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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Pomalidomide suppresses cerulein-induced acute pancreatitis in mice

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Abstract
Background An overproduction of proinflammatory mediators in severe acute pancreatitis contributes to the systemic inflammatory response, which may lead to multiorgan damage and even death. Thus, inflammatory cytokines, e.g., tumor necrosis factor (TNF)-α and interleukin (IL)-1β, may be novel targets for the treatment of acute pancreatitis. The aim of this study was to investigate the therapeutic effects of pomalidomide (or CC-4047), a thalidomide analog and immunomodulatory agent, in acute pancreatitis.
Methods Acute pancreatitis was induced in C57BL/6 mice by intraperitoneal administration of cerulein (100 μg/kg/h × 8). Pomalidomide was administered (0.5 mg/kg orally) 1 h before the first or 1 h after the last cerulein administration. The severity of the acute pancreatitis was evaluated biochemically and morphologically.
Results Pretreatment with pomalidomide significantly reduced the plasma levels of amylase and lipase; the histological injury; and the expression of TNF-α, IL-1β, monocyte chemotactic protein-1 (MCP-1), and inducible nitric oxide synthase (iNOS) in cerulein-induced acute pancreatitis. Post-treatment with pomalidomide also decreased the cerulein-induced elevation of plasma amylase and lipase and decreased the pancreatic damage.
Conclusions Treatment with pomalidomide ameliorated the severity of cerulein-induced acute pancreatitis in mice. Our data suggest that pomalidomide may become a new therapeutic agent in future clinical trials for the treatment of acute pancreatitis.

Keywords Cerulein · Pancreatitis · Pomalidomide · CC-4047 · Cytokines

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Introduction

Acute pancreatitis, an inflammatory disease of the pancreas, is the second most common inpatient gastrointestinal disease diagnosed in the United States, with around 210,000 patients admitted annually [1]. The worldwide incidence of acute pancreatitis is around 10–80 cases per 100,000 population/year. Although in most individuals with mild pancreatitis the disease has a self-limiting course, in 20% of them it may develop into severe acute pancreatitis, with an overall mortality rate of up to 38% [2]. Currently, the treatment of acute pancreatitis still depends on supportive measures—pain control, intravenous fluid supplement, and fasting. No effective pharmacologic agents have been

identified in treating acute severe pancreatitis in large clinical trials [3].

Recently, evidence has suggested that the upregulation of proinflammatory mediators, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-12, and monocyte chemotactic protein-1 (MCP-1), may reflect a local response of pancreatic acinar cell damage and that intra-acinar enzyme activation may trigger the development of acute pancreatitis [4–6]. Proinflammatory mediators, once released into the circulation from the local inflammatory reaction, may progress to a systemic inflammatory response and eventually multiple organ dysfunctions may develop. So the targeting of these proinflammatory mediators has been proposed as a novel strategy for the treatment of acute pancreatitis.

Thalidomide, an effective sedative and anti-emetic drug marketed during the mid-1950s, was withdrawn from the market in 1961 because of its teratogenic effects [7]. In 1965, Sheskin [8] succeeded serendipitously in using thalidomide to treat erythema nodosum leprosum, a potentially life-threatening inflammatory complication of leprosy; this was the first report to demonstrate the anti-inflammatory property of thalidomide. Around 3 decades after the drug was banned, it was discovered that thalidomide inhibited the synthesis of TNF-α by increasing TNF-α mRNA degradation [9]. Subsequently thalidomide was used to treat diseases associated with increased TNF-α production, such as rheumatoid arthritis, chronic graft-versus-host disease, and Crohn's disease [10–12]. Since 1996, chemists have synthesized new structural analogs of thalidomide with improved ability to inhibit TNF-α and decreased or lack of teratogenic effects; new immunomodulatory derivatives such as pomalidomide (or CC-4047) and lenalidomide (or CC-5013) have been developed [13–15].

Pomalidomide has been shown to be approximately 20,000 times more potent than thalidomide as a TNF-α inhibitor in vitro [14, 15]. Moreover, it has also been demonstrated to inhibit other proinflammatory cytokines, such as IL-1β, IL-6, and IL-12, and enhance the production of the anti-inflammatory cytokine IL-10, by lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMCs) [16, 17]. Although many clinical trials have been carried out with pomalidomide to treat advanced multiple myeloma, myelodysplastic syndrome, and metastatic prostate cancer, with acceptable adverse effects, there are no studies using pomalidomide to treat inflammation-associated diseases. In this study, we investigated the effects of pomalidomide in an established mouse model of cerulein-induced acute pancreatitis. Our results indicate that pretreatment with pomalidomide attenuated the severity of the cerulein-induced acute pancreatitis by reducing TNF-α, IL-1β, monocyte chemotactic protein-1 (MCP-1), and inducible nitric oxide synthase (iNOS) expression, and attenuating histological damage. Post-treatment with pomalidomide was less effective. This study suggests that pomalidomide may be a potential agent for the prevention and treatment of acute pancreatitis.

Materials and methods

Animals

Male C57BL/6 mice, purchased from the National Laboratory Animal Center (Taipei, Taiwan), were housed at the Laboratory Animal Center, Tzu Chi University, under standard conditions. Male mice weighing 20–25 g were used in this study. All experimental procedures were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee of Tzu Chi University. Every effort was made to minimize the suffering of the animals and the number of animals used.

Acute cerulein-induced pancreatitis and pomalidomide treatment

Acute pancreatitis was induced by 8 doses (first dose at zero time followed by 7 hourly administrations) of i.p. injection of cerulein (100 μg/kg, dissolved in saline solution). In the pretreatment study, mice were randomly divided into the following four groups ($n = 6$–10 for each group). (1) The saline + vehicle group: mice were treated with 8 hourly i.p. injections of saline (0.9% NaCl, 5 μl/g). One hour before the first dose of saline injection, the vehicle (10 μl/g), olive oil with 1% dimethyl sulfoxide (DMSO), was given orally. (2) The cerulein + vehicle group: treatment was the same as that in the first group of animals, except that saline was replaced with cerulein. (3) The cerulein + pomalidomide group: mice received one dose of pomalidomide (dissolved in vehicle) orally 1 h before the first administration of cerulein. (4) The saline + pomalidomide group: the protocol was the same as that for the saline + vehicle group, except that pomalidomide was used instead of vehicle.

For the examination of histological changes and plasma enzyme assays of acute pancreatitis, the mice were sacrificed at 4, 8, or 24 h after the first administration of saline or cerulein. For the time course measurement of inflammatory cytokines and their mRNA expression, the mice were sacrificed at 30 min, and at 2, 4, 8, and 24 h following the first cerulein injection. Mice were sacrificed under chloral hydrate (10%, 10 μl/g, i.p.) anesthesia. Blood was collected by direct cardiac puncture. A portion of the pancreas was preserved in 10% formalin for histological examination, and the remainder was immediately stored at −80°C for further analysis.

To determine the maximum therapeutic effect, serial doses of pomalidomide (0.1, 0.5, 1, 5, 10, and 50 mg/kg) were used to treat acute pancreatitis and the dose–response was evaluated, using plasma amylase and lipase levels as the indicators. The efficacy of post-treatment with pomalidomide was also examined. In the post-treatment experiment, the study groups were the same as those in the pretreatment experiment except that pomalidomide or vehicle was given 1 h after the last administration of cerulein. The post-treatment mice were sacrificed at 24 or 30 h after the first administration of saline or cerulein and the histological changes and plasma enzymes were examined in a manner similar to that employed for the pretreatment experiment.

Histological grading of acute pancreatitis

Hematoxylin/eosin staining was performed on 5-μm sections from the paraffin-embedded pancreas samples. The severity of acute pancreatitis was graded by a semi-quantitative assessment of edema, acinar cell necrosis, and inflammatory cell infiltration described previously [18]. Briefly, edema was scored as 0, absent; 1, focal increase between lobules; 2 diffuse increase between lobules; and 3, acini disrupted and separated. Inflammatory cell infiltrate was scored as 0, absent; 1, in ducts (around ductal margins); 2, in the parenchyma (in <50% of the lobules); and 3, in the parenchyma (in >50% of the lobules). Acinar necrosis was scored as 0, absent; 1, periductal necrosis (5%); 2, focal necrosis (5–20%); and 3, diffuse parenchymal necrosis (20–50%). The total histological scores, representing the sums of the scores for edema, cell necrosis, and inflammatory cell infiltration, were compared between control and treatment groups.

Measurement of cytokines and western blotting for iNOS

Pancreas tissue was homogenized in PRO-PREP protein extraction solution (iNtRON Biotechnology, Seongnam, South Korea). Homogenates were centrifuged at 13,000 rpm (Allegra 21R centrifuge; Beckman Coulter, Brea, CA, USA) at 4°C for 5 min. The supernatant was removed and stored at −80°C until further assay. The protein concentration of the supernatant was measured with a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA). A mouse cytokine antibody array (R&D Systems, Minneapolis, MN, USA) was used to detect the expression of 40 different inflammatory cytokines in pancreas tissue in the different groups at 24 h after the administration of cerulein. In another experiment, the levels of TNF-α in plasma and pancreas were determined with commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems). All the procedures were performed according to the manufacturer's instructions. The level of iNOS from the pancreatic tissue at 24 h after cerulein administration was also quantified by western blot. Briefly, 40 μg of protein extract from pancreatic tissue was separated on 10% sodium dodecylsulfate (SDS) polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 2 h with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST), and subsequently probed with a specific antibody against iNOS (1:500; Abcam, Cambridge, UK) at 4°C overnight. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (1:2000; KPL, Gaithersburg, MD, USA) for 1 h at room temperature, and immunoreactivities were visualized using an enhanced chemiluminescence detection system. To ensure equal amounts of protein loading, membranes were also incubated with a mouse anti-actin antibody (1:2000; Millipore, Temecula, CA, USA) as the internal control. The relative expression of protein bands was analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA) after scanning the radiographic film into a computer.

Amylase and lipase assays

Plasma levels of amylase and lipase have been routinely used to estimate the severity of pancreatitis. These levels were measured at 24 and 30 h after the first dose of cerulein administration by the clinical laboratory of Tzu Chi General Hospital (Hualien, Taiwan). Results were expressed as international units per liter.

Immunohistochemical staining

At 24 h after the first dose of cerulein or saline injection, the pancreas tissues were fixed in 10% formalin. Five-micrometer sections of paraffin-embedded pancreas tissues were processed according to the conventional method for immunohistochemical staining. Sections were incubated overnight at 4°C with anti-iNOS antibody (1:100 in phosphate-buffered saline [PBS]) (BD Biosciences, San Jose, CA, USA). Subsequently, horseradish peroxidase-conjugated secondary antibody was added for 45 min (Novolink-polymer, Leica Microsystem, Newcastle upon Tyne, UK), and substrate 3,3′-diaminobenzidine (DAB; Dako, Glostrup, Denmark) was added for 5 min. Finally, the sections were washed, counterstained with hematoxylin, dehydrated, and sealed for visualization.

RNA isolation and real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA (1 μg), extracted from small pieces of pancreas using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), was reverse-transcribed to synthesize cDNA, using a ReverTra Ace-α-reverse transcription kit (Toyobo, Osaka, Japan) and oligo(dT) as a primer. Real-time PCR was performed using Power Syber Green PCR Master mix (Applied Biosystems, Warrington, UK) in an ABI 7300 Real-Time PCR System (Applied Biosystems). Each reaction was performed in duplicate, and the dissociation curves were constructed to ensure that only a single product was amplified. The following specific primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-CGACTTCAACAGCAACTCCCACTCTTCC-3′ (sense) and 5′-TGGGTGGTCCAGGGTTTCTTACTCC TT-3′ (antisense); TNF-α, 5′-GGGCCACCACGCTCTTC TGTCT-3′ (sense) and 5′-GCCACTCCAGCTGCTCCTCC AC-3′ (antisense); and IL-1β, 5′-TCGCTCAGGGTCAC AAGAAA-3′ (sense) and 5′-CCATCAGAGGCAAGGAG GAA-3′ (antisense). The signals were normalized to GAPDH mRNA, as an internal control, and the data were expressed as a fold-change over the saline control group at 30 min after the induction of pancreatitis.

Materials

Cerulein was purchased from Sigma-RBI (Taipei, Taiwan). Pomalidomide was synthesized according to the published methods [14, 19]. It was used only in the present study.

Statistical analysis

All the data in the Figures are expressed as means ± standard error of mean (SEM). The results were analyzed by one-way or two-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test and the Bonferroni post-test, respectively, depending on the analytic data. $P < 0.05$ was considered statistically significant.

Results

Dose–response effect of pomalidomide on cerulein-induced elevation of plasma amylase and lipase levels

Acute pancreatitis was induced by 8 doses of i.p. administration of cerulein. The plasma levels of amylase and lipase were increased by 4- and 8-fold, respectively, 24 h after the first dose of cerulein (Fig. 1a, b) To determine the dose–response effect of pomalidomide on acute pancreatitis and to determine its maximum therapeutic dose, pretreatment with serial doses (0.1, 0.5, 1, 5, 10, and 50 mg/kg) was carried out, and the maximum inhibitory effect in suppressing the cerulein-induced elevation of plasma amylase and lipase levels was found to be 0.5 mg/kg pomalidomide (Fig. 1a, b). A similar inhibitory effect was found with 0.1 mg/kg pomalidomide. However, the inhibitory effect decreased gradually and appeared to level off as the dose of pomalidomide was increased to 50 mg/kg, a 100-fold increase over the maximum effective dose. Therefore, 0.5 mg/kg of pomalidomide was chosen for the subsequent experiments, and the number of animals in each group was increased to 10. These results are summarized in Fig. 1c, d. Pretreatment with pomalidomide (0.5 mg/kg) reduced the cerulein-induced elevation of plasma levels of amylase and lipase by 44 and 52% (compared to cerulein injection alone), respectively. Pomalidomide (0.5 mg/kg) alone had no effect on the levels of amylase and lipase.

Effects of pomalidomide on the histological changes of cerulein-induced pancreatitis

The histological examination of pancreas sections at 24 h after the first cerulein injection revealed extensive tissue damage, characterized by interstitial edema (Fig. 2c); inflammatory cell infiltration (Fig. 2c, g), including neutrophils and macrophages (Fig. 2g inset); and acinar cell necrosis (Fig. 2g). Pretreatment with pomalidomide (0.5 mg/kg) markedly reduced the cerulein-induced histological features of pancreatic injury (Fig 2d, h), and the features appeared nearly as normal as those in the saline + vehicle or saline + pomalidomide groups (Fig. 2a, b, e, f). The semi-quantitative histological scoring to assess the severity of acute pancreatitis is shown in Fig. 2i. The total histological scores (edema + inflammatory cell infiltration + cell necrosis) were already significantly increased 4 h after cerulein administration, and the scores continued to increase up to 24 h. Pomalidomide alone was without significant effect. Pretreatment with 0.5 mg/kg pomalidomide significantly decreased the total scores at 8 and 24 h after the first cerulein injection. A trend toward decrease, but without statistical significance, was observed at 4 h following cerulein dosing. Pretreatment with one dose of 0.5 mg/kg pomalidomide almost completely reversed the cerulein-mediated pancreatic injury to normal.

Effects of pomalidomide on cerulein-induced cytokine protein and TNF-α and IL-1β mRNA expression

A cytokine array was used to screen the expression of proinflammatory cytokines 24 h after the induction of pancreatitis. Among the 40 cytokines surveyed, only the expression of MCP-1 was elevated, and its expression

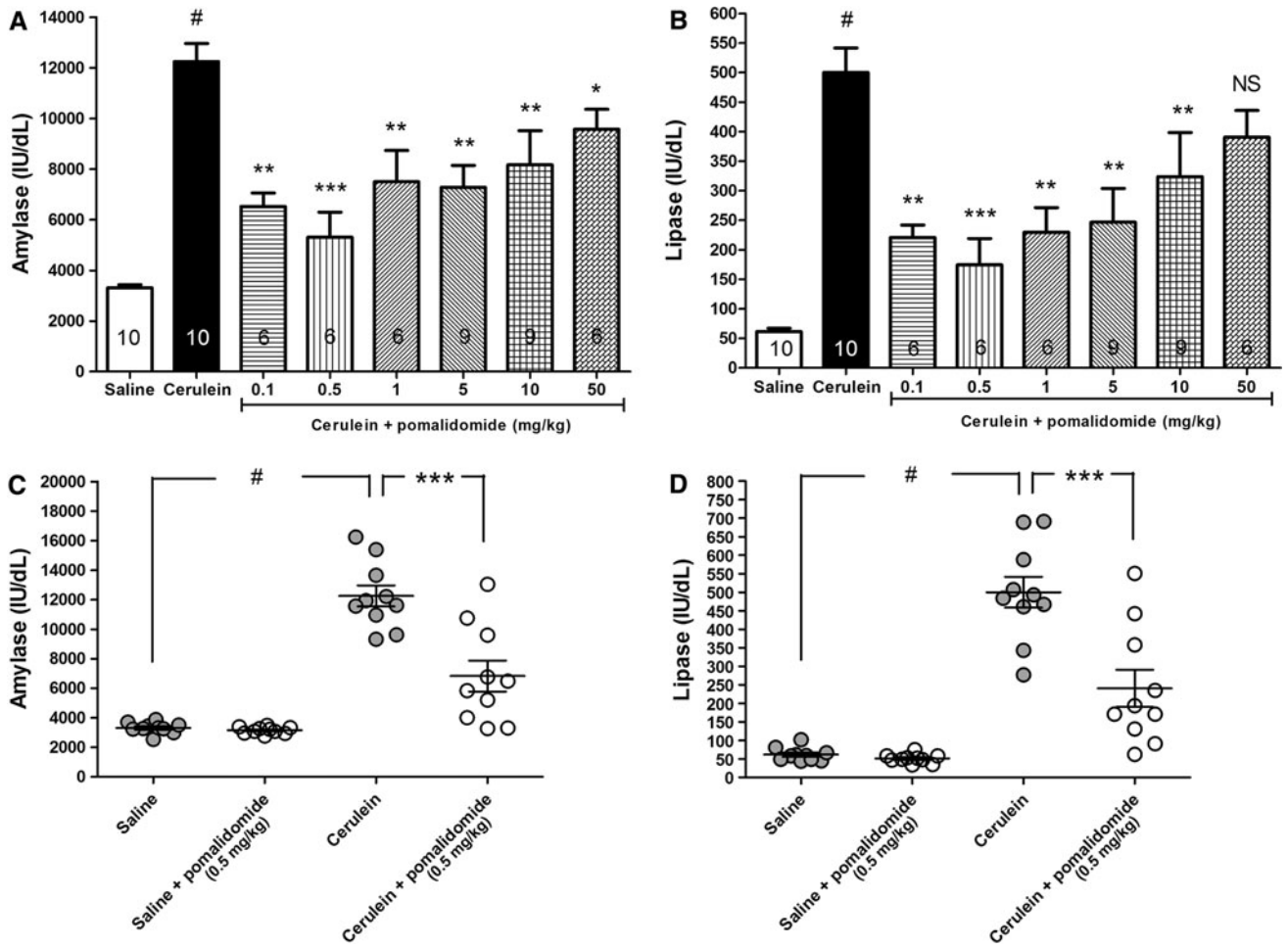

Fig. 1 Dose–response effects of pomalidomide and summary of the effect of pomalidomide (0.5 mg/kg) on the plasma levels of amylase and lipase. Pretreatment with various doses (0.1–50 mg/kg) of pomalidomide significantly reduced the plasma levels of amylase (**a**) and lipase (**b**) in male C57BL/6 mice that received intraperitoneal injections of cerulein (100 μg/kg × 8 doses); however, the lipase level was not reduced by pomalidomide treatment at a dose of 50 mg/kg. The most effective dose (0.5 mg/kg) of pomalidomide for suppressing elevated levels of plasma amylase (**c**) and lipase (**d**) is shown. Data are expressed as means ± SEM. The number of animals used is shown at the *bottom of each column* in **a** and **b**; $n = 10$ in **c** and **d**. $^{\#}P < 0.0001$ versus saline group; $^{*}P < 0.05$ versus cerulein group; $^{**}P < 0.01$ versus cerulein group; $^{***}P < 0.0001$ versus cerulein group; *NS* not significant

returned to the control level after pretreatment with 0.5 mg/kg pomalidomide (Fig. 3). In contrast, other inflammation-associated cytokines, such as TNF-α, IL-1α, IL-1β, IL-6, and IL-12, did not show significant alterations (Fig. 3). To clarify the role of TNF-α, the time courses of its protein expression in the serum and pancreas tissue were measured at 30 min, and 2, 4, 8, and 24 h after the first dose of cerulein. The results did not show significant alteration in the expression levels in either the serum or the pancreas tissue among control, cerulein-treated, or pomalidomide-pretreated animals at any time point examined (results not shown).

We further measured the mRNA expression of TNF-α and IL-1β, using real-time RT-PCR, in pancreas tissue at 30 min and 2, 4, 8, and 24 h after the first dose of cerulein. Low levels of TNF-α and IL-1β mRNA expression were observed in the saline control and the saline + pomalidomide group at the five time points examined. In contrast, TNF-α mRNA expression started to increase at 30 min and reached the peak level at 2 h after the induction of pancreatitis (Fig. 4a). Pretreatment with pomalidomide significantly decreased the expression of TNF-α mRNA. The maximal expression of IL-1β mRNA also occurred at 2 h after the induction of pancreatitis, and pretreatment with pomalidomide significantly reduced IL-1β mRNA expression compared to that in the cerulein + vehicle group (Fig. 4b).

Effects of pomalidomide on iNOS expression

The correlation between acute pancreatitis and oxidative-nitrosative stress has been documented [20–23]. Inducible

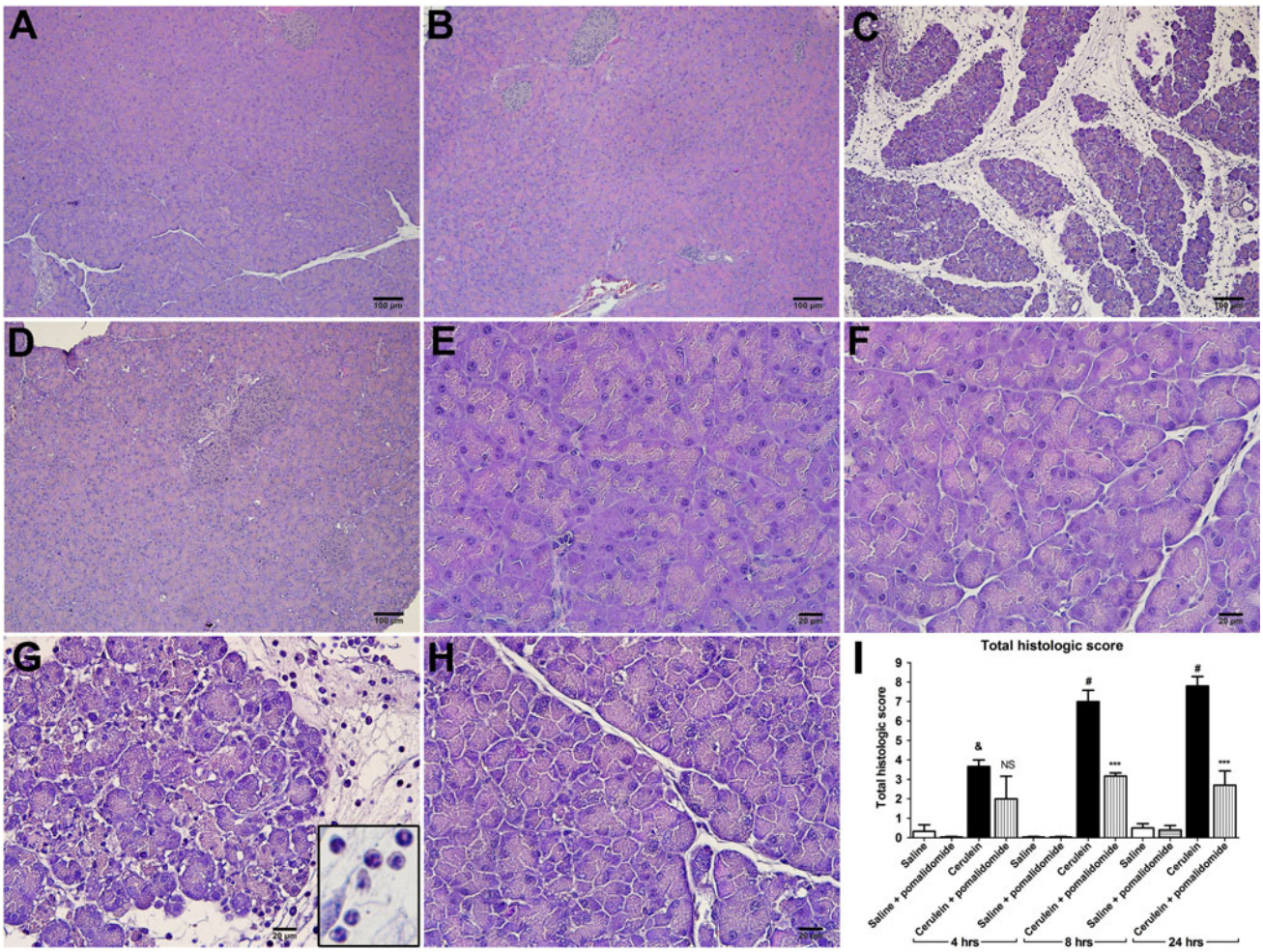

Fig. 2 Effects of pretreatment with pomalidomide on the morphological changes of cerulein-induced pancreatitis. Representative micrographs (×100 for **a**–**d** and ×400 for **e**–**h**) of hematoxylin and eosin-stained pancreas sections 24 h after the induction of pancreatitis are shown. **a**, **e** Sections from saline-vehicle and **b**, **f** sections from saline-pomalidomide-treated mice demonstrated normal architecture. The pancreas sections from mice with cerulein-vehicle treatment (**c**, **g**) demonstrated morphological derangement, with the appearance of acinar cell necrosis, separation of acinar cells due to marked interstitial edema, and inflammatory cell infiltration. *Inset* in **g** shows enlarged infiltrating cells consisting mainly of polymorphonuclear neutrophils and a macrophage. Pretreatment with one dose of pomalidomide (0.5 mg/kg) reversed the cerulein-induced structural derangement to near normal morphology (**d**, **h**). Pretreatment with 0.5 mg/kg pomalidomide significantly decreased the total histological scores (sum of interstitial edema, inflammatory cell infiltration, and acinar cell necrosis) at 8 and 24 h after the first cerulein injection (**i**). Data are expressed as means ± SEM ($n = 3$–10 per group). $^{\&}P < 0.05$ versus saline-vehicle group; $^{\#}P < 0.0001$ versus saline-vehicle group; $^{***}P < 0.0001$ versus cerulein-vehicle group; *NS* not significant

nitric oxide synthase (iNOS) is a marker of oxidative-nitrosative stress and is involved in the stimulation of inflammation. The expression of iNOS was markedly enhanced in the cerulein-treated mice at 24 h after the induction of pancreatitis (Fig. 5e, f) and iNOS was expressed mainly in the cytosol of the infiltrated inflammatory cells (Fig. 5c, d). Pretreatment with pomalidomide significantly reduced the expression of iNOS (Fig. 5b, e, f) to near the level of the saline-pomalidomide group (Fig. 5a, e, f).

Effect of post-treatment with pomalidomide on cerulein-induced pancreatitis

We have demonstrated that pretreatment with 0.5 mg/kg pomalidomide effectively reduced cerulein-induced acute pancreatitis. We further investigated the post-treatment effect of pomalidomide (0.5 mg/kg), which was given 1 h after the last dose of cerulein (the 8th dose). Sixteen hours (i.e., 24 h following the induction of pancreatitis) after post-treatment with pomalidomide, the plasma levels of

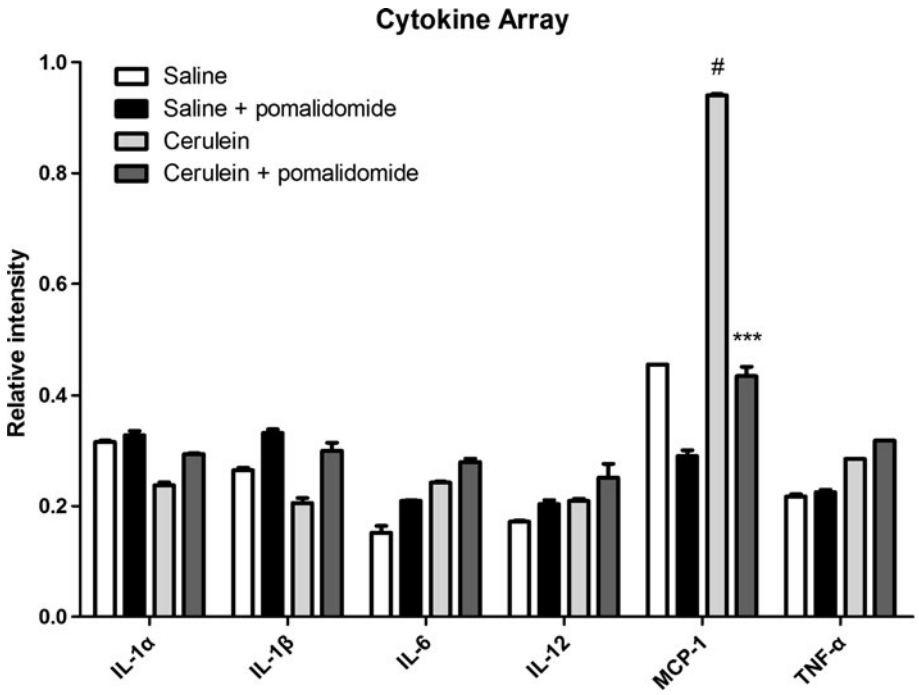

Fig. 3 The effect of pomalidomide on inflammatory cytokine expression in the pancreas. A cytokine array panel was carried out 24 h after the induction of pancreatitis. The expression of monocyte chemotactic protein-1 (*MCP-1*) was significantly higher in cerulein-treated mice, and its elevated expression was reduced after pretreatment with pomalidomide (0.5 mg/kg). The data are expressed as the relative intensity of the cytokine signal from treatment relative to the standard positive control signal on the panel. $n = 2$, $^{\#}P < 0.0001$ versus saline-vehicle group; $^{***}P < 0.0001$ versus cerulein-vehicle group. *TNF-α* tumor necrosis factor-α, *IL-1β* interleukin-1β

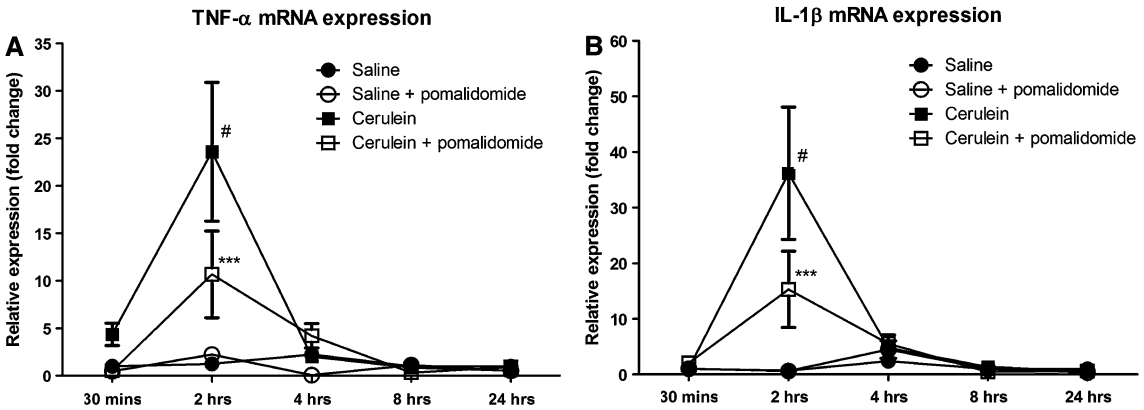

Fig. 4 The effect of pomalidomide (0.5 mg/kg) on the TNF-α and IL-1β mRNA levels in the pancreas. The levels of mRNA for TNF-α (**a**) and IL-1β (**b**) in the pancreas tissue were determined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and expressed as the fold-change relative to the values of saline-vehicle-treated mice at 30 min after the induction of pancreatitis. Data are expressed as means ± SEM. ($n = 3$–6 per group). $^{\#}P < 0.0001$ versus saline-vehicle and saline-pomalidomide groups; $^{***}P < 0.0001$ versus cerulein-vehicle group

amylase and lipase were significantly decreased when compared with those in the cerulein-vehicle group without pomalidomide treatment (Fig. 6a, b). However, at 22 h (i.e., 30 h following the induction of pancreatitis) after treatment with pomalidomide, the amylase level showed a significant decrease, while the lipase level only demonstrated a trend toward decrease (Fig. 6c, d). We also found that the elevation of amylase and lipase levels at 30 h was not as high as those at 24 h after the initiation of pancreatitis, indicating that spontaneous resolution of acute pancreatitis occurred in this model.

Morphological examination of pancreas tissue sections showed that the gross tissue damage induced by cerulein was effectively reduced 16 or 22 h post-pomalidomide treatment (Fig. 7c, g). The total histological scores were elevated by cerulein and significantly decreased by pomalidomide treatment at 24 h, while the scores were not significantly different between cerulein administration and pomalidomide treatment at 30 h (Fig. 7d, h). The total histological scores were higher at 24 h than those at 30 h.

Discussion

Pomalidomide has mostly been used to treat hematological malignancies and advanced solid tumors with metastasis,

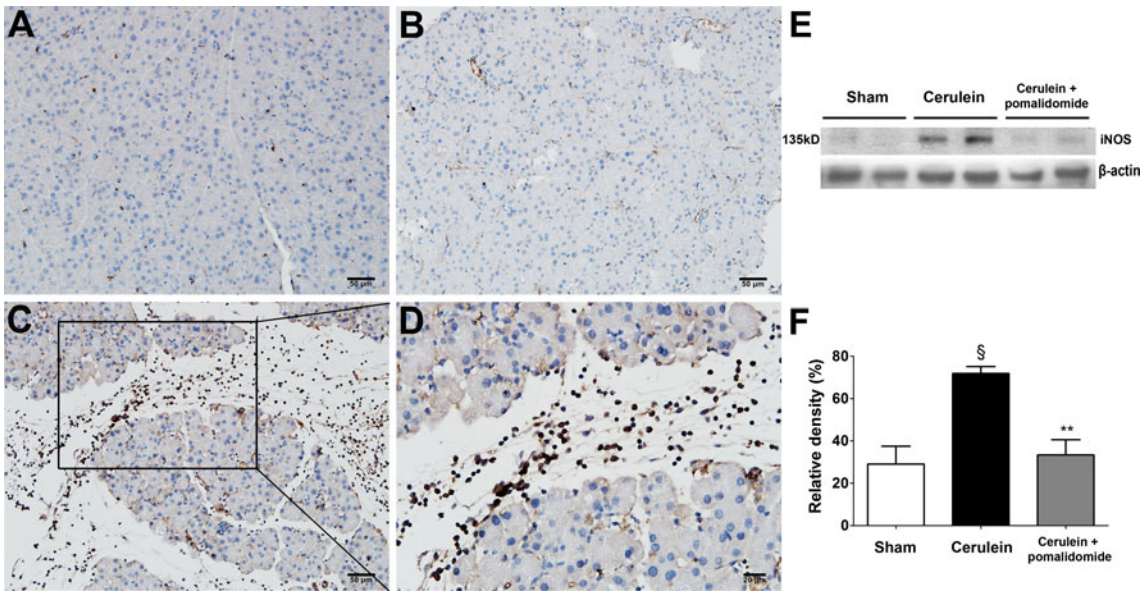

Fig. 5 The effect of pomalidomide on inducible nitric oxide synthase (*iNOS*) expression in the pancreas at 24 h after the induction of pancreatitis. No positive staining for iNOS was observed in the pancreas sections from saline-pomalidomide-treated mice (**a**, ×100). On the contrary, intense positive staining for iNOS was found in the pancreas sections from cerulein-treated mice (**c**, ×100). At higher magnification *of the inset in* **c** (×400), iNOS was found to be expressed mainly in the cytosol of infiltrated inflammatory cells (**d**). The immunostaining for cerulein-induced iNOS was significantly reduced when animals had been pretreated with pomalidomide (0.5 mg/kg) (**b**, ×100). Western blotting analysis from pancreas lysate showed significant expression of iNOS in cerulein-treated mice, and reduced expression when mice had been pretreated with pomalidomide (0.5 mg/kg) (**e**). Quantification of western blotting results shown in **f**. Data are presented as relative density (%) which the iNOS signal was normalized to the actin signal. Data are expressed as means ± SEM. ($n = 4$ per group). $^{\S}P < 0.01$ versus saline-pomalidomide group; $^{**}P < 0.01$ versus cerulein-vehicle group. *Sham* saline-pomalidomide group

through its immunomodulatory effects [24]. There has been no study using pomalidomide to treat inflammation-associated diseases. In the present study, we demonstrated for the first time that pomalidomide attenuated the development of a murine model of cerulein-induced acute pancreatitis via reducing the expression of TNF-α and IL-1β mRNAs and MCP-1 and iNOS proteins in the pancreas tissue.

Morphological evidence of pancreatitis, e.g., interstitial edema and inflammatory cell infiltration, started to appear after the 4th dose (or about 4 h after the induction of pancreatitis) of cerulein. Acute pancreatitis was successfully and consistently induced in C57BL/6 mice following the administration of 8 doses of cerulein. Because pretreatment with pomalidomide effectively decreased the severity of pancreatitis, we therefore examined the expression of TNF-α, which plays a crucial role in the pathogenesis of acute pancreatitis, in the cerulein-treated animals. Although TNF-α and IL-1β proteins in pancreas tissue were not elevated following cerulein administration, an early short-lived increase of TNF-α and IL-1β mRNAs and a delayed increase of MCP-1 and iNOS proteins were found. The infiltration of inflammatory cells, neutrophils and macrophages, was also observed at 4 h after cerulein administration. Therefore, our observations substantiate the pivotal role of TNF-α in the activation of inflammatory genes, cell death, and the recruitment of immune cells in acute pancreatitis [5, 6]. Furthermore, the significant reduction of TNF-α and IL-1β mRNAs, as well as the attenuation of morphological changes by pomalidomide pretreatment indicated that the amelioration of acute pancreatitis exerted by this drug is due to its strong anti-inflammatory effect.

The expression of MCP-1 has been found to be a poor prognostic marker for severe acute pancreatitis in humans [25, 26]. Studies using rat and mouse models of acute pancreatitis have demonstrated that blocking MCP-1 activity attenuates the severity of acute pancreatitis [27, 28]. In the present study, we found that treatment with pomalidomide reduced MCP-1 expression in mice with cerulein-induced pancreatitis. The ability of pomalidomide to inhibit MCP-1 expression has not been reported before. This ability may result from the direct inhibition of MCP-1 expression or from indirect inhibition through blocking the activation of nuclear factor (NF)-κB, the downstream signal mediator of TNF-α receptor activation. Nitric oxide (NO) and other free radicals are involved in oxidative stress, which plays important roles in the pathogenesis of acute pancreatitis and the systemic inflammatory response [23]. Treatment with anti-oxidant agents showed beneficial effects in humans and in animal models of acute pancreatitis [22, 29, 30]. Inducible NOS (iNOS) is a marker of

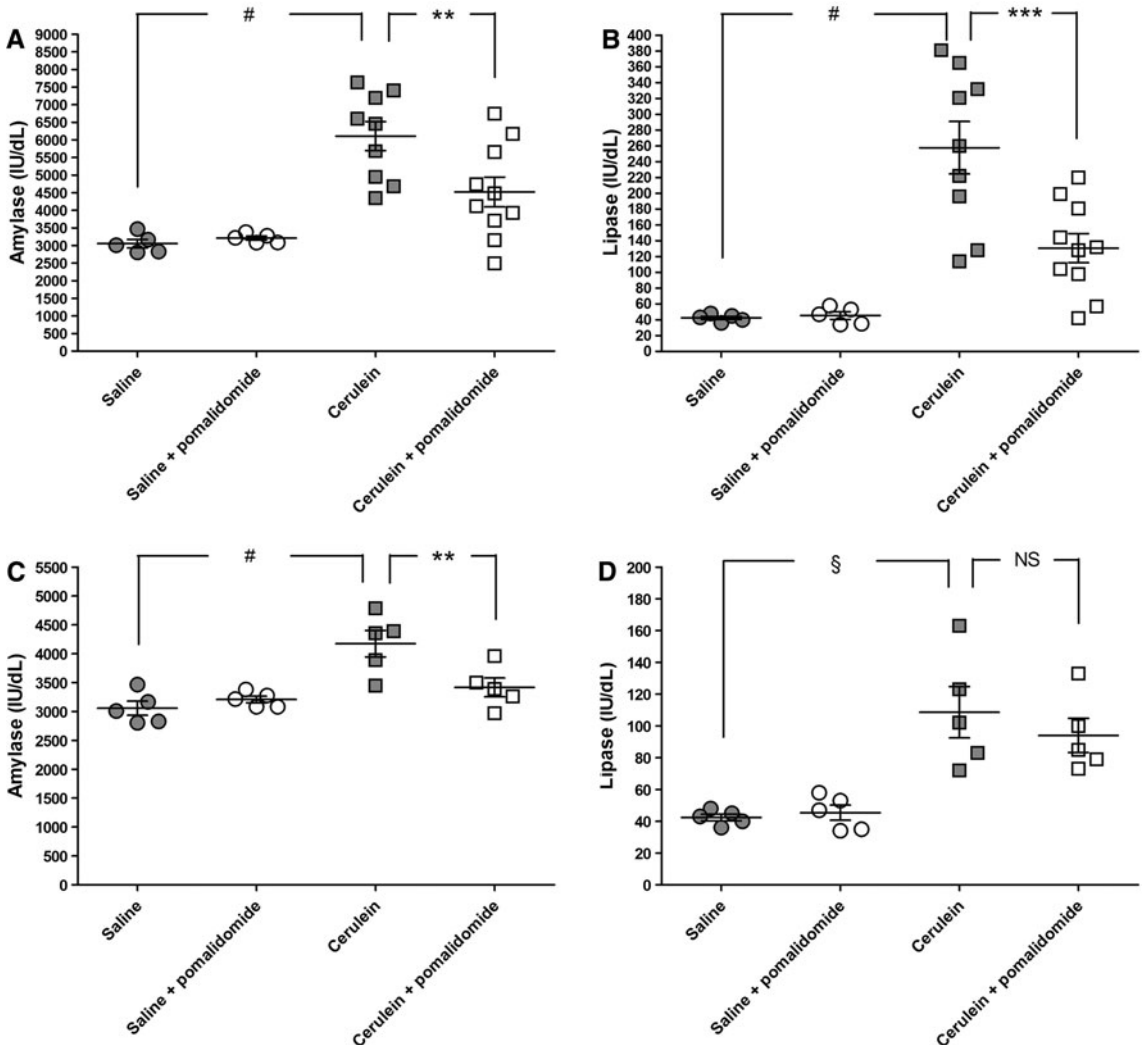

Fig. 6 The effects of post-treatment with pomalidomide on the plasma levels of amylase and lipase after the induction of pancreatitis. The plasma levels of amylase and lipase in cerulein-treated mice were significantly higher than those in the saline-vehicle group both at 24 h (**a**, **b**) and 30 h (**c**, **d**) after the induction of pancreatitis. But the elevation of amylase and lipase induced by cerulein at 30 h was not as high as that induced at 24 h after the initiation of pancreatitis. The administration of pomalidomide (0.5 mg/kg) at 1 h after the last injection of cerulein (the 8th dose) in mice significantly blunted the elevation of the amylase (**a**) and lipase (**b**) levels at 24 h and the amylase (**c**), but not the lipase level (**d**) at 30 h after the induction of pancreatitis. Data are expressed as means ± SEM. $^{\#}P < 0.0001$ versus saline-vehicle group; $^{\S}P < 0.01$ versus saline-vehicle group; $^{***}P < 0.0001$ versus cerulein group; $^{**}P < 0.01$ versus cerulein group; *NS* not significant; $n = 5$–10

oxidative-nitrosative stress and a downstream product of NF-κB activation. In the present study, pomalidomide attenuated the expression of iNOS, which was expressed mainly in the cytosol of infiltrated inflammatory cells in pancreas tissue in mice with pancreatitis.

An interesting finding was that the dose–response effect of pomalidomide was U-shaped. A small gradual decrease of the inhibitory effect, more obvious on plasma amylase activity, was observed when doses of pomalidomide greater than 0.5 mg/kg were used. The reason for this finding may be that, in addition to the inhibitory effects exhibited on the production of TNF-α, IL-1β, IL-6, and IL-12 in the stimulated macrophages, pomalidomide also exhibits a co-stimulatory effect on stimulated $CD4^+$ and $CD8^+$ T cells and dendritic cells [16, 31–33]. Treatment with pomalidomide in stimulated $CD4^+$ or $CD8^+$ T cells also increased the production of IL-2, TNF-α, and interferon (IFN)-γ [31]. The co-stimulatory effect of pomalidomide on the stimulated T cells was IL-2-mediated and dose-dependent. As the dose of pomalidomide increased, the IL-2 production from T cells increased [32]. Greater production of IL-2 would lead to more T-cell activation and greater induction of the release of inflammatory cytokines such as TNF-α and IFN-γ. Consequently, the reduced anti-inflammatory effects of higher doses of pomalidomide may result from its co-stimulatory effects on T cells.

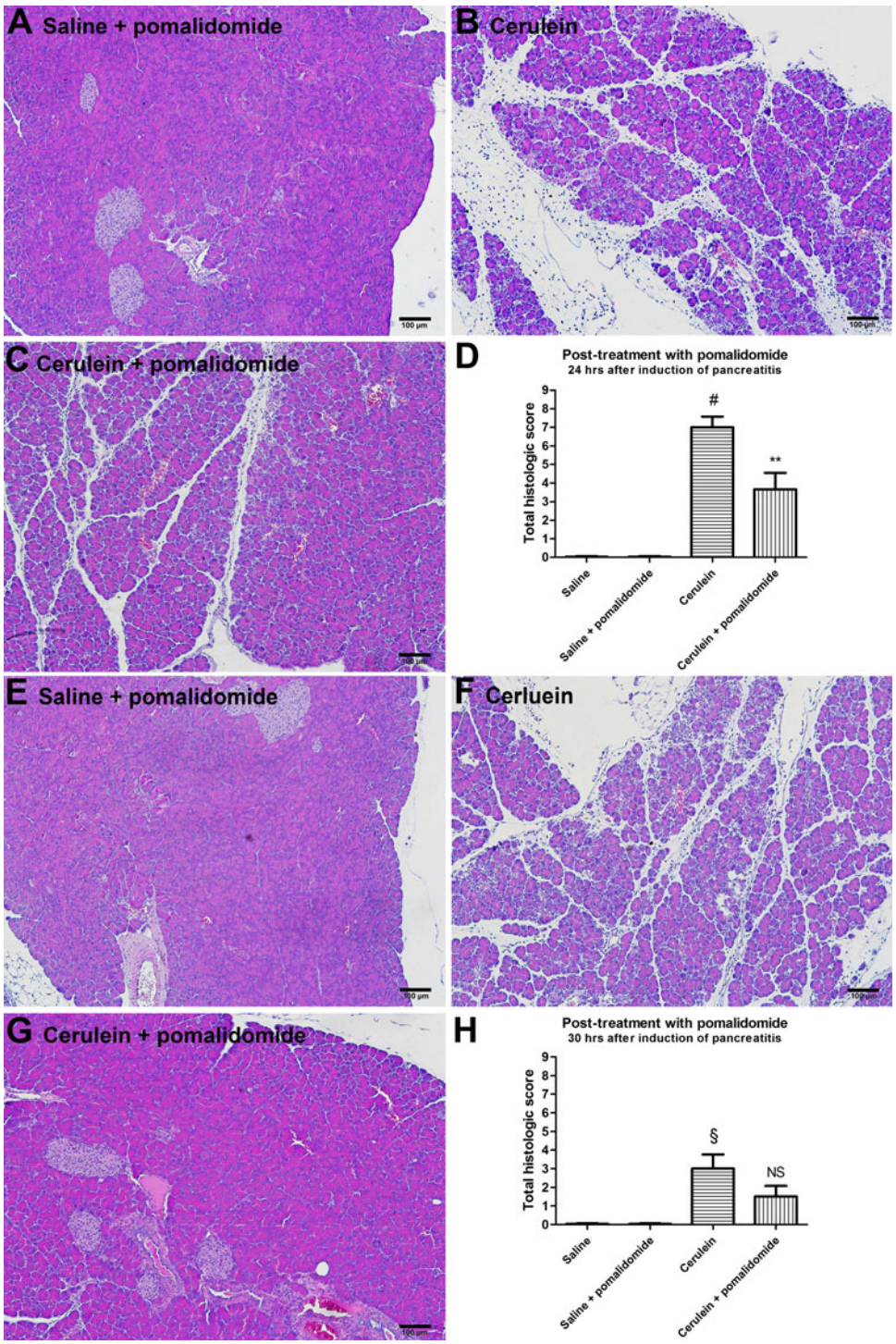

Fig. 7 Effects of post-treatment with pomalidomide on the morphological changes of cerulein-induced pancreatitis. Representative micrographs (×100) of hematoxylin and eosin-stained pancreas sections at 24 h (**a**, **b**, **c**) and 30 h (**e**, **f**, **g**) after the induction of pancreatitis are shown. **a**, **e** Sections from saline-pomalidomide-treated mice exhibited normal architecture. **b**, **f** Sections from cerulein-vehicle-treated mice demonstrated pancreatic injury characterized by interstitial edema, acinar cell necrosis, and inflammatory cell infiltration. The severity of pancreatitis, evaluated by the total histological scores (sum of interstitial edema, inflammatory cell infiltration, and acinar cell necrosis) is shown in mice sacrificed at 24 h (**d**) and at 30 h (**h**) after the induction of pancreatitis. Data are expressed as means ± SEM. $^{\#}P < 0.0001$ versus saline-vehicle group; $^{\S}P < 0.01$ versus saline-vehicle group; $^{**}P < 0.01$ versus cerulein group; *NS* not significant; $n = 3$

Pharmacokinetic data in humans showed that pomalidomide was well absorbed orally, with peak plasma concentrations occurring 2.5–4 h after dosing, and a median half-life of 7 h [34]. The well-tolerated dose of pomalidomide in humans according to the results of current clinical trials is 2 mg/day, equal to 0.04 mg/kg in a person with a body weight of 50 kg [34]. Pharmacokinetic data of pomalidomide in mice are still lacking, but we can expect that the elimination rate of pomalidomide in the mouse would be at least several-fold higher than that in humans, due to the higher metabolic rate in mice. According to published in vivo studies, the dose of pomalidomide used alone to treat mouse models of metastatic colon-rectal cancer and childhood acute lymphoblastic leukemia was 50 mg/kg [35, 36]. This is obviously higher than the best therapeutic dose (0.5 mg/kg) for treating acute pancreatitis

in the present study. Moreover, in this study, the dose of 0.1 mg/kg was still observed to have a therapeutic effect. If pomalidomide can be used to treat acute pancreatitis in humans in the future, only low doses may be needed to exert therapeutic effects, and there will be fewer side effects.

In the present study, we have shown that pretreatment with pomalidomide ameliorated cerulein-induced pancreatitis. We have also demonstrated that the protective effect of pomalidomide appears to be mediated by suppressing the production of inflammatory mediators including TNF-α, IL-1β, iNOS, and MCP-1. Although a direct inhibitory effect of pomalidomide on the cerulein-induced activation of intra-acinar proenzymes cannot be ruled out, the reduction of the severity of pancreatitis by pomalidomide in the post-treatment experiment, at the time point when pancreatitis had been induced successfully, does not support a major direct effect of pomalidomide on intra-acinar proenzyme activation.

The time point of post-treatment with pomalidomide in the present study was 8 h after the induction of pancreatitis. However, in this study, we observed that TNF-α mRNA expression was upregulated early, at 2 h, and returned to the basal level at 4 h after the induction of pancreatitis, which means that the therapeutic effects of post-treatment with pomalidomide may have involved other anti-inflammatory effects in addition to the ability of pomalidomide to inhibit TNF-α. Apart from the immunomodulatory effects shown by pomalidomide on the inhibition of TNF-α, IL-1β, IL-6, and IL-12 and the enhancement of IL-10 production, pomalidomide may have other unknown anti-inflammatory effects. More investigations will be needed to disclose the complete mechanisms of pomalidomide's actions.

In conclusion, the present study has found that pomalidomide is a potent therapeutic agent for the treatment of cerulein-induced acute pancreatitis in C57BL/6 mice. The beneficial effects appear to result from the inhibition of TNF-α, IL-1β, MCP-1, and iNOS expression. Because the protocol used to induce acute pancreatitis in mice is relatively mild, other animal models with more severe pancreatitis should be investigated to assess the potential utility of pomalidomide for the treatment of severe pancreatitis in humans.

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