Low Methoxyl Pectin Protects against Autoimmune Diabetes and Associated Caecal Dysfunction

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Scope: This study aims to examine the protective effects of specific low-methoxyl pectin (LMP) on the development of type 1 diabetes (T1D).

Methods and results: Female non-obese diabetic (NOD) mice are weaned onto either control or 5% LMP supplemented diets for up to 22 weeks of age. T1D incidence, gut barrier function, and pancreatic-gut immune responses are analyzed. LMP supplementation significantly dampened the onset of T1D in NOD mice. LMP supplementation induces caecal homeostasis, as indicated by the increasing SCFAs production, higher expression of tight junction proteins claudin 1, zonula occludens-2 in caecum. Furthermore, LMP-mediated caecal homeostasis impacts gut-pancreatic immunity, as evidenced by increased regulatory T cell population, modulated inflammatory cytokine expression, and suppressed NOD like receptor protein 3 (NLRP3) inflammasome activation in both caecum and pancreas.

Conclusion: The data demonstrate that LMP limits T1D development by inducing caecal homeostasis to shape pancreatic immune environment, providing a scientific basis for using LMP as a novel functional supplementation to intervene T1D.

1. Introduction

Type 1 diabetes (T1D) is a severe chronic autoimmune disease characterized by autoreactive T-cell-mediated selective destruction of pancreatic beta-cells in genetically predisposed individuals.[3] Despite its genetic attribution, accumulating evidence indicates that impaired intestinal homeostasis and subsequent translocation of diabetogenic antigens are crucial for T1D development.[2–4] Indeed, increasing dietary fibers intake has been recommended to maintain gut homeostasis and is protective against T1D, as fibers can be fermented by gut microbiota to produce health-promoting SCFAs, enhance intestinal barrier function, and shape the gut immunological environment.[5,6] However, our earlier studies have shown that only dietary fibers with specific chemical compositions have these beneficial effects, while others are ineffective.[6,7] Therefore, finding dietary fibers with efficacy in preventing intestinal barrier dysfunction and immune disorder is highly needed to suppress T1D development.

Originated from fruit and vegetables, pectin is a family of structural carbohydrates with an α (1-4)-linked galacturonic acid polysaccharide backbone and can differ in degree of methyl esterification. Low-methoxyl pectin (LMP) refers to pectin in

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which the acid units in the backbone are methyl-esterified for less than 50%. The health effects of pectins correlate with their physicochemical properties, Popov and his colleagues reported the anti-inflammatory property of pectin is correlated with the degree of methyl-esterification, and LMP, but not high methoxyl pectin, has immune-modulatory effects on blood leukocytes.[8]

Our previous studies also found that pectin, especially LMP, exerts direct anti-inflammatory effects in the intestine, and also reduces intestinal permeability.[9,10] In addition, Dietary effects of pectin have been shown to be effective against a number of autoimmune diseases such as inflammatory bowel disease,[11] allergic airway inflammations,[12] and atopic dermatitis.[13,14] However, the immunological role of LMP on T1D remains to be explored.

Here, we evaluated the protective effect of LMP on T1D in non-obese diabetic (NOD) mice. In particular, regulatory effects of LMP on intestinal SCFAs production and barrier function, regulatory T cell (Treg) infiltration, and NOD like receptor protein 3 (NLRP3) inflammasome activation in pancreas and intestine were examined to reveal whether LMP mediated intestinal homeostasis contributes to T1D prevention.

2. Experimental Section

2.1. Mice and Diets

Female NOD/LtJ mice were purchased from Su Pu Si Biotechnology Co., Ltd (Suzhou, Jiangsu, China) and maintained in SPF environment at the Animal Housing Unit of Jiangnan University under a controlled temperature and a 12-h light/12-h dark cycle. All animal experiments were carried out according to protocols approved by the Institutional Animal Ethics Committee of Jiangnan University (JN. No 20150331–0410). Four-week old female NOD mice were fed AIN-93G purified diets with or without 5% (wt/wt) LMP until sacrificed. Mice were sacrificed by a lethal dose of pentobarbital sodium when they developed T1D (22th week in the study), and then pancreas, caecum, colon, MLN, and PLN samples were harvested for subsequent assays.

2.2. Dosage Information

Four-week old female NOD mice were fed AIN-93G purified diets with or without 5% (wt/wt) LMP until sacrificed. The dosage was chosen based on previous studies,[9,15,16] and this dose is within the reported range of LMP for nutritional supplementation (3.3–15%) in literature.[17–19]

2.3. Blood Glucose Measurement

Mice were fasted for 6 h and blood glucose levels were monitored and determined with an Accu-chek glycosometer (Roche Diagnostics, Almere, The Netherlands). A blood glucose level > 14.0 mmol L$^{-1}$ on two consecutive readings was considered to indicate a diabetic state.

2.4. Histology

Pancreatic tissues were fixed, sectioned (5 µm thick) and stained with hematoxylin and eosin (H&E). The degree of insulitis was determined from multiple nonsequential slides from six individual mice. Slides were scanned by Pannormaic at 25x magnification. 30 islets per mice were scored for the evaluation of insulitis and assigned a score by evaluating the degree of immune cell infiltration and categorized as follows: 0—no insulitis, 1—peri-insulitis, 2—invasive insulitis with <50% infiltration of islets, 3—invasive insulitis with >50% in filtration of islets. For cecal and colonic section, the segment of the tissues was immediately fixed with 4% paraformaldehyde and embedded in paraffin, 5 µm sections were cut, and stained with hematoxylin and eosin. The length of villus was measured by a Digital slice scanner (PANNORMIC MIDI, 3DHISTECH, Hungary). Briefly, the length of representative villi was manually drawn with blue lines which was done in a blind manner and measured by software automatically.

2.5. RNA Isolation and Quantitative PCR (qPCR)

Total RNA was extracted from caecal and pancreatic tissues using Trizol (TaKaRa Biotechnology, Dalian, China) according to manufacturer’s protocol. Complementary DNA was prepared by reverse transcription of 2 µg total RNA using the PrimeScript RT Master Mix (TaKaRa). The mRNA expression level of target genes were determined using SYBR-Green Quantitative PCR kit (TaKaRa) by iCycler iQ system (Bio-Rad, Hercules, CA, USA) according to the previous study.[9] Detailed primers sequences are shown in Table S1, Supporting Information.

2.6. Western Blot

Tissue sample preparation and Western blot were performed as previously described.[6] Primary antibodies against NLRP3 (Cat:15101), apoptosis-associated speck-like protein containing a CARD (ASC) (Cat: 67824), cleaved IL-1β (Cat: 63124), NLRP3 (Cat: 5174), cleaved IL-18 (Cat:52718) (all from Cell Signaling Technology, Beverly, MA, USA), caspase-1-p20 (Cat: sc-1597), ZO-2 (Cat: sc-11448), claudin-1 (Cat: sc-17658), and occludin (Cat: sc-8144) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied.

2.7. Isolation of Immune Cells from Pancreas, Pancreatic Lymph Node (PLN), and Mesenteric Lymph Node (MLN) of NOD Mice

Immune cells were freshly prepared from pancreas, PLN and MLN as previously described[20] by passing the freshly isolated organs through a 70 µm nylon mesh. Cells were then washed with cold PBS and resuspended in PBS for staining.

2.8. Flow Cytometry

The single cells isolated from pancreas and lymph nodes were washed and resuspended in PBS. Surface staining was performed with the following mAbs: anti-CD45, -CD4, and -CD25

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Figure 1. Low-methoxyl pectin (LMP) supplementation protects against Type 1 diabetes (T1D) in non-obese diabetic (NOD) mice. A) Illustration of LMP treatment on NOD mice from 4 to 22 weeks of age. B) Diabetes incidence of NOD mice treated with or without 5% LMP supplementation from 4 to 22 weeks of age. n = 20. C) The fasting blood glucose of non-diabetic NOD mice at 22 weeks of age. n = 10. D) H&E staining of pancreatic islets from NOD mice at 22 weeks of age. Insulitis score was quantified. n = 6 Scale bar, 100 μm. Bars represent means ± SEM; *p < 0.05 and **p < 0.01.

at room temperature for 30 min. Detailed antibody information was shown in Table S2, Supporting Information. Cells were then fixed and permeabilized. Intracellular detection of Foxp3 was performed using anti-Foxp3 at 4 °C for 50 min. Gating strategy was shown in Figure S1, Supporting Information.

2.9 Lipopolysaccharide (LPS)

Plasma LPS was measured by ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (GenScript, USA Inc.) according to the manufacturer’s instructions.

2.10 Analysis of SCFAs

Caecal content samples (50 mg) were collected under sterile conditions and were stored in liquid nitrogen. Concentrations of SCFA were measured by GC coupled to the MS detector of GCMS-QP2010 (Shimadzu, Japan) as described previously.[21]

2.11 Statistical Analyses

Diabetes incidence was plotted according to the Log-rank test. Statistical significance was determined using a two-tailed Student’s t-test. p-value of < 0.05 was considered statistically significant. Statistical analyses were carried out using GraphPad Prism 7 software (GraphPad, La Jolla, CA, USA).

3. Results

3.1 LMP Protects against T1D in NOD Mice

To examine the protective effects of LMP supplementation on T1D development, female NOD mice were fed AIN-93G diet with or without 5% LMP from weaning until up to 22 weeks of age (Figure 1A). Incidence of T1D was significantly reduced in mice fed with LMP (Figure 1B). Among non-diabetic mice of both groups at 22-week age, fasting glucose levels of LMP-fed mice were significantly lower than control NOD mice (Figure 1C).
Figure 2. LMP shapes the pancreatic immune environment in NOD mice. A,B) The frequency and number of CD4⁺CD25⁺Foxp3⁺ T cells gated from CD4⁺ T cells in pancreas (A) and PLN (B) were shown. \( n = 5 \). C) TGF-\( \beta \)1, TNF-\( \alpha \) and IL-6 mRNA expression in pancreas were detected by qPCR. \( n = 3 \). D) NLRP3, Casp-1-p20, cleaved IL-1\( \beta \), and cleaved IL-18 in pancreas were measured by Western blot analysis. Quantification was determined by densitometry analysis. E) NLRP3, Caspase-1, IL-1\( \beta \), and IL-18 mRNA expression in pancreas were measured by qPCR. Data are representative of three independent experiments. Bars represent means ± SEM; *\( p < 0.05 \) and **\( p < 0.01 \).

Accordingly, less immune cell infiltration and insulitis in pancreatic islets were observed in LMP-supplemented mice (Figure 1D).

3.2. LMP Exerts Immune-Modulatory Effects in Pancreas

As shown in Figure 2, the frequencies of Treg both in pancreas (Figure 2A) and PLN (Figure 2B) were enhanced by LMP supplementation. Consistent with the increasing Treg populations, higher mRNA level of immune modulatory cytokine TGF-\( \beta \)1 and lower expression of the pro-inflammatory cytokines TNF-\( \alpha \) and IL-6 in pancreas were found when NOD mice were fed LMP (Figure 2C), indicating auto-immuno-attenuating effects of LMP in T1D. In addition, the modulatory effect of LMP on pancreatic immune responses during T1D was further confirmed by its suppressive effect on NLRP3 inflammasome activation (Figure 2D) and decreased mRNA levels of NLRP3, caspase-1, IL-1\( \beta \), and IL-18 in the pancreas (Figure 2E).

3.3. LMP Prevents Barrier Dysfunction in Caecum of NOD Mice

Disruption of gut homeostasis is considered as an early pathological hallmark of T1D.\(^{[19,22,23]}\) Next, we assessed the effect of LMP on intestinal integrity in the caecum and colon to reveal the potential mechanisms of LMP on T1D attenuation. In the caecum, LMP upregulated expression of claudin-1 and ZO-2 both at protein and mRNA levels (Figure 3A,B). We did not observe similar effects of LMP on claudin-1 nor occludin in the colon (Figure 3C). Consistent with enhanced tight junction protein expression in the caecum, LMP supplementation reduced intestinal permeability as indicated by decreased serum LPS level (Figure 3D). Accordingly, LMP supplementation significantly improved morphology
of the caecum with enhanced barrier integrity and increased villus length rather than the colon in NOD mice (Figure 3E,F).

3.4. LMP Promotes the Immune Balance in Caecum of NOD Mice

The SCFAs, as microbial metabolic products of dietary fiber, are crucial to gut barrier integrity,\(^{[24]}\) and also prevent T1D by promoting intestinal immune homeostasis through Treg induction.\(^{[25]}\) Consequently, we next determined acetate, propionate, butyrate, and total SCFA levels in caecum and colon. The data showed that LMP significantly increased caecal acetate, propionate, butyrate, and total SCFA production in caecum compared with the control NOD mice (Figure 4A,B), which was not observed in the colon (Figure 4C), indicating that most of the LMP is fermented before it reaches the colon to exert its effect. Consistent with the increased SCFAs in caecum, LMP enhanced frequencies of Treg in MLN (Figure 4D), and increased expression of TGF-β1 and
Figure 4. LMP induces caecal immune homeostasis in NOD mice. A) Acetate, propionate, butyrate in caecal content were analyzed by GC-MS. B) Total short chain fatty acids in caecal content were analyzed. C) Acetate, propionate, butyrate in colonic content were analyzed by GC-MS. D) The frequency of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells gated from CD4<sup>+</sup> T cells in mesenteric lymph nodes (MLN) was shown. E) TGF-β1 and TNF-α mRNA expression in caecum were analyzed by qPCR. F) NLRP3, Casp-1-p20, cleaved IL-1β, and cleaved IL-18 in caecum were measured by Western blot. Quantification was measured by densitometry analysis. G) NLRP3, Caspase-1, IL-1β, and IL-18 mRNA expression in caecum were analyzed by qPCR. Each dot represents a biological replicate from three independent experiments. Bars represent means ± SEM; *p < 0.05 and **p < 0.01.

4. Discussion

Our study demonstrated that dietary supplementation with LMP limits T1D development in NOD mice, which is mediated by the enhanced Treg frequencies and inhibited NLRP3 inflammasome activation in pancreas. Furthermore, LMP enhanced production of immune modulating bacterial products SCFAs and improved decreased TNF-α in caecum (Figure 4E), pointing to a role for LMP in mediating intestinal immune balance by SCFAs to attenuate T1D. In addition, LMP supplementation markedly suppressed the expression of NLRP3 and associated proteins in caecum (Figure 4F,G), suggesting that its immune-regulatory effects are associated with inhibited NLRP3 inflammasome activation.
intestinal integrity in the caecum, which shaped pancreatic immune environment and contributed to T1D amelioration. To the best of our knowledge, no previous study has investigated the potential effects of LMP on T1D, heralding a novel dietary approach with LMP to prevent autoimmune diabetes.

Impairments in Treg numbers, function and induction critically contribute to autoimmune destruction in T1D, and increasing frequencies of Treg is considered as frontier strategy to treat T1D.[11,26–28] Dietary fibers have been demonstrated to exhibit immunomodulatory effects by regulating Treg generation, trafficking, and function through their fermentation products such as SCFAs.[29] On the one hand, SCFAs elevate the number and function of induced Treg cells in the colon and peripheral blood.[30,31] Furthermore, SCFAs also influence gene transcription in Treg cells[32] via inhibition of histone-deacetylase (HDAC) activity or activation of G protein coupled receptors.[33] Consistent with these earlier data, significant increase of acetate, propionate, and butyrate in caecum, accompanied with higher frequencies of CD4+CD25+ Foxp3+ T cells in pancreas, PLN, and MLN were observed in LMP-treated NOD mice, which explained LMP-induced Treg expansion and T1D attenuation. However, although our data clearly indicate caecal immune balance induced by LMP positively influenced pancreatic autoimmunity, the exact mechanism underlying caecum-pancreas axis that how Treg cells are induced from caecum and migrate or influence pancreatic immune environment is still unclear and deserve further investigations.

In addition to immune dysregulation, impaired intestinal barrier with increased permeability is also a crucial factor for T1D due to the increasing passage of intestinal diabetogenic antigens,[34] and modification of intestinal permeability via prebiotics is a promising approach to prevent or treat autoimmune diabetes.[23] In our study, dietary LMP supplementation improved caecal barrier as shown by the enhanced claudin-1 and ZO-2 protein expression. Dietary fibers including pectin can be consumed by gut microbiota and degraded to SCFAs, and SCFAs has been reported to strengthen intestinal barrier via promoting tight junction,[35,36] which protects against the onset of T1D.[37] In accordance with these findings, we found higher levels of acetate, propionate, and butyrate in caecum after LMP treatment, indicating SCFAs may be the effector metabolites of LMP on T1D attenuation with their barrier-reinforcing effects on caecum. Besides, a pro-inflammatory intestinal cytokine environment affects tight junction functionality and subsequently increases intestinal permeability.[23,38,39] Here, the LMP supplementation increased the anti-inflammatory TGF-β, and decreased pro-inflammatory TNF-α expression levels in caecum. The LMP therefore creates an anti-inflammatory cytokine milieu in the caecum, which might also improve intestinal barrier function. More interestingly, our study demonstrated that the protective effect of dietary fibers on the development of T1D was mediated by caecal homeostasis rather than colon. This location of action in the large intestine may be attributed to the low esterified chemical composition of LMP that causes its rapid fermentation in the caecum. It has been suggested that the degree of esterification in the structure of pectin affects the fermentation location, product and health-promoting effects conferred.[10,40] Another possible reason for the preferential effect of LMP on caecum is the differential microbial distribution across the intestinal segments.[41] Pectin of different structure has been reported to increase the abundance of probiotic Bifidobacterium and Lactobacillus and subsequent SCFA production.[42] Therefore, future sequencing analysis of the gut microbiota composition in caecum and colon will shed light on the prebiotic mechanism of LMP on different intestinal segments.

The beneficial effects of dietary fiber on epithelial integrity and intestinal homeostasis are shaped by SCFAs-NLRP3 inflammasome axis.[43,44] Herein, we further determined the regulatory effects of LMP on NLRP3 inflammasome in caecum, and found that LMP suppressed NLRP3 inflammasome activation in mice. The possible reason is that LMP increased caecal SCFAs levels, since SCFAs has been reported to inhibit NLRP3 inflammasome activation by acting as HDACs inhibitors.[45] However, a dual role of NLRP3 inflammasome is unraveled in the study of Macia et al.[46] where NLRP3 inflammasome activation promoted health benefits of dietary fiber on colitis through SCFAs-sensing receptors GPR43 and GPR109A. The different roles of NLRP3 in regulating intestinal homeostasis may be partly ascribed to different experimental models and settings. In addition, the role of NLRP3 inflammasome in T1D has also been increasingly recognized.[45,46] Genetic ablation of NLRP3 protects mice from the development of T1D by suppressing T-cell activation, Th1 differentiation, and migration of pathogenic T cells to pancreatic islets.[46] In our observation, LMP supplementation also suppressed NLRP3 activation in pancreas, which further explained the protective role of LMP on T1D. However, a detailed mechanistic study on how LMP modulates NLRP3 inflammasome activation in pancreas remained to be explored.

In summary, we demonstrate that a novel dietary fiber, low methoxyl pectin, can ameliorate T1D by increasing SCFAs production in caecum. The increasing SCFAs act as effector of LMP to suppress NLRP3 inflammasome activation, promote gut integrity, and Treg cells induction in the gut and pancreas, which prevent the development of T1D.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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