume of normal saline using a 30 gauge needle, followed by the injection 100 µl of air to ensure distal delivery of acid. Sixteen hours thereafter, *Klebsiella* pneumonia was induced exactly as described[16, 17]. In short, *K. pneumoniae* (serotype 2; ATCC 43816, Rockville, MD) was cultured for 16 hours at 37°C in 5% CO_2 in Luria Broth. This suspension was diluted 1:100 in fresh medium and grown for 3 hours to midlogarithmic phase. Bacteria were harvested by centrifugation at 1500 x g for 15 minutes, washed twice in pyrogen-free 0.9 % NaCl, and resuspended in 10 ml 0.9 % NaCl. Before inoculation, mice were lightly anesthetized with inhaled isoflurane (Forene, Abott, Queensborough, Kent, UK). An inoculum of 50 µl of the bacterial solution, containing 9000 CFU, was administered intranasally (i.n.). Control mice were inoculated with 50 µl pyrogenic-free isotonic saline alone. The number of bacteria was confirmed retrospectively by serial dilution of the inoculum in sterile isotonic saline and culture on blood agar plates for 16 hours. In one of these studies, a neutralizing anti-mouse TNFα monoclonal antibody (TN3, 0.5 mg in 200 µl saline) was administered intraperitoneally 30 minutes before induction of *Klebsiella* pneumonia. TN3 is a well characterized antibody that at the dose given here effectively neutralized endogenous TNF-α in a variety of mouse models[18-21], including pneumonia [22]. In other experiments, *Klebsiella* lipopolysaccharide (LPS, 10 µg in 50 µl saline; Sigma) or saline was administered i.n. 16 hours after i.t. injection of HCl or saline. In a first series of experiments mice were sacrificed 4, 8, 16 or 24 hours after i.t. instillation of HCl or saline. In subsequent experiments, in which the effect of previous exposure to HCl on the responsiveness to *Klebsiella* was investigated, mice were killed immediately before administration of *K. pneumoniae* or *Klebsiella* LPS (*i.e.* 16 hours after HCl administration), or 24 hours after induction of pneumonia or 6 hours after i.n. delivery of LPS for evaluation of the parameters described below. These time points were chosen since they are considered to be representative for an adequate assessment of lung inflammation in these models[16, 17, 23].

Tissue handling

At the time of sacrifice, mice were anesthetized with FFM and blood was collected from the vena cava inferior in heparin coated vacutainer tubes. The lungs were removed and processed as described previously[16, 17, 24, 25]. Lung weight was expressed as mg of lung per gram of mouse to obtain relative organ weight. In the pneumonia studies, lungs were homogenized at 4^0C in 5 volumes of sterile 0.9% NaCl using a tissue homogenizer (Biospec Products, Bartlesville, OK) that was carefully cleaned and desinfected with 70% alcohol after each homogenization. Serial 10-fold dilutions in sterile isotonic saline were made of these homogenates, and 50 µl volumes were plated onto sheep-blood agar plates and incubated at 37^0C and 5% CO_2. CFU were counted after 24 hours. For measurement of cytokines and chemokines, lung homogenates were lysed in lysisbuffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl, 2 mM Triton (X-100), Pepstatin A, Leupeptin, Aprotinin (20ng/ml), pH 7.4) and

spinned at 1500 x g at 4^0C for 15 minutes; the supernatant was frozen at -20^0C.

Bronchoalveolar lavage

The trachea was exposed through a midline incision and cannulated with a sterile 22-gauge Abbocath-T catheter (Abbott, Sligo, Ireland). Bronchoalveolar lavage (BAL) was performed by instilling two 0.5 ml aliquots of sterile isotonic saline. 0.9-1 ml of lavage fluid was retrieved per mouse, and total cell numbers were counted from each sample in a haemocytometer. BAL fluid (BALF) differential cell counts were carried out on cytospin preparations stained with modified Giemsa stain (Diff-Quick; Baxter, McGraw Park, IL)

Histologic examination

Lungs were fixated in 4% paraformaldehyde in phosphate buffered saline and embedded in paraffin; 4 µm thick sections were stained with haematoxylin and eosin. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters as described previously[26]: interstitial inflammation, intra-alveolar inflammation, edema, endothelialitis, bronchitis, pleuritis, and thrombi formation. Each parameter was graded on a scale from 0 to 3 as follows: 0, absent; 1, mild; 2, moderate; and 3, severe. The total lung inflammation score was expressed as the sum of the scores for each parameter, the maximum being 21.

Assays

TNF-α, Interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1 and IL-10 were measured by Cytometric Bead Array according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Macrophage inflammatory protein (MIP)-2, keratinocyte derived chemokine (KC) and IL-1β were measured by ELISA's according to the manufacturer's recommendations (R&D systems, Minneapolis, MN). Protein levels in BALF were measured using the BCA protein kit according to the manufacturers instructions (Pierce, Rockford, IL).

Ex vivo stimulation of alveolar macrophages

Ex vivo stimulation of alveolar macrophages was performed as described previously with minor modifications[26]. In short, alveolar macrophages were harvested from mice 16 hours after saline or acid aspiration by BAL (n = 8 per group). Samples of two mice were pooled and total cell numbers were counted using a hemocytometer. Immediately thereafter cell counts were differentiated using Giemsa stained cytospins. Considering the total cell count and the number of alveolar macrophages in the samples, 1×10^5 alveolar macrophages per ml in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, penicillin, streptomycin, and 10% FCS were allowed to adhere in 96 wells plates (Greiner, Alphen a/d Rijn, The Netherlands) for two hours and washed with RPMI 1640 to remove nonadherent cells. Ad-

herent cells were stimulated with *K. pneumoniae* LPS for 16 h. Supernatants were collected and stored at -70°C until assayed for TNF-α.

Statistical analysis
All data are expressed as means ± standard error (SE). Comparisons between groups were conducted using analysis of variance and Mann Whitney U test where appropriate. Significance was set at $P < 0.05$.

Results

Time course of acid aspiration lung injury
I.t. instillation of HCl is a frequently used model to study aspiration pneumonitis in rodents. Although the majority of these experimental studies only determined the short term effects of acid aspiration, some investigations demonstrated that the inflammatory response to i.t. HCl is transient in nature, peaking during the first 6 hours[14, 15, 27, 28]. In order to establish the severity of the model in our hands, we first performed time course experiments in which we followed animals for 24 hours after i.t. administration of HCl or saline. Acid aspiration was not associated with lethality. Total cell counts in BALF showed a transient rise peaking after 4 hours, which was exclusively determined by a strong influx of neutrophils (Figures 1A and B). In addition, acid aspiration resulted in transient increases in cytokine concentrations in BALF peaking after 8 hours (shown for TNF-α and IL-6 in Figures 1C and D). Based on these results, we chose a 16-hour interval between acid aspiration and induction of pneumonia to study the effect of previous acid aspiration on the immune response to respiratory tract infection, considering that at this time point the inflammatory response to HCl was clearly lessening although still detectable (Figure 1 and Table 1). With this we sought to avoid eliciting additive inflammation due to simultaneous administration or administration shortly after each other of two inflammatory stimuli. Of note, histological analysis showed normal lung tissue 16 hours after acid aspiration, without any sign of inflammation, confirming the mild severity of this model (data not shown).

Acid aspiration increases pulmonary inflammation and bacterial outgrowth during *K. pneumoniae* pneumonia
Klebsiella pneumonia induced 16 hours after i.t. administration of HCl was associated with a strongly enhanced inflammatory response in the lung when compared to that observed in animals not previously exposed to acid. Indeed, lung concentrations of TNF-α, IL-1β and IL-6 were on average 10 times higher in mice that had received HCl, whereas chemokine levels were approximately twice as high in these animals; the pulmonary levels of the anti-inflammatory cytokine IL-10 were not different between groups (Table 2). Additionally, mice previously exposed to HCl demonstrated a stronger influx of neutrophils into their

BALF, increased pulmonary edema and higher BALF protein levels (Figure 2), and more pronounced inflammation upon histological analysis (Figure 3). Semiquantitative histology scores were significantly higher in HCl/pneumonia mice than in saline/pneumonia mice (13 ± 2 and 6 ± 2 respectively; P<0.05). The enhanced inflammatory reaction in these mice was accompanied by an increased outgrowth of *K. pneumoniae*: the bacterial load in mice administered with HCl was $2.4 \pm 0.9 \times 10^8$ per ml lung homogenate versus $7.6 \pm 0.3 \times 10^5$ per ml lung homogenate in mice that had not received HCl (P < 0.05).

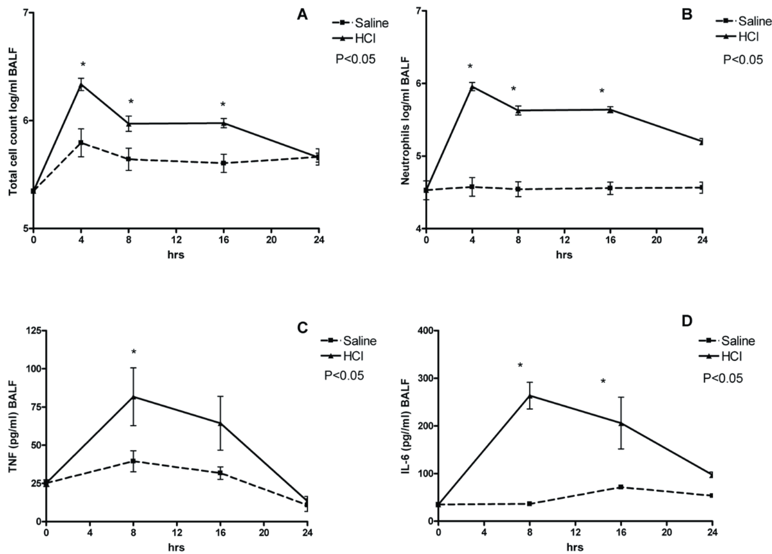

Figure 1. Cell and neutrophil counts and TNF-α and IL-6 levels in broncho alveolar lavage fluid after saline or acid aspiration. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Mice were sacrificed 0, 4, 8, 16 and 24 hours after aspiration. Data are mean ± SE (n = 5 mice per group). P value indicates curve comparison using two way ANOVA; * P<0.05 at indicated time point versus saline aspiration.

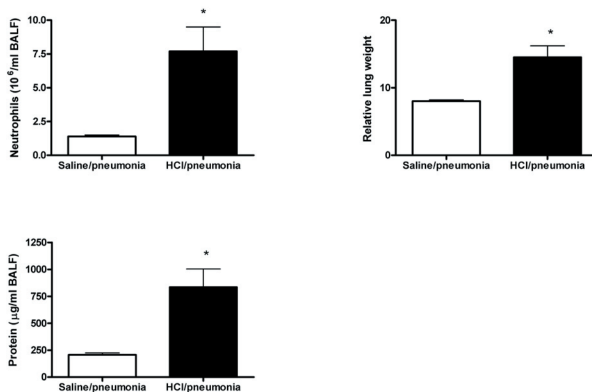

Figure 2. Aspiration pneumonitis increases neutrophil influx, pulmonary edema and BALF protein levels iduring pneumonia. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours later mice were i.n. inoculated with 9000 CFU *K. pneumoniae*. All mice were sacrificed 24 hours postinfection. Data are mean ± SE (n = 8 mice per group). *P<0.05 versus saline/pneumonia mice.

	Saline	HCl
Cytokines/chemokines (pg/ml)		
TNF-α	13 ± 1	32 ± 8*
IL-1β	193 ± 12	296 ± 46*
MCP-1	298 ± 14	714 ± 197*
IL-6	6 ± 1	119 ± 149*
IL-10	ND	495 ± 87*
KC	235 ± 36	495 ± 87*
MIP-2	266 ± 104	2820 ± 56*

Table 1: Lung cytokine and chemokine concentrations 16 hours after acid or saline aspiration. Mice received either 50 µl 0.1 N HCl or an equivalent volume of saline i.t. Cytokine and chemokine concentrations were measured in lung homogenates. Data are mean ± SE (n = 8 mice per group). ND = non detectable. *P < 0.05 versus saline.

Acid aspiration increases pulmonary inflammation induced by *Klebsiella* LPS

During bacterial pneumonia the bacterial load provides a potent proinflammatory stimulus in the lungs[12, 13]. Hence, the higher bacterial loads in the lungs of mice previously exposed to HCl could at least in part have been responsible for the exaggerated lung inflammation during *Klebsiella* pneumonia in these animals. To explore this possibility we administered *Klebsiella* LPS i.n. 16 hours after i.t. administration of HCl or saline, and evaluated the lung inflammatory reaction 6 hours later. Although the differences between the HCl and saline groups were less profound as compared with the pneumonia investigations described above, mice previously exposed to HCl displayed significantly more lung inflammation upon i.n. challenge with *Klebsiella* LPS. Indeed, lung TNF-α, IL-6, MIP-2 and KC levels were higher in mice administered with HCl (Table 3), and these mice demonstrated a stronger influx of neutrophils into their BALF, increased pulmonary edema and higher BALF protein levels (Figure 4), and more inflammation upon histological analysis (Figure 5). Histology scores were higher in HCl/LPS mice as compared to saline LPS mice, 11 ± 1 versus 8 ± 1 respectively, although this did not reach statistical significance (P=0.09). Together these data suggest that the increased inflammation during *Klebsiella* pneumonia in mice challenged with acid likely is caused by both priming of cells in the pulmonary compartment for a subsequent proinflammatory stimulus and the presence of a stronger proinflammatory stimulus due to the higher bacterial load.

	Saline/pneumonia	HCl/pneumonia
Cytokines/chemokines (pg/ml)		
TNF-α	67 ± 6	734 ± 278*
IL-1β	2014 ± 585	21786 ± 5786*
MCP-1	268 ± 17	856 ± 112*
IL-6	88 ± 7	837 ± 214*
IL-10	207 ± 41	216 ± 32
KC	898 ± 118	2078 ± 344*
MIP-2	1257 ± 295	2507 ± 331*

Table 2.: Aspiration pneumonitis increases lung cytokine and chemokine levels during *K. pneumoniae* pneumonia. Mice received either 50 µl 0.1 N HCl or an equivalent volume of saline i.t. Sixteen hours later mice were i.n. inoculated with 9000 CFU K. pneumoniae. All mice were sacrificed 24 hours postinfection. Cytokine and chemokine concentrations were measured in lung homogenates. Data are mean ± SE (n = 8 mice per group). *P < 0.05 versus saline/pneumonia.

Acid aspiration primes alveolar macrophages for enhanced TNF-α release

Alveolar macrophages are a major source for cytokines in the bronchoalveolar space[12, 13]. We therefore considered it of interest to determine the influence of acid aspiration on the production of cytokines by alveolar macrophages. For this mice received either HCl or saline i.t.; after 16 hours their alveolar macrophages were harvested and stimulated “ex vivo” with *Klebsiella* LPS. In line with the in vivo data described above, alveolar macrophages obtained from mice exposed HCl released significantly more TNF-α than alveolar macrophages harvested from mice administered with saline (Figure 6).

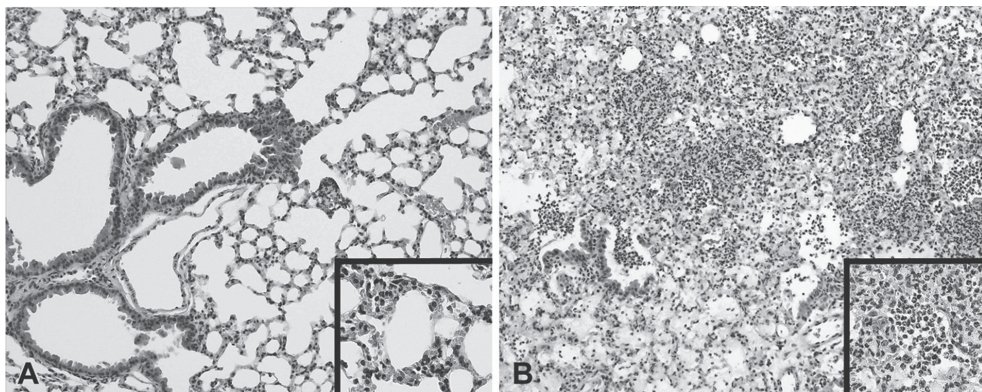

Figure 3. Aspiration pneumonitis increases pulmonary inflammation during pneumonia. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours later mice were i.n. inoculated with 9000 CFU *K. pneumoniae*. All mice were sacrificed 24 hours postinfection. Representative lung histology slides from saline/pneumonia (A) and HCl/pneumonia (B) mice are shown (n = 8 mice per group). H& E staining, magnification x 10, insert x 40.

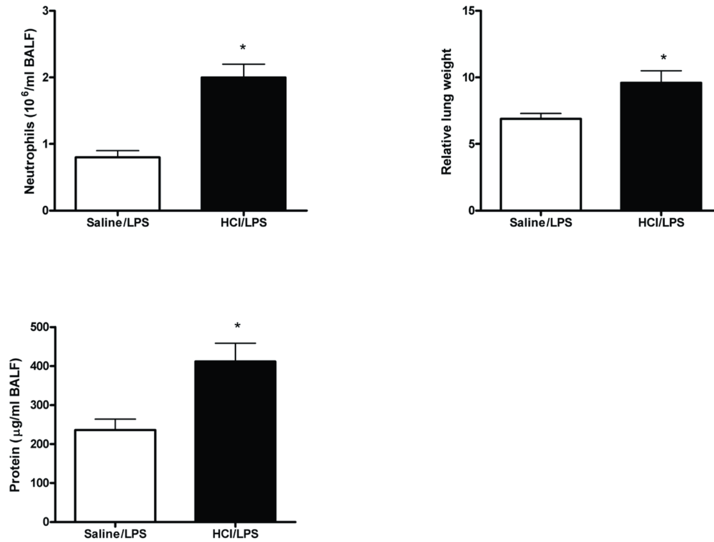

Figure 4. Aspiration pneumonitis increases neutrophil influx, pulmonary edema and BALF protein levels during *K. pneumoniae* LPS induced lung injury. Aspiration pneumonitis was induced by i.t. injection of 50 ul of 0.1 N HCl, control mice received saline. Sixteen hours later mice were i.n. inoculated with 10 µg *K. pneumoniae* LPS. All were sacrificed 6 hours after LPS inhalation. Data are mean ± SE (n = 8 mice per group). *P<0.05 versus saline/LPS mice.

Role of TNF-α in lung inflammation and bacterial outgrowth during primary and secondary *Klebsiella* pneumonia

TNF-α has been implicated as an important mediator in the innate immune response to *Klebsiella* pneumonia. Inhibition of TNF-α by a soluble TNF-α receptor-immunoglobulin construct has been found to reduce lung inflammation while facilitating bacterial outgrowth during *K. pneumoniae* pneumonia in previously healthy mice[29]. We here investigated whether this role for TNF-α is maintained during *Klebsiella* pneumonia preceded by acid aspiration. For this mice administered i.t with HCl or saline 16 hours before i.n. infection with *K. pneumoniae* received a neutralizing anti-mouse TNF-α antibody 30 minutes prior to induction of pneumonia. In mice that had received saline, anti-TNF-α treatment increased the inflammatory response during *Klebsiella* pneumonia, as reflected by higher IL-6, MIP-2 and KC concentrations in lung homogenates, higher relative lung weights (Table 4), and more inflammation upon histological analysis (Figure 7, A versus B) with histology scores of 6 ± 1 in control and 13 ± 2 in anti TNF-α treated mice (P<0.05). The increased inflammation in anti-TNF-α treated mice was accompanied by an approximately 100-fold higher bacterial load in the lungs of these animals ($3.0 \pm 1.0 \times 10^5$ versus $375 \pm 166 \times 10^5$ per ml lung homogenate respectively, P <0.05). By contrast, in animals previously exposed to HCl, anti-TNF-α did neither influence lung inflammation (Table 4, Figure 7, C versus D), histol-

ogy scores 14 ± 2 in control and 13 ± 2 in anti TNF-α treated mice (P>0.05), nor bacterial outgrowth (8 ± 4 x 10^7 per ml lung homogenate in anti-TNF-α treated mice versus 13 ± 6 x 10^7 per ml lung homogenate respectively in control mice, P = non significant). Of note, in these experiments acid aspiration increased lung inflammation during *Klebsiella* pneumonia in mice not treated with anti-TNF-α, confirming the results presented in Table 2 and Figures 2 and 3.

Cytokines/chemokines (pg/ml)	Saline/LPS	HCl/LPS
TNF-α	186 ± 21	245 ± 22*
IL-1β	2365 ± 411	3251 ± 649
MCP-1	443 ± 38	501 ± 56
IL-6	154 ± 13	313 ± 27*
IL-10	186 ± 22	282 ± 42
KC	2947 ± 154	5117 ± 898*
MIP-2	2965 ± 605	10623 ± 1144*

Table 3.: Aspiration pneumonitis increases lung cytokine and chemokine concentrations induced by Klebsiella LPS. Mice received either 50 µl 0.1 N HCl or an equivalent volume of saline i.t. Sixteen hours later mice were i.n. inoculated with 10 µg *K. pneumoniae* LPS; all mice were sacrificed 6 hours after LPS administration. Cytokine and chemokine concentrations were measured in lung homogenates. Data are mean ± SE (n = 8 mice per group). *P < 0.05 versus saline/LPS.

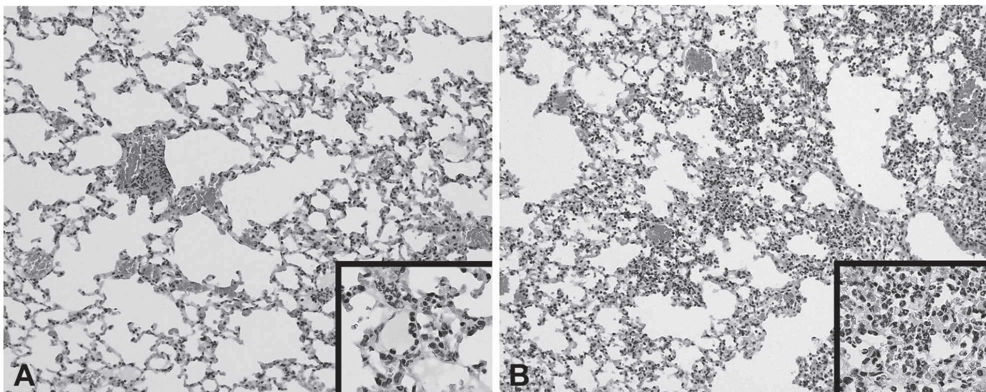

Figure 5. Aspiration pneumonitis increases pulmonary inflammation during *K. pneumoniae* LPS induced lung injury. Aspiration pneumonitis was induced by i.t. injection of 50 ul of 0.1 N HCl, control mice received saline. Sixteen hours after aspiration mice were i.n. inoculated with 10 ug *K. pneumoniae* LPS. All were sacrificed 6 hours after LPS inhalation. Representative lung histology slides from saline/LPS (A) and HCl/LPS (B) mice are shown (n = 8 mice per group). H& E staining, magnification x 10, insert x 40.

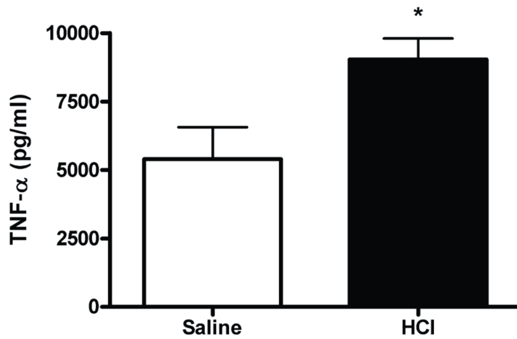

Figure 6. Aspiration pneumonitis primes alveolar macrophages for enhanced TNF-α release. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours later mice were subjected to broncho alveolar lavage. Freshly isolated AM e (n = 8 per group: cells from two mice were pooled yielding four samples per group) were incubated with *K. pneumoniae* LPS (10 µg/ml) for 16 h before measurement of TNF-α. *P<0.05 versus saline aspiration mice.

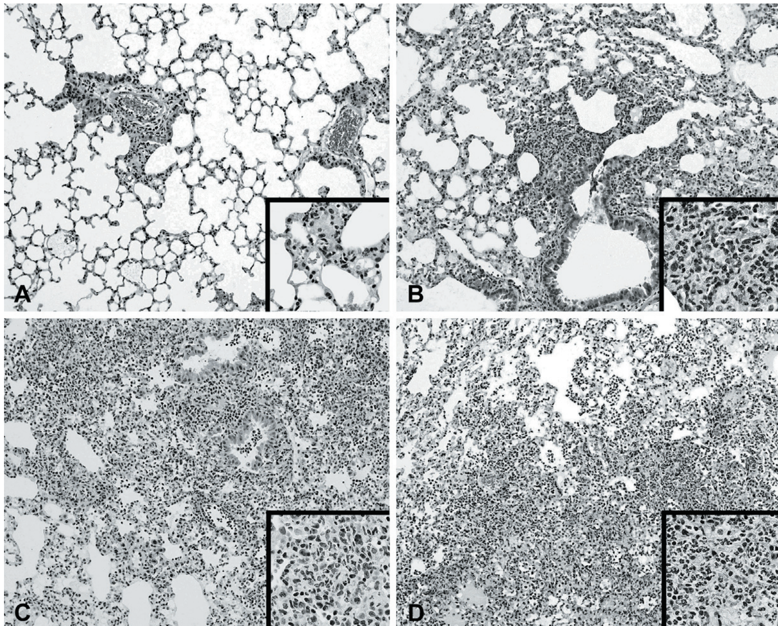

Figure 7. Anti TNF-α treatment increases inflammation during pneumonia preceded by saline aspiration but not during pneumonia preceded by aspiration pneumonitis. Aspiration pneumonitis was induced by i.t. injection of 50 µl of 0.1 N HCl, control mice received saline. Sixteen hours after aspiration mice were i.n. inoculated with 9000 CFU K. pneumoniae. Groups of saline/pneumonia (A and B) and HCl/pneumonia (C and D) mice were pretreated 30 minutes before induction of pneumonia with a neutralizing anti-TNF antibody (B and D). All mice were sacrificed 24 hours postinfection. H& E staining, magnification x 10, insert x 40.

Discussion

Nosocomial pneumonia is a common and severe complication in critically ill patients. Although some patients with nosocomial pneumonia do not suffer from preexisting lung disease, many display an inflammatory reaction in their pulmonary compartment, caused by various clinical conditions such as ventilator-induced lung injury, ARDS and aspiration pneumonitis, prior to developing pneumonia. The main objective of this study was to determine whether a mild and transient form of aspiration pneumonitis, induced by a single i.t. administration of HCl, influences the host response to subsequent *K. pneumoniae* pneumonia. We here show that previous exposure to HCl results in an exaggerated inflammatory reaction during pneumonia caused by both an increased bacterial outgrowth (resulting in a stronger proinflammatory stimulus) and priming of alveolar macrophages for the effect of *Klebsiella*. Inhibition of TNF-α, generally considered an important mediator of lung injury, did not reduce pulmonary inflammation in mice with pneumonia that had inhaled acid and even enhanced lung inflammation in mice with primary *K. pneumoniae* pneumonia.

Two earlier experimental studies investigated the pathogenesis of pneumonia in the host with aspiration pneumonitis[8, 9]. Mitsushima et al.[8] infected mice with *Pseudomonas aeruginosa* five minutes after i.t. delivery of HCl or saline and found an increased bacterial load in the lungs of acid treated animals 24 hours later; the associated lung inflammatory response was not evaluated in that study. Rotta et al.[9] infected rats with *Escherichia coli* one minute after i.t. administration of gastric aspirate and observed higher bacterial counts and more lung injury in these animals 24 hours later when compared with rats that had received saline earlier. Our study differs from these earlier investigations not only in that we used a different respiratory pathogen, but in particular in that we chose an interval of 16 hours between the acid and bacterial challenges. It is not unexpected that concurrent administration of HCl [8] or gastric aspirate[9] with bacteria results in an enhanced inflammatory response when compared with administration of bacteria only. Indeed, acid aspiration perse causes neutrophil recruitment and proinflammatory cytokine release into the bronchoalveolar space [9, 27, 28, 30, 31] (and the present study) and it can be anticipated that the simultaneous instillation of another proinflammatory stimulus (*i.e*. bacteria) will elicit even more lung inflammation. In the study by Rotta et al. [9] the severity of the lung injury and the extent of neutrophil influx into BALF in rats challenged with gastric aspirate and bacteria was not or barely different from that observed in animals challenged with gastric aspirate only, indicating that the aspiration pneumonitis rather than the bacterial pneumonia was the major determinant of the pulmonary response. This markedly differs from our study, in which the acid-induced inflammatory response was waning at the time pneumonia was induced and lung histology was normal. We consider this timing of subsequent challenges closer to the clinical scenario and to provide more insight in the pathogenesis of secondary pneumonia in the host with pre-

existing aspiration pneumonitis. Our data show that even if the introduction of bacteria into the respiratory tract is delayed for 16 hours after induction of a mild aspiration pneumonitis, the lungs respond with an exaggerated inflammatory response. Considering that previous acid exposure resulted in a much stronger increase in lung inflammation after infection with live *K.pneumoniae* than after instillation of *Klebsiella* LPS, and considering that bacterial loads in mice administered with HCl were 1000-fold higher than in saline treated mice, it is likely that the enhanced lung inflammation during post-aspiration pneumonitis pneumonia is caused by a combination of the presence of a stronger proinflammatory stimulus (*i.e*. more bacteria) and triggering for an increased inflammatory reaction to a given stimulus. At least part of this augmented inflammatory response is caused by priming of alveolar macrophages; indeed, our results suggest that aspiration pneumonitis renders alveolar macrophages more sensitive for subsequent inflammatory stimuli.

The mechanisms by which aspiration pneumonitis results in a reduced resistance against respiratory pathogens have not been fully elucidated. Likely, direct damage of the epithelial barrier lining the respiratory tract by a caustic effect of acid plays a role herein. In addition, acid exposure may stimulate the adherence of bacteria to airway epithelial cells[8, 32]. It should be noted that an adequate inflammatory response in the lung to invading bacteria is important for an effective host defense. Indeed, inhibition of the activity of proinflammatory cytokines has been found to facilitate the outgrowth of *K. pneumoniae* and other respiratory pathogens in mouse models of primary pneumonia[12, 13]. Apparently, the overexuberant inflammation in mice with preexisting aspiration pneumonitis does not help the host in mounting a stronger defense against infection.

TNF-α has been implicated as an important factor in the pathogenesis of various inflammatory lung diseases[10-13]. TNF-α concentrations have been found elevated in BALF of patients with ARDS or pneumonia[33-37], and in experimentally induced aspiration pneumonitis inhibition of TNF-α attenuated lung inflammation[38, 39]. Moreover, several lines of evidence indicate that this pluripotent cytokine is of eminent importance for an adequate host response to *K. pneumoniae* pneumonia. In mice with primary *Klebsiella* pneumonia, inhibition of endogenously produced TNF-α with a soluble TNF receptor-immunoglobulin construct resulted in an enhanced outgrowth of bacteria[29]. Moreover, transient transgenic expression of TNF-α within the murine respiratory tract, using a recombinant adenoviral vector containing TNF-α cDNA, enhanced the clearance of *K. pneumoniae* from the lung[40]. Interestingly, the beneficial effect of transgenic TNF-α expression was dose dependent and high doses of the adenoviral vector enhanced lethality due to *Klebsiella* pneumonia[40]. In our current investigation we found > 10-fold higher TNF-α concentrations in the lungs of mice with *K. pneumoniae* pneumonia and preexisting aspiration pneumonitis when compared with mice with primary pneumonia. In light of the pivotal role of TNF-α in lung inflammation [10, 11] and

the “double-edged” role of TNF-α in *Klebsiella* pneumonia, we considered it of interest to examine the function of TNF-α in respiratory tract infection by *K. pneumoniae* following aspiration pneumonitis. In line with the study by Laichalk et al.[29], anti-TNF-α treatment strongly enhanced the outgrowth of *K. pneumoniae* in mice that received saline i.t. 16 hours before induction of pneumonia, which was accompanied by increased lung inflammation. However, in mice in which *K. pneumoniae* infection followed aspiration pneumonitis, anti-TNF-α did neither influence lung inflammation nor bacterial outgrowth. Together these data suggest that the relatively low TNF-α concentrations detected after primary *Klebsiella* pneumonia are of utmost importance for an appropriate immune response to the invading bacteria, and that inhibition of TNF-α in this setting facilitates bacterial multiplication and as a consequence thereof inflammation. In contrast, in mice with preexisting aspiration pneumonitis the strongly elevated TNF-α during pneumonia is part of an exaggerated overall proinflammatory response in the lung and by itself TNF-α does not impact on inflammation or antibacterial defense. This finding is in line with earlier observations with transgenic overexpression of TNF-α in murine *K.pneumoniae* pneumonia cited above.

In conclusion, we here demonstrate that aspiration pneumonitis triggers the host to an enhanced inflammatory response to *K. pneumoniae* concurrently facilitating bacterial outgrowth. Although TNF-α plays a pivotal role in host defense against primary *Klebsiella* pneumonia, in the host with preexisting aspiration pneumonitis the strongly elevated TNF-α concentrations in the lungs are not involved in the enhanced lung inflammation or the increased bacterial outgrowth. Whereas most preclinical studies on nosocomial pneumonia are performed in previously healthy animals, animal models in which pneumonia is preceded by a primary injury such as aspiration pneumonitis likely reflect the clinical setting more accurately. The different roles of TNF-α in primary and secondary *K. pneumoniae* pneumonia underline the need to study the pathogenesis of pneumonia and potential new treatment targets not only in healthy animals but also in animals with preexisting disease.

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chapter 8

che central and autonomous nervous system; essential regulators of the immune response

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Introduction

"The brain is the last and grandest biological frontier, the most complex thing we have yet discovered in our universe. The brain boggles the mind."
James D. Watson (from *Discovering the Brain*, National Academy Press, 1992)

Of all the organs in our body, the brain is the most versatile and poses the greatest mystery. It contains hundreds of billions of cells interlinked through trillions of connections which generate our thoughts, houses our "soul", makes us sense, feel and move through the somatic and sensory nervous system and orchestrates essential life functions through the autonomic nervous system. The brain consists of extensively connected subsystems, such as the brain stem, limbic system, hypothalamus and cerebral cortex and many others which are all directly or indirectly connected to sensory (afferent) and effector (efferent) pathways reaching virtually any location in the body. The autonomic nervous system relays input on all vital processes in the body to the brain and controls our heart rate, respiratory rate, blood pressure, gut motility, body temperature and virtual any essential involuntary process. One could say that in any vital process that takes place there is "always a brain attached". Although it is widely accepted that virtually any vital process is supervised and controlled by the central nervous system (CNS), our innate immune response has long been regarded as a totally peripheral system regulated by the interaction of immune competent cells with pathogens and with each other. In this article, we will review data that suggest that communication between the immune, nervous and endocrine systems is in fact an essential homeostatic system that regulates the innate immune response. First we will discuss the classic regulation of innate immunity and the physiological anatomy of the autonomic nervous system; second we will discuss new insights in immune to brain and brain to immune communication and finally focus on the cholinergic anti inflammatory pathway and the important physiological insights and therapeutic opportunities that arise from the concept that activation of innate immunity is, at least in part, regulated in the CNS.

Classic regulation of innate immunity, the balance between pro and anti inflammation

Inflammation is a physiologic response of the host to either invasion of micro organisms or local injury. The innate immune system is the first line of defense against invading pathogens. Invading pathogens activate the cells of the innate immune system by a variety of, pathogen class specific, stimuli[1]. As a result of these triggers immune competent cells are activated to release a plethora of pro-inflammatory soluble mediators, such as cytokines (e.g. tumor necrosis factor (TNF-α)-α, Interleukin (IL)-1) and chemokines (e.g. IL-8)[2].

Goal of this inflammatory reaction is to restrain and localize the infection to the infected compartment of the body and ultimately clear the pathogen and remove the injury.

However, the release of all these mediators can be detrimental to the host[3]. Inflammatory reactions must be fine-tuned and regulated in a precise manner since exaggerated inflammation may lead to tissue damage and morbidity. Indeed, in recent years many common diseases have been recognized as “inflammatory conditions”, including atherosclerosis, ischemia reperfusion injury, rheumatoid arthritis, inflammatory bowel disease and fulminant sepsis[2, 4]. In these diseases, there is an induction of activation of the innate immune system, activation and migration of neutrophils and the cytokine network. To keep the potentially detrimental effects of the pro-inflammatory system in check an anti-inflammatory cytokine system (e.g. IL-10, IL-4) counterbalances the pro inflammatory systems. In health and disease, the balance between pro and anti inflammatory systems is essential to maintain a delicate homeostasis which ensures an adequate host defense with minimal collateral damage due to over aggressive responses of the innate immune system. On the basis of this concept, new therapies have been developed in recent years. Elimination of TNF-α by monoclonal antibodies restores the inflammatory balance and efectively treats “pro inflammatory diseases” such as Crohn’s disease and rheumatoid arthritis [5].

Physiological anatomy of the autonomic nervous system

The autonomic nervous provides constant and extremely rapid control of the visceral functions, such as arterial pressure, gastrointestinal motility and secretion, urinary bladder emptying, sweating, body temperature and many other activities of which some are totally and others partially controlled by the autonomic nervous system. The autonomic nervous system consists of sensory neurons and motor neurons that run between the CNS (especially the hypothalamus and medulla oblongata) and internal organs. It differs from the sensory-somatic system in using two groups of motor neurons to stimulate the effectors instead of one. Preganglionic neurons arise in the CNS and run into a ganglion where they synapse with a postganglionic neuron that runs all the way into the effector region. The autonomic nervous system has two subdivisions that usually act reciprocally of each other; the sympathetic and the parasympathetic nervous system.

The motor neurons of the sympathetic nervous system arise in the spinal cord and pass into two large chains of perivertrebral ganglia. Here the preganglionic neuron can synapse directly with a postganglionic neuron, travel up or down the chain to synapse in a ganglion at another level with postganglionic neurons or travel up or downward and terminate in one of the prevertebral ganglia. Either way, from the ganglion the postganglionic fibers travel

all the way to their destination in the various organs where norepinephrine (NE) is released by the postganglionic neuron. One exception is the sympathetic innervation of the adrenal medulla. Here, preganglionic fibers travel all the way to the medulla without synapsing and end directly on neuronal cells that secrete epinephrine and NE into the blood stream. The release of NE results in stimulation of the heartbeat, a raise in blood pressure, dilatation of the bronchi, a shunt of blood away from non-vital organs and an inhibition of bladder and gastrointestinal peristalsis. Activation of the sympathetic nervous system results in generalized responses since preganglionic neurons usually synapse with many postganglionic neurons and also because the release of epinephrine by the adrenal medulla ensures that even body sites that are not reached by postganglionic sympathetic neurons will be drenched with epinephrine. In short, stimulation of the sympathetic branch of the autonomic nervous system prepares the body for emergency: which are usually referred to as “fight or flight” reactions.

About 75% of parasympathetic nerves fibers arise form the tenth cranial nerves, the vagus nerves. Other sympathetic innervation comes from cranial nerves III, VII and IX and from the second and third sacral spinal nerves. The two vagus nerves originate in the medulla oblongata and wander all the way through the cervical area (where one vagus nerve travels right and the other left of the trachea) and the thoracic and abdominal regions of the body. At several locations both vagus nerves meet and share fibers with each other. The vagus nerve supplies parasympathetic innervation to the heart, the lungs, the esophagus, the small intestine, the proximal half of the colon, the liver, the pancreas, the ureters and the spleen. Just as the sympathetic nervous system the vagus nerve has both preganglionic and postganglionic neurons. However, the preganglionic fibers travel uninterrupted to their destination and synapse with postganglionic fibers inside the target organ where short postganglionic fibers spread throughout the organ. Parasympathetic stimulation causes slowing of the heartbeat, lowering of blood pressure and activation of gastrointestinal peristalsis. In short, parasympathetic innervation returns the body to its resting state after it has been activated by the sympathetic nervous system.

Sympathetic and parasympathetic neurons all secrete one of the two synaptic neurotransmitters, acetylcholine (Ach, cholinergic neurons) or NE (adrenergic neurons). All preganglionic neurons as well as the postganglionic neurons of the parasympathetic division are cholinergic whereas (except for sweat glands) postganglionic sympathetic neurons are adrenergic. Before Ach or NE can exert effects on the target organ they need to bind to specific receptors. Usually binding of a neurotransmitter to a receptor results in either a change in cellular membrane permeability or in the alteration of intracellular enzymes, such as in the case of NE binding where cyclic AMP (cAMP) is formed in response to receptor binding[6]. Ach activates two different receptors, muscarinic and nicotinic Ach receptors.

Muscarinic receptors are found in all effector cells stimulated by postganglionic neurons whereas nicotinic receptors are found in the synapses between pre and postganglionic neurons. Adrenergic receptors can be divided in α and β receptors. Stimulation of α and β receptors have sometimes contrary effects on target organs, implicating that the effects of epinephrine and NE on organs is dependent on the type of receptors present in the particular organ.

Interactions between the CNS and the immune system

The CNS and the immune system have several important features in common. Both systems are designed to constantly survey the body for danger and mount an appropriate response to these treats. In contrast to classical thinking, these two systems act together in orchestrating the immune response in response to infection or injury. Stimulation or ablation of several regions of the brain can alter immune responses; secondly, inflammatory processes can alter the firing rate of CNS neurons. Thus, there is a cross talk between inflammatory cells and the CNS that can go towards as well as from an inflamed site of the body.

Immune to brain communication

The CNS receives sensory input from the immune system through both humoral and neural routes (Figure 1). IL-1β, TNF-α, and other immunological active mediators, can signal the brain in circumscript areas[7]. These so called circumventricular organs include specific sites in the hypothalamus as well as the dorsal vagal complex (DVC)[8]. The DVC consists of the nucleus tractus solitarius (NTS), the dorsal motor nucleus of the vagus (DMN) and the area postrema (AP) (Figure 1)[9]. The DMN is the major site of origin of efferent vagal neurons whereas the main portion of vagal sensory input is received by neurons in the NTS. The AP, which lacks a tight blood-brain barrier, is an important circumventricular organ and site for humoral immune-to-brain communication[7]. In fact, reversible inactivation of the DVC completely blocks endotoxin induced behavioral changes and expression of c-FOS (neuronal activation marker) in forebrains regions of endotoxemic animals[8]. Exactly how cytokines are able to cross the blood brain barrier at these sites and activate the CNS is a matter of debate. Some studies suggest there is active transport of cytokines across the blood brain barrier[6]. Others implicate receptors for cytokines and for bacterial fragments that are constitutively expressed in cells within circumventricular organs and upregulated during inflammation[7]. Binding of cytokines to these receptors induces responses including changes in electrical activity of neurons, induction of transcription factors leading to modifications in gene expression during inflammation and to a localized release of secondary signal molecules which are able to cross the blood brain barrier[7].

Neural pathways, predominantly the vagus nerve, also signal the brain for danger (Figure 1)[10]. Cytokines and bacterial products such as endotoxin stimulate afferent neural fibers in

the vagus nerve that are processed in the brain and result in the initiation of an acute phase response, induction of fever and upregulation of IL-1β in the brain [10-12].
Immunogenic stimuli activate vagal afferents either directly by cytokines released by inflammatory cells at the site of infection or injury or indirectly through chemoreceptive cells located in vagal paraganglia[13]. After stimulation by cytokines, vagal afferent fibers transmit signals to the DVC[13, 14] where most sensory information is relayed to the NTS.

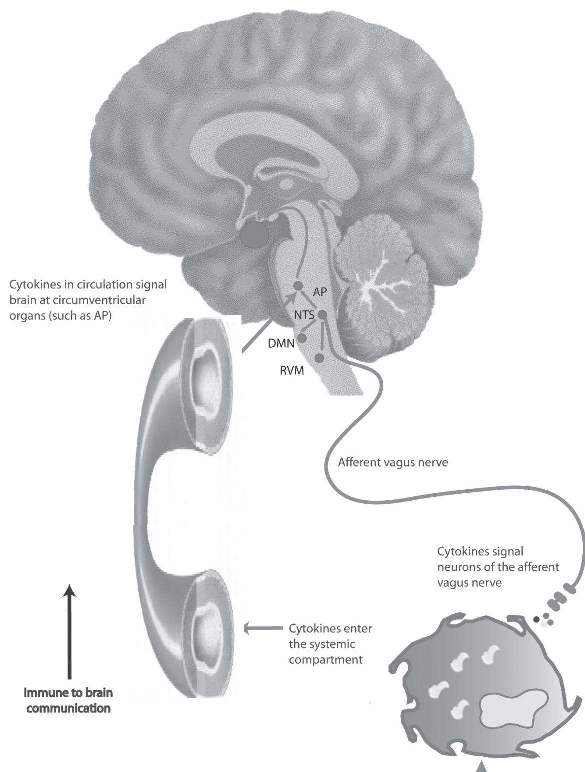

Figure 1: Immune to brain communication. The brain receives information from the immune system by humoral as well as neural pathways. Cytokines elicit signaling in the afferent vagus nerve which reach the nucleus tractus solitarius (NTS). From here, projections lead via the area postrema (AP) to the hypothalamus and subsequently the pituitary gland , and also to the dorsal motor nucleus (DMN) of the vagus nerve and the rostro ventrolateral medulla (RVM). All these nuclei are involved in generating responses of the brain back to the immune system (see figure 2). Via the humoral route cytokines can signal through circumventricular organs such as the AP.

Whether humoral or neural pathways are essential in relaying information on the presence of inflammation to the brain is largely dependent on the magnitude of the inflammatory response. In experimental studies it has been shown that when the level of inflammation is low, such as when a low dose of endotoxin is injected intraperitoneally, vagotomy inhibits the stimulation of the hypothalamus-pituitary-adrenal (HPA) axis and the induction of IL-1 in the brain[10, 11] whereas high doses of endotoxin induce responses by the brain independent of the vagus nerve[15, 16]. This implicates that neural pathways are essential in the relay of localized inflammation whereas information about severe systemic inflammation reaches the brain predominantly through humoral pathways.

Brain to immune communication

Neuro-endocrine pathways

When notified of ongoing inflammation, either by humoral or neural pathways as described above, the brain excerts strong anti inflammatory effects through activation of the HPA axis (Figure 2). Information received via the afferent vagus in the NTS is relayed to the hypothalamus which may induce release of α-melanocyte stimulating hormone (α-MSH) and corticotropin releasing hormone (CRH). CRH induces adrenocorticotropin hormone rlease (ACTH) by the pituitary gland and activates a neural-endocrine anti inflammatory pathway[11, 13, 17]. Upon ACTH stimulation, cortisol is released by the adrenal medulla. Cortisol inhibits pro inflammatory gene expression by immune cells by binding to an intracellular receptor and subsequent suppression of nuclear factor kappa B (NFκB) activity as well as activation of transcription of anti inflammatory genes[18].

Hard wired connections

The CNS and the immune system are directly linked through the autonomic nervous system (Figure 2). Direct contact between postganglionic neurons of the autonomic nervous system and immune cells, either with immune cells in lymphoid organs or with residential or migrated immune cells located in an inflamed area, provides a direct hard wired link for the CNS to modulate inflammatory responses in vivo. Lymphoid organs are innervated by the parasympathetic nervous system as well as the sympathetic nervous system[19]. Furthermore, lymphocytes, granulocytes and macrophages have been shown to carry receptors for Ach[20, 21] as well as NE[22] but also for various other substances released by neurones such as vaso intestinal peptide[23], α-MSH[24] and leptin[24].

Sympathetic nervous system, stimulation of beat receptors on immune cells

The autonomic nervous system is activated upon detection of inflammation either directly or via activation of the NTS through the afferent vagus nerve. With regard to the sympathetic nervous system, there are connections of the NTS with nuclei, such as the rostro ventrolateral medulla (RVM), that activate the sympathetic nervous system (Figure 2)[25]. Activation of preganglionic neurons of the sympathetic nervous system induces the release of epinephrine by the adrenal medulla into the bloodstream, converting a neural pathway into an endocrine anti-inflammatory pathway, since in response to catecholamines monocytes release less pro inflammatory mediators and are stimulated to produce IL-10[22, 26, 27] [6]. The major importance of this pathway is shown by experiments were the infusion of β agonists in humans and animals reduces inflammation during experimental endotoxemia whereas β receptor antagonists stimulate pro-inflammatory responses[22]. The importance of

the sympathetic nervous system in inhibiting the immune response is illustrated by several elegant experiments. In a mouse model of stroke, the hypothesis that a stroke-induced immunodeficiency increases the susceptibility to bacterial infections was tested. Indeed, mice developed spontaneous pneumonia within three days after the induction of stroke. Administration of the β adrenoreceptor antagonist propranolol drastically reduced the incidence of pneumonia, the defect in lymphocyte activation and mortality after stroke[27]. This suggests that immunosuppression during stroke is actually catecholamine-mediated. Furthermore, NE is released by postganglionic neurons which directly affects nearby immune cells through β receptors (Figure 2).

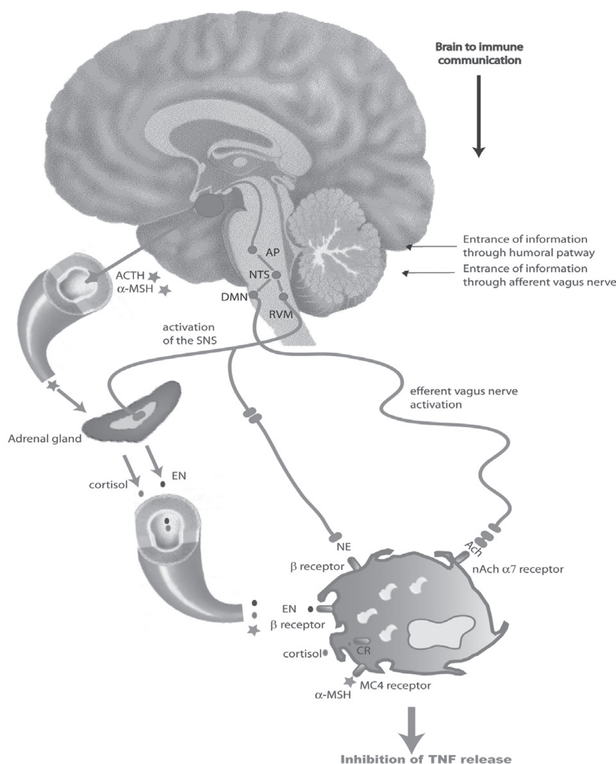

Figure 2: Brain to immune communication. Brains to immune pathways are activated by signals reaching the brain though the systemic compartment and the vagus nerve. The brain responds by activating a neuro-endocrine pathway through the release of adrenocorticotropin releasing hormone (ACTH), which activates cortisol release by the adrenal medulla as well as a-melanocyte stimulating hormone (a-MSH). Cortisol inhibits immune cells through intracellular receptors, a-MSH through the MC4 receptor. Also, vagal efferent activity is activated through the dorsal motor nucleus (DMN), which results in the release of Acetylcholine (Ach) at vagal post synaptic neurons, inhibiting immune cells through the nicotinic Ach α 7 receptor. Furthermore the sympathetic nervous system (SNS) is activated and epinephrine (EN) is released by the adrenal cortex and norepinephrine (NE) at postsynaptic SNS neurons. Both EN and NE inhibit immune cells through b-receptors.

Parasympathetic nervous system; the cholinergic anti-inflammatory pathway

Upon activation of the NTS, projections to the AP stimulate the HPA axis and projections to the RVM the sympathetic nervous system (Figure 3). However, direct connections between the NTS and the DMN ensure that when signals reach the NTS vagal efferent activity is stimulated as well (Figure 3). Release of Ach by postganglionic neurons of the vagus nerve inhibits the release of pro inflammatory cytokines by immune cells (Figure 3).

In vitro studies have shown that immune cells are susceptible to Ach. When macrophages are exposed to Ach, the principle parasympathetic neurotransmitter, these cells are effectively deactivated[20]. This Ach-induced deactivation is characterized by a dose-dependent reduction in the release of a series of proinflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-18, by macrophages stimulated with endotoxin[20]. Ach acts through two types of receptors: muscarinic and nicotinic. In addition to the brain and "wire-innervated" peripheral structures, these Ach receptor subtypes are also expressed by immune cells[17, 21, 28-30]. Evidence indicates that the anti-inflammatory effects of Ach are mediated by nicotinic Ach receptors, and in particular by the α7 subunit of the nicotinic Ach receptor[21]. In vitro studies have shown that Ach and nicotine inhibit endotoxin-induced proinflammatory cytokine release by macrophages; this Ach effect can be prevented by nicotine receptor antagonists, and macrophages deficient for the α7 subunit of the nicotinic Ach receptor can not be inhibited with regard to cytokine release by Ach or nicotine[20, 21]. In vivo studies in endotoxemia and other models of inflammation have shown that macrophages are directly influenced by vagus nerve derived Ach, suggesting that the vagus nerve provides a hard wired anti inflammatory pathway called the "cholinergic anti inflammatory pathway" (Figure 3)[28]. In these studies, electrical stimulation of the efferent vagus nerve inhibits TNF release induced by injection of endotoxin into rats and mice and prevents shock; however, electrical stimulation of the vagus nerve in mice deficient for the α7 subunit of the nicotinic Ach receptor does not result in a reduced cytokine release upon endotoxin administration[20]. Besides inflammation induced by endotoxin, the cholinergic anti-inflammatory pathway can also inhibit other types of inflammation in vivo. Direct stimulation of the vagus nerve diminished shock and proinflammatory cytokine synthesis in liver and heart obtained from animals subjected to ischemia-reperfusion injury induced by transient aortic occlusion[31]. Furthermore, in hypovolemic hemorrhagic shock in rats, stimulation of the vagus nerve increased survival time, reverted hypotension, blunted NF-κB activity in the liver and reduced TNF levels[32]. Localized inflammation is also affected by the cholinergic anti inflammatory pathway, as shown in experimental murine arthritis induced by carrageenan where vagus nerve stimulation inhibited the inflammatory response and suppressed the development of paw swelling [33]. In, as of yet unpublished, studies by our group we have shown that the severity of experimental pancreatitis is dependent on nicotinic Ach receptors and the vagus nerve and that the cholinergic anti inflammatory pathway regulates host defense and the inflammatory response during experimental gram negative sepsis. Another line of evidence comes from studies in which vagus nerve activity was stimulated centrally. CNI-1493, a tetravalent guanylhydrazone, has been shown to induce efferent vagus nerve firing when injected intracerebroventrically[33]. CNI-1493 significantly suppressed carrageenan-induced paw edema, even in doses at least 6-logs lower than those required for a systemic effect. Bilateral cervical vagotomy or atropine blockade abrogated the anti-inflammatory effects of CNI-1493 indicating that the intact vagus nerve is required for CNI-1493 activity.

Taken together, activation of efferent vagus nerve activity provides the CNS with a fast and powerful anti inflammatory pathway that is mediated by the release of Ach by postganglionic vagal neurons which inhibits the release of pro inflammatory mediators by immune cells in the area of inflammation.

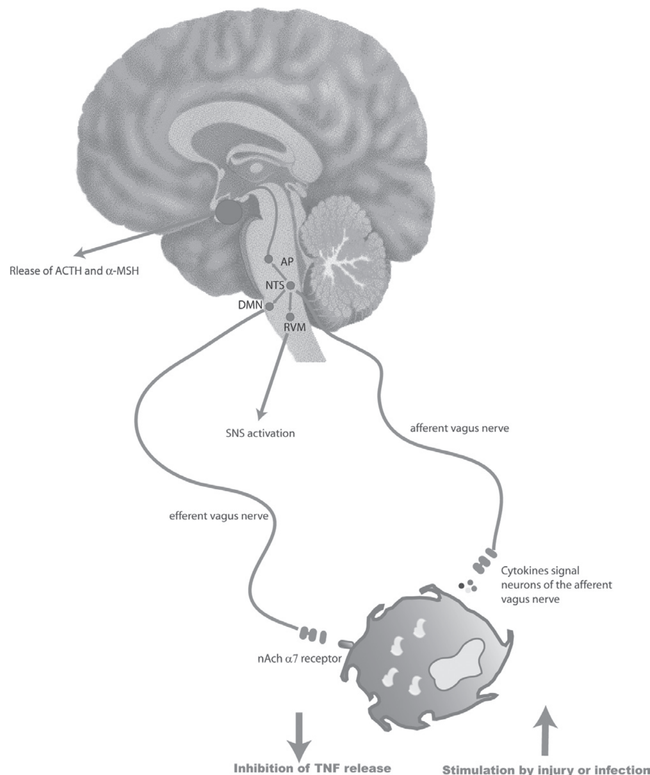

Figure 3: The cholinergic anti-inflammatory pathway. The afferent vagus nerve is stimulated by cytokines. Information is relayed to nucleus tractus solitarius (NTS). From here, projections lead to the area postrema (AP); this nucleus connects to the hypothalamus which activates the HPA axis. Second, projections to the rostro ventrolateral medulla (RVM) activate the sympathetic nervous system and epinephrine (EN) is released by the adrenal cortex and norepinephrine (NE) at postsynaptic SNS neurons. Finally, through the dorsal motor nucleus (DMN) vagal efferent activity is activated through the dorsal motor nucleus (DMN), which results in the release of acetylcholine (Ach) at vagal post synaptic neurons, inhibiting immune cells via nicotinic receptors.

Therapeutic Implications

We have reviewed data that show that the CNS and the immune system are actually two tightly linked systems. The CNS is informed about ongoing inflammation by humoral and neural networks and responds in a reflex like manner by the release of hormones and through activation of the autonomic nervous system. This “inflammatory reflex” is a powerful endogenous system designed to restrain the potential detrimental effects of excessive inflammatory responses to the body. The identification of these links between the CNS and the nervous system provides an opportunity to study new therapeutic approaches for diseases in which unrestrained inflammation is essential. Most current strategies for treatment of unrestrained inflammation are based on direct suppression of pro-inflammatory cytokines or cytokine activity. The identification of the cholinergic anti-inflammatory pathway now suggests several new approaches to modify cytokines and inflammatory responses to therapeutic advantage. Such potential new approaches include electrical stimulation of the vagus

nerve which may represent a novel strategy to inhibit the production of TNF and to protect against pathological inflammation. In this regard it is important to realize that permanently implanted vagus nerve stimulators are clinically approved devices for treatment of epilepsy and depression[34-37]. So far, more than 15.000 patients have been implanted with a vagus nerve stimulator for these indications with only moderate side effects[38]. It is conceivable to treat patients with inflammatory diseases, severe infections and overshoot inflammatory syndromes, such as sepsis and the systemic inflammatory response syndrome, with vagus nerve stimulation. Especially in TNF-α mediated diseases, such as Crohn's disease, rheumatoid arthritis and sepsis vagus nerve stimulation alone or as a supplemented to treatment with anti TNF-α strategies might be a valuable treatment. Since the anti inflammatory effects of the vagus nerve are carried out through nicotinic Ach receptors of the α 7 subtype, pharmacological stimulation of the α7 subunit of the nicotinic Ach receptor may be another approach to modulate inflammatory disorders. We have obtained proof of principle that compounds specifically stimulating the α7 subunit of the nicotinic Ach receptor can inhibit endotoxin-induced TNF release by macrophages in vitro and in mice in vivo. A third target might be the development of small molecules, such as CNI-1493, that stimulate proximal components of the cholinergic anti-inflammatory pathway in the CNS and induce vagal efferent firing. Of note, many anti inflammatory drugs such as aspirin, indomethacin and ibuprofen have also been shown to increase vagus nerve activity which may indeed contribute to their mode of action[39]. Finally, it is intriguing to note that the functions of the autonomic nervous system are carried out involuntary and often in a reflex like manner, however, due to the connections of the autonomic nervous system with the cerebral cortex a certain amount of conscious control is possible. An elegant example for this is the control certain individuals can exert over their heart rate and blood pressure in deep meditation, which exceeds the amount of maximal change observed during sleep or hypnosis. Knowledge of the link between the vagus nerve and regulation of the inflammatory response makes it conceivable that inhibition of vagal activity, which for example may be associated with chronic stress, contributes to the development of mild inflammatory syndromes. On the other hand, one might postulate that behavioral techniques that induce vagal activity could be effective as an anti inflammatory treatment.

Conclusion

The CNS and the immune system are tightly linked through humoral, endocrine and hard wired connections. The autonomous nervous system provides the CNS with real time information on the status of immunological activation in the body and the CNS responds to this information by generating a series of generalized behavioral and endocrine (fever, anorexia, ACTH release) as well as hard wired responses. These hard wired responses include the release of epinephrin and NE through the sympathetic nervous system as well as through activating efferent activity in the vagus nerve. Both systems function to suppress inflam-

mation in order to prevent inflammatory responses to become generalized. Especially the vagus nerve is an essential neural circuit for immunomodulation since its efferent activity is stimulated upon the detection of inflammation and subsequently inflammation is controlled in a reflex like manner by the anti inflammatory effects of Ach on immune cells. Knowledge of these newly discovered connections between the nervous system and the immune system, and especially of the cholinergic anti-inflammatory pathway, provides new insights in the regulation of the immune response and may pave the way for new options for the treatment of inflammatory diseases.

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chapter 9

the cholinergic anti-inflammatory pathway regulates the host response during septic peritonitis

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Introduction

Innate immunity is the first line of defense against invading pathogens [1]. The innate immune system is tightly regulated and consists of a plethora of cell-associated receptors, cytokines, chemokines and other mediators, that orchestrates the early response to infection [2-4]. Upon the first encounter with pathogens, the host seeks to ensure an adequate inflammatory reaction to combat infection, but at the same time tries to prevent collateral damage to tissues due to excessive immune activation. Failure to control inflammation during infection may result in the clinical syndrome of sepsis, characterized by a damaging systemic inflammatory response and distant organ injury. As such, limiting the acute inflammatory response to an infection is an important task of the immune system, and several counter-regulatory mechanisms exist to accomplish this, including the release of anti-inflammatory cytokines, soluble cytokine inhibitors and stress hormones [2-4].

Recently, the cholinergic nervous system was identified as a pathway that reflexively monitors and modifies the inflammatory response [5, 6]. The most compelling evidence for a role of the cholinergic nervous system in the regulation of inflammation is derived from studies in rodents challenged with endotoxin (LPS), the proinflammatory component of the outer membrane of Gram-negative bacteria [7, 8]. In experimental endotoxemia in rats, surgical dissection of the vagus nerve lead to enhanced systemic tumor necrosis factor (TNF)-α production and accelerated the development of shock; in turn electrical stimulation of the vagus nerve downregulated TNF-α production and protected the animals from hypotension [7]. Vagus nerve stimulation also inhibited the acute inflammatory response to acute hypovolemic hemorrhagic shock [9]. The vagus nerve exerts anti-inflammatory effects by its major neurotransmitter acetylcholine, which interacts with nicotinic acetylcholine receptors on macrophages resulting in inhibition of LPS-induced release of TNF-α and other proinflammatory cytokines [7, 8]; the acetylcholine receptor α7 subunit is required for this effect [8]. Hence, this “cholinergic anti-inflammatory pathway” provides the host with a powerful mechanism to “sense” inflammation via sensory pathways that relay information to the brain, and to counteract excessive inflammation in a very fast, discrete and localized way through acetylcholine released by the efferent vagus nerve.

Knowledge of the role of the anti-inflammatory cholinergic pathway during infection is not available. Therefore, in the present study we sought to determine whether this anti-inflammatory pathway regulates host responses during experimental abdominal sepsis induced by intraperitoneal injection with live Escherichia (E.) coli. We studied the host response to infection in mice in which this pathway was disrupted by vagotomy, and in animals in which the peripheral part of this pathway, nicotinic acetylcholine receptors on macrophages, was

stimulated by pretreatment with nicotine.

Methods

Mice

Female C57BL/6 mice (Harlan, Horst, the Netherlands), 8-10 weeks old, were used in all experiments. The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Experimental groups

Study 1: In a first study we evaluated the role of the vagus nerve and nicotinic receptors on the initial host response during septic peritonitis. Mice were subjected to sham surgery, unilateral cervical vagotomy, nicotine pretreatment or a combination of vagotomy and nicotine pretreatment. To inhibit the cholinergic anti-inflammatory pathway, mice were subjected to unilateral left-sided cervical vagotomy or sham surgery four days before induction of peritonitis as described previously [7]. For this mice were anesthetized by intraperitoneal injection of 0.07 ml/g FFM mixture (Fentanyl (0.315 mg/ml)-Fluanisone (10 mg/ml) (Janssen, Beersen, Belgium), Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands). A ventral cervical midline incision was used to expose the left cervical vagus trunk, which was ligated with 4–0 silk sutures and divided. Subsequently, the skin was closed with 3 sutures. In sham-operated animals the left vagus nerve was exposed and isolated from surrounding tissue but not transected. A unilateral vagotomy was chosen since early experiments showed that bilateral cervical vagotomy is lethal in mice (data not shown). In initial experiments we compared the effect of left-sided and right-sided vagotomy and found no major differences (see Results). The peripheral part of the cholinergic anti-inflammatory pathway (nicotinic acetylcholine receptors on macrophages) was stimulated by pretreatment of mice with nicotine (Sigma) added to the drinking water (100 µg/ml) from four days before induction of peritonitis [10, 11]; control mice received normal drinking water. All mice were sacrificed 6 hours postinfection. Hence, four groups of mice were studied: normal drinking water + sham surgery, normal drinking water + vagotomy, nicotine pretreatment + sham surgery and nicotine pretreatment + vagotomy (N = 8 per group).

Study 2: In a separate study, the effects of vagotomy on host defense and organ damage were evaluated during more established sepsis. In this study mice (N = 8 per group) were subjected to sham surgery or vagotomy as described above and sacrificed 24 hours postinfection. In addition, in separate groups of mice (N = 12 per group) survival was monitored for 3 days.

Study 3: In a separate study, the effects of nicotine pretreatment on host defense and organ

damage were evaluated during more established sepsis. In this study mice (N = 8 per group) were subjected to control or nicotine pretreatment as described above and sacrificed 24 hours postinfection. In addition, in separate groups of mice (N = 12 per group) survival was monitored for 3 days.

Induction of peritonitis

Peritonitis was induced as described previously [12-14]. In brief, E. coli O18:K1 was cultured in Luria Bertani medium (LB, Difco, Detroit, MI) at 37°C, harvested at mid-log phase and washed twice before inoculation. Mice were injected intraperitoneally with approximately 1-5 x 10^4 CFU E. coli in 200µl sterile saline. The inoculum was plated on blood agar plates to determine the exact number of viable counts (in retrospect 1 x 10^4 CFU in study 1 and 2 and 5 x 10^4 CFU in study 3). Mice were euthanized 6 or 24 hours after infection; at this time point mice were anesthetized by inhalation of isoflurane and peritoneal lavage was performed with 5ml of sterile isotonic saline using an 18-gauge needle. Peritoneal lavage fluid was collected in sterile tubes and put on ice. After collection of peritoneal lavage fluid deeper anesthesia was induced by intraperitoneal injection of 0.07ml/g FFM (as described above). After opening of the abdomen blood was drawn from the vena cava inferior and collected in sterile tubes containing heparin and immediately placed on ice. Livers were subsequently harvested for histological analysis.

Cell counts and differentials

Cell counts, determined on each peritoneal lavage sample, were determined in a hemocytometer (Türck counting chamber). The cells were then diluted to a final concentration of 10^5 cells/ml and differential cell counts were performed on cytospin preparations stained with Giemsa.

Assays

Cytokines and chemokines (TNF-**α**, IL-1β, IL-6, cytokine-induced neutrophil chemoattractant (KC) were measured using specific ELISA's (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The detection limits were 31 pg/ml for TNF-**α**, 16 pg/ml for IL-1β, 16 pg/ml for IL-6, and 12 pg/ml for KC. Alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were determined with commercially available kits (Sigma) using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions.

Histology

Livers for histology were harvested at 24 hours after infection, fixed in 10% formaline and embedded in paraffin. 4 µm sections were stained with hematoxylin and eosin (H&E), and analyzed by a pathologist who was blinded for groups. To score liver injury, the follo-

wing parameters were analyzed: formation of thrombi, hepatocellular necrosis and portal inflammation. Each parameter was graded on a scale of 0 to 3 with 0: absent, 1: occasional 2: mild, 3: moderate, 4: severe. The total injury score was expressed as the sum of the score for all parameters, the maximum being 12. Granulocyte staining was performed as described previously [15, 16]. Briefly, slides were deparaffinized and endogenous peroxydase activity was quenched by a solution of methanol/0.03% H_2O_2 (Merck, Darmstadt, Germany). After digestion with a solution of pepsin 0.25% (Sigma) in 0.01M HCl, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to FITC-labelled anti-mouse Ly-6-G mAb (Pharmingen, San Diego, CA). Slides were then incubated with a rabbit anti-FITC antibody (Dako) followed by a further incubation with a biotinylated swine anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidin-ABC solution (Dako) and developed using 1% H_2O_2 and 3.3'-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. After light counterstaining with methylgreen, the sections were mounted in glycerin gelatin and analyzed. Active caspase 3 staining was used to detect apoptotic bodies as described previously [15]. In brief, deparaffinized slides were boiled 2 x 5 min. in citrate buffer (pH 6.0). Non-specific binding and endogenous peroxydase activity were blocked, followed by incubation with a rabbit anti-human active caspase 3 polyclonal antibody (Cell signalling, Beverly, MA), followed by incubation with a biotinylated swine anti-rabbit antibody (Dako). The slides were further developed as described above in the Ly-6 protocol. All antibodies were used in concentrations recommended by the manufacturers. The intensities of the granulocyte and active caspase 3 staining were scored on a semiquantative scale (0 = absent, 1 = few positive cells , 2 = moderate staining , 3 = frequent staining, 4 = abundant staining).

Enumeration of bacteria and monitoring of survival

Liver lobes were harvested and homogenized at 4^0C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFU's were determined from serial dilutions of peritoneal lavage fluid, liver and blood, plated on blood agar plates and incubated at 37°C for 16 hours before colonies were counted. All culture plates revealed pure cultures of E. coli O18:K1 only. Of note, nicotine did not influence the growth of E. coli in vitro. In survival studies mortality was assessed every 2 hours for 72 hours.

Statistical analysis

Differences between groups were calculated by Mann-Whitney U test or by one way analysis of variance followed by Tukey's post test when more than two groups were compared. For survival analysis, Kaplan-Meier analysis followed by log rank test was performed. Values are expressed as mean ± SEM unless indicated otherwise. A P-value < 0.05 was considered statistically significant.

Results

Left versus right cervical vagotomy

All mice tolerated unilateral cervical vagotomy well; besides a transient weight loss (maximal 10-15%) during the first 3 days after the procedure, no sickness behavior or mortality occurred in any animal up to several weeks thereafter. Sham operated mice also demonstrated a transient weight loss (5-10%) during the first two days. At the time peritonitis was induced all mice had regained their original body weight. Left and right unilateral cervical vagotomy influenced host responses during peritonitis (described below) in a similar way. To simplify the figures and data presentation, only the results obtained after left unilateral vagotomy are given.

	Control		**Nicotine**	
	Sham	**VGX**	**Sham**	**VGX**
Cells (10^5/ml)				
Total cell count	6.7 ± 0.6	6.9 ± 1.1	7.0 ± 0.8	6.3 ± 0.9
Granulocytes	4.8 ± 0.4	6.9 ± 1.1	4.7 ± 0.6	4.1 ± 0.6
Macrophages	1.7 ± 0.2	1.7 ± 0.3	2.1 ± 0.3	2.2 ± 0.3
Cytokines and chemokines (pg/ml)				
TNF-α	677 ± 61	1131 ± 132*	334 ± 52*	456 ± 32*
IL1-β	278 ± 41	412 ± 21*	105 ± 21*	142 ± 19*
IL6	2478 ± 478	4878 ± 512 *	1228 ± 216*	1218 ± 412*
KC	568 ± 32	705 ± 102	532 ± 62	612 ± 58

Table I:Vagotomy enhances early cytokine release (determined 6 hours post infection) whereas nicotine inhibits early cytokine release independent of the integrity of the vagus nerve. Peritonitis was induced by intraperitoneal injection of 104 CFU *E. coli*. Four days before infection mice were subjected to sham surgery, left-sided cervical vagotomy (VGX), pretreatment with drinking water supplemented with nicotine (100 µg/ml) or a combination of vagotomy and nicotine pretreatment. Data are means ± SEM of 8 mice per group at 6 hours postinfection. Notably, cell counts are shown as 105/ml. Differences between groups were calculated by one way analysis of variance.*P < 0.05 vs sham operated mice with peritonitis not treated with nicotine.

Initial cytokine release during septic peritonitis is regulated by nicotinic receptors and the vagus nerve

Intraperitoneal injection of E. coli results in a strong and rapid inflammatory response within the abdominal cavity characterized by the release of inflammatory mediators and the recruitment of leukocytes into the peritoneal lavage fluid [12, 14]. First we evaluated whether the initial inflammatory response during septic peritonitis is mediated by the vagus nerve and nicotinic receptors. Six hours postinfection mice subjected to unilateral vagotomy showed increased levels of TNF-α, IL-6 and IL-1β in peritoneal lavage fluid (Table I, P<0.05)

and plasma (not shown) as compared to sham operated mice. Conversely, mice pretreated with nicotine displayed lower levels of these mediators in peritoneal lavage fluid (Table I, P<0.05) and plasma (not shown). Interestingly, the pro inflammatory effects of vagotomy were reversed in mice subjected to vagotomy who were concurrently treated with nicotine (Table I, P<0.05). Of note, at this early time point the number of neutrophils (Table I) and CFU recovered from the peritoneal lavage fluid, blood and the liver (Figure 1) were comparable in all groups.

	Sham surgery peritonitis	Vagotomy peritonitis
Cells (10^6/ml)		
Total cell count	5,5 ± 0,4	27,2 ± 7,3*
Granulocytes	4,1 ± 0,3	22,7 ± 1,9*
Macrophages	1,3 ± 0,2	4,0 ± 0,9*
Lymphocytes	0,1 ± 0,1	0,3 ± 0,1
Cytokines and chemokines (pg/ml)		
TNF-α	200 ± 109	685 ± 111*
IL1-β	309 ± 41	488 ± 43*
IL6	4070 ± 1015	7875 ± 1207
KC	684 ± 92	1057 ± 122*

Table II : Vagotomy increases cell influx and cytokine and chemokine levels in peritoneal lavage fluid (determined 24 hours post infection). Peritonitis was induced by intraperitoneal injection of 104 CFU E. coli. Four days before infection mice were subjected to left-sided cervical vagotomy or sham surgery. Data are means ± SEM of 8 mice per group at 24 hours postinfection. Notably, cell counts are shown as 106/ml. Differences between groups were calculated by Mann-Whitney U test.*P < 0.05 vs sham operated mice with peritonitis.

Vagotomy exaggerates, whereas nicotine attenuates the inflammatory response to established septic peritonitis

Unilateral cervical vagotomy before induction of septic peritonitis was associated with a significantly enhanced influx of leukocytes into the peritoneal fluid 24 hours postinfection (P < 0.05 versus sham operated mice), which was almost exclusively caused by an increased invasion of neutrophils (Table II). In addition, vagotomy resulted in higher local concentrations of TNF-α, IL-1β, IL-6 and KC during peritonitis when compared with infected sham operated mice (all P < 0.05 for the difference between groups; Table II). In contrast, nicotine treatment was associated with a decreased influx of neutrophils and lower concentrations of cytokines and chemokines in peritoneal lavage fluid during peritonitis when compared to mice with peritonitis that did not receive nicotine (all P < 0.05 for the difference between groups; Table III). Plasma cytokine levels were influenced by vagotomy and nicotine in a similar manner as peritoneal lavage fluid concentrations (data not shown).

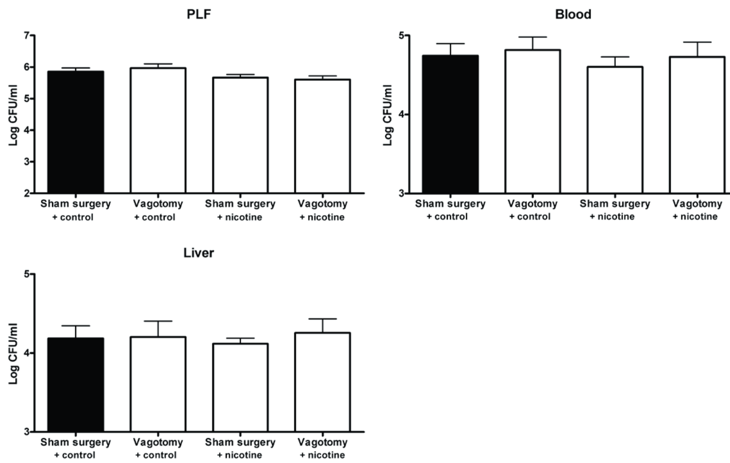

Figure 1: Interference with the cholinergic anti inflammatory pathway does not influence early bacterial outgrowth. Peritonitis was induced by intraperitoneal injection of E. coli. Four days before infection mice were subjected to left-sided cervical vagotomy, nicotine pretreatment (100 µg/ml added to drinking water) or by a combination of vagotomy and nicotine pretreatment . Data are means ± SEM of 8 mice per group at 6 hours postinfection. Differences between groups were calculated by one way analysis of variance. .Differences between groups were not significant. PLF = peritoneal lavage fluid.

	Control + Peritonitis	Nicotine + peritonitis
Cells (10^6/ml)		
Total cell count	6.4 ± 0.8	2.9 ± 0.4*
Granulocytes	5.1 ± 0.6	1.9 ± 0.3*
Macrophages	1.2 ± 0.2	0.9 ± 0.2
Cytokines and chemokines (pg/ml)		
TNF-α	687 ± 270	238 ± 128*
IL1-β	652 ± 132	230 ± 35*
IL6	10719 ± 5413	6643 ± 937*
KC	1487 ± 356	953 ± 156*

Table III. Nicotine reduces cell influx and cytokine and chemokine levels in peritoneal lavage fluid (determined 24 hours post infection). Peritonitis was induced by intraperitoneal injection of 5 x 104 CFU E. coli. From four days before infection mice received either normal drinking water of drinking water supplemented with nicotine (100 µg/ml). Data are means ± SEM of 8 mice per group at 24 hours postinfection. Notably, cell counts are shown as 106/ml. Differences between groups were calculated by Mann-Whitney U test. *P < 0.05 vs control mice with peritonitis.

Vagotomy increases, whereas nicotine attenuates liver injury

Liver injury is one of the hallmarks of distant organ damage in experimental peritonitis and is related to the extent of systemic inflammation [12-14]. Mice not injected with *E. coli* did not display evidence of necrosis or apoptosis in their livers irrespective of whether they were subjected to vagotomy or nicotine treatment (not shown). Peritonitis in mice in which the cholinergic system was not manipulated by either vagotomy or nicotine was accompanied by histopathological evidence of liver necrosis, inflammation, thrombosis and apoptosis 24 hours after infection (Figures 2A, 2C, 3A and 3C). In vagotomized mice, the extent of both liver damage (Figure 3B) and apoptosis (Figure 3D) was markedly increased during peritonitis ($P < 0.05$ versus sham operated mice). Conversely, nicotine treatment prevented liver damage after intraperitoneal infection with E. coli ($P < 0.05$ versus control mice, Figure 3B). In addition, the extent of apoptosis was decreased in nicotine treated mice during peritonitis although the difference with infected control mice did not reach statistical significance in the semi-quantitative analysis ($P = 0.10$; Figure 3D). Granulocyte stainings of liver specimens revealed that vagotomy was also associated with an increased influx of neutrophils into the liver 24 hours after induction of peritonitis ($P < 0.05$ versus sham operated mice, Figure 2E and 2F). Nicotine treatment tended to diminish hepatic neutrophil accumulation during peritonitis (not significant; Figure 3E and 3F).

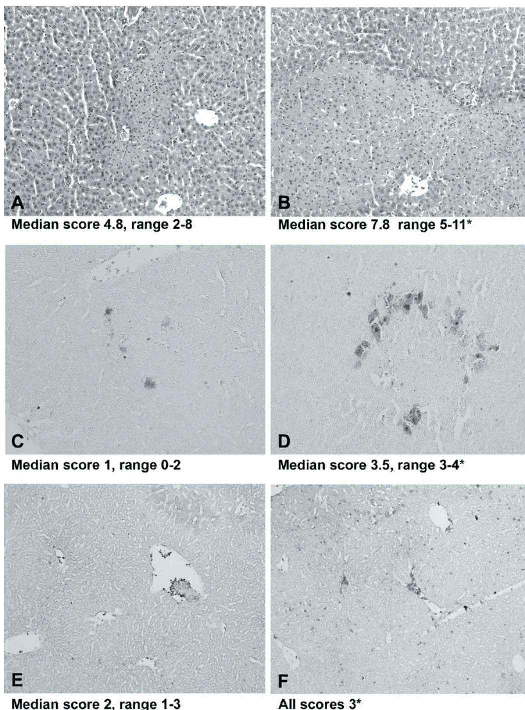

Figure 2: Vagotomy increases histopathological damage to the liver during septic peritonitis. Peritonitis was induced by intraperitoneal injection of *E. coli*. Four days before infection mice were subjected to left-sided cervical vagotomy (right panels: B, D, F) or sham surgery (left panels: A, C, E). Representative liver sections, obtained 24 hours after infection, are shown, with medians and ranges derived from semiquantative analyses (see Methods) of 8 mice per group indicated below each picture. A+B: H&E stainings, x 10; C+D: anti active caspase 3 staining, x 20; E + F: anti Ly-6 (granulocyte) staining, x 10. Differences between groups were calculated by Mann-Whitney U test.* $P < 0.05$ versus sham surgery.

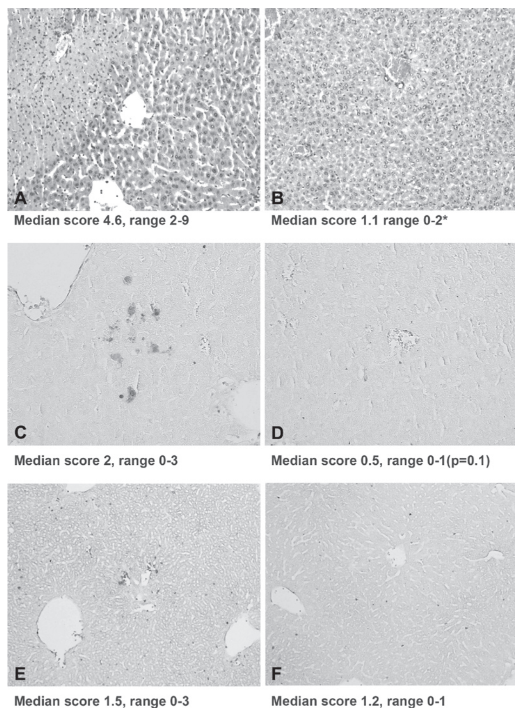

Figure 3: Nicotine attenuates histopathological damage to the liver during septic peritonitis. Peritonitis was induced by intraperitoneal injection of *E. coli*. From four days before infection mice received either drinking water supplemented with nicotine (100 µg/ml)(right panels: B, D, F) or normal drinking water (left panels: A, C, E). Representative slides, obtained 24 hours after infection, are shown, with medians and ranges derived from semiquantative analyses (see Methods) of 8 mice per group indicated below each picture. A+B: H&E stainings x 10; C+D: anti-active caspase 3 staining, x 20; E + F: anti Ly-6 (granulocyte) staining, x 10. Differences between groups were calculated by Mann-Whitney U test.* $P < 0.05$ versus control.

Clinical chemistry can be used to evaluate hepatocellular injury in a quantitative way in this model [12-14]. Thus, we measured the plasma concentrations of ALAT and ASAT at 24 hours postinfection. Peritonitis was associated with elevated ALAT and ASAT levels, confirming our previous studies [12-14]. Vagotomy resulted in even higher plasma ALAT and ASAT concentrations, although the differences with sham operated mice were not significant due to a relatively large interindividual variation (Figure 4). Nicotine pretreated mice showed a marked reduction in the plasma transaminase levels ($P<0.05$ versus control mice, Figure 5).

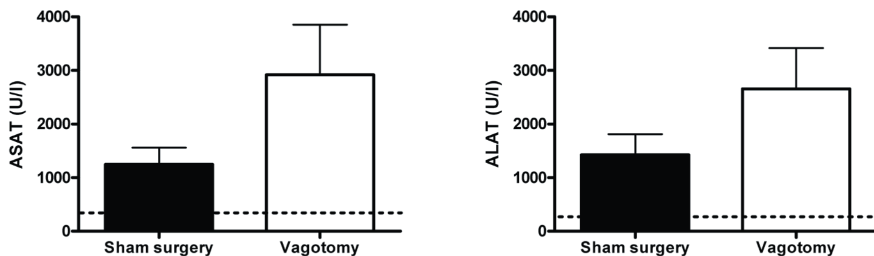

Figure 4: Effect of vagotomy on plasma transaminase levels. Peritonitis was induced by intraperitoneal injection of *E. coli*. Four days before infection mice were subjected to left-sided cervical vagotomy or sham surgery. Data are means ± SEM of 8 mice per group at 24 hours postinfection. Dotted line represents transaminase levels in uninfected mice. Differences between groups were calculated by Mann-Whitney U test. Differences between groups were not significant.

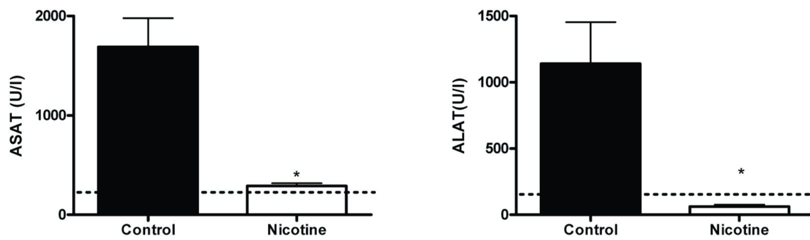

Figure 5: Nicotine reduces plasma transaminase levels. Peritonitis was induced by intraperitoneal injection of *E. coli.* From four days before infection mice received either normal drinking water or drinking water supplemented with nicotine (100 µg/ml). Data are means ± SEM of 8 mice per group at 24 hours postinfection. Dotted line represents transaminase levels in uninfected mice. Differences between groups were calculated by Mann-Whitney U test.*P < 0.05 vs control mice with peritonitis.

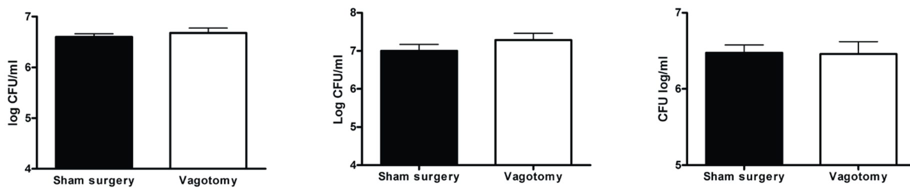

Figure 6: Vagotomy does not influence bacterial clearance. Peritonitis was induced by intraperitoneal injection of *E. coli*. Four days before infection mice were subjected to left-sided cervical vagotomy or sham surgery. Data are means ± SEM of 8 mice per group at 24 hours postinfection. Differences between groups were calculated by Mann-Whitney U test.Differences between groups were not significant. PLF = peritoneal lavage fluid.

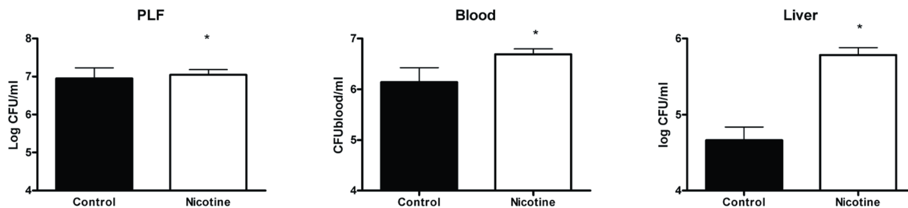

Figure 7: Nicotine impairs bacterial clearance. Peritonitis was induced by intraperitoneal injection of *E. coli.* From four days before infection mice received either normal drinking water of drinking water supplemented with nicotine (100 µg/ml). Data are means ± SEM of 8 mice per group at 24 hours postinfection. Differences between groups were calculated by Mann-Whitney U test.*P < 0.05 vs control mice with peritonitis. PLF = peritoneal lavage fluid.

Nicotine pretreatment impairs bacterial clearance and survival during peritonitis

An adequate local inflammatory response is important for mounting an effective antibacterial response during peritonitis [12-14]. Therefore, we determined the consequences of the effect of vagotomy and nicotine on the host inflammatory reaction to E. coli infection on

the bacterial loads in the peritoneal lavage fluid (the site of the infection), blood (to evaluate to which extent the infection became systemic), and liver at 24 hours after infection (i.e. shortly before the first deaths occurred). Vagotomy did not influence the number of E. coli CFU's recovered from these three body sites (Figure 6). However, nicotine treatment facilitated the outgrowth of E. coli in peritoneal fluid, blood and liver (all $P < 0.05$ versus control mice, Figure 7). Finally, we determined the effect of vagotomy and nicotine treatment on mortality. Consistent with their influence on the outgrowth of E. coli during the infection, vagotomy did not alter mortality during septic peritonitis (data not shown), whereas nicotine significantly accelerated mortality ($P < 0.05$ versus control mice, Figure 8). Notably, the relatively modest adverse effect of nicotine on survival in this fulminant model of sepsis was reproduced in two separate additional experiments (data not shown).

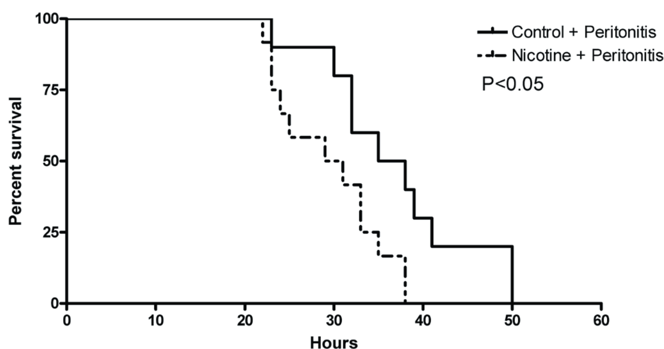

Figure 8: Nicotine accelerates mortality during septic peritonitis. Peritonitis was induced by intraperitoneal injection of *E. coli*. From four days before infection mice received either normal drinking water of drinking water supplemented with nicotine (100 µg/ml). N = 12 mice per group. P value indicates the difference between groups by log-rank test.

Discussion

The efferent vagus nerve has been implicated as an important anti-inflammatory pathway through an interaction of its principal neurotransmitter acetylcholine with nicotinic cholinergic receptors, in particular the α7 subunit, on resident macrophages. Whereas the function of this cholinergic anti-inflammatory pathway has been well established in models of sterile inflammation [7-9, 17, 18], the present study is the first to investigate its role in the innate immune response to infection with live bacteria. We here demonstrate that inhibition of the cholinergic anti-inflammatory pathway by cervical vagotomy results in an enhanced early and late inflammatory response to septic peritonitis induced by intraperitoneal injection of E. coli, characterized by increased cytokine release, an enhanced influx of inflammatory cells to the site of infection and the occurrence of extensive liver damage. Conversely, activation of the peripheral component of the pathway by oral administration of

nicotine attenuated early and late inflammation, as reflected by decreased cytokine production and neutrophil recruitment and prevention of liver damage. These data suggest that the cholinergic anti-inflammatory pathway plays an essential role in the regulation of inflammatory responses during septic peritonitis.

The currently reported effects of vagus nerve manipulation by vagotomy or nicotine are in line with earlier investigations that examined the influence of vagus nerve activity on sterile inflammation. Previous studies have identified an important role for the afferent vagus nerve in the detection of inflammation by the central nervous system. Whether humoral or neural pathways are essential in relaying information to the brain is largely dependent on the magnitude of the inflammatory response. Experimental studies it have shown that when the level of inflammation is low, such as when a low dose of endotoxin is injected intraperitoneally, vagotomy inhibits the stimulation of the hypothalamus-pituitary-adrenal (HPA) axis and the induction of IL-1 in the brain[19, 20] whereas high doses of endotoxin induce responses by the brain independent of the vagus nerve[21, 22]. This implicates that neural pathways are essential in the relay of localized inflammation whereas information about severe systemic inflammation reaches the brain predominantly through humoral pathways. The vagus nerve is not only essential in the detection of inflammation but also provides an important route for the central nervous system to respond. In experimental endotoxemia, direct electrical or chemical vagus nerve stimulation reduced serum TNF-α levels and prevented shock [7, 18], whilst cervical vagotomy augmented serum TNF-α levels and sensitized animals to the lethal effects of LPS [7, 8]. In other models of systemic inflammation, induced by either ischemia reperfusion injury or hypovolemic hemorrhagic shock, stimulation of the vagus nerve decreased serum TNF-α levels and prevented the development of hypotension [9, 17]. Previous, as of yet unpublished, studies by our group in endotoxemic mice have shown that the anti inflammatory effects of electrical vagus nerve stimulation are relatively short lived and wean two to four hours after stimulation. On the basis of these results, given the duration of the septic peritonitis model used here, we decided not to use vagus nerve stimulation in the present study. It should be noted that nicotine was administered via the drinking water beginning from four days prior to induction of peritonitis. Due to this route of administration and due to the acute nature of the model used, we were not able to examine the effect of postponed treatment with nicotine.

Our data confirm the anti-inflammatory potential of the vagus nerve in a well established model of abdominal sepsis. We first show that the initial inflammatory response, which has been shown to be essential for host defense in this model [13, 23], during septic peritonitis is regulated by the vagus nerve and nicotinic receptors. Six hours postinfection mice subjected to unilateral vagotomy showed increased levels of pro inflammatory cytokines as compared to sham operated mice. Conversely, mice pretreated with nicotine displayed lower levels of

these mediators. Interestingly, the pro inflammatory effects of vagotomy were overturned in mice subjected to vagotomy concurrently treated with nicotine confirming that nicotine acts on the peripheral part of the cholinergic anti inflammatory pathway (that is independent of the integrity of the vagus nerve). In subsequent studies mice were sacrificed 24 hours postinfection and we investigated the effects of vagotomy and nicotine pretreatment on host defense and liver damage during more established septic peritonitis. Interference with the function of the vagus nerve strongly influenced not only the proinflammatory cytokine response to E. coli peritonitis but also the migration of leukocytes to the site of the infection, one of the hallmarks of the early immune reaction to invading pathogens. Moreover, our study documented a protective role of the intact vagus nerve against liver injury accompanying experimental E. coli peritonitis. We specifically focused on hepatic injury and inflammation, since we previously documented hepatocellular damage in this infection model [12-14], and since the liver is richly innervated by the vagus nerve [24]. We used nicotine to chemically stimulate the peripheral part of the cholinergic anti-inflammatory pathway. Previous studies have shown that nicotine inhibits LPS-stimulated TNF-α release by human, as well as mouse, macrophages in vitro via a specific interaction with the α7 subunit of nicotinic acetylcholine receptors [8]. These findings were corroborated by in vivo studies using LPS challenged α7-deficient mice, in which the anti-inflammatory effect of electrical stimulation of the vagus nerve was abolished [8]. Together with our finding that nicotine added to drinking water reduced TNF-α concentrations in mice challenged with live E. coli in vivo, we consider it likely that nicotine exerts its anti-inflammatory effects through an interaction with the α7 subunit of nicotinic acetylcholine receptors on macrophages. Of note, the same scheme and route of nicotine administration has been reported to reduce colonic damage during spontaneous colitis in IL-10 gene deficient mice [10, 11, 25]. Unfortunately, we were not able to confirm and expand our results using α7-deficient mice, since these mice do not breed well (information by Jackson Laboratories) and our own prolonged efforts to breed these mice in our institution did not result in a colony large enough to perform in vivo experiments.

Interruption or stimulation of the vagus nerve had a profound impact on the recruitment of neutrophils to the infected peritoneal cavity. Since the local release of the neutrophil attracting chemokine KC during peritonitis was enhanced by vagotomy and decreased by nicotine, it is conceivable that the alterations in neutrophil migration to the site of infection are, at least in part, mediated by KC. Alternatively, these results can also be explained by a direct effect of vagotomy or nicotine on neutrophils. Previous studies have shown that a variety of nicotinic Ach receptors is present on neutrophils and that stimulation of nicotinic receptors inhibits neutrophil migration which is, at least in part, mediated by inhibition of adhesion molecule expression on both endothelial cell surface and neutrophils [26].

Whereas the inflammatory response to septic peritonitis was increased after vagotomy and reciprocally decreased by nicotine treatment, bacterial clearance and survival were altered by nicotine only. A possible explanation could be that unilateral vagotomy induces only a partial interference with the cholinergic anti-inflammatory pathway. Notably, the effect of bilateral vagotomy could not be investigated since this procedure is lethal in mice. After nicotine pretreatment, bacterial clearance and survival were significantly reduced. Since host defense in peritonitis is a delicate balance between pro-inflammatory pathways intended to eliminate bacteria and anti-inflammatory pathways intended to prevent systemic inflammation, any imbalance in pro- or anti-inflammatory mediators might prove harmful. Indeed, our laboratory recently demonstrated that elimination of the anti-inflammatory cytokine IL-10 in septic peritonitis resulted in an uncontrolled systemic inflammatory response syndrome and lethality in spite of the fact that IL-10 deficiency facilitated the clearance of bacteria from the peritoneal cavity [12]. In the current study, pretreatment with nicotine resulted in a reduction of local and systemic inflammation but increased lethality due to a decrease in bacterial clearance and enhanced dissemination of bacteria. Taken together, these findings illustrate the delicacy of the balance between pro- and anti-inflammation during septic peritonitis.

Excessive activation of coagulation plays an important role in the pathogenesis of severe sepsis [27] and the model used here is associated with profound activation of the coagulation system [28]. Of note, in the current studies we did not find consistent effects of either vagotomy or nicotine on the procoagulant response to abdominal sepsis, as measured by thrombin-antithrombin levels in peritoneal lavage fluid, and fibrin staining of liver sections (data not shown).

It should be noted that variation existed in some of the endpoints measured in the respective control groups of study 2 (sham surgery) and study 3. The exact number of viable bacteria used to inoculate mice can only be quantified retrospectively; in study 2 the inoculum contained 1 x 10^4 CFU whereas in study 3 5 x 10^4 E. coli were injected. Although an effect of sham surgery can not be excluded, we consider it most likely that the larger bacterial challenge given in study 3 explains the somewhat higher control values in this experiment. Importantly, in both separate studies control and intervention groups were injected with exactly the same inoculum at the same time.

Peritonitis is a common cause of sepsis [29] and E. coli remains one of the most frequently isolated pathogens in intraperitoneal infections [30]. Intraperitoneal administration of live E. coli results in a paradigm that resembles a clinical condition commonly associated with septic peritonitis [31]. By using this model we here demonstrate for the first time that the cholinergic anti-inflammatory pathway is an essential regulator of the innate immune response to

a severe bacterial infection. We further show that stimulation of the cholinergic anti-inflammatory pathway by nicotine impairs bacterial clearance and survival during E. coli induced septic peritonitis. The cholinergic anti-inflammatory pathway may be a future target for the modulation of the host inflammatory response to sepsis.

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chapter 10

the vagus nerve and nicotinic receptors mediate experimental pancreatitis severity in mice

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Introduction

Acute pancreatitis is a common sterile inflammatory condition that carries a significant risk of morbidity and mortality. It is characterized by premature activation of digestive enzymes and a subsequent local and systemic inflammatory response mediated by the release of pro-inflammatory cytokines and the activation and migration of neutrophils (1). Communication between the immune, nervous and endocrine systems is essential for the regulation of the inflammatory response. Through humoral and neural pathways, predominantly the vagus nerve, the central nervous system is notified of ongoing inflammation (2). Cytokines and bacterial products such as endotoxin stimulate afferent neural fibers in the vagus nerve that are processed in the brain and result in the initiation of an acute phase response and induction of fever [(2,3,4)]. Furthermore, the central nervous system responds by initiating several anti-inflammatory pathways, designed to prevent the detrimental effects of the uncontrolled release of inflammatory mediators. One of these pathways involves an increased activity in the efferent vagus nerve, called the "nicotinic anti-inflammatory pathway" which reflexively modifies the inflammatory response [(5,6)]. The most compelling evidence for a role of the cholinergic nervous system in the regulation of inflammation is derived from studies in rodents challenged with endotoxin, the pro-inflammatory component of the outer membrane of Gram-negative bacteria [(7,8)]. Vagotomy lead to enhanced systemic tumor necrosis factor (TNF)-α production and accelerated the development of shock; in turn electrical stimulation of the efferent vagus nerve down regulated TNF-α production and protected animals from hypotension (7). Further studies showed that the anti-inflammatory properties of the efferent vagus nerve are mediated through its major neurotransmitter acetylcholine (Ach), which interacts with nicotinic Ach receptors on macrophages resulting in inhibition of endotoxin-induced responses [(7,8)] and that the nicotinic Ach receptor α 7 subunit is required for this effect (8).

In the present study we sought to determine whether this anti-inflammatory pathway regulates host responses during experimental pancreatitis. We studied the inflammatory response and the severity of experimental pancreatitis in mice in which this pathway was disrupted by vagotomy. In a separate study, pancreatitis was induced in mice in which the peripheral part of this pathway, nicotinic Ach α 7 receptors on immune competent cells, was stimulated with a selective agonist, 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21) [(9,10)]. In other mice the reciprocal approach was undertaken and nicotinic receptors were blocked by using mecamylamine (11). Our results show that the vagus nerve and nicotinic receptors are part of an important anti-inflammatory mechanism that limits the severity of experimental pancreatitis.

Methods

Animals

Female C57Bl/6 mice (Harlan, Horst, the Netherlands), 8-10 weeks old, were used in all experiments. The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Animal model and experimental groups

Pancreatitis was induced by hourly 12 intraperitoneal (i.p.) injections of cerulein (Research Plus, Manasquan, NJ) in 200 µl sterile saline (12). A supramaximal stimulating dose was used for all injections (50 µg/kg); control mice received 12 i.p. saline injections. All mice were sacrificed one hour after the last injection.

In study 1, mice were anesthetized with hypnorm (Janssen Pharmaceutics, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands) and subjected to left cervical vagotomy or sham surgery three days before induction of pancreatitis as described previously (7). A ventral cervical midline incision was used to expose the left cervical vagus trunk, which was ligated with 4–0 silk sutures and divided. Subsequently, the skin was closed with 3 sutures. In sham-operated animals the nerve was exposed and isolated from surrounding tissue but not transected.

In study 2, pancreatitis was induced in mice treated with the non selective nicotinic receptor antagonist mecamylamine (11) (Sigma, St. Louis, MO) (1 mg/kg) or with the selective α 7 nicotinic receptor agonist 3-(2,4-dimethoxybenzylidene)anabaseine (GTS-21)(Critical Therapeutics, Lexington, MA) [9,10] (4 mg/kg), both administered by i.p. injection in 200 µl of saline 30 minutes prior to induction of acute pancreatitis; control mice received saline.

Tissue handling

One hour after the last cerulein injection, mice were anesthetized with hypnorm (Janssen Pharmaceutics, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the *vena cava inferior* in heparin coated vacutainer tubes. Whole blood was collected and spinned for 10 minutes at 3600 rpm and plasma was subsequently removed and stored at -20^0 C. Pancreatic and lung edema were estimated by expressing relative organ weight (mg of organ/g of mouse) as described previously [13,14]. Longitudinal dissected parts of the pancreas were removed and frozen in liquid nitrogen to prevent degradation; other parts were fixed in 10% formalin and embedded in paraffin for histological analysis. Lungs were removed and frozen in liquid nitrogen until homogenized for myeloperoxidase (MPO) quantification.

Histologic examination

Four µm sections were stained with hematoxylin and eosin. All specimes were scored by a pathologist unaware of the origin of the specimens. Pancreatitis severity was scored as previousy described [14,15]. Briefly, necrosis, inflammation and edema were scored on a 0-3 scale. Each score is shown independently, a total severity score was also calculated (maximum= 9 points). To quantify the number of neutrophils in the pancreas, neutrophils were stained using a naphtol AS-D chloroacetate esterase staining method (usually referred to as: "Leder" staining) as described elsewhere [16,17]. Numbers of neutrophils are expressed as the number of positive cells per high power field (X400).

Assays

Plasma amylase and lipase were determined with a commercially available kit (Sigma), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Results are expressed in international units (U) per ml (amylase) and U/l (lipase). Interleukin (IL)-6 and macrophage inflammatory protein (MIP)-2 were measured by ELISA according to the manufacturers recommendations (R&D systems, Minneapolis, MN). Lung homogenates were prepared as previously described (18). MPO content was measured in pancreas and lung homogenates, as described elsewhere (18). MPO activity is expressed as percentage of control compared with control animals.

Statistical analysis

All data are expressed as means ± standard error (SE). Comparisons between groups were conducted using one way analysis of variance followed by the Neuman-Keuls multiple comparison test. Significance was set at $P < 0.05$.

Results

Outcome and timing of unilateral cervical vagotomy

All mice tolerated unilateral left cervical vagotomy well; besides a transient weight loss during the first 3 days after the procedure, no sickness behavior or mortality occurred in any animal up to several weeks thereafter.

Previous vagotomy exaggerates pancreatitis

Twelve hourly injections of cerulein elicited pancreatitis, as indicated by profound increases in the plasma concentrations of amylase and lipase, an increase in pancreatic weight and histopathologic examination of the pancreas demonstrating marked edema, infiltration of neutrophils and focal areas of necrosis. Left cervical vagotomy resulted in an increased severity of pancreatitis. Left cervical vagotomy significantly increased the plasma concentrations of amylase and lipase (both $P < 0.05$, Figure 1). In addition, pancreas weights were

higher in vagotomized animals (P < 0.05, Table 1), and their histology revealed more severe pancreatitis (P < 0.05, Table 1 with representative slides shown in Figure 2). Considering that neutrophils play an important role in cerulein-induced acute pancreatitis [19,20], we further examined neutrophil influx into the pancreas by measuring MPO in pancreas homogenates and by counting the number of Leder positive cells in pancreas tissue. Both parameters strongly increased upon induction of acute pancreatitis, confirming the conventional hematoxylin and eosin pancreas slides. However, vagotomy resulted in higher MPO pancreas concentrations and more Leder positive cells in pancreas tissue (both P < 0.05, Table 1). Finally, we measured the plasma concentrations of IL-6 since these have been found to correlate with disease severity in patients in pancreatitis (21). Cerulein-induced pancreatitis was associated with strongly elevated levels of plasma IL-6, which were even higher in mice that had underwent vagotomy (P < 0.05, Table 1).

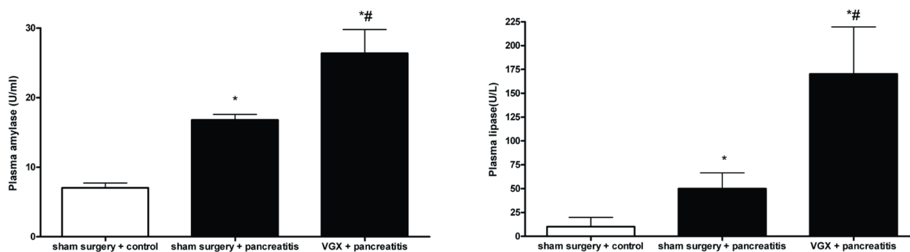

Figure 1: Vagotomy increases plasma amylase and lipase levels during pancreatitis. Three days before induction of pancreatitis mice were subjected to sham surgery or left cervical vagotomy (VGX). Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 i.p. saline injections. Plasma was harvested one hour after the 12th injection. Plasma amylase and lipase levels are shown. * P < 0.05 versus sham surgery + sham, † P < 0.05 versus sham surgery + pancreatitis.

Blockade of nicotinic receptors augments whereas stimulation of nicotinic receptors attenuates pancreatitis

Having established that vagotomy exaggerates the severity of cerulein-induced pancreatitis, we next set out to determine whether pharmacological manipulation of nicotinic receptors influences pancreatitis severity. Thus, in a separate study, pancreatitis was induced after i.p. injection with saline, mecamylamine (a nicotinic receptor antagonist) (11) or GTS-21 (a selective agonist for the nicotinic Ach α 7 receptor) [9,10]. In line with the results obtained after vagotomy, mecamylamine augmented the severity of pancreatitis. Indeed, when compared with mice administered with saline, mecamylamine treated mice displayed higher plasma levels of amylase and lipase (both P < 0.05, Figure 3), higher relative pancreas weights (P < 0.05, Table 2), and higher pancreatitis histology scores (all P < 0.05, Table 2, with representative slides shown in Figure 4). Moreover, mecamylamine treatment resulted in a more profound neutrophil influx into the pancreas, as reflected by higher MPO concentrations in

pancreas homogenates and more (Leder positive) neutrophils in pancreas tissue (both $P < 0.05$ versus saline injected mice, Table 3). Mecamylamine also augmented systemic IL-6 release, resulting in higher plasma IL-6 levels when compared to saline injected animals ($P < 0.05$, Table 2). In the same experiment we used GTS-21 to stimulate nicotinic Ach α 7 receptors, considering that this receptor subtype has been found to signal the anti-inflammatory effects of Ach on macrophages (8). In line with the results obtained with the nicotinic receptor antagonist mecamylamine, GTS-21 treatment reduced the severity of cerulein-induced pancreatitis: when compared to saline administered mice, GTS-21 treated animals had lower plasma amylase and lipase levels (both $P < 0.05$, Figure 3), lower relative pancreas weights ($P < 0.05$, Table 2), and lower pancreatitis histology scores (all $P < 0.05$, Table 2, with representative slides shown in Figure 4). Moreover, GTS-21 inhibited neutrophil influx into the pancreas, as indicated by lower pancreas MPO concentrations and less Leder positive cells in pancreas tissue (both $P < 0.05$ versus saline injected mice, Table 2). GTS-21 treatment was also associated with lower plasma IL-6 concentrations when compared to saline injected animals ($P < 0.05$, Table 2).

	sham surgery + control	**sham surgery + pancreatitis**	**vagotomy + pancreatitis**
Relative pancreas weight	5.7 ± 0.2	7.8 ± 0.2*	8.9 ± 0.3*†
Histology score (total)	0.7 ± 0.2	3.2 ± 0.1*	4.9 ± 0.3*†
Edema	0.5 ± 0.2	1.5 ± 0.2*	2 ± 0.1*
Inflammation	0.1 ± 0.1	0.9 ± 0.2*	1.6 ± 0.2*†
Necrosis	0.1 ± 0.1	0.8 ± 0.1*	1.3 ± 0.1*†
MPO (% of sham surgery + control)	100	172 ± 21*	238 ± 12*†
Leder positive cells	0 ± 0	32 ± 6*	61 ± 8*†
Plasma Il-6 (pg/ml)	43 ± 9	408 ± 44*	743 ± 86*†

Table 1: Mice underwent either sham surgery or left cervical vagotomy three days before induction of pancreatitis. Pancreatitis was induced by 12 i.p. injections of cerulein (50 µg/kg); control mice received saline i.p. Mice were sacrificed one hour after the 12th injection. Data are means ± SE of 8 mice per group. High power field (HPF) represents X400 magnification. * $P < 0.05$ versus control, † $P < 0.05$ versus sham surgery + pancreatitis.

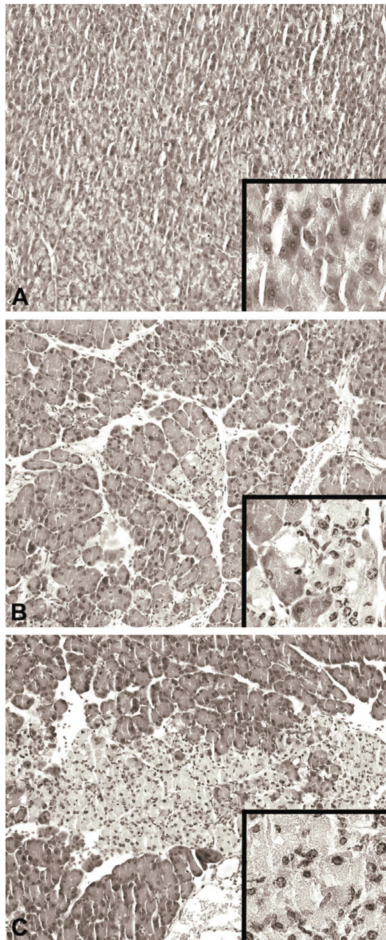

Figure 2: Pancreatitis severity is enhanced by vagotomy. Three days before induction of pancreatitis mice were subjected to sham surgery or left cervical vagotomy. Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 i.p. saline injections. Pancreas was harvested one hour after the 12th injection. Representative pancreas histology slides (from a total of 8 mice per group) are shown from sham surgery + control mice (A), sham surgery+ pancreatitis mice (B) and vagotomy + pancreatitis mice (C). H&E staining, magnification x 10, insert x 40.

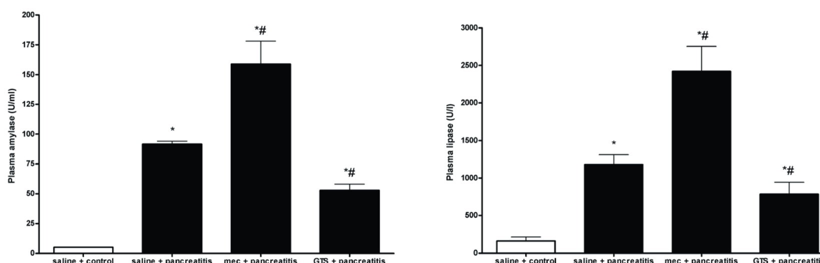

Figure 3: Blockade of nicotinic receptors augments whereas stimulation of nicotinic receptors attenuates plasma amylase and lipase levels during pancreatitis. Thirty minutes before induction of pancreatitis mice were i.p. injected with saline, mecamylamine (mec) or GTS-21 (GTS). Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 i.p. saline injections. Plasma was harvested one hour after the 12th injection. Plasma amylase and lipase levels are shown. * P < 0.05 versus saline + sham, † P < 0.05 versus saline + pancreatitis.

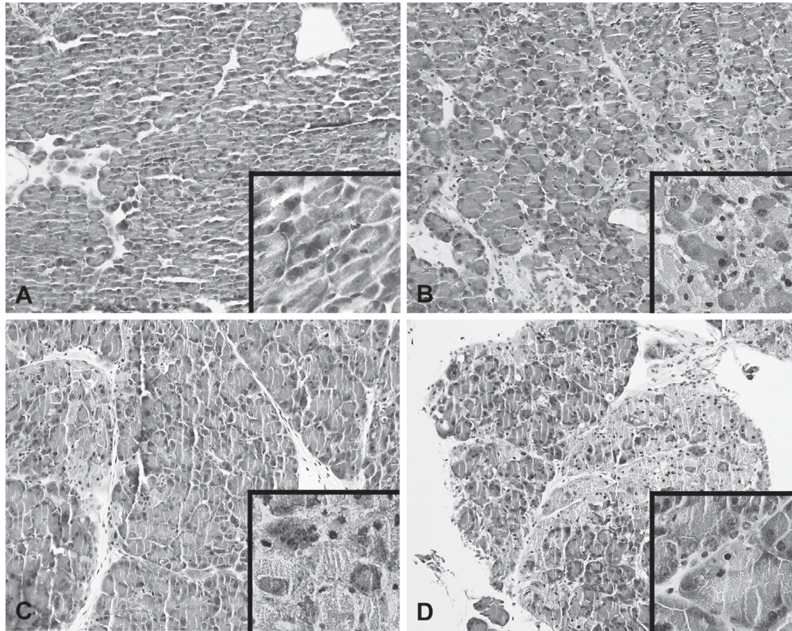

Figure 4: Pancreatitis severity is influenced by nicotinic receptors. Thirty minutes before induction of pancreatitis mice were i.p. injected with saline, mecamylamine or GTS-21. Pancreatitis was induced by 12 hourly i.p. injections of cerulein; sham mice received 12 i.p. saline injections. Pancreas was harvested one hour after the 12th injection. Representative pancreas histology slides (from a total of 8 mice per group) are shown from saline + control mice (A), saline + pancreatitis mice (B), mecamylamine + pancreatitis mice (C) and GTS-21 + pancreatitis mice (D). H&E staining, magnification x 10, insert x 40.

	saline + control	saline + pancreatitis	mecamylamine + pancreatitis	GTS-21 + pancreatitis
Relative pancreas weight	5.5 ± 0.3	8.9 ± 0.3*	9.9 ± 0.3*††	7.3 ± 0.3*†
Histology score (total)	0.5 ± 0.2	4.9 ± 0.5*	8.8 ± 0.5*†	2.4 ± 0.4*†
Edema	0.1 ± 0.1	1.5 ± 0.2*	2.8 ± 0.3*†	0.8 ± 0.3*†
Inflammation	0.4 ± 0.2	1.6 ± 0.3*	2.9 ± 0.1*†	1.6 ± 0.1*†
Necrosis	0.0 ± 0.0	1.8 ± 0.2*	2.9 ± 0.1*†	0.7 ± 0.1*†
MPO (% of sham surgery + control)	100	203 ± 34*	272 ± 22*†	148 ± 18*†
Leder positive cells	0 ± 0	42 ± 8*	68 ± 12*†	23 ± 4*†
Plasma Il-6 (pg/ml)	86 ± 11	512 ± 52*	816 ± 92*†	317 ± 32*†

Table 2: Blockade of nicotinic receptors augments whereas stimulation of nicotinic receptors attenuates pancreatitis severity. Mice were injected i.p. with saline, mecamylamine or GTS-21 thirty minutes before induction of pancreatitis. Pancreatitis was induced by 12 i.p. injections of cerulein (50 µg/kg); control mice received saline i.p. Mice were sacrificed one hour after the 12th injection. Data are means ± SE of 8 mice per group. High power field (HPF) represents X400 magnification. * $P < 0.05$ versus control, † $P < 0.05$ versus saline + pancreatitis.

Neither vagotomy nor pharmacological manipulation of nicotinic receptors influence pancreatitis associated lung inflammation

Cerulein-induced acute pancreatitis is associated with a mild inflammatory response in the lungs, which is characterized by modest increases in lung MPO content, edema and MIP-2 levels [22,19,14]. In line, we here found modest but significant increases in lung weights, lung MPO levels and lung MIP-2 concentrations after repeated cerulein injections (Tables 3 and 4). In contrast to the marked effects of vagotomy, mecamylamine and GTS-21 on the severity of pancreatitis, neither vagotomy (Table 3) nor pharmacological manipulation of nicotinic receptors (Table 4) influenced pancreatitis associated lung inflammation.

	sham surgery + control	**sham surgery + pancreatitis**	**vagotomy + pancreatitis**
Lung MPO (% of sham surgery + control)	100	169 ± 9*	182 ± 7*
Relative lung weight	6.6 ± 0.2	7.3 ± 0.2*	7.4 ± 0.2*
MIP-2 lung (pg/ml)	62.1 ± 6.2	298.2 ± 14.4*	303.0 ± 15.6*

Table 3: Pancreatitis associated lung inflammation develops independently of the vagus nerve. Mice underwent either sham surgery or left cervical vagotomy three days before induction of pancreatitis. Pancreatitis was induced by 12 i.p. injections of cerulein (50 µg/kg); control mice received saline i.p. Mice were sacrificed one hour after the 12th injection. Markers for pancreatitis associated pulmonary inflammation are shown. Data are means ± SE of 8 mice per group. * $P < 0.05$ versus control, † $P < 0.05$ versus sham surgery + pancreatitis.

	saline + control	**saline + pancreatitis**	**mecamylamine + pancreatitis**
Lung MPO (% of sham surgery + control)	100	177 ± 8*	189 ± 9*
Relative lung weight	6.5 ± 0.2	7.3 ± 0.2*	7.4 ± 0.2*
MIP-2 lung (pg/ml)	58 ± 5	268 ± 11*	298 ± 17*

Table 4: Pancreatitis associated lung inflammation develops independently of nicotinic receptors. Mice were injected i.p.with saline, mecamylamine or GTS-21 thirty minutes before induction of pancreatitis. Pancreatitis was induced by 12 i.p. injections of cerulein (50 µg/kg); control mice received saline i.p. Mice were sacrificed one hour after the 12th injection. Markers for pancreatitis associated pulmonary inflammation are shown. Data are means ± SE of 8 mice per group. * $P < 0.05$ versus control, † $P < 0.05$ versus sham surgery + pancreatitis.

Discussion

The efferent vagus nerve, has recently been implicated as an important anti-inflammatory pathway through an interaction of its principal neurotransmitter Ach with nicotinic receptors, in particular the α7 subunit, on resident macrophages [8,23]. We here show, for the first time, that inhibition of the nicotinic anti-inflammatory pathway by cervical vagotomy or by blockade of nicotinic receptors by the administration of mecamylamine (11), results in enhanced severity of pancreatitis which is associated with an increase in the migration of neutrophils towards the inflamed pancreas. Conversely, activation of the peripheral component of the pathway by administration of the selective nicotinic receptor agonist GTS-21 [9,10] attenuated pancreatitis severity which was associated with a decrease in pancreatic neutrophil accumulation. These data suggest that a nicotinic anti-inflammatory pathway, consisting of the vagus nerve and nicotinic receptors, plays an essential role in the regulation of inflammatory responses during experimental pancreatitis.

The results of this study are in agreement with previous investigations that examined the influence of vagus nerve activity and Ach on sterile inflammation. In addition to the brain and "wire-innervated" peripheral structures, Ach receptor subtypes are also expressed by immune cells [5,24,25,23,8]. In vitro studies have shown that immune cells are susceptible to Ach. When macrophages are exposed to Ach these cells are effectively deactivated (7). This Ach-induced deactivation is characterized by a dose-dependent reduction in the release of a series of pro-inflammatory cytokines by macrophages stimulated with endotoxin (7). Further evidence has implicated the α7 subunit of the nicotinic Ach receptor, since the inhibitory effects of Ach and nicotine on macrophages could not be reproduced in macrophages deficient for this receptor [8,23]. In experimental endotoxemia, direct electrical or chemical vagus nerve stimulation reduced serum TNF-α levels and prevented shock [7,26], whilst cervical vagotomy augmented serum TNF-α levels and sensitized animals to the lethal effects of endotoxin [7,8]. In the cecal ligation and puncture model, aspecific cholinergic agonists improve survival (23). In other models of systemic inflammation, induced by either ischemia reperfusion injury or hypovolemic hemorrhagic shock, stimulation of the vagus nerve decreased serum TNF-α levels and prevented the development of hypotension [27,28]. Our data extend the anti-inflammatory potential of the vagus nerve and nicotinic receptors in a well established model of experimental pancreatitis and show, for the first time, that selective pharmacological stimulation of the α 7 nicotinic Ach receptor exerts anti-inflammatory effects in vivo.

The objective of this study was to evaluate the therapeutic potential of the nicotinic anti-inflammatory pathway during experimental pancreatitis. To inhibit this pathway we used two approaches; we either sectioned the vagus nerve to interfere with the hard wired component

of the pathway or we blocked the peripheral part of the pathway, nicotinic Ach receptors, using mecamylamine. Indeed, both approaches enhanced pancreatic edema, levels of plasma hydrolases and histologic abnormalities in the pancreas. Additionally, plasma IL-6 levels (which are raised in patients with acute pancreatitis and correlate with disease severity (21)) were increased in both groups of mice as compared to untreated pancreatitis animals. These data indicate that the vagus nerve and nictonic receptors limit pancreatitis severity in vivo. Alternatively, mecamylamine might have influenced pancreatitis severity through an interaction with central nicotinic receptors. However, since the dose of mecamylamine used predominantly antagonizes peripheral receptors (11) and mecamylamine treatment exerted similar affects as vagotomy it is likely that the observed effects are mediated through the vagus nerve and peripheral nicotinic receptors. Since the anti-inflammatory effects of the vagus nerve are mediated through nicotinic Ach α 7 receptors (8) we used a selective ligand for these receptors, GTS-21 [(9,10)], to stimulate the nicotinic anti-inflammatory pathway. Indeed, stimulation of these receptors attenuated all markers of pancreatitis severity. This is the first time it has been shown that selective pharmacological activation of nicotinic Ach α 7 receptors can influence inflammatory responses in vivo.

Acute pancreatitis is characterized by the premature activation of digestive enzymes in the pancreas, followed by a massive immunological response resulting in autodigestion of the gland, local and subsequent systemic inflammation (1). This second, inflammatory phase in the pathophysiology of acute pancreatitis is characterized by the involvement of cytokines and chemokines as well as activation and influx of neutrophils (29). This is of pathogenetic significance not only since neutrophil depletion ameliorates the severity of experimental acute pancreatitis (30) but also because neutrophils are themselves capable of secondary activation of digestive enzymes in the pancreas and therefore initiate a positive feedback loop of pancreatic inflammation (20). Interfering with the nicotinic anti-inflammatory pathway not only reduced the severity of pancreatic injury but also influenced the migration of neutrophils towards the pancreas. Sectioning the vagus nerve, or blocking nicotinic receptors with mecamylamine, increased the number of neutrophils that migrated towards the pancreas whereas stimulating α 7 nicotinic receptors with GTS-21 attenuated pancreatic neutrophil migration. Previous studies have shown that a variety of nicotinic Ach receptors is present on neutrophils and that stimulation of nicotinic receptors inhibits neutrophil migration which is, at least in part, mediated by inhibition of adhesion molecule expression on both endothelial cell surface and neutrophils (31). Experimental cerulein induced pancreatitis is associated with the development of mild pulmonary inflammation. In contrast to pancreatitis severity, the extent of pulmonary inflammation was unchanged in experiments in which the nicotinic anti-inflammatory pathway was either inhibited or stimulated. This is in line with previous studies which showed that during endotoxemia central and electrical stimulation of the vagus nerve abolished TNF-α release in the systemic compartment, the

liver, the heart and the spleen but not the lung (26). This finding is not completely explained but is most likely associated with the unique features of alveolar macrophages which, in contrast to all other residential macrophages, show an anti-inflammatory phenotype and are not susceptible to the nicotinic anti-inflammatory pathway [(26,32)].

We here demonstrate for the first time that the nicotinic anti-inflammatory pathway is an essential regulator of inflammation during experimental pancreatitis. We further show, for the first time, that pharmacological stimulation of the peripheral part of the nicotinic anti-inflammatory pathway, α 7 nicotinic Ach receptors, attenuates inflammation in vivo. The nicotinic anti-inflammatory pathway may be a future target for the treatment of pancreatitis.

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chapter 11

acute vagotomy activates the cholinergic anti-inflammatory pathway.

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With great interest we have read the paper by Francis etal (2004 Aug;287(2):H791-7). In this study, the role of the sympathetic and parasympathetic nervous system in the proinflammatory cytokine response was studied in a rat model of myocardial infarction. The authors suggest that the release of proinflammatory cytokines in the brain after myocardial infarction is independent of blood-borne cytokines and that cardiac sympathetic afferent nerves activated by myocardial ischemia signal the brain to increase cytokine production. Secondary, the authors suggest that an intact vagus nerve is required for the full expression of proinflammatory cytokines in the injured heart as well as in the circulation, since vagotomy resulted in a marked decrease of systemic and cardiac cytokine release. In the discussion the authors acknowledge that this is quite surprising, since previous reports have suggested that the vagus nerve excerpts anti inflammatory effects during inflammatory syndromes. We believe that this surprising finding can be explained by the design of the experiments and would like to propose this explanation to the authors and suggest additional experiments to evaluate whether this is indeed the case.

Recently, it has been shown that the vagus nerve provides a "cholinergic anti inflammatory pathway" during endotoxemia[1]. In these experiments, vagus nerve stimulation resulted in decreased cytokine release whereas vagotomy resulted in an increase in cytokine release. The vagus nerve downregulates inflammation by decreasing the release of tumor necrosis factor (TNF)-α by macrophages. In studies in which vagus nerve activity was stimulated by intra cerebroventricular injection of CNI-1493, systemic but also cardiac TNF-α release was inhibited during subsequent endotoxemia[2]. In line with this, as yet unpublished data from our group shows that the same cardiac TNF-α release is also inhibited during electrical VNS in this model suggesting that this pathway is present in the heart as well. These anti-inflammatory effect of vagus nerve stimulation are mediated by an interaction of acetylcholine, the principle neurotransmitter of the vagus nerve, with macrophage nicotinic acetylcholine receptors expressing the α7 subunit[3]. In the above mentioned studies, vagotomy was performed several days before endotoxemia was induced, the reason for this is that when vagotomy is performed and the vagus nerve is manipulated, the nerve dumps acetylcholine at distal nerve endings. In fact, experiments by our group show that when a vagotomy is performed 30 minutes before endotoxemia this results in a decrease in subsequent TNF-α release (Figure 1). In contrast, performing a vagotomy 3 days before endotoxemia results in an increase of TNF-α release as described above (Figure 1).

Since the authors performed a vagotomy one hour before the myocardial infarction it is therefore conceivable that the effects observed by vagotomy might be overshadowed by the effects of the release of acetylcholine by the vagus nerve induced by manipulating and cutting the nerve. In our opinion it cannot be ruled out that the release of acetylcholine and not

the effects of the absence of vagus nerve activity during experimental myocardial infarction were studied. We would suggest to repeat the vagotomy experiments by prolonging the time between vagotomy and experimental myocardial infarction.

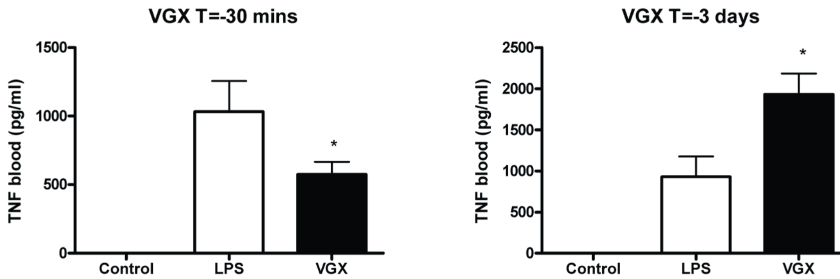

Figure 1: Difference in inflammatory phenotype due to timing of vagotomy previous to endotoxemia. Endotoxemia was induced in male Lewis rats by intravenous injection of 10 mg/kg of E. coli 0111:B4 LPS, controls received saline. Either 30 minutes or three days earlier, a unilateral left cervical vagotomy was performed. Rats were sacrificed two hours after LPS injection, plasma TNF-α levels are shown of control rats as well as endotoxemic rats subjected to sham surgery (not involving manipulation of the vagus nerve, LPS) or vagotomy (VGX). N = 8 rats per group. *P<0.05 vs LPS.

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chapter 12

vagus nerve stimulation inhibits activation of coagulation and fibrinolysis during endotoxemia in rats

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Introduction

Sepsis results in the concurrent activation of several host mediator systems including inflammation and coagulation [1]. Virtually every patient with sepsis displays evidence for activation of the coagulation system, which in its most extreme form can result in the well-known syndrome of disseminated intravascular coagulation [2, 3]. The tendency toward fibrin deposition and microvascular thrombosis in severe infection is increased further by concurrent inhibition of anticoagulant mechanisms. Indeed, sepsis and endotoxemia are associated with an impaired function and/or synthesis of natural anticoagulants such as antithrombin (AT) and activated protein C, and with inhibition of the fibrinolytic system primarily due to enhanced release of plasminogen activator inhibitor type I (PAI-1) [2, 3]. Interestingly, tight and bidirectional interactions exist between inflammation and hemostasis during sepsis and excessive activation of both pathways likely contributes to the lethality of sepsis [1, 4].

Recently, the cholinergic nervous system has been implicated in the regulation of inflammatory responses [5]. In particular, electrical stimulation of the efferent vagus nerve prevented the development of shock and reduced the release of the proinflammatory cytokine tumor necrosis factor (TNF)-α in rats [6, 7], an effect that appeared to be mediated by an effect of acetylcholine, the principal neurotransmitter of the vagus nerve, on α7 cholinergic receptors on macrophages [8]. Electrical vagus nerve stimulation (VNS) also inhibited the acute inflammatory response to acute hypovolemic hemorrhagic shock [9], splanchnic artery occlusion shock [10] and intestinal inflammation during experimentally induced ileus [11]. Moreover, chemical stimulation of the α7 cholinergic receptor attenuated systemic inflammation and improved the outcome of mice with polymicrobial abdominal sepsis [12], reduced cytokine release during bacterial peritonitis [13], diminished the severity of experimental pancreatitis [14] and suppressed endothelial cell activation during the localized Shwartzman reaction [15]. Knowledge of the effect of the efferent vagus nerve on the hemostatic mechanism is not available. Considering the eminent role of coagulation activation in the host response to severe infection the primary objective of the present study was to determine the effect of electrical VNS on activation of coagulation and fibrinolysis after injection of lipopolysaccharide (LPS) into rats.

Methods

Animals:

Male Lewis rats (Harlan, Horst, the Netherlands), 200-250 grams in weight, were used in

all experiments. The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Design:
Rats were anesthetized by intraperitoneal injection of 0.07 ml/g FFM mixture (Fentanyl (0.315 mg/ml)-Fluanisone (10 mg/ml) (Janssen, Beersen, Belgium), Midazolam (5 mg/ml) (Roche, Mijdrecht, The Netherlands) and thereafter kept under anesthesia by isoflurane (Upjohn, Ede, The Netherlands). A cervical midline incision was used to expose the left cervical vagus trunk, which was isolated and placed loosely in the jaw of a bipolar electrode (Harvard Apparatus, Holliston, Massachusetts). The vagus nerve was electrically stimulated for 10 minutes before and 10 minutes after LPS injection with predefined settings (5 V, 2 ms, 1Hz) as described previously [6]. In sham stimulated animals the left vagus nerve was exposed and placed in the bipolar electrode but not stimulated. LPS (from Escherichia coli 0111:B4; Sigma, St Louis, MI; 10 mg/kg) was administered via the penal vein. Rats (N=8 per group at each time point) were killed before, and 2, 4 and 6 hours after LPS injection and blood was collected in tubes containing heparin or citrate. In addition, at 0, 2 and 4 hours spleens were collected and homogenized at 40C in 5 volumes of sterile isotonic saline with a tissue homogenizer (Biospect Products, Bartlesville, OK). These homogenates were diluted 1:2 in lysis buffer (150 mM NaCl, 15 mM Tris, 1 mM MgCl.H2O, 1 mM CaCl2, 1% Triton X-100, 100 μg of pepstatin A/ml, leupeptin, aprotinin, pH 7.4) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1,500 x g for 15 min, after which the supernatants were stored at -20°C until measurements of cytokines.

Assays:
Thrombin-antithrombin complexes (TATc, Dade Behring, Marburg, Germany) and D-dimer (Diagnostica Stago, Roche, Almere, the Netherlands) were measured by ELISA. AT [16] and plasminogen activator (PA) activity [17] were measured by automated amidolytic techniques. Tissue-type plasminogen activator (tPA) antigen was measured with an ELISA. Briefly, microtiter plates are coated with rabbit anti-rat t-PA polyclonal antibodies (American Diagnostica, Greenwich, CT) at a concentration of 4 μl/ml. The plates were incubated with samples that were diluted in phosphate-buffered saline containing casein (5 mg/ml). Bound t-PA antigen was subsequently quantitated with biotinylated rabbit anti-rat tPA antibodies, followed by avidin-peroxidase and tetramethylbenzimidine. As a standard recombinant rat JMI-229 tPA was used [18]. PAI-1 activity was determined with an automated coagulation analyser (Behring Coagulation System) with reagents and protocols from Dade Behring. TNF-α, Interleukin (IL)-6, IL-1β and IL-10 were measured by Luminex (Bio-rad laboratories, Hercules, CA). Cytokine-induced neutrophil chemoattractant (CINC)-1 and CINC-3 were measured by ELISA (R & D systems, Abingdon, United Kingdom).

Statistical analysis:

All values are means ± SEM. Differences between groups were analyzed by two way analysis of variance (interaction between treatment and time); the “overall” P value obtained with this test is depicted in the figures. In case this analysis yielded a $P < 0.05$, Bonferroni post tests were done to determine the significance at individual time points (depicted as asterisks in the figures). A P-value < 0.05 was considered statistically significant.

Results

VNS inhibits LPS-induced coagulation activation

Intravenous injection of LPS resulted in a brisk activation of the coagulation system as indicated by increases in the plasma concentrations of TATc and D-dimer, both peaking after 4 hours (31 ± 2 µg/L and 51 ± 5 µg/L respectively; Figure 1A and 1B). This procoagulant response was accompanied by a modest but significant decrease in plasma AT levels after injection of LPS reaching a nadir after 4 hours (92 ± 2%; Figure 1C). Electrical VNS strongly inhibited LPS-induced coagulation activation: peak TATc levels were only 17 ± 2 µg/L in rats subjected to VNS, whereas plasma D-dimer only reached values of 31 ± 2 µg/L (both $P < 0.001$ versus LPS only). Moreover, VNS completely prevented the decline in AT levels ($P < 0.001$ versus LPS only).

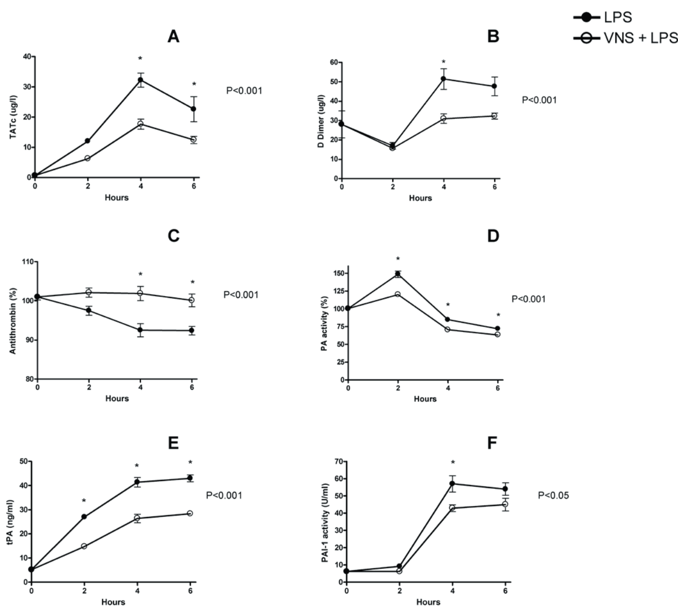

Figure 1: Vagus nerve stimulation inhibits the coagulant and fibrinolytic response during endotoxemia. Rats were subjected to electrical vagus nerve stimulation (VNS, open symbols) or sham stimulation (closed symbols) as described in the Methods. All animals received a bolus intravenous injection of LPS (10 mg/kg) at t = 0. A: thrombin-antithromin complexes (TATc). B: D-dimer. C: antithrombin. D: plasminogen activator (PA) activity. E: tissue type plasminogen activator (tPA). F: plasminogen activator inhibitor type I (PAI-1). Data are means +/- SEM of 8 rats per group at each time point. P values given in graphs indicate difference between groups by two way analysis of variance. * indicates $P < 0.05$ at the specified time point by Bonferroni post test.

VNS attenuates the LPS-induced changes in the fibrinolytic system
LPS induced a rapid activation of the fibrinolytic system as reflected by an early rise in plasma PA activity, peaking after 2 hours (148 ± 5 %; Figure 1D). The increase in PA activity was accompanied by a concurrent increase in the plasma levels of tPA antigen, which continued to rise beyond 2 hours after LPS, peaking after 6 hours (43 ± 1 µg/L; Figure 1E). The decrease in PA activity from 2 hours onward closely followed the increase in plasma PAI-1 activity, which reached a plateau between 4 and 6 hours post LPS (4 hours: 57 ± 5 U/mL; Figure 1F). VNS attenuated these biphasic changes in the fibrinolytic system: it inhibited the early rises in PA activity and tPA antigen (peak levels 120 ± 13 % and 28 ± 10 µg/L respectively, both $P < 0.001$ versus LPS only), as well as the delayed increase in PAI-1 activity (peak levels 45 ± 8 U/mL, $P < 0.05$ versus LPS only).

Effect of VNS on plasma and splenic cytokine levels
The effect of VNS on LPS-induced TNF-α release has been documented previously [6-8]. We sought to expand these earlier data by measuring the plasma and splenic concentrations of other cytokines with a known effect on the hemostatic mechanism (Figure 2). LPS injection induced profound rises in the concentrations of TNF-α, IL-1β, IL-6 and IL-10 in plasma and spleen homogenates, all peaking 2 hours after LPS administration. Electrical VNS most potently inhibited the LPS-induced rises in plasma and splenic TNF-α concentrations: whereas LPS elicited peak TNF-α levels of 10 ± 2 ng/mL in plasma and 12 ± 3 ng/mL in spleen homogenates, peak concentrations in rats subjected to VNS were 3 ± 1 ($P < 0.001$) and 6 ± 2 ng/mL ($P < 0.05$) respectively (Figure 2A and B). In addition, VNS attenuated the LPS-induced increase in splenic IL-1β levels (Figure 2D, $P < 0.05$), whereas the inhibition of LPS-induced IL-1β release into the circulation did not reach statistical significance (Figure 2C). VNS reduced IL-6 levels in both plasma and spleen homogenates after LPS injection (Figure 2E and F, both $P < 0.05$ versus LPS only). By contrast, VNS did not influence the increases in plasma and splenic IL-10 during endotoxemia (Figure 2G and H).

Effect of VNS on CXC chemokine release
To obtain further insight into how broad the anti-inflammatory effect of VNS is during endotoxemia, we measured the plasma levels of two CXC chemokines: CINC-1 and CINC-3. LPS injection was associated with transient rises in the plasma concentrations of both mediators, with peak levels reached after 4 hours (CINC-1: 33 ± 7 ng/mL; CINC-3: 32 ± 7 ng/mL). VNS did not influence LPS-induced CINC-1 release (Figure 3A), whereas it attenuated the rise in plasma CINC-3 concentrations with peak levels reaching only 19 ± 4 ng/mL (Figure 3B, $P < 0.05$ versus LPS only).

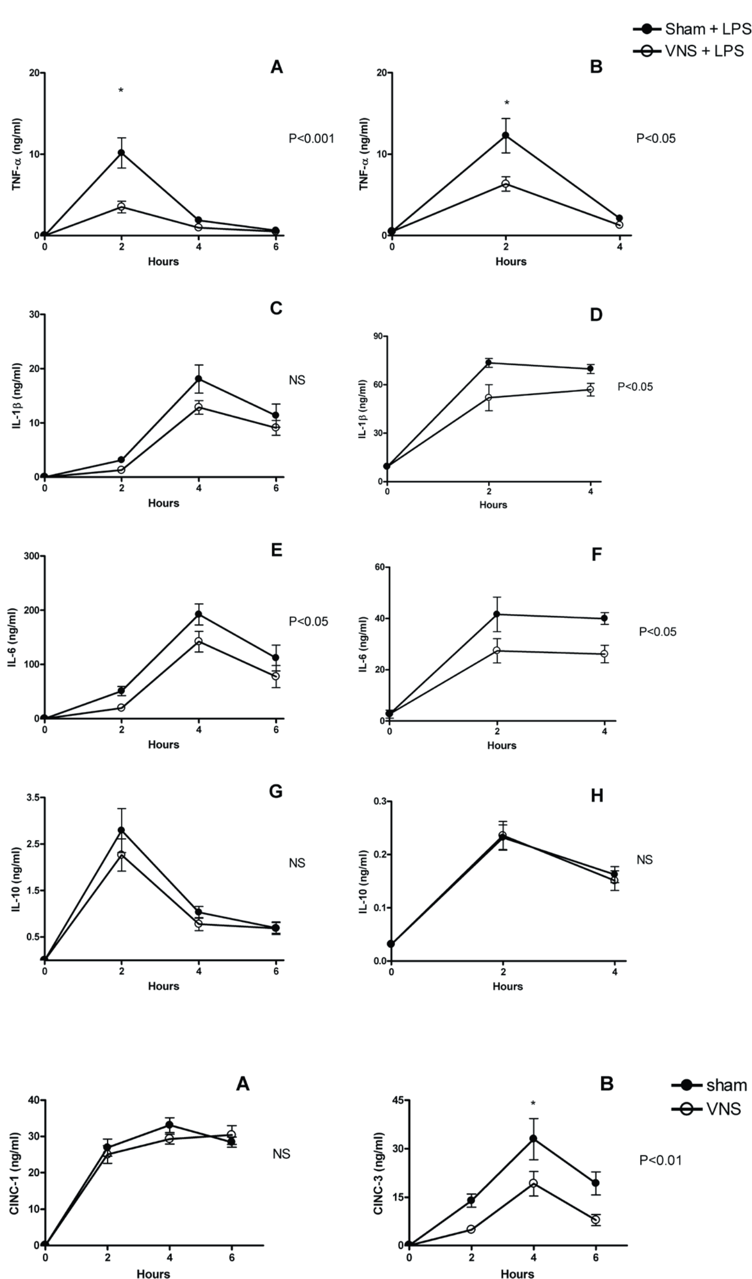

Figure 2: Vagus nerve stimulation inhibits TNF-α and IL-6 release during endotoxemia. Rats were subjected to electrical vagus nerve stimulation (VNS, open symbols) or sham stimulation (closed symbols) as described in the Methods. All animals received a bolus intravenous injection of LPS (10 mg/kg) at t = 0. Left panels: plasma concentrations. Right panels: splenic concentrations A + B: TNF-α. C + D: IL-1 β. E + F: IL-6. G + H: IL-10. Data are means +/- SEM of 8 rats per group at each time point. P values given in graphs indicate difference between groups by two way analysis of variance. * indicates $P < 0.05$ at the specified time point by Bonferroni post test. NS = non significant.

Figure 3: Vagus nerve stimulation inhibits CINC-3 release during endotoxemia. Rats were subjected to electrical vagus nerve stimulation (VNS, open symbols) or sham stimulation (closed symbols) as described in the Methods. All animals received a bolus intravenous injection of LPS (10 mg/kg) at t = 0. A: CINC-1; B: CINC-3. Data are means +/- SEM of 8 rats per group at each time point. P values given in graphs indicate difference between groups by two way analysis of variance. * indicates $P < 0.05$ at the specified time point by Bonferroni post test. NS = non significant.

Discussion

The cholinergic anti-inflammatory pathway is a physiological mechanism that regulates host inflammatory responses via the local release of acetylcholine, the primary neurotransmitter secreted by the vagus nerve [5]. Electrical stimulation of the vagus nerve has been reported to inhibit inflammation in several models including endotoxemia [6-8], ischemia-reperfusion injury [10, 19] and hypovolemic hemorrhagic shock [9]. The most prominent anti-inflammatory effect of VNS in these models was inhibition of the release of the omnipotent cytokine TNF-α. In light of the increased interest in the interplay between inflammation and coagulation in sepsis [1, 3, 4], we here investigated the effect of electrical VNS on coagulation and fibrinolysis during endotoxemia in rats. The main finding of our study was that VNS attenuated both LPS-induced activation of the coagulation system and the characteristic biphasic changes in fibrinolysis involving early activation followed by a relatively delayed inhibition. These results suggest for the first time that the cholinergic anti-inflammatory pathway, besides manipulating cytokine production, influences the hemostatic mechanism in vivo which may be of considerable importance for our understanding of the beneficial effects of VNS during systemic inflammatory response syndromes such as occurs during severe sepsis.

The coagulation and fibrinolytic response to intravenous LPS has been studied extensively in healthy humans [20-22]. We here demonstrate that LPS induces similar changes in the hemostatic mechanism of rats. Indeed, LPS injection resulted in activation of the coagulation system, as indicated by increases in the plasma concentrations of TATc and D-dimer. In addition, LPS administration elicited an early activation of the fibrinolytic system as reflected by an early rise in plasma PA activity and tPA antigen levels, followed by a delayed increase in plasma PAI-1 activity. Electrical VNS blunted all of these responses, which should not go without notice, since studies in humans have revealed that the activation of coagulation and fibrinolysis induced by intravenous LPS proceeds independently from one another [22, 23]. What could be the common underlying mechanism by which VNS inhibits both coagulation and fibrinolysis? In this respect the recent study by de Jonge et al. is of particular interest [11]. These authors found that the cholinergic agonist nicotine exerts anti-inflammatory effects in macrophages in vitro by activation of the transcription factor STAT3. As such, engagement of macrophage cholinergic receptors resulted in activation of a molecular route that strongly resembles the signaling pathway of the IL-10 receptor without the requirement for IL-10 itself [11]. Moreover, the anti-inflammatory effect of electrical VNS during ileus produced by intestinal manipulation in mice totally relied on activation of macrophage STAT3 [11]. Interestingly, our group previously showed that administration of recombinant IL-10 inhibits both coagulation and fibrinolysis in humans

exposed to low dose LPS [24], thereby mimicking the effects of VNS described here. Together we consider it conceivable that VNS influences coagulation and fibrinolysis via the signaling cascade described by de Jonge et al. [11], which is also used by IL-10 to affect the hemostatic response. Considering that activation of coagulation is strictly dependent on tissue factor in this model [23, 25, 26], it is likely that VNS acts on tissue factor expression to inhibit coagulation. This possibility is supported by the fact that triggering of the IL-10 receptor results in inhibition of tissue factor expression on mononuclear cells [27, 28]. The modest inhibition of IL-6 release is, in our opinion, of less importance, considering that complete elimination of this cytokine failed to [29] or only partially [30] inhibited LPS-induced coagulation in vivo. On the other hand, the inhibition of LPS-induced TNF-α release by VNS is the most probable mechanism by which this procedure attenuated the fibrinolytic response to LPS. Indeed, all of these responses are strictly dependent on TNF-α [31, 32] and also IL-10 induced inhibition of fibrinolysis likely proceeds via inhibition of TNF-α [24].

A limitation of our study is that it does not provide direct insight into the effect of VNS on tissue factor expression and the balance between tissue factor and its major inhibitor tissue factor pathway inhibitor. This inhibitor can be released upon activation of the vascular endothelium; although nicotine has been found to attenuate endothelial cell activation in mice in vivo [15], it remains to be established whether VNS influences the vascular endothelium and/or the tissue factor/tissue factor pathway inhibitor balance.

The effect of VNS on LPS-induced TNF-α release into the circulation has been documented previously [6-8]. We expanded these earlier data by measuring the plasma and splenic concentrations of other cytokines with a known effect on the hemostatic mechanism. Of these, TNF-α and IL-6 were significantly inhibited by VNS, albeit the latter to a modest extent. IL-1β release was also slightly inhibited by VNS, significantly so in the spleen. In contrast, the release of the anti-inflammatory cytokine IL-10 was not influenced by VNS. These data are in line with the described in vitro effects of acetylcholine in macrophage cultures, wherein acetylcholine inhibited the release of TNF-α, IL-1β and IL-6 without influencing IL-10 secretion [6]. Similarly, nicotine reduced LPS-induced TNF-α and IL-6 release by murine peritoneal macrophages in vitro without influencing IL-10 [11].

VNS inhibited the LPS-induced rise in plasma CINC-3 levels. This finding is in line with the effect of VNS in ileus in mice, where electrical stimulation of the vagus nerve reduced the secretion of not only TNF-α and IL-6 in peritoneal lavage fluid, but also of macrophage inflammatory protein 2 (the murine homolog of CINC-3)[11]. It remains to be established why VNS selectively affects CINC-3 without altering CINC-1 release.

The present report focuses on the effect of electrical stimulation of the vagus nerve. Several investigations have documented the potential of chemical VNS. Nicotine, which stimulates peripheral α7 cholinergic receptors, inhibited systemic inflammation during murine endotoxemia and abdominal sepsis [12, 13]. Similarly, CNI-1493, a tetravalent guanylhydrazone molecule that induces vagus nerve firing via a centrally mediated intracerebral effect, inhibited TNF-α release induced by LPS in rats [7]. A melanocortin peptide (adrenocorticotropin 1-24) suppressed NFκB dependent systemic inflammation and shock triggered by hemorrhage in rats by central activation of the cholinergic anti-inflammatory pathway [33]. In addition, we recently showed that pretreatment with the selective α7 cholinergic receptor agonist 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21) markedly reduced the severity of acute pancreatitis induced by repeated injections of cerulein in mice [14]. Further research is warranted to establish whether such interventions influence the hemostatic mechanism.

We here describe a previously unrecognized effect of VNS on the hemostatic response to a sublethal injection of LPS in a rat model that resembles the well characterized human endotoxemia model. Activation of the hemostatic mechanism as a consequence of inflammation can be considered instrumental in containing inflammatory activity to the site of infection. However, uncontrolled activation may cause harm and give rise to a vicious cycle, eventually leading to dramatic events such as manifested in severe sepsis and disseminated intravascular coagulation. Whereas the ability of VNS to inhibit the production of the potent proinflammatory cytokine TNF-α during endotoxemia had been documented previously [6-8], we demonstrate for the first time that VNS also inhibits activation of coagulation and fibrinolysis. Stimulation of the cholinergic anti-inflammatory pathway may therefore not only impact on inflammation but also on the coagulant-anticoagulant balance during sepsis and endotoxemia.

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chapter 13

stimulation of alpha 7 cholinergic receptors inhibits lipopolysaccharide-induced neutrophil recruitment by a tumor necrosis factor-alpha independent mechanism

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Introduction

In recent years it has become apparent that the cholinergic nervous system plays an important role in limiting inflammatory responses (1). In the so-called cholinergic anti-inflammatory pathway enhanced efferent activity of parasympathetic nerve endings results in inhibition of the release of proinflammatory cytokines such as tumor necrosis factor (TNF)-α by macrophages. Disruption of this neural-based system by vagotomy renders animals more vulnerable to toxicity elicited by the administration of lipopolysaccharide (LPS), the proinflammatory component of the outer membrane of gram-negative bacteria. Indeed, in rats surgical dissection of the vagus nerve led to exaggerated release of TNF-α and accelerated the development of hypotensive shock after intravenous injection of LPS (2). In line, vagotomy also enhanced the local and systemic inflammation accompanying bacterial peritonitis (3) and cerulein-induced acute pancreatitis (4). Conversely, stimulation of the cholinergic anti-inflammatory pathway results in inhibition of inflammation. In particular, electrical stimulation of the efferent vagus nerve prevented the development of shock and reduced the release of TNF-α in endotoxemic rats (2), attenuated the acute inflammatory response to acute hypovolemic hemorrhagic shock (5) and diminished intestinal inflammation during experimentally induced ileus (6).

A series of elegant studies established that acetylcholine is the principal mediator of the anti-inflammatory effects of vagus nerve stimulation (VNS); acetylcholine secreted by VNS binds to α7 cholinergic receptors on macrophages to suppress proinflammatory cytokine production (7). Stimulation of α7 cholinergic receptors by specific agonists, such as nicotine, attenuated systemic inflammation and improved the outcome of mice with polymicrobial abdominal sepsis (8), reduced proinflammatory cytokine release during Escherichia coli peritonitis (3) and suppressed endothelial cell activation during the localized Shwartzman reaction (9). Knowledge of the effect of pharmacologic stimulation of cholinergic receptors during endotoxemia is limited. Administration of nicotine inhibited the release of high mobility group box 1 (HMGB1) triggered by high dose LPS in mice and protected against lethality in this model; nicotine did not significantly influence interleukin (IL)-6 release in endotoxemic mice, whereas the effect on other cytokines were not reported (8). CNI-1493, a tetravalent guanyl-hydrazone molecule that induces vagus nerve firing via a centrally mediated intracerebral effect, inhibited TNF-α release induced by LPS in rats (10).

We recently showed that pretreatment with the selective α7 cholinergic receptor agonist 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21) markedly reduced the severity of acute pancreatitis induced by repeated injections of cerulein in mice (4). We here sought to determine the effect of GTS-21 on cytokine and chemokine release and neutrophil influx after intraperitoneal administration of LPS.

Materials and Methods

Mice:
Female C57BL/6 mice were purchased from Harlan (Horst, the Netherlands). Experiments were performed with mice that were 8 weeks old. The protocol was approved by the Institutional Animal Care and Use Committee of the Academic Medical Center.

Design:
In a first series of experiments mice received an intraperitoneal injection (total volume 200 µl) with either vehicle (sterile normal saline) or GTS-21 (4 mg/kg; Critical Therapeutics Inc, Lexington, MA); one hour later mice received an intraperitoneal injection with 200 µg LPS (from Escherichia coli, serotype 0111:B4; Sigma, St. Louis, MO; in 200 µl saline). Animals were killed 2, 6 or 24 hours after LPS injection (8 per group at each time point). At these time points mice were first anesthetized by inhalation of 2% isoflurane (Abbott Laboratories Ltd., Kent, UK) / 2 liters of O2. A peritoneal lavage was then performed with 5 ml of sterile isotonic saline using an 18-gauge needle, and peritoneal lavage fluid (PLF) was collected in sterile tubes (Plastipack [Becton-Dickinson, Mountain View,). After collection of peritoneal fluid, deeper anesthesia was induced by intraperitoneal injection of 0.07 ml of FFM mixture (fentanyl [0.315 mg/ml]-fluanisone [10 mg/ml] [Janssen, Beersen, Belgium], midazolam [5 mg/ml] [Roche, Woerden, the Netherlands]) per g. The abdomen was opened, and blood was drawn from the vena cava inferior into a sterile syringe, transferred to tubes containing heparin, and immediately placed on ice. In a second set of experiments mice received vehicle (saline), GTS-21 (4 mg/kg) and/or etanercept (Enbrel, Wyeth Pharmaceuticals, Madison, NJ; 2 mg/kg) in a total volume of 200 µl; one hour later, mice received an intraperitoneal injection with 200 µg LPS (from E. coli, serotype 0111:B4; Sigma, in 200 µl saline). Animals were killed 1, 3 or 6 hours after LPS injection (8 per group at each time point) and PLF and blood were harvested as described above. GTS-21 is a selective α7 cholinergic receptor agonist (11, 12) that given at the dose used here strongly reduced the severity of acute pancreatitis induced by repeated cerulein injections (4). Etanercept is a TNF-α blocking protein containing the extracellular ligand-binding domain of the p75 TNF-α receptor; etanercept effectively neutralizes mouse TNF-α in vivo (13-15) and given at the dose used here markedly reduced LPS-induced lethality in mice (16).

Cell counts and differentials
Cell counts were determined in PLF using a hemacytometer (Beckman Coulter, Fullerton, CA). Subsequently, the pellet was diluted in phosphate-buffered saline to a final concentration of 105 cells/ml and differential cell counts were performed on cytospin preparations stained with a modified Giemsa stain (Diff-Quick; Dade Behring AG, Düdingen, Switzer-

land).

Assays

TNF-α, IL-6, IL-10 and monocyte chemoattractant protein (MCP)-1 levels were determined using a commercially available cytometric beads array multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer's recommendations. Macrophage inflammatory protein (MIP)-2 and keratinocyte derived chemokine (KC) levels were measured using commercially available ELISA kits (R&D systems, Abingdon, UK).

Statistical analysis:

All values are means ± SEM. Differences between groups were analyzed by two way analysis of variance followed by Bonferroni post tests to determine the significance at individual time points where appropriate. A P-value < 0.05 was considered statistically significant.

Results

Effect of GTS-21 on the local and systemic release of cytokines

Intraperitoneal injection of LPS induced a brisk increase in the concentrations of all cytokines measured in both PLF (Figure 1, left panels) and plasma (Figure 1, right panels). At 24 hours after LPS administration all cytokine levels had returned to baseline; therefore Figure 1 only shows data obtained 2 and 6 hours post LPS. In general, plasma concentrations were much higher than simultaneously measured PLF levels, most likely due to the dilution of the latter samples (please note the different scales used in Figure 1 for PLF and plasma concentrations). The most consistent effect of GTS-21 on LPS-induced cytokine release was the inhibition of TNF-α secretion in both PLF and plasma (both $P < 0.05$ versus LPS only). GTS-21 very modestly but significantly reduced LPS-induced IL-6 release in plasma ($P < 0.05$ versus LPS only) but not in PLF. MCP-1 or IL-10 release was not influenced by GTS-21 in either PLF or plasma.

GTS-21 inhibits LPS-induced neutrophil influx into the peritoneal cavity

Intraperitoneal LPS injection resulted in a strong influx of neutrophils into the peritoneal cavity reaching a plateau between 6 and 24 hours (Figure 2). This inflammatory response was attenuated by GTS-21 ($P < 0.05$ versus LPS only).

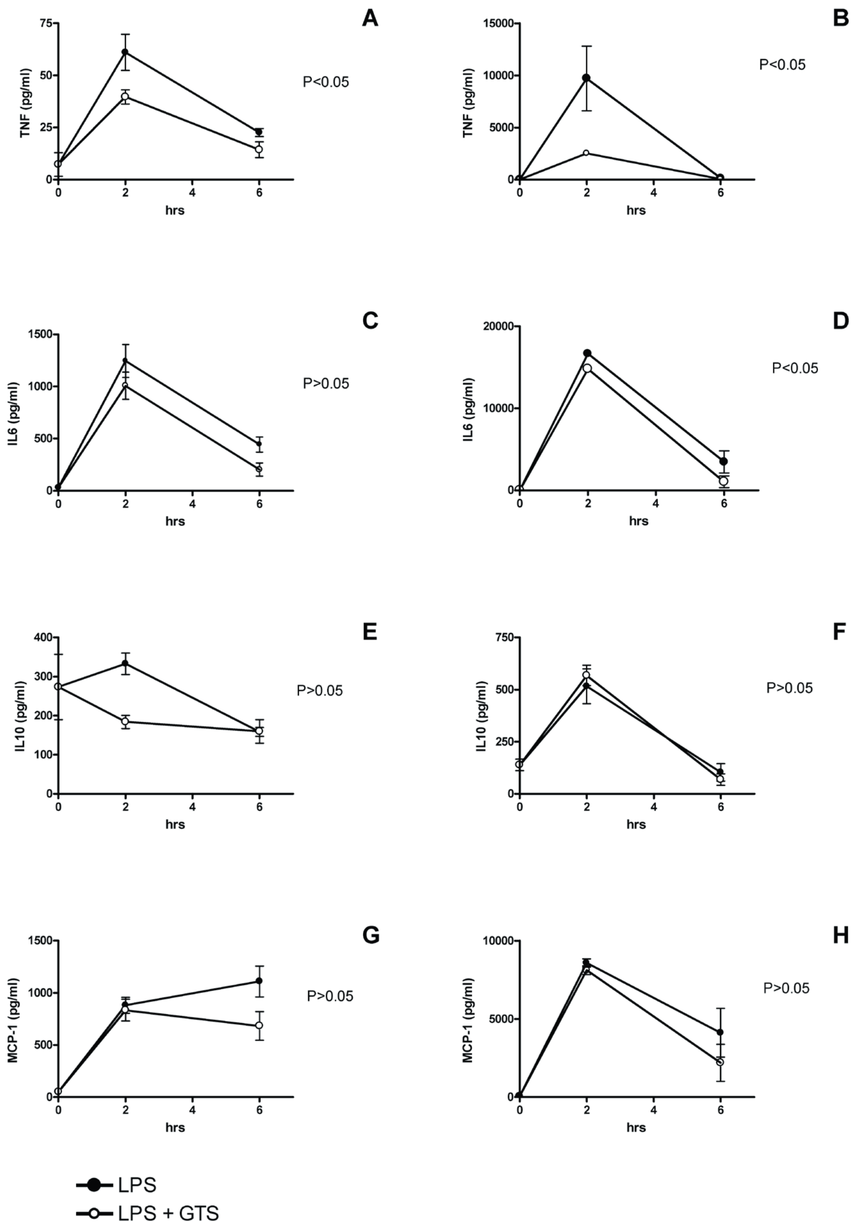

Figure 1: GTS-21 inhibits LPS-induced TNF-α release. Mice received an intraperitoneal injection with either sterile normal saline or GTS-21 (4 mg/kg); one hour later mice received an intraperitoneal injection with LPS (200 μg). Cytokine levels were measured in peritoneal lavage fluid (left panels) and plasma (right panels). TNF-α (A,B), IL-6 (C,D), IL-10 (E,F) and MCP-1 (G, H). Data are means +/- SEM of 8 mice per group at each time point. P values given in graphs indicate difference between groups by two way analysis of variance.

The effect of GTS-21 on neutrophil recruitment does not depend on inhibition of TNF-α

TNF-α is considered a pivotal mediator of LPS-induced inflammation (17). Having established that GTS-21 inhibits TNF-α release and neutrophil influx, we next wished to determine whether the GTS-21 induced inhibition of neutrophil recruitment was the consequence of the reduction in TNF-α levels. Therefore, we administered mice with the TNF-α neutralizing compound etanercept at a dose that protected mice against LPS-induced lethality (16), and compared its effect on neutrophil influx with that of GTS-21. Etanercept strongly reduced TNF-α levels in plasma after LPS administration (Figure 3A, $P < 0.05$ versus LPS only); in this experiment, GTS-21 also attenuated LPS-induced TNF-α release (Figure 3A, $P < 0.05$), confirming the data presented in Figure 1. As expected (considering that TNF-α is an important intermediate factor in LPS-induced IL-6 release (17)), etanercept diminished IL-6 levels upon LPS administration (Figure 3B, $P < 0.05$); in this experiment the effect of GTS-21 on LPS-induced IL-6 secretion into the circulation was not significant. Remarkably, etanercept did not influence the LPS-induced influx of neutrophils into PLF (Figure 4A), whereas, in accordance with the experiment shown in Figure 2, GTS-21 attenuated this inflammatory response. The combined treatment with etanercept and GTS-21 did not result in a further reduction in neutrophil recruitment. The GTS-21 induced inhibition of neutrophil influx was not caused a reduction in the local concentrations (i.e. in PLF) of the neutrophil attracting CXC chemokines MIP-2 and KC. Indeed, whereas etanercept did not alter LPS-induced MIP-2 and KC release into PLF, GTS-21 enhanced KC release while not affecting MIP-2 concentrations (Figure 4B and C).

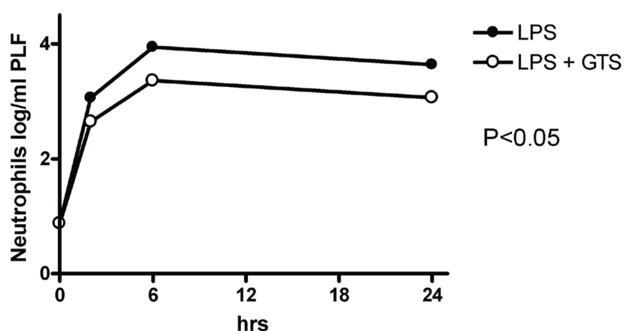

Figure 2: GTS-21 inhibits LPS-induced neutrophil recruitment. Mice received an intraperitoneal injection with either sterile normal saline or GTS-21 (4 mg/kg); one hour later mice received an intraperitoneal injection with LPS (200 μg). Neutrophil numbers were determined in peritoneal lavage fluid. Data are means +/- SEM of 8 mice per group at each time point. P values given in graphs indicate difference between groups by two way analysis of variance.

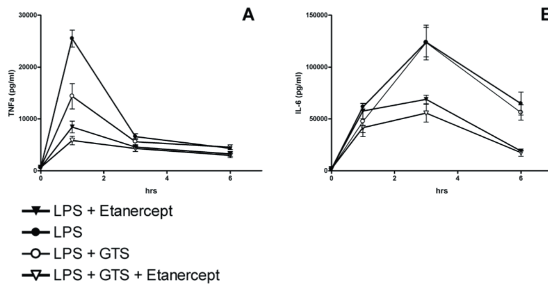

Figure 3: Effect of etanercept on TNF-α and IL-6 levels. Mice received an intraperitoneal injection with either sterile normal saline, GTS-21 (4 mg/kg) and/or etanercept (2 mg/kg); one hour later mice received an intraperitoneal injection with LPS (200 µg). Cytokine levels were measured in plasma. TNF-α (A) and IL-6 (B). Data are means +/- SEM of 8 mice per group at each time point. P values given in graphs indicate difference between groups by two way analysis of variance.

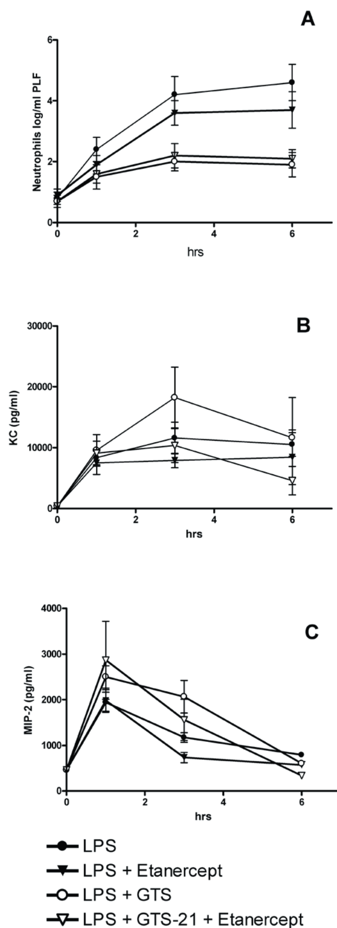

Figure 4: GTS-21 inhibits neutrophil recruitment by a mechanism that does not rely on inhibition of TNF-α or CXC chemokine activity. Mice received an intraperitoneal injection with either sterile normal saline, GTS-21 (4 mg/kg) and/or etanercept (2 mg/kg); one hour later mice received an intraperitoneal injection with LPS (200 µg). Neutrophil counts (A), and the levels of MIP-2 (B) and KC (C) were determined in peritoneal lavage fluid. Data are means +/- SEM of 8 mice per group at each time point. P values given in graphs indicate difference between groups by two way analysis of variance.

Discussion

Recruitment of neutrophils to the site of an infection is a crucial first step in the host response to invading bacteria. We here demonstrate that GTS-21, a compound with selective agonist activity on α7 cholinergic receptors (11, 12), inhibits the influx of neutrophils into the peritoneal cavity upon intraabdominal injection of LPS. The reduction of neutrophil migration by GTS-21 was not the consequence of GTS-21 induced inhibition of TNF-α release: elimination of endogenous TNF-α by etanercept did not influence LPS-induced neutrophil influx either in the presence or in the absence of GTS-21 treatment. These data identify a novel anti-inflammatory effect of chemical α7 cholinergic receptor stimulation that is independent from its well known capacity to inhibit TNF-α production.

The current data are in line with our recent study on the effects of GTS-21 during experimentally induced acute pancreatitis, wherein GTS-21 inhibited neutrophil influx into the pancreas as measured by intrapancreatic myeloperoxidase activity and the number of Leder-positive cells in pancreas tissue (4). In addition, we recently studied the effect of nicotine on the inflammatory response to E. coli peritonitis (3). In that study nicotine did not influence the early (6 hours) recruitment of neutrophils to the peritoneal cavity after intraperitoneal administration of live bacteria, whereas at this time point nicotine did reduce intraabdominal TNF-α concentrations. During more established peritonitis, 24 hours after infection, nicotine treatment was associated with not only reduced peritoneal TNF-α levels, but also with a clearly diminished recruitment of neutrophils (3). In contrast with our present investigation with GTS-21, nicotine reduced KC levels in PLF 24 hours after bacterial infection, suggesting that during E. coli peritonitis nicotine at least in part modulated neutrophil migration via an effect on this neutrophil attracting chemokine (3). It should be noted that in the experiments presented in Figure 4 GTS-21 potentiated LPS-induced KC release. Although an explanation for this unexpected finding is lacking, it is clear that reduced CXC chemokine secretion upon LPS administration does not play a role in the inhibiting effect of GTS-21 on neutrophil influx. Neutrophils express several nicotinic receptors, including the α7 cholinergic receptor (18), and stimulation of these receptors has been shown to inhibit neutrophil migration by a mechanism that involves inhibition of adhesion molecule expression on both the endothelial surface and neutrophils (19). Together these data suggest that GTS-21 may inhibit neutrophil migration at least partially through a direct effect on neutrophils that does not rely on indirect effects via reduced production of either TNF-α or CXC chemokines such as KC and MIP-2.

GTS-21 modestly reduced IL-6 release in the first experiments (Figure 1) but not in the second studies (Figure 3). Notably, GTS-21 treatment did diminish plasma IL-6 concentrations in mice with acute pancreatitis (4), which may however simply have been a reflection

of a less severe disease in GTS-21 treated animals. The release of the anti-inflammatory cytokine IL-10 was not influenced by GTS-21. This finding is in line with the described in vitro effects of acetylcholine in macrophage cultures, wherein acetylcholine inhibited the release of TNF-α without influencing IL-10 secretion (2). Similarly, nicotine reduced LPS-induced TNF-α release by murine peritoneal macrophages in vitro without influencing IL-10 (6). GTS-21 did also not inhibit the release of MCP-1, KC or MIP-2 upon intraperitoneal LPS injection. Hence, with respect to inhibition of the release of cytokines and chemokines during endotoxemia, GTS-21 apparently has a relatively selective effect on TNF-α.

Several α7 cholinergic receptor agonists have been developed in the recent past in light of their potential as novel therapeutics for the treatment of schizophrenia (20). Our current finding that one of these agents, GTS-21, inhibits two major inflammatory responses (TNF-α release and neutrophil recruitment) in the well established model of LPS-induced peritonitis adds to the now accumulating evidence that such compounds exert anti-inflammatory effects that may prove beneficial in inflammatory diseases. As such, further research is warranted to evaluate the effects of specific α7 cholinergic agonists in models of variable diseases such as overwhelming sepsis, ischemia/reperfusion injury, inflammatory bowel disease and rheumatoid arthritis.

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chapter 14

therapeutic effects of troglitazone in experimental chronic pancreatitis in mice

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Introduction

Chronic pancreatitis is characterized by progressive destruction of parenchymal tissue ultimately leading to exocrine and endocrine function loss. Clinical symptoms include abdominal pain, steatorrhea and diabetes mellitus. The incidence of chronic pancreatitis varies from region to region, from 7-15 per 100.000 per year, and is rising [(1)]. Risk factors are chronic alcohol abuse as well as genetic factors such as mutations in the cystic fibrosis gene, cationic trypsinogen gene and serine protease inhibitor-1 [(2)]. Knowledge of the pathophysiology of chronic pancreatitis is limited . Chronic pancreatitis is considered to result from chronic repetitive inflammation within the pancreas due to alcohol abuse or recurrent bouts of even minor events of pancreatic inflammation, resulting in recurrent repair of pancreatic damage and ultimately in activation of a profibrotic cascade. Fibrosis formation in the pancreas is initiated by differentiation and activation of pancreatic stellate cells (PSC) which produce collagen as a result [(3)]. PSC can be activated directly by alcohol or by transforming growth factor (TGF)-β which is produced locally in case of repetitive inflammation [(4,5,6)].

Peroxisome Proliferator Activated Receptor (PPAR)-γ is a member of the nuclear receptor family of transcription factors [(7)]. Considerable evidence indicates that PPAR-γ agonists inhibit inflammatory responses during inflammatory diseases [(8,7,9,10)]. Furthermore, PPAR-γ decreases TGF-β1 production and may therefore inhibit PSC activation and fibrosis formation [(11,12)]. Taken together, PPAR-γ ligands may have anti-inflammatory and antifibrotic properties which both may exert a beneficial effect on the development and course of chronic pancreatitis [(13,9)]. In the present investigation we to determined the therapeutic potential of troglitazone (a member of the glitazone family and a synthetic ligand for PPAR-γ) in a mouse model of experimental chronic pancreatitis [(12,9)].

Materials and methods

Animals

Female C57BL/6 mice (Harlan, Horst, the Netherlands), 10-12 weeks old, were used in all experiments. The Institutional Animal Care and Use Committee of the Academic Medical Center approved the protocol.

Induction of chronic pancreatitis

Chronic pancreatitis was induced by repeated intraperitoneal (i.p.) injections of the cholecystokinin analogue cerulein (Research Plus, Manasquan, NJ), as described [(14)]. A supramaximal stimulating dose of cerulein was used for all injections (50 μg/kg). Five groups

of mice (n = 10 each) were studied (Figure 1). All mice received 6 hourly i.p. injections, 3 times a week for 6 weeks; groups A and B received saline injections (no induction of pancreatitis), groups C, D and E received cerulein injections (induction of pancreatitis). Groups A and C received normal chow throughout the entire 7-week study period. Groups B and D received chow mixed with troglitazone 0.2% (Sankyo Pharma, Tokyo, Japan) for a total of 6 weeks (weeks 1-6). This dose and administration route of troglitazone has been previously described [15]. Group E received normal chow during the first 3 weeks (weeks 1-3) and chow mixed with 0.2% troglitazone for the next 3 weeks (weeks 4-6). All groups received normal chow in the final (7th) week, after which mice were killed. Water was administered ad libitum to all mice.

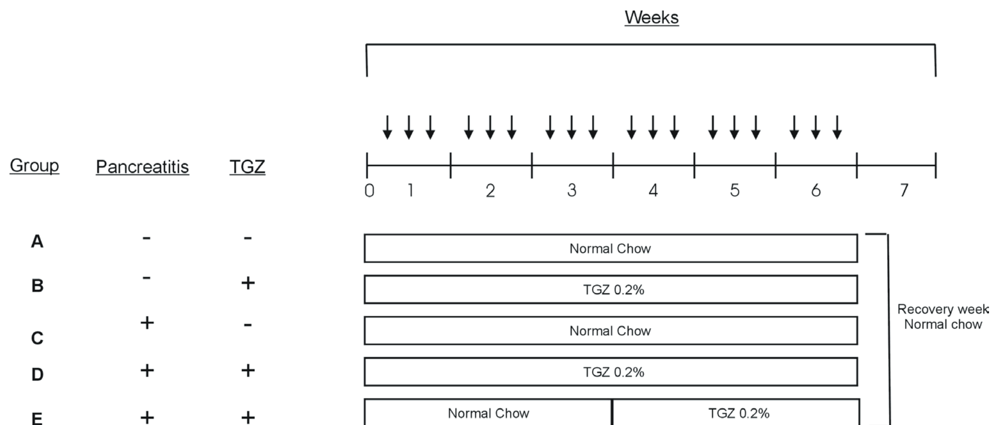

Figure 1. Experimental design. Five groups of mice (n = 10 each) were studied. All mice received 6 hourly i.p. injections, 3 times a week for 6 weeks; groups A and B received saline injections (no induction of pancreatitis), groups C, D and E received cerulein injections (50 µg/kg; induction of pancreatitis). Groups A and C received normal chow throughout the entire 7-week study period. Groups B and D received chow mixed with troglitazone (TGZ) 0.2% for a total of 6 weeks (weeks 1-6). Group E received normal chow during the first 3 weeks (weeks 1-3) and chow mixed with 0.2% TGZ for the next 3 weeks (weeks 4-6). All groups received normal chow in the final (7th) week, after which mice were killed. Arrows indicate six i.p. injections with saline or cerulein.

Tissue handling

Seven weeks after the first i.p. injection mice were anesthetized with Hypnorm (Janssen Pharmaceutics, Beerse, Belgium) and midazolam (Roche, Mijdrecht, the Netherlands), and blood was collected from the vena cava inferior in heparin coated vacutainer tubes. The pancreas was removed, and one longitudinal dissected part was frozen in liquid nitrogen to prevent degradation. Pancreas tissue was stored at –80^{0}C until further assays were performed.

Histologic examination

For histopathologic examination remaining pancreatic tissue was fixed in 10% buffered

formalin and embedded in paraffin; 4 µm sections were stained with hematoxylin and eosin. All specimes were scored by a pathologist (SF) unaware of the origin of the specimens. Evaluation of the pancreas was performed as previously described [14]. Briefly, areas of abnormal architecture were defined and quantified as 0: absent, 1: rare, 2: minimal (<10%), 3: moderate (10-50%) or 4: severe. Within these areas the presence of glandular atrophy, fibrosis and pseudotubular complexes were each scores as 0: absent, 1: minimal (<10%), 2: moderate (10-50%) and 3: severe. For all parameters, three pancreas sections were randomly selected from each mouse. These sections were scored and a median score was calculated. In addition, the content of inflammatory cells (mainly neutrophils) and edema were scored on a 0-4 scale as described elsewhere [16]. Furthermore, to investigate the extent of pancreatic destruction, we quantified the number of acinar cells by counting the number of acinar cells of at least three high power fields (HPF, x 400 maginification) per pancreatic specimen. A mean number was calculated for each mouse.

Intrapancreatic collagen quantification

The amount of intrapancreatic collagen was quantified using image analysis of Sirius red stained pancreatic sections as well as by quantifying hydroxyproline content. Quantitative analysis of collagen was performed by morphometric analysis [14]. Three digitized pictures of each pancreatic section (10 mice per group), viewed through a Olympus BX60 microscope (Olympus, Zoeterwoude, Netherlands) equipped with a ×20 objective lens, were transmitted by a coolsnap video camera (Roper scientific, Vianen, Netherlands) to a Dell 300-MHz PC equipped with Image Pro Plus software (Media cybernetics, Gleichen, Germany). The total amount of collagen stained on each submitted section was calculated by the computer via the digitalized image as follows. In the first step, pancreas was distinguished from the background according to a difference in light density, and a measurement of the total pancreatic tissue area was performed. In the second step, the amount of collagen (stained in red) was measured and was finally expressed as a percentage of the total pancreatic surface. Furthermore, total collagen was assessed by measuring hydroxyproline content as described elsewhere [17]. In brief, pancreatic samples were hydrolyzed in 12 M HCl at 110°C for 16 hours. The samples were resuspended in 2 ml deionized water and 1 mL of chloramine T dissolved in5 M sodium acetate/10% isopropanol. Next, 3 mmol/L perchloric acid and 1 mL of Ehrlich's reagent (ICN Biochemicals, Aurora, OH) were added, mixed and incubated at 65 ^{0}C for 15 minutes. Finally absorbance was measured at 550 nm and values were compared with serial dilutions of trans-4-hydroxy-L-proline. All reagents for the hydroxyproline assay were purchased from Sigma.

Laminin and α-SMA stainings

For laminin, and α-SMA, stainings slides were deparaffinized and endogenous peroxydase activity was quenched by a solution of methanol/0.03% H_2O_2 (Merck, Darmstadt, Ger-

many). After digestion with a solution of pepsine 0.25% (Sigma, St Louis, MO, USA) in 0.01M HCl for laminin, and with 10 mM sodium citrate solution, pH 6.0 for 10 minutes at 98°C in microwave oven for α-SMA, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to a rabbit anti-laminin antibody (Abcam, Cambridge, MA) or a mouse IgG2a anti-α-SMA antibody (Dako), respectively. After washes, slides stained for laminin were probed with a goat anti-rabbit poly-HRP (Powervision, Immunologic, Duiven, NL). Slides stained for α-SMA were incubated with a HRP-labelled goat anti-mouse IgG2a antibody (Southern Biotech, Birmingham, AL). Slides were finally developed using 1% H_2O_2 and 3.3'-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were mounted in glycerin gelatin without counter staining. Laminin stainings were quantified using image analysis as described above. Alfa-SMA stainings were quantified by counting the number of α-SMA positive cells in at least three HPF per section, positive cells located in or near blood vessel walls were ignored. Data are expressed as the number of α-SMA positive cells per 50 acinar cells to correct for the variance in the number of cells per HPF between the groups.

Assays

For measurements of intrapancreatic amylase and active TGF-β1 concentrations, pancreas samples were homogenized in 5 volumes of sterile saline with a standard tissue homogenizer (Biospec Products, Bartlesville, OK). Homogenates were lysed in lysisbuffer (300 mM NaCl, 15 mM Tris, 2 mM MgCl, 2 mM Triton (X-100), Pepstatin A, Leupeptin, Aprotinin (20ng/ml), pH 7.4) and spinned at 1500 x g at 4^0C for 15 minutes; the supernatant was frozen at -20^0C until analysis. Protein levels in homogenates were measured using the BCA protein kit according to the manufacturers instructions (Pierce, Rockford, IL). Levels of pancreatic amylase and TGF-β were normalized by equal protein loading. Amylase levels in pancreas homogenates were determined with a commercially available kit (Sigma, St. Louis, MO), using a Hitachi analyzer (Boehringer Mannheim, Mannheim, Germany).. Active TGF-β1 levels were measured in pancreas homogenates by ELISA according to the instructions of the manufacturer, this ELISA measures the active, immunoreactive, form of TGF-β1 only (R&D Systems, Minneapolis, MN). Myeloperoxidase (MPO) content was measured in pancreas homogenates, as described elsewhere [(18,19,20)]. The plasma concentrations of IL-6 (Pharmingen, San Diego, CA) and soluble tumor necrosis factor receptor type 1 (TNFR1)(R & D Systems) were measured by ELISA in accordance with the instructions of the manufacturers.

Statistical analysis.

All data are expressed as means ± standard error (SE). Comparisons between groups were conducted using one way ANOVA followed by Dunn's post test. Significance was set at $P < 0.05$.

Results

Troglitazone treatment attenuates pancreatic damage and fibrosis
Chronic pancreatitis was induced by 6 hourly i.p. cerulein injections, 3 times a week for 6 weeks. Troglitazone was given either during the whole 6-week period, or from week 4 to 6 (Figure 1). All mice treated with repeated cerulein injections (Groups C, D and E) displayed histopathologic signs of chronic pancreatitis at the time of sacrifice (week 7), as reflected by abnormal architecture, glandular atrophy pseudotubular complexes, fibrosis, edema and inflammatory cell infiltrate (Table 1 and Figure 2; all $P<0.05$ for the comparisons with Groups A and B). In mice in which chronic pancreatitis was induced and that were treated with troglitazone from either week 1 to 6 (Group D) or from week 4-6 (Group E) all markers of pancreatic damage were significantly attenuated (Table 1 and Figure 2; all $P<0.05$ for the comparisons with Group C). Notably, the effects of early and postponed troglitazone treatment were similar. To evaluate the degree of fibrosis in the pancreas, Sirius red stained pancreas sections were analyzed using compute assisted digital image analysis and pancreatic hydroxyproline content was quantified (Figure 3, Table 2). Pancreatic collagen content dramatically increased after induction of chronic pancreatitis ($P<0.05$ for the comparison between Groups A and B versus Groups C, D and E). However, the increase in pancreatic collagen content was diminished by troglitazone, irrespective of the treatment schedule (Figure 3, Table 2, $P<0.05$ for the comparison between Group C versus Groups D and E). Furthermore, pancreas laminin content was quantified by image analysis of laminin. As expected, laminin accumulated during chronic pancreatitis induction. Independently of the treatment schedule, troglitazone treatment reduced the accumulation of laminin (Table 2, $P<0.05$ A and B versus C as well as C versus D and E). α-SMA immunohistochemistry was performed to quantify the number of stellate cells, the putative cells responsible for fibrosis, in the pancreas. In line, the number of stellate cells increased during pancreatitis induction and this increase in stellate cells was attenuated by troglitazone (Table 2, $P<0.05$ A and B versus C, as well as C versus D and E). This effect was similar in mice receiving early or delayed troglitazone treatment.

Troglitazone reduces the increase in intrapancreatic MPO content
MPO activity in pancreas homogenates were determined as a measure for neutrophil accumulation within the organ (Figure 4). Mice that received repeated cerulein injections demonstrated a profound rise in pancreatic MPO concentrations ($P<0.05$ for the comparison between Groups A and B versus Groups C, D and E). Troglitazone diminished this increase in pancreatic MPO levels in mice with pancreatitis irrespective of the treatment schedule ($P<0.05$ for the comparison between Group C versus Groups D and E).

	A	B	C	D	E
Abnormal architecture	0 ± 0	0 ± 0	2.0 ± 0.4#	0.9 ± 0.3*#	0.7 ± 0.2*#
Glandular atrophy	0 ± 0	0 ± 0	2.7 ± 0.2#	1.4 ± 0.3*#	1.4 ± 0.2*#
Pseudotubular complexes	0 ± 0	0 ± 0	2.6 ± 0.3#	1.6 ± 0.2*#	1.8 ± 0.2*#
Fibrosis	0 ± 0	0 ± 0	2.9 ± 0.1#	2.2 ± 0.2*#	2.2 ± 0.2*#
Total score of above	0 ± 0	0 ± 0	10.0 ± 0.6#	5.7 ± 0.9*#	6.0 ± 0.3*#
Edema	0 ± 0	0 ± 0	2.4 ± 0.3#	1.5 ± 0.2*#	1.7 ± 0.1*#
Inflammation	0 ± 0	0 ± 0	2.3 ± 0.3#	1.3 ± 0.4*#	1.2 ± 0.1*#

Table 1: Troglitazone reduces the severity of experimental chronic pancreatitis. For the description of Groups A to E see Figure 1. For the description of the histopathology score see the Methods. Data are mean ± SE (n = 10 mice per group). #P<0.05 vs A and B, *P<0.05 vs

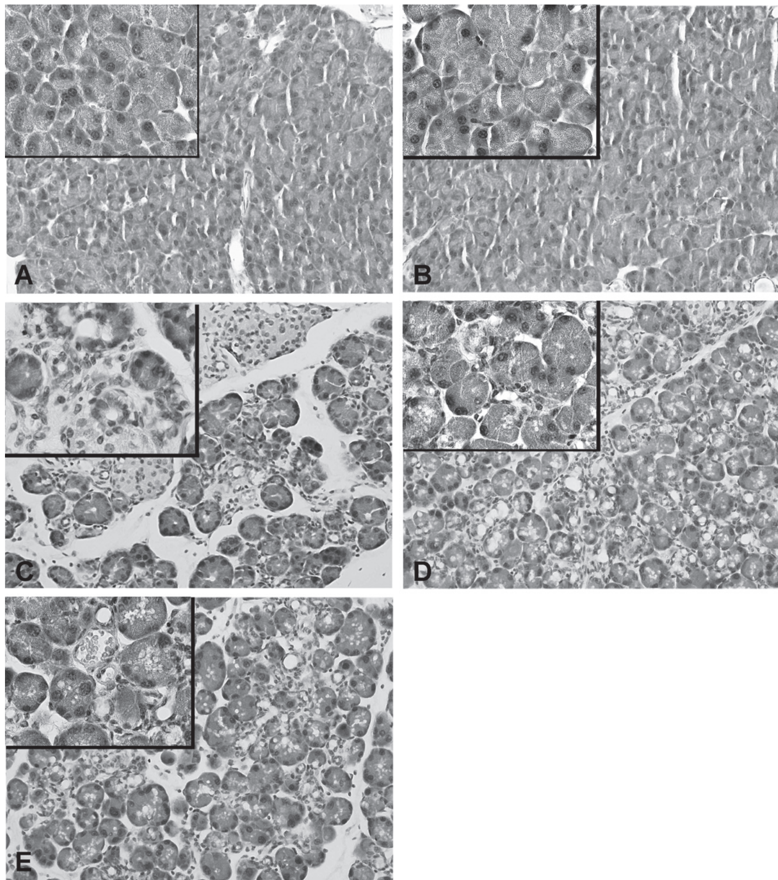

Figure 2. Troglitazone treatment reduces pancreatic damage. Representative H&E stained pancreas histology slides from a total of 10 mice per group. For the description of Groups A to E see Figure 1. For data derived from scoring H&E stained pancreas specimens see table 1. H& E staining, magnification x 10, insert x 40.

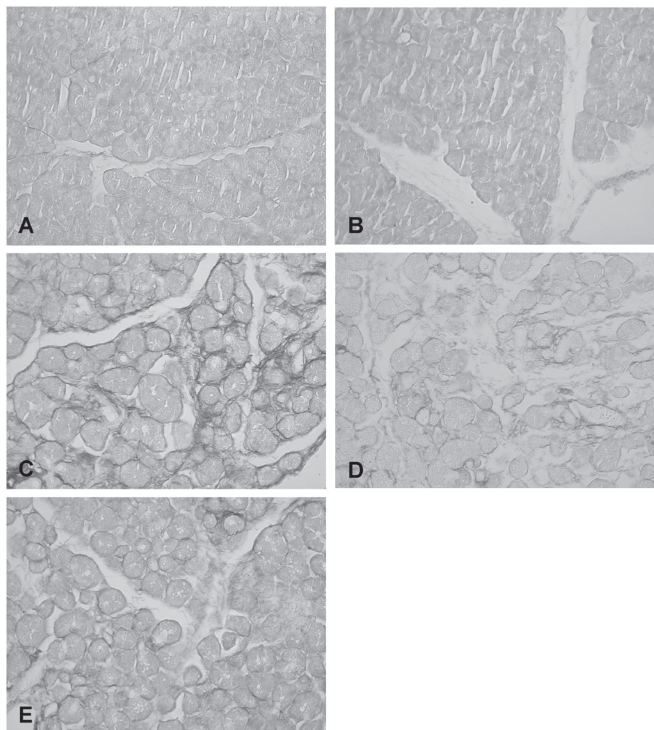

Figure 3. Troglitazone treatment reduces pancreatic collagen content. Representative Sirius red stained pancreas sections from a total of 10 mice per group used to quantify tissue collagen content. For the description of Groups A to E see Figure 1. For data derived from image analysis of Sirius red stained specimens see table 2. H& E staining, magnification x 10, insert x 40.

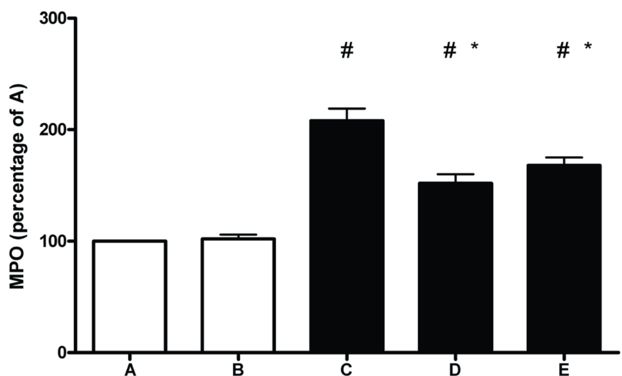

Figure 4. Troglitazone treatment reduces MPO content. Pancreas MPO activity is expressed as a percentage of Group A. Open bars represent saline injected mice (without pancreatitis); closed bars represent cerulein injected mice (with pancreatitis). Data are mean ± SE of 10 mice per group. For the description of Groups A to E see Figure 1. #P<0.05 vs A and B, *P<0.05 vs C.

Troglitazone partially prevents intrapancreatic acinar cell depletion

Chronic pancreatitis is associated with a decrease in acinar cells and therefore a loss in exocrine function [14,2]. To obtain insight into pancreas acinar cell content in our study, we counted the number of acinar cells per HPF and measured amylase concentrations in pancreas homogenates. The number of acinar cells per HPF as well as levels of amylase were

markedly decreased in mice with experimentally induced chronic pancreatitis as compared to control mice (Figure 5; P < 0.05 for the comparison of Groups A and B versus Groups C, D and E). Troglitazone partially prevented this decrease in intrapancreatic acinar cells as well as amylase concentration in mice with pancreatitis (P<0.05 for the comparison between Group C versus Groups D and E). This effect was similar in mice receiving early or postponed troglitazone treatment.

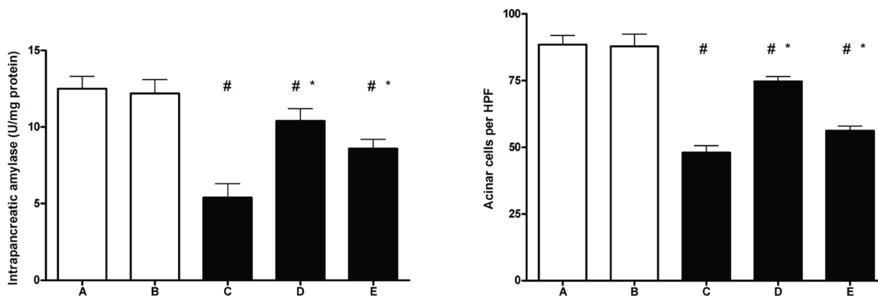

Figure 5. Troglitazone treatment reduces loss in pancreas acinar cell content. Open bars represent saline injected mice (without pancreatitis); closed bars represent cerulein injected mice (with pancreatitis). Data are mean ± SE of 10 mice per group. For the description of Groups A to E see Figure 1. #P<0.05 vs A and B, *P<0.05 vs C.

Troglitazone treatment prevents the increase in pancreatic active TGF-β1 concentrations

TGF-β1 has been implicated as an important mediator in the development of fibrosis in chronic pancreatitis [21,22]. Since TGF-β1 is synthesized and released as a latent form that requires activation before it manifests biological activity, and the activation step appears to be as important as synthesis in determining TGF-β1 activity we measured active TGF-β1 in these studies. Induction of chronic pancreatitis was associated with an increase in active TGF-β1 concentrations in pancreas homogenates (Figure 6; P<0.05 for the comparison between Groups A and B versus Groups C). Remarkably, troglitazone completely prevented the rise in pancreatic active TGF-β1 levels in mice administered repeated cerulein injections (P<0.05 for the comparison between Group C versus Groups D and E). This effect was similar in mice that received early or postponed troglitazone treatment.

Troglitazone attenuates signs of systemic inflammation

Several plasma markers have been found to correlate with the degree of pancreas inflammation in patients, including IL-6 and soluble TNF receptors [23]. Therefore, to obtain insight in the occurrence of systemic inflammation we measured the plasma concentrations of IL-6 and soluble TNF receptor type 1 (Figure 7). Chronic pancreatitis was accompanied by a rise in the plasma levels of both mediators (P<0.05 for the comparison between Groups A

and B versus Groups C, D and E). Troglitazone attenuated these increases (P<0.05 for the comparison between Group C versus Groups D and E).

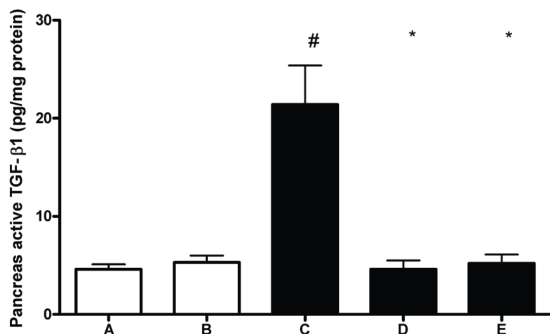

Figure 6. Troglitazone reduces pancreatic active TGF-β1 levels during experimental chronic pancreatitis. Open bars represent saline injected mice (without pancreatitis); closed bars represent cerulein injected mice (with pancreatitis). Data are mean ± SE of 10 mice per group and are expressed as pg/mg protein. For the description of Groups A to E see Figure 1. #P<0.05 vs A and B, *P<0.05 vs C.

	A	B	C	D	E
Hydroxyproline content (pg/mg protein)	2.9 ± 0.6	2.7 ± 0.5	15.2 ± 3.1#	6.5 ± 2.1#*	8.1 ± 2.3#*
Sirius red staining (%)	0.8 ± 0.2	0.9 ± 0.2	10.8 ± 0.7#	4.6 ± 0.5#*	5.5 ± 0.7#*
Laminin content (%)	8.1 ± 1.1	8.3 ± 1.1	31.2 ± 1.9#	16.5 ± 2.3#*	23.1 ± 1.4#*
α-SMA positive cells (/50 acinar cells)	1.2 ± 0.4	1.2 ± 0.4	11.8 ± 0.8#	5.4 ± 0.6#*	7.3 ± 0.5#*

Table 2: Troglitazone reduces fibrosis formation and the number of activated stellate cells during experimental chronic pancreatitis. For the description of Groups A to E see Figure 1. Fibrotic parameters are shown. For a detailed description of the methods, systems and setting see the Methods section. Data are mean ± SE (n = 10 mice per group, 3 sections analysed per mouse). #P<0.05 vs A and B, *P<0.05 vs C.

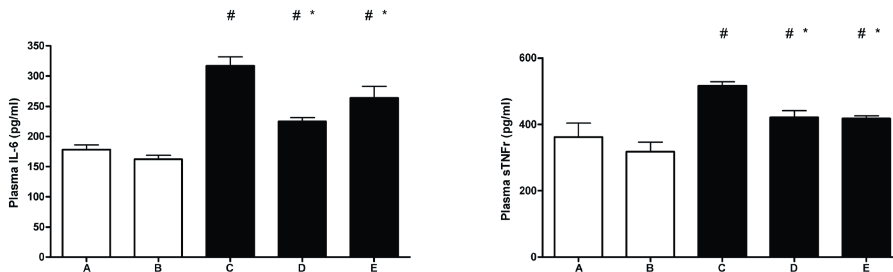

Figure 7. Troglitazone reduces plasma IL-6 and soluble TNFR-1 during experimental chronic pancreatitis. Open bars represent saline injected mice (without pancreatitis); closed bars represent cerulein injected mice (with pancreatitis). Data are mean ± SE of 10 mice per group. For the description of Groups A to E see Figure 1. #P<0.05 vs A and B, *P<0.05 vs C.

Discussion

In the present investigation, we induced chronic pancreatitis in mice using a scheme in which animals received six hourly cerulein injections three times a week for six weeks. Mice that received cerulein injections developed chronic pancreatitis as evidenced by severe histological damage, fibrosis and loss in acinar cell content. Mice treated with the PPAR-γ ligand troglitazone, either for the whole study period or during week 4-6 only, showed a significant attenuation of all histopathologic parameters of chronic pancreatitis as well as a reduction in the loss of acinar cells. Furthermore, markers of inflammation within the pancreas (MPO) and in the circulation (IL-6, soluble TNFR1), as well as pancreatic active TGF-β1 were all decreased in troglitazone treated mice. Therefore, troglitazone reduces pancreatic inflammation, damage and fibrosis in experimental chronic pancreatitis and is even beneficial in a therapeutic setting when given after initial damage has been established.

The etiology of the development of chronic pancreatitis is largely unknown. Current evidence indicates that chronic pancreatitis is the result of repetitive episodes of pancreatic inflammation and necrosis, inducing repetitive episodes of regeneration and ultimately leading to activation of PSC by TGF-β1 and the induction of fibrosis. This "necrosis-fibrosis" hypothesis identifies two major components in the development of fibrosis: first recurrent episodes of inflammation and second the activation of PSC by TGF-β1.

PPAR-γ is a member of the nuclear receptor family of transcription factors that mediates growth, differentiation, and inflammation [(7)]. Natural ligands of PPAR-γ are fatty acids, arachidonic acid metabolites and prostaglandins; synthetic ligands of PPAR-γ are non-steroidal anti-inflammatory drugs and a family of antidiabetic drugs called glitazones, among which troglitazone. Upon binding of a ligand, the PPAR-γ receptor forms a heterodimer with the retinoid X receptor and becomes activated. This heterodimer can interfere with mitogen activated protein (MAP)-kinase and the Nuclear Factor κ (NFκ)-B proinflammatory pathways [(7,9)]. Glitazones have been used clinically in the treatment of Type II diabetes [(24,25)]. The antidiabetic properties of glitazones are contributed to the fact that these compounds, by activating PPAR-γ, counter effect the insulin resistance generating effects of TNF-α [(26)]. Considerable evidence indicates that PPAR-γ agonists may have beneficial effects in other diseases as well, due to their anti-inflammatory properties. PPAR-γ agonists negatively regulate proinflammatory cytokine production by mononuclear cells as well as adhesion molecule expression on endothelial cells [(8,7,9,10)]. In this study, we observed that mice treated with troglitazone, either from the beginning of pancreatitis induction or from week 4-6 only, showed an attenuation of inflammation in the pancreas, as reflected by a reduction of MPO activity and neutrophil content estimated by semi quantitative analyses of histologi-

cal sections. In the systemic compartment, markers of inflammatory activation, IL-6 and soluble TNFR1 were also diminished by troglitazone treatment, likely due to a reduction in inflammation in the pancreas itself. Indeed, the circulating levels of both mediators have been shown to correlate directly with the degree of pancreatic inflammation [27].

In this study we show that inhibition of inflammatory responses by troglitazone reduced pancreatic inflammation, regeneration and finally pancreatic damage during the bouts of repetitive inflammation induced by cerulein. It is conceivable that reduction of inflammation is due to an anti-inflammatory effect of troglitazone during the repetitive acute components of the model, indeed, previous studies have shown that PPAR-γ agonists inhibit inflammation during cerulein induced acute pancreatitis [28,29]. The positive effects of inhibition of inflammation on the development of pancreatic damage in this model are in line with another previous study, showing that mice lacking the anti-inflammatory cytokine IL-10 experienced a significant exacerbation of pancreatic damage which was related to an increase in pancreatic inflammation and a reduction of pancreatic regeneration [14].

TGF-β1 is a regulator of extracellular matrix remodeling in the pancreas, and may (upon activation from the latent inactive form) be an important promoting factor in the pathogenesis of chronic pancreatitis. This hypothesis is supported by findings of enhanced TGF-β1 expression in human chronic pancreatitis and development of fibrosis and inflammation in pancreata of transgenic mice over expressing TGF-β1 [30,31]. In vitro data suggests that PPAR-γ agonists inhibit the activation of PSC, cells that have been implicated in the formation and progression of fibrosis, during chronic pancreatitis by inhibiting the PSC activating effects of TGF-β [9]. In this study we show that indeed the PPAR-γ ligand troglitazone reduces the levels of active TGF-β1 in the pancreas as well as the number of PSC which ultimately results in an impairment of fibrosis formation. These data are in line with studies in experimental hepatic fibrosis, in which glitazone treatment reduced hepatic stellate cell (HSC) activation, TGF-β1 levels and fibrosis [32,10] and with a recent paper in which spontaneous chronic pancreatitis severity was reduced in male Wistar Bonn/ Kobori rats by treatment with an angiotensin converting enzyme inhibitor which attenuated TGF-β1 expression in the pancreas, resulting in the prevention of PSC activation and pancreatic fibrosis [33]

Our study shows that troglitazone inhibits inflammation, pancreatic damage and fibrosis during chronic pancreatitis. These data are in line with a previous study in WBN/Kob rats with spontaneous chronic pancreatitis in which troglitazone decreased chronic pancreatitis severity [34]. In WBM/Kob rats pancreatitis develops spontaneously in the aging rat due to an incompletely understood mechanism. These changes are not restricted to the exocrine pancreas, since they are accompanied by several additional lesions such as severe endocrine pancreatic failure and diabetes, myocardiopathy, neuropathy and bone loss [35,36,37]. In

this study, we used a different model of chronic pancreatitis which specifically reflects the development of exocrine chronic pancreatitis according to the necrosis-fibrosis hypothesis. An important finding in this study is that the positive effects of troglitazone are maintained if the treatment is delayed up until halfway through the seven week model. At this point, mice already had experienced nine episodes of acute pancreatitis and subsequent regeneration. This approach, in which treatment was started after initial pancreatic damage had been established, was almost as effective as treatment during the whole period of pancreatitis induction. In additional experiments mice were sacrificed after three weeks during this model (data not shown). These experiments revealed that the fibrosis response was already present but relatively mild at this stage, suggesting that adding troglitazone after three weeks did block the progression of fibrosis formation rather than reversing it. However, it should be noted that the model used here is less suitable to determine whether troglitazone is able to reverse pancreas fibrosis, since the "recovery week" (i.e. the week without cerulein injections after 6 weeks of cerulein treatment) is essential for the development of a strong fibrotic response [38]. Reversal of fibrosis might in theory be possible since studies in liver fibrosis have provided a substantial body of evidence indicating that liver fibrosis is a dynamic process that can progress and regress over time [39]. Following treatment of hepatitis C with Pegylated-interferon and ribavirin, it has been shown that when the virus is cleared the rate of fibrosis progression can be reversed [40]. In human and rat HSC as well as in established experimental liver fibrosis, gliotoxin, an agent which induces apoptosis in inflammatory cells, upregulates apoptosis in HSC which results in a regression of liver fibrosis [41]. Therefore, it is conceivable that fibrosis should be regarded as a dynamic process which can be stopped and possibly even reversed. Since PSC and HSC share many functional characteristics, there is reason to believe that in pancreatic fibrosis similar mechanisms exist. In line with these data, we postulate that treatment with troglitazone after initial fibrosis has been established results in inhibition of PSC which stops fibrosis progression but might also in part enhance the resolution of pancreatic fibrosis indicating that glitazones may be a valuable therapy for chronic pancreatitis.

As this is the first time that any anti-inflammatory/antifibrotic strategy has been shown to be effective in a therapeutic setting during experimental chronic pancreatitis, further studies evaluating the efficacy of glitazones during human chronic pancreatitis are warranted.

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chapter 15

summary

David J. van Westerloo

Part I: pancreatitis and immunopathology during critical illness

In the first part of this thesis specific components of the inflammatory response during pancreatitis and infection during critical illness are investigated. Since an animal model of acute pancreatitis is used throughout the thesis a review of the current literature on the pathophysiology of acute pancreatitis is given in Chapter 2. Furthermore, in Chapter 3 a clinical study is presented that adds to the understanding of the pathophysiology of acute pancreatitis and suggests a potential new molecular target for its prophylaxis or treatment. In Chapter 3 we evaluate whether prophylactic administration of Semapimod, a small molecule inhibiting mitogen activated protein (MAP) kinase pathways, is effective in the prevention of post ERCP pancreatitis. Since inflammatory activation is an early event during the pathophysiology of acute pancreatitis and MAP kinase pathways are critical intracellular pathways in the inflammatory response we postulated that inhibition of MAP kinase pathways lower the incidence of pancreatitis after an ERCP. For this, 260 patients undergoing an ERCP in our hospital were included in a randomized double blind trial comparing semapimod with placebo. This study shows that prophylactic administration of Semapimod lowers the incidence of post ERCP hyperamylasemia and also the incidence of post ERCP pancreatitis although this latter end point did not reach statistical significance.

Chapter 4 illustrates the interaction of one disease with another. In this study we investigate whether patients with end stage renal disease requiring hemodialysis or peritoneal dialysis are at risk for the development of pancreatitis (Chapter 4). This study builds on a case report of two patients with end stage renal failure who developed acute pancreatitis during continuous ambulatory peritoneal dialysis which raised the question whether patients with end stage renal failure undergoing dialysis have an increased risk for acute pancreatitis. Indeed, the study indicates that especially peritoneal dialysis is a risk factor for the development of acute pancreatitis. Since most patients presented with several risk factors associated with acute pancreatitis a single responsible risk factor among dialysis patients could not be identified.

The remainder of the first part of this thesis focuses on the immune response to infection in animals already suffering from a previous injury. Many severe infections do not develop in previously healthy patients but in patients with a pre-existing disease such as a trauma, vascular occlusive disease, pancreatitis or sterile lung injury. This clinical situation is of major clinical relevance since most serious infections in an intensive care setting do not occur "out of the blue" in healthy subjects but in those already suffering from another ongoing disease. In the current literature concerning the immunopathology of serious infections this "two hit" phenomenon is overlooked since most studies evaluating the inflammatory response to infection are performed in healthy animals. We chose to develop two distinct two event models, one in which infection is preceded by sterile inflammation in a different

organ (pancreatitis followed by pneumonia or sepsis) and one in which infection is preceded by inflammation in the same organ (acid aspiration lung injury followed by pneumonia). Using these models we evaluated the effects of local and systemic inflammation on the inflammatory response to subsequent infection.

In cases of severe acute pancreatitis, the outcome is determined by the development of severe systemic inflammation as well as secondary infectious complications in the pancreas and in extra pancreatic organs(1;2). To evaluate the effects of pre existent pancreatitis on subsequent extra pancreatic infection "two hit" experiments were designed in which cerulein induced pancreatitis was followed by pneumonia (Chapter 5) or septic peritonitis (Chapter 6). In Chapter 5 pancreatitis was followed by *Pseudomonas aeruginosa* pneumonia, since this pathogen is frequently associated with nosocomial pneumonia in the intensive care setting. We demonstrate that experimental pancreatitis leads to a reduced bacterial clearance in the pulmonary compartment during *P. aeruginosa* pneumonia. Vice versa, pneumonia prolongs the course of pancreatitis which is associated with a localization of bacteria in the inflamed pancreas and an enhanced influx of neutrophils. These data provide an interesting concept that adds to the understanding of the fact that patients with pancreatitis and complicating infections have a poor prognosis. In Chapter 5 we address not only the interaction between pancreatitis and septic peritonitis (caused by the injection of *Escherichia coli*), but also evaluate the role of Toll like receptor (TLR) 4 in pancreatitis and in the host response to pancreatitis and subsequent septic peritonitis. This is of interest since TLRs have been identified as crucial mediators of the innate immune response to infection. TLRs are pattern recognition receptors among which TLR4 is the signaling receptor for lipopolysaccharide (LPS)(3-5). The adequate "sensing" of LPS by the host has been implicated as an important early event in the innate immune response to Gram-negative bacteria(6). We demonstrated that TLR4 is indeed an essential mediator of the immune response during septic peritonitis in previously healthy animals. However, in animals subjected to previous pancreatitis this important role of TLR4 is no longer present. The discrepancy between the role of TLR4 during primary *E. coli* sepsis and E. coli sepsis complicating pancreatitis underlines the need to study the regulation of the innate immune response and potential new targets in the treatment of sepsis in animal models closely resembling the clinical situation of intensive care patients.

Since all experimental studies described above involve the interaction of a primary disease with an infection located in another organ we next studied the effects of a primary sterile inflammatory stimulus on an infection in the same body compartment. In this study, reported in Chapter 7, an animal model mimicking aspiration pneumonitis caused by the aspiration of gastric acid in the lung is used. Aspiration of gastric contents occurs in various hospitalized patients, in particular those with a reduced consciousness(7;8). The associated

lung injury, commonly referred to as aspiration pneumonitis, is primarily caused by gastric acid(9). Although the exact underlying mechanisms for this association is unclear, it has been suggested that acid aspiration primes the lung for an enhanced inflammatory response to a subsequent challenge(10). In Chapter 7, we induce pneumonia by intranasal inoculation with *Klebsiella pneumoniae*, a common nosocomial respiratory pathogen(2), in mice with or without preceding acid aspiration. In addition, arguing that tumor necrosis factor (TNF)-α has been implicated as an important mediator in various inflammatory lung diseases(11-14), we also investigate the role of this pluripotent cytokine in lung inflammation during *Klebsiella pneumonia* in mice with or without preexisting aspiration pneumonitis. Our study demonstrates that aspiration pneumonitis triggers the host to an enhanced inflammatory response to *K. pneumoniae* concurrently facilitating bacterial outgrowth. Although TNF-α plays a pivotal role in host defense against primary *Klebsiella pneumonia*, in the host with preexisting aspiration pneumonitis the strongly elevated TNF-α concentrations in the lungs are not involved in the enhanced lung inflammation or the increased bacterial outgrowth. In line with data shown in Chapter 6 the different roles of TNF-α in primary and secondary *K. pneumoniae* pneumonia underline the need to study the pathogenesis of pneumonia and potential new treatment targets not in healthy animals but in animals with preexisting disease.

Part II: The cholinergic anti-inflammatory pathway as a regulator of the inflammatory response to critical illness

Recently, the vagus nerve and nicotinic acetylcholine (Ach) receptors have been identified as crucial mediators of the inflammatory response. In vitro studies have shown that immune cells are susceptible to Ach, the principle parasympathetic neurotransmitter. When macrophages are exposed to Ach these cells are effectively deactivated(15-20). Evidence indicates that the anti-inflammatory effects of Ach are mediated by nicotinic Ach receptors, and in particular by the α7 subunit of the nicotinic Ach receptor(20). In vivo studies in endotoxemia and other models of inflammation have shown that macrophages are directly influenced by vagus nerve derived Ach, suggesting that the vagus nerve provides a hard wired anti-inflammatory pathway called the "cholinergic anti-inflammatory pathway"(16). In these studies, electrical stimulation of the efferent vagus nerve inhibited TNF release induced by injection of endotoxin into rats and mice and prevented shock; however, electrical stimulation of the vagus nerve in mice deficient for the α7 subunit of the nicotinic Ach receptor did not result in a reduced cytokine release upon endotoxin administration(15).

A comprehensive review on neuro immune interactions and the cholinergic anti-inflammatory pathway is reported in Chapter8. In Chapter 9 evaluates whether this pathway

regulates host responses during experimental abdominal sepsis induced by intraperitoneal injection with Escherichia coli. The host response to infection was studied in mice in which this pathway was disrupted by vagotomy, and in animals in which the peripheral part of this pathway, nicotinic acetylcholine receptors on macrophages, was stimulated by pretreatment with nicotine. Chapter 9 shows for the first time that the cholinergic anti-inflammatory pathway is an essential regulator of the innate immune response to a severe bacterial infection. Furthermore it shows that stimulation of the cholinergic anti-inflammatory pathway by nicotine impairs bacterial clearance and survival during E. coli induced septic peritonitis. Chapter 10 evaluates whether this anti-inflammatory pathway regulates host responses during experimental pancreatitis as well. The inflammatory response and the severity of experimental pancreatitis in mice in which this pathway was disrupted by vagotomy are evaluated. In a separate study, pancreatitis was induced in mice in which the peripheral part of this pathway, nicotinic Ach α 7 receptors on immune competent cells, was stimulated with a selective agonist, 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21)(21;22). In other mice the reciprocal approach was undertaken and nicotinic receptors were blocked by using mecamylamine (23). In line with data shown in Chapter 9 this chapter shows that the nicotinic anti-inflammatory pathway is an essential regulator of inflammation during experimental pancreatitis as well. We further show, for the first time, that pharmacological stimulation of the peripheral part of the nicotinic anti-inflammatory pathway, α 7 nicotinic Ach receptors, attenuates inflammation in vivo. Taken together, the nicotinic anti-inflammatory pathway may be a future target for the treatment of sepsis as well as pancreatitis.

Chapter 11 describes the effects of acute vagotomy in rats and shows that timing of vagotomy in experiments is crucial to be able to interpret the results adequately. In Chapter 12 the effect of electrical vagus nerve stimulation (VNS) on coagulation and fibrinolysis during endotoxemia was investigated. Activation of the hemostatic mechanism as a consequence of inflammation can be considered instrumental in containing inflammatory activity to the site of infection. However, uncontrolled activation may cause harm and give rise to a vicious cycle, eventually leading to dramatic events such as manifested in severe sepsis and disseminated intravascular coagulation. We here describe a previously unrecognized effect of VNS on the hemostatic response to a sublethal injection of LPS. Whereas the ability of VNS to inhibit the production of the potent proinflammatory cytokine TNF-α during endotoxemia had been documented previously, we demonstrate for the first time that VNS also inhibits activation of coagulation and fibrinolysis. Stimulation of the cholinergic anti-inflammatory pathway therefore not only impacts on inflammation but also on the coagulant-anticoagulant balance during sepsis and endotoxemia.

In Chapter 13 the capacity of the selective α7 cholinergic receptor agonist GTS-21 to inhibit LPS-induced inflammatory responses in mice during endotoxemia is investigated. In this

paper we specifically investigate the mechanisms by which chemical α7 cholinergic receptor stimulation influences the inflammatory response. We report on the finding that GTS-21 inhibits two major inflammatory responses (TNF-α release and neutrophil recruitment) during LPS-induced peritonitis which adds to the evidence that such compounds exert anti-inflammatory effects that may prove beneficial in inflammatory diseases.

Part III: The inflammatory response to chronic pancreatitis

Chronic pancreatitis is characterized by progressive destruction of parenchymal tissue ultimately leading to exocrine and endocrine function loss. Clinical symptoms include abdominal pain, steatorrhea and diabetes mellitus(24). Knowledge of the pathophysiology of chronic pancreatitis is limited. Chronic pancreatitis is considered to result from chronic repetitive inflammation within the pancreas due to alcohol abuse or recurrent bouts of even minor events of pancreatic inflammation, resulting in recurrent repair of pancreatic damage and ultimately in activation of a profibrotic cascade. This hypothesis is usually referred to as the necrosis fibrosis hypothesis. Fibrosis formation in the pancreas is initiated by differentiation and activation of pancreatic stellate cells (PSC) which produce collagen as a result(25). In this part of the thesis we report a study which investigates the a potential new treatment target for the treatment of chronic pancreatitis. Chronic pancreatitis is a disease which is especially hard to study since the organ is inaccessible for sampling in humans. Therefore, we used a chronic pancreatitis model with repetitive cerulein hyperstimulation since this model adheres to the necrosis fibrosis hypothesis of chronic pancreatitis. In Chapter 14 we investigate the therapeutic effects of troglitazone, a ligand for Peroxisome Proliferator Activated Receptor (PPAR)-γ, in this model(26). Considerable evidence indicates that PPAR-γ agonists inhibit inflammatory responses during inflammatory diseases(26-29). Furthermore, PPAR-γ decreases TGF-β1 production and may therefore inhibit PSC activation and fibrosis formation(30;31). We show that troglitazone indeed reduces inflammation and fibrinogenesis during experimental chronic pancretitis. This beneficial effect of troglitazone is even present if the treatment is commenced after initial damage and fibrosis has been established. As this is the first time that any anti-inflammatory/antifibrotic strategy has been shown to be effective in a therapeutic setting during experimental chronic pancreatitis, further studies evaluating the efficacy of glitazones during human chronic pancreatitis are warranted.

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nederlandse samenvatting voor minder ingewijden

David van Westerloo

Deel I: Studies naar pancreatitis en de afweerrespons tegen infectie bij ernstig zieke patienten

Het eerste deel van dit proefschrift onderzoekt aspecten van de afweerrespons tegen infectie tijdens ernstige ziekte. Een paar keer wordt daarbij gebruik gemaakt van een diermodel van acute pancreatitis, een ernstige steriele (dus niet bacteriële of virale) ontsteking van de alvleesklier.

Bij wijze van introductie geeft Hoofdstuk 2 een overzicht van de verschijningsvormen van deze ziekte en van de gangbare denkbeelden over de ontstaanswijze. In de volgende hoofdstukken, Hoofdstuk 3 en 4, presenteren wij twee eigen klinische studies op het gebied van acute pancreatitis. De klinische studie in Hoofdstuk 3 evalueert de vraag of het zinvol is patiënten die een zogenaamde ERCP ondergaan, een onderzoek van de alvleesklier en galwegen, profylactisch te behandelen met semapimod om irritatie van de alvleesklier te voorkomen. Semapimod is een remmer van zogenaamde MAP kinasen die een belangrijke rol spelen bij de regulering van de ontstekingsrespons. Deze studie, een onderzoek bij 260 patiënten, laat zien dat toediening van semapimod inderdaad de irritatie van de alvleesklier na ERCP voorkomt, alhoewel in deze studie nog geen statistisch significante afname van het aantal acute pancreatitis gevallen optrad. De klinische studie in Hoofdstuk 4 onderzoekt of acute pancreatitis vaker voorkomt bij patiënten die een dialyse ondergaan. Dat blijkt inderdaad het geval. De verklaring daarvoor blijkt te vinden in de additionele werking van de meerdere risicofactoren voor pancreatitis die dialyse patiënten meestal hebben.

Vanaf hoofdstuk 5 richt dit deel van het proefschrift zich op de afweerrespons tegen infectie bij dieren die ziek gemaakt werden met een eerdere, niet infectieuze, aandoening. Dit onderzoek is van belang omdat ernstige infecties meestal niet ontstaan bij gezonde personen, maar vooral bij patiënten in een intensieve zorgsetting die al ziek zijn ten gevolge van een trauma, een pancreatitis of een andere ernstige aandoening. Om zinvol experimenteel onderzoek te doen naar de afweer tegen infecties bij ernstig zieke patiënten, is het van groot belang om adequate diermodellen te ontwikkelen die met zulke eerdere ziektes rekening houden.

Aan het onderzoek ligt ten grondslag de ontwikkeling van zogenaamde “twee hit” modellen. In deze diermodellen werd in muizen eerst pancreatitis geïnduceerd waarna in tweede instantie dezelfde muizen een bloedvergiftiging (sepsis) of longontsteking(zie Hoofdstuk 5 en 6) ondergingen. In een andere studie werd in muizen eerst een steriele longontsteking opgewekt welke enkele uren later werd gevolgd door een bacteriële longontsteking (zie Hoofdstuk 7).

Uit deze onderzoeken blijkt dat infecties in dieren die al ziek zijn van een eerdere stimulus een heel ander (ernstiger) beloop hebben dan in niet eerder ziek gemaakte dieren. In deze

dieren trad niet alleen een veel heftiger ontstekingrespons op maar ook een ongebreidelde bacteriële groei. Ook bleek dat concepten die van groot belang worden geacht in de afweer tegen infecties niet van belang zijn tijdens infectie in een twee hit setting. Zo vonden wij bijvoorbeeld dat een receptor (TLR4) waarvan het idee is dat deze cruciaal is voor de afweer tegen bloedvergiftiging hoegenaamd geen rol speelt tijdens bloedvergiftiging in onze twee hit diermodellen. Dit onderzoek is dan ook van groot belang om de waarde van eerdere bevindingen in conventionele (één hit) diermodellen te relativeren. Het is derhalve van het grootste belang dat in het vervolg onderzoek naar ernstige ziekten wordt uitgevoerd in diermodellen die lijken op de echte klinische situatie. Alleen dan kunnen er conclusies getrokken waar men in de praktijk iets aan heeft.

Deel II: De nervus vagus en de cholinerge anti-inflammatoire pathway

Het tweede deel van dit proefschrift onderzoekt de relatie tussen het centrale zenuwstelsel en de afweer respons. Recentelijk werd aangetoond dat de nervus vagus, een zenuw die de hersenen met het gehele lichaam verbindt en die een belangrijke rol speelt bij het reguleren van allerlei processen in het lichaam (bloeddruk, ademhaling, hartslag, darmbeweging), tevens een rol speelt bij de regulering van de ontstekingsrespons. De gedachte is dat deze zenuw aan het eind van zijn zenuwbanen een stof vrijgeeft (acetylcholine) die in staat is afweer respons cellen rustig, dat wil zeggen anti-inflammatoir te maken. Deze nieuw ontdekte zenuw naar immuun pathway werd vanwege de naam van deze stof en haar werking de "cholinerge anti-inflammatoire pathway" genoemd. Dit eerdere onderzoek suggereerde dat prikkeling van deze zenuw (met elektrische impulsen) en toediening van acetylcholine-achtige stoffen een belangrijke nieuwe manier zou kunnen zijn het afweer systeem te remmen. Dit zou van groot belang kunnen zijn aangezien de afweer respons tijdens ernstige ziekte eigenlijk veel te sterk werkt en dan nadelig is voor de kansen op overleving na een ernstige steriele ontsteking (bijvoorbeeld pancreatitis) of na een infectie (bijvoorbeeld bloedvergiftiging). Goede dierstudies die bewijzen dat deze zenuw inderdaad dit effect heeft tijdens een ernstige ontsteking of infectie waren echter niet voorhanden.
Hoofdstuk 8 presenteert om te beginnen een overzicht van wat er tot nu toe bekend is over deze cholinerge anti-inflammatoire pathway. In de volgende hoofdstukken brengt dit proefschrift verslag uit van enkele vernieuwende studies op dit gebied.
Hoofdstuk 9 laat zien dat deze pathway inderdaad een belangrijke rol speelt bij de regulering van afweer respons tijdens bloedvergiftiging. Bij dieren, behandeld met nicotine, een acetylcholine achtige stof, trad een zeer sterke remming van de afweer respons op. Maar als de nervus vagus, voorafgaand aan de bloedvergiftiging, chirurgisch was doorgenomen trad een heftiger afweer respons op. Daarmee toont dit onderzoek voor het eerst aan dat deze pathway in vivo ook echt bestaat en dat manipuleren ervan effect heeft op de respons tegen ernstige infectie. Hoofdstuk 10 doet verslag van een soortgelijk onderzoek bij dieren met

een acute pancreatitis. Ook nu versterkt remming van de pathway (door doornemen van de zenuw of door een blokker van acetylcholine) de ontsteking, terwijl stimulatie van het effect van acetylcholine, dit keer met een door ons herontdekte en voor het gewenste effect hele selectieve acetylcholine achtige stof die GTS-21 heet, de ontsteking sterk vermindert. Deze pathway zou niet alleen gebruikt kunnen worden voor de ontwikkeling van nieuwe therapeutische interventies tegen ernstige infecties, maar ook tegen steriele ontstekingen.

Ontwikkeling van selectieve acetylcholine achtige stoffen zoals GTS-21 voor behandeling van ernstige infecties of van steriele ontstekingen is derhalve een hele zinvolle onderneming die kan leiden tot nieuwe effectieve medicijnen voor ziekten als bloedvergiftiging, pancreatitis maar ook voor ziekten als reuma en de ziekte van Crohn. Hoofdstuk 12 toont aan dat elektrische stimulatie van de nervus vagus, naar analogie van de eerder genoemde acetylcholine achtige stoffen, ook heel sterke afweer remmende effecten heeft tijdens ernstige ontsteking, in dit geval gesimuleerd door de injectie van bacteriële bestanddelen in het lichaam (zogenaamde endotoxine shock) van muizen. Dit betekent dat er ook potentie is voor de ontwikkeling van elektrische nervus vagus stimulatoren voor de behandeling van ernstige ontstekingsziekten. Het betreft hier een zeer reële optie, aangezien dergelijke implanteerbare stimulatoren thans al gebruikt worden voor de behandeling van therapie refractaire depressie en epilepsie. Het onderzoek bracht tevens een nieuw effect van nervus vagus stimulatie aan het licht, namelijk een duidelijke afname van de stolling activatie zoals die bij ernstige ontsteking in het kader van bloedvergiftiging in het lichaam voorkomt. Aangezien activatie van stolling een belangrijke bijdrage levert aan de kans om aan een bloedvergiftiging te overlijden houdt deze bevinding belangrijke therapeutische implicaties in. Hoofdstuk 13 onderzoekt hoe het eerder genoemde GTS-21 haar ontstekingremmende effecten feitelijk uitoefent. De werking van GTS-21 berust op het feit dat TNF- α erdoor geremd wordt, wat een belangrijke boodschappereiwit (cytokine) is dat als doel heeft ontstekingscellen te activeren en naar de plaats van ontsteking te dirigeren. Bovendien blijkt GTS-21 de migratie van witte bloedcellen naar het ontstekingsgebied ook onafhankelijk van TNF -α te remmen.

Deel III: Chronische pancreatitis

Het derde en laatste deel van dit proefschrift gaat over chronische pancreatitis, een chronische ontsteking van de alvleesklier. Deze ziekte kenmerkt zich door ontsteking en secundair verbindweefseling (fibrose vorming) van de alvleesklier. Daardoor gaan functies van de alvleesklier verloren, met als gevolg het ontstaan van suikerziekte en een verminderde opname van voedingsstoffen uit de darm. Chronische pancreatitis is een slecht bestudeerde ziekte omdat het orgaan bij mensen moeilijk te benaderen is voor sampling en omdat er weinig werkzame diermodellen voorhanden zijn. Hoofdstuk 14 beschrijft, mede op basis

van eerdere studies, de ontwikkeling van een wél werkzaam diermodel voor deze ziekte. De studie laat een dramatische reductie zien van de ernst van chronische pancreatitis bij muizen behandeld met troglitazone, een remmer van het zogenaamde Peroxisome Proliferator Activated Receptor (PPAR)-γ. Aangezien dit de eerste studie is die een positief effect laat zien van welk medicijn dan ook op de ontwikkeling van chronische pancreatitis in vivo lijkt deze belangrijke bevinding zeker navolging te verdienen in verdere klinische humane studies.

Samenvattend zijn de conclusies van dit proefschrift:
Acute pancreatitis na ERCP kan mogelijk voorkomen worden door profylactische toediening van semapimod.
Acute pancreatitis komt frequenter voor bij dialyse patiënten aangezien zij meerdere risicofactoren blijken te hebben voor de ontwikkeling ervan.
Infecties bij patiënten die al ziek zijn op basis van een eerder event verlopen heftiger (meer ontsteking en hogere bacteriële load) en de immunopathologie van infectie in deze patiënten is significant anders dan bij een spontane infectie in een eerder gezonde patiënt.
De nervus vagus reguleert ontsteking processen zowel als stolling activatie in vivo. Manipulatie van deze “cholinergische anti-inflammatoire pathway” middels medicijnen (GTS-21) of elektrische zenuw stimulatie zijn waardevolle potentiële nieuwe therapeutische opties in de behandeling van ernstige infecties en ontstekingsziekten.
Troglitazone remt fibrose vorming en ontsteking in een diermodel van chronische pancreatitis.

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