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IFN-γ Protects Cerulein-Induced Acute Pancreatitis by Repressing NF-κB Activation

Takahito Hayashi,* Yuko Ishida,* Akihiko Kimura,* Yoichiro Iwakura, † Naofumi Mukaida,‡ and Toshikazu Kondo*2*

We explored the pathophysiological roles of IFN-γ in cerulein-induced acute pancreatitis. In wild-type (WT) mice, cerulein injection caused acute pancreatitis as evidenced by increased serum amylase levels and pathological changes such as interstitial edema, vacuolization, acinar cell necrosis, and neutrophil infiltration in pancreas. Concomitantly, cerulein treatment augmented intrapancreatic gene expression of TNF-α, KC/CXCL1, MIP-2/CXCL2, cyclooxygenase-2 (COX-2), and IFN-γ in WT mice. In situ hybridization combined with immunofluorescence analyses demonstrated that infiltrating neutrophils expressed IFN-γ mRNA. Unexpectedly, IFN-γ−/− mice exhibited exacerbated cerulein-induced pancreatic injury, with enhanced neutrophil recruitment. Moreover, intrapancreatic gene expression of TNF-α, KC/CXCL1, MIP-2/CXCL2, and COX-2, in pancreas of cerulein-treated WT mice as evidenced by the increases in nuclear amount and DNA-binding activity of NF-κB p65. In comparison with WT mice, IFN-γ−/− mice exhibited exaggerated and prolonged NF-κB activation, probably due to reduced acetylation of Stat1, a main signal transducer of IFN-γ, because acetylated Stat1 can inhibit NF-κB activation. Indeed, IFN-γ-activated Stat1 and reciprocally reduced NF-κB activation and COX-2 expression in neutrophils. Finally, even when administered 4 h after the first cerulein injection, IFN-γ remarkably attenuated acute pancreatitis in both WT and IFN-γ−/− mice, with reduced NF-κB activation and COX-2 expression. Thus, IFN-γ can have anti-inflammatory effects on acute pancreatitis by depressing the proinflammatory consequences of NF-κB activation. The Journal of Immunology, 2007, 178: 7385–7394.

Acute pancreatitis is a common inflammatory disease of the abdomen, with an annual incidence rate of ~10–20 cases per 100,000 people in Western countries (1, 2). Acute pancreatitis is characterized by interstitial edema, vacuolization, and parenchymal necrosis with a concomitant massive infiltration of leukocytes, mainly composed of neutrophils (3, 4). Acute pancreatitis is commonly caused by excessive ethanol consumption, biliary tract disease, certain medications, and invasive procedures of the biliary and pancreatic ducts (3, 4). The treatment of acute pancreatitis consists mainly of supportive medical therapeutic modalities such as fluid and nutrient replenishment, and inhibition of pancreatic proteases. However, it still has a high mortality ranging from 15–25% even at present (3, 5). Therefore, the development of a novel therapy is required, based on the molecular clarification of the pathogenesis.

Pancreatic damage is initiated by autodigestion of the pancreas by acinar cell-derived proteases, followed by a massive infiltration of leukocytes such as neutrophils and macrophages (6). Infiltration of macrophages and neutrophils is an early event of acute pancreatitis and is presumed to be involved in the removal of necrotic cells, which are generated by autodigestion mediated by acinar cell-derived proteases (6). Simultaneously, the infiltrating leukocytes produce various proinflammatory cytokines, such as IL-1 and TNF-α, and release proteinases and reactive oxygen species (7, 8). Thus, the net effect of leukocyte infiltration is to aggravate acute pancreatitis. This conclusion is supported by the observations that depletion of neutrophils attenuated tissue injuries observed in acute murine pancreatitis models (9).

IFN-γ is mainly produced by NK cells and CD4-positive Th1 cells and has multiple effects on macrophages, NK cells, and T lymphocytes (10, 11). Thus, IFN-γ is presumed to have a crucial role in various immune responses, particularly those mediated by Th1 cells. Both insulin promoter-specific overexpression of IFN-γ by the pancreas and genetic deletion of suppressor of cytokine signaling-1, an antagonistic signal transducer of IFN-γ, leads to spontaneous development of severe chronic pancreatitis with a predominant infiltration of lymphocytes (12, 13). These observations suggest that IFN-γ can induce pancreas tissue injury mainly by activating T lymphocytes in these mice.

Several lines of evidence indicate the essential involvement of IFN-γ in models of acute inflammatory diseases by influencing neutrophil infiltration (14–16). Demols et al. (17) demonstrated that the administration of CD4-positive T cells into nude mice partially exaggerated the severity of cerulein-induced acute pancreatitis, presumably due to IFN-γ production.
by adaptively transferred cells, but they did not clarify the effects of the adoptive transfer on neutrophil infiltration. Moreover, accumulating evidence indicates the essential contribution of cyclooxygenase-2 (COX-2) to cerulein-induced acute pancreatitis, because inhibition of the COX-2 pathway also ameliorates pancreatitis (18). Furthermore, we observed that IFN-γ, a potential inducer of COX-2 gene expression, was detected in neutrophils infiltrating the pancreas in the same disease model. To address the role of IFN-γ, we compared pathological consequences of cerulein-induced pancreatitis in wild-type (WT) and IFN-γ-deficient (IFN-γ−/−) mice. In contrast with our expectation, we discovered protective roles of IFN-γ in cerulein-induced acute pancreatitis by repressing the activation of NF-κB, an indispensable transcription factor for COX-2 gene expression, in neutrophils. Finally, exogenous IFN-γ attenuated cerulein-induced acute pancreatitis by repressing NF-κB activation and subsequent COX-2 gene expression, even when administered after the start of cerulein injection.

Materials and Methods
Reagents and Abs
Cerulein and a selective COX-2 inhibitor, NS-398, were purchased from Sigma-Aldrich. Recombinant murine IFN-γ and LPS were obtained from PeproTech and Difco, respectively. A hybridoma clone, RB6-8C5, which secretes a rat anti-mouse Ly6G mAb (19, 20), was a gift from Dr. R. Coffman (DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA). The anti-Ly6G Ab was purified as described previously (15). An examiner without prior knowledge of the experimental procedures, enumerated the numbers of infiltrating neutrophils, macrophages, or NK cells on 10 randomly chosen visual fields at 400 magnification, and the average of the 10 selected microscopic fields was calculated.

Table I. Sequences of the primers used for RT-PCR

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<th>Cycle</th>
<th>Product Size (bp)</th>
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a(F), Forward primer; (R), reverse primer.

Measurements of pancreatic water content
Pancreas was obtained at 12 h after the first cerulein injection to weigh a wet tissue weight. The tissues were then freeze-dried overnight and reweighed to determine a dry tissue weight. Pancreatic water content was calculated as the difference between the wet and dry tissue weights and expressed as a ratio of the wet tissue weight.

Histopathological and immunohistochemical analyses
Pancreatic tissues were removed at the indicated time intervals after the first cerulein injection and fixed in 4% formaldehyde buffered with PBS (pH 7.2), embedded in paraffin, and cut to obtain 4-μm-thick sections. Thereafter, after the sections were stained with H&E, histopathological changes such as vacuolization and necrosis of pancreatic acinar cells were evaluated by an examiner without prior knowledge of the experimental procedures, based on the following criteria: 0, none; 1, <25%; 2, 25–50%; 3, 50–75% and 4, 75–100%. A part of the pancreatic tissue was stained with anti-MPO pAb, anti-F4/80 Ab, or anti-pan NK cells Ab, as described previously (15). An examiner without prior knowledge of the experimental procedures, enumerated the numbers of infiltrating neutrophils, macrophages, or NK cells on 10 randomly chosen visual fields at ×400 magnification, and the average of the 10 selected microscopic fields was calculated.

Determination of apoptotic cells
Deparaffinized sections were treated with proteinase K solution for 10 min at 37°C, after endogenous peroxidase was inactivated with 0.3% H2O2 in
A double-color immunofluorescence analysis

Deparaffinized sections were incubated with PBS containing 1% normal donkey serum and 1% BSA to reduce nonspecific reactions as previously described (16). Thereafter, the sections were further incubated in the combination of Abs. All Abs were used at a concentration of 1 μg/ml. After the incubation with fluorochrome-conjugated secondary Abs (15 μg/ml) at room temperature for 30 min, the sections were observed under fluorescence microscopy.

ELISA for IFN-γ

Pancreas samples were homogenized with 0.3 ml of PBS containing Complete Protease Inhibitor Mixture (Roche) at the indicated time intervals. The homogenates were centrifuged at 12,000 × g for 15 min. IFN-γ levels and total protein contents in the supernatant were measured using a specific ELISA kit (BioSource International) and a BCA protein assay kit (Pierce), respectively. The data were expressed as picograms of IFN-γ per milligram of total protein for each sample.

Extraction of total RNAs and RT-PCR

Semi-quantitative RT-PCR analyses with Taq polymerase (Nippon Gene) were conducted on 5 μg of total RNAs extracted from pancreatic tissues or isolated neutrophils (1 × 10⁷) using the specific sets of primers with the optimal cycles consisting of 94°C for 1 min, optimal annealing temperature for 1 min, and 72°C for 1 min, followed by the incubation at 72°C for 3 min (Table I), as described previously (15, 16). The PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. The band intensities were measured using Image Analysis software (Scion Image) and the ratios of each band to β-actin were calculated.

Isolation of peritoneal neutrophils

Peritoneal neutrophils were isolated as described previously (15). Briefly, WT mice were i.p. injected with 2 ml of 3% proteose peptone (Difco) in MEM. Eight hours later, the peritoneal cavity was washed with MEM and the abdominal lavage fluid was collected. Thereafter, polymorphonuclear neutrophils were isolated from the pellets of the abdominal lavage by gradient density centrifugation with Percoll (50/75%), and cultured in MEM containing 5% FBS. The purity of the isolated neutrophils was ~95%, as assessed by microscopic examination with Giemsa staining (data not shown). Then, LPS (10 μg/ml) or LPS plus murine IFN-γ (10³ U/ml) was added to culture medium. At 12 h later, the cells were collected for subsequent analyses. In some experiments, isolated neutrophils were incubated with the pancreatic extracts, as described previously (23). Briefly, at 12 h after the first cerulein injection, pancreas was removed from WT mice, and homogenized on ice in PBS, followed by the centrifugation at 4,000 × g for 5 min. After further centrifugation at 12,000 × g for 15 min, cell-free supernatant was incubated with isolated neutrophils. Twelve hours later, total RNA was extracted for RT-PCR analyses.

Immunoprecipitation and Western blot analysis

Pancreatic tissues or isolated neutrophils (1 × 10⁷) were homogenized with a lysis buffer (20 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Triton, 1 mM EDTA) containing Complete Protease Inhibitor Mixture (Roche), and centrifuged to obtain lysates. Transcription factors were analyzed, as described previously (24). Briefly, the extracts immunoprecipitated with anti-Stat1 Ab, were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. After being immersed with the blocking buffer (10 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl, 5% skim milk, and 0.5% Tween 20), the membranes were incubated with mouse anti-acetyl lysine (AcLys)- mAb or anti-Stat1 mAb at 4°C overnight. The Ag-Ab complexes were detected by using the ECL-Western blotting detection system (Amersham Bioscience Japan), according to the manufacturer’s instructions. Thereafter, the membranes were reprobed with anti-Stat1 Ab. For Western blotting for NF-κB p65, cell lysates from neutrophils were further separated into cytosol and nuclear fractions using the NE-PER method (Pierce) as described previously (25). Each fraction was subjected to Western blotting using anti-NF-κB p65.

Measurement of NF-κB p65 DNA-binding activity

Nuclear proteins were extracted from the obtained cell pellets using the NE-PER method (Pierce) as described previously (25). Subsequently, the
were obtained from WT and IFN-γ−/− mice at the indicated time intervals after the first cerulein injection (n = 8). All values represent the mean ± SEM. **, p < 0.01, IFN-γ−/− vs WT. b–i, Histopathological stained sections of the pancreas from WT and IFN-γ−/− (H&E staining, original magnification, ×200). Representative results from eight animals at each time point are shown here. Histopathological alterations such as pancreatic acinar cell vacuolization (j) and necrosis (k) were scored at the indicated time intervals in WT and IFN-γ−/− mice as described in Materials and Methods (n = 8). All values represent the mean ± SEM. **, p < 0.01, IFN-γ−/− vs WT. l, Analysis of pancreatic water content in WT and IFN-γ−/− mice at 12 h after the first cerulein injection (n = 8). All values represent the mean ± SEM. **, p < 0.01, IFN-γ−/− vs WT. m and n, Immunohistochemical analyses of neutrophil recruitment into the pancreas after cerulein challenge. The samples were obtained from WT and IFN-γ−/− mice at the indicated time intervals, and immunostained with anti-MPO pAbs. Samples obtained at 12 h from WT and IFN-γ−/− mice are shown in m and n, respectively. Representative results from eight animals are shown here (original magnification, ×200). The numbers of neutrophils per a high-power microscopic field were counted and are shown in o. All values represent the mean ± SEM (n = 8). **, p < 0.01, IFN-γ−/− vs WT.

**FIGURE 2.** a, Determination of serum amylase levels in WT and IFN-γ−/− mice at the indicated time intervals after the first cerulein injection (n = 8). All values represent the mean ± SEM. **, p < 0.01, IFN-γ−/− vs WT. b–i, Histopathological stained sections of the pancreas from WT and IFN-γ−/− (H&E staining, original magnification, ×200). Representative results from eight animals are shown here. Histopathological alterations such as pancreatic acinar cell vacuolization (j) and necrosis (k) were scored at the indicated time intervals in WT and IFN-γ−/− mice as described in Materials and Methods (n = 8). All values represent the mean ± SEM. **, p < 0.01, IFN-γ−/− vs WT. l, Analysis of pancreatic water content in WT and IFN-γ−/− mice at 12 h after the first cerulein injection (n = 8). All values represent the mean ± SEM. **, p < 0.01, IFN-γ−/− vs WT. m and n, Immunohistochemical analyses of neutrophil recruitment into the pancreas after cerulein challenge. The samples were obtained from WT and IFN-γ−/− mice at the indicated time intervals, and immunostained with anti-MPO pAbs. Samples obtained at 12 h from WT and IFN-γ−/− mice are shown in m and n, respectively. Representative results from eight animals are shown here (original magnification, ×200). The numbers of neutrophils per a high-power microscopic field were counted and are shown in o. All values represent the mean ± SEM (n = 8). **, p < 0.01, IFN-γ−/− vs WT.

multiwell chemiluminescent assay was performed to detect NF-κB p65 protein using the EZ-Detect Transcription Factor kit (Pierce), according to the manufacturer’s instructions. The p65 contents were expressed as relative light units divided by total protein contents for each sample.

Chromatin immunoprecipitation (ChIP) assay

Isolated neutrophils (1 × 107) were applied to ChIP assay as described previously (26). After neutrophils were incubated with LPS (10 μg/ml) or LPS plus murine IFN-γ (10 U/ml) for 12 h, the cells were cross-linked with 1% formaldehyde for 15 min followed by resuspension in SDS-lysis buffer. After the sonication, immunoprecipitation was conducted using anti-NF-κB p65 Abs. Then, PCR analysis was performed using specific primers for the promoter region of the mouse COX-2 gene (forward: 5′-CTAATT CCACCAGTACAGATG-3′; reverse: 5′-ACTAGGCGAGACTCAGCGAG-3′). The PCR products were analyzed on a 1% agarose gel. This primer set covers the COX-2 promoter segment from −540 to −265, which contains NF-κB-binding sites.

In situ hybridization (ISH)

Digoxigenin-labeled IFN-γ sense and antisense probes were obtained by using the DIG RNA Labeling kit (Boehringer Mannheim Biochemicals) according to the manufacturer’s instructions. The sense probe was used as a negative control. Deparaffinized sections were fixed with 4% paraformaldehyde in PBS for 15 min and incubated with 10 μg/ml protease K in TE buffer (10 mM Tris-HCl and 1 mM EDTA) at 37°C for 10 min. After washing with 5× SSC at room temperature for 15 min, the sections were prehybridized at 55°C for 1 h with a buffer containing 50% deionized formamide, 5× SSC, and 40 μg/ml salmon sperm DNA. Then, the RNA probes were added to the prehybridization buffer to 400 ng/ml and the slides were incubated under a cover at 55°C for 16 h in a moist chamber. The section was incubated with anti-digoxigenin Abs and positive signals were visualized with a color-substrate solution containing NBT salt and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt for 16 h (27). After ISH, the slides were further processed to immunofluorescence analysis using anti-MPO pAb (28).
Statistical analysis

The means and SEMs were calculated for all parameters determined in this study. Statistical significance was evaluated by using ANOVA or Mann-Whitney's U test. A value of $p < 0.05$ was accepted as significant.

Results

Intrapancreatic IFN-$\gamma$ expression in WT mice after cerulein challenge

Cerulein injection markedly enhanced IFN-$\gamma$ mRNA expression in pancreas, starting at 4 h and reaching a peak by 7 h after the injection (Fig. 1, a and b). Concomitantly, intrapancreatic IFN-$\gamma$ protein levels were progressively increased from 4 h on after the first cerulein injection (Fig. 1c). In situ hybridization analysis detected IFN-$\gamma$ mRNA in tissues containing infiltrated inflammatory cells from 4 h on after the first cerulein injection (Fig. 1d). Controls using sense probes did not give rise to any signals, indicating the specificity of the reaction (Fig. 1e). Immunohistochemical analysis detected IFN-$\gamma$ protein in inflammatory cells recruited into the pancreas following cerulein challenge (data not shown). Moreover, ISH combined with immunofluorescence staining revealed that most Ly6G-positive granulocytes expressed IFN-$\gamma$ mRNA later than 7 h after the first cerulein injection (Fig. 1f). At 12 h after the first cerulein injection, IFN-$\gamma$ was detected in a small proportion of F4/80-positive macrophages, and NK cells, in addition to Ly6G-positive granulocytes (data not shown). However, the infiltrating neutrophils outnumbered the infiltrating macrophages.
and NK cells, 3- and 17-fold, respectively (data not shown). Moreover, when mice were rendered granulocytopenic with anti-Ly6G Ab, intrapancreatic IFN-γ gene expression was significantly attenuated (data not shown). Furthermore, coincubation with cerulein-treated pancreatic extracts significantly augmented IFN-γ gene expression in isolated neutrophils (Fig. 1, h and i). Collectively, these observations suggest that granulocytes were the major source of IFN-γ in this model.

**Cerulein exacerbated pancreatitis and enhanced COX-2 gene expression in IFN-γ−/− mice**

The essential contribution of COX-2 to pancreatitis has been indicated by the observation that COX-2-deficient mice exhibited attenuated cerulein-induced pancreatitis (18, 29). Moreover, IFN-γ can augment COX-2 gene expression in several types of cells (30–32). Thus, we postulated that enhanced IFN-γ expression may promote pancreatitis by up-regulating COX-2 gene expression. To test this hypothesis, we compared cerulein-induced pancreatitis in IFN-γ−/− mice treated with WT mice. There were no significant differences in serum amylase levels between untreated WT and IFN-γ−/− mice (Fig. 2a). Both WT and IFN-γ−/− mice exhibited progressive increases in serum amylase levels from 4 h on, decreasing by 24 h after the first cerulein injection. The elevations in serum amylase were significantly greater in IFN-γ−/− than WT mice (Fig. 2a).

There were no apparent morphological differences in the pancreas of untreated WT and IFN-γ−/− mice (Fig. 2, b and c). The interstitial tissues became moderately edematous at the earlier phase after cerulein challenge and vacuolization and necrosis of pancreatic acinar cells became apparent along with leukocyte infiltration after 7 h in WT mice (Fig. 2, d, f, and h). In IFN-γ−/− mice, vacuolization and necrosis were more severe than in WT mice (Fig. 2, e, g, i, and k). Moreover, pancreatic water content due to edema was significantly higher in IFN-γ−/− than in WT mice (Fig. 2l). The inflammatory cells infiltrated the pancreas (Fig. 2, b–i) and neutrophil infiltration was more apparent in IFN-γ−/− mice than in WT mice (Fig. 2, m–o). However, macrophages were recruited to...
a similar extent in both WT and IFN-γ−/− mice (data not shown) and only a few NK cells infiltrated into the pancreas of WT and IFN-γ−/− mice (data not shown). Moreover, cerulein treatment augmented pancreatic COX-2 gene expression in WT and IFN-γ−/− mice beginning at 7 h after injection and the enhancement was significantly greater in IFN-γ−/− than WT mice (Fig. 3, a and b). COX-2 proteins were detected in Ly6G-positive neutrophils by two-color stained immunofluorescence analysis (Fig. 3c). Furthermore, a COX-2 inhibitor, NS-398, markedly reduced cerulein-induced increases in serum amylase levels, when administered to IFN-γ−/− mice (Fig. 3d). Collectively, these observations suggest that COX-2 expression is enhanced in the absence of IFN-γ and results in exacerbating cerulein-induced pancreatitis.

**Enhanced gene expression of TNF-α, KC/CXCL1, and MIP-2/CXCL2 in IFN-γ−/− mice**

Neutrophil recruitment was regulated by coordinate actions of CXC chemokines (21) and TNF-α (33). Hence, we examined intrapancreatic gene expression of TNF-α, KC/CXCL1, and MIP-2/CXCL2 in WT and IFN-γ−/− mice. Under the experimental conditions used, the gene expression of these molecules was faintly detected in unchallenged WT and IFN-γ−/− mice (Fig. 4a). Cerulein challenge enhanced gene expression of TNF-α, KC/CXCL1, and MIP-2/CXCL2 and their increments were exaggerated in IFN-γ−/− mice, compared with WT mice (Fig. 4). Thus, enhanced intrapancreatic neutrophil recruitment in IFN-γ−/− mice may be ascribed to enhanced expression of TNF-α and these chemokines.

**Attenuated apoptosis of pancreatic acinar cells in IFN-γ−/− mice**

Only a few apoptotic acinar cells were observed in untreated WT and IFN-γ−/− mice. Cerulein increased the number of apoptotic acinar cells in WT mice, with concomitantly enhanced gene expression of Fas and Fasl (Fig. 4c). Increases in acinar cell apoptosis, and Fas and Fasl, expression, were attenuated in IFN-γ−/− mice compared with WT mice (Fig. 5a). Because acinar cell apoptosis can be

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**FIGURE 7.** a–d, Western blotting analyses of NF-κB p65 in cytosol and nuclear extracts from peritoneal neutrophils after LPS and/or IFN-γ treatment. Representative results of cytosol extracts and those of nuclear extracts from six independent experiments are shown in a and c, respectively. The ratios of NF-κB p65 to β-actin in cytosol and nuclear extracts are shown in b and d, respectively. e, DNA-binding activity of the nuclear NF-κB p65 in peritoneal neutrophils after LPS and/or IFN-γ treatment. All values represent the mean ± SEM (n = 6 experiments in each group). ***, p < 0.01, vs vehicle.**

f, Acetylated Stat1 was detected as described in Materials and Methods. Whole extracts of peritoneal neutrophils were immunoprecipitated with anti-Stat1 Ab and then were subjected to Western blotting analysis with an Ab recognizing AcLys. The same membrane was reprobed with anti-Stat1 Ab. Representative results from six independent experiments are shown here. g, The ratios of acetylated Stat1 to total Stat1 were calculated and are shown here. Each value represents mean ± SEM (n = 6). ***, p < 0.01, vs vehicle. h and i, RT-PCR analysis of COX-2 gene expression in peritoneal neutrophils after LPS and/or IFN-γ treatment. Representative results from six independent experiments are shown in h, i. The ratios of COX-2 to β-actin were calculated and shown here. Each value represents mean ± SEM (n = 6). ***, p < 0.01, LPS treatment vs LPS + IFN-γ treatment. j and k, ChIP assay was performed with anti-NF-κB p65 Ab, followed by PCR analysis of the associated DNA using specific primers of the COX-2 promoter. Representative results from six independent experiments are shown here. The ratios of NF-κB p65 to β-actin in input were calculated and are shown in k. All values represent the mean ± SEM (n = 6). ***, p < 0.01, vs vehicle.
Aberrant activation of NF-κB and reciprocal attenuation of Stat1 acetylation in IFN-γ−/− mice

Accumulating evidence indicates that the activation of a transcription factor, NF-κB, is indispensable for the gene transcription of COX-2 (35) as well as TNF-α, KC/CXCL1, and MIP-2/CXCL2 (36–38). To address the possibility that augmented NF-κB activation in IFN-γ−/− mice can enhance the expression of these genes, we determined the activation state of the NF-κB p65 proteins in the pancreas. There was no significant difference in the activity of NF-κB p65 in the pancreas between unchallenged WT and IFN-γ−/− mice. In WT mice, the DNA-binding activity of NF-κB p65 started to increase at 4 h, reached a peak at 7 h, and returned to baseline level by 24 h after the first cerulein injection (Fig. 6a). The activity of NF-κB p65 was significantly higher in IFN-γ−/− mice at 7 and 12 h than WT mice. Several lines of evidence suggest that IFN-γ can acetylate its major signal transducer, Stat1, and thereby repress NF-κB activation in vitro (24). Hence, we examined the acetylation states of Stat1. There was no significant difference in the total amount of Stat1 in the pancreas of WT mice and IFN-γ−/− mice during the entire course of acute pancreatitis (Fig. 6b). To the contrary, Stat1 acetylation was augmented in WT but not IFN-γ−/− mice (Fig. 6b and c). Thus, these observations suggest that the absence of IFN-γ reduced Stat1 acetylation and reciprocally prolonged NF-κB activation even in vivo.

Suppression of NFκB activation by IFNγ in neutrophils

In the course of pancreatitis, COX-2 was mainly expressed by infiltrating neutrophils (Fig. 3c) and we detected Stat1 in infiltrating neutrophils (Fig. 6d). Thus, it is probable that IFN-γ can act on infiltrating neutrophils and eventually modulate COX-2 expression. To address this possibility, we examined the effects of IFN-γ on LPS-induced NF-κB activation in murine peritoneal neutrophils. LPS treatment markedly increased the intranuclear level of NF-κB p65 and enhanced its DNA-binding activity in neutrophils (Fig. 7, a–c). IFN-γ treatment reversed the nuclear level and DNA-binding activity of NF-κB p65 to basal levels (Fig. 7, a and e), but reciprocally induced Stat1 acetylation (Fig. 7, f and g), resulting in the suppression of LPS-induced COX-2 gene expression by IFN-γ (Fig. 7, h and i). ChIP assay consistently demonstrated that LPS increased the amount of NF-κB p65 bound to the COX-2 promoter region and that IFN-γ reversed the effects of LPS (Fig. 7, j and k). Moreover, during the whole course of this experiment, IFN-γ barely influence the viability of isolated neutrophils (data not shown). Thus, it is probable that IFN-γ can diminish NF-κB bound to the COX-2 promoter region by Stat1 acetylation, thereby suppressing COX-2 gene expression in neutrophils.

Alleviation of cerulein-induced pancreatic injury by IFNγ administration

We finally examined the effects of the administration of exogenous IFN-γ on cerulein-induced pancreatic injury in WT mice. Treatment with rIFN-γ (10^4 U) markedly reduced the elevation in serum amylase levels, even when administered 4 h after the initiation of injury.
cerulein injection (Fig. 8a). Concomitantly, IFN-γ injection markedly attenuated cerulein-induced elevation in NF-κB activation and subsequent COX-2 expression in pancreas (Fig. 8, b–d). Moreover, the administration of IFN-γ (104 U) alleviated cerulein-induced acute pancreatitis also in IFN-γ−/− mice (Fig. 8e). Collectively, these observations would indicate that IFN-γ could attenuate cerulein-induced expression of COX-2, an essential mediator of pancreatitis, by repressing NF-κB activation, and thereby alleviate pancreatic injury.

**Discussion**

Several lines of evidence suggest the detrimental roles of proinflammatory cytokines in the pathogenesis of experimental pancreatitis (7, 8). Due to its pleiotropic biological effects on macrophages and NK cells (10, 11), IFN-γ is presumed to be involved in various kinds of inflammatory diseases (14–16) and therefore frequently classified as a proinflammatory cytokine. Indeed, we observed that cerulein treatment augmented the intrapancreatic expression of both IFN-γ mRNA and protein, along with pancreatic tissue damage and massive neutrophil infiltration. IFN-γ is presumed to be produced mainly by NK cells, Th1 cells, and macrophages (10, 11). In contrast, IFN-γ production by neutrophils has been documented in various inflammatory conditions (15, 39, 40). In line with these observations, infiltrating neutrophils appeared to be the major source of IFN-γ during cerulein-induced acute pancreatitis as evidenced by ISH and ISH combined with immunofluorescence staining. This notion was further supported by our present experiments using isolated neutrophils or neutrophenic WT mice.

COX-2 and its products are crucially involved in various types of inflammatory responses (41, 42). Evidence is accumulating based on the anti-inflammatory effects of inhibitors that PGE2, a product of COX-2, is indispensable for the development of acute pancreatitis (18, 29). Supporting this notion, cerulein-induced acute pancreatitis was markedly attenuated in COX-2-deficient mice (18, 29). Moreover, IFN-γ can enhance COX-2 gene expression in various types of cells including macrophages (30–32). Furthermore, Demols et al. (17) claimed that activated CD4+ T cell-derived IFN-γ could be one of the injurious factors in acute pancreatitis. These observations favor the prediction that neutrophil-derived IFN-γ can aggravate cerulein-induced pancreatitis, by up-regulating COX-2 gene expression. Contrary to our expectation, IFN-γ−/− mice exhibited exaggerated cerulein-induced acute pancreatitis as evidenced by augmented elevation in serum amylase levels and greater histopathological changes, compared with WT mice. Moreover, COX-2 gene expression was augmented in IFN-γ−/− mice, compared with WT mice. These observations would indicate that neutrophil-derived IFN-γ has a protective rather than pathogenic role in cerulein-induced acute pancreatitis.

The essential involvement of neutrophil infiltration in acute pancreatitis has been based on the observations that depletion of neutrophils (our unpublished data) or inhibition of neutrophil-derived elastase attenuated acute pancreatitis in rodents (9, 43). Infiltrating neutrophils can contribute to the pathogenesis of acute pancreatitis by producing various proinflammatory cytokines (44, 45) and/or releasing proteases. Indeed, COX-2, a pathogenic mediator of cerulein-induced pancreatitis, was detected predominantly in neutrophils. In particular, mouse neutrophils exhibit potent chemotactic responses to ELR(Glu-Leu-Arg)-motif-positive CXC chemokines such as KC/CXCL1 and MIP-2/CXCL2 and, by using a specific receptor, CXCR2 (21, 36, 37). IFN-γ−/− mice have been reported to exhibit attenuated neutrophil infiltration with reduced CXC chemokine expression in several disease models (14–16). In our study, neutrophil infiltration and the expression of CXC chemokines were enhanced in cerulein-treated IFN-γ−/− mice, as compared with WT mice. These discrepancies can be explained by the observations that IFN-γ exhibited in vitro either inhibitory or stimulatory effects on the expression of these ELR-motif-positive CXC chemokines (46–49), in a context-dependent manner.

Cerulein itself can markedly activate NF-κB (50), and NF-κB p50-deficient mice were resistant to cerulein-induced acute pancreatitis (22). Moreover, NF-κB activation is indispensable for COX-2 gene expression (33). Thus, we postulated that the enhanced expression of COX-2 in IFN-γ−/− mice might result from augmented NF-κB activation. Supporting this assumption, IFN-γ−/− mice exhibited prolonged NF-κB activation in neutrophils, which are a major cell type expressing COX-2. The activation of NF-κB, a master transcription factor for various proinflammatory genes, is tightly regulated to prevent its aberrant activation, which can lead to pathological changes. Thus, this raises a question as to how NF-κB activation was prolonged in the pancreas of IFN-γ−/− mice treated with cerulein. Stat1, a major signal transducer of IFN-γ, can regulate the transcription of target genes after phosphorylation of its tyrosine residues by JAKs (51), while in vitro, acetylation but not tyrosine phosphorylation of Stat1 is indispensable for its interaction with NF-κB p65, thereby impairing the nuclear translocation of NF-κB p65 (24, 52, 53). Here, we demonstrated that IFN-γ deficiency reduced the amount of acetylated Stat1 with a reciprocal enhancement of NF-κB activity in cerulein-treated pancreas. Conversely, IFN-γ treatment increased the amount of acetylated Stat1 and reciprocally diminished NF-κB bound to the COX-2 promoter in LPS-stimulated neutrophils. Thus, during acute pancreatitis, IFN-γ-mediated acetylation of Stat1 can repress NF-κB activation and subsequent COX-2 gene expression in neutrophils in an autocrine and/or paracrine manner.

In several disease models (14, 16), the lack of IFN-γ diminished Fas-mediated apoptosis. Consistently, the cerulein-induced increase in apoptotic acinar cell number was attenuated in IFN-γ−/− mice with a diminished gene expression of Fas and FasL, compared with WT mice. Fas-deficient mice exhibited exaggerated cerulein-induced acute pancreatitis together with attenuated apoptosis of acinar cells (34). These observations would indicate that Fas-mediated acinar cell apoptosis is protective for cerulein-induced pancreatitis. Thus, reduced acinar cell apoptosis may synergically contribute to exaggeration of cerulein-induced pancreatitis in IFN-γ−/− mice.

At present, the treatment of acute pancreatitis is largely a supportive one consisting of rehydration, nutritional support, and/or the use of antibiotics (3, 4). Several protease inhibitors are administered but with little effects on improvement in mortality and/or morbidity (54, 55). Thus, the development of a novel type of treatment is urgently required. Here, we provided definitive evidence of the protective roles of endogenously-produced IFN-γ for cerulein-induced acute pancreatitis. Moreover, IFN-γ markedly alleviated cerulein-induced acute pancreatitis, even when administered 4 h after the first cerulein injection. Although IFN-γ is generally well-tolerated by patients with various malignancies and viral hepatitis, it exhibits dose-dependent toxicity (56–58). Thus, our present observations suggest that IFN-γ therapy may be feasible for a grave disorder, acute pancreatitis, after determining its optimal dose and schedule to avoid adverse effects.

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