Children With Islet Autoimmunity and Enterovirus Infection Demonstrate a Distinct Cytokine Profile

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Cytokines are upregulated in prediabetes, but their relationship with Enterovirus (EV) infection and development of islet autoimmunity is unknown. Cytokines (n = 65) were measured using Luminex xMAP technology in a nested case-control study of 67 children with a first-degree relative with type 1 diabetes: 27 with islet autoantibodies (Ab^+) and 40 age-matched persistently autoantibody negative (Ab^-) control subjects. Of 74 samples, 37 (50%) were EV-PCR^+ in plasma and/or stool (EV^+) and the remainder were negative for EV and other viruses (EV^-). Fifteen cytokines, chemokines, and growth factors were elevated ($P \leq 0.01$) in Ab^+ versus Ab^- children (interleukin [IL]-1β, IL-5, IL-7, IL-12(p70), IL-16, IL-17, IL-20, IL-21, IL-28A, tumor necrosis factor-α, chemokine C-C motif ligand [CCL]13, CCL26, chemokine C-X-C motif ligand 5, granulocyte-macrophage colony-stimulating factor, and thrombopoietin); most have proinflammatory effects. In EV^+ versus EV^- children, IL-10 was higher (P = 0.005), while IL-21 was lower (P = 0.008). Cytokine levels did not differ between Ab^+EV^+ and Ab^+EV^- children. Heat maps demonstrated clustering of some proinflammatory cytokines in Ab^+ children, suggesting they are coordinately regulated. In conclusion, children with islet autoimmunity demonstrate higher levels of multiple cytokines, consistent with an active inflammatory process in the prediabetic state, which is unrelated to coincident EV infection. Apart from differences in IL-10 and IL-21, EV infection was not associated with a specific cytokine profile. ***Diabetes*** **61:1500–1508, 2012**

Type 1 diabetes is characterized by selective pancreatic β-cell destruction, accompanied by an inflammatory response within the islets (insulitis), which has a patchy distribution (1). Cytokines and chemokines play an integral role in the stimulation, regulation, and intercellular signaling of immune cells and are important mediators of insulitis and β-cell death (2). They are upregulated in prediabetes (3,4) and may provide additional surrogate markers of disease.

Genetic susceptibility to type 1 diabetes is conferred primarily by HLA DRB1, DQA1, and DQB1 (5); however, the prevalence of high-risk HLA genotypes in new-onset cases has decreased in recent years (6,7). Furthermore, the recent rise in childhood type 1 diabetes incidence (8,9) has occurred too rapidly to be explained by genetic factors alone. Of the putative etiological agents implicated in the complex interplay between genes and the environment, Enterovirus (EV) infections are probably the most extensively studied.

Many human EV genotypes demonstrate β-cell tropism (10); their specificity for β-cells is evidenced by detection of the Coxsackie adenovirus receptor, a major EV receptor, in the islets but not the exocrine pancreas (11). In a recent meta-analysis, we reported that EV infections were significantly associated with onset of type 1 diabetes (odds ratio [OR] ~10) and islet autoimmunity (OR ~4) (12). However, viral infections can also protect from diabetes, possibly by an immunoregulatory or "bystander suppression" effect (13). EV infections may contribute to type 1 diabetes by causing direct cell lysis or through bystander activation whereby infection stimulates recruitment of immune cells and cytokines, leading to β-cell destruction, release of sequestered autoantigens, and activation of autoreactive T cells, triggering autoimmunity (14).

It has been proposed that an imbalance between T-helper (Th)1 cytokines with proinflammatory effects (e.g., tumor necrosis factor [TNF]-β and interferon [IFN]-γ), anti-inflammatory cytokines produced by Th2 cells (e.g., interleukin [IL]-4 and IL-10), and regulatory T (Treg) cells (e.g., IL-10 and transforming growth factor [TGF]-β) underlies type 1 diabetes pathogenesis (4). A third effector pathway involving Th17 cells has also been associated with autoimmune disease, including type 1 diabetes (15). However, it is becoming increasingly clear that a single mechanism is unlikely and multiple pathways for β-cell damage lead to type 1 diabetes (2).

Despite evidence for upregulation of proinflammatory cytokines prior to (4) and at diabetes onset (16,17), findings are inconsistent across studies. Furthermore, there are limited data examining the interplay between EV infection and cytokines, and the relationship between EV infection, islet autoimmunity, and cytokines has not been studied prior to disease onset. Therefore, the primary hypothesis for this study was that children with islet autoimmunity (Ab^+) demonstrate a proinflammatory cytokine response compared with autoantibody negative (Ab^-) children and that contemporaneous EV infection modifies their cytokine response. We also sought to understand whether overall cytokine profiles can differentiate Ab^+ versus Ab^- children, with or without EV infection, using multivariate models.

RESEARCH DESIGN AND METHODS

Study protocol. The study sample was drawn from a cohort of 245 infants and children who have one or more first-degree relatives with type 1 diabetes participating in a prospective cohort study examining the association between

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viral infections and development of autoimmunity/type 1 diabetes: the Viruses in the Genetically at Risk (VIGR) study. Recruitment began in 2004; we initially included children with low-risk HLA genotypes who were ineligible for the TRIGR (Trial to Reduce IDDM in the Genetically at Risk) study (n = 67), but subsequent recruitment was independent of TRIGR, and participants were included irrespective of HLA genotype. Plasma, stool, and/or throat swabs were collected at clinic visits scheduled every 3 to 12 months. Samples were frozen at −80°C until testing. Informed consent was obtained from all participants and/or their parents.

Ab testing. Serum samples were tested for Ab to insulinoma-associated protein 2 (IA2) antigen (IA-2A), GAD, and insulin (IAA). The IA-2A Ab and GAD Ab testing was performed with a radioimmunoassay using ^{125}I-labeled IA-2A and GAD. Antigen-Ab complexes were recovered using protein-A agarose. Precipitates were recovered after centrifugation at 3,000*g*, with the amount of radioactivity proportional to IA-2A and GAD concentrations in the test sample expressed as nU/mL of serum. IAA was measured using a radioimmunoassay with displacement of unlabeled insulin and precipitation of polyethylene glycol. Displaced percentage binding was calculated for each sample by subtracting the counts with excess unlabeled insulin from those with labeled insulin alone. This difference was multiplied by the amount of unlabeled insulin added to the tubes to give the amount of insulin bound by the serum, expressed as nU/mL of serum. The cutoff levels for Ab positivity in these assays were 0.6 units/mL for GAD, 0.8 units/mL for IA-2A, and 53.0 nU/mL for IAA.

Viral detection. Plasma samples were tested for cytomegalovirus, varicella zoster virus, herpes simplex virus, Epstein Barr virus, and EV RNA with multiplex PCR (mPCR), as previously described (18). Stool samples were tested using the same mPCR as well as for norovirus and rotavirus and using a nested multiplex RT-PCR. Serum samples were also tested for cytomegalovirus, varicella zoster virus, herpes simplex virus, Epstein Barr virus, IgM, and IgG using commercial enzyme immunosorbent assay and for EV by complement fixation (19) at the South Eastern Area Laboratory Services at Prince of Wales Hospital.

HLA genotyping. High-resolution sequencing-based HLA typing of the DRB1 and DQB1 alleles was performed at the Tissue Typing Laboratories of the Australian Red Cross Blood Service, Sydney, Australia.

Cytokine measurement. The concentrations of 65 cytokines and chemokines were measured in plasma using Luminex xMAP technology color-coded microspheres, according to the manufacturer's instructions (Millipore, Billerica, MA). Cytokines measured were IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-16, IL-17, IL-20, IL-21, IL-23, IL-28A, IL-33, IL-1 receptor antagonist (IL-1ra), soluble IL-2 receptor-α (sIL-2Rα), IFN-α2, IFN-γ, TNF-α, TNF-β, TGF-α, chemokine C-C motif ligand (CCL)1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL17, CCL21, CCL22, CCL24, CCL26, CCL27, chemokine C-X-C motif ligand (CXCL)1, CXCL5, CXCL8, CXCL10, CXCL12, CXCL13, chemokine C-X3-C ligand (CX3CL)1, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage (GM)-CSF, epidermal growth factor (EGF), leukemia inhibitory factor (LIF), thrombopoietin (TPO), TNF-related apoptosis–inducing ligand (TRAIL), vascular endothelial growth factor (VEGF), stem cell factor (SCF), thymic stromal-derived lymphopoietin (TSLP), fibroblast growth factor 2 (FGF-2), platelet-derived growth factor (PDGF)-AA, PDGF-AA/BB, soluble CD40 ligand (sCD40 L), and FMS-like Tyr kinase 3 (Flt3) ligand. Concentrations were calculated using the StatLIA Immunoassay Analysis Software. IL-21 levels were below the detection range of the multiplex assay in 55 (74%) of the samples, so they were retested using a Human ELISA MAX Deluxe set (BioLegend, San Diego, CA).

Statistical analysis. For statistical purposes, out-of-range cytokine levels were assigned an arbitrary value corresponding to the minimum (or maximum) detectable concentration. If an extrapolated value below the minimum detectable concentration was present, out-of-range levels were assigned an arbitrary concentration 0.1 pg/mL below the lowest value. This was necessary to account for the low cytokine concentrations in those samples and to reduce the risk of a type II error.

Data not normally distributed were log transformed, and descriptive statistics are reported as mean ± SD. Differences in cytokine concentrations in Ab^+ versus Ab^- and EV^+ versus EV^- children were analyzed using generalized estimating equations (GEEs) to allow for intraindividual correlation where there was more than one sample from the same child. HLA risk was included in the models to account for its possible confounding effect on cytokine concentrations. Samples were further divided into subgroup 1: Ab^+/EV^+ (n = 16); subgroup 2: Ab^-/EV^+ (n = 21); subgroup 3: Ab^+/EV^- (n = 18); and subgroup 4: Ab^-/EV^- (n = 19); between-group differences were analyzed using one-way ANOVA. Data were analyzed using SPSS Statistics (Version 19, IBM Corporation, Armonk, NY).

The Bonferroni adjustment is often used to account for the increased chance of a significant finding when multiple independent tests are performed on the same subject group. However, when there are a large number of comparisons, this would make the P value somewhat overconservative. We have therefore elected to present only those findings that were significant at a level of $P \leq 0.01$.

We hypothesized that the correlation in expression of the 65 cytokines would be influenced by the presence of islet autoimmunity. The nonparametric equivalent of the Pearson correlation coefficient, the Spearman correlation coefficient, was used to calculate the pairwise correlation in cytokine concentrations between every pair of cytokines. These created a matrix of correlation values, which describes the similarity in concentration between each pair of cytokines, and this matrix was visualized using a heat map. First, hierarchical clustering was used to determine groups of cytokines with similar expression patterns in Ab^+ patients. The complete linkage method was used to generate the clusters because this groups the data points that are most similar together. Second, to understand whether cytokine expression patterns reflected Ab status, we used heat maps and hierarchical clustering to group the patients based on the expression of 65 cytokines. For this analysis, subgroup 2 (Ab^-/EV^+) patients were excluded to limit classification to risk of islet autoimmunity. The correlation in cytokine profiles between each pair of patient samples was calculated using Spearman correlation coefficient. R statistical analysis software (version 2.13.1) was used for correlation analyses and for creating heat maps.

RESULTS

To date, 19 of 245 children (7.8%) have developed persistent islet autoimmunity, defined as being positive for at least one Ab at two or more visits at least 6 months apart, and of these, 11 have at least two positive Ab (4.5% of total cohort). Mean follow-up from first seroconversion is 3.7 years. One child progressed to type 1 diabetes at age 5.6 years; the sample from first seroconversion at age 2.7 years after an EV infection was included in the analysis. A further 42 children (17.1%) developed transient Ab, defined as either positive at only one visit or positive at multiple visits but subsequently negative for at least two visits, beyond 12 months of age. The remaining 184 children (75%) have been consistently Ab^-.

In the present nested case-control analysis, we included samples from 67 children (32 male) selected on the basis of Ab seroconversion and EV-PCR results (Table 1); samples were selected to enable comparison of cytokine profiles by

TABLE 1
Characteristics of participants stratified by islet Ab status

Characteristic	Ab^+ (n = 27)	Ab^- (n = 40)	P value
Male, n (%)	12 (44)	20 (50)	0.66
Samples (n)	34	40	
Mean age at time of sampling (SD)	2.5 (1.8)	2.6 (2.8)	
Mean age at first seroconversion (SD)	2.1 (1.7)	—	—
High-risk HLA genotype, n (%)	7 (26)	6 (15)	0.29
EV-PCR$^+$ samples, n (%)	16 (47)	21 (53)	0.64
Previous EV infections (EV-PCR$^+$) (n)			0.39
0	15	15	
1	14	15	
2	3	8	
3	2	1	
4	0	1	
Relative with type 1 diabetes, n (%)			0.28
Mother	16 (59)	17 (43)	
Father	8 (30)	16 (40)*	
Sibling	3 (11)	9 (23)*	

*Two children had both a father and a sibling with type 1 diabetes.

islet Ab status (Ab^+/Ab^-) and EV infection status (EV^+/EV^-). Of 34 samples from Ab^+ children (26 persistent, 8 transient), 16 (47%) were EV^+ (negative for other viruses on mPCR) and the remainder were negative for all viruses. Of 40 samples from 40 aged-matched control subjects, who were consistently Ab^- throughout the study, 21 (53%) were EV^+ (negative for other viruses on mPCR) and the remainder were negative for all viruses. Most EV^+ samples were in stool (30 of 37, 81%), 3 were positive in plasma and stool (8%), and 4 were positive in plasma only (11%). Neither sex (OR 1.2 [95% CI 0.4–3.4]; $P = 0.8$) nor high-risk HLA genotype (2.7 [0.7–9.8]; $P = 0.13$) were significantly associated with development of islet autoimmunity.

Cytokine levels: Ab^+ versus Ab^-. The plasma concentrations of 14 cytokines were significantly ($P \leq 0.01$) higher in Ab^+ compared with Ab^- children. These were a mixture of proinflammatory cytokines IL-1β, IL-5, IL-7, IL-12(p70), IL-16, IL-17, IL-21, and TNF-α; anti-inflammatory cytokines IL-20 and IL-28A; chemokines CCL13, CCL26, and CXCL5; and one growth factor, GM-CSF. Results of univariate analyses, including mean (SD) values for all cytokines in Ab^+ versus Ab^- children, are shown in Supplementary Tables 1 and 2. After adjusting for HLA status, TPO (a growth factor) became significant; results of multivariate GEE analysis for the 15 of 65 (23%) cytokines significantly associated with islet autoimmunity are shown in Table 2. When the eight children with transient Ab^+ were excluded from analysis, all cytokines remained significantly ($P \leq 0.01$) associated with Ab^+, with the exception of TPO (OR 1.3 [95% CI 1.1–1.6]; $P = 0.017$).

EV^+ versus EV^-. Comparing the samples based on presence of EV infection, only the concentration of IL-10 ($P = 0.005$) was significantly higher in EV^+ versus EV^- samples, while IL-21 was lower in EV^+ samples ($P = 0.008$). There was no association between EV infection and HLA status ($P = 0.52$). Mean (SD) values for all cytokines in EV^+ versus EV^- children and between-group differences are shown in Supplementary Tables 3 and 4.

Ab and/or EV positive versus negative. The concentrations of 12 cytokines (IFN-α2, IL-5, IL-12(p70), IL-16, IL-20, IL-28A, LIF, TNF-α, CCL13, CCL26, GM-CSF, and VEGF) were significantly different across the four subgroups (Ab^+/EV^+, Ab^-/EV^+, Ab^+/EV^-, and Ab^-/EV^-), with the highest levels found predominantly in the Ab^+/EV^- subgroup (Figs. 1*A* and *B*). Healthy control subjects (Ab^-/EV^-) had the lowest cytokine concentrations, with 16 undetectable in >50% of their samples. Comparing Ab^+/EV^+ and Ab^+/EV^-, no cytokine concentrations were significantly different. There was some evidence for higher IL-10 ($P = 0.05$) and lower IL-21 ($P = 0.05$) levels, consistent with differences in EV^+ versus EV^- samples in the total population. Results of ANOVA for all 65 cytokines are shown in Supplementary Tables 5 and 6.

Heat map analyses. Heat maps were used to visualize the correlation in expression between each possible pair of the 65 cytokines in Ab^+ patients (Fig. 2). Hierarchical clustering generated four small groups of cytokines, with similar patterns of expression within each group. Most proinflammatory cytokines grouped together (e.g., IL-1β, IL-5, IL-7, IL-15, IL-33, LIF, and CCL-26) (Fig. 2, top cluster), as did many chemokines; however, anti-inflammatory cytokines (IL-4, IL-10, IL-13, IL-20, and IL-28A) and growth factors (GM-CSF and TPO) were spread across the clusters.

Heat maps and hierarchical clustering were also used to classify Ab status (Ab^+ vs. Ab^-) based on the expression of 65 cytokines (Fig. 3); this generated four clusters of cytokines. In the first cluster (Fig. 3, top), 61% (16 of 26) of samples were from subgroup 4 (Ab^-/EV^-), representing 84% (16 of 19) of subgroup 4 patients. All 12 samples in the second cluster were from Ab^+ children; subgroups 1 and 3 (Ab^+/EV^+ and Ab^+/EV^-). In the third and fourth clusters, 6 of 8 (75%) and 7 of 11 (64%) of samples were from Ab^+ children, respectively. This suggests that cytokine expression profiles have some capacity to distinguish Ab^+ versus Ab^- children but have limited capacity to classify Ab^+ case subjects based on EV infection status. This is consistent with the finding of no significant difference in individual cytokine concentrations between Ab^+/EV^+ and Ab^+/EV^- children.

TABLE 2
ORs for cytokines in children with islet autoimmunity versus persistently negative Ab

	OR*	95% CI	*P* value†
Proinflammatory cytokines			
IFN-α2	2.7	1.2–6.2	0.02
IFN-γ	1.5	1.0–2.2	0.03
IL-1α	1.4	1.1–2.0	0.02
IL-1β	**1.6**	**1.2–2.1**	**0.002**
IL-2	1.4	1.0–1.8	0.03
IL-5	**1.6**	**1.2–2.4**	**0.007**
IL-7	**1.7**	**1.2–2.6**	**0.008**
IL-12(p70)	**1.8**	**1.2–2.6**	**0.003**
IL-15	1.3	1.0–1.6	0.04
IL-16	**2.1**	**1.4–3.0**	**<0.001**
IL-17	**3.1**	**1.4–7.1**	**0.006**
IL-21	**2.4**	**1.3–4.7**	**0.009**
IL-23	1.3	1.1–1.6	0.012
IL-33	**1.3**	1.0–1.6	0.03
LIF	1.3	1.1–1.7	0.02
sCD40 L	2.2	1.2–4.1	0.02
TNF-α	**4.1**	**1.5–10.9**	**0.005**
TNF-β	1.4	1.0–2.0	0.04
Anti-inflammatory cytokines			
IL-13	1.3	1.1–1.7	0.018
IL-20	**1.7**	**1.1–2.6**	**0.01**
IL-28A	**1.8**	**1.2–2.7**	**0.004**
Chemokines			
CCL1 (I-309)	2.4	1.2–4.7	0.02
CCL4 (MIP1-β)	3.2	1.2–8.6	0.02
CCL7 (MCP-3)	1.4	1.1–1.9	0.02
CCL8 (MCP-2)	2.3	1.2–4.5	0.02
CCL13 (MCP-4)	**8.7**	**2.2–34.4**	**0.002**
CCL21 (Ckine6)	2.4	1.1–5.2	0.03
CCL26 (Eotaxin-3)	**1.6**	**1.2–2.1**	**0.002**
CXCL5 (ENA-78)	**2.7**	**1.4–5.5**	**0.006**
CXCL8 (IL-8)$^+$	2.3	1.2–4.5	0.02
CXCL12 (SDF-1α+β)	2.8	1.1–7.1	0.03
Growth factors			
EGF	2.1	1.2–3.7	0.014
Flt3 ligand	2.2	1.2–4.0	0.012
GCSF	1.02	1.0–1.04	0.04
GM-CSF	**1.6**	**1.1–2.4**	**0.008**
PDGF-AA	1.9	1.1–3.3	0.02
PDGF-AABB	1.8	1.1–3.1	0.03
TGF-α	1.4	1.1–1.8	0.02
TPO	**1.3**	**1.1–1.6**	**0.01**
VEGF	2.3	1.2–4.4	0.02

*Analysis performed using GEEs, adjusting for high-risk HLA genotype status. †Cytokines significantly associated with islet autoimmunity at $P \leq 0.01$ are shown in boldface.

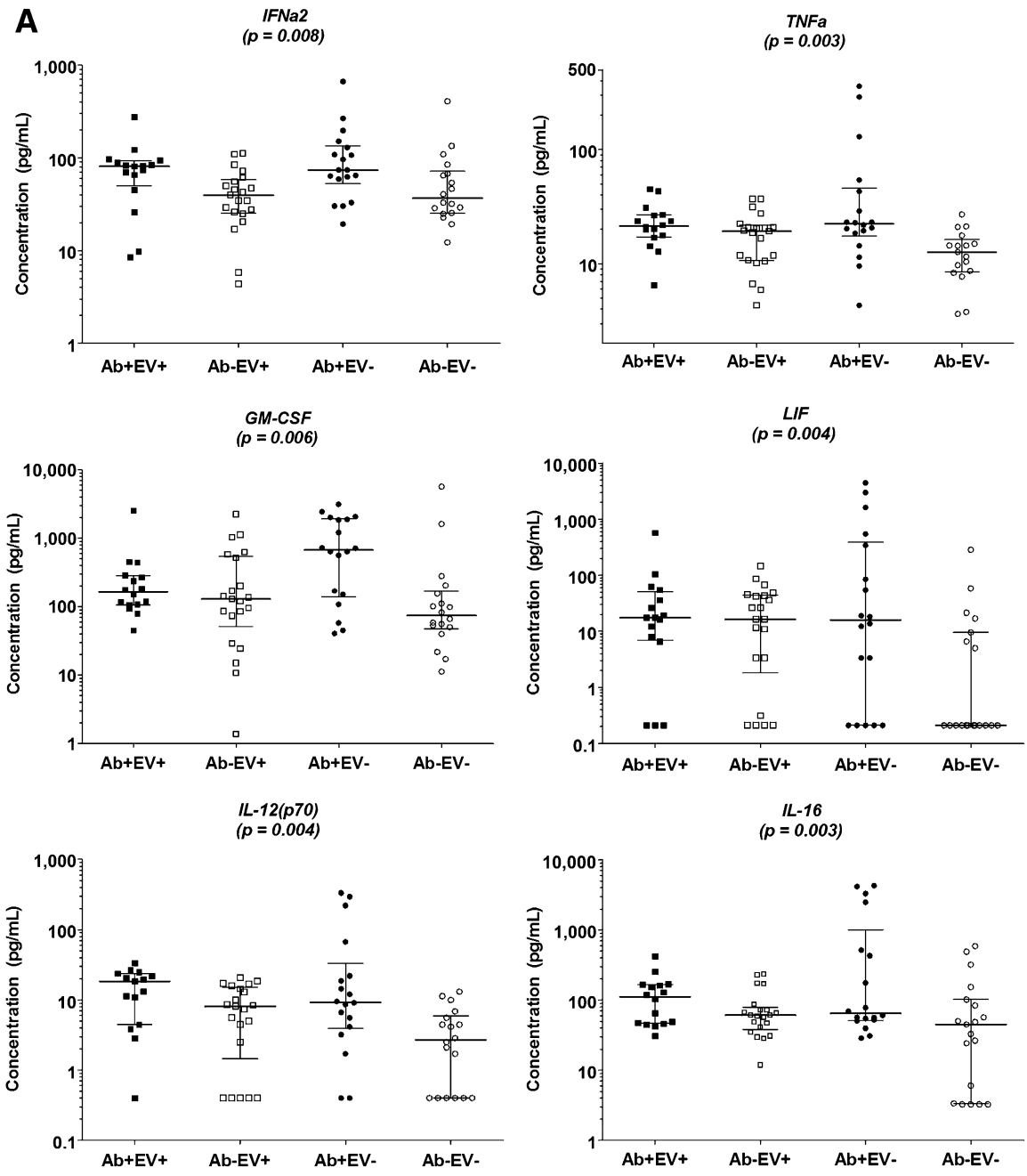

FIG. 1. *A*: **Proinflammatory cytokine concentrations across the four subgroups of islet Ab and/or EV infection.** *B*: **Pro- and anti-inflammatory cytokine, chemokine, and growth factor concentrations across the four subgroups of Ab and/or EV infection. Statistical significance defined as $P \leq 0.01$.**

DISCUSSION

This prospective cohort study provides the first comprehensive analysis of multiple cytokines in association with islet autoimmunity and/or EV infection. We discovered a predominantly proinflammatory cytokine milieu in Ab^+ at-risk children whose cytokine profiles did not differ by EV infection. Using hierarchical cluster analysis, we found some evidence for grouping of cytokines by their function in Ab^+ children, suggesting coordinate regulation of cytokines in the prediabetic phase. Of the 15 of 65 (23%) significantly elevated cytokines, chemokines, and growth factors, 8 cytokines (IL-1β, IL-5, IL-7, IL-12(p70), IL-16, IL-17, IL-21, and TNF-α) are proinflammatory. The chemokines CCL13, CCL26, and CXCL5 are chemotactic for a range of immune cells, including T cells and monocytes, and play a role in driving inflammation. In contrast, IL-7 and GM-CSF have been associated with protection from type 1 diabetes (20,21). It is now recognized that many cytokines do not fit into dichotomous categories of proinflammatory or anti-inflammatory but have multiple and complex roles (2). Our findings support the concept of diverse immunological networks in the development of islet autoimmunity and type 1 diabetes; indeed, a further 29 cytokines were elevated if a less conservative $P < 0.05$ is considered. We also identified possible new contributors, such as IL-16 and IL-20.

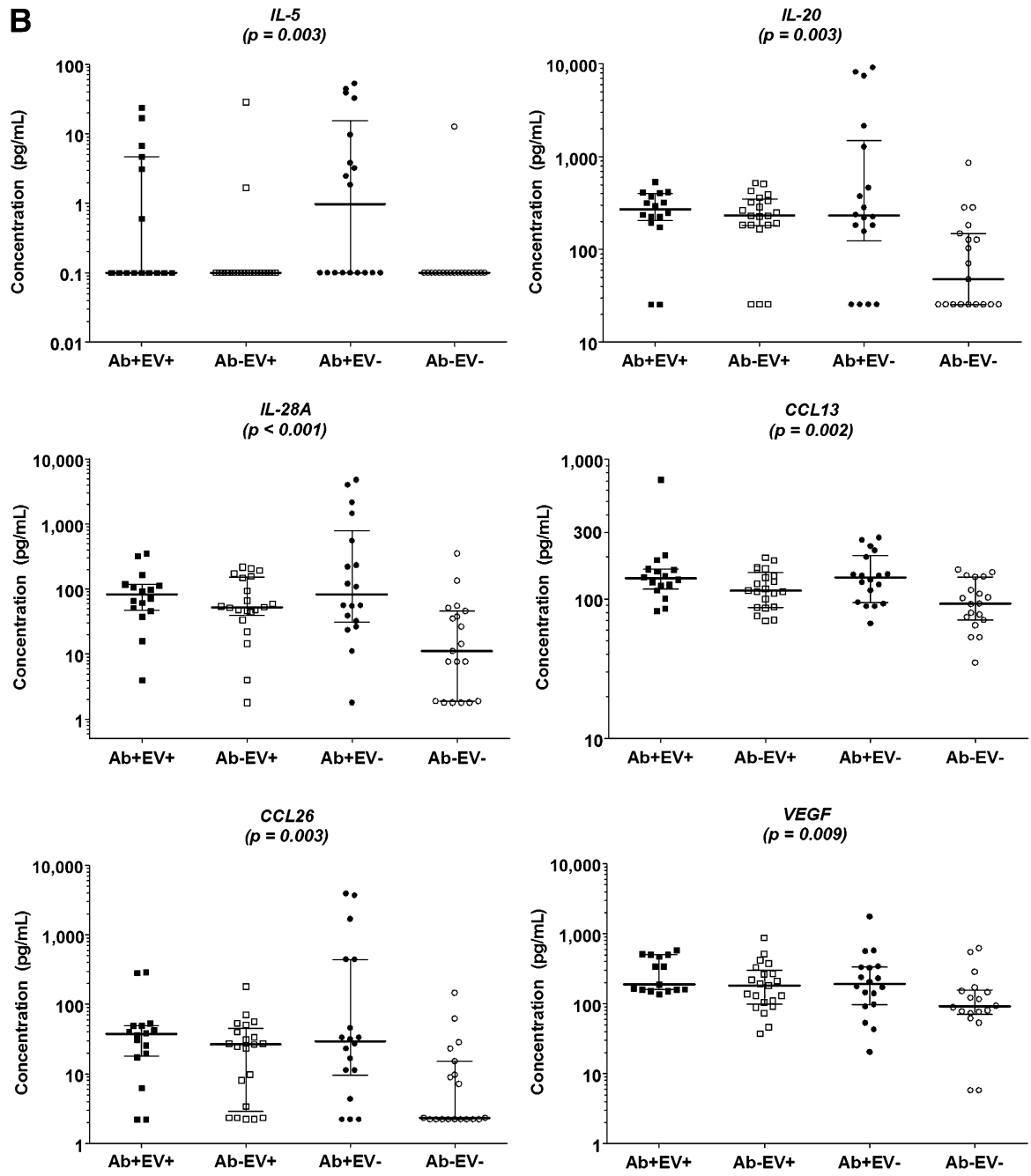

FIG. 1. Continued.

It has been suggested that there is a greater Th1 bias in prediabetes compared with at disease onset (4,22). We did not find higher levels of Th1 cytokines, such as IL-2, IFN-γ, and TNF-β, although there was some evidence for an increase in the latter two (IFN-γ: OR 1.5, $P = 0.03$; TNF-β: 1.4, $P = 0.04$). In contrast, cell-based studies show a stronger Th2 response to islet autoantigens in prediabetes—specifically, increased IL-4 and IL-5 expression (23); IL-5 was significantly increased in our children (1.6, $P = 0.007$), while there was a trend for elevated IL-4 (1.7, $P = 0.06$). In a longitudinal study of at-risk individuals with multiple Ab, the chemokines CCL4 and CCL5 were upregulated; neither were significantly increased in our population, although there was some evidence for elevated CCL4 (3.2, $P = 0.02$). We did not find elevation of cytokines that have been observed at disease onset, including IL-2 and IL-6; Th2 cytokines IL-4 and IL-13; Th3 cytokine IL-10; and chemokines CCL2, CCL4, CCL5, and CXCL10 (4,16,17,24,25). The inconsistent findings across studies of cytokine profiles in islet autoimmunity versus type 1 diabetes may relate to differences in study population characteristics; timing of sample collection; methodological differences, including sample size and methods of analysis; and of import, the heterogeneity of the disease process.

IL-1β is a proinflammatory Th1 cytokine produced after T-cell activation. Therefore, its elevation in the current study is not surprising. In contrast, IL-1β was not detected in cases of new-onset type 1 diabetes (16,26), suggesting that it may

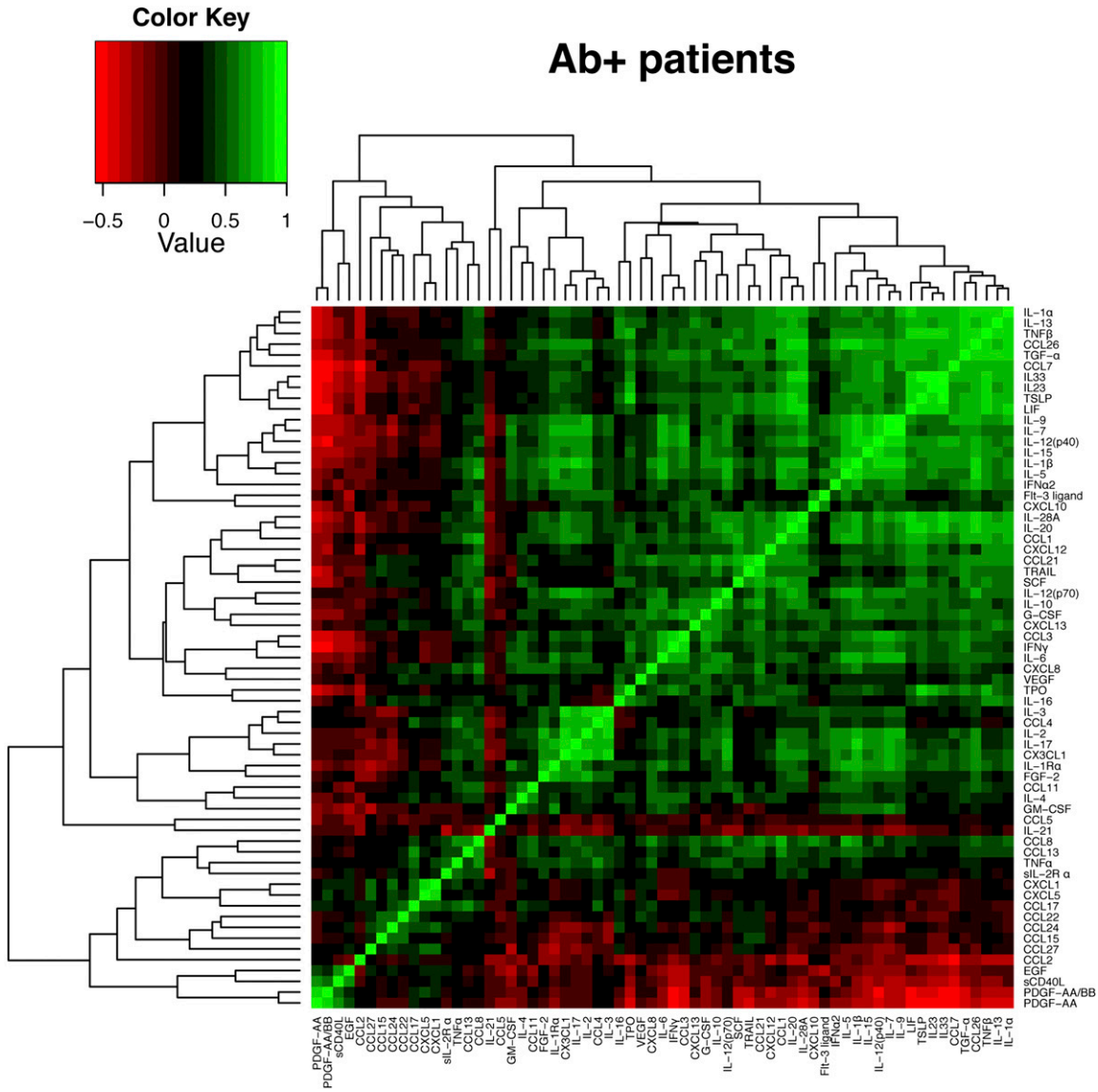

FIG. 2. Heat map and cluster analysis of cytokines in Ab$^+$ children. The heat map is a visual representation of the correlation values between each pair of cytokines, where the corresponding pair of cytokines are labeled at each row and column of the matrix. The matrix is symmetrical about the bottom-left to upper-right diagonal axis. Green represents positive correlation, black represents low correlation, and red represents negative correlation, as shown in the color key. Cytokines with highly correlated expression profiles are grouped together using hierarchical clustering, and the clusters are represented in the dendrogram. Many proinflammatory cytokines grouped together (e.g., the top cluster contains IFN-α2, IL-1β, IL-7, IL-15, IL-33, and LIF), as did many chemokines; however, anti-inflammatory cytokines and growth factors were distributed across different groups.

contribute in the early phase of β-cell damage. IL-12, secreted by macrophages and dendritic cells, activates Th1 CD4$^+$ cells and promotes the effector functions of CD8$^+$ T cells and NK cells (27,28). It stimulates IFN-γ and TNF-α production and is a key cytokine in the inflammatory destruction of β-cells (29). In contrast to our findings, IL-12 was not elevated in patients with multiple islet Ab (3). Given that our population is younger and earlier in the disease course, we speculate that IL-12 may contribute to induction of autoimmunity rather than its progression.

IL-16, which recruits and activates multiple immune cells, including T cells, monocytes, and dendritic cells, previously has not been associated with human type 1 diabetes; however, neutralization of IL-16 protected nonobese diabetic mice from developing autoimmune diabetes (30). IL-17 is the principal Th17 cytokine, causing severe tissue inflammation and inducing expression of other cytokines, including IL-1β, GM-CSF, TNF-α (also elevated in our population), IL-6, and chemokines, such as CXCL10 (31). The higher levels of IL-17 in Ab$^+$ children are in keeping with two studies showing increased IL-17 secretion by T cells in new-onset type 1 diabetes (15,32), suggesting an important role for this cytokine in disease pathogenesis resulting from its effects on inflammation and apoptosis.

IL-20, which belongs to the IL-10 family of cytokines, has been linked to other autoimmune diseases, such as rheumatoid arthritis (33). A genome-wide association study identified the IL-20 gene as a possible new candidate gene in type 1 diabetes (34); however, its role as a mediator of islet inflammation has not been examined. IL-28A, also elevated in Ab$^+$ children, inhibits viral replication, upregulates major

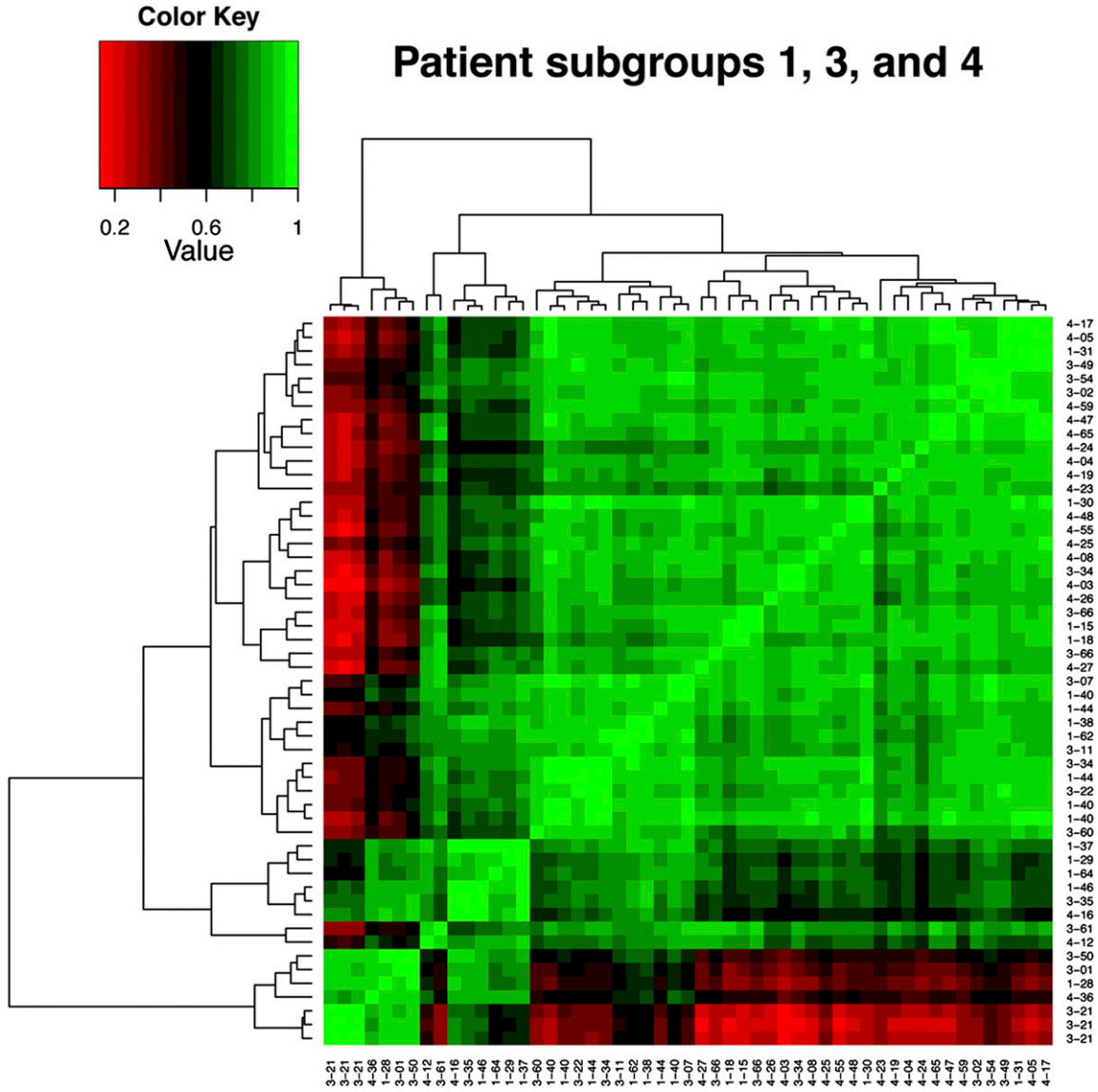

FIG. 3. Heat map and cluster analysis of cytokines in clinical samples. The heat map is a visual representation of the correlation values between each pair of samples denoted by the corresponding row and columns of the matrix. The label represents the subgroup (1: Ab$^+$/EV$^+$; 3: Ab$^+$/EV$^-$; 4: Ab$^-$/EV$^-$) and sample number; samples with EV infection (Ab$^-$/EV$^+$) were excluded. The matrix is symmetrical about the bottom-left to upper-right diagonal axis. Green represents positive correlation, black represents low correlation, and red represents negative correlation, as shown in the color key. Samples with correlated cytokine profiles are grouped together by hierarchical clustering, and the clusters are represented in the dendrogram. The figure shows that cytokine profiles have some capacity to distinguish Ab$^+$ vs. Ab$^-$ children but do not differentiate Ab$^+$ cases based on EV infection status.

histocompatibility complex I expression, and potentiates CD8$^+$ T-cell expansion (35). Our findings suggest these cytokines may contribute to the immunological network of type 1 diabetes, and their functional role in type 1 diabetes development warrants further investigation.

TNF-α stimulates production of other proinflammatory cytokines and can directly cause β-cell apoptosis through activation of various signaling pathways (2). Lower levels of TNF-α were found in children with newly diagnosed type 1 diabetes compared with healthy control subjects (36), suggesting an impaired immune response, whereas levels were higher in children at diabetes onset compared with high-risk children (4). In contrast, TNF-α levels were fourfold higher in Ab$^+$ versus Ab$^-$ children, supporting its pathogenic importance early in disease development.

It is of interest that Ab$^+$ children had elevated levels of two cytokines (IL-7 and GM-CSF) that may protect against type 1 diabetes (37). IL-7 and GM-CSF promote the survival and function of Treg cells via tolerogenic dendritic cells (20,21). Their elevation may reflect upregulation of Treg cells in response to islet inflammation in the early stages of prediabetes. They may also have proinflammatory actions. Furthermore, overexpression of IL-7 has been linked to the development of autoimmunity (38), and higher levels were found in children at diabetes onset (4).

Elevation of IL-21 is a novel finding in prediabetes in humans. Produced by activated CD4$^+$ T cells, IL-21 is a Th2/Th17 that counteracts the effects of Treg cells and is necessary for type 1 diabetes in NOD mice (39). It is interesting that IL-21 is an important factor in the control of persistent viral infections and may contribute to the CD4$^+$ T cell–mediated immune response to viral infections (40). In Ab$^+$ children, IL-21 was elevated (OR 2.4, P = 0.009); in

contrast, it was lower in EV^+ versus EV^- children, with some evidence for lower levels in Ab^+/EV^+ versus Ab^+/EV^- children ($P = 0.05$). It is clear that the role of this cytokine in EV-associated islet autoimmunity and type 1 diabetes requires further investigation.

IL-10 was the only other cytokine significantly elevated in EV^+ versus EV^- samples. IL-10 is an anti-inflammatory cytokine, which inhibits synthesis of Th1 and macrophage-derived cytokines and suppresses the activity of antigen-presenting cells (41). However, IL-10 augments Coxsackievirus B4–induced pancreatitis by disrupting Treg cell and effector T-cell responses in mouse models (42). We speculate that elevated IL-10 could play a role in persistent EV infection, leading to development of islet autoimmunity. We have previously shown that EV infection increases mRNA expression of IFN-γ, IFN-α, IFN-β, TNF-α, CXCL10, CCL5, and CCL2 in a rodent insulinoma cell line (43), and increased gene expression of proinflammatory cytokines and chemokines was observed in EV-infected human islets (44,45). However, these studies examined mRNA expression in vitro, whereas we evaluated serum cytokine concentrations in vivo; plasma levels may not be sensitive enough to detect changes in local cytokine production in the pancreatic islets.

When the samples were divided into four subgroups based on both Ab and EV infection status, the levels of 12 cytokines were significantly different (Fig. 1*A* and *B*), with evidence of a heightened proinflammatory cytokine response in nonvirally mediated autoimmunity. Cytokine expression profiles, in association with hierarchical clustering, classified samples belonging to subgroup 4 (Ab^-/EV^-), with 84% of samples clustered together, but it was not possible to separate patient samples from subgroups 1 (Ab^+/EV^+) or 3 (Ab^+/EV^-). Furthermore, the temporal relationship between EV infection and islet autoimmunity cannot be elucidated fully without examination of frequently obtained longitudinal samples. Alternatively, it is possible that the inflammatory process in the development of islet autoimmunity is similar, regardless of the environmental trigger. Of interest, duplicate samples in the hierarchical clusters tended to be in close proximity to each other (Fig. 3), suggesting repeatability of cytokine expression in the same individual.

There are several potential limitations to our findings. The study population included some children who may be at lower risk of progression to type 1 diabetes, including positivity for only one Ab and eight case subjects with transient Ab. However, after excluding the latter case subjects, the association with 14 of 15 cytokines remained. We did not exclude children with low-risk HLA genotypes, who may also be at lower risk of development of type 1 diabetes; however, the findings were consistent after adjusting for HLA genotype. Because we examined islet autoimmunity as the study outcome, the significance of these findings to development of type 1 diabetes is unclear. The selection of control subjects from the cohort of children with first-degree relatives with type 1 diabetes may have introduced bias, since genetic predisposition may influence cytokine levels (3); however, this would tend to reduce rather than augment any observed differences.

In conclusion, we have shown that children with islet autoimmunity demonstrate a marked and primarily proinflammatory cytokine profile, along with some immunoregulatory and anti-inflammatory effects (e.g., IL-20 and IL-28A). Nevertheless, the contributory role, if any, of many cytokines in the disease pathway remains to be defined. While in vitro experimental studies are required to clarify the mechanism of pathogenesis, more detailed longitudinal studies of individuals who progress to diabetes will aid in delineating the time course and significance of multiple cytokines and their interactions in the disease process. Such information may also assist in developing combination therapies targeting the immune response to prevent type 1 diabetes.

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W.-C.G.Y. analyzed data and wrote the manuscript. A.A.-S. performed laboratory testing and edited the manuscript. C.N.I.P. and M.R.W. performed hierarchical cluster analysis and edited the manuscript. J.C. coordinated study recruitment and sample collection. N.J.H. contributed to study recruitment. W.D.R. contributed to study design and edited the manuscript. M.E.C. was responsible for study design and recruitment, contributed to sample collection and data analysis, and reviewed and edited the manuscript. M.E.C. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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