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Oral delivery of single-chain insulin (SCI-59) analog by bacterium-like particles (BLPs) induces oral tolerance and prevents autoimmune diabetes in NOD mice

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ABSTRACT

Oral tolerance, induced by oral administration of autoantigens, is a promising therapeutic approach to treat type 1 diabetes mellitus (T1DM). However, the degradation of antigens passing through the gastrointestinal tract (GIT) leads to low induction efficiency. Based on our previous study, a single-chain insulin (SCI-59) analog, bound to the surface of lactic acid bacteria (LAB) bacterium-like particles (BLPs), was more stable in the simulated gastric fluid, compared to free SCI-59 and insulin. Based on the analysis of diabetes progression, a significant decrease in the incidence of diabetes was observed in mice fed BLPs-SCI-59. Oral administration of BLPs-SCI-59 can enhance glucose tolerance in NOD mice and this effect may result from the protection of pancreatic islet beta cells, as compared to the free SCI-59 group and BLPs group. Oral administration of BLPs-SCI-59 can significantly reduce insulitis and preserve the ability of insulin secretion in treated mice. Oral vaccination with BLPs-SCI-59 induced SCI-59 specific T cell tolerance in treated mice, which may due to the repair of Th1/Th2 imbalance and increased $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs). These results show that oral vaccination with BLPs-SCI-59 is a promising way to prevent T1DM in NOD mice.

1. Introduction

As an autoimmune disease, the primary characterization of type 1 diabetes mellitus (T1DM) is the hyperglycaemia as a result of the insulin deficiency [1]. Although it can be diagnosed at different ages, T1DM is most often diagnosed in childhood or adolescence and it continues for the life of the patient [2]. $CD4^+$ and $CD8^+$ T lymphocytes and proinflammatory cytokines mediated specific disruption of the pancreatic islet beta cells is considered to be the final step to develop T1DM [3]. Typical beta cell autoantibodies associated with T1DM are those targeting multiple autoantigens, such as insulin, 65 kDa glutamic acid decarboxylase (GAD-65), 60 kDa heat shock protein (HSP60), znic transporter 8 (ZNT8) or insulinoma-associated protein 2 (IA-2) [4,5].

Oral tolerance means the specific inhibition of immunological responses to orally administrated antigens. It is considered as a promising treatment for inflammatory and autoimmune diseases, including T1DM [6]. Oral administration of those above autoantigens has shown positive effect in suppressing T1DM in non-obese diabetic (NOD) mice [7–12] and in children who have a genetic predisposition to T1DM [13].

Requiring complicated post-translational modifications from proinsulin, mature insulin molecular includes two separated chains, which are connected by three disulfide linkages [14]. To improve the oral bioavailability, a large dose of insulin is needed [6,13] and its clinical application may be limited due to the complicated and costly purification processes during insulin production. Alternatively, a single-chain insulin (SCI) analog, using short peptides to connect the B chain's C-terminus with the A chain's N-terminus directly, may be a better choice [15,16]. Using GGGPRR as the linker peptide, chemical synthesized SCI-57 analog has a similar conformation and bioactivity to wild-type insulin [17]. At the physiological pH, SCI-57 secreted by *Lactococcus lactis* still maintains its biological activity [18].

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In our previous study, we designed a new SCI-59 analog containing an eight-residue linker peptide (RSRGLPFR) between the insulin B- and A-chain. In addition to the secretory expression of SCI-59 in the recombinant L. *lactis* NZ3900 strains, surface display of SCI-59 by lactic acid bacteria (LAB) bacterium-like particles (BLPs) was also realized. Both forms of SCI-59 have ability to bind and activate insulin receptor on the surface of 3T3-L1 adipocytes, indicating that the synthesized SCI-59 has a similar conformation to wild-type insulin [19]. Compared with the free SCI-59 and insulin, SCI-59 bound to BLPs (BLPs-SCI-59) is more stable in the simulated gastric fluid, indicating that LAB BLPs may provide protective effects for SCI-59 in vivo [19]. As a further study, we aimed to evaluate whether oral administration of BLPs-SCI-59 can induce antigen specific oral tolerance and prevent T1DM in NOD mice.

2. Materials and methods

2.1. Bacterial strains and vaccine production

As described in our previous studies [19,20], 50 ng/μl nisin was added to the culture of recombinant *L. lactis* NZ3900 (pMRF5018) and *L. lactis* NZ3900 (pMRF5019), which were constructed on the basis of *L. lactis* NZ3900 (NIZO Food Research), to induce the secretory expression of SCI-59 and SCI-59-3LysM, respectively. At stationary phase, cells of *L. lactis* MG1363 were collected, washed by phosphate buffer saline (PBS, pH 7.0) and then boiled in 10% trichloroacetic acid (TCA) solution to produce MG1363 BLPs. After washing, MG1363 BLPs were resuspended in PBS (2.5×10^{10} BLPs/ml). 2.5×10^9 MG1363 BLPs were added to 100 μl 20-fold concentrated acellular supernatant including SCI-59-3LysM and incubated for 1 h at room temperature to produce the vaccine BLPs-SCI-59, which can be collected, washed and resuspended in PBS.

2.2. Laboratory animals

Provided by Shanghai Slaccas Experiment Animal Limited Company (Shanghai, China), female NOD/LtJ mice (four weeks old) were maintained under controlled environmental conditions (22 ± 3 °C, 50% ± 10% humidity) on a 12-h light-dark schedule. Standard chow and sterilized drinking water were offered. Animal care and all experiments were allowed by the Ethics Committee on Experimental Animal Using of Wuhan University.

2.3. Grouping, vaccination and estimate of diabetes

NOD mice were grouped as follows (n = 15 per group). Control mice were fed 2.5×10^9 MG1363 BLPs in 100 μl PBS (BLPs group). The second group was orally administrated 100 μl cell-free supernatant (20-fold concentrated) containing SCI-59 (SCI-59 group). The last group was orally administrated 2.5×10^9 MG1363 BLPs carrying SCI-59 from 100 μl cell-free supernatant (20-fold concentrated) containing SCI-59-3LysM (BLPs-SCI-59 group). All mice were orally administrated once daily for the first week and then three times weekly until 20 weeks of age.

Collected every four weeks, treated mouse serum was stored at −80 °C until further analysis. Blood glucose levels and body weight were monitored and recorded weekly. Once two consecutive measured values were higher than 11.1 mmol/L and symptoms including polyuria, weight loss occurring, diabetes was confirmed.

2.4. Quantification of serum antibody subtypes

At 20 weeks of age, ELISA was applied to analyze anti-SCI-59 antibodies in mouse serum. Using purified SCI-59 as the blocking antigen, serially diluted sera were added to the microtiter plate wells. The secondary antibody including horseradish peroxidase (HRP)-conjugated anti-mouse IgG, IgG1, IgG2a antibody (Proteintech, China) were added respectively. The absorption value (450 nm) was obtained in a microplate reader (Bio-Rad, USA). The titer was defined as described previously [8].

2.5. Oral glucose tolerance test (OGTT)

At 21 weeks of age, all undiseased mice in each group were selected for OGTT. Basal blood glucose levels were recorded after an overnight fast. And then, each mouse was given glucose (2 g/kg) intraperitoneally. Blood glucose levels at 0, 15, 30, 60, 90, and 120 min were measured and recorded.

2.6. Pancreatic islet histopathological analysis

When the observation period finished (40 weeks), three mice per group were sacrificed to collect pancreas for evaluation of insulitis by hematoxylin-eosin (HE) staining as described previously [21]. The evaluation of insulitis was performed in accordance with the following criteria: 1, no insulitis (infiltration is not detected); 2, peri-insulitis (infiltration is only detected around the islets); 3, mild insulitis (infiltrated area < 50%); 4, severe insulitis (infiltrated area ≥ 50%) [22]. At least twenty islets were evaluated and scored from each mouse in each group.

2.7. C-peptide levels assay

To evaluate the effects of oral immunization on pancreatic beta cells function during developing of T1DM, dynamic changes in serum C-peptide levels were monitored using an ELISA kit (Sigma, USA). The ELISA process was carried out according to the product manual.

2.8. Spleen lymphocyte proliferation assay and analysis of cytokine

Splenic cell suspensions were collected from the aforementioned NOD mice used for insulitis evaluation and erythrocytes were removed by cytolysis in NH_4Cl (2 min, 37 °C). Afterwards, 1×10^6 cells/well were cultured in 96-well plates and stimulated with medium, bovine serum albumin (BSA, 10 μg/ml, Sangon, China), concanavalin A (ConA, 2.5 μg/ml, Sangon, China) or purified SCI-59 (10 μg/ml). After a 72 h incubation, proliferation was analyzed using WST-8 test (Beyotime, China) at 570 nm (using 630 nm as the reference wavelength) with the help of a microplate reader (Bio-Rad, USA). The definition of stimulation index (SI) was the ratio of the average OD value of samples incubated with different stimulant to that of samples incubated with medium. And then, cytokine production was analyzed in supernatants collected by centrifugation. Contents of interleukin (IL)-2, IL-4, IL-10 and interferon (IFN)-γ were analyzed by ELISA (Beyotime, China) according to the product manual.

2.9. Tregs analysis

Lymphocytes of the aforementioned NOD mice used for insulitis evaluation were collected from the pancreatic lymph nodes (PLN) by using the mouse lymphocyte separation medium (Solarbio, China). The assay was carried out with the help of a mouse Tregs labeling kit including APC-antiCD3, FITC-antiCD4, PE-antiCD25 and PE-cy5-antiFoxP3 antibodies (Invitrogen, USA), under the guidance of the product manual. Stained Tregs were analyzed by flow cytometry with the help of a FACScan flow cytometer (BD Biosciences, USA).

2.10. Statistical analysis

All statistical analysis was carried out with the help of Graphpad Prism 6 software (La Jolla, CA, USA). Difference in the incidence of diabetes were evaluated using the Mantel-Cox log-rank test. Data are shown as the mean ± SD. Statistical comparisons of means were

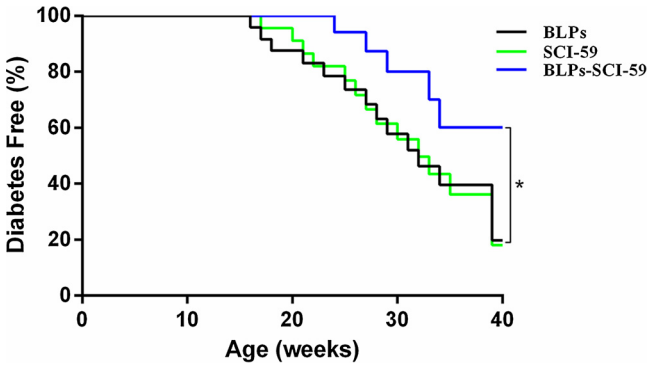

Fig. 1. The frequency of diabetes-free mice over time shows that oral vaccination with BLPs-SCI-59 induces remission of diabetes in NOD mice. For each group (n = 15 per group), all mice were orally administrated one time a day for the first week and then three times weekly until the mice were 20 weeks old. Diabetes mellitus was diagnosed when glucose values are higher than 11.1 mmol/L. * $p < 0.05$.

carried out using ANOVA followed by Student's *t*-text. *, $p < 0.05$ and **, $p < 0.01$ were used as the definition of statistical significance.

3. Results

3.1. Oral administration of BLPS-SCI-59 prevented T1DM

During the observation period (4–40 weeks), the incidence of T1DM in treated mice was analyzed among groups (Fig. 1). This incidence reduced significantly in BLPs-SCI-59 group compared with the group fed free SCI-59 or BLPs. Diabetes onset was delayed in the BLP-SCI-59 group (24 weeks of age), as compared to the SCI-59 group (17 weeks of age) and the BLPs group (16 weeks of age). At 40 weeks of age, only 6 out of 15 (40%) mice in the BLPs-SCI-59 group were diagnosed as diabetes. However, 12 out of 15 (80%) mice in the SCI-59 group and 13 out of 15 (87%) mice in the BLPs group developed diabetes (Fig. 1, $p < 0.05$). Therefore, these results showed that oral vaccination with BLPs-SCI-59 possessed a preventative effect against T1DM in NOD mice.

3.2. Oral vaccination with BLPs-SCI-59 improved glucose tolerance

In order to assess the preventative ability of BLPs-SCI-59 against T1DM, an OGTT was performed in 21-week-old NOD mice (Fig. 2). Blood glucose levels in all mice were similar after an overnight fast (Fig. 2A). After glucose injection, it was detected that blood glucose levels in all groups rose to peak values at 20 min and decreased subsequently (Fig. 2A). At 15 min post-injection, blood glucose levels were significantly lower in mice fed BLPs-SCI-59, as compared to those in mice of SCI-59 and BLPs group (Fig. 2B, $p < 0.05$). At 120 min, blood glucose values in all mice had returned to normal. However, the maintenance time of hyperglycemia in mice fed BLPs-SCI-59 was much shorter than those in mice fed free SCI-59 and BLPs (Fig. 2A).

3.3. Oral administration of BLPs-SCI-59 reduced insulitis

To analyze the effects of oral vaccination with BLPs, SCI-59, BLPs-SCI-59 on suppressing insulitis, comparative analysis of insulitis among mice in different groups was performed. At 40 weeks of age, their pancreatic tissues were analyzed and scored by HE staining (Fig. 3). The SCI-59 group and BLPs group featured more evident inflammation in pancreatic islets compared with the BLPs-SCI-59 group (Fig. 3A-C). About 24% of pancreatic islets in the BLPs-SCI-59 group showed severe insulitis, compared with 60% and 68% in the SCI-59 group and BLPs group, respectively (Fig. 3D). A significant reduction in insulitis was observed in mice fed BLPs-SCI-59 compared with the free SCI-59- or

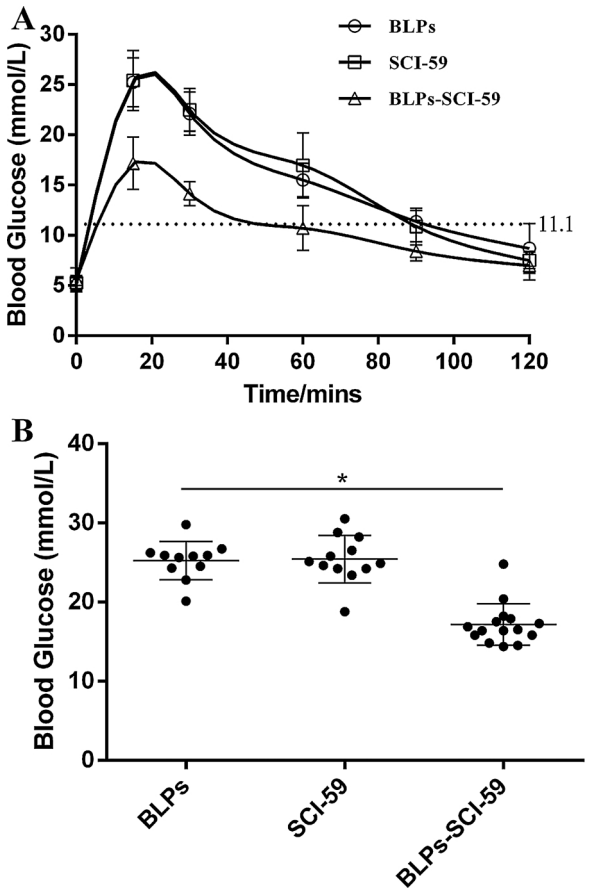

Fig. 2. Oral vaccination with BLPs-SCI-59 improves glucose tolerance. At 21 weeks of age, mice that were free of diabetes in each group (BLPs group, n = 11; SCI-59 group, n = 12; BLPs-SCI-59 group, n = 15) were selected for glucose tolerance test. (A) After glucose injection, blood glucose was measured at different time points. (B) Comparative analysis of blood glucose values in different groups at 15 min. Data are shown as means ± SD. * $p < 0.05$.

BLPs-fed mice (Fig. 3E, $p < 0.05$). Therefore, it showed that the islets from the BLPs-SCI-59 group were more intact and less inflammation compared to the SCI-59 group and BLPs group.

3.4. Oral administration of BLPs-SCI-59 preserved C-peptide secretion

The dynamic changes in C-peptide were monitored for assessing pancreatic beta cell function among different groups. As shown in Fig. 4, C-peptide contents in mice fed BLPs-SCI-59 were significantly higher ($p < 0.05$) than those in mice fed free SCI-59 and BLPs in the second half of the observation period (25–40 weeks). Mice fed BLPs-SCI-59 showed superior maintenance of C-peptide secretion, as compared with those in the SCI-59 group and BLPs group.

3.5. Oral administration of BLPs-SCI-59 induced SCI-59-specific Th2-like humoral immune response

In order to study the mechanism leading to the suppressive effects on insulitis, we analyzed serum SCI-59-specific antibodies among different groups. As shown in Fig. 5, mice fed BLPs-SCI-59 produced higher titers of anti-SCI-59 IgG. While no anti-SCI-59 IgG was detected in both the SCI-59 group and BLPs group. The IgG subtype analysis showed that the serum anti-SCI-59 IgG1 levels were significantly higher ($p < 0.05$) in the BLPs-SCI-59 group than those in the SCI-59 group and BLPs group. For anti-SCI-59 IgG2a, no significant difference was observed among various groups. Therefore, these results indicated that mice fed BLPs-SCI-59 induced SCI-59-specific Th2-type (IgG1), other

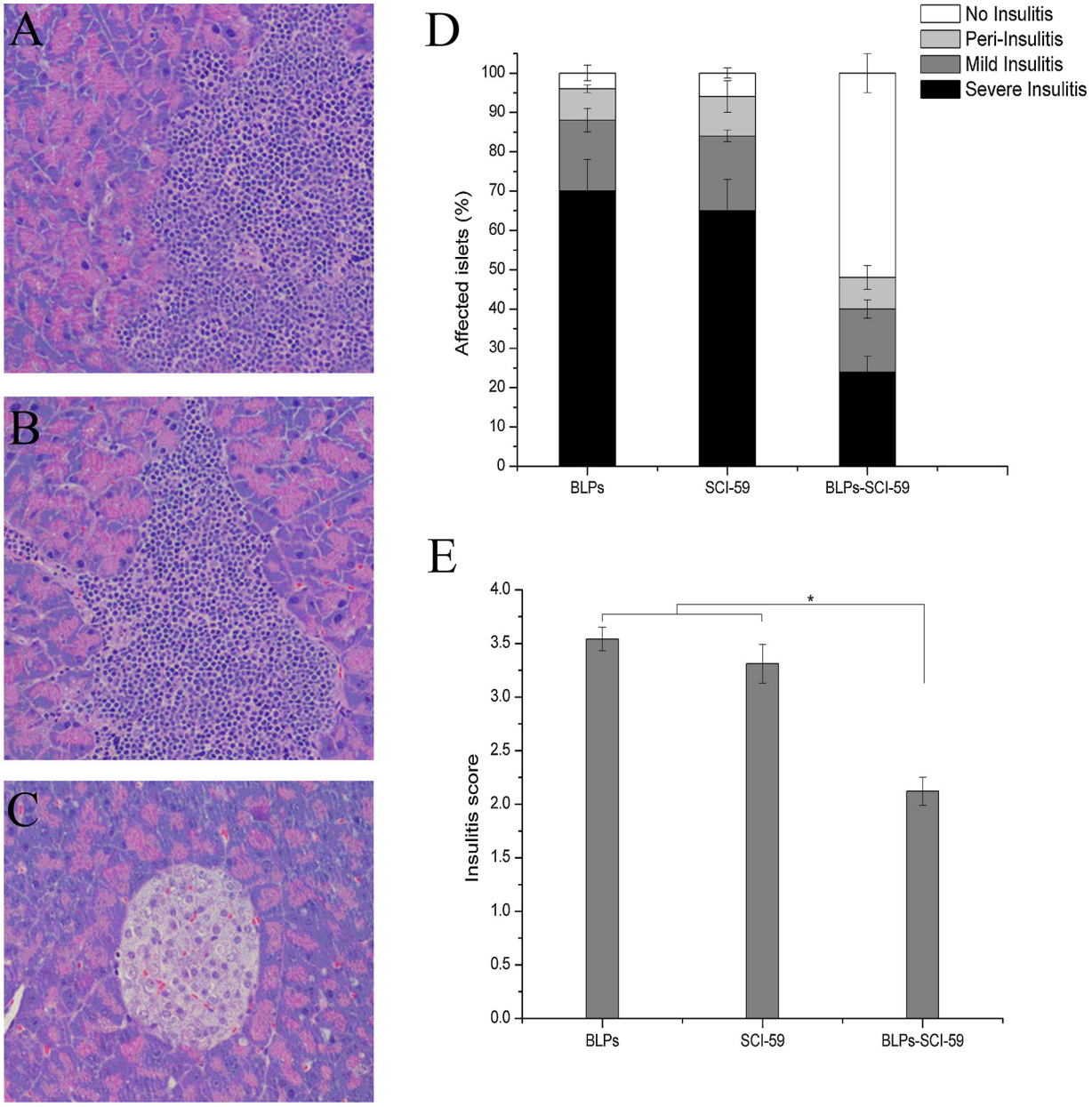

Fig. 3. Oral vaccination with BLPs-SCI-59 protects pancreatic islets. 40-week-old mice (n = 3 per group) were sacrificed and the pancreas were collected for HE staining. A, B, C, the representative pancreatic islet from the BLPs group (A), SCI-59 group (B) and BLPs-SCI-59 group (C) (× 400). (D and E) Comparative analysis of insulitis among groups. At least 60 islets were analyzed for each group. Data are shown as means ± SD. * $p < 0.05$.

than Th1-type (IgG2a), humoral immune responses.

3.6. Oral administration of BLPs-SCI-59 induced antigen-specific suppression of splenocyte proliferation and a shift in Th1/Th2

To evaluate whether the immune tolerance was stimulated, splenocyte proliferation test was carried out. The results (Fig. 6) showed that ConA enhanced splenocyte proliferation in all tested mice, while BSA made no difference to splenocyte proliferation. Significant proliferation stimulated by SCI-59 was detected in splenocytes collected from both the SCI-59 group and BLPs group. Oppositely, cell proliferation SI stimulated by SCI-59 in the BLPs-SCI-59 group was significantly lower than those of the SCI-59 group and BLPs group ($p < 0.05$).

Based on the analysis results of cytokine production upon SCI-59 stimulation (Fig. 7), secretion of Th1-type cytokines (IL-2 and IFN-γ) by splenocyte was significantly lower in the BLPs-SCI-59 group than in the SCI-59 group and BLPs group (Fig. 7A and B, $p < 0.01$). Conversely, oral administration of BLPs-SCI-59 significantly increased Th2-type cytokines (IL-4 and IL-10) production as compared to the SCI-59 group and BLPs group (Fig. 7C and D, $p < 0.01$).

3.7. Oral vaccination with BLPs-SCI-59 enhanced Tregs differentiation

In order to test whether oral administration of BLPs-SCI-59 induces tolerance through Tregs, flow cytometry was performed to analyze the $CD4^+CD25^+FoxP3^+$ Tregs in the peripheral lymph system. A significantly higher content of such Tregs in the PLN $CD4^+$ T cell compartment was detected in mice fed BLPs-SCI-59 compared with the SCI-59 group and BLPs group (Fig. 8, $p < 0.05$).

4. Discussion

Mucosal immune tolerance has been considered as a safe and

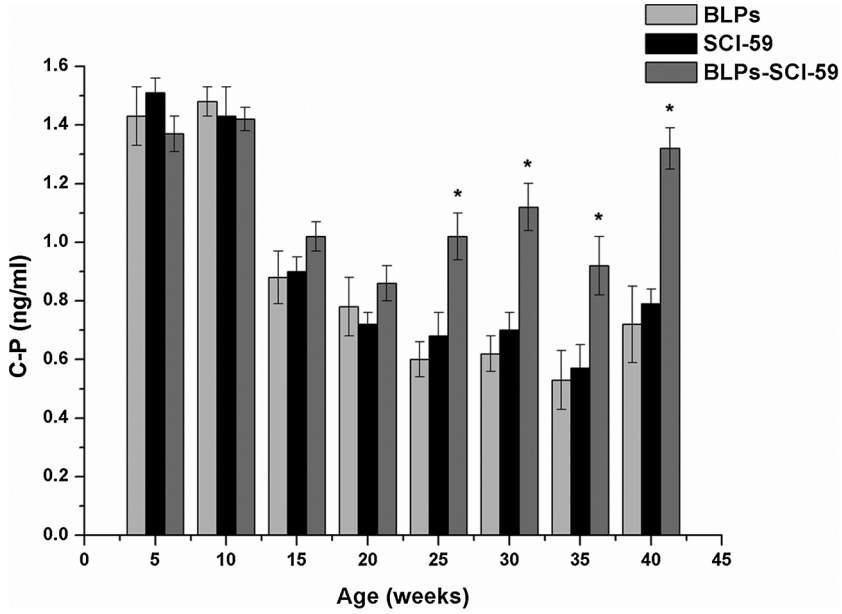

Fig. 4. Monitoring of C-peptide serum levels among all groups. Results are shown as means ± SD. * $p < 0.05$.

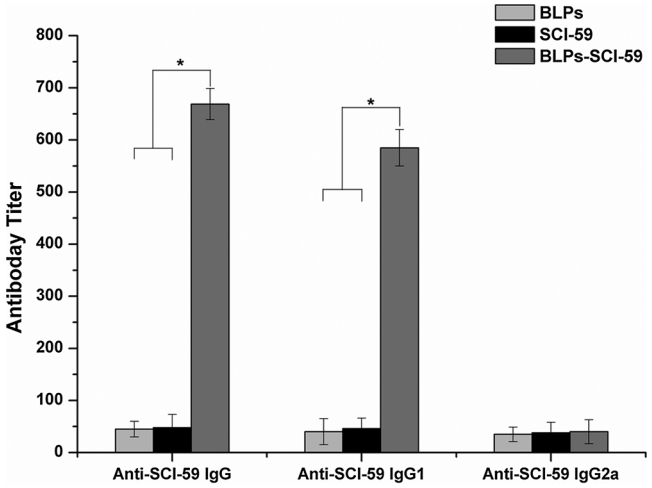

Fig. 5. Oral vaccination with BLPs-SCI-59 induces antigen-specific antibodies. 20-week-old mice (n = 10 per group) serum were quantified for anti-insulin antibodies and antibody subtypes. Data are shown as means ± SD. * $p < 0.05$.

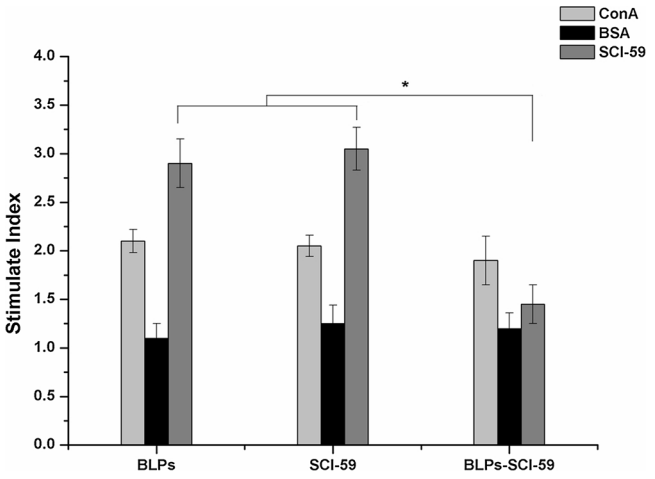

Fig. 6. Oral vaccination with BLPs-SCI-59 induces antigen-specific suppression of splenocyte proliferation. At 40 weeks of age, splenic cells were isolated from NOD mice for T cell proliferation assay by stimulating with ConA, BSA and SCI-59. Three mice per group were individually tested in three independent experiments. Data are presented as means ± SD. * $p < 0.05$.

effective way to suppress inflammatory or autoimmune diseases, as the agents applied in these cures have less toxicity, can be administered conveniently, and promote antigen-specific immune responses [23–25]. However, the degradation of autoantigens within the intestinal tract may be a challenge for this immunotherapy [13,26–28]. In order to protect oral antigens from degradation passing through the GIT, the method of delivery is a key factor to consider. Resulting from natural LAB cells, BLPs have been used to improve the treatment effect of existing vaccine and deliver subunit antigens by binding them on their surface [29]. Belonging to microparticles [30], LAB BLPs may be used as an ideal platform for oral delivery of SCI-59 as described previously [19].

Using MG1363 BLPs as negative control, we compared the continuous effects of oral vaccination with the free SCI-59 and BLPs-SCI-59 on the prevention of T1DM. Consequently, the incidence of disease in BLPs-SCI-59 group (40%) decreased significantly compared with the SCI-59 group (80%) and the BLPs group (87%). No significant difference of the incidence between the SCI-59 group and BLPs group was detected. This may be due to the rapid degradation of the free SCI-59 passing through the GIT [19,28] and little autoantigen arrives to the tolerogenic microenvironment of the gut mucosa, which can present antigens to the immune system to stimulate the formation of tolerance efficiently [7]. However, SCI-59 delivered by BLPs (BLPs-SCI-59) may be protected from degradation and retain the proper form in the gut. This affords for the significant suppression of diabetes in mice fed BLPs-SCI-59.

In order to analyze the ability of BLPs-SCI-59 to regulate glucose levels in tested mice, an OGTT was performed. Compared to the SCI-59 group and BLPs group, a stronger ability towards regulation of glucose levels was detected by oral administration of BLPs-SCI-59. This suggests that inflammatory responses around islets had already happened at this time in the SCI-59 group and BLPs group, resulting to a reduction of the sensitivity of pancreatic islet beta cells to glucose [31]. This postulation can be supported by a higher incidence of disease in the SCI-59 group and BLPs group as described above and the insulitis evaluation of the tested mice when the observation period finished. Compared to the SCI-59 group and BLPs group, more intact islets and less inflammation were

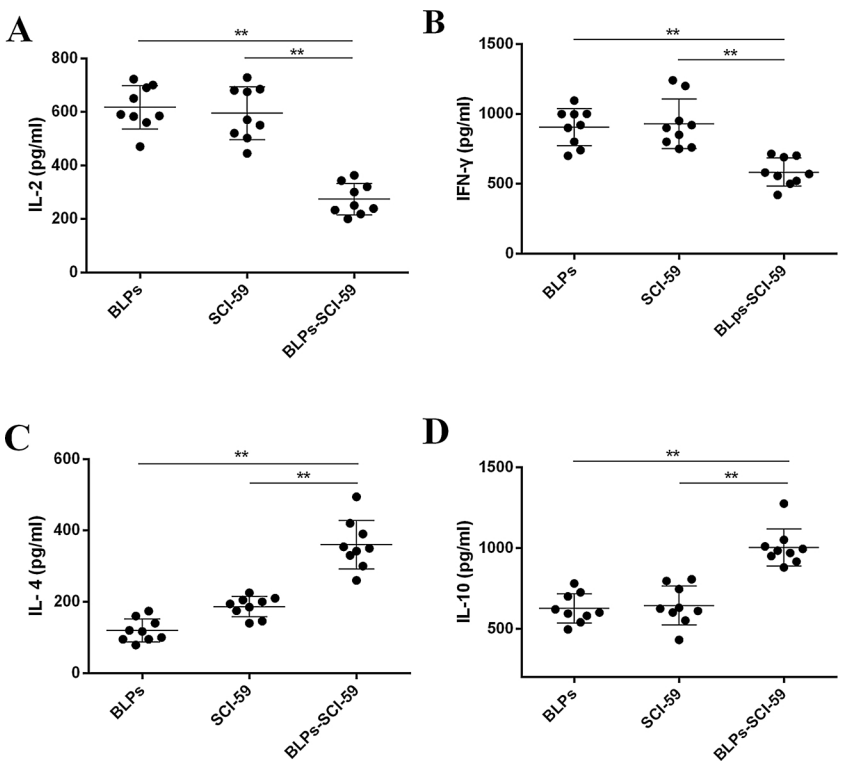

Fig. 7. Cytokine production assay shows that oral vaccination with BLPs-SCI-59 induces an increased Th2 shift from the Th1 profile. IL-2 (A), IFN-γ (B), IL-4 (C) and IL-10 (D) were quantified in splenic cell suspensions incubated with SCI-59. Three mice per group were individually tested in three independent experiments. Means and SDs are presented as lines. ** $p < 0.01$.

detected in the BLPs-SCI-59 group and it shows that BLPs-SCI-59 has an effect on preventing insulitis. This protective effect of pancreatic islet beta cells induced by oral administration of BLPs-SCI-59 can be further supported by the analysis of the dynamic changes in C-peptide levels, which reflects pancreatic islet beta cells function directly [32]. Compared to the SCI-59 group and BLPs group, a superior maintenance of C-peptide secretion was detected in mice fed BLPs-SCI-59, indicating that there was more insulin released in the pancreatic islets of mice fed BLPs-SCI-59 than in those of the SCI-59 group and BLPs group.

To investigate the immune mechanisms leading to the development

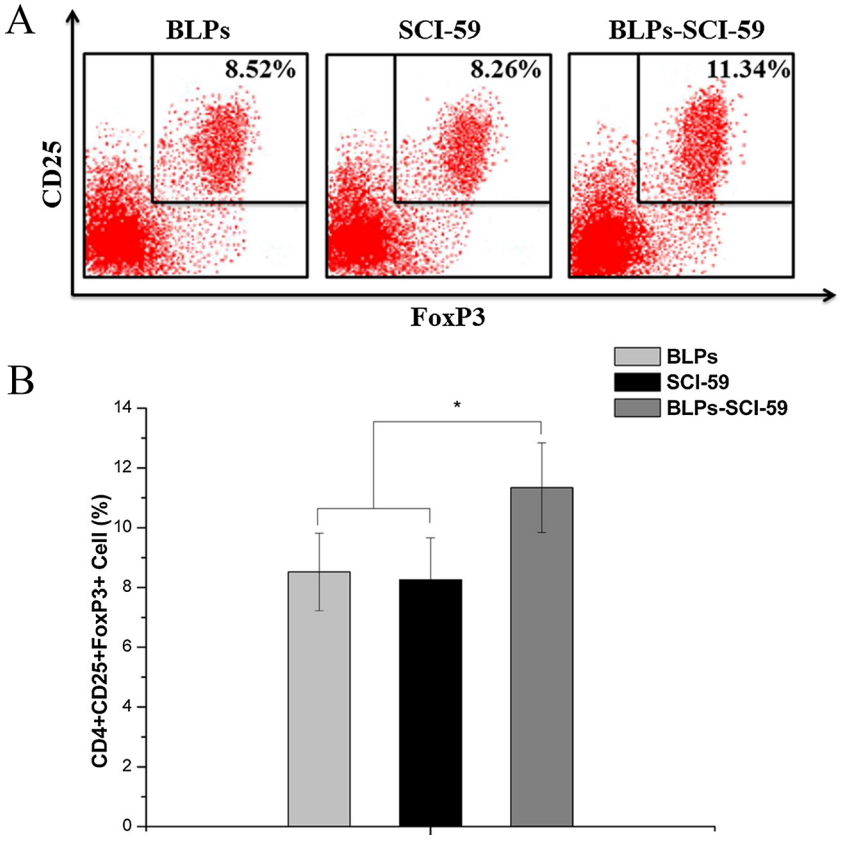

Fig. 8. Oral vaccination with BLPs-SCI-59 enhances regulatory T cell differentiation. $CD4^+CD25^+FoxP3^+$ Tregs in the PLN were analyzed using flow cytometry (A) and comparison among different groups (B). Three mice per group were individually tested in three independent experiments. Data are presented as means ± SD. * $p < 0.05$.

of oral tolerance in mice fed BLPs-SCI-59, we first analyzed serum specific antibodies among different groups. We show that oral administration of BLPs-SCI-59 may induce a Th2-type (IgG1) of SCI-59-specific antibody subclasses, instead of a Th1-type (IgG2a). This is consistent with previous studies that oral vaccination with T1DM associated autoantigens could induce antigen-specific Th2-associated antibody subclasses [8,33–36]. Then, this effect was further supported by the analysis of splenocyte proliferation in vitro. Spleen cells from the SCI-59 group and BLPs group showed rapid proliferation by stimulation with SCI-59. However, spleen cells from the BLPs-SCI-59 group were significantly suppressed by SCI-59. T cells collected from all mice presented similar reactivates to ConA, suggesting that no general inhibition of T cell response was stimulated by oral vaccination with BLPs-SCI-59. Therefore, prevention of T1DM by oral vaccination with BLPs-SCI-59 was related to inhibition of spontaneous proliferative T cell responses to SCI-59. The cytokines analysis showed that IL-4 and IL-10 secretion enhanced and IL-2 and IFN-γ secretion decreased, indicating the Th1-associated autoimmune response shifted toward Th2-associated autoimmune response. As the cytokine environment affect the differentiation of Th1/Th2 cells [37], more IL-4 and IL-10 and less IL-2 and IFN-γ environment induced by BLPs-SCI-59 stimulate more naïve T cell to Th2 cells, accompanied by the suppression of the differentiation of Th1 cells. Therefore, oral administration of BLPs-SCI-59 can restore the unbalance of Th1/Th2, which may lead to the occurrence and development of T1DM [32,38]. In contrast, no antigen-specific humoral immune response was induced by oral vaccination with the free SCI-59 and BLPs, accounting for the little effect of protection against T1DM. $CD4^+CD25^+FoxP3^+$ Tregs play a key role in suppressing T cells differentiate into inflammatory cells, including Th17 lymphocytes [39,40]. Therefore, the protection against T1DM observed in mice fed BLPs-SCI-59 may result from the significant increasing of $CD4^+CD25^+FoxP3^+$ Tregs compared to the SCI-59 group and BLPs group.

In summary, our study demonstrated that oral vaccination with BLPs-SCI-59 can prevent T1DM in NOD mice and stimulate antigen specific oral tolerance. This beneficial effect is achieved through an improved regulatory immune response to repair Th1/Th2 imbalance. These results indicate that LAB BLPs may be applied as a potential vector for delivering autoantigens to gut mucosa to induce tolerance.

Declaration of Competing Interest

The authors have declared that there is no competing interest.

Acknowledgements

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