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The IL-33/ST2 Pathway Controls Coxsackievirus B5–Induced Experimental Pancreatitis

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Coxsackievirus B (CVB) is a common cause of acute and chronic infectious myocarditis and pancreatitis. Th1 cells producing IFN-γ and TNF-α are important for CVB clearance, but they are also associated with the pathogenesis of inflammatory lesions, suggesting that the modulation of Th1 and Th2 balance is likely important in controlling CVB-induced pancreatitis. We investigated the role of IL-33, which is an important recently discovered cytokine for induction of Th2-associated responses, in experimental CVB5 infection. We found that mice deficient in IL-33R, T1/ST2, significantly developed more severe pancreatitis, had greater weight loss, and contained higher viral load compared with wild-type (WT) mice when infected with CVB5. Conversely, WT mice treated with rIL-33 developed significantly lower viral titers, and pancreatitis was attenuated. Mechanistic studies demonstrated that IL-33 enhances the degranulation and production of IFN-γ and TNF-α by CD8^{+} T and NK cells, which is associated with viral clearance. Furthermore, IL-33 triggers the production of IL-4 from mast cells, which results in enhanced differentiation of M2 macrophages and regulatory T cells, leading to the attenuation of inflammatory pancreatitis. Adoptively transferred mast cells or M2 macrophages reversed the heightened pancreatitis in the T1/ST2^{−/−} mice. In contrast, inhibition of regulatory T cells exacerbated the disease in WT mice. Together, our findings reveal an unrecognized IL-33/ST2 functional pathway and a key mechanism for CVB5-induced pancreatitis. These data further suggest a novel approach in treating virus-induced pancreatitis, which is a major medical condition with unmet clinical needs.   *The Journal of Immunology*, 2013, 191: 000–000.

Coxsackieviruses are nonenveloped, icosahedral viruses with a single-stranded positive RNA genome that belong to the *Picornaviridae* family and enterovirus genus. Coxsackieviruses are highly diverse and circulate worldwide. There are 23 known serotypes of coxsackieviruses A (CVA1-23) and 6 serotypes of coxsackieviruses B (CVB1-6) (1).

CVBs are highly cytolytic and are one of the most common causes of acute infectious myocarditis (2, 3). CVBs also infect pancreatic acinar cells, often leading to pancreatic deficiency (4). Both mild and severe forms of acute pancreatitis are characterized by inflammation and edema of the exocrine pancreas, which constitutes ∼85% of the pancreas, and in the most severe form, tissue necrosis

is also observed (5). A single attack of acute pancreatitis, which results in tissue damage and the loss of acinar cells, is normally followed by the recovery of the structure and function of the exocrine pancreas, suggesting that the pancreas has regenerative capacity after limited damage and tissue loss. If pancreatitis is not completely resolved, however, the disease may progress to a chronic state, causing pancreatic deficiency and resulting in malnutrition and weight loss (4). Coxsackievirus persistence has been related to chronic cardiomyopathy and pancreatitis. Although the morphological damage is reversible in acute pancreatitis as a result of tissue repair, the changes observed in chronic disease are irreversible. Currently, there is no treatment for chronic pancreatitis.

The role of T lymphocytes in protection and disease induced by coxsackieviruses is complex and not well understood. Although T lymphocyte responses are required for survival in the early stages of acute pancreatitis, CD4^{+} T cells can also exacerbate pancreatitis (6). Thus, Th1 cells that produce IFN-γ have been associated with the pathogenesis of CVB5-induced pancreatitis (7). In CVB3 infection, the resistance of female mice to myocarditis is associated with the induction of type II cytokines and alternatively activated macrophages (M2) (7). We have previously reported that the recently discovered cytokine, IL-33, is a potent inducer of type II cytokines and M2 (8, 9). We therefore investigated the role of IL-33 in CVB5-induced pancreatitis.

IL-33 is a member of the IL-1 family and promotes CD4^{+} T cell differentiation to an atypical Th2 phenotype that is characterized by the expression of IL-5 and IL-13 (10). IL-33 also stimulates mast cell (11–14), basophil (15, 16), and eosinophil (15, 17, 18) responses. IL-33 is mainly produced by epithelial and endothelial cells in response to tissue damage, viral infection, allergens, and inflammation. IL-33 binds to the receptor T1/ST2, which exists in two isoforms: a soluble form (sST2) that acts as a decoy receptor, and a membrane-bound form presented as a heterodimer with IL-

1R–associated protein (IL-1RAcP) in the membrane of mast cells, Th2 cells, dendritic cells, basophils, and macrophages (19, 20).

We demonstrated that the production of IL-33 in the pancreas is markedly elevated post CVB5 infection, whereas the production of sST2 is reduced. T1/ST2$^{-/-}$ mice developed an exacerbated form of pancreatitis, whereas the treatment of wild-type (WT) mice with IL-33 significantly attenuated the disease and reduced the viral load. Mechanistic analysis revealed that the IL-33/ST2 pathway activates mast cells resulting in the production of IL-4, which polarizes macrophages to the M2 phenotype, and the enhancement of Foxp3$^+$ regulatory T cells (Tregs) in a Stat6-dependent manner. T1/ST2 signaling also led to an increase in CD8$^+$ and NK cell populations. Furthermore, adoptively transferred M2 and mast cells suppressed inflammatory pancreatitis. Our findings therefore reveal an unrecognized role of the IL-33/ST2 pathway in attenuating CVB5-induced pancreatitis and further suggest that IL-33 may be a potential therapeutic agent against Coxsackievirus-induced pathology.

Materials and Methods

Mice

BALB/c and Stat6$^{-/-}$ mice on the BALB/c background were purchased from The Jackson Laboratory. T1/ST2$^{-/-}$ mice on the BALB/c background were generated as described previously (21).

Ethics statement

The animal protocol used was approved by the Ethical Commission of Ethics in Animal Research of the University of São Paulo, Ribeirão Preto School of Medicine (protocol no. 029/2011). This commission is part of the National Brazilian College of Animal Experimentation.

Virus titration and infection

The original stock of CVB5, kindly provided by Prof. Roger Loria (Medical College of Virginia, Richmond, VA), was propagated in a Vero cell monolayer in MEM (Life Technologies) supplemented with 2% FBS and antibiotics at 37˚C. The virus was titrated by limiting dilutions and inoculated in quadruplicates to determine the 50% cytopathic effect of the cellular monolayer (TCID$_{50}$). BALB/c, ST2$^{-/-}$, and Stat6$^{-/-}$ mice were injected i.p. with either 10^7 TCID$_{50}$ CVB5 in 100 μl medium or Vero cell supernatants alone as a negative control. The animals were sacrificed at 3 or 7 d postinfection (p.i.).

Histology and scores

The pancreas from mice was fixed and embedded in paraffin. The slides were prepared and stained with H&E. The inflammatory and edema/necrosis scores were quantified as follows: 1, <25% of the tissue affected; 2, 25–50% of the tissue affected; and 3, >50% of the tissue affected.

Treatment of mice and cell transfer

Mice were treated i.p. with 200 ng/day IL-33 (BioLegend) on days −1, 0, 1, and 2 p.i. Mast cell degranulation was inhibited by the i.p. injection of 100 mg/kg cromolyn sodium (Sigma Aldrich) in PBS twice a day (22). The anti–glucocorticoid-induced TNF receptor (GITR) Ab was obtained from hybridomas (DTA-1) as previously described (23). The mice were injected i.p. with 500 μg of the Ab 2 d p.i. The control mice received 500 μg normal mouse IgG. Bone marrow–derived macrophages were differentiated in culture for 7 d with 20% L929 supernatant and polarized to M2 for 2 d with 5 ng/ml M-CSF (BioLegend), 30 ng/ml IL-33, and 30 ng/ml IL-4 (Invitrogen). The polarization to M2 was assessed by CD206 (mannose receptor) expression by FACS. M2 macrophages (1 × 10^6) were transferred i.v. to the recipient mice 2 d p.i. Mast cells were obtained by culturing the bone marrow cells with 100 ng/ml stem cell factor and 20 ng/ml IL-3 (PeproTech) for 21 d (24). The cells were analyzed by FACS for the expression of CD117e and FcεRI. The mast cells (5 × 10^5) were transferred i.v. to the recipient mice 2 d p.i.

Macrophage infection

Macrophages were differentiated from bone marrow cells for 7 d in media containing 20% of L929 supernatants. The cells were infected with CVB5 at a multiplicity of infection of 5 for 24 h. The cytokines in the supernatants were measured by ELISA.

Detection of cytokines by ELISA

The culture supernatants and pancreas homogenates were examined for TNF-α, IL-12p40, IL-4, IL-5, IL-13, IL-10, IFN-γ, IL-33, and sST2 according to the manufacturer's instructions (R&D Systems).

RNA extraction and real-time PCR

Total RNA from the pancreas was extracted using the illustra RNAspin Kit (GE Healthcare) according to the manufacturer's instructions and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR (qPCR) was performed targeting the 5′ untranslated region of CVB5, which is a region that is conserved in all enteroviruses. In brief, the reaction was performed in triplicates in a final volume of 10 μl containing 150 ng template cDNA, 5 μl TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol/l of each primer, and 150 nmol/l of probe. The primer and probe sequences are as follows: HEV-F: 5′-GCGGAACCGACTACTTTGGG-3′, HEV-R: 5′-CTCAATTG TCACCATAAGCAGCC-3′, HEV-probe: Fam-TCCGTGTTTCCTTTTATTCTTATA-MGB. The amplification reactions were performed on a 7300 Real Time PCR system (Applied Biosystems). A standard curve was produced using serial 10-fold dilutions of the plasmid pGEM-HEV that contained a 200-nt fragment from the 5′ untranslated region of CVB5. Viral loads were determined as the number of copies of viral RNA per gram of pancreas tissue. The expression of *iNos*, *Arginase-1*, *Fizz-1*, *Tnfα*, *Foxp3*, *Id2*, *Areg*, and *Gapdh* were analyzed by qPCR using the SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: *iNos* forward: 5′-CGAAACGCTTCACTTCCAA-3′, reverse: 5′-TGAGCCTATATTGCTGTGGCT-3′; *Arginase-1* forward: 5′-GTTCCCAGATGTACCAGGATTC-3′, reverse: 5′-CGATGTCTTTGGCA-GATATGC-3′; *Fizz-1* forward: 5′-TCCCAGTGAATACTGATGAGA-3′, reverse: 5′-CCACTCTGGATCTCCCAAGA-3′; Tnfα forward: 5′-TGTGCT-CAGAGCTTTCAACAA-3′, reverse: 5′-CTTGATGGTGGTGCATGAGA-3′; *Foxp3* forward: 5′-ACAACCTGAGCCTGCACAAGT-3′, reverse: 5′-GCC-CACCTTTTCTTGGTTTTG-3′; *Id2* forward: 5′-CCTGAACACGGACA-TCAGC-3′, reverse: 5′-CACAGAGTACTTTGCTATCATTCG-3′; *Areg* forward: 5′-GCGAATGCAGATACATCGAGAA-3′, reverse: 5′-TCCAC-ACCGTTCACCAAAGTAA-3′; *Gapdh* forward: 5′-TGCAGTGGCAA-AGTGGAGAT-3′, reverse: 5′-CGTGAGTGGAGTCATACTGGAA-3′. The results were analyzed using the ΔCT method (cycle threshold of test − cycle threshold of endogen control) (25).

Flow cytometry

To measure intracellular cytokine production, we incubated pancreatic lymph node cells with PMA (500 μg/ml), ionomycin (50 μg/ml), and brefeldin (GolgiStop; BD Biosciences) for 6 h at 37˚C. Both stimulated and unstimulated cell suspensions were incubated with Fc block (BD Biosciences) for 45 min. PE-, PE-Cy7–, FITC-, allophycocyanin-, allophycocyanin-Cy7–, or PerCP-conjugated mAbs against CD3, CD49b, CD19, CD4, CD8, CD25, CD107a, CD117, FcεRI, and ST2 (BD Biosciences) were added to the cell suspension and incubated for 30 min at 4˚C. The cells were then fixed and permeabilized with the Mouse Foxp3 Buffer Set (BD Biosciences) followed by incubation with Alexa Fluor 647–anti-Foxp3, or fixed and permeabilized with Cytoperm/Cytofix (BD Biosciences) followed by incubation with PE- or allophycocyanin-conjugated mAbs against IFN-γ and TNF-α according to the manufacturer's instructions. The following Abs were used to evaluate innate lymphoid cells: FITC-conjugated mAbs against CD3, CD19, CD11b, CD11c, CD49b, allophycocyanin–c-kit, PE–Sca-1, allophycocyanin-Cy-CD90, PE-Cy7-CD25, and PE-CF594-CD127. The cells were analyzed on a flow cytometer (FACSCantoII; BD Biosciences). Data analysis was performed using the FlowJo software (Tree Star, Ashland, OR).

Urea quantification

The urea concentration was determined by adding 25 α-isonitroso–propiophenone dissolved in 100% ethanol to the pancreas homogenate and incubating at 100˚C for 45 min. After 10 min in the dark, the OD at 540 nm was determined in a microplate reader (BioRad) using 200-laliquotsin nonsterile microculture plates. A calibration curve was prepared with increasing amounts of urea between 15 and 30 μg and 400 μl of the acid mixture. A total of 25 μl α-isonitroso–propiophenone was added to 100 μl of the urea solution.

Ab titrations

The concentrations of serum anti-CVB5 IgG and IgM Abs were determined by ELISA. In brief, 96-well plates were coated with 8 μg inactivated CVB5 overnight at 4˚C. The plates were blocked with PBS containing 1% BSA

for 2 h; then serum (1:100 dilution) was added and incubated for 3 h at room temperature. Anti-mouse IgG or IgM (Zymed) was added with the secondary Abs conjugated to peroxidase and incubated at 37°C for 1 h. The reaction was developed with tetramethylbenzidine (BD Bioscience) for 10 min and stopped with H_2SO_4 2N. The absorbance at 450 nm was measured. Neutralizing Abs were analyzed in Vero cell cultures. Sera were complement inactivated at 56°C for 1 h and then incubated with 100 $TCID_{50}$ CVB5 for 1 h at 37°C. CVB5 alone or preincubated with serum was inoculated in quadruplicates to Vero cell cultures. The cytopathic effect in 50% of cellular monolayers ($TCID_{50}$) was then determined.

Statistical analysis

The data were analyzed using the Student t test or the ANOVA followed by the Tukey–Kramer test. A p value <0.05 was considered statistically significant.

Results

IL-33 is produced post CVB5 infection and is essential for the recovery of pancreatitis

We first investigated whether IL-33 and T1/ST2 expression were induced by CVB5 infection. BALB/c mice were infected i.p. with 10^7 $TCID_{50}$ of CVB5, and the production of IL-33 and sST2 in the pancreas was determined by ELISA. IL-33 levels were significantly elevated on day 7 p.i., whereas sST2, the decoy receptor for IL-33, was markedly reduced by day 3 (Fig. 1A). These results suggest that IL-33 may be associated with CVB5 infection. Infection also in-

duced the emergence of T1/ST2$^+$ cells in the pancreatic lymph nodes, with numbers reaching $\sim 4 \times 10^4$ cells/organ. Total T1/ST2$^+$ lymphocytes, T1/ST2$^+$CD3$^+$CD4$^+$ T cells, T1/ST2$^+$CD3$^+$CD8$^+$ T cells, and T1/ST2$^+$CD19$^+$ B cells were also increased in the pancreas p.i. (Fig. 1B). However, T1/ST2$^+$CD49b$^+$ NK cells and T1/ST2$^+$CD3$^+$CD49b$^+$ NKT cells were present in low numbers and did not significantly change. We then investigated the potential role of IL-33 by comparing the disease development in T1/ST2$^{-/-}$ and WT mice infected with CVB5. WT and T1/ST2$^{-/-}$ mice had similar levels of acinar cell destruction 3 d p.i. However, although the acinar cells of the WT mice began to regenerate and recover their morphology by day 7 p.i., T1/ST2$^{-/-}$ mice contained even more severe pancreatic damage and tissue necrosis on day 7 p.i. than on day 3 (Fig. 1C). Pancreatitis in the T1/ST2$^{-/-}$ mice persisted for at least 21 d p.i. (data not shown). T1/ST2$^{-/-}$ mice also had a significantly reduced weight gain and pancreas weight as a percentage of body weight as compared with WT mice (Fig. 1D). Viral RNA analysis revealed that the viral load peaked at day 3. By day 7, ST2$^{-/-}$ mice had an increased viral load in the pancreas compared with WT mice (Fig. 1E). We next investigated the effect of rIL-33 on CVB5 infection in WT mice. Mice treated with IL-33 showed markedly reduced acinar cell destruction by day 3 (Fig. 1F) and a significantly reduced viral titer (Fig. 1G). Together, these results indicate that IL-33 plays a protective role against CVB5-induced pathology.

FIGURE 1. T1/ST2 protects mice from CVB5-induced pancreatitis. BALB/c mice were infected i.p. with 10^7 $TCID_{50}$ of CVB5. (**A**) IL-33 and sST2 levels in the pancreas were determined by ELISA up to 7 d p.i. (**B**) The number of T1/ST2$^+$ cells in the pancreatic lymph nodes was analyzed by flow cytometry (FACS) 7 d p.i. (**C**) The pancreatic histology of WT and T1/ST2$^{-/-}$ mice on days 0, 3, and 7 p.i. was analyzed by H&E staining. Scale bars, 100 μm. Inflammatory and edema/necrosis scores were quantified. (**D**) The body weight of WT and T1/ST2$^{-/-}$ mice during CVB5 infection, and the ratio of the pancreas to body weight on day 7 p.i. (**E**) Pancreatic viral RNA was determined by qPCR on days 3 and 7 p.i. (**F** and **G**) WT BALB/c mice were infected with CVB5 and injected i.p. with IL-33 or PBS on days −1, 0, 1, and 2 p.i. Mice were sacrificed on day 3 p.i. (F) The pancreatic histology (H&E) was analyzed, and inflammatory and edema/necrosis scores were quantified. Scale bars, 100 μm. (G) The viral titer and RNA levels in the pancreas were determined by virus titration and qPCR, respectively. All of the data represent the mean ± SE, $n = 6–7$, from two to three independent experiments. Asterisks indicate statistically significant differences with $p < 0.05$. NI, Not infected; ST2, T1/ST2.
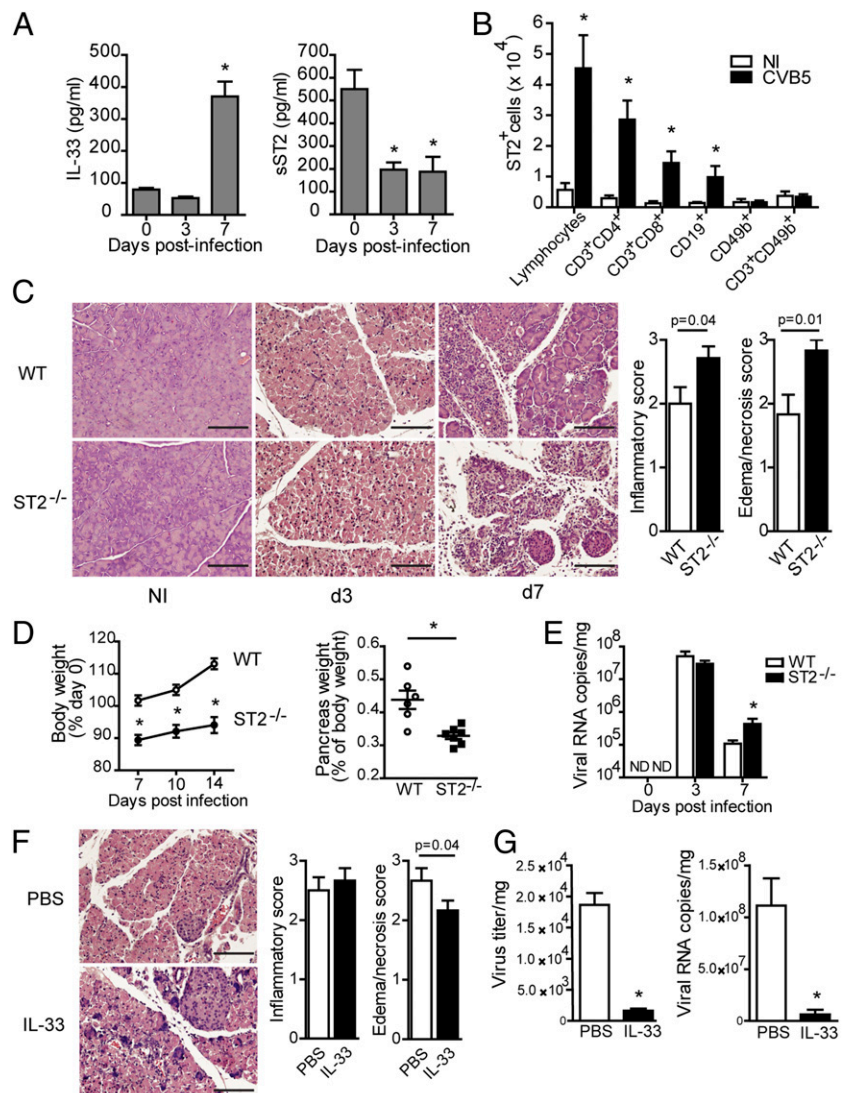
Because Abs are essential for viral clearance during enteroviral infection (26, 27), we first determined the levels of CVB5-specific Abs in infected WT and T1/ST2$^{-/-}$ mice. We found that T1/ST2$^{-/-}$ mice produced more serum anti-CVB5 IgG and IgM than WT mice (Supplemental Fig. 1A). However, there was no difference in the concentration of serum neutralizing Abs against CVB5 between the two strains of mice (Supplemental Fig. 1B). Therefore, the protective effect of the IL-33/ST2 pathway is unlikely to be due to viral neutralizing Abs.

The role of T cells and NK cells during CVB5 infection

We next examined the cellular composition of the pancreatic lymph nodes. Seven days p.i., T1/ST2$^{-/-}$ mice had a decreased percentage of CD4$^+$ T and CD49b$^+$ NK cells compared with the WT mice, but both strains of mice had an equal frequency of CD8$^+$ T, B, and NKT cells (Supplemental Fig. 2). However, T1/ST2$^{-/-}$ mice had a significantly reduced percentage and total number of multifunctional CD8$^+$ T cells, as indicated by the decreased percentage of CD107a$^+$IFN-γ$^+$, CD107a$^+$TNF-α$^+$, and CD107a$^+$TNF-α$^+$IFN-γ$^+$ cells in the pancreatic lymph nodes (Fig. 2A). Moreover, degranulating NK cells, as well as double-positive CD107a$^+$IFN-γ$^+$ and CD107a$^+$TNF-α$^+$ cells, were also diminished in the absence of T1/ST2 (Fig. 2B). These results therefore suggest that the antiviral effect of IL-33 may be mediated via the induction of CD8$^+$ and NK cells, which have well-established antiviral roles.

Mast cells are associated with protection against CVB5-induced pancreatitis

We then investigated the mechanism by which IL-33 attenuates pancreatitis. Mast cells express a high density of cell-surface T1/ST2 (28), and we have previously reported that mast cells play an important role in IL-33–mediated rheumatic disease (13, 29). We therefore examined the potential role of mast cells in CVB5-induced pancreatitis. At 7 d p.i., T1/ST2$^{-/-}$ mice infected with CVB5 contained markedly less FcεRI$^+$CD117$^+$ mast cells and produced significantly reduced levels of IL-4 in the pancreas compared with the WT mice (Fig. 3A). To directly demonstrate a role for mast cells in this model of pancreatitis, we passively transferred mast cells into WT or T1/ST2$^{-/-}$ mice. The mast cells were generated from the bone marrow of naive WT mice (Fig. 3B) and were adoptively transferred (5 × 10^5 cells) into WT or T1/ST2$^{-/-}$ mice on day 2 p.i. Although viral load was not changed

after mast cell transfer (Fig. 3C), the T1/ST2$^{-/-}$ mice that received the mast cells recovered from pancreatitis by 7 d p.i. (Fig. 3D, 3E). Moreover, the T1/ST2$^{-/-}$ recipients of the mast cells contained increased RNA levels of *Foxp3* (a Treg marker; Fig. 3G), *Arginase-1*, and *Fizz-1* (M2 markers), but decreased levels of *iNos* and *Tnfα* (M1 markers) in the pancreas at 7 d p.i. compared with the T1/ST2$^{-/-}$ mice that did not receive mast cells (Fig. 3F). To confirm the role of mast cells in the impairment of pancreatitis, we inhibited mast cell degranulation by treating WT mice with cromolyn sodium, as previously described (22). Cromolyn induced more severe edema and necrosis in the pancreas (Fig. 3H, 3I). In addition, Foxp3 expression was reduced in the pancreas of treated mice (Fig. 3J), whereas the expression of M1 and M2 markers was modestly but not significantly changed (Fig. 3K). Neither the transfer of mast cells to the T1/ST2$^{-/-}$ mice nor the treatment of WT mice with cromolyn changed the number of CD8$^+$ T cells or viral clearance (Fig. 3D, 3L). These results demonstrate that mast cells play an important protective role in IL-33/ST2–mediated protection against CVB5-induced pancreatitis. These results also suggest a potential role for Foxp3$^+$ Tregs and M2 macrophages in this protection.

Stat6 is associated with the protection against CVB5-induced pancreatitis

Because IL-4 is a major product of mast cells activated with IL-33 (30), and Stat6 phosphorylation is a key downstream event for IL-4–mediated signaling, we investigated the potential role of Stat6 in CVB5-induced pancreatitis. p.i., T1/ST2$^{-/-}$ mice expressed lower levels of Stat6 mRNA in the pancreas compared with WT mice. In addition, the treatment of WT mice with cromolyn sodium also reduced the expression of this molecule (Fig. 4A). Therefore, Stat6$^{-/-}$ and WT mice were infected with CVB5, and pancreatic histology was examined 7 d p.i. Whereas the pancreas from the WT mice had recovered from the pancreatitis, the pancreas from the Stat6$^{-/-}$ mice remained highly inflamed with intense edema and necrosis (Fig. 4B, 4C). There was no difference in the number of mast cells between the infected WT and Stat6$^{-/-}$ mice (Fig. 4D). However, the pancreas from the infected Stat6$^{-/-}$ mice contained significantly higher levels of *iNos* RNA and reduced levels of *Arginase-1* and *Fizz-1* RNA compared with the WT mice (Fig. 4E). The pancreas from the Stat6$^{-/-}$ mice also contained less CD4$^+$Foxp3$^+$ Tregs than the WT mice (Fig. 4F). Furthermore, the percentage of IFN-γ–producing and the total

FIGURE 2. T1/ST2 induces CD8$^+$ T and NK cells after CVB5 infection. BALB/c and T1/ST2$^{-/-}$ mice were infected i.p. with 10^7 TCID$_{50}$ of CVB5. Pancreatic lymph node cells were harvested 7 d p.i., and stimulated with PMA and ionomycin in the presence of brefeldin. The number of CD8$^+$ T (**A**) and NK cells (**B**) that were degranulating, CD107a$^+$ and producing IFN-γ and TNF-α was analyzed by flow cytometry (FACS). All of the data represent the mean ± SE of seven mice per group and are representative of two experiments. ST2, T1/ST2.
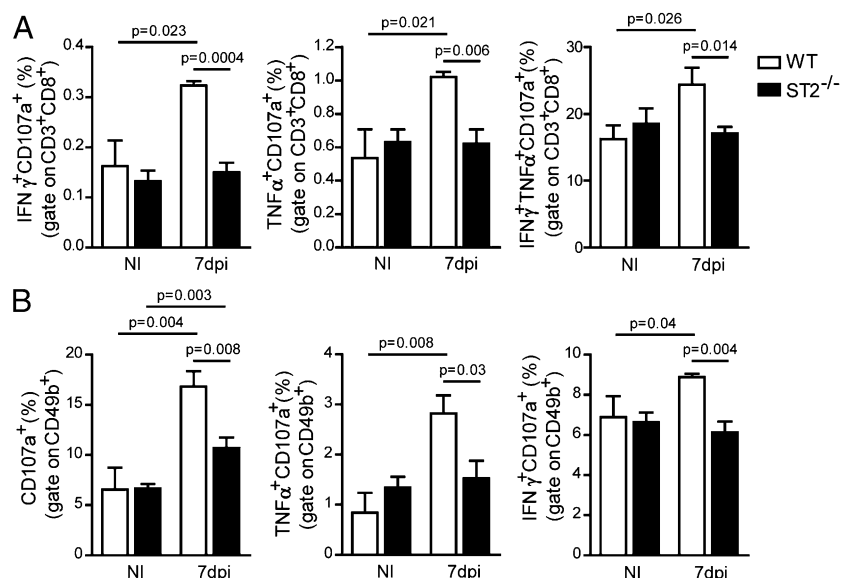
FIGURE 3. Mast cells are required for the protection against pancreatitis. (**A**) BALB/c WT and T1/ST2$^{-/-}$ mice were infected i.p. with 10^7 TCID$_{50}$ of CVB5, and the presence of mast cells and the concentration of IL-4 in the pancreas on day 7 were determined by FACS and ELISA, respectively. (**B**) Mast cells were differentiated in vitro by culturing bone marrow cells for 21 d and analyzed by FACS. (**C**) The viral RNA levels in the pancreas were determined by qPCR. (**D**) The histology (H&E) of the pancreas was examined on day 7 p.i. Scale bars, 100 μm. (**E**) Inflammatory and edema/necrosis scores were quantified. The expression of M1 (*iNos*, *Tnfα*) and M2 (*Arginase-1*, *Fizz-1*) markers (**F**) and Foxp3 (**G**) in the pancreas of T1/ST2$^{-/-}$ recipients were determined by qPCR 7 d p.i. BALB/c mice were infected i.p. with 10^7 TCID$_{50}$ of CVB5 and treated twice a day with cromolyn sodium to inhibit mast cell function. (**H**) The histology (H&E) of the pancreas was examined on day 7 p.i. Scale bars, 100 μm. (**I**) Inflammatory and edema/necrosis scores were quantified. The expression of *Foxp3* (**J**), *iNos*, and *Arginase-1* (**K**) in the pancreas was determined by qPCR 7 d p.i. (**L**) The number of CD8$^+$ T and NK cells that were degranulating, CD107a$^+$, and producing IFN-γ and TNF-α was analyzed by flow cytometry (FACS). All of the data represent the mean ± SE of five to seven mice per group and are representative of two experiments. Asterisks indicate statistically significant differences with *p* < 0.05. NT, not transferred; ST2, T1/ST2.
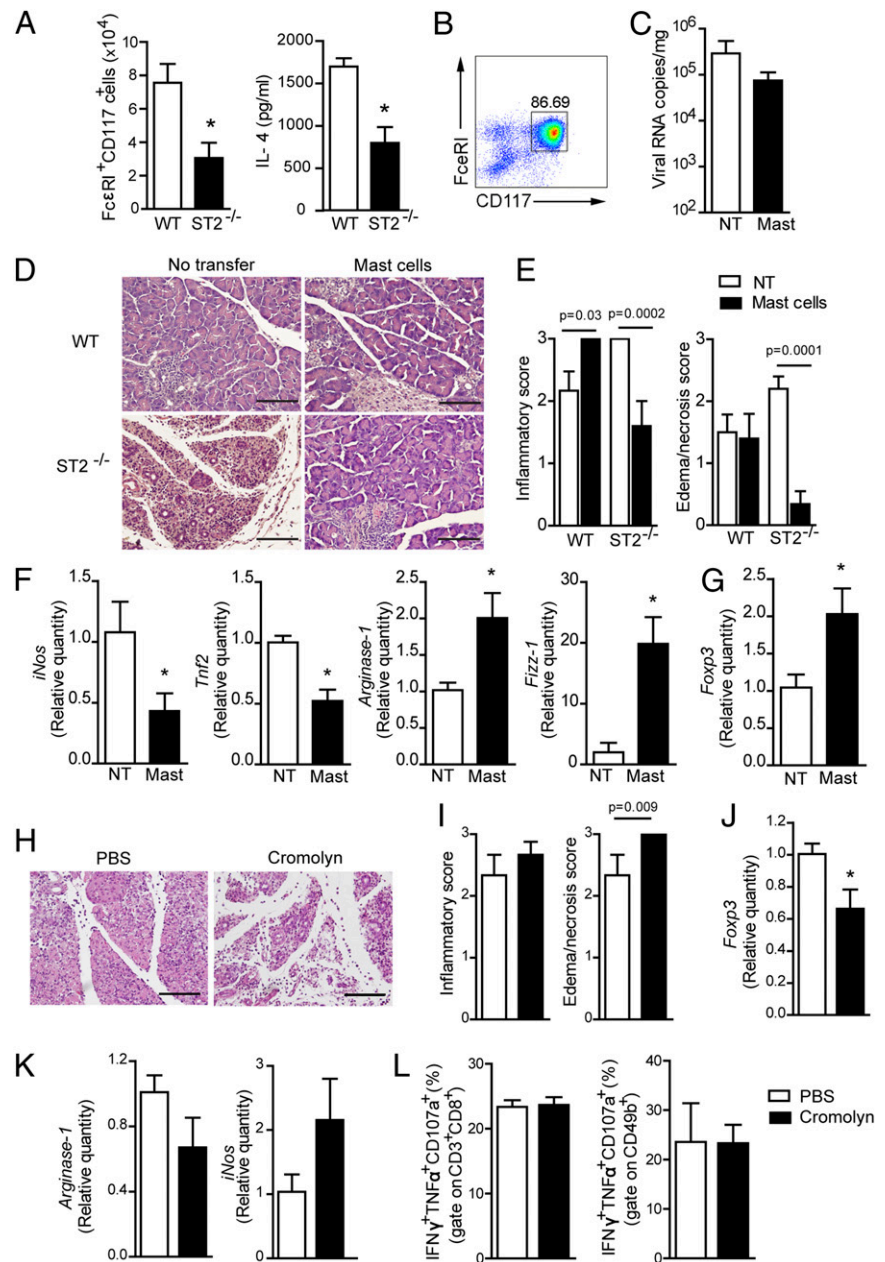
number of CD8$^+$ cells were reduced in the pancreatic lymph nodes from Stat6$^{-/-}$ mice (Fig. 4G). These reductions correlated with a significant increase in pancreatic CVB5 RNA (Fig. 4H). The percentages of IFN-γ–producing and degranulating NK cells were not changed in the absence of Stat6 (data not shown). Together, these results demonstrate that Stat6$^{-/-}$ mice recapitulate the phenotype of the T1/ST2$^{-/-}$ mice during CVB5 infection. Furthermore, Stat6 not only acts after mast cell activation, leading to increased levels of M2 and Tregs cells, but also acts to increase CD8$^+$ T cells and reduce viral load.

IL-33 stimulates M2 macrophages and innate lymphoid cells after CVB5 infection

IL-33 is a potent inducer of M2 (8), most likely via IL-4 and Stat6. The results with mast cells and Stat6$^{-/-}$ mice strongly indicate that M2 could play an important role in the IL-33–mediated protection against CVB5-induced pancreatitis. We therefore analyzed the pancreas of T1/ST2$^{-/-}$ and WT mice infected with CVB5 for M1 and M2 markers. The pancreas from T1/ST2$^{-/-}$

mice expressed more *Tnfα* RNA, the same levels of *iNos* RNA, but less *Arginase-1* and *Fizz-1* RNA compared with the WT mice (Fig. 5A). The pancreatic cells from the infected T1/ST2$^{-/-}$ mice also produced less urea, which is a marker of M2 activity (Fig. 5B). In addition, bone marrow–derived macrophages from T1/ST2$^{-/-}$ mice produced more TNF-α and IL-12p40 than cells from the WT mice 24 h after CVB5 infection in vitro (Fig. 5C). These results indicate that the IL-33/ST2 pathway is important for the induction of M2 by CVB5 in vivo and in vitro. We then directly tested the role of M2 in CVB5-induced pancreatitis. Bone marrow–derived macrophages were differentiated to M2 in vitro with IL-33, IL-4, and M-CSF (Fig. 5D), and adoptively transferred to WT or T1/ST2$^{-/-}$ mice 2 d p.i. Whereas the T1/ST2$^{-/-}$ mice developed massive pancreatitis 7 d p.i., the T1/ST2$^{-/-}$ mice given the M2 cells were protected from pancreatitis (Fig. 5E, 5F), although viral load was not changed (Fig. 5G). These results therefore demonstrate that the induction of M2 by the IL-33/ST2 pathway plays a key role in protecting mice against CVB5-induced pancreatitis.
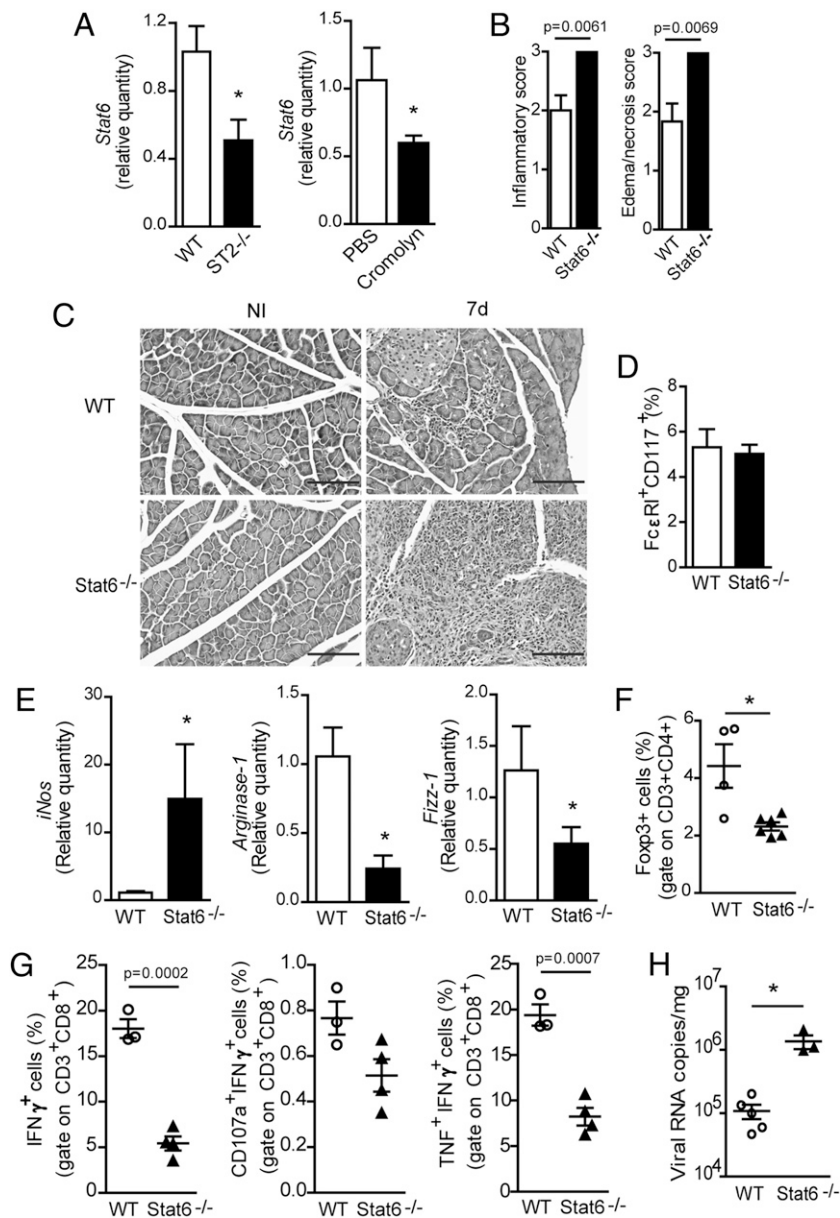
FIGURE 4. Stat6 is required for the protection against CVB5-induced pancreatitis. (**A**) The expression of Stat6 in the pancreas of WT, T1/ST2$^{-/-}$, and WT treated with cromolyn sodium mice. (**B** and **C**) The histology (H&E) of the pancreas from BALB/c WT and Stat6$^{-/-}$ mice 7 d p.i. with CVB5. (B) Inflammatory and edema/necrosis scores were quantified. (C) Representative images. Scale bars, 100 μm. (**D**) The percentage of mast cells (FcεRI$^+$ CD117$^+$) in the pancreatic lymph nodes 7 d p.i. was analyzed by FACS. (**E**) M1 (*iNos*) and M2 (*Arginase-1*, *Fizz-1*) markers in the pancreas were quantified by qPCR day 7 p.i. (**F**) The frequency of CD4$^+$Foxp3$^+$ T cells in the pancreatic lymph nodes 7 d p.i. was analyzed by FACS. (**G**) Lymph node cells were harvested 7 d p.i. and stimulated with PMA and ionomycin in the presence of brefeldin for 6 h at 37°C. The number of CD8$^+$ T degranulating, CD107a$^+$, and IFN-γ– and TNF-α–producing cells were analyzed by flow cytometry (FACS). (**H**) Viral RNA load in the pancreas 3 d p.i. All of the data are the mean ± SE of five to six mice per group and are from two independent experiments. Asterisks indicate statistically significant differences with $p < 0.05$. NI, Not infected; ST2, T1/ST2.

IL-33 was also reported to induce tissue repair through stimulation of innate lymphoid type 2 cells (ILCs) (31). We then investigated whether these cells are induced by IL-33 after CVB5 infection. We found that CVB5 infection of WT mice induced an increase on the percentage of lineage-negative (Lin$^-$) cells that do not express markers of T cells, B cells, NK cells, macrophages, and dendritic cells, but express CD90 and CD25. T1/ST2$^{-/-}$ naive mice presented lower percentage of these cells than WT, and no change was observed after CVB5 infection (Fig. 6A), showing that the induction of ILCs after CVB5 infection is dependent on IL-33 signaling. Lin$^-$CD90$^+$CD25$^+$ cells expressed the same quantity of Sca-1, c-kit, and CD127 in the absence of T1/ST2 (Fig. 6B), and the main transcription factor expressed by ILCs (Id2) was induced by infection; however, it was not dependent of IL-33 signaling because its expression was similar in T1/ST2$^{-/-}$ mice (Fig. 6C). Thus, we quantified the expression of amphiregulin (Areg), which is produced by ILCs and is responsible for lung remodeling during influenza virus infection (31). We were not able to detect Areg expression in pancreatic lymph nodes from naive mice; however, after CVB5 infection, Areg was stimulated, and in the absence of

T1/ST2 this expression was significantly diminished (Fig. 6C). These findings indicate that ILCs are induced by CVB5 infection in an IL-33 signaling–dependent mechanism.

Tregs are associated with resistance to CVB5-induced pancreatitis

M2 macrophages have been associated with an increase in the number of Tregs at the site of CVB infection and are implicated in the protection against CVB3-induced myocarditis (7). Our results strongly suggest that Tregs also play an important role in the IL-33–mediated protection against CVB5-induced pancreatitis. We therefore determined whether Tregs played a direct role in our experimental model. The pancreas from T1/ST2$^{-/-}$ mice infected with CVB5 expressed less *Foxp3* mRNA compared with the WT mice (Fig. 7A). The pancreatic lymph nodes also contained a significantly lower frequency of CD4$^+$Foxp3$^+$ Tregs (Fig. 7B). Furthermore, CVB5-infected WT mice treated with an anti-GITR Ab, which inhibits subsets of Tregs (32), developed massive cellular infiltration and inflammation compared with the IgG-treated control mice, which had normal pancreas histology 7 d p.i. (Fig.

FIGURE 5. M2 macrophages attenuate pancreatitis in T1/ST2$^{-/-}$ mice. BALB/c WT and T1/ST2$^{-/-}$ mice were infected i.p. with CVB5 and sacrificed 7 d p.i. (**A**) The expression of M1 and M2 macrophage markers in the pancreas was determined by qPCR. (**B**) Urea production in the pancreas was also determined. (**C**) Bone marrow–derived macrophages were infected with CVB5 and cultured for 24 h. The concentrations of the cytokines in the supernatants were measured by ELISA. (**D**) Bone marrow–derived macrophages were differentiated to M2 macrophages for 2 d, and the expression of the M2 marker CD206 was determined by FACS. (**E** and **F**) M2 macrophages (1 × 10^6) were transferred i.v. into mice infected 2 d previously with CVB5. (**E**) Inflammatory and edema/necrosis scores were quantified. (**F**) The pancreatic histology was observed on day 7 p.i. Scale bars, 100 μm. (**G**) The viral RNA levels in the pancreas were determined by qPCR. All of the data are the mean ± SE, n = 5–6 from two experiments. Asterisks indicate statistically significant differences with p < 0.05. ST2, T1/ST2.
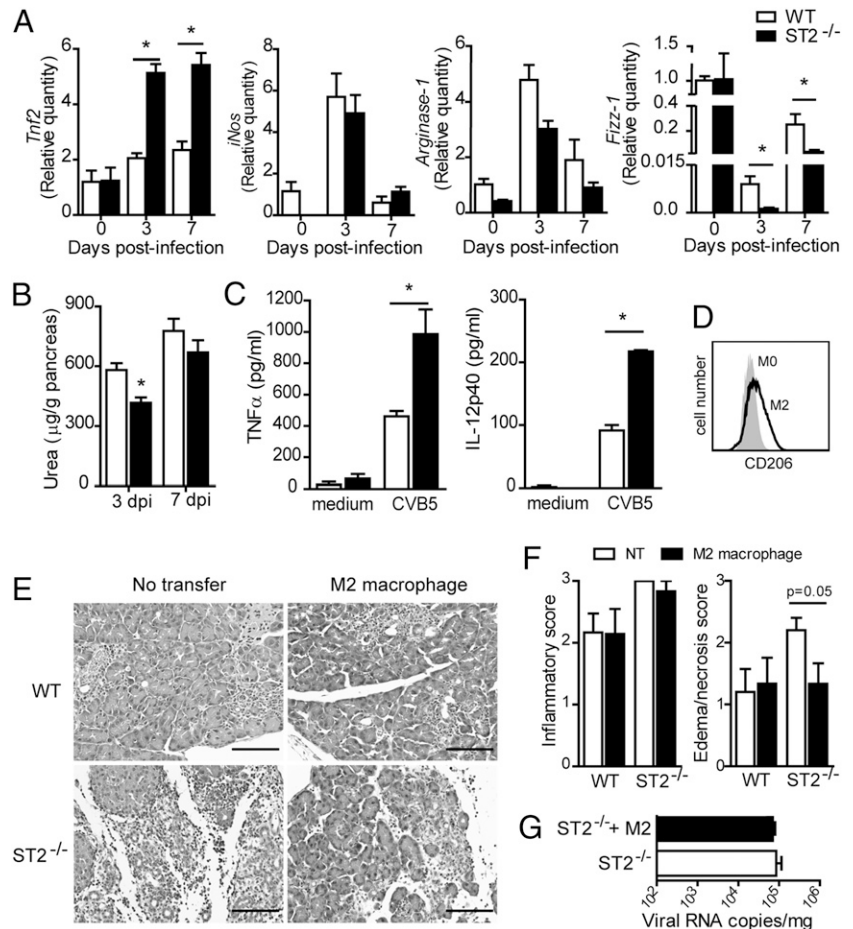
7C, 7D). These results therefore demonstrate that, aside from the effects of T1/ST2 on the CVB5 controls, it is also important to recruit Tregs to the lesion site. Because Tregs are essential for the protection against pancreatic lesions, they may contribute to the IL-33/ST2–mediated protection against CVB5-induced pancreatitis.
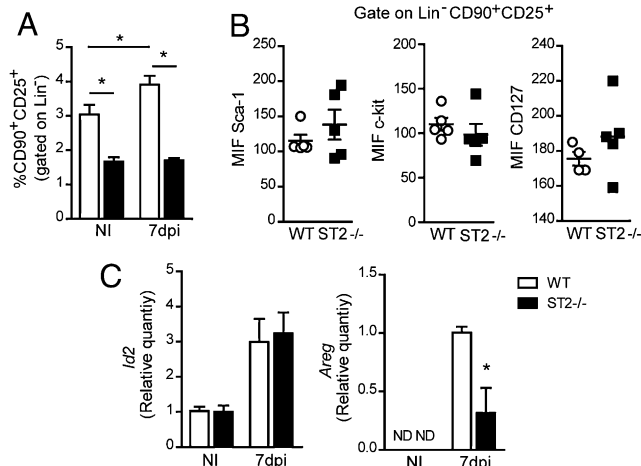
FIGURE 6. T1/ST2 induces innate lymphoid cells and Areg expression after CVB5 infection. (**A**) BALB/c WT and T1/ST2$^{-/-}$ mice were infected i.p. with CVB5, and percentage of (Lin$^-$CD90$^+$CD25$^+$) cells was evaluated on day 7 d by FACS. (**B**) Median of fluorescence intensity of Sca-1, c-kit, and CD127 was analyzed on Lin$^-$CD90$^+$CD25$^+$ gate. (**C**) The expression of Id2 and Areg were quantified by qPCR. All of the data are the mean ± SE, n = 6 from two experiments. Asterisks indicate statistically significant differences with p < 0.05. ST2, T1/ST2.

Discussion

The data presented in this article reveal an unrecognized pathway that controls Coxsackievirus-induced pancreatitis, an important disease with unmet clinical needs. We showed that mice lacking the IL-33R T1/ST2 developed severe CVB5-induced pancreatitis. Moreover, treatment with rIL-33 effectively reduced the virus load and ameliorated pancreatitis in WT mice. We demonstrated that protective IL-33 effects paradoxically involved the activation of mast cells, which are normally associated with proinflammatory responses. We show that the activated mast cells may produce IL-4, which activates the anti-inflammatory M2 macrophages and Tregs in a Stat6-dependent manner. In addition, we show that IL-33 induces viral clearance, which is associated with stimulation of CD8$^+$ T cells and NK cells.

The antiviral effects of IL-33 are unlikely due to neutralizing Abs, because there was no difference in Ab levels between the infected WT and T1/ST2$^{-/-}$ mice. Although we found that the levels of total CD3$^+$CD8$^+$ T cells were similar in the two strains of mice post CVB5 infection, we showed that in the absence of T1/ST2, there was a significant reduction in the number of IFN-γ– and TNF-α–producing CD3$^+$CD8$^+$ T cells, which have been implicated in a range of antiviral activities (33, 34). This is consistent with a recent report indicating that IL-33 could drive protective antiviral CD8$^+$ T cell responses during LCMV infection in mice (35). NK cells were also thought to be important in controlling CVB5 infection (36). We observed a decrease in the total number of NK cells in T1/ST2$^{-/-}$ mice, which may also account for the higher viral load in these animals.

In addition, T1/ST2$^{-/-}$ mice had significantly reduced levels of mast cells, and the adoptive transfer of mast cells markedly

FIGURE 7. Tregs are associated with resistance to CVB5-induced pancreatitis. BALB/c WT and T1/ST2$^{-/-}$ mice were infected i.p. with CVB5, and (**A**) the expression of *Foxp3* RNA in the pancreas 3 and 7 d p.i. was determined by qPCR. (**B**) The frequency of CD4$^+$Foxp3$^+$ T cells in the pancreatic lymph nodes was analyzed by FACS. (**C** and **D**) WT BALB/c mice were injected i.p. with 500 μg anti-GITR Ab or normal IgG as a control 2 d p.i. (C) Inflammatory and edema/necrosis scores were quantified. (D) The pancreatic histology (H&E) was examined 7 d p.i. Scale bars, 100 μm. All of the data are the mean ± SE, *n* = 5–6 from two experiments. Asterisks indicate statistically significant differences compared to non-infected (NI) with *p* < 0.05. ST2, T1/ST2.
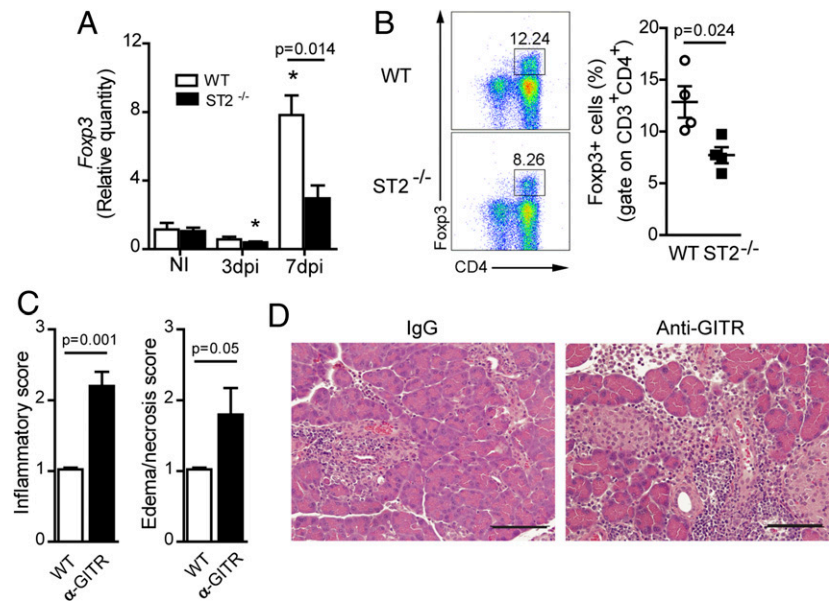
protected WT mice from pancreatitis. Mast cells express a high density of T1/ST2 (23) and are normally associated with proinflammatory functions (29). However, in the present system, mast cells play an important anti-inflammatory role in protecting mice against CVB5-induced inflammatory pancreatitis, as indicated by enhanced pancreatitis after mast cell inhibition. Because the adoptive transfer of mast cells into T1/ST2$^{-/-}$ mice attenuated pancreatitis, it is likely that mast cells function as a result of IL-33 treatment. Our findings are consistent with a number of recent reports indicating a protective role for mast cells against acute inflammation (37, 38). In particular, mast cells have been shown to induce Tregs to mediate their anti-inflammatory functions (37). In our model, T1/ST2$^{-/-}$ mice that contained adoptively transferred mast cells developed increased levels of *Foxp3* RNA in the pancreas, which is consistent with the notion that mast cells directly induce Tregs. Furthermore, CVB5-infected WT mice treated with cromolyn expressed significantly reduced levels of *Foxp3* RNA in the pancreas. Moreover, an anti-GITR Ab markedly increased pancreatitis in CVB5-infected WT mice. Together, these results suggest that IL-33–activated mast cells may suppress inflammatory pancreatitis via the induction of Tregs.

The pancreas from T1/ST2$^{-/-}$ mice that were given mast cells also expressed reduced levels of *iNos* and *Tnfα* RNA, which is typical of M1 macrophages, and elevated levels of *Arginase-1* and *Fizz-1* RNA, which is typical of M2 macrophages. Our findings are consistent with an earlier report (39) that analyzed gene expression in the pancreas from mice infected with Coxsackievirus B4. The levels of M2/Th2 RNA were altered in these mice. We now indicate one molecular and cellular mechanism for the preferential induction of M2 macrophages and the potential therapeutic control of viral-induced pancreatitis. M2 are associated with anti-inflammatory functions (40, 41). We have previously reported that the preferential induction of M2 in vivo contributed significantly to the anti-inflammatory functions of IL-33 (8, 42). Furthermore, a recent report shows that M2 macrophages are able to promote Treg differentiation in CVB3-induced myocarditis (7). Moreover, it has been shown that Tregs can also induce M2 maturation (43). Thus, it is likely that in our system, mast cells play a central role in the IL-33–mediated attenuation of pancreatitis via the induction of M2 and Tregs. Tregs seem to inhibit inflammatory pancreatitis, whereas M2 macro-

phages may induce acinar cell proliferation and tissue repair, because M2 are known to be involved in tissue regeneration in several models (44–46). In addition, ILCs have also been reported to respond to IL-33 through T1/ST2 expression. ILCs are also important for containing lung injury (31) and airway hyperreactivity (47) induced by influenza virus infection. We found that CVB5 increases the percentage of ILCs in pancreatic lymph node and stimulates the expression of Areg in an IL-33 signaling–dependent manner. Therefore, it is likely that these cells may also be involved in the protection against CVB5-induced pancreatitis by stimulating tissue remodeling.

We have also explored the mechanism by which mast cells induce the differentiation of M2 and Tregs. Cytokine profile analysis indicated that IL-4 was the major cytokine substantially reduced in the CVB5-infected T1/ST2$^{-/-}$ mice compared with the similarly infected WT mice. We therefore investigated the role of Stat6, the major downstream transcription factor for IL-4. Stat6 expression p.i. was reduced in the absence of T1/ST2 and after the inhibition of mast cell degranulation. In addition, Stat6$^{-/-}$ mice recapitulated the phenotypes of the T1/ST2$^{-/-}$ mice during CVB5 infection. Thus, Stat6$^{-/-}$ mice developed markedly more severe pancreatitis and had reduced frequency of Tregs and M2 macrophages. Moreover, Stat6$^{-/-}$ mice had reduced numbers of CD8$^+$ T cells and a higher viral load. Importantly, the Stat6-mediated increase in M2 macrophages and Tregs is likely to depend on mast cells, whereas the Stat6-mediated induction of multifunctional CD8$^+$ T cells and viral clearance is mast cell independent. Therefore, it is possible that IL-33 may act directly on CD8$^+$ T cells as previously shown in an LCMV model (35).

The mouse model of CVB5-induced pancreatitis shares many features with human diseases, which are often debilitating and may necessitate organ transplant. Chronic pancreatitis is also a major risk factor for pancreatic cancer, one of the most intractable conditions (48, 49). Our findings revealing a molecular and cellular mechanism behind the reduction in CVB5 load and attenuation of pancreatitis upon IL-33 treatment represent a significant advance in our knowledge in this important medical condition. Our findings also reveal an unrecognized role of IL-33 in a key disease area. A recent report shows that patients with acute pancreatitis exhibited elevated levels of sST2 (the decoy receptor of

IL-33) early during pancreatitis, and these elevated levels correlated with the parameters of severity (50), suggesting that IL-33 may also play an important protective role in clinical pancreatitis. Therefore, our findings indicate that IL-33 may be an important potential option for treating this debilitating disease.

Disclosures
The authors have no financial conflicts of interest.

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