Immunoproteomic Profiling of Antiviral Antibodies in New-Onset Type 1 Diabetes Using Protein Arrays

The rapid rise in the incidence of type 1 diabetes (T1D) suggests the involvement of environmental factors including viral infections. We evaluated the association between viral infections and T1D by profiling antiviral antibodies using a high-throughput immunoproteomics approach in patients with new-onset T1D. We constructed a viral protein array comprising the complete proteomes of seven viruses associated with T1D and open reading frames from other common viruses. Antibody responses to 646 viral antigens were assessed in 42 patients with T1D and 42 age- and sex-matched healthy control subjects (mean age 12.7 years, 50% males). Prevalence of antiviral antibodies agreed with known infection rates for the corresponding virus based on epidemiological studies. Antibody responses to Epstein-Barr virus (EBV) were significantly higher in case than control subjects (odds ratio 6.6; 95% CI 2.0–25.7), whereas the other viruses showed no differences. The EBV and T1D association was significant in both sex and age subgroups (≤12 and >12 years), and there was a trend toward early EBV infections among the case subjects. These results suggest a potential role for EBV in T1D development. We believe our innovative immunoproteomics platform is useful for understanding the role of viral infections in T1D and other disorders where associations between viral infection and disease are unclear.

Type 1 diabetes (T1D) is a chronic heterogeneous disease characterized by the progressive autoimmune destruction of pancreatic β-cells. The incidence of T1D is rising by an average of 3–5% in recent years, which cannot be fully explained by genetic predisposition alone (1). Moreover, the concordance rate for developing T1D among monozygotic twins is ~66%, lower than that for type 2 diabetes (2). Hence, it is likely that environmental factors play a significant role during T1D development (3). Among various environmental factors considered relevant to T1D are those of nutrition and psychosocial factors; yet, viral infections have attracted particular interest (4,5).

Although there are a number of studies indicating viral effects on T1D pathogenesis, the exact mechanistic explanations for how viruses contribute to T1D etiology are still unknown. Viral infection or presence may act as a longitudinal factor during the induction of a single islet antibody, the simulation from a single islet antibody to multiple islet antibodies, or the progression from β-cell autoimmunity to clinical onset of T1D (6). Several studies reported that both the initial development of autoantibodies (AAbs) and the progression to multiple AAbs occurred at an early age. Subsequently, individuals progress to clinical T1D at different paces during which viral infections may act as an accelerator (7,8). For example, enterovirus infection was shown to increase progression to clinical onset in the Diabetes and Autoimmunity Study in the Young (DAISY) study (9). As the complex role of viral infections in T1D remains elusive, it would be valuable to address this important scientific question by assessing immune responses to many viruses and their antigens using many samples collected longitudinally from birth to disease onset.

Many viruses have been implicated in T1D in both animal models and humans with varying levels of evidence.
Historically, the prevalence of viral infections in T1D was explored either by genomic approaches (which work if the viral nucleic acids remain present at the time of assay) or immunological approaches that only evaluated one viral protein or one type of virus at a time (10,11). Viral DNA or mRNA were detected by PCR or in situ hybridization in a relatively low-throughput manner (12,13). At the protein level, immunohistochemical staining and electron microscopy have been used to stain and observe viral proteins (14,15). Both in situ hybridization and immunohistochemical require the use of pancreatic sections from rare pancreatic tissue followed by tedious sample processing procedures. Many serological studies investigated the presence of antibodies to viruses. M-antibody ELISA has been a classic way to profile immunoglobulin (Ig)M antibodies in T1D patients (11). The plaque assay, which measures the presence of neutralization antibodies against the whole virus, is another method to profile serological antibodies to specific viral serotypes (16,17). The complement fixation test uses complement activation and the lysis of red blood cells to indicate the presence of certain viruses (10). Recent advances in next-generation sequencing technology have opened new venues for studying the role of viral infection in T1D development (18).

Despite these efforts, we still do not have a clear understanding of the association between viral infections and T1D development. A lack of quantitative and high-throughput technologies has limited the ability to study the role of viral infections in this disease comprehensively. Conflicting reports have stemmed from observations based on limited sample sizes (4). Previous studies focusing on a single viral protein or a single viral species have failed to provide a complete picture of infection history and their antibody responses at the systems level. Protein microarrays provide an ideal tool for multiplexed screening of specific antibodies in sera against thousands of different viral proteins printed on a standard microscope slide.

The aim of this study was to assess the prevalence of antiviral antibodies to 646 viral proteins from 23 T1D-related and other common viruses in patients with new-onset T1D and age- and sex-matched healthy control subjects. By examining antibody responses to hundreds of individual viral antigens at the proteome level, we hope to provide a complete picture of infection at a dimension never achieved before. Antibody-positive rates of studied viruses were determined and compared between T1D case and control subjects. Specific antibody responses on the array were validated by a confirmatory ELISA. The past onset nature of T1D samples may prevent us from drawing conclusions of whether the virus is pathological in inducing β-cell autoimmunity or accelerating clinical T1D. Nonetheless, this is the first comprehensive study of antibody response to a large number of viral species at the individual viral protein level in T1D. We believe the successful application of our platform to a large number of samples collected longitudinally before or after seroconversion will provide a better understanding of viral infection and T1D development.

**RESEARCH DESIGN AND METHODS**

**Serum Samples**

All samples were collected at clinics overseen by the University of Florida, with subjects emanating from that geographic region. They were collected with written informed consent and with the approval of institutional review boards at the University of Florida. Serum samples were obtained from patients with new-onset T1D within 3 months of diagnosis. Control samples were prepared in a fashion identical to that for the T1D samples and were selected to be matched with regard to age, sex, time of sampling, and geographic area. Control subjects were relatives of case subjects. Control samples were tested to be negative for major known T1D AAbs (islet antigen 2 [IA-2] antibody [IA-2A], GAD antibody [GADA], and zinc transport 8 antibody [ZnT8A]). Peripheral blood samples were drawn from the antecubital vein, and serum was prepared and stored as aliquots at −80°C. All individuals were free of other autoimmune diseases at the time of collection. The sample information is shown in Table 1.

**Selection of Viral Strains**

Based on literature mining, we identified viruses from seven different genera that have been implicated in T1D, which include enterovirus (coxsackievirus B [CVB]4), human endogenous retrovirus K (IDDMK1, 2–22 strain), rubivirus (rubella virus), rubulavirus (mumps virus [MuV]), rotavirus (rotavirus A), cytomegalovirus (HCMV), and lymphocryptovirus (Epstein-Barr virus [EBV]). These viruses encompassed a variety of genome types (ssRNA+, ssRNA−, dsRNA, and dsDNA). Selection of viral strain from each genus was based on their relevance to T1D as well as the availability of viral genome template (10,12,13,19–22). We also obtained additional viral genes from other common viruses to further enrich our collection (Table 2).

**Viral Gene Cloning**

All viral genes were first cloned into the pDONR221 Gateway compatible donor vector (Life Technologies, Carlsbad, CA). Different cloning approaches were used based on the available resources (viral genome type, gene

| Table 1—Characteristics of patients with new-onset T1D and healthy control subjects |
|-----------------------------------------------|----------------|----------------|
| Patients with new-onset T1D (n = 42)      | Healthy control subjects (n = 42)   |
| Age | 11.5 | 12 |
| Median | 11.5 | 12 |
| Range | 4–31 | 4–31 |
| Male sex, n (%)| 21 (50) | 21 (50) |
| AAb status, n (%) | GADA positive | 33 (78.6) | 0 |
| IA-2A positive | 18 (54.5) | 0 |
| ZnT8A positive | 20 (47.6) | 0 |
characteristics, etc.). Viral genes were directly amplified by PCR if the viral DNA or cDNA templates were available. Viruses of ssRNA+ genome type, such as coxsackievirus, were first reverse transcribed into cDNA before PCR amplification. Some viral genes are not continuous across the viral genome, for example, UL89 from HCMV. Each gene fragment was amplified separately and then fused together by PCR elongation to obtain full-length viral genes. Genes without available templates, such as the superantigen gene from the IDDMK1, 22 strain, were produced using de novo gene synthesis. We are also grateful for the many Gateway-compatible viral entry clones obtained from the scientific community (Fig. 1). Viral genes in the pDONR221 vector were transferred to a T7 promoter–based in vitro expression vector pANT7_cGST by LR reaction (23). All viral genes cloned in this vector have a COOH-terminal glutathione S-transferase (GST) fusion tag in frame with the protein. The sequence verified clones were stored in and are available from the plasmid repository (http://dnasu.org/DNASU/) (24).

### Nucleic Acid Programmable Protein Array Production
With use of the standard protocol (25,26), 1,200 ng/μL plasmid DNA was coprinted with rabbit anti-GST polyclonal antibody (GE Healthcare, Pittsburgh, PA), BSA, and bissulfosuccinimidyl suberate protein cross-linker (Thermo Fisher Scientific, Waltham, MA) as printing mix using Genetix QArrayer (Genetix, New Milton, U.K.). Each viral gene was printed in duplicate on each slide. Human IgG, IgA, and IgM were printed to show successful detection by secondary antibodies. Plasmid encoding a known T1D autoantigen (IA-2 protein) was printed as a positive control for T1D samples. Empty spots, spots printed with printing buffer without plasmid DNA, spots printed with printing buffer with plasmid encoding a hemagglutinin-fusion protein and anti-GST capture antibody were negative controls. Hemagglutinin-fusion protein can be expressed but not captured by anti-GST capture antibody served as a negative control for nonspecific capturing. Nucleic acid programmable protein array (NAPPA) has been successfully applied in AAb biomarker discovery and protein posttranslational modifications studies (27,28).

### Array Quality Assessment
As previously described (29,30), plasmid DNA on the array was stained by a Quant-iT PicoGreen dsDNA Assay kit (Life technologies, CA) to verify successful printing. For protein display, groups of four slides were blocked in 30 mL SuperBlock Blocking Buffer (Thermo Fisher Scientific) at room temperature (RT) for 1 h on the shaker. Then slides were rinsed five times with deionized (DI)
water and placed in a metal slide rack (Amazon, Seattle, WA) for drying by centrifugation at 1,000 rpm for 3 min at RT. A 160 μL human HeLa cell lysate–based protein expression system (Thermo Fisher Scientific) was injected into HybriWell (Grace Bio-Labs, Bend, OR) sealed slides and incubated in the oven (EchoTherm, Carlsbad, CA) at 30°C for 1.5 h for protein expression and 15°C for 30 min for protein capture. Viral proteins were produced with a COOH-terminal GST fusion tag and captured in situ by coprinted anti-GST capture antibody on the array. The use of a tag at the COOH terminus ensures that captured proteins have been fully translated. Each slide was incubated with 5% milk PBS with 0.2% Tween-20, 3 mL of 1:200 diluted anti-GST monoclonal antibody (Cell Signaling Technology, MA), and 3 mL of 1:500 diluted horseradish peroxidase–labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at RT on the shaker, respectively. Slides were washed three times with 5% milk PBST, with 5 min each time in between. Finally, slides were washed with PBST and rinsed with deionized water. Each slide was incubated with 500 μL 1:50 diluted tyramide signal amplification buffer (PerkinElmer, MA) to generate fluorescent signals. Then slides were washed, dried, and scanned by Tecan scanner (Tecan Group LTD, Männedorf, Switzerland).

**Antiviral Antibody Profiling**

Antiviral antibodies were profiled on the HS 4800 Pro hybridization station (Tecan, Männedorf, Switzerland). Proteins were expressed as previously described. Expressed slides were placed in the hybridization chambers and programmed with 1 h of blocking with 5% milk PBST and 16 h of incubation with 160 μL of 1:50 diluted serum at 4°C followed by 1 h of detection with 160 μL of 1:500 diluted Alexa Fluor 647 goat anti-human IgG or 160 μL 1:300 diluted Alexa Fluor 647 goat anti-human IgA and DyLight 549–conjugated goat anti-human IgM (Jackson ImmunoResearch Laboratories) at RT. Slides were washed, dried, and scanned by Tecan scanner. A pooled sample was prepared by mixing equal volumes of individual samples. The pooled sample was run on every serum screening day to show day-to-day technical reproducibility.

Strong antibody responses resulted in saturated signals of the local spot with diffusion to the neighboring spots.
Serum dilution was determined by the optimal sensitivity with minimum diffusion. The presence of diffusion was defined as a ring. To determine the signal intensities for the rings, we quantified the median intensity in between the ring spot and the neighboring spot as the raw ring intensity. We further quantified the median intensity in between the ring spot and the second closest spot (excluding the spot area) as the background ring intensity. The net ring intensity was calculated by subtracting the background ring intensity from the raw ring intensity. The data were only extracted for the spots with rings as ring positive net intensity data. The maximum of the ring positive net intensity for duplicates of each viral antigen was used in the quantitative analysis.

**Rapid Antigenic Protein In Situ Display ELISA**

Ninety-six-well ELISA plates (Corning Life Sciences, Salt Lake City, UT) were coated with 50 μL of 10 ng/mL anti-GST antibody (GE Healthcare) in coating buffer (0.5 mol/L carbonate bicarbonate buffer, pH 9.6) overnight at 4°C. On the next day, coated plates were washed five times with 100 μL PBST and blocked with 100 μL of 5% milk PBST for 1.5 h. Meanwhile, 40 ng/μL viral protein encoding plasmid was expressed in the human HeLa cell lysate-based protein expression system at 30°C for 1.5 h in the oven. Viral antigen was diluted in milk PBST at 1:200. Diluted antigen (50 μL) was captured in each well at RT for 1 h on a shaker at 500 rpm. Plates were washed five times with PBST. Each well was incubated with 50 μL of 1:1,000 diluted serum at RT for 1 h, washed again, and incubated with 50 μL of 1:10,000 diluted horseradish peroxidase–labeled anti-human secondary antibody (Jackson ImmunoResearch Laboratories) at RT for 1 h. Plates were incubated on a shaker at 500 rpm. Finally, the plates were washed and incubated with 50 μL 1-Step Ultra TMB ELISA Substrate for 10 min (Thermo Fisher Scientific) for detection and 50 μL of 2 mol/L sulfuric acid to stop the reaction. OD$_{450}$ was measured by Envision Multilabel Reader (PerkinElmer, Waltham, MA).

**Statistical Analysis**

Sample information is presented as proportions and medians and ranges. A positive antibody response to a viral antigen was defined by the presence of a ring in at least one of the replicates of that protein. An individual was considered to have a positive antibody response to a virus if he or she had an antibody response to at least one viral antigen from the viral proteome. Odds ratios (ORs) and exact 95% CIs were calculated for viruses and T1D association using conditional likelihood estimation and Fisher exact test (31). Exact Wilcoxon rank sum tests were applied to the maximum of the ring positive net intensity data to determine the significance of antibodies to individual viral protein proteins between cases and control subjects. The Benjamini-Hochberg procedure was used to adjust $P$ values to account for multiple testing. These tests were performed using the coin package in R 3.0.3. Exploratory subgroup analyses of the association between antibody positivity to viral proteins and the three known T1D autoantigens (GADA, IA-2A, or ZnT8A), sex, and age subgroups ($\leq12$ and $>12$ years) were performed. $P$ values $<0.05$ were considered significant. Heatmaps were generated in MultiExperiment Viewer, version 4.9 (http://www.tm4.org/mev.html). Graphs and plots were generated in GraphPad Prism 6.

**RESULTS**

**Viral Protein Array Production and Quality Assessment**

We have established a high-throughput pipeline (Fig. 1) and cloned 292 open reading frames (ORFs) from seven viruses reported to be associated with T1D and 354 ORFs from other common viruses into our protein array–compatible expression vector (Table 2). Among these, for 15 out of 23 viral strains we obtained 100% ORFs from the viral genome. The high coverage of viral ORFs and the diversity of viral strains would help us to fulfill the goal of a systematic survey of antiviral antibodies in patients with new-onset T1D.

For confirmation of printing quality, plasmids on the array were stained by PicoGreen that showed uniform DNA staining across the slide (Supplementary Fig. 1). The fluorescent signals of protein display were pseudocolored with a rainbow color scheme. The correlation coefficient of signal intensities of protein display as detected by the anti-GST tag antibody between two slides was $>0.92$ (Supplementary Fig. 1).

**Profiling of Antiviral Antibodies**

Quality-assured slides were challenged with serum samples from 42 patients with new-onset T1D and 42 age- and sex-matched healthy control subjects, with median ages of 11.5 and 12 years, respectively. IgG was profiled on a set of 84 slides, and IgA and IgM were profiled simultaneously on another slide set. Arrays were regularly probed with the pooled sample to assess intra- and interarray reproducibility (see RESEARCH DESIGN AND METHODS). The interspot correlation coefficients for IgG and IgA profiling of duplicate spots on the same array were 0.94 and 0.91, respectively (Supplementary Fig. 2). The interarray correlation coefficients of IgG, IgA, and IgM profiling of duplicate arrays on different days ranged from 0.92 to 0.94 (Fig. 2). The high interspot precision within slides and day-to-day reproducibility on different slides demonstrated the robustness of our serum screening practice and good control of assay quality.

We used rapid antigenic protein in situ display (RAPID) ELISA to confirm the antibody reactivity observed on the viral protein arrays. RAPID ELISA is an established in-house–developed immunoassay. Like NAPPA and several well-established clinical assays for T1D AAbs, it relies on a cell-free system to produce antigens. RAPID ELISA uses the same viral antigen-encoding plasmids used on the arrays. The intra-assay and interassay reproducibility is typically 0.99 (Supplementary Fig. 3). The ease in developing a RAPID ELISA to any antigen in the collection allowed us to confirm the performance of a smaller number of candidate antigens with many samples reproducibly, quickly, and affordably. RAPID ELISA data were consistent
with clinical radioimmunoprecipitation assay and the luciferase immunoprecipitation systems assay data (data not shown). Given its easy adaptability, high reproducibility, and comparability with established clinical assays, it was chosen as a confirmatory platform to validate the signals on the array. The ring positive net intensities of two EBV antigens, BFRF3 and BLRF2, obtained on the viral protein arrays agreed well with ELISA, further proving the validity of our array platform in profiling antiviral antibodies (Supplementary Fig. 4).

Differential Antiviral Antibody Response on the Arrays
A protein array approach enables the assessment of different antibody reactivity patterns for all proteins from the same virus. Heatmaps of IgG and IgA reactivity to viral proteins from the seven viruses previously associated with T1D were generated using the ring positive net intensities (Fig. 3). IgG antibody response to VP1 from CVB, nucleoprotein from MuV, and influenza A virus were detected in almost every individual in both case and control groups, which suggests they are immunodominant antigens from those viruses during infection. IgA reactivity was generally a subset of the IgG-responsive antigens albeit weaker than IgG reactivity for the same viral protein; however, VP3 and VP4 from CVB were both more reactive for IgA response than IgG. Although our secondary antibodies clearly detected both IgA and IgM control spots simultaneously on our arrays (Supplementary Fig. 5), we did not observe any positive serological IgM reactivity. This may suggest that none of the individuals had active infections at the time of blood sampling and possibly low IgM concentration in the serum.

Higher Frequency of Antibody Response to EBV in T1D Patients
We compared the humoral immune responses for each virus between the case and the control groups. As a sensitive criterion, a positive antibody response to a virus in each subject was defined by a positive antibody response to at least one viral antigen from the viral proteome. By this criterion, the prevalence of viral antibody responses obtained on the array is shown in Table 3. The historical rates of infection determined by epidemiology studies in which sera were sampled are shown in Supplementary Table 1. These two agreed well. For example, in our study subjects, the prevalence of HPV infection was low while the frequencies of influenza A viruses were high, which was in agreement with epidemiological data (Supplementary Table 1).
Notably, we found that antibody responses to EBV antigens were more frequent in patients with new-onset T1D than in healthy control subjects (88% vs. 52%; OR 6.6, 95% CI 2.0–25.7; P value 0.018), whereas none of the other viruses showed differences between the two groups (Table 3). Responses against EBV were higher in case than in control subjects among both sexes and in both commonly used age subgroups (≥12 and <12 years) (Supplementary Table 2).

We detected many immunodominant EBV proteins based on antibody-mediated immunity such as BFRF3 and BLRF2 (viral capsid antigen [VCA]), BZLF1 (early antigen [EA]), and EBNA1 (nuclear antigen). On average, each case had antibody responses to 8 EBV proteins whereas each control had antibody responses to 6 EBV proteins.

We then assessed the association of antibody responses to individual viral proteins with subgroups of patients with T1D. HERK, human endogenous retrovirus K; RUBA, rubella virus; RV, rotavirus.

**Figure 3**—Heat maps of IgG and IgA reactivity to viral proteins from the seven viruses previously associated with T1D. HERK, human endogenous retrovirus K; RUBA, rubella virus; RV, rotavirus.
T1D. IgG antibody response to IA-2 was statistically significantly higher in patients with T1D ($P = 0.006$), confirming the detection of known T1D AAbs on our protein array platform. Although no antibody to a single protein alone was a sufficient predictor, we found that a more sensitive viral response criterion could be defined as a positive antibody response to at least one viral antigen from the viral proteome. Next, we tested the association of antibodies against each viral protein against the responses to the three known T1D autoantigens (IA-2, GAD65, and ZnT8). No antibody responses to individual viral proteins exhibited statistically significant associations with any of the three known T1D autoantigens or sex. Only one protein, IgG antibody response to the NP protein from influenza A virus (H1N1), was significantly ($P = 0.007$) higher in one age subgroup (>12 years) compared with the age ≤12 years subgroup of patients with T1D (data not shown).

**DISCUSSION**

To our knowledge, this is the first study to examine antiviral antibodies to individual viral proteins at the proteomic level in new-onset T1D patients. We accomplished this by using an innovative and flexible protein array platform, resulting in a study with several unique strengths including the ability to display virtually any viral protein, proteins produced just-in-time for study, and proteins produced using human ribosomes and chaperone proteins. A key finding here was a higher EBV-specific immune response in T1D. By examining antibody responses to hundreds of individual viral antigens, we analyzed the role of viral infections in T1D development at a dimension never achieved before. Previously, publications focused on the detection of antibodies to whole viruses or a limited number of viral proteins (10,11). Such studies might underrepresent the prevalence of viral infections because some patients may not produce antibodies that respond to the tested antigens. Even some commercial ELISA kits cannot achieve 100% sensitivity in detecting viral infections (32). Indeed, in our study, responses to viral antigens were quite heterogeneous from individual to individual. The examination of all proteins for each virus

### Table 3—ORs for the association of antibody responses to viruses between T1D case and healthy control subjects

| Virus/protein controls | T1D (n) | T1D (%) | HC (n) | HC (%) | OR    | 95% CI     | $P$   |
|------------------------|---------|---------|--------|--------|-------|------------|-------|
| HCMV*                  | 24      | 57.1    | 23     | 54.8   | 1.1   | 0.4–2.8    | 1.000 |
| EBV*                   | 37      | 88.1    | 22     | 52.4   | 6.6   | 2.0–25.7   | 0.018 |
| CVB*                   | 42      | 100.0   | 42     | 100.0  | 0.0   | 0.0 to Inf | 1.000 |
| RUBA*                  | 22      | 52.4    | 25     | 59.5   | 0.8   | 0.3–1.9    | 1.000 |
| MuV*                   | 41      | 97.6    | 40     | 95.2   | 2.0   | 0.1–123.8  | 1.000 |
| HERK*                  | 3       | 7.1     | 2      | 4.8    | 1.5   | 0.2–19.3   | 1.000 |
| RV*                    | 27      | 64.3    | 32     | 76.2   | 0.6   | 0.2–1.6    | 1.000 |
| HBV                    | 1       | 2.4     | 0      | 0.0    | Inf   | 0.0 to Inf | 1.000 |
| HPV16                  | 2       | 4.8     | 1      | 2.4    | 2.0   | 0.1–123.8  | 1.000 |
| HPV18                  | 4       | 9.5     | 0      | 0.0    | Inf   | 0.7 to Inf | 1.000 |
| CHIKV                  | 1       | 2.4     | 0      | 0.0    | Inf   | 0.0 to Inf | 1.000 |
| SFV                    | 1       | 2.4     | 0      | 0.0    | Inf   | 0.0 to Inf | 1.000 |
| SINV                   | 0       | 0.0     | 0      | 0.0    | 0.0   | 0.0 to Inf | 1.000 |
| Influenza A virus (H1N1)| 41    | 97.6    | 42     | 100.0  | 0.0   | 0.0–39.0   | 1.000 |
| Influenza A virus (H3N2)| 37  | 88.1    | 40     | 95.2   | 0.4   | 0.0–2.5    | 1.000 |
| VZV                    | 31      | 73.8    | 27     | 64.3   | 1.6   | 0.6–4.5    | 1.000 |
| SV40                   | 1       | 2.4     | 0      | 0.0    | Inf   | 0.0 to Inf | 1.000 |
| VACV                   | 1       | 2.4     | 2      | 4.8    | 0.5   | 0.0–9.8    | 1.000 |
| YF                     | 0       | 0.0     | 0      | 0.0    | 0.0   | 0.0 to Inf | 1.000 |
| MeV, vaccine strain    | 14      | 33.3    | 14     | 33.3   | 1.0   | 0.4–2.7    | 1.000 |
| MeV, WT strain         | 31      | 73.8    | 35     | 83.3   | 0.6   | 0.2–1.8    | 1.000 |
| Adenovirus             | 34      | 81.0    | 36     | 85.7   | 0.7   | 0.2–2.6    | 1.000 |
| Tioman virus           | 0       | 0.0     | 0      | 0.0    | 0.0   | 0.0 to Inf | 1.000 |
| IA-2p                  | 16      | 38.1    | 0      | 52.924 | 3.1   | 3.1–919.6  | 0.006 |
| GST controln           | 0       | 0.0     | 0      | 0.0    | 0.0   | 0.0 to Inf | 1.000 |

T1D, subjects with new-onset T1D; HC, health control subjects. Expansions for all abbreviations can be found in Table 1. Inf, infinite.

*The seven viruses epidemiologically associated with T1D. **IA-2 protein used as a positive control. **Plasmid expressing only the GST-tag protein as a negative control.
affords the opportunity to look for associations not only with viral infection but also with immune responses to specific viral antigens. By focusing on the host immune response, we avoided missing events due to the transient nature of detecting viral DNA/RNA, which may only be present briefly during infection (18).

High-throughput and low sample consumption are great advantages of our array platform over conventional one-antigen-at-a-time immunoassays. With use of our platform, only 3.2 μL sera were needed to probe thousands of antigens on the slide. On average, each antigen consumes as little as 0.002 μL of serum sample, which is significantly less than individual assays such as enzyme immunoassay and radioimmunoprecipitation assay, which consume several microliters per antigen per assay.

Our array platform enables the multiplexed detection of different immunoglobulin classes and the flexibility to include any genes of interest into the study design. Typically, IgG is considered the most common and durable response, providing information of historical infections. IgA associates with mucosal immunity and IgM correlates with acute infection. It will be useful to consider expanding the content of these viral protein arrays. The emergence of next-generation sequencing techniques provides powerful flexibility of a systematic evaluation that examines every sample against all viral proteins. Similarly, immunoassays based on the whole viral particles only detect responses against viral capsid antigens.

The relationships among age, EBV infection, and T1D are interesting. By dividing our subjects into two commonly used age-groups for such studies (≥12 and <12 years), we observed that there was a statistically higher prevalence of EBV infection among the T1D cases for both subgroups. However, as nearly all adults eventually develop antibody responses to EBV (41), we wondered if there was a relationship between age and the occurrence of antibodies to EBV antigens in patients compared with control subjects. To address this, we binned the individuals into different age-groups (4–7, 8–11, 12–15, and >15 years) and evaluated the response rate to at least one EBV protein. These subgroups were too small to evaluate statistically; however, as shown in Supplementary Fig. 6, for individuals <15 years, patients with T1D trended toward a greater likelihood of having been infected with EBV at a younger age than control subjects. This may suggest the possibility that an early age of EBV infection might contribute to the risk of developing T1D, although we cannot rule out the possibility that individuals with high T1D risk might be more prone to EBV infection at an earlier age. This question could be best addressed with a longitudinal study of individual’s serum responses before and after development of T1D.

Connections between EBV and T1D date back to 1974 (44). Earlier studies focused on detecting low-grade temporary viral infections using either PCR or serological antibody responses in studies with a small sample size (45,46). Elliott and Pilcher reported that EBV infection was not associated with islet cell and insulin Ab seroconversion (46). Hyöty et al. (47) profiled antibody levels to VCA and EA using a commercial ELISA kit, which examined responses against the combined protein antigen and found lower VCA IgG class antibody levels in T1D patients. This apparent difference from our findings may arise from differences in the methods for determining viral infections. Our study profiled antibodies to all 85 EBV proteins tested individually, whereas the earlier studies focused on antibody responses to the combination of only several EBV proteins. Notably, if we had considered responses to the several proteins from VCA or EA from our data, none would have reached significance (Supplementary Table 3). There were also important differences in the sample source: our samples were obtained in U.S. compared with Finland for the previous study. Viral infections are both seasonal and regional. Differences of viral infections in the general population may be different based on sample locations.

The mechanism through which EBV might contribute to the pathogenesis of T1D remains uncertain. However, several possible scenarios can be envisioned. First, EBV may be spread from circulating infected B cells to pancreatic tissue, resulting in local antiviral immune responses that damage β-cells. Second, EBV infection induces the release of cytokines, which promotes the maturation of immune cells to enhance their cytotoxicity (48,49). Third, EBV infection may trigger a cross-reactive autoimmune response.
through molecular mimicry of viral antigens and host proteins (50). These hypotheses need further evaluation. Recent evidence showed that viral infection may play a role in accelerating the progression from β-cell autoimmunity to clinical T1D (9). However, it is also possible the higher EBV responses may be a consequence of T1D but not a cause (51). The antibody responses detected in our work reflect past infections. As the samples used in our study were from patients with T1D postonset, investigation of longitudinal samples is needed to determine whether infection is important during seroconversion or during progression to clinical diagnosis of T1D. The successful application of this multiplexed viral proteome platform would facilitate this investigation.

Historically, coxsackievirus has been the most frequently cited T1D-related virus. We selected the E2 strain of CVB in our study because it was first isolated from the pancreas of a child with diabetic ketoacidosis (19). However, we did not find a difference in the apparent history of infection (OR 1, P value 1.000), which is consistent with the recent work by Oikarinen et al., who used a neutralizing antibody assay to investigate 41 different enterovirus serotypes (16). The other five viruses previously reported to be related with T1D also did not show prevalence difference between case and control subjects in our study.

Despite its virtues, the current study has some limitations. We did not have HLA data on our samples; however, an association between specific HLA genotypes and antiviral antibody responses in T1D has not been clearly established. Conflicting conclusions were drawn from studies on the same virus (52–55). The association between HLA genotypes and the humoral immune response is likely to be virus specific. The findings reported here specifically relate to early seroconversion of children to responsiveness to EBV. The overwhelming majority of adults display antibody responses to EBV (>90%) (41,56,57), making it unlikely that responses to EBV are significantly restricted by HLA. In this study, we found a higher rate of antiviral antibodies to EBV in young T1D patients compared with the children of similar ages, which suggests a potential role of EBV infection in T1D development.

To make the best possible comparisons with these samples, for which there were no HLA data available, we carefully matched our case and control subjects for age, sex, and time and geographic area of sampling. Furthermore, the control subjects were relatives of case subjects, which would not guarantee HLA matching but would ensure that the control subjects came from the same HLA pool and would have similar risk of T1D (11,58). This strategy has been used successfully to detect other associations between viruses and T1D, even in the absence of HLA matching (16).

In summary, we successfully demonstrated the use of our viral protein array platform in profiling antiviral antibodies and completed a preliminary study that found a potential link between EBV infection at an early age and T1D development. The success of this work established the utility of a flexible high-throughput multiplexed platform to profile a large number of longitudinal samples including time points surrounding “seroconversion” and clinical diagnosis. A comprehensive understanding of antibody responses to thousands of viral antigens in longitudinal samples and samples from HLA-matched healthy control subjects would greatly improve our knowledge of the role of viral infection in T1D development.

Acknowledgments. The authors thank Roivainen Merja (National Institute of Health and Welfare, Helsinki, Finland), Tom Hobman (University of Alberta, Edmonton, AB, Canada), Biao He (University of Georgia), Marc Vidal (Harvard Medical School), David E. Hill (Dana-Farber Cancer Institute), James M. Pipas (University of Pittsburgh), Jürgen Haas (University of Edinburgh, Edinburgh, U.K.) and Vincent Lotteau (Université de Lyon, Lyon, France) for kindly providing viral templates/clones. The authors thank Kristi Barker (Arizona State University) for critically reading this manuscript and Karen Anderson (Arizona State University) for providing a sample from a subject with clinically confirmed acute EBV infections.

Funding. This study was supported by JDRF (5-2012-537) and National Institutes of Health (AI42288).

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. X.B. collected viral genes, performed serum screening, and wrote the manuscript. G.W. was responsible for the accuracy of data analysis. A.D., A.T., J.S., and X.Y. helped with viral gene cloning. J.W. helped with RAPID ELISA experiments. C.W., D.S., and M.A. provided serum samples and valuable discussions. J.O. and J.L. contributed to experiment design, data generation, and analysis and reviewed and edited the manuscript. X.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 74th Scientific Sessions of the American Diabetes Association, San Francisco, CA, 13–17 June 2014.

References
1. Onkamo P, Väänänen S, Karvonen M, Tuomilehto J. Worldwide increase in incidence of Type I diabetes—the analysis of the data on published incidence trends. Diabetologia 1999;42:1395–1403
2. Redondo MJ, Jeffrey J, Fain PR, Eisenbarth GS, Orban T. Concordance for islet autoimmunity among monozygotic twins. N Engl J Med 2008;359:2849–2850
3. Eringsmark Regnell S, Lernmark A. The environment and the origins of islet autoimmunity and type 1 diabetes. Diabet Med 2013;30:155–160
4. Craig ME, Nair S, Stein H, Rawlinson WD. Viruses and type 1 diabetes: a new look at an old story. Pediatr Diabetes 2013;14:149–158
5. Roivainen M, Klingel K. Virus infections and type 1 diabetes risk. Curr Diab Rep 2010;10:350–356
6. Schneider DA, von Herrath MG. Potential viral pathogenic mechanism in human type 1 diabetes. Diabetologia 2014;57:2009–2018
7. Parikka V, Näntö-Salonen K, Saarinen M, et al. Early seroconversion and rapidly increasing autoantibody concentrations predict prepubertal manifestation of type 1 diabetes in children at genetic risk. Diabetologia 2012;55:1926–1936
8. Chmiel R, Giannopoulou EZ, Winkler C, Achenbach P, Ziegler AG, Bonifacio E. Progression from single to multiple islet autoantibodies often occurs soon after seroconversion: implications for early screening. Diabetologia 2015;58:411–413
9. Stene LC, Roivainen S, Hylöty H, et al. Enterovirus infection and progression from islet autoimmunity to type 1 diabetes: the Diabetes and Autoimmunity Study in the Young (DAISY). Diabetes 2010;59:3174–3180
10. Gamble DR, Kinsley ML, FitzGerald MG, Bolton R, Taylor KW. Viral antibodies in diabetes mellitus. BMJ 1969;3:627–630
11. Banatvala JE, Bryant J, Schernthaner G, et al. Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia. Lancet 1985;1:1409–1412

12. Foy CA, Quirke P, Lewis FA, Futers TS, Bodansky HJ. Detection of common viruses using the polymerase chain reaction to assess levels of viral presence in type 1 (insulin-dependent) diabetic patients. Diabet Med 1995;12:1002–1008

13. Pak CY, Eun HM, McArthur RG, Yoon JW. Association of cytomegalovirus infection with autoimmune type 1 diabetes. Lancet 1988;2:1–4

14. Ylipaasto P, Klingel K, Lindberg AM, et al. Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells. Diabetologia 2004;47:225–239

15. Dotta F, Censi S, van Halteren AG, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A 2007;104:5115–5120

16. Olkarien S, Taurinaen S, Hober D, et al.; VirDiab Study Group. Virus antibody survey in different European populations indicates risk association between coxsackievirus B1 and type 1 diabetes. Diabetes 2014;63:655–662

17. Laitinen OHHH, Honkanen H, Pakkanen O, et al. Coxsackievirus B1 is associated with induction of β-cell autoimmunity that portends type 1 diabetes. Diabetes 2014;63:446–455

18. Lee HS, Bries T, Winkler C, et al.; TEDDY study group. Next-generation sequencing for viruses in children with rapid-onset type 1 diabetes. Diabetologia 2013;56:1705–1711

19. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med 1979;300:1173–1179

20. Conrad B, Weissmahr RN, Böni J, Arcari R, Schüpbach J, Mach B. A human endogenous retroviral superantigen as candidate autoimmune gene in type 1 diabetes. Cell 1997;90:303–313

21. Honeyman MC, Coulson BS, Stone NL, et al. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. Diabetes 2000;49:1319–1324

22. Forrest JM, Menser MA, Harley JD. Diabetes mellitus and congenital rubella. Pediatrics 1969;44:445–447

23. Festa F, Steele J, Bian X, Labaer J. High-throughput cloning and expression library creation for functional proteomics. Proteomics 2013;13:1381–1399

24. Seiler CY, Park JG, Sharma A, et al. DNA/SU plasmid and PSI-Biology-Materials repositories: resources to accelerate biological research. Nucleic Acids Res 2014;42:D1253–D1260

25. Miersch S, LaBaer J. Nucleic acid programmable protein arrays: versatile tools for array-based functional protein studies. Curr Protoc Protein Sci 2011; Chapter 27:Unit27.2

26. Miersch S, Bian X, Wallstrom G, et al. Serological autoantibody profiling of type 1 diabetes by protein arrays. J Proteomics 2013;94:486–496

27. Takulapalli BR, Qiu J, Magee DM, et al. High density diffusion-free nanowell arrays. J Proteome Res 2012;11:4382–4391

28. Yu X, Woolery AR, Luong P, et al. Copper-catalyzed azide-alkyne cycloaddition (click chemistry)-based detection of global pathogen-host AMPylation on self-assembled human protein microarrays. Mol Cell Proteomics 2014;13:3164–3176

29. Ramachandran N, Raphael JV, Hainsworth E, et al. Next-generation high-density self-assembling functional protein arrays. Nat Methods 2008;5:535–538

30. Qiu J, LaBaer J. Nucleic acid programmable protein array a just-in-time multiplexed protein expression and purification platform. Methods Enzymol 2011; 500:151–163

31. Breslow NE, Day NE. Statistical methods in cancer research. Volume I - The analysis of case-control studies. IARC Sci Publ 1980;5:338

32. Tramper-Stranders GA, van der Ent CK, Sliker MG, et al. Diagnostic value of serological tests against Pseudomonas aeruginosa in a large cystic fibrosis population. Thorax 2006;61:689–693

33. Mejia-Leon ME, Petrosino JF, Ajami NJ, Dominguez-Bello MG, de la Barca AM. Fecal microbiota imbalance in Mexican children with type 1 diabetes. Sci Rep 2014;4:3814

34. Vogetaeder W, Dumfahrt A, Meyersbach P, Schanzke W, Dierich MP. Antibodies in human sera recognizing a recombinant outer membrane protein encoded by the envelope gene of the human endogenous retrovirus K. AIDS Res Hum Retroviruses 1993;9:687–694

35. Eisenhut M, Schwarz TF, Hegenscheid B. Seroprevalence of dengue, chikungunya and Sindbis virus infections in German aid workers. Infection 1999;27:82–85

36. Nwanegbo E, Vardas E, Gao W, et al. Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States. Clin Diagn Lab Immunol 2004;11:351–357

37. Storas SA, Dollard SC, Radford KW, Flanders WD, Pass RF, Cannon MJ. Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. Clin Infect Dis 2006;43:1143–1151

38. Dunne EF, Unger ER, Stemberg M, et al. Prevalence of HPV infection among females in the United States. JAMA 2007;297:813–819

39. Weinbaum CM, Williams I, Mast EE, et al.; Centers for Disease Control and Prevention (CDC). Recommendations for identification and public health management of persons with chronic hepatitis B virus infection. MMWR Recomm Rep 2008;57:1–20

40. Butel JS. Patterns of polyomavirus SV40 infections and associated cancers in humans: a model. Curr Opin Virol 2012;2:508–514

41. Balfour HH Jr, Sifakis F, Silman JA, Knight JA, Schmeling DO, Thomas W. Age-specific prevalence of Epstein-Barr virus infection among individuals aged 6-19 years in the United States and factors affecting its acquisition. J Infect Dis 2013;208:1286–1293

42. Cherry JD, Harriman KH. Why do vaccine-preventable disease outbreaks occur in the United States? [article online], 2012. Available from http://www.idse.net/download/VPD_IDSE12_WM.pdf. Accessed October 2012

43. Latner DR, McGrew M, Williams NJ, Sowers BW, Bellini WJ, Hickman CJ. Estimates of mumps seroprevalence may be influenced by antibody specificity and serologic method. Clin Vaccine Immunol 2014;21:286–297

44. Burgess JA, Kirkpatrick KL, Menser MA. Fulminant onset of diabetes mellitus during an attack of infectious mononucleosis. Med J Aust 1974;2:706–707

45. Foy CA, Quirke P, Williams DJ, et al. A search for candidate viruses in type 1 diabetic pancreas using the polymerase chain reaction. Diabet Med 1994;11:564–569

46. Elliott RBP, Pilcher CC. Epstein-Barr Virus infection is not associated with islet cell and insulin auto-antibody seroconversion. Diabetologia 1995;38:1130

47. Hyltö H, Räsänen L, Hiltunen M, Lehtinen M, Huupponen T, Leinikki P. Increased prevalence of Epstein-Barr virus infection among individuals aged 6-19 years in the United States and factors affecting its acquisition. J Infect Dis 2013;208:1286–1293

48. Hornef MW, Wagner HJ, Kruse A, Kirchner H. Cytokine production in a whole-blood assay after Epstein-Barr virus infection in vivo. Clin Diagn Lab Immuno 1991;99:359

49. Hyöty H, Räsänen L, Hiltunen M, Lehtinen M, Huupponen T, Leinikki P. Decreased antibody reactivity to Epstein-Barr virus capsid antigen in type 1 (insulin-dependent) diabetes mellitus. APMS 1991;99:359–363

50. Homel MW, Wagner HJ, Kruse A, Kirchner H. Cytokine production in a whole-blood assay after Epstein-Barr virus infection in vivo. Clin Diagn Lab Immunol 1995;2:209–213

51. Williams H, McAulay K, Macsween KF, et al. The immune response to primary EBV infection: a role for natural killer cells. Br J Haematol 2005;129:266–274

52. Parkkonen P, Hyötty H, Ilonen J, Reijonen H, Ylä-Herttuala S, Leinikki P; Childhood Diabetes in Finland Study Group. Antibody reactivity to an Epstein-Barr virus BERF4-encoded epitope occurring also in Asp-57 region of HLA-DQβ beta chain. Clin Exp Immunol 1994;95:287–293

53. Schernthaner G, Banatvala JE, Scherbaum W, et al. Coxsackie-B-virus-specific IgM responses, complement-fixing islet-cell antibodies, HLA DR antigens,.
and C-peptide secretion in insulin-dependent diabetes mellitus. Lancet 1985;2:630–632
54. Field LL, McArthur RG, Shin SY, Yoon JW. The relationship between Coxsackie-B-virus-specific IgG responses and genetic factors (HLA-DR, GM, KM) in insulin-dependent diabetes mellitus. Diabetes Res 1987;6:169–173
55. Çekin Y, Özkaya E, Gülkesen H, Akçurin S, Çolak D. Investigation of enterovirus infections, autoimmune factors and HLA genotypes in patients with T1DM. Minerva Endocrinol 2014;39:67–74
56. Anderson KS, Ramachandran N, Wong J, et al. Application of protein microarrays for multiplexed detection of antibodies to tumor antigens in breast cancer. J Proteome Res 2008;7:1490–1499
57. Anderson KS, Sibani S, Wallstrom G, et al. Protein microarray signature of autoantibody biomarkers for the early detection of breast cancer. J Proteome Res 2011;10:85–96
58. Salvatoni A, Baj A, Bianchi G, Federico G, Colombo M, Toniolo A. Intrafamilial spread of enterovirus infections at the clinical onset of type 1 diabetes. Pediatr Diabetes 2013;14:407–416