Ethylenecarbodiimide-fixed splenocytes carrying whole islet antigens decrease the incidence of diabetes in NOD mice via down-regulation of effector memory T cells and autoantibodies

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Abstract. Type 1 diabetes mellitus (T1DM) is a syndrome of loss of glucose homeostasis caused by the loss of β cell chronic autoimmunity against islet cells. Islet-specific epitopes coupled antigen presenting cells by Ethylenecarbodiimide (ECDI) is a promising strategy to induce antigen-specific tolerance. However, single epitope induced tolerance is insufficient to prevent the onset of T1DM. The aim of this study is to evaluate the efficacy of whole islet antigens in preventing the onset and progression of T1DM and identify the underlying immune mechanism in NOD mice. In this study, the whole islet antigens, derived from islet lysate isolated from BALB/c mice, were coupled to splenocytes of BALB/c mice by ECDI fixation (SP-Islet lysate), and then intravenously administrated to NOD mice. The results showed that, compared with control group, SP-Islet lysate treated mice had reduced insulitis score and autoantibody levels, and improved glucose tolerance and insulin/glucagon production. Furthermore, the effector memory T cells (T EMs ) were downregulated and regulatory T cells (Tregs) were upregulated by the SP-Islet lysate treatment, with reduced populations of Th1&Th17 cells. In conclusion, ECDI-fixed splenocytes carrying whole islet antigens effectively prevented the onset of T1DM in NOD mice, via suppressing the production of autoantibodies and inducing anergy of autoreactive T cells.

Key words: Type 1 diabetes mellitus, Whole islet antigen, Autoantibody, Effector memory T cell, Regulatory T cell

TYPE 1 DIABETES MELLITUS (T1DM) is a chronic autoimmune disease, mainly characterized with cell-mediated autoimmune destruction of the pancreatic β-cells in predisposed individuals [1]. The autoimmunity in T1DM is predisposed by the genetic susceptibility, which is known to lead to loss of immunological tolerance to certain β-cell antigens. Autoreactive T cells specifically attack the insulin producing β cells, and both NOD mice and T1DM patients exhibit mostly an effector/memory phenotype consistent with ongoing β cell autoimmunity due to antigen-experienced in vivo proliferation history which contributes to the difficulty in preventing disease progression of T1DM [2, 3]. Regulatory T cells (Tregs) have an essential role in the maintenance of immunological tolerance and participate in regulating the onset and progression of T1DM [4]. Autoantibodies such as anti-insulin autoantibodies (IAA), anti-glutamic acid decarboxylase autoantibodies (GADA), anti-islet cell antibodies (ICA), anti-islet antigen-2 antibodies (IA-2A), and anti-zinc transporter autoantibodies (ZnT8A) are likely de-
Therefore, therapies containing multiple islet-specific epitopes, coupled to APCs by ECDI fixation, are needed to be developed and evaluated for the prevention and treatment of autoimmune T1DM.

In this context, we proposed that ECDI-fixed splenocytes carrying whole islet antigens might effectively inhibit the autoimmune. In this study, splenocytes were fixed with islet lysate containing the whole islet antigens by ECDI and then intravenously infused to NOD mice, aiming to evaluate the efficacy of this treatment in inducing immunological tolerance and preventing the onset of T1DM.

**Methods**

**Mice**

Female NOD mice aged 8 weeks and male BALB/c mice aged 7 weeks were purchased from Beijing Huafukang Biosciences (Beijing, China), and housed in a specific pathogen-free facility at a cycle of 12:12 light/dark, constant temperature and humidity at Key Laboratory for Critical Care Medicine of the Ministry of Health. The female NOD strain in this study derived from the outbred Jcl:ICR line of mice through repetitive brother-sister mating [18]. This sub-strain of NOD mice spontaneously presents a diabetic incidence of around 60%. Animal bodyweight was measured at least every two days. NOD mice were considered diabetic when random blood glucose levels were ≥250 mg/dL for two consecutive days measured from tail bleeding using Roche glucometer. All animal studies were approved by the Institutional Animal Care and Ethics Committee of Tianjin First Center Hospital.

**Tolerance induction**

Spleen tissues from BALB/c mice were grinded with a frosted surface of a slide in RPMI 1640 (Solarbio, Beijing, China), filtrated through a 70 μm-pore size mesh Cellstrainer™ (Falcon, BD Biosciences, Heidelberg, Germany) and washed in phosphate buffered saline (PBS) to create a single cell suspension. Tris-NH₄Cl (Solarbio, Beijing, China) was used to eliminate the red blood cells. The cells were washed in PBS with centrifuge (400 g, 5 min) and resuspended in PBS to create a single cell suspension. Pancreases of BALB/c mice were digested by collagenase P (0.5 mg/mL, Roche, Indiana, USA) at 37°C for 15 min, and purified by density gradient (Histopaque 1077, Sigma, Louis, USA). Islet lysate was prepared by ten freeze-thaw cycles of islets from BABL/c mice. Splenocytes (3.2 × 10⁸ cells/mL) were incubated with islet lysates (lysate of 1,600 islets/mL) in the presence of ECDI (150 mg/mL) on ice for 1 hour with intermittent shaking. The splenocytes coupled with cell-free islet lysate were washed with PBS and filtrated to remove cell clumps, and then resuspended in PBS. Unconjugated lysates and free ECDI had been eliminated by above washing and filtration. A total of 1 × 10⁸ splenocytes coupled with

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islet lysate from about 500 islets were injected intravenously to female NOD mice firstly at the age of 10 weeks and secondly at 11 weeks, and sham injection with equal volume of saline was performed in the control group.

**Oral glucose tolerance test**

Prior to sacrifice, oral glucose tolerance test (OGTT) was performed. NOD mice were fasted for 16 hours, and then administrated with glucose (50% W/V, 2 g/Kg) by intragastric gavage. Blood glucose levels were determined at 0, 15, 30, 45, 60, 90, and 120 minutes after glucose administration. By using the fasting blood glucose level as baseline, area under the curve (AUC) was calculated.

**H&E and immunohistochemical staining**

Pancreatic tissue was fixed in formaldehyde, embedded in paraffin blocks, and sectioned into 3 μm slices. Tissue slides were deparaffinized, rehydrated and stained with Hematoxylin and eosin (H&E), anti-mouse insulin (1:200, Abcam, Cambridge, UK) and anti-mouse glucagon (1:200, Abcam, Cambridge, UK), as previously described [19, 20]. At least 10 nonadjacent sections were analyzed per pancreas. At least 20 islets from each mouse pancreas were scored according to the following criteria: 0, no infiltration; 1, intact islets but with immune cells surrounding the islets (peri-isulitis); 2, immune cells infiltration in less than 50% of the area; 3, immune cells infiltration in more than 50% of the area; 4, islets that are completely destroyed.

**Flow cytometry (FCM)**

The NOD mice were scarified at the 10 weeks after first injection of SP-Islet lysate. At the same time, pancreatic draining lymph node cells and splenocytes were collected and assessed immediately. For analysis of memory T cells, after being washed with ice-cold PBS, cells were surface labeled with CD3-PE-Cyanine7, CD4-FITC, CD44-APC and CD62L-PE. For analysis of Treg cells, the samples were surface labeled with CD3-PE-Cyanine7, CD4-FITC and CD25-PE, and intracellularly stained with Foxp3-APC, following the manufacturer’s instruction (eBioscience™ Mouse Regulatory T cell Staining kit, eBioscience, San Diego, California, USA). For analysis of Th1 and Th17 cells, the samples were suspended at a density of 5 × 10^6 cells/mL in complete culture medium (RPMI 1640 supplemented with 1% Penicillin-Streptomycin Solution, and 10% FBS), and were stimulated for 5 hours using 50 ng/mL of phorbol myristate acetate (PMA, Sigma, St. Louis, Missouri, USA) and 1 μg/mL ionomycin (Solarbio, Beijing, China) at 37°C and 5% CO_2_. Washed with ice-cold PBS, the cells were surface labeled with CD3-PE-Cyanine7, CD4-FITC. After fixation and permeabilization, the cells were stained with IL-17-PE and IFN-γ-APC. CD4-FITC, CD3-PE-Cyanine7, CD44-APC, CD62L-PE antibodies were purchased from eBioscience (San Diego, California, USA), and IFN-γ, and IL-17 antibodies from Biolegend (Dedham, Massachusetts, USA). All samples were analyzed using a CANTOII flow cytometer (BD Biosciences, Franklin, New Jersey, USA) and FlowJo cytometry analysis software (FlowJo, San Francisco, California, USA).

**Autoantibody detection**

IAA, ICA, GADA, IA-2A in sera from different treatment groups were measured with mouse autoantibodies ELISA kit (IAA, ICA, GADA, IA-2A) (Neoscientific, Boston, Massachusetts, USA) according to the manufacturer’s instructions.

**Statistical analysis**

T1DM incidence and survival rate were analyzed by Kaplan-Meier plot with a log-rank test. Differential autoantibodies levels, and percentage of lymphocyte subsets were analyzed by Student t test, and represented as mean ± SEM. AUC in the OGTT was calculated by SPSS 17 (Armonk, New York, USA). Other statistical analyses were performed using Graphpad Prism 7.2 (GraphPad, La Jolla, California, USA). p values <0.05 were considered as statistically significant.

**Results**

**SP-Islet lysate treatment prevents the onset and progression of T1DM**

Female NOD mice aged ≥10 weeks are in the effector phase of autoimmunity, and generally assumed as a clinically relevant model for the investigation of the potential efficacy of novel treatments for T1DM [21]. To assess the efficacy of SP-Islet lysate in preventing T1DM, BALB/c islet lysate was used as a soluble form of whole islet antigens, and coupled to the splenocytes isolated from BALB/c mice by ECDI fixation (SP-Islet lysate). SP-Islet lysate was intravenously injected into the recipient female NOD mice firstly at the age of 10 weeks and secondly at 11 weeks (SP-Islet lysate group, n = 18,
Fig. 1A). Our preliminary study has compared the effect of ECDI-SP with saline on NOD mice. The results showed that ECDI-SP and saline had no obvious difference on the protection of diabetes incidence (Fig. S1), as well as tolerance induction (Fig. S2) in NOD mice. Thus, the present study adopted saline injection as control treatment ($n = 10$) to avoid non-necessary animal sacrifice. In our study, 60% of the mice became diabetic at 20 weeks of age in the control group, while only 16.7% of mice developed T1DM in the SP-Islet lysate group ($p < 0.05$, log rank test, Fig. 1B), with better survival rate ($p < 0.01$, log-rank test, Fig. 1C). These results indicate that SP-Islet lysate treatment decreased the incidence of T1DM in NOD mice.

Autoantibodies, such as GADA, IAA, ICA, IA-2A, are considered as the typical markers for autoimmunity in T1DM [22]. To determine the effect of SP-Islet lysate on the autoimmunity development, the levels of GADA, IAA, ICA, IA-2A in mice blood serum were detected by ELISA in 20-week-old mice (10 weeks after treatment).
The level of GADA, IAA, IA-2A of the mice in treatment group were significantly lower than those in control group, with a relative decrease of 80.2% ($p < 0.01$), 53.1% ($p < 0.01$), and 63.3% ($p < 0.01$), respectively (Fig. 1D). However, there was no significant decrease in ICA level after SP-Islet lysate treatment ($p = 0.49$, Fig. 1D).

The immune cells specifically damage pancreatic β-cells and the presence of inflammation peri-islet predicts the progression of T1DM disease in NOD mice. To further elucidate the effect of SP-Islet lysate treatment, we analyzed the immune cells infiltration by H&E staining and insulitis scoring. Results showed that insulitis score was reduced in the SP-Islet lysate treated group (Fig. 1E & D). These results suggest that SP-Islet lysate treatment can inhibit the stimulation of autoreactive T$_{EMs}$ but not the T$_{CMs}$.

**SP-Islet lysate treatment maintains islet function**

Insulitis seriously influences islet function. The impairment of glucose tolerance in T1DM predicts the impairment of islet function. OGTT results demonstrated that the SP-Islet lysate treated mice had better glucose tolerance, evidenced by the greater AUC ($p < 0.05$, Fig. 2A & B). This implies that SP-Islet lysate treatment prevented impairment of glucose tolerance. Concomitantly, the IHC result showed that the SP-Islet lysate treatment significantly ameliorated the islet function, with significant increase of insulin positive cells (72.92 ± 5.014 for the treated group, 37.76 ± 8.804 for the control group, $p < 0.05$), as well as glucagon positive cells (27.54 ± 2.926 for the treated group, 14.44 ± 2.642 for the control group, $p < 0.05$), while islet function was well maintained in the treated mice (Fig. 2C & D). These results indicate that SP-Islet lysate treatment maintained the islet function of NOD mice.

**SP-Islet lysate treatment decreases the frequency of autoreactive effector memory T-cells and Th1&Th17 cells**

The percentage of memory T cells was reported to be increased in NOD mice when developed T1DM [23], and autoreactive memory T cells contributes to the difficulty in preventing disease progression in new-onset T1DM [2]. As shown in Fig. 3A, effector memory T cells (T$_{EMs}$) were represented by CD4$^{+}$CD44$^{hi}$CD62L$^{-}$ T cell populations. SP-Islet lysate treatment downregulated T$_{EMs}$ both in the pancreatic draining lymph nodes (11.70 ± 1.93% for the treatment group, 34.06 ± 3.63% for the control group, $p < 0.001$, Fig. 3B) and in the spleens (13.7 ± 0.96% for the treatment group, 17.95 ± 1.09% for the control group, $p < 0.05$, Fig. 3B). However, there is no obvious change for the central memory T cells ($p = 0.52$ for the T$_{CMs}$ in pancreatic draining lymph nodes, and $p = 0.53$ for the splenic T$_{CMs}$, Fig. 3C & D). These results suggest that SP-Islet lysate treatment can inhibit the stimulation of autoreactive T$_{EMs}$ but not the T$_{CMs}$.

Th1 and Th17 have been implicated in the pathogenesis of T1D in mice and humans due to secreting pro-inflammatory cytokines such as IL-17, IL-21, IL-27, IFN-$\gamma$ and TGF-$\beta$ [24]. In our study, the IFN-$\gamma$CD4$^{+}$ T cells (Th1) and IL-17CD4$^{+}$ T cells (Th17) were both downregulated by SP-Islet treatment in both pancreatic draining lymph nodes ($p < 0.05$) and spleens ($p < 0.01$, Fig. 3B).

**SP-Islet lysate treatment upregulates the frequency of Tregs**

Previous studies displayed that an increasing loss of Tregs function conduces to autoimmunity in the NOD-mouse models of T1DM [25, 26]. Tregs have been shown to suppress the functions of effector T cells through multiple mechanisms including (but not limited to) production of inhibitory cytokines (IL-10, TGF-$\beta$), IL-2 sequestration, and blockade of the co-stimulatory molecule [27]. In our study, FCM was used to analyze the frequency of CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ Treg cells in pancreatic draining lymph nodes and spleens (Fig. 4). The results showed that the percentage of CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ Tregs of the pancreatic lymph nodes in the SP-Islet lysate treatment group was significantly higher than that in control group (8.04 ± 0.43% for treatment group, 5.48 ± 0.55% for the control group, $p < 0.01$, as shown in Fig. 4B). Similarly, the splenic Tregs population also presents a significant upregulation (4.78 ± 1.18% for treatment group, 2.42 ± 0.35% for the control group, $p < 0.05$, as shown in Fig. 4B). These results indicated that SP-Islet lysate treatment upregulated the frequency of CD4$^{+}$CD25$^{+}$Foxp3$^{+}$ Tregs in NOD mice.
Discussion

T1DM occurs as a result of an immune regulation disorder, including the formation of T1DM-associated autoantibodies, the expansion of autoreactive CD4+ and CD8+ T cells, and the imbalance of Tregs and Th1&Th17 cells. These factors collaborate to destroy β-cells in islets [28]. ECDI-fixed splenocytes coupled with special antigens can induce antigen-specific tolerance [13], and this method is currently receiving much attention for its potential to serve as a clinical therapeutic strategy for autoimmune diseases. In this study, we detected the effect of SP-Islet lysate on the incidence of T1DM in NOD mice. The results revealed that this treatment effectively provided protection against the onset and progression of T1DM in NOD mice.

Islet autoantibodies are considered as the markers of T1DM-related autoimmunity, and are associated with the onset of T1DM [29]. The present study found that SP-Islet lysate treatment significantly reduced the levels of GADA, IA-2A, IAA in serum, indicating a general inhibition on humoral autoimmunity. Interestingly, no
Fig. 3  SP-Islet lysate treatment decreases the frequency of autoreactive T_{EMs} and Th1/Th17. (A) Representative FCM dot plots of memory T-cells in pancreatic draining lymph nodes and spleens from NOD mice in control group and SP-Islet lysate treated group aged 20 weeks. Each sample was stained with CD3, CD4, CD44, and CD62L antibodies. CD3 and CD4 were used to gate CD4+ T-cells. CD44 and CD62L were used to gate memory T-cells. (B) Quantitative analysis of the frequency of T_{EMs} (CD4+ CD44^{high} CD62L^{low}) and T_{CMs} (CD4+ CD44^{high} CD62L^{+}). (C) FCM analysis of Th1 and Th17 lymphocyte subsets in pancreatic draining lymph nodes and spleens from 20-week NOD mice treated by SP-Islet lysate and control. Each sample was stained with CD3, CD4, IFN-γ and IL-17 antibodies, CD3 and CD4 was used to gate CD4+ T cells. IFN-γ and IL-17 were used to gate Th1 and Th17. (D) The proportions of Th1 cells (CD4+ IFN-γ^- IL17^-), Th17 cells (CD4+ IL17^+ IFN-γ^-) in each group. *p < 0.05, **p < 0.01, ***p < 0.001. Experiments were repeated three times independently.
significant decrease of ICA levels was observed in the SP-Islet lysate treatment group in this study. This may be due to the amount of islet antigens in SP-Islet lysate system exceeded the proper antigen amount for tolerance induction.

Accumulated studies in NOD mice have demonstrated that T1DM occurred as a consequence of the β-cell destruction by the β-cell-autoreactive CD4\(^+\) and CD8\(^+\) T cells [4, 27, 30-32]. In our study, similar to the changing trend of autoantibodies, the frequency of T\(_{EMs}\) also presented a significant decrease in the SP-Islet lysate group compared with control group. These results indicate that the ECDI fixed splenocytes carrying whole islet antigens can potently induce antigen-specific tolerance. Two possible reasons might collectively explain this phenomenon. At first, β-cell antigens existed in ill-defined environmental cues can not only directly stimulate the production of autoantibodies, but also govern the islets infiltration and the activation of naive CD4\(^+\) T cells to antigen-responsive memory T cells [27, 33]. Secondly, autoantibodies and autoreactive memory T cells can mutually stimulate each other and further amplify the autoimmunity. Heninger et al. found that the activation of memory CD4\(^+\) T cells could further promote the maturation of B cells and hence stimulate the secretion of autoantibodies [34]. The existence of autoantibodies can, in turn, induce the activation and proliferation of antigen-responsive CD4\(^+\) T cells, reported by two recent studies [35, 36]. Taken together, ECDI-fixed splenocytes carrying whole islet antigens can effectively inhibit both humoral and cellular autoimmunity involved in T1DM, and hence confer protection against the onset and progression of T1DM in NOD mice. Nonetheless, in the tolerance induced by SP-Islet lysate treatment, the relationship of humoral and cellular autoimmunities still needs to be elucidated in future.

Imbalance of Th1&Th17 and Tregs is crucial for generation of increasing T\(_{EMs}\) in T1DM [37]. While some studies have shown that tolerance-inducing therapies can lead to an increase in Tregs in lymphoid and non-lymphoid target tissue, conversely, it is difficult to inhibit T\(_{EMs}\) formation in autoimmunity diseases [38]. In our

![Fig. 4](image-url)  

**Fig. 4**  
SP-Islet lysate treatment upregulates the Tregs frequency. (A) Representative FCM panels of Tregs in pancreatic draining lymph nodes and spleens harvested from NOD mice in control group and SP-Islet lysate treated group aged 20 weeks. Each sample was stained with CD3, CD4, CD25, and Foxp3 antibodies. CD3 and CD4 were used to gate CD4\(^+\) T cells. CD25 and Foxp3 were used to gate Tregs. (B) Quantitative analysis of the frequency of Tregs (CD4\(^+\)CD25\(^+\)Foxp3\(^+\)). *p < 0.05, **p < 0.01. Experiments were repeated three times independently.
study, the treatment of SP-Islet lysate had an anticipated effect in promoting Tregs, what’s more important, the frequency of TEMs and Th1/Th17 also presented a significant decrease in the SP-Islet lysate group compared with control group. Therefore, another reason for the reduction of TEMs by SP-Islet lysate treatment might be partially attributed to the preservation of the balance between Th1&Th17 and Tregs.

In summary, the treatment of ECDI-fixed splenocytes carrying whole islet antigens effectively cut down the morbidity of T1DM in NOD mice, and maintained the islet functions. This protective effect is mediated by reduced response of memory T cells and secretion of autoantibodies in SP-Islet lysate treated NOD mice. Our findings imply that this treatment has a potential to be employed as an immunotherapy to prevent the clinical onset of T1DM in genetically predisposed individuals. In future, elegant studies are needed for the clinical translation of this treatment.

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Disclosure

The authors declare that they have no conflict of interest to disclose.

Reference


