# A type I interferon transcriptional signature precedes autoimmunity in children genetically at-risk of type 1 diabetes.

**Running title:** Type I interferon signature in preclinical T1D

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# ABSTRACT

Diagnosis of the autoimmune disease type 1 diabetes (T1D) is preceded by the appearance of circulating autoantibodies to pancreatic islets. However, almost nothing is known about events leading to this islet autoimmunity. Previous epidemiological and genetic data have associated viral infections and anti-viral type I interferon (IFN) immune response genes with T1D. Here, we first used DNA microarray analysis to define an IFN-inducible transcriptional signature in peripheral blood mononuclear cells from a group of active systemic lupus erythematosus patients (N=25) in order to investigate its expression in cohorts of healthy controls (N=87), T1D patients (N=64) and a large longitudinal birth cohort of children genetically predisposed to T1D (N=109; 454 microarrayed samples). Expression of the IFN signature was increased in genetically-predisposed children prior to the development of autoantibodies (*P*=0.0012), but not in established T1D patients. Upregulation of IFN-inducible genes was transient, temporally associated with a recent history of upper respiratory tract infections (*P*=0.0064) and marked by increased expression of the SIGLEC-1 (CD169) receptor by CD14$^+$ monocytes. DNA variation in IFN-inducible genes altered T1D risk (*P*=0.007), as exemplified by *IFIH1*, one of the genes in our IFN signature and which increased expression is a known risk factor. These findings identify transient increased expression of type I IFN genes in pre-clinical diabetes as a risk factor for autoimmunity in children with a genetic predisposition to T1D.

Type 1 diabetes (T1D) is an autoimmune disease characterized by the T-lymphocyte mediated destruction of the insulin-producing β cells in the pancreatic islets. Seroconversion to islet autoantibody positivity occurs in the first years of life, often many years before insulin dependence and disease diagnosis (1, 2). Although first characterized in the 1970's, islet autoantibodies remain the only established disease-specific markers. However, the precise mechanisms that lead to the break in tolerance and to infiltration of the pancreas by autoreactive T cells remain poorly understood in humans. Genetic studies have implicated type I interferon (IFN) signaling and antiviral immune responses in the etiology of T1D, through the association of several candidate genes in this biological pathway (*IFIH1*, *TLR7/TLR8*, *FUT2* and *GPR183*) (1, 3). These data support the hypothesis that viral infections could be an environmental factor for T1D (4, 5). Furthermore, IFN-α and increased HLA class I can be readily detected in the islet β cells of patients with T1D, which is consistent with a model involving direct cytotoxic T-cell killing of β cells in the pathogenesis of the disease (6, 7). These findings are remarkably consistent with the data from the spontaneous mouse model of diabetes (NOD), in which increased IFN production is directly involved in the pathogenesis of the disease (8, 9).

An IFN-inducible transcriptional signature, originally characterized in systemic lupus erythematosus (SLE) patients, and found to correlate with increased disease severity (10, 11) has now been identified in the peripheral blood of patients with other autoimmune and infectious diseases (12, 13). Here, we investigated the expression of an IFN signature in the onset of autoimmunity and progression to a clinical diagnosis of T1D in a large prospective birth cohort of children at high risk of developing T1D (14). Our findings provide novel evidence for the role of an exacerbated IFN response in the earlier stages of the autoimmune response in T1D and show for the first time that a disease-associated transcriptional signature can be detected in genetically-predisposed children prior to the development of autoantibodies.

# RESEARCH DESIGN AND METHODS

**Subjects.** All samples and information were collected with written and signed informed consent. Study participants included 49 recently-diagnosed T1D patients (disease duration <= 3 years); 15 adult long-standing T1D patients; 93 adult healthy volunteers; 25 adult SLE patients with active disease; and 109 children genetically predisposed to T1D enrolled in the BABYDIET study (14). All BABYDIET children were prospectively measured for four T1D-specific autoantibodies: IAA, GADA, IA2A and ZnT8, from age 3 months (further details on the BABYDIET study design and patient selection are provided in Supplementary Data).

Recently-diagnosed T1D patients were enrolled in the Diabetes-Genes, Autoimmunity and Prevention (D-GAP) study. The D-GAP study was approved by the Royal Free Hospital & Medical School research ethics committee; REC (08/H0720/25). Adult long-standing T1D patients and healthy volunteers were enrolled in the Cambridge BioResource (CBR; www.cambridgebioresource.org.uk). The study was approved by the local Peterborough and Fenland research ethics committee (05/Q0106/20).

SLE patients attended or were referred to the Addenbrooke's Hospital (UK) specialist vasculitis unit between July 2004 and May 2008 meeting at least four ACR SLE criteria (15). All presented with active disease and immunosuppressive therapy was commenced or increased. The SLE patients were recruited under the following two ethics applications: Cambridge/Hinxton Centre for translational research in autoimmune disease, approved Jan 23, 2004 by the Cambridge LREC (Ref: 04/023); Cambridge/Hinxton Centre for translational research in autoimmune disease 2, approved Jun 27, 2008 by the Cambridgeshire 3 REC (Ref: 08/H0306/21).

A workflow diagram of the study design is depicted in **Fig. 1** and baseline characteristics and demographics of all study participants and cohorts are summarized in **Fig. 2** and **Table 1.**

**PBMC preparation and RNA isolation**. Peripheral blood mononuclear cells (PBMCs) were isolated from whole-blood by density gradient centrifugation using Lympholyte

(Cedarlane). Total RNA was isolated from $10^6$ PBMCs using the phenol-chloroform method according to the manufacturer's instructions. RNA concentrations were measured by NanoDrop (Thermo Scientific) and RNA integrity was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). All RNA samples analysed in this study were isolated from PBMCs collected directly into TRIZOL reagent (Life Technologies) and stored at -80ºC within less than 24 hours of venepuncture to avoid alterations of the gene expression profile caused by freezing and resuscitation of viable PBMCs. The duration of storage of stable denatured PBMC lysates in TRIZOL prior to RNA isolation varied according to the sample cohort: (i) BABYDIET - median 90.4 months, min 57.1 months, max 136.9 months; (ii) CBR T1D patients - median 6.8 months, min 1.8 months, max 15.7 months; (iii) SLE patients - median 12.9 months, min 2.1 months, max 58.8 months). The investigators were blinded to sample group allocation during sample preparation.

**Ex-vivo stimulation of PBMCs.** Two million PBMCs from six adult healthy donors (see **Table 1**) were cultured in X-VIVO medium (Lonza) containing 1% heat-inactivated human AB serum (Sigma-Aldrich) in the presence or absence of IFN-β (100 U/ml; Peprotech) on flat-bottom 24-well plates (BD). Cells were harvested at 2, 6 and 18 h post-stimulation and were immediately stored in TRIZOL reagent (Life Technologies) at -80ºC. In addition, resting PBMCs from each donor (unstimulated; time 0 h) were also stored in TRIZOL reagent at -80ºC.

**cDNA synthesis microarray hybridization.** Single-stranded cDNA was synthesized from 200 ng total RNA using the Ambion whole-transcript expression kit (Ambion) according to the manufacturer's instructions. 3.44 µg cDNA was fragmented and labelled using the GeneChip terminal labelling and hybridization kit and hybridized to 96-sample Titan Affymetrix Human Gene 1.1 ST arrays, which provide comprehensive whole-transcriptome coverage with over 750,000 unique 25-mer oligonucleotide probes interrogating more than 28,000 annotated genes (median 22 probes/gene). All gene expression data generated in this study are deposited with ArrayExpress (http://www.ebi.ac.uk/arrayexpress/, accession no. E-MTAB-1724).

**PBMC immunostainings and flow cytometry.** Surface expression of SIGLEC-1, IL-15R,

PD-L1, TRAIL, CD69 and CD38 was measured in cryopreserved PBMCs from three T1D patients showing upregulated expression of IFN-inducible genes and three age- and sex-matched patients with low expression of the IFN-inducible genes. Aliquots of $5\times10^6$ cryopreserved PBMCs, from the same preparation used for microarray hybridization, were thawed as described previously (16) and were stained for 1 h at 4°C. The investigators were blinded to the sample group allocation during the experimental procedure and data analysis.

Expression of these six IFN-inducible surface proteins was also measured in fresh PBMCs from one healthy donor after in-vitro stimulation with IFN-α (10 ng/ml), IFN-β (10 ng/ml) (Peprotech) or culture medium (unstimulated) for 3, 6, 24, 72, 120 and 168 h. Cells were harvested at 3, 6, 24, 72, 120 and 168 h post-stimulation, and stained for 1 h at 4°C, as described previously (16).

Antibodies used in this study are summarized in **Supplementary Table 1**. Positivity for SIGLEC-1 was determined on the basis of the upper one percentile of the of the isotype control (PE-conjugated mouse IgG1k; BD Biosciences) immunostaining in the respective donor. Immunostained samples were analyzed using a BD Fortessa (BD Biosciences) flow cytometer with FACSDiva software (BD Biosciences). Flow cytometry data were exported in the format 3.0 and analyzed using FlowJo (Tree Star, Inc.). Doublet exclusion was performed for all assessed populations.

**Gene expression data analysis.** Data were summarized by exon-level probesets and normalized using variance stabilizing normalization (17), separately, for BABYDIET samples and other samples. In addition, we downloaded public microarray data from ArrayExpress as detailed in **Supplementary Data**.

Seventy of 524 BABYDIET hybridizations were omitted from the analysis based on large median normalized unscaled standard errors (suggestive of poor RNA quality) and principal component (PC) analysis indicating outliers. Of these, 62 samples belonged to a single batch, in which RNA isolation was compromised due to contaminated chloroform leading to inaccurate gene expression measurement in these samples. Hierarchical clustering was performed in R (http://www.R-project.org/) (18) using the function hclust and PCs were used

to summarize gene expression signatures, either directly (SLE and independent datasets) or by projection onto PCs defined in SLE patients (T1D and control samples) as detailed in **Supplementary Data**. A sample's projection on the first PC (PC1), which correlated with upregulation of IFN-inducible genes, was used to quantify their IFN signature. The R package "wGSEA" (http://cran.r-project.org/web/packages/wgsea/index.html) was used to perform gene-set enrichment analysis to assess whether IFN-inducible genes were enriched for variants associated with T1D, compared to a control set of probesets generated by ten to one matching according to the coefficient of variation in the unstimulated (time 0 h) PBMC samples from the same donors. Probesets were assigned to genes using Ensembl (Version 67), and to capture potential regulatory variation, genic regions were extended by +/-200 kb centred on the most 5-prime transcriptional start site (19).

Paired and unpaired moderated t-tests (20) were used to test for expression differences at individual probesets between cells cultured in the presence or absence of IFN-β at each timepoint and between sub-groups of SLE patients, respectively. For longitudinal datasets we tested the association between the IFN signature and factors of interest by fitting a linear mixed model, using a random intercept to allow for within-individual correlation and including age and sex as covariates.

**Correlation between infectious events and the expression of the IFN signature in the BABYDIET cohort.** At each visit, parents completed a detailed questionnaire on their children's history of infections, fever and medication. Specifically, they were asked about fever, infectious symptoms (such as diarrhea, vomiting, constipation and allergies) and the name of administered pharmaceutical agents or their active ingredient with starting date and duration of infections and medication. Infectious disease was defined as an acute event according to the ICD-10 Code or by a symptom indicating an infectious genesis. Infectious events were assigned to a specific time interval by their date of onset. We defined three categories of infectious diseases: a) infections of the respiratory tract, ear, nose, throat and the eye (if inflammatory symptoms of the respiratory tract were reported), b) gastrointestinal infections (if the main symptoms were diarrhea and/or vomiting), c) other infections (e.g. with symptoms of skin or mucosa lesions). For the current analysis, gastrointestinal and other infections were considered as non-respiratory tract infections. Other disease events such as

allergies or accidents were not considered as infectious diseases. Separate infectious diseases of one category were defined as one infectious event if there were less than six days of potential remission between the respective infections, as these seemed likely to be caused by the same infectious agent. Infectious events that could be matched to microarray samples were included for analysis. Because we wanted to maximize the power to investigate if the expression of IFN-inducible genes was correlated with the occurrence a direct cause of the an infectious event, we only included in the analysis infectious events that were measured within 1.5 months of the microarray measurements. 48 microarray measurements were excluded from the analysis because they were taken more than 1.5 months after the infection report. Incidences of infections were subdivided according to whether they were respiratory or not. Samples with 2 or more recent infections were grouped as "2+". Counts of both non-respiratory and respiratory infections were fitted in a single model, including age, sex, and a random intercept to allow for within-individual correlation, and either type of infection was examined individually, stratifying by the count of the other infection.

# RESULTS

**Characterization of a type I IFN transcriptional signature in PBMCs.** We identified 1,111 probesets mapping to 225 unique genes that were differentially expressed (false discovery rate, FDR<0.001) at one or more time points in PBMCs isolated from six healthy donors in response to IFN-β stimulation for 2, 6 and 18 h (**Supplementary Table 2**). IFN-α and IFN-β, the two main type I IFN, are known to regulate a very similar set of genes. Expression of these 225 genes in PBMCs stimulated with IFN-α or IFN-β was strongly correlated in a public dataset (21) (**Supplementary Fig. 1**, ρ=0.86) and we therefore describe them as type I IFN-inducible. We then used p values from a GWAS of 7,514 cases and 9,045 controls (3) to examine the evidence for association between these genes and T1D. Three IFN-inducible genes, *IFIH1, CD69* and *IL2RA*, have been previously characterized as candidate causal genes in known T1D susceptibility loci (www.T1DBase.org). A gene-set enrichment analysis showed that variants near these 225 type I IFN-inducible genes showed stronger association with T1D than variants near a control set of genes (p=4x10$^{-5}$ overall, and p=0.007, excluding the MHC region).

An IFN-inducible transcriptional signature has been previously identified in whole-blood from SLE patients (10, 11). We clustered 25 SLE patients based on expression of the IFN-inducible probesets and found that patients clustered into two distinct groups, with 21 (84%) showing an upregulation of the IFN-inducible genes (**Fig. 3**). Increased expression was seen across the vast majority of IFN-inducible genes and was specific to these genes (**Supplementary Fig. 2** and **Supplementary Table 2**). Our summary IFN signature measure, represented by an individual's projection of the 225 identified IFN-inducible genes onto to the first principal component, explained over 60% of variation in expression and correlated strongly with increased expression of the IFN-inducible genes. An IFN signature can also be induced by treatment with recombinant IFN-β and infection, which we demonstrated using public data from multiple sclerosis (MS) patients and controls (22) (**Supplementary Fig. 3**) and two large cohorts of healthy donors followed longitudinally after vaccination against influenza (23) (**Supplementary Fig. 4**). Post vaccination, the IFN induction is rapid (seen within 24 hours) and transient, returning to baseline within 14 days.

**Expression of the IFN signature in pre-diabetes is transient and precedes**

**seroconversion.** We found evidence for an IFN signature in a subset of the 64 T1D patient and 87 healthy control samples (**Fig. 3**), but at a lower frequency than in SLE patients. While the T1D patient samples could clearly be separated into two groups (three samples showed strong upregulation of the IFN-inducible genes), there was no clear grouping of controls, and it is possible that IFN signature genes were not strongly upregulated in any control samples. Transient expression of the IFN signature was also observed in 454 longitudinal measurements from 109 unique BABYDIET children (**Supplementary Fig. 5**). In contrast to the SLE patients, there was no clear grouping of the samples (**Supplementary Fig. 6**). Instead, the pattern resembled that found in MS patients (22), with a large group of samples with relatively homogeneous expression, and a smaller group showing variable expression (**Supplementary Fig. 3**).

Compared with T1D patients, the expression of the IFN signature was increased in BABYDIET children, but we cannot separate the effects of disease stage and age in this comparison. Instead, we compared the 22 individuals who seroconverted during the course of the study to the 87 subjects who did not (**Fig. 2**), and observed that the mean expression of the IFN signature was highest in samples collected before seroconversion and lowest in those from children who did not seroconvert during the course of the study (**Fig. 4***A*; p=0.0012). The IFN signature in children was correlated with recent self-reported incidence of respiratory infections (p=0.0064; **Fig. 4***B*) but not with age, recent self-reported incidence of non-respiratory infections or fever (p=0.444, p=0.524 and p=0.478, respectively; **Supplementary Fig. 7**). Similarly, a correlation was observed only with respiratory infection when stratifying by non-respiratory infections, and not vice versa (**Supplementary Fig. 8**). We found no evidence for an effect of time of gluten exposure in the children's diet and the development of an IFN signature in peripheral blood (p=0.211, data not shown).

In order to obtain independent support for our results, we analysed published data from two autoantibody-positive children before clinical diagnosis of T1D, three children who developed T1D during the course of the study and three age-matched controls in a longitudinal birth cohort from Finland (24). Although the limited sample size precludes formal statistical analysis, visual inspection provides obvious support for our results in BABYDIET: expression of the IFN signature was again highest in samples before

seroconversion, lowest in control samples, and intermediate in samples taken post-seroconversion and from children with clinical T1D (**Fig. 4*C*** and **Supplementary Fig. 9**).

**SIGLEC-1 expression by monocytes marks the IFN signature.** We identified six genes encoding for surface proteins among the 225 IFN-inducible genes: *SIGLEC-1, IL15R, PD-L1, TRAIL, CD69* and *CD38*. We assessed the expression levels of these proteins on the major subsets of circulating leukocytes by flow cytometry in all three of the T1D patients in whom we found evidence for the upregulation of IFN-inducible genes (**Fig. 2**) and in three age- and sex-matched patients with low expression of these genes. Expression of SIGLEC-1 (CD169, sialoadhesin) was increased on the surface of $CD14^+$ monocytes of the three patients with an IFN signature (**Fig. 5*A***) and correlated strongly with the expression levels of IFN-inducible genes, defined by PC1 ($\rho$=0.88; **Fig. 5*B***). Furthermore, we found a strong correlation between the frequency of SIGLEC-1$^+$ $CD14^+$ monocytes and *SIGLEC-1* mRNA expression levels in PBMCs ($\rho$=0.91; **Fig. 5*C***) measured by microarray in these six donors, suggesting that mRNA expression in whole blood is a good surrogate for the surface expression of the protein on monocytes. In SLE, *SIGLEC-1* mRNA expression was on average 7.4 fold higher in the 21 IFN signature-positive patients than in the four IFN signature-negative patients ($p=1.6 \times 10^{-4}$; **Fig. 5*D***), which further supports the specificity of the expression of this protein in individuals with increased IFN signalling in the peripheral blood.

We found no evidence for differential expression of the other five surface markers in any of the assessed immune subsets 24-72 hours after stimulation. In contrast, SIGLEC-1 expression on monocytes increased linearly with IFN-$\alpha$ or IFN-$\beta$ stimulation up to 7 days of culture (**Fig. 6**), suggesting that expression of this protein is sustained for a long period of time following an IFN response.

# DISCUSSION

We have shown for the first time that expression of a type I IFN-inducible transcriptional signature is increased in the peripheral blood prior to the development of islet autoimmunity. The results from our study are supported by data from the accompanying study by Kallionpaa *et al* (25), which also reports increased expression of IFN-inducible genes in Finnish children at increased risk of T1D prior to the onset of autoimmunity and seroconversion. Together, these two independent datasets provide convincing evidence linking an activated innate immune response with the development of islet autoimmunity, which is currently the strongest known predictive risk factor for T1D. Other gene expression studies have implicated the IL-1B signaling pathway in the onset of clinical diabetes (26), and increased type I IFN signaling, oxidative phosphorylation (27) and a systematic suppression of the immune response (24) prior to the onset of the disease. However, to date, such studies have been limited in sample size, not reproduced independently, and have not been able to investigate transcriptional changes associated with the course of the disease, particularly in the earliest stages, preceding the first autoimmune manifestations, which remain poorly characterized. One of the greatest strengths of this study is the access to samples taken longitudinally from children before and after the first clinical signs of T1D. This has enabled us to depict not only the transient nature of IFN signature expression, but also to demonstrate the temporal correlation of the expression of the IFN signature with recent respiratory infections and its correlation with future seroconversion.


Increased expression of type I IFN is a normal response to both viral and bacterial infections and can occur in any healthy individual, and particularly in children, who are more likely to be exposed to viral infections. Comparison with a recent study of adult MS patients and controls (22) shows that the pattern of upregulation in MS, T1D, healthy controls and children at risk of T1D is distinct and often less extreme to that found in SLE, an autoimmune disease characterized by systemic and chronic inflammation. Instead, there is a range of expression of the signature, which in MS correlates with IFN treatment. Increased expression of the IFN signature could reflect a previous history of IFN responses but the pattern of expression suggests that individuals in the population may have variable sensitivities to IFN stimulation, with hypersensitive subjects being at higher risk of

developing a more pronounced type I IFN response. Our results also suggest that the frequency of circulating SIGLEC-1-expressing monocytes, which correlates with the presence of an IFN signature in peripheral blood, could be a marker of a recent anti-viral immune response. In support of this hypothesis, expression of SIGLEC-1 was previously shown to be increased in other diseases associated with chronic IFN signaling, such as SLE, systemic sclerosis and MS, and suggested to be a potential biomarker of disease activity (28-30).

The increased expression of SIGLEC-1 observed on monocytes from individuals with an IFN signature provides evidence for the secretion of at least some products of the IFN-inducible genes. Consistent with this observation, increased serum concentration of IFN-α in SLE patients has been well established (31, 32) and, more recently, this serum IFN-α is bioactive and increased in the serum of SLE patients with an active IFN signature (33). Furthermore, expression of the IFN receptor and phosphorylation of the respective downstream signaling molecule STAT1 have also been shown in MS patients treated with IFN-β (34). Although in this study, we were not able to validate the secretion of additional proteins encoded by IFN signature genes, these data suggest a strong direct correlation between this transcriptional signature and the secretion of the products encoded by the IFN-inducible genes.

In mice, activation of the innate IFN immune response has been associated with class switching of IgM antibodies to the IgG isotype (35) and to the development of pathogenic IgG autoantibodies in murine SLE (36). Similarly, it is plausible that an increased innate IFN immune response in pre-diabetic individuals can lead to the class switching of naturally occurring IgM autoantibodies into IgG islet-specific autoantibodies that characterise T1D patients. The average increase in IFN signature in children who subsequently seroconvert could reflect an increased frequency, duration or strength of IFN upregulation, or some combination thereof. The transient expression of the IFN signature shown here suggests the presence of flares of IFN signaling, which would be in agreement with a relapsing-remitting model of T1D etiology (37, 38).

There is a long-held belief that viral infection is an environmental factor in the etiology of T1D (4, 5). An increased incidence of respiratory infections during childhood has recently been shown to be associated with the development of islet autoimmunity in the BABYDIET study (5). Consistent with this observation, we observed an enrichment of samples from AAB+ children collected before seroconversion with 2 or more reported respiratory infections (p=0.0128; **Fig. 4B**). Upper tract respiratory infections are mainly caused by common viral infection targeting the upper respiratory tract and, therefore, can be used as a surrogate marker for a viral infection. In this study, in the same children, we found evidence for an increased expression of type I IFN-inducible genes in children with a recent history of respiratory infections, which supports the hypothesis that common viral infections could be a key environmental factor in T1D. One potential weakness of the study was the recording of respiratory infection incidence, which was self-reported in daily diaries. While the prospective nature of recording protects against recall bias, there may be variability between families in interpretation of symptoms. Further, data were aggregated into quarterly incidence before analysis, while our analysis of the influenza vaccination cohort suggests the upregulation of IFN-inducible genes is likely to persist for only days or weeks. Although neither factor can plausibly induce a spurious relationship with the IFN signature, together, they should dilute any relationship between infection and gene upregulation. In light of these limitations, we cannot infer a direct causal link between respiratory infections and seroconversion. This hypothesis will need to be validated in larger prospective studies, using more sensitive assays for the screening of specific viral infections, designed to investigate the occurrence of respiratory infections and the development of T1D-specific autoantibodies. Importantly, together with the data from Kallionpaa *et al* (25), our findings implicate heightened IFN responses, manifested by an IFN signature, in the development of islet autoimmunity. In addition, we also show evidence for a correlation (p=0.0064) between respiratory infections and increased IFN response and previously have shown a correlation between respiratory infections in early life and seroconversion (5). ~~Our results also agree with previously published work, which show that a type I IFN signature is induced by common respiratory infection (39, 40) and provide the first evidence that an IFN signature can be correlated with common viral infections in T1D.~~ We thus hypothesize that IFN upregulation could represent a mechanistic link for the increased risk of islet autoimmunity with increased incidence of respiratory infections, which is consistent with a recent report

establishing a pathogenic role for the enterovirus Coxsackievirus B1 in the induction of β-cell autoimmunity (41).

In support of this hypothesis, a recent study has characterized the whole blood transcriptional signature associated with three common viral respiratory infections: respiratory syncytial virus (RSV), influenza and human rhinovirus (42). Notably, there was a strong overlap between the genes that define the IFN signature described in our study and the three gene expression modules associated with IFN response that were found to be modulated upon viral infection (M1.2 = 83.33%, M3.4 = 84.09% and M5.12 = 57.45%; Supplementary Table 3), even though these modules were defined from whole blood RNA and our IFN signature was obtained from PBMCs. Interestingly, the transcriptional changes caused by RSV infection remained altered even after one month after acute infection (42), suggesting that virally induced IFN signatures may persist in the periphery for some time after the viral infection has been cleared. RSV is the most common cause of respiratory infections in children and may thus represent an important cause of chronic IFN signalling in humans, particularly in young children.

The expression of the IFN signature was strongest in BABYDIET children who went on to develop T1D-specific autoantibodies before seroconversion. Previous studies (24, 26) have failed to find statistical support for increased IFN signaling in patients after T1D diagnosis compared to healthy controls. This likely reflects both the relationship between upregulation of IFN-inducible genes and infection, which is more frequent in children and the inability to capture a transient transcriptional signature in a cross-sectional patient population. Nevertheless, the genetic association of T1D risk with polymorphisms near IFN-inducible genes and the previously reported genetic association with an IFN regulatory factor 7 (IRF7) driven inflammatory transcriptional network (iDIN) in monocytes (43) suggest an intriguing possibility: sensitivity to an anti-viral immune response could be genetically-determined, such that an increased sensitivity to initiate an overt IFN response predisposes to T1D. Such a model is supported by the established T1D candidate gene *IFIH1*, which is amongst the IFN-inducible genes we identified here and encodes the major receptor for viral double-stranded RNA. Its binding to RNA induces type I IFN production, which is a key

requirement for CD8^{+} T-cell responses (44, 45).  Furthermore, expression of IFIH1 (or MDA5) correlates with increased T1D risk (46).  However, even larger, longitudinal studies of adults and children will be required to properly address both these questions, such as TEDDY (http://teddy.epi.usf.edu/), which is already underway.

It is important to note that the transient nature of the IFN signature precludes at this time an obvious clinical application as a diagnostic biomarker of anti-islet autoantibody seroconversion or progression to T1D.  Nevertheless, our findings clearly implicate increased type I IFN signaling in the pathogenesis of T1D and advance our understanding of the interaction between genes and environmental risk factors in disease etiology.  Consistent with this hypothesis, in the NOD mouse model, IFN signaling has been shown to be critical for the initiation of the disease (8, 9). In this disease model, IFN-α production in the pancreas could be detected as early as 3 weeks, leading to the regulation of a IFN signaling network (9, 47), and preceded the recruitment of diabetogenic T cells to the pancreas. Interestingly, blockade of IFN signaling was sufficient to delay or even prevent disease onset in this model (8, 9). In humans, the detection of IFN-α in the pancreas of T1D patients has been well documented (7), and patients undergoing therapy with recombinant IFN-α/β for MS or hepatitis have been reported to be at increased risk of developing autoantibodies and secondary autoimmune diseases, such as SLE and T1D (48-50).  These paradoxical effects of IFN-β in MS underlie the complex nature of type I IFN signaling and highlight the immunoregulatory potential of these molecules, which can have both beneficial and detrimental effects *in vivo* (51-53). Although the mechanism for the therapeutic effect of IFN-β in MS is unclear, its role in promoting autoimmunity is thought to involve the activation of dendritic cells leading to increased uptake and cross-presentation of self-antigen from apoptotic cells, which is critical for the activation of autoreactive cytotoxic CD8^{+} T cells (54). In T1D, HLA class I hyper-expression in the pancreatic islet β cells in response to IFN signaling is a hallmark of disease (7).  These observations provide support for direct killing of β cells by cytotoxic CD8^{+} T cells via the recognition of HLA class I molecules with bound peptides from β-cell antigens on the β-cell surface by autoreactive T cell receptors.  In addition, a recent study indicates that IFN-α can inactivate T regulatory cell function (55), which based on genetic (1) and immunological (56) results, is a central pathway in the pathogenesis of T1D.

Our findings, and those reported in the accompanying study by Kallionpaa *et al* (25) implicate the activation of innate IFN immune pathways in the initiation of islet autoimmunity ~~and support the long-established hypothesis that viral infections could be major environmental risk factors in T1D~~. <u>They support the long-established hypothesis that viral infections are major environmental risk factors in T1D and suggest IFN upregulation as a mechanistic link.</u> Together these two independent longitudinal cohorts provide a unique insight into the pre-clinical stage of T1D and show for the first time that temporal changes in the expression of type I IFN-inducible genes are associated with the earliest stages of disease pathogenesis.

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## Author contributions

## Competing financial interests

The authors declare no competing financial interests.

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**FIGURE LEGENDS**

**Fig. 1. Workflow of the study's experimental design.** Workflow diagram depicting the different stages of the study design and main outcomes from each stage.

**Fig. 2. Summary diagram of the BABYDIET cohort.** Shown is a diagram representing the division of the 109 BABYDIET children and microarray samples according to seroconversion and progression to T1D. N, number of individuals; AAB, T1D-specific autoantibodies (see **Research Design and Methods** for details); MA, number of microarray measurements.

**Fig. 3. An IFN signature can be detected in peripheral blood of systemic lupus erythematosus (SLE) and type 1 diabetes (T1D) patients. (A)** Plots depicting the two first principal components (PC) obtained from the expression of the 1,111 IFN-inducible probesets defined in this study in SLE patients (n=25). The percentage of variance explained by each PC is shown on the respective axis. **(B)** Plots depicting the two first PC obtained from the projection of the expression of the IFN-inducible probesets in subjects from cross-sectional cohorts of T1D patients (n=64; left panel) and adult healthy controls (n=87; right panel) onto the PC axes defined in the analysis of the SLE patients (see **Research Design and Methods** for detail). The cross-sectional cohorts of T1D patients include 15 adult long-standing T1D patients (median 13 years since diagnosis; median age 31 years) enrolled from the CBR and 49 recently diagnosed patients (median 1.4 years since diagnosis; median age 12 years) recruited from the D-GAP study. Samples that show clear evidence of upregulation of IFN-inducible genes by hierarchical clustering are depicted in red.

**Fig. 4. Expression of the IFN signature is increased prior to seroconversion and correlates with a recent history of respiratory infections. (A)** A quantitative summary IFN signature score (IFN signature; depicted in the y-axis) was obtained by the individual's projection of the 225 identified IFN-inducible genes onto the first principal component defined in the SLE group. The IFN signature is shown for all 109 BABYDIET children (454 longitudinally microarrayed samples), stratified according to time to seroconversion, including 305 microarray measurements from 87 donors that did not seroconvert ("never"), 89 microarray measurements from 22 AAB+ donors taken before seroconverstion ("before

AAB+") and 60 microarray measurements from the same 22 AAB+ donors taken after seroconversion ("after AAB+").  The number of microarray measurements taken before and after seroconversion from each of the 22 AAB+ donors is depicted in **Supplementary Fig. 5**.  The p value represents a two-sided two degrees of freedom test comparing the average IFN signature of the three groups.  **(B)** IFN signature is shown for all 109 BABYDIET children, stratified according to number (0, 1 or 2+ of self-recorded episodes of respiratory infections in the three month period nearest to, and within 1.5 months of, the date of collection of the blood sample used for microarray analysis.  Samples from AAB+ children collected before seroconversion are marked in red.  The p value represents a two-sided one degree of freedom test treating Incidence as a quantitative variable and comparing the average IFN signature of the three groups using a linear mixed model.  **(C)** Expression of the IFN signature was measured in an independent longitudinal cohort comprised of two T1D-specific autoantibody-positive (AAB+) Finnish children (19 samples) compared to three T1D patients (25 samples) and three matched control children (16 samples). Samples from the AAB+ children were further stratified according to whether they were before (n=4) or after (n=15) seroconvertion (SC). The IFN signature in these samples was defined based on the expression of the 54 most discriminatory genes for the clustering of the SLE samples, shown in **Supplementary Table 2**.  n, number of microarray measurements in each group.


**Fig. 5.  SIGLEC-1$^+$ expression by CD14$^+$ monocytes is a marker of increased IFN responses.**  **(A)** Frequency of SIGLEC-1$^+$ CD14$^+$ monocytes was measured by flow cytometry in cryopreserved PBMCs from three T1D patients with increased expression of IFN-inducible genes (IFN+) and three age- and sex-matched T1D patients with low expression of IFN-inducible genes (IFN-).  Histograms for the isotype control immunostainings are depicted in grey immediately below the respective volunteer.  Positivity for SIGLEC-1 is defined on the basis of the upper one percentile of the respective isotype control (illustrated by the vertical black bar in one representative example).  The percentage of SIGLEC-1$^+$ CD14$^+$ monocytes is indicated for each volunteer.  **(B)** Correlation between the frequency of SIGLEC-1$^+$ CD14$^+$ monocytes and the expression of the IFN inducible genes IFN signature in peripheral blood of the same six T1D patients, as measured by the projection of the expression of the IFN-inducible genes of from each sample onto the first principal component (PC1) defined in the SLE group.  **(C)** Correlation between the frequency of SIGLEC-1$^+$ CD14$^+$ monocytes measured by flow cytometry and the normalized *SIGLEC-1* mRNA expression in PBMCs from the six assessed T1D patients, measured by DNA

microarray.  **(D)** Scatter plot (mean ± SD) depicting the normalised *SIGLEC-1* mRNA expression in PBMCs isolated from 25 SLE patients stratified by the presence (IFN+; depicted by red squares) or absence of an IFN signature (IFN-; depicted by blue circles).  The p value was calculated using a two-sided Wilcoxon test.  ρ, correlation coefficient.

**Fig. 6.  Time-course of the expression of six IFN-inducible proteins on the surface of CD14$^+$ monocytes.**  Surface expression of six IFN-inducible surface-proteins (SIGLEC-1, IL-15R, PD-L1, TRAIL, CD69 and CD38) was measured by flow cytometry on CD14$^+$ monocytes.  PBMCs isolated from a healthy donor were cultured with IFN-α (10 ng/ml; depicted by a red line), IFN-β (10 ng/ml; depicted by a blue line) or culture medium (unstimulated; depicted by a black line).  Protein expression was quantified on CD14$^+$ monocytes at 3 h, 6 h, 24 h, 72 h, 120 h and 168 h post-stimulation.  MFI, median fluorescence intensity.

# TABLES

**Table 1. Baseline characteristics of study participants**

| Cohort | Individuals (N) | MA (N) | Age (years) | | Male N (%) | Disease duration (years) | |
|---|---|---|---|---|---|---|---|
| | | | Median | Range | | Median | Range |
| T1D patients (D-GAP)[*] | 49 | 49 | 12 | 6-34 | 29 (59%) | 1.4 | 0-3 |
| T1D patients (CBR)[†] | 15 | 15 | 31 | 22-35 | 5 (33%) | 13 | 0-23 |
| T1D patients (Combined) | 64 | 64 | 13 | 6-35 | 34 (53%) | 1.7 | 0-23 |
| Healthy Controls (IFN-β stimulation, CBR)[‡] | 6 | 6 | 35 | 22-37 | 3 (50%) | N/A | N/A |
| Healthy Controls (IFN signature, CBR)[§] | 87 | 87 | 42 | 22-52 | 27 (31%) | N/A | N/A |
| SLE patients | 25 | 25 | 43 | 19-61 | 5 (20%) | N/A | N/A |
| BABYDIET[‖] | 109 | 454 | 1.5 | 0.2-9.1 | 45 (41%) | N/A | N/A |

*Additional information for BABYDIET*

| Seroconverters[¶] (N) | Median age of seroconversion (years) | Progressors to T1D (N) | Median age at diagnosis (years) |
|---|---|---|---|
| 22 | 2.1 | 9 | 6.2 |

Baseline characteristics for the study participants stratified by the study cohorts. [*]Newly diagnosed T1D patients (duration of disease <= 3 years) enrolled in the Diabetes - Genes, Autoimmunity and Prevention (D-GAP) study. [†]Long-standing adult T1D patients enrolled from the Cambridge BioResource (CBR). [‡]Healthy donors selected from the CBR for the IFN-β stimulation assay. [§]Healthy donors selected from the CBR for the microarray assays. [‖]BABYDIET is a prospective birth cohort of genetically-predisposed T1D children, with at least one first-degree relative diagnosed with T1D and carrier of the high-risk HLA-DRB1*03 and/or HLA-DRB1*04 alleles. [¶]BABYDIET children that developed at least one of four T1D-specific autoantibody persistently during the course of the study (ie. IAA, GAD, IA2A and ZnT8).  MA, microarray measurements; N/A, not applicable.