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Expression of interleukin 1-like cytokine interleukin 33 and its receptor complex (ST2L and IL1RAcP) in human pancreatic myofibroblasts

Atsushi Nishida,1 Akira Andoh,1 Hirotugu Imaeda,1 Osamu Inatomi,1 Hisanori Shiomi,2 Yoshihide Fujiyama1

ABSTRACT

Objective Interleukin 33 (IL33) is a cytokine belonging to the IL1 family and it binds to a complex of the ST2L/IL1 receptor accessory protein (IL1RAcP). To define the role of IL33 in fibrogenesis of the pancreas, the expression of IL33, ST2L and IL1RAcP was examined in chronic pancreatitis tissues. The effects of IL33 on the functions of human pancreatic myofibroblasts were also investigated.

Methods Tissue samples were obtained surgically. The expression of IL33, ST2L and IL1RAcP was examined by standard immunohistochemical procedures. Messenger RNA expression for IL33, ST2L and IL1RAcP was analysed by northern blotting and real-time PCR analyses, and protein expression was assessed by western blotting and ELISA. Cell proliferation and migration were assessed by a 3H-thymidine incorporation assay and the modified Boyden chamber assay, respectively.

Results IL33, ST2L and IL1RAcP were expressed by α-SMA-positive myofibroblasts in the fibrosis of chronic pancreatitis. In human pancreatic myofibroblasts, IL33 was weakly immunoexpressed without any stimuli, and this was markedly enhanced by IL1β, tumour necrosis factor α (TNFα) and lipopolysaccharide (LPS) via the mitogen-activated protein kinase (MAPK)-dependent AP-1 activation pathway. ST2L mRNA was weakly detected in unstimulated cells, and IL4 and interferon γ (IFNγ) strongly enhanced ST2L expression via STAT6 and STAT1 signalling, respectively. IL33 rapidly induced the phosphorylation of MAPKs and IκBα, and enhanced the expression of inflammatory mediators (IL6, IL8, IP-10, Gro-α, Gro-β and MCP-1) in IL4- or IFNγ-pretreated cells. IL33 stimulated the proliferation and migration of pancreatic myofibroblasts.

Conclusions IL33 and its receptor complex (ST2L and IL1RAcP) constitute a novel signalling system which may play an important role in the pathogenesis of chronic pancreatitis.

INTRODUCTION

Interleukin 33 (IL33) is a cytokine belonging to the IL1 family, which was recently identified as a ligand for the IL1 receptor (IL1R) family member ST2.1 Like IL1 and IL18, IL33 can be proteolytically cleaved in vitro by caspase-1 to generate a mature form of the protein. The injection of IL33 in mice induces broad proinflammatory effects, inducing eosinophilia, splenomegaly, goblet cell hyperplasia and mucous production at mucosal surfaces, and increased serum levels of IL5 and IgE.1 Based on these in vivo effects, IL33 has been considered to play a role in Th2-mediated immune responses.

ST2L exists as a full-length, membrane-spanning molecule as well as a soluble ST2 decoy variant that can neutralise the function of IL33.2 IL33 binds to ST2L, and this complex engages the IL1 receptor accessory protein (IL1RAcP), a key molecule of IL35 signalling.3 ST2L has been reported to be constitutively expressed by mast cells as well as Th2 lymphocytes in both mice and humans.1 2 4 After binding to ST2L and following IL1RAcP-mediated signalling, IL33 stimulates the production of IL6, IL8 and IL10.

Pancreas
IL1β and tumour necrosis factor α (TNFα) by mast cells, and enhances IL5, IL13 and interferon γ (IFNγ) production in Th2-polarised T cells.

Fibrosis of the pancreas is one of the representative histopathological findings in cases of chronic pancreatitis. Previous studies have suggested that pancreatic myofibroblasts play an important role in the progression of pancreatic fibrosis. In a recent study, IL33 and ST2L were shown to participate in a paracrine signalling system between cardiac fibroblasts and cardiomyocytes to modulate cardiac hypertrophy and fibrosis. The discovery of IL33 production in cardiac fibroblasts and the modulation of cardiac fibrosis moved us to explore the role of IL33/ST2L signalling in pancreatic myofibroblasts. We hypothesised that IL33/ST2L signalling may contribute to fibrogenesis in the pancreas through the modulation of myofibroblast function. We found that pancreatic myofibroblasts secrete IL33 and express ST2L in response to inflammatory mediators. Furthermore, IL33 stimulated the proliferation and migration of pancreatic myofibroblasts. These indicate that IL33/ST2L signalling is involved in the pathophysiology of chronic pancreatitis.

MATERIALS AND METHODS

Reagents

Recombinant human IL1β, IL17 and IFNγ were purchased from R&D Systems (Minneapolis, Minnesota, USA), and the other cytokines were obtained from PeproTech (Rocky Hill, New Jersey, USA). Anti-human IL53, ST2L and IL1RACP antibodies were purchased from R & D Systems. All other reagents were purchased from Cell Signalling Technology (Beverly, Massachusetts, USA). Anti-human IL33, ST2L and IL1RAcP antibodies were purchased from R&D Systems (Minneapolis, Minnesota, USA), and the other cytokines were obtained from PeproTech (Rocky Hill, New Jersey, USA). Anti-human IL33 EIA kit was purchased from R&D Systems. A human IL33 EIA kit was purchased from Takara Bio (Otsu, Japan).

Tissue samples and immunochemistry

Human pancreatic tissue was obtained from five patients with clinically diagnosed chronic pancreatitis (four alcoholic and one idiopathic) with surgery for intractable back pain or significant suspicion of pancreatic cancer. Normal pancreatic tissues were obtained from patients who underwent total gastrectomies due to gastric cancer, according to the methods previously described. The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). All culture media were supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. Over 98% of the cells were immunopositive for α-SMA, a marker for myofibroblasts. The studies were performed on passages two to six of myofibroblasts isolated from four resection specimens.

Reverse transcription-PCR and real time-PCR

The expression of mRNA in the samples was assessed by reverse transcription PCR (RT-PCR) and real-time PCR analyses. The RT-PCR was performed according to the methods described in our previous report. The oligonucleotide primers used in this study are shown in table 1. The real-time PCR was performed using a LightCycler 2.0 system (Roche Applied Science, Tokyo, Japan). The PCR products were then ligated into TA cloning vectors (Fromega, Madison, Wisconsin, USA) and sequenced. The PCR was performed using a SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California, USA). The data were normalised versus β-actin.

Northern blot analyses

Total cellular RNA was isolated by the acid guanidinium thio- cyanate—phenol—chloroform method. Northern blotting was performed according to a previously described method. The hybridisations were performed with 32P-labelled human probes generated by a random primed DNA labelling kit (Amersham, Arlington Heights, Illinois, USA), and were evaluated by autoradiography.

Western blot analyses

For the analysis of IL33, ST2L and IL1RACP protein expression, the cells were exposed to cytokines for predetermined periods of time. The cells were then washed with PBS and lysed in SDS sample buffer containing 100 μmol/l 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.

Table 1. Oligonucleotides used in this study

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<th>Gene name</th>
<th>Primer</th>
<th>Reference (first author)</th>
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Adenovirus-mediated gene transfers

We used a recombinant adenovirus expressing a stable mutant form of \( \text{I} \kappa \text{B} \alpha \) (Ad-I\( \kappa \text{B} \alpha \)D\( \text{N} \)).\(^{24}\) a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun)\(^{25}\) and a recombinant adenovirus containing bacterial \( \beta \)-galactosidase cDNA (Ad-LacZ). The stable mutant form of I\( \kappa \text{B} \alpha \) (I\( \kappa \text{B} \alpha \)D\( \text{N} \)) lacks the 54 NH\(_2\)-terminal amino acids of wild-type I\( \kappa \text{B} \alpha \), and is neither phosphorylated nor proteolysed in response to signal induction, but fully inhibits nuclear factor kappa B (NF-\( \kappa \text{B} \) ) 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 \( \beta \)-galactosidase (data not shown). The recombinant adenovirus was transferred into the cells, and the cells were made quiescent for 48 h before being assessed for the effects of the transferred gene.

STAT1 and 6 mRNA interference (RNAi) experiments

The siRNA for human STAT1/6 and a control siRNA were purchased from Santa Cruz Biotechnology. The cells were cultured in complete medium that did not contain antibiotics for 2 days. The cells were then seeded onto six-well plates 1 day prior to the transfection, and cultured to 60–70% confluence on the following day. For the RNAi experiments, Lipofectamine LTX and 2.5 \( \mu \)l of PLUS Reagent (Invitrogen, Carlsbad, California, USA) were used. STATs expression was determined by RT-PCR and western blotting.

Nuclear extracts and electrophoretic gel mobility shift assays

Nuclear extracts were prepared from cells exposed to the cytokines for 1.5 h. The consensus oligonucleotides for AP-1 (5’ CGCTTGATGAGTCAGCCGGAA) were purchased from Promega (Madison, Wisconsin, USA). The oligonucleotides were 5’ end-labelled with T4 polynucleotide kinase (Promega) and \( [\gamma-\text{32P}] \text{ATP} \) (Amersham). The binding reactions were performed according to previously described methods.\(^{14}\)

\( ^{3} \text{H} \)-thymidine incorporation and chemotactic assay

The \( ^{3} \text{H} \)-thymidine incorporation assay was performed according to the method described previously.\(^{10} \) \( ^{3} \text{H} \)-thymidine incorporation performed for 12 h was analysed. Cell migration was evaluated by the modified Boyden chamber assay using a BioCoat Matrigel Invasion Chamber (BD Bioscience, Franklin Lakes, NJ). A cell suspension (5×10\(^{4} \) cells/ml) was added to the culture inserts equipped with 8-\( \mu \)m pores in a 24-well companion plate. The lower chamber included serum-free medium with IL33. The cells were incubated for 24 h at 37°C in a 5% CO\(_{2} \) atmosphere. The cells on the lower surface of the membrane

![Figure 1](http://example.com/image.png)

**Figure 1** Representative immunohistochemical expression of IL33, ST2L and IL1RAcP in the pancreas. (A) IL33 immunostaining in normal pancreatic tissue and chronic pancreatitis; (B) ST2L immunostaining in normal pancreatic tissue and chronic pancreatitis; and (C) IL1RAcP immunostaining in normal pancreatic tissue and chronic pancreatitis. (D) IL33, ST2L and IL-RAcP staining in chronic pancreatitis. Magnification, ×200. IL, interleukin; IL1RAcP, interleukin 1 receptor accessory protein.
were stained with Diff-Quik stain (Sysmex, Kobe, Japan). The invading cells were counted in several fields at a magnification of ×100.

Statistical analyses
The statistical significance of the differences was 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
Immunohistochemical studies of IL33 expression in human pancreas
There was no IL33 immunostaining in the normal human pancreas tissue (figure 1A). In contrast, IL33 was detectable in the fibrosis of chronic pancreatitis (figure 1A D), whereas isotype control with goat IgG antibody showed negative staining (supplementary figure 1A). To characterise the IL33-expressing cells, double immunostaining for IL33 and α-smooth muscle actin (α-SMA) was performed in the chronic pancreatitis samples. As shown in figure 1A right panel, the IL33/α-SMA double immunopositive cells were detected as yellow, and the IL33-immunopositive cells coincided with part of the α-SMA-immunopositive cells. These findings are compatible with observations in cardiac fibroblasts,12 and indicate that α-SMA-immunopositive myofibroblasts are a source of IL33 in chronic pancreatitis.

Immunostaining for ST2L was also performed. As shown in figure 1B ST2L was not detected in the normal pancreas. On the other hand, ST2L was detectable in the fibrosis of chronic pancreatitis (figure 1B D). Immunostaining for ST2L was also performed. As shown in figure 1B ST2L was not detected in the normal pancreas. On the other hand, ST2L was detectable in the fibrosis of chronic pancreatitis (figure 1B D). Immunostaining for ST2L was also performed. As shown in figure 1B ST2L was not detected in the normal pancreas. On the other hand, ST2L was detectable in the fibrosis of chronic pancreatitis (figure 1B D).

To investigate the regulatory mechanisms underlying IL33 induction, human pancreatic myofibroblasts were stimulated with various cytokines for 12 h, and then IL33 mRNA expression was analysed by northern blot analyses (figure 2A). IL33 mRNA was weakly expressed without any stimuli, and IL1β, TNFα, and lipopolysaccharide (LPS) markedly enhanced IL33 mRNA expression. Dose- and time-dependent effects of these factors were confirmed (supplementary figure 2). We also evaluated IL33 mRNA expression in quiescent pancreatic stellate cells (PSCs) isolated by the method described by Apte et al.26 As shown in supplementary figure 1B, IL33 mRNA expression was not detected in quiescent PSCs.

Similar results were observed at the protein level. The cells were stimulated for 24 h with IL1β, TNFα and LPS, and then the IL33 protein expression was analysed by ELISA (figure 2B). IL1β, TNFα and LPS stimulated IL33 protein secretion in pancreatic myofibroblasts. As reported by Schmitz et al1 western blot analyses revealed that IL33 was detected as an intracellular molecule of 30 kDa, and an extracellular molecule of 22 kDa (supplementary figure 3A).

Next, we tested the effects of combinations of IL1β, TNFα and LPS (figure 2C,D). Real-time-PCR analysis and ELISA showed that combinations of IL1β plus TNFα, IL1β and LPS, and/or TNFα plus LPS additively enhanced IL33 mRNA expression and IL33 secretion.

Figure 2 IL33 mRNA and protein expression in human pancreatic myofibroblasts. (A) The cells were stimulated with cytokines (100 ng/ml) for 12 h. IL33 mRNA expression was analysed by northern blotting. Ribosomal RNA, stained by ethidium bromide, is shown in the lower panel. (B) The cells were stimulated with cytokines (100 ng/ml) for 12 h. IL33 mRNA expression was analysed by real-time PCR. (C) Combined effects of cytokines on IL33 mRNA expression. The cells were stimulated with IL1β (100 ng/ml), TNFα (100 ng/ml), LPS (100 ng/ml) and combinations of these factors for 12 h, and then IL33 mRNA expression was determined by real-time PCR. The data from the real-time PCR were expressed as the IL33 mRNA expression relative to β-actin mRNA expression (mean±SD from four different experiments). **p<0.01 versus medium only. (D) Combined effects of cytokines on IL33 mRNA expression. The cells were stimulated with IL1β (100 ng/ml), TNFα (100 ng/ml), LPS (100 ng/ml) and combinations of these factors for 24 h, and then IL33 protein secretion was determined by EIA Kit (mean±SD from four different experiments). **p<0.01 versus medium only. IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; TGF, transforming growth factor; TNFα, tumour necrosis factor α.
**AP-1 activation is required for IL33 mRNA induction**

The promoter sequences analysed by the UCSC Genome Browser created by the Genome Bioinformatics Group of UC Santa Cruz (University of California, Santa Cruz, California, USA) showed several consensus binding sites for AP-1 in the promoter regions of the human IL33 gene. To assess the role of the transcription factor AP-1, we evaluated the effects of a recombinant adenovirus containing a dominant negative mutant of c-Jun (Ad-DN-c-Jun) on IL33 mRNA expression. As shown in figure 3 upper sections, pancreatic myofibroblasts were incubated with medium alone, IL1β (100 ng/ml), TNFα (100 ng/ml) or LPS (100 ng/ml) for 12 h. IL1β (100 ng/ml), TNFα (100 ng/ml) and LPS (100 ng/ml) significantly increased IL33 mRNA expression, but LY294002 had no effects. The activation of p42/44 and p38 MAPK in response to IL33 mRNA induction, we evaluated the effects of p42/44 MAPK inhibitors (PD98059 (100 ng/ml)) in the presence or absence of MEK inhibitors (PD98059 (100 ng/ml), U0126) and a PI3K inhibitor (SB203580) on IL33 mRNA expression. As shown in the middle sections of figure 3 PD98059, U0126 and SB203580 significantly suppressed cytokine-induced IL33 mRNA induction, but LY294002 had no effects. The activation of p42/44 and p38 MAPK in response to IL1β, TNFα and LPS were confirmed (supplementary figure 4).

Next, we investigated the effects of MAPK inhibitors on AP-1 activation. As shown in the lower sections of figure 3 the electrophoretic gel mobility shift assays (EMSA) showed that the MAPK inhibitors suppressed IL1β-, TNFα- and LPS-induced IL33 mRNA expression.

**ST2L expression in pancreatic myofibroblasts**

We investigated the expression of the IL33 receptor complex, ST2L and IL1RαCp, in human pancreatic myofibroblasts. As shown in figure 4A IL1RαCp mRNA was constitutively expressed by these cells. On the other hand, ST2L mRNA was weakly detected in unstimulated myofibroblasts, and the addition of IL4 and IFNγ strongly increased ST2L mRNA expression. Significant effects of IL4 and IFNγ on ST2L mRNA expression were also confirmed by real-time-PCR (figure 4B). On the other hand, ST2L mRNA was not detected in quiescent PSCs (supplementary figure 1B), suggesting that activation of PSCs is required for responsiveness to IL33.

The effects of IL4 and IFNγ were also evaluated at the protein level. Western blot and immunostaining analyses showed that IL4 and IFNγ actually induced ST2L protein expression in pancreatic myofibroblasts (figure 4C). On the other hand, IL1RαCp was constitutively expressed in these cells. As shown in figure 4D IL4 and IFNγ additively increased ST2L mRNA expression.

**Role of STATs activation in ST2L induction**

The molecular mechanisms underlying ST2L induction were analysed. Signal transducer and activator of transcription 6 (STAT6) is activated in response to IL4, and mediates the various biological actions of IL4.51 As shown in figure 5A IL4 rapidly induced STAT6 phosphorylation in pancreatic myofibroblasts, and a knockdown of STAT6 by STAT6-specific siRNA significantly downregulated IL4-induced ST2L mRNA expression (the

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**Figure 3** Effects of AP-1 and MAPK inhibition on IL33 mRNA expression. Upper part: Human pancreatic myofibroblasts were infected with an adenovirus expressing the DN-c-Jun, and at 48 h after infection the cells were stimulated with IL1β (100 ng/ml), TNFα (100 ng/ml) or LPS (100 ng/ml) for 12 h. IL33 mRNA expression was determined by real-time-PCR. An adenovirus expressing LacZ was used as the negative control. Middle part: Effects of MAPK inhibitors and a PI3K inhibitor on IL33 mRNA expression. The cells were stimulated with each cytokine (IL1β (100 ng/ml), TNFα (100 ng/ml) and LPS (100 ng/ml)) in the presence or absence of MEK inhibitors (PD98059 (10 μmol/l) and U0126 (10 μmol/l)), p38 inhibitor (SB203580 (10 μmol/l)), and PI3K inhibitor (LY294002 (10 μmol/l)) for 12 h, and then the IL33 mRNA expression was determined by real-time PCR. Lower part: Effects of MAPK inhibitors on AP-1 activation. The cells were incubated with medium alone, IL1β (100 ng/ml), TNFα (100 ng/ml) or LPS (100 ng/ml) with or without MAPK inhibitors (10 μmol/l) for 1.5 h, and the AP-1 DNA binding activities were evaluated by electrophoretic gel mobility shift assays (EMSA). The data from the real-time PCR were expressed as the IL33 mRNA expression relative to β-actin mRNA expression (mean±SD from four different experiments). **p<0.01 versus absence of inhibitors. IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; TNFα, tumour necrosis factor α.
effective knockdown of STAT6 expression is shown in supplementary figure 5). Similarly, the molecular mechanisms underlying IFNγ-induced ST2L expression was also analysed. STAT1 plays a central role in the various biological effects of IFNγ. As shown in figure 5B IFNγ rapidly induced STAT1 phosphorylation, and a knockdown of STAT1 by STAT1-specific siRNA significantly downregulated IFNγ-induced ST2L mRNA expression (the effective knockdown of STAT1 expression is shown in supplementary figure 5).

Furthermore, both IL4- and IFNγ-induced ST2L mRNA expression were blocked by the presence of dominant-negative c-Jun AP-1, but not by a stable mutant form of IκBα (Ad-IκBΔN) (lower sections of figure 5A,B). As demonstrated in supplementary figure 6, IL4 and IFNγ-induced ST2L mRNA expression was also blocked by the addition of p42/44 MAPK inhibitors (PD98059 and U0126), but inhibitors of p38 MAPK and PI3K had no effects. These observations support a role for STAT6- and STAT1-mediated signalling in IL4- and IFNγ-induced ST2L mRNA expression, respectively. In addition, the activation of c-Jun AP-1 and p42/44 MAPK also play a role in both IL4- and IFNγ-mediated ST2L mRNA induction.

IL33 induces MAPK and IκB activation in pancreatic myofibroblasts
As shown in figure 6A stimulation with IL33 rapidly induced the phosphorylation of p42/44 MAPK, p38 MAPK, JNK, and IκBα in pancreatic myofibroblasts.

In order to enhance ST2L expression, the cells were pretreated with IL4, IFNγ and/or IL4 plus IFNγ for 24 h. The cells were then washed, and stimulated with IL33. As shown in figure 6B pretreatment of the cells with IL4, IFNγ and/or IL4 plus IFNγ for 24 h additively enhanced MAPK and JNK activation.

IL33 enhances the expression of inflammatory mediators in IL4- or IFNγ-pretreated cells
We investigated which molecules were induced by IL33 stimulation in pancreatic myofibroblasts. As shown in figure 7 without pretreatment with IL4 and/or IFNγ, IL33 did not stimulate any mRNA expression of the inflammatory mediators (IL6, IL8, IP-10, Gro-α, Gro-β and MCP-1). In contrast, in cells pretreated with IL4 and/or IFNγ for 24 h, IL33 significantly enhanced the mRNA expression of these mediators (figure 7). On the other hand, IL33 did not stimulate TGFβ1 mRNA expression, and reduced PDGF mRNA expression (supplementary figure 7A).

Figure 4 ST2L and IL1RAcP expression in human pancreatic myofibroblasts. (A) The cells were stimulated with cytokines (100 ng/ml) for 12 h. ST2L and IL1RAcP mRNA expression were then analysed by RT-PCR. (B) The cells were stimulated with cytokines (100 ng/ml) for 12 h. ST2L mRNA expression was analysed by real-time PCR. (C) The cells were stimulated with each cytokine (IL4 (100 ng/ml) and IFNγ (100 ng/ml)) for 24 h, and ST2L and IL1RAcP was detected by an immunocytochemical procedure. (D) Combined effects of cytokines on ST2L mRNA expression. The cells were stimulated with IL4 (100 ng/ml), IFNγ (100 ng/ml) and combinations of these factors for 12 h, and then ST2L mRNA expression was determined by real-time PCR. The data from the real-time PCR were expressed as the ST2L mRNA expression relative to β-actin mRNA expression (mean±SD from four different experiments). **p<0.01. IFN, interferon; IL1RAcP, interleukin 1 receptor accessory protein.
**Effects of IL33 on cell proliferation and cell migration**

To investigate the role of IL33 in fibrogenesis in the pancreas, a 3H-thymidine incorporation assay was performed. IL33 dose-dependently stimulated 3H-thymidine incorporation (figure 8A), and a combination of IL33 plus IL4 or IL33 plus IFNγ significantly enhanced 3H-thymidine incorporation as compared to IL33 alone (figure 8B). Pretreatment of the cells with IL4, IFNγ or IL4 plus IFNγ significantly increased 3H-thymidine incorporation (figure 8C). Furthermore, IL33 stimulation for 24 h dose-dependently enhanced the migration of these cells (figure 8D).

As shown in figure 7 IL33 stimulated expression of MCP-1, which mediates the proliferation and migration of pancreatic myofibroblasts. To rule out the possibility that the secreted MCP-1 affected the proliferation of pancreatic myofibroblasts, effects of IL33 were evaluated in cells transfected with MCP-1 siRNA. As shown in supplementary figure 7B, effects of IL33 on proliferation of pancreatic myofibroblasts were not affected by the presence of MCP-1 siRNA, indicating that IL33-induced MCP-1 did not participate in the IL33-induced proliferation responses. In addition, as shown in supplementary figure 7A, IL33 also did not stimulate the expression of transforming growth factor β1 (TGFβ1) and platelet-derived growth factor (PDGF), which are regarded as stimulators of proliferation of myofibroblasts. These results suggest that IL33 is a direct stimulator of the proliferation of pancreatic myofibroblasts.

Finally, we evaluated the effects of IL33 on collagen synthesis. As shown in figure 8E IL33 had no effects on procollagen type I secretion, although IFNγ significantly reduced it. IL33 also did not stimulate the mRNA expression of TGFβ1, a potent stimulator of collagen synthesis (supplementary figure 7A). Thus, there was no evidence supporting that IL33 stimulates collagen synthesis in pancreatic myofibroblasts.

**DISCUSSION**

IL33 is a newly described cytokine from the IL1 family, which also includes IL1α, IL1β and IL18. IL33 is broadly expressed in many tissues, but its receptor molecule ST2L is mainly expressed by immune cells such as mast cells and polarised Th2 cells. To date, the precise molecular mechanisms underlying IL33 and ST2L induction remain unclear. In the present study, we investigated IL33 and its receptor expression in the pancreas. Here, we report for the first time that: (1) human pancreatic myofibroblasts are a source of IL33, and express the IL33 receptor complex ST2L/IL1RaCp; (2) IL1β, TNFα and LPS are potent inducers of IL33 secretion via a MAPK-dependent AP1 pathway; (3) IL4 and IFNγ induce ST2L expression via STAT6.
and STAT1 activation, respectively; (4) IL33 enhances the expression of the inflammatory mediators in IL4- and IFNγ-pretreated cells; and (5) IL33 stimulates the proliferation and migration of these cells. Current observations suggest that pancreatic myofibroblasts play an important role in inflammatory responses and fibrosis via the expression of IL33 and its receptor ST2L/IL1RACP.

Schmitz et al reported the relatively restricted expression of IL33 mRNA at the level of cell type, which includes smooth muscle cells, epithelial cells, dermal fibroblasts, keratinocytes and dendritic cells. In the present study, we showed that unstimulated pancreatic myofibroblasts express and secrete modest levels of IL33 mRNA and protein, and that IL1β, TNFα and LPS induced a marked increase in the expression of IL33 mRNA and protein. Since the promoter region of the human IL33 gene has multiple consensus binding sites for AP-1, we evaluated the effects of a recombinant adenovirus expressing a dominant negative mutant of c-Jun (Ad-DN-c-Jun). The addition of Ad-DN-c-Jun markedly suppressed IL1β-, TNFα- and LPS-induced IL33 mRNA expression, indicating a role for AP-1 in IL33 mRNA induction. Furthermore, p42/44 MAPK-inhibitors (PD98059 and U0126) and p38 MAPK inhibitor (SB203580) significantly inhibited IL1β-, TNFα- and LPS-induced IL33 mRNA expression. These inhibitors also suppressed IL1β-, TNFα- and LPS-induced AP-1 activation. Combined with the previous reports that AP-1 acts downstream of evolutionarily conserved signalling pathways, such as MAPK, we concluded that p42/44 and p38 MAPK pathway-dependent AP-1 activation plays a role in IL1β-, TNFα- and LPS-induced IL33 mRNA expression.

IL33 binds to ST2L, and this complex engages IL1RACP as a co-receptor. ST2L expression has been reported in a limited number of cell types, such as Th2 cells, NK cells, mast cells and basophils. Cardiomyocytes also express ST2L. However, in other cell types, ST2L expression has not been identified. In addition, the precise mechanisms underlying ST2L induction are still largely unknown, although IL3 has been reported to be an inducer of ST2L expression in mast cells. In this study, we demonstrated the expression of ST2L and IL1RACP in human pancreatic myofibroblasts. Although IL1RACP was constitutively expressed, ST2L was inducible in response to IL4 (Th2 cytokine) and IFNγ (Th1 cytokine). Since the biological actions of IL4 and IFNγ have been reported to depend on STAT activation, we evaluated the effects of a knockdown of the STAT genes. In pancreatic myofibroblasts, IL4 and IFNγ induced STAT6 and STAT1 phosphorylation, and STAT6- and STAT1-specific siRNA completely inhibited the IL4- and IFNγ-induced ST2L expression. These findings indicate that IL4- and IFNγ-induced ST2L expression is mediated by STAT6 and STAT1, respectively. Furthermore, experiments using Ad-DN-c-Jun showed that AP-1 is a common mediator of IL4- and IFNγ-induced ST2L expression. The activation of p42/44 MAPK was also involved in ST2L induction. Thus, it becomes clear that in pancreatic myofibroblasts, ST2L induction was mediated by T cell-derived cytokines (IL4 and IFNγ), and that the mechanism controlling ST2L expression may be different from that of IL33 induction.

IL33 has been reported to induce activation of the NF-κB pathway, as well as MAPKs via binding to ST2L. Mast cells respond to IL33 by producing pro-inflammatory cytokines, such as IL6 and chemokines. Similar responses were investigated in pancreatic myofibroblasts. We found that recombinant IL33 actually induces the phosphorylation of MAPKs and JκB. The activation of MAPKs was strongly enhanced by the pre-incubation of these cells with IL4 and IFNγ, suggesting the contribution of enhanced signalling through IL4- and IFNγ-induced ST2L expression. Furthermore, in IL4- and IFNγ-pretreated cells, IL33 caused a significant increase in the mRNA expression of pro-inflammatory mediators, such as IL6, IL8, IP-10, Gro-α, Gro-β and MCP-1. These observations indicated that the responses reported in mast cells, IL33 signalling plays a role in the expression of inflammatory mediators.
These suggest a sequential amplification of IL-4 mRNA expression, which is then followed by IL-8 mRNA expression, and finally by the induction of GRO-α mRNA. In this study, we demonstrated that IL-33 is a potent stimulator of IL-4 synthesis in pancreatic myofibroblasts.33 40 It stimulates mesenchymal stem cells to the tissue, and activating the inflammatory cascade via stimulating the proliferation and migration of pancreatic myofibroblasts.33 40 IL-33 signalling in pancreatic myofibroblasts is crucial for the accumulation of inflammatory mediators, such as the expression of pro-inflammatory cytokines (IL1β and TNFα) derived from immune cells such as monocytes and lymphocytes, as well as bacterial factor (LPS), which induce IL-33 secretion from pancreatic myofibroblasts. The secreted IL-33 then stimulates the surrounding immune cells (mast cells, Th2 cells and NK cells) to produce IL-4 and IFNγ. Next, the myofibroblasts respond to the secreted IL-4 and IFNγ, and enhance ST2L expression. IL-33 signalling through enhanced expression of ST2L induces IL-6 chemokines via autocrine and/or paracrine pathways, resulting in the accumulation and activation of immune and inflammatory cells (macrophages, lymphocytes and granulocytes) in the pancreas. These responses further stimulate myofibroblasts to secrete IL-33, and amplify the inflammatory responses.

We demonstrated that IL-4 and IFNγ are critical factors for IL-33 signalling in pancreatic myofibroblasts, since sufficient biological activity of IL-33, such as the expression of pro-inflammatory mediators, requires the pretreatment of the cells with IL-4 and IFNγ. In a recent study by Smithgall et al., IL-33 stimulated IL-4 and IFNγ production from mast cells, Th2 cells and NK cells.2 These suggest a sequential amplification cascade of the inflammatory responses elicited by IL-33 between pancreatic myofibroblasts and immune cells. The activation of quiescent PSCs to myofibroblasts has been reported to occur in response to inflammatory mediators, ethanol and oxidative stress.5 11 26 Following activation, the proinflammatory cytokines (IL1β and TNFα) derived from immune cells such as monocytes and lymphocytes, as well as bacterial factor (LPS), induce IL-33 secretion from pancreatic myofibroblasts. The secreted IL-33 then stimulates the surrounding immune cells (mast cells, Th2 cells and NK cells) to produce IL-4 and IFNγ. Next, the myofibroblasts respond to the secreted IL-4 and IFNγ, and enhance ST2L expression. IL-33 signalling through enhanced expression of ST2L induces IL-6 and chemokines via autocrine and/or paracrine pathways, resulting in the accumulation and activation of immune and inflammatory cells (macrophages, lymphocytes and granulocytes) in the pancreas. These responses further stimulate myofibroblasts to secrete IL-33, and amplify the inflammatory responses.

The proliferation and migration of pancreatic myofibroblasts are crucial responses in the progression of pancreatic fibrosis. It has been reported that PDGF is a potent stimulator of PSC proliferation, and TGFβ and activin A also play a role in myofibroblast-mediated fibrogenesis through autocrine and paracrine loops.6 In this study, we demonstrated that IL-33 is...
a novel factor which directly stimulated the proliferation and migration of pancreatic myofibroblasts. IL33 did not stimulate collagen synthesis, but promoted the proliferation of unstimulated pancreatic myofibroblasts. These responses were significantly enhanced by pre-treatment with IL4 and IFNγ. As mentioned above, IL33 stimulated MCP-1 expression in these cells, suggesting that the secreted MCP-1 indirectly mediated the actions of IL33. However, a direct stimulatory effect for IL33 was supported by two observations: (1) blockade of MCP-1 actions of IL33. However, a direct stimulatory effect for IL33 expression by MCP-1-specific siRNA did not affect the effects of IL33; and (2) IL33 did not stimulate mRNA expression of TGFβ and PDGF, potent stimulators of myofibroblast proliferation. Recent studies suggested a role for IL33 in cardiac and hepatic fibrosis, but the direct effects of IL33 at the cellular level have not been identified. Our observations on IL33 functions in this study, such as induction of MCP-1 expression and direct stimulatory effect on proliferation, indicate that IL33 is a new factor accounting for the progression of fibrosis in the pancreas.

In conclusion, we demonstrated that IL33 and its receptor are expressed in human pancreatic myofibroblasts. Pancreatic myofibroblasts responded to IL33 by the expression of pro-inflammatory mediators, and increased activities in terms of proliferation and migration. Thus, IL33 is considered to be a novel factor involved in the pathogenesis of chronic pancreatitis. Further elucidation of the details of the functional roles of IL33 and its ST2L will enable us to evaluate their potential as useful targets in the therapeutic strategies for chronic pancreatitis.

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**REFERENCES**


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