Association of HLA-dependent islet autoimmunity with systemic antibody responses to intestinal commensal bacteria in children

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Microbiome sequence analyses have suggested that changes in gut bacterial composition are associated with autoimmune disease in humans and animal models. However, little is known of the mechanisms through which the gut microbiota influences autoimmune responses to distant tissues. Here, we evaluated systemic antibody responses against cultured human gut bacterial strains to determine whether observed patterns of antimicrobial antibody (ACAb) responses are associated with type 1 diabetes (T1D) in two cohorts of pediatric study participants. In the first cohort, ACAb responses in sera collected from participants within 6 months of T1D diagnosis were compared with age-matched healthy controls and also with patients with recent onset Crohn’s disease. ACAb responses against multiple bacterial species discriminated among these three groups. In the second cohort, we asked whether ACAb responses present before diagnosis were associated with later T1D development and with HLA genotype in participants who were discordant for subsequent progression to diabetes. Serum IgG2 antibodies against Roseburia faecis and against a bacterial consortium were associated with future T1D diagnosis in an HLA DR3/DR4 haplotype–dependent manner. These analyses reveal associations between antibody responses to intestinal microbes and HLA-DR genotypes and islet autoantibody specificity and with a future diagnosis of T1D. Further, we present a platform to investigate antibacterial antibodies in biological fluids that is applicable to studies of autoimmune diseases and responses to therapeutic interventions.

INTRODUCTION

The increased incidences of autoimmune and inflammatory diseases in developed countries over the past 50 years suggest a causal role for environmental factors associated with improved hygiene and reduced microbial exposures in early life (1). Despite epidemiological support for this “hygiene hypothesis” (1), a mechanistic connection between the gut microbial ecosystem and autoimmunity that targets sites distant from the gut remains unclear. The composition and function of the gut microbial community (the microbiome) have been associated with risk of autoimmune and inflammatory diseases, including type 1 diabetes (T1D), rheumatoid arthritis (RA), and spondyloarthritis (2–4). The complex interactions between the gut microbiome and the adjacent mucosal immune system are dysregulated in Crohn’s disease (CD) and ulcerative colitis (UC), where inflammation targets the gastrointestinal tissue and compromises intestinal permeability. However, it is not clear whether immune responses to gut microbiota are associated with autoreactivity to host tissues outside the gut.

T1D results from autoimmune destruction, largely executed by T cells, of the insulin-producing pancreatic β cells. Genetic factors, most notably the human leukocyte antigen (HLA) class II haplotypes that govern antigenic peptide presentation to T cells, are key contributors to T1D risk (5). Although the disease can present at any age, it is most common in childhood and has displayed earlier ages at onset over the past several decades (6, 7), again suggesting an impact of dynamic environmental factors. To address the increase in T1D incidence (8), recent studies have sought to define associations between gut microbiome composition and either disease onset or the appearance of islet autoantibodies, a hallmark of prediabetes (4, 9, 10). A recurrent finding is that the fecal microbiota of individuals with multiple islet autoantibodies or recent onset T1D displayed reduced taxonomic diversity, although associations with specific genera have varied between the studies (4, 10, 11).

We reasoned that an influence of the gut microbiome on islet autoimmunity would require immune responses detectable outside the gut mucosa. To address this idea, we have investigated patterns of antibody responses detectable in the blood against human fecal commensal (nonpathogenic) microbes. We hypothesized that the patterns of antimicrobial antibody (ACAb) responses might reveal interactions between HLA genotype and systemic immune response to gut microbes in the context of pancreatic autoimmunity.

We developed a platform to define and compare the titers and isotypes of ACAb responses, adapted from an assay used to detect antibodies against bacterial pathogens in immunized mice (12). We characterized ACAb response patterns in two pediatric cohorts: those with recent onset CD or T1D compared with healthy controls (HCs) and a prospective cohort of children at risk for, and prior to, onset of T1D. We observed that patterns of ACAb responses distinguished patients with CD from patients with T1D and from HCs. ACAb responses against specific commensals measured before T1D diagnosis differentiated individuals with islet autoimmunity and HCs in an HLA haplotype–dependent manner. Moreover, we identify an
HLA-dependent association of ACAb against specific commensals with the specificities of anti-islet autoantibodies before diabetes development. Our results indicate that distinct HLA genotype–associated immune response patterns against gut commensals accompany pre-diabetes, and we provide an approach to evaluate the role of the gut microbiome in immune-mediated diseases affecting extramucosal tissues.

RESULTS
Anticommensal bacterial antibodies (ACAb) have been reported in mice harboring mutations in innate immune sensing pathways (13) and in adults with HIV infection or inflammatory bowel disease [CD and UC; (14)]. Whereas HIV patient and HC sera displayed equivalent levels of antibody binding to gut- and skin-derived bacteria, the patients with CD and patients with UC displayed elevated antibody responses to three bacterial isolates, suggesting that gut barrier compromise results in heightened immune priming to intestinal bacteria. Here, we evaluated the titers and isotopes of ACAb to ask whether these patterns are associated with T1D, an autoimmune disease where the target tissue is distant from the gut and is not characterized by clinical enteropathy.

We measured ACAb responses against a panel of bacterial strain pools representative of genera found in healthy human gut microbiota (table S1). Given the extensive strain-level genetic variation in human gut bacterial species (15), multiple strains were used for each species to maximize potential antigenic diversity. Also included was a pre-gut bacterial species (15), as well as IgA against Enterococcus faecalis, as well as IgA against Enterococcus faecalis, which was observed in patients with CD compared with HC (Fig. 2A). To visualize the variation in ACAb responses by all the samples to each bacterial target, we performed an unsupervised principal components analysis (PCA) (Fig. 2B). ACAb responses from CD patient and HC samples clustered separately by two PC that captured 70% of the variance.

A linear discriminant analysis (LDA) was conducted to determine whether these ACAb responses could discriminate CD from HC samples (Fig. 2C). We used variance inflation factor (VIF)–based (VIF threshold = 10) variable selection to remove responses that could be represented by combinations of other (collinear) responses and then performed an LDA with 100× repeated twofold cross-validation on the remaining variables (Materials and Methods). The resulting linear discriminant values correctly classified 78.8% [95% confidence interval (CI), 70.3 to 87.5%] of all randomly selected samples, suggesting that the VIF-selected set of ACAb responses could discriminate between most CD and HC samples.

Because a substantial proportion of NET individuals were in or beyond the pubertal period when the serum samples were obtained, we tested for effects of sex using linear regression models with sex and clinical status as covariates. Sex differences were observed for IgA responses against Bacteroides fragilis, C. perfringens, E. faecalis, and MET-2 and total Ig responses against C. perfringens. For IgA responses against B. vulgatus and E. faecalis, we found a significant interaction between sex and status [analysis of variance (ANOVA) for interaction coefficients, false discovery rate (FDR)–adjusted q < 0.12]. ACAb responses were higher in female compared with male CD cases, but this pattern was not observed in HC individuals (fig. S2).

ACAab responses distinguish patients with T1D from HC or healthy control individuals
Multiple studies have reported correlations between the taxonomic composition and inferred functional capacity of host microbiota and autoimmune diseases in human study participants. However, we lack an understanding of the mechanisms that link these mucosal microbes with systemic immune responses and autoimmune targeting of distant tissues. In contrast to CD, the target of many autoimmune diseases is outside the gut. To examine immune responses to gut commensal bacteria in this setting, we examined serum samples from pediatric study participants in the NET study that had been recently diagnosed with T1D compared with age-matched HC (Fig. 1D). To enhance the scope of the analysis, we added bacterial species belonging to genera reported to be differentially abundant in children with ≥2 islet autoantibodies compared with autoantibody-negative age-, sex- and HLA-matched children or children with new onset T1D (9, 10). Patients with recent onset T1D and HC sera displayed differences in both Ig and IgA ACAb responses against Anaerotruncus colihominis, Bifidobacterium animalis, B. fragilis, B. vulgatus, C. perfringens, and Lactobacillus acidophilus, as well as total Ig against Gemmiger formicilis and R. faecis (Fig. 3A). Unsupervised PCA of all ACAb responses also displayed these differences between T1D and HC individuals (Fig. 3B), suggesting that the patterns of ACAb responses differed between these two groups of study participants.
Fig. 1. Overview of ACAb assay and pediatric cohorts in this study. (A) Schematic representation of the ACAb assay. In a first step, bacteria targets of a specific strain pool are incubated with four serial dilutions of the serum sample, allowing serum antibodies to bind bacteria surface antigens. Next, fluorescently labeled, secondary anti-isotype antibodies (anti-IgA, IgG1, IgG2, and total Ig) are added, allowing the visualization of the bacteria through flow cytometry. (B) An example of the signal measured by flow cytometry for one bacterial pool and four serial dilutions of a serum sample. The first four panels show contour plots of the anti-isotype intensity versus forward scatter (FSC-A) parameter. The last panel shows a histogram overlay of the anti-isotype signal intensities. (C) Schematic representation of the response index calculation. For every serum sample, bacterial strain pool, and isotype, the dilution curve of the MFI versus dilution factor is generated on a log-log scale (left, gray lines). The mean dilution curve is generated using the average MFI values for all serum samples (left, black line). For every sample, the difference between its dilution AUC and the mean dilution AUC is calculated ($\Delta$AUC; right). The resulting response index is calculated as $2^{\Delta$AUC}. (D) Schematic representation of the NET and TrialNet cohort participants analyzed in this study. Pediatric serum samples (age ≤ 18 years) from the NET study were collected from patients with recent onset CD ($n = 32$), patients with recent onset T1D ($n = 49$), and age-matched HCs ($n = 90$). Serum samples were also obtained from TrialNet individuals ≤18 years of age. Samples defined as cases ($n = 68$) were collected before T1D diagnosis from individuals with 2 or more islet autoantibodies who developed T1D during the study follow-up. Samples defined as controls ($n = 62$) were collected from age-, sex-, and HLA-matched individuals who did not receive a T1D diagnosis within the follow-up period.
class II genotypes in T1D risk and reported associations between gut microbiome composition and disease (4, 9, 10), there is no evidence for HLA association with human gut microbial composition. The TrialNet metadata included HLA genotype, allowing us to address the possibility that ACAb responses display associations with HLA haplotypes with and without T1D onset. First, we evaluated ACAb responses without inclusion of HLA haplotype as a variable and observed no association with a future diagnosis of T1D (fig. S6A). In addition, neither PCA (fig. S6B) nor LDA (fig. S6C) revealed differential ACAb patterns between prediabetic case and control samples. Thus, we next analyzed the ACAb responses using HLA-DR haplotypes as a covariate.

A multivariate linear regression analysis including clinical status and HLA haplotypes as covariates revealed robust associations of certain ACAb responses with a future T1D diagnosis in an HLA class II–dependent manner (Fig. 4, A and B; comparisons for all ACAb responses are shown in fig. S7). The interaction between a future T1D diagnosis and the presence of high-risk haplotypes DR3 and DR4 was associated with IgG2 ACAb responses against MET-2 and R. faecis (ANOVA, P = 0.009 and 0.01, respectively; q = 0.16; Fig. 4, C and D). Samples from cases lacking both DR3 and DR4 displayed higher MET-2 and R. faecis responses, those with either DR3 or DR4 had intermediate responses, and cases with both DR3/DR4 haplotypes had lowest responses (Fig. 4, C and D). In contrast, samples from controls displayed the opposite response pattern to both bacterial groups. Total Ig responses against the same bacterial species also showed a significant HLA-DR3 and DR4–dependent association with clinical status (fig. S8, A to D). Ig and IgG2 responses against the same bacterial target were highly correlated (fig. S9, A and B) suggesting that IgG2 responses were the major signals captured against the bacterial targets, although correlations between total Ig, IgA, and IgG1 suggest that IgA and IgG1 responses may also contribute (fig. S9, C to F).

Longitudinal microbiome analyses in children at risk for T1D have revealed notable age-dependent, developmental patterns in microbiota diversity, composition, and function (21–23). These findings, together with the wide age range in the TrialNet study participants (fig. S4), prompted us to investigate whether their antibody responses against gut commensals were also age dependent. IgG2 and total Ig responses against MET-2 and IgG2 responses against R. faecis were correlated with age (ANOVA, P < 0.02, q < 0.07; Fig. 4, E and F, and fig. S8, E and F). Moreover, when the analysis was adjusted for the age effect, the HLA–dependent associations with future T1D diagnosis of IgG2 and total Ig antibodies against MET-2 and R. faecis reported above were stronger (ANOVA, P values before and after age adjustment shown in Fig. 4, C and D, and fig. S8, C and D). Therefore, the HLA–dependent association of ACAb responses with T1D development was robust to age variation in the TrialNet cohort.

Thus, inclusion of HLA haplotypes in the analysis was critical to reveal associations of ACAb responses and T1D, and these associations were strengthened by analysis that removed age effects. The HLA dependence of the relationship between ACAb responses and future T1D development suggests a critical role of this genetic risk factor in modifying the interactions between immune responses to gut microbes and diabetes development.

**Association of ACAb responses with islet autoantibody specificity**

Assessment of islet autoantibody titers is the best available biomarker for predicting the development of T1D in genetically susceptible

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**Table 1. Characteristics of NET cohort study participants.**

<table>
<thead>
<tr>
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<th>CD (n = 32)</th>
<th>T1D (n = 49)</th>
<th>Controls (n = 90)</th>
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<tbody>
<tr>
<td><strong>Sex (% males)</strong></td>
<td>n = 17 (53%)</td>
<td>n = 31 (62%)</td>
<td>n = 30 (33%)</td>
</tr>
<tr>
<td><strong>Age at diagnosis</strong> (years; mean ± SD)</td>
<td>12.30 (±3.26)</td>
<td>10.68 (±3.95)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Age at ACAb test</strong> (years; mean ± SD)</td>
<td>12.96 (±3.03)</td>
<td>11.08 (±3.98)</td>
<td>14.91 (±2.10)</td>
</tr>
<tr>
<td><strong>Time between ACAb test and diagnosis</strong> (days; mean ± SD)</td>
<td>241 (±480)</td>
<td>135 (±94)</td>
<td>N/A</td>
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individuals (24, 25). Moreover, islet autoantibody antigen specificity displays HLA class II haplotype dependence. Anti-GAD65 autoantibodies (GADA) have been associated with DR3 and anti-insulin autoantibodies (IAA) and islet cell autoantibodies (ICA) with DR4 haplotype (26, 27). We tested these associations in the TrialNet cases using longitudinal measures of GADA, IAA, tyrosine phosphatase-protein IA-2 autoantibodies (IA2A), and ICA. Recapitulating previous studies, we found that DR3-positive individuals had a higher frequency of positive GADA measures before T1D diagnosis ($\chi^2 = 15.73, P = 5 \times 10^{-4}$; fig. S10A), and DR4-positive individuals displayed a higher frequency of positive IAA measures compared with alternative DR3/DR4 genotypes (fig. S10B; $\chi^2 = 6.0, P = 0.014$). Thus, HLA class II haplotype associations with islet autoantibody specificity in this TrialNet cohort accorded with those previously reported in independent groups of individuals (26, 27).

Given the observed HLA-dependent associations of some ACAb responses with future T1D diagnosis (Fig. 4) and the well-established association of autoantibody seroconversion with progression to T1D, we investigated the relationship between ACAb responses and islet autoantibody seroconversion. A canonical correlation analysis (CCA) between the ACAb responses and autoantibody specificities revealed relationships between these two datasets (see Materials and Methods.
The key finding was that the isotype of ACAb responses clustered with islet autoantibody specificity. Specifically, IgG1 ACAb responses were negatively correlated with ICA, and IgG2 ACAb responses were positively correlated with IA2A and negatively correlated with IAA in seroconverted individuals. In contrast to these IgG responses, IgA isotype ACAb did not display strong correlations with any islet autoantibody specificity in these prediabetic individuals.

The CCA revealed the correlations between ACAb isotype responses and autoantibody specificities (Fig. 5). Our data (fig. S10) and previous studies (26, 27) demonstrate associations between HLA haplotypes and islet autoantibody specificity. Therefore, we tested the HLA dependence of the associations between islet autoantibody specificities and ACAb responses. Pairwise regression models were used to identify the main effects of autoantibody specificity on ACAb responses. We identified six ACAb-autoantibody associations with a nominal $P < 0.05$ and $q < 0.5$ (Fig. 6A and fig. S11A), which were further analyzed for dependency on HLA-DR3 or -DR4 haplotypes. Three of these associations did not reach statistical significance after correction for multiple tests (fig. S11). For three other ACAb, we observed significant associations with islet autoantibody specificity ($q < 0.2$; Fig. 6). The association between IAA and IgG2 against Commensal strain pool
R. faecis and that between IA2A and IgG2 against Streptococcus gallolyticus were both more robust with inclusion of HLA haplotype. Thus, the presence of DR3 and IAA specificity displayed an additive effect on the R. faecis IgG2 response (ANOVA, \( P = 0.01 \), \( q = 0.12 \); Fig. 6B). Similarly, the association between IA2A specificity and IgG2 against S. gallolyticus was only observed in the absence of DR4 (ANOVA, \( P = 0.0003 \); \( q = 0.06 \); Fig. 6C). The latter observation indicates that the relationship between the islet autoantibody specificity and this ACAb response is modulated by HLA-DR4 haplotype (interaction ANOVA, \( P = 0.03 \); \( q = 0.25 \); Fig. 6D). In contrast, the relationship between ICA-specific autoantibodies and the B. fragilis IgG1 response (ANOVA, \( P = 0.005 \); \( q = 0.17 \); Fig. 6A) was independent of HLA-DR (ANOVA, \( P > 0.06 \); Fig. 6, B and C). Thus, as presented above, for association of ACAb responses with future progression to T1D (Fig. 4), the relationship between certain ACAb responses and islet autoantibody specificities was also HLA-DR haplotype dependent.

### DISCUSSION

The rapid increase in inflammatory and autoimmune diseases in developed countries over the past 60 years (28) has focused attention on environmental factors, and their potential impact on the gut microbiome, to explain this epidemiology. In T1D, 16S ribosomal RNA gene and shotgun metagenomic sequencing studies have investigated the composition and functionality of gut microbiota and its relationship to anti-islet autoimmunity in human cohorts (4, 21, 29–33). One recurrent finding from these studies is lower diversity in bacterial composition in children who became diabetic compared with those who did not, but these differences were not evident in islet autoantibody seroconverters who remained diabetes free (4). However, microbiome sequence analyses have not produced a consensus on the relationship between abundance of specific bacteria taxa and either future disease development or islet autoantibody seroconversion. A recent longitudinal analysis of \( n = 10,903 \) fecal metagenomes from \( n = 783 \) children in The Environmental Determinants of Diabetes in the Young (TEDDY) study who progressed to autoantibody seroconversion or T1D, with matched controls, reported that “...most of the taxonomic and functional signals we detected in case-control comparisons were modest in effect size and statistical significance” (22). Given the size and analytical scope of this study, the results demonstrate that heterogeneity in microbiome composition presents a significant challenge to resolving patterns that are associated with immune responses against a distant target tissue. Here, we have queried immune responses to intestinal bacteria in pediatric participants at risk for T1D and provide evidence that, in contrast to microbiome composition, these responses are associated with the development of T1D and the specificity of anti-islet autoimmunity in an HLA genotype–dependent manner.

Compared with age-matched HCs, we found that the titers of serum IgA and total Ig against specific commensals were higher in patients with recent onset pediatric CD (Fig. 1A), consistent with previous reports (34–36). These heightened anticommensal immune responses likely reflected impaired intestinal barrier function observed in CD (37, 38), which allowed translocation of gut bacterial antigens and subsequent priming of B cells in extramucosal lymphoid organs. In contrast, most human studies suggest that, in T1D, gut barrier disruption can be detected after, but not prior to, disease onset (39–41). We found that levels of serum total Ig and IgA against most commensal bacteria tested were lower in NET study participants recently diagnosed with T1D compared with healthy matched controls (Fig. 2A). A longitudinal study of children at risk for allergic diseases reported that decreased IgG seroreactivity to a group of commensal antigens during infancy was associated with allergy development later in life (42), suggesting a protective role of adaptive immune response to the microbiota against immune-mediated diseases in susceptible individuals. The decreased ACAb titers in patients with new onset T1D indicate that the disease may be inversely correlated with systemic immune stimulation by gut commensals. Consistent with this idea, fecal microbiome analysis of infants with high-risk HLA haplotypes for T1D has suggested that sources of lipopolysaccharide (LPS) during early life distinguished Finnish and Estonian individuals from those in a neighboring region of Russia (21). Relative to the former two countries, this Russian region has a far lower prevalence of T1D and allergic disease. Perhaps early-life exposure to some microbial components protects from autoimmunity. Our analyses discriminated both CD and T1D cases from HCs (Figs. 1, B and C, and 2, B and C), demonstrating that anticommensal response patterns reflect the distinct phenotypes of the two diseases.

In the NET study participants with T1D, disease-related metabolic abnormalities may influence immune function, specifically antibody responses to commensal bacteria. To mitigate this possibility, we examined ACAb responses in serum samples collected months before diagnosis in the independent TrialNet prospective cohort. Whereas ACAb response patterns discriminated patients with recent onset T1D from HC NET participants, the prediabetic TrialNet cases were indistinguishable from matched controls (fig. S6). However, inclusion of the individuals’ HLA genotype in the analysis revealed robust association between ACAb responses and a future T1D diagnosis (Fig. 4). The observed HLA-DR dependence of ACAb responses identified a relationship between immune responses to gut bacteria and the most impactful inherited risk factor for this disease (Fig. 7).

Specific ACAb responses displayed a DR3/DR4-dependent association with disease progression in highest–genetic risk TrialNet participants carrying both DR3 and DR4 haplotypes. These individuals

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**Table 2. Characteristics of TrialNet cohort study participants.**

<table>
<thead>
<tr>
<th></th>
<th>Cases (n = 68)</th>
<th>Controls (n = 62)</th>
</tr>
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<tr>
<td><strong>Sex (% males)</strong></td>
<td>n = 36 (53%)</td>
<td>n = 32 (52%)</td>
</tr>
<tr>
<td><strong>Age at T1D diagnosis (years; mean ± SD)</strong></td>
<td>11.61 (±3.76)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Age at ACAb test (years; mean ± SD)</strong></td>
<td>10.67 (±3.82)</td>
<td>10.92 (±3.65)</td>
</tr>
<tr>
<td><strong>Time between ACAb test and diagnosis (days; mean ± SD)</strong></td>
<td>344 (±120)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Follow-up time (days; mean ± SD)</strong></td>
<td>1045 (±711)</td>
<td>2646 (±1051)</td>
</tr>
<tr>
<td><strong>HLA-DR3 positive/DR4 negative</strong></td>
<td>n = 20 (29%)</td>
<td>n = 18 (29%)</td>
</tr>
<tr>
<td><strong>HLA-DR3 negative/DR4 positive</strong></td>
<td>n = 22 (32%)</td>
<td>n = 21 (34%)</td>
</tr>
<tr>
<td><strong>HLA-DR3 positive/DR4 positive</strong></td>
<td>n = 12 (18%)</td>
<td>n = 10 (16%)</td>
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</table>
Fig. 4. ACAb response indices displaying interaction effects between HLA haplotype and clinical status in TrialNet samples (cases, red; controls, blue). (A) Box-and-whiskers plot of anti–MET-2 IgG2 response indices (y axis), segregated by HLA haplotype (x axis). (B) Box-and-whiskers plots of anti–R. faecis IgG2 response indices (y axis), segregated by HLA haplotype (x axis). (C) Interaction plot of anti–MET-2 IgG2 response indices. (D) Interaction plot of anti–R. faecis IgG2 response indices. (E) Linear regression of anti–MET-2 IgG2 response indices by age. (F) Linear regression of anti–R. faecis IgG2 response indices by age. (A and B) The horizontal line represents the median, and the rectangle represents the interquartile range (25th to 75th percentile). Whiskers extend to the minimum and maximum values that were not outliers. Outliers were defined as points outside of median ± 1.5× interquartile range. (C and D) Data are shown as means ± SE, and P and q values for the interaction effect significance are shown for the responses without (IgG2 MET-2: P = 0.009, q = 0.16; IgG2 R. faecis: P = 0.01, q = 0.16) and with (IgG2 MET-2: P = 0.005, q = 0.11; IgG2 R. faecis: P = 0.007, q = 0.11) age adjustment. (E and F) Regression lines for cases and controls as well as age effect–associated P and q values are shown (IgG2 MET-2: P = 0.0004, q = 0.005; IgG2 R. faecis: P = 0.01, q = 0.06).
HLA haplotypes are strongly associated with multiple human autoimmune and autoinflammatory diseases (5). Several studies in RA have provided evidence that disease-related HLA haplotypes may also be associated with immune responses to microbes. In patients with RA, peptides derived from the gut commensal Prevotella copri and presented by HLA-DR can stimulate inflammatory responses. Serum IgA and IgG antibodies to P. copri were also associated with titers of anti-citrullinated protein antibodies (43), a biomarker of RA onset (44).

Here, we report correlation between specific islet autoantibodies and IgG1 and IgG2, likely T cell–dependent, ACAb responses (Fig. 5). An important limitation of our study is that the bacterial strains used to reveal ACAb are proxies for those that triggered the initial B cell responses in these study participants. These findings raise the possibility that some antibodies against commensals may cross-react with self-antigens. A characterization of the reactivity profile of intestinal plasma blasts isolated from healthy participants revealed that 25% of intestinal IgA and IgG antibodies were polyreactive with diverse foreign and self-antigens, including insulin and bacterial LPS (45). In patients with RA or autoimmune hepatitis, antibodies against mucosal pathogens were correlated with disease-associated autoantibodies (46, 47). A recent study in the nonobese diabetic mouse demonstrated molecular mimicry between an integrase peptide derived from the common gut commensal Bacteroides and an islet antigen. In this model, colonization with a Bacteroides encoding the integrase

Fig. 5. Correlations between isotype-specific ACAb responses and islet autoantibody–positive status in TrialNet case samples (n = 68). Correlations between isotype-specific ACAb responses (columns, bottom) and islet autoantibody–positive status (rows, left) are shown in heatmap colors. ACAb responses and islet autoantibodies were each clustered by similarity by hierarchical clustering (top and right dendrograms, respectively). ACAb isotypes are as follows: IgA, blue; IgG1, green; IgG2, purple.
from the humans of interest will provide optimal targets for immune response analyses and to identify a spectrum of relevant microbial antigens.

Our study identifies a robust relationship between immune responses to intestinal bacteria, a future T1D diagnosis, antibody responses to islet autoantigens, and high-risk HLA haplotypes. Additional studies in independent cohorts of individuals are required to determine whether patterns of ACAb responses reported here may be predictive of progression to islet autoimmunity and diabetes. Our approach provides an “immune system’s view” of the microbiome and complements

Fig. 6. ACAb responses displaying an association with islet autoantibody-positive status in TrialNet case samples (n = 68). (A) Box-and-whiskers plots of isotype-specific anticommensal response indices (y axis), separated by autoantibody statuses (x axis). (B) Box-and-whiskers plot of the isotype-specific anticommensal response indices (y axis), autoantibody status, and HLA-DR3 genotype status (x axis). (C) Box-and-whiskers plot of the isotype-specific anticommensal response indices (y axis), autoantibody status, and HLA-DR4 genotype status (x axis). (D) Interaction plots for isotype-specific anticommensal response indices with significant HLA-DR3/DR4 genotype status additive effect. (A to C) The horizontal line represents the median, and the rectangle represents the interquartile range (25th to 75th percentile). The whiskers extend to the minimum and maximum values that were not outliers. Outliers are defined as points outside of median ± 1.5× interquartile range. (A) P values were generated by a linear regression model with only the autoantibody positivity as the explanatory variable and indicate the significance of the autoantibody main effect. (B and C) P values were generated by a linear regression model with autoantibody positivity and DR3/DR4 genotype as the explanatory variables and indicate the significance of the autoantibody effect in the HLA-adjusted model. (D) Data are shown as means ± SE. P values were generated by ANOVA comparing the additive linear model to the model with an interaction term (see Materials and Methods).
The distribution of ages at serum collection varied from 3.1 to 7.89 years from enrollment (cases, for ≥2 islet autoantibodies and developed T1D within the study period (ranging from 0.78 to 7.89 years from enrollment; cases, n = 68). The distribution of ages at serum collection varied from 3.1 to 48.1 years. Samples collected from individuals ≤18 years of age were included in the present study.

**Human commensal bacterial collections**

The bacterial strains used in this study were isolated and archived by E. Allen-Vercoe either as part of the Human Microbiome Project reference genome collection or as part of a study to characterize the microbiota of a healthy human donor for metabolomic assessment (16). *L. acidophilus* was isolated from a probiotic preparation, VSL#3 (Alfasigma Inc.). The strains are shown in table S1.

**Bacterial preparation**

Bacteria were resuscitated from axenic, frozen stocks on fastidious anaerobe agar (Neogen Corporation) supplemented with 5% defibrinated sheep’s blood (Hemostat Laboratories), under anaerobic conditions (85% N2, 10% H2, and 5% CO2) at 37°C for 48 to 72 hours. Representative biomass was scraped from freshly grown plates and resuspended in 1 ml of sterile PBS, washed twice with 10 ml of PBS (13,000 rpm for 10 min at 4°C) and resuspended at 10⁷ bacteria/ml. For assessment of antibody responses to the 32-strain MET-2 consortium, the concentration of each cultured strain was then adjusted to 10⁵ bacteria/ml and mixed in equal proportions.

**ACAb assay**

Serially diluted samples (50 µl) were incubated with 50 µl of bacteria (~5 × 10⁵ bacteria) at 4°C for 1 to 2 hours and then washed twice with wash buffer (13,000 rpm for 10 min at 4°C). Bacteria were then incubated (30 min at 4°C) with 50 µl of fluorophore-conjugated anti-isotype antibodies [goat anti-human IgG (DyLight 405 conjugated; Jackson ImmunoResearch Laboratories Inc.), mouse anti-human IgG1 (phycoerythrin conjugated, SouthernBiotech), mouse anti-human IgG2 (AF647 conjugated, SouthernBiotech), or mouse anti-human IgA (fluorescein isothiocyanate conjugated, Jackson ImmunoResearch Laboratories Inc.) (Fig. 1). Bacteria were washed twice with PBS and resuspended in 200 µl of 2% paraformaldehyde (Canemco Inc.). The samples were analyzed using an LSRFortessa (Becton Dickinson) with settings preoptimized for bacterial cell detection. Flow cytometry analysis of serum samples was performed using FlowJo software (Treestar Inc.).

**Calculation of response indices**

Human serum concentrations (four per serum sample) and their corresponding mean fluorescence intensity (MFI) values were log₂ transformed and linearly regressed using Prism (GraphPad Prism, GraphPad Software, La Jolla, CA, USA) to produce one regression line for each sample/isotype/bacteria target combination. The area under the curve (AUC) was calculated for each regression line. These AUCs were directly proportional to serum ACAb responses and were compared with the average AUC of all samples for each bacterial target and antibody isotype combination. The differences between sample AUC and the average AUC were expressed as ΔAUC. Response indices are the 2ΔAUC values, such that the average of all
AUCs becomes 1 and that sample responses are positive values and either greater than or less than 1 (Fig. 1).

**Islet autoantibodies**

In TrialNet case control samples, the ICA status was the seropositivity status of the serum sample used in the ACAb assay. For antibodies against biochemically defined antigens (IAA, IA2A, and GADA), the autoantibody status was considered positive if the participant had seroconverted and negative if the participant had never seroconverted or had reverted at the time of ACAb serum sample collection. Seroconversion was defined as two repeat measures of the same autoantibody. Reversion was defined as two or more autoantibody negative measures following seroconversion (50).

**Statistical analysis**

All statistical analyses were performed in R (www.R-project.org/). PCA was performed on ACAb responses using the prcomp function in the base package. To account for variable collinearity that can produce unstable LDA discriminators, collinear variables were removed using stepwise VIF selection (VIF threshold, 10). LDA on VIF-selected ACAb response indices was performed using the MASS, cars, and base packages. To assess the discrimination power of the LDA function, repeated (100x) twofold cross-validation was performed using the MASS and base packages. To account for unbalanced case versus HC numbers, cross-validation was performed using samples bootstrap-rarefied to the condition with least samples. Linear regression of ACAb responses against various explanatory variables (clinical status, HLA haplotypes, islet autoantibody specificity, and age) was performed using the base and phi R packages. The significance of the interaction term in the linear model was assessed using the anova function comparing the additive model to the model with an interaction term. Age adjustment was performed by adding age as a covariate in the linear regression model. Multiple testing correction was performed by controlling the FDR for the family of tests performed using the p.adjust function in R. The association of islet autoantibodies and ACAb responses was investigated using a stepwise hypothesis testing approach. 1) The main effect of every autoantibody specificity was tested for every ACAb response. Nominally significant associations (P < 0.05, q < 0.5) were further investigated at the next step. 2) For nominally significant autoantibody-ACAb associations from step 1, the regression model was adjusted by addition of the HLA haplotype DR3 or DR4 to test whether the autoantibody specificity effect was HLA dependent. Tests performed at step 1 and step 2 were corrected for multiple testing by controlling the FDR. Autoantibody-ACAb response associations with q < 0.2 either at step 1 or step 2 were considered significant and are reported in Fig. 6.

The regularized CCA was performed using the mixOmics R package on the ACAb responses and autoantibody positivity data to extract relationships between these two multidimensional data-sets. Specifically, CCA maximizes the correlation between linear combinations of variables from the two datasets (i.e., ACAb responses and autoantibody positivity) while adjusting for the within-dataset correlations and is therefore a more powerful approach compared with performing pairwise correlations between the variables.

The cor.test function was used to test correlation between pairs of ACAb responses. Plots were generated using the ggplot2 and ggrepel packages.

**SUPPLEMENTARY MATERIALS**

immunology.sciencemag.org/cgi/content/full/4/32/eaau8125/DC1

Fig. S1. Age of NET participants at time of serum sample collection.

Fig. S2. Sex effects on ACAb responses in CD (n = 32) and HC (n = 68).

Fig. S3. Sex effects on ACAb responses in T1D (n = 32) and HC (n = 68).

Fig. S4. Sex effects on ACAb responses in fatty liver disease (n = 32) and HC (n = 68).

Fig. S5. Sex effects on ACAb responses in hepatitis C (n = 32) and HC (n = 68).

Fig. S6. Comparison of linear regression of ACAb responses and autoantibody positivity while adjusting for the within-dataset correlations and is therefore a more powerful approach compared with performing pairwise correlations between the variables. The cor.test function was used to test correlation between pairs of ACAb responses. Plots were generated using the ggplot2 and ggrepel packages.

**REFERENCES AND NOTES**


Acknowledgements: We acknowledge serum samples from the CIHR/MSSC NET study in Clinical Autoimmunity Co-Principal Investigators (A. Bar-Or, B. Banwell, A. Griffiths, M. Silverberg, C. Piccirillo, C. Polychronakos, and P. Sherman) and from the TrialNet Biorepository through an ancillary study to the Type 1 Diabetes TrialNet Pathway to Prevention study (TN-01). The use of samples from the NET and Pathway to Prevention Trial Net studies for this work performed in this paper was approved by The Hospital for Sick Children. Funding: J.S.D. acknowledges support from the Canadian Institutes of Health Research (CIHR), the Juvenile Diabetes Research Foundation (JDRF), the Hospital for Sick Children Research Foundation and the Anne and Max Tanenbaum Chair, University of Toronto. E.A.-V. acknowledges funding from the Natural Sciences and Engineering Research Council of Canada. A.P. was supported by fellowships from the JDRF and CIHR. TrialNet TN-01 is supported by NIH grants U01 DK061010, U01 DK061034, U01 DK061042, U01 DK061058, U01 DK085465, U01 DK085461, U01 DK085466, U01 DK085476, U01 DK085499, U01 DK085509, U01 DK103180, U01 DK103153, U01 DK103266, U01 DK103282, U01 DK106984, U01 DK106994, U01 DK107013, U01 DK107014, and UC4 DK106993 and the JDRF. Author contributions: J.S.D. and P.P. designed the study. J.S.D. supervised the study. L.M. and S.M. performed ACAb assays. E.A.-V. and M.C.D. provided bacterial strains and advice on their culture. A.B.-O. provided access to NET serum samples and helpful comments. A.P., C.Y., and S.M.-T. performed the data analysis. A.P., C.Y., and J.S.D. wrote the manuscript. P.P., E.A.-V., and A.B.-O. commented on the manuscript. Competing interests: The authors declare that they have no competing interests. Data and materials availability: Raw data including flow cytometry files are available by request.

Submitted 20 July 2018 Accepted 13 December 2018 Published 1 February 2019 10.1126/sciimmunol.aau8125

Association of HLA-dependent islet autoimmunity with systemic antibody responses to intestinal commensal bacteria in children

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DOI: 10.1126/sciimmunol.aau8125

Prediabetic microbiome

Studies in humans and mice have identified associations between intestinal microbiome composition and development of autoimmune diseases, including type 1 diabetes (T1D). How these microbes exert influence on immune responses in distant tissues is unknown. By studying pediatric cohorts, Paun et al. examined whether serum antibody responses to intestinal commensal bacteria were associated with T1D status. They report that antibody responses against specific commensals measured before T1D diagnosis distinguished individuals with islet autoantibodies from healthy controls in a human leukocyte antigen haplotype-dependent manner. Their results link immune responses to gut microbes with later T1D development and suggest that they may be predictive of T1D when analyzed with genetic risk factors.