主論文

Acquired immunity plays an important role in the development of murine experimental pancreatitis induced by alcohol and lipopolysaccharide

Shinji Nakayama, MD, Akiyoshi Nishio, MD, PhD, Masao Yamashina, MD, PhD, Takashi Okazaki, MD, Yutaku Sakaguchi, MD, PhD, Katsunori Yoshida, MD, PhD, Toshiro Fukui, MD, PhD, Kazushige Uchida, MD, PhD, Kazuichi Okazaki, MD, PhD.

Third Department of Internal Medicine, Kansai Medical University, Moriguchi, Japan

Running title: Acquired immunity in murine pancreatitis

Correspondence to: Akiyoshi Nishio, MD, PhD.
Third Department of Internal Medicine, Kansai Medical University,
10-15 Fumizono-cho, Moriguchi, 570-8507, Japan.
Phone: +81-6-6993-9465, Fax: +81-6-6996-4874
E-mail: nishioa@takii.kmu.ac.jp

Disclosure

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Abstract

Objective: Although chronic alcohol ingestion is the major cause of chronic pancreatitis, less than 10% of alcohol abusers develop this disease. To address this issue, we created a murine model of pancreatitis induce by alcohol and lipopolysaccharide (LPS) and analyzed its immune responses.

Methods: C57BL/6 mice were administered 20% ethanol (AL) in their drinking water and then injected intraperitoneally with LPS twice weekly for four weeks. Severe combined immunodeficient (SCID) mice were reconstituted with splenocytes, $CD4^+$ cells, or $CD8^+$ T cells isolated from wild-type mice and then treated similarly. The severity of pancreatitis was graded histologically and serum cytokine levels were measured.

Results: AL alone did not cause pancreatitis. However, the administration of AL+LPS or LPS alone induced pancreatitis. The histological scores were higher in mice treated with AL+LPS than in those treated with LPS. Serum levels of interleukin 1β, interferon γ and tumor necrosis factor α were elevated in the AL+LPS-treated mice. SCID mice developed pancreatitis only after their reconstitution with splenocytes, $CD4^+$ cells, or $CD8^+$ T cells.

Conclusions: Repeated stimulation of the innate immune system is necessary, but not sufficient, to cause pancreatitis. The participation of the acquired immune response is essential for the development of the disease.

Key Words: murine pancreatitis, alcohol, lipopolysaccharide, Toll-like receptor, innate immunity, acquired immunity

Introduction

Chronic pancreatitis is a progressive fibroinflammatory disease in which the exocrine pancreatic parenchyma is largely destroyed and replaced by fibrous tissue, eventually leading to varying degrees of exocrine and, in the later stages, endocrine pancreatic insufficiency [1]. Although chronic pancreatitis has been considered a different entity from acute pancreatitis, it is now acknowledged that acute, recurrent acute, and chronic pancreatitis are parts of the same disease continuum [2]. The most commonly reported cause of chronic pancreatitis is the heavy consumption of ethanol (AL) [3]. However, although past studies have reported that 70%–90% of cases of chronic pancreatitis are attributable to alcohol, only about 50% of chronic pancreatitis has been attributed to alcohol in recent studies [4, 5].

Interestingly, alcohol alone is not sufficient to cause chronic pancreatitis because less than 10% of long-term alcoholics ever develop the disease [6]. Experimental studies have shown that the metabolites of AL injure the pancreatic acinar cells and activate stellate cells in vitro, although the pancreas processes AL efficiently via oxidative and nonoxidative pathways [7, 8]. However, the prolonged administration of AL does not induce chronic pancreatitis in vivo [9, 10]. Therefore, it has been proposed that alcohol may sensitize individuals to pancreatitis, which is triggered by causative environmental and genetic factors [10].

Recent studies have reported that transient endotoxemia may occur following acute alcohol consumption in healthy volunteers and in alcoholics without advanced alcoholic liver disease [11, 12]. Plasma endotoxin concentrations in patients with alcoholic liver disease were higher than those in patients with non-alcoholic liver disease [13]. These findings support the inference that alcohol ingestion facilitates the translocation of bacterial toxins to the blood circulation [12]. The source of the increased endotoxin concentration in the blood is considered to be intestinal Gram-negative bacteria because bacterial overgrowth in the small intestine has been reported in long-term alcohol abusers [14, 15]. Alcohol can also directly damage the

mucosa of the upper gastrointestinal tract and enhance the permeability of the gut mucosa. Evidence of increased intestinal permeability caused by alcohol ingestion has been reported in both animal experiments and human studies using various permeability probes [14].

The role of lipopolysaccharide (LPS), the main constituent of endotoxin, in the induction of alcoholic pancreatitis has been studied in rat models. Fortunato et al. reported that although neither alcohol nor LPS alone caused overt pancreatic damage, the administration of LPS to alcohol-fed rats resulted in the necrosis and inflammation of the pancreas, suggesting that alcohol sensitizes the pancreatic gland to the toxic effects of otherwise innocuous doses of LPS [16, 17]. Vonlaufen et al. showed that the administration of LPS initiates overt pancreatic injury in alcohol-fed rats and that repeated exposure to LPS causes progressive pancreatic damage in these animals, resulting in acinar atrophy and fibrosis, which are the hallmarks of chronic pancreatitis [18].

The Toll-like receptors (TLRs) are pathogen-binding receptors that recognize the pathogen-associated molecular patterns of a diverse range of microbial pathogens, and play an important role in the innate immune system [19]. The recognition of microbial components by TLRs activates signal transduction pathways, inducing the expression of various genes. The products of these genes trigger the innate immune responses and further instruct the acquired immune responses [20]. Because LPS is a ligand of TLR4, endotoxemia in alcohol abusers activates the innate immune response and subsequently the acquired immune response via TLR-mediated signaling. Indeed, moderate numbers of inflammatory cells, including lymphocytes, plasma cells, and macrophages, are present in the pancreas in alcoholic chronic pancreatitis (ACP) [21]. However, the immune responses in alcoholic pancreatitis are not understood fully, partly because no appropriate animal model is available.

To clarify the role of the immune responses in the pathogenesis of ACP, we created a murine model, induced by the administration of AL and LPS, and analyzed the role of the innate

and acquired immune responses in the development of the disease.

Materials and Methods

Mice

Eight-week-old C57BL/6 wild-type (WT) mice were purchased from Japan SLC (Shizuoka, Japan). Syngeneic severe combined immunodeficient (SCID) mice were purchased from RIKEN (Tsukuba, Japan). The mice were kept under specific-pathogen-free conditions on a 12-hour light/dark cycle with free access to drinking water and a normal diet.

Induction of Pancreatitis

We modified the procedure reported by Li et al [9]. The mice were administered increasing concentrations of AL in their drinking water: 10% AL for one week, then 15% AL for one week, and finally 20% AL until the completion of the LPS administration period. One week after the commencement of treatment with 20% AL, the mice were injected intraperitoneally with peptidoglycan (TLR2 ligand) at a dose of 5 mg/kg body weight, polyinosinic polycytidylic acid (poly I:C, TLR3 ligand; 5 mg/kg), or LPS (*Escherichia coli* 0111:B4; 4 mg/kg) twice weekly for four weeks (n = 7, each group; all reagents were from Sigma Chemical Co., St Louis, MO, USA). The controls consisted of phosphate-buffered saline (PBS) or LPS-treated mice without AL administration and those treated with AL alone (n = 7, each group). All experiments were conducted with the approval of the Ethics Committee for the Use of Animals of Kansai Medical University.

Reconstitution of SCID Mice with Splenic T Cells

Splenocytes (2×10^7) obtained from WT mice were injected intraperitoneally into syngeneic SCID mice twice at two-weekly intervals (n = 5). The $CD4^+$ and $CD8^+$ T cells were isolated from the splenocytes of WT mice using magnetic bead isolation kits (Miltenyi Biotec, Tokyo, Japan). The purity of each T-cell subset was assessed by flow-cytometric analysis before cell transfer and exceeded 95%. Contamination of the $CD8^+$ T cells with $CD4^+$ T cells and of the $CD4^+$ T cells with $CD8^+$ T cells were both less than 1%. $CD4^+$ or $CD8^+$ T cells (5×10^6) were

injected intraperitoneally into the SCID mice twice at two-weekly intervals (n = 3, each group). The SCID mice reconstituted with whole splenocytes, $CD4^+$ T cells, or $CD8^+$ T cells were treated with AL and LPS for four weeks, as for the WT mice.

Histopathological Examination

The AL-fed WT mice were killed 24 hours after a single injection of LPS or after treatment for four weeks with various TLR ligands. The SCID mice were killed after treatment for four weeks with AL and LPS. Blood samples were collected and the sera were stored at –30 °C until analysis. Pancreatic tissues were excised for histopathological examination. The tissues were fixed in 10% phosphate-buffered formaldehyde (pH 7.2), embedded in paraffin, and sectioned at 3.0 μm thickness, then stained with hematoxylin and eosin and examined under light microscopy. The severity of pancreatitis was graded with a scoring system described previously [19, 20]. Within each pancreatic section, the area of the total parenchyma affected by abnormal pancreatic tissue architecture (percentage involvement) was graded as follows: 0 = absent, 1 = rare, 2 = minimal (<10%), 3 = moderate (<50%), and 4 = major (>50%). Within these areas, the grade of glandular atrophy, pseudotubular complex formation, and fibrosis were scored as follows: glandular atrophy (0 = absent, 1 = minimal, 2 = moderate, 3 = severe), presence of pseudotubular complexes (0 = absent, 1 = minimal, 2 = moderate, 3 = severe), and fibrosis (0 = absent, 2 = only within areas, 4 = diffuse). Each final score was the product of the grade multiplied by its percentage involvement. The total score was calculated as the sum of each final score.

Measurement of Serum Amylase and Cytokine Levels

Serum amylase levels were measured by a commercial laboratory (SRL Inc., Osaka, Japan). Serum levels of interleukin 1β (IL-1β), IL-4, IL-10, IL-12p70, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) were measured using Bio-Plex Pro cytokine bead assay kits (Bio-Rad Laboratories, Tokyo, Japan).

Immunohistochemical Examination

Formalin-fixed paraffin-embedded pancreatic tissue sections were stained immunohistochemically. The sections were deparaffinized in xylene, rehydrated in a graded series of alcohol, and washed in PBS. The endogenous peroxidase activity was blocked with 1% H_2O_2 for 30 minutes. For antigen retrieval, the sections were pretreated in Target Retrieval Solution (Dako, Tokyo, Japan) in a pressure cooker at 100 °C for 10 minutes. After the sections were blocked with ProTaqs (Quartett Immunodiagnostika und Biotechnologie GmbH, Berlin, Germany) for 10 minutes, they were incubated overnight at 4 °C with one of the following primary antibodies: rat anti-mouse CD4 monoclonal antibody (mAb; 1:100 dilution); rat anti-mouse CD8 mAb (1:200); rat anti-mouse Ly-6G mAb (Gr1; 1:100); or rat anti-mouse CD45R mAb (B220; 1:200) (all from eBioscience, San Diego, CA). After the sections were rinsed briefly, they were treated with biotinylated rabbit anti-rat IgG secondary antibody (1:100; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, rinsed, and incubated with peroxidase-conjugated avidin–biotin complex (ABC Elite Kit; Vector Laboratories) for 30 minutes at room temperature. The immunoreactive deposits were visualized by the application of a fresh mixture of 3,3′-diaminobenzidine and 0.005% H_2O_2 in Tris-buffered saline (0.05 M, pH 7.6). The sections were counterstained with hematoxylin, dehydrated, cleared, and mounted.

Statistical Analysis

One-way analysis of variance, followed by Fisher's protected least significant difference test, was used to evaluate the differences between multiple groups using StatView software (Version 5; SAS Institute, NC). A two-tailed P value of less than 0.05 was considered significant.

Results

Pancreatitis in WT Mice

The administration of 20% AL alone for four weeks did not induce pancreatitis in WT mice (Fig. 1B). Moreover, the coadministration of peptidoglycan or poly I:C did not cause pancreatitis in the AL-fed mice (data not shown). In contrast, pancreatitis developed after the administration of LPS, with or without AL treatment. After the mice were injected with LPS for four weeks, mild inflammatory cell infiltration and the destruction of the pancreatic acini were observed (Fig. 1C). The administration of LPS coupled to the ingestion of AL induced intense infiltration of inflammatory cells, associated with the destruction of the acini, the formation of pseudotubular complexes, and irregular fibrosis in the pancreas (Fig. 1D). The histological scores for glandular atrophy, pseudotubular complex formation, and the total scores for pancreatitis were significantly greater in the mice treated with AL and LPS than in the mice treated with LPS alone ($P<0.01$; Fig. 2). The histological scores for fibrosis were greater in the mice treated with AL and LPS than in the mice treated with LPS alone, but the difference was not significant. However, a single injection of LPS with or without the ingestion of AL caused no apparent changes in the pancreas (data not shown).

Serum Amylase and Cytokine Levels

Serum amylase levels were significantly elevated in the mice treated with AL and LPS (1589 ± 471 IU/mL; mean $\pm$ standard deviation) compared with those in mice treated with PBS (713 ± 95) or AL alone (647 ± 57) ($P<0.01$; Fig. 3). The elevation of serum amylase was greater in the mice treated with LPS alone (1110 ± 531) than in those treated with PBS or AL alone, but these differences were not significant. To examine the role of proinflammatory and anti-inflammatory cytokines in the induction of pancreatitis, we compared the serum cytokine levels of the control, AL-, LPS-, and AL+LPS-treated mice. The serum levels of IL-1β, IFN-γ, and TNF-α were significantly elevated in the AL+LPS-treated mice compared with those of all

other groups ($P < 0.01$; Fig. 4A, 4E, 4F). Similar elevation of IL-10 was observed in the mice treated with LPS alone or with AL and LPS, and IL-10 was significantly higher than that of the control and AL-treated mice ($P < 0.05$; Fig. 4C). There were no significant differences in the IL-4 and IL-12p70 levels of the LPS- and AL+LPS-treated mice (Fig. 4B, 4D).

Immunohistochemical Staining of the WT Pancreas

An immunohistochemical analysis showed intense infiltration of Gr1-positive neutrophils, accompanied by $CD4^+$ cells, $CD8^+$ T cells, and $B220^+$ B cells, in the pancreases of WT mice treated with AL and LPS (Fig. 5C, 5D). These inflammatory cells infiltrated the periductal areas and pancreatic parenchyma. Immunohistochemical staining of mice treated with LPS alone showed similar inflammatory cell infiltration, although it was less prominent than in the AL+LPS-treated mice (data not shown).

Pancreatitis in SCID Mice

The administration of AL and LPS did not induce pancreatitis in SCID mice (Fig. 6A). However, pancreatitis developed in the mice treated with AL and LPS after their immune systems were reconstituted with whole spleen cells obtained from WT mice (Fig. 6B). The SCID mice that were reconstituted with $CD4^+$ or $CD8^+$ T cells also developed pancreatitis, although its severity was weaker than that in the SCID mice transfused with complete spleen cells (Fig. 6C, 6D). All the histological scores for glandular atrophy, pseudotubular complex formation, and fibrosis were significantly greater in the SCID mice reconstituted with whole spleen cells than in those reconstituted with $CD4^+$ T cells ($P < 0.05$) or $CD8^+$ T cells ($P < 0.01$), except the fibrosis score, which did not differ between the mice treated with whole splenocytes and those treated with $CD4^+$ T cells (Fig. 7A–D). The histological scores for glandular atrophy and fibrosis were greater in the mice reconstituted with $CD4^+$ T cells than in those reconstituted with $CD8^+$ T cells, but the difference was only significant for the glandular atrophy scores ($P < 0.05$).

Discussion

Accumulating evidence has suggested that alcohol promotes chronic pancreatic damage through the toxic effects of its metabolites and oxidative stress, and by facilitating the activation of pancreatic stellate cells (PSC) [22]. However, the pathogenesis of alcoholic chronic pancreatitis (ACP) remains elusive. A major obstacle to understanding the mechanism of ACP is the lack of a suitable animal model, because administering AL to most animal species does not cause overt pancreatic injury [9, 10]. In attempts to produce a model of ACP, AL administration has been combined with the administration of cerulein [10, 23], cholecystokinin [24], and chemical toxins, such as trinitrobenzenesulfonic acid [25], and invasive manipulations, such as pancreatic duct ligation [26]. However, the involvement of these causal factors is unlikely in the clinical situation.

LPS, the main component of endotoxin, has been considered a candidate compound that accelerates the development of ACP because serum endotoxin levels are increased in alcoholics [11, 12]. LPS was shown experimentally to induce apoptosis in rat pancreatic acinar cells [27]. Furthermore, single or repeated administration of LPS to AL-fed rats causes pancreatic injury, with the infiltration of inflammatory cells, and ultimately chronic pancreatitis [16–18]. However, rat models do not seem to be appropriate for the analysis of the immune responses in ACP, partly because genetically modified rats are not readily available. To resolve this issue, we used mice to create a model of ACP and found that the administration of LPS alone or in association with AL caused pancreatic damage. Importantly, it is possible to use a variety of mouse strains, including immunodeficient mice, for the analysis of the immune responses in ACP.

The hypothesis of a "necrosis–fibrosis" sequence in chronic pancreatitis is now well supported by clinical and experimental studies [10, 28]. We investigated whether acute pancreatitis was induced in AL-fed mice by a single treatment with LPS. In contrast to previous

studies based on rat models [16–18], a single treatment with LPS did not cause acute pancreatitis, suggesting that pancreatic injury progresses gradually, with no overt attack of acute pancreatitis in our model. This finding is consistent with a previous study that showed that alcoholic acute pancreatitis rarely occurs after a single binge [1] and a recent hypothesis that ACP progresses to irreversible pancreatic damage as a consequence of recurrent acute pancreatitis, which may remain subclinical [29].

Increasing evidence supports the involvement of the immune system in the pathogenesis of chronic pancreatitis. Mononuclear inflammatory cell infiltration is a common finding in the pancreases of patients with chronic pancreatitis [30]. A recent study reported the infiltration of T lymphocytes in murine models of chronic pancreatitis [31]. In a rat model of dibutyltin-dichloride-induced chronic pancreatitis, Sparmann et al. reported that $CD4^+$ T cells predominated among these infiltrating lymphocytes, but there was an increase in $CD8^+$ T cells during the chronic process, resulting in a reduced CD4/CD8 ratio [32]. However, few immunological analyses have been performed in experimental models of ACP. In this study, we demonstrated the intense infiltration of neutrophils, accompanied by $CD4^+$, $CD8^+$, and $B220^+$ cells, in the inflamed pancreas. This is consistent with a previous study in which the infiltration of $CD4^+$, $CD8^+$, $CD45^+$, and $CD68^+$ inflammatory cells was observed in patients with ACP [33].

To further investigate the role of the innate and acquired immune responses in the pathogenesis of pancreatitis, we examined whether treatment with AL and LPS causes pancreatitis in immunodeficient mice. The combined administration of AL and LPS induced pancreatitis in WT mice, but not in SCID mice, which lack an acquired immune system. However, pancreatitis developed after reconstitution of the SCID mice with $CD4^+$ or $CD8^+$ T cells isolated from WT mice. These findings indicate that the innate immune response may be involved, but is insufficient to induce pancreatitis, and that the participation of the acquired

immune response is necessary for the development of the disease. In our study, there was no significant difference in the disease severity between mice reconstituted with $CD4^+$ T cells and those reconstituted with $CD8^+$ T cells. The different roles of $CD4^+$ and $CD8^+$ T cells has been reported in a murine model of autoimmune hepatitis; $CD4^+$ T cells play a role in the initiation of the disease and $CD8^+$ T cells contribute to its progression [34]. The roles of $CD4^+$ and $CD8^+$ T cells in the pathogenesis of ACP should be clarified in a future study.

The LPS-mediated stimulation of the innate immune response is mandatory because treatment with AL alone did not cause pancreatitis as reported previously [9, 10]. Because LPS is a ligand of TLR4, we investigated whether other TLR ligands are involved in the development of ACP. In this study, we used peptidoglycan and poly I:C because the activation of TLR2 by peptidoglycan has been implicated in the pathogenesis of autoimmune pancreatitis [35] and poly I:C, a synthetic double-stranded RNA and a TLR3 ligand, is known to induce autoimmune phenomena in some mouse strains [35, 36]. However, the administration of neither peptidoglycan nor poly I:C caused pancreatitis in untreated or AL-treated mice. Therefore, the administration of LPS may specifically induce pancreatitis in mice treated with AL.

Recent animal and human studies have shown that TLR4 polymorphisms or mutations can impair the individual's capacity to respond to LPS, resulting in altered susceptibility to infectious or inflammatory diseases, including necrotic infection in acute pancreatitis [37, 38]. In contrast to the protective role of TLR4-mediated signaling, its augmentation by repeated stimulation with LPS led to the development of pancreatitis. In our study, serum levels of IL-1β, IFN-γ, and TNF-α were elevated in mice with pancreatitis, and were significantly higher in mice treated with AL and LPS than in those treated with LPS only. LPS is known to promote proinflammatory cytokine release [14] and alcohol and its metabolites also affect cytokine production in pancreatic acinar cells [39]. However, the elevation of IL-10 was similar in mice treated with LPS alone and those treated with AL and LPS. These findings were consistent with

a previous study in which in vitro experiments using human monocytes demonstrated that while treatment with LPS alone increased both proinflammatory and anti-inflammatory cytokines, administration of LPS in alcohol-fed mice further increased proinflammatory cytokine production with no increase in IL-10 production [40]. Endogenous IL-10 has been shown to suppress glandular atrophy and fibrosis in experimental chronic pancreatitis [41]. Our findings suggest that stronger Th-1 cytokine predominance was induced by the combined administration of AL and LPS than was induced by LPS alone. We have previously reported that the levels of proinflammatory and anti-inflammatory cytokine production induced by TLR signaling may differ among various TLR ligands, and suggested that this imbalance between proinflammatory and anti-inflammatory cytokine production induces autoimmune-mediated pancreatitis in mice [42]. Therefore, we infer that the coadministration of AL and LPS induced strong Th-1 cytokine predominance and the consequent activation of the acquired immune response, leading to the development of ACP.

Lipopolysaccharide has been shown to activate PSC and inhibit PSC apoptosis, leading to the synthesis of excess extracellular matrix proteins and the development of pancreatic fibrosis. Importantly, alcohol and LPS together exert synergistic effects on PSC activation and apoptosis. In addition to the activation of PSC by the cytokines produced during pancreatic necroinflammation, LPS activates them directly by interacting with the TLR4 expressed on their surfaces [18]. Furthermore, the upregulation of TLR4 in the pancreatic ductal epithelium, vascular endothelium, and islets is reported during the inflammatory processes of pancreatitis [43]. Toll-like receptor 4 signaling mediates the intracellular signal transduction of the inflammatory responses and triggers the activation of nuclear factor kappa B, which controls the release of cytokines. This enhanced cytokine production in the pancreas may contribute to the activation of the innate and acquired immune responses, resulting in the development of ACP.

In conclusion, we have shown that the administration of LPS induced pancreatitis in AL-fed mice and that the accumulation of subtle pancreatic injury induced by exposure to LPS led to overt pancreatic damage and fibrosis. We have also shown that acquired immunity is essential for the development of alcoholic pancreatitis. Our mouse model will allow the detailed clarification of the immune responses involved in the pathogenesis of this disease.

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Figure Legends

Figure 1. Histopathological analysis of the pancreases of WT mice treated with AL and LPS. Representative pancreatic sections stained with hematoxylin and eosin: after treatment for four weeks with PBS (A), treatment for four weeks with AL alone (B), treatment for four weeks with LPS alone (C), and treatment for four weeks with AL+LPS (D). After treatment for four weeks with LPS, mild inflammatory cell infiltration and destruction of the acini were observed. The administration of LPS to AL-fed mice induced intense infiltration of inflammatory cells, associated with the destruction of the acini, pseudotubular complex formation, and irregular fibrosis in the pancreas. Bars indicate 100 μm.

Figure 2. Histological scoring of chronic pancreatitis in WT mice treated with AL and LPS. The severity of chronic pancreatitis was graded with a semiquantitative system: glandular atrophy (A), pseudotubular complex formation (B), fibrosis (C), and total score (D). All histological scores were higher in the mice treated with AL+LPS than in those of the other groups (**$P < 0.01$), except for the fibrosis scores of mice treated with AL+LPS and those treated LPS alone. The results are expressed as means ± standard deviations (SD).

Figure 3. Serum amylase levels in WT mice treated with AL and LPS. Amylase levels were significantly elevated in mice treated with AL and LPS compared with those in mice treated with PBS or AL alone (**$P < 0.01$). The results are expressed as means ± SD.

Figure 4. Serum cytokine levels in WT mice treated with AL and LPS: IL-1β (A), IL-4 (B), IL-10 (C), IL-12p70 (D), IFN-γ (E), and TNF-α (F). Serum levels of IL-1β, IFN-γ, and TNF-α were significantly elevated in AL+LPS-treated mice compared with those treated with PBS, AL, or LPS (**$P < 0.01$). Similar IL-10 elevation was observed in mice treated with LPS alone and AL+LPS-treated mice, and IL-10 was significantly higher than that of the control and AL-treated mice (*$P < 0.05$).

Figure 5. Immunohistochemical staining of the pancreases of WT mice treated with AL and

LPS. Representative pancreatic sections stained with hematoxylin and eosin (A, B) and immunohistochemical staining for CD4 (C), CD8 (D), B220 (E), and Gr1 (F). Intense infiltration of Gr1-positive neutrophils, accompanied by $CD4^+$ T cells, $CD8^+$ T cells, and B cells, was observed in the pancreatic parenchyma. Bars indicate 100 μm.

Figure 6. Histopathological analysis of the pancreases of SCID mice treated with AL and LPS. SCID mice were reconstituted with whole splenocytes, $CD4^+$ T cells, or $CD8^+$ T cells isolated from WT mice, and then treated with AL and LPS for four weeks. Representative pancreatic sections stained with hematoxylin and eosin: mice without cell transfer (A), mice with whole splenocyte transfer (B), mice with $CD4^+$ T-cell transfer (C), and mice with $CD8^+$ T-cell transfer (D). After treatment for four weeks, the SCID mice did not develop pancreatitis, whereas the mice that were reconstituted with splenocytes, $CD4^+$ T cells, or $CD8^+$ T cells developed pancreatitis. Bars indicate 100 μm.

Figure 7. Histological scoring of chronic pancreatitis in SCID mice treated with AL and LPS. The severity of chronic pancreatitis was graded with a semiquantitative system: glandular atrophy (A), pseudotubular complexes (B), fibrosis (C), and total score (D). The scores for glandular atrophy, pseudotubular complex formation, and fibrosis, and the total scores were greater in mice reconstituted with whole splenocytes than in mice reconstituted with $CD4^+$ (**$P < 0.01$) or $CD8^+$ T cells (*$P < 0.05$), except for the fibrosis scores of mice treated with whole splenocytes and those treated with $CD4^{+\ T\ cell}$. The scores for glandular atrophy and pseudotubular complexes and the total scores tended to be higher in the mice reconstituted with $CD4^+$ T cells than in those reconstituted with $CD8^+$ T cells. The results are expressed as means ± SD.

Fig. 1 Histopathological analysis of the pancreases of WT mice treated with ethanol and LPS

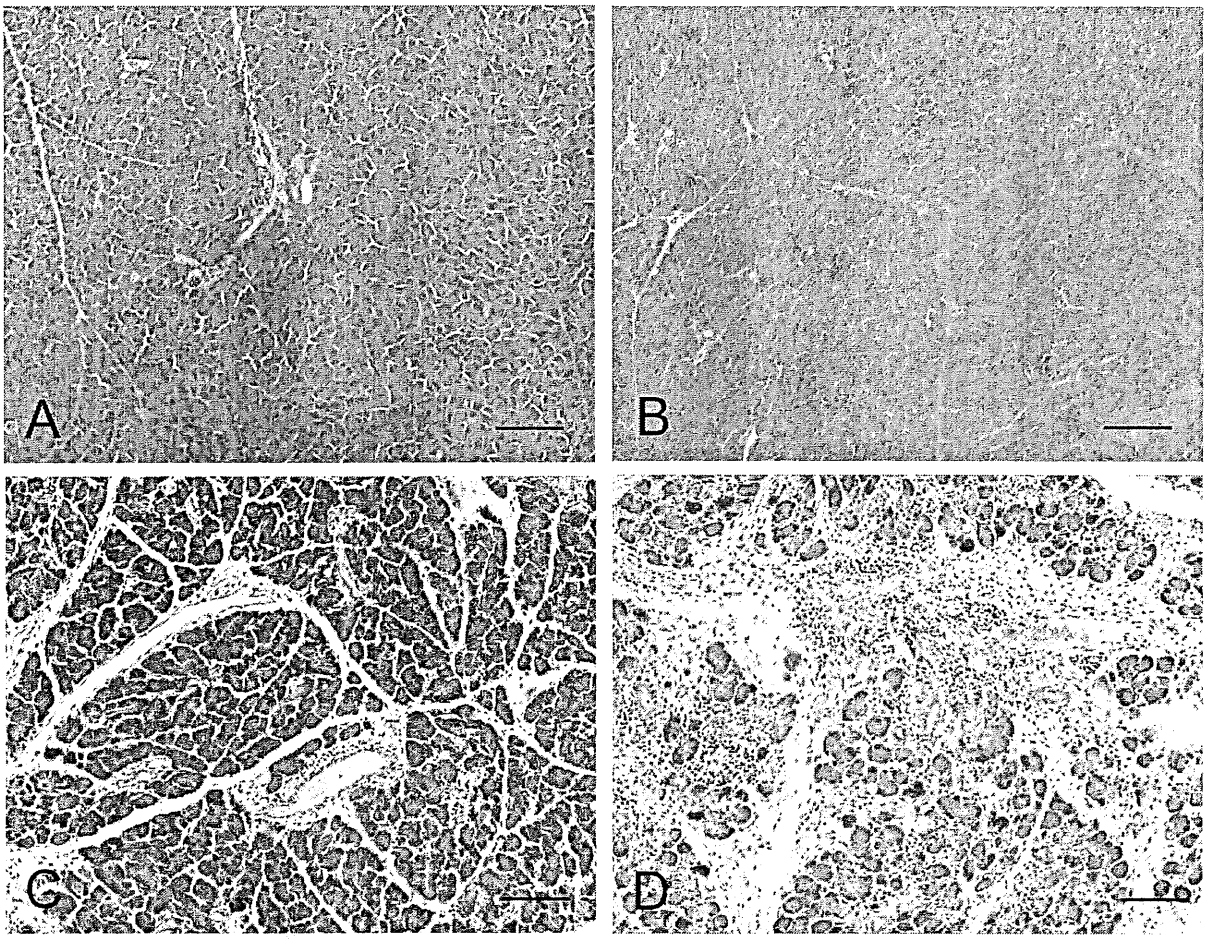


Fig. 2 Histological scoring of chronic pancreatitis in WT mice treated with ethanol and LPS

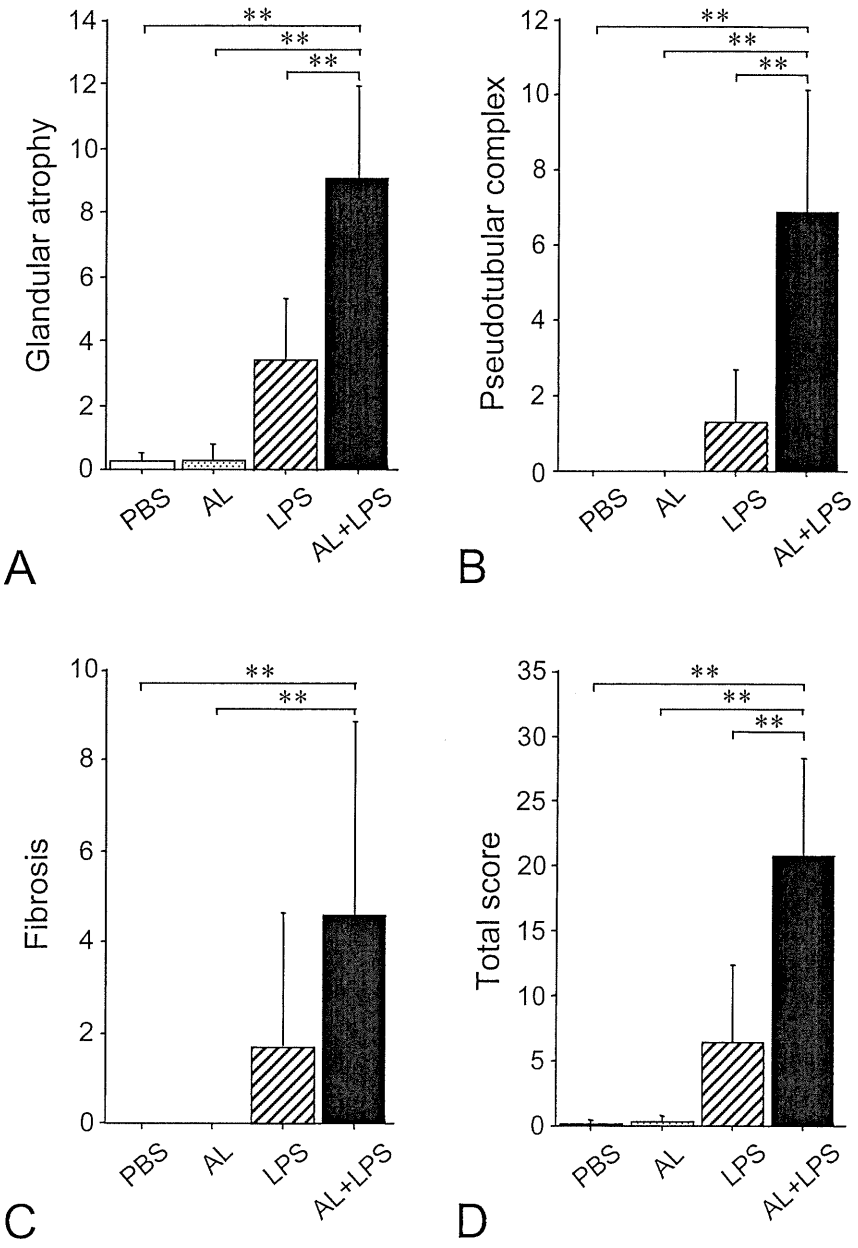


Fig.3 Serum Amylase levels in WT mice treated with ethanol and LPS

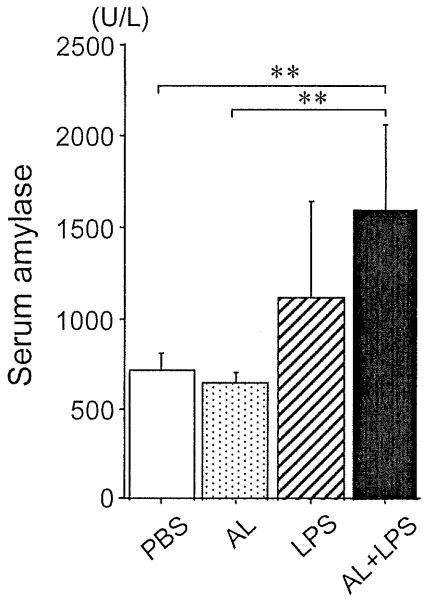


Fig.4 Serum cytokine levels in WT mice treated with ethanol and LPS

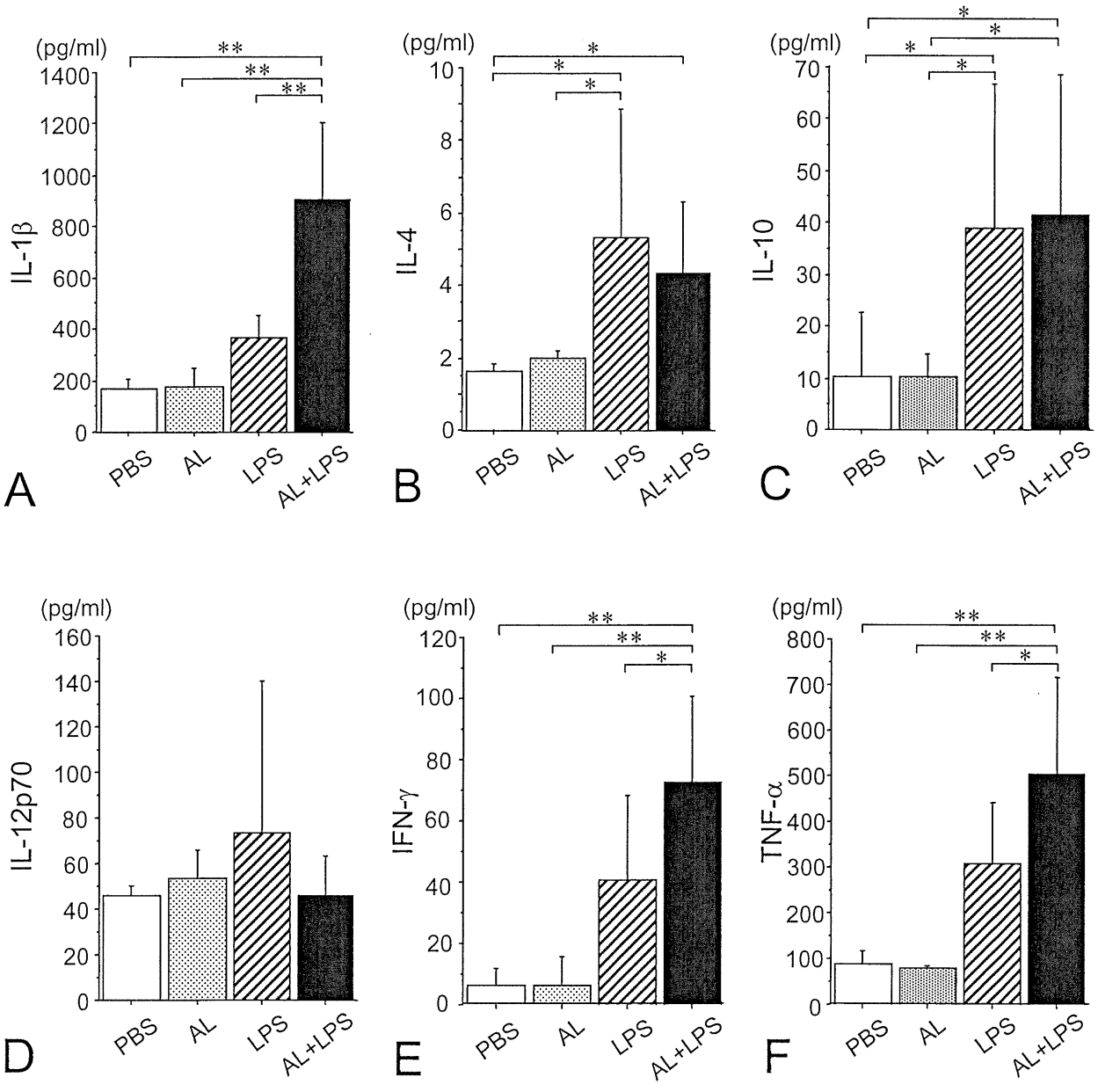


Fig. 5 Immunohistochemical staining of the pancreases of WT mice treated with ethanol and LPS

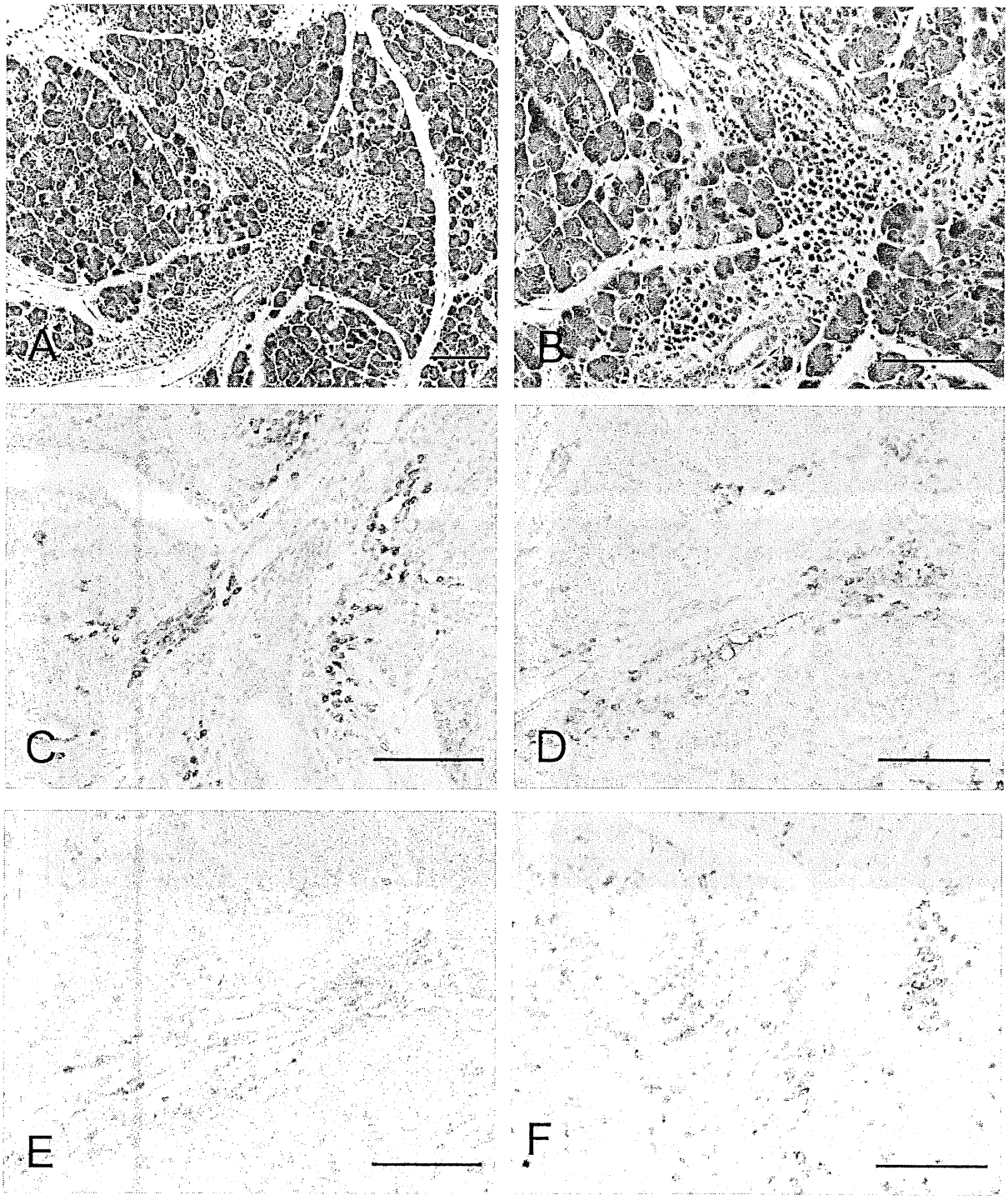


Fig. 6 Histological analysis of the pancreases of SCID mice treated with ethanol and LPS

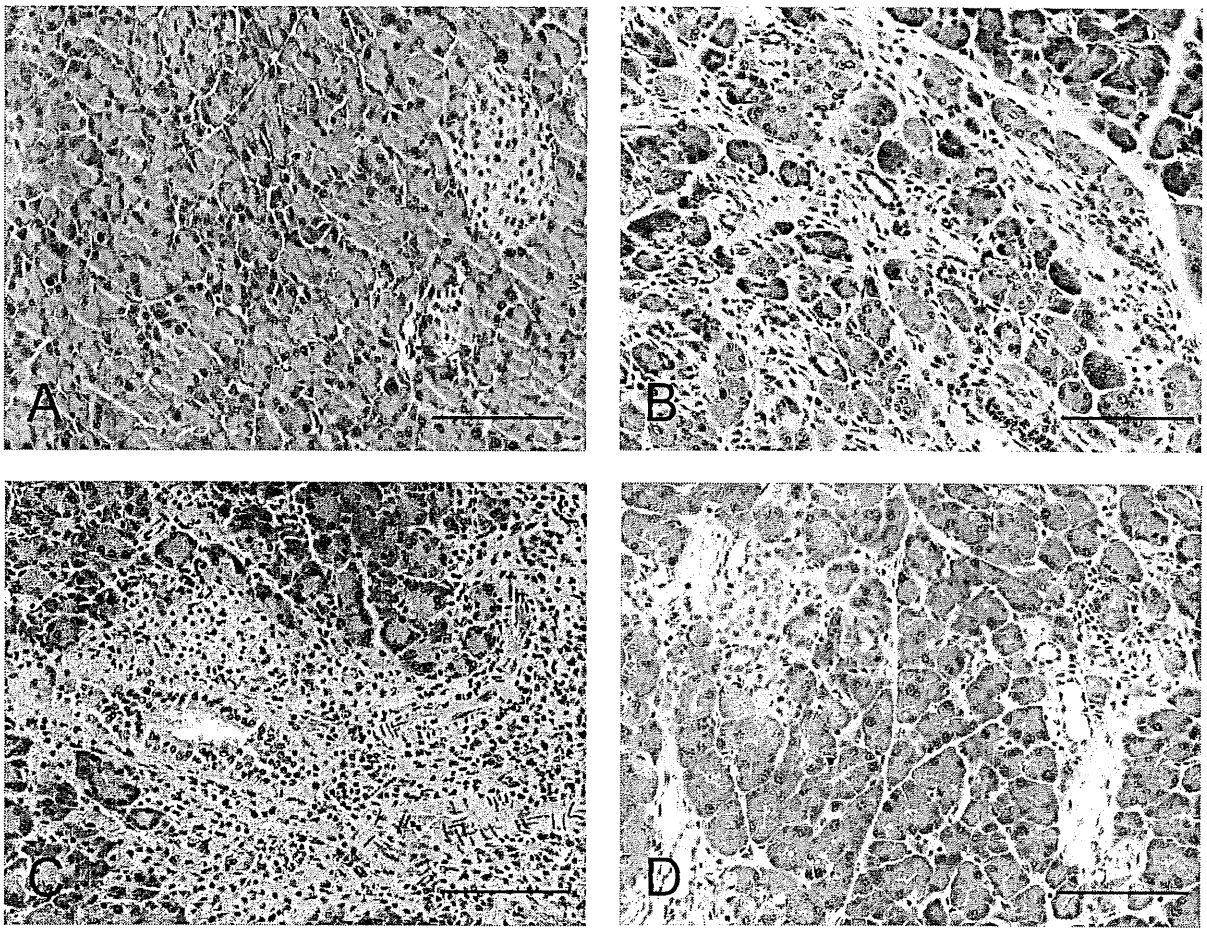
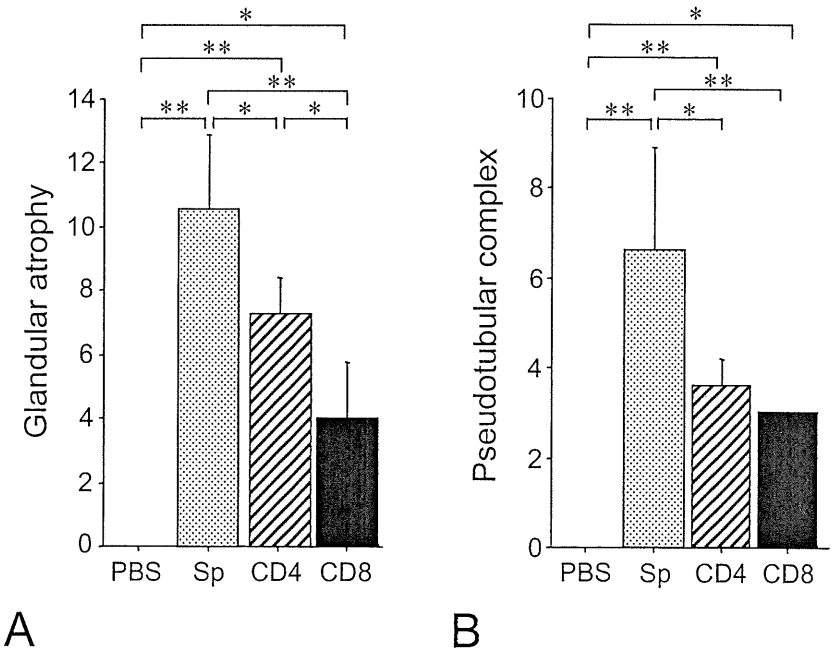
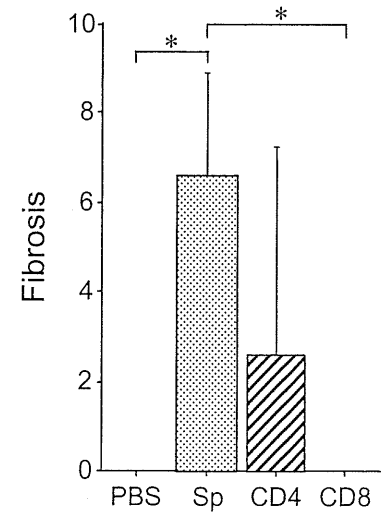


Fig.7 Histological scoring of chronic pancreatitis in SCID mice treated with ethanol and LPS

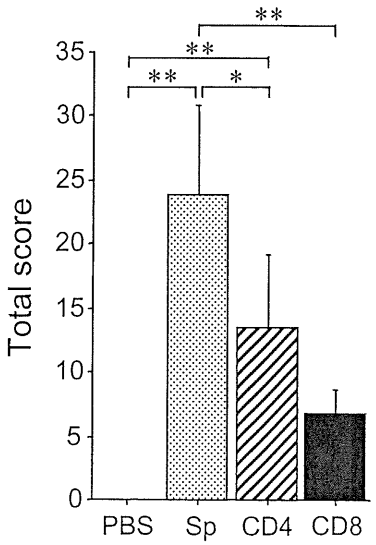


A

B



C



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