Altered systemic and intestinal IgA immune responses in individuals with type 1 diabetes

Short title: Altered IgA immunity in individuals with T1D

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Abbreviations: Antiphospholipid syndrome (APS); Follicular B cells (FoB); Glutamic acid decarboxylase autoantibody (GADA); Inflammatory bowel disease (IBD); Insulinoma-associated antigen-2 autoantibody (IA-2A); Lamina propria (LP); Marginal zone B cells (MZB); Non-obese diabetic (NOD); Not significant (NS); Peripheral blood mononuclear cells (PBMCs); Systemic lupus erythematosus (SLE); Zinc-transporter 8 autoantibody (ZnT8A).

Abstract

Objective: Increasing evidence supports the observation that IgA exerts a critical effect on the susceptibility to autoimmunity by modulating gut homeostasis and subsequent host immunity. We hypothesized that the IgA immunity is altered in individuals with type 1 diabetes. To test our hypothesis, we investigated intestinal, oral and peripheral IgA immune responses in individuals with type 1 diabetes.

Methods: We collected stool, oral cavity, and blood samples from subjects diagnosed with type 1 diabetes (within one year and over one year) and healthy control individuals. Serum islet autoantibody titers were detected by radio ligand assays. IgA-bound-bacteria and IgA-expressing B cells were studied by flow cytometry. Oral free IgA level was measured by ELISA. Serum and stool free IgA concentrations were determined by immune-turbidimetry method.

Results: Individuals diagnosed with type 1 diabetes within one year had an increased proportion of stool IgA-bound-bacteria, compared with healthy control individuals. The proportion of stool IgA-bound-bacteria was positively associated with the glutamic acid decarboxylase autoantibody (GADA) titer. Moreover, individuals with a longer duration of disease displayed a higher level of IgA-bound-bacteria than those diagnosed within one year. In contrast to healthy control individuals, type 1 diabetes patients had increased serum IgA concentrations.

Conclusions: Individuals with type 1 diabetes display altered IgA immunity, especially increased stool IgA-bound-bacteria, which is likely to contribute to beta-cell autoimmunity and the disease

development, and thus, might be considered as a novel therapeutic target for the treatment of type 1

diabetes.

Introduction

Type 1 diabetes is an immune-associated disease caused by the destruction of pancreatic beta cells (1), which involves complex interplay between immune cells and beta cells (2). Whilst adaptive immune lymphocytes such as $CD4^+$ and $CD8^+$ T cells have been demonstrated to play dominant roles in beta-cell damage (2-6), B cells are critical in facilitating T cell-mediated autoimmunity (7). Using animal models of human type 1 diabetes, previous studies found that B cell-deficient ($\mu MT^{-/-}$) NOD mice, which were also impaired in generation of immunoglobulins, were protected from type 1 diabetes development, suggesting that B cells and antibody produced by B lymphocytes are important in the immuno-pathogenesis of type 1 diabetes (7-10). In addition, we have shown increased marginal zone B cells (MZB) but decreased follicular B cells (FoB) in newly-diagnosed individuals with type 1 diabetes, which are closely related to altered beta-cell function and glucose level, indicating that B cells play a role in loss of self-tolerance to beta cells (11).

Among immunoglobulins secreted by B cells, IgA is known to play an important role in the homeostasis of mucosal immunity by restraining the translocation and/or the attachment of bacterial pathogens to gut epithelial sites (12-15). It was demonstrated that IgA reactive to gut commensal bacteria can bind to intestinal microbiota and then modify the composition and function of gut microbiota, which in turn regulates host immunity (16-19). In healthy human individuals, early studies showed that a significant proportion of gut microbiota are bound by IgA and are stably maintained in homeostatic conditions (20). Recently, aberrant IgA secretion and IgA binding to bacteria have been reported to be associated with increased susceptibility to autoimmune diseases, including inflammatory bowel disease (IBD),

antiphospholipid syndrome (APS), and allergies as well (21-23). Moreover, 16S rRNA sequencing of sorted IgA-bound-bacteria revealed that the taxonomy of gut IgA-bound-bacteria in individuals with these autoimmune and inflammatory disorders was very different from that in healthy control individuals (21,24,25). Interestingly, IgA-bound-bacteria have a stronger pathogenic effect on the induction of colitis than non-IgA-bound-bacteria (15). These studies suggest that IgA plays an important role in maintaining gut homeostasis and in mediating the susceptibility to autoimmunity. However, little is known about the role of mucosal IgA immunity in type 1 diabetes. We hypothesized that altered IgA immune responses including those at mucosal sites may contribute to the development of type 1 diabetes.

To comprehensively investigate the role of IgA immunity in type 1 diabetes, we assessed mucosal and systemic IgA immunity in individuals with type 1 diabetes. To study mucosal IgA immunity, we investigated IgA-bound-bacteria proportion in stool and the oral cavity, as well as free IgA level in these two mucosal sites. To study systemic IgA immunity, we investigated the proportion of IgA-expressing B cells in peripheral blood mononuclear cells (PMBCs) and the level of circulating IgA in the serum from individuals with type 1 diabetes and gender- and age-matched healthy control participants. Our results revealed novel information about the potential role of IgA in the development of type 1 diabetes in humans.

Materials and Methods

Study subjects

Twenty three subjects with type 1 diabetes diagnosed within one year (the disease average duration was 5.5 months and the range was one day to 12 months), together with twenty five gender- and age-matched healthy control individuals were recruited from Chinese population from the Second Xiangya Hospital of Central South University (Table 1). Thirty three individuals with similar age but diagnosed with type 1 diabetes over one year (the disease average duration was 4 years and the range was about 13 months to 12 years) were also recruited to further investigate the IgA-bound bacteria level in patients with longer disease duration. The average age of the study participants was 11-13 years old (Table 1). Type 1 diabetes was diagnosed in accordance with American Diabetes Association criteria (26). Exclusion criteria were as follows: 1) individuals who had received treatment with antibiotic(s) within three months prior to study enrollment; 2) individuals who used probiotics in the week before enrollment; 3) individuals who had acute or chronic inflammatory condition; 4) individuals who were receiving any medication other than treatment for diabetes with insulin during the study and/or before enrollment; 5) individuals who had a known history of immunological conditions including allergy, asthma, systemic lupus erythematosus (SLE), and Crohn's disease; 6) individuals who had a known history of malignancy or other health issues including gastrointestinal tract disease, severe cardiovascular or cerebrovascular disease, liver and kidney disease; 7) individuals who had mental disabilities. Individuals who didn't collect samples according to our instructions were also excluded. All the participants and their legal guardians gave informed consent. Oral, stool and blood samples were collected from newly-diagnosed participants (diagnosed within one year), and we also collected stool samples from individuals

diagnosed more than one year previously. The study was conducted in agreement with the Declaration of Helsinki and was approved by the Ethics Committee of the Second Xiangya Hospital.

Sample collection

Oral sample collection was carried out by rinsing the oral cavity with 10 ml sterile saline in the early morning before any oral hygiene practice, eating and drinking. Following collection, the samples were spun at 12,000 g for 5 minutes to pellet bacteria. Oral bacteria were stored in glycerol at a final concentration of 25% and were frozen at -80°C before assay. The supernatant was frozen at -20°C for "free" IgA measurement. Fresh stool samples were collected using sterile procedures either in the hospital or the hotel near the hospital. The samples, which were kept on ice, were immediately transported to the laboratory, where they were aliquoted and frozen at -80°C within two hours from the collection. Blood specimens were drawn after overnight fasting. Whole blood samples were collected into blood collection tubes, containing either EDTA as an anticoagulant for peripheral blood mononuclear cells (PBMCs) isolation or a separating gel for serum collection. Blood samples were processed within 2 hours after collection. PBMCs isolated by density gradient centrifugation were suspended in freezing medium (40% FCS, 10% DMSO, and 50% RPMI-1640 medium) and were frozen in liquid nitrogen before use. Serum samples were collected from blood centrifuged at 3000 rpm for 5 minutes and stored at -20°C before test.

HbA1c, glucose, and C-peptide assays

Glycosylated hemoglobin (HbA1c) was measured by using a BioRad VARIANT II Hemoglobin Testing System (BioRad, Hercules, California, USA). A Hitachi 7170 analyser (Hitachi, Holliston, Massachusetts, USA) was used to determine the plasma glucose level. C peptide level was tested using Adiva Centaur System (Siemens, Munich, Germany). As previously reported, the coefficients of variation were 3.7%-4.1% for inter-assay, and 1.0%-3.3% for intra-assay (27).

Islet autoantibody assays

Titers of autoantibodies including anti-glutamic acid decarboxylase autoantibody (GADA), anti-insulinoma-associated antigen-2 autoantibody (IA-2A), and anti-zinc-transporter 8 autoantibody (ZnT8A) were quantified by radio ligand assays. The sensitivities of the GADA, IA-2A, and ZnT8A assays were 82%, 76%, and 72%, respectively. The specificities for these assays were 98%, 100%, and 100%, respectively. The inter- and intra-assay coefficients of variation were 8.9% and 11.2% for GADA testing. These results were evaluated in the 2016 Islet Autoantibody Standardization Program as previously described (28).

Immunoglobulin assays

IgA concentration in the oral cavity rinse was detected using ELISA reagents purchased from Southern Biotechnology. Briefly, the plates were coated with the standards or the oral samples, overnight at 4°C. After washing and blocking, the plates were incubated with AP-conjugated goat anti-human IgA at room temperature for 2 hours, followed by further washing and addition of the phosphate substrate (Sigma). The enzymatic color change was determined by micro-plate reader (Perkin Elmer, Waltham,

Massachusetts, USA) at the recommended optical density. Serum IgM, IgG, and IgA immunoglobulins were measured by turbidimetric inhibition immunoassay following the manufacturer's instructions (Beckman Coulter Life Sciences). Sensitivity is defined as the lowest measurable concentration which can be distinguished from zero with 95% confidence. Sensitivity for serum IgG determination is 33.3 mg/dL. Sensitivity for serum IgM determination is 4.2 mg/dL. Sensitivity for serum IgA determination is 108 mg/L.

Cellular Staining

PBMCs were thawed in 37 °C water bath properly, washed with complete media, and incubated with Fc blocker at room temperature for 20 minutes. The cells were then stained with monoclonal antibodies (mAbs) to surface molecules. For internuclear transcription factor staining, cells were fixed (1 hour) and permeabilized with True-Nuclear™ Transcription Factor Buffer Set (BioLegend) prior to internuclear antibody staining. Cells were stained with antibodies to the following markers: PE anti-human IgA (RRID: AB_2727738), FITC anti-human IgG (RRID: AB_1163674), FITC anti-human CD185 (CXCR5) (RRID: AB_ 2561896), FITC anti-human CD40 (RRID: AB_1186034), APC anti-human IgM (RRID: AB_ 493012), APC anti-human CD86 (RRID: AB_2721448), Alexa Fluor® 647 anti-Pax-5 (RRID: AB_2562425), APC/Cyanine7 anti-human CD19 Antibody (RRID: AB_314248), and PE/Cy7 anti-human IgD antibody (RRID: AB_10680462). The stained cells were analyzed on a BD FACSCanto II flow cytometer (BD, Franklin Lakes, New Jersey, USA), and the results were analyzed with FlowJo 8.8.6 (Tree Star, Ashland, Oregon, USA). Lymphocytes were gated based on their FSC-A/SSC-A properties. Single cells and subsequent live cells were gated on the basis of their

FSC-A/FSC-H properties and 7-AAD viability staining, respectively. The viability of the thawed samples was routinely 90-96%.

Bacterial staining

Stool samples were defrosted and re-suspended in sterile PBS (1 g/ml) and homogenized by vortexing vigorously for 30 seconds. After centrifugation (300 g, 1 minute, room temperature) to remove the large debris, 300 μl supernatant was used for "free" IgA measurement and 100 μl supernatant was further spun at 12,000 g for 5 minutes to pellet bacteria for IgA-bound-bacterial staining. Oral samples were quickly thawed in a 37°C water bath and spun at 12,000 g for 5 minutes to pellet bacteria. The fecal or oral bacterial pellets were washed and re-suspended in 50 μl of 1% BSA/PBS for 15 minutes prior to incubation with anti-human IgA-PE antibody (Miltenyi, RRID: AB_2727738) for 30 minutes at room temperature. Samples were then washed and resuspended in PBS for flow cytometric analysis. An isotype antibody was used as a control.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software Version 8 for Mac (GraphPad Software, San Diego, California, US). Data were expressed as mean (lower 95% CI of mean, upper 95% CI of mean), mean ± SD, or median (25th-75th percentile). A normality test was performed on the data before analysis, and statistical differences between the groups were analyzed by a two-tailed Student's *t*-test (if data normally distributed), or a two-tailed Mann-Whitney test (if data not normally distributed). For multiple group comparison, analysis of differences was assessed using a one-way ANOVA test

followed by multiple *t* tests with Tukey's correction. Differences between the expressions of surface

markers on B cells and the concentrations of serum Igs were determined using multiple *t*-tests with

Holm-Sidak correction. Correlations and regressions were analyzed using a two-tailed *Pearson* or

Spearman nonparametric correlation coefficient test and linear regression. Power calculations using G*

Power software indicated that the sample size of 23 would give 80% power at an effect size of 0.53,

two tailed, $P = 0.05$. Exact group size and statistical tests performed for each experiment are described

in the figure legends. $P < 0.05$ was considered significant.

Results

Individuals with type 1 diabetes displayed altered systemic IgA immunity

We firstly assessed the phenotype of the B cells from the peripheral blood of individuals with newly diagnosed type 1 diabetes. Although no significant difference was found in the percentages of CD19+ B cells (Data not shown), B cells from individuals with newly diagnosed type 1 diabetes showed more activated phenotypes compared with those from healthy control participants, indicated by increased expression of CD40 [97.01% (95% CI: 95.84%, 98.19%) vs 95.08% (95% CI: 93.71%, 96.44%), P = 0.0322, Figure 1a], increased expression of CD86 [1.855% (95% CI: 1.483%, 2.228%) vs 1.249% (95% CI: 0.9253%, 1.572%), P = 0.0293, Figure 1b], and increased expression of CXCR5 [93.30% (95% CI: 92.28%, 94.31%) vs 90.66% (95% CI: 88.97%, 92.34%), P = 0.0275, Figure 1c] among the eight activation markers test. Moreover, the expression of the transcription factor PAX-5, which plays an important role in B cell differentiation, was also increased in individuals with newly diagnosed type 1 diabetes compared with that in healthy control participants [94.54% (95% CI: 91.97%, 97.11%) vs 86.39% (95% CI: 81.99%, 90.79%), P = 0.0028, Figure 1d]. Additionally, we found that people with newly diagnosed type 1 diabetes had increased expressions of surface immunoglobulins on B cells when compared with healthy control individuals, including IgM [29.50% (95% CI: 25.63%, 33.37%) vs 22.34% (95% CI: 19.10%, 25.58%), P = 0.0197], and IgG [5.46% (95% CI: 4.458%, 6.462%) vs 3.63% (95% CI: 2.702%, 4.571%), P = 0.0248]. Although no significant difference was found in the expression of IgA [22.70% (95% CI: 19.62%, 25.78%) vs 17.96% (95% CI: 14.70%, 21.22%), P = 0.0679], there is an obvious trend (P = 0.0346 before statistical adjustment), while IgD expression was comparable (Figure 1e-f). To know the immunoglobulin release in the circulation, we measured the concentrations

of serum immunoglobulins. Interestingly, there were no significant differences in the secreted circulating IgM or IgG concentrations (Figure 1g), whereas circulating IgA level was higher in individuals with newly diagnosed type 1 diabetes than that in healthy control participants [1.353 g/l (95%CI: 1.119 g/l, 1.587 g/l) vs 0.8812 g/l (95%CI: 0.5995 g/l, 1.163 g/l), $P = 0.0337$, Figure 1g]. These data showed that not only were B cells generally more activated, the IgA-expressing B cells had reinforced capacity to release immuno-globulins in individuals with newly diagnosed type 1 diabetes, compared to their healthy control counterparts.

Individuals with type 1 diabetes showed normal oral IgA immunity

It is known that the majority of IgA-producing B cells are located in mucosal sites in the oral cavity and gut as well (29-31). We then assessed the mucosal IgA immunity by investigating that in oral mucosal site firstly in individuals with newly diagnosed type 1 diabetes and healthy control donors. To determine oral IgA immunity, we tested IgA binding to oral bacteria and non-bacteria-bound free IgA levels in oral samples collected from individuals with newly diagnosed type 1 diabetes and control subjects. Unlike different systemic IgA immunity, no significant difference was found in the proportion of IgA-bound-bacteria in the oral cavity between individuals with newly diagnosed type 1 diabetes and healthy control individuals (Figure 2a-b). Additionally, the level of free IgA in oral samples from individuals with type 1 diabetes was similar to that in healthy control participants (Figure 2c). Therefore, our results showed that individuals with newly diagnosed type 1 diabetes didn't show abnormal oral IgA immunity.

Individuals with type 1 diabetes showed distinct intestinal IgA immunity

Most mucosal IgA is mainly produced in the lamina propria (LP) of small intestine, and is known to play an important role in the gut immune response (18). Next, we assessed IgA immunity in the intestinal mucosa by assessing the level of IgA binding to bacteria and the concentration of free IgA, as we had done for the oral mucosal site. Interestingly, individuals with newly diagnosed type 1 diabetes had a higher proportion of IgA-bound-bacteria than healthy control participants [29.71% (95% CI: 21.28%, 38.14%) vs 17.38% (95% CI: 12.96%, 21.79%), P = 0.0086, Figure 3a-b]. No significant difference was found when we compared females and males within the type 1 diabetes group or within healthy control group (Data not shown), indicating that sex is not associated with the difference. Our further analysis showed that the level of IgA-bound-bacteria was non-statistically significantly negatively correlated with the islet function indicated by the concentration of fasting C-peptide (FCP) (r = -0.3776, P = 0.0756, Figure 3c), but it was significantly positively correlated with the titer of anti-GAD autoantibody (GADA, r = 0.5098, P = 0.0386, Figure 3d). However, no positive correlations were found between the level of stool IgA-bound-bacteria and anti-IA-2 or anti-ZnT8 autoantibody titer (Data not shown). In addition to IgA binding to gut bacteria, we also determined the concentration of non-bacteria-bound free IgA in the stool samples. In contrast to the high level of IgA-bound-bacteria, the concentration of free IgA in individuals with type 1 diabetes was not significantly different from that in healthy control subjects (Figure 3e). To further investigate whether the level of intestinal IgA-bound-bacteria is related to the severity of islet beta-cell destruction, we recruited patients with similar age but diagnosed with type 1 diabetes over one year (1 year to 12 years) and collected stool samples from these participants. Stool IgA-bound-bacteria proportions were measured and compared with the proportions in individuals diagnosed with type 1 diabetes within one year and healthy control subjects

(as shown in Figure 3b). Interestingly, the frequency of IgA-bound-bacteria appeared to be closely

associated with the disease duration, as individuals with longer duration of type 1 diabetes displayed a

higher level of IgA-bound-bacteria than healthy control donors [52.13% (95% CI: 44.94%, 59.32%) vs

17.38% (95% CI: 12.96%, 21.79%), $P < 0.0001$, Figure 3f] and individuals diagnosed with type 1

diabetes within one year as well [52.13% (95% CI: 44.94%, 59.32%) vs 29.71% (95% CI: 21.28%,

38.14%), $P < 0.0001$, Figure 3g]. Therefore, altered IgA mucosal immunity from the gut but not oral

cavity is more likely to participate in beta-cell autoimmunity and disease development.

Discussion

IgA is known to play an essential role in maintaining the integrity of mucosal barrier by clearing potentially harmful pathogens and molecules (13,32). Moreover, mucosal IgA can regulate commensal bacterial function and gene expression, which in turn affects the host immune system (16-18). Recently, aberrant IgA responses to the gut microbiota have been found in adults with inflammatory bowel disease (15), antiphospholipid syndrome (21) and some infants prior to developing asthma and allergy (22). Therefore, IgA exerts a critical effect on mediating the susceptibility to autoimmune diseases. In this study, we found that individuals with type 1 diabetes showed an higher proportion of IgA-bound-bacteria in the stool, which was positively associated with the GADA titer and the disease duration, indicating that intestinal IgA-bound-bacteria are closely related with the islet beta-cell autoimmunity and the underlying autoimmune process. In contrast to intestinal IgA-bound-bacteria, no significant difference was found in oral mucosal IgA-bound-bacteria between individuals with type 1 diabetes and healthy control participants. Therefore, our results from two mucosal sites suggest that the altered gut mucosal IgA immune response might play an important role in the progression of type 1 diabetes.

A recent mouse model study showed that the modulation of intestinal microbial composition changed serum IgA concentration and the number of IgA-secreting plasma cells in the bone marrow (33). Moreover, gut microbiota were found to activate the mucosal immune system and induce IgA production (34), which can be enhanced by the TLR3 agonist poly I:C and the TLR5 agonist flagellin (35,36). Therefore, gut microbiota play an important role in modulating the host IgA immune responses. In this study, we found that not only were peripheral B cells more activated, but also there was increased

serum IgA concentration in the individuals with type 1 diabetes, the latter being consistent with previous studies (37). Increasing numbers of studies have demonstrated that individuals with type 1 diabetes have different compositions of gut microbiota compared with healthy control participants (38-40). Importantly, we recently found, in a different study, that gut bacteria from individuals with type 1 diabetes induced distinct IgA immune responses in germ-free non-obese diabetic (NOD) mice, which is likely to be due to deficiency of gut microbial metabolites that can protect against type 1 diabetes (41), supporting that altered gut microbiota in individuals with type 1 diabetes might contribute to the altered IgA responses. Interestingly, Paun and colleagues recently reported that serum IgA from individuals with type 1 diabetes has a differential ability to bind specific bacteria compared with that from healthy individuals (42). It is possible that the activated peripheral B cells found in our study are responsible for the increased systemic IgA; however, whether these activated B cells and increased serum IgA also contribute to the increased gut mucosal IgA that binds to bacteria and the role of gut IgA-bound-bacteria in the immuno-pathogenesis of type 1 diabetes require further elucidation.

Taken together, our studies have revealed a previously unidentified immune-pathogenic pathway in which IgA-associated gut microbiota may be involved in beta-cell autoimmunity and the progression of disease. There are some limitations in our study. First, our study is a cross-sectional not a longitudinal study, thus we do not have longitudinal information about the dynamic change(s) in IgA immune response in the same study subjects. Second, we did not study subjects who are at a high risk of developing type 1 diabetes including the first degree relatives; it is, therefore, not clear whether the altered IgA immunity seen in individuals with type 1 diabetes occurs at pre-diabetic phase. Our future

directions will address these limitations. However, these caveats do not detract from our novel findings that individuals with type 1 diabetes displayed altered systemic and intestinal IgA immune responses, especially increased stool IgA-bound-bacteria, which is likely to contribute to beta-cell autoimmunity and the disease development, and thus, may be a novel therapeutic target for the treatment of type 1 diabetes.

Data availability: The datasets generated and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

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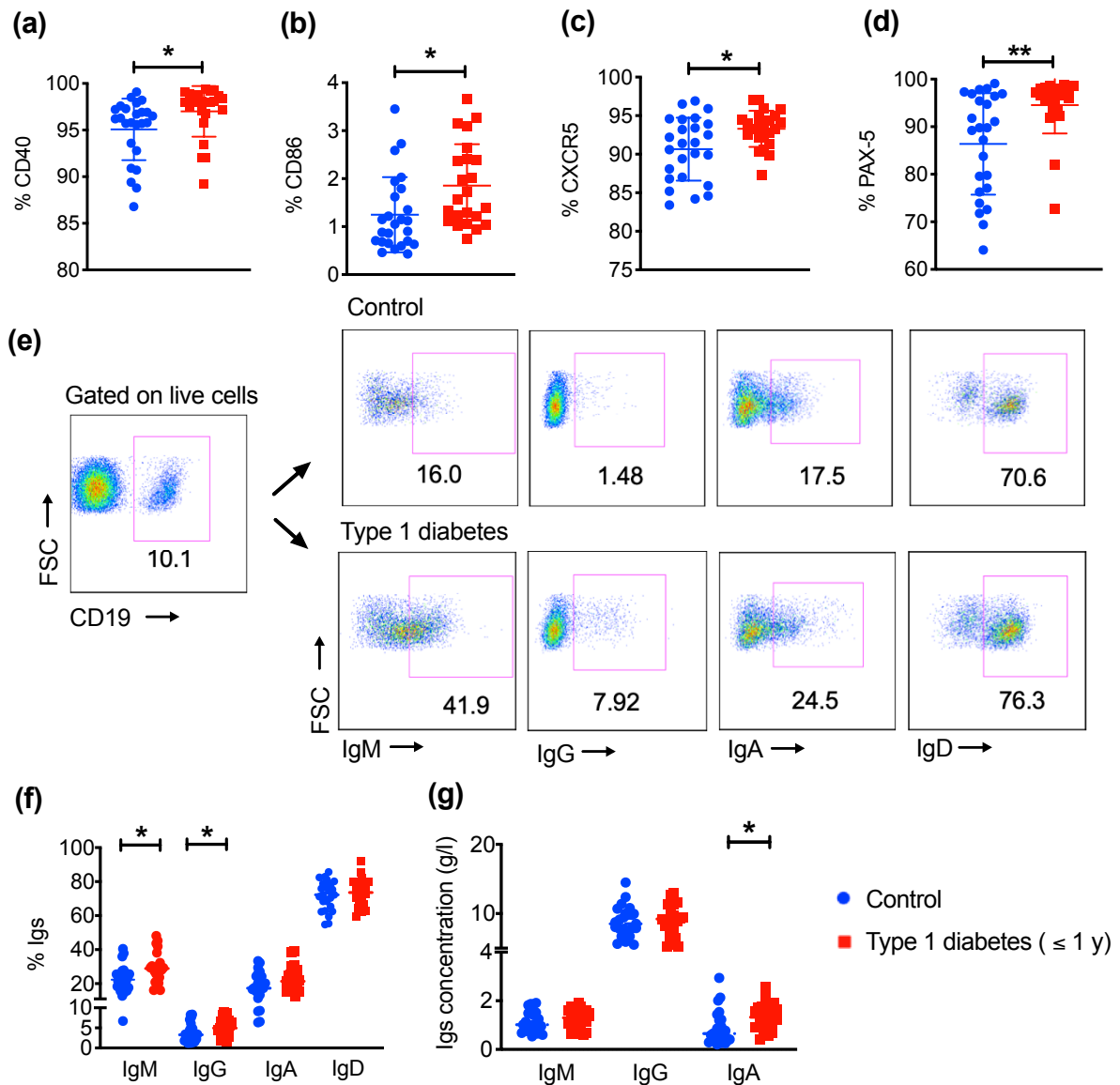
Figures and figure legends

Figure 1. Systemic IgA immune responses in the peripheral blood of individuals with newly diagnosed type 1 diabetes and healthy control participants. (*a-c*) Expression of activation markers on CD19$^+$ B cells from individuals with newly diagnosed type 1 diabetes (n = 23) and control participants (n = 25). These were CD40 (a), CD86 (b), and CXCR5 (c). (*d*) Expression of transcription factor PAX-5 in CD19$^+$ B cells from individuals with newly diagnosed type 1 diabetes (n = 23) and control participants (n = 25). (*e-f*) Expression of cell surface immunoglobulins on CD19$^+$ B cells from

individuals with newly diagnosed type 1 diabetes (n = 23) and control participants (n = 25). Representative flow cytometric plots of IgM, IgG, IgA, and IgD on B cells (e), and the summarized proportions (f). (***g***) Concentrations of serum IgM, IgG and IgA in individuals with newly diagnosed type 1 diabetes (n = 23) and control participants (n = 25). Data are expressed as mean ± SD (a-d, and f-g), and multiple *t*-tests with Holm-Sidak correction or a two-tailed Mann-Whitney test was used for statistical analysis. *$P < 0.05$, **$P < 0.01$.
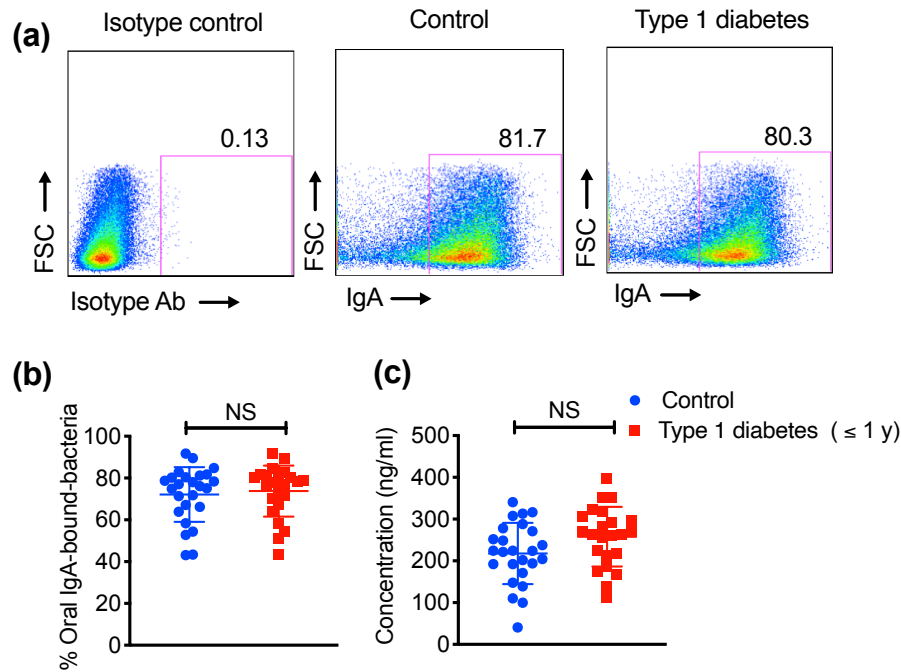
Figure 2. Oral IgA-bound-bacteria proportion and free IgA concentration in individuals with newly diagnosed type 1 diabetes and healthy control participants. (*a-b*) Proportion of oral IgA-bound-bacteria from donors with newly diagnosed type 1 diabetes and healthy control participants. Representative flow cytometric gating of an isotype control, and oral IgA-bound-bacteria gating from a healthy control participant and an individual with newly diagnosed type 1 diabetes (a). Summarized proportions of oral IgA-bound-bacteria (b) in individuals with newly diagnosed type 1 diabetes (n = 23) and healthy control participants (n = 25). (*c*) Concentration of oral IgA in individuals with newly diagnosed type 1 diabetes (n = 23) and healthy control participants (n = 25). Data are presented as mean ± SD and were assessed for statistical significance using a two-tailed Mann-Whitney test in (b), or a two-tailed Student's *t*-test in (c). NS, not significant.
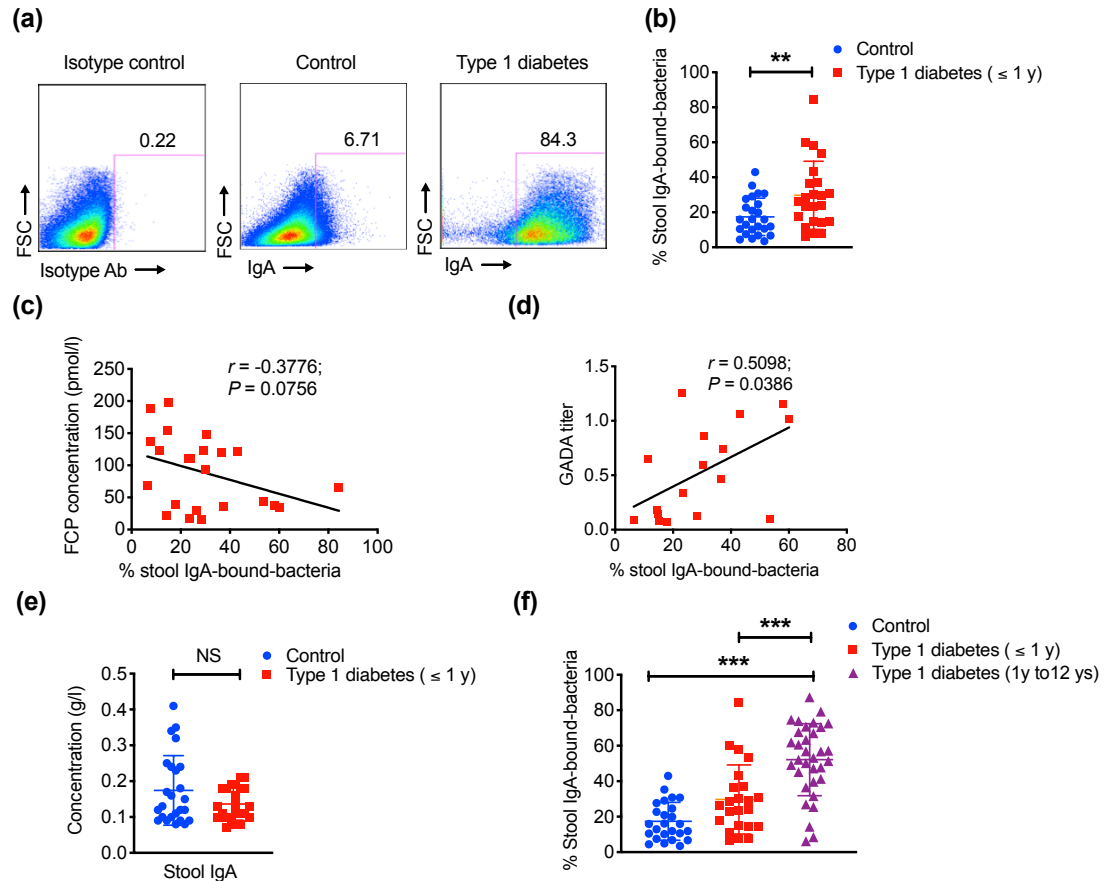
Figure 3. Stool IgA-bound-bacteria proportion and free IgA concentration in individuals with type 1 diabetes and healthy control participants. (*a-b*) Stool IgA-bound-bacteria proportion from donors with newly diagnosed type 1 diabetes and healthy control subjects. Representative flow cytometric profiles of stool IgA-bound-bacteria (a) in a healthy control participant (middle plot) and an individual with type 1 diabetes (right plot), compared with an isotype control (left plot). Summarized proportions of IgA-bound-bacteria (b) in individuals with type 1 diabetes, diagnosed within one year (n = 23), and healthy control participants (n = 25). (*c*) Correlation of the proportion of IgA-bound-bacteria with the level of fasting C peptide (FCP) in people with newly diagnosed type 1 diabetes (n = 23). (*d*) Correlation of the proportion of IgA-bound-bacteria with the titer of GADA (d, n = 17) in individuals with newly diagnosed type 1 diabetes. (*e*) Secretory IgA concentration in donors with newly diagnosed type 1 diabetes (n = 23) and healthy control participants (n = 25). (*f*) Stool IgA-bound-bacteria from

individuals with newly diagnosed type 1 diabetes (n = 23), individuals with longer type 1 diabetes duration (1 year to 12 years, n = 33), and healthy control participants (n = 25). Data are presented as mean ± SD in (b, and e-f) . Data were analyzed with a two-tailed unpaired t-test in (b), a two-tailed Mann-Whitney test in (e), or a one-way ANOVA test followed by multiple t tests with Tukey's correction in (f). Data were analyzed using a two-tailed *Pearson* coefficient test and linear regression in (c), or a two-tailed *Spearman* nonparametric coefficient test and linear regression in (d). NS, not significant, **$P < 0.01$, ***$P < 0.001$.

Table

Table 1. Demographics and clinical information[1]

	Healthy individuals	Participants with Type 1 diabetes ($\leq$ 1 year)	Participants with Type 1 diabetes (1 y to 12 ys)
N (Females/males)	25 (12/13)	23 (12/11)	33 (15/18)
Age (years)	11 (9.0-17.0)	12 (10.0-18.0)	13.0 (10.5-15.75)
Fasting glucose (mmol/L)	4.62 (4.19-5.06)	6.49 (5.38-8.40)***	6.85 (5.95-10.24)***
HbA1c (mmol/mol)	N/A[a]	74 (58-105) mmol/mol	69 (52-83) mmol/mol
HbA1c (%)	N/A	8.90 (7.50-11.80)%	8.50 (6.95-9.70)%
FCP[b] (pmol/L)	N/A	121.5 (34.0-183.9)	14.8 (12.6-77.2) †††
2hPCP[c] (pmol/L)	N/A	281.8 (173.7-528.4)	21.2 (14.1-180.0)†††

[1]Data are presented as median (25th-75th percentile); a. N/A, not available. ***$P < 0.001$ compared with healthy control participants. †††$P < 0.001$ compared with patients diagnosed with type 1 diabetes within one year. b. FCP, fasting C-peptide; c. 2hPCP, 2h postprandial C-peptide.