Gene Expression Analysis of the Pre-Diabetic Pancreas to Identify Pathogenic Mechanisms and Biomarkers of Type 1 Diabetes

Linda Yip*, Rebecca Fuhlbrigge, Reem Alkhataybeh and C. Garrison Fathman

Division of Immunology and Rheumatology, Department of Medicine, Stanford University, Stanford, CA, United States

Type 1 Diabetes (T1D) occurs as a result of the autoimmune destruction of pancreatic β-cells by self-reactive T cells. The etiology of this disease is complex and difficult to study due to a lack of disease-relevant tissues from pre-diabetic individuals. In this study, we performed gene expression analysis on human pancreas tissues obtained from the Network of Pancreatic Organ Donors with Diabetes (nPOD), and showed that 155 genes were differentially expressed by ≥2-fold in the pancreata of autoantibody-positive (AA+) at-risk individuals compared to healthy controls. Only 48 of these genes remained changed by ≥2-fold in the pancreata of established T1D patients. Pathway analysis of these genes showed a significant association with various immune pathways. We were able to validate the differential expression of eight disease-relevant genes by QPCR analysis: A significant upregulation of CADM2, and downregulation of TRPM5, CRH, PDK4, ANGPL4, CLEC4D, RSG16, and FCGR2B was confirmed in the pancreata of AA+ individuals versus controls. Studies have already implicated FCGR2B in the pathogenesis of disease in non-obese diabetic (NOD) mice. Here we showed that CADM2, TRPM5, PDK4, and ANGPL4 were similarly changed in the pancreata of pre-diabetic 12-week-old NOD mice compared to NOD.B10 controls, suggesting a possible role for these genes in the pathogenesis of both T1D and NOD disease. The loss of the leukocyte-specific gene, FCGR2B, in the pancreata of AA+ individuals, is particularly interesting, as it may serve as a potential whole blood biomarker of disease progression. To test this, we quantified FCGR2B expression in peripheral blood samples of T1D patients, and AA+ and AA- first-degree relatives of T1D patients enrolled in the TrialNet Pathway to Prevention study. We showed that FCGR2B was significantly reduced in the peripheral blood of AA+ individuals compared to AA- controls. Together, these findings demonstrate that gene expression analysis of pancreatic tissue and peripheral blood samples can be used to identify disease-relevant genes and pathways and potential biomarkers of disease progression in T1D.

Keywords: gene expression, type 1 diabetes, auto-antibody positive, non-obese diabetic mice, pancreas, biomarker, FCGR2B
INTRODUCTION

Type 1 Diabetes (T1D) results from the autoimmune and chronic destruction of pancreatic β-cells by self-reactive T cells. It is the most common autoimmune disease in children, affecting approximately 1 in 500 in the United States, and the incidence is growing each year (1). The etiology of this disease is complex, and involves a strong genetic component and a number of possible environmental triggers. Genome-wide association studies (GWAS) have identified >60 polymorphisms that contribute to the genetic susceptibility of T1D (2, 3) and various environmental triggers have been shown to play a role in disease onset (4–6). The pathogenesis of this disease, however, remains unclear due to the difficulty in identifying pre-diabetic individuals and in obtaining biological specimens from such individuals.

Animal models of T1D have been crucial in understanding the mechanisms involved in the pathogenesis of T1D, but there are notable differences in human disease development. In humans, β-cell destruction occurs months to years before the onset of hyperglycemia. Individuals are asymptomatic, and develop autoantibodies (AAs) against various islet antigens including glutamic acid decarboxylase (GAD), zinc transporter 8 (ZnT8), insulin, and islet antigen 2 (IA-2) during the pre-hyperglycemic stage of disease. The presence and number of AAs detected in the serum is currently the most reliable biomarker for assessing the disease risk. However, there is a need for additional early, robust and reproducible biomarkers of disease progression.

The pancreata of at-risk AA+ individuals are significantly smaller than those of healthy individuals (7, 8), and analysis of pancreas sections demonstrate increased CD4+ and CD11c+ immune cell infiltration in the exocrine pancreas of these individuals (9). The islets of at-risk individuals differ functionally and morphologically from healthy controls (10), but insulin is only detected in a small percentage of AA+ individuals (11, 12). In contrast, the islets of non-obese diabetic (NOD) mice, a well-established mouse model of T1D, develop large peri-insulitic lesions beginning at 4 weeks of age. Destructive insulitis occurs at ~12 weeks of age, and hyperglycemia follows at ~16 to 25 weeks of age (13).

We and others have shown that the pancreata and islets of rodent models of T1D differ from those of healthy controls, and that these differences are reflected in the gene expression profile during the progression of disease (13–15). Gene expression data support the upregulation of glucagon and insulin secretion, β-cell hyperactivation and/or ER stress playing a role during different stages of disease development (13–15). Currently, it is unclear if similar changes in gene expression are observed in the pancreata of pre-diabetic humans after seroconversion preceding hyperglycemia.

Human pancreatic tissues from healthy controls, AA+ and T1D patients were available through the Juvenile Diabetes Research Foundation Network for Pancreatic Organ Donors with Diabetes (JDRF nPOD; www.jdrfnpod.org). These samples are collected from deceased donors and processed under well-defined standard operating procedures. However, due to the high level of pancreatic ribonucleases and tissue recovery time, RNA degradation occurs (16–18). Nevertheless, studies have shown that the impact of RNA degradation on the assessment of gene expression by microarray analysis is minimal, and largely offset by biological differences between samples (19).

In this study, we obtained pancreas samples of at-risk AA+ individuals, T1D patients, and healthy controls from nPOD and performed gene expression analyses. We then compared the genes that were differentially expressed in pre-diabetic AA+ individuals with those that were differentially expressed in the pancrea of NOD mice. The overlapping genes may be pathogenic and play a role in the development of T1D and NOD disease. We identified a number of biologically relevant genes that were changed in the pancrea of AA+ individuals. One gene, in particular, is expressed in leukocytes and could serve as a potential biomarker of disease progression that may be monitored in blood samples of individuals with a genetic risk for developing T1D. This gene, FCGR2B, encodes the CD32B receptor, an inhibitory low affinity receptor for the Fc region of IgG complexes (20, 21). Changes to FCGR2B/CD32B expression have been associated with multiple autoimmune diseases in humans (22–25), and have been suggested to underlie the autoimmune susceptibility of several strains of mice, including NOD mice (26, 27).

In this study, we also examined the potential of FCGR2B as a candidate biomarker of disease progression, by comparing FCGR2B expression in peripheral blood RNA samples of T1D patients, AA+ individuals who later developed T1D (AA+ progressors), and AA-first-degree relatives of T1D patients (AA- FDRs) obtained from the National Institute of Health TrialNet repository.

MATERIALS AND METHODS

Human Samples

Pancreas

Human pancreas samples were obtained through the JDRF network for Pancreatic Organ Donors with Diabetes (nPOD). Patient information for the final samples used for this study is shown on Tables 1A and B, and standard operating procedures for tissue processing are available at https://www.jdrfnpod.org/for-investigators/standard-operating-procedures/. Tissues were obtained from cadaveric donors that died of various causes, and AA+ individuals were identified by autoantibody screening after death. Thus, the length of islet autoimmunity in AA+ individuals is unknown. The pancreatic tissues used for this study were minced, placed in cryovials containing RNAlater, incubated at room temperature for 15 min and then snap frozen and stored at -80°C until shipment. RNA was extracted as described below.

Whole Blood Samples

Whole blood RNA samples were provided by TrialNet. Samples from AA- FDRs (n=15) and AA+ FDRs who later progressed to hyperglycemia (AA+ progressors, n=20) were from individuals enrolled in the TrialNet Pathway to Prevention Study. Samples from T1D patients (n=10) were obtained from individuals.
### TABLE 1A | Patient information for nPOD pancreata samples.

| nPOD ID | Serum AA* | Region | Age† | Sex | BMI | Ethnicity | Cause of Death†† | C-peptide (ng/ml) | Pancreas Weight (g) | RNA RIN |
|---------|-----------|--------|------|-----|-----|-----------|-----------------|------------------|-------------------|---------|
| Normal Controls | | | | | | | |
| 6102 | Negative | body | 45 | F | 35.1 | Caucasian | CVS | 0.55 | 87.32 | 4.6 |
| 6172 | Negative | tail | 19 | F | 32.4 | Caucasian | CVS | 8.02 | 59.7 | 5.8 |
| 6179 | Negative | tail | 21 | F | 20.7 | Caucasian | HT | 2.74 | 72.4 | 5.2 |
| 6227 | Negative | body | 17 | F | 26.4 | Caucasian | CVS | 2.75 | 60.4 | 5.6 |
| 6229 | Negative | body | 31 | F | 26.9 | Caucasian | HT | 6.23 | 45.6 | 7 |
| 6234 | Negative | body | 20 | F | 25.6 | Caucasian | HT | 6.89 | 49.86 | 3.7 |
| 6253 | Negative | body | 19 | F | 34.3 | African American | HT | 7.22 | 102.31 | 5.3 |
| AA+ subjects | | | | | | | |
| 6027 | ZnT8A+ | tail | 18 | M | 19.9 | Caucasian | n/a | n/a | 52.9 | 4.4 |
| 6090 | GADA+ | head | 2 | M | 18.8 | Hispanic/Latino | HT | 5.34 | 19.3 | 5.5 |
| 6123 | GADA+ | head | 23 | F | 17.6 | Caucasian | HT | 2.01 | 48.34 | 7.6 |
| 6170 | GADA+ | head | 34 | F | 36.9 | African American | A | 4.29 | 110.3 | 7.6 |
| 6184 | GADA+ | body | 47 | F | 27 | Hispanic/Latino | HT | 3.42 | 71.8 | 4.4 |
| 6197 | GADA+ IA-2A+ | tail | 22 | M | 28.2 | African American | HT | 17.48 | 73.3 | 8.1 |
| Established T1D subjects | | | | | | | |
| 6088 | GADA+ IA-2A+ ZnT8A+ mIAA+ | head | 31 | M | 27 | Caucasian | HT | <0.05 | 32 | 5.5 |
| 6143 | IA-2A+ mIAA+ | head | 32 | F | 26.1 | Caucasian | A | <0.05 | 35.7 | 8.5 |
| 6148 | GADA+ mIAA+ | head | 17 | M | 23.9 | Caucasian | A | 0.06 | 26.36 | 5.0 |
| 6161 | IA-2A+ mIAA+ | head | 19 | F | 36.1 | Caucasian | CVS | <0.05 | 39.8 | 5.0 |
| 6180 | GADA+ IA-2A+ ZnT8A+ mIAA+ | body | 27 | M | 25.9 | Caucasian | HT | <0.05 | 36.7 | 4.0 |
| 6241 | mIAA+ | body | 33 | M | 18.4 | Caucasian | CVS | <0.05 | 24.92 | 8.0 |
| 6245 | GADA+ IA-2A+ | body | 22 | M | 23.2 | Caucasian | HT | <0.05 | 31.58 | 7.2 |
| 6258 | mIAA+ | body | 39 | F | 28.7 | Caucasian | HT | <0.05 | 32.56 | 5.4 |
| 6263 | Negative | body | 34 | M | 23.5 | Hispanic/Latino | CVS | 3.18 | 51.59 | 7.8 |
| 6266 | GADA+ IA-2A+ mIAA+ ZnT8A+ | body | 30 | M | 27.1 | Caucasian | A | <0.05 | 51.66 | 6.6 |

*GAD, Glutamic acid decarboxylase; IA2+, Insulnine-associated protein 2; mIAA+, micro-Insulin auto-antibody; ZnT8, Zinc transporter 8 as determined by nPOD. Methods used for AA+ detection are available at: http://www.jdrfnpod.org/for-investigators/standard-operating-procedures/.

†For T1D samples, years with T1D are indicated.
††Cause of Death: HT, Head trauma; CVS, Cerebrovascular/Stroke; A, Anoxia.
enrolled in the Glutamic Acid Decarboxylase (GAD) Vaccine trial. IRB approval was obtained at the institution where samples were collected. All participants provided written informed consent. Before use, all RNA samples were repurified using the Qiagen RNeasy mini kit. Briefly, 100 µl of RNA (<100 µg) was combined with 350 µl of buffer RLT, mixed, added to 250 µl ethanol, and applied to a RNeasy mini column to bind the RNA. The RNA was washed and eluted according to manufacturer’s instructions. Re-extracted RNA concentrations were measured by Nanodrop and RNA quality was determined by Bioanalyzer analysis (Agilent Technologies) and listed in Table 2.

Animals
Female NOD/LtJ (NOD) and NOD.B10Sn-H2b/J (NOD.B10) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were maintained under pathogen-free conditions at the Stanford School of Medicine Animal facility, according to institutional guidelines under approved protocols. Pancreas tissues were isolated from 12-week-old NOD and NOD.B10 mice (8 animals per group) and immediately homogenized in Trizol reagent on ice, and stored at -80°C. RNA was extracted as described below. See Supplementary Figure 1 for blood glucose levels, weight of mice, and bioanalyzer traces of RNA samples.

RNA Extraction From Pancreas Tissue
Total RNA was extracted using Trizol reagent and the Qiagen RNeasy mini kit, as previously described (28, 29). For human pancreas tissues, RNA was extracted from at least 14 samples per group (controls, AA+ and T1D). Pancreatic samples were thawed on ice, removed from RNAlater, and homogenized in Trizol reagent (100 mg tissue/ml Trizol) on ice, and stored at -80°C. RNA was extracted as described below. See Supplementary Figure 1 for blood glucose levels, weight of mice, and bioanalyzer traces of RNA samples.

TABLE 1B | HLA Information for nPOD pancreata samples.

| HLA Symbol | Description |
|------------|-------------|
| DRB1*03:01 | DRB1*04:01 DQA1*03:01, 05:01 DQB1*02:01, 03:01 (DR3, DR4) |
| DRB1*04:01 | 13:03 DQA1*01:01, 05:01 DQB1*03:03:01 (DR3) |
| DRB1*01:01 | HLA information not available for 6179, 6227, 6229, 6234 and 6253 |
| DRB1*08:01 | 01:11 DQA1*04:01, 05:01 DQB1*03:01:04:02 (DR4) |
| DRB1*04:01 | 13:03 DQA1*02:01, 03:01 DQB1*02:03:03:01 (DR4) |
| DRB1*04:04 | 07:04 DQA1*03:01, 03:01 DQB1*03:03:02:02 (DR4, DR4) |
| DRB1*01:01 | 03:02:07:01 DQA1*02:01, 04:01 DQB1*02:02:04:02 (DR3) |

AA+ subjects

| HLA Symbol | Description |
|------------|-------------|
| DRB1*03:01 | 01:15 DQA1*01:02, 05:01 DQB1*02:01, 06:02 (DR3) |
| DRB1*04:04 | 01:02, 03:01 DQB1*03:02:06:02 (DR3) |
| DRB1*01:01 | 08:11 DQA1*04:01, 05:01 DQB1*03:01:04:02 (DR4) |
| DRB1*04:01 | 13:03 DQA1*02:01, 03:01 DQB1*02:03:03:01 (DR4) |
| DRB1*04:04 | 07:04 DQA1*03:01, 03:01 DQB1*03:03:02:02 (DR4, DR4) |
| DRB1*03:02 | 07:01 DQA1*02:01, 04:01 DQB1*02:02:04:02 (DR3) |

Established T1D subjects

| HLA Symbol | Description |
|------------|-------------|
| DRB1*01:01 | 03:03 DQA1*01:01, 05:01 DQB1*02:01, 05:01 (DR3) |
| DRB1*03:01 | 04:04 DQA1*03:01, 05:01 DQB1*02:01, 03:01 (DR3, DR4) |
| DRB1*03:01 | 04:04 DQA1*03:01, 05:01 DQB1*02:01, 03:01 (DR3) |
| DRB1*04:01 | 07:01 DQA1*02:01, 03:01 DQB1*02:02:03:02 (DR4) |
| DRB1*01:01 | 03:01 DQA1*01:01, 05:01 DQB1*02:01, 05:01 (DR3) |

The aqueous phase was removed, mixed with an equal volume of 70% ethanol and bound to a Qiagen RNeasy mini-column. The column was washed and RNA was eluted according to manufacturer’s instructions. Total RNA concentrations were measured by Nanodrop and quality was determined by Bioanalyzer analysis (Agilent Technologies, Santa Clara, CA). Bioanalyzer traces for human and mouse samples are shown in Figure 1 and Supplementary Figure 1, respectively. The human samples with the highest RNA integrity numbers (RIN; ≥6 per group) were selected for gene expression analysis studies.

Microarray Analysis

Human Samples
One-color microarrays were performed by the Stanford Human Immune Monitoring Center to measure gene expression in individual pancreas samples of seven controls, six AA+, and 10 T1D patients (Table 1) using the SurePrint G3 Human GE 8x60K microarray kit (Agilent Technologies). One microgram of total RNA was labeled with Cy3 using the low RNA input fluorescence linear amplification kit (Agilent Technologies). Hybridization was performed using the gene expression hybridization kit (Agilent Technologies) and the microarray hybridization chamber (Agilent Technologies), according to the manufacturer. Microarray chips were scanned using the DNA microarray scanner (Agilent Technologies) and data were processed with Feature Extraction Software (Agilent Technologies), and analyzed using GeneSpring GX 12.6.1 Software (Agilent Technologies). All microarray data have been submitted to the Gene Expression Omnibus (GEO) Database at NCBI (GEO series accession number: GSE72492), and are accessible at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72492. Samples were filtered for entities that were detected in at least six out of 23 samples, and entities with annotated gene symbols. Because of the gender imbalance between the groups, genes that were significantly changed by ≥5 fold between male and female subjects were removed from the analysis (Supplementary Table 1). A final list of 29,438 entities was used for further analysis. Moderated T-tests were performed to identify differentially expressed genes (p<0.05), and pathway analysis of differentially expressed genes was performed using Ingenuity Pathway Analysis (IPA; Qiagen Inc; https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis).

Mouse Samples
Two-color microarrays were performed to measure gene expression in the pancreas of individual 12-week-old NOD mice against a pool of age-matched NOD.B10 mice (n=8 animals per group) using the Whole Mouse Genome Microarray Kit, 4x44K 2-color arrays (Agilent Technologies) as previously described (29). Data were analyzed using GeneSpring (version 12.6.1, Agilent Technologies). Samples were filtered for entities with annotated gene symbols that are expressed (minimum raw expression ≥ 25) in at least four of the eight NOD samples, have equivalent human homologues, and are expressed in the human pancreas samples. The resultant 25,283 entities were analyzed. Data are available at GEO (Series accession number: GSE154739, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154739). T-test and Benjamini-Hochberg multiple testing correction were performed
### TABLE 2 | Patient information for TrialNet whole blood RNA samples.

#### AA- First Degree Relatives

| ID | HLA (DRB1:DQA1:DQB1)* | Serum AA† | Age | Sex | Years to Onset | RNA RIN |
|----|------------------------|-----------|-----|-----|---------------|--------|
| 1  | HLAa: 1401:0101:0503, HLAB: 1102:0501:0201 | Negative | 13  | F   | –             | 8.1    |
| 2  | HLAa: 1501:0102:0602, HLAB: 1301:0103:0603 | Negative | 9   | F   | –             | 8.5    |
| 3  | HLAa: 0701:0201:0202, HLAB: 0401:0301:0302 (DR4) | Negative | 15  | M   | –             | 8.2    |
| 4  | HLAa: 1201:0501:0301, HLAB: 1302:0501:0301 | Negative | 18  | M   | –             | 7.8    |
| 5  | HLAa: 0101:0101:0501, HLAB: 0102:0101:0501 | Negative | 12  | F   | –             | 7.5    |
| 6  | HLAa: 0401:0301:0302 (DR4), HLAB: 0701:0201:0202 | Negative | 18  | F   | –             | 8.3    |
| 7  | No data | Negative | 24  | F   | –             | 5.6    |
| 8  | HLAa: 0701:0201:0202, HLAB: 0401:0301:0301 (DR4) | Negative | 17  | M   | –             | 7.1    |
| 9  | HLAa: 0701:0201:0202, HLAB: 0701:0201:0202 | Negative | 7   | F   | –             | 8.1    |
| 10 | HLAa: 0401:0301:0302 (DR4), HLAB: 0301:0501:0201 (DR3) | Negative | 12  | M   | –             | 7.8    |
| 11 | HLAa: 0408:0301:0301, HLAB: 0301:0501:0201 (DR3) | Negative | 45  | M   | –             | 6.2    |
| 12 | No data | Negative | 34  | M   | –             | 7.7    |
| 13 | HLAa: 1302:0102:0604, HLAB: 0101:0101:0502 | Negative | 9   | M   | –             | 8.4    |
| 14 | HLAa: 1104:0501:0301, HLAB: 0301:0501:0201 (DR3) | Negative | 43  | F   | –             | 7.4    |
| 15 | HLAa: 0401:0301:0301 (DR4), HLAB: 0401:0301:0302 (DR4) | Negative | 10  | M   | –             | 8.8    |

Average ± SE: **19.1 ± 3.1** - **7.7 ± 0.2**

#### AA+ First Degree Relatives that later progressed to T1D

| ID | HLA (DRB1:DQA1:DQB1)* | Serum AA† | Age | Sex | Years to Onset | RNA RIN |
|----|------------------------|-----------|-----|-----|---------------|--------|
| 1  | HLAa: 0101:0101:0501, HLAB: 0401:0301:0302 (DR4) | ICA512+ | 5.5  | M   | 3.0           | 7.1    |
| 2  | HLAa: 0301:0501:0201 (DR3), HLAB: 0404:0301:0302 (DR4) | GAD65+ | 43.6 | F   | 3.9           | 8.5    |
| 3  | HLAa: 0301:0501:0201 (DR3), HLAB: 0401:0301:0302 (DR4) | GAD65+ | 11.8 | M   | 2.1           | 9.3    |
| 4  | HLAa: 0401:0301:0302 (DR4), HLAB: 0401:0301:0302 (DR4) | ICA+ | 23.4  | M | 1.8 | 9.2 |
| 5  | HLAa: 0401:0301:0302 (DR4), HLAB: 0404:0301:0302 (DR4) | GAD65+ | 40.6 | F | 3.7 | 8.8 |
| 6  | HLAa: 0404:0301:0302 (DR4), HLAB: 0801:0401:0402 | GAD65+ GAD65H+ | 10.0 | M | 3.3 | 9.4 |
| 7  | HLAa: 0301:0501:0201 (DR3), HLAB: 1501:0102:0602 | ICA512+ | 13.5 | M | 2.6 | 6.1 |
| 8  | HLAa: 0301:0501:0201 (DR3), HLAB: 0301:0501:0201 (DR3) | GAD65+ ICA+ | 41.7 | F | 3.0 | 7.0 |
| 9  | HLAa: 0901:0301:0303, HLAB: 0301:0501:0201 (DR3) | GAD65+ GAD65H+ | 50.0 | M | 1.1 | 7.2 |
| 10 | No data | GAD65+ | 4.9  | F   | 2.0           | 10     |
| 11 | HLAa: 0401:0301:0302 (DR4), HLAB: 0801:0401:0402 | GAD65+ ICA+ | 8.3  | M | 3.8 | 9.8 |
| 12 | No data | GAD65+ | 12.6 | F | 3.4 | 9.7 |
| 13 | HLAa: 0401:0301:0302 (DR4), HLAB: 0301:0501:0201 (DR3) | GAD65+ ICA+ | 11.4 | M | 2.4 | 9 |
| 14 | HLAa: 0301:0501:0201 (DR3), HLAB: 0404:0301:0302 (DR4) | GAD65+ ICA+ | 8.5  | M | 5.5 | 8.7 |
| 15 | HLAa: 0301:0501:0201 (DR3), HLAB: 1302:0102:0604 | GAD65+ | 45.5 | M | 3.1 | 8.7 |

(Continued)
to identify differentially expressed genes in the pancreata of NOD vs. NOD.B10 mice with a fold change ≥2-fold (corrected \(p<0.01\)).

**Validation by QPCR**

We identified a number of biologically relevant genes among those that were changed in the pancreata of AA+ individuals vs. controls. These include genes that have previously been associated with T1D, T2D, metabolism or obesity, and genes with biological functions that may be involved in the pathogenesis of T1D. These genes and their significance are listed in Table 3.

QPCR was performed to validate differential gene expression. cDNA was synthesized and QPCR was performed as previously described (28, 29). Briefly, first strand cDNA was generated from 1 μg of total RNA using Superscript III (Invitrogen) and random hexamers according to manufacturer’s instructions. QPCR was performed to measure human INS, BCL2L15, ERAP1, CADM2, ERAP1, ETV5, KANK1, SIM1, ABCB9, PGC, CEACAM6, CALB1, S100B, SMPD3, IL33, SCIMP, SEZ6L, PLD1, GNYL, CRTAM, CD180, IL4R, PLXNA4, PDK4, FGFR2B, TRPM5, CRH, ANGPTL4, CLEC4D, RGS16, ICOSLG, CD19, CD20, CD11C, and housekeeping genes GAPDH, ACTB, B2M, and 18S rRNA using commercially available Taqman gene expression assays (Applied Biosystems). cDNA was preamplified using the Taqman PreAmp Mastermix (Applied Biosystems) prior to QPCR for measurements that initially gave threshold cycle (Ct) values of >30. The 7900HT Fast Real Time PCR System (Applied Biosystems) and the Taqman Gene Expression Mastermix (Applied Biosystems) were used, according to manufacturer’s instructions. The comparative Ct method for relative quantification (\(ΔΔCt\)) was used. QPCR was also performed to measure the expression of FCGR2B, CD19, CD20, CD11C, and ACTB in whole blood RNA samples obtained from TrialNet. For these experiments, cDNA was synthesized (from 100 ng of total RNA) and QPCR was performed to described above.

**TABLE 2 | Continued**

| AA+ First Degree Relatives that later progressed to T1D | Serum AA* | Age | Sex | Years after Onset | RNA RIN |
|---|---|---|---|---|---|
| ID HLAA: 0103:0101:0501 HLAb: 0401:0301:0302 (DR4) | ICA512+ | 23.4 | M | 3.0 | 7.5 |
| ID HLAA: 0103:0101:0501 HLAb: 1101:0501:0301 | GAD65+ | 41.6 | F | 3.0 | 6.9 |
| ID HLAA: 0301:0501:0201 (DR3) HLAb: 0401:0301:0302 (DR4) | GAD65+ | 8.9 | F | 2.9 | 9.1 |
| ID HLAA: 0301:0501:0201 (DR3) HLAb: 0301:0501:0201 (DR3) | GAD65+ ICA+ | 6.4 | F | 3.3 | 8.4 |
| ID HLAA: 0301:0501:0201 (DR3) HLAb: 0101:0101:0501 | GAD65+ ICA+ | 24.7 | M | 3.3 | 7.5 |
| **Average ± SE:** | **21.7 ± 3.6** | **3.2±0.2** | **8.4 ± 0.2** |

**T1D subjects**

| ID HLAA: 1301:0103:0608 HLAb: 0901:0301:0303 | GAD65+ ICA512+ mIAA+ ICA+ | 23.2 | M | 2.3 | 7.4 |
| HLAA: 0401:0301:0302 (DR4) HLAb: 0103:0101:0501 | GAD65+ ICA512+ mIAA+ | 23.8 | F | 2.3 | 8.4 |
| HLAA: 0901:0301:0303 HLAb: 1201:0501:0301 | GAD65+ mIAAA+ | 26.8 | F | 2.3 | 7.8 |
| HLAA: 0404:0301:0302 (DR4) HLAb: 0404:0301:0302 (DR4) | GAD65+ ICA512+ mIAA+ | 13.1 | F | 2.3 | 8.5 |
| HLAA: 0404:0301:0302 (DR4) HLAb: 0405:0301:0201 (DR4) | GAD65+ ICA512+ mIAA+ | 40.8 | M | 2.2 | 8.6 |
| HLAA: 0401:0301:0302 (DR4) HLAb: 0101:0101:0501 | GAD65+ mIAA+ | 18.2 | F | 2.3 | 8.2 |
| HLAA: 0401:0301:0302 (DR4) HLAb: 0301:0501:0201 (DR3) | GAD65+ ICA512+ mIAA+ | 18.6 | M | 2.2 | 7.6 |
| HLAA: 0301:0501:0201 (DR3) HLAb: 1601:0102:0502 | GAD65+ mIAA+ | 14.7 | F | 2.2 | 7.6 |
| HLAA: 0401:0301:0302 (DR4) HLAb: 0301:0501:0201 (DR3) | ICA512+ mIAA+ ICA+ | 16.7 | F | 2.2 | 7.3 |
| HLAA: 0401:0301:0301 (DR4) HLAb: 0301:0501:0201 (DR3) | GAD65+ ICA512+ mIAA+ | 16.2 | M | 2.3 | 8.3 |
| **Average ± SE:** | **20.7 ± 2.5** | **2.8 ± 0.2** | **8.2 ± 0.2** |

*HLAα and HLAβ, HLA Haplotype α and HLA Haplotype β shown as DRB1:DQA1:DQB1. **GAD65 - GAD65 standard (TN local) assay; Positive >0.032, ICA512 - ICA512/IA2 standard (TN local) assay; Positive ≥10, GAD65H - GAD65 harmonized assay; Positive ≥20, IA-2H - ICA512/IA2 harmonized assay; Positive >5, MIAA - mIAA; Positive >0.1, ICA - Islet Cell Antibody; Positive >10. Serum autoantibody information were provided by TrialNet.
Statistical Analysis
Statistical analyses for QPCR experiments were performed using the two-tailed Mann-Whitney test (Prism 8, GraphPad Software Inc. San Diego, CA). A P-value of $\leq 0.05$ was considered significant.

RESULTS
Gene Expression Analysis in Human Pancreata Samples
Total RNA was initially isolated from $\geq 14$ subjects per group (healthy controls, AA+ and T1D subjects). Bioanalyzer analysis showed considerable RNA degradation in some samples, with RIN (RNA integrity number) values ranging from 2.0 to 8.5 (Table 1, Figure 1). The majority of samples had a RIN $\geq 5$, and samples with RIN values of $\geq 3.7$ were used for microarray analysis (n=7 controls, 6 AA+, and 10 T1D samples). Microarray and QPCR results showed that the expression of housekeeping genes ACTB, GAPDH did not significantly differ between groups and did not strongly correlate with RIN values (Figure 2). As expected, INS (Insulin) expression was found to be significantly reduced in the pancreata of T1D patients compared to controls, and pre-diabetic AA+ individuals (Figure 2).

Microarray analysis was performed on 29,168 entities representing 20,813 genes that were expressed in at least six of the 23 human pancreata. Because of the gender imbalance between groups, we eliminated genes from our analysis that were significantly changed by $>5$-fold in male vs. female subjects (Supplementary Table 1). The expression of certain genes, however, may still be influenced by age or sex.

We showed that 155 genes (59 upregulated and 96 downregulated) were changed by $\geq 2$-fold in the pancreata of AA+ compared to controls (Figure 3A; Supplementary Table 2), and 645 genes (252 genes upregulated and 393 downregulated) were changed by $\geq 2$-fold in the pancreata of T1D patients compared to controls (Supplementary Table 3). Among the 155 genes changed by $\geq 2$-fold in the pancreata of AA+ individuals (Figure 3A), 48 genes were similarly and significantly changed by $\geq 2$-fold in T1D patients (Figure 3B), 19 were significantly changed by $<2$-fold in T1D patients (Figure 3C), and 88 were not significantly changed in T1D patients (Figure 3D). Another 28 genes were found to be significantly changed by 1.5 to 2-fold in AA+ vs. controls, but $>2$-fold in T1D vs. controls (Figure 3E). These data indicate that 107 genes may be transiently changed after seroconversion, but no longer changed after the establishment of hyperglycemia, while 76 genes may be changed after seroconversion and during T1D. Heatmaps of gene expression for the 183 genes (represented in Figures 3A, E) are shown in Figure 3F (upregulated genes) and Figure 3G (downregulated genes).

Pathway Analysis of Differentially Expressed Genes in At-Risk AA+ vs. Controls
IPA pathway analysis was performed on the 183 genes that are differentially expressed in the pancreata of AA+ individuals compared to controls. A significant association of genes with various immune response functions was observed (Table 4A), with the strongest association seen with “function of helper T lymphocytes” ($p = 1.93x10^{-6}$). Upstream regulator analysis using IPA identified several cytokines including IL-1β that may drive the observed changes in gene expression (Table 4B). The top upstream regulator was found to be CNR1 (canniboid receptor 1,
**TABLE 3** | Biologically relevant genes that are significantly changed in the pancreata of AA+ vs. control (Microarray experiments).

**Genes associated with T1D, T2D, metabolism, or obesity**

| Gene     | Description                                    | Significance                                                                 | Reference                   |
|----------|------------------------------------------------|-----------------------------------------------------------------------------|------------------------------|
| CADM2    | cell adhesion molecule 2                      | SNP associated with risk of obesity, regulates insulin sensitivity            | Speliotes et al. (30);      |
|          |                                                |                                                                             | Dorapoo, 2012;               |
|          |                                                |                                                                             | Rathjen et al. (31);        |
|          |                                                |                                                                             | Yan, 2018                   |
| BCL2L15  | BCL2-like 15                                  | Gene located in a T1D-associated loci.                                      | T1Dbase.org                 |
| ERAP1    | endoplasmic reticulum aminopeptidase 1        | Gene located in a T1D-associated loci.                                      | Fung et al. (32)            |
| ETV5     | ets variant 5                                 | SNP located near ETV5 gene linked to obesity.                               | Thorleifsson et al. (33)    |
| KANK1    | KN motif and ankyrin repeat domains 1         | Variants associated with fasting proinsulin & insulinogenic index            | Huyghe et al. (34)          |
| SIM1     | single-minded family bHLH transcription factor | Gene in an obesity-associated loci.                                         | Meyre et al. (35)           |
| PLXNA4   | plexin A4                                     | Gene associated with T2D.                                                   | Saxena et al. (36)          |
| TRPM5    | transient receptor potential cation channel,   | TRPM5 variants associated with pre-diabetic phenotypes.                     | Briel et al. (37);          |
|          | subfamily M, member 5                         | Regulates insulin secretion and loss of expression leads to a pre-diabetic    | Ketterer et al. (39)        |
| ABCB9    | ATP-binding cassette, sub-family B, member 9   |                               |                              |
| PGC      | Progastricsin (pepsinogen C)                  |                               |                              |
| CEACAM6  | carcinoembryonic antigen-related cell adhesion | Involved in insulin homeostasis and T cell proliferation, and serves as a    | Kuespert, 2006              |
| molecul  | receptor for host-specific viruses and bacteria.|                               |                              |
| CALB1    | calbindin 1, 28kDa                            | Ovreexpression reduces glucose-stimulated insulin secretion by modulating    | Lee et al. (41)             |
| S100B    | S100 calcium binding protein B                | An inflammatory protein that serves as a ligand for the receptor of         | Chen et al. (42)            |
|          |                                                | advanced glycation end products that are involved in the development of      |                              |
|          |                                                | pathogenic T cells and apotosis of beta cells.                              |                              |
| SMPD3    | sphingomyelin phosphodiesterase 3, neutral    | Can mediate cellular responses to IL-1b and TNFa.                           | Rutkte et al. (43)          |
| membrane | membrane 33                                   | May promote the development of inflammatory autonimmune T cells in           | Milovanovic et al. (44)    |
| IL33     | interleukin 33                                 |                               |                              |
| ETV5     | ets variant 5                                 | Controls differentiation of TH17 cells.                                     | Pham et al. (45)            |
|          |                                                |                               |                              |
| CRH      | corticotropin releasing hormone               | expressed in beta cells. Plays a role in insulin secretion and beta cell    | Kanno et al. (46);          |
|          |                                                | proliferation.                                                             | Huising et al. (47)         |
| PDK4     | pyruvate dehydrogenase kinase, isozyme 4      | A key modulator of glucose homeostasis. Regulates expression of the          | Dateki et al. (48);         |
|          |                                                | transcription factor Pfkta.                                                 | Zhang, 2014                 |
| ANGPTL4  | angioptinen-like 4                            | Involved in glucose homeostasis, lipid metabolism, and insulin sensitivity. | Xu et al. (49);             |
|          |                                                | Regulates islet morphology.                                                 | Kim et al. (50)             |
| SCIMP    | SLP adaptor and CSK interacting membrane       | Expresed on B cells and involved in signal transduction after MHCII         | Draber et al. (51)          |
|          | protein                                       | stimulation.                                                               |                              |
| SEZ6L    | seizure related 6 homolog-like                | A substrate for BACE2, an inhibitor of beta cell proliferation.             | Stutzer et al. (52)         |
| ICOSLG   | inducible T-cell co-stimulator ligand         | Provides co-stimulation through ICOS, and may be involved in the           | Martin-Orozco et al. (53)   |
|          |                                                | expansion of activated Tregs.                                               |                              |
| PLD1     | phospholipase D1, phosphatidylinositol-specific| Phosphatidic acid formation on the granule membrane by PLD1 is essential   | Ma et al. (54)              |
|          |                                                | for glucose-stimulated insulin secretion.                                   |                              |
| TRPM5    | transient receptor potential cation channel,  | TRPM5 variants associated with pre-diabetic phenotypes.                     | Briel et al. (37);          |
|          | subfamily M, member 5                         | Regulates insulin secretion and loss of expression leads to a pre-diabetic   | Ketterer et al. (38)        |
| QNL1     | granulysin                                    | An antimicrobial peptide. Reduced QNL1 expression has been observed in the    | Jin et al. (55)             |
| CRTAM    | cytotoxic and regulatory T cell molecule      | Involved in establishing late phase T cell polarity, and allows increased   | Yeh et al. (56)             |
|          |                                                | production of IFNγ and IL22.                                                |                              |
| CD180    | CD180 molecule (RP105)                        | An important modulator of Toll-like receptor 4 signaling that is involved   | Divanovic et al. (57)       |
|          |                                                | in activating innate and adaptive immune responses.                        |                              |
| FCGR2B   | Fc fragment of IgG, low affinity Iib, receptor | Impaired FCGR2B function leads to abberant B cell activation and the         | Jiang et al. (26);          |
|          |                                                | development of autoimmunity. Reduced Fcgr2b linked to autoimmune susceptibility in NOD mice. | Xu et al. (27);             |
| CLEC4D   | C-type lectin domain family 4, member D       | Plays a non-redundant role in protecting against anti-mycobacterial and      | Wilson et al. (59)          |
| IL4R     | interleukin 4 receptor                        | IL4 can prevent autoimmune diabetes in rodent models of T1D.               | Ko et al. (59)              |
| RGS16    | regulator of G-protein signaling 16           | Key factor in G-protein mediated activation of lymphocytes. Plays a role in | Villasenor et al. (60);     |
|          |                                                | beta-cell development, proliferation, and insulin secretion.                | Vivot et al. (61)           |

The following references are included in Table 3 (26, 31–37, 39–46, 48, 50–59, 61).
also known as the CB1 receptor; $p < 2.55 \times 10^{-5}$), a receptor that has been linked to β-cell dysfunction and islet inflammation (62).

**QPCR Validation of Biologically Relevant Genes That Are Changed in AA+ Individuals**

Among the 183 genes that are significantly changed in the pancreata of AA+ individuals vs. controls, we identified 9 genes that are located in genetic loci that have previously been associated with T1D, T2D, metabolism or obesity, by GWAS studies. In addition, we identified another seven upregulated genes, and 15 downregulated genes, that have functions biologically relevant for disease pathogenesis (Table 3). The expression of these genes was verified by QPCR analysis. We were able to validate changes in eight out of 29 genes examined (Figure 4, Supplementary Figure 2, and Table 5). A significant upregulation of CADM2, and downregulation of TRPM5, CRH, PDK4, ANGPTL4, CLEC4D, RGS16, and FCGR2B was confirmed in the pancreata of AA+ vs. controls. Fold-change differences were higher when gene expression was measured by QPCR vs. microarray analysis. The QPCR cycle threshold (Ct) values for these genes are shown in Supplementary Table 5. RNA RIN values of the samples did not appear to influence the expression of these 8 genes when measured by microarray or QPCR analysis (Supplementary Figures 3 and 4).
Our inability to validate additional genes may be due to inherent differences in the quantification methods used. QPCR data is normalized to house-keeping gene expression and may be affected by the choice of house-keeping gene. The Taqman assays used for QPCR may also contain primers and probes that target a different region of the gene compared to the probes on the microarray. Thus, differential expression of gene transcripts or alternative splicing of genes could result in differences between microarray and QPCR data.

FIGURE 3 | Differentially expressed genes in the pancreata of AA+ individuals compared to controls. 155 genes (59 upregulated and 96 downregulated) were significantly changed by at least 2-fold in the pancreata of AA+ individuals vs. controls (A). Among these, 48 were changed by >2-fold (B), 19 were changed by <2-fold (C), and 88 were unchanged (D) in the pancreata of T1D patients vs. controls. An additional 28 genes were significantly changed by 1.5 to 2-fold in AA+ vs. controls and >2-fold in T1D vs. controls (E). Gene expression heatmaps of the upregulated (F) and downregulated (G) genes from panels A and E are shown. These genes were further analyzed for biological function (See Supplementary Table 2 and Table 3).
TABLE 4A | Immune functions associated with differentially expressed genes in AA+ vs. control identified by IPA.

| Diseases or Functions Annotation | p-Value* | Genes |
|---------------------------------|----------|-------|
| function of helper T lymphocytes | 1.93E-06 | CRTAM, DUSP1, ICOSLG, IL33, IL4R, NPY1R, RGS16 |
| proliferation of B-1 lymphocytes | 1.71E-04 | FCGR2B, IL33, IL4R |
| function of Th1 cells | 2.89E-04 | CRTAM, DUSP1, NPY1R, RGS16 |
| function of Th2 cells | 3.69E-04 | ICOSLG, IL33, IL4R, RGS16 |
| quantity of IgG2a | 8.03E-04 | CD180, FCGR2B, ICOSLG, IL4R, NPY1R |
| degranulation of leukocytes | 9.07E-04 | FCGR2B, PLD1, PSH, PTPNB |
| accumulation of Th2 cells | 1.30E-03 | IL33, RGS16 |
| function of T lymphocytes | 1.41E-03 | CRTAM, DUSP1, ERAP1, ICOSLG, IL33, IL4R, NPY1R, RGS16 |
| quantity of IgG2b | 1.75E-03 | CD180, FCGR2B, ICOSLG, NPY1R |
| induction of leukocytes | 2.44E-03 | CRH, IL33, IL4R, MBP |
| degranulation of mast cells | 3.14E-03 | CRH, DUSP1, FCGR2B, IL4R, PLD1 |
| quantity of interleukin | 3.97E-03 | CLEC4D, CRH, DUSP1, IL4R, NPY1R |
| stimulation of mast cells | 4.12E-03 | CRH, IL33 |
| Th2 immune response | 4.24E-03 | ICOSLG, IL33, IL4R |
| function of immune system | 4.68E-03 | CLEC4D, FCGR2B, ICOSLG, IL4R, NPY1R |
| activation of basophils | 6.07E-03 | FCGR2B, IL33 |
| function of blood cells | 6.67E-03 | CRH, CRTAM, DUSP1, ERAP1, FCGR2B, ICOSLG, IL33, IL4R, NPY1R, PLD1, RGS16, TRPM5 |
| induction of helper T cells | 6.80E-03 | IL33, MBP |
| accumulation of T lymphocytes | 6.82E-03 | DUSP1, ICOSLG, IL33, RGS16 |
| induction of lymphocytes | 7.28E-03 | CRH, IL33, MBP |
| activation of blood cells | 8.05E-03 | C10TIF1, CD180, CEACAM6, CRH, CRTAM, DUSP1, ERAP1, FCGR2B, GNLY, ICOSLG, IL33, IL4R, MBP, NPY1R |
| infiltration of cells | 8.45E-03 | CRH, DUSP1, EGLN3, FCGR2B, IL33, IL4R, IPX2, MBP, S100B |
| stimulation of phagocytes | 8.78E-03 | CRH, IL33, IL4R |
| function of leukocytes | 8.85E-03 | CRH, CRTAM, DUSP1, ERAP1, FCGR2B, ICOSLG, IL4R, NPY1R, RGS16, TRPM5 |
| quantity of IgG | 9.17E-03 | CD180, CLEC4D, FCGR2B, ICOSLG, IL4R, NPY1R |
| activation of leukocytes | 1.01E-02 | ICOSLG, IL33 |
| stimulation of leukocytes | 1.08E-02 | CRH, ICOSLG, IL33, IL4R, MBP |
| quantity of immunoglobulin | 1.09E-02 | CD180, CLEC4D, FCGR2B, ICOSLG, IL4R, NPY1R |
| quantity of dendritic cells | 1.25E-02 | CLEC4D, FCGR2B, GNLY, TRPM5 |
| hypersensitive reaction | 1.26E-02 | CLEC4D, DUSP1, FCGR2B, ICOSLG, IL33, IL4R, MBP, NPY1R, PDK4 |
| stimulation of lymphocytes | 1.35E-02 | CRH, ICOSLG, IL33, MBP |
| quantity of transitional B cells | 1.61E-02 | CD180, NPY1R |
| quantity of lymph follicle | 1.65E-02 | CD180, FCGR2B, ICOSLG, NPY1R |
| inflammation of pancreas | 1.72E-02 | FCGR2B, IL33 |
| maturation of bone marrow-derived dendritic cells | 1.72E-02 | FCGR2B, NPY1R |
| infiltration of leukocytes | 1.77E-02 | CRH, DUSP1, EGLN3, FCGR2B, IL33, IL4R, IPX2, S100B |
| delayed hypersensitive reaction | 1.77E-02 | CLEC4D, FCGR2B, IL4R, NPY1R |
| quantity of cytokine | 1.79E-02 | CLEC4D, CRH, DUSP1, IL4R, NPY1R, SIM1 |
| development of Th2 cells | 1.83E-02 | ICOSLG, IL4R |
| proliferation of mast cells | 1.83E-02 | FCGR2B, IL33 |
| quantity of IgA | 1.88E-02 | FCGR2B, ICOSLG, NPY1R |
| activation of lymphocytes | 1.98E-02 | CD180, CEACAM6, CRTAM, DUSP1, FCGR2B, ICOSLG, IL33, MBP, NPY1R |

*Performed using Fisher’s exact test to measure likelihood that the association between the dysregulated gene set and a related function is due to random association.

Among the genes that we validated by QPCR, the change in FCGR2B expression is particularly interesting. This gene has been shown to play a role in multiple autoimmune diseases and a role in the development of NOD disease (26). FCGR2B encodes the inhibitory CD32B receptor that is mainly expressed in B-cells (20). To examine if the change in FCGR2B expression may simply restate a change in the abundance of immune cells, we measured CD19, CD20 and CD11C expression, and found no significant difference in expression between controls, AA+ and T1D patients (Figure 5).

Comparison of Differentially Expressed Genes in At-Risk AA+ Individuals and Pre-Diabetic NOD Mice

To determine if similar genes are altered in the pancreata of AA+ individuals and pre-diabetic NOD mice, we performed microarray analysis on pancreas samples of 12-week-old NOD mice compared to age-matched congenic non-diabetes-prone NOD.B10 mice. Female NOD mice develop insulitic lesions starting at 4 weeks of age, and the onset of destructive insulitis occurs at 12 weeks age, followed by hyperglycemia at ~16 weeks of age (13). For these experiments, pancreatic tissues of 12-week-old NOD and NOD.B10 mice were immediately isolated and homogenized in Trizol reagent. RNA prepared from these samples showed little RNA degradation, with RIN values exceeding 8 for all samples (Figure S1). 25,283 entities representing 15,119 genes expressed in NOD pancreas samples overlap with genes on the human microarray. Among these, 2,043 entities representing 15,119 genes expressed in NOD pancreas samples overlap with genes on the human microarray. Among these, 2,043 entities representing 1880 genes were differentially expressed by at least 2-fold (p<0.01) in the pancreata of NOD vs. NOD.B10 mice. Only 12 genes were found to be similarly
changed in the pancreata of NOD mice and human AA+ individuals (Figure 6A). Interestingly 4 of the 12 genes were among those that we successfully validated by QPCR (Figure 4). We showed that Cadm2 was significantly increased while Trpm5, Pdk4 and Angptl4 were significantly reduced in the pancreata of NOD vs. NOD.B10 mice. No changes in housekeeping genes Actb and Gapdh were observed (Figure 6A). Crh was not present in the mouse microarray. The other three genes, Fcgr2b, Clec4d,
and Rgs16, that were reduced in the pancreata of AA+ individuals, were not changed in the pancreata of NOD mice (Figure 6B). These genes are abundantly expressed on B-cells, T-cells, DCs, and/or macrophages (20, 63–65). Thus their expression in the pancreas is impacted by the presence of immune cells in the insulitic lesion of NOD mice. Accordingly, we found significantly elevated expression of various leukocyte markers including Cd3e and CD28 (T-cell markers), Cd20 and B220 (B-cell markers), Cdi1c (Itgax; dendritic cell marker), and Cd11b (Itgam; macrophage marker) in the pancreata of NOD vs. NOD.B10 mice (Figure 6C).

**FCGR2B Gene Expression in Whole Blood Samples**

Since FCGR2B is highly expressed on B-cells, we asked whether the expression of this gene could serve as a potential biomarker of disease progression in peripheral blood cells. QPCR was performed to measure FCGR2B expression in whole blood samples of AA- first-degree relatives of T1D patients (AA- FDRs; n=15), AA+ FDRs who later progressed to hyperglycemia (AA+ progressors, n=20), and established T1D patients. Remarkably, we showed that FCGR2B expression was significantly lower in the whole blood of AA+ progressors and T1D patients compared to

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**TABLE 5 | Microarray and QPCR data for biologically relevant genes that are differentially expressed in the pancreata of AA+ and T1D patients vs. controls.**

**GWAS genes changed in AA+ vs. controls**

| Gene            | Microarray | QPCR          |
|-----------------|------------|---------------|
| Fold change     |            |               |
| AA+ vs. Controls|            |               |
| T1D vs. Controls|            |               |
| CADM2           | 3.1*       | 2.3*          |
| BCL2L15         | 2.7*       | 1.4           |
| ERAP1           | 2.1*       | 1.1           |
| ET5            | 1.8*       | 2.4*          |
| KANK1           | -2.5*      | -1.4          |
| SIM1            | -2.4*      | -2.2*         |
| PLXNA4          | -2.2*      | 1.2           |
| TRPM5           | -2.2*      | -1.8          |
| ABCB9           | -2.2*      | -2.1*         |

Biologically relevant genes: upregulated in AA+ vs. controls

| Gene            | Microarray | QPCR          |
|-----------------|------------|---------------|
| Fold change     |            |               |
| AA+ vs. Controls|            |               |
| T1D vs. Controls|            |               |
| PGC             | 4.8*       | 1.8           |
| CEACAM6         | 2.9*       | 1.9           |
| CALB1           | 2.6*       | 1.7           |
| ST00B           | 2.5*       | 2.7*          |
| SMPD3           | 2.3*       | 1.7           |
| ET5             | 1.8*       | 2.4*          |
| IL33            | 1.7*       | 2.9*          |

Biologically relevant genes: downregulated in AA+ vs. controls

| Gene            | Microarray | QPCR          |
|-----------------|------------|---------------|
| Fold change     |            |               |
| AA+ vs. Controls|            |               |
| T1D vs. Controls|            |               |
| CRH             | -4.2*      | -2.2          |
| PDK4            | -4.0*      | -1.6          |
| ANGPTL4         | -3.6*      | -2.6*         |
| SCIMP           | -3.6*      | -2.0          |
| SEZ6L           | -3.0*      | -1.9          |
| ICOSLG          | -2.6*      | -1.6          |
| PLD1            | -2.5*      | -1.4          |
| TRPM5           | -2.2*      | -1.6          |
| GNLY            | -2.2*      | 1.8           |
| CRTAM           | -2.2*      | 1.4           |
| CD180           | -2.2*      | 1.1           |
| FCGR2B          | -2.1*      | 1.2           |
| CLEC4D          | -2.1*      | -1.3          |
| IL4R            | -2.0*      | -1.9*         |
| RGS16           | -1.7*      | -2.4*         |

**FCGR2B Gene Expression in Whole Blood Samples**

Since FCGR2B is highly expressed on B-cells, we asked whether the expression of this gene could serve as a potential biomarker of disease progression in peripheral blood cells. QPCR was performed to measure FCGR2B expression in whole blood samples of AA- first-degree relatives of T1D patients (AA- FDRs; n=15), AA+ FDRs who later progressed to hyperglycemia (AA+ progressors, n=20), and established T1D patients. Remarkably, we showed that FCGR2B expression was significantly lower in the whole blood of AA+ progressors and T1D patients compared to
AA+ FDRs (Figure 7A). CD19, CD20, and CD11c expression was not changed in AA+ progressors, while CD19 and CD20 expression was significantly elevated in peripheral blood cells of T1D patients vs. AA+ FDRs (Figures 7B–D). When FCGR2B is expressed as a ratio of the B-cell markers CD19 or CD20, the differences observed between AA+ progressors and T1D vs. AA+ FDRs were more significant (Figures 7E, F). These data suggest that a loss of FCGR2B expression may occur in the peripheral blood, possibly in B-cells, during the development and progression of T1D.

DISCUSSION

A number of gene expression analysis studies have previously been performed to examine disease pathogenesis in T1D. The majority of studies, however, have been carried out using peripheral blood samples of AA+ at-risk and early onset T1D patients, while relatively few have been performed in the pancreata of these individuals, where insulitis, and islet destruction occur (66–72). This is partly due to the lack of pancreas samples available for study, and the difficulty in extracting high-quality RNA from these tissues. High levels of endogenous RNAses, DNases and proteases present in the pancreas result in the rapid autolysis of tissues following tissue damage, and lead to varying levels of RNA degradation (16, 73, 74). This is frequently observed in tissues collected post-mortem, when extensive tissue processing times are unavoidable. Pancreatic tissues collected from live individuals yield high quality RNA, but are difficult to obtain. In the Diabetes Virus Detection (DiViD) study, pancreatic biopsies were collected from live recent onset T1D patients, however, enrollment was halted when serious complications developed in 3 of the 6 individuals enrolled in the study (75, 76).

In this study, we utilized pancreatic tissue samples from JDRF nPOD for gene expression analysis. This repository contains one of the largest collections of tissues recovered from at-risk AA+ and T1D patients. Pancreatic tissues collected by nPOD are routinely minced and treated with RNAlater to increase RNA stability (73). The quality of the RNA extracted from pancreatic samples stored at nPOD has previously been assessed, and found to vary based on several factors including cause of death. Samples from head trauma victims had lower RIN values (18). This was also observed in our set of samples (Table 1). While appreciable levels of RNA degradation occurred in some of the samples, we were still able to identify a number of biologically relevant genes that were differentially expressed in AA+ and T1D patients compared to controls. Others have already shown that degraded RNA can be used successfully for transcriptome analysis and that the biological differences between groups outweigh the effects of RNA degradation on gene expression (19).

This study is the first to show that a distinct subset of genes is differentially expressed in the pancreata of at-risk AA+ individuals compared to T1D patients and to healthy controls. Of the 155 genes changed by >2-fold in the pancreata of AA+ individuals, only one-third remained changed in the pancreata of established T1D patients, reflecting the transient nature of events that are active during the early stages of disease development. After the establishment of hyperglycemia, the number of differentially expressed genes increased by ~4-fold to 645 genes. This includes genes such as INS that are lost as a result of β-cell death (Figure 2).

Changes in the gene expression profile of AA+ individuals reflect the activation of various immune pathways (Table 4A), and are consistent with the increased CD4+ and CD11c+ cell infiltration previously observed in the pancreata of AA+ patients (9). A number of cytokines including IL-1β, IL-10, TNSF13B (BAFF), TNF, and IL-6 were identified by IPA analysis to be upstream regulators of various differentially expressed genes (Table 4B). These cytokines have already been shown to play a role in the development of T1D (77–82).

To identify novel mechanisms that may contribute to disease pathogenesis, we focused on biologically relevant genes that were changed in the pancreata of AA+ individuals, and successfully validated the differential expression of 8 genes (CADM2, TRPM5, PDK4, ANGPTLA, CRH, CLECAD, RGS16, and FCGR2B) by QPCR. We also performed gene expression analysis on 12-week-old NOD mice, during the onset of destructive insulitis.
to identify genes or disease mechanisms that may be similarly changed in the pancreata of pre-diabetic NOD mice and “pre-diabetic” AA+ at-risk individuals. NOD disease shares many similarities with human T1D, however, there are striking differences in disease progression, especially in the pancreas where relatively mild insulitis is observed in humans, and large insulitic lesions and highly infiltrated islets are observed in NOD mice (83). As a result, the most differentially expressed genes observed in the pancreata of pre-diabetic NOD mice vs. NOD.B10 control mice are those expressed in the infiltrating leukocytes (See GEO series GSE154739).

We showed that only 12 genes were similarly changed in the pancreata of NOD mice and AA+ individuals (Figure 6A). Surprisingly, four of these genes were among the eight disease-relevant genes that we validated to be changed in the pancreata of AA+ individuals. These four genes, Cadm2, Trpm5, Pdk4, and Angptl, are not highly expressed in leukocytes and thus, their expression is not significantly impacted by the presence of infiltrating cells in the NOD pancreas. The other three genes validated to be reduced in the pancreata of AA+ individuals, Clec4d, Rgs16, and Fcgr2b, are abundantly expressed on leukocytes, thus, their expression in the NOD pancreas would be significantly influenced by the high level of insulitis. The Crh gene was not present on the mouse microarray.

Our data suggest that increased expression of CADM2, and reduced expression of TRPM5, PDK4, and/or ANGPTL4 could...
play a role in the development of both human T1D and NOD disease by altering mechanisms that control insulin sensitivity, insulin secretion, β-cell and pancreas development, and/or islet cell organization. The CADM2 gene encodes cell adhesion molecule 2, a mediator of synaptic signaling. GWAS studies have identified SNPs near CADM2 that are associated with higher expression of CADM2, and the development of obesity (30, 84). In animal models, increased Cadm2 expression is associated with obesity, while knockout or loss of Cadm2 prevents obesity, improves insulin sensitivity and protects mice from developing diabetes (31, 84). Interestingly, Cadm2 expression is reduced in obese and insulin-resistant mice by treatment with leptin (84), an adipose hormone that has been suggested as a potential treatment for T1D (85).

The TRPM5 gene, encodes the transient receptor potential cation channel, subfamily M, member 5, a calcium-activated non-selective channel expressed in β-cells that is an indispensable regulator of insulin secretion (86, 87). In humans, genetic variation within the TRPM5 locus associates with pre-diabetic phenotypes in subjects who are at risk for developing T2D (38). In mice, loss of Trpm5 expression results in compromised glucose-stimulated insulin secretion, and maintained elevated blood glucose levels following a glucose challenge (37). TRPM5 has also been shown to mediate insulin secretion by L-arginine and to potentiate glucose-induced insulin secretion by glucagon-like peptide 1 (88, 89).

The ANGPTL4 gene, encodes angiopoietin-like 4, a protein that is critically involved in maintaining normal glucose and lipid metabolism (49). In the islets, Angptl4 is expressed in glucagon-secreting alpha cells, and enhances glucose-stimulated insulin secretion. Mice deficient in Angptl4 have impaired glucose tolerance, secreting significantly lower levels of insulin in response to glucose-stimulation, and have dysmorphic islets with abnormally distributed alpha cells (50). Interestingly, we had previously observed disorganization of alpha cell distribution within the islets of 12-week-old NOD mice (14). It is possible that this may result from the significant loss of Angptl4 expression in the pancreas.

The PDK4 gene encodes pyruvate dehydrogenase kinase 4. In the pancreas, PDK4 may induce the expression of the pancreas-specific transcription factor 1a (PTF1A) (48). In mice, Ptf1a is involved in pancreatic development and function. Reduced Ptf1a levels leads to pancreatic hypoplasia and glucose intolerance. Ptf1a levels are a determinant of pancreatic size (90). This is interesting, as reduced pancreatic size has been observed in T1D patients, AA+ individuals, and AA- first-degree relatives of T1D patients (7, 8, 91, 92). Ptf1a also activates expression of Pdx1 (Pancreas/duodenum homeobox protein 1) (93, 94), an important transcription factor that regulates β-cell proliferation, differentiation, and function (95). It is possible that reduced levels of PDK4 lead to loss of PTF1A, and subsequently, a loss of PDX1 expression in the pancreata of AA+ individuals and NOD mice.

The CRH gene encodes corticotropin-releasing hormone, an important regulator of the hypothalamic-pituitary-adrenal (HPA) axis. In islets, CRH activates corticotropin-releasing hormone receptors on β-cells to stimulate insulin secretion, and promote islet development and proliferation (46, 47, 96). Reduced levels of CRH have been observed in the plasma of T2D patients (97). Reduced pancreatic expression of CRH may increase the risk for developing T1D in AA+ individuals.

We observed significantly reduced expression of 3 other genes, RGS16, CLEC4D, and FCGR2B, in the pancreata of AA+ individuals, but not in that of NOD mice. These genes are enriched in leukocytes, and loss of their expression may contribute to the development of T1D by promoting inflammation, activating T cells and B-cells, and altering β-cell development. The RGS16 gene encodes regulator of G-protein signaling 16, and is abundantly expressed in immune cells and pancreatic islets (61, 98–100). Increased RGS16 expression inhibits T cell migration in response to various chemokines, and downregulates pro-inflammatory cytokine production in monocytes (99, 100). In the pancreas, RGS16 is highly enriched in β-cells, where it is involved in the cell development and proliferation (60), and in the regulation of insulin secretion (61).

The CLEC4D gene encodes a C-type lectin mycobacterial receptor that is highly expressed in macrophages and myeloid cells. CLEC4D plays a crucial role in anti-mycobacterial host defense. In mice, loss of Clec4d expression results in elevated inflammation, increased mycobacterial levels, and increased mortality (58). In humans, a polymorphism in the CLEC4D gene that results in reduced expression, is associated with increased susceptibility to pulmonary tuberculosis (58). T1D patients have a reduced capacity to produce pro-inflammatory cytokines in response to Mycobacterium tuberculosis (101), and have a higher risk of developing tuberculosis (102). This may involve a loss of CLEC4D expression.

The FCGR2B gene encodes CD32B, an inhibitory low affinity receptor for the Fc region of IgG complexes that is highly expressed on B-cells. This receptor contains an immunoreceptor tyrosine-based inhibitory motif that negatively regulates the BCR complex and its signaling threshold (20). Activation of CD32B in B-cells ultimately leads to diminished BCR-dependent cell activation and antibody production, and loss of CD32B expression can lead to hyper-responsiveness, proliferation and maturation of B-cells. Diminished CD32B expression has already been observed in a number of autoimmune diseases. The autoimmune susceptibility of various strains of mice including NOD mice has also been attributed to polymorphisms in the FCGR2B gene that result in reduced CD32B expression in germinal center B-cells (26, 27). Loss of FCGR2B gene expression, and reduced surface expression of CD32B have been observed in activated B-cells of NOD mice, and human systemic lupus erythematosus patients (24–27), while a lower frequency of CD32B+ B-cells, and lower mean fluorescent intensity of CD32B expression have been observed in B-cells of rheumatoid arthritis patients compared to controls (23). Reduced FCGR2B gene expression has also been observed in the PBMCs of patients with active Graves’ disease (92).

Here, we showed that FCGR2B gene expression was significantly reduced in the pancreas and peripheral blood of AA+ progressors and T1D patients compared to controls.
CD20, and CD11c expression was not significantly changed in AA+ progressors. However, when FCGR2B is expressed relative to CD19 or CD20 expression, differences between the AA+ progressors vs. AA-FDRs were more significant. This was also observed in T1D patients, suggesting that FCGR2B expression may be diminished in B-cells of “pre-diabetic” AA+ individuals and T1D patients. There is abundant evidence suggesting a role for B-cells in T1D (103–106). A loss of CD32B expression may lead to deficient negative feedback regulation of the BCR resulting in intrinsic hyper-responsiveness, proliferation and maturation of B-cells, and contribute to the onset of T1D. Since significantly reduced expression of FCGR2B may be seen in AA+ individuals years before the onset of T1D (Table 2), this gene could potentially serve as an early biomarker of disease progression, and be used to predict disease onset. Flow cytometry experiments are currently in progress at our lab to study the expression of CD32B in longitudinal blood samples collected from AA+ individuals and healthy controls.

Together, our data show that different pathological events driven by changes in CADM2, TRPMS, PDK4, ANGPTLA, CRH, CLEC4D, RGS16, and FCGR2B gene expression may contribute to the development of T1D. These genes are involved in various functions including pancreatic development, β-cell proliferation, insulin sensitivity and secretion, inflammation, and/or immune cell function. Changes in the expression of these genes were mainly observed in the pancreata of AA+ individuals, during the active development of disease, and overlapped well with changes observed in the pancreata of pre-diabetic NOD mice. These findings emphasize the importance of utilizing tissues collected from pre-diabetic individuals, rather than established T1D patients, in understanding the pathogenesis of disease and in identifying early biomarkers of disease progression. Biorepositories such as nPOD and TrialNet play a crucial role in providing access to these rare and important samples.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE72492; https://www.ncbi.nlm.nih.gov/geo/, GSE154739.

ETHICS STATEMENT

The animal study was reviewed and approved by Stanford University Institutional Animal Care and Use Committee (IACUC).

AUTHOR CONTRIBUTIONS

LY performed the microarray and QPCR experiments, performed the data analysis, prepared the manuscript, and composed the figures and tables. RF performed RNA extraction, bioanalyzer, and QPCR analysis. RA performed QPCR experiments. CF and LY were involved in the planning and direction of this work. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.609271/full#supplementary-material
REFERENCES

1. Atkinson MA. The pathogenesis and natural history of type 1 diabetes. Cold Spring Harb Perspect Med (2012) 2. doi: 10.1101/chpmed.a007641

2. Concannon P, Rich SS, Nepom GT. Genetics of type 1A diabetes. N Engl J Med (2009) 360:1646–54. doi: 10.1056/NEJMra0808284

3. Noble JA, Erlich HA. Genetics of type 1 diabetes. Cold Spring Harb Perspect Med (2012) 2:a007732. doi: 10.1101/chpmed.a007732

4. Ronningen KS. Type 1 diabetes: prospective cohort studies for identification of the environmental trigger. Arch Immunol Ther Exp (Warsz) (2013) 61:459–68. doi: 10.1007/s00005-013-0247-9

5. Knip M, Simell O. Environmental triggers of type 1 diabetes. Cold Spring Harb Perspect Med (2012) 2:a007690. doi: 10.1101/chpmed.a007690

6. Barbeau WE. What is the key environmental trigger in type 1 diabetes—is it viruses, or wheat grain, or both? Autoimmun Rev (2012) 12:295–9. doi: 10.1016/j.autrev.2012.05.003

7. Campbell-Thompson M, Wasserfall C, Montgomery EL, Atkinson MA, Kaddis JS. Pancreas organ weight in individuals with disease-associated autoantibodies at risk for type 1 diabetes. JAMA (2012) 308:2337–9. doi: 10.1001/jama.2012.15008

8. Williams AJ, Thrower SL, Sequiños IM, Ward A, S Bickerton AS, Triay JM, et al. Pancreatic volume is reduced in adult patients with recently diagnosed type 1 diabetes. J Clin Endocrinol Metab (2012) 97:E2109–2113. doi: 10.1210/jc.2012-1815

9. Rodriguez-Calvo T, Ekelow O, Ammiranti N, Zapardiel Gonzalez J, van Herrath MG. Increased immune cell infiltration of the exocrine pancreas: a possible contribution to the pathogenesis of type 1 diabetes. Diabetes (2014) 63:3880–90. doi: 10.2337/db14-0549

10. Atkinson MA. Losing a grip on the notion of beta-cell specificity for immunity responses in type 1 diabetes: can we handle the truth? Diabetes (2014) 63:3572–4. doi: 10.2337/db14-1069

11. Campbell-Thompson M, Fu A, Kaddis JS, Wasserfall C, Schatz DA, Pugliese A, et al. Insulitis and beta-Cell Mass in the Natural History of Type 1 Diabetes. Diabetes (2016) 65:719–31. doi: 10.2337/db15-0779

12. Izt Veld P, Lievens D, De Grijs J, Ling Z, Van der Auwer A, Pipeleers-Marichal M, et al. Screening for insulitis in adult autoantibody-positive organ donors. Diabetes (2007) 56:2400–4. doi: 10.2337/db07-0416

13. Yip L, Fathman CG. Type 1 diabetes in mice and men: gene expression profiling to investigate disease pathogenesis. Immunol Rev (2014) 58:340–50. doi: 10.1007/s12026-014-0850-8

14. Yip L, Taylor C, Whiting CC, Fathman CG. Diminished adenosine A1 receptor expression in pancreatic alpha-cells may contribute to the pathology of type 1 diabetes. Diabetes (2013) 62:4208–19. doi: 10.2337/db13-0614

15. Prokopec KE, Prokopec K, Rhen DI, Serrano D, Wang GS, Patrick C, Morgan BS, Pare MF, et al. Changes in insulin, glucagon and ER stress precede immune activation in type 1 diabetes. J Endocrinol (2018) 239:181–95. doi: 10.1530/JEO-18-0328

16. Azevedo-Pouly AC, Elgamal OA, Schmittgen TD. RNA isolation from pancreatic lymph nodes during type 1 diabetes. Nat Immunol (2009) 10:1026–33. doi: 10.1038/ni.1773

17. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU, et al. Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet (2010) 42:937–48.

18. Rathjen T, Yan X, Kononenko NL, Ku MC, Song K, Ferrarese L, et al. Regulation of body weight and energy homeostasis by neuronal cell adhesion molecule 1. Nat Neurosci (2017) 20:1096–103. doi: 10.1038/nn.4590

19. Fung EY, Smyth DJ, Howson JM, Cooper JD, Walker NM, Stevens H, et al. Analysis of 17 autoimmune disease-associated variants in type 1 diabetes identifies 6q23/TNFAIP3 as a susceptibility locus. Genes Immun (2009) 10:1088–91. doi: 10.1038/genes.2008.99

20. Thorleifsson G, Walters GB, Guðjardóttir DS, Steinthorsdottir V, Sulem P, Helgadottir A, et al. Genome-wide association analysis in 6,486Danese at increased risk for type 2 diabetes. JAMA (2015) 313:2405–13. doi: 10.1001/jama.2015.7337

21. Saxena R, Saleheen D, Been LE, Garfiovo MP, Braun T, Bjønnes A, et al. Genome-wide association study identifies a novel locus contributing to type 2 diabetes susceptibility in Sikhs of Punjabi origin from India. Diabetes (2015) 64:1706–15. doi: 10.2337/db15-0677

22. Beezel LR, Montell-Peller Zoller MK, Ingrandbrett CS, Fleig A, Penner ER, Enklaar T, et al. TRPM5 regulates glucose-stimulated insulin secretion. Pflugers Arch (2010) 460:69–76. doi: 10.1007/s00424-010-0835-z

23. Ketterer C, Mussig K, Henn M, Duidiazi K, Randrianaivo E, Wagner R, et al. Genetic variation within the TRPM5 locus associates with pre-diabetic phenotypes in subjects increased risk rate for type 2 diabetes. Metabolism (2011) 60:1325–33. doi: 10.1016/j.metabol.2011.02.002

24. Harder MN, Appel EV, Grarup N, Sorensen ES, Jorgensen T, et al. The type 2 diabetes risk allele of TMEM154-rs6813195 associates with decreased beta cell function in a study of 6,486 Danes. PLoS One (2015) 10: e1012089. doi: 10.1371/journal.pone.0101289

25. Hassan MI, Toor A, Ahmad F. Progastriscin: structure, function, and its role in tumor progression. J Mol Cell Biol (2010) 2:2118–27. doi: 10.1093/jmcb/mjq001
41. Lee D, Obukhov AG, Shen Q, Liu Y, Dhaban P, Nowczky MC, et al. Calbindin-D28k decreases L-type calcium channel activity and modulates intracellular calcium homeostasis in response to K+ depolarization in a rat beta cell line RINm16-38. Cell Calcium (2006) 39:475–85. doi: 10.1016/j.cca.2006.01.010

42. Chen Y, Yan SS, Colgan J, Zhang HP, Luban J, Schmidt AM, et al. Blockade of late stages of autoimmune diabetes by inhibition of the receptor for advanced glycation end products. J Immunol (2004) 173:1399–405. doi: 10.4049/jimmunol.173.2.1399

43. Rutkute K, Karakashian AA, Giltiay NV, Dobierzewska A, Nikolova-Mileva I, Arsenijevic N, et al. IL-33/ST2 axis in inflammation and immunopathology. Mol Immunol (2012) 52:89–99. doi: 10.1016/j.molimm.2012.02.038

44. Milovanovic M, Volarevic V, Radosavljevic G, Jovanovic I, Pejnovic N, Arsenijevic N, et al. IL-33/ST2 axis in inflammation and immunopathology. Mol Immunol (2012) 52:89–99. doi: 10.1016/j.molimm.2012.02.038

45. Divanovic S, Trompette A, Petiniot LK, Allen JL, Flick LM, Belkaid Y, et al. Combined administration of plasmids encoding IL-4 and IL-10 prevents the development of autoimmune diabetes in nonobese diabetic mice. Mol Ther (2001) 4:431–6. doi: 10.1006/mthe.2001.0459

46. Villasenor A, Wang ZY, Rivera LB, Ocal A, Astorhelum JW, Scherer PE, et al. Rgs16 and Rgs8 in embryonic endocrine pancreas and mouse models of diabetes. Dis Model Mech (2010) 3:567–80. doi: 10.1245/dmm.003210

47. Vivot K, Moulle VS, Zarrouki B, Tremblay C, Mancini AD, Maechi H, et al. The regulator of G-protein signaling RGS16 promotes insulin secretion and beta-cell proliferation in rodent and human islets. Mol Metab (2016) 5:988–96. doi: 10.1016/j.molmet.2016.08.010

48. Gonzalez-Mariscal I, Montoro RA, Doyle ME, Liu QR, Rouse M, O’Connell JF, et al. Absence of cannabinoid 1 receptor in beta cells protects against high-fat/high-sugar diet-induced beta cell dysfunction and inflammation in murine islets. Diabetologia (2018) 61:1470–83. doi: 10.1007/s00125-018-4576-4

49. Graham LM, Gupta V, Schauer G, Reid DM, Kinberg M, Denney KM, et al. The C-type lectin receptor CLEC8F/S (CLEC4D) is expressed by myeloid cells and triggers cellular activation through Syk kinase. J Biol Chem (2017) 292:25964–74. doi: 10.1074/jbc.M117.816414

50. Shankar SP, Wilson MS, DiVietro JA, Mentink-Kane MM, Xie Z, Wynn TA, et al. RGS16 attenuates pulmonary Th2/Th17 inflammatory responses. J Immunol (2010) 188:6347–54. doi: 10.4049/jimmunol.1103781

51. Wang KL, Tai JJ, Wong WC, Han H, Sem X, Yeap WH, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood (2011) 118:e16–31. doi: 10.1182/blood-2010-12-326355

52. Klak M, Urban S, Gomolka M, Cichon J, Ambroziewicz F, Berman A, et al. Changes in Gene Expression of Selected Genes in Patients with Type 1 Diabetes and Pancreas Transplant in Peripheral Blood. Transplant Proc (2019) 51:2787–92. doi: 10.1016/j.transproceed.2019.04.086

53. Planas R, Carrillo J, Sanchez A, de Villa MC, Nunez F, Verdaguer J, et al. Gene expression profiles for the human pancreas and purified islets in type 1 diabetes: new findings at clinical onset and in long-standing diabetes. Clin Exp Immunol (2010) 159:23–44. doi: 10.1111/j.1365-2318.2009.04053.x

54. Richardson SJ, Rodriguez-Calvo T, Gerling JC, Mathews CE, Kaddis JS, Russell MA, et al. Islet cell hyperexpression of HLA class I antigens: a defining feature in type 1 diabetes. Diabetologia (2016) 59:2448–58. doi: 10.1007/s00125-016-4067-4

55. Padmos RC, Schloot NC, Beyan H, Ruwhof C, Staal FJ, Ridder D, et al. Distinct monocyte gene-expression profiles in autoimmune diabetes. Diabetes (2008) 57:2768–73. doi: 10.2338/db08-0496

56. Panarina M, Kisdan K, Alnek K, Heilman K, Peet A, Uibo R. Interferon and interferon-inducible gene activation in patients with type 1 diabetes. Scand J Immunol (2014) 80:283–92. doi: 10.1111/sij.12204

57. Han D, Cai X, Wen J, Matheson D, Skylar JS, Kenyon NS, et al. Innate and adaptive immune gene expression profiles as biomarkers in human type 1 diabetes. Clin Exp Immunol (2012) 170:131–8. doi: 10.1111/j.1365-2249.2012.04650.x

58. Han D, Levy A, Matheson D, Mineo D, Messinger S, Blomberg MB, et al. Immune profiling by multiple gene expression analysis in patients at-risk and with type 1 diabetes. Clin Immunol (2011) 139:290–301. doi: 10.1016/j.clim.2011.02.016

59. Jun E, Oh J, Lee S, Jun HR, Seo EH, Jang JY, et al. Method Optimization for Biobanking of human pancreas cancer tissue: impact of ex-vivo biobanking on gene expression. Frontiers in Endocrinology (2020) 11: 802. doi: 10.3389/fendo.2020.00802

60. Wilson GJ, Marakalalai MJ, Hoving JC, van Laarhoven A, Drummond RA, Kerscher B, et al. The C-type lectin receptor CLEC8F/S/CLEC4D is a key component of anti-CD1d expression on pancreatic beta cells, promotes beta cell proliferation. Cell Host Microbe (2015) 17:252–9. doi: 10.1016/j.chom.2015.01.004

61. Ko KS, Lee M, Koh JJ, Kim SW. Combined administration of plasmids encoding IL-4 and IL-10 prevents the development of autoimmune diabetes in nonobese diabetic mice. Mol Ther (2001) 4:431–6. doi: 10.1006/mthe.2001.0459

62. Villasenor A, Wang ZY, Rivera LB, Ocal A, Astorhelum JW, Scherer PE, et al. Rgs16 and Rgs8 in embryonic endocrine pancreas and mouse models of diabetes. Dis Model Mech (2010) 3:567–80. doi: 10.1245/dmm.003210

63. Krovgold L, Edwin B, Buenes T, Ludvigsson J, Korsgren O, Hyoty H, et al. Pancreatic biopsy by minimal tail resection in live adult patients at the onset of type 1 diabetes: experiences from the DVID study. Diabetologia (2014) 57:1341–3. doi: 10.1007/s00125-013-3155-y

64. Anquetil F, Sabouri S, Thivolet C, Rodriguez-Calvo T, Zapardiel-Gonzalo J, Amiran N, et al. Alpha cells, the main source of IL-1beta in human pancreas. J Autoimmun (2017) 81:68–73. doi: 10.1016/j.jaut.2017.03.006
Yip et al. Pre-Diabetic Pancreatic Gene Expression

78. Lu J, Liu J, Li L, Lan Y, Liang Y. Cytokines in type 1 diabetes: mechanisms of action and immunotherapeutic targets. *Clin Transl Immunol* (2020) 9:e1122. doi: 10.1016/j.cti.2020.02.125

79. Parackova Z, Klooger A, Rataj M, Kaysrova J, Zentsova I, Sumnik Z, et al. Alteration of B cell subsets and the receptor for B cell activating factor (BAFF) in paediatric patients with type 1 diabetes. *Immunol Lett* (2017) 189:94–100. doi: 10.1016/j.imlet.2017.04.009

80. Qiao YC, Shen J, Hong XZ, Liang L, Bo CS, Sui Y, et al. Changes of regulatory T cells, transforming growth factor-beta and interleukin-10 in patients with type 1 diabetes mellitus: A systematic review and meta-analysis. *Clin Immunol* (2016) 170:61–9. doi: 10.1016/j.clim.2016.08.004

81. Wang Q, Racine JJ, Ratiu JJ, Wang S, Eitinger T, Rassweiler C, et al. Transient BAFF Blockade Inhibits Type 1 Diabetes Development in Nonobese Diabetic Mice by Enriching Immunoregulatory B Lymphocytes Sensitive to Deletion by Anti-CD20 Cotreatment. *J Immunol* (2017) 199:3757–70. doi: 10.4049/jimmunol.1700882

82. Wilson CA, Jacobs C, Baker P, Baskin DG, Dower S, Lernmark A, et al. IL-1beta modulation of spontaneous autoimmune diabetes and thyroiditis in the BB rat. *J Immunol* (1990) 144:3784–8.

83. In’t Veld P. Insulins in human type 1 diabetes: a comparison between patients and animal models. *Semim Immunopathol* (2014) 36:569–79. doi: 10.1007/s00281-014-0438-4

84. Yan X, Wang Z, Schmidt V, Gauert A, Willnow TE, Heinig M, et al. GLP-1 stimulates insulin secretion by PKC-dependent TRPM4 and rapid changes in [Ca2+]i. *Proc Natl Acad Sci USA* (2018) 107:4813–8. doi: 10.1073/pnas.1715322110

85. Wang MY, Chen L, Clark GO, Lee Y, Stevens RD, Ilkayeva OR, et al. Leptin regulates body weight and energy homeostasis in mice. *Proc Natl Acad Sci USA* (2010) 107:15156–161. doi: 10.1073/pnas.0913244106

86. Colsoul B, Vennekens R, Nilius B. Transient receptor potential cation channels in pancreatic beta cells. *Rev Physiol Biochem Pharmacol* (2011) 161:87–110. doi: 10.1007/978-3-7091-0152-1_2

87. Pravitt D, Monteill-Zoller MK, Brixel L, Spangenberg C, Zabel B, Fleig A, et al. TRPM5 is a transient Ca2+-activated cation channel responding to rapid changes in [Ca2+]. *Proc Natl Acad Sci USA* (2003) 100:15166–71. doi: 10.1073/pnas.0334624103

88. Krishnan K, Ma Z, Bjorklund A, Islam MS. Role of transient receptor potential melastatin-like subtype 5 channel in insulin secretion from rat beta-cells. *Pancreas* (2014) 43:597–604. doi: 10.1097/MPA.0000000000000108

89. Shigeto M, Ramacheya R, Tarasov AI, Cha CY, Chibalina MV, Hastoy B, et al. Patients with type 1 diabetes mellitus have impaired IL-1 beta modulation of spontaneous autoimmune diabetes and thyroiditis in the BB rat. *J Immunol* (1990) 144:3784–8.

90. In’t Veld P. Insulins in human type 1 diabetes: a comparison between patients and animal models. *Semim Immunopathol* (2014) 36:569–79. doi: 10.1007/s00281-014-0438-4

91. Yan X, Wang Z, Schmidt V, Gauert A, Willnow TE, Heinig M, et al. GLP-1 stimulates insulin secretion by PKC-dependent TRPM4 and rapid changes in [Ca2+]i. *Proc Natl Acad Sci USA* (2018) 107:4813–8. doi: 10.1073/pnas.1715322110

92. Wilson CA, Jacobs C, Baker P, Baskin DG, Dower S, Lernmark A, et al. IL-1beta modulation of spontaneous autoimmune diabetes and thyroiditis in the BB rat. *J Immunol* (1990) 144:3784–8.

93. Int’Veld P. Insulins in human type 1 diabetes: a comparison between patients and animal models. *Semim Immunopathol* (2014) 36:569–79. doi: 10.1007/s00281-014-0438-4

94. Wiebe PO, Kormish JD, Roper VT, Fujitani Y, Alston NI, Zaret KS, et al. Ptf1a binds to and activates area III, a highly conserved region of the Pdx1 promoter that mediates early pancreas-wide Pdx1 expression. *Mol Cell Biol* (2007) 27:4093–104. doi: 10.1128/MCB.01978-06

95. Fujimoto K, Polonsky KS. Pdx1 and other factors that regulate pancreatic beta-cell survival. *Diabetes Obes Metab* (2009) 11 Suppl 4:3–7. doi: 10.1111/j.1463-1326.2009.01211.x

96. O’Carroll AM, Howell GM, Roberts EM, Lolait SJ. Vasopressin potentiates corticotropin-releasing hormone-induced insulin release from mouse pancreatic beta-cells. *J Endocrinol* (2008) 197:231–9. doi: 10.1677/JOE-07-0645

97. Hashimoto K, Nishioka T, Takao T, Numata Y. Low plasma corticotropin-releasing hormone concentration (CRH) levels in patients with non-insulin dependent diabetes mellitus (NIDDM). *Endocr J* (1993) 40:705–9. doi: 10.1507/endocrj.40.705

98. Kveberg L, Ryan JC, Rolstad B, Ingjerdingen M. Expression of regulator of G protein signalling proteins in natural killer cells, and their modulation by Ly49A and Ly49D. *Immunology* (2005) 115:358–65. doi: 10.1111/j.1365-2567.2005.02174.x

99. Lippert E, Yowe DL, Gonzalez JA, Justice JP, Webster JM, Fedyk ER, et al. Role of regulator of G protein signaling 16 in inflammation-induced T lymphocyte migration and activation. *J Immunol* (2003) 171:1542–55. doi: 10.4049/jimmunol.171.3.1542

100. Suurvali J, Pahimta M, Saar R, Palme V, Natt A, Titvel T, et al. RGS16 restricts the pro-inflammatory response of monocytes. *Scand J Immunol* (2015) 81:23–30. doi: 10.1111/sij.12253

101. Lachmandas E, Thiem K, van den Heuvel C, Hijmans A, de Galan BE, Tack CJ, et al. Patients with type 1 diabetes mellitus have impaired IL-1beta production in response to Mycobacterium tuberculosis. *Eur J Clin Microbiol Infect Dis* (2018) 37:371–80. doi: 10.1007/s10096-017-3145-y

102. Shen TC, Lin CL, Wei CC, Liao WC, Chen WC, Chen CH, et al. Increased risk of tuberculosis in patients with type 1 diabetes mellitus: results from a population-based cohort study in Taiwan. *Medicine* (2014) 93:e66. doi: 10.1097/MD.0000000000000096

103. Smith MJ, Simmons KM, Cambier JC. B cells in type 1 diabetes mellitus and diabetic kidney disease. *Nat Rev Nephrol* (2017) 13:72–20. doi: 10.1038/nrneph.2017.138

104. Himman RM, Cambier JC. Role of B lymphocytes in the pathogenesis of type 1 diabetes. *Curr Diabetes Rep* (2014) 14:543. doi: 10.1007/s11892-014-0543-8

105. Stechova K, Kolar M, Blatny R, Halbluber Z, Vcelakova J, Hubackova M, et al. Healthy first degree relatives of patients with type 1 diabetes exhibit significant differences in basal gene expression pattern of immunocompetent cells compared to controls: expression pattern as predeterminant of autoimmune diabetes. *Scand J Immunol* (2011). doi: 10.1111/j.1365-3083.2011.02637.x

106. Deng C, Xiang Y, Tan T, Ren Z, Cao C, Huang G, et al. Altered Peripheral B-Lymphocyte Subsets in Type 1 Diabetes and Latent Autoimmune Diabetes in Adults. *Diabetes Care* (2015). doi: 10.23736/s0149-1998.15-1765

**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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