Life Sciences 221 (2019) 301–310

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Ameliorating gut microenvironment through staphylococcal nuclease-mediated intestinal NETs degradation for prevention of type 1 diabetes in NOD mice

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ARTICLE INFO

Keywords:
Type 1 diabetes
Neutrophil extracellular traps
Staphylococcal nuclease
Intestinal microenvironment

ABSTRACT

Aims: Recent studies have revealed that neutrophil extracellular traps (NETs) provide negative feedback in the progression to chronic inflammation and contribute to the pathogenesis of multiple autoimmune diseases including type 1 diabetes (T1D). In addition, accumulating evidences suggest that gut immunity play a key role in T1D pathogenesis. Our study aimed to evaluate whether staphylococcal nuclease (SNase) targeting intestinal NETs can ameliorate the intestinal inflammatory environment and protect against T1D development in non-obese diabetic(NOD) mice.

Main methods: Degradation of NETs with SNase in *vitro* was examined using SYTOX green assay. NOD/LtJ mice were oral administration of *Lactococcus lactisl* (*L. lactis*) pCYT: SNase and blood glucose levels were monitored weekly. Several biomarkers of NETs formation, gut leakage and inflammation were determined using a commercial ELISA kit. T Cell phenotypes in peripheral immune system were analyzed in flow cytometry and fecal samples were isolated to investigate intestinal microbiota.

Key findings: The oral delivery of SNase by *L. lactis* can decrease the NETs levels and ameliorate inflammation both in the intestine and pancreatic islets and finally effectively regulate the blood glucose levels of NOD mice. Meanwhile, zonulin and lipopolysaccharide levels also reduced in SNase-fed NOD mice, suggesting SNase could improve gut barrier function via intestinal NETs degradation. Furthermore, the abundances of the intestinal microbiota and butyrate-producing gut bacteria were also increased with SNase treatment.

Significance: SNase shows potential for intestinal NETs to prevent T1D based on the gut-pancreas axis.

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease caused by the cell-mediated immune destruction of pancreatic cells, which leads to an absolute insulin deficiency, disturbing glucose metabolism [1]. Many researches have indicated that several factors can contribute to T1D, including HLA gene susceptibility, microenvironmental inflammation, aberrant gut microbiota and immune system disorders. The pancreas and the gut belong to the same intestinal immune system, and evidence of a link between T1D and the gut has accumulated [2]. From early reports of a strong influence of diet on T1D, it was evident that gut immunity must play a central role in T1D pathogenesis [3]. Recently, increased intestinal permeability and mucosal inflammation in small intestinal biopsies from patients with T1D have been demonstrated [4]. In addition, recent evidences from animal models, such as non-obese diabetic(NOD) mice and Bio Breeding diabetes-prone (BB-DP) rats, as well as five human studies, indicated that the gut microbiota is strongly associated with diabetes development [5,6].

NETs were originally recognized as a host defense mechanism in which neutrophils release their nuclear and granular contents to contain and kill pathogens [7]. However, it was also discovered that the improper formation or elimination of NETs may contribute directly to tissue damage and autoimmune disease initiation, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and diabetes [8,9]. Neutrophils mediate the pro-inflammatory response to tissue injury or infection by releasing cytokines that recruit and stimulate other immune cells and promote the activation of adaptive immunity [10]. On the other hand, it was discovered that proteins such as citrullinated histone (H3Cit) that are released into the circulatory system during NETosis are a source of autoantigens in autoimmune disease

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https://doi.org/10.1016/j.lfs.2019.02.034
Received 17 January 2019; Received in revised form 13 February 2019; Accepted 15 February 2019

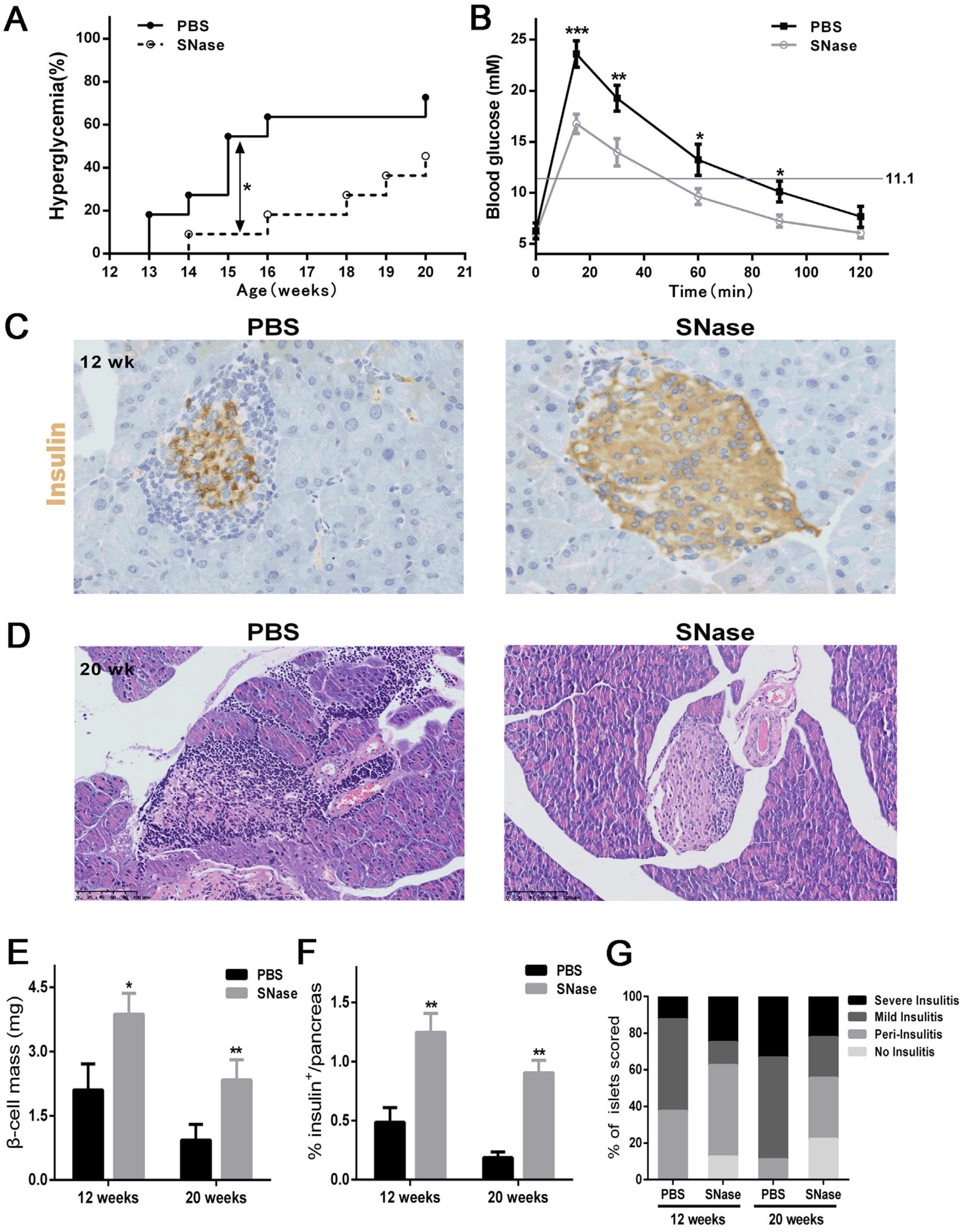

Fig. 1. Effects of oral administration of recombinant *L. lactis* expressing SNase on T1D in NOD mice.
A: The natural development of diabetes in PBS and SNase treated NOD mice ($n = 15$ per group). *B*: An oral glucose tolerance test was performed in 18-week-old NOD mice that were fasted overnight. Blood glucose was measured at the indicated time points after glucose injection ($n = 5$ mice/group). *C*: Representative images of insulin immunohistochemistry (brown) of pancreatic sections from 12-week-old NOD mice (Original magnification 400 ×). *D*: Representative images of HE staining from the pancreatic islet at 20 weeks of age (Original magnification 200 ×). *E*: Analysis of β-cell mass by immunohistochemistry (n = 5/group) at 12 and 20 weeks of age. *F*: Insulin-positive cells in the pancreas in the two groups at different times. *G*: Insulitis scoring of NOD mice at different ages (n = each). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[11]. Recent evidence suggests that circulating protein levels and enzymatic activities of neutrophil elastase (NE) and proteinase 3 (PR3), both of which can inactivate the anti-inflammatory mediator progranulin [12], are markedly elevated in patients with T1D [13].

DNA is the major structural component of NETs coated with histones and microbicidal proteases [14]. Thus, nuclease can serve as a weapon to destroy the DNA scaffold of NETs. Staphylococcal nuclease (SNase) secreted by *Staphylococcus aureus* (*S. aureus*) is a non-specific phosphodiesterase that has a strong ability to degrade this scaffold to single- or double-stranded DNA and RNA. We have proved that SNase

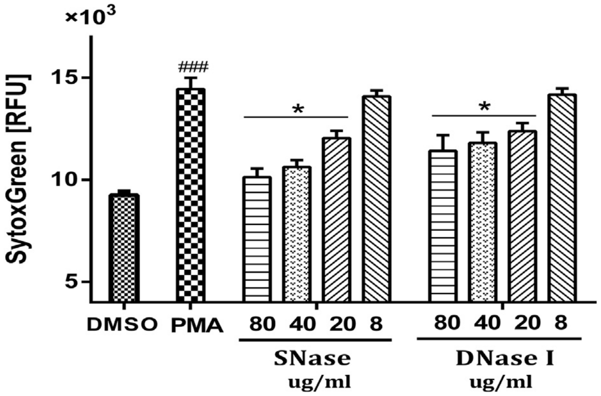

Fig. 2. SNase effectively degrades NETs *in vitro*

Measurement of PMA-induced NETs formation by SYTOX green fluorescence in non-diabetic NOD mouse-derived neutrophils treated with different concentrations of SNase or DNase I at week 8. RFU: relative fluorescence units; PMA: phorbol 12-myristate 13-acetate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

possesses a strong enzymatic ability in environments with pH scale between 4.8 and 9.2 [15]. Our study indicated that the oral delivery of SNase by L. *lactis* effectively regulates blood glucose levels, postpones the onset time of diabetes, and significantly downregulates cf-DNA/NETs, NE and PR3 in the serum compared with controls [16].

However, whether SNase can affect pancreas islet inflammation and T1D development by ameliorating the intestinal immune microenvironment remains unclear. Hence, we examined the inflammation, permeability and NETs level of the intestinal mucosa, the changes in the intestinal microbiota, and the levels of systemic inflammatory factors and subgroups of T lymphocytes, which aimed to explore the mechanism of SNase in T1D development.

2. Materials and methods

2.1. Degradation of NETs with SNase in vitro

Peripheral heparinized blood was collected from NOD mice and treated with neutrophil separation reagent (Tianjing Haoyang Bioscience, China). The harvested neutrophils were diluted with RPMI-1640 medium (HyClone, GE, USA) containing 10% fetal bovine serum (FBS) (ScienCell, USA) at a concentration of 2×10^5 cells/well in a volume of 200 μL and then stimulated with PMA (80 μM, prepared with DMSO) or DMSO (solvent control) and placed in a humidified incubator at 37 °C with CO_2 (5%) for 120 min. Then, the PMA-incubated neutrophils were subjected to SNase/DNase I at four concentrations (80, 40, 20, and 8 μg/mL; prepared with PBS) and PBS (solvent control) for 10 min. The DMSO-incubated neutrophils were treated with PBS (20 μL) for 10 min. NETs were examined using the fluorescence-based SYTOX green assay (Nanjing KeyGEN BioTECH, China) as described previously [16]. SYTOX green (1 mM) was added to the cultures after specific periods of incubation, and the cultures were observed after a 15-min reaction in the dark. Fluorescence intensity (RFU, *Ex*: 485/20, *Em*: 528/20) was detected with a fluorescence microplate reader (BioTek Synergy 2, USA).

2.2. Mice and strains

NOD mice represent a strain with an elevated susceptibility in developing type 1 diabetes (IDDM) [17]. Thirty 4-week-old female NOD/LtJ mice were provided by Beijing HFK Bioscience Co., Ltd. Housing of all mice occurred under specific pathogen-free conditions at the Pharmaceutical Animal Experimental Center of China Pharmaceutical University, and the animals were fed sterile food and water ad libitum with a 12-h dark/light cycle. The protocol for this study was approved by the Animal Ethics Committee of China Pharmaceutical University. Animal studies are reported in compliance with the ARRIVE guidelines [18,19].

2.3. Oral feeding and assessment of diabetes

Bacterial strains were grown and harvested as previously described [16]. NOD mice were randomly divided into the following two groups: the *L. lactis* pCYT: SNase group (SNase group) and the PBS group (the control group). After one week of adaptation, oral administration of *L. lactis* pCYT: SNase (4×10^9 CFU/mouse) or the same dose of PBS was carried out every day during the first week and then three times per week until the mice were 12 weeks old. After the administration of SNase by *L. lactis* and at the end of the experiment (20 weeks old), the mice were killed humanely for the relevant analyses.

2.4. Oral glucose tolerance test(OGTT)

At the end of the observation period, the non-diabetic mice in each group ($n = 5$) were selected for oral glucose tolerance test (OGTT). After 8-hour fasting overnight, the basal blood glucose was detected followed by injecting i.p. each mouse with glucose solution (2 g·kg^{-1}). Then blood glucose was monitored at the following time points: 0 min, 15 min, 30 min, 60 min, 90 min and 120 min [16].

2.5. Histologic examination

The mice ($n = 5$ in each group) were sacrificed at 12 and 20 weeks to harvest the pancreas and small intestinal tissue. The samples were fixed with 4% paraformaldehyde, followed by dehydration with alcohol gradients, clearing with xylene, embedding in paraffin and sectioning. After deparaffinization, the sections were subjected to hematoxylin and eosin (HE) staining and then systematically scanned with a NanoZoomer 2.0 RS (Hamamatsu, Japan) to score insulitis.

Insulin secreted by β-cells and the levels H3Cit were analyzed by immunohistochemistry. Briefly, the specimens were stained with mouse antibodies to insulin (Servicebio, Cat. No. GB13121, China) and anti-histone H3 (Abcam, Cat. No. ab5103) and then incubated with HRP-conjugated goat anti-mouse IgG antibodies (KPL, USA). The immunostained sections were examined with a Nikon microscope (Nikon Ts2R, Japan). The percentage of β-cell area per whole pancreas was then calculated, and the β-cell mass was calculated by multiplying the β-cell area by the whole pancreas weight [20].

2.6. Dynamic monitoring of the serum levels of zonulin, LPS, MPO, IL-18 and IL-1β by ELISA

Blood samples were collected from the orbit of each mouse every two weeks and then centrifuged at 4000 rpm for 5 min to harvest the serum. Detection was conducted by ELISA, according to the protocol provided by the manufacturer of the kits (Jiangsu Yutong Biotech, China).

2.7. Flow cytometry

Single-cell suspensions of the mesenteric lymph nodes (MLN), pancreatic lymph nodes (PLN) and spleens from two groups ($n = 5$ in each group) were prepared by mechanical disruption at 12 and 20 weeks age. The cells were incubated with fluorochrome-conjugated monoclonal antibodies specific for CD4 (RM5), CD25 (PC61) and matching isotype controls (all BioLegend). After surface staining, intracellular staining was performed using the Fix/Perm and Perm/Wash reagents (BD Biosciences) according to the manufacturer's recommendations. Intracellular staining (ICS) Abs against T-bet (4B10

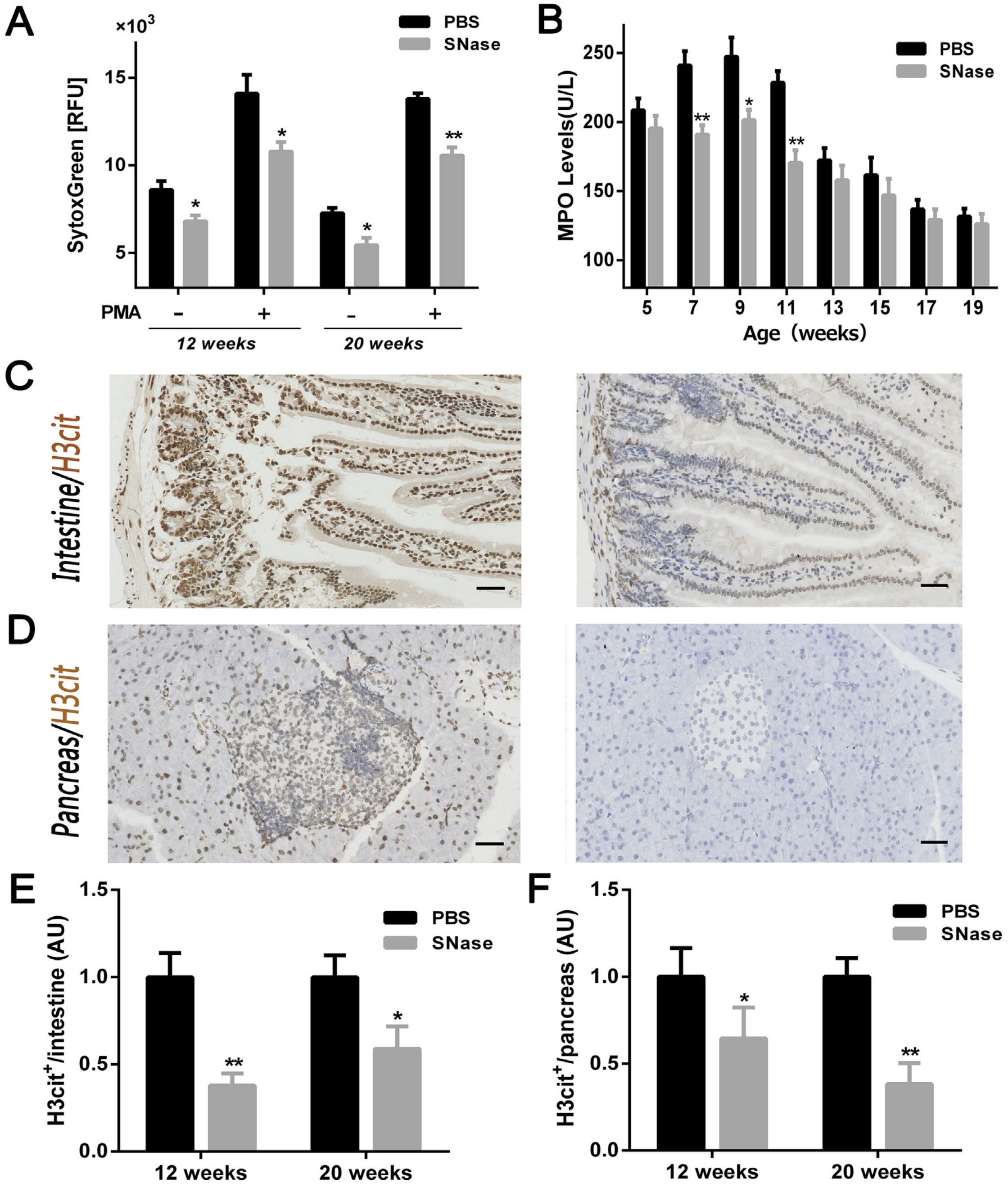

Fig. 3. SNase effectively degrades NETs *in vivo*

A: Spontaneous (incubated without PMA) and PMA-induced NETs formation was measured by SYTOX Green fluorescence of neutrophils from PBS and SNase treated NOD mice at different ages. *B:* Dynamic monitoring of the serum levels of MPO in the groups by ELISA. *C:* Representative images of immunohistochemical staining for H3Cit (brown) collected from the intestine (*C*) and pancreas (*D*) of PBS- and SNase-treated NOD mice at 12 weeks of age. Scale bars: 50 μm. "H3Cit$^+$/intestine" (*E*) and "H3Cit$^+$/pancreas" (*F*) levels were measured by immunohistochemical mean integrated optical density (IOD) of eight randomly chosen fields of each tissue section from two groups. 5 sections of each group ($n = 5$ each group) had been analyzed. At each time point, H3Cit-positive cells in the respective tissue from the PBS group were normalized to 1 for clarity. AU: arbitrary units. $^*P < 0.05$, $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

BioLegend), GATA3 (16E10A23 BioLegend), Foxp3 (R16-715 BD Biosciences) and RORγt (Q31-378 BD Biosciences) or matching isotype controls were used according to the manufacturer's instructions. Samples were acquired on a BD Accuri™ C6 flow cytometer, and data were analyzed with FlowJo software version X 10.0.7.

2.8. Fecal microbiota analysis

At 17 weeks of age, fecal samples were collected from NOD mice in a sterile environment and rapidly frozen. DNA extraction, amplification, 16S rRNA sequencing and taxonomic classification were performed by the Shanghai Personal Biotechnology Company. Briefly, total genomic DNA was extracted from all fecal samples in a sterile environment. A library of the V3-V4 region of the 16S rRNA gene was prepared from the isolated microbial DNA samples for Illumina sequencing. The DNA library was subjected to paired-end sequencing on the Illumina Miseq platform. The acquired sequences were analyzed for Operational Taxonomy Unit (OTU) delineation with QIIME software,

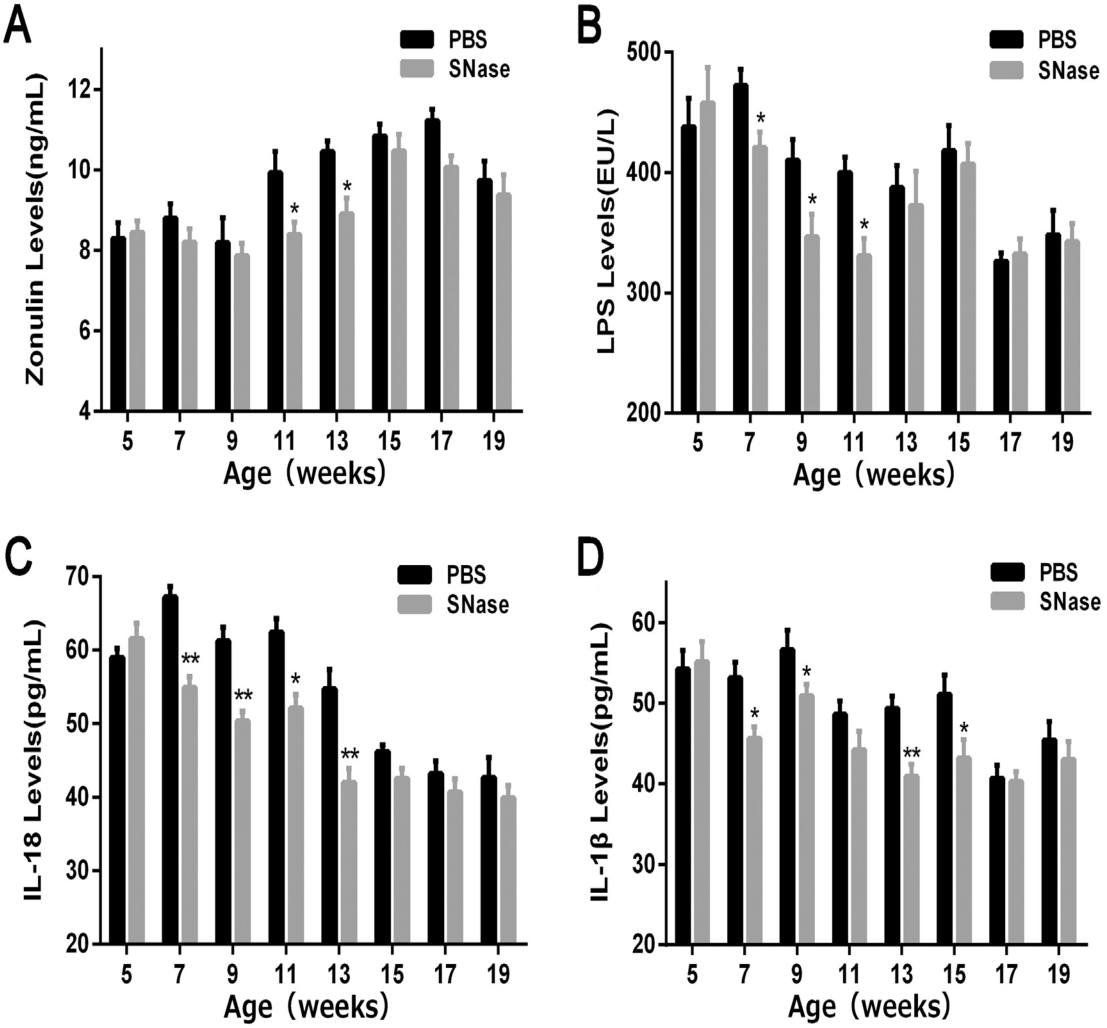

Fig. 4. SNase ameliorates the inflammatory microenvironment in NOD mice.
A–D: Intergroup comparison of the serum levels of zonulin (*A*), LPS (*B*), IL-18 (*C*) and IL-1β (*D*) throughout the observation period. $^*P < 0.05$, $^{**}P < 0.01$.

and sequences with 97% similarity were assigned to the same OTU. To compare diversity, the OTU table was rarified, and three metrics were calculated: Chao1, Observed species and Shannon index. The OTUs were converted to relative abundances by normalizing to total OTU clustering to analyze the composition of the gut microbiota and the variations in functional genera.

2.9. Statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology [21]. The statistical analysis was carried out using SPSS 22.0 software. Unless stated otherwise, all measurement data was presented as the mean ± SEM. The intergroup comparisons of the cumulative morbidity rate were carried out with the Mantel-Cox log-rank test. For the in vitro degradation assay, the intergroup comparison of fluorescence intensity was conducted by one-way ANOVA. The rest of the statistical analysis performed in this study was carried out with the Student's *t*-test. $P < 0.05$ indicates statistical significance.

3. Results

3.1. SNase prevents T1D in NOD mice

To study the effect of SNase delivered by *L. lactis* on the development of T1D, 4-week-old female NOD mice were orally administered genetically modified *L. lactis* pCYT: SNase to observe the natural history of disease development. As shown in Fig. 1*A*, the incidence of diabetes in the control group started to rise at 13 weeks and reached 54.5% at 15 weeks, while the morbidity was only 9.09% in the SNase group, indicating a significantly postponed onset.

An OGTT was performed in 18-week-old NOD mice to investigate β-cell function. The blood glucose level of the SNase group was significantly higher at both the 15-min and 30-min (Fig. 1*B*) time points after glucose injection. Moreover, it took much longer for the PBS group to normalize blood glucose than the SNase group. This result suggested that NOD mice treated with SNase showed enhanced glucose tolerance. We also examined β-cell mass and insulin-immunostained cells in pancreatic sections from SNase-treated mice compared with controls, and the results revealed an increased β-cell mass and proportion of insulin-positive cells in the pancreas at 12 and 20 weeks (Fig. 1*C*,*E*,*F*). At 12 weeks of age, histological insulitis scoring of the two groups showed no significant differences, with the SNase treatment showing a minor decrease in islets with insulitis (Fig. 1*G*). At the end of the observational period (20 weeks), only 11.1% of islets from the PBS group exhibited no insulitis or peri-insulitis, compared with 55.5% of islets in the SNase group (Fig. 1*D*, *G*).

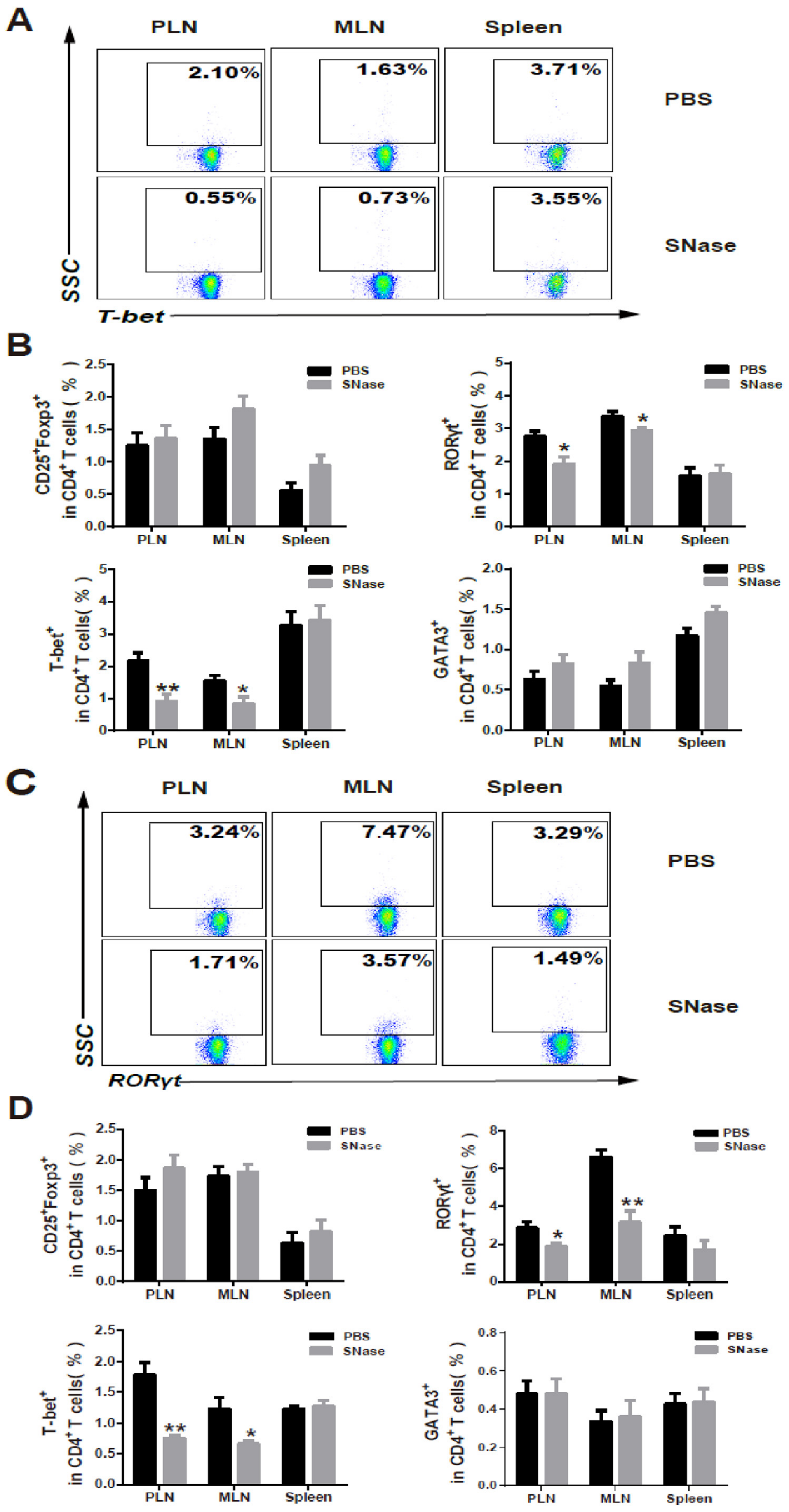

Fig. 5. SNase downregulates Th17 and Th1 cells in the peripheral immune system of NOD mice *A*: Representative flow cytometry plots showing Th1 cells among $CD4^+$ T cells from the PLN, MLN and spleen infiltrates of PBS and SNase treated 12-week-old NOD mice. *B,D:* Summary of the proportion of Treg ($CD25^+$ $Foxp3^+$ cells among $CD4^+$ T cells), Th17 ($ROR\gamma t^+$ cells among $CD4^+$ T cells), Th1 ($T\text{-}bet^+$ cells among $CD4^+$ T cells) and Th2 ($GATA3^+$ cells among $CD4^+$ T cells) cells from the PLN, MLN and spleen in PBS and SNase treated NOD mice at 12 (*B*) and 20 (*D*) weeks of age were analyzed by flow cytometry. *C*: Representative flow cytometry plots showing RORγt positive Th17 lymphocytes in the peripheral immune system of PBS and SNase treated NOD mice at 20 weeks of age. Bar graphs represent the mean of duplicates from 5 mice per group. $^*P < 0.05$, $^{**}P < 0.01$.

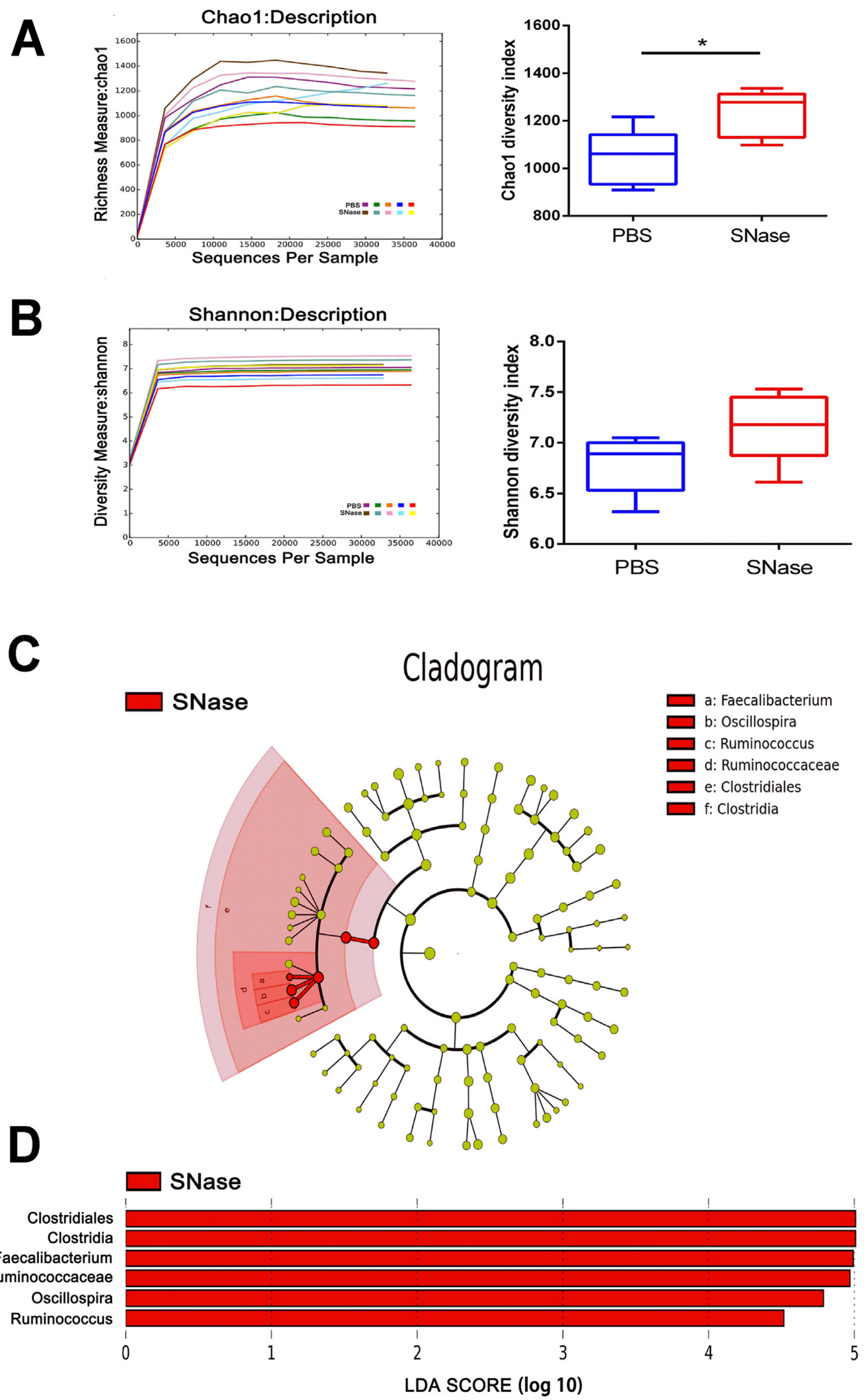

Fig. 6. SNase alters the species richness and composition of the gut microbiota in NOD mice.
A–B: At 17 weeks of age, fecal samples were isolated, and 16S rRNA genes were sequenced. The species richness (Chao1) (*A*) and the species diversity (Shannon) (*B*) of the gut microbiota. *C-D*: Cladogram generated from linear discriminant analysis (LDA) effect size (LEfSe) analysis (*C*) and the LDA score (*D*) showing the most significant differentially abundant taxa enriched in the microbiota from SNase (red, $n = 5$) groups. $^{*}P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. SNase effectively degrades NETs in vitro and vivo

PMA (phorbol 12-myristate 13-acetate), a potent mitogen and robust NETs inducer, has been shown to prime neutrophils to produce NETs *ex vivo* [22]. rhDNase is able to functionally destroy murine NETs derived from activated neutrophils *in vitro* [23]. Thus, we tested whether SNase could reduce the NETs production in the PMA-stimulated neutrophils. As shown in Fig. 2, PMA-treated neutrophils served as a positive control, while the solvent control (DMSO) failed to display a stimulatory effect. Compared with the positive control, the cells treated with high concentrations ($>2.5\,\mu g/\mu L$) of SNase and DNase I had a significantly lower NETs-dependent relative fluorescence unit (RFU) value, as measured by the SYTOX green assay. These findings indicated that similar to DNase I, SNase can also degrade NETs effectively *in vitro* in a concentration-dependent manner.

Increased NETs formation has been implicated in patients with T1D and in NOD mice [13]. Therefore, we also analyzed the influence of PBS and SNase treatment on spontaneous and induced NETs formation in neutrophils from NOD mice. At 12 and 20 weeks, SNase administration led to a significant decrease in spontaneous or PMA-induced neutrophil-derived NETs formation in the cellular level compared with PBS group (Fig. 3*A*). The levels of MPO, which is released from activated neutrophils during NETosis, were significantly lower in the SNase group than in the control group during the treatment period (Fig. 3*B*). H3Cit (a NETs marker) immunohistochemistry indicated that SNase can reduce H3Cit levels in the intestine (Fig. 3*C*, *E*) and pancreas (Fig. 3*D*, *F*) of NOD mice. In general, these results suggested that SNase can disrupt NETs effectively *in vitro* and *vivo*.

3.3. SNase ameliorates intestinal permeability and inflammatory microenvironment in NOD mice

Excessive NETs formation and impaired clearance of NETs have been shown to contribute to inflammation, which has a broad and important role in the pathogenesis of T1D [24]. Hence, we tested the influence of SNase-mediated NETs degradation on the inflammatory microenvironment in NOD mice. The SNase group had a relatively low serum zonulin and LPS levels compared with the control, which suggested that SNase treated NOD mice showed well-controlled intestinal permeability and inflammation (Fig. 4*A*, *B*). IL-18 and IL-1β levels are strongly associated with NETs formation and the pathogenesis of autoinflammatory and autoimmune diseases [25]. Compared with the control, both IL-18 and IL-1β serum levels in the SNase group were significantly lower during the treatment period indicating that SNase can effectively prevent the release of pro-inflammatory cytokines in NOD serum (Fig. 4*C*, *D*).

3.4. SNase downregulates Th17 and Th1 cells in the peripheral immune system of NOD mice

To uncover the influences of NETs degradation via SNase on the peripheral immune system of NOD mice, we examined cell phenotypes in the spleen, PLN and MLN in 12- and 20-week-old NOD mice respectively. We found that after the administration of SNase the percentagesof Th1 and Th17 cells in the MLN and PLN, both in 12- and 20-week-old NOD mice, were significantly decreased (Fig. 5*B*, *D*). Meanwhile, increase of Foxp3 + Tregs and Th2 cells were also found in the three peripheral immune organs in SNase group of 12-week although there were no significant differences (Fig. 5*B*). But the results showed that the difference of the frequencies of Tregs and Th2 cells in 20-week-old NOD mice tends to decrease (Fig. 5*D*).

3.5. SNase alters the species richness and composition of the gut microbiota in NOD mice

To investigate whether the oral delivery of SNase by *L. lactis* affects the composition of the intestinal microbiota, the 16S rRNA gene was sequenced using the MiSeq platform. Compared with the control mice, SNase-treated mice exhibited a relative increase in species richness (Chao1) and a trend toward increased species diversity (non-significant) of the gut microbiota (Fig. 6*A*, *B*). Moreover, SNase-treated mice exhibited significantly increased species richness in the class *Clostridia*, which is composed of the genera *Oscillospira*, *Faecalibacterium* and *Ruminococcus* (Fig. 6*C*, *D*). Collectively, our data clearly indicate that the oral delivery of SNase by *L. lactis* increases the abundance of the intestinal microbiota and alters the composition of the gut microbiota.

4. Discussion

Many recent discoveries support the hypothesis that abnormal NETs formation and impaired clearance of NETs are involved in the pathogenesis of T1D. In young NOD mice, NETs formation was observed in the pancreatic islets as early as 2 weeks after birth [26]. NETs also contribute to the pathogenesis of autoimmunity by exposing cryptic autoantigen, which may facilitate the generation of autoantibodies in autoimmune diseases [10]. In addition, NETs structural integrity needs to maintain the antigenicity of cytoplasmic proteins because DC uploading and autoimmunity induction are prevented by NETs treatment with DNase [27]. Our previous study showed that SNase can be successfully delivered into the small intestinal mucosa of NOD mice by recombinant *L. lactis* and remain for at least 3 days. The mice treated with non-genetically modified *L. lactis* and PBS shared a similar pharmacodynamic results which provided that *L. lactis* could not reverse or prevent the progression of T1D. In current study, PBS-treated mice were served as a control group. Firstly, we have verified that SNase can effectively degrade NETs *in vitro* and *vivo*, and demonstrated that early treatment with SNase to disorganize NETs in the gut plays an inhibitory role in the development of autoimmune diabetes in NOD mice.

Compared with our previous study, NOD mice were orally administered recombinant *L.lactis* at a higher frequency and for a shorter period in this study (3 times per week until 12 weeks vs. once per week until 20 weeks), and we obtained similar effective results. There was a statistically significant difference between the morbidity of the two groups at 15 weeks (vs. 21 weeks in the previous study). Together, these results showed that pharmacodynamic results can be improved by optimizing the frequency and the duration of administration. HE staining and islet immunostaining revealed that SNase prevented immune cell infiltration in the pancreatic islets and significantly increased β-cell mass compared to PBS-treated NOD mice. OGTT suggested that the NOD mice treated with SNase were more capable of regulating blood glucose than the control mice.

In addition, our study showed a significant reduction of neutrophil-derived spontaneous and PMA-induced NETs formation in SNase-treated NOD mice. The serum levels of MPO, also like cf-DNA/NETs, NE, and PR3 detected in our early study, was significantly lower in the SNase group during the treatment period. Moreover, the decreased pancreatic H3Cit levels indicated that the level of NETs in the pancreas decreased in the SNase group, which was consistent with the observed increases in the β-cell mass and the proportion of insulin-positive cells in the pancreas. These data suggested that NETs are somehow associated with β-cell autoimmunity and T1D development. However, what interested us more is how SNase delivered from the gut affects systemic NETs formation and ultimate inflammation in the pancreas.

Recently, there has been an explosion of interest in surrounding the role of the gut in T1D. The "gut-pancreas axis" theory associates T1D development with an abnormal deregulated intestinal microenvironment, implying an altered gut microbiota composition, a pro-inflammatory microenvironment in the intestine and altered mucosal immunity [6,28]. In this study, we focused on the influence of SNase treatment on the intestinal inflammatory microenvironment and mucosal immunity in NOD mice. In SNase-treated mice, the lower levels of H3Cit were found in the intestinal mucosa than in the control. The gut-associated lymphatic tissue (GALT) and the MLN, the largest lymph node in the body, are considered the primary inductive sites of adaptive immune responses [13].

Imbalance of subsets of $CD4^+$ T cell which is an important factor in maintaining the balance of inflammation and anti-inflammatory may be a contributor to the onset and development of T1D [29]. Some degree of immune imbalance occurs in patients with T1D, such as the balance between Th1 and Th2 types of cells [30]. In this study, we examined four subsets of CD4$^+$T cell to explore the influences of NETs degradation via SNase on the peripheral immune system of NOD mice. It is clear that Th1 and Th17 cells are both inflammatory cells which promote the progression of T1D, while Th2 cells or Tregs are inflammation-suppressing cells which inhibits Th1 or Th17 cells differentiation. Our study showed that SNase can significantly downregulate the Th17/Th1 cells differentiation in the peripheral immune system of NOD mice (mainly in MLN and PLN), but not significantly upregulates the Tregs/Th2 cells. Also the variation of local T cell differentiation can be changed to a less inflammatory state by reducing pro-inflammatory cytokine, such as IL-1β and IL-18 secretion, ultimately countering the systemic inflammatory immune imbalance.

Many observations confirmed a direct link between the loss of intestinal barrier function, abnormal antigen delivery, an inflammatory intestinal microenvironment and progression toward T1D [31,32]. The impairment of normal barrier function may allow the passage of noxious molecules, microorganisms and orally delivered antigens, causing excessive activation of neutrophils to undergo NETosis [33], or even worse, inflammation and a higher risk of autoimmunity or insulitis [34]. A large proportion of T1D patients have prolonged/persistent enterovirus infection associated with an inflammation process in the gut mucosa [35,36]. We found that when compared with the control, serum levels of zonulin (a critical biomarker for evaluating intestinal barrier function) and LPS (a classic stimulant of NETs formation) in the SNase group were significantly lower during the treatment period, which suggested decreased intestinal permeability and improved intestinal barrier function. Therefore, we concluded that SNase treatment can preserve the intestinal barrier function, which further decreases systemic inflammation and NETs formation and limits the risk of forming a cycle that accelerates the development of T1D.

Accumulating evidences support the idea that the intestinal microbiome is involved in T1D pathogenesis through the gut-pancreas axis [34,37]. It has been reported that the human infant gut microbiome change along with specific alterations that precede T1D onset [38]. It is possible that altered microbiota are not the cause but promoters of T1D [39]. In this study, we found that oral delivery of SNase by *L. lactis* clearly altered the gut microbiota composition and significantly increased the microbial species richness. Moreover, the species richness of the genera *Oscillospira*, *Faecalibacterium* and *Ruminococcus* was remarkably increased in SNase-treated mice. These genera are obligate anaerobes that reduce the oxidative state of the gut [40]. Moreover, these genera can produce butyrate, which is important for host glucose control [41,42], insulin sensitivity regulation, insulin signaling and intestinal gluconeogenesis [43]; In addition, these organisms represent potent immune modulators, being involved in peripheral regulatory T-cell generation [44]. and in the regulation of pro-inflammatory cytokine production [45]. Hence, we concluded that small intestine-colonized SNase, via NETs degradation, can repair an impaired gut immune system and contribute to intestinal homeostasis.

5. Conclusions

In summary, our study showed that SNase targeting intestinal NETs can ameliorate the intestinal inflammatory microenvironment and regulate mucosal immunity, protecting against T1D development in NOD mice. Thus, we propose that intestinal NETs, as an important environmental trigger, may contribute to the initiation and progression of T1D. Although the explicit role of intestinal NETs in the pathogenesis of autoimmune diabetes remains in question and requires further investigation, our study suggests that therapeutic interventions aimed at the gut-pancreas axis may prove promising for future T1D prevention or treatment approaches.

Conflict of interest statement

The Authors declare that there are no conflicts of interest.

Acknowledgements

The English language of this article was edited by American Journal Experts.

Funding: This work was supported by the National Natural Science Foundation of China [grant number 81673340]; the Jiangsu Provincial Natural Science Foundation of China [grant number BK20161462]; and the "Double First-Class" University project [grant numbers CPU2018GF/GY16,CPU2018GF/GY17].

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