Phosphatidylinositol 3-Kinase/Akt Signaling Mediates Interleukin-32α Induction in Human Pancreatic Periacinar Myofibroblasts

Short title: IL-32α and PI3K/Akt pathway


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Abbreviations:

PI3k, phosphatidylinositol 3-kinase; NF-κB, nuclear factor-κB; AP-1, activated protein-1; IL, interleukin; TNF, tumor necrosis factor; RT-PCR, reverse transcription-polymerase chain reaction; AGPC, acid guanidium thiocyanate-phenol-chloroform, IFN, interferon.
Abstract

(Background and aim) Interleukin (IL)-32 is a recently described proinflammatory cytokine, characterized by the induction of nuclear factor (NF)-κB activation. We studied IL-32α expression in human pancreatic periacinar myofibroblasts, which play important roles in the regulation of extracellular matrix metabolism and inflammatory responses in the pancreas. (Methods) IL-32α protein expression was evaluated by Western blot analyses, and IL-32 mRNA expression was analyzed by Northern blot and real-time PCR analyses. (Results) IL-32α mRNA was weakly expressed without a stimulus, and its expression was markedly enhanced by IL-1β, IFN-γ and TNF-α. IL-1β, IFN-γ and TNF-α enhanced intracellular accumulation of IL-32α protein, but IL-32α was not detected in supernatants. Each cytokine dose- and time-dependently induced IL-32α mRNA expression. An inhibitor of phosphatidylinositol 3-kinase (LY294002) significantly suppressed IL-1β-, IFN-γ- and TNF-α-induced IL-32α mRNA expression, although MAPK inhibitors had no effect. Akt activation in response to these cytokines was confirmed by Western blot. Furthermore, LY294002 suppressed both IL-1β- and TNF-α-induced NF-κB activation and IL-1β-, TNF-α- and IFN-γ-induced AP-1 activation. Blockade of NF-κB and AP-1 activation by an adenovirus expressing a stable mutant form of IκBα and a dominant negative mutant of c-Jun markedly suppressed IL-1β-, IFN-γ- and/or TNF-α-induced IL-32α mRNA expression. (Conclusions) Human pancreatic periacinar myofibroblasts expressed IL-32α in response to IL-1β, TNF-α and IFN-γ. IL-32α mRNA expression is dependent on interactions between the PI3K/Akt-pathway and the NF-κB/AP-1 system.

(Key words) pancreatitis, inflammation, cytokine
Introduction

Interleukin (IL)-32 is a recently described cytokine produced by T lymphocytes, natural killer (NK) cells, monocytes, and epithelial cells (20, 23). Although IL-32 was first reported as a transcript in IL-2 activated NK and T cells, it appears that tissue cells are a dominant and widespread source for IL-32 (11). The gene encoding IL-32 is located on human chromosome 16p13.3 and is organized into eight exons (7). There are four splice variants (IL-32α, IL-32β, IL-32δ and IL-32γ), and IL-32α is the most abundant transcript. Of particular importance, IL-32 is prominently induced by interferon (IFN)-γ in lung epithelial cells and monocytes (20).

IL-32 exhibits several properties typical of proinflammatory cytokines (20, 23). For example, it stimulates the secretion of IL-1β, TNF-α, IL-6 and IL-8 by means of the activation of nuclear factor (NF)-κB and p38 mitogen-activated protein kinases (MAPKs) (20, 23). Recently, Netea et al. demonstrated that IL-32 augments the production of IL-1β and IL-6 induced by muramyl dipeptide (MDP), a peptidoglycan fraction of bacteria, by means of the nucleotide-binding oligomerization domain proteins (NOD1 and NOD2) through a caspase-1-dependent mechanism (23). NODs are a family of intraerytoplasmic bacterial sensors, and the recognition of bacterial peptidoglycans subsequently induces NF-κB activation (27).

IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis (5, 11, 18, 32), mycobacterium tuberculosis infections (21, 24) and inflammatory bowel disease (31). However, IL-32 expression in cells of pancreas origin remains unclear. Furthermore, precise molecular mechanisms controlling IL-32 expression also remain unclear. In this study, we investigated IL-32α expression in human pancreatic periacinar myofibroblasts, which are located in the periacinar regions of normal human pancreas (29). These cells are characterized by the expression of extracellular matrixes (ECMs) and α-smooth muscle actin (α-SMA). They play important roles in the regulating ECM metabolism and inflammatory responses in the pancreas (17, 30).

Materials and Methods

Reagents

Recombinant human IL-1β, IL-17 and IFN-γ were purchased from R&D Systems (Minneapolis, MN), and other cytokines were obtained from PeproTech (Rocky Hill, NJ). Anti-human IL-32α antibodies were purchased from R & D Systems. All other reagents were purchased from Sigma Chemical Co. (St Louis, MO).
**Human Pancreatic Myofibroblast Cultures**

Primary cultures of pancreatic periacinar myofibroblasts were isolated according to the methods previously described (29). Cells were cultured in DMEM containing 10% FBS. All culture media were supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin. Above 98% of the cells were positive for α-SMA. Studies were performed on passage 2-6 of myofibroblasts isolated from 6 resection specimens. The study design was approved by the ethics committee of Shiga University of Medical Science. Informed consent was obtained from all patients prior to sample collection.

**Real-Time Polymerase Chain Reaction**

Expression of human IL-32α mRNA in samples was assessed by real-time PCR analyses. Real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan) with the following primers specific for human IL-32α; 5'
AGCTGGAGGACGACTTCAAA (nucleotides 192-211, Gene bank accession No. BC018782) (34) and 3' AGGTGGTGTCAGTATCTTCA (nucleotides 642-623). PCR products were ligated into TA cloning vectors (Promega, Madison, WI) and sequenced. PCR was conducted using a SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA). Data were normalized versus β-actin for human IL-32.

**Northern Blot Analyses**

Total cellular RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (8). Northern blots were performed according to a previously described method (2). Hybridizations were performed with 32P-labeled human probes, generated by a random primed DNA labeling kit (Amersham, Arlington Heights, IL), and evaluated by autoradiography.

**Western Blot Analyses**

For analysis of IL-32α protein expression, cells were exposed to cytokines for predetermined periods of time. Cells were then washed with PBS and lysed in SDS sample buffer containing 100 µM orthovanadate. For Western blotting, 10 µg of protein from each sample was subjected to SDS-PAGE on a 4-20% gradient gel under reducing conditions (30). Biotinylated anti-human IL-32α antibodies were purchased from R&D Systems (Minneapolis, MN) and peroxidase-conjugated streptavidine was purchased from Dako Japan (Kyoto, Japan). Subsequently, detection was performed using the enhanced chemiluminescence Western blotting system (Amersham).

For Akt phosphorylation analyses, cells were exposed to cytokines for predetermined
periods of time. Antibodies directed against phosphorylated- and total Akt were purchased from Cell Signaling Technology (Beverly, MA), and peroxidase-conjugated second antibodies were purchased from Amersham (Arlington Heights, IL).

**Adenovirus-Mediated Gene Transfers**

We used a recombinant adenovirus expressing a stable mutant form of IκBα (Ad-IκBΔN) (25), a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) (38) and a recombinant adenovirus containing bacterial β-galactosidase cDNA (Ad-LacZ). The stable mutant form of IκBα (IκBΔN) lacks 54 NH2-terminal amino acids of wild type IκBα, and is neither phosphorylated nor proteolyzed in response to signal induction, but fully inhibits NF-κB activation. The dominant negative mutant c-Jun (TAM67) lacks the transactivational domain of amino acids 3 to 122 of wild type c-Jun, but retains the DNA-binding domain. In preliminary experiments, Ad-LacZ infections of colonic myofibroblasts with a multiplicity of infection (MOI) of 10 showed a maximal expression (85 % positive) of β-galactosidase. The recombinant adenovirus was transferred into the cells, and cells were made quiescent for 48 h before being assessed for the effects of the transferred gene.

**Nuclear Extracts and Electrophoretic Gel Mobility Shift Assays (EMSAs)**

Nuclear extracts were prepared from cells exposed to cytokines for 1.5 h by the method of Dignam and Roeder (10). Consensus oligonucleotides for NF-κB (5’ AGTTGA-GGGGACTTTCCCCAGCC) and AP-1 (5’ CGCTTGATGAGTGCCGGAA) were purchased from Promega (Madison, WI). The consensus binding sequence is underlined within each sequence. Oligonucleotides were 5’ end-labeled with T4 polynucleotide kinase (Promega) and [γ-32P]ATP (Amersham). Binding reactions were performed according to previously described methods (4). In supershift experiments, 1 µl of antibody to each transcription factor was added to the binding mixture in the absence of labeled probe. Antisera specifically recognizing each transcriptional factor were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The experiments with unlabeled oligonucleotides used a 100-fold molar excess relative to the radiolabeled oligonucleotide.

**Statistical Analyses**

Statistical significances of differences were determined by the Mann-Whitney U test (Statview Version 4.5). Differences resulting in P values less than 0.05 were considered to be statistically significant.
Results

**Induction of IL-32α in human pancreatic myofibroblasts**

To investigate regulatory mechanisms underlying IL-32α induction in human pancreatic periacinar myofibroblasts, cells were stimulated with various cytokines for 12 h and IL-32α mRNA expression was detected by Northern blot analyses (Fig. 1A). In these cells, IL-32α mRNA was weakly expressed without any stimulus, and IL-1β, IFN-γ and TNF-α markedly enhanced IL-32α mRNA expression. TNF-α and IFN-γ effects were stronger than those induced by IL-1β.

Similar results were observed at the protein level. Cells were stimulated for 24 h with IL-1β, IFN-γ and TNF-α, and IL-32α protein expression was analyzed by Western blots. IL-32α was detected as a molecular weight protein of 25 kDa, which is comparable with a previous report (23). Stimulation with IL-1β, IFN-γ and TNF-α enhanced intracellular accumulation of IL-32α protein (Fig. 1B). As observed at mRNA level, TNF-α and IFN-γ effects were stronger than those induced by IL-1β. In contrast, we could not detect secreted forms of IL-32α in supernatants.

Next, we tested the effects of combinations of IL-1β, IFN-γ and TNF-α (Fig. 1C). Northern blot analyses showed that combinations of IL-1β plus TNF-α, IL-1β plus IFN-γ and/or TNF-α plus IFN-γ synergistically enhanced IL-32α mRNA expression.

**Effects of IL-1β, TNF-α and IFN-γ**

Effects of IL-1β, TNF-α and IFN-γ on IL-32 mRNA expression were examined more precisely. Human pancreatic myofibroblasts were incubated for 12 h with increasing concentrations of IL-1β, TNF-α and IFN-γ, and the IL-32 mRNA expression was analyzed by Northern blotting. As shown in Fig. 2A-C, these cytokines dose-dependently up-regulated IL-32 mRNA expression. The IL-1β effect was detected at as low as 0.01 ng/ml, and reached a maximum at 10 ng/ml. The TNF-α effect was observed at as low as 0.1 ng/ml, and reached a maximum at 100 ng/ml. The IFN-γ effect was also detected as low as 0.1 ng/ml, and gradually increased to 500 ng/ml.

The kinetics of IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression were evaluated (Fig. 2D-F). Cells were stimulated with IL-1β (10 ng/ml), TNF-α (100ng/ml) or IFN-γ (100 ng/ml), and sequential changes in IL-32α mRNA expression were determined by Northern blotting. IL-1β induced an increase in the accumulation of IL-32α mRNA, and this reached a maximum 12 h after stimulation. Thereafter, the induced-IL-32α mRNA levels decreased. TNF-α and/or IFN-γ also induced a gradual but continuous increase in the accumulation of IL-32α mRNA for 24 h.
Effects of MAP-kinase inhibitors and PI3K inhibitors

The MAP-kinase and phosphatidylinositol 3-kinase (PI3K)/Akt pathways are implicated in cytokine signaling in various cell types. To investigate molecular mechanisms underlying IL-32\(\alpha\) induction in human pancreatic myofibroblasts, we evaluated the effects of following inhibitors: p42/44 MAP kinase inhibitors (PD98059 and U0216) (1, 13), a p38 MAPK inhibitor (SB203580) (9) and a phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) (12). Real-time PCR demonstrated that treatment with MEK inhibitors (PD98059 and U0216) or a p38 MAPK inhibitor (SB203580) had no effect on IL-1\(\beta\)-, TNF-\(\alpha\)- and/or IFN-\(\gamma\)-induced IL-32\(\alpha\) mRNA (Fig. 3A, B and C). Contrary to these findings, a PI3K inhibitor, LY294002 (35) significantly blocked the effect of IL-1\(\beta\)-, TNF-\(\alpha\)- and/or IFN-\(\gamma\) on IL-32\(\alpha\) mRNA expression (Fig. 3A, B and C). The effects of LY294002 were confirmed by Northern blotting (Fig. 3D), and Wortmannin (12), another PI3K inhibitor, also blocked IL-1\(\beta\)-, TNF-\(\alpha\)- and/or IFN-\(\gamma\)-induced IL-32\(\alpha\) mRNA expression (Fig. 3E). These results suggest that PI3K activation is involved in IL-1\(\beta\)-, TNF-\(\alpha\)- and IFN-\(\gamma\)-induced IL-32\(\alpha\) mRNA expression in human pancreatic myofibroblasts, although MEK and p38 MAPK pathways are dispensable.

Akt activation in response to cytokines

In human pancreatic myofibroblasts, the induction of Akt phosphorylation by IL-1\(\beta\), TNF-\(\alpha\) and IFN-\(\gamma\) was evaluated by Western blotting. As shown in Fig. 4, IL-1\(\beta\), TNF-\(\alpha\) and IFN-\(\gamma\) induced the Akt phosphorylation as early as 5 min after the stimulation. These data indicate that Akt, a protein kinase recruited by PI3K activation, is rapidly activated by IL-1\(\beta\), TNF-\(\alpha\) and IFN-\(\gamma\) in human pancreatic myofibroblasts.

Effects of inhibition of NF-kB and AP-1 signaling

Promoter sequences analyzed by the UCSC Genome Browser created by the Genome Bioinformatics Group of UC Santa Cruz (the University of California, Santa Cruz, CA) showed consensus binding sites for NF-\(\kappa\)B (at bp -638 to -649) and AP-1 (at bp -230 to -242) in promoter regions of human IL-32\(\alpha\) gene. To assess the role of transcription factors NF-\(\kappa\)B and AP-1, we evaluated the effects of a recombinant adenovirus containing a stable mutant form of I kB\(\alpha\) (Ad-I kB\(\alpha\)S N) and a dominant negative mutant of c-Jun (Ad-DN-c-Jun) on cytokine-induced IL-32\(\alpha\) mRNA expression. As shown in Fig.5, cells were infected with recombinant adenovirus, and were cultured for 48 h. Cells were stimulated for 12 h with IL-1\(\beta\) (10ng/ml), TNF-\(\alpha\) (100ng/ml) and IFN-\(\gamma\) (100ng/ml), and the expression of IL-32\(\alpha\) mRNAs was determined by Northern blots. Ad-I kB\(\alpha\)N inhibited the effects of both IL-1\(\beta\) and TNF-\(\alpha\) on IL-32\(\alpha\) mRNA expression, and Ad-DN-c-Jun also suppressed the effects of both
IL-1β, TNF-α and IFN-γ on IL-32α mRNA expression. Inhibitory effects were not induced by the Ad-LacZ gene, which was used as a negative control. These suggest that NF-κB and AP-1 play a role in IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression.

Effects of PI3K-inhibitor (LY294002) on NF-κB/AP-1 activation

The PI3K/Akt pathway is an upstream activators for NF-κB and AP-1 in various cell types (19, 22, 26, 28, 35). To investigate the possibility that in human pancreatic myofibroblasts the PI3K/Akt pathway contributes to NF-κB/AP-1 activation, we investigated the effects of LY294002 on NF-κB and AP-1 activation in these cells. As shown in Fig. 6A, EMSAs showed that PI3K inhibition by LY294002 suppressed both IL-1β- and TNF-α-induced NF-κB activation. The specificity of this reaction was confirmed by the addition of cold oligo-DNA, which abolished the reactive band (Fig. 6C). The addition of antibodies directed against a 50,000 MW-subunit (p50) and a 65,000 MW-subunit (p65) of NF-κB induced supershifts of the binding complexes, indicating that this binding complex was a heterodimer consisting of p50 and p65 subunits (Fig. 6C).

Similarly, LY294002 blocked IL-1β-, TNF-α- and IFN-γ-induced AP-1 activation (Fig. 6B). The specificity of this reaction was confirmed by the addition of cold oligo-DNA, which abolished the reactive band (Fig. 6D). The addition of antibodies directed against Fos/Jun induced supershifts, indicating that this binding complex was a heterodimer consisting of p50 and p65 subunits (Fig. 6D).

Totally, in human pancreatic myofibroblasts IL-1β-, TNF-α- and IFN-γ-induced PI3K/Akt and following NF-κB and AP-1 activation contributes to IL-32α mRNA induction.

Discussion

IL-32 is a recently reported cytokine, expressed by T lymphocytes, natural killer (NK) cells, monocytes, and epithelial cells (20, 23). However, IL-32 expression by mesenchymal cells has not been identified. Furthermore, IL-32 expression by cells of pancreatic origin remains unclear. In the present study, we demonstrate several findings: (a) human pancreatic myofibroblasts are a source of IL-32α; (b) IL-1β, TNF-α and IFN-γ are potent stimulators for IL-32α induction; and (c) PI3K/Akt pathway-dependent NF-κB/AP-1 activation plays a crucial role in IL-32α induction.

Previous studies defined that proinflammatory cytokines such as IL-1β, IL-12, IL-18 and IFN-γ are stimulators for IL-32 expression (20, 31). However, these observations are limited in genetically-engineered cells or in transformed cells, and molecular mechanisms underlying
IL-32α induction remains unclear. In the present study, we showed that in human pancreatic myofibroblasts IL-1β, TNF-α and IFN-γ are potent inducers of IL-32α mRNA expression. To address the molecular mechanism contributing to IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression, we evaluated the effects of MAPK inhibitors and PI3K inhibitor on IL-32α induction. Although we have previously shown that MAPKs play a crucial role in inducing proinflammatory cytokines such as IL-6 and IL-8 in human pancreatic and colonic myofibroblasts (3, 16, 30), p42/44 MAPK-inhibitors (PD98059 and U0216) and p38 MAPK-inhibitor (SB203580) did not affect IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression. In contrast, a PI3K-inhibitor (LY294002) effectively suppressed IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression. A similar suppression was also confirmed by another PI3K inhibitor, Wortmannin. Wortmannin and LY294002 have different structures and bind to different PI3K epitopes, and inhibitory effects observed with both Wortmannin and LY294002 provide a good indication for PI3K involvement (36). Furthermore, in these cells IL-1β, TNF-α and IFN-γ induced phosphorylation of Akt, a protein kinase immediately recruited by PI3K activation (6). These observations indicate, for the first time, that the PI3K/Akt pathway contributes to proinflammatory cytokine-induced IL-32α mRNA expression in human pancreatic myofibroblasts. Recent studies showed that the PI3K/Akt pathway plays an important role in pancreatic regenerative responses (37), acinar cell functions (15) and endocrine pancreas (39). In addition, the PI3K/Akt pathway regulates trypsinogen activation during acute pancreatitis (33). Besides these functions, our findings suggest a new aspect of the PI3K/Akt pathway in inflammatory and immune responses in the pancreas.

Many cytokine-inducible responses are mediated by the important DNA binding proteins such as NF-κB and AP-1. The promoter region of the human IL-32α gene has consensus binding sites for NF-κB (at bp -638 to -649) and AP-1 (at bp -230 to -242), suggesting an involvement of NF-κB and AP-1 activation in IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression. To confirm this possibility, we used a recombinant adenovirus expressing a stable mutant form of IκBα (Ad-IκBΔN) (25) and a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) (38). Successful infection of Ad-IκBΔN and/or Ad-DN-c-Jun fully inhibits NF-κB and AP-1 activation. As shown in Fig. 6, pretreatment with Ad-IκBΔN blocked IL-1β- and TNF-α-induced IL-32α mRNA expression, and treatment with Ad-DN-c-Jun also suppressed IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression. These data indicate that NF-κB and AP-1 activation play a role in IL-32α
mRNA induction in our system.

Recent studies indicate that the PI3K/Akt pathway regulates activation of transcription factors, such as NF-κB and AP-1, in some cell types (19, 22, 26, 28, 35). These studies implicated several mechanisms of transcription factor regulation by PI3K, which may act in a cell-specific manner. Based on this notion, we assumed crosstalk between the PI3K/Akt pathway and NF-κB/AP-1 activation in cytokine-induced IL-32α mRNA expression. EMSAs showed that IL-1β and TNF-α induced NF-κB and AP-1 activation, and IFN-γ stimulated AP-1 activation in human pancreatic myofibroblasts. LY294002 potently suppressed NF-κB and/or AP-1 activation, indicating a role for the PI3K/Akt pathway in IL-1β-, TNF-α- and IFN-γ-induced NF-κB and AP-1 activation. Thus, combined with inhibitory effects of LY294002 on IL-1β-, TNF-α- and IFN-γ-induced IL-32α mRNA expression, it is likely that the sequential activation of PI3K/Akt-induced NF-κB/ AP-1 pathways may be crucial in cytokine-induced IL-32α mRNA induction in human pancreatic myofibroblasts.

Experiments using recombinant IL-32α suggest that IL-32α is a proinflammatory cytokine, which is characterized by inducing the release of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and chemokines) through NF-κB and p38 MAPK activation pathways (11, 20). However, it remains unclear whether IL-32α exerts its biological effects as a secretory cytokine, since the IL-32α protein does not possess a typical hydrophobic signal peptide in its N terminus which is a typical feature of secreted cytokines (7). In Cos7 cells transfected with IL-32α cDNA, intracellular IL-32α was approximately 7-fold abundant as compared to secreted IL-32α (20). In the aforementioned model, there is a possibility that IL-32α in supernatants was released by apoptotic cells or during cell disruption. In this study, we could not detect IL-32α secretion by immunoprecipitation in human pancreatic myofibroblasts. This was also confirmed in human colonic myofibroblasts (data not shown). In contrast to IL-32α, in Cos7 cells transfected with IL-32β cDNA, the abundance of IL-32β was comparable in supernatants and lysates (20). Although it is unclear which of the IL-32 isoform is effectively secreted from particular cell types, it may be that IL-32α plays a role as a cytoplasmic protein. Recently, Goda et al. demonstrated that overexpression of intracellular IL-32β induced apoptosis in HeLa cells, which was blocked by interference of IL-32β transcription (14). These data suggest a role for cytoplasmic IL-32 in cell turnover. Apoptosis functions to delete damaged cells and restore tissue architecture, and IL-32α may induce apoptosis in damaged cells at inflammatory sites such as pancreatitis. IL-32α might function as a mediator bridging apoptosis and inflammation.

In conclusion, we demonstrated that IL-32α is expressed in human pancreatic
myofibroblasts. IL-32α was induced by IL-1β, IFN-γ and TNF-α, and was mediated by interactions between the PI3K/Akt-pathway and the NF-κB/AP-1 system. Interestingly, IL-32α was not secreted by human pancreatic myofibroblasts. The role of the cytoplasmic accumulation of IL-32α in human pancreatic myofibroblasts should be further investigated.

References


29. Saotome T, Inoue H, Fujiyama M, Fujiyama Y, and Bamba T. Morphological and immunocytochemical identification of periacinar fibroblast-like cells derived from


**Figure Legends**

**Fig. 1.** IL-32α mRNA and protein expression in human pancreatic myofibroblasts.

(A) IL-32α mRNA expression. Cells were stimulated with cytokines [IL-1β (50
ng/ml) and other cytokines (200 ng/ml) for 12h. IL-32α mRNA expression was analyzed by Northern blotting. Control; cells cultured in medium alone. (B) Intracellular IL-32α protein expression. Cells were stimulated with each cytokine [IL-1β (10 ng/ml), TNF-α (100 ng/ml) and IFN-γ (100 ng/ml)] for 48h, and then lysed with lysis buffer. IL-32α protein was analyzed by Western blotting. (C) Combined effects of cytokines on IL-32α mRNA expression. Cells were stimulated with IL-1β (10 ng/ml), TNF-α (100 ng/ml), IFN-γ (100 ng/ml) and combinations of these cytokines for 12h, and then IL-32α mRNA expression was determined by Northern blotting.

**Fig. 2.** Effects of IL-1β, TNF-α and IFN-γ on IL-32α mRNA expression.

(A-C) Dose-dependent induction of IL-32α mRNA in human pancreatic myofibroblasts. Cells were incubated with different doses of each cytokine, and IL-32α mRNA expression was determined by Northern blots. Ribosomal RNA, stained by ethidium bromide, is demonstrated in the lower panel. Control; cells cultured in medium alone.

(D-F) Kinetics of IL-32α mRNA expression in human pancreatic myofibroblasts. Cells were stimulated with cytokines [IL-1β (10 ng/ml), TNF-α (100 ng/ml) and IFN-γ (100 ng/ml)] for pre-determined times, and IL-32α mRNA expression was sequentially analyzed by Northern blots.

**Fig. 3.** Effects of MAPK inhibitors and a PI3K inhibitor on IL-32α mRNA expression.

(A-C) Cells were stimulated with each cytokine [IL-1β (10 ng/ml), TNF-α (100 ng/ml) and IFN-γ (100 ng/ml)] in the presence or absence of MEK inhibitors [PD98059 (20 µM) and U0216 (12.5 µM)], p38 inhibitor [SB203580 (25 µM)], and PI3K inhibitor [LY294002 (25 µM)] for 12h, and then IL-32α and β-actin mRNA expression was determined by real-time PCR. Relative IL-32α mRNA expression to β-actin mRNA expression was initially calculated, and data were expressed as a fold-increase compared to control (mean ± SD of 4 different experiments). Control; cells cultured in medium alone. **P < 0.01.

(D and E) Cells were stimulated with each cytokine [IL-1β (10 ng/ml), TNF-α (100 ng/ml) and IFN-γ (100 ng/ml)] in the presence or absence of PI3K inhibitors [LY294002 (25 µM) or Wortmannin (5 nM)] for 12h, and then IL-32α mRNA expression was determined by Northern blots.
**Fig. 4.** Kinetics of Akt activation in human pancreatic myofibroblasts. Cells were stimulated with cytokines [IL-1β (10 ng/ml), TNF-α (100ng/ml) and IFN-γ (100 ng/ml)], and phosphorylated (p-) and total Akt were sequentially detected by Western blotting.

**Fig. 5.** Effects of NF-κB and/or AP-1 inhibition on IL-32α mRNA expression. Cells were infected with an adenovirus expressing the IκBΔN or DN-c-Jun, and after 48h after infection cells were stimulated with IL-1β (10 ng/ml), TNF-α (100ng/ml) or IFN-γ (100 ng/ml) for 12 h. IL-32α mRNA expression was determined by Northern blot analyses. Adenovirus expressing LacZ were used as negative controls. Control; cells cultured in medium alone.

**Fig. 6.** Electrophoretic gel mobility shift assays (EMSA) for NF-κB and/or AP-1 DNA-binding activities (A and B). Cells were incubated with medium alone (control), IL-1β (10 ng/ml), TNF-α (100ng/ml) or IFN-γ (100 ng/ml) with or without LY294002 (25µM) for 1.5 h, and then nuclear extracts were prepared. The specificity of reactive bands was confirmed by the addition of clod oligo-DNA (C and D). Supershift experiments indicated heterodimer complexes of NF-κB (p50/p65) and AP-1 (Fos/Jun) (C and D).
Fig. 1 Nishida

(A) β-actin mRNA

Control  IL-1β  IL-4  IL-6  IL-10  IFN-γ  TNF-α  TGF-β

IL-32 mRNA

(B) IL-32α

Control  IL-1β  IFN-γ  TNF-α

36kDa → 22kDa →

IL-32α

(C) β-actin mRNA

Control  IL-1β  IFN-γ  TNF-α  IL-1β + IFN-γ  IL-1β + TNF-α  TNF-α + IFN-γ

IL-32 mRNA

Control  IL-1β  IFN-γ  TNF-α  IL-1β + IFN-γ  IL-1β + TNF-α  TNF-α + IFN-γ

β-actin mRNA
Fig. 2 Nishida

(A) IL-1β (ng/ml)

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(C) IFN-γ (ng/ml)

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(D) IL-1β-induced

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(E) TNF-α-induced

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(F) IFN-γ-induced

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**Fig. 3 Nishida**

(A) Bar graph showing the fold increase in gene expression for various conditions. The x-axis represents different treatments: Control, IL-1β, U0126, PD98059, SB203580, and LY294002. The y-axis represents the fold increase.

(B) Similar to (A) but with IFN-γ as the treatment.

(C) Similar to (A) but with TNF-α as the treatment.

(D) Western blot showing IL-32 mRNA expression under different conditions. The treatments include Control, IL-1β, IFN-γ, and TNF-α with LY294002.

(E) Western blot showing β-actin mRNA expression under different conditions. The treatments include Control, IL-1β, IFN-γ, and TNF-α with Wortmannin.

**Notes:**
- **IL-1β**
- **IFN-γ**
- **TNF-α**
- **LY294002**
- **β-actin**

**Legend:**
- **Control**
- **IL-1β**
- **IFN-γ**
- **TNF-α**
- **LY294002**
- **Wortmannin**
Fig. 4 Nishida

(A) Time (min)  
0 5 15 30 60  

IL-1β-induced  
P-Akt  
Total Akt  

(B) Time (min)  
0 5 15 30 60  

TNF-α-induced  
P-Akt  
Total Akt  

(C) Time (min)  
0 5 15 30 60  

IFN-γ-induced  
P-Akt  
Total Akt
Fig. 5 Nishida

(A)

Control  IL-1β  Ad-kBΔN  IFN-γ  TNF-α

IL-32 mRNA

28S rRNA

(B)

Control  IL-1β + Ad-LacZ  TNF-α

IL-32 mRNA

28S rRNA
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