Nuclear expression of interleukin-33 in pancreatic stellate cells

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Masamune A, Watanabe T, Kikuta K, Satoh K, Kanno A, Shimosegawa T. Nuclear expression of interleukin-33 in pancreatic stellate cells. Am J Physiol Gastrointest Liver Physiol 299: G821–G832, 2010. First published August 5, 2010; doi:10.1152/ajpgi.00178.2010.—Activated pancreatic stellate cells (PSCs) play a pivotal role in pancreatic fibrosis in chronic pancreatitis and pancreatic cancer. Recent studies have suggested a role of IL-33, a newly identified IL-1 family member, in fibrosis. We here examined the expression of IL-33 and the IL-33-mediated regulation of cell functions in PSCs. PSCs were isolated from human and rat pancreas tissues. The expression of IL-33 was examined by Western blotting, PCR, ELISA, and immunostaining. The roles of IL-33 in the regulation of PSC functions were examined by using recombinant IL-33 and small interfering RNA. Activated PSCs expressed IL-33 in the nucleus, and the expression was increased by IL-1β, TNF-α, PDGF-BB, and IFN-γ, but not TGF-β1. Nuclear IL-33 expression was also observed in the pancreatic acinar and ductal cells. IL-1β induced IL-33 expression mainly through the activation of NF-κB and ERK pathways and partially through that of p38 MAP kinase, whereas PDGF-BB induced IL-33 expression mainly through the activation of ERK pathway. PSCs expressed soluble ST2, ST2L, and IL-1RAcP, but the expression level of ST2L was relatively low. Recombinant IL-33 did not stimulate key cell functions of PSCs. Decreased IL-33 expression by small interfering RNA resulted in decreased proliferation in response to PDGF-BB. In conclusion, activated PSCs expressed IL-33 in the nucleus. IL-33 might regulate the PDGF-induced proliferation in PSCs.

IL-33 is a newly identified cytokine of the IL-1 family, which also includes IL-1α, IL-1β, and IL-18 (4, 13, 15, 29, 35). IL-33 is synthesized as a ~30-kDa precursor protein and is cleaved in vitro by caspase-1 to form a mature, secreted 18-kDa protein (4, 13, 15, 28, 34). IL-33 is a dual-function protein that may act as both an extracellular cytokine and an intracellular nuclear factor with transcriptional regulatory properties (9). IL-33 was initially described as “nuclear factor from high endothelial venules” because it resided in the nucleus of high endothelial cells exhibiting transcriptional repressor properties (7). Thereafter, IL-33 was identified as an extracellular ligand for the IL-1 receptor-related protein ST2, which signals in conjunction with IL-1 receptor accessory protein (IL-1RAcP) (1, 10, 37). The ST2 gene encodes three isoforms of ST2 protein including ST2L, a transmembrane form, and soluble ST2, a secreted form that can serve as a decoy receptor for IL-33 (15, 35, 39). Binding of IL-33 to the ST2L on mast cells and T helper 2 (Th2) lymphocytes has been shown to induce the production of proinflammatory and Th2 cell-associated cytokines including IL-4, IL-5, and IL-13 (1, 10, 35). Thus exogenous IL-33 seems to act more as an immunoregulatory cytokine that participates in the control of Th2 immunity rather than as a proinflammatory cytokine. IL-33 is broadly expressed in various tissues including stomach, lung, spinal cord, brain, and skin, as well as in cells including smooth muscle cells, epithelial cells, fibroblasts, endothelial cells, and activated macrophages (35). Recent studies have suggested a role of IL-33 in various diseases. Treatment of mice with IL-33 induced expression of IL-4, IL-5, and IL-13 in vivo, resulting in severe mucosal inflammation in the lung, esophagus, and small intestine (35). Inhibition of IL-33 signaling attenuated the severity of experimental arthritis (33). In addition, a role of IL-33 in fibrotic diseases has been suggested (2, 20, 34). Heart failure and cardiac fibrosis were associated with increased IL-33 production (34). In a mouse model of liver fibrosis, injection of the fusion protein ST2-Fc increased Th2 cytokine secretion and enhanced liver fibrosis (2). Overexpression of IL-33 was associated with liver fibrosis in mice and humans (20). But the expression of IL-33 and IL-33-mediated cell functions in PSCs remain largely unknown.

METHODS

Materials and Animals

Collagenase P, recombinant IL-1β, recombinant TNF-α, and recombinant IFN-γ were purchased from Roche Diagnostics (Mannheim, Germany). Recombinant IL-33 was from Peprotech (Rocky Hill, NJ). Rabbit antibody against human IL-33 was from MBL (Nagoya, Japan). Goat antibody against mouse IL-33, which reacts with rat IL-33, was from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against type I collagen and ST2 were from Abcam (Cambridge, MA). Recombinant PDGF-BB, recombi-
nant TGF-β1, and rabbit anti-GAPDH antibody were from R&D Systems (Minneapolis, MN). BAY11–7082, U0126, SB203580, SP600125, and wortmannin were from Calbiochem-Novabiochem (San Diego, CA). Other reagents were from Sigma-Aldrich (St. Louis, MO) unless specifically described.

**Cell Culture**

Human PSCs were isolated from the resected pancreas tissues of patients undergoing operation for pancreatic cancer or CP as previously described (22), under the approval by the Ethics Committee of Tohoku University School of Medicine. Experiments were performed using human PSCs from five independent preparations and those between passages 3 and 9 after isolation. Rat PSCs were prepared as previously described (21) from the pancreas tissues of male Wistar rats (Japan SLC, Hamamatsu, Japan) using Nycodenz solution (Nycomed Pharma, Oslo, Norway) after perfusion with 0.03% collagenase P. All animal procedures were performed in accordance with the National Institutes of Health Animal Care and Use Guidelines. The experimental protocol was reviewed and approved by the Animal Experiment Committee of Tohoku University School of Medicine. Freshly isolated rat PSCs and those between passages 2 and 4 were used. Cells were maintained in Ham’s F-12/DMEM (1:1) supplemented with 10% heat-inactivated FBS, penicillin sodium, and streptomycin sulfate. Unless specifically described, we incubated PSCs in serum-free medium for 24 h before addition of the experimental reagents. For some experiments, inhibitors of signal transduction pathways were added at 30 min before the addition of IL-1β or PDGF-BB.

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Basel, Switzerland) and maintained in the endothelial cell growth medium provided (EGM-2 Bulletkit; Takara Bio, Otsu, Japan). Experiments were performed using cells of passage 2.

**Expression of IL-33**

**Real-time PCR and reverse transcription PCR.** Total RNA was prepared using RNeasy total RNA preparation kit (Qiagen, Valencia, CA). The level of IL-33 mRNA was quantified by real-time PCR as previously described (26). Total RNA (~100 ng) was reverse transcribed in a volume of 20 μl. The resultant cDNA (2 μl) was subjected to real-time PCR with the LightCycler-FastStart DNA

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**Table 1. Primer sequences used for reverse transcription-PCR and real-time PCR**

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human IL-33</td>
<td>CTGCCCTGTAAACAACAGTCTT</td>
<td>CTGGTCTGGCAGTGGTTTT</td>
<td>398</td>
</tr>
<tr>
<td>Human α-SMA</td>
<td>TCCCTCCTGAGAAAGAGGTAC</td>
<td>CTGAGAAGGATTTCTGAGAT</td>
<td>202</td>
</tr>
<tr>
<td>Human GAPDH</td>
<td>ACCATCTCTGGCAGATCCT</td>
<td>GGGGATTCGCTGGTGAAAGCT</td>
<td>371</td>
</tr>
<tr>
<td>Human ST2</td>
<td>AGGCTTTTCCTGATTTTCTGAATG</td>
<td>CAGTGACAGGAGGAGGATCTAAATGGA</td>
<td>659</td>
</tr>
<tr>
<td>Human ST2L</td>
<td>AGGCTTTTCCTGATTTTCTGAATG</td>
<td>GGGGATTCGCTGGTGAAAGCT</td>
<td>454</td>
</tr>
<tr>
<td>Human IL-1RaP</td>
<td>AGAAGGCAAGGAGAAGAGAGA</td>
<td>CTGAGAAGGAGAAGAGAGAGA</td>
<td>431</td>
</tr>
<tr>
<td>Rat IL-33</td>
<td>GTGCGGAGAAGAAGAAGAAGA</td>
<td>TGCGGCTACATTAAGAAAGAG</td>
<td>347</td>
</tr>
<tr>
<td>Rat α-SMA</td>
<td>TGGAGCTGACTGAGATG</td>
<td>GATGACGCTGAGCTGAG</td>
<td>292</td>
</tr>
<tr>
<td>Rat GAPDH</td>
<td>ACATCACTCTGAGCCCTCTCGC</td>
<td>GGGAGGTGCTGCTGCTGCTGTTTG</td>
<td>258</td>
</tr>
<tr>
<td>Rat ST2</td>
<td>GTGCGGAGAAGAAGAAGAGA</td>
<td>AAAGGAAAGTCTCCAGAAGAGG</td>
<td>235</td>
</tr>
<tr>
<td>Rat ST2L</td>
<td>TGGAGGAGAAGAAGAGAAGA</td>
<td>AAAGAAGTCTCCAGAAGAGG</td>
<td>331</td>
</tr>
<tr>
<td>Rat IL-1RaP</td>
<td>TGAGAAGGAGAAGAAGAGAGAGA</td>
<td>TTGCGGCTATGACTTTTCTGTTTCC</td>
<td>302</td>
</tr>
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</table>

IL-1RaP, IL-1 receptor accessory protein; α-SMA, α-smooth muscle actin.
Master SYBR Green I kit (Roche Diagnostics), using a LightCycler instrument (Roche Diagnostics). Specific primer sets are shown in Table 1. Reactions were performed in a volume of 20 μl containing 0.5 μM primers and 2.5 mM MgCl₂. The PCR protocol consisted of an initial denaturation step at 95°C for 10 min and 50 cycles of denaturation (95°C for 15 s), annealing (60°C for 10 s), and extension (72°C for 10 s). For each step, the temperature transition rate was 20°C/s. Melting curve analyses were performed to confirm the PCR product identity and to differentiate specific amplification from non-specific products by denaturation (95°C for 10 s), annealing (65°C for 10 s), and slow heating to 95°C (temperature transition rate, 0.1°C/s), combined with continuous fluorescence measurement at 0.2°C increments. After completion of the PCR, the copy number of the target molecules was calculated by plotting fluorescence vs. cycle number. As a standard curve, we used the linear regression line based on the data of standard crossing points (threshold cycle) vs. the logarithm of standard sample concentrations. The expression level of IL-33 was evaluated by the ratio to GAPDH mRNA.

Expression of IL-33 and its receptor system was also examined by reverse transcription-PCR. The primer sequences and expected sizes of the PCR products are shown in Table 1. The primer set for ST2 specifically amplified ST2, but not ST2L, whereas the primer set for ST2L specifically amplified ST2L, but not ST2 (8, 19). The cycle conditions were as follows: preheating at 94°C for 2 min, followed by 30 (for GAPDH) or 35 (except for GAPDH) cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and then a final extension at 72°C for 10 min. Five of the 30 μl of PCR products were separated by 1.5% agarose gel electrophoresis and visualized under ultraviolet after staining with ethidium bromide.

Western blotting. Western blotting was performed as previously described (24). Briefly, cells were lysed in SDS buffer, and total cellular proteins (~100 μg) were fractionated on 10% or 20–20% gradient SDS-polyacrylamide gels (Bio-Rad; Hercules, CA). They were transferred to a nitrocellulose membrane, and the membrane was incubated with rabbit anti-IL-33 antibody (at 1:1,000 dilution) overnight at 4°C. After incubation with peroxidase-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technologies, Beverly, MA), proteins were visualized by use of an ECL kit (GE Healthcare, Chalfont St. Giles, UK). The level of GAPDH was determined in a similar manner.

ELISA. Cell culture supernatants were harvested and stored at −80°C until use. The level of IL-33 in the culture supernatants was determined by ELISA using a commercial kit (Peprotech). The ELISA detection sensitivity was ≥32 pg/ml.

Immunohistochemical staining. The pancreas tissues were removed from patients undergoing operation for CP or pancreatic cancer and fixed by immersion in 4% paraformaldehyde overnight at 4°C. The specimens were embedded in regular paraffin wax and cut into 4-μm sections. Normal pancreatic tissues were taken from areas of peripheral tissue away from the tumor. Immunohistochemical staining for IL-33 was performed as previously described (22) by use of a streptavidin-biotin-peroxidase complex detection kit (Histofine Kit; Nichirei, Tokyo, Japan). Briefly, tissue sections were deparaffinized and rehydrated in PBS. Following antigen retrieval with the target retrieval solution (Dako, Glostrup, Denmark), endogenous peroxidase
activity was blocked by incubation with 0.3% hydrogen peroxide. After immersion in 10% normal goat serum, the sections were incubated with rabbit anti-IL-33 antibody (at 1:200 dilution) overnight at 4°C. The slides were incubated with biotinylated goat anti-rabbit IgG antibody, followed by peroxidase-conjugated streptavidin. Finally, the color was developed by incubating the slides for several minutes with diaminobenzidine (Dojindo, Kumamoto, Japan). For negative controls, primary antibody was replaced with nonspecific rabbit IgG, and nuclei were counterstained with hematoxylin.

Immunofluorescent staining. Immunofluorescent staining was performed as previously described (24). PSCs were directly plated on μ-slides (ibidi, Munich, Germany) and fixed in methanol for 10 min at −20°C. After blocking with 10% normal goat serum, cells were incubated with rabbit anti-IL-33 antibody (1:100 dilution) overnight at 4°C. After washing, the cells were incubated with Alexa Fluor488-labeled goat anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA) for 1 h. After washing, the cells were analyzed for fluorescence via an all-in-one type fluorescent microscope (BioZero BZ-8000; Keyence, Osaka, Japan). Nuclear counterstaining was performed with 4,6-diamidino-2-phenylinodole (DAPI).

Double immunofluorescent staining for IL-33 and α-SMA was performed as previously described (41). Briefly, tissue sections were deparaffinized and rehydrated in PBS. Following antigen retrieval with the target retrieval solution, the slides were blocked with 3% BSA and incubated with rabbit anti-IL-33 antibody (at 1:200 dilution) and mouse anti-α-SMA antibody (at 1:200 dilution) overnight at 4°C. After washing, the slides were incubated for 1 h with Alexa Fluor546-labeled goat anti-rabbit IgG antibody (at 1:200 dilution; Invitrogen) and Alexa Fluor488-labeled donkey anti-mouse IgG antibody (at 1:200 dilution; Invitrogen). After washing, the slides were analyzed for fluorescence by fluorescent microscopy. Nuclear counterstaining was performed with DAPI.

Cell Proliferation Assay

Cell proliferation was assessed using a commercial kit (Cell proliferation ELISA, BrdU; Roche Diagnostics) according to the manufacturer’s instructions. This is a colorimetric immunoassay based on the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation during DNA synthesis. Serum-starved PSCs were treated with IL-33 or PDGF-BB (at 25 ng/ml) overnight and labeled with BrdU for 3 h. Cells were fixed and incubated with peroxidase-conjugated anti-BrdU antibody. Then the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine was added, and BrdU incorporation was quantified by optical density (OD)370–OD492.

Cell Migration Assay

Cell migration was examined by the modified Boyden’s chamber assay using a commercial kit (QCM chemotaxis colorimetric cell migration assays; Millipore, Bedford, MA). Briefly, the cell suspension (2 ×
105 cells/well) was added to the culture inserts (8-µm pore) coated with type I collagen in a 24-well companion plate. The lower chamber included PDGF-BB (at 25 ng/ml) or IL-33 at the indicated concentrations. After 24-h incubation, the cell suspension in the upper chamber was aspirated, and the upper surface of the filter was carefully cleaned with cotton plugs. Cells that migrated through the polycarbonate membrane were stained, lysed, and quantified on a standard microplate at OD560.

**Cytokine Assay**

After 24-h incubation, cell culture supernatants were harvested and stored at −80°C until use. The levels of IL-8 and monocyte chemoattractant protein-1 in the culture supernatants were determined by ELISA via commercial kits (R&D Systems).

**Collagen Assay**

Collagen expression was determined by procollagen type I C-peptide (PICP) assay. PSCs were plated into 24-well plates and grown to confluence. After 48-h incubation with IL-33, the culture supernatants were harvested and stored at −80°C until use. The level of PICP was determined by use of an ELISA kit (Takara Bio).

**RNA Interference**

Human PSCs were plated on 96-well culture plates 1 day before transfection so that they would reach 60–70% confluence at the time of transfection. Cells were treated with a mixture of 60 pmol small interfering RNA (siRNA) (sc-75333; Santa Cruz Biotechnology) and 6 µl X-tremeGENE siRNA transfection reagent (Roche Diagnostics) in a volume of 150 µl according to the manufacturer’s instruction. Scramble siRNA (Santa Cruz Biotechnology) at the same concentration served as a control. Twenty-four hours after the transfection, cells were serum starved overnight and treated with PDGF-BB in serum-free medium for 16 h. Cells were then labeled with BrdU for 3 h, and cell proliferation was examined by using a commercial kit as described before.

**Statistical Analysis**

The results were expressed as means ± SD. Experiments were performed at least three times and similar results were obtained. Representative luminograms are shown. Differences between the groups were evaluated by ANOVA, followed by Fisher’s test for post hoc analysis. A P value less than 0.05 was considered statistically significant.

**RESULTS**

**Activated PSCs Expressed IL-33**

Human PSCs, isolated from the pancreas of patients undergoing operation for CP or pancreatic cancer, expressed typical activation markers including α-SMA, vimentin, type I collagen, and fibronectin (Fig. 1). We examined the expression of IL-33 in a primary culture of PSCs. Reverse transcription-PCR showed that human PSCs, cultured in serum-containing medium, expressed IL-33 mRNA (Fig. 2A). IL-33 mRNA expression was detectable but low in freshly isolated, quiescent rat PSCs. The IL-33 mRNA expression was increased in culture-activated PSCs (passage 2) (Fig. 2A). As previously reported (16), HUVECs expressed IL-33 mRNA. Western blotting of the whole cell lysates showed the IL-33 protein expression at ~30 kDa, corresponding to the size of pro-IL-33, in human PSCs (Fig. 2B). Immunofluorescent staining showed IL-33 expression in the nucleus of activated human and rat PSCs, as well as of HUVECs (16), cultured in serum-containing medium (Fig. 2C). Faint nuclear expression of IL-33 was noted in freshly isolated rat PSCs, which also expressed desmin, a marker of PSCs (25, 27, 32). These in vitro findings prompted us to examine whether PSCs expressed IL-33 in vivo. Immunohistochemical staining showed the expression of IL-33 in spindle-shaped cells in the area of pancreatic fibrosis in patients with CP or pancreatic cancer (Fig. 3). In agreement with the previous reports (7, 29), IL-33 expression was noted in the nucleus of endothelial cells (arrow in Fig. 3B). In addition, nuclear IL-33 expression was noted in the acinar and ductal cells in the resected normal pancreatic tissues (Fig. 4). Immunofluorescent staining showed IL-33 expression in the nucleus of α-SMA-positive cells (shown in purple) (Fig. 5). Thus activated PSCs expressed IL-33 in the nucleus both in vitro and in vivo.

**Proinflammatory Cytokines and Growth Factors Stimulated IL-33 Expression**

We next examined whether IL-33 expression was stimulated by proinflammatory cytokines and growth factors in PSCs. IL-1β, TNF-α, IFN-γ, and PDGF-BB, but not TGF-β1, increased IL-33 expression in pancreatic acinar and ductal cells. Immunohistochemical staining for IL-33 was performed in the resected normal pancreatic tissues taken from areas of the peripheral tissue away from the tumor. Nuclear expression of IL-33 was observed in pancreatic acinar (A) and ductal (C) cells. The control staining in a serial section of A and C using nonspecific rabbit IgG as a primary antibody is presented as B and D, respectively. Nuclei were counterstained with hematoxylin in B and D. Original magnification: ×200 (A and B) or ×400 (C and D).
expression as shown by Western blotting and real-time PCR in human PSCs (Fig. 6, A and B). IL-33 expression was induced by these proinflammatory cytokines and growth factors regardless of the origin of PSCs (isolated from CP or from pancreatic cancer). IL-1β, TNF-α, IFN-γ, and PDGF-BB, but not TGF-β1, increased IL-33 expression in rat PSCs, too (Fig. 6C). By immunofluorescent staining, faint IL-33 expression was observed in the nucleus of serum-starved control cells, and the expression was increased by IL-1β, TNF-α, and PDGF-BB (Fig. 6D). In our experimental setting, release of IL-33 into the culture media was below the detection level (≥32 pg/ml) of ELISA even after treatment with IL-1β, TNF-α, IFN-γ, or PDGF-BB, alone or in combination, for up to 72 h (data not shown).

Roles of Signaling Pathways in IL-1- and PDGF-Induced IL-33 Expression

Previous studies have shown that IL-1β and PDGF-BB induced the activation of a variety of signal transduction pathways including NF-κB, ERK, p38 MAP kinase, JNK, and phosphatidylinositol 3 kinase-Akt pathways (Ref. 25 and references therein). To clarify the roles of these signal transduction pathways in IL-33 expression, we employed specific inhibitors of these signaling pathways. We employed Bay11–7082 (at 5 μM, an inhibitor of NF-κB), U0126 (at 5 μM, an inhibitor of ERK pathway), SB203580 (at 25 μM, an inhibitor of p38 MAPK), SP600125 (at 10 μM, an inhibitor of JNK pathway), and wortmannin (at 100 nM, an inhibitor of phosphatidylinositol 3-kinase-Akt pathway). It has been previously shown that these inhibitors effectively inhibited the respective target pathways in PSCs at the concentrations used in this study (25). IL-1-induced IL-33 mRNA expression in human PSCs was almost completely inhibited by Bay 11–7082 and U0126, and partially by SB203580, whereas SP600125 and wortmannin were ineffective (Fig. 7A) Western blotting mirrored the results of real-time PCR (Fig. 7B). On the other hand, PDGF-induced IL-33 expression was almost completely inhibited by U0126, and partially by wortmannin, whereas SB203580, Bay11–7082, and SP600125 were ineffective (Fig. 7, C and D). Similarly, IL-1-induced IL-33 expression was almost completely inhibited by Bay 11–7082 and U0126 in rat PSCs, whereas PDGF-induced IL-33 expression was abolished by U0126 (data not shown). Collectively, IL-33 induction was differentially regulated depending on the type of stimuli (IL-1β and PDGF-BB).

Recombinant IL-33 Did Not Stimulate Proliferation or migration of PSCs

We then examined whether recombinant IL-33 affected the cell functions of PSCs. Recombinant IL-33 did not induce proliferation or migration in human PSCs, whereas PDGF-BB did (Fig. 8, A and B). Recombinant IL-33 did not stimulate the production of IL-8, monocyte chemoattractant protein-1, or
PICP (Fig. 8, C and D), whereas fibrinogen did as previously reported (22). IL-33 did not induce these cell functions regardless of the origin of PSCs (isolated from CP or from pancreatic cancer). IL-33 did not induce these cell functions in rat PSCs (data not shown). In addition, IL-33 did not induce the transformation of freshly isolated rat PSCs into myofibroblast-like cells (data not shown). Thus both of activated and quiescent rat PSCs did not respond to IL-33.

Fig. 6. Cytokines and growth factors increased IL-33 expression. Serum-starved human or rat PSCs were left untreated [control (Cont)], or treated with IL-1β (IL, at 2 ng/ml), TNF-α (TNF, at 10 ng/ml), PDGF-BB (PD, at 25 ng/ml), IFN-γ (IFN, at 100 ng/ml), or TGF-β (TGF, at 10 ng/ml) in serum-free medium. A and C: after 24 h, total cell lysates (~100 μg) were prepared from human (A) or rat (C) PSCs, and the levels of IL-33 and GAPDH were examined by Western blotting. B: after 6-h incubation, total RNA was prepared from human PSCs, and the levels of IL-33 and GAPDH were determined by real-time PCR. **P < 0.01 vs. control. C: after 24-h incubation, the expression of IL-33 in human PSCs was examined by immunofluorescent staining. Nuclei were counterstained with DAPI (blue), and merged images are also presented. Original magnification: ×200.

Fig. 7. Roles of signaling pathways in IL-33 expression. Serum-starved human PSCs were left untreated or treated with IL-1β (at 2 ng/ml) or PDGF-BB (at 25 ng/ml) in the absence or presence of inhibitors of signaling pathways. We employed Bay11–7082 (Bay, at 5 μM), U0126 (U, at 5 μM), SB203580 (SB, at 25 μM), SP600125 (SP, at 10 μM), and wortman-nin (W, at 100 nM). A and C: after 6-h incubation, total RNA was prepared, and the levels of IL-33 and GAPDH were determined by real-time PCR. *P < 0.05, **P < 0.01 vs. IL-1 or PDGF-BB only. B and D: after 24-h incubation, total cell lysates (~100 μg) were prepared, and the levels of IL-33 and GAPDH were examined by Western blotting.
Expression of ST2L, a Functional IL-33 Receptor, Is Low in PSCs

Lack of responsiveness to recombinant IL-33 might be due to the absence of a functional IL-33 receptor (ST2L) in human PSCs. We therefore examined the expression of the IL-33 receptor system in PSCs. Reverse transcription-PCR showed that both human and rat activated PSCs expressed mRNAs for ST2, and IL-1RAcP (Fig. 9A). ST2L mRNA expression could be detected in human PSCs by reverse transcription-PCR, but the level was lower compared with that in HUVECs, which express significant levels of ST2L (17). Western blotting revealed only a single band at ~52 kDa (Fig. 9B), which corresponds to the size of soluble ST2 in a reducing condition (18, 44). Thus the predominant ST2 isoform expressed in PSCs was soluble ST2, but not ST2L.

Pancreatic Acinar and Ductal Cells Expressed ST2

We also examined the expression of ST2 in the resected normal pancreatic tissues taken from areas of the peripheral tissue away from the tumor. ST2 expression was observed in pancreatic acinar, endothelial, and ductal cells (Fig. 10).

Knocking Down of IL-33 Expression by siRNA Resulted in Decreased PDGF-Induced Proliferation

We finally examined the role of IL-33 in PDGF-induced proliferation in PSCs. Transfection of IL-33 siRNA effectively inhibited PDGF-induced IL-33 expression (Fig. 11A). PDGF-induced proliferation was significantly decreased in IL-33 siRNA-transfected cells compared with cells transfected with control scramble siRNA (Fig. 11B). Thus IL-33 might regulate PDGF-induced proliferation in PSCs.

DISCUSSION

In this study, we examined the expression of IL-33, a recently identified member of the IL-1 gene family, in PSCs. We here showed that human and rat activated PSCs expressed IL-33, predominantly in the nucleus, in a similar manner to endothelial cells as previously reported (9, 16). IL-33 expression was increased by IL-1β, TNF-α, PDGF-BB, and IFN-γ, but not by TGF-β1. IL-33 expression was low in freshly isolated, quiescent rat PSCs but was increased upon activation, indicating that IL-33 induction was associated with the transformation to an α-SMA-positive myofibroblastic phenotype. This is along with previous findings that mechanical stress induced IL-33 expression in rat cardiac fibroblasts during their conversion into myofibroblasts (34). Similarly, IL-33 expression was not detected in fibroblasts from normal human tissues, but in α-SMA-positive fibroblastic reticular cells of lymphoid tissue (29).

Expression of the known IL-33 receptors ST2 and IL-1RAcP was additionally detected in PSCs, implying a role for IL-33 signaling within the pancreas in autocrine and/or paracrine manners. However, it should be noted that ST2L expression in PSCs was much lower than that in HUVECs, at least at the mRNA level. Western blotting using anti-ST2 antibody, which could detect all of soluble ST2, ST2L, and ST2V, demonstrated only a single band corresponding to the size of soluble ST2, indicating the low expression of ST2L in PSCs at both mRNA and protein levels. It has been shown that the amount
of ST2L expression is a determinant factor of the responsiveness to IL-33 (28), and recombinant IL-33 failed to affect key cell functions of PSCs in this study. In addition, we could not detect the IL-33 protein by ELISA in the culture supernatants even after the stimulation with cytokines and growth factors. This is in agreement with previous studies showing that IL-33 protein was poorly released into the culture supernatant (20, 42). The IL-33 level in the culture medium of TNF-α-stimulated adipocytes was less than 5 pg/ml (42). The IL-33 level in the culture supernatant of hepatic stellate cells stimulated with the combination of TNF-α, IL-1β, IL-6, and IFN-γ for 24 h was reportedly about 30 pg/ml (20), which was below the detection level of the ELISA used in our study. These results suggest that if IL-33 is secreted from PSCs (at levels below the sensitivity of the ELISA) it is likely to act locally, a scenario similar to that of another member of the IL-1 family, IL-18 (36). We here showed that pancreatic acinar and ductal cells expressed ST2, suggesting that IL-33 released from PSCs might act locally on these neighboring cells. But it remains unknown whether the ST2 expressed on these neighboring cells is functional, because the anti-ST2 antibody recognizes all the isoforms of ST2 including both functional (ST2L) and nonfunctional IL-33 receptors. Alternatively, IL-33 may predominantly act in an “intracrine” nonsecreted manner as a nuclear factor (9, 12). The predominant expression of IL-33 in the nucleus in PSCs supports this concept. The nuclear localization of IL-33 might be consistent with previous studies showing that IL-33 may act in a way analogous to alarmins IL-1α or HGMB1 (9, 13). IL-33 had previously been described in high endothelial venule endothelial cells as a nuclear protein and was thus called a nuclear factor from high endothelial venules (7). Nuclear IL-33 might exert unique biological activities independent of caspase cleavage and cell surface receptor binding (7, 9). Like other members of the IL-1 family such as IL-1α, IL-1β, and IL-18, IL-33 is expressed as a propeptide and lacks a typical leader sequence for protein secretion (4). The functions of nuclear IL-33 remain largely unknown, but accumulating evidence suggests that IL-33 may act as a nuclear repressor of the transcription of proinflammatory genes, thus promoting Th2 and anti-inflammatory phenotypes (13). Knocking down of IL-33 expression by siRNA resulted in decreased proliferation in response to PDGF-BB, suggesting that nuclear IL-33 expression might regulate
of liver fibrosis by CCl4, blocking of the IL-33/ST2 signaling with liver fibrosis in mice and humans (20). In a mouse model it has been shown that IL-33 overexpression was associated with fibrosis, but this system might exert profi-

vation of ERK pathway. PDGF-BB induced IL-33 expression mainly through the activation of ERK and NF-

factor expression is a determinant factor of the responsiveness to IL-33 (42). Given the important roles of IL-1β, TNF-α, IFN-γ, and PDGF in the development of pancreatic fibrosis (3, 5, 25, 27, 32), increased IL-33 expression in PSCs by these cytokines is likely to play a role in pancreatic fibrosis. IL-33 might play a role in CP through its actions on mast cells (43), an important mediator of the pathogenesis of CP (11). It has been shown that the total number of mast cells was significantly higher in CP than in the normal pancreas and positively correlated with the extent of fibrosis and the intensity of inflammation (11). Obviously, further studies, especially those using experimental models of pancreatitis, are required to clarify the pathophysiological roles of IL-33 in the course of pancreatitis.

During the preparation of this manuscript, Nishida et al. (31) reported that human pancreatic myofibroblasts expressed IL-

33, ST2L, and IL-1RαCP. But several of their results were different from those of our study. First, IL-33 expression was observed mainly in the cytoplasm and IL-33 protein was secreted in the study by Nishida et al., whereas in the present study IL-33 expression was predominantly found in the nucleus. Second, ST2L protein expression was significantly observed both in vivo and in vitro in the previous study, but the level of ST2L expression both at the mRNA and protein levels was low in our study. Third, mRNA expression of ST2L and IL-33 in quiescent rat PSCs was observed in the present study, but not in the previous study. Last, recombinant IL-33 induced proliferation and migration of human pancreatic myofibro-

blasts in the previous study, but IL-33 was ineffective in ours. The reasons for these discrepancies are unknown, but different preparations and characteristics of PSCs (termed “myofibro-

blasts” in the study by Nishida et al.) might be one explanation.

PDGF-induced proliferation in PSCs. Obviously, further studies are necessary to establish the functional roles of IL-33 in PSCs.

Little is yet known about the regulation of IL-33 induction, but the effects of cytokines vary depending on the cell types. IL-1α, IL-1β, IFN-γ, and TNF-α have been reported to increase IL-33 expression in synovial fibroblasts (43), hepatic stellate cells (20), leukocytes (35), and adipocytes (42), but not in cardiac fibroblasts (34). IL-1β and TNF-α did not induce IL-33 expression in human monocytic THP-1 cells (30), IL-1β, TNF-α, and VEGF downregulated IL-33 expression in HUVECs (7). In addition, we here showed for the first time that PDGF-BB, one of the most potent mitogens for PSCs in vitro (21), increased IL-33 expression in PSCs. Interestingly, the underlying molecular mechanisms for IL-33 induction were different between IL-1β and PDGF-BB: IL-1β induced IL-33 expression mainly through the activation of ERK and NF-κB, and partially through that of p38 MAP kinase, whereas PDGF-BB induced IL-33 expression mainly through the activation of ERK pathway.

Recent studies have described the association of the IL-33/ST2 system with fibrosis, but this system might exert profibrotic or antifibrotic effects according to the models examined. It has been shown that IL-33 overexpression was associated with liver fibrosis in mice and humans (20). In a mouse model of liver fibrosis by CCl4, blocking of the IL-33/ST2 signaling with the fusion protein ST2-Fc resulted in increased production of Th2 cytokines and enhanced liver fibrosis (2). Both ST2 and TGF-β levels were increased in bleomycin-induced lung fibro-

sis (38). IL-33/ST2 signaling was mechanically activated in cardiac fibroblasts, and recombinant IL-33 treatment reduced cardiac fibrosis and improved survival following pressure overload by transverse aortic constriction (34). Importantly, the overall functional consequences of the IL-33/ST2 signaling are complicated by the altered expression of ST2. Although soluble ST2 acts as an antagonistic decoy receptor for IL-33 and could inhibit IL-33 signaling (14, 34), the amount of ST2L expression is a determinant factor of the responsiveness to IL-33 (42). Given the important roles of IL-1β, TNF-α, IFN-γ, and PDGF in the development of pulmonary fibrosis (3, 5, 25, 27, 32), increased IL-33 expression in PSCs by these cytokines is likely to play a role in pancreatic fibrosis. IL-33 might play a role in CP through its actions on mast cells (43), an important mediator of the pathogenesis of CP (11). It has been shown that the total number of mast cells was significantly higher in CP than in the normal pancreas and positively correlated with the extent of fibrosis and the intensity of inflammation (11). Obviously, further studies, especially those using experimental models of pancreatitis, are required to clarify the pathophysiological roles of IL-33 in the course of pancreatitis.

To avoid preparation-dependent variance, we used PSCs from five independent preparations, all of which expressed typical activation markers of PSCs. Although there is much indirect evidence for an immunological role of IL-33 in vitro and in vivo, only a limited number of reports have described the detection of soluble, endogenous IL-33 (31, 34). Another explanation might be the different antibodies used in the respective studies. Of note, the anti-ST2L antibody used in the previous study is not specific to ST2L; it recognizes all three isoforms of ST2 including soluble ST2, ST2L, and ST2V. But several of their results were different from those of our study. First, IL-33 expression was observed mainly in the cytoplasm and IL-33 protein was secreted in the study by Nishida et al., whereas in the present study IL-33 expression was predominantly found in the nucleus. Second, ST2L protein expression was significantly observed both in vivo and in vitro in the previous study, but the level of ST2L expression both at the mRNA and protein levels was low in our study. Third, mRNA expression of ST2L and IL-33 in quiescent rat PSCs was observed in the present study, but not in the previous study. Last, recombinant IL-33 induced proliferation and migration of human pancreatic myofibro-

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Nuclear IL-33 expression was observed in pancreatic cancer cells as well as in PSCs. In addition, we have found that pancreatic cancer cell lines including BXPC3 expressed IL-33 (A. Masamune et al., unpublished observations). These findings suggest an unrecognized role of IL-33 in the progression of pancreatic cancer. On the other hand, elevated soluble ST2 levels have been found in sera of patients with systemic lupus...
erythematous, progressive systemic sclerosis, Wegener’s granulomatosis, and Behcet’s disease, suggesting a role for the IL-33/ST2 system in autoimmune diseases (18). It is of particular interest to see a role of the IL-33/ST2 system in autoimmune pancreatitis. Experiments along these lines are ongoing in our laboratory.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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