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# Interleukin-33 prevents the development of autoimmune diabetes in NOD mice

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ABSTRACT

IL-33/ST2 signal is important for the generation of forkhead box P3 (Foxp3)$^+$ regulatory (Treg) cells, which contribute to immune homeostasis in the context of diseases. The aim of this study was to determine whether targeting IL-33/ST2 signal could establish immunological tolerance and prevent type 1 diabetes (T1D) in non-obese diabetic (NOD) mice. Female NOD mice treated with IL-33 for 4 weeks decreased the incidence and delayed the onset of autoimmune diabetes, whereas IL-33 did not revert blood glucose concentration and disease development in mice with new-onset diabetes. IL-33 reduced immune cell infiltration, increased the number of insulin-positive islet cells, as well as increased antiapoptosis molecule *Bcl2* and reduced proapoptosis molecules *Caspase3* at mRNA levels in the pancreas. IL-33 increased the expression of phosphorylated-Akt and phosphorylated-PI3K in the pancreas. Systemic administration of IL-33 increased the number of $CD4^+CD25^+Foxp3^+$Treg cells and induced expression of Treg cell-associated molecules ST2 and GATA3 in splenic lymphocytes, and increased *Foxp3*, *Ctla4*, and *Gata3* at the -mRNA level in pancreatic lymph nodes of NOD mice. IL-33 signaling stimulated activation of phosphorylation of p44/42 (Erk) and p38 MAPK, as well as CD39 in the spleen. Our results showed that IL-33 prevents disease development in prediabetic NOD mice, and highlight IL-33/ST2 as a potential therapeutic target to prevent T1D.

## 1. Introduction

Type 1 diabetes (T1D) is a tissue-specific autoimmune disease caused by the breakdown of T lymphocyte tolerance to islet cell antigens, which leads to the destruction of insulin-producing β cells in the pancreas [1]. Regulatory T cells (Treg cells) that express the Foxp3 transcription factor play a central in controlling the disease progression in T1D via maintaining immunologic tolerance, which potently suppresses the activation and effector function of other immune cells [2]. Studies from mice and patients indicate that Treg cell depletion led to accelerated diabetes [3], whereas the increase in Treg cell numbers by transfer or antibody treatment ameliorated disease progression [4–6]. Thus, strategies that increase the number or functional capacity of Treg cells are seen as a potential therapeutic approach.

IL-33 has been recognized as an important cytokine for Treg cells activation and differentiation by binding to its specific receptor ST2 [7,8]. IL-33 promoted Foxp3 and ST2 expression through direct transcriptional regulation, which provide a feedback loop accelerating Treg cell differentiation [9]. IL-33–mediated anti-inflammatory effects are involved in T-cell–mediated immune response and have been reported to regulate the inflammatory process in the treatment of immune-mediated diseases [10–12]. Importantly, recent studies are expanding its functions, showing that administration of IL-33 rescued islet function in the context of obesity- and streptozotocin-induced β cell stress [13,14]. We hypothesized that IL-33 might allow Treg cells to establish peripheral tolerance to stop immune destruction of β cells, and prevent disease development in the nonobese diabetic (NOD) mice and, as a consequence, activation of IL-33/ST2 signals may be of therapeutic benefit in T1D.

## 2. Method

### 2.1. Mice and treatments

Wild-type female NOD/LtJ mice were provided by Beijing HFK Bio-Technology Co. Ltd. (Beijing, China) and kept under specific pathogen-free conditions with free access to food and water. All mice were fed ad libitum with standard murine chow. All mouse experiments were performed using protocols approved by the Animal Ethics Committee of Zhengzhou University. At 7 weeks of age, the mice were randomized

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into groups receiving intraperitoneally either 0.25 μg/mouse recombinant mouse IL-33 (rmIL-33) or an equivalent volume of phosphate buffered saline (PBS) at twice- weekly intervals for 4 consecutive weeks. To further explore whether IL-33 could reverse disease development, mice with new-onset diabetes were treated with IL-33 for 4 weeks. Blood levels were measured using an Accu-Chek Active glucometer (Roche Diagnostics GmbH, Mannheim, Germany). Mice with three consecutive blood glucose concentrations that exceeded 13.9 mmol/L were considered to have diabetes. Intraperitoneal glucose tolerance test (IPGTT) analyses were performed at 12 weeks of age as previously described [15].

### 2.2. Reagents

Recombinant mouse IL-33 (rmIL-33), mouse CD39 antibody, and flow cytometry mouse lyse buffer were purchased from R&D systems (Minneapolis, MN, USA). RNeasy Mini Kit was from Qiagen (Venlo, Netherlands). iScript complementary DNA (cDNA) synthesis kit was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Phospho-p44/42 (Erk1/2) Thr202/Tyr204 Rabbit mAb, phospho-Akt serine 473 Rabbit mAb, phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) Rabbit mAb, phospho-p38 MAPK Thr180/Tyr182 Rabbit mAb, and insulin antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). CD3 antibody and MPO antibody were purchased from Abcam (Cambridge, UK). Mouse IL-33 monoclonal Antibody was purchased from OriGene Technologies (Rockville, MD, USA). ST2 polyclonal antibody was purchased from Proteintech Group (Rosemont, IL, USA). FITC-CD4, APC-CD25, PE-Foxp3, and Alexa Fluor 405-ST2/IL-33R antibody were purchased from R&D systems (Minneapolis, MN, USA). GATA3-PE-Cyanine 7 antibody was purchased from eBioscience (San Diego, CA, USA).

### 2.3. Western blot

Pancreas and splenic lymphocytes were isolated from mice at 13 to 14 weeks of age. The tissues were lysed to yield total protein lysates, which were separated by sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes. Membranes were incubated with anti-p-Akt, anti-p-PI3K, anti-p-p38, p-p44/42, anti-IL-33, anti-ST2, anti-CD39, anti-actin, anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (1:1000 dilutions) overnight at 4 °C. Appropriate horseradish peroxidase-conjugated secondary antibodies were applied for 1.5 h at room temperature, and detected with chemiluminescent reagents covered. The protein bands that were normalized against β-actin or GAPDH were quantitated densitometrically using Image Lab software.

### 2.4. RNA isolation and quantitative real-time polymerase chain reaction

Total RNA of pancreas and pancreatic lymph node was isolated with the RNeasy Mini Kit according to the manufacturer's instructions. The RNA was reverse transcribed with iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time polymerase reaction (qRT-PCR) was carried out in an ABI StepOne Real-Time PCR System (Table 1). mRNA levels of target genes were normalized to those of β-actin. For quantitative polymerase chain reaction analysis, all specimens were repeated three times with consistent results. The following genes were measured: Clta4, Foxp3, Gata3, Klrg1, Lag3, Bcl2l1, Caspase3, Caspase6, Mafa, Pdx1, Neurod1.

### 2.5. Histology

Pancreata from NOD mice treated with IL-33 or PBS for 4 weeks were sectioned at 13 to 14 weeks of age. The tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and stained. Insulitis score was analyzed under double-blinded conditions. The degree of insulitis was determined as follows: 0 = no insulitis; 1 = peri-insulitis; 2 = invasive insulitis with < 50% islet area affected; 3 = invasive insulitis with > 50% islet area affected. Standard immunohistochemistry staining methods were used to assess the levels of insulin, lymphocyte infiltration (CD3 antibody, Abcam, Cambridge, UK), and neutrophils infiltration (MPO antibody, Abcam, Cambridge, UK).

**Table 1**
Mouse primers for qRT-PCR.

| Gene | Primer sequence e (5′–3′) |
|---|---|
| Actin | CTGAGAGGGAAATCGTGCGT; R: CCACAGGATTCCATACCCAAGA |
| Clta4 | F: AATGCCTCATTCCTGAGACCA; R: ATCCTACCATCTGCTGCTTCC |
| Foxp3 | F: AAAGGAGAAGCTGGGAGCTATG; R: GGAAGAACTCTGGGAAGGAACT |
| Gata3 | F: CATTACCACCTATCCGCCCTAT; R: GTTGCCCCGCAGTTCACA |
| Klrg1 | F: TGGGGCTTTTGACTGTGATTC; R: GTGGCTACCATTCCTCGTCC |
| Lag3 | F: TCACGTTGGCGGTCATCA; R: CGTACACTGTCGCTCCAAGAA |
| St2 | F: TCCCTGGAATATGACTGTCTGG; R: CGTTCCGGGTTTTGTAAGGT |
| Il33 | F: AGCAAGACCAGGTGCTACTACG; R: TCAGCTTCTTCCCATCCACA |
| Mafa | F: TGCTCCTCGGTGCCCTCT; R: GTCCTCCGGCGTCAGGTT |
| Pdx1 | F: TGAAATCCACCAAAGCTCACG; R: TGTAGGCAGTACGGGTCCTCT |
| Neurod1 | F: GCAGAAGGCAAGGTGTCCC; R: TCTTGTCTGCCTCGTGTTCC |
| Bcl2 | F: GGATGGAGTAAACTGGGGTCG; R: GGTGGTCATTCAGATAGGTGGC |
| Caspase3 | F: AGTCTGACTGGAAAGCCGAAAC; R: GACTGGATGAACCACGACCC |
| Caspase6 | F: TACGCATACGACGCCAAAA; R: ACGTTGTCCAGCTTGTCTGTCT |

### 2.6. Flow cytometry

Spleens from NOD mice treated with IL-33 or PBS for 4 weeks were removed at 13–14 weeks of age. The tissues were homogenized and lysed in flow cytometry mouse lyse buffer using standard procedures. Single-cell suspensions were stained with specific antibodies: FITC-CD4, APC-CD25, PE-Foxp3, Alexa Fluor 405-ST2/IL-33R, and GATA3-PE-Cyanine 7 antibody. Analysis was performed on a FACSCanto cytometer using FlowJo software (Ashland, OR, USA).

### 2.7. Statistical analyses

Statistical analyses were performed using GraphPad Prism 5 software. Data were presented as mean ± standard error of the mean (SEM). Statistical significance was determined using the two-tailed unpaired *t*-test. The occurrence of diabetes was assessed by Kaplan–Meier curves and statistically compared by log-rank test. A value of $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. IL-33 ameliorates insulitis in NOD mice

Mice were treated with IL-33 or vehicle from 7 weeks of age, when insulitis has begun but the viable islet mass remains. We used anti-CD3 and MPO mAb staining to identify immune cells infiltrating the islets. Representative data from 13 to 14-week-old NOD mice are shown in Fig. 1. After 4 weeks' treatment, most islets in the PBS-treated NOD mice had extensive immune cell infiltration, as demonstrated by the $CD3^+$ T lymphocytes and $MPO^+$ neutrophils infiltration in stained section (Fig. 1A). In contrast, mice treated with IL-33 exhibited reduced percentage of severely infiltrated islets and more islets free of immune cell infiltration. Scoring the infiltration levels of the islets revealed that, at 13–14 weeks of age, only 15% of the IL-33-treated islets showed a score of 3, and most (65%) scored 0 or 1, whereas 25% of the PBS-treated islets demonstrated a score of 3, and most (70%) scored 2 or 3 (Fig. 1B).

We stained islet cells with insulin antibody to determine the number of β cells. By 13–14 weeks of age, as the proportion of immune cells infiltration increased, the proportion of β cells declined in PBS-treated

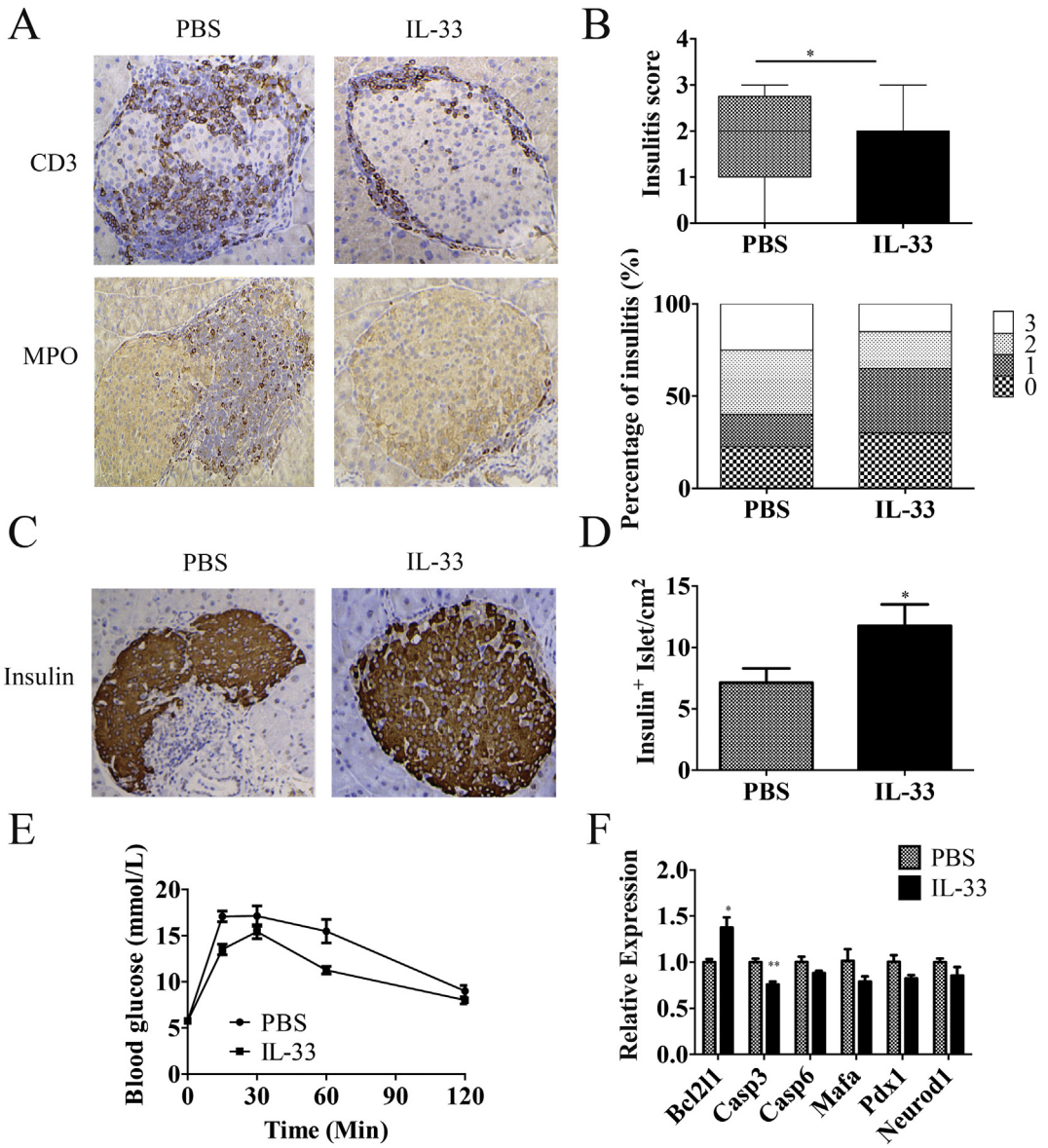


**Fig. 1.** IL-33 ameliorates insulitis in NOD mice. IL-33 (0.25 μg/mouse intraperitoneally) was administered twice weekly for 4 consecutive weeks at 7 weeks of age in NOD mice. (A) Representative pancreas immunohistochemistry staining for $CD3^+$ T cells and $MPO^+$ neutrophils. (B) Insulitis score and percentage of insulitis (n = 80 islets). (C) Representative pancreas immunohistochemistry staining for insulin-positive islets. (D) Number of islets per area (n = 6). (E) IPGTT test analyses were performed at 12 weeks of age (n = 6). (F) The expression of *Bcl2*, *caspase3*, *caspase6*, *Mafa*, *Pdx1*, *Neurod1* in pancreas was analyzed by qRT-PCR (n = 6). All data were expressed as mean ± SEM in each group. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

mice. IL-33–treated mice had an increased number of insulin-positive islets compared to PBS-treated NOD mice (Fig. 1C, D). Residual β cell function, as determined by IPGTT, reflect a significant improvement after administration of IL-33 (Fig. 1E). Notably, IL-33 increased mRNA expression of *Bcl2* that acts as an antiapoptotic molecule, whereas proapoptotic molecules of *Caspase3* were decreased (Fig. 1F). However, the mRNA levels of critical transcription factors of insulin, including *Pdx1* (pancreatic and duodenal homeobox 1), *Mafa* (MAF bZIP transcription factor A), and *Neurod1* (neuronal differentiation 1), were not affected by IL-33 treatment (Fig. 1F).

### 3.2. IL-33 prevents the development of autoimmune diabetes in NOD mice

We next determined whether IL-33 treatment has long-term protective effects in NOD mice. Consistent with reduced prediabetes insulitis, we found that diabetogenesis was also ameliorated in IL-33–treated NOD mice (Fig. 2A). IL-33 significantly decreased diabetes incidence to 50% (8/16) and delayed onset to 18 weeks of age; 50% IL-33-treated mice (8/16) remained free of diabetes up to 40 weeks after cessation of treatment. In contrast, diabetes incidence was 75% (12/16) by 29 weeks of age and disease onset at age of 17 weeks in PBS-treated mice. We also found that IL-33 failed to revert blood glucose concentration and disease development in mice with new-onset diabetes (Fig. 2B). These results show that IL-33 inhibited the progression of diabetes in previously unestablished diabetic NOD mice.

### 3.3. IL-33/ST2 activates PI3K/Akt pathways in pancreas of NOD mice

The levels of IL-33 and its receptor ST2 in isolated pancreas from NOD mice at age 13–14 weeks were determined by western blot analysis. Our results showed that compared to PBS treatment, IL-33–treated mouse pancreas had a high expression of IL-33 and its receptor ST2 (Fig. 3A). We also found an increase in *St2* at the mRNA level, whereas the expression of *Il33* was not significantly affected in IL-33-treated

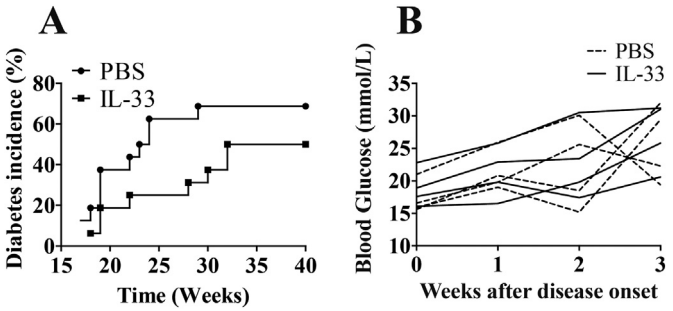


**Fig. 2.** IL-33 prevents disease development in prediabetic NOD mice. (A) Kaplan-Meier analysis of diabetes incidence in NOD mice. Female NOD mice at 7 weeks of age were treated with 0.25 μg/mouse intraperitoneally. IL-33 or PBS was administered twice weekly for 4 weeks. Diabetes incidence was monitored up to 40 weeks of age (n = 16). (B) Blood glucose concentrations in mice with new-onset diabetes that had not reversed after 4 weeks of IL-33 treatment (n = 4).

NOD mice (Fig. 3B). We further analyzed PI3K/Akt signaling in the pancreas. As shown in Fig. 3, IL-33 increased the expression of phosphorylated-Akt and phosphorylated-PI3K in the pancreas of NOD mice (Fig. 3C).

### 3.4. IL-33 increases ST2$^+$GATA3$^+$ Treg cells and Treg-associated molecules in NOD mice

We further analyzed whether IL-33 could increase the number of Tregs in splenic lymphocytes by flow cytometry. Compared to PBS treatment, IL-33 led a significant increase in CD4$^+$CD25$^+$Foxp3$^+$T cell (Treg cell) frequencies in NOD mice (Fig. 4A, B). In addition, analysis of GATA3 expression revealed that GATA3$^+$Treg cells were enriched in IL-33-treated NOD mice (IL-33 vs PBS: ~21% vs ~14%) (Fig. 4A, B). IL-33 treatment results in an increase of ST2$^+$Treg cells from ~10% in PBS-treated mice to 20% in the IL-33-treated mice by 4 weeks of treatment (Fig. 4A, B). Especially, ST2$^+$Treg cells that were enriched in IL-33–treated NOD mice expressed high levels of GATA3 compared to PBS-treated NOD mice (IL-33 vs PBS: ~11% vs ~4%) (Fig. 4A, B). We also analyzed CD39 expression that has functional relevance of immunosuppression of Treg cells [16] and found that IL-33 increased expression of CD39 in splenic lymphocytes (Fig. 4C). Consistently, IL-33 increased mRNA expression of molecules and transcription factors important for Treg cell function, including *Foxp3*, *Ctla4*, and *Gata3* in pancreatic lymph nodes (Fig. 4D).

### 3.5. IL-33/ST2 activates p-38/Erk pathway in the spleen of NOD mice

We further investigated whether IL-33 could activate MAPK pathway in splenic lymphocytes. The results showed that IL-33 increased phosphorylation of p44/42 (Erk) and p38 in splenic lymphocytes of NOD mice. Consistently, the expression of IL-33 and ST2 also increased in IL-33–treated mice (Fig. 5).

## 4. Discussion

Previous studies using obese mice have identified IL-33 as an important cytokine in the controlling inflammatory response of adipose tissues and rescuing pancreatic β cells [13,17]. Here we show the effect of IL-33 on preventing the development of autoimmune diabetes in NOD mice and expand the existing knowledge on the effects of IL-33/ST2 signals. IL-33 dramatically reduced insulitis by reducing immune cell infiltration of islets such as CD3$^+$T cells and MPO$^+$ neutrophils, which suggests that IL-33 may impede immune cell migration into or proliferation within the islet. We also found that IL-33 activated Akt2/PI3K pathways in the pancreas. This pathway is the downstream of insulin-like growth factor (IGF-1) [18], fibroblast growth factor (FGF) [19], incretin hormones glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) [20] signals, which are important for islet development and function [21]. Consistently, prediabetic NOD mice with IL-33 treatment increased the number of insulin-positive islets. Thus, the results suggested that IL-33 could prevent or delay β cell destruction at least in the early stages of diabetes.

In this context, early administration of IL-33 to NOD mice can prevent diabetes development and confer long-lasting protection against T1D. However, later administration fails to reverse disease progression in mice with new-onset diabetes. Additionally, we found IL-33 increased the expression of *Bcl2* and decreased the expression of *caspase3*, but IL-33 did not alter the expression of critical transcription factors of insulin such as *Mafa*, *Pdx1*, *Neurod1*. The results suggested that IL-33 might attenuate the process of cell apoptosis in the pancreas. That may be why residual β cells are important for IL-33 to be effective in NOD mice.

Actually, IL-33 as an important immune modulator with pleiotropic activities can target distinct immune cell subsets [9,22]. The dichotomous roles of IL-33, which can possess protective and proinflammatory functions, are dependent on the immunologic status of the

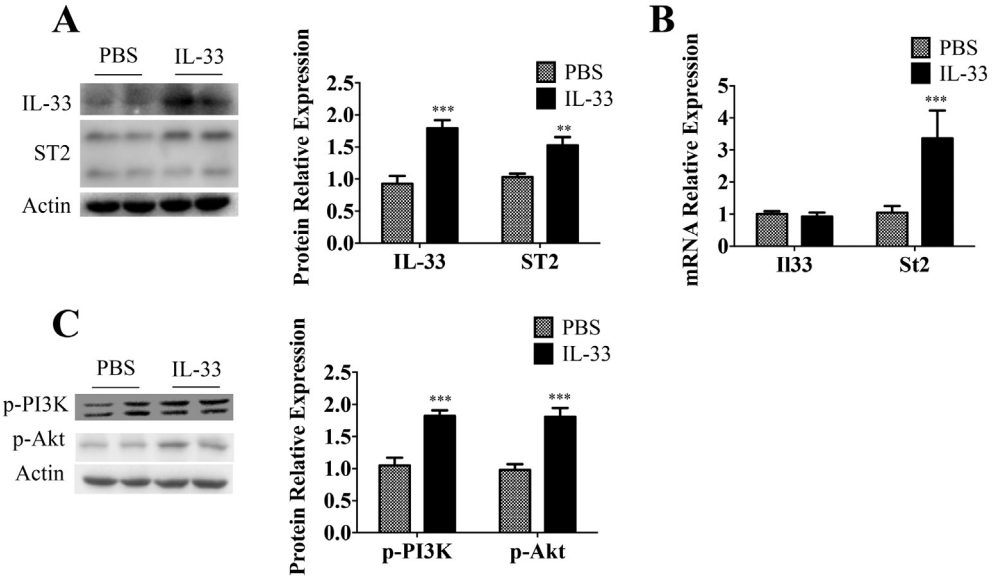


**Fig. 3.** IL-33/ST2 activates PI3K/Akt pathways in the pancreas of NOD mice. (A) The expression of IL-33, ST2 in pancreas was analyzed by western blot (n = 6). (B) The expression of *Il33* and *St2* in pancreas was analyzed by qRT-PCR (n = 6). (C) The expression of p-PI3K and p-Akt in pancreas was analyzed by western blot (n = 6). All data were expressed as mean ± SEM in each group. (n = 6). (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

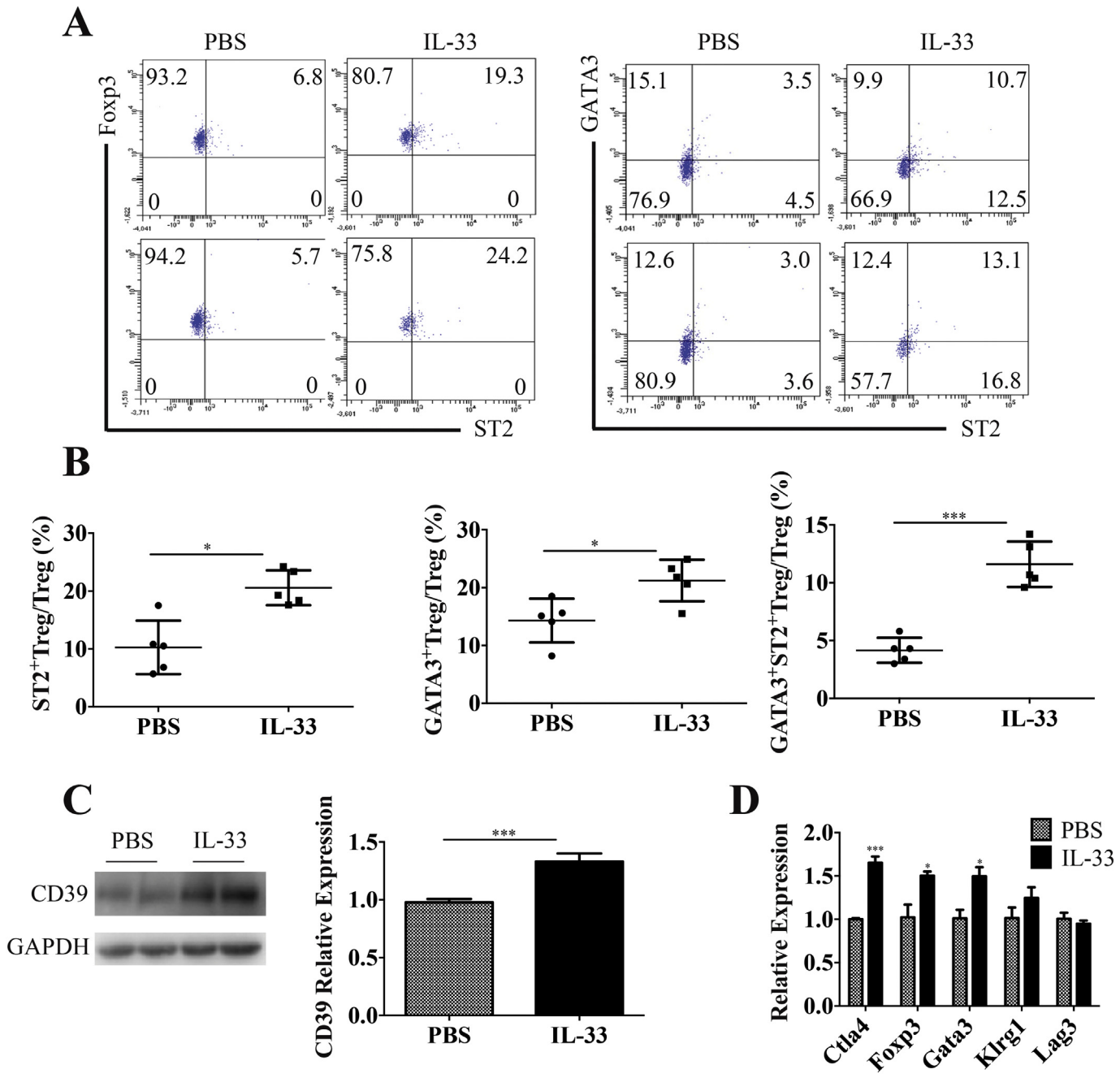


**Fig. 4.** I IL-33 increases ST2$^+$GATA3$^+$Treg cells and Treg-associated molecules in NOD mice. Splenic lymphocytes were isolated at 13 weeks of age and the percentage of Treg cells was determined by FACS. (A) Two representative plots for two individual mice are gated to CD4$^+$CD25$^+$T cells and CD4$^+$CD25$^+$Foxp3$^+$T cells. (B) Proportion of ST2$^+$Treg cells, GATA3$^+$Treg cells, and GATA3$^+$ST2$^+$Treg cells presented in the splenic lymphocytes of NOD mice at 13 weeks of age (n = 5). (C) The expression of CD39 in splenic lymphocytes was analyzed by western blot (n = 6). (D) The expression of *Foxp3*, *Ctla4*, *Gata3*, *Lag3*, *Klrg1* was analyzed by qRT-PCR (n = 6). All data were expressed as mean ± SEM in each group. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

host or the type of immune cells [7,23,24]. In order to minimize the risk of generalized immune suppression or boosting immune response, we used low-dose IL-33 therapy (0.25 μg/dose eight times), which is lower than the previously reported dose but the total doses were greater in a diabetic mouse model [13,14]. The results showed that IL-33 at 0.25 μg was sufficient to activate and expand Tregs in splenic lymphocytes and increased expression of Treg cell-associated molecules such as *Foxp3*, *Ctla4*, *Gata3* at the mRNA level in pancreatic lymph nodes of NOD mice. The results are consistent with a previous study that IL-33 not only boosts Foxp3 production but also enhances the expression of its own receptor via induction of GATA3 phosphorylation [7]. Thus, we can speculate that IL-33-mediated Treg cells may contribute to the inhibition of autoimmune attack leading to the destruction of β cells.

Additionally, we demonstrated that IL-33 signals promoted the expression of ectonucleotidases CD39 in splenic lymphocytes. CD39 contributes to suppressive activity of Treg cells by promoting the generation of adenosine; consequently, Treg cells from CD39-null mice showed impaired suppressive properties in vitro and in vivo [25–27]. Regarding other immune cells, CD39 also contributes to suppressive activity of type 1 regulatory T cells in cooperation with CD73 expressed by responder T cells and antigen-presenting cells [28]. CD39$^{-/-}$ DCs showed adenosine triphosphate unresponsiveness and impaired antigen-presenting capacity [29]. The coordinated effects of CD39 on suppressive T cells and antigen-presenting cells generate

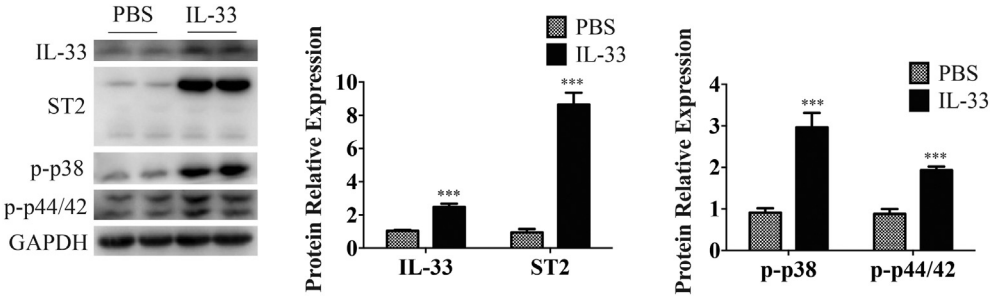


**Fig. 5.** IL-33/ST2 activates p-38/Erk pathway in the spleen of NOD mice. The expression of IL-33, ST2, p-p44/42 (Erk1/2), p-p38 in splenic lymphocytes was analyzed by western blot. All data were expressed as mean ± SEM in each group (n = 6). (* P < 0.05; ** P < 0.01; *** P < 0.001).

immunosuppressive loops, indicating roles of CD39 in the inhibitory function of immune cells. However, this finding needs to be addressed in more detail in the IL-33–mediated protective effect in NOD mice.

Our data and that from other studies support a role for IL-33 signaling to stimulate events downstream includes phosphorylation of Erk1/2 and p38 MAPK [30]. The results are consistent with the fact that IL-33–mediated stimulation of ST2 expression on Treg cells activated p38 MAPK, which induced expansion of the $ST2^+$ Treg subset [31]. In addition, P38-dependent signal pathway is also required for the expansion and suppressive function of Tregs [32,33]. Thus, the results suggested MAP kinase pathway may be involved in the IL-33-mediated effects in the T1D.

In conclusion, we show that IL-33 administration in prediabetic mice can prevent the development of autoimmune diabetes, which may be associated with an increased number of $ST2^+GATA3^+$Treg cells to maintain immune homeostasis. Our data described that IL-33 may be a novel therapeutic immune target in NOD mice.

## Declaration of interest

The authors declare that there is no duality of interest associated with this manuscript.

## Acknowledgements

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