Acute pancreatitis (AP) is an inflammatory disease in which the regulatory pathways are not clearly elucidated. Activation of interleukin 1β (IL-1β) and immunomodulation via MyD88, the first signaling molecule in the ST2 pathway, seem to be involved. Because IL-33, the ST2 ligand, is an IL-1 family member and acts as an alarmin, we explored the ST2 pathway in human and mouse AP. Soluble ST2 was assayed by enzyme-linked immunosorbent assay (ELISA) in plasma of 44 patients admitted for AP. The levels of soluble ST2 increased early during AP and correlated with parameters of severity. Under two different experimental models of AP (ie, choline-deficient-ethionine-supplemented diet and cerulein injections), ST2-deficient mice (Il1rl1−/−) presented with more severe disease than wild-type mice, with increased activation of mast cells. In vitro, Il1rl1−/− bone-marrow-derived mast cells exhibited exacerbated degranulation, compared with the wild type. Flow cytometry identified mast cells as the main peritoneal population expressing ST2. Using immunohistochemistry and ELISA, we showed constitutive expression of IL-33 in murine pancreas and its release during experimental AP. Correlated with AP severity, increased soluble ST2 levels evoke involvement of the ST2 pathway in human AP. Furthermore, our experimental data suggest a protective role for ST2 during AP, highlighting the potential regulatory role of mast cells and the possibility of the ST2 pathway as a new therapeutic target in AP. (Am J Pathol 2012, 180:2330–2339; http://dx.doi.org/10.1016/j.ajpath.2012.03.009)
two isoforms of ST2 proteins: ST2L, a transmembrane form, and soluble ST2 (sST2), a secreted form that can serve as a decoy receptor by binding IL-33 and thus inhibiting its signaling through ST2L. Along with promoting a Th2 response, ST2 has been shown to sequester MyD88, the first signaling molecule activated by TLR4 and IL-1R1. This effect has identified ST2 as a key regulator in endotoxin tolerance.

Given that the IL-1 cytokine family is activated early in the course of AP, that speculation exists concerning secretion of alarmins during AP necrosis, and that MyD88 might regulate the AP inflammatory storm, we investigated whether the IL-33-ST2 pathway might be involved in AP in humans and in mice.

Materials and Methods

Patients

Plasma from 44 patients admitted to our institution for an episode of AP between 2005 and 2008 was studied. Inclusion criteria were, first, the taking of the blood sample less than 24 hours after onset of symptoms, and then at least two of the following: typical pain, amylase and lipase concentrations at least thrice the upper normal limit, and compatible modifications observed under imaging. Clinical characteristics of these patients are given in Table 1.

The etiologies of AP were biliary for 61% of the patients, alcoholic for 21%, and miscellaneous (including hypertriglyceridemia, unknown or familial etiology, and intraductal papillary mucinous tumor) for 18%. Patients with chronic pancreatitis were excluded. Necrotizing pancreatitis was defined by the absence of enhancement of pancreatic tissue on contrast-injected computed tomography or magnetic resonance imaging. Severe AP was defined using the Atlanta classification, involving the presence of one of the following at any time during the AP course: i) failure of one or more organ systems (respiratory, renal, gastrointestinal, circulatory), disseminated intravascular coagulation, or hypocalcemia ≥ 7.5 mg/dL; ii) occurrence of one or more local complications (pancreatic necrosis, pseudocyst, or abscess); or iii) a score of ≥ 3 on Ranson’s criteria or a score of ≥ 8 on the Acute Physiology and Chronic Health Evaluation II scoring system (APACHE II). Blood samples were taken on the day of admission and were repeated 1, 2, 7, and 30 days later. Sixteen healthy volunteers with no history of pancreatic disease were recruited and served as control. Plasma was stored at −20°C until the assay. The study was performed after approval of the Erasmus Hospital ethics committee. Written informed consent was obtained from each participant (see NCT01315613 at http://www.clinicaltrials.gov).

Mouse Models of Acute Pancreatitis

Wild-type (WT) and ST2-deficient (Il1rl1−/−) mice backcrossed with the BALB/c strain for six generations were housed in conventional facilities. For the first AP model, after overnight fasting, 6-week-old female mice were fed a choline-deficient ethionine-supplemented (CDE) diet (MP Biomedicals, Solon, OH) for 72 hours. For the second pancreatitis model, female mice were injected hourly with 50 µg/kg cerulein i.p. (Sigma-Aldrich, St Louis, MO) or vehicle for 10 hours. At the end of the experiments or at 0, 48, or 72 hours for the CDE diet, mice were sacrificed by cervical dislocation. Blood was sampled, and sera were stored at −20°C until assays (hydrolyase measurements in both models; assessment of plasmatic levels of trypsin, IL-33, and IL-6 in the CDE model). Pancreatic glands were excised and fixed in formaldehyde for histological assessment.

Assessment of Severity of Experimental Acute Pancreatitis

Paraffin-embedded pancreatic sections were stained with H&E and examined in a blinded manner by two independent investigators (R.O. and P.D.) The severity of AP was graded by a semiquantitative assessment of edema, inflammatory cell infiltrate, acinar necrosis, and vacuolization. The scoring system used in the cerulein model (Table 2) is adapted from Moreno et al. In the CDE diet model, the grading refers to a five-point scale ranging from 0 (absent) and 1 (minimal) to 4 (maximal). For necrosis and intracellular vacuolization, this grading

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample size</td>
<td>n = 44</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54 (17–86)</td>
</tr>
<tr>
<td>Sex, male/female (%)</td>
<td>43/57</td>
</tr>
<tr>
<td>BMI (kg·m−2)</td>
<td>26.8 (18.6–59)</td>
</tr>
<tr>
<td>Etiology [no. (%)]</td>
<td></td>
</tr>
<tr>
<td>Biliary</td>
<td>27 (61)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>9 (21)</td>
</tr>
<tr>
<td>Other</td>
<td>8 (18)</td>
</tr>
<tr>
<td>Time of inclusion after onset of symptoms (hours)</td>
<td>16 (6–24)</td>
</tr>
<tr>
<td>Time from onset of symptoms to CT/MRI (hours)</td>
<td>15 (2–120)</td>
</tr>
<tr>
<td>Necrotizing/interstitial AP (%)</td>
<td>20/80</td>
</tr>
<tr>
<td>CTSI or MRSI</td>
<td>3 (0–10)</td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>33</td>
</tr>
<tr>
<td>&lt;30%</td>
<td>4</td>
</tr>
<tr>
<td>30% to 50%</td>
<td>1</td>
</tr>
<tr>
<td>&gt;50%</td>
<td>2</td>
</tr>
<tr>
<td>Not available</td>
<td>4</td>
</tr>
<tr>
<td>MOF [no. (%)]</td>
<td>1 (2)</td>
</tr>
<tr>
<td>SIRS [no. (%)]</td>
<td>22 (50)</td>
</tr>
<tr>
<td>Ranson’s score* at 48 hours</td>
<td>2 (0–7)</td>
</tr>
<tr>
<td>CRP at admission (mg/dL)</td>
<td>1.2 (0–24)</td>
</tr>
<tr>
<td>CRP at 24 hours (mg/dL)</td>
<td>13.5 (0.1–35)</td>
</tr>
<tr>
<td>CRP at admission [no. (%)]</td>
<td></td>
</tr>
<tr>
<td>ICU admission</td>
<td>8 (18)</td>
</tr>
<tr>
<td>Length of hospitalization (days)</td>
<td>7 (3–65)</td>
</tr>
<tr>
<td>Death [no. (%)]</td>
<td>1 (2)</td>
</tr>
<tr>
<td>Classification† mild/severe [no. (%)]</td>
<td>17/27 (38.6/61.4)</td>
</tr>
</tbody>
</table>

Data are presented as the median (range) or percent of total. A severity of acute pancreatitis according to the Atlanta classification. BMI, body mass index; CRP, C-reactive protein; CT, computed tomography; CTSI, CT severity index; ICU, intensive care unit; MOF, multiple organ failure; MRI, magnetic resonance imaging; MRSI, MRI severity index; SIRS, systemic inflammatory response syndrome.
refers to an approximate percentage of cells involved: 0 = absent; 1 = 5% to 15%; 2 = 15% to 35%; 3 = 35% to 50%; and 4 = 50%. Severity items were calculated as mean score value/10 pancreatic fields at 200 magnification.

Amylase and lipase levels were quantified using automated chromogenic assays with commercially available kits (Roche/Hitachi, Roche Diagnostics, Mannheim, Germany).

**Immunohistochemistry and Mast Cell Staining**

Formalin-fixed, paraffin-embedded pancreas tissue sample sections (5 μm thick) were deparaffinized in xylene. Epitope retrieval was achieved by boiling the sections in EDTA buffer (pH 9) in a microwave oven (600 W; 2 × 5 minutes with 5 minutes rest between the two). Endogenous peroxidases were quenched with 30% H2O2 (Merck, Darmstadt, Germany) in methyl alcohol for 30 minutes. Nonspecific background staining was inhibited by 10 minutes incubation with 5% horse serum (Vector Laboratories, Burlingame, CA). Avidin-biotin blocking reagents were used for 30 minutes to block endogenous biotin. Sections were incubated for 1 hour at room temperature with polyclonal goat anti-human IL-33 antibody (5 μg/mL) (AF3626; R&D systems, Abingdon, UK). Thereafter, sections were incubated with biotin-conjugated secondary antibody (anti-goat IgG, 1:200; Vector Laboratories). Immunoreactivity was visualized by avidin-biotin-peroxidase complex (ABC) kit reagents (Vector Laboratories) and 3,3′-diaminobenzidine (BioGenex, Fremont, CA) in 50 mmol/L Tris-buffer (pH 7.6) containing 0.3% H2O2. Finally, sections were weakly counterstained with hematoxylin. Normal goat IgG was used as a negative control.

Toluidine blue staining was used to identify mast cells. Consecutive pancreas tissue samples were deparaffinized in xylene and stained with a solution containing 0.1% toluidine blue (Sigma-Aldrich), 4% formaldehyde, and 1% acetic acid (pH 2.8) for 20 minutes. After a washing, slides were incubated in ethanol 95% until mast cells appeared deep red.

**RNA Extraction and RT-PCR**

Total RNA was extracted from freshly lysed pancreas using a commercially available kit (TriPure; Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. Preparation of cDNA and PCR for ST2, IL33, and hypoxanthine-guanine phosphoribosyltransferase (HPRT) genes were performed using standard procedures. Reactions were incubated in a DNA thermal cycler for 33 cycles for ST2 and IL33 (denaturation, 20 seconds at 94°C; annealing, 20 seconds at 58°C; extension, 30 seconds at 72°C). Thereafter, PCR products were loaded onto a FlashGel system (Lonza, Rockland, ME). Sense and antisense PCR primer sequences were as follows: HPRT, 5′-ATGGACAGGACTGAAAGA-3′ and 5′-AATGACACAAACGTGATTC-3′; ST2, 5′-GGTGAAGCAAGAATTCAAGAAG-3′ and 5′-CAGG-GGTGAAGCAAGAATTCAAGAAG-3′.

### Table 2. Histological Scoring System Used to Assess the Severity of Acute Pancreatitis Induced by Cerulein Injection

<table>
<thead>
<tr>
<th>Scoring</th>
<th>Indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edema</td>
<td>0 Absent</td>
</tr>
<tr>
<td></td>
<td>1 Focally increased between lobules</td>
</tr>
<tr>
<td></td>
<td>2 Diffusely increased between lobules</td>
</tr>
<tr>
<td></td>
<td>3 Acini disrupted and separated</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory cell infiltrate</td>
<td>0 Absent</td>
</tr>
<tr>
<td></td>
<td>1 Rare or around ductal margins</td>
</tr>
<tr>
<td></td>
<td>2 In the parenchyma (&lt;50% of the lobules)</td>
</tr>
<tr>
<td></td>
<td>3 In the parenchyma (&gt;50% of the lobules)</td>
</tr>
<tr>
<td>Necrosis</td>
<td>0 Absent</td>
</tr>
<tr>
<td></td>
<td>1 Architectural changes, pyknotic nuclei</td>
</tr>
<tr>
<td></td>
<td>2 Focal necrosis (&lt;10% of the parenchyma)</td>
</tr>
<tr>
<td></td>
<td>3 Diffuse parenchymal necrosis (&gt;10% of the parenchyma)</td>
</tr>
</tbody>
</table>

Adapted from the scoring system of Moreno et al.18
TAACAGGTCTCTCCATA-3’; and IL-33, 5’-GGGTAC- 
CAAGCATGAAGA-3’ and 5’-TGCATTCAATGAAA- 
CAAGTC-3’.

Peritoneal Cell Cytologic Analysis

Six WT and five Il1rl1−/− mice were injected with 10 mL 
i.p. of saline solution, and the cell suspension was col-
clected for flow cytometry analysis of ST2 membranous 
expression. In addition, 2 × 10^6 cells were centrifuged 
ono a glass slide for toluidine blue staining and confir-
mation of the presence of mast cells as deep red cells 
under light microscopy. Surface staining was performed 
using fluorescein-isothiocyanate-labeled anti-ST2 (clone 
DJ8), allopocyanin-labeled anti-c-kit (clone 2B8), 
anti-CD4 (clone RM4-5), anti-CD11b (clone M1/70), and 
phycoerythrin-labeled anti-FcεRI (clone MAR-1), anti-
CD3 (clone 145-2C11), anti-Ly6G (clone 1A8), and anti-
F4/80 (clone BM8). Fluorochrome-conjugated antibodies 
were purchased from MD Biosciences (Zürich, Switzer-
land), eBioscience (Vienna, Austria), or BD Biosciences 
(Erembodegem, Belgium). Cell acquisition was per-
fomed using a FACS Calibur flow cytometry system, and 
analyses were done with CellQuest Pro software 4.0 (BD 
Biosciences).

Bone-Marrow-Derived Mast Cells

Bone-marrow-derived mast cells (BMMCs) were isolated 
according to a previously published protocol.21 Briefly, 
BMMCs were obtained by culturing femoral bone marrow 
cells from WT and Il1rl1−/− mice in Iscove’s modified 
Dulbecco’s medium (Lonza) containing 10% FBS, 100 
IU/mL penicillin, 100 μg/mL streptomycin, 250 ng/mL 
amphotericin B (Gibco; Life Technologies, Paisley, UK), 
and 5 × 10−5 mol/L β-mercaptoethanol. Nonadherent 
cells were cultured for 6 to 12 weeks in the presence of 
IL-3 (5 ng/mL) and stem cell factor (SCF) (50 ng/mL; 
Invitrogen-Life Technologies, Paisley, UK). Thereafter, 
cell purity was controlled by flow cytometry, revealing

Figure 2. CDE diet model in WT mice and ST2- 
deficient Il1rl1−/− mice (ST2−/−). Serum amy-
lase (A) and lipase (B) levels were measured in 
mice exposed to the CDE diet for 0, 48, and 72 
hours. Data are presented as means ± SEM of 
pooled data of 10 (baseline) to 24 (CDE group) 
mice per group from seven independent exper-
iments. C: H&E staining for histological assess-
ment of the pancreas after 72 hours of CDE diet 
in WT and Il1rl1−/− mice. Original magnifica-
tion, ×200. D: Semiquantification of histological 
severity of pancreatitis in WT (n = 24) and 
Il1rl1−/− (n = 15) mice fed a CDE diet for 72 
hours. E, edema; I, inflammation; N, necrosis; V, 
vacuolization. Data are presented as pooled 
scores from five independent experiments. E: 
IL-6 levels assessed by ELISA in serum of WT and 
Il1rl1−/− mice during CDE diet-induced AP. 
Data are presented as means ± SEM of pooled 
data of 15 (baseline) to 33 (CDE group) mice per 
group from nine independent experiments. 
*P < 0.05 versus baseline; †P < 0.01 WT versus 
Il1rl1−/− at 72 hours (A and B). *P < 0.05 WT 
versus Il1rl1−/−; **P < 0.01 WT versus 
Il1rl1−/− (D). †P = 0.001 WT versus Il1rl1−/− 
at 72 hours (E).
>90% of c-kit<sup>high</sup> FcεRI<sup>high</sup> cells, considered as differentiated mast cells. For <i>in vitro</i> experiments, 10<sup>6</sup> BMMCs/well were cultured for 24 hours with lipopolysaccharide (LPS) (1 μg/mL; <i>E. coli</i> serotype 055:B5; Sigma-Aldrich) or with mouse rIL-33 (10 ng/mL; R&D Systems, Minneapolis, MN). Tryptase activity in BMMC culture supernatants and in mouse sera was assayed with a mast cell degranulation assay kit (Merck Millipore, Darmstadt, Germany) according to the manufacturer’s protocol, in which calcium ionophore was used as an inducer of degranulation and protamine as an inhibitor of it.

**Pancreas Cell Preparation**

Acinar cells were isolated as described previously, with slight modifications. In overnight-fasted WT mice not receiving any treatment, pancreata were dissected free of mesenteric fat, minced in Hank’s buffered saline solution with 5 mmol/L EDTA solution at 37°C, and centrifuged for 2 minutes at 500 × g twice to eliminate fat. The tissues were rinsed in Waymouth’s medium (Invitrogen-Life Technologies) containing penicillin and streptomycin (0.1 mg/mL; Lonza), 20% FBS, and 0.01% soybean trypsin inhibitor (Sigma-Aldrich) and were digested by means of two cycles of shaking at 25 cycles/minute in a solution of 6.25 U/mL collagenase in Hank’s buffered saline solution containing 0.2% bovine serum albumin at 37°C. Nondigested tissue was eliminated by 70-μm pore filtration, and isolated cells were washed twice in complete Waymouth’s medium. Thereafter, acinar cells were isolated on 60%/20% Ficoll gradient centrifugation and washed twice in Ham’s F-12K (Kaighn’s) medium (Invitrogen-Life Technologies) containing 10% FCS, 0.5% bovine serum albumin, 0.01% soybean trypsin inhibitor, and 0.1 mg/mL penicillin and streptomycin, leading to an acinar cell suspension of small aggregates. Acinar cells (5 × 10<sup>6</sup> cells/mL) from WT mice were sonicated, and IL-33 was measured by ELISA in the lysed cell suspension.

**Statistical Analysis**

Data are expressed as median and range (minimum to maximum). Multiple comparisons were performed with the Kruskal-Wallis test. The Mann-Whitney <i>U</i>-test was used for post hoc analysis. Nonparametric correlations were calculated with the Spearman’s test; comparisons between paired data were calculated with the Wilcoxon test. Calculations were performed with SPSS 15.0 software (IBM SPSS, Chicago, IL). The level of statistical significance was set at <i>P</i> < 0.05.

**Results**

**In Humans, sST2 Levels Increase Early during AP and Correlate with AP Severity**

On the day of admission (day 0), sST2 levels were dramatically increased in the plasma of patients with AP (2924 pg/mL; range, 87 to 2 × 10<sup>6</sup>), compared with healthy subjects (56.5 pg/mL; range, 0 to 546; <i>P</i> < 0.001). These levels remained elevated during the early AP period and decreased to near-normal ranges 30 days after the acute episode in patients (119 pg/mL; range, 0 to 1267; <i>P</i> = 0.06 versus healthy subjects) (Figure 1).

On the first day after admission (day 1), plasma sST2 levels were higher in patients who developed necrotizing pancreatitis than in those with edematous AP (5584 pg/mL; range, 215 to 2 × 10<sup>6</sup>) versus 1147 pg/mL (range, 15 to 2 × 10<sup>6</sup>; <i>P</i> < 0.05). Furthermore, sST2 plasma levels on day 1 correlated significantly with the need for intensive
care unit admission, length of hospital stay, and the presence of severe AP ($p = 0.316, 0.313,$ and $0.358$, respectively).

**ST2-Deficient Mice Present More Severe AP**

Given that we had demonstrated activation of the ST2 pathway in humans during AP, and to gain further insight into its possible role in AP, we submitted WT and Il1rl1 mice to two experimental models of AP. Severity of AP was evaluated both in the CDE diet-induced AP model (Figure 2) and in the cerulein model (Figure 3).

After 72 hours of a CDE diet, Il1rl1 mice had significantly higher levels of hydrolases than WT mice [amylase, 19,620 IU/L (range, 7272 to 55,132) versus 7740 IU/L (range, 1300 to 19,492) ($p < 0.001$); lipase, 1180 IU/L (range, 280 to 4884) versus 128 IU/L (range, 32 to 1352) ($p < 0.001$)] (Figure 2, A and B). Pancreatic histological assessment revealed more severe necrosis, inflammation, and edema in Il1rl1 mice than in WT mice (Figure 2, C and D). In addition, 72 hours after the CDE diet, IL-6 concentrations were significantly higher in sera of Il1rl1 mice than in WT mice (Figure 2E).

In line with these results, Il1rl1 mice also presented more severe disease after 10 hourly cerulein injections. Their hydrolase levels were significantly higher than those of WT mice [amylase, 17,424 IU/L (range, 6336 to 49,196) versus 8856 IU/L (range, 4080 to 36860) ($p < 0.01$); lipase, 626 IU/L (range, 128 to 3260) versus 186 IU/L (range, 64 to 2260) ($p < 0.01$)] (Figure 3, A and B). Il1rl1 mice presented higher edema, inflammation, and necrosis histological scores than WT mice (Figure 3, C and D).

**Peritoneal Mast Cells Express ST2**

In normal murine pancreas, expression of ST2 mRNA was at the limit of detection, compared with clear expression of its ligand IL-33 (Figure 4A). We therefore looked outside the pancreas for the presence of the ST2 receptor. Peritoneal cells were obtained by peritoneal lavage of WT and Il1rl1 mice and assessed for ST2 expression by flow cytometry. We looked for ST2 expression on WT peritoneal cells using Il1rl1 cell suspension as negative control. We observed that 1.9% (range, 1.1% to 2.2%) of all peritoneal cells were ST2+. Furthermore, gating on this ST2+ cell population, we could not detect any CD11b$^+$ F4/80$^+$ Ly6G$^+$ neutrophils, nor any CD11b$^+$ F4/80$^+$ Ly6G$^+$ macrophages. However, a small minority (1.8%; range, 1.7% to 1.8%) was CD3$^+$ CD4$^+$ (T lymphocytes) but a large majority (98.0%; range, 97.4% to 98.4%) was c-kit$^+$ FceRI$^+$ (mast cells) (Figure 4B).

**Mast Cells Are Activated during AP, and Their ST2 Receptor Is Involved in the Degranulation Process**

With toluidine blue staining, we confirmed the presence of a few mast cells in pancreatic interlobular spaces at baseline and their increase in peripancreatic peritoneal tissue during the course of CDE diet-induced AP (Figure 5A). To assess mast cell activation during experimental AP, we measured the tryptase concentration in sera of mice submitted to the CDE diet model.23 Tryptase levels

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**Figure 4. ST2 and IL-33 mRNA expression. A: HPRT (housekeeping gene), ST2, and IL-33 mRNA constitutive pancreatic expression was detected by classical RT-PCR on freshly isolated WT and Il1rl1 mice. Each analysis was performed in duplicate. B: Flow cytometry analysis of peritoneal cells isolated from mice focusing on c-kit$^+$ FceRI$^+$ cells (mast cells) (left panel) shows their ST2 expression in WT mice but not in the negative control Il1rl1 mice (right panel). FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allophycocyanin.**
increased during AP in WT mice, but the increase was significantly greater in \textit{Il1rl1} \textsuperscript{+/+} mice (Figure 5B), confirming mast cell activation in AP.

Given that mast cell activation is higher in \textit{Il1rl1} \textsuperscript{+/+} mice, we studied their degranulation potential by measuring tryptase in the supernatants of BMMCs generated from WT and \textit{Il1rl1} \textsuperscript{+/+} mice. Flow cytometry analysis of isolated BMMCs confirmed their mast cell phenotype (c-kit/Flt3/PE, phycoerythrin; APC, allophycocyanin). Tryptase activity was measured in the supernatants of BMMCs. Degranulation was either stimulated (S) by calcium ionophore (10 nmol/L) or inhibited (I) by protamine (100 nmol/L). Data are presented as mean ± SEM of pooled data from four independent experiments. *P < 0.01 versus baseline; †P < 0.05 WT versus \textit{Il1rl1} \textsuperscript{+/+} mice (B).

Recent reports suggest a regulatory role for IL-33 in the mast cell degranulation process.\textsuperscript{24,25} In the present in vitro experiments, however, we could not observe this effect, because incubation of BMMCs with IL-33 did not modify tryptase levels detected in supernatants either in \textit{Il1rl1} \textsuperscript{+/+} BMMCs or in WT BMMCs (data not shown). Nevertheless, because degranulation and production of proinflammatory cytokines by stimulated BMMCs are two independent processes,\textsuperscript{9,26} we confirmed their normal activity by using LPS or IL-33 as stimulators and positive controls. In agreement with previous findings,\textsuperscript{26} LPS did induce IL-6 and IL-13 secretion by mast cells independent of ST2. Moreover, as Moulin et al\textsuperscript{27} reported, IL-33 alone was able to induce proinflammatory cytokine production by WT BMMCs, but not by \textit{Il1rl1} \textsuperscript{+/+} BMMCs (Figure 6, C and D).

\textbf{In Mice, IL-33, the ST2 Ligand, Is Expressed in the Pancreas at Baseline and Is Released during AP}

Both WT and \textit{Il1rl1} \textsuperscript{+/−} pancreas exhibited constitutive IL-33 mRNA expression (Figure 4A) without any quantitative difference. Immunohistochemistry confirmed constitutive pancreatic IL-33 expression localized in the cytoplasm of acinar cells (Figure 6A). Furthermore, after isolation and sonication of \(5 \times 10^6\) acinar cells/mL, we measured IL-33 in the lysed cell suspension by ELISA at 37 pg/mL, confirming acinar cell IL-33 production at the protein level.

After 48 and 72 hours of the CDE diet leading to AP in mice, IL-33 concentrations rose significantly in the serum [control diet, 43.3 pg/mL (range, 0 to 169.2); 48-hour CDE diet, 128.6 pg/mL (range, 53.1 to 397.3); 72-hour CDE diet, 427 pg/mL (range, 18 to 1707); P < 0.001 versus control] (Figure 6B).

In the present in vitro experiments, however, we could not observe this effect, because incubation of BMMCs with IL-33 did not modify tryptase levels detected in supernatants either in \textit{Il1rl1} \textsuperscript{+/−} BMMCs or in WT BMMCs (data not shown). Nevertheless, because degranulation and production of proinflammatory cytokines by stimulated BMMCs are two independent processes,\textsuperscript{9,26} we confirmed their normal activity by using LPS or IL-33 as stimulators and positive controls. In agreement with previous findings,\textsuperscript{26} LPS did induce IL-6 and IL-13 secretion by mast cells independent of ST2. Moreover, as Moulin et al\textsuperscript{27} reported, IL-33 alone was able to induce proinflammatory cytokine production by WT BMMCs, but not by \textit{Il1rl1} \textsuperscript{+/−} BMMCs (Figure 6, C and D).
Discussion

The present study reports for the first time the involvement of the ST2 receptor in the severity of acute pancreatitis. The ST2 pathway is activated early in the course of AP in humans and correlates with severity of the disease. Furthermore, ST2-deficient mice developed more severe pancreatitis in two independent experimental models, suggesting a protective role of the pathway during AP. In addition, we showed that mast cells express ST2 and that the receptor seems to be involved in their degranulation process. Finally, we showed that IL-33, the ST2 ligand, is expressed by pancreatic acinar cells and is released during AP.

High levels of sST2 are described in several human illnesses. Soluble ST2 is a powerful predictor of mortality in heart failure and myocardial infarction, and is proposed as a novel biomarker in cardiovascular diseases. Although the biological function of elevated sST2 in patients with AP is still uncertain, our in vivo murine data suggest a protective pathogenic role for ST2, rather than simply that of a biomarker.

ST2 exists in two isoforms and both can exert protective functions. The use of sST2-Fc fusion protein protects mice in several inflammatory experimental models, suggesting an anti-inflammatory role for sST2 as a decoy receptor binding IL-33. On the other hand, Brint et al identified the transmembrane form as a negative regulatory component of IL-1R/TLR4 signaling, inhibiting LPS-induced production of proinflammatory cytokines. In the present study, we also observed a protective role for ST2 in AP; ST2-deficient mice exhibited more severe disease than WT in two different experimental models. Pancreatic expression of TNF-α, IL-1β, IL-6, and IL-13 did not differ between WT and Il1rl1−/− mice in the course of AP (data not shown). In addition to hydrolase measurements, pancreatic histological scoring, and serum IL-6 levels indicating greater severity of pancreatitis in Il1rl1−/− mice, we also observed elevated serum concentrations of tryptase in these knockout mice. Given that tryptase is a reflection of mast cell activation, and that these cells are known to be involved in the physiopathology of pancreatitis, it seems unlikely that these levels are simply another marker of severity; rather, they probably indicate activation of mast cells during our experimental model of AP, and even more in Il1rl1−/− mice. Moreover, mast cells are known to express ST2. In the present study, we not only identified the peripancreatic location of mast cells, but also showed that they were the main cell population in the peritoneal cavity to express ST2.

Mast cell degranulation, associated with tryptase release, is an early event in AP in humans and in rodent models. Several reports have suggested a regulatory role for IL-33, the ST2 ligand, in this process. In the present work, however, and in accord with reports of others, we were unable to demonstrate this regulatory role, although BMMCs were normally responsive to IL-33 in terms of cytokine production. Nonetheless, the constitutive presence or absence of

Figure 6. Pancreatic and plasma expression of IL-33. A: Immunohistochemical expression of IL-33 in the pancreas of control WT mice. IL-33 (left) and control isotype (right) immunostaining reveals acinar cell IL-33 expression. Original magnification, ×200. B: ELISA quantification of serum IL-33 levels after the indicated time of CDE diet in mice. C and D: Inflammatory activity of BMMCs isolated from WT and Il1rl1−/− mice was evaluated by ELISA quantification of IL-6 (C) and IL-13 (D) production in supernatants of BMMCs nonstimulated (NS) or stimulated by LPS or rIL-33. Data are presented as mean ± SEM of pooled values of seven independent experiments, each comprising three to nine mice per group (B), or of duplicate data representative of three independent experiments (C and D). **P < 0.001 versus control group. †P < 0.05 48 hours versus 72 hours of CDE diet.
ST2 itself seemed to be implicated in mast cell activation, because we demonstrated that \( l11\text{rt}^{+/−} \) BMMCs degranulate more than WT BMMCs. In addition, these data were consistent with our in vivo experiments, in which the trypase concentration increased in sera of mice during CDE-induced AP, and significantly more in \( l11\text{rt}^{+/−} \) mice. We therefore speculate that ST2 plays a key role in regulation of the severity of pancreatitis.

IL-33 has been reported to be expressed mainly by endothelial cells, fibroblasts, adipocytes, synovium, and more recently and specifically, by pancreatic myofibroblasts and cardiac fibroblasts. In the present study, we showed that pancreatic acinar cells constitutively express IL-33 mRNA and protein, and that AP is associated with an increase in the serum concentration of IL-33 in mice. In humans, however, IL-33 was detectable in only 9 of the 44 patients and did not correlate with AP severity (data not shown). Functions and secretion mechanisms of this newly identified cytokine are not yet fully elucidated. In asthma, arthritis, and anaphylactic shock, exogenous IL-33 was shown to exacerbate the disease through ST2 activation. In contrast, activation of ST2 leading to sequestration of MyD88 or inhibition of cardiomyocyte hypertrophy suggests a protective role for IL-33. It has been described as a double agent, acting in the nucleus as an inhibitor of NF-κB and an inhibitor of NF-kB-dependent gene expression, or in a paracrine manner as an alarmin.

Given that IL-33 is constitutively expressed in the pancreas and that its levels increase in serum during AP, our results suggest that IL-33 is implicated in AP and is more likely to act as an endogenous danger signal, like high mobility group protein B1 (HMGB1). Recently, Enoksson et al. identified mast cells as important contributors to early cell injury responses and acute inflammation. They confirmed the central role played by IL-33 released by necrotizing cells in mast cell proinflammatory activation. Moreover, two recent reports strongly implicate IL-33 involvement in human chronic pancreatitis. One study showed that pancreatic stellate cells express IL-33 and ST2, and the other reported the expression of these molecules by pancreatic myofibroblasts. Because we demonstrated that the ST2 pathway is protective in the acute phase of pancreatitis, we speculate that, as the disease evolves to a chronic form, this pathway might become noxious by promoting fibrosis, but this will require confirmation in vivo.

In conclusion, we have demonstrated for the first time the involvement of the IL-33-STAT2 pathway in the pathophysiology of AP in humans, with a rise in sST2 levels in human serum during AP and its correlation with disease severity. In mice, we show that ST2 plays a protective role in AP and is expressed by peritoneal mast cells, which are activated during AP. Finally, we demonstrate that IL-33, the ST2 ligand, is expressed by murine pancreatic acinar cells and is released during AP. Thus, the IL-33–ST2 pathway might constitute a new therapeutic target for modulating immune activation during the AP inflammatory storm.

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References