Correction

IMMUNOLOGY AND INFLAMMATION

Correction for "T-cell responses to hybrid insulin peptides prior to type 1 diabetes development," by Angela M. Mitchell, Aimon A. Alkanani, Kristen A. McDaniel, Laura Pyle, Kathleen Waugh, Andrea K. Steck, Maki Nakayama, Liping Yu, Peter A. Gottlieb, Marian J. Rewers, and Aaron W. Michels, which published February 4, 2021; 10.1073/pnas.2019129118 (*Proc. Natl. Acad. Sci. U.S.A.* **118**, e2019129118).

The authors note that, due to a printer's error, Figs. 3 and 4 appeared incorrectly. The corrected figures and their legends appear below. The online version has been corrected.

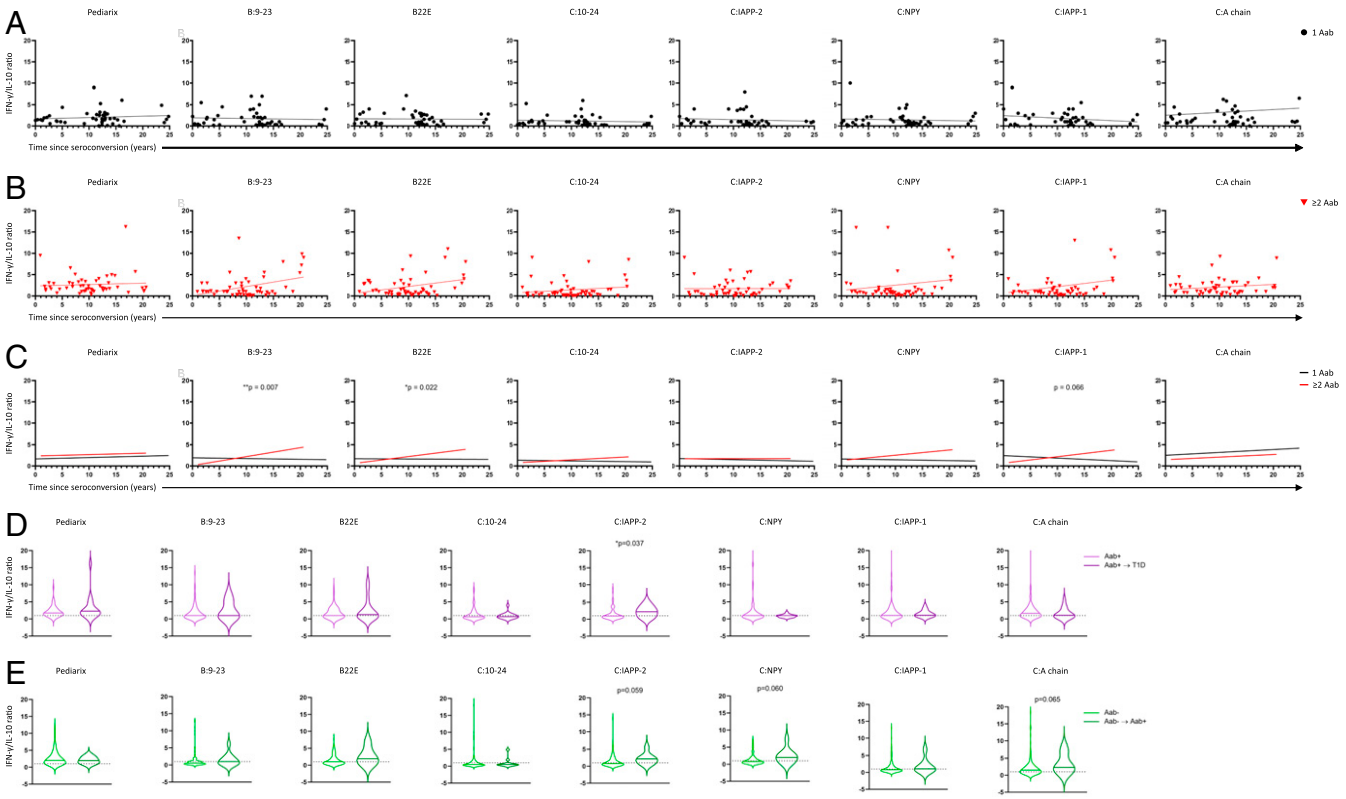

Fig. 3. Individuals progressing to clinical disease show distinct inflammatory responses to specific HIPs. IFN-γ/IL-10 ratios from ELISPOT assays are plotted against the number of years since seroconversion for Aab^+ individuals. Graphs with linear regression lines showing all visits from (*A*) individuals with one Aab at the initial visit ($n = 12$) and (*B*) two or more Aabs ($n = 13$) displayed. (*C*) The linear regression lines from *A* and *B* are overlaid for each stimulus. *P* values depicted using linear regression analyses. Due to very elevated IFN-γ/IL-10 ratio, one point is not displayed on each of the following graphs: C:A chain in *A* (12.1 y, ratio 64), C:NPY in *B* (13.2 y, ratio 26), and C:IAPP-1 in *B* (8.6 y, ratio 21); however, these values were included to determine linear regression lines and statistical comparisons. (*D*) Graphs depicting IFN-γ/IL-10 ratios for all study visits from Aab^+ (purple) and (*E*) Aab^- (green) individuals. Darker colored lines represent responses from Aab^+ individuals who progressed to clinical T1D ($n = 5$) and Aab^- individuals who developed an islet Aab ($n = 3$), while lighter colored lines display responses from those not progressing to T1D ($n = 20$) or developing an islet Aab ($n = 35$). Dotted black lines are at 1.0, with values greater than 1.0 indicating a predominantly inflammatory response, and values less than 1.0 an anti-inflammatory response. *P* values depicted using a Mann–Whitney *u* test.

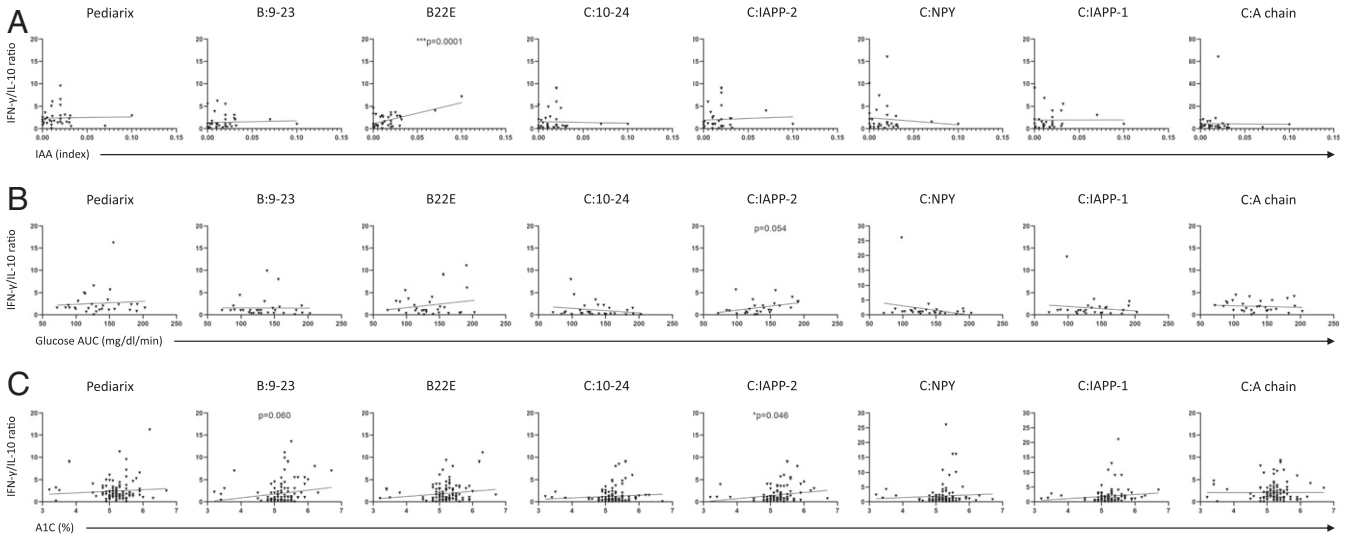

Fig. 4. IAA levels and measures of glucose metabolism correlate with responses to HIPs during progression to T1D. (*A*) IFN-γ/IL-10 ratios from PBMC ELISPOT assays using native insulin B:9–23, B22E HIP, native C:10–24, and the C:IAPP-2 HIP are plotted against IAA levels in individuals having a positive IAA value from at least one visit (n = 9). (*B*) Glucose AUC measurements following a 2-h OGTT are plotted versus the corresponding IFN-γ/IL-10 ratios at matched visits for Aab^+ participants (n = 13). (*C*) Hemoglobin A1C levels, which is a measure of average blood glucose over the preceding 3 mo, are graphed versus the corresponding IFN-γ/IL-10 ratios at matched visits for all participants that had A1C measurements (n = 30). *P* values depicted using linear regression analysis.

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T-cell responses to hybrid insulin peptides prior to type 1 diabetes development

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T-cell responses to posttranslationally modified self-antigens are associated with many autoimmune disorders. In type 1 diabetes, hybrid insulin peptides (HIPs) are implicated in the T-cell–mediated destruction of insulin-producing β-cells within pancreatic islets. The natural history of the disease is such that it allows for the study of T-cell reactivity prior to the onset of clinical symptoms. We hypothesized that CD4 T-cell responses to posttranslationally modified islet peptides precedes diabetes onset. In a cohort of genetically at-risk individuals, we measured longitudinal T-cell responses to native insulin and hybrid insulin peptides. Both proinflammatory (interferon-γ) and antiinflammatory (interluekin-10) cytokine responses to HIPs were more robust than those to native peptides, and the ratio of such responses oscillated between pro- and antiinflammatory over time. However, individuals who developed islet autoantibodies or progressed to clinical type 1 diabetes had predominantly inflammatory T-cell responses to HIPs. Additionally, several HIP T-cell responses correlated to worsening measurements of blood glucose, highlighting the relevance of T-cell responses to posttranslationally modified peptides prior to autoimmune disease development.

autoimmunity | T cell | type 1 diabetes | antigen | posttranslational modification

Type 1 diabetes (T1D) is a prototypical organ-specific autoimmune disease that develops in stages (1, 2). The stages are marked by the presence of islet autoantibodies directed against insulin and other β-cell proteins, followed by impaired glucose tolerance, and finally clinical diabetes marked by hyperglycemia and the need for insulin treatment (3). The T1D disease course provides a defined preclinical period and the ability to measure immune responses prior to clinical symptoms.

Self-reactive T cells target pancreatic β-cells in both murine models of spontaneous autoimmune diabetes and human T1D (4), with a number of antigens implicated as T-cell epitopes (5, 6). Recently, posttranslationally modified (PTM) epitopes have been characterized as novel autoantigens that may lead to a break in tolerance, thus resulting in T-cell–mediated immunity to pancreatic islets. PTM of antigens is well-described in autoimmune diseases, such as celiac disease (gluten sensitivity), in which tissue transglutaminase mediates deamidation of glutamine to glutamic acid within gliadin to create immunogenic CD4 T-cell epitopes (7–10). In rheumatoid arthritis, citrullinated peptides form epitopes from cartilage proteins that both elicit antibody responses and activate CD4 T cells (11). Similarly, a novel class of epitopes within T1D are hybrid insulin peptides (HIPs) that are formed within lysozymes of β-cells through a covalent bond between an insulin peptide fragment and another β-cell peptide, thereby generating a neo-epitope (12, 13).

Recent studies provide strong evidence for the role of HIPs in the development of diabetes in the nonobese diabetic (NOD) mouse model of spontaneous autoimmune diabetes (12, 14–16). Notably, the antigen for the well-studied "diabetogenic" BDC2.5 T-cell clone and transgenic mouse model is a HIP formed between a peptide fusion of C-peptide and a cleavage product of chromogranin A, termed WE14 (12, 17, 18). C-peptide is cleaved from the A and B chains of insulin prior to secretion from the β-cell. In the NOD mouse, HIP-reactive CD4 T cells have a proinflammatory phenotype, can be detected prior to the onset of diabetes, and their frequency increases as the disease progresses (15). Another CD4 T-cell epitope critical for NOD diabetes development is a fragment of the insulin B chain, consisting of amino acids 9 to 23 (B:9–23) (19–21). A strongly stimulating T-cell epitope is very likely a HIP consisting of a fragment of this insulin B-chain peptide with a portion of C-peptide fused to the C-terminal end (22). HIP-reactive CD4 T cells have also been studied in the context of human T1D, with multiple CD4 T-cell clones and lines grown from the residual pancreatic islets of T1D organ donors subsequently responding to these neo-epitopes (12, 23, 24). A number of HIP-reactive T cells have also been measured from the peripheral blood in newly diagnosed T1D patients (25–30); however, the timing of when these T cells appear in the disease course and whether these responses directed at PTM peptides precede those toward native antigens remains to be addressed. We hypothesized that HIP T-cell responses precede clinical diabetes development and are more robust than responses to native insulin peptides.

In this study, we longitudinally collected peripheral blood mononuclear cells (PBMCs) from genetically at-risk individuals and measured reactivity to a panel of HIPs and native antigens using sensitive enzyme-linked immunospot (ELISPOT) assays, which have previously been used to identify CD4 T-cell responses in

Significance

Immune responses to modified self-proteins are involved in the pathogenesis of a number of autoimmune diseases. Type 1 diabetes (T1D) is a prototypical organ-specific autoimmune disease that results from the T-cell–mediated destruction of insulin-producing β-cells within pancreatic islets. The stages of T1D development allow for the measurement of immune responses prior to clinical symptom onset. In our study, we measured T-cell responses from genetically at-risk individuals to both naturally occurring insulin and hybrid insulin peptides, novel neo-epitopes implicated in T1D pathogenesis. Our results have important implications to stratify the risk of developing T1D and identifying individuals who may benefit from immune intervention studies.

Author contributions: A.K.S., M.N., L.Y., P.A.G., M.J.R., and A.W.M. designed research; A.M.M., A.A.A., K.A.M., and K.W. performed research; A.M.M., L.P., and A.W.M. analyzed data; and A.M.M., A.K.S., M.N., L.Y., P.A.G., M.J.R., and A.W.M. wrote the paper.

Competing interest statement: P.A.G. and A.W.M. are inventors on a patent, "Insulin Mimotopes and Methods of Using the Same" (US patent number 10,363,288).

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T1D (31). We show that PBMCs respond to native insulin peptides, but the cells respond more robustly to specific HIPs, including the insulin B chain B:9–23 HIP (B22E) and two C-peptide–derived HIPs (C-peptide/islet amyloid polypeptide-2 [C:IAPP-2] and C:A chain). We demonstrate that T-cell responses fluctuate between pro- and antiinflammatory during the preclinical phase prior to T1D development. Interestingly, individuals who progressed to clinical disease or who seroconverted to islet autoantibody positivity during the course of the study had a distinct polarization toward proinflammatory responses to specific HIPs. Remarkably, the T-cell response to the C:IAPP-2 HIP correlated with worsening measures of blood glucose. Overall, the data support a pathogenic role for PTM epitopes in the preclinical stage of T1D, and the fluctuating nature of the T-cell responses has implications for timing therapies to prevent T1D and potentially other autoimmune disorders.

Results

Individuals Genetically At Risk for T1D. We collected PBMC samples every 6 mo for 2 y from islet autoantibody-positive (Aab^+; $n = 25$) and Aab^- ($n = 38$) participants enrolled in the Diabetes AutoImmunity Study in the Young (DAISY). DAISY is an established prospective birth cohort study following genetically at-risk children for the development of islet Aabs and clinical T1D (32). Both groups were well-matched in terms of age, sex, race, and duration of follow-up, with a median of four visits per participant (Table 1). As expected, the vast majority of individuals carried one or two T1D human leukocyte antigen (HLA) class II risk alleles (i.e., DQ8 and/or DQ2), with no significant differences between the two groups. Testing was performed for all four islet Aabs (those directed against insulin, glutamic acid decarboxylase (GAD), insulinoma antigen-2 [IA-2], and zinc transporter 8 [ZnT8]) at each visit (Datasets S1 and S2). Additionally, the Aab^+ participants and those that developed Aabs during the study had measures of average blood glucose over the preceding 3 mo (hemoglobin A1C) at most visits, and oral glucose tolerance tests were performed in a subset of Aab^+ individuals (Dataset S1).

Table 1. Demographic and immunologic characteristics of study participants

	Aab^+ ($n = 25$)	Aab^- ($n = 38$)	P value*
Age, y			
Mean (SD)	18.1 (3.8)	18.7 (3.6)	0.51
Median	17.4	18.7	
Range	12.1–25.6	14.2–24.2	
Gender			
Female, % (No.)	56 (14)	53 (20)	1.0
Race, % (No.)			0.27
White	100 (25)	92 (35)	
African American	0 (0)	8 (3)	
Other	0 (0)	0 (0)	
Ethnicity, % (No.)			0.75
Hispanic or Latino	24 (6)	18 (7)	
Not Hispanic or Latino	76 (19)	82 (31)	
Duration of follow-up, mo			
Mean (SD)	19.5 (6.2)	21.9 (4.1)	0.08
Median	20.0	23.4	
Range	6.7–32.2	11.9–27.6	
No. of visits			
Median	4.0	4.0	0.91
T1D Aabs at enrollment, % (No.)			
1	48 (12)	NA	
2	32 (8)	NA	
3	20 (5)	NA	
HLA-DQ genotype, % (No.)			0.50
DQ8/X†	52 (13)	39 (15)	0.44
DQ2/X‡	16 (4)	11 (4)	0.70
DQ2/8	28 (7)	37 (14)	0.59
DQX/X	4 (1)	13 (5)	0.39

NA, not applicable.
*P values for continuous variables use t test, categorical variables use Fisher's exact test.
†DQ8 consists of the alleles DQA1*03:01-DQB1*03:02.
‡DQ2 consists of the alleles DQA1*05:01-DQB1*02:01 or DQA1*02:01-DQB1*02:02.

Robust Cytokine Responses to HIPs. We tested a panel of epitopes, including both HIPs and the corresponding native insulin peptides, that are known to activate CD4 T cells derived from the residual pancreatic islets of T1D organ donors, using cytokine ELISPOT assays (Fig. 1*A*). Regardless of Aab status, T cells from at-risk individuals responded to HIPs via production of interferon-γ (IFN-γ) and interleukin-10 (IL-10), prototypical proinflammatory and antiinflammatory cytokines, respectively (Fig. 1*B* and *SI Appendix*, Fig. S1*A*). The HIPs eliciting the most robust responses relative to no antigen stimulation were C-peptide/insulin A chain (C:A chain), insulin B chain B:9–23 (B22E), and C:IAPP-2 (*SI Appendix*, Fig. S1 *B* and *C*). As expected, native peptides (C-peptide 10–24 [C:10–24] and B:9–23) did induce cytokine responses; however, the magnitudes of those responses were lower compared to HIPs (Fig. 1 *C* and *D*).

To assess whether the cells responded in a pro- or antiinflammatory manner to the peptides, the IFN-γ/IL-10 ratio was determined for both Aab^+ and Aab^- individuals. A ratio above 1.0 is indicative of a proinflammatory response, while less than 1.0 is antiinflammatory. In general, PBMCs from the Aab^+ group responded to the HIPs in a proinflammatory manner, whereas the response was primarily antiinflammatory in PBMCs from Aab^- individuals with median IFN-γ/IL-10 ratios <1.0 for six of the seven tested HIPs and native peptides (Table 2 and *SI Appendix*, Fig. S2). The IFN-γ/IL-10 ratio to the native insulin B:9–23 peptide was predominantly <1.0 and lower in the Aab^- group compared to Aab^+, indicating those without Aabs have a more antiinflammatory response to this peptide ($P = 0.008$) (*SI Appendix*, Fig. S2). In addition to measuring cytokine responses by ELISPOT, we also assessed cytokine secretion into the culture supernatant via ELISA assays in a subgroup of participants. The trends from ELISA measurements were similar to the ELISPOT results; however, ELISPOT was more sensitive for detecting low-level responses, as many of the ELISA measurements for both IFN-γ and IL-10 were below the lower limits of detection (*SI Appendix*, Fig. S3). Taken together, these results indicate that HIP-reactive T-cell responses are present in those at-risk of T1D, and the magnitude of response is greater for HIPs comprised of C-peptide with IAPP, C-peptide with insulin A chain, and the B:9–23 (B22E) epitope compared to the corresponding native insulin peptides.

Temporal Changes in IFN-γ/IL-10 Ratios to HIPs. While it is important to understand the generalized responses of PBMCs to HIPs in T1D at-risk individuals, it is well-established that symptoms related to rheumatologic and neurologic autoimmune diseases can wax and wane over time (33, 34). Therefore, we sought to determine whether HIP responses varied when measured in a given individual over time. Although the IFN-γ and IL-10 responses to HIPs did fluctuate slightly over time, the IFN-γ/IL-10 ratio varied dramatically, often switching from a proinflammatory to an antiinflammatory response between two consecutive 6-mo visits (Fig. 2 *A* and *B*). This phenomenon was true for native peptides as well (B:9–23 and C:10–24), although these peptides tended to elicit more of an antiinflammatory response (Fig. 2 *C* and *D*). As expected, responses to Pediarix (childhood vaccine containing five immunogens) were predominantly proinflammatory and

A

Peptide	Abbreviation	Sequence
Insulin B chain B:9-23 (native)	B:9-23	SHLVEALYLVCGE-RG
Insulin B chain B:9-23	B22E	SHLVEALYLVCGE-EG
Proinsulin C-peptide C:10-24 (native)	C:10-24	GQVELGGG-PGAGSLQ
C-peptide/islet amyloid polypeptide-2	C:IAPP-2	GQVELGGG-NAVEVLK
C-peptide/neuropeptide Y	C:NPY	GQVELGGG-SSPETLI
C-peptide/islet amyloid polypeptide-1	C:IAPP-1	GQVELGGG-TPIESHQ
C-peptide/insulin A chain	C:A chain	GQVELGGG-GIVEQCC

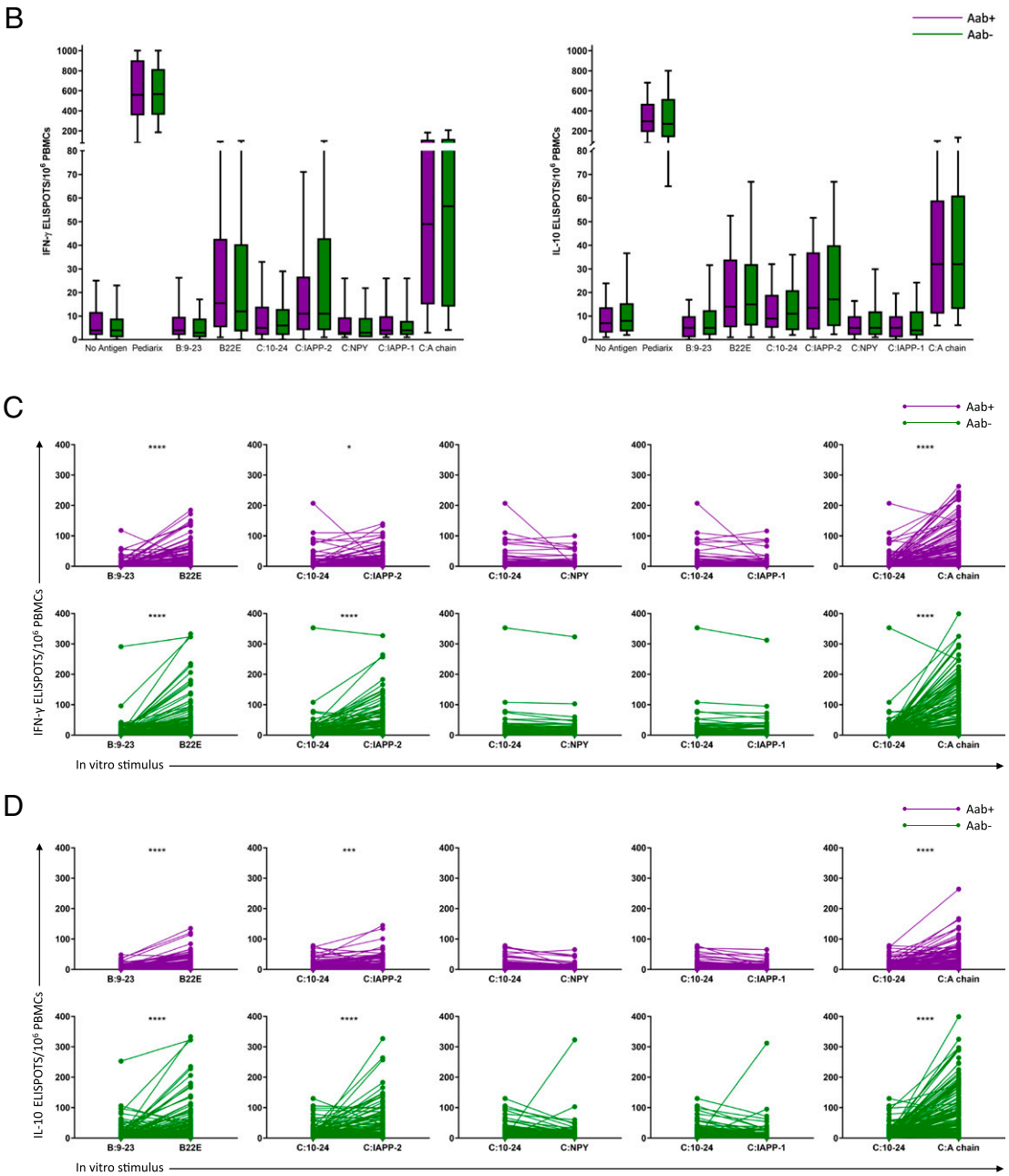

Fig. 1. Individuals at risk of T1D have robust cytokine responses to HIPs relative to native peptides. (*A*) Amino acid sequences for five HIPs and two native peptides are shown, with the fusion peptides depicted by left-side/C-terminal amino acid sequences from insulin B chain or C-peptide indicated in black and right-side/N-terminal amino acid sequences from C-peptide, IAPP, NPY, or insulin A chain indicated in red. (*B*) In each case, freshly isolated PBMCs were cultured in the presence or absence of the peptide for 48 h, washed, and then cells transferred to an IFN-γ or IL-10 monoclonal antibody-coated plate for overnight culture followed by development and enumeration of ELISPOTs. Depicted are IFN-γ and IL-10 production in response to the tested peptides from islet Aab$^+$ (purple) and Aab$^-$ (green) participants from all visits. Median responses are denoted by black lines within boxes, and 25% and 75% intervals are denoted by whiskers. Values indicate the total number of ELISPOTs per 10^6 PBMCs for each cytokine. A control response to Pediarix (childhood vaccine containing five immunogens) was performed in each assay. Graphs comparing (*C*) IFN-γ and (*D*) IL-10 ELISPOT responses between native and HIP peptides for Aab$^+$ (purple) and Aab$^-$ (green), with lines connecting paired samples from an individual. *$P < 0.05$, ***$P < 0.001$, and ****$P < 0.0001$ using a two-tailed paired *t* test.

Table 2. Median cytokine ELISPOT values for each tested antigen

	IFN-γ		IL-10		IFN-γ/IL-10	
Antigen	Aab$^+$	Aab$^-$	Aab$^+$	Aab$^-$	Aab$^+$	Aab$^-$
None	4	4	7	8	—	—
Pediarix	560	567	296	270	1.8	2.0
B:9–23	4	3	5	5	1.0	0.5
B22E	15.5	12	14	15	1.1	0.8
C:NPY	3	3	5	5	1.0	0.8
C:IAPP-1	4	4	5	4	1.1	0.8
C:IAPP-2	11	11	13.5	17	0.8	0.8
C:A chain	49	56.5	32	32	1.4	1.6
C:10–24	5	6	9	11	0.6	0.5

$n = 100$ assays from 25 Aab$^+$ participants and $n = 153$ assays from 38 Aab$^-$ participants.

remained as such over time (Fig. 2 *C* and *D*). In contrast to T-cell responses, islet Aabs did not fluctuate as dramatically over the same time period (*SI Appendix*, Fig. S4). Our results demonstrate a relapsing and remitting nature of T-cell responses to HIPs during the preclinical T1D period.

Distinct Responses in Those Progressing to Clinical Disease. The presence of multiple islet Aabs confers a higher risk of developing clinical T1D compared to a single Aab (35), and this was observed in our study with 4 of 13 (31%) multiple Aab$^+$ individuals developing clinical T1D compared to 1 of 12 (8%) with a single Aab. We compared immune responses to native peptides and HIPs between these two subsets of individuals based upon time from Aab seroconversion (Fig. 3 *A*–*C*). Higher IFN-γ/IL-10 ratios correlated to time from Aab seroconversion for both insulin B:9–23 and the B22E HIP in the multiple Aab$^+$ group (Fig. 3*C*), and this was statistically significant when compared to the single Aab$^+$ individuals ($P = 0.007$ for B:9–23 and $P = 0.022$ for B22E HIP). Interestingly, the slope of response was similar between native B:9–23 and the B22E HIP (Fig. 3*C*), indicating that T-cell responses to both peptides progress following the development of islet Aabs. Correlations to higher IFN-γ/IL-10 ratios from the time of seroconversion were not apparent for the other tested peptides.

One individual with a single islet Aab directed against GAD converted to multiple Aabs at a single study visit (Aab$^+$ #12 in Dataset S1, one to three Aabs). This individual had increased IFN-γ ELISPOTs to the tested HIPs and native B:9–23, but not to native C:10–24 compared to the preceding study visits, along with decreased IL-10 ELISPOTs. The responses to HIPs were more robust compared to the native peptides after the individual developed additional Aabs. This finding indicates a role for proinflammatory T-cell responses at the time of developing additional Aabs directed to insulin and zinc transporter 8 in this individual.

Next, we examined the five Aab$^+$ participants that developed new-onset clinical T1D, requiring treatment with exogenous insulin. This rate of T1D development (~10% per year) is in line with other large prospective studies following at-risk individuals with multiple Aabs (35). Additionally, three Aab$^-$ individuals developed an islet Aab during the study (i.e., islet autoimmunity). Given that these eight individuals progressed along the continuum of T1D development, we examined their immune responses more closely, with all responses from the five Aab$^+$ individuals being measured prior to T1D development (Fig. 3*D*). In those Aab$^+$ individuals that progressed to clinical T1D, the C:IAPP-2 HIP more frequently induced a proinflammatory response compared to those that did not progress to T1D ($P = 0.037$) (Fig. 3*D*). Notably, participants who progressed to T1D did not seroconvert to Aab positivity more recently than those who did not develop T1D and were of a similar age to those not progressing to T1D (*SI Appendix*, Fig. S5). When examining the participants that developed islet autoimmunity, the C:IAPP-2, C:NPY (neuropeptide Y), and C:A chain HIPs induced more frequent proinflammatory T-cell responses compared to those that did not develop Aabs, with a trend toward statistical significance (Fig. 3*E*).

Taken together, these results provide evidence that specific HIP T-cell responses may be involved in both the transition to islet autoimmunity and driving progression to clinical disease after islet Aabs develop.

Specific HIP Responses Correlate with Insulin Autoantibodies and Measures of Glucose Metabolism. As all of our tested HIPs contained at least one fragment of insulin, and CD4 T cells undergo cognate interactions with B cells that can lead to antibody production, we analyzed whether there was a correlation between T-cell responses and insulin autoantibody (IAA) levels. There was a statistically significant correlation between B22E HIP IFN-γ/IL-10 responses and IAA levels ($P = 0.0001$), whereas no correlations were observed between IAA levels and responses to other HIPs or native peptides (Fig. 4*A*).

Next, we determined whether HIP responses correlated with measures of glucose metabolism. A 2-h oral glucose tolerance test (OGTT), which measures blood glucose levels following a glucose challenge, was performed at multiple visits in 13 Aab$^+$ participants. When comparing the glucose area under the curve (AUC) to IFN-γ/IL-10 ratios, the C:IAPP-2 HIP response trended toward significance ($P = 0.054$), indicating that a more inflammatory response corresponds to higher blood glucose levels (Fig. 4*B*). Hemoglobin A1C measurements reflect the average blood glucose over the preceding 3 mo and were performed more frequently than OGTTs. A1C values correlated with C:IAPP-2 HIP IFN-γ/IL-10 ratios ($P = 0.046$), with a trend toward significance for the native insulin B:9–23 response ($P = 0.060$) (Fig. 4*C*). Overall, our data demonstrating that Aab$^+$ participants have more inflammatory responses to specific HIPs and that these responses correlate with worsening measures of blood glucose implicate a link between HIP-specific T-cell responses and progression to clinical T1D onset.

Discussion

An altered balance between effector T-cell (proinflammatory) and regulatory T-cell (antiinflammatory) responses has been implicated in multiple autoimmune diseases (36–39), and our study provides evidence that this imbalance occurs prior to clinical T1D onset. Previous studies demonstrated the presence of insulin-specific T cells in both T1D patients and HLA-matched nondiabetic individuals, but the autoreactive T cells from patients were polarized toward a proinflammatory phenotype, while those from controls were biased toward antiinflammatory (26, 31). Similarly, we previously demonstrated that PBMCs from new-onset T1D patients respond to the B22E HIP via robust IFN-γ production, while nondiabetic controls produce a predominant IL-10 response, especially in those with T1D-protective HLA-DQ alleles (25). In our present study, the individuals at risk of T1D had peripheral blood immune responses to three specific HIPs that were more robust than the corresponding native proinsulin peptides, as measured by IFN-γ/IL-10 ratios, and these responses tended to fluctuate between pro- and antiinflammatory when examined at 6-mo intervals over 2 y. These data provide a basis for relapsing and remitting T-cell autoimmunity during the preclinical T1D period, which is not observed in levels of islet Aabs.

Our data also reveal that genetically predisposed individuals have T-cell responses to HIPs independent of islet Aab status, indicating that their presence may not be sufficient to cause

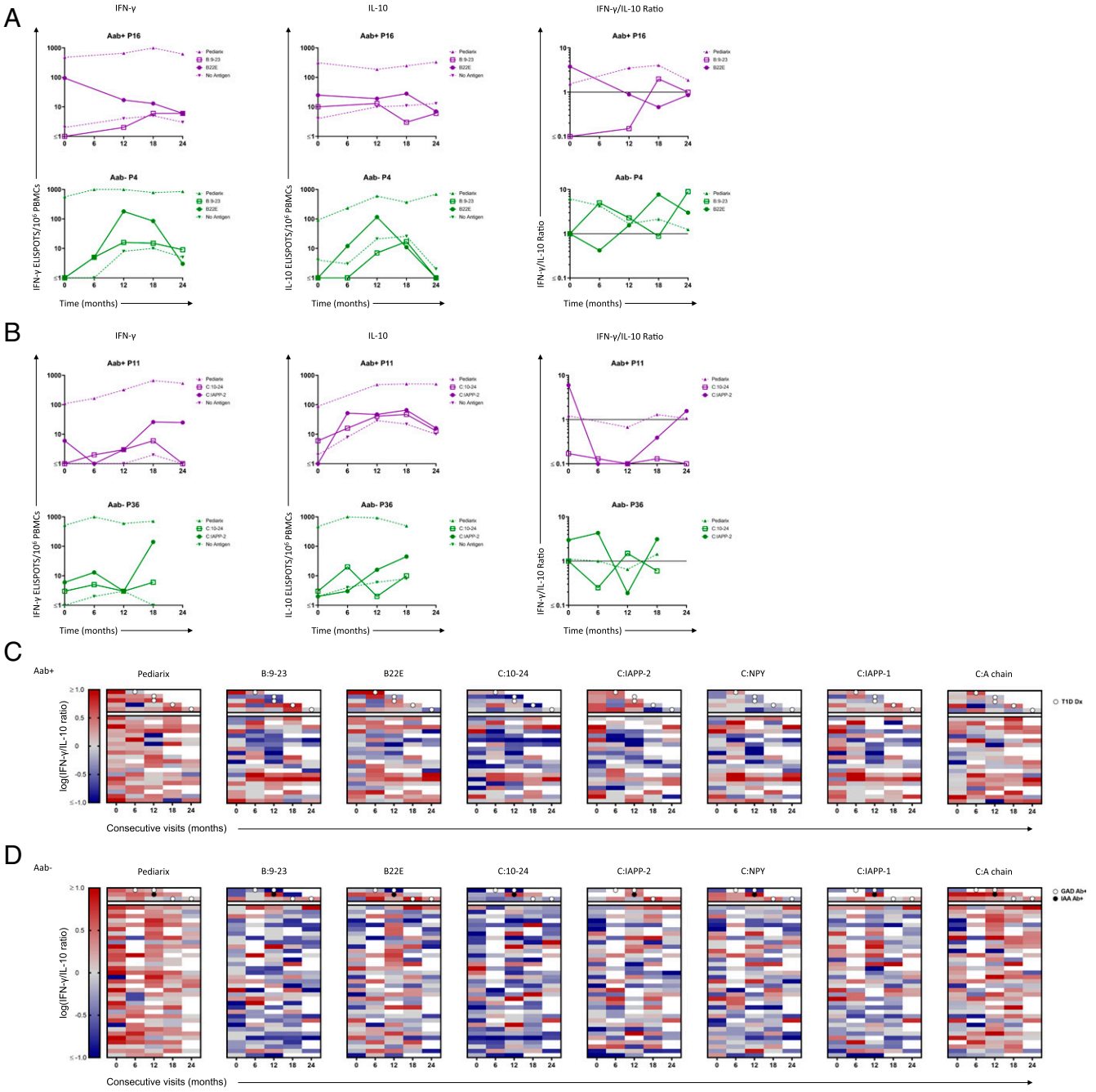

Fig. 2. Temporal changes in IFN-γ/IL-10 ratios to HIPs in individuals at risk of T1D. ELISPOT responses over time from a single individual displaying the IFN-γ (*Left*) and IL-10 (*Center*) ELISPOT responses with IFN-γ/IL-10 ratios (*Right*) at each consecutive visit in response to Pediarix, no antigen, and (*A*) native insulin B:9–23 and the B22E HIP or (*B*) native C:10–24 and the C:IAPP-2 HIP. Heat maps depicting IFN-γ/IL-10 ratios at each 6-mo visit from all (*C*) Aab$^+$ ($n = 25$) and (*D*) Aab$^-$ ($n = 38$) individuals in response to a given antigen stimulus. Red coloring indicates a predominantly inflammatory response, and blue coloring indicates a predominantly antiinflammatory response. White coloring indicates no data or there was not a visit at that time point. The visits at or just prior to clinical T1D onset for five Aab$^+$ individuals are marked with open white circles in (*C*), and visits for islet Aab seroconversion for three Aab$^-$ individuals are labeled with open white dots (GAD Aabs) or closed black dots (IAA) in *D*.

disease. Notably, the IFN-γ/IL-10 ratio to native insulin B:9–23 was statistically different between those with and without Aabs, showing a more antiinflammatory response in those without islet Aabs. When comparing individuals with single versus multiple Aabs, higher IFN-γ/IL-10 ratios correlated to time from Aab seroconversion for insulin B:9–23 and the B22E HIP in the multiple Aab group. We also detected a distinct polarization toward higher IFN-γ responses compared to IL-10 among individuals who progressed to clinical T1D or seroconverted to initial islet Aab positivity, which suggests that active T-cell autoimmunity directed toward HIPs occurs during these critical time points in disease progression. However, our data regarding islet Aab seroconversion should be interpreted with caution, as the three individuals developed only a single Aab, and the presence of multiple Aabs confers a higher risk of clinical T1D development (35).

In addition to an imbalance between pro- and antiinflammatory responses to self-antigens, PTM of autoantigens is a potential mechanism that leads to a loss of tolerance in a number

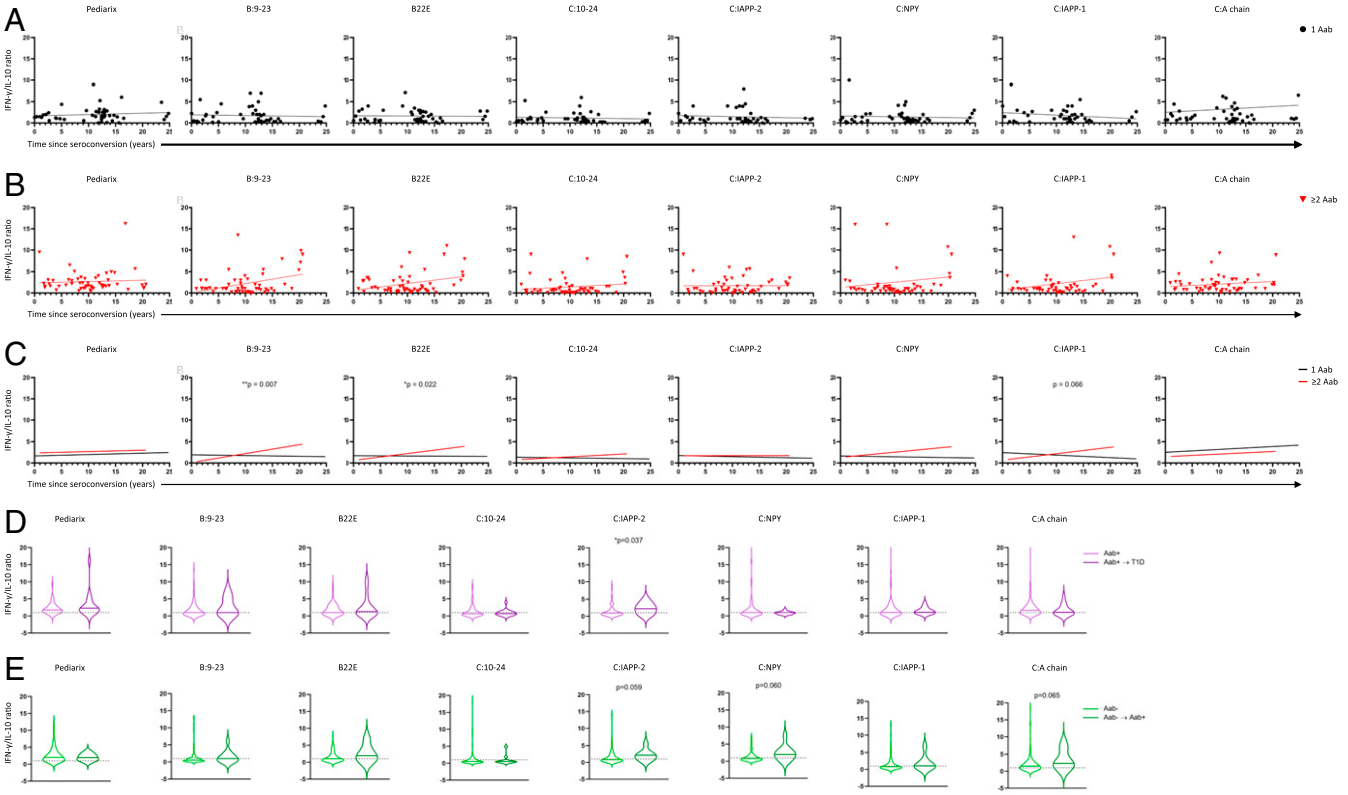

Fig. 3. Individuals progressing to clinical disease show distinct inflammatory responses to specific HIPs. IFN-γ/IL-10 ratios from ELISPOT assays are plotted against the number of years since seroconversion for Aab$^+$ individuals. Graphs with linear regression lines showing all visits from (*A*) individuals with one Aab at the initial visit (n = 12) and (*B*) two or more Aabs (n = 13) displayed. (*C*) The linear regression lines from *A* and *B* are overlaid for each stimulus. *P* values depicted using linear regression analyses. Due to very elevated IFN-γ/IL-10 ratio, one point is not displayed on each of the following graphs: C:A chain in *A* (12.1 y, ratio 64), C:NPY in *B* (13.2 y, ratio 26), and C:IAPP-1 in *B* (8.6 y, ratio 21); however, these values were included to determine linear regression lines and statistical comparisons. (*D*) Graphs depicting IFN-γ/IL-10 ratios for all study visits from Aab$^+$ (purple) and (*E*) Aab$^-$ (green) individuals. Darker colored lines represent responses from Aab$^+$ individuals who progressed to clinical T1D (n = 5) and Aab$^-$ individuals who developed an islet Aab (n = 3), while lighter colored lines display responses from those not progressing to T1D (n = 20) or developing an islet Aab (n = 35). Dotted black lines are at 1.0, with values greater than 1.0 indicating a predominantly inflammatory response, and values less than 1.0 an antiinflammatory response. *P* values depicted using a Mann–Whitney *U* test.

of autoimmune diseases, including citrullinated peptides in rheumatoid arthritis (11), deamidated peptides in celiac disease (7–10), and HIPs in T1D (12, 13, 28, 29). Within T1D, a number of studies have identified other islet antigen modifications leading to the generation of CD4 T-cell epitopes, including deamidation of GAD, proinsulin, and IA-2, along with citrullination of GAD (40–43). Additionally, CD8 T cells have been found to respond to peptides derived from an out-of-frame alternate start codon within the proinsulin gene (44), and fusion peptides within IAPP are also recognized by islet-derived CD8 T cells (45). Both CD4 and CD8 T-cell responses to these translational and posttranslationally modified islet antigens warrant future studies in those progressing along the continuum of T1D development.

In the NOD mouse model of autoimmune diabetes, CD4 T cells enumerated with fluorescent HIP/MHC tetramers increase in frequency within the peripheral blood as the disease progresses (15), and our data now provide a basis for HIP-reactive T-cell responses prior to disease onset in human T1D. We demonstrate that responses can be detected prior to disease onset in genetically at-risk T1D individuals. Our finding that robust inflammatory T-cell responses to several HIPs were detected in Aab$^+$ individuals who subsequently developed T1D implies that these responses may contribute to disease progression. Furthermore, we observed that worsening glucose measurements correlated with reactivity to the C:IAPP-2 HIP, indicating that immune responses to PTM antigens might reflect changes in β-cell function during T1D pathogenesis.

Due to the potential pathogenic role of inflammatory HIP-reactive T cells prior to T1D onset, targeting these cells with immune therapy may delay or prevent disease onset. Recently, a randomized placebo-controlled trial using T-cell–targeted immunotherapy with an anti-CD3 monoclonal antibody delayed diabetes onset by an average of 2 y in individuals at risk of T1D that had both islet Aabs and impaired glucose metabolism (46). This clinically meaningful result indicates that immune intervention prior to disease onset can alter T1D progression. Our findings demonstrate that HIP-specific inflammatory T-cell responses are present in at-risk individuals at a similar point in disease progression as to when anti-CD3 therapy was administered. Monitoring T-cell responses to PTM antigens prior to T1D onset holds promise for determining which individuals are likely to progress to clinical disease and may benefit from immune intervention to prevent T1D onset.

Materials and Methods

Study Subjects. Subjects were recruited from DAISY, which is an ongoing prospective birth cohort study following genetically at-risk individuals for development of islet Aabs and clinical T1D (32). Written informed consent was obtained from each participant and guardian when the participant was less than 18 y of age, and the Colorado Multiple Institutional Review Board approved the study. Study visits occurred every 6 to 12 mo to collect peripheral blood for islet Aab measurements and ELISPOT assays. Additionally, glucose measurements were performed in the Aab$^+$ cohort.

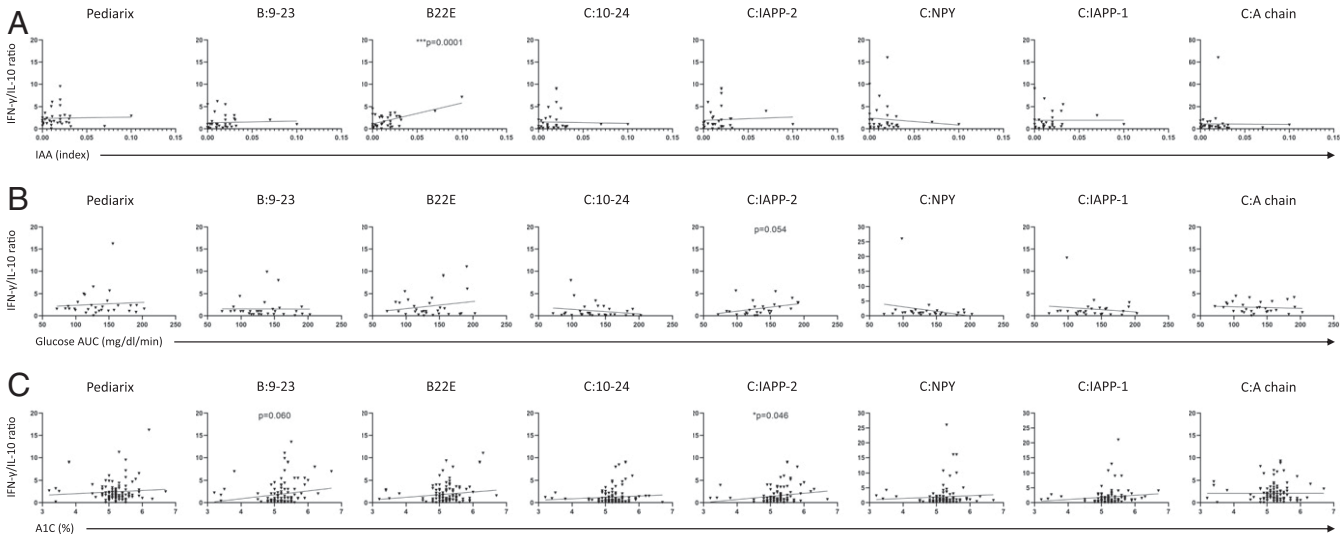

Fig. 4. IAA levels and measures of glucose metabolism correlate with responses to HIPs during progression to T1D. (*A*) IFN-γ/IL-10 ratios from PBMC ELISPOT assays using native insulin B:9–23, B22E HIP, native C:10–24, and the C:IAPP-2 HIP are plotted against IAA levels in individuals having a positive IAA value from at least one visit ($n = 9$). (*B*) Glucose AUC measurements following a 2-h OGTT are plotted versus the corresponding IFN-γ/IL-10 ratios at matched visits for Aab^+ participants ($n = 13$). (*C*) Hemoglobin A1C levels, which is a measure of average blood glucose over the preceding 3 mo, are graphed versus the corresponding IFN-γ/IL-10 ratios at matched visits for all participants that had A1C measurements ($n = 30$). *P* values depicted using linear regression analysis.

Islet Aab Measurements and HLA Genotyping. Serum obtained from peripheral blood was used to measure islet Aabs to insulin, GAD65, IA-2, and ZnT8 by fluid-phase radio-binding assays, as previously described (47, 48). Previously collected and stored DNA samples were used to type HLA-DRB1, DQA1, and DQB1 alleles using oligonucleotide probes, as previously described (49).

Antigens. The native and hybrid insulin peptides listed in Fig. 1*A* were obtained from Genemed Synthesis at >95% purity. Pediarix (GlaxoSmithKline), a childhood vaccine containing five different immunogens, was used as a positive control for each cytokine ELISPOT assay.

Cytokine ELISPOT Assays. PBMCs were isolated from heparinized blood using Ficoll-Paque Plus (GE Health Sciences). Freshly isolated PBMCs were used to perform IFN-γ and IL-10 ELISPOT assays (UCyTech Biosciences), as previously described (50). Briefly, PBMCs (1×10^6) were cultured in 250 μL of serum-free AIM-V Medium (Invitrogen) in a 48-well polystyrene culture plate with 10 μg/mL of peptide dissolved in phosphate buffered saline (PBS) at 37 °C with 5% CO_2. Cells were harvested at 48 h and washed. The cells were then resuspended in 300 μL medium and transferred as three 100-μL aliquots into 96-well clear polystyrene culture plates coated with a cytokine capture monoclonal antibody (separate for IFN-γ and IL-10). Seventeen hours later, the cells were removed by decanting, and the wells were washed three times with PBS containing 0.05% Tween-20. Spots were then developed by sequential incubations with the biotinylated second site anti–IFN-γ or anti-IL-10 antibody, gold-labeled goat anti-biotin antibody, and a precipitating silver substrate. Spots were enumerated with a Bioreader 4000 Pro X (BIOSYS). For each assay, negative control wells received no antigen, and positive control wells received the Pediarix vaccine (1 μL). IFN-γ/IL-10 ratios were calculated using the total number of ELISPOTs per 10^6 PBMCs for each cytokine.

To assess the reproducibility of the assay, fresh blood samples from three individuals were split into two samples. Each sample had PBMCs separately isolated and used in an IFN-γ and IL-10 ELISPOT assay. The variance was calculated between each condition in assays 1 and 2 for each patient that was above background (no antigen). The average variance for IFN-γ was 11%, and IL-10 was 16% in these paired assays.

Cytokine ELISAs. After 48 h of culturing PBMCs with or without antigen for the ELISPOT assays, the plate was centrifuged at 500 × *g* for 5 min, and the supernatant was removed and frozen at −80 °C until IFN-γ and IL-10 were measured using a highly sensitive ELISA (V-PLEX, Meso Scale Diagnostics).

Glucose Measurements. An OGTT (1.75 g/kg body weight of glucose with a maximum of 75 g) was administered, and whole blood was collected at time 0, 15, 30, 60, 90, and 120 min for glucose measurements using a Glucose 201 DM Analyzer (HemoCue). A 2-h glucose AUC was then calculated. Glycated hemoglobin A1C was measured using a DCA2000 Analyzer (Siemens Healthcare Diagnostics).

Statistical Analyses. Statistical analyses were performed using SAS 9.4 (SAS Institute) and GraphPad Prism 8.4 software (GraphPad Software). The statistical tests used for each experiment are indicated in the corresponding table or figure legend. $P < 0.05$ was considered significant. Continuous variables (age and duration of follow up) were compared using *t* tests, and a Fisher's exact test was used for categorical variables (sex, race, ethnicity, and HLA-DQ genotype). To account for repeat measurements within participants over time, linear mixed models with a random intercept (to account for correlation of measures within an individual) were used to test the relationship between different groups (Aab^+ and Aab^-). Paired *t* tests and Mann–Whitney *U* tests were used to compare ELISPOT responses between peptides, while linear regression analyses were used to compare ELISPOT responses following time from islet Aab seroconversion, to IAA levels, and glucose measurements.

Data Availability. All study data are included in the article and/or supporting information.

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